รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของกระบวนการเชื่อมโยงข้ามต่อคุณสมบัติทางกายภาพและชีวภาพของแผ่นฟิล์มโปรตีนกาวไหม

โดย รองศาสตราจารย์ ดร. พรอนงค์ อร่ามวิทย์ และคณะ

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คณะผู้วิจัย	สังกัด
1. รองศาสตราจารย์ ดร. พรอนงค์ อร่ามวิทย์	คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
2. รองศาสตราจารย์ ดร. ธีระพล ศรีชนะ	คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์
3. ผู้ช่วยศาสตราจารย์ ดร. โศรดา กนกพานนท์	คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Executive Summary

โครงการ ผลของกระบวนการเชื่อมโยงข้ามต่อคุณสมบัติทางกายภาพและชีวภาพของแผ่นฟิล์มโปรตีนกาวไหม

สืบเนื่องจากงานวิจัยที่แสดงให้เห็นว่า โปรตีนกาวไหมหรือที่เรียกว่าเซริซินมีคุณสมบัติทางชีวภาพที่เหมาะสมต่อการนำมาใช้ประโยชน์โดยเฉพาะการนำมาประยุกต์เป็นวัสดุที่ใช้ในการปิดบาดแผล ผู้วิจัยจึงได้ทำฟิล์มปิดบาดแผลจากโปรตีนกาวไหม โดยมีการผสมโพลิเมอร์พบว่าอัตราส่วนของโปรตีนกาวไหม:polyvinyl alcohol:glycerin ในขนาด 3:2:1 จะให้แผ่นฟิล์มที่มีคุณลักษณะทางกายภาพเหมาะสม มีความยืดหยุ่นดีเหมาะแก่การนำไปใช้ประโยชน์ แต่ฟิล์มดังกล่าวสามารถละลายน้ำได้ง่ายทำให้ไม่สามารถปกป้องบาดแผลได้ดีเท่าที่ควร จึงจำเป็นต้องผ่านกระบวนการเชื่อมโยงข้ามซึ่งอาจเป็นกระบวนการทางกายภาพหรือทางเคมี เพื่อทำให้การละลายของแผ่นฟิล์มลดน้อยลง

ผลจากการศึกษาพบว่ากระบวนการเชื่อมโยงข้ามด้วยกายภาพอันได้แก่ การใช้ความร้อน การใช้ความร้อนภายใต้ความดัน การใช้รังสียูวี การใช้รังสีแกมมา ไม่สามารถปรับปรุงคุณสมบัติของแผ่นฟิล์มโปรตีนกาวไหมได้อย่างมีประสิทธิภาพ แต่กระบวนการเชื่อมโยงข้ามทางเคมีโดยใช้ genipin ซึ่งเป็นสารสกัดจากดอกพุดและมีความเป็นพิษน้อยกว่า glutaraldehyde ถึง 10,000 เท่าในความเข้มข้นต่าง ๆ ตั้งแต่ร้อยละ 0.01-0.1 พบว่ามีประสิทธิภาพดี โดยความเข้มข้นของ genipin ที่เพิ่มมากขึ้นส่งผลให้มีร้อยละของการเชื่อมโยงข้ามและยังทำให้ค่าการต้านทานแรงดึงและร้อยละของการยืดตัว ณ จุดขาดของแผ่นฟิล์มโปรตีนกาวไหมเพิ่มสูงขึ้นโดยเฉพาะที่ genipin ความเข้มข้นสูง ๆ นอกจากนี้ genipin ยังทำให้ค่าการพองตัว (swelling) ของแผ่นฟิล์มโปรตีนกาวไหมลดลงแต่ยังสามารถดูดความชื้นและพองตัวได้ไม่ต่ำกว่า 3 เท่าของน้ำหนักซึ่งเพียงพอต่อการนำไปใช้ประโยชน์ทางการแพทย์โดยเฉพาะสำหรับบาดแผลที่มีสารคัดหลั่งมาก นอกจากนี้แผ่นฟิล์มที่ผลิตขึ้นและผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ยังสามารถปลดปล่อยโปรตีนกาวไหมออกจากแผ่นฟิล์ม ซึ่งโปรตีนกาวไหมที่ถูกปลดปล่อยออกมา จะสามารถกระตุ้นการสร้างคอลลาเจนซึ่งเป็นปัจจัยสำคัญประการหนึ่งต่อการหายของบาดแผล โดยแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นสูง (ร้อยละ 0.1) สามารถปลดปล่อยโปรตีนกาวไหมออกมาได้ต่ำที่สุด แต่สามารถทำให้การปลดปล่อยออกมาในลักษณะเนิ่นนานได้ซึ่งเป็นคุณลักษณะที่ต้องการ อีกทั้งยังถูกย่อยสลายได้ด้วยเอนไซม์ต่ำที่สุด เมื่อนำมาทดสอบกับเซลล์ผิวหนังพบว่า แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นสูง ๆ จะเพิ่มการยึดเกาะของเซลล์ทำให้เซลล์มีการเจริญเติบโตที่ดี เซลล์สามารถสร้างสาร nitric oxide ซึ่งเป็นสารที่กระตุ้นการสร้างโปรตีนของเซลล์ รวมถึงสามารถสร้างคอลลาเจนได้สูงสุดเมื่อเทียบกับแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นต่ำ ๆ ด้วยเหตุนี้จึงอาจกล่าวได้ว่า แผ่นฟิล์มโปรตีนกาวไหมที่มีองค์ประกอบของ โปรตีนกาวไหม:polyvinyl alcohol:glycerin ในอัตราส่วน 3:2:1 และ genipin ในความเข้มข้น 0.1% มีความเหมาะสมต่อการนำไปใช้ทางการแพทย์เพื่อกระตุ้นการซ่อมแซมและเจริญเติบโตของเซลล์ที่มีบาดแผลต่อไป

บทคัดย่อ

โปรตีนกาวไหมเป็นวัสดุที่เหลือใช้ทางการเกษตรซึ่งจากการศึกษาพบว่า โปรตีนกาวไหมที่ได้จากไหมสายพันธุ์จุล 1/1 ซึ่งมีรังไหมสีขาว เมื่อนำมาสกัดด้วยความร้อนภายใต้ความดัน จะทำให้ได้โปรตีนกาวไหมที่มีคุณสมบัติในการกระตุ้นการสร้างคอลลาเจนได้ดี เนื่องจากโปรตีนกาวไหมสามารถเกิดเป็นเจลได้ที่ความเข้มข้นสูง ดังนั้นจึงสามารถนำมาทำเป็นแผ่นฟิล์มได้ แต่อย่างไรก็ดี โปรตีนกาวไหมเพียงชนิดเดียวเมื่อนำมาทำเป็นแผ่นฟิล์มจะมีลักษณะเปราะ ไม่เหมาะสมต่อการนำมาใช้ แต่เมื่อนำมาผสมร่วมกับโพลิเมอร์ชนิดอื่น ๆ เช่น polyvinyl alcohol จะให้แผ่นฟิล์มที่มีความยืดหยุ่นดี และมีคุณสมบัติทางกายภาพที่เหมาะสม นอกจากนี้หากมีการใส่สารกลุ่ม plasticizer เช่น glycerin ร่วมด้วยก็จะทำให้มีคุณสมบัติดียิ่งขึ้น โดยอัตราส่วนของโปรตีนกาวไหม:polyvinyl alcohol:glycerin ที่เหมาะสมคือ 3:2:1 โดยน้ำหนัก ซึ่งจะทำให้แผ่นฟิล์มที่ได้มีความคงตัว ยืดหยุ่นดี สามารถนำมาใช้ประโยชน์ทางการแพทย์ต่อไปได้ แต่เนื่องจากแผ่นฟิล์มส่วนใหญ่ที่นำมาใช้เป็นแผ่นปิดบาดแผล นอกจากจะมีความคงตัวและยืดหยุ่นได้ดีแล้ว ยังต้องมีคุณสมบัติที่คงตัวเมื่อสัมผัสกับน้ำหรือของเหลวที่หลั่งออกมากับบาดแผล อีกทั้งยังต้องทนต่อการย่อยสลายของเอนไซม์จากบาดแผล ด้วยเหตุนี้แผ่นฟิล์มโปรตีนกาวไหมที่พัฒนาขึ้นยังต้องผ่านกระบวนการเชื่อมโยงข้าม (cross-linking) เพื่อให้ทนต่อสภาวะดังที่กล่าวมาแล้วได้

กระบวนการเชื่อมโยงข้ามสามารถแบ่งได้เป็น 2 กลุ่มใหญ่คือ กระบวนการเชื่อมโยงข้ามด้วยกายภาพ อันได้แก่ การใช้ความร้อน การใช้ความร้อนภายใต้ความดัน การใช้รังสียูวี การใช้รังสีแกมมา เป็นต้น หรือกระบวนการเชื่อมโยงข้ามทางเคมีได้แก่การใช้สารเคมีเช่น glutaraldehyde หรือ genipin เมื่อทดสอบกระบวนการเชื่อมโยงข้ามทางกายภาพกับแผ่นฟิล์มโปรตีนกาวไหมพบว่า กระบวนการเชื่อมโยงข้ามทางกายภาพไม่ทำให้เกิดความเสียหายแก่แผ่นฟิล์ม เช่น ไม่ก่อให้เกิดรูพรุน แต่การเชื่อมโยงข้ามแผ่นฟิล์มโปรตีนกาวไหมด้วยรังสียูวีหรือรังสีแกมมาทำให้แผ่นมีความขรุขระมากขึ้น แต่กระบวนการเชื่อมโยงข้ามทางกายภาพยังไม่สามารถปรับปรุงคุณสมบัติของแผ่นฟิล์มโปรตีนกาวไหมได้อย่างมีประสิทธิภาพ ดังจะเห็นได้จากค่า contact angle และค่า surface density ที่ไม่เปลี่ยนแปลงไปจากเดิมมากนัก ด้วยเหตุนี้จึงมีการทดสอบกระบวนการเชื่อมโยงข้ามทางเคมี โดยสารเคมีที่เลือกใช้ได้แก่ genipin ซึ่งเป็นสารสกัดจากดอกพุดที่มีความเป็นพิษน้อยกว่า glutaraldehyde ถึง 10,000 เท่า

เมื่อนำสาร genipin ในความเข้มข้น 0.01, 0.025, 0.05 และ 0.1% มาผสมในแผ่นฟิล์มโปรตีนกาวไหม พบว่า แผ่นโปรตีนกาวไหมมีสีที่เข้มขึ้นส่งผลให้การแพร่ผ่านของแสงลดลงและมีร้อยละของการเชื่อมโยงข้าม (% cross-linking) ที่เพิ่มขึ้นตามความเข้มข้นของสาร genipin ที่ใส่เข้าไป อย่างไรก็ตาม genipin ไม่ทำให้ค่า surface density และ contact angle ของแผ่นฟิล์มโปรตีนกาวไหมเปลี่ยนแปลงแต่อย่างใด ในขณะเดียวกัน สาร

genipin กลับทำให้ค่าการต้านทานแรงดึงและร้อยละของการยืดตัว ณ จุดขาดของแผ่นฟิล์มโปรตีนกาวไหมเพิ่มสูงขึ้นโดยเฉพาะที่ genipin ความเข้มข้นสูง ๆ นอกจากนี้ genipin ยังทำให้ค่าการพองตัว (swelling) ของแผ่นฟิล์มโปรตีนกาวไหมลดลงแต่ยังสามารถดูดความชื้นและพองตัวได้ไม่ต่ำกว่า 3 เท่าของน้ำหนักซึ่งเพียงพอต่อการนำไปใช้ประโยชน์ทางการแพทย์โดยเฉพาะสำหรับบาดแผลที่มีสารคัดหลั่งมาก

คุณสมบัติที่สำคัญประการหนึ่งของแผ่นฟิล์มจากโปรตีนกาวไหมคือคุณสมบัติในการปลดปล่อยโปรตีนกาวไหมออกจากแผ่นฟิล์ม ซึ่งโปรตีนกาวไหมที่ถูกปลดปล่อยออกมา จะสามารถกระตุ้นการสร้างคอลลาเจนซึ่งเป็นปัจจัยสำคัญประการหนึ่งต่อการหายของบาดแผล พบว่า แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นสูง (ร้อยละ 0.1) สามารถปลดปล่อยโปรตีนกาวไหมออกมาได้ต่ำที่สุด แต่สามารถทำให้การปลดปล่อยออกมาในลักษณะเนิ่นนานได้ซึ่งเป็นคุณลักษณะที่ต้องการ อีกทั้งยังถูกย่อยสลายได้ด้วยเอนไซม์ต่ำที่สุด เมื่อนำมาทดสอบกับเซลล์ผิวหนังพบว่า แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นสูง ๆ จะเพิ่มการยึดเกาะของเซลล์ทำให้เซลล์มีการเจริญเติบโตที่ดี เซลล์สามารถสร้างสาร nitric oxide ซึ่งเป็นสารที่กระตุ้นการสร้างโปรตีนของเซลล์ รวมถึงสามารถสร้างคอลลาเจนได้สูงสุดเมื่อเทียบกับแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นต่ำ ๆ ด้วยเหตุนี้จึงอาจกล่าวได้ว่า แผ่นฟิล์มโปรตีนกาวไหมที่มีองค์ประกอบของโปรตีนกาวไหม:polyvinyl alcohol:glycerin ในอัตราส่วน 3:2:1 และ genipin ในความเข้มข้น 0.1% มีความเหมาะสมต่อการนำไปใช้ทางการแพทย์เพื่อกระตุ้นการซ่อมแซมและเจริญเติบโตของเซลล์ที่มีบาดแผลต่อไป

คำสำคัญ: โปรตีนกาวไหม, ฟิล์ม, กระบวนการเชื่อมโยงข้าม

Abstract

Silk sericin, a degumming protein, is considered as waste product in textile manufacturing. Silk sericin extracted by high pressure, high temperature degumming technique from Chul 1/1 strain, a white cocoon shell, exhibits the highest collagen promotion activity. Since silk sericin forms gel easily at high concentration, it can form a film for further applications. However, silk sericin itself forms fragile material which is not suitable for medical uses. Mixing silk sericin with other polymers such as polyvinyl alcohol generates flexible film with good physical properties. Plasticizer such as glycerin also can improve the properties of silk sericin film. The optimum ratio for making a stable and flexible film is silk sericin : polyvinyl alcohol : glycerin at 3:2:1 by weight of each component. Besides the stability and flexibility of film, it also needs to be stable after contact with pus from wounds and resistant to enzyme degradation. Cross-linking process will help improving these properties.

Cross-linking process can be divided into 2 groups which are physical cross-linking by heat, heat under vacuum, UV irradiation, gamma irradiation and chemical cross-linking by using glutaraldehyde or genipin. Physical cross-linking processes do not damage silk sericin film but UV as well as gamma irradiation made rough films. However, all physical cross-linking process cannot improve the silk sericin film effectively which indicated by significantly unchanged in contact angle and surface density value. Due to these results, chemical cross-linking has been further explored by using genipin which is an extract from *Gardenia jasminoides* and 10,000 times less toxic than glutaraldehyde.

Adding genipin at concentrations 0.01, 0.025, 0.05 and 0.1% into silk sericin film makes the darker color films and less light transmission as well as increase the degree of cross-linking. However, genipin does not change the surface density and contact angle of the film. At the same time, cross-linking by genipin increases the tensile strength and percent elongation of the film but decreases the swelling property. Nevertheless, sericin film after cross-linking with genipin can still absorb humidity and performs good swelling property, at least 3 times of its weight which is suitable for high secretion wounds.

The main property of silk sericin film is to be able to release sericin which can activate collagen production from cells while maintain its stability. The result shows that silk sericin film composed of genipin at high concentration (0.1% w/v) can release the lowest amount of silk sericin in sustained-release manner compared with genipin at lower concentrations. After testing the film with fibroblast cells, silk sericin film

containing high concentration of genipin increases cell viability and promotes cell adhesion resulting in higher amount of nitric oxide and collagen produced from cells. We can conclude that silk sericin film containing sericin:polyvinyl alcohol:glycerin at the ratio of 3:2:1 with genipin at 0.1% w/v can promote the wound healing process and activate cell growth which is suitable for medical applications.

Abstract: Sericin, film, cross-linking

สัญญาเลขที่ DBG 5380039

โครงการ “ผลของกระบวนการเชื่อมโยงข้ามต่อคุณสมบัติทางกายภาพและชีวภาพของแผ่นฟิล์มโปรตีนกาวไหม”

รายงานฉบับสมบูรณ์

เนื้อหางานวิจัย

.....

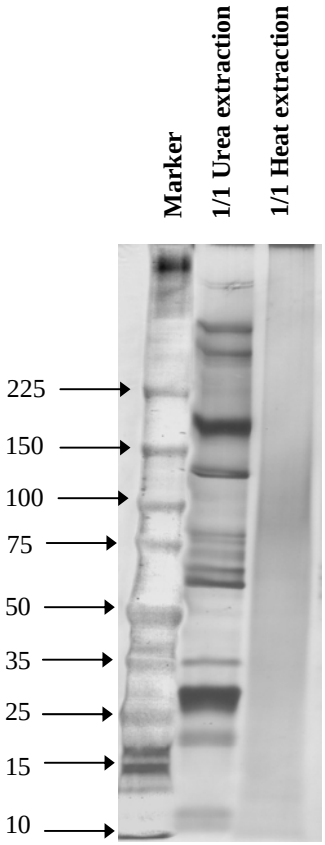
สืบเนื่องจากงานวิจัยจำนวนมากทั้งในและต่างประเทศได้แสดงให้เห็นว่า โปรตีนกาวไหมหรือที่เรียกว่าเซริซินมีคุณสมบัติทางชีวภาพที่เหมาะสมต่อการนำมาใช้ประโยชน์มากมายเช่น มีคุณสมบัติในการต้านอนุมูลอิสระ สามารถป้องกันการเจริญเติบโตของแบคทีเรียได้ และเนื่องจากเซริซินเป็นสารที่สามารถดูดซึมน้ำได้ดีจึงสามารถทำให้ผิวหนังชุ่มชื้นอันเป็นคุณสมบัติที่ดีในการนำใช้กับบาดแผลเนื่องจากจะทำให้ผู้ป่วยเกิดความเจ็บปวดน้อยลง นอกจากนี้ยังได้มีงานวิจัยพบว่าเซริซินสามารถกระตุ้นการสร้างและเพิ่มการยึดเกาะกันของเซลล์สร้างเส้นใยผิวหนังของมนุษย์ได้ (human skin fibroblasts) อีกทั้งยังพบอีกว่าเซริซินสามารถเพิ่มการสร้างคอลลาเจนทำให้บาดแผลในหนูทดลองหายได้รวดเร็วขึ้น จากผลการศึกษาโครงการ คุณสมบัติทางกายภาพและชีวภาพของฟิล์มปิดแผลที่ผลิตจากโปรตีนกาวไหม (ทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย สัญญาเลขที่ DBG5180017) พบว่าไหมสายพันธุ์ไทยมีคุณสมบัติไม่ด้อยไปกว่าไหมสายพันธุ์ต่างประเทศเนื่องจากมีคุณสมบัติในการกระตุ้นการเจริญเติบโตของเซลล์ผิวหนังและกระตุ้นการสร้างคอลลาเจนได้ โดยเฉพาะอย่างยิ่งไหมสายพันธุ์จุล 1/1 จากบริษัทจุลไหมไทย จำกัด ซึ่งเป็นไหม bivoltine มีรังไหมสีขาว (ภาพที่ 1) พบว่ามีคุณสมบัติในการกระตุ้นการเจริญเติบโตของเซลล์และการสร้างคอลลาเจนได้สูงกว่าไหมสายพันธุ์ไทยอื่น ๆ ในขณะเดียวกันยังกระตุ้นการสร้าง proinflammatory cytokines อันได้แก่ interleukin-1β (IL-1β) และ tumor necrosis factor-α (TNF-α) ได้ต่ำที่สุด ดังนั้นในการศึกษานี้จะนำโปรตีนกาวไหมจากไหมสายพันธุ์จุล 1/1 มาใช้ผลิตแผ่นฟิล์มและทดสอบต่อไป

ภาพที่ 1 รังไหมไทยสายพันธุ์จุล 1/1

อย่างไรก็ตาม คุณสมบัติของโปรตีนกาวไหมทั้งทางด้านกายภาพและชีวภาพยังขึ้นอยู่กับกรรมวิธีในการสกัดด้วย จากการวิจัยพบว่าโปรตีนกาวไหมที่สกัดด้วยความร้อนจะมีคุณสมบัติในการกระตุ้นการเจริญเติบโตของเซลล์ผิวหนังและกระตุ้นการสร้างคอลลาเจนได้สูงสุด ซึ่งกระบวนการสกัดโปรตีนกาวไหมด้วยความร้อนมีขั้นตอนดังนี้

นำรังไหมมาตัดให้มีขนาดประมาณ 5 ตารางมิลลิเมตร ใส่น้ำบริสุทธิ์ในอัตราส่วน 1:30 นำไป autoclave ที่ 120 องศาเซลเซียสนาน 60 นาที หลังจากนั้นทำการกรองกากออก และนำสารละลายที่ได้ไปทำให้แห้งโดยวิธี freeze-drying

เพื่อควบคุมคุณภาพของโปรตีนกาวไหมที่สกัดได้จึงจำเป็นต้องมีการตรวจวัดน้ำหนักโมเลกุลและความเข้มข้นของโปรตีนกาวไหมในสารละลายซึ่งเป็นคุณสมบัติเบื้องต้นก่อน ซึ่งการตรวจวัดน้ำหนักโมเลกุลทำได้โดยใช้ gel electophoresis (SDS-PAGE gradient gel 5-20%) และใช้ silver staining ผลได้ดังภาพที่ 2



ภาพที่ 2 การตรวจวัดน้ำหนักโมเลกุลของโปรตีนกาวไหมสายพันธุ์จุล 1/1 ที่สกัดด้วยสารละลายยูเรียและความร้อน

จะเห็นได้ว่าวิธีการสกัดโปรตีนกาวไหมด้วยสารละลายยูเรียเป็นวิธีที่ให้โปรตีนกาวไหมที่มีน้ำหนักโมเลกุลต่าง ๆ แยกเป็นแถบชัดเจน มีน้ำหนักโมเลกุลตั้งแต่ 10 ถึง > 225 KDa ในขณะที่โปรตีนกาวไหมจากการสกัดด้วยความร้อนจะให้น้ำหนักโมเลกุลเป็นแถบต่อเนื่องกัน มีน้ำหนักโมเลกุลตั้งแต่ 25-150 KDa เมื่อทำการสกัดซ้ำไม่ต่ำกว่า 3 ครั้งพบว่าให้ผลเหมือนกันทุกครั้งกล่าวคือ กระบวนการสกัดด้วยความร้อนจะให้แถบต่อเนื่องบน gel electrophoresis และน้ำหนักโมเลกุลใกล้เคียงกันทุกครั้ง

<u>การตรวจวัดความเข้มข้นของโปรตีนกาวไหม</u>

การตรวจวัดความเข้มข้นของโปรตีนกาวไหมสามารถทำได้โดยนำสารละลายโปรตีนกาวไหมหลังการกรองเอากากออก มาวิเคราะห์ปริมาณโปรตีนด้วย BCA kit (ภาคผนวกที่ 1)

จากการตรวจวัดความเข้มข้นของโปรตีนกาวไหมจากการสกัดในแต่ละครั้งให้ผลการทดลองดังแสดงในตารางที่ 1

ตารางที่ 1 ความเข้มข้นของโปรตีนกาวไหมจากการสกัดไหมสายพันธุ์จุล 1/1 ด้วยความร้อน

ครั้งที่	ความเข้มข้นของโปรตีน (มิลลิกรัม/มิลลิลิตร)
1	10.87
2	9.80
3	9.70
4	10.26
5	10.76
ค่าเฉลี่ย $\pm$ SD	10.28 ± 0.53

จะเห็นได้ว่า ความเข้มข้นของโปรตีนกาวไหมที่สกัดได้ในแต่ละครั้งไม่แตกต่างกันมากนัก อย่างไรก็ตามก่อนที่จะนำโปรตีนกาวไหมที่สกัดได้มาใช้ในการทำแผ่นฟิล์มเพื่อทดสอบต่อไป ผู้วิจัยได้นำสารละลายที่ได้มาวัดปริมาณโปรตีนก่อนใช้ด้วยทุกครั้ง

<u>การคัดเลือกโพลิเมอร์และความเข้มข้นของโพลิเมอร์ที่เหมาะสมในการเป็นสารก่อฟิล์ม</u>

จากการนำโปรตีนกาวไหมมาทำเป็นแผ่นด้วยวิธี casting พบว่าแผ่นที่ได้มีลักษณะเปราะ ไม่สามารถลอกออกมาเป็นแผ่นได้จึงจำเป็นต้องผสมร่วมกับโพลิเมอร์อื่น ๆ โพลิเมอร์ที่ได้รับการรับรองจากสำนักงานคณะกรรมการอาหารและยาของสหรัฐอเมริกา (FDA) เพื่อนำมาใช้ในการทำแผ่นเนื้อเยื่อในมนุษย์ได้แก่

1. Polyvinyl alcohol
2. Poloxamer (Pluronic®)
3. Agarose

ซึ่งได้นำโพลิเมอร์ชนิดต่าง ๆ มาผสมกับโปรตีนกาวไหมเพื่อขึ้นรูปเป็นแผ่นฟิล์มในอัตราส่วนต่าง ๆ กัน พบว่าจากคุณสมบัติทางกายภาพ polyvinyl alcohol เป็นโพลิเมอร์ที่เหมาะสมที่สุดที่จะนำมาใช้ทำแผ่นฟิล์มเนื่องจากให้แผ่นที่มีความยืดหยุ่นเหมาะสม เป็นเนื้อเดียวกัน ไม่มีรอยแตกบนแผ่น ดังนั้นจึงได้ศึกษาอัตราส่วนของโปรตีนกาวไหมและ polyvinyl alcohol ที่เหมาะสมต่อการนำมาทำเป็นแผ่นฟิล์ม

<u>วิธีการทดลอง</u>

1. นำ polyvinyl alcohol มาละลายในน้ำให้มีความเข้มข้นร้อยละ 5 และนำไปอุ่นที่ 37 องศาเซลเซียส นาน 5 ชั่วโมง

2. นำสารละลายของโปรตีนกาวไหมมาผสมกับสารละลายในข้อ 1) เพื่อให้ได้ความเข้มข้นต่าง ๆ กัน
3. เทสารละลายที่ได้ลงใน petri dish และตั้งทิ้งไว้ที่อุณหภูมิห้องนาน 48 ชม.

จากการทดสอบผสมสารละลาย polyvinyl alcohol กับโปรตีนกาวไหมในอัตราส่วนต่าง ๆ ได้แก่

2% polyvinyl alcohol

1% sericin + 2% polyvinyl alcohol

2% sericin + 2% polyvinyl alcohol

3% sericin + 2% polyvinyl alcohol

4% sericin + 2% polyvinyl alcohol

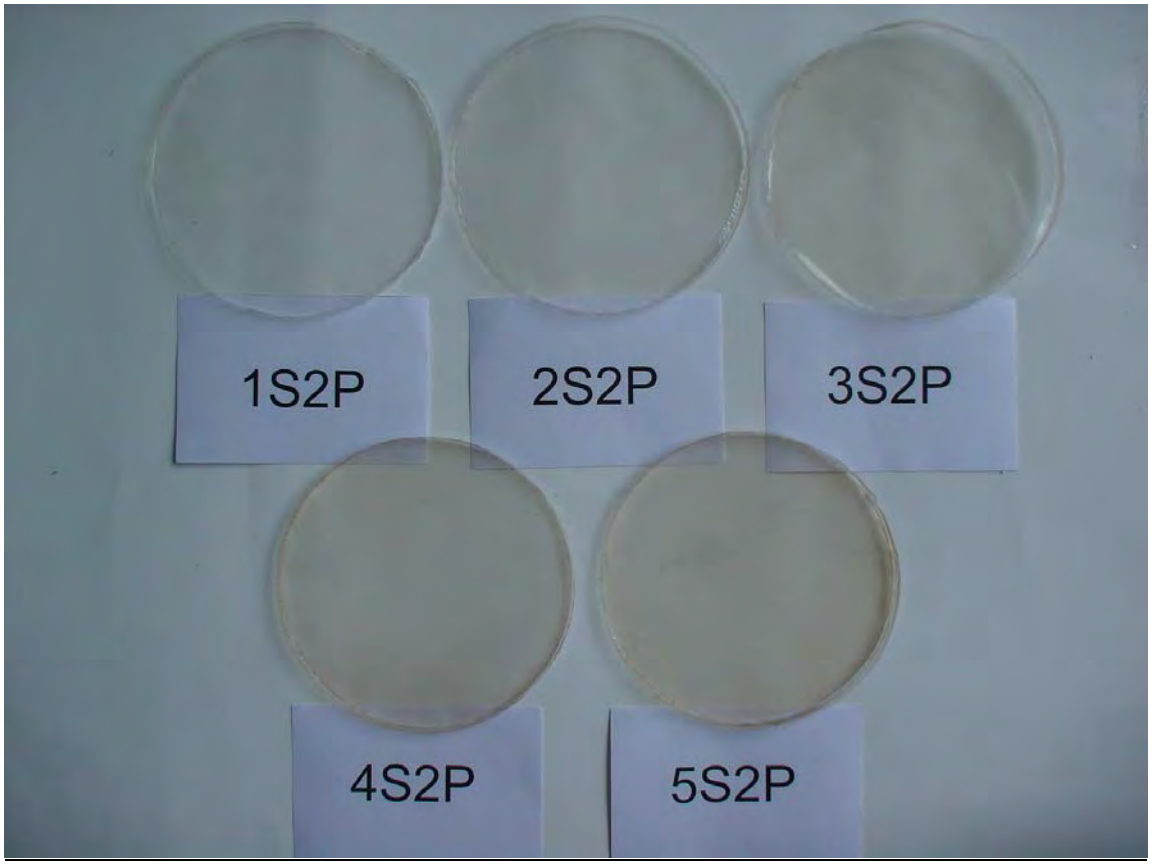
5% sericin + 2% polyvinyl alcohol

ได้แผ่นฟิล์มที่มีลักษณะทางกายภาพดังที่แสดงในตารางที่ 2 และภาพที่ 3-4

ตารางที่ 2 ลักษณะทางกายภาพของแผ่นฟิล์มที่มีส่วนประกอบของโปรตีนกาวไหมและโพลิเมอร์ในอัตราส่วนต่าง ๆ กัน

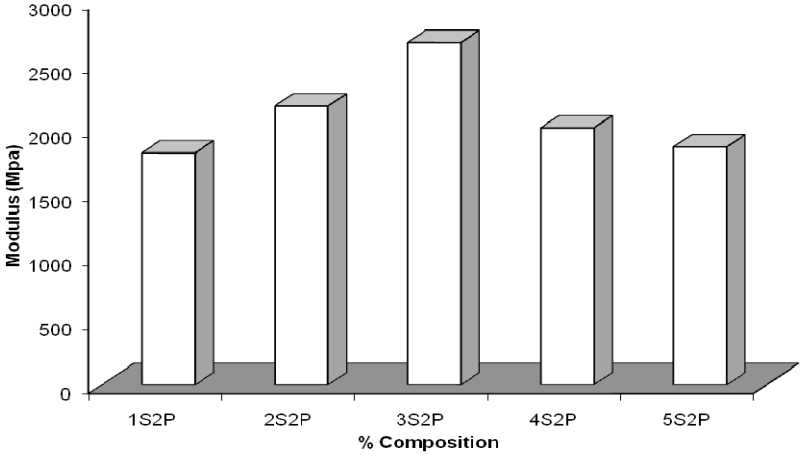
ส่วนประกอบของแผ่นฟิล์ม	**ลักษณะทางกายภาพ**
2% Polyvinyl alcohol	เปราะบาง แตกง่าย ไม่เป็นแผ่นเรียบเนียน
1% Sericin + 2% Polyvinyl alcohol	แผ่นเปราะง่าย เรียบเนียนขึ้นเล็กน้อยแต่ไม่มีความยืดหยุ่น
2% Sericin + 2% Polyvinyl alcohol	เปราะเล็กน้อย มีการแยก phase (สีไม่สม่ำเสมอ)
3% Sericin + 2% Polyvinyl alcohol	แผ่นเรียบเนียน ยืดหยุ่นค่อนข้างดี ไม่มีการแยก phase
4% Sericin + 2% Polyvinyl alcohol	แผ่นเปราะเล็กน้อย มีสีเข้มและไม่ค่อยเป็นเนื้อเดียวกัน
5% Sericin + 2% Polyvinyl alcohol	แผ่นเปราะมาก มีการแยกชั้นและไม่เป็นเนื้อเดียวกัน สีเข้ม

ภาพที่ 3 แสดงแผ่นฟิล์มจาก 2% polyvinyl alcohol



ภาพที่ 4 แสดงแผ่นฟิล์มจาก 2% polyvinyl alcohol ร่วมกับโปรตีนกาวไหมเซริซินที่มีความเข้มข้นต่าง ๆ ตั้งแต่ 1-5%

จากการทดสอบทำแผ่นฟิล์มโปรตีนกาวไหมร่วมกับ polyvinyl alcohol ที่ความเข้มข้นต่าง ๆ พบว่าความเข้มข้นของโปรตีนกาวไหมและ polyvinyl alcohol ในสัดส่วนร้อยละ 3 และ 2 ตามลำดับ จะให้แผ่นฟิล์มที่มีลักษณะทางกายภาพเหมาะสมที่สุด เนื่องจากแผ่นที่ได้มีความเรียบเนียน ไม่เปราะและไม่มีการแยก phase นอกจากนี้สียังไม่เข้มจนเกินไป นอกจากนี้ยังได้ศึกษาความยืดหยุ่นของแผ่นฟิล์มดังกล่าว ผลได้ตามภาพที่ 5

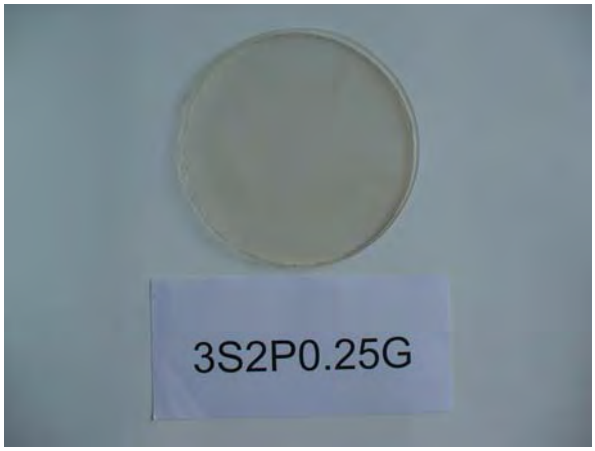


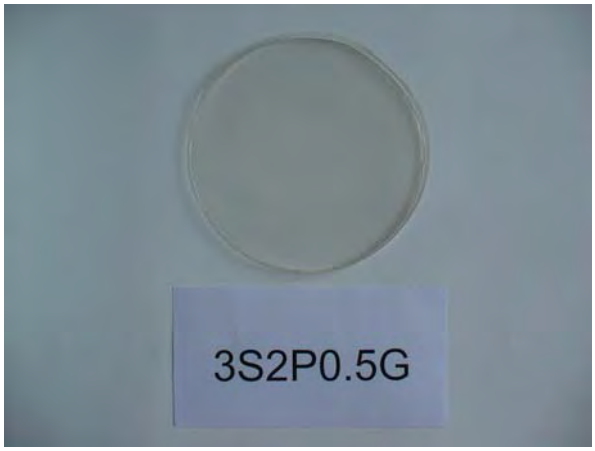
ภาพที่ 5 ค่าแสดงความยืดหยุ่นของแผ่นฟิล์มที่ประกอบด้วย polyvinyl alcohol ร้อยละ 2 และโปรตีนกาวไหมร้อยละ 1-5

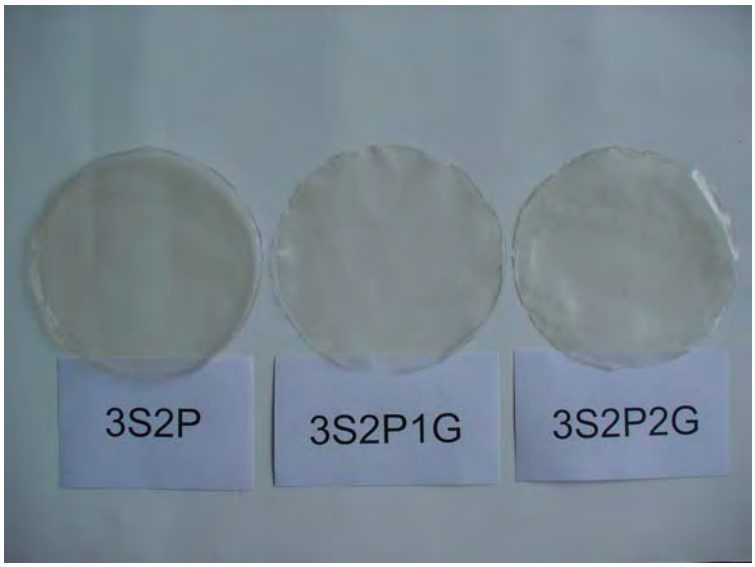
จากผลแสดงให้เห็นว่าแผ่นฟิล์มจากโปรตีนกาวไหมร้อยละ 3 และ polynivyl alcohol ร้อยละ 2 จะให้ความยืดหยุ่นดีที่สุด เมื่อพิจารณาร่วมกับคุณสมบัติทางกายภาพทำให้ฟิล์มดังกล่าวมีความเหมาะสมที่สุดที่จะนำมาพัฒนาต่อ แต่อย่างไรก็ตาม แผ่นฟิล์มที่ได้ยังมีความยืดหยุ่นน้อย ด้วยเหตุนี้ผู้วิจัยจึงได้ทดสอบโดยการเติมสาร plasticizer ซึ่งมีคุณสมบัติดูดความชื้นอันได้แก่ glycerin เข้าไปเพื่อเพิ่มความยืดหยุ่น โดยเติม glycerin เข้าไปในสัดส่วนร้อยละ 0.25-2 พบว่าลักษณะทางกายภาพที่ได้แสดงในตารางที่ 3 และภาพที่ 6

ตารางที่ 3 ลักษณะทางกายภาพของแผ่นฟิล์มที่ประกอบด้วยโปรตีนกาวไหม polyvinyl alcohol และ glycerin

ส่วนประกอบของแผ่นฟิล์ม	ลักษณะทางกายภาพ
3% Sericin + 2% Polyvinyl alcohol + 0.25% Glycerin	แผ่นยังมีความเปราะ ไม่เรียบเนียน
3% Sericin + 2% Polyvinyl alcohol + 0.5% Glycerin	เปราะเล็กน้อย เรียบเนียนขึ้น
3% Sericin + 2% Polyvinyl alcohol + 1% Glycerin	แผ่นเรียบเนียน ยืดหยุ่นดี เป็นเนื้อเดียวกัน
3% Sericin + 2% Polyvinyl alcohol + 1.5% Glycerin	แผ่นเหนอะหนะเล็กน้อย ไม่ค่อยเรียบเนียน
3% Sericin + 2% Polyvinyl alcohol + 2% Glycerin	แผ่นเหนอะหนะมาก ไม่เรียบเนียน







ภาพที่ 6 แสดงแผ่นฟิล์มจาก 3% sericin + 2% polyvinyl alcohol ร่วมกับ glycerin ที่มีความเข้มข้นต่าง ๆ ตั้งแต่ 0.25-2%

จากผลดังกล่าว จะเห็นได้ว่าแผ่นฟิล์มที่ประกอบด้วย 3% sericin + 2% polyvinyl alcohol + 1% glycerin มีคุณสมบัติดีที่สุดที่จะนำมาใช้เป็นแผ่นฟิล์มปิดแผล อย่างไรก็ตามเมื่อนำแผ่นฟิล์มดังกล่าวมาแช่ในน้ำ พบว่า แผ่นฟิล์มละลายหมดภายในเวลาน้อยกว่า 3 ชั่วโมง ซึ่งจำเป็นต้องนำแผ่นฟิล์มมาผ่านกระบวนการเชื่อมโยงข้ามเพื่อให้คุณสมบัติในการละลายน้อยลง

กระบวนการเชื่อมโยงข้ามสามารถแบ่งออกได้เป็น 2 ประเภทคือ

1. กระบวนการเชื่อมโยงข้ามทางกายภาพ วิธีนี้จะทำให้เกิดกระบวนการเชื่อมโยงข้ามที่มีประสิทธิภาพต่ำ แต่ไม่ก่อให้เกิดสารตกค้าง จึงเหมาะกับแผ่นที่มีลักษณะบางและมีวัตถุประสงค์เพื่อใช้ในสภาวะที่มีความชื้นไม่สูงมากนัก
2. กระบวนการเชื่อมโยงข้ามทางเคมี วิธีนี้จะทำให้เกิดกระบวนการเชื่อมโยงข้ามที่มีประสิทธิภาพสูง แผ่นที่ได้จะมีความแข็งแรงเพิ่มขึ้นอย่างมีนัยสำคัญ แต่อาจก่อให้เกิดปัญหาจากการตกค้างของสารที่ใช้ในกระบวนการเชื่อมโยงข้ามโดยเฉพาะในกรณีที่สารเชื่อมโยงข้ามอาจก่อให้เกิดพิษต่อเซลล์

ในการศึกษานี้จะใช้กระบวนการเชื่อมโยงข้ามทั้ง 2 วิธีเปรียบเทียบกันเพื่อคัดเลือกวิธีที่มีประสิทธิภาพสูงสุดในการเชื่อมโยงข้ามแผ่นฟิล์มจากโปรตีนกาวไหม

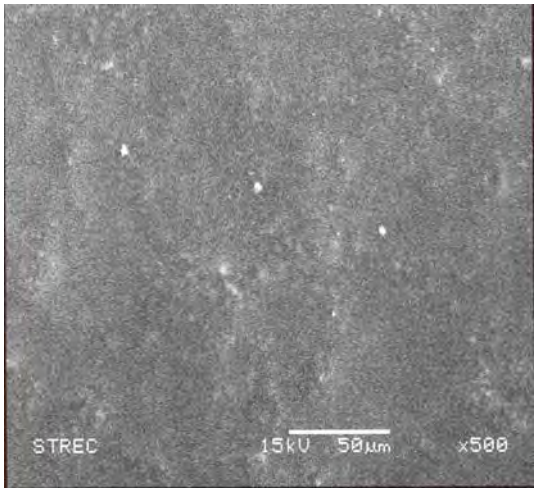
กระบวนการเชื่อมโยงข้ามทางกายภาพที่ใช้ได้แก่

1. การใช้ความร้อน
2. การใช้ความร้อนภายใต้ภาวะ vacuum
3. การใช้แสง UV
4. การใช้รังสีแกมมา

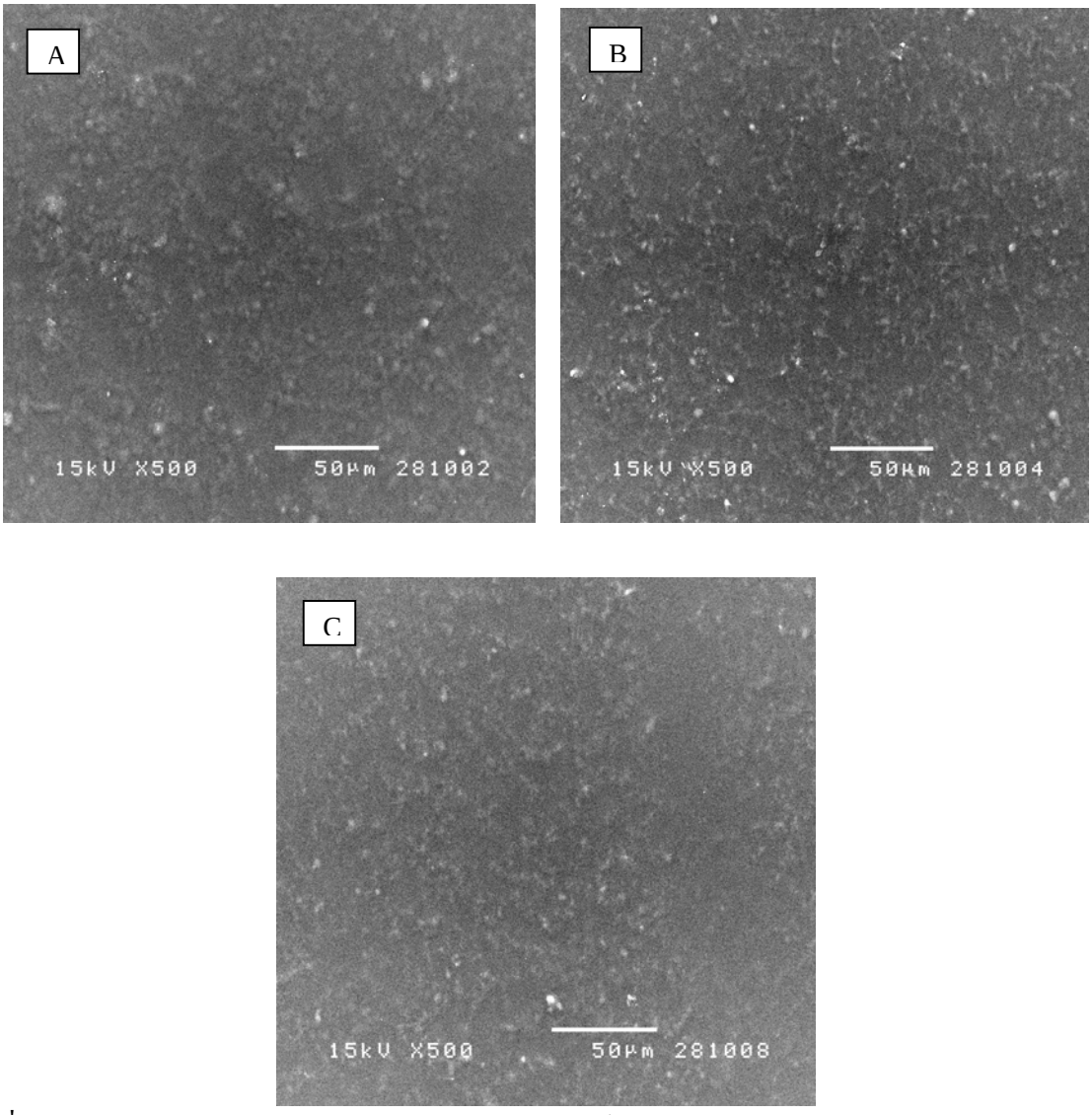
กระบวนการเชื่อมโยงข้ามทางเคมีที่ใช้ได้แก่

1. การใช้สาร genipin ซึ่งเป็นสารเชื่อมโยงข้ามที่ได้จากธรรมชาติ (จากลูกพุด) [1] และมีงานวิจัยที่แสดงให้เห็นว่า genipin มีความเป็นพิษน้อยกว่า glutaraldehyde ถึง 10,000 เท่า [2, 3]
2. การใช้สาร glutaraldehyde

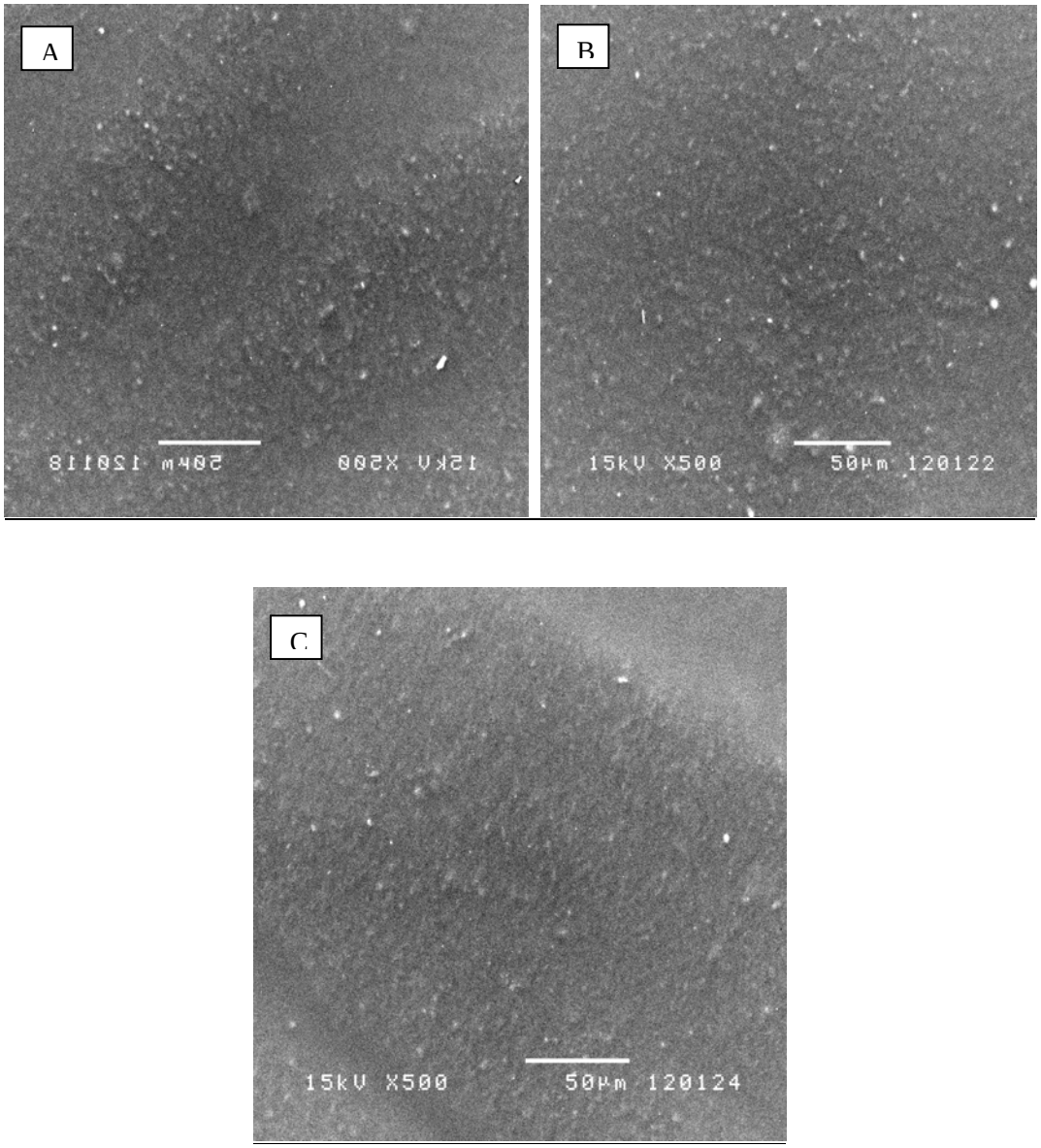
ในเบื้องต้นได้ทดสอบกระบวนการเชื่อมโยงข้ามทางกายภาพ ผลจากการเชื่อมโยงข้ามด้วยความร้อนโดยใช้การอบในตู้อบที่อุณหภูมิ 60 และ 120 องศาเซลเซียสพบว่า แผ่นฟิล์มโปรตีนกาวไหมเมื่อนำไปอบที่ 60 องศาเซลเซียสไม่ส่งผลให้เกิดความแตกต่างจากแผ่นที่ไม่อบแต่อย่างใด แผ่นฟิล์มยังละลายหลังจากแช่ในน้ำไม่เกิน 3 ชั่วโมงเช่นเดิม ในขณะที่แผ่นฟิล์มที่ผ่านการอบที่ 120 องศาเซลเซียสมีลักษณะม้วนงอ ไม่เป็นแผ่น จนไม่สามารถนำไปใช้ประโยชน์ต่อไปได้ ดังนั้นจึงมีการนำแผ่นฟิล์มมาอบในเตาอบภายใต้ vacuum ที่อุณหภูมิ 105 องศาเซลเซียสซึ่งเป็นอุณหภูมิที่เครื่องตั้งไว้ถาวรโดยอบนาน 24, 48 และ 72 ชั่วโมงตามลำดับ ผลจากการอบพบว่าแผ่นที่ได้มีลักษณะทางกายภาพดี ไม่มีการม้วนงอ ไม่แข็งกรอบ แม้ว่าจะมีสีเข้มขึ้นเล็กน้อย อีกทั้งระยะเวลาในการอบไม่มีผลต่อคุณสมบัติทางกายภาพของแผ่นฟิล์ม ภาพที่ 7 และ 8 แสดงภาพจาก Scanning Electron Microscope (SEM) ของแผ่นฟิล์มที่ไม่ผ่านการอบภายใต้สุญญากาศและที่ผ่านการอบนาน 24, 48 และ 72 ชั่วโมงตามลำดับ



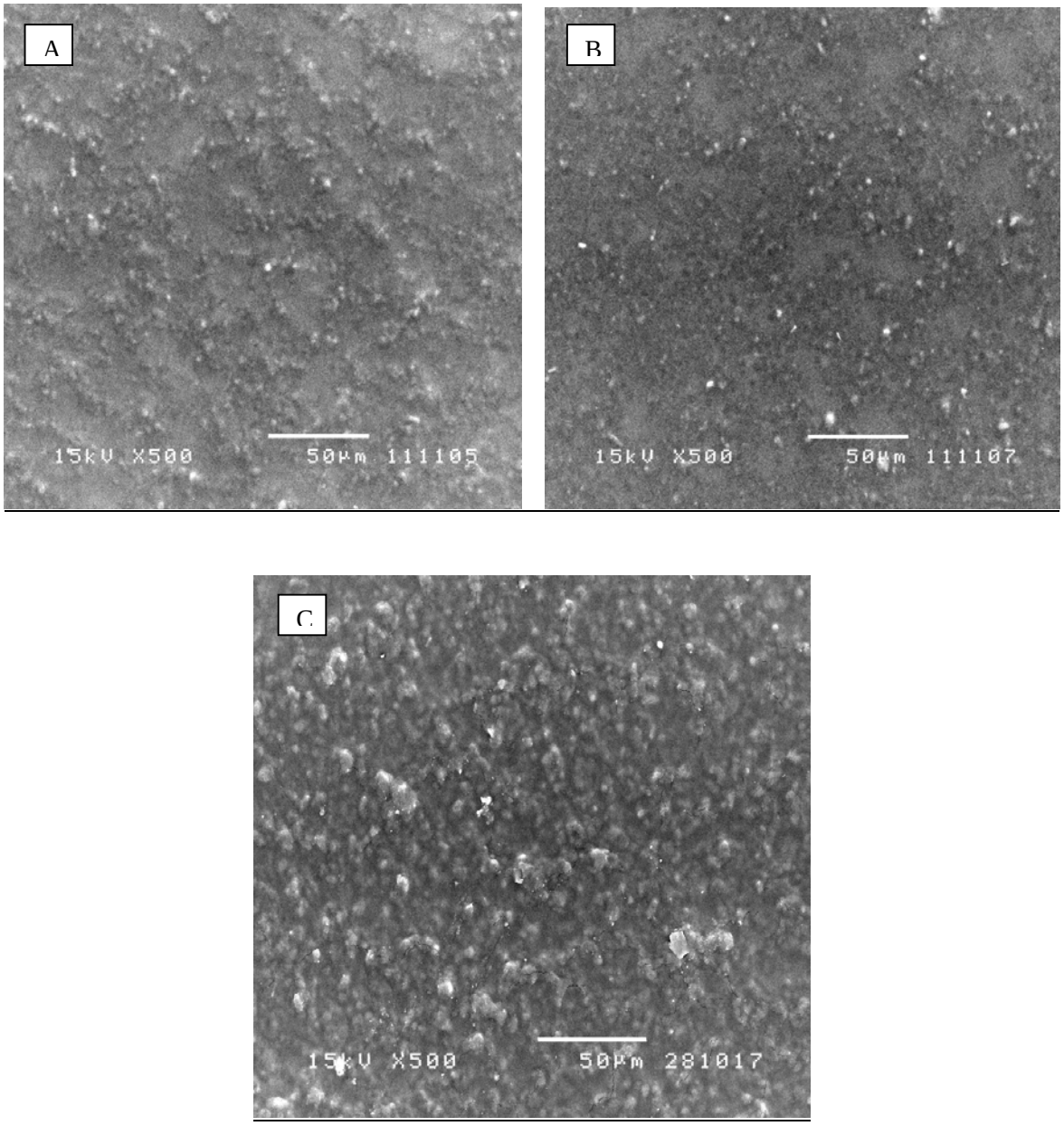
ภาพที่ 7 ภาพ Scanning Electron Microscope (SEM) แผ่นฟิล์มจาก 3% sericin + 2% polyvinyl alcohol ร่วมกับ glycerin ที่มีความเข้มข้น 1%



ภาพที่ 8 ภาพ Scanning Electron Microscope (SEM) แผ่นฟิล์มจาก 3% sericin + 2% polyvinyl alcohol ร่วมกับ glycerin ที่มีความเข้มข้น 1% ผ่านการอบที่อุณหภูมิ 105 องศาเซลเซียสภายใต้ vacuum นาน 24 (A), 48 (B) และ 72 (C) ชั่วโมง ตามลำดับ

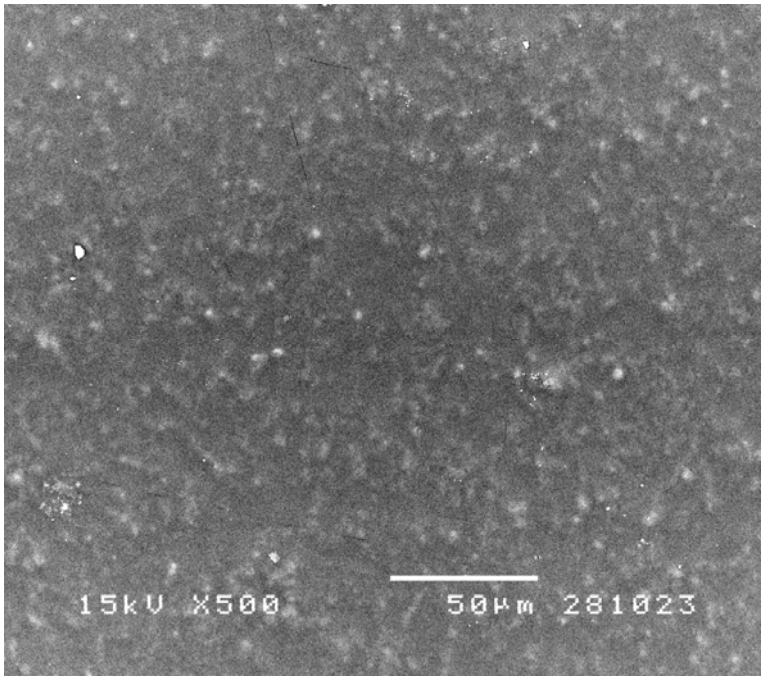


ภาพที่ 9 ภาพ Scanning Electron Microscope (SEM) แผ่นฟิล์มจาก 3% sericin + 2% polyvinyl alcohol ร่วมกับ glycerin ที่มีความเข้มข้น 1% ผ่านการอบที่อุณหภูมิ 105 องศาเซลเซียสนาน 24 (A), 48 (B) และ 72 (C) ชั่วโมง ตามลำดับ



ภาพที่ 10 ภาพ Scanning Electron Microscope (SEM) แผ่นฟิล์มจาก 3% sericin + 2% polyvinyl alcohol ร่วมกับ glycerin ที่มีความเข้มข้น 1% ผ่านการอบด้วยแสง UV นาน 30 (A), 45 (B) และ 60 (C) นาทีตามลำดับ

หมายเหตุ: นำแผ่นฟิล์มเข้าตู้อบฉายรังสี UV (GS Gene Linker® UV Chamber, Bio-rad, California, USA) ด้วยพลังงาน 559.28 mJ/m^2



ภาพที่ 11 ภาพ Scanning Electron Microscope (SEM) แผ่นฟิล์มจาก 3% sericin + 2% polyvinyl alcohol ร่วมกับ glycerin ที่มีความเข้มข้น 1% ผ่านการฉายรังสีแกมมาขนาด 9 kGy/hr นาน 6 วินาที

หมายเหตุ: ตู้อบรังสีแกมมา มี specification ของ gamma chamber 500 (BRIT, Board of Radiation & Isotrope Technology, India) ดังนี้

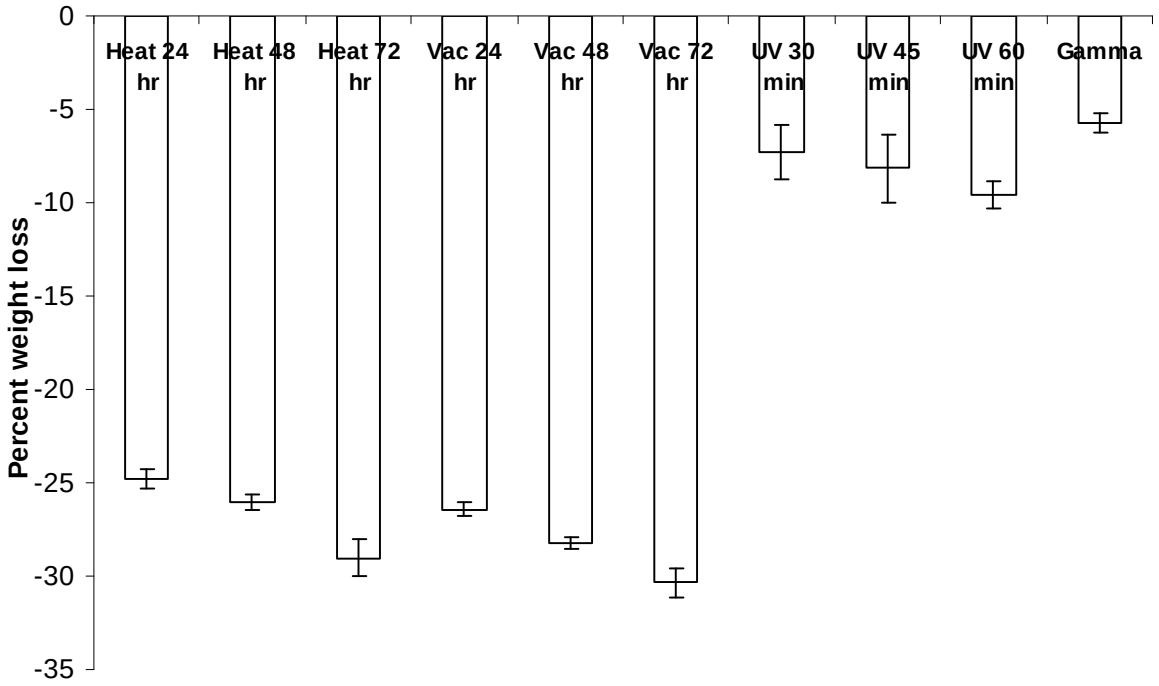
Maximum Co-60 source capacity	518 TBq (14000 Ci)
Dose rate at max capacity	9 kGy/hr (0.9 Mega Rad/hr)
Dose rate uniformity	+25% or better radially; -25% or better axially
Irradiation volume	5000 cc approx.
Timer range	6 seconds onwards

จากภาพ SEM ของแผ่นฟิล์มจะเห็นได้ว่าแผ่นฟิล์มที่ผ่านการอบด้วยความร้อน, ความร้อนภายใต้ vacuum, ผ่านการอบรังสี UV และรังสีแกมมา ไม่มีผลให้เกิดรูพรุนที่แผ่น เช่นเดียวกับแผ่น control ที่ไม่ผ่านกระบวนการเชื่อมโยงข้ามใด ๆ อย่างไรก็ตาม แผ่นที่ผ่านกระบวนการเชื่อมโยงข้ามบางชนิดอาจมีผลต่อลักษณะทางกายภาพ ของฟิล์มอันจะเห็นได้จากแผ่นฟิล์มมีความขรุขระเพิ่มขึ้น เช่น การอบด้วยรังสี UV และรังสีแกมมาจะทำให้แผ่นฟิล์มโปรตีนกาวไหมมีความขรุขระเพิ่มขึ้น โดยเฉพาะแผ่นที่ผ่านการอบรังสี UV นาน 60 นาที

<u>คุณสมบัติทางกายภาพของแผ่นฟิล์มโปรตีนกาวไหม</u>

น้ำหนักที่เปลี่ยนแปลงไปของแผ่นฟิล์มเมื่อผ่านกระบวนการเชื่อมโยงข้ามต่าง ๆ

คำนวณจากร้อยละของน้ำหนักแผ่นฟิล์มที่เปลี่ยนแปลงไป เปรียบเทียบกับน้ำหนักของแผ่นฟิล์มก่อนผ่านกระบวนการเชื่อมโยงข้าม

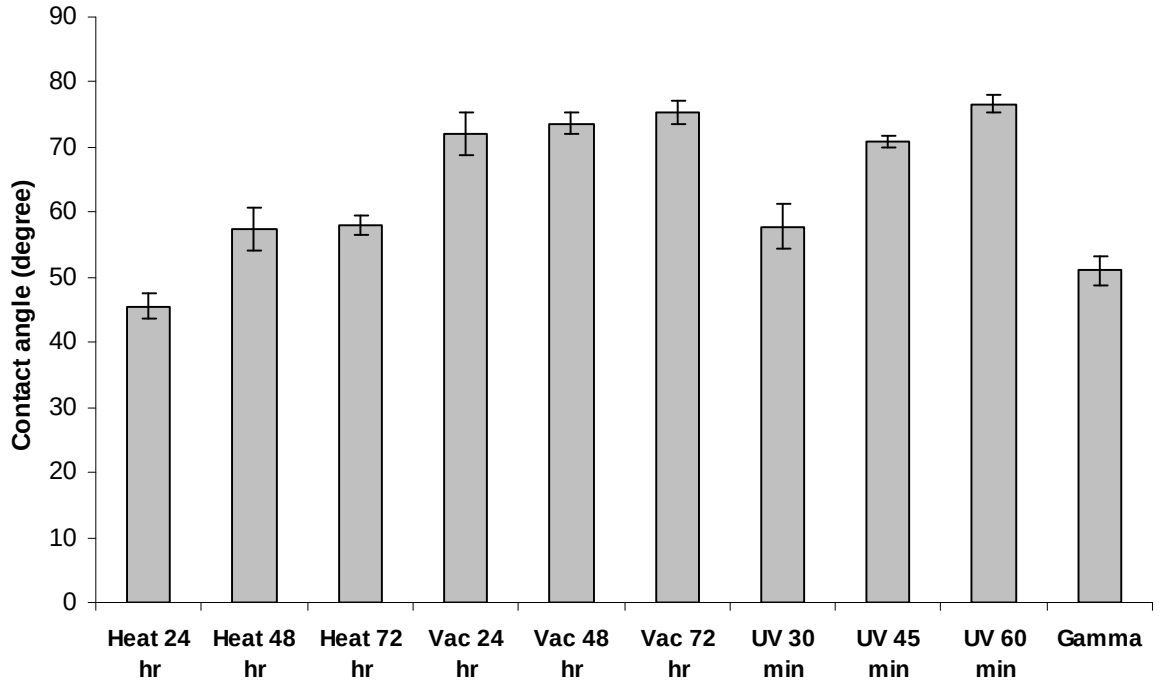


ภาพที่ 12 ร้อยละของน้ำหนักแผ่นฟิล์มที่เปลี่ยนแปลงไปหลังจากผ่านกระบวนการเชื่อมโยงข้ามวิธีต่าง ๆ กันในระยะเวลาต่าง ๆ กัน

จากผลการทดลองจะเห็นได้ว่า แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยการอบภายใต้ vacuum จะสูญเสียน้ำหนักสูงที่สุดเมื่อเปรียบเทียบกับวิธีอื่น ๆ โดยสูญเสียน้ำหนักไปถึงประมาณร้อยละ 25 นอกจากนี้ระยะเวลาในการอบที่นานขึ้น ยังส่งผลให้การสูญเสียน้ำหนักของแผ่นฟิล์มเพิ่มสูงขึ้นด้วย โดยกระบวนการเชื่อมโยงข้ามด้วยรังสีแกมมาจะทำให้แผ่นฟิล์มมีการสูญเสียน้ำหนักน้อยที่สุด

Contact angle ของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามต่าง ๆ

เนื่องจากการดูดซับน้ำของแผ่นฟิล์มโปรตีนกาวไหมมีผลต่อคุณสมบัติที่ดีของแผ่นปิดแผล contact angle เป็นค่าหนึ่งที่แสดงถึงความชอบน้ำของตัวอย่างที่ทำการทดสอบ เนื่องจากในกระบวนการเชื่อมโยงข้ามของแผ่นฟิล์มโปรตีนกาวไหมอาจมีการสูญเสียน้ำและส่งผลต่อ contact angle ของตัวอย่างได้ ดังนั้นผู้วิจัยจึงได้ทำการวิเคราะห์ค่า contact angle ของตัวอย่างได้ผลดังภาพที่ 13



ภาพที่ 13 Contact angle ของแผ่นฟิล์มหลังจากผ่านกระบวนการเชื่อมโยงข้ามวิธีต่าง ๆ กัน ในระยะเวลาต่าง ๆ กัน

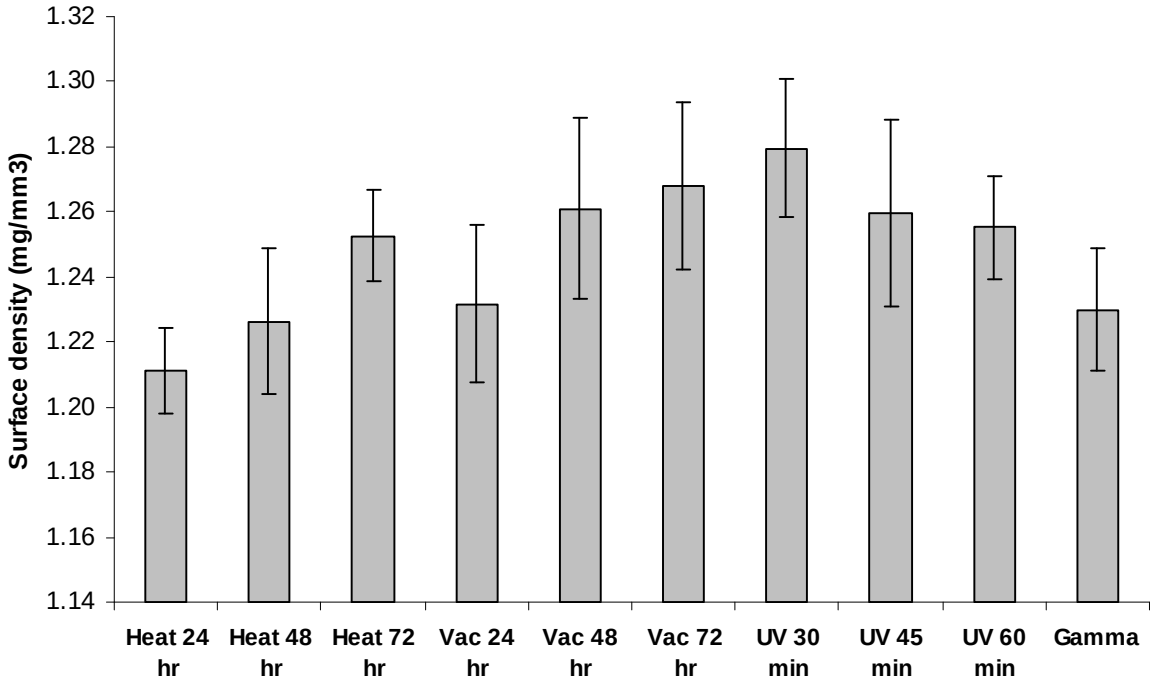
จากผลการทดลองจะเห็นได้ว่า แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยความร้อนและการฉายรังสีแกมมาจะมี contact angle ต่ำกว่าเมื่อเทียบกับแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามอื่น ๆ โดยแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยความร้อนในทุกระยะเวลาและการฉายรังสีแกมมาจะมีความแตกต่างของ contact angle จากแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยความร้อนภายใต้ vacuum อย่างมีนัยสำคัญทางสถิติในทุกระยะเวลา และโดยทั่วไปเมื่อหยดน้ำถูกหยดลงบนแผ่นที่มีความเป็น hydrophilic สูงจะส่งผลให้มี contact angle ที่ต่ำ ซึ่งแสดงให้เห็นว่า แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยความร้อนเพียง 24 ชั่วโมง และการอบด้วยรังสีแกมมาจะมีความเป็น hydrophilic สูงสุด ในขณะที่แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยความร้อนภายใต้ vacuum จะมี hydrophilicity ต่ำที่สุด

Surface density ของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามต่าง ๆ

เนื่องจากกระบวนการเชื่อมโยงข้ามอาจมีผลทำให้น้ำหนักของแผ่นฟิล์มโปรตีนกาวไหมเปลี่ยนแปลงไป อีกทั้งกระบวนการเชื่อมโยงข้ามที่มีความร้อนเข้ามาเกี่ยวข้อง อาจส่งผลต่อการหดหรือขยายตัวของแผ่นฟิล์มโปรตีนกาวไหมได้ ด้วยเหตุนี้ผู้วิจัยจึงศึกษา surface density ของแผ่นฟิล์มโปรตีนกาวไหมแต่ละชนิดเพื่อ เปรียบเทียบกันด้วย

<u>วิธีการทดลอง</u>

1. ตัดฟิล์มให้มีความยาวเท่ากันคือ 1.5 X 4.5 เซนติเมตร
2. ชั่งน้ำหนัก และวัดความหนาของแผ่นฟิล์มแต่ละแผ่นอย่างน้อย 5 จุดต่อ 1 แผ่น
3. คำนวณหา surface density โดยใช้อย่างน้อย 6 ตัวอย่างในแต่ละชนิดของฟิล์ม



ภาพที่ 14 Surface density ของแผ่นฟิล์มหลังจากผ่านกระบวนการเชื่อมโยงข้ามวิธีต่าง ๆ กัน ในระยะเวลาต่าง ๆ กัน

จากผลการทดลองจะเห็นได้ว่า แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยความร้อนจะมี surface density ต่ำที่สุด (เมื่อเปรียบเทียบกับระยะเวลาที่ใช้ในการเชื่อมโยงข้ามที่เท่ากัน) ในขณะที่กระบวนการเชื่อมโยงข้ามด้วยรังสี UV จะให้แผ่นฟิล์มโปรตีนกาวไหมที่มีค่า surface density สูงที่สุด อย่างไรก็ตาม ระยะเวลาในกระบวนการเชื่อมโยงข้ามด้วยความร้อนและด้วยความร้อนที่มี vacuum ที่นานขึ้น ส่งผลให้แผ่นฟิล์มนั้น ๆ มีค่า surface density ที่เพิ่มขึ้น ในขณะที่ แผ่นฟิล์มที่ผ่านกระบวนการเชื่อมโยงข้ามด้วยรังสี UV เมื่อใช้เวลาอบ UV ที่นานขึ้นกลับส่งผลให้ค่า surface density ของแผ่นฟิล์มลดลง

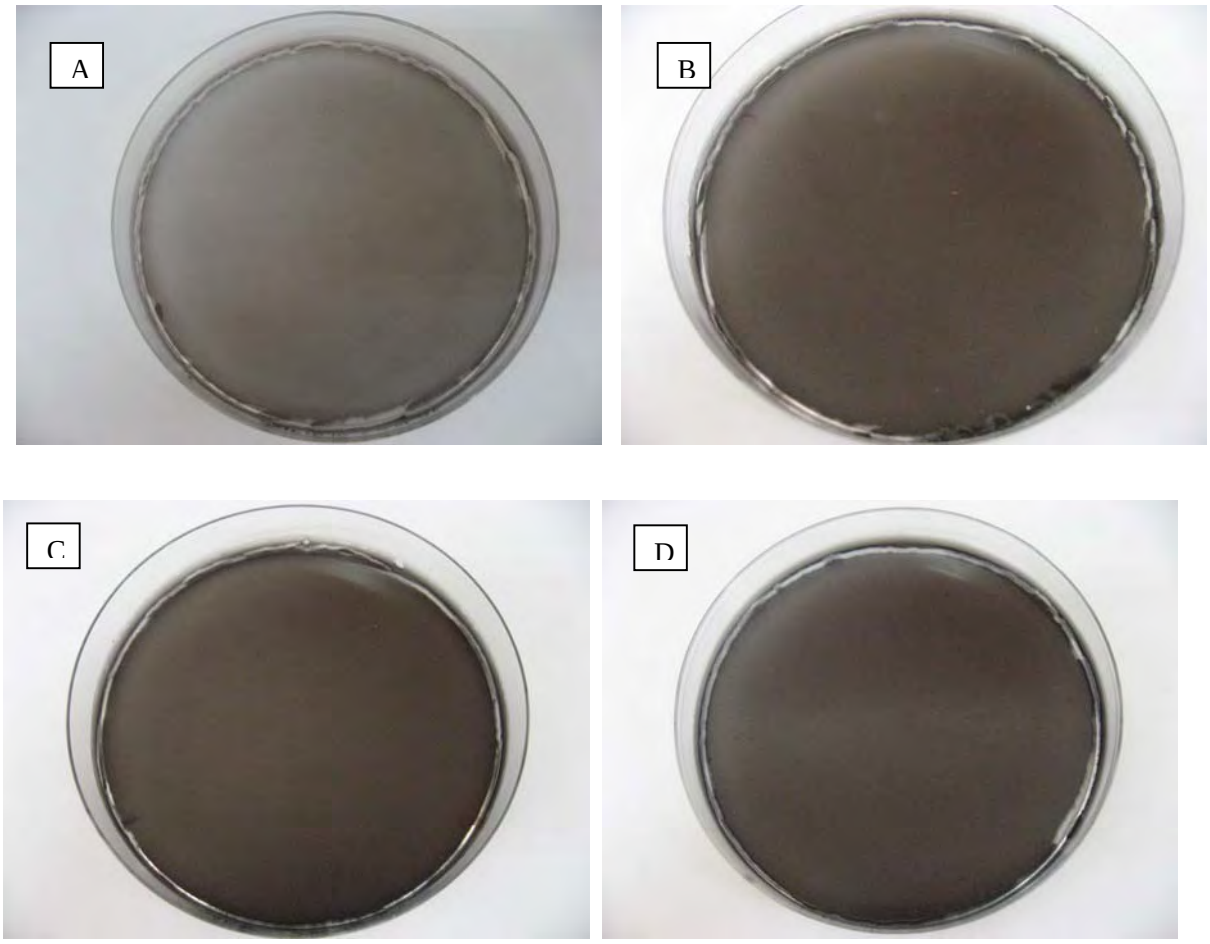
การเตรียมแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

เพื่อทดสอบกระบวนการเชื่อมโยงข้ามทางเคมี สาร genipin ซึ่งเป็นสารธรรมชาติที่สกัดได้จากดอกพุด และมีรายงานว่าสารนี้มีความเป็นพิษต่ำกว่า glutaraldehyde ถึง 10,000 เท่า นอกจากนี้ยังก่อให้เกิดการอักเสบในสัตว์ทดลองน้อยกว่า glutaraldehyde ด้วยเช่นกัน ดังนั้น genipin จึงถูกนำมาใช้เป็นสารเพื่อให้เกิดกระบวนการเชื่อมโยงข้ามทางเคมีในแผ่นฟิล์มโปรตีนกาวไหม

วิธีการทดลอง

1. นำ polyvinyl alcohol มาละลายในน้ำให้มีความเข้มข้นร้อยละ 5 และนำไปอุ่นที่ 37 องศาเซลเซียส นาน 5 ชั่วโมง
2. นำสารละลายของโปรตีนกาวไหมและ glycerin มาผสมกับสารละลายในข้อ 1)
3. ละลาย genipin ใน ethyl alcohol และผสมลงในสารละลายในข้อ 2) ให้มีความเข้มข้นตั้งแต่ 0.01-0.1% ตามลำดับ
4. เทสารละลายที่ได้ลงใน petri dish และตั้งทิ้งไว้ที่อุณหภูมิห้องนาน 48 ชม.

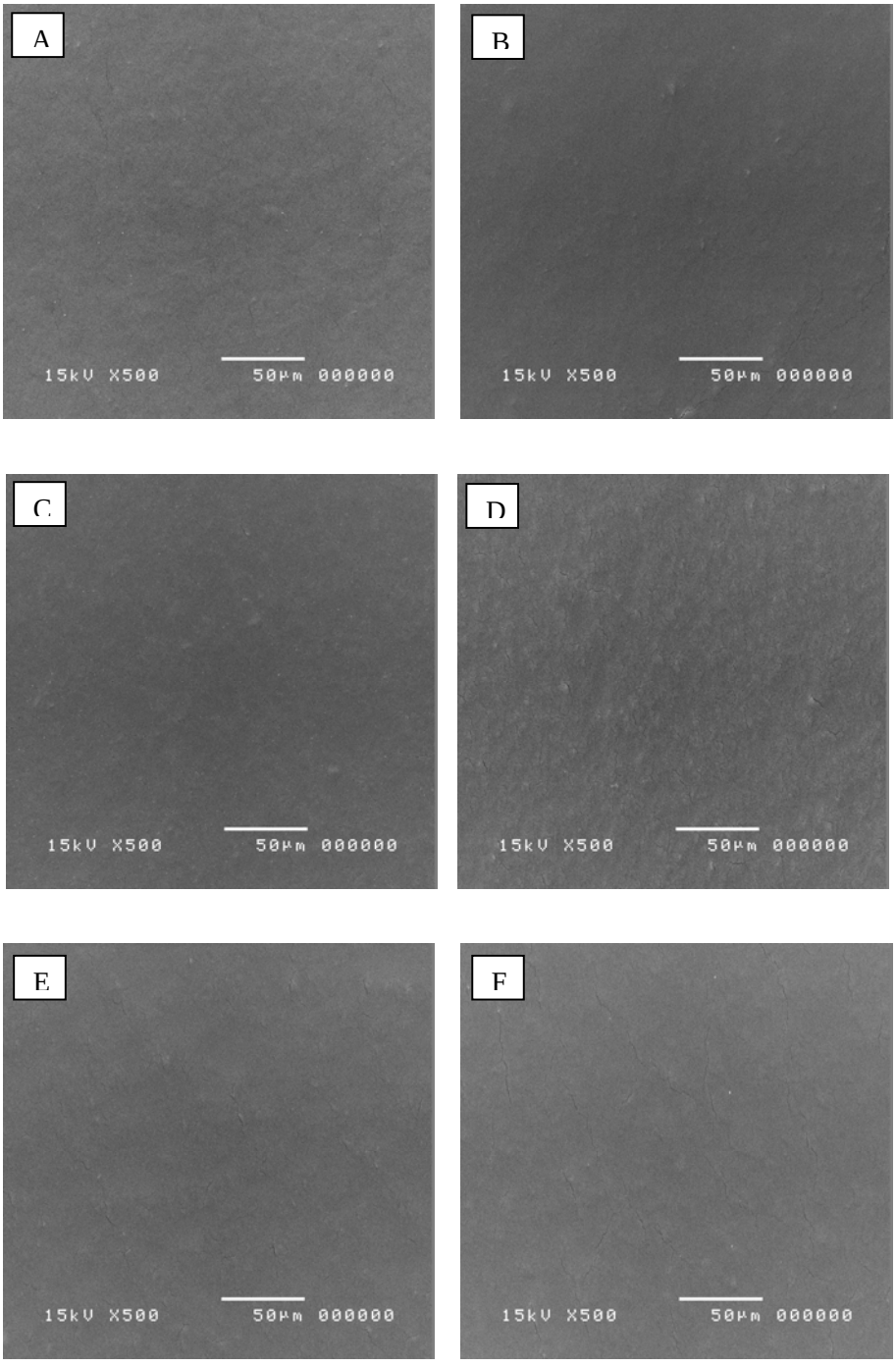
ผลจาการเตรียมได้แผ่นฟิล์มดังภาพที่ 15



ภาพที่ 15 ภาพแผ่นฟิล์มโปรตีนกาวไหมที่ประกอบด้วย 3% sericin + 2% polyvinyl alcohol + 1% glycerin ร่วมกับ genipin ที่ความเข้มข้น 0.01% (A), 0.025% (B), 0.05% (C) และ 0.1% (D) ตามลำดับ

ความเข้มข้นของ genipin ที่เพิ่มขึ้น ส่งผลให้แผ่นฟิล์มโปรตีนกาวไหมมีสีที่เข้มขึ้นด้วย นอกจากนี้ แผ่นฟิล์มที่ได้ยังไม่สามารถลอกออกจาก petri dish ได้ง่ายเมื่อตั้งทิ้งไว้เป็นเวลานาน จะต้องลอกออกทันทีเมื่อแผ่นแห้งสนิท อย่างไรก็ตาม แผ่นดังกล่าวจะถูกนำมาใช้ทดสอบในขั้นต่อไป

เมื่อนำแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin มาทำการวิเคราะห์ด้วย Scanning Electron Microscope (SEM) พบว่าแผ่นฟิล์มยังมีลักษณะเรียบเนียนในทุกความเข้มข้นของ genipin ที่เติมเข้าไป ซึ่งแตกต่างจากกรณีที่ผ่านกระบวนการเชื่อมโยงข้ามทางกายภาพที่แผ่นฟิล์มจะมีพื้นผิวขรุขระ แต่ในกรณีที่ความเข้มข้นของ genipin เพิ่มสูงขึ้นจะส่งผลให้มีรอยแตกเล็ก ๆ บนแผ่นฟิล์มเพิ่มมากขึ้น อย่างไรก็ตามรอยแตกเหล่านั้นมีขนาดเล็กมากจนไม่ส่งผลให้เกิดการเปราะแตกของแผ่นฟิล์ม ดังแสดงในภาพที่ 16



ภาพที่ 16 ภาพ Scanning Electron Microscope (SEM) แผ่นฟิล์มจาก 3% sericin + 2% polyvinyl alcohol ร่วมกับ glycerin ที่มีความเข้มข้น 1% (A) ร่วมกับ genipin ที่ความเข้มข้น 0.01% (B), 0.025% (C), 0.05% (D) 0.075% (E) และ 0.1% (F) ตามลำดับ

การศึกษาร้อยละของการเชื่อมโยงข้าม (% cross-linking) ของแผ่นฟิล์มที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

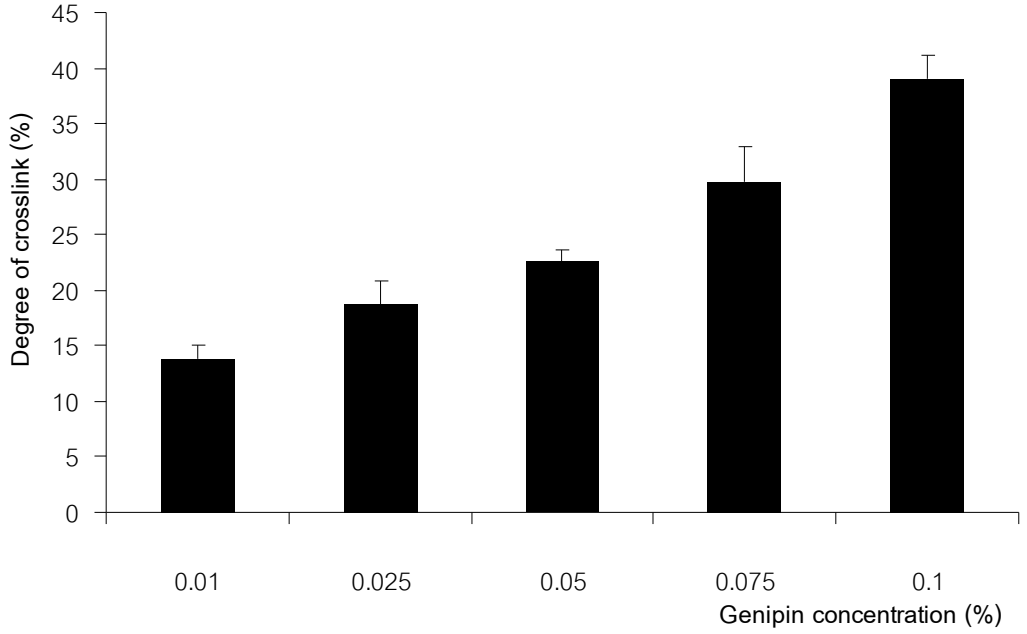
เนื่องจากปริมาณ genipin ที่แตกต่างกันอาจมีผลต่อร้อยละของการเชื่อมโยงข้ามที่แตกต่างกัน ทำการศึกษาโดยใช้วิธีของ Bubnis et al. (1992) [4] ซึ่ง free amino groups ของโปรตีนกาวไหมเซริซินจะทำปฏิกิริยากับ 2,4,6-trinitrobenzene sulfonic acid (TNBS) เกิดเป็นสารมีสีซึ่งสามารถวัดการดูดกลืนแสงได้ด้วย UV spectroscopy ร้อยละของการเชื่อมโยงข้ามสามารถคำนวณได้จากค่าความแตกต่างระหว่างค่าการดูดกลืนแสงของแผ่นฟิล์มที่ไม่ได้ผ่านกระบวนการเชื่อมโยงข้ามและแผ่นที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

<u>วิธีการทดลอง</u>

1. แผ่นฟิล์มขนาด 5 มิลลิกรัมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ถูกนำไปใส่ในหลอดทดลอง
2. เติม 1 มิลลิลิตรของ 0.5% TNBS
3. เติม 1 มิลลิลิตรของ 4% sodium hydrogen carbonate ($NaHCO_3$, pH 8.5)
4. นำไปอุ่นที่ 40 องศาเซลเซียสนาน 2 ชั่วโมง
5. เติม 2 มิลลิลิตร 12.24 N hydrochloric acid ที่ 70 องศาเซลเซียสนาน 2 ชั่วโมง
6. วัดค่าการดูดกลืนแสงที่ 415 nm โดยร้อยละของการเชื่อมโยงข้ามสามารถคำนวณได้จาก

$$\text{Degree of cross-linking (\%)} = \frac{1 - \text{Abs ของฟิล์มที่ cross-link}}{\text{Abs ของฟิล์มที่ไม่ได้ cross-linked}} \times 100$$

ผลการทดลองแสดงในภาพที่ 17 จะเห็นได้ว่า ความเข้มข้นของ genipin ที่เพิ่มสูงขึ้นส่งผลให้ % cross-linking เพิ่มสูงขึ้นด้วยเช่นกัน โดยแผ่นฟิล์มที่มี genipin ในความเข้มข้น 0.1% มี % cross-linking แตกต่างจากแผ่นฟิล์มที่มี genipin ในความเข้มข้น 0.01%, 0.025%, 0.05% อย่างมีนัยสำคัญทางสถิติ



ภาพที่ 17 ร้อยละของการเชื่อมโยงข้าม (% cross-linking) ของแผ่นฟิล์ม
ที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

การแพร่ผ่านของแสง (% transmission) ผ่านแผ่นฟิล์มโปรตีนกาวไหมที่เชื่อมโยงข้ามด้วย genipin

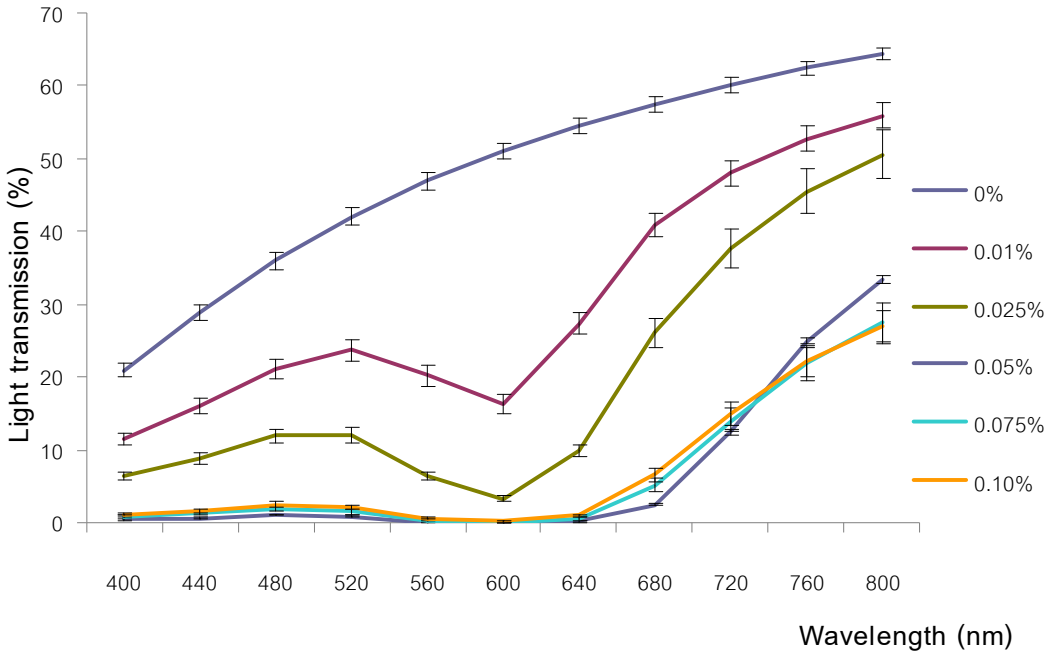
เนื่องจากการแพร่ผ่านของแสงเป็นปัจจัยหนึ่งที่สำคัญต่อการนำแผ่นฟิล์มโปรตีนกาวไหมไปประยุกต์ใช้ทั้งทางด้านการแพทย์และการนำมาใช้ในอุตสาหกรรมอาหาร แผ่นฟิล์มที่สามารถให้แสงผ่านได้ดีจะทำให้สามารถสังเกตบาดแผลได้ง่ายหากนำมาใช้ทำแผ่นปิดแผล และสามารถสังเกตสภาวะของอาหารที่เก็บภายในแผ่นฟิล์มได้

<u>วิธีการทดลอง</u>

1. ตัดแผ่นฟิล์มโปรตีนกาวไหมที่มีความกว้าง 1 X 2 เซนติเมตร
2. นำแผ่นดังกล่าวใส่ใน cuvette โดยใส่ให้แนบกับผนังด้านข้าง หลีกเลี่ยงฟองอากาศ
3. วัดการผ่านของแสง (% transmission) โดยใช้เครื่อง UV spectroscopy

ผลการศึกษาพบว่าแผ่นฟิล์มโปรตีนกาวไหมที่มีความเข้มข้นของ genipin สูงขึ้นจะทำให้การแพร่ผ่านของแสงลดลงซึ่งสอดคล้องกับลักษณะทางกายภาพเนื่องจากแผ่นฟิล์มที่มีgenipin เป็นองค์ประกอบสูงจะมีสีเข้ม

ส่งผลให้การแพร่ผ่านของแสงลดลง อย่างไรก็ตาม เมื่อความเข้มข้นของ genipin เพิ่มสูงกว่า 0.05% จะไม่พบความแตกต่างของการแพร่ผ่านของแสง ดังแสดงในภาพที่ 18



ภาพที่ 18 การแพร่ผ่านของแสง (% transmission) ผ่านแผ่นฟิล์มโปรตีนกาวไหมที่เชื่อมโยงข้ามด้วย genipin

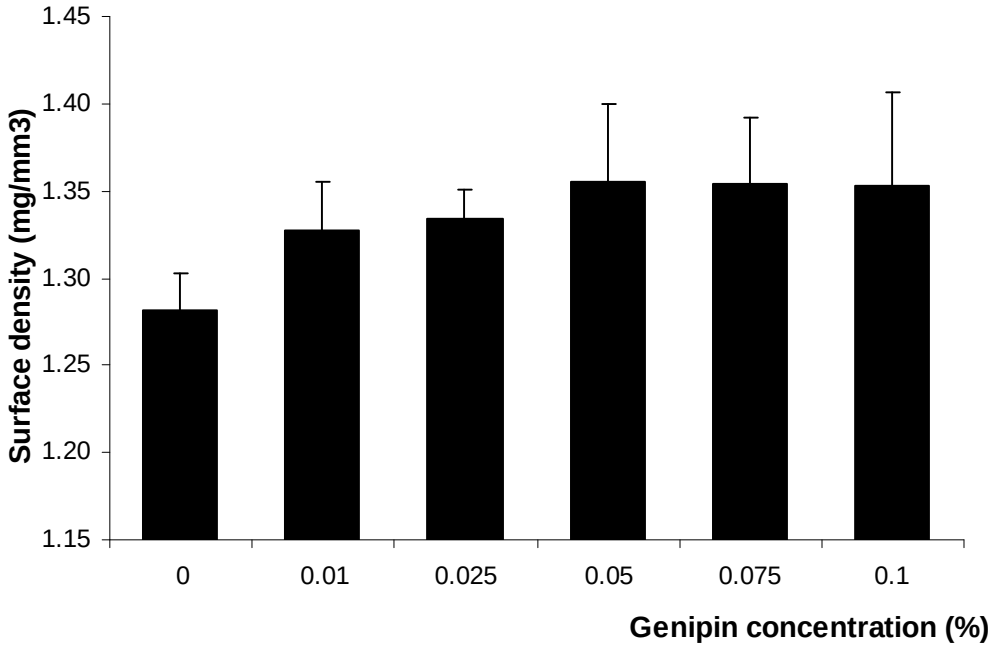
ค่า surface density ของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

ความหนาแน่นของพื้นผิว (surface density) ของแผ่นฟิล์มมีผลต่อคุณสมบัติทางกายภาพ เนื่องจากแผ่นที่มีความหนาแน่นมากมักจะมีคุณสมบัติในการแพร่ผ่านของอากาศลดลง

<u>วิธีการทดลอง</u>

1. ตัดแผ่นฟิล์มโปรตีนกาวไหมให้มีขนาด 1X2 เซนติเมตร โดยหลีกเลี่ยงการใช้มือจับแผ่นเพื่อป้องกันน้ำหนักแผ่นที่อาจเปลี่ยนแปลง
2. นำแผ่นดังกล่าวไปชั่งน้ำหนักอย่างแม่นยำ
3. คำนวณหาค่า surface density โดยใช้น้ำหนัก (มิลลิกรัม)/ขนาด (ลูกบาศก์มิลลิเมตร)

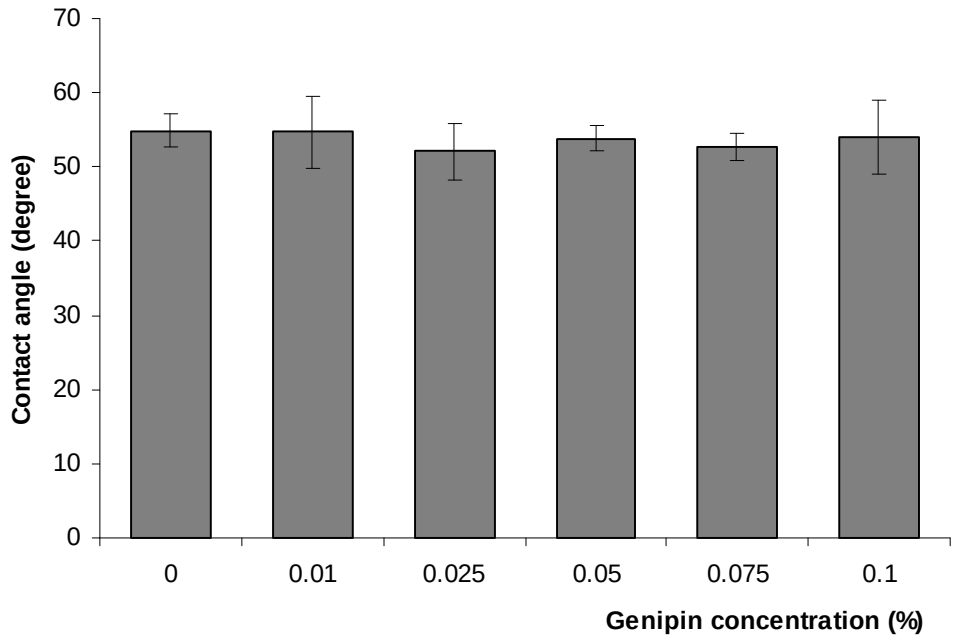
ผลการทดลองแสดงดังภาพที่ 19 จะเห็นได้ว่าเมื่อมีการนำ genipin ผสมเข้าไปในแผ่นฟิล์มโปรตีนกาวไหมจะทำให้ค่า surface density ของแผ่นเพิ่มสูงขึ้น อย่างไรก็ตามความเข้มข้นของ genipin ที่สูงขึ้นไม่ได้ทำให้ค่า surface density ของแผ่นฟิล์มเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ



ภาพที่ 19 ค่า surface density ของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

Contact angle ของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

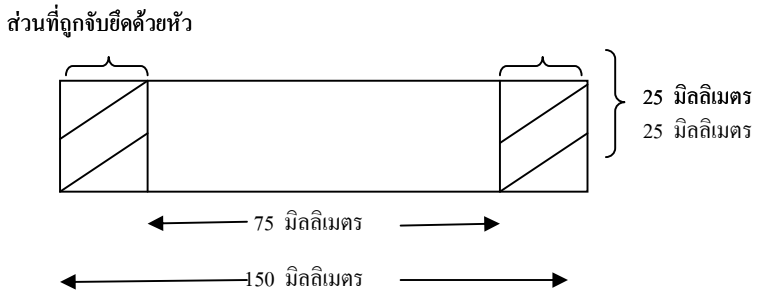
ค่า contact angle แสดงให้เห็นว่าแผ่นฟิล์มมีคุณสมบัติชอบน้ำมากน้อยเพียงใด โดยค่า contact angle ที่สูงแสดงให้เห็นว่าน้ำชอบเกาะกลุ่มเป็นหยดหรืออาจกล่าวได้ว่าแผ่นฟิล์มดังกล่าวเปียกน้ำได้ยาก จากการศึกษาพบว่า แผ่นฟิล์มที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กัน มีค่า contact angle ไม่แตกต่างกันและไม่แตกต่างจากแผ่นที่ไม่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin หรืออาจกล่าวได้ว่า คุณสมบัติในการเปียกน้ำของแผ่นฟิล์มไม่แตกต่างกัน ผลการทดลองแสดงในภาพที่ 20



ภาพที่ 20 ค่า contact angle ของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

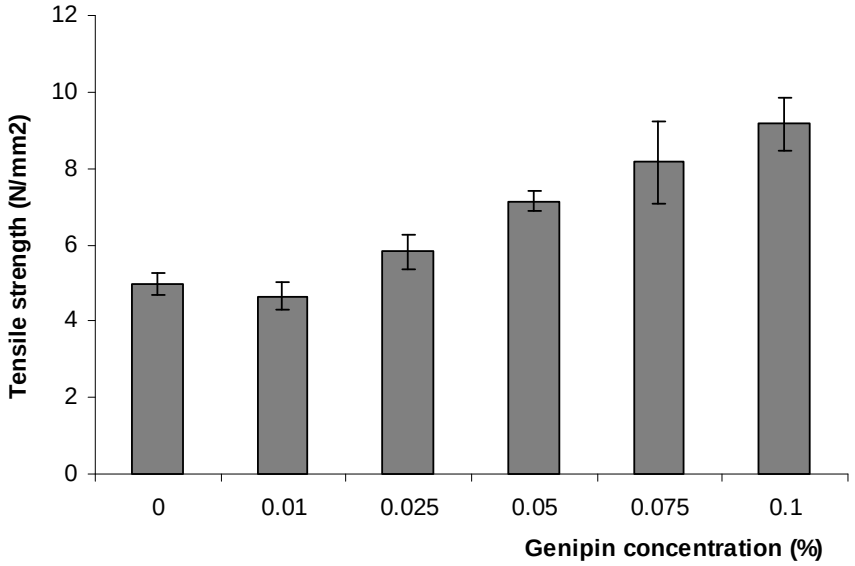
ความแข็งแรงทางกลของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin

เนื่องจากความแข็งแรงเชิงกลเป็นคุณสมบัติที่สำคัญประการหนึ่งของแผ่นฟิล์มที่จะนำมาประยุกต์ใช้ โดยการวัดความแข็งแรงทางกลของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นต่าง ๆ สามารถวัดได้จากค่า tensile strength และร้อยละของการยืดตัว (percents of elongation) โดยใช้เครื่อง Universal testing machine (Instron 5567) ทำการทดสอบแรงดึงตาม ASTM D5035 (Standard Test Method for Breaking Force and Elongation of Textile Fabrics) โดยนำแผ่นฟิล์มโปรตีนกาวไหมมาตัดให้มีขนาดความกว้าง 25 มิลลิเมตร และความยาวอย่างน้อย 150 มิลลิเมตร มีระยะห่างระหว่างที่จับด้านบนและด้านล่างเท่ากับ 75 มิลลิเมตร (ภาพที่ 21) หลังจากนั้นนำแผ่นฟิล์มมาวัดความสามารถในการทนแรงดึงด้วยเครื่อง Universal testing machine โดยใช้อัตราการการดึงที่ 300 มิลลิเมตรต่อนาทีจนขาด และทดสอบตัวอย่างละ 3 ซ้ำ เพื่อนำค่าผลการทดสอบมาวิเคราะห์หาค่าเฉลี่ย

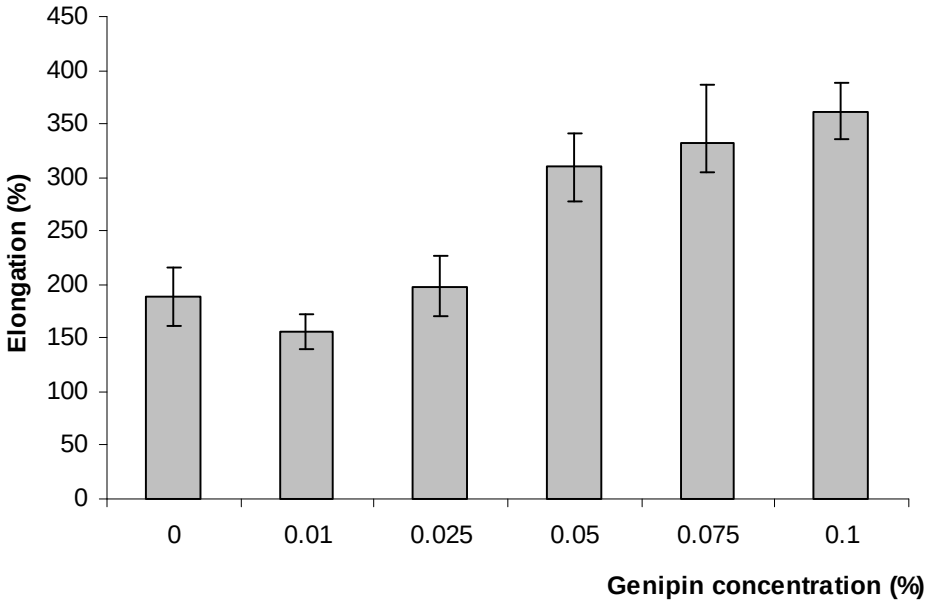


ภาพที่ 21 แสดงภาพตัวอย่างขนาดชิ้นงานที่ใช้ทดสอบความต้านทานต่อการดึง ซึ่งในการทดสอบนี้จะทดสอบกับแผ่นฟิล์มทั้งในขณะเปียกและขณะแห้ง

ผลการทดลองพบว่าความแข็งแรงเชิงกลของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ขณะแห้งได้ผลดังภาพที่ 22 และ 23 ตามลำดับ โดยแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้น 0, 0.01 และ 0.025% มีค่าการต้านทานแรงดึง (tensile strength) และค่าร้อยละของการยืดตัว ณ จุดขาด (% elongation) ต่ำกว่าแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้น 0.05, 0.075 และ 0.1% อย่างมีนัยสำคัญทางสถิติ

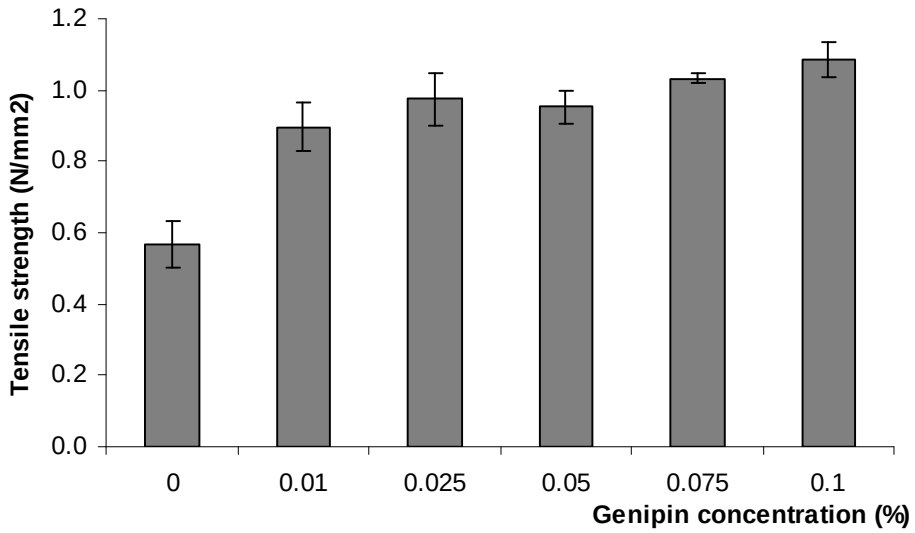


ภาพที่ 22 แสดงการต้านทานแรงดึงของแผ่นฟิล์มโปรตีนกาวไหมขณะแห้งที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กัน

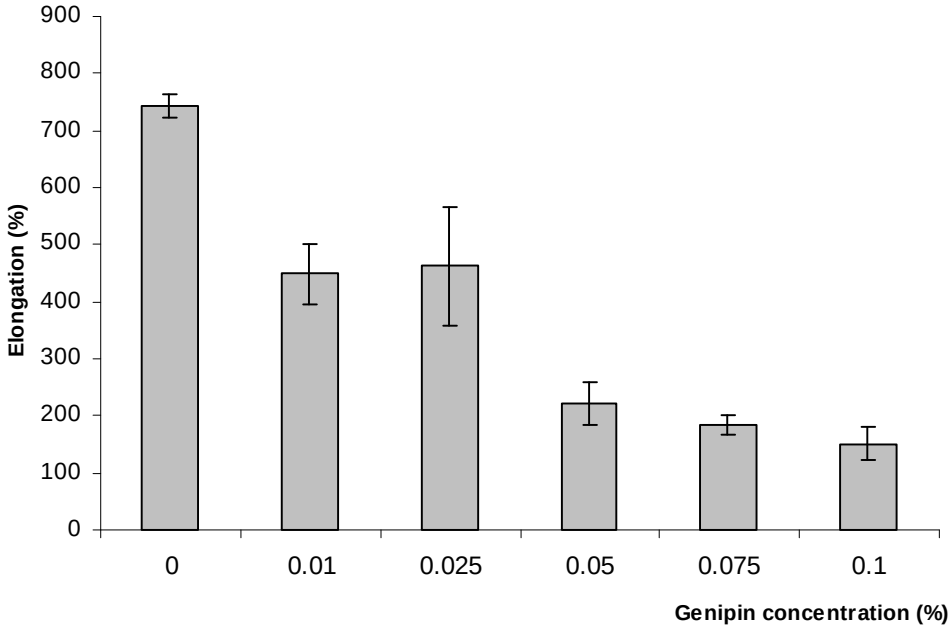


ภาพที่ 23 แสดงค่าร้อยละของการยืดตัว ณ จุดขาดของแผ่นฟิล์มโปรตีนกาวไหมขณะแห้งที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กัน

เนื่องจากคุณสมบัติเชิงกลของแผ่นฟิล์มอาจมีความแตกต่างกันขณะเปียก จึงได้นำแผ่นฟิล์มโปรตีนกาวไหมไปแช่ในน้ำสะอาดนาน 5 นาทีก่อนนำมาทดสอบคุณสมบัติในการต้านทานแรงดึงและร้อยละของการยืดตัว ณ จุดขาด อีกครั้งหนึ่ง ซึ่งผลแสดงในภาพที่ 24 และ 25 จะเห็นได้ว่าการต้านทานแรงดึงของแผ่นฟิล์มโปรตีนกาวไหมขณะเปียกมีค่าต่ำกว่าการต้านทานแรงดึงของแผ่นฟิล์มโปรตีนกาวไหมขณะแห้งในทุกความเข้มข้นของ genipin ในขณะที่ค่าร้อยละของการยืดตัว ณ จุดขาดของแผ่นฟิล์มโปรตีนกาวไหมจะแตกต่างกันเมื่อเปียกและแห้งกล่าวคือ ในขณะแห้ง แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นสูง ๆ จะมีค่าร้อยละของการยืดตัว ณ จุดขาดสูงด้วย แต่ในขณะเปียก ความเข้มข้นของ genipin ที่เพิ่มสูงขึ้นจะทำให้ค่าร้อยละของการยืดตัว ณ จุดขาดต่ำลง ซึ่งแสดงให้เห็นว่าแผ่นฟิล์มโปรตีนกาวไหมขณะแห้งเหมาะสำหรับนำมาใช้ในทางการแพทย์โดยไม่จำเป็นต้องนำไปทำให้เปียกก่อนใช้เนื่องจากคุณสมบัติในการยืดตัวจะลดลงขณะเปียก



ภาพที่ 24 แสดงการต้านทานแรงดึงของแผ่นฟิล์มโปรตีนกาวไหมขณะเปียกที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กัน



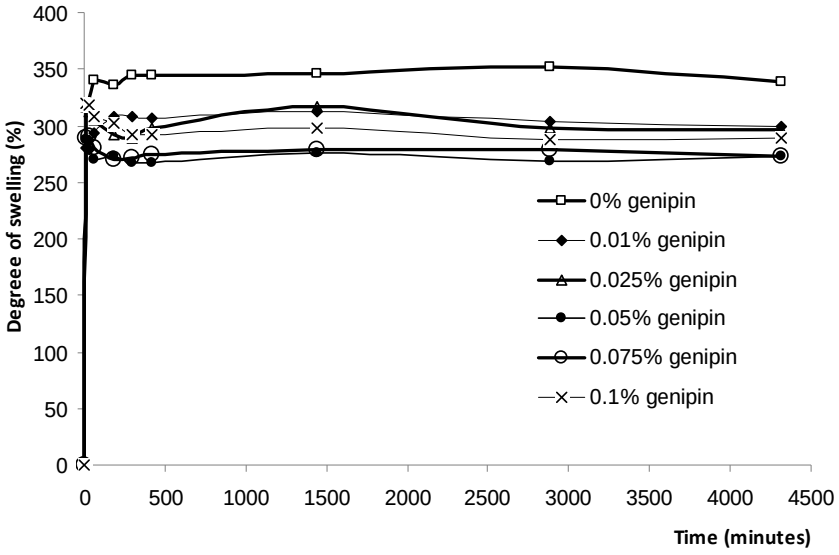
ภาพที่ 25 แสดงค่าร้อยละของการยืดตัว ณ จุดขาดของแผ่นฟิล์มโปรตีนกาวไหมขณะเปียกที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กัน

คุณสมบัติในการดูดความชื้นของแผ่นฟิล์มโปรตีนกาวไหม

<u>วิธีการทดลอง</u>

1. นำแผ่นฟิล์มโปรตีนกาวไหมมาตัดให้มีน้ำหนักใกล้เคียงกัน
2. นำตัวอย่างบรรจุในตะกร้อโลหะที่มีลักษณะโปร่งและน้ำผ่านได้
3. จุ่มตัวอย่างในข้อ 2) ลงในน้ำบริสุทธิ์
4. นำตัวอย่างขึ้นมาที่เวลาต่าง ๆ กัน ซับน้ำภายนอกตะกร้อโลหะให้แห้ง และชั่งน้ำหนักที่เปลี่ยนแปลงไป
5. คำนวณร้อยละของน้ำหนักที่เปลี่ยนแปลงไปที่ช่วงเวลาต่าง ๆ

ผลการทดลองแสดงในภาพที่ 26 ซึ่งแสดงให้เห็นว่าแผ่นฟิล์มโปรตีนกาวไหมที่ไม่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin จะมีความสามารถในการดูดซับความชื้นได้สูงสุดและสามารถพองตัวได้สูงสุดเช่นเดียวกัน ซึ่งความสามารถในการดูดซับความชื้นและความสามารถในการพองตัวนี้จะแตกต่างจากแผ่นฟิล์มโปรตีนกาวไหมที่มี genipin เป็นองค์ประกอบอย่างมีนัยสำคัญทางสถิติ ในขณะที่แผ่นฟิล์มโปรตีนกาวไหมที่มี genipin เป็นองค์ประกอบในความเข้มข้นตั้งแต่ 0.01-0.1% ไม่มีความแตกต่างของการดูดซับความชื้นและความสามารถในการพองตัว อย่างไรก็ตาม แผ่นฟิล์มโปรตีนกาวไหมที่มีและไม่มี genipin เป็นองค์ประกอบสามารถดูดความชื้นและพองตัวได้ไม่ต่ำกว่า 3 เท่าของน้ำหนัก ซึ่งอาจกล่าวได้ว่าแผ่นฟิล์มโปรตีนกาวไหมมีคุณสมบัติในการดูดซับความชื้นและพองตัวได้ดี เหมาะสมต่อการนำไปใช้ในทางการแพทย์โดยเฉพาะสำหรับการดูแลบาดแผลที่มีสารคัดหลั่งมาก



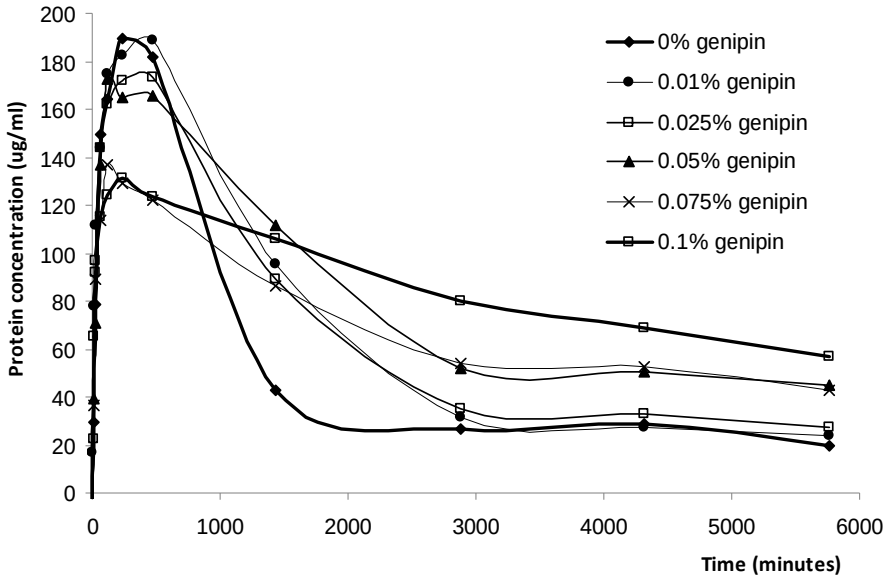
ภาพที่ 26 แสดงค่าร้อยละของการพองตัวของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กันเมื่อจุ่มลงในน้ำ

การปลดปล่อยของโปรตีนกาวไหมจากแผ่นฟิล์ม

<u>วิธีการทดลอง</u>

1. นำแผ่นฟิล์มมาตัดให้มีขนาดเท่ากับอุปกรณ์บรรจุตัวอย่างสำหรับเครื่อง dissolution apparatus
2. ใส่ตัวอย่างเข้าไปในในเครื่อง dissolution apparatus ที่มีการบรรจุ isotonic PBS buffer pH 7.0 ที่คนอยู่ตลอดเวลา
3. เก็บตัวอย่างที่เวลาต่าง ๆ กันและนำมาวิเคราะห์ปริมาณโปรตีนด้วย BCA assay kit

ผลการทดลองแสดงในภาพที่ 27 ซึ่งแสดงให้เห็นว่ามีการปลดปล่อยโปรตีนออกมาจากแผ่นฟิล์มโปรตีนกาวไหมตั้งแต่นาทีที่ 5 และชั่วโมงที่ 8 จะมีการปลดปล่อยโปรตีนกาวไหมออกมาจากตัวอย่างสูงสุด โดยแผ่นฟิล์มโปรตีนกาวไหมที่มี genipin เป็นส่วนประกอบที่ความเข้มข้น 0.075 และ 0.1% จะมีการปลดปล่อยโปรตีนออกมาต่ำสุดและแตกต่างจากแผ่นฟิล์มโปรตีนกาวไหมที่ไม่มี genipin และมี genipin ที่ความเข้มข้น 0.01-0.05% อย่างมีนัยสำคัญทางสถิติ ซึ่งสอดคล้องกับผลการศึกษาในเบื้องต้นที่แสดงให้เห็นว่า ความเข้มข้นของ genipin ที่เพิ่มขึ้นทำให้ degree of cross-linking เพิ่มขึ้น ส่งผลให้ปริมาณโปรตีนที่ถูกปลดปล่อยออกมาลดลงไปด้วย



ภาพที่ 27 แสดงความเข้มข้นของโปรตีนที่ถูกปลดปล่อยออกมาจากแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กัน

อย่างไรก็ตาม ปริมาณของโปรตีนกาวไหมที่มีผลดีต่อเซลล์ (คือเพิ่มจำนวนการรอดชีวิตของเซลล์และสามารถกระตุ้นการสร้างคอลลาเจนได้อย่างมีประสิทธิภาพ) ควรเป็นความเข้มข้นที่ไม่สูงมากและค่อย ๆ ถูกปลดปล่อยอย่างต่อเนื่อง โดยความเข้มข้นที่สูงเกินไปก็อาจเป็นพิษต่อเซลล์ได้เช่นเดียวกัน ดังนั้นความเข้มข้นของ genipin ที่เหมาะสมซึ่งจะทำให้เกิดการปลดปล่อยโปรตีนกาวไหมจากแผ่นฟิล์มอย่างช้า ๆ ในความเข้มข้นต่ำอย่างสม่ำเสมอควรมีความเข้มข้นตั้งแต่ 0.05% ขึ้นไป

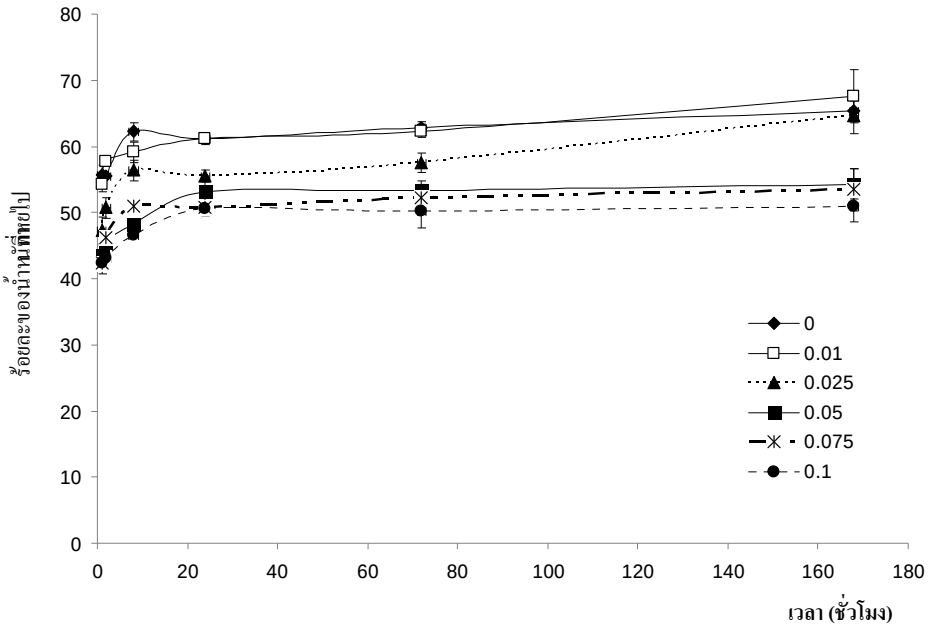
การย่อยสลายของแผ่นฟิล์มในสารละลายเอนไซม์

เนื่องจากแผ่นฟิล์มเมื่อนำไปใช้ปิดบาดแผล อาจมีโอกาสสัมผัสกับเอ็นไซม์ที่ถูกปลดปล่อยออกมาจากเลือดหรือน้ำหนองของบาดแผล จึงมีโอกาสทำให้แผ่นฟิล์มย่อยสลายได้ ซึ่งการย่อยสลายของแผ่นฟิล์มจะส่งผลให้คุณสมบัติในการปกป้องบาดแผลลดลง ด้วยเหตุนี้อัตราการย่อยสลายในสารละลายเอนไซม์จึงควรเหมาะสม

<u>วิธีการทดลอง</u>

1. นำตัวอย่างแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นต่าง ๆ กันแช่ใน lysozyme ความเข้มข้น 1.6 ไมโครกรัม/มิลลิลิตร ที่ละลายใน PBS pH 7.4
2. Incubate ที่อุณหภูมิ 37 องศาเซลเซียส และเปลี่ยนสารละลายเอนไซม์ทุกวัน
3. เมื่อครบเวลา นำตัวอย่างไปทำให้แห้งโดยการ freeze drying
4. คำนวณหาอัตราการย่อยสลายเป็นร้อยละของน้ำหนักตัวอย่างที่เปลี่ยนแปลงไปจากน้ำหนักเริ่มต้น

ผลการทดลองแสดงในภาพที่ 28 จะเห็นได้ว่าเมื่อแผ่นฟิล์มถูกเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นเพิ่มสูงขึ้นจะทำให้การย่อยสลายภายใต้เอนไซม์ลดน้อยลง จะเห็นได้ชัดเจนเมื่อความเข้มข้นของ genipin สูงกว่า 0.05% ซึ่งผลการทดลองแสดงให้เห็นว่าความเข้มข้นของ genipin ที่เพิ่มสูงขึ้นส่งผลให้ degree of cross-linking เพิ่มขึ้นและแผ่นฟิล์มมีอัตราการย่อยสลายที่ลดลง เหมาะสมต่อการนำไปใช้ปิดบาดแผลเนื่องจากแผ่นฟิล์มจะสามารถติดอยู่ได้นานแม้ว่าจะสัมผัสกับเอนไซม์ที่หลั่งจากบาดแผลก็ตาม



ภาพที่ 28 แสดงน้ำหนักที่เปลี่ยนไปของแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กันเมื่อนำมาแช่ในเอนไซม์

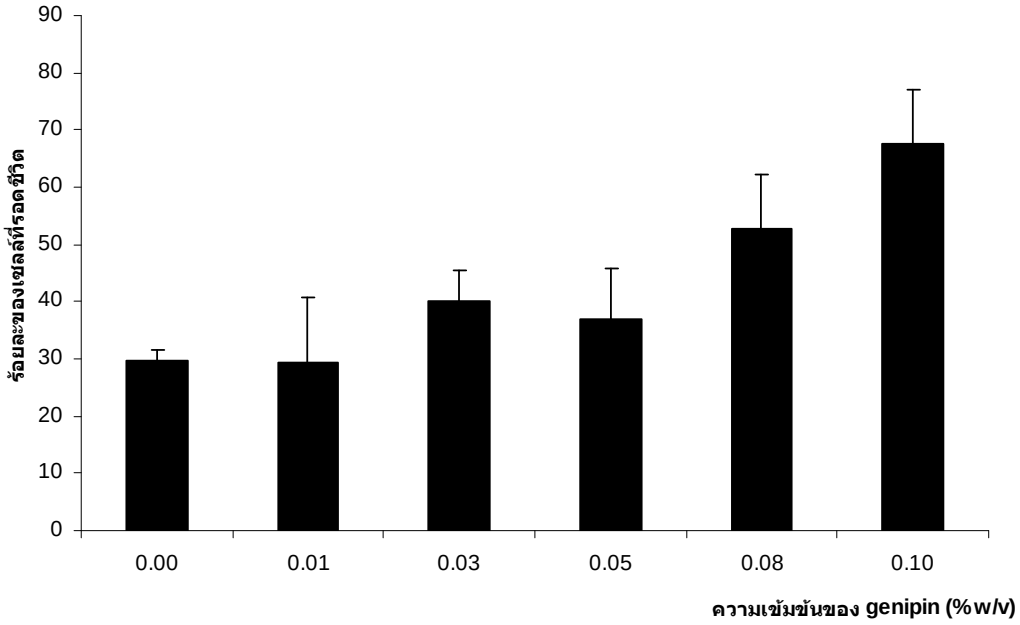
ความเข้ากันได้ของแผ่นฟิล์มที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin กับเซลล์ผิวหนัง

<u>วิธีการทดลอง</u>

1. เตรียมตัวอย่างแผ่นฟิล์มจากโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นต่าง ๆ กัน ในขนาดที่พอดีกับหลุม 96-well plate
2. นำไปฆ่าเชื้อด้วยการอบก๊าซ ethylene oxide
3. Seed mouse fibroblast cell line (L929) ในความเข้มข้น 5×10^5 เซลล์/หลุมและ incubate นาน 24 ชั่วโมง
4. นำมาตรวจสอบความเป็นพิษต่อเซลล์ของตัวอย่างโดยเติม MTT reagent แล้ววัดค่า absorbance ที่ความยาวคลื่น 570 nm
5. นำค่า absorbance ที่วัดได้มาคำนวณค่าเป็น % relative cell viability เปรียบเทียบกับ control ซึ่งเป็นแผ่นฟิล์มโปรตีนกาวไหมที่ไม่ผ่านกระบวนการเชื่อมโยงข้ามใด ๆ (ไม่มี genipin)

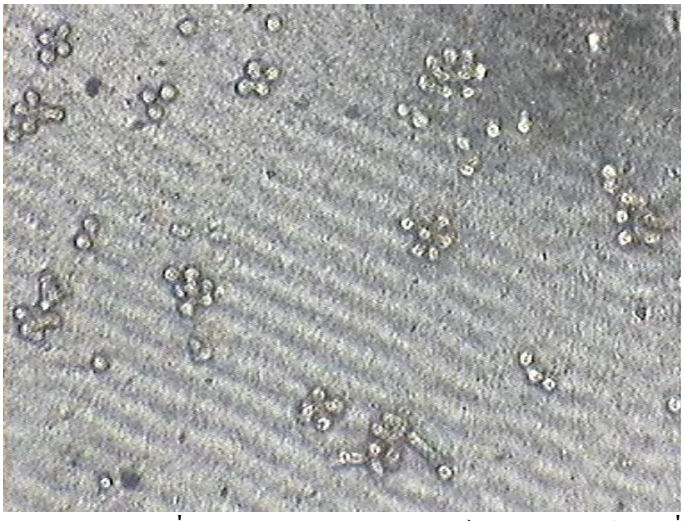
ผลการทดลองแสดงในภาพที่ 29 จะเห็นได้ว่าเมื่อความเข้มข้นของ genipin เพิ่มสูงขึ้นจะส่งผลให้อัตราการรอดชีวิตของเซลล์เพิ่มมากขึ้น เมื่อทำการวิเคราะห์จะเห็นได้ว่า ในแผ่นฟิล์มที่ไม่มีสารเชื่อมโยงข้าม

(genipin) อยู่ เซลล์จะไม่สามารถยึดเกาะได้ส่งผลให้การเจริญเติบโตและการรอดชีวิตของเซลล์ไม่ดี ในขณะที่แผ่นฟิล์มที่ผ่านการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นสูงจะทำให้เซลล์สามารถยึดเกาะกับพื้นผิวของแผ่นฟิล์มได้ มีอัตราการเจริญเติบโตและการรอดชีวิตที่สูง

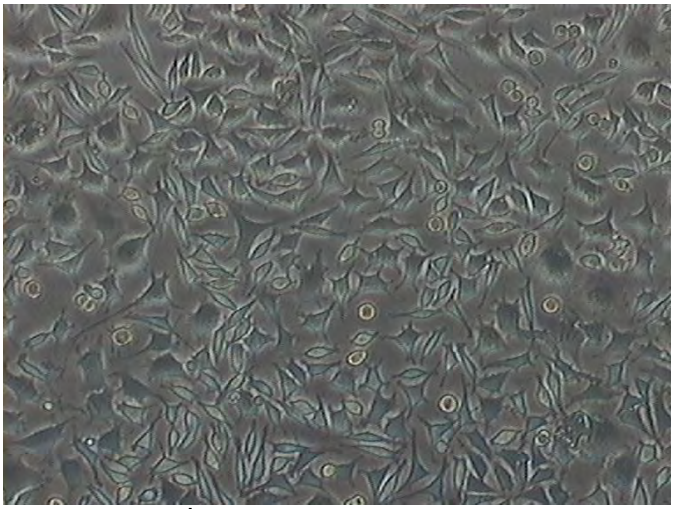


ภาพที่ 29 แสดงร้อยละของเซลล์ผิวหนังที่มีชีวิตเมื่อ incubate กับแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นต่าง ๆ กัน

ภาพที่ 30 และ 31 แสดงภาพของเซลล์ผิวหนังที่เลี้ยงบนแผ่นฟิล์มโปรตีนกาวไหม โดยภาพที่ 30 แสดงภาพเซลล์ที่เลี้ยงบนแผ่นฟิล์มโปรตีนกาวไหมที่ไม่ผ่านกระบวนการเชื่อมโยงข้าม (ไม่มี genipin) ในขณะที่ภาพที่ 31 แสดงภาพของเซลล์ผิวหนังที่เลี้ยงบนแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้น 0.10% จะเห็นได้ว่า เมื่อไม่มี genipin เซลล์ไม่สามารถยึดเกาะกับแผ่นฟิล์มได้ดี เซลล์ยังมีลักษณะเป็นทรงกลม ไม่เกิดการขยายเป็น pseudopodia ในขณะที่เซลล์บนแผ่นฟิล์มที่มี genipin 0.01% มีการเพิ่มจำนวนจนแน่นเกือบเต็มพื้นที่ และมีลักษณะเป็น pseudopodia แสดงถึงการเจริญเติบโตที่ดีของเซลล์



ภาพที่ 30 แสดงเซลล์ผิวหนัง (L929) ที่เจริญเติบโตอยู่บนแผ่นฟิล์มโปรตีนกาวไหมที่ไม่มีสารเชื่อมโยงข้าม



ภาพที่ 31 แสดงเซลล์ผิวหนัง (L929) ที่เจริญเติบโตอยู่บนแผ่นฟิล์มโปรตีนกาวไหมที่มีสารเชื่อมโยงข้าม genipin 0.10%

การศึกษาคุณสมบัติในการกระตุ้นการสร้าง nitric oxide จากแผ่นฟิล์มโปรตีนกาวไหม

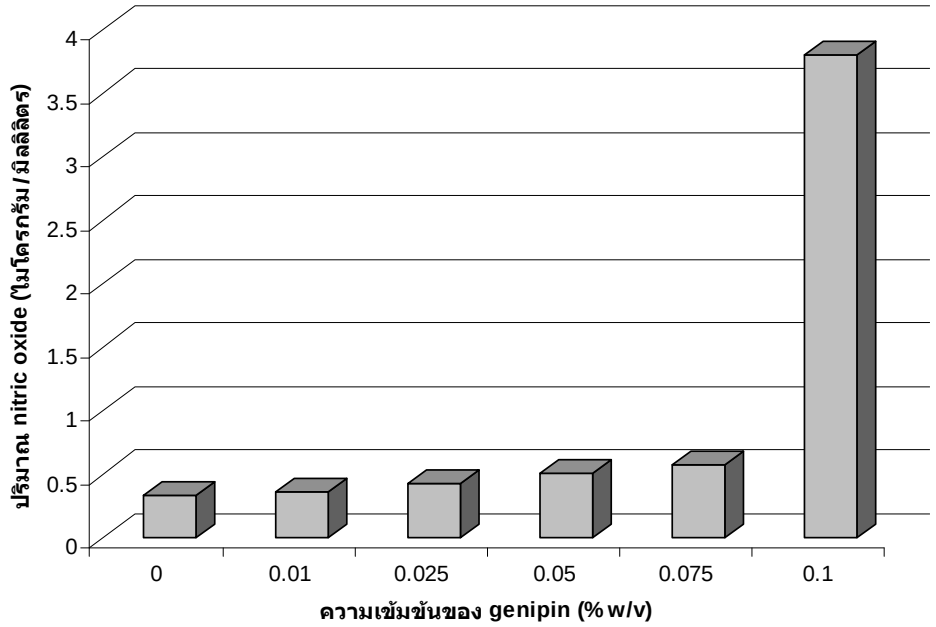
<u>วิธีการทดลอง</u>

1. เตรียมตัวอย่างแผ่นฟิล์มจากโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นต่าง ๆ กัน ในขนาดที่พอดีกับหลุม 96-well plate
2. นำไปฆ่าเชื้อด้วยการอบก๊าซ ethylene oxide
3. นำเซลล์ที่ต้องการทดสอบ ซึ่งในที่นี้ใช้ L929 mouse fibroblast cells ในความเข้มข้นเริ่มต้น 5 x 10^5/หลุมมาใส่ในถาดหลุมขนาด 96 หลุม/plate ซึ่งมี Dulbecco's Modified Eagle Medium

(DMEM) ร่วมกับ fetal bovine serum (FBS) เป็นองค์ประกอบมาเลี้ยงใน plate ที่มีแผ่นฟิล์มโปรตีนกาวไหมที่มีสารเชื่อมโยงข้าม (genipin) ในความเข้มข้นต่าง ๆ เคลือบอยู่ โดย plate ที่เป็น control จะใช้แผ่นฟิล์มโปรตีนกาวไหมที่ไม่มีสารเชื่อมโยงข้าม (genipin)

4. หลังจากเลี้ยงนาน 24 ชั่วโมง สารละลายในส่วนของ supernatant จะถูกนำมาทดสอบปริมาณของ nitric oxide ด้วย Griess reaction ซึ่งเป็นการนำ 100 μL Griess reagent (1% N-(1-Napthyl)-ethylenediamine dihydrochloride และ 1% sulfanilamide ใน 2.5% phosphoric acid) ในปริมาณที่เท่ากับสารละลายส่วนของ supernatant มาผสมให้เข้ากัน วัดค่าการดูดกลืนแสงที่ 570 nm เปรียบเทียบกับ standard curve ของ NaNO

ผลการทดลองแสดงในภาพที่ 32 จะเห็นได้ว่าปริมาณ nitric oxide ที่ถูกสร้างขึ้นจากแผ่นฟิล์มโปรตีนกาวไหมที่มีความเข้มข้นของสารเชื่อมโยงข้าม (genipin) ในความเข้มข้นต่ำ ๆ หรือไม่มีสารเชื่อมโยงข้ามเลยจะมีปริมาณน้อย ในขณะที่แผ่นฟิล์มโปรตีนกาวไหมที่ประกอบด้วย genipin ในความเข้มข้นสูง (0.10%) จะสามารถกระตุ้นการสร้าง nitric oxide จากเซลล์ได้มาก



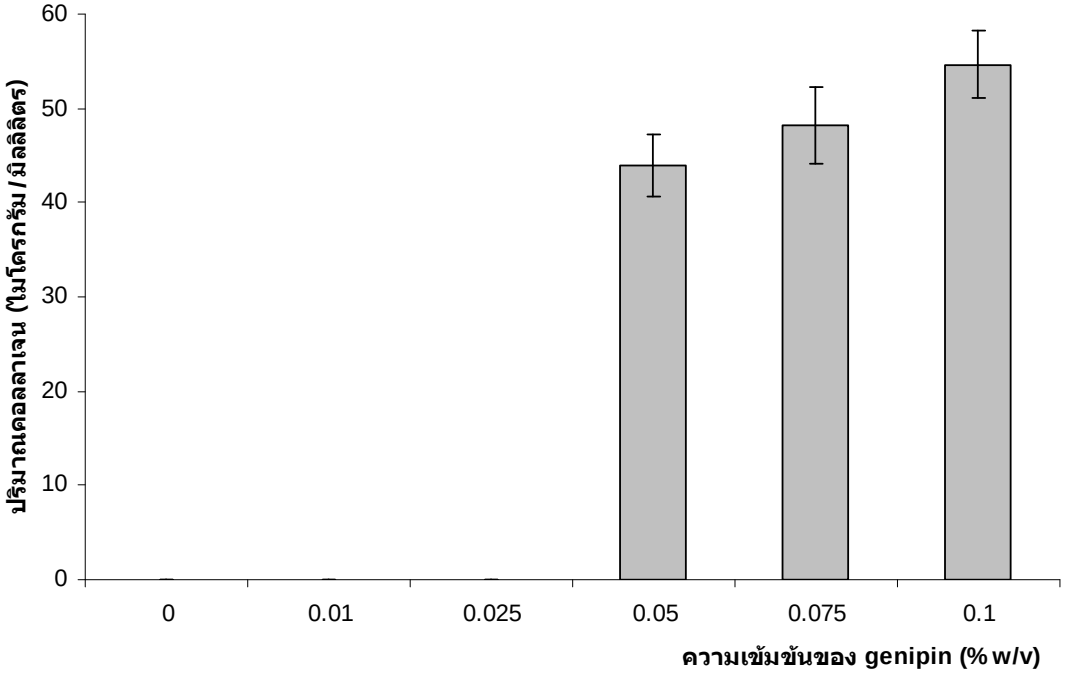
ภาพที่ 32 แสดงปริมาณ nitric oxide ที่เซลล์ผิวหนัง (L 929) สร้างขึ้นหลังจากสัมผัสกับแผ่นฟิล์มโปรตีนกาวไหมที่มีสารเชื่อมโยงข้าม (genipin) ในความเข้มข้นต่าง ๆ

Nitric oxide เป็นสารที่สามารถบ่งบอกการเกิดภาวะการอักเสบของเซลล์ได้ โดยปริมาณ nitric oxide ที่สูงแสดงให้เห็นว่าเซลล์อาจอยู่ในภาวะที่ต้องการการซ่อมแซม อย่างไรก็ตามปริมาณ nitric oxide ที่ต่ำ ๆ (น้อยกว่า 400 ไมโครโมล) จะเป็นตัวที่กระตุ้นให้เซลล์ผิวหนังมีการสร้างคอลลาเจนและโปรตีนได้มากขึ้น ดังนั้นในกรณีของแผ่นฟิล์มโปรตีนกาวไหมที่ทำการทดสอบซึ่งปริมาณ nitric oxide ที่ถูกสร้างขึ้นไม่สูงมากนักแสดงให้เห็นว่าจะเป็นผลดีต่อเซลล์และบาดแผล ส่งผลให้มีการสร้างคอลลาเจนเพิ่มสูงขึ้น ซึ่งในกรณีนี้แผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin 0.10% น่าจะสามารถกระตุ้นการสร้างคอลลาเจนได้สูงสุด

คุณสมบัติในการกระตุ้นการสร้างคอลลาเจนของแผ่นฟิล์มโปรตีนกาวไหม

<u>วิธีการทดลอง</u>

1. เตรียมตัวอย่างแผ่นฟิล์มจากโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นต่าง ๆ กัน ในขนาดที่พอดีกับหลุม 96-well plate
2. นำไปฆ่าเชื้อด้วยการอบก๊าซ ethylene oxide
3. นำเซลล์ที่ต้องการทดสอบ ซึ่งในที่นี้ใช้ L929 mouse fibroblast cells ในความเข้มข้นเริ่มต้น 5 x 10^5/หลุมมาใส่ในถาดหลุมขนาด 96 หลุม/plate ซึ่งมี Dulbecco's Modified Eagle Medium (DMEM) ร่วมกับ fetal bovine serum (FBS) เป็นองค์ประกอบมาเลี้ยงใน plate ที่มีแผ่นฟิล์มโปรตีนกาวไหมที่มีสารเชื่อมโยงข้าม (genipin) ในความเข้มข้นต่าง ๆ เคลือบอยู่
4. โดย plate ที่เป็นแผ่นฟิล์มโปรตีนกาวไหมที่ไม่มีสารเชื่อมโยงข้าม (genipin) จะจัดเป็น negative control พร้อมกันนั้นในส่วนที่เป็น positive control จะมีการเติม melittin ซึ่งเป็นเปปไทด์ที่ได้จากพิษของผึ้งเข้าไปในอาหารเลี้ยงเซลล์ด้วย
5. หลังจากเลี้ยงนาน 24 ชั่วโมง สารละลายในส่วนของ supernatant จะถูกนำมาทดสอบปริมาณของคอลลาเจนชนิดที่ 1 ด้วย Sircol® collagen assay kit (Biocolor Ltd., Northern Ireland, UK) (ภาคผนวกที่ 2) วัดค่าการดูดกลืนแสงที่ 500 nm เปรียบเทียบกับ standard curve ของคอลลาเจนชนิดที่ 1 ที่สกัดจากวัว



ภาพที่ 33 แสดงปริมาณ collagen ที่เซลล์ผิวหนัง (L 929) สร้างขึ้นหลังจากสัมผัสกับแผ่นฟิล์มโปรตีนกาวไหมที่มีสารเชื่อมโยงข้าม (genipin) ในความเข้มข้นต่าง ๆ

จากผลการทดลองจะเห็นได้ว่าแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นตั้งแต่ 0.05% w/v สามารถกระตุ้นการสร้างคอลลาเจนจากเซลล์ได้ ทั้งนี้อาจเนื่องมาจากแผ่นฟิล์มที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ที่ความเข้มข้นสูงสามารถเพิ่มการยึดเกาะและการเพิ่มจำนวนของเซลล์ได้ เมื่อปริมาณเซลล์มากขึ้นจึงส่งผลให้โปรตีนกาวไหมที่ถูกปลดปล่อยออกมาสามารถกระตุ้นการสร้างคอลลาเจนของเซลล์ได้ด้วย

สรุปผลการศึกษา

จากผลการศึกษาพบว่าโปรตีนกาวไหมซึ่งมีคุณสมบัติในการกระตุ้นการสร้างคอลลาเจนได้ดี สามารถนำมาขึ้นรูปเป็นแผ่นฟิล์มได้ อย่างไรก็ตาม แผ่นฟิล์มที่ผลิตขึ้นจำเป็นต้องมีการเติมสารโพลิเมอร์อื่น ๆ เพื่อปรับปรุงคุณสมบัติทางกายภาพ จากการศึกษาพบว่า การใช้ polyvinyl alcohol ในอัตราส่วนร้อยละ 2 ร่วมกับโปรตีนกาวไหมในอัตราส่วนร้อยละ 3 และสาร glycerin ในอัตราส่วนร้อยละ 1 (อัตราส่วนน้ำหนักเปียก) สามารถขึ้นรูปเป็นแผ่นโปรตีนกาวไหมที่มีคุณสมบัติเหมาะสม สามารถนำไปประยุกต์ใช้ได้ต่อไป เพื่อให้การสลายตัวของแผ่นฟิล์มและการปลดปล่อยโปรตีนกาวไหมเป็นไปอย่างช้า ๆ และต่อเนื่อง อีกทั้งเพื่อปรับปรุง

คุณสมบัติด้านความยืดหยุ่นของแผ่นฟิล์มดังกล่าว กระบวนการเชื่อมโยงข้ามจัดเป็นกระบวนการที่มีความสำคัญ โดยกระบวนการเชื่อมโยงข้ามทางกายภาพ ไม่สามารถปรับปรุงคุณสมบัติทางกายภาพของแผ่นฟิล์มโปรตีนกาวไหมได้ดีเท่าที่ควร แต่กระบวนการเชื่อมโยงข้ามทางเคมีโดยการใช้ genipin ในความเข้มข้นร้อยละ 0.1 โดยน้ำหนักต่อปริมาตรของแผ่นฟิล์มโปรตีนกาวไหม สามารถเชื่อมโยงข้ามได้อย่างมีประสิทธิภาพ โดยให้แผ่นฟิล์มโปรตีนกาวไหมที่มีความคงตัว ยืดหยุ่นดี สามารถปลดปล่อยโปรตีนกาวไหมได้อย่างช้า ๆ และสม่ำเสมอ ไม่มีความเป็นพิษต่อเซลล์ อีกทั้งยังสามารถกระตุ้นการสร้าง nitric oxide จากเซลล์ได้ซึ่งสาร nitric oxide มีความสัมพันธ์กับการสร้างโปรตีนเพื่อซ่อมแซมเมื่อเซลล์ได้รับบาดเจ็บ นอกจากนี้ยังสามารถกระตุ้นการสร้างคอลลาเจนจากเซลล์ได้ในปริมาณสูงเมื่อเปรียบเทียบกับแผ่นฟิล์มโปรตีนกาวไหมที่ผ่านกระบวนการเชื่อมโยงข้ามด้วย genipin ในความเข้มข้นอื่น ๆ

ดังนั้นอาจสรุปได้ว่า แผ่นฟิล์มโปรตีนกาวไหมที่ประกอบด้วยสารอื่น ๆ ในความเข้มข้น 3% โปรตีนกาวไหม + 2% polyvinyl alcohol + 1% glycerin และ 0.1% genipin ซึ่งใช้เป็นสารเชื่อมโยงข้าม มีคุณสมบัติทางกายภาพและชีวภาพที่เหมาะสม สามารถนำมาใช้ในประโยชน์ด้านอื่น ๆ ทางการแพทย์ได้ต่อไป

เอกสารอ้างอิง

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4. Bubnis, W.A. and Ofner III, C.M., *The determination of ε-amino group in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid*. Anal Biochem, 1992. 207: p. 129–133.

ภาคผนวกที่ 1

INSTRUCTIONS

PIERCE
3747 N. Meridian Road
P.O. Box 117
Rockford, IL 61105

BCA™ Protein Assay Kit

23225 23227 1296.3

Number	Description
23225	**BCA™ Protein Assay Kit**, sufficient reagents for 500 test tube or 5,000 microplate assays
23227	**BCA™ Protein Assay Kit**, sufficient reagents for 250 test tube or 2,500 microplate assays

Kit Contents:

BCA™ Reagent A, 1,000 ml (in Product No. 23225) or 500 ml (in Product No. 23227), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide

BCA™ Reagent B, 25 ml, containing 4% cupric sulfate

Albumin Standard Ampules, 2 mg/ml, 10 x 1 ml ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide

Storage: Upon receipt store at room temperature. Product shipped at ambient temperature.

Note: If either Reagent A or Reagent B precipitates upon shipping in cold weather or during long-term storage, dissolve precipitates by gently warming and stirring solution. Discard any kit reagent that shows discoloration or evidence of microbial contamination.

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Introduction

The BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid.[1] The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/ml). The BCA™ method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.[2] Studies with di-, tri- and tetrapeptides suggest that the extent of color formation caused by more than the mere sum of individual color-producing functional groups.[2] Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are

prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve. If precise quantitation of an unknown protein is required, it is advisable to select a protein standard that is similar in quality to the unknown; for example, a bovine gamma globulin (BGG) standard (see Related Pierce Products) may be used when assaying immunoglobulin samples.

Two assay procedures are presented. Of these, the Test Tube Procedure requires a larger volume (0.1 ml) of protein sample; however, because it uses a sample to working reagent ratio of 1:20 (v/v), the effect of interfering substances is minimized. The Microplate Procedure affords the sample handling ease of a microplate and requires a smaller volume (10-25 µl) of protein sample; however, because the sample to working reagent ratio is 1:8 (v/v), it offers less flexibility in overcoming interfering substance concentrations and obtaining low levels of detection.

Preparation of Standards and Working Reagent (required for both assay procedures)

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1 ml ampule of 2.0 mg/ml Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/ml)			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank
Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250 µg/ml)			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	700 µl	100 µl of Stock	250 µg/ml
B	400 µl	400 µl of vial A dilution	125 µg/ml
C	450 µl	300 µl of vial B dilution	50 µg/ml
D	400 µl	400 µl of vial C dilution	25 µg/ml
E	400 µl	100 µl of vial D dilution	5 µg/ml
F	400 µl	0	0 µg/ml = Blank

B. Preparation of the BCA™ Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   (# standards + # unknowns) x (# replicates) x (volume of WR per sample) = total volume WR required

   Example: for the Standard Test Tube Protocol with 3 unknowns and 2 replicates of each sample:

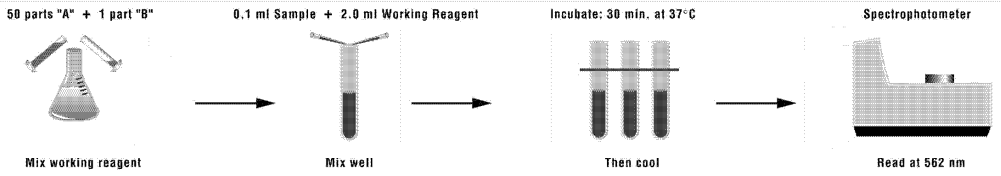
   (9 standards + 3 unknowns) x (2 replicates) x (2 ml) = 48 ml WR required

   Note: 2.0 ml of the WR is required for each sample in the Test Tube Procedure, while only 200 µl of WR reagent is required for each sample in the Microplate Procedure.

2. Prepare WR by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1, Reagent A:B). For the above example, combine 50 ml of Reagent A with 1 ml of Reagent B.

   Note: When Reagent B is first added to Reagent A, a turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

Procedure Summary (Test Tube Procedure, Standard Protocol)



Test Tube Procedure (Sample to WR ratio = 1:20)

1. Pipette 0.1 ml of each standard and unknown sample replicate into an appropriately labeled test tube.
2. Add 2.0 ml of the WR to each tube and mix well.
3. Cover and incubate tubes at selected temperature and time:
   - Standard Protocol: 37°C for 30 minutes (working range = 20-2,000 µg/ml)
   - RT Protocol: RT for 2 hours (working range = 20-2,000 µg/ml)
   - Enhanced Protocol: 60°C for 30 minutes (working range = 5-250 µg/ml)

   Notes:
   - Increasing the incubation time or temperature increases the net 562 nm absorbance for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
   - Use a water bath to heat tubes for either Standard (37°C incubation) or Enhanced (60°C incubation) Protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.
4. Cool all tubes to RT.
5. With the spectrophotometer set to 562 nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

   Note: Because the BCA™ Assay does not reach a true end point, color development will continue even after cooling to RT. However, because the rate of color development is low at RT, no significant error will be introduced if the 562 nm absorbance measurements of all tubes are made within 10 minutes of each other.
6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25 µl of each standard or unknown sample replicate into a microplate well (working range = 20-2,000 µg/ml).

   Note: If sample size is limited, 10 µl of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2,000 µg/ml.
2. Add 200 µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT.
5. Measure the absorbance at or near 562 nm on a plate reader.

   Notes:
   - Wavelengths from 540-590 nm have been used successfully with this method.
   - Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562 nm measurements are desired, increase the incubation time to 2 hours.

- Increasing the incubation time or ratio of sample volume to WR increases the net 562 nm measurement for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful.

6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm measurements of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze, desalt, or dilute sample Increase copper concentration in working reagent (e.g., use 50:2, Reagent A:B) Remove interfering substances from sample using Product No. 23215
Blank absorbance is OK, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze, desalt, or dilute sample
	Color measured at the wrong wavelength	Measure the absorbance at 562 nm
Color of samples appears darker than expected	Protein concentration is too high	Dilute sample
	Sample contains lipids or lipoproteins	Add 2% SDS to the sample to eliminate interference from lipids[3] Remove interfering substances from sample using Product No. 23215
All tubes (including blank) are dark purple	Buffer contains a reducing agent	Dialyze or dilute sample Remove interfering substances from sample using Product No. 23215
	Buffer contains a thiol	
	Buffer contains biogenic amines (catecholamines)	
Need to measure color at a different wavelength	Spectrophotometer or plate reader does not have 562 nm filter	Color may be measure at any wavelength between 540 nm and 590 nm, although the slope of standard curve and overall assay sensitivity will be reduced

A. Interfering substances

Certain substances are known to interfere with the BCA™ Assay including those with reducing potential, chelating agents, and strong acids or bases. Because they are known to interfere with protein estimation at even minute concentrations, avoid the following substances as components of the sample buffer:

Ascorbic Acid	EGTA	Iron	Impure Sucrose
Catecholamines	Impure Glycerol	Lipids	Tryptophan
Creatinine	Hydrogen Peroxide	Melibiose	Tyrosine
Cysteine	Hydrazides	Phenol Red	Uric Acid

Other substances interfere to a lesser extent with protein estimation using the BCA™ Assay, and these have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Standard Test Tube Protocol are listed in Table 2 (see last page of Instructions). Substances were compatible at the indicated concentration in the Standard Test Tube Protocol if the error in protein concentration estimation caused by the presence of the substance in the sample was less than or equal to 10%. The substances were tested using WR prepared immediately before each experiment. Blank-corrected 562 nm absorbance measurements (for a 1,000 µg/ml BSA standard + substance) were compared to the net 562 nm measurements of the same standard prepared in 0.9% saline. In the Microplate Procedure, where the sample to WR ratio is 1:8 (v/v), maximum compatible concentrations will be lower.

B. Strategies for eliminating or minimizing the effects of interfering substances

The effects of interfering substances in the BCA™ Protein Assay may be eliminated or overcome by one of several methods.

- Remove the interfering substance by dialysis or gel filtration.
- Dilute the sample until the substance no longer interferes. This strategy is effective only if the starting protein concentration is sufficient to remain in the working range of the assay upon dilution.
- Precipitate the proteins in the sample with acetone or trichloroacetic acid (TCA). The liquid containing the substance that interfered is discarded and the protein pellet is easily solubilized in ultrapure water or directly in the alkaline BCA™ WR.[4] A protocol for performing this on samples to be assayed with BCA™ Protein Assay Reagent is available at the Pierce web site. Alternatively, Product No. 23215 may be used (see Related Pierce Products).
- Increase the amount of copper in the WR (prepare WR as 50:2 or 50:3, Reagent A:B), which may eliminate interference by copper chelating agents.

Note: For greatest accuracy, the protein standards must be treated identically to the sample(s).

Related Pierce Products

23209	**Albumin Standard Ampules, 2 mg/ml**, 10 x 1 ml ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide
23208	**Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set**, 7 x 3.5 ml aliquots in the range of 125-2,000 µg/ml
23212	**Bovine Gamma Globulin Standard, 2 mg/ml**, 10 x 1 ml ampules
23213	**Pre-Diluted Protein Assay Standards**, Bovine Gamma Globulin Fraction II (BGG) Set, 7 x 3.5 ml aliquots in the range of 125-2,000 µg/ml
23221	**BCA™ Reagent A**, 1,000 ml
23223	**BCA™ Reagent A**, 250 ml
23224	**BCA™ Reagent B**, 25 ml
23235	**Micro BCA™ Protein Assay Kit**, working range of 0.5-20 µg/ml
23236	**Coomassie Plus™ Protein Assay Kit**, working range of 1-1,500 µg/ml
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**, sufficient reagents to pre-treat 500 samples to remove interfering substances before total protein quantitation

Additional Information

A. Please visit the Pierce web site for additional information on this product including the following items:

- Frequently Asked Questions
- Tech Tip protocol: Eliminate interfering substances from samples for BCA™ Protein Assay
- Tech Tip protocol: Shorten BCA™ Protein Assay incubation using a microwave oven

B. Response characteristics for different proteins

Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins. These differences relate to amino acid sequence, pI, structure and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response. Most protein assay methods utilize BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined (Figure 1). However, if great accuracy is required, the standard curve should be prepared from a pure sample of the target protein to be measured.

Table 3 shows typical BCA™ Protein Assay protein-to-protein variation in color response. All proteins were tested at a concentration of 1,000 µg/ml using the 30-minute/37°C Test Tube Protocol. The average net color response for BSA was normalized to 1.00 and the average net color response of the other proteins is expressed as a ratio to the response of BSA.

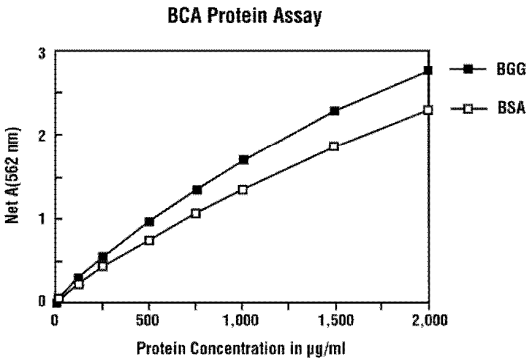


Figure 1: Typical color response curves for BSA and BGG using the Standard Test Tube Protocol (37°C/30-minute incubation).

Table 3. Protein-to-Protein Variation. Absorbance ratios (562 nm) for proteins relative to BSA using the Standard Test Tube Protocol.

Ratio = (Avg "test" net Abs.) / (avg. BSA net Abs.)

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.85
α-Chymotrypsinogen, bovine	1.14
Cytochrome C, horse heart	0.83
Gamma globulin, bovine	1.11
IgG, bovine	1.21
IgG, human	1.09
IgG, mouse	1.18
IgG, rabbit	1.12
IgG, sheep	1.17
Insulin, bovine pancreas	1.08
Myoglobin, horse heart	0.74
Ovalbumin	0.93
Transferrin, human	0.89
Average ratio	**1.02**
Standard Deviation	**0.15**
Coefficient of Variation	**14.7%**

C. Alternative Total Protein Assay Reagents

If interference by a reducing substance or metal-chelating substance contained in the sample cannot be overcome, try the Coomassie Plus™ Protein Assay Kit (Product No. 23236), which is less sensitive to such substances.

D. Cleaning and Re-using Glassware

Exercise care when re-using glassware. All glassware must be cleaned and given a thorough final rinse with ultrapure water.

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1. Smith, P.K., *et al.* (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76-85.
2. Wiechelman, K., Braun, R. and Fitzpatrick, J. (1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal Biochem.* **175**:231-7.
3. Kessler, R. and Fanestil, D. (1986). Interference by lipids in the determination of protein using bicinchoninic acid. *Anal. Biochem.* **159**:138-42.
4. Brown, R., Jarvis, K. and Hyland, K. (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* **180**:136-9.

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Fischer, T., *et al.* (1999). Clathrin-coated vesicles bearing GAIP possess GTPase-activating protein activity in vitro. *Proc. Nat. Acad. Sci.* **96:**6722-7.

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Roberts, K.P., Ensrud, K.M. and Hamilton, D.W. (2002). A comparative analysis of expression and processing of the rat epididymal fluid and sperm-bound forms of proteins D and E. *Biology of Reproduction* **67:**525-33.

Table 2. Compatible Substance Concentrations in the BCA™ Protein Assay (see text for details).

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	25 mM
Ammonium sulfate	1.5 M
Asparagine	1 mM
Bicine, pH 8.4	20 mM
Bis-Tris, pH 6.5	33 mM
Borate (50 mM), pH 8.5 (#28384)	undiluted
B-PER® Reagent (#78248)	undiluted
Calcium chloride in TBS, pH 7.2	10 mM
Na-Carbonate/Na-Bicarbonate (0.2 M), pH 9.4 (#28382)	undiluted
Cesium bicarbonate	100 mM
CHES, pH 9.0	100 mM
Na-Citrate (0.6 M), Na-Carbonate (0.1 M), pH 9.0 (#28388)	1:8 dilution*
Na-Citrate (0.6 M), MOPS (0.1 M), pH 7.5 (#28386)	1:8 dilution*
Cobalt chloride in TBS, pH 7.2	0.8 mM
EPPS, pH 8.0	100 mM
Ferric chloride in TBS, pH 7.2	10 mM
Glycine•HCl, pH 2.8	100 mM
Guanidine•HCl	4 M
HEPES, pH 7.5	100 mM
Imidazole, pH 7.0	50 mM
MES, pH 6.1	100 mM
MES (0.1 M), NaCl (0.9%), pH 4.7 (#28390)	undiluted
MOPS, pH 7.2	100 mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	10 mM
PBS; Phosphate (0.1 M), NaCl (0.15 M), pH 7.2 (#28372)	undiluted
PIPES, pH 6.8	100 mM
RIPA lysis buffer; 50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	undiluted
Sodium acetate, pH 4.8	200 mM
Sodium azide	0.2%
Sodium bicarbonate	100 mM
Sodium chloride	1 M
Sodium citrate, pH 4.8 or pH 6.4	200 mM
Sodium phosphate	100 mM
Tricine, pH 8.0	25 mM
Triethanolamine, pH 7.8	25 mM
Tris	250 mM
TBS; Tris (25 mM), NaCl (0.15 M), pH 7.6 (#28376)	undiluted
Tris (25 mM), Glycine (192 mM), pH 8.0 (#28380)	1:3 dilution*
Tris (25 mM), Glycine (192 mM), SDS (0.1%), pH 8.3 (#28378)	undiluted
Zinc chloride in TBS, pH 7.2	10 mM

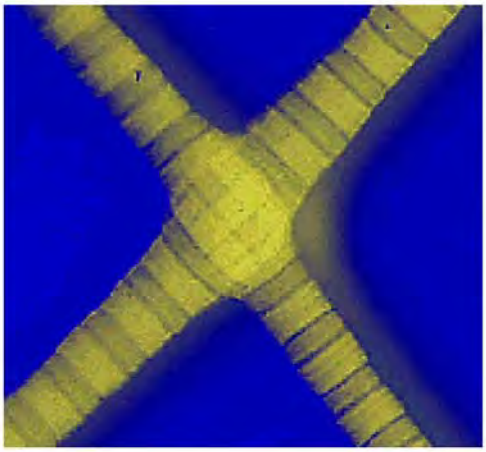
Substance	Compatible Concentration
Detergents**	
Brij®-35	5.0%
Brij®-56, Brij®-58	1.0%
CHAPS, CHAPSO	5.0%
Deoxycholic acid	5.0%
Octyl β-glucoside	5.0%
Nonidet P-40 (NP-40)	5.0%
Octyl β-thioglucopyranoside	5.0%
SDS	5.0%
Span® 20	1.0%
Triton® X-100	5.0%
Triton® X-114, X-305, X-405	1.0%
Tween®-20, Tween®-60, Tween®-80	5.0%
Zwittergent® 3-14	1.0%
Chelating agents	
EDTA	10 mM
EGTA	--------
Sodium citrate	200 mM
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	10 mM
Ascorbic acid	--------
Cysteine	--------
Dithioerythritol (DTE)	1 mM
Dithiothreitol (DTT)	1 mM
Glucose	10 mM
Melibiose	--------
2-Mercaptoethanol	0.01%
Potassium thiocyanate	3.0 M
Thimerosal	0.01%
Misc. Reagents & Solvents	
Acetone	10%
Acetonitrile	10%
Aprotinin	10 mg/L
DMF, DMSO	10%
DMSO	10%
Ethanol	10%
Glycerol (Fresh)	10%
Hydrazides	--------
Hydrides (Na_2BH_4 or $NaCNBH_3$)	--------
Hydrochloric Acid	100 mM
Leupeptin	10 mg/L
Methanol	10%
Phenol Red	--------
PMSF	1 mM
Sodium Hydroxide	100 mM
Sucrose	40%
TLCK	0.1 mg/L
TPCK	0.1 mg/L
Urea	3 M
o-Vanadate (sodium salt), in PBS, pH 7.2	1 mM

* Diluted with ultrapure water; ** Detergents were tested using Pierce high-purity Surfact-Amps™ Products, which have low peroxide content;
-- Dashed-line entry indicates that the material is incompatible with the assay.

ภาคผนวกที่ 2

Sircol™

Soluble Collagen Assay



biocolor
life science assays

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Assay Reference Images

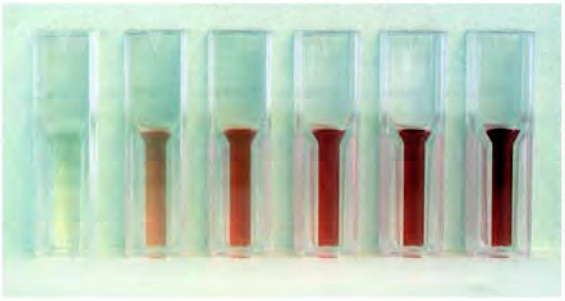
+30min

Mix Sircol reagent and collagen reference standard

+40min

Centrifuge and remove supernatant carefully

+50min

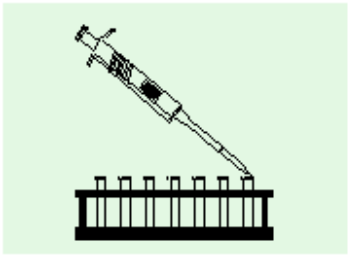


Add alkali reagent
(picture obtained using 0-50µg collagen reference standard)

email: info@biocolor.co.uk

Sircol Soluble Collagen Assay

Time Req: 1 hour **Detection Limit:** 2.5µg*



Set up assay:

Label a set of 1.5 ml microcentrifuge tubes.

If sufficient material is available, run duplicate samples.

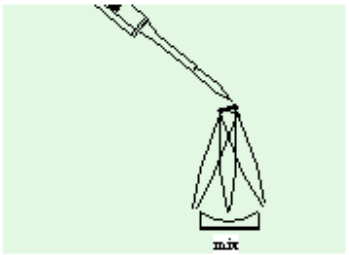
Prepare;

[1] Reagent blanks: (100 µl of distilled water or the test sample buffer).

[2] Collagen standards: (aliquots containing 5, 10, 25, 50 µg).

[3] Test samples, (volumes: 10 to 100 µl *). Adjust the contents of all tubes to 100 µl with distilled water or appropriate buffer.

To each tube add 1 ml Sircol Dye reagent and cap all of the tubes; mix contents by inverting.



Mixing:

Place tubes in a mechanical shaker for 30 minutes, (or manually shake at 5 minute intervals).

During this time period the Sircol Dye will bind to soluble collagens.

The dye reagent is designed so that the collagen-dye complex will precipitate out of solution.

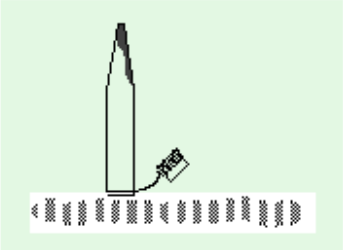
Centrifuging:

Transfer the tubes to a micro centrifuge and spin the tubes at >10,000 x g for a 10 minute period*.

It is important to firmly pack the insoluble pellet of the collagen-dye complex at the bottom of the tubes, so as to avoid any loss during draining.

Draining:

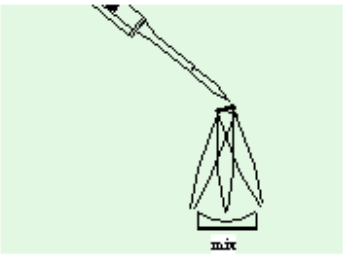
The unbound dye solution is removed by carefully inverting and draining the tubes.



Any remaining droplets can be removed from the tubes by *gently* tapping the inverted tube on a paper tissue or a cotton wool bud can be used for removing droplets of dye from the rim of the tubes.

Do not attempt to physically remove any fluid that is in close contact to the deposit.

Release of bound dye:



To each tube add 1 ml of the Alkali reagent.

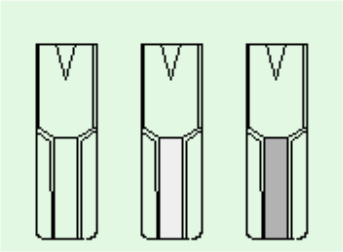
Re-cap the tubes and release the bound dye into solution. A vortex mixer is suitable.

When the bound dye has been dissolved, usually within 10 minutes, the samples are ready for measurement.

The colour is light stable, but should be read within 2 to 3 hours.

Keep the tubes capped until ready for measurement.

Measurement:



(a) Spectrophotometer, set wavelength to 540 nm.
Use semi-micro glass, or plastic disposable cuvettes.

(b) Colorimeter, set using a blue-green filter.
Use semi-micro cuvettes or tubes.

(c) Multiwell plate reader, set using a blue-green. Transfer 200 µl aliquots of samples from tubes to the wells of a 96 well, multiwell plate.

Set the above instruments to zero using water. Measure absorbance of reagent blanks, collagen standards and the test samples.

Subtract the reagent blank reading from the standard and test sample readings. Check duplicates are within ±10%.

Plot standards on graph and use the graph to calculate the collagen content of the test samples. (see manual)

Sircol™
Soluble Collagen
Assay

TECHNICAL INFORMATION

The *Sircol* Assay has been designed
for *in vitro* research work only

Handle the
Sircol Assay Kit
using

GOOD LABORATORY PRACTICE

Read Manual Before Use

5th Edition 2007

***Sircol* Manual**

Published by
Biocolor Ltd.
67 Church Road, Newtownabbey BT36 7LS
Northern Ireland.
www.biocolor.co.uk

***Sircol* Assay**

Manual

Intended Applications:

The Sircol Collagen Assay is a quantitative dye-binding method designed for the analysis of acid-soluble collagens extracted from mammalian tissues and collagens released into culture medium by mammalian cells during *in vitro* culture.

Collagen forms that can be measured:

[i] salt-soluble collagens (1 M NaCl in 0.05M Tris, pH 7.5.)

[ii] acid-soluble collagens (0.01 to 1.0 M acetic acid)

[iii] pepsin-soluble collagens (0.5 M acetic acid + pepsin)

[iv] total soluble collagens (composed of [i],[ii] & [iii])

Collagen Types that can be measured:

[1] Mammalian collagens, Types I to V, can be measured

[2] Collagen Types VI to XIV can be assayed, but have not been calibrated due to insufficient purified material available for the preparation of standard curves

[3] Sircol dye binding does not discriminate between collagen types

[4] The dye reagent binds specifically to the [Gly-X-Y]n helical structure found in all collagens

[5] Sircol dye binding decreases, gradually with the thermal denaturation of collagen (collagen to gelatin)

[6] Non-vertebrate collagens bind less dye, due in part to lower denaturation temperature and less hydroxyproline residues, and would require species matched purified preparations for standard calibration curves

Sircol Assay Kit components:

[1] The dye reagent contains Sirius Red in picric acid and has been formulated for specific binding to collagen under the conditions defined in the Sircol Assay Manual.

[2] Alkali reagent contains 0.5 M sodium hydroxide, for releasing Sircol Dye from the collagen-dye complex.

[3] Salt soluble collagen precipitating reagent; contains L-lysine monohydrochloride.

[4] Collagen standard, acid soluble Type I, supplied as a sterile solution in 0.5 M acetic acid within a sealed vial. Concentration: 1 mg/ml.
This is sterile bovine skin collagen that has been imported from the USA, and was obtained from disease free animals.
In countries that forbid the importation of bovine derived material, a rat tail collagen standard, acid soluble Type I, is supplied as a sterile solution in 0.5 M acetic acid within a sealed vial. Concentration: 0.5 mg/ml.

[5] This Sircol Assay Manual; (also available as a pdf, see www.biocolor.co.uk)

Other components required, but not supplied:

[a] Capped 1.5ml capacity microcentrifuge tubes

[b] Variable volume micropipettors and pipette tips

[c] A mechanical mixer for the microcentrifuge tubes. Any equipment that provides consistent shaking, rolling or rotation of the tubes is suitable

[d] A centrifuge with a 1.5ml tube rotor head and capable of at least 10,000 x g, to firmly pack the collagen-dye pellet.

[e] A spectrophotometer, a colorimeter or a microwell plate reader with a blue-green filter.

Recommended storage conditions for components:

Unopened;

All components have been prepared for long term stability (at least 12 months), when stored at room temperature.

Do not freeze as complete re-solubilisation may not occur.

Opened;

The assay components will retain their shelf-life, providing the glass vials of collagen standard and lysine solution are:

(i) stored at +4ºC when not in use

(ii) the metal seal is not removed

The contents of both vials are best sampled as follows:

Remove the centre metal disc only from the vial tops. Obtain aliquots from the vials, when required, using a plastic syringe fitted with a sterile hypodermic needle. The butyl rubber seal on the vial has a thin centre disc, suitable for needle insertion into the vial. Discard vial if solution contents become turbid.

Mode of action of the Sircol dye reagent with soluble collagens:

The Sircol dye reagent contains Sirius Red. The Colour Commission name is Direct Red 80. The molecular structure of the dye is shown below (Fig. 1).

Mechanism by which the dye reacts with collagen:

Sirius Red is an anionic dye with sulphonic acid side chain groups. These groups react with the side chain groups of the basic amino acids present in collagen.

The specific affinity of the dye for collagen, under the assay conditions is due to the elongated dye molecules becoming aligned parallel to the long, rigid structure of native collagens that have intact triple helix organisation.

Dye affinity is much reduced when collagen is heat denatured (>45ºC to the form of random chains of gelatin) and the triple helix unwinds.

Dye binding is gradually lost when collagen (and gelatin) are exposed to bacterial collagenases.

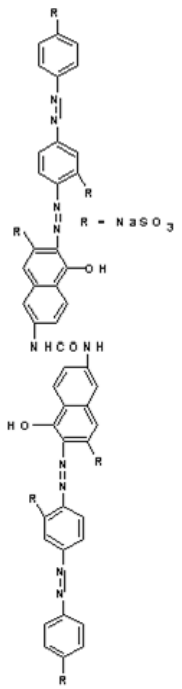


Fig. 1

The molecular structure of the Sircol Dye

Test sample compositional requirements:

As the Sircol Assay is a colorimetric procedure, samples for analysis should be free of any particulate material (cell debris and insoluble extracellular matrix).

[1] The test sample can be in a salt buffer solution, acetic acid or culture medium.

[2] If a surfactant has been used during tissue extraction, it is recommended that this extraction solution is Sircol tested with the Reference Collagen Standard to check that it has no adverse effects on collagen-dye binding.

[3] The presence of soluble proteins in samples, including proteoglycans, tropoelastin and other soluble ECM materials, does not interfere with the Sircol Assay.

[4] Cell culture medium with foetal calf serum supplements of up to 5% does not interfere with the collagen assay. When higher serum supplements have been used, the increasing bulk of serum proteins, relative to the amount of collagen present, can cause problems.

In this latter case the following options could be examined:

[a] reduce the serum supplement to 5%, either after cell attachment has occurred, or reduce serum to 5% for the culture medium that will be collected and used for assay;

[b] selectively remove the bulk of the serum albumin from the test sample by affinity chromatography (Blue-Sepharose CL-6B);

[c] precipitate the collagen out of solution, adding NaCl to a concentration of 4 M for samples with neutral pH; or 2 M NaCl for samples in acetic acid. Centrifuge, drain well and re-solubilize the collagen pellet in 0.5 M acetic acid.

Assay of test solutions containing < 25 µg/ml collagen, without prior concentration:

Method

The Sircol Dye Reagent contains sufficient dye to permit test sample volumes to be increased up to 200 µl, before adding 1.0ml of Dye Reagent.

Note 1

It is not recommended that more than 200 µl of sample volumes be used with 1.0 ml of Dye Reagent. Excessive dye dilution, by increasing sample volumes above 200 µl, can result in loss of collagen dye saturation.

Note 2

For test samples with collagen levels less than 25 µg/ml, but with more than 5 µg/ml, it is possible to directly obtain assay results without sample concentration by *increasing* both sample and dye volumes, using larger volume conical centrifuge tubes.

Add 1000 µl of test solution, followed by 5.00ml of Dye Reagent, contained in a 15 ml capacity conical centrifuge tube. The collagen bound dye complex, recovered after centrifugation, is then solubilised in 1.00 ml of the Alkali Reagent.

No multiplication factor is required, when using 1.00 ml test samples/5.00 ml dye volumes, to express the results as µg/ml.

Set up assay:

To duplicate 1.5 ml microcentrifuge tubes, add sample volumes of between 10 and 100 µl:

(a) collagen standards

(b) test samples and reagent blanks (100 µl of 0.5 M acetic acid, extraction buffer or fresh unused tissue culture medium).
Adjust the volume in all tubes to 100 µl.

Working Standards:

It is recommended that the collagen standard is initially run, in duplicate, at three concentrations; using 12.5, 25 and 50 µl aliquots of the supplied Reference Collagen Standard.

The standards, with the reagent blanks, are used to produce a calibration curve with the selected spectrophotometer, colorimeter or microwell plate reader.

In subsequent assay batches a minimum requirement is duplicates of a mid-range collagen standard and reagent blanks.

In repeated assays these secondary standard and reagent blanks should give absorption values, at 540 nm, to within ± 5% of that defined by the initial standard curve.

Test samples:

With test samples, where the approximate collagen concentrations are as yet unknown (collagen absorption at 280 nm is very low due to the limited number of aromatic amino acids and, therefore, cannot be used as a guide), 50 µl aliquots are suggested for the first run.

If required use more or less, in the next batch of assays to bring all test sample readings within the concentration range covered by the standard curve.

Dye reagent and mixing:

Add 1.0 ml Sircol dye reagent to all tubes. Cap tubes and mix contents. First by inversion, as the density/viscosity difference between the sample and the dye reagent can differ considerably. Then gently mix tube contents at room temperature for 30 ± 5 minutes.

The same mechanical mixer, at the same setting, should if possible be used for all assay batches to minimise experimental variations.

Centrifuge to recover collagen-dye complex:

Transfer tubes to a microcentrifuge and centrifuge at 10,000 x g for 10 minutes. This operation is to pack the collagen-dye pellet at the bottom of the tubes.

A minimum RCF of 10,000 x g is required; higher values, if available, can be used as this will force more unbound dye out of the pellet. This also reduces the risk of any pelleted material being lost when the tube contents are decanted.

Removal of unbound dye:

Remove tubes from centrifuge and uncap. The supernatants are drained off and discarded. The collagen bound dye remains as a pellet at the bottom of the tubes.

While the tube is still inverted, use an absorbent paper tissue or a cotton bud to remove any dye solution from the top end of the tube wall. Do not remove any beads of fluid close to the collagen-dye precipitate on the side wall of the tube.

The Sircol dye reagent contains a surfactant to aid draining from the non-wettable plastic tubes. A wash step is **not** recommended, but may be considered necessary for low concentration test samples. Wash with 500 µl of ethanol (99% pure; and methanol free).

Do not wash with water. An ethanol wash step can reduce reagent blank readings close to zero, but increases the experimental variation between duplicate samples to above that obtainable without the wash step. This is due to the dye precipitate not packing as firmly after the ethanol wash, which causes a loss of some bound dye during the second draining step.

Recovery of collagen bound dye:

To the collagen-dye pellet add 1.0 ml of the Alkali reagent and then cap the tubes. Bring the collagen bound dye back into solution; a vortex tube mixer is most convenient, but holding the top of the tube in one hand while flicking the bottom of the tube with a finger of the other hand is also effective.

The dye should be in solution within 5 minutes. Pellets that are centrifuged at a high RCF may take a little longer. Gelatin samples can require several mixing operations to fully release gelatin-bound dye.

Measurement of collagen bound dye:

The alkaline dye solution is stable to indoor light, but should be measured within 3 hours. Ensure tubes remain capped, to avoid loss due to evaporation, until ready for reading.

Spectrophotometer;

Use semi-micro glass, or plastic disposable cuvettes. Set the instrument wavelength to 540 nm and use water to set the absorbance reading to zero. Read and record the absorbance values of assay blanks, standards and test solutions.

Colorimeter;

The instrument should have a sample cell or cuvette suitable for reading test volumes within 1 ml, and a 1 cm light path length.

The range of filters in colorimeters varies. A blue-green filter, often labelled 500, 510 or 550 nm, will usually be found suitable. To confirm that the filter selected is suitable, use the three concentrations of collagen standards. Ensure that these readings produce a straight line standard curve, that passes through zero.

Microwell plate colorimeter;

Transfer 200 µl aliquots of the alkali dye solutions from the assay tubes to the wells of a microwell plate.

The selection and testing of a suitable colour filter should follow the recommendations given for the colorimeter above.

Calculation of collagen concentration in test samples:

Subtract the reagent blank reading from all the standard and test readings. The reagent blank value should be between 0.15 and 0.18 (a guide range only – based on the equipment used). Higher values (>0.25) indicate that the draining and removal of unbound dye technique could be improved.

When low reagent blank values are obtained consistently, it may be more convenient to set the measuring instrument to zero with the reagent blank; thus avoiding the need to subtract the background value from the test samples.

Monitor the variation in absorbance readings between duplicate samples. Initially some wide variations may occur. Assuming that this is not due to a pipetting error, the most likely source of error is the draining step. A little practice with draining and drying of the top of the tubes leads to a consistent mode of practice, and the ability to bring experimental error of duplicate samples to within ± 5% of the mean.

Using a computer spreadsheet with graphical output, plot the three Collagen Reference Standard absorbance means against their known collagen concentrations. On joining the points, these should produce a straight line graph which can then be extended to pass through zero (absorbance and concentration).

Test sample absorbance values can now be read off the graph to determine their collagen concentration. Readings below 0.05 and higher than 1.00 are unreliable and should be re-assayed – after either concentration or dilution of the test material.

Where test material contains uncommonly used salts, detergents or biological molecules, it may be necessary to initially evaluate their suitability with the assay. This can be performed by adding known amounts of the Collagen Reference Standard to the test samples and checking collagen recovery, following completion of the assay.

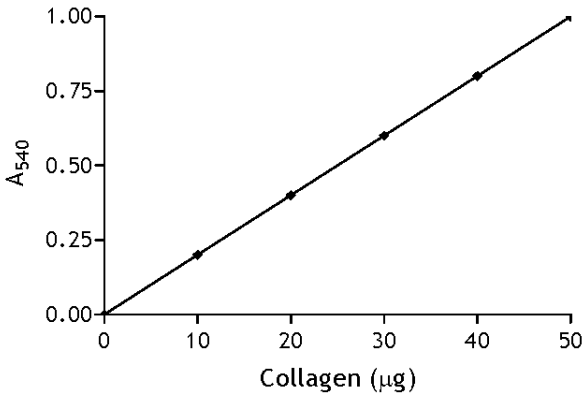


Fig. 2 A typical straight line calibration curve for dye bound by acid soluble collagen.

The above calibration graph was prepared using aliquots of the collagen standard solution; according to the assay procedure outlined on the inside cover.

TEST SAMPLE PREPARATION

General comments

Collagen is ubiquitous to all animals and is found within, or surrounding, all tissues and organs. Collagen is the most abundant animal protein, accounting for about one third of the total protein of mammals.

The range of collagen types and their functional roles ensures that they play a key role in all aspects of growth, maturity, pregnancy and ageing. Because of their widespread occurrence, changes to collagens occur in most chronic and some acute diseases.

Given the diversity of animal species and tissue material that are used to study the above processes, there can be no single universal sample preparation procedure. Some of the more commonly used preparations and extractions are described below. A further valuable source of information is in published research papers (see coloured insert for recent research papers that have used the Sircol Assay).

IN VITRO STUDIES

Application for low collagen concentrations in cell culture medium:

Common practice during cell culture is to employ the culture medium at a volume of ~0.25 ml/cm^2 surface area of the selected culture flask, dish or plate. This culture medium ratio has been found to provide an acceptable balance between providing sufficient medium to meet the cells nutritional requirement, while avoiding excessive concentrations of cellular metabolites that accumulate during culture. The 0.25 ml/cm^2 ratio also ensures that culture medium volume, above anchorage dependent cells, does not unduly restrict exchange of CO_2 and O_2.

When monitoring the secretion of collagen the 0.25 ml/cm^2 culture medium ratio can frequently produce collagen concentrations of ~ 5 µg/100 µl (50 µg/ml) in the culture medium.

The amount and rate of production of collagen can vary considerable from this *'average'* value under various test conditions; collagen synthesis is often found to increase as the cell population nears confluence.

Examination of the 'standard curve' in the Sircol manual reveals that for test samples with collagen concentrations of ~5 µg/100 µl the absorbance reading is too close to the reagent blank absorbance value to provide confidence in the collagen value obtained.

The need to concentrate test samples, prior to the assay, would cause a substantial delay and additional work before results can be obtained. A delay that detracts from a major benefit of using the Sircol Assay – the convenience of obtaining results in one hour.

For direct procedures to measure test samples with collagen concentrations below 25 µg/ml, and more than 5 µg/ml, see page 4.

IN VIVO **STUDIES**

Extraction of soluble collagens from tissue, cartilage and organs:

Samples for analysis should be collected under aseptic conditions, where possible. Material sampled post-mortem should be collected as soon after death as possible. Briefly wash the external surface with sterile water or saline to remove any debris and blood stains. If the sample contains attached adipose tissue this should also be trimmed off using a scalpel.

If extraction is not to be carried out immediately, then the samples should be placed into labelled, sealable, plastic envelopes and frozen as quickly as possible (weigh prior to freezing). Do not store at 0 to 5ºC, even if extraction is to be performed the following day. The major risk at this early stage of preparation is proteases; these are released by dead cells and by contaminating bacterial enzymes (many active at low temperatures).

Stored frozen samples are best 'thawed-out', in the plastic envelope, within a refrigerator at 5ºC. Decide if collagen content of the test samples is to be expressed as 'dry weight' or 'wet weight'. If dry weight, it will be necessary to take a representative sample, obtain its wet weight and then dry the sample in a heated, or un-heated, drying cabinet containing drying granules. The samples are weighed daily until a constant dry weight value is obtained; most tissues and cartilages are ~ 70% water.

To optimise collagen extraction the tissue sample should be 'diced' into small cubes, using a sharp scalpel. Avoid producing cubes of less than 2 to 3 mm as the 'squeezing' of these small tissue particles can result in fluid being lost from the cut surfaces. Weigh the prepared samples into sterile flasks or beakers. Use as large a weight sample as possible. You cannot have too much collagen and larger sample sizes also reduce variation, due to non-homogeneous collagen distribution within the tissue.

[1] Salt soluble collagens

The salt soluble collagen fraction represents the most recent collagen secreted by the cell. Within a few hours in the extracellular matrix (or in the cell culture medium) this salt soluble collagen (tropocollagen monomers) will 'crystallise' into collagen fibrils and become salt insoluble. The salt soluble collagen fraction will be small, needing a large sample weight to produce Sircol detectable amounts of collagen (>2.5 µg). The salt soluble collagen fraction is also the most vulnerable to protease degradation.

Extraction: Salt soluble collagen solvent is a 0.05 M Tris buffer, pH 7.5, containing 1.0 M sodium chloride. This solvent should also include a 'Protease Inhibitor Cocktail' (ready to use cocktail mixtures are available from Sigma-Aldrich).

Beware if preparing a DIY cocktail from dry components, as many of these agents are **toxic**.

Extraction solvent volumes will depend on the material being extracted. A 10 volumes of solvent to wet tissue weight ratio is suggested. The sample should be stirred overnight at 0 to 5ºC. To obtain a transparent solution, containing the salt soluble collagen, centrifuge at 15000 x g for 60 minutes. As the Sircol Assay is a colorimetric assay, turbid or translucent extracts are not suitable for analysis. If centrifugation does not produce a transparent supernatant, consider filtering this solution through a 0.4 or 0.8 µm filter unit. Initial trials should be performed on non-essential tissue; to determine test sample weights, weight to solvent ratio and whether a second extraction of the residue is required for quantitative extraction.

[2] Acid soluble collagens

Dilute acetic acid (0.5 M) solubilises non-cross linked, and some cross linked forms of collagen. The pH of 0.5 M acetic acid is ~3.0, so as with salt extraction a protease inhibitor cocktail is recommended. The solvent to tissue ratio and extraction times are also similar to salt extraction.

This method of extraction represents the quickest and simplest procedure for recovering the recently synthesised collagen pool from tissues. The yield of acid soluble collagen recovered will be dependent on the age of the animal (more collagen is synthesised during early growth).

In adult animals most of the collagens have long term stability, usually exceeding the life span of the animal. Increases in acid soluble collagens, however, are found in various disease processes; where the extracellular matrix is being destroyed or collagen is being laid down to replace cell loss in tissues and organs.

[3] Pepsin soluble collagens

This extraction procedure is usually the 'method of choice' for recovering the recently synthesised collagen pool from tissues. It produces a larger yield of collagen than when 0.5 M acetic acid is used alone.

This is due to pepsin cleaving off part of the *C*- terminal, non-helical region of the alpha-chains that make up the triple helix of tropocollagen. This *C*- terminal non-helical region contains the initial covalent cross link between the alpha-chains. This cross-link aligns the three tightly wound left handed helices, permitting them to form the right handed super helix of tropocollagen. In the ECM further cross-linking occurs between adjacent alpha-chains and other tropocollagen molecules that are packed into the forming collagen fibrils.

The pepsin (EC 3.4.23.1) should have good activity, and is dissolved in 0.5 M acetic acid. As a general rule, use about a 1:10 ratio of pepsin: tissue wet weight. Aseptic conditions should apply during overnight extraction at room temperature. Stir vigorously during this time period.

Treatment of Extracts prior to Assay:

The Sircol Assay is a colorimetric assay and it is essential that test samples are transparent. Opalescence or turbidity will result in the non-specific attachment of Sircol Dye to suspended material. Coloured transparent solutions are suitable. It is not uncommon to have reddish-brown extracts due to the presence of haemoglobin and/or myoglobin. These soluble proteins do not cause interference with the Sircol Assay.

When turbidity occurs, it should be removed prior to assay. High speed centrifugation is often effective. Filtering a small representive aliquot of the test solution through a 0.4 or 0.8 μm filter unit (attached to a 2 or 5 ml syringe) is also usually effective.

Final Note: Beware of microbial protease activity; assay samples as soon as possible following extraction.

Cross-linked insoluble collagen:

The fraction of collagen remaining in the tissue residue after salt, acid and pepsin extraction is insoluble, covalently cross-linked collagen. Extraction treatments that can solubilise this collagen do so by either causing peptide cleavage into fragments or by denaturation of the collagen to gelatin.

Gelatin can be measured by the Sircol assay, but the degree of protein denaturation effects how much dye will be bound. If heat is used to solubilise the insoluble collagen, then providing the temperature/time are standardized, and similarly treated insoluble collagen standards are included as controls, the assay can be used to measure insoluble collagen.

Extraction procedure:

Insoluble collagen when suspended in water and heated at temperatures above 60ºC will be gradually converted into water soluble gelatin. The time required will depend, in part, on the nature and frequency of cross-links and the collagen: water ratio. However, the major factor that the time will depend on is the temperature used for extraction. The higher the temperature the shorter the time required, but also the more collagen that is denatured. The more collagen that is denatured the less Sircol dye that can be bound by the gelatin.

The time-temperature effect on dye binding by gelatin is shown below. A temperature of 80ºC is recommended, as this permits accurate temperature control by using a water-bath with a thermostat and a lid to reduce evaporation loss. It is important to ensure that all of the insoluble collagen has been solubilised from the test samples. Use small samples of test material and run similar weights of insoluble collagen standards as controls. Insoluble collagens are readily available from biochemical suppliers at low cost.

The heat extraction procedure, although prolonged, can after the initial calibrations be performed with limited supervision.

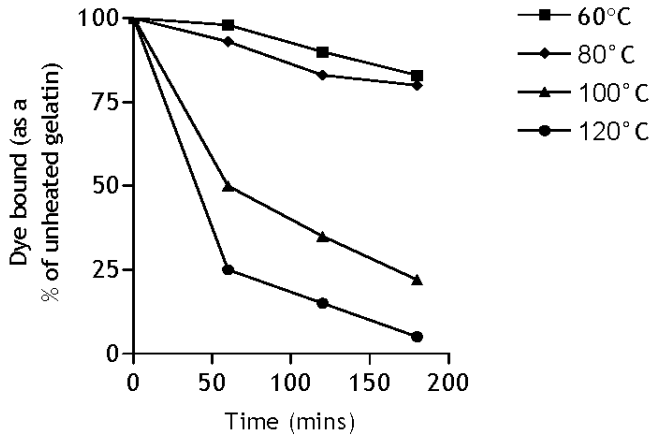


Fig. 3

The effect of temperature/time on gelatin, and the subsequent dye-binding of the Sircol Dye

Collagenase assays:

These assays aim to examine test material for the presence of collagenase activity or the presence of activators/inhibitors of collagenase. Studies to determine the amount of collagen remaining at known time intervals, or at a fixed time periods with increasing concentrations of the test agent, can usually be completed within one working day.

The collagen standard supplied with the Sircol Assay Kit can be used as a collagenase substrate. If larger quantities are required, a convenient source is rat tail tendons. Some commercial sources of 'acid-soluble' collagens may be found to be only ~40% acid-soluble; this appears to be due to the acid-soluble collagen 'ageing' (in the presence of a trace of moisture) during storage. Freshly prepared acid-soluble collagen is a preferable substrate.

Collagenase activity assay:

To dried, formaldehyde vapour fixed, collagen and/or gelatin films on microwell plates add test solutions, buffer blanks, reference enzymes etc. Adjust all test volumes to a common value e.g. 100 µl. Seal the wells by covering the plate in 'cling film' or cover the top surface with clear plastic adhesive tape strips.

Incubate at the optimum temperature for the enzyme(s) under investigation. The time required for 20-50% digestion of the substrate(s) will need to be determined. With bacterial collagenase of ~10 U, a 3 hour period at 20ºC, aided by the use of a rocking type mixer, can digest ~ 40% of the collagen film (this data is offered as a starting guide, results can vary considerably due to differences in enzyme purity and activity). On completion of the incubation period remove the plastic film from the plate.

Drain the contents from the wells into a sink. The plate is then inverted and allowed to drain dry on a paper towel. Tap the plate firmly on to the towel so as to dislodge any fluid remaining within the wells. Replace the plate, open-well side up, and add 200 µl Sircol dye reagent to each well. Carefully cover the fluid-full wells and leave for 30 minutes, then drain the dye from the plate.

To measure the amount of bound dye present, and thus the amount of collagen remaining, add 100 µl Sircol Alkali reagent to each well. If possible place the plate on a mechanical rocker; otherwise periodically move the plate in a rotational manner, without lifting it of the bench surface. A 15 minute extraction period is sufficient to bring the bound dye into solution. Do not drain the fluid contents from the wells. The samples are now ready for inspection and/or measurement.

Visual inspection:

Place the microwell plate on a light-box and examine the well staining pattern. A map of the results can be made for permanent record (a blank map is provided) or photographed.

Quantitative Measurement:

Place the plate into a multiwell plate colorimeter. Select a suitable colour filter (see collagen assay for advice on filter selection). Read the absorbance of each well and obtain a printout of the absorbance readings.

The collagen plus water/buffer samples will have the highest absorbance readings as no collagen was removed/digested.

The wells in column 12 will have the lowest reading as no collagen was present.

The test wells values can be expressed as a percentage of the 100% standard or if a range of collagen concentrations were used the quantity (mg or moles) of collagen digested by the test samples can be calculated.

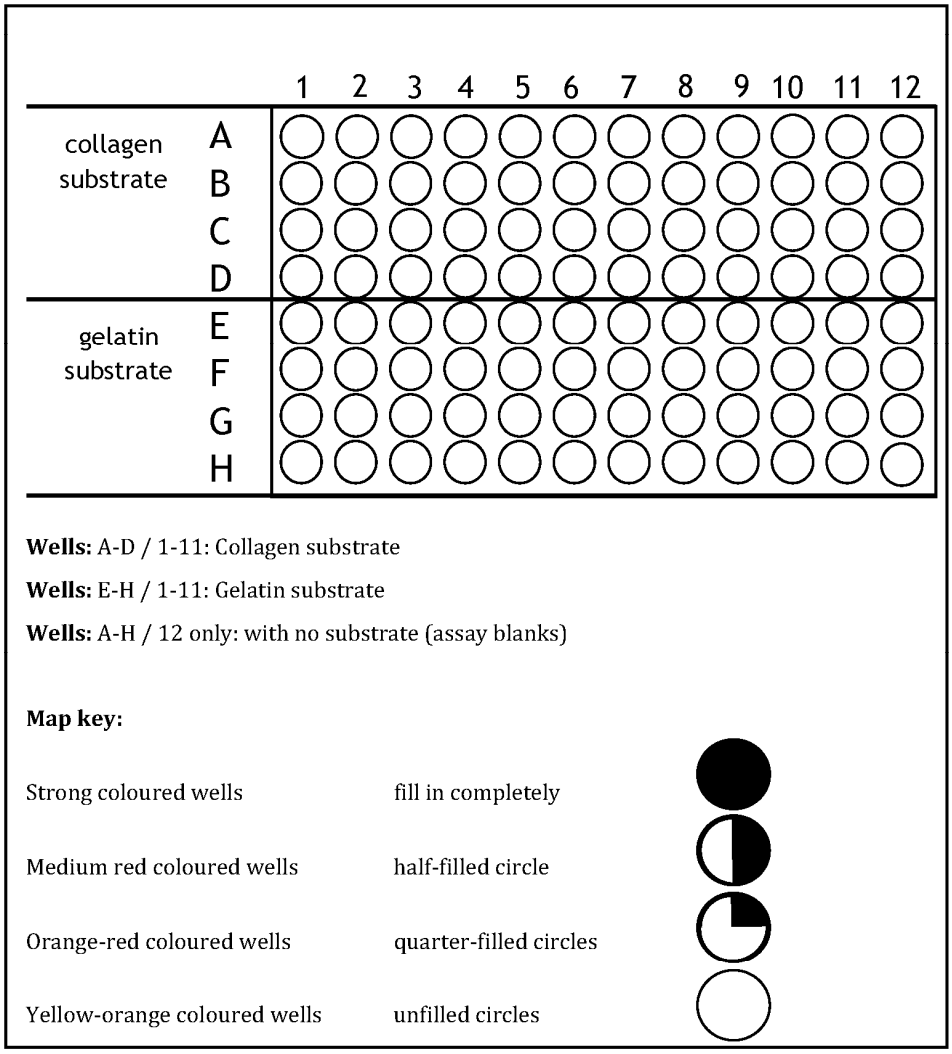


Fig. 4

Design of a microplate assay for protease detection and quantification

Other methods for the measurement of collagen:

Where the analyst has been using other, indirect, methods for measuring collagen it is useful to run comparison duplicates with the Sircol dye-binding assay. The results from earlier work will then be comparable with planned future work.

The collagen standards used in the previous method and the collagen standard supplied in the Sircol assay kit should be measured by both methods. In case the test material contains any interfering substance(s), a selected number of test samples (with and without added collagen) should also be assayed by both methods; with as small a time difference between the two assay methods as is practical.

Likewise method comparisons should be obtained, where necessary, to be able to compare collagen assayed by the Sircol dye-binding method with earlier published work that has used amino acid analysis, hydroxyproline determinations or dpm measurements of H^3/C^{14} labelled proline.

The initial effort reuired in running these correlation trials will be rewarded by the substantial savings in time, labour and cost upon subsequent conversion to the Sircol collagen assay.

A major advantage of the Sircol assay is that it can be carried out in a 'non-biochemical' laboratory. The correlation of the Sircol collagen dye-binding assay with the hydroxyproline method is shown in Fig. 5.

Collagen measurement by hydroxyproline determination:

Many methods and modifications for the determination of hydroxyproline have been published. The procedure can be both difficult and unreliable.

The assay procedure can be subdivided into four steps:

[1] The collagen containing sample is placed into a glass ampoule, and sufficient concentrated HCl added to provide a final acid concentration of 6 M. The ampoules are sealed by melting the glass neck in a hot flame.
The sealed ampoules, including ampoules containing known amounts of collagen and hydroxyproline standards, are placed in a heating block, or a sand bath and maintained at 110ºC for 18 hours.

[2] Remove and allow cooling, before opening the ampoules. All the protein that had been present, including collagen, has been digested to a mixture of free amino acids. The excess HCl needs to be removed, either by titrating with strong NaOH (add a drop of methyl red) or by evaporation of the contents to dryness.

The former method produces a high concentration of NaCl which may interfere in the subsequent steps. The drying method requires a fan extracted fume cupboard and heat/time to drive off the HCl vapour. Some of the residues formed can be difficult to redissolve. Where the test material contained much carbohydrate or glycoprotein a brown/black residue can form that interferes in subsequent steps.

[3] Aliquots of the hydolysed protein, containing an estimated hydroxyproline concentration of between 1 and 5 mg, are added to labelled glass stoppered tubes (~15 x 150mm).

Standards, collagen digests, hydroxyproline standards (hydrolysed treated and untreated) and reagent blanks are similarly treated.

To all tubes add 1ml of a freshly prepared solution of Chloramine-T (0.05 M) [the Chloramine-T should be of analytical quality and the solid sealed and stored from moisture/light]. Stopper all tubes, mix and leave for 10 minutes.

To all tubes add 1 ml *p*-dimethylaminobenzaldehyde (20%) cap tubes and ensure good mixing of this dense addition with the tube contents.

Transfer to a 60ºC water bath and maintain at this temperature for 20 minutes, then remove and allow cooling to room temperature.

Measure absorbance at 560 nm within one hour.

Plot standard curve and determine the hydroxyproline content of the test samples, within the linear range of the curve. Test samples below 1 mg, or above 5 mg, will require either concentration or dilution before being re-assayed.

[4] Conversion of hydroxyproline concentration to collagen concentration:

Collagens are frequently cited to contain 14% hydroxyproline by weight; this value is based on mammalian type I collagens, other collagens will contain more or less hydroxyproline. Foetal collagens can contain much less hydroxyproline, as indeed do many non-mammalian collagens.

The 14% hydroxyproline value should therefore be used as an estimate for comparison with the actual value obtained from the test standard collagen; which should be the same species and type as that present in the test samples.

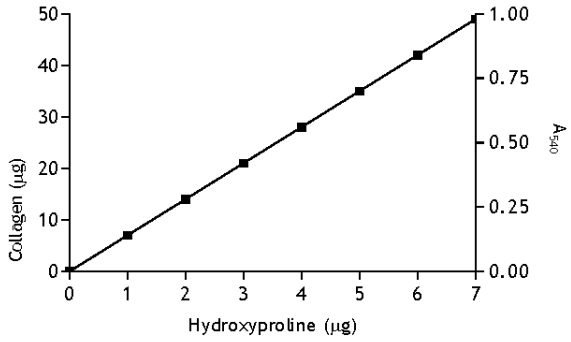


Fig. 5

Assay correlation;

Sircol dye binding versus hydroxyproline. (bovine skin collagen)

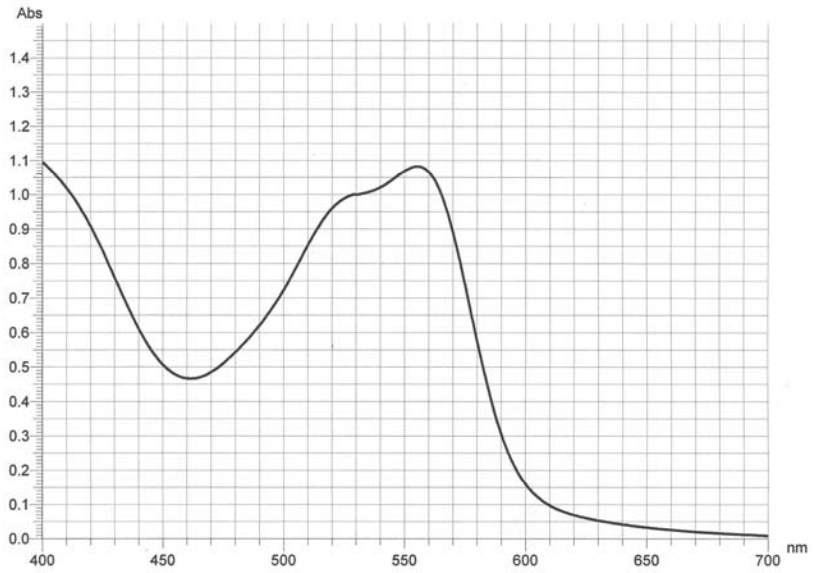


Fig. 6

Absorption spectrum for the Sircol Dye in Alkali Reagent

Abs max @ 555nm

COLLAGEN SOURCE REFERENCES

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Sircol™

Collagen

Assay Kit

Options

Standard Assay Kit ***Product Code:*** **S1000**

Components of the assay kit: **(120 assays)**

Sircol Dye Reagent 120 ml

Alkali Reagent 120 ml

Salt-soluble collagen Precipitating Reagent 15 ml

Collagen [Type I] 5ml

1mg/ml standard in 0.5 M acetic acid,

(Bovine; produced from USA disease free animals)

Sircol Assay Manual 24 pages

(also available to download from our website)

Assay Large Ecomony Pack ***Product Code:*** **S5000**

Components of this assay kit, as S1000 **(475 assays)**

Supplementary Unit Packs

Sircol Dye Reagent only **[3 x 120 ml]**

Product Code: **S1005**

Collagen Standard **[3 x 5 ml ampoules]**

Product Code: **S1010**

Sterile bovine acid-soluble collagen; [Type I]

Concentration: 1 mg/ml in 0.5M acetic acid.

Collagen Standard **[3 x 5 ml ampoules]**

Product Code: **S1011**

Sterile rat tail acid-soluble collagen; [Type I]

Concentration: 0.5 mg/ml in 0.5 M acetic acid.

Other Assays available from Biocolor

APOPercentage Apoptosis Assay

For mammalian cells that have a conventional phospholipid composition membrane. Assay is not recommended for non-mammalian cells and is not suitable for neural cells. Requires an inverted microscope, (magn. x 100), for detection and a microplate reader for measurement. Digital microphotograph analysis option using Adobe Photoshop.

Assay sensitivity; single apoptotic cell ***Assay run time; 1 hour***

Fastin Elastin Assay

Suitable for *in-situ* tissue insoluble cross-linked elastin as found in elastic cartilages and in soft tissues, such as the aorta and skin. Elastin produced during *in-vitro* cell culture. Hot oxalic acid extraction is required prior to analysis to bring the elastin into solution.

Assay sensitivity; 5ug ***Assay run time; 4 hours***

Blyscan Sulfated Glycosaminoglycans Assay

For analysis of sulfated glycosaminoglycan components of proteoglycans including decorin, biglycan, fibromodulin, aggrecan, syndecan, betaglycan. ***Galactosaminoglycans:*** chondroitin sulfates, dermatan sulfate. ***Glucosaminoglycans:*** heparan sulfate, heparin, keratan sulfate. Test material; cell culture medium, amniotic fluid, urine, synovial fluid and tissue extracts.

Assay sensitivity; 0.5ug ***Assay run time; 1 hour***

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Output ที่ได้จากโครงการ

นอกเหนือจากองค์ความรู้เกี่ยวกับโปรตีนกาวไหมและการแปรรูปโปรตีนกาวไหมให้เป็นแผ่นฟิล์มและผ่านกระบวนการเชื่อมโยงข้ามจนได้แผ่นฟิล์มที่เหมาะสมแล้ว ผลจากการศึกษาวิจัย คณะผู้วิจัยได้เผยแพร่ผลงานที่ได้ในวารสารต่าง ๆ ทั้งในประเทศและระดับนานาชาติดังนี้

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BMC Nephrology

RESEARCH ARTICLE Open Access

Sericin cream reduces pruritus in hemodialysis patients: a randomized, double-blind, placebo-controlled experimental study

Pornanong Aramwit[1*], Orathai Keongamaroon[1], Tippawan Siritientong[1], Nipaporn Bang[1] and Ouppatham Supasyndh[2]

Abstract

Background: Uremic pruritus (UP) is a significant complication in ESRD patients and substantially impairs their quality of life. UP is considered to be a skin manifestation of chronic inflammation. Because sericin can suppress the release of pro-inflammatory cytokines, the purpose of this study was to investigate the short-term safety and efficacy of sericin cream for treating UP in hemodialysis patients.

Methods: This study used a double-blind design to investigate the effects of random topical administration of sericin cream and cream base (placebo) on either the right or left extremities of hemodialysis patients for 6 weeks. Skin hydration, irritation and pigmentation were evaluated every 2 weeks using Skin Diagnostic SD27. The visual analog scale for itching was also evaluated every 2 weeks, and the Kidney Disease Quality of Life Short Form was performed on the day of each patient's enrollment and after 6 weeks of treatment.

Results: Fifty dialysis patients were enrolled, 47 of which completed the study. The hydration of the skin of the patients' extremities increased significantly after administration of sericin cream; significant differences were found between sericin treatment and control after 6 weeks of treatment ($p = 0.041$ for arms and $p = 0.022$ for legs, respectively). Moreover, a significant difference was also found in skin irritation between the two treatments ($p = 0.013$ for arms and $p = 0.027$ for legs, respectively). At the end of the study, the skin pigmentation level was significantly reduced on both the arms ($p = 0.032$) and legs ($p = 0.021$) of the sericin-treated side compared with the side treated with cream base. The mean itching score decreased significantly from moderate to severe at the time of enrollment to mild pruritus after 6 weeks of treatment ($p = 0.002$). A better quality of life was found in all domains tested although statistically significant differences before and after treatment was found only in the patients' pain scores, the effect of kidney disease on daily life, sleep quality and symptoms or problems related to kidney disease.

Conclusions: We conclude that sericin cream has a high potential for reducing UP in hemodialysis patients. The trial registration number of this study is ISRCTN16019033; its public title is "sericin cream reduces pruritus in hemodialysis patients".

Keywords: Pruritus, Hemodialysis, Sericin, Hydration, Inflammation, Pigmentation

* Correspondence: aramwit@gmail.com
[1]Bioactive Resources for Innovative Clinical Applications Research Unit and Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
Full list of author information is available at the end of the article

Background

Itching associated with end-stage renal disease (ESRD) or uremic pruritus (UP) affects between 20 and 50% of renal failure patients [1-3] who have no primary skin disease or systemic or psychological dysfunction that might cause pruritus. In addition, approximately 80% of patients undergoing hemodialysis were found to be affected by UP [4-6]. Unfortunately, dialysis has only a slight impact on pruritus [7].

Pruritus is an unpleasant symptom that negatively impacts a patient's quality of life. The impact of moderate/severe uremic pruritus on the mortality of patients with ESRD seems to be associated with sleep disturbance rather than with uremic pruritus *per se* [8,9]. Moreover, in the recent Dialysis Outcomes and Practice Patterns Study II (DOPPS II) trial, pruritus was associated with depression, sleep disturbance and increased mortality risk [8,10]. The pathophysiology of UP is still unclear; many hypotheses have been proposed to explain its occurrence, including xerosis and hypohidrosis (a condition in which the skin is usually atrophic and dry), the presence of pruritogenic cytokines (histamine, kallikrein, interleukin [IL]-2, acetylcholine and other substances that are released by histamine-mediated mast cell stimulation and may lower the UP threshold), secondary hyperparathyroidism, immune-inflammatory reactions in which sericin cream is thought to play a central role in producing an anti-inflammatory effect, the uremic neuropathy hypothesis and the opioid hypothesis (k-opioid receptor understimulation and overexpression of μ-opioid receptors) [7,8].

Sericin, a biopolymer with a high molecular weight, is a water-soluble protein that is obtained from the silkworm (*Bombyx mori*) [11]. Sericin is characterized by the presence of 32% serine, which is the main amino acid of the natural moisture factor (NMF) in human skin; therefore, sericin has excellent moisturizing properties that may be helpful for treating hypohidrosis. Sericin also shows many biological activities and has been widely studied for potential use in medicines and biomaterials [12-16]. In 2009, Aramwit *et al.* showed that sericin significantly decreased the levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in sericin-treated wounds in rats 7 days after an injury compared with the levels found in normal saline-soaked wounds and cream base-treated wounds [17]. As previously mentioned, the immune-inflammatory hypothesis considers UP a dermatologic manifestation of chronic inflammation and treats the condition as a possible result of derangements in the immune system that are based on a pro-inflammatory pattern. Based on this reasoning, sericin may help to relieve UP.

At present, there is no widely accepted treatment for UP. Topical products, such as moisturizers and emollients, are typically used to alleviate symptoms. Because sericin can suppress the release of pro-inflammatory cytokines, the present study was designed to investigate the short-term safety and efficacy of sericin cream for treating UP in ESRD patients. The quality of life of ESRD patients after using sericin cream was also evaluated.

Results

Molecular weight and amino acid composition of sericin

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of sericin prepared by the high-temperature and high-pressure degumming technique showed continuous bands with molecular weights ranging from 50–150 kDa. The highest-intensity band had an apparent molecular weight of approximately 100 kDa. The amino acid composition of sericin extracted with this method is given in Table 1.

Study population

Fifty hemodialysis patients with ESRD were enrolled in this study; however, only 47 subjects completed the treatment (3 patients were withdrawn due to relocation). Thirty of the 47 subjects were female (63.83%), and 17 were male (36.17%); the mean age was 49.6 ± 11.2 years. The average duration of dialysis was 24.6 ± 3.1 months. Table 2 shows the characteristics and biochemical parameters of the study population. Most of the biochemical parameters, such as calcium (9.87 ± 1.32 mg/dL), phosphorus (4.35 ± 1.02 mg/dL), albumin (3.96 ± 0.62 g/dL),

Table 1 Amino acid composition of sericin

Amino acid	Amount (%)
Serine (Ser)	33.63
Aspartic acid (Asp)	15.64
Glycine (Gly)	15.03
Threonine (Thr)	8.16
Glutamic acid (Glu)	4.61
Alanine (Ala)	4.10
Tyrosine (Tyr)	3.45
Methionine (Met)	3.39
Valine (Val)	2.88
Arginine (Arg)	2.87
Lysine (Lys)	2.35
Histidine (His)	1.06
Leucine (Leu)	1.00
Isoleucine (Ile)	0.56
Proline (Pro)	0.54
Cysteine (Cys)	0.44
Phenylalanine (Phe)	0.28

These mean values were obtained by triplicate analysis.

Table 2 Baseline characteristics of subjects (N = 47)

	Characteristics		
Gender (M:F)	17:30		
Age (years)	49.6 ± 11.2		
Dialysis duration (months)	24.6 ± 3.1		
Hematocrit (%)	30.12 ± 3.55		
BUN (mg/dL)	57.89 ± 12.65		
Creatinine (mg/dL)	10.98 ± 2.25		
Calcium (mg/dL)	9.87 ± 1.32		
Phosphorus (mg/dL)	4.35 ± 1.02		
Albumin (g/dL)	3.96 ± 0.62		
ALT (IU/L)	14.23 ± 5.98		
AST (IU/L)	15.32 ± 5.12		
Alkaline phosphatase (IU/L)	96.58 ± 40.32		
Total bilirubin (mg/dL)	0.32 ± 0.11		
Itching (scale 1–10)	7.05 ± 2.17		
Skin parameters	**Sericin cream treatment**	**Cream base treatment**	***p*-value**
Skin hydration			
Arm	28.67 ± 7.11	27.55 ± 7.84	0.819
	(13.90-44.90)	(13.36-44.34)	
Leg	25.10 ± 7.67	23.29 ± 7.37	0.982
	(13.98-49.70)	(7.74-38.88)	
Skin irritation			
Arm	409.34 ± 232.09	318.50 ± 484.26	0.892
	(177.60-1,441.00)	(217.00-2,558.40)	
Leg	364.74 ± 120.20	308.22 ± 116.14	0.857
	(177.00-685.40)	(158.80-818.20)	
Skin pigmentation			
Arm	373.56 ± 142.32	360.35 ± 249.29	0.834
	(155.60-729.80)	(145.80-1,400.00)	
Leg	435.94 ± 178.06	402.25 ± 154.53	0.901
	(199.00-1,182.80)	(196.00-811.00)	

BUN = blood urea nitrogen, ALT = alanine transaminase, AST = aspartate aminotransferase.

total bilirubin (0.32 ± 0.11 mg/dL) and liver enzyme (alanine transaminase (ALT) 14.23 ± 5.98 IU/L, aspartate aminotransferase (AST) 15.32 ± 5.12 IU/L and alkaline phosphatase 96.58 ± 40.32 IU/L) levels, were within the normal range. The baseline characteristics of each parameter, including skin hydration, skin irritation and skin pigmentation on the right and left extremities, showed no significant differences ($p = 0.819$ and 0.982 for skin hydration on arms and legs, $p = 0.892$ and 0.857 for skin irritation on arms and legs, $p = 0.834$ and 0.901 for skin pigmentation on arms and legs, respectively). The average itching score of the subjects using the visual analogue scale (VAS) at baseline was 7.05, which was considered moderate to severe (VAS < 4.0 is considered to reflect mild pruritus, while VAS 4.0 - 6.9 indicates moderate pruritus, and VAS > 7.0 is considered to indicate severe pruritus [10]). At baseline, both the arms and legs of the subjects showed some degree of skin irritation. None of the subjects reported any allergy or dermatological symptoms caused by the sericin or the cream base.

The level of skin hydration in the patients' extremities increased after treatment with either sericin or cream base. The same patients received sericin and placebo (cream base) treatment and that application of each compound were confined to one side of the body. On the sericin-treated side of the body, the skin hydration of the arms was 28.67 ± 7.11 at baseline, and it increased to 33.62 ± 6.93 after treatment for 6 weeks ($p = 0.047$), while the skin hydration of the legs, which was 25.10 ±

7.67 at baseline, increased to 29.05 ± 7.74 after 6 weeks of treatment ($p = 0.025$). On the cream-base-treated side of the body, the skin hydration of the arms was 27.55 ± 7.84 at baseline, and it increased to 29.40 ± 4.92 after treatment for 6 weeks ($p = 0.593$); the skin hydration of the legs, which was 23.29 ± 7.37 at baseline, changed to 26.02 ± 6.47 after 6 weeks of treatment ($p = 0.276$). The skin hydration changes were significantly higher on the side that received the sericin cream than on the side that received the cream base, and a significant difference between the sericin cream and cream base treatments was found in the level of skin hydration in both the arms and legs during the sixth week of treatment ($p = 0.041$ for arms and $p = 0.022$ for legs, respectively). Table 3 shows the skin parameters for hydration (measured by the Corneometer) and for irritation and pigmentation (measured by the Mexameter) of the subjects' extremities at weeks 2, 4 and 6 after treatment. Figures 1 and 2 illustrate the percent changes in the parameters of the skin that received the sericin cream or the cream base during weeks 2–6 compared to the baseline. Six weeks after treatment, the average level of skin hydration on the side of the body that received the sericin cream was significantly increased compared to the baseline ($p = 0.047$ for arms and $p = 0.025$ for legs), while the side that received the cream base showed no significant difference ($p = 0.593$ for arms and $p = 0.276$ for legs) in skin hydration. The arms of the patients who were treated with the sericin cream showed significant differences in the level of skin hydration four weeks after the treatment compared to baseline ($p = 0.022$).

Skin irritation was measured based on the redness of the skin; both the sericin cream and the cream base reduced the level of skin irritation throughout the study period. As shown in Table 3, the arms and legs that were treated with sericin cream showed statistically significantly reduced irritation or redness from the second week after treatment until the end of the study compared to the baseline ($p = 0.031$ for arms and $p = 0.040$ for legs). The degree of redness of the legs of the patients who were treated with the cream base during weeks 4 ($p = 0.048$) and week 6 ($p = 0.036$) was also significantly reduced compared to the baseline. In addition, a significant difference between treatment with sericin cream and cream base was found in the level of skin irritation of both arms ($p = 0.013$) and legs ($p = 0.027$) after 6 weeks of treatment.

The use of sericin cream significantly reduced the darkness of the skin on both the arms and legs of the patients, as shown in Table 3. In addition, in the sixth week of treatment, there was a significant difference in the level of skin pigmentation in the arms and legs treated with the sericin cream compared to those treated with the cream base (Figures 1 and 2, $p = 0.032$ for arms and $p = 0.021$ for legs, respectively). The cream base showed no statistically significant effect on the level of skin pigmentation, and no significant difference compared to baseline was found in the skin pigmentation of the patients' arms and legs after use of the cream base for 6 weeks ($p = 0.082$ for arms and $p = 0.067$ for legs, respectively).

The mean VAS scores for itching at baseline and after 2, 4 and 6 weeks of treatment are shown in Figure 3; the scores shown are based on the overall symptoms without differentiating between the sericin cream and cream base treatments. The mean pruritus score gradually decreased from the beginning of the study and with increasing weeks of treatment. The mean pruritus score at baseline was 7.05 ± 2.17 (range 1–10, median 8), which is indicates moderate to severe pruritus [10,18]; the mean score decreased to 2.23 ± 1.73 (range 0–6, median 2), indicating mild pruritus [10,18], after 6 weeks of treatment ($p = 0.008$).

Table 4 shows the mean score assigned to each domain of the Kidney Disease Quality of Life Short Form (KDQOL-SF) by the hemodialysis patients on the

Table 3 Skin parameters of hydration, irritation and pigmentation on the subjects' extremities after treatment

Parameters	Baseline		2nd Week		4th Week		6th Week	
	SS cream	Cream base	SS cream	Cream base	SS cream	Cream base	SS cream	Cream base
Hydration								
Arm	28.67±7.11	27.55±7.84	29.59±6.07	28.00±5.73	32.41±7.10*	29.36±6.01	33.62±6.93*	29.40±4.92
Leg	25.10±7.67	23.29±7.37	25.72±5.74	24.16±5.95	27.24±6.23	26.43±5.90	29.05±7.74*	26.02±6.47
Irritation								
Arm	409.34±232.09	318.50±484.26	283.85±77.33*	302.17±74.87	276.53±66.67*	286.29±77.26	260.71±69.30*	291.24±82.68
Leg	364.74±120.20	308.22±116.14	295.59±75.21*	283.82±75.04	267.93±78.03*	255.42±74.78*	261.54±79.77*	256.46±69.06*
Pigmentation								
Arm	373.56±142.32	360.35±249.29	308.09±108.37*	342.73±130.47	302.62±111.02*	349.71±132.26	285.42±116.99*	338.88±115.54
Leg	435.94±178.06	402.25±154.53	374.51±131.43*	386.38±141.47	347.51±139.57*	378.88±115.54	320.72±142.50*	375.03±133.28

* indicates a significant difference compared with the same treatment at baseline ($p < 0.05$).
SS = silk sericin.

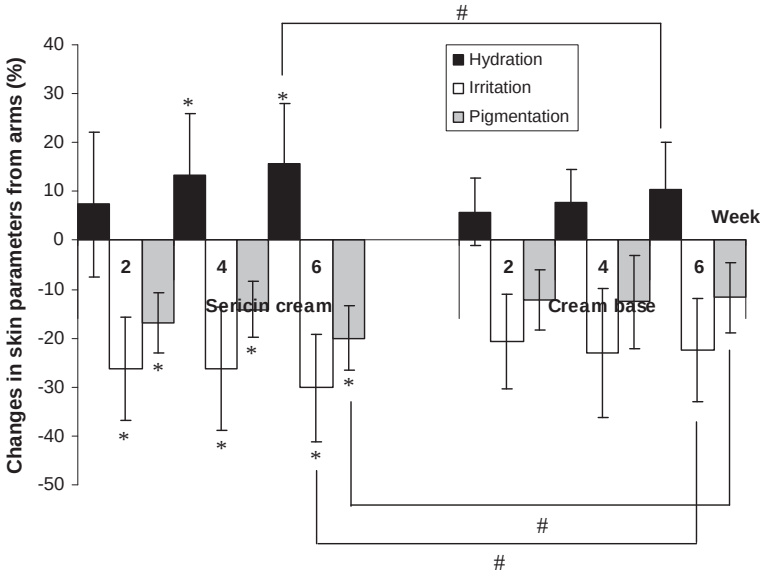

Figure 1 Changes in skin parameters of hydration, irritation and pigmentation on the arms. Detailed legend: The changes in the skin parameters of hydration (measured by Corneometer), irritation and pigmentation (measured by Mexameter) on patients' arms treated with sericin cream or with cream base at weeks 2, 4 and 6 of treatment compared to baseline (* indicates significant differences compared to baseline; # indicates significant differences between treatments) ($p < 0.05$).

enrollment day and after 6 weeks of treatment. As above, the quality-of-life score was based on the patients' overall symptoms without differentiating between the sericin cream and cream base treatments. On the enrollment day, the mean scores ranged from 43.05 for general health to 83.84 for the domain related to dialysis staff encouragement. After 6 weeks of treatment, the mean scores ranged from 44.21 for kidney disease burden to 87.50 for encouragement by the dialysis staff. We found a better quality of life in all the measured domains, including sleep and mood/emotional distress, after the treatment period. When the mean score on the enrollment day was compared with the mean score on the day after completion of treatment, significant

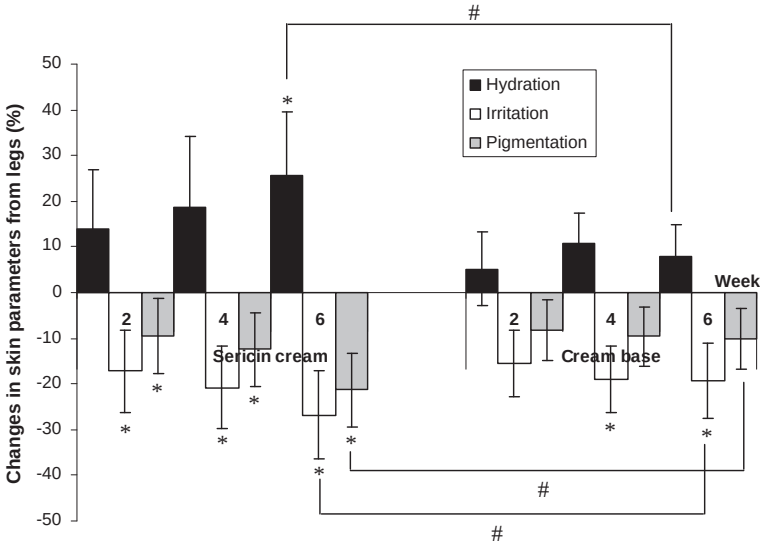

Figure 2 Changes in skin parameters of hydration and irritation and pigmentation on the legs. Detailed legend: The changes in the skin parameters of hydration (measured by Corneometer), irritation and pigmentation (measured by Mexameter) on patients' legs treated with sericin cream or cream base at weeks 2, 4 and 6 of treatment compared to baseline (* indicates significant differences compared to baseline; # indicates significant differences between treatment) ($p < 0.05$).

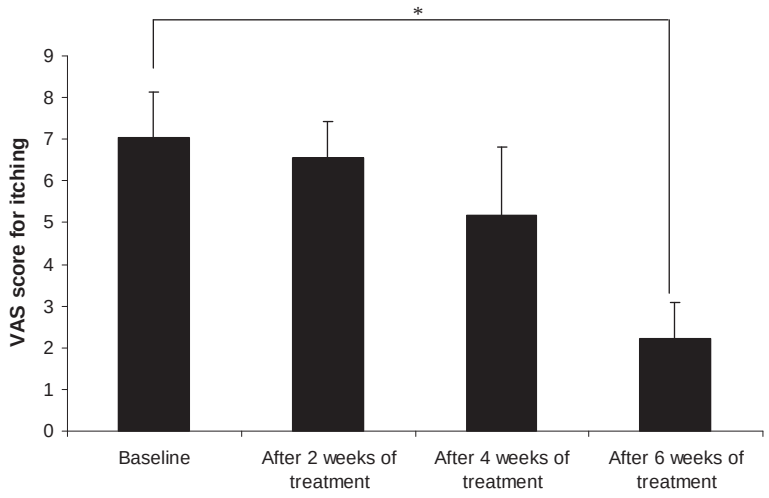

Figure 3 The mean VAS score for itching. Detailed legend: The mean VAS score for itching at baseline and after 2, 4 and 6 weeks of treatment (* indicates significant difference, $p < 0.05$).

differences were found in some domains, including pain ($p = 0.001$), the symptoms/problems list in kidney disease ($p = 0.000$), the effect of kidney disease on daily life ($p = 0.008$) and sleep, the most relevant parameter for itching ($p = 0.014$). The overall score increased from 60.00 at the time of enrollment to 61.95 after 6 weeks of treatment, although this difference was not statistically significant ($p = 0.091$).

Discussion

This study has shown that the use of sericin cream can reduce UP in hemodialysis patients according to their VAS scores. Sericin cream can also significantly increase skin hydration and reduce skin irritation and skin pigmentation in patients. The molecular weight analysis of the sericin used in this study gave results that agree with those reported by Sprague, who indicated that sericin is a mixture of at least 15 different polypeptide chains ranging in size from 20–220 kDa [19]. Similar to the earlier report by Kato *et al.*, serine was the most abundant amino acid found in sericin [20].

Although antihistamines, anti-allergic agents and topical corticosteroids are commonly used to treat UP in ESRD patients, their effectiveness is sometimes limited [2,8,21,22]. Alternative and effective treatments for intractable pruritus that generate low levels of adverse reactions, particularly in sensitive patients such as the ESRD group, must therefore be developed. This clinical study is the first to suggest that a sericin cream is beneficial for treating pruritus in hemodialysis patients. The benefits of this treatment apparently result from increases in skin hydration and suppression of pro-inflammatory cytokines, as shown in our earlier study [17,23], thereby resulting in less skin irritation without any allergic reactions.

Pruritus associated with chronic kidney disease has been shown to be associated with elevated levels of C-reactive protein and other inflammatory cytokines. This evidence suggests that there is an inflammatory component in this form of pruritus [24]. Some authors consider UP to be a skin manifestation of chronic inflammation [25]. Recently, the "persistent microinflammation" theory, in which it is suggested that inflammation may be related to the genesis of UP, was proposed [26]. The skin of ESRD patients with UP contains an increased number of mast cells, and these cells can release various substances such as histamine, interleukins (IL) and tumor necrosis factor (TNF) [27-30].

Pruritic skin can appear normal except for dryness; because the severity of pruritus is closely correlated with skin dryness [31], xerosis treatment normally begins with a topical agent such as a gentle moisturizing cream. Because sericin is well known for its moisturizing effect and its ability to reduce the generation of pro-inflammatory cytokines by fibroblasts, this study assessed the usefulness of sericin cream in treating UP. Evaluating the degree of pruritus was difficult because it is a subjective symptom; therefore, functional measurements of skin conditions known to be associated with pruritus, such as skin hydration and skin irritation, were considered to be objective indices. We found that both sericin cream and the cream base used to prepare the sericin cream increased the moisture content of the stratum corneum; however, the level of hydration was significantly higher in the skin treated with sericin cream. The side of the body treated with sericin cream had significantly lower levels of post-treatment dryness compared to pretreatment dryness and lower total dermatological severity scores in parameters such as skin pigmentation.

Table 4 Mean scores for each domain of the KDQOL-SF in hemodialysis patients on the enrollment day and after 6 weeks of treatment (N = 47)

	Enrollment Day	After treatment for 6 weeks
Physical component score (PCS)		
Physical functioning	73.90 ± 18.99	77.93 ± 18.57
Role limitations-physical	50.00 ± 42.20	51.83 ± 44.16
Pain	48.29 ± 16.96	74.27 ± 17.36*
General health	43.05 ± 24.08	47.32 ± 22.17
Mental component score (MCS)		
Energy/fatigue	50.73 ± 18.93	56.59 ± 17.62
Social function	69.82 ± 20.53	78.35 ± 22.54
Role limitations-emotional	56.10 ± 42.45	61.79 ± 41.87
Emotional well-being	61.17 ± 15.60	64.98 ± 16.51
Kidney disease component score (KDCS)		
Symptoms/problems list	64.33 ± 9.98	78.51 ± 10.44*
Effect of kidney disease on daily life	59.91 ± 20.46	68.22 ± 16.33*
Burden of kidney disease	43.45 ± 29.38	44.21 ± 25.29
Cognitive function	65.53 ± 18.25	69.27 ± 19.43
Work status	60.98 ± 41.10	68.29 ± 39.93
Sexual function	71.74 ± 33.12	75.00 ± 23.62
Quality of social interaction	70.08 ± 18.80	76.91 ± 16.06
Sleep	43.41 ± 22.08	52.74 ± 18.22*
Social support	70.33 ± 22.52	76.02 ± 25.01
Dialysis staff encouragement	83.84 ± 13.76	87.50 ± 12.18
Patient satisfaction	70.73 ± 20.34	78.05 ± 17.65
Overall score	60.00 ± 21.68	61.95 ± 19.90

* Indicates significant differences compared to baseline, $p < 0.05$.

The pathogenesis of ESRD itself is associated with inflammation and oxidative stress, as reflected in studies of plasma biochemistry, including the levels of the cytokines IL and TNF-α [32-34], classic markers of inflammatory processes that are strongly affected by pathological conditions [35]. These cytokines are upregulated in the plasma of ESRD patients [32,36]. The skin inflammation associated with the release of IL and TNF-α is further complicated by the inflammatory lesions that occur secondary to scratching [37]. Our results indicate that improvement in skin hydration and suppression of the release of pro-inflammatory cytokines in skin might be the mechanism by which sericin improves UP. Skin irritation, which can be caused by either skin inflammation or by scratching, was substantially reduced in the sericin group compared with the cream base group after 6 weeks of treatment. This result confirmed that sericin reduces skin irritation in patients with UP and suggested that it might be due to suppression of the inflammatory cytokines in skin. Similar results have been obtained in animal studies [23].

Secondary skin lesions that can result from the itch-scratch cycle include excoriations, hyperpigmentation, lichenification, prurigo nodules and scars [38]. Chronic renal failure is usually accompanied by a variety of cutaneous manifestations; dermal manifestations, including hyperpigmentation, have also been reported, and these can exact a considerable toll on the quality of life [39-41]. Although skin pigmentation is not related to specific clinical symptoms, it has an important effect on patient satisfaction. Sericin has been reported to have activity against tyrosinase [15], an enzyme related to melanin production; as this study shows, treatment with sericin results in significant reduction in the skin color of patients.

The pruritus/mortality relationship may be strongly attributed to sleep disturbances, as previously mentioned in the DOPPS [8,9,42]. Many previous studies have found a strong association between inflammation, as measured by C-reactive protein or inflammatory cytokines, and sleep disturbances in dialysis patients [43-45]. The significant effects of pruritus on sleep, mood and social functioning require further investigation with the goal of improving the available treatments for this serious ESRD complication. We found that reduction in itching intensity from moderate to severe pruritus at the time of enrollment to mild pruritus after 6 weeks of treatment was associated with a better quality of life in all of the measured domains, including the mental, physical and kidney disease components. The KDQOL-SF score in the ESRD patients indicated improvements in several domains regarding the patients' quality of life, particularly pain and sleep, which are relevant to itching. A better quality of sleep may reflect some degree of relief from itching achieved through the treatments.

This study has certain limitations. First, the study design was an in-subject controlled study; it cannot be determined whether the improvements in the itching evaluation and the quality of life were from the sample (sericin cream) or the placebo (cream base). Moreover, due to the in-subject controlled design, the biochemical parameters could not be evaluated at the end of the study. Second, the study included a small number of patients, and these were followed for a relatively short time. A long-term study with a larger sample size is necessary. In addition to studies of hemodialysis patients, similar studies of other ESRD patients, such as peritoneal dialysis and kidney transplant patients, are also necessary to confirm the findings presented here.

Conclusions

Our study shows that sericin reduces pruritus in patients with UP. The use of sericin cream significantly increased the level of skin hydration after 6 weeks of treatment

compared to baseline and to the use of the cream base. The use of sericin cream also significantly reduced the level of skin irritation and pigmentation after 6 weeks of treatment compared to baseline, while use of the cream base reduced skin pigmentation slightly but not significantly. The results of this study suggest that sericin cream may be a good choice for treating pruritus in hemodialysis patients.

Methods

Preparation of silk sericin cream

Because commercial sericin cream is unavailable, the sericin cream used in this study was prepared from raw materials. *Bombyx mori* cocoons were purchased from Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). The cocoons were extracted with purified water (1 g of dry silk cocoon: 30 mL of water) using a high temperature and pressure degumming technique in an autoclave (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 121°C and 15 psi for 60 min. This technique has been shown to be safe for the preparation of material used on keratinocyte and fibroblast cells and can activate high collagen production related to wound healing [46]. After filtration through a membrane (Whatman filter paper No. 1, Whatman PLC, Kent, United Kingdom) to remove fibroin, sericin powder was obtained by freezing and lyophilizing the sericin solution with a Heto LL3000 lyophilizer (Allrod, Denmark). Petroleum jelly, mineral oil, lanolin, glycerin, bisabolol, triethanolamine stearate, propylparaben and methylparaben were used to formulate a cream base. For an 8% sericin cream, a concentration that has been shown to be safe and effective in the treatment of second-degree burn wounds, the sericin powder was dissolved in warm water and then mixed with the other ingredients during the cream-forming process.

Molecular weight determination of sericin

To determine the molecular weight of sericin, SDS-PAGE was performed as previously described, with some modifications [47]. Briefly, samples were prepared for SDS-PAGE by adding an equal volume of sample buffer (0.25 M Tris–HCl, pH 7.0 containing 4% SDS, 10% sucrose, 10% 2-mercaptoethanol and 0.025% bromophenol blue) to each protein solution. Each sample was then incubated at 98°C for 2–3 min and loaded onto a 5%-20% gradient gel (Atto Corporation, Tokyo, Japan). Electrophoresis was performed in 125 mM Tris base with 0.96 M glycine and 0.5% SDS, and the polypeptide bands were detected using silver staining.

Amino acid analysis of sericin

The amino acid composition of sericin was determined using an amino acid analyzer (Hitachi L-8500A, Tokyo, Japan). Samples for analysis were hydrolyzed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) at 100°C for 24 h under vacuum. The experiments were performed in triplicate.

Study design

Skin hydration may be related to pruritus, and this parameter is sensitive to relative humidity, personal activities and diet. Therefore, an in-subject control using a split-body biometrological assessment (each patient received both treatments but on different sides of the body) was used to evaluate the safety and efficacy of the sericin cream for the treatment of UP in hemodialysis patients. Moreover, the distribution of pruritus between the patients was highly variable whereas the manifestation of mirror symmetry was an attribute they all shared [48]. An in-subject, randomized, double-blind, placebo-controlled experimental study was designed to investigate the effects of the sericin cream versus the cream base (placebo) in reducing the symptoms of UP (itching, dryness and redness) and skin pigmentation in stable maintenance hemodialysis patients. Each of the parameters, including skin hydration, skin irritation, skin pigmentation and itching score, was evaluated at baseline and at 2, 4 and 6 weeks after treatment intervention. The sericin cream and the cream base were identical in texture and scent. All of the products were packaged in containers that were label-free except for the treatment code number, and the packages were identical in shape, size and color; therefore, the treatment assignment remained unknown to the participants, the study investigators and the medical personnel. The subjects were recruited from December 2010 to February 2011, and the study was conducted between March 2011 and December 2011 at the Division of Nephrology, Phramongkutklao Hospital and at Priest Hospital, Thailand. Signed informed consent was obtained from all subjects after a thorough discussion of the protocol, its rationale and the potential risks. This study was approved by the Ethics Committee of the Institute Review Board at Phramongkutklao Hospital, Thailand and ended after the last participant completed the intervention.

Study population

Inclusion criteria

All ESRD patients at Phramongkutklao and Priest Hospitals over 18 years of age who had received hemodialysis for at least 3 months were screened for this study. Having mild to severe pruritus as measured by the VAS during the previous 6 weeks was also an inclusion criterion. The patients were required to refrain from using any antipruritic treatment (oral or topical) for a period of not less than 2 weeks prior to the start of the study. Patients of both genders, regardless of

comorbidities or prescribed medications, were eligible. Any medication that had an antipruritic effect was discontinued 2 weeks before the study. No changes in the patients' prescription medications were required during this study with the exception of a concomitant antipruritic treatment.

Exclusion criteria

Pruritus caused by other skin diseases or medication was excluded by careful clinical assessment. Patients with a history of silk protein allergy, who were allergic to any compounds in the formula, or who had biliary atresia, liver problems, cancer, metabolic disorders or other diseases related to systemic pruritus were also excluded. Patients who had skin problems or rashes on their extremities (arms or legs) were also excluded from this study. Participants left the project when they could not comply with the treatment, when they were unwilling to continue with the study, or when the physician opined that other treatments were needed to relieve the symptoms. After reviewing patient profiles and explaining the protocol, 27 patients were excluded due to liver problems (N = 4), cancer (N = 3), metabolic disorder (N = 7), rashes on their extremities (N = 6) and refusal to participate in the study (N = 7). The remaining patients (N = 50) were enrolled in the study.

Study treatment

In 2004, Okada and Matsumoto [49] evaluated the effect of an emollient containing a high water content on mild uremic pruritus; based on this study, the number of samples needed for a dependent sample was approximately 50 subjects. Split-body biometrological assessments were performed. The physician investigator enrolled the subjects into this study, and using a computer-generated block of four, another investigator generated the random allocation sequence that divided the patients into two groups. The identities of the patients in each group were concealed from both the investigators and the patients. The on-duty nurses assigned the participants to the intervention. The participants, investigators and those assessing the outcomes were blinded after assignment to the interventions. The patients in the first group received the sericin cream on their left extremities (left arm and left leg), while the other side of the body received the cream base. The patients in the second group received both the sericin cream and the cream base, but on opposite sides of the body from the first group. All of the patients were shown how to topically apply the assigned treatment evenly over the area indicated twice daily for a period of 6 weeks after showering.

Measurement and outcome

The level of skin hydration on the arms and legs was assessed using a Corneometer® (KOKO Kosmetikvertrieb GmbH & Co., Leichlingen, Deutschland). The Corneometer® registers the moisture content in the surface layers of the skin as deep as 10–20 μm; the presence of capillary blood vessels and superficial skin fat do not influence this measurement. Skin irritation or erythema (measured by the redness of the skin) and skin pigmentation (measured by the melanin content) were assessed using a Mexameter linked to a Skin Diagnostic SD27 (Courage + Khazaka electronic GmbH, Köln, Germany). The measurement of melanin and the erythema readings are based on a light source with three specific wavelengths; the radiation is absorbed by the skin and reflected diffusely. A photodetector was used to analyze the diffuse reflection from the skin. The same measuring probe is used to quantify the skin redness (erythema) and to determine the skin pigmentation or the degree of skin darkness (melanin). The irritating effects of substances and the soothing effects of active agents can also be recorded by the investigator. Skin hydration, irritation and pigmentation values have no units because they are computer- generated based on the different dielectric constants of water (80.10 at 20°C) and other substances (typically < 7). The measuring capacitor shows changes of capacitance according to the moisture content of the samples (for hydration) and skin color (for irritation and pigmentation). Each parameter was measured at least three times in the same randomized area at patients' extremities, and the mean value was used for the analysis. During the study, the patients were advised to consume similar types and amounts of food and beverages. Activities such as longer exposure to the sunlight and traveling were to be avoided to reduce any confounding factors. The percent changes in each parameter were calculated by subtracting the baseline score from the post-treatment scores at weeks 2, 4 and 6 according to the following equation:

$$\%\text{changes in each parameter} = [(P_t - P_0)/P_0] \times 100,$$

where P_0 is the value of each parameter at baseline (at the time of enrollment) and P_t is the value of each parameter during the follow-up period (2, 4 or 6 weeks). All of the measurements were performed in triplicate.

Because itching is a systemic symptom and likely to be generalized, most patients could not identify whether the itching occurred primarily on the right or left side of the body; therefore, itching was scored as an overall symptom. The severity of itching was systemically assessed on both the arms and legs of all patients using VAS on the enrollment day and every 2 weeks after treatment began. We used a VAS that consisted of a 10-cm

horizontal line with no scale markings. The patients were asked to mark the intensity of their itching on the scale, with the strongest possible level of itching or unbearable pruritus marked on the right end of the line (10 cm) and no itching marked on the left end (0 cm) [50].

Patients' quality of life

The patients' quality of life was assessed using the Thai version of the KDQOL-SF Version 1.3 [51]. Quality of life was evaluated on the enrollment day and after 6 weeks of treatment. The mean scores for the individual domain scores and for the three composite summary scores, which include the mental component score (MCS), the physical component score (PCS) and the kidney disease component score (KDCS), were compared as shown in Table 4. For the Hayes algorithm [52], the raw data obtained from the patients were first transformed into a pre-coded numeric value of 0–100; a higher transformed score reflected a better quality of life [51,53].

Safety monitoring

The occurrence of allergic reactions during treatment with sericin cream was regularly evaluated by two dermatologists during each visit. The Naranjo algorithm was used to determine the likelihood of whether an adverse drug reaction was actually caused by the sericin cream or by other factors.

Statistical analysis

The results are expressed as the mean ± SD unless otherwise indicated. Statistical analysis was performed using SPSS version 10.0 (SPSS Inc., Chicago, Illinois, USA). A bidirectional α-level of significance was set at $p = 0.05$ for all of the measurements. From the baseline to weeks 2, 4 and 6, the VAS score changes and the levels of skin hydration, irritation and pigmentation were computed for each patient within the treatment group using a repeated measure analysis of variance (ANOVA). The paired t-test was used to analyze changes in the patients' quality of life between baseline and 6 weeks after treatment. The differences in each parameter for the patients receiving the sericin cream and the cream base were compared at each time point using Student's t-test.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PA had full access to all of the data in the study, accepts responsibility for the integrity of the data and affirms that everyone who contributed significantly to the work has been listed. PA and OS developed the clinical study design and the experimental design. OK, TS and NB recruited the subjects and conducted the skin evaluation, patient consent, analysis and data interpretation. PA drafted the manuscript. All authors read the manuscript, provided critical input and approved the final version.

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Author details

[1]Bioactive Resources for Innovative Clinical Applications Research Unit and Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand. [2]Division of Nephrology, Department of Medicine, Phramongkutklao Hospital and College of Medicine, Bangkok, Thailand.

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A Novel Silk Sericin/Poly (Vinyl Alcohol) Composite Film Crosslinked with Genipin: Fabrication and Characterization for Tissue Engineering Applications

T. Siritientong[1,a], P. Aramwit[1,b,*]

[1] Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

[a]tippawan.siri@gmail.com, [b]aramwit@gmail.com

Keywords: Silk sericin, Poly vinyl alcohol, Film, Genipin

Abstract. Silk sericin, a gumming protein from silk cocoons, has been a considerable natural protein-based biopolymer for fabrication of desired constructs for potential tissue engineering applications. This study investigated the formulation of a novel biopolymeric silk sericin/poly (vinyl alcohol) film with genipin as crosslinking agent and its physical properties. Silk sericin itself forms a fragile material, adding other polymers such as poly (vinyl alcohol) and glycerin, a plasticizer, resulting in a strong and flexible matrix. The results indicated that at higher concentration of genipin (0.1% w/v), the percentages of crosslinking in sericin/poly (vinyl alcohol) films was significantly higher. The matrices also exhibited higher tensile modulus value and higher elasticity at higher genipin concentration which can be inferred to higher integrity of the structure compared to matrices with genipin at low concentration (0.01% w/v). On the other hand, the reverse patterns were found in percentages of light transmission and the releasing profile of sericin from the composite films. Adding genipin into the matrices resulted in a lower percentage of light transmission indicated the increase in opacity. The releasing profile of sericin from the films showed that high genipin concentrations reduced the peak of protein released and trended to provide the sustain-released profile of protein. These findings indicated that silk sericin film can be formed and the concentrations of crosslinking agents really affect its physical properties.

Introduction

Silk sericin, a glue-like protein from silk cocoon from the silkworm *Bombyx mori*, composes of a wide range of beneficial properties that make it attractive for various applications. It is now used as a main cosmetic ingredient due to its antioxidation and moisturizing effect [1]. Silk sericin has been found to possess wound healing properties through enhancing collagen synthesis and skin fibroblast attachment without causing any inflammation [2]. From these benefits, silk sericin can be used as a biomaterial in various forms including film. However, a cast film from intact sericin is very fragile and unable to handle due to its brittleness; thus, other polymers need to be added in order to solve this problem. Polyvinyl alcohol (PVA), a synthetic polymer with excellent film-forming properties and glycerin, a well-known plasticizer, are able to blend with silk sericin to obtain a stable composite film [3,4]. In addition, the crosslinking of polymers, both physical and chemical, constitutes a useful method for the improvement or modification of the physicochemical properties of biopolymeric materials. The obvious limitation of physical crosslinking processes such as ultraviolet treatment or dehydrothermal treatment is low degree of crosslinking due to the crosslinking process occurring only at the surface of the material whereas chemical crosslinking methods can be reacted with all the bulk of the matrix [5]. Genipin is a natural crosslinking agent extracted from the gardenia fruit that overcomes the toxic characteristic in commonly used synthetic crosslinker, glutaraldehyde [6]. Recent studies have used genipin as a crosslinking agent with gelatin [6], resulting in deep blue-colored materials with desirable properties. The objective of this study was to investigate the physical properties of silk sericin/PVA composite film crosslinked with genipin at various concentrations to explore a novel biopolymeric material for tissue engineering applications.

Material and Methods

Preparation of Silk Sericin/PVA Composite Film: The cocoons of silkworm (Chul Thai Agro-Industries Co. Ltd., Thailand) were mixed with purified water and autoclaved at 121°C for 60 minutes and then filtered to provide silk sericin solution. The extracted sericin solution was mixed with PVA and glycerin. Genipin (Wako, Japan) at various concentrations ranged from 0.01-0.10%w/v was added into the mixture to obtain the unique blue-colored solution. It was casted onto the dishes and left at the room temperature overnight followed by drying at 50°C for 24 hours.

Degree of Crosslinking: Following reaction with 0.5% trinitrobenzene sulfonic acid (TNBS) and 4% $NaHCO_3$, the film was heated at 40°C for 2 h. 12.24 N HCl was added and then heated at 70°C for 2 h. The absorbance of the solutions was determined at 415 nm. This property was calculated by [7]:

$$\text{Degree of crosslinking (\%)} = \frac{1 - \text{Absorbance of crosslinked film}}{\text{Absorbance of uncrosslinked film}} \times 100$$

Determination of Mechanical Properties: Tensile modulus and elongation at break of the film were determined by the Universal Testing Machine (Hounsfield H10KM, UK) equipped with a 10 N load cell at 25°C. at a constant cross-head speed of 30 mm/min.

Determination of Light Transmission: Percentages of light transmission of the films were measured in wavelength between 400 and 800 nm at 40 nm intervals using UV/Vis spectrophotometer (PerkinElmer Ltd., Germany).

Release of Sericin from the Composite Films: The known-weigh samples were placed into PBS (pH 7.4) at room temperature with continuous stirring in a closed-container. The fractions of the solution were collected at curtain intervals and immediately determined the amount of protein using a BCA Protein Assay Reagent (Pierce, USA).

Results and Discussions

Genipin is a natural crosslinking agent that reveals remarkable reaction with primary amine group of amino acids such as lysine and arginine of certain proteins which can be found in silk sericin [8,9]. The degree of crosslinking of silk sericin/PVA composite film has been calculated by the relative difference between the absorbance values of uncrosslinked and crosslinked samples after primary amino groups reacted with TNBS, a UV chromophore. The higher in the differences means the higher percent of free amino groups lost after crosslinking with genipin. Table 1 shows the degree of crosslinking in the film with various concentrations of genipin from 0.01 to 0.1% w/v. The result indicates that the degree of crosslinking significantly increases at higher concentration of genipin.

Table 1. Percentages of crosslinking in silk sericin/PVA composite film with various concentrations of genipin compared with the film without genipin, expressed in mean ± standard deviation.

Genipin concentration [%w/v]	Percentages of crosslinking [%]
0.010	13.84 ± 2.59
0.025	18.82 ± 4.00
0.050	22.64 ± 1.89
0.075	29.75 ± 6.46
0.100	38.97 ± 4.51

Mechanical Properties. To be used as a biomaterial, the mechanical properties of the composite film are important. The integrity and elasticity of the structures should be suitable enough to be handle and easy to apply. Genipin concentration above 0.05% w/v provokes a significant increase in

the tensile modulus and elongation at break as shown in Table 2. The results indicate that genipin-crosslinked films are more elastic, more integrity, and have better mechanical properties compared to uncrosslinked one, similar to the results found by others [8].

Table 2 Tensile modulus and elongation at break of silk sericin/PVA composite film with various concentrations of genipin, expressed in mean ± standard deviation

Genipin concentration [%w/v]	Tensile modulus [N/mm^2]	Elongation [%]
0.000	4.97 ± 0.59	188.54 ± 55.22
0.010	4.66 ± 0.72	156.50 ± 31.39
0.025	5.82 ± 0.91	198.68 ± 55.64
0.050	7.14 ± 0.57	309.60 ± 63.36
0.075	8.17 ± 2.15	331.98 ± 55.02
0.100	9.17 ± 1.37	361.98 ± 53.85

Percentages of Light Transmission. Crosslinking significantly decreases the percentages of light transmission, even at the low genipin concentration. With higher genipin composition, it can visually notice the dark blue-colored of the film compared to the uncrosslinked transparent yellow one. Figure 1 demonstrates the extent of light transmission decreases when genipin concentration increases. The previous study reported that the dark blue appearance was associated with the oxygen radical-induced polymerization of genipin as well as its reaction with amino groups in protein [10].

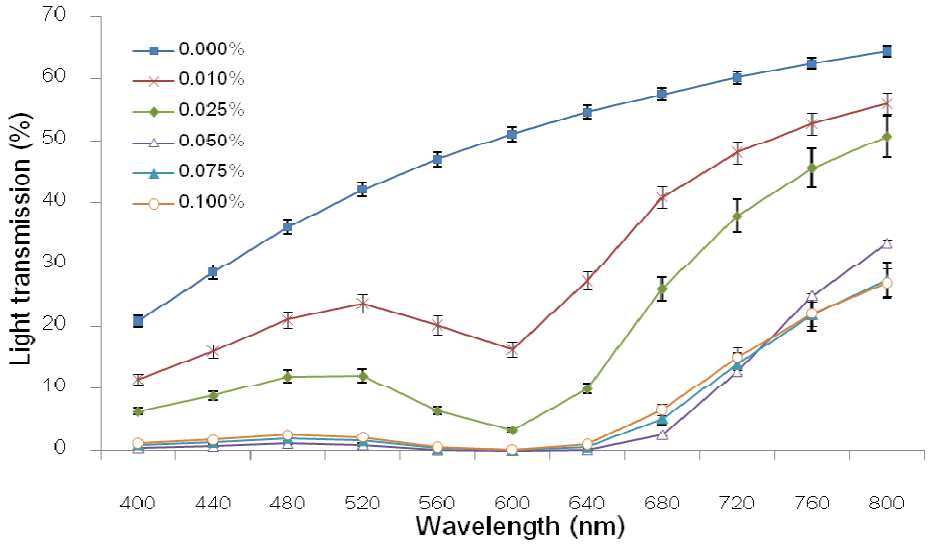

Fig.1. Percentages of light transmission of silk sericin/PVA composite film with various concentrations of genipin

Release of Sericin. The amount of sericin released from the composite film with various concentrations of genipin is shown in Figure 2. The maximum of protein released from all samples were obtained at about 8 hours. The uncrosslinked film control leached out the highest amount of sericin similar to that of 0.01% w/v genipin concentration. However, the observable reduction of the peak of protein released came together with higher genipin concentration which indicated the higher degree of crosslinking. Moreover, at 0.1% w/v genipin concentration, the fraction of protein release trended to provide the sustain-released profile which may be appropriate for further applications since no fluctuation and longer duration of protein released.

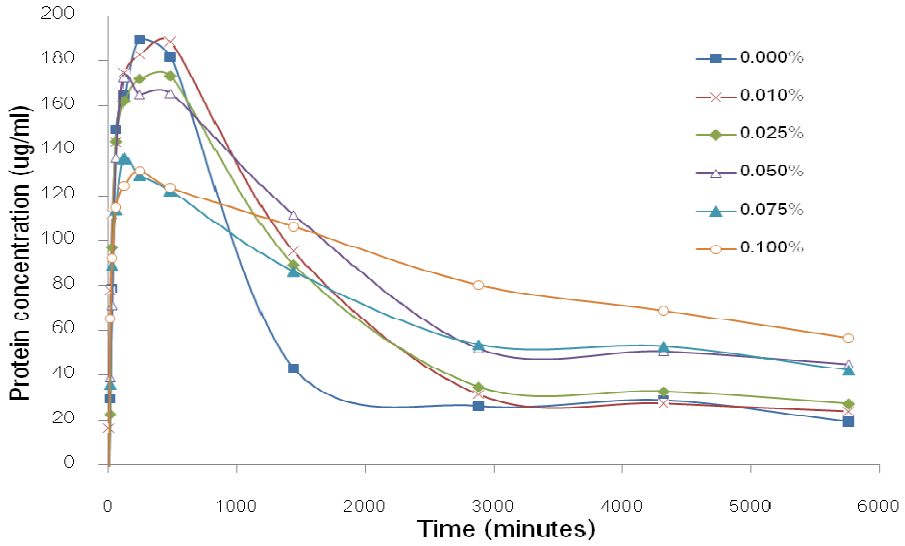

Fig.2. Amount of protein released from the silk sericin/PVA composite film with various concentrations of genipin

Conclusions

Stable sericin/PVA film has been obtained by crosslinking with genipin. The increase in genipin concentration from 0.01% to 0.1% w/v significantly increases in degree of crosslinking which significantly improves the physical properties as the significance increase in tensile modulus and elongation a break of the films. The advantage in higher stability of the matrix is beneficial in practical applications of these composite films as a biomaterial. Compared with the transparent yellow uncrosslinked sericin/PVA film, the genipin crosslinked composite film becomes dark blue colored and reduces in the percentages of light transmission as genipin composition increased. Moreover, the peak of sericin released from the scaffold is lower in genipin-crosslinked composite film and shows the sustain-released profile which is a desired characteristic in tissue engineering application. The results obtained in this study indicate that genipin can be a considerably effective naturally occurring crosslinking agent to improve the properties of the silk sericin/PVA composite film as a biopolymeric material for biomedical applications.

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Potential applications of silk sericin, a natural protein from textile industry by-products

Pornanong Aramwit[1], Tippawan Siritientong[1] and Teerapol Srichana[2]

Abstract

Silk is composed of two major proteins, fibroin (fibrous protein) and sericin (globular, gumming protein). Fibroin has been used in textile manufacturing and for several biomaterial applications, whereas sericin is considered a waste material in the textile industry. Sericin has recently been found to activate the proliferation of several cell-lines and has also shown various biological activities. Sericin can form a gel by itself; however, after mixing with other polymers and cross-linking it can form a film or a scaffold with good characteristics that can be used in the cosmetic and pharmaceutical industries. Sericin is proven to cause no immunological responses, which has resulted in a more acceptable material for biological applications.

Keywords

Biomaterials, immunological responses, protein cross-linking, silk sericin

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Silk protein and its use

Silk derived from the silkworm *Bombyx mori* is composed of two major proteins, fibroin and sericin. Fibroin is a fibrous protein, present as a delicate twin thread linked by disulfide bonds, enveloped by successive sticky layers of sericin that help in the formation of a cocoon. Sericin or silk glue is a globular protein (Ki et al., 2007; Poza et al., 2002; Wu et al., 2007), that constitutes 25 to 30% of silk proteins. It consists of 18 amino acids (Takasu et al., 2002b), most of which have strong polar side chains such as hydroxyl, carboxyl and amino groups (Zhang, 2002). Its high hydrophilicity arises from the high content of serine and aspartic acid, approximately 33.4 and 16.7% of sericin, respectively (Tsubouchi et al., 2005; Zhang, 2002; Zhaorigetu et al., 2001).

Silk fibres made from fibroin have many uses in textiles, medical and industrial applications, mainly because of its unique properties such as water absorbency, dying affinity, thermo-tolerance, lustre and insulation properties. It is also a raw material for producing precious fabrics, parachutes, tyre lining materials, artificial blood vessels and surgical sutures (Mondal et al. 2007). To manufacture lustrous silk from the dried cocoons of silkworm, fibroin is separated from sericin – the other major component of the cocoon – by a degumming process and the sericin is mostly discarded in the wastewater. It is estimated that out of the 1 million tons (fresh weight) of cocoon production worldwide, or about 400 000 tons of dry cocoon, approximately 50 000 tons of sericin could be recovered from the waste solution (Kim, 2007; Zhang, 2002). Sericin is at present an unutilized by-product of the textile industry and the discarded degumming wastewater also ultimately leads to environmental contamination due to the high oxygen demand for its degradation by microbes (Fabiani et al., 1996). It has been reported that sericin waste solution in Thailand has a biochemical oxygen demand value of 4840 mg L^{-1}, a chemical oxygen demand of 8870 mg L^{-1} and nitrogen content (0.11%). If sericin was recovered, perhaps it could be used as a 'value added' product for many sericin-derived products and purposes (Vaithanomsat and Kitpreechavanich, 2008) and this would also be beneficial in terms of the economy and the environment.

[1]Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
[2]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla, Thailand

Corresponding author: Pornanong Aramwit, Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
Email: aramwit@gmail.com

Silk sericin structure and extraction methods

Polarization microscopy shows that silk sericin forms three layers surrounding a fibroin fibre and sericin can be fractionated into at least four components (Tokutake, 1980). Seventy percent of the total amino acid content in sericin is mainly of polar amino acids, especially serine and aspartic acid. A circular dichroism spectrum and infrared absorption spectrum show that the molecular configuration of sericin is mainly random crimp (Kweon et al., 2000; Sheng et al., 2000). X-ray diffraction analysis and differential thermal analysis indicate that the assembled structure of sericin powder is an amorphous structure (Sheng et al., 2000), however, the amorphous sericin transforms into a β-structure in the presence of water (Takasu et al., 2002a).

The secondary structure of sericin varies depending on the ways in which it is prepared (Kim, 2007). It can remain in a partially unfolded state, with 35% β-sheet and 63% random coil, with no α-helix content (Tsukada and Bertholon, 1981). A sericin solution turns into a gel state after a period of time and can return to a solution upon heating. During the gelation of a sericin solution, certain amounts of random coil are changed into a β-sheet. This sol–gel state transition is one characteristic of sericin that is different to fibroin (Kim, 2007). The gelation of sericin was first investigated in 1994 by Zhu et al. (1995). They found that gelation was rapid at a low temperature of 10°C and at low pH, approximately 6–7. The strength of the sericin gel increased, whereas the surface tension decreased. Kweon et al. (2000) reported that the gelation time of sericin decreased with increasing poloxamer concentration because the hydrophilic parts of the poloxamer absorb the water surrounding sericin.

There are several methods to remove sericin in the so-called degumming process of cocoons. Degumming by heat or heat under pressure has an advantage because it results in no impurity. However, this is not usually applied in the silk industries because the treatment is too long, and causes significant fibroin damage. Extraction under pressure is difficult to control, and using acids for sericin removal is relatively hazardous compared with using an alkaline solution. Almost all industrial removal methods now involve, extraction with soaps and detergents is now the most widely used industrial technique since it causes no fibre degradation and is a relatively simple process compared to its removal by enzymes, heat or pressure (Freddi et al., 2003). However, this process makes it very difficult to gain high quality sericin for further studies or applications. Alkali, soaps and detergent impurities need to be first removed before further applications.

As sericin is normally present in silk industry wastewater, the recovery of sericin is also an important environmental issue to be investigated. Wu et al. (2007) found that ethanol can successfully precipitate sericin from silk wastewater and the yield was gradually enhanced as the ethanol concentration was increased. However, this technique does not sound economically and environmental friendly when applied on an industrial scale. Other techniques have been applied for sericin recovery such as enzymatic hydrolysis (Vaithanomsat and Kitpreechavanich, 2008), freeze- and tray-drying (Vaithanomsat and Kitpreechavanich, 2008) and membrane filtration (Capar et al., 2008; Fabiani et al., 1996; Vaithanomsat and Kitpreechavanich, 2008). Membrane filtration may sound to have more advantages compared with other techniques since it could provide an opportunity to separate sericin from other impurities. Moreover, it offers the advantages of concentrating sericin which would decrease its loss in a post-precipitation step (Caper et al., 2009). However, more than one technique may be required for certain sericin applications such as cosmetics, manufacturing silk materials requires both ultrafiltration and enzymatic hydrolysis (Vaithanomsat and Kitpreechavanich, 2008) whereas a single nanofiltration is required for biomaterial applications (Caper et al., 2008). Hence, the silk sericin recovery technique may need to be handled individually in order to determine the most suitable process for each of its possible applications.

Sericin is a mixture of proteins with different molecular properties. It exists in a wide range of molecular weights, from 10 to over 400 kDa (Kato et al., 1998; Takasu et al., 2002b), depending on the extraction methods, temperature, pH, and processing time (Freddi et al., 2003; Vaithanomsat and Kitpreechavanich, 2008; Zhang, 2002). Figure 1 represents the schematic of a degumming process to isolate fibroin and sericin from silk cocoons. Sericin extracted by different methods can even provide different amino acid compositions (Aramwit et al., 2010a). Heat and acid extraction give sericin a molecular weight of from 35–150 kDa, whereas sericin extracted by alkaline solution has a molecular weight of from 15–75 kDa. Sericin extracted by heat, acid and alkaline solution normally show broad, disperse bands using sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, whereas sericin extracted using urea is the only method to provide distinct bands between 10 to >225 kDa. Sericin with a low molecular weight, commonly less than 20 kDa, is soluble in cold water and can be recovered during the early stages of raw silk production, whereas higher molecular weight sericin is soluble in hot water and can be obtained from the later stages.

Biological activities of silk sericin

Even though sericin has received much less attention than fibroin mainly due to sericin being a multi-component protein with an indefinite structure, sericin itself exhibits several biological activities and has proven to be a biocompatible agent. Serine-rich sequences in sericin, responsible, together with aspartic acid, for its hydrophilicity, are sensitive to

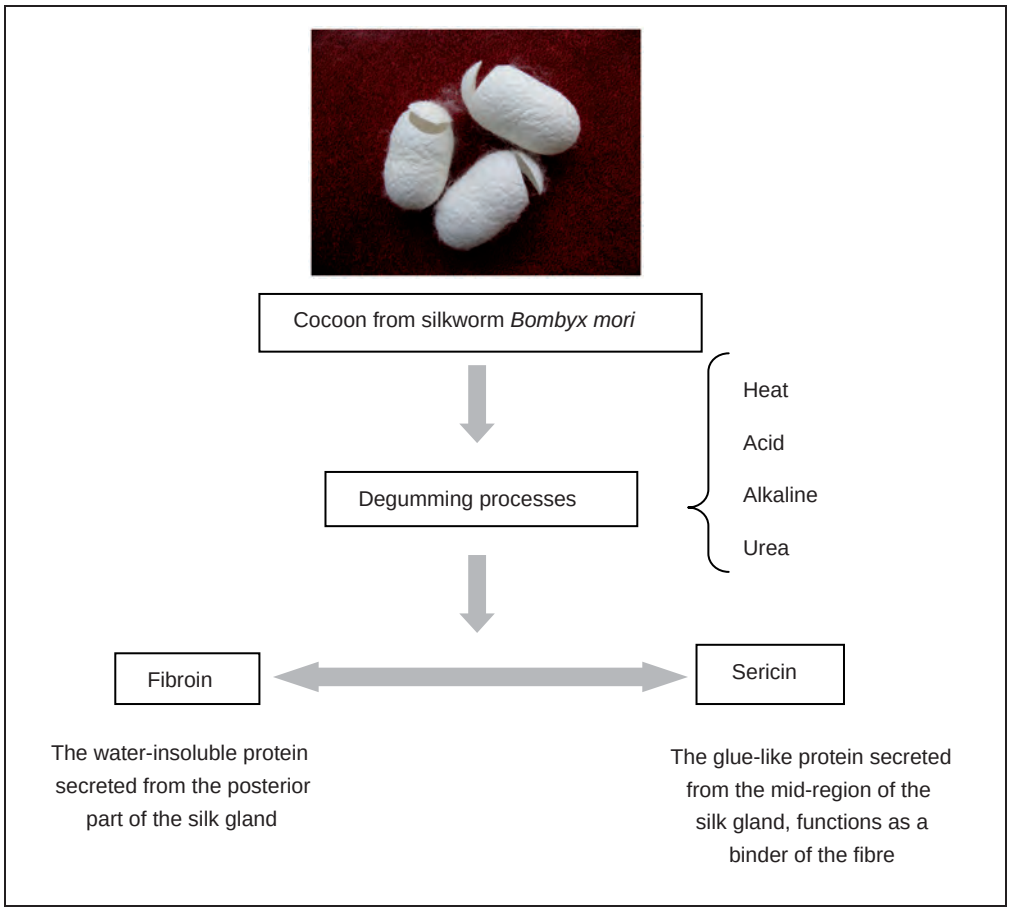

Figure 1. The schematic of a degumming process to isolate fibroin and sericin from silk worm cocoons.

chemical modifications. Because of its unique biochemical and biophysical properties, sericin has been studied for various potential applications. These characteristics include biocompatibility, biodegradability, antibiotic-antibacterial activity, antioxidant behaviour, anti-tyrosinase activity, anticarcinogenic effects, UV protective properties, and coagulant and moisturizing capabilities in cosmetic preparations for example in the cosmetics industry (Aramwit et al., 2010a; Kato et al., 1998; Tamada et al., 2004; Zhaorigetu et al., 2001, 2003).

Due to its high content of serine and glycine, sericin can be used as a moisturizer in the cosmetics industry (Kato et al., 1998). Sericin gel has shown moisturizing properties, has prevented transepidermal water loss and restored natural moisturizing factors (Voegeli et al., 1993). It also enhances the elasticity of the skin and has anti-wrinkle and anti-aging effects via its collagen promoting activity (Aramwit and Sangcakul, 2007; Ogawa and Yamada, 1999; Voegeli et al., 1993, Yamada et al., 1998). Since sericin shows more hydrophilicity than fibroin and can activate collagen synthesis in dermal tissue, it was used to form a scaffold and be a good candidate for tissue engineering applications such as neural tissue engineering or skin substitution (Hsieh et al., 2007; Mandal et al., 2009; Ren et al., 2009) while fibroin, which has higher tensile strength than sericin is normally used to form scaffolds for bone or vascular tissue engineering (Park et al., 2010; Zhou et al., 2010).

In the biomedical field, sericin has been shown to be a promising biomaterial for cell culture because it promotes cell proliferation in a serum-free medium (Table 1) (Ogawa et al., 2004; Takahashi et al., 2003; Terada et al., 2005; Tsubouchi et al., 2005). Terada et al. (2005) showed that sericin-S, with a molecular weight ranging from 5–100 kDa, effectively improved serum-free mammalian cell cultures. It was also found to enhance the attachment of cultured human skin fibroblasts (Tsubouchi et al., 2005). In 2005, Tsubouchi et al. also reported that living fibroblast cells increased to 250% of the control after 72 h. It was, therefore, considered for a role in the healing process of skin lesions (Tsubouchi et al., 2005). However, the extraction methods and optimum sericin concentrations for promoting cell proliferation need to be optimized (Aramwit et al., 2010b). Sericin obtained by heat extraction produced the highest cell viability compared with that obtained by acid, alkali or urea extraction. Moreover, sericin at a low concentration ($<10\,\mu g\,mL^{-1}$) seemed to work better to promote cell proliferation and increase cell viability. Sericin at a concentration higher than $100\,\mu g\,mL^{-1}$ shows toxicity to cells, especially sericin obtained by the urea extraction method. Ogawa et al. used sericin as a supplement to culture rat islets for the treatment of diabetes. The results indicated that sericin accelerated the proliferation of the rat insulinoma cell line RIN-5F (Ogawa

Table 1. Study in silk sericin properties for biomedical applications

Comments	Authors
Improve serum-free mammalian cell culture	Terada et al. (2005)
Enhance culture human skin fibroblast attachment and play an important role in the wound healing process	Tsuboushi et al. (2005)
Supply the rat islets culture for treatment of diabetes	Ogawa et al. (2004)
Enhance wound healing by sericin cream	Aramwit et al. (2007)
Suppress UVB-induced and chemical-induced acute damage and tumor promotion in mouse skin	Zhaorigetu et al. (2003)

et al., 2004). The serum-free cell culture has overcome one of the disadvantages of the conventional cell culture method. Although serum is an effective factor for cell growth, it is frequently contaminated with viruses despite claims that they could be removed by filtration (Freshney, 2000).

Despite its ability to promote cell proliferation, Kato et al. (1998) reported that sericin could suppress *in vitro* lipid peroxidation, and provided the first evidence that it had an antioxidant action. In 2003, sericin was found to inhibit UVB-induced acute cell damage and tumours by reducing oxidative stress on the skin of a hairless mouse (Zhaorigetu et al., 2003). It was further found to inhibit tyrosinase activity, and suggestions were made for its possible applications in the food and cosmetics industries (Aramwit et al., 2010a; Kato et al., 1998). Sericin obtained by different extraction methods and from different silk strains can inhibit tyrosinase activity differently (Aramwit et al., 2010a). Coloured silk cocoons, which contain flavonoids and carotenoids, exhibit higher anti-tyrosinase activity than white-shelled cocoons. Consumption of sericin can reduce serum lipids, ameliorate glucose tolerance and elevate serum adiponectin in rats fed with a high-fat diet (Limpeanchob et al., (2010); Okazaki et al., 2010) and it has also shown protective effect against alcohol-mediated liver damage in mice (Li et al., 2008).

Bioconjugation of sericin with polymers has provided new drug delivery methods with reduced immunogenicity and increased drug stability (Mandal and Kundu, 2009). It can be covalently linked to produce conjugates via its –OH, –COOH and NH_2 active groups on its surface (Kundu et al., 2008). The sericin's surface can also be modified by grafting vinyl monomers. Graft modifications have been found to overcome some drawbacks such as its instability in water, poor solubility in organic solvents and low resistance to microbial attack (Nakamura and Hatakeyama, 1984; Wei et al., 1989; Yao et al., 2003).

In 2004, Zhang et al. found that microparticles of silk sericin can act as a protein vector for L-asparaginase immobilization (Zhang et al., 2004). The immobilized enzyme can reduce its immunity, toxicity and has a greatly improved tolerance to proteolysis which allows for its use as an orally administered medicine in animals. Sericin films, forming gels and sponges can also be used in drug delivery systems since it can be produced to exhibit a sustained-release profile for a charged protein–fluorescein isothiocyanate–albumin both *in vitro* and *in vivo* (Nishida et al., 2011).

Biomaterials from silk-sericin

In recent years, several studies have suggested sericin's potential as a wound dressing agent (Aramwit and Sangcakul, 2007; Teramoto et al., 2008; Zhang, 2002). Its hydrophilicity assists to maintain a moist environment and to absorb excess exudates from wounds. Both two-dimensional (films, membranes) and three-dimensional (hydrogel and porous scaffolds) matrices from sericin have been reported. Not only in dressing form, but used as a sericin cream has exhibited good wound healing properties without causing allergic reactions in rats (Aramwit and Sangcakul, 2007). Wounds treated with sericin have shown fewer inflammatory effects and wound size reduction was faster than the control. The surface of the epidermis recovered more quickly to its normal thickness with increased collagen.

Different blending methods have resulted in different properties of films suitable for a variety of applications (Table 2). Teramoto et al. (2008) developed a sericin gel film and characterized it as a wound dressing. A sericin solution was gelled with ethanol, and a film was prepared by drying a sheet-shaped sericin hydrogel. The resulting film was stable against swelling with highly flexible and had good handling properties. It showed no toxicity and had low cell adhesion, which was also a requirement for a wound dressing that would not destroy regenerated tissues when peeled off. The optical transparency of the gel film might be an additional advantage since it allows for the direct observation of wounds. Sericin can also form a film via casting. The main differences between a gel film and a cast film are the preparation methods. A gel film is prepared via gelation, whereas a cast film is prepared directly from the solution, which is further casted on a plastic or glass plate and the film obtained after evaporation of solvent. This dry sericin gel film was brittle and difficult to peel off but it became flexible and was peeled off easily when wetted. In

Table 2. Exploitation of silk sericin properties as a biomaterial

Material	Advantages	Authors
Blending protein with other materials		
Polyurethane; foam	Good moisture absorbing/deabsorbing properties	Nomura et al. (1995)
Polyvinyl alcohol (PVA); film	Highly interface of PVA/sericin complex and excellent fracture strain	Ishikawa et al. (1987)
PVA; hydrogel	Good moisture absorbing/deabsorbing properties	Yoshii et al. (2000)
Improving by the cross-linking of protein		
Dimethylolurea	High strength and water permeability cross-linked sericin membranes	Gimenes et al. (2007)
UV-radiation	High yield of cross-linked sericin in short time	Kim et al. (2002)
Polyethylene glycol diglycidyl ether	Good mechanical properties and cytocompatibility sericin films	Xie et al. (2007)
Genipin	Good mechanical properties and sericin can be released efficiently for promoting collagen production	Aramwit et al. (2010c)

the wet state, its planar dimension remained unchanged but its thickness increased by fivefold. In contrast, when the cast film was wetted, it was difficult to handle, resulting from a large deformation (i.e. the film expanded fourfold in the planar dimension) (Teramoto et al., 2008). These differences arise mainly from the gelation process (Teramoto and Miyazawa, 2005; Teramoto et al., 2005; 2008). Pure sericin is not easily made into a membrane or film that is sufficiently strong and elastic. Membranes and films made from sericin are found to be fragile, especially in the dry stage, probably because of the broad distribution of its molecular weight (Gimenes et al., 2007). Moreover, they swell in aqueous solution and even dissolve at elevated temperatures (Gimenes et al., 2007). To solve these problems, an increase in cohesion between protein polypeptide chains was thought to be effective for the improvement of the barrier properties of the film cross-linking (Sabato et al., 2001). For instance, the cross-linking of proteins by means of chemical or physical treatments was reported to improve its permeability and mechanical properties. Attempts have been made to stabilize native proteins by inducing the formation of hydrogen, electrostatic and covalent bonds (Whitaker, 1977). Blending proteins with other materials, especially synthetic polymers, has also been shown to be an effective approach to improve the properties (Mondal et al., 2007). Environmentally friendly biodegradable polymers can be obtained by blending sericin with other resins. Nomura et al. showed that polyurethane foam incorporating sericin had excellent moisture absorbing/desorbing properties as well as good mechanical and thermal properties (Nomura et al., 1995). The resulting polymers can be further made into films, fibres and moulded objects. They are also inexpensive because they contain a significant amount of waste sericin.

Sericin blends well with water-soluble polymers, especially polyvinyl alcohol (PVA), which is one of the few vinyl polymers that are water-soluble as well as being susceptible to ultimate biodegradation in the presence of suitably adapted micro-organisms. Its ability to produce highly resistant film and its hydrophilic character are accounted for by the improved mechanical properties of natural polymers including sericin (Chiellini et al., 2003). In 1987, Ishikawa et al. (1987) investigated the fine structure and physical properties of a film blend composed of silk sericin and PVA. The film was prepared by mixing extracted sericin with PVA, casting the solution on a plastic plate and drying at room temperature for a day. This film had a microphase-separated structure. The interface between the two phases consisted of a PVA/sericin complex. The resulting film had excellent fracture strain and showed little elongation at elevated temperatures. A sericin/PVA hydrogel is said to have good elasticity and moisture absorbing/desorbing properties that arise from the high hydrophilicity of both sericin and PVA (Yoshii et al., 2000).

Cross-linking silk sericin

In 2007, Gimenes et al. produced a sericin/PVA blend membrane for the pervaporation separation of ethanol/water mixtures (Gimenes et al., 2007). The membranes were prepared by blending sericin and PVA, followed by chemical cross-linking with dimethylolurea (DMU), a common chemical cross-linker (Nagura et al., 2001). It was demonstrated that the blended membranes are preferentially permeable to water and have higher sorption selectivity than membranes made of either sericin or PVA alone. In addition, sericin/PVA

membranes cross-linked chemically and thermally were compared. Heating favours protein cross-linking by disrupting the protein structure and exposing the sulfhydryl and hydrophobic groups, resulting in the formation of disulfide linkages (Gennadios and Weller, 1991; Kinsella et al., 1985). By contrast, DMU achieved cross-linking by condensation reactions with OH groups on adjacent amino acids (Sloan, 1997). The data showed that thermal cross-linking resulted in membranes with a lower selectivity than those cross-linked chemically by DMU. In spite of its benefit, chemical cross-linking can impair the native characteristics of sericin and can cause toxicity due to chemical residues (Teramoto et al., 2008). Genipin, a natural compound extracted from gardenia fruit, can also be successfully cross-linked to sericin to form a scaffold and exhibited good physical properties (Aramwit et al., 2010c). A higher temperature and a longer heating time could increase the degree of cross-linkage, but the membranes become too brittle and hard to retain their integrity. Furthermore, excessive heat causes protein denaturation. Thus, the thermal cross-linking of sericin-containing materials seems to be an inappropriate approach (Gimenes et al., 2007).

Fancy and Kodadek (1999) studied protein cross-linking reactions triggered by visible light. High yields of cross-linked products were obtained in less than a second. With a small amount of cross-linking agent and benzophenone as a photoinitiator, the cross-linking reaction was accomplished within 3 min. By considering these two experiments, the use of long wavelength light might be attractive for sericin cross-linking because the reaction is rapid and few biomolecules absorb light out of the UV region.

Sericin film is not only applicable for wound healing; it is also an edible film that can be used in the food industry as a substitute for traditional plastic films. In the preparation of a sericin film, ethanol is commonly used as a solvent. It is generally acknowledged that alcohol changes the structure of proteins. For sericin, it induces β-sheet formation. Although methanol can also induce structural changes in the same manner, ethanol is less toxic and is beneficial for applications of sericin as a biomaterial (Teramoto et al., 2008). After Mase et al. (2006) developed a new strain of *B. mori* named sericin-hope in 2006, it was widely used in sericin studies because the larvae of sericin-hope produced spin fibres comprised exclusively of sericin, making it possible to obtain intact sericin. This intact sericin forms fibres or gels with better mechanical properties than those made from degraded sericin (Teramoto et al., 2005). This could be used to prepare a cast film and a hydrogel without chemical modification such as cross-linking (Teramoto et al., 2008). Furthermore, the formation of an elastic hydrogel requires intact sericin because its properties are sensitive to the denaturation of sericin (Kundu et al., 2008; Teramoto et al., 2005). However, with the aim of recovering and making use of sericin from degumming waste, blending and cross-linking methods for preparing sericin-contained biomaterials still need further in-depth investigation.

Immunological responses to silk sericin

Even though silk sericin has been shown in many studies to be a good candidate for biomedical and cosmetic applications (Aramwit and Sangcakul, 2007; Kato et al., 1998; Kundu et al., 2008; Modal et al., 2007; Zhang, 2002), there have been some reports on the immune responses to silk proteins (Celedon et al., 2001; Soong and Kenyon, 1984; Zaoming et al., 1996). Soong and Kenyon reported adverse reactions to virgin silk sutures in cataract surgery in 12 patients (Soong and Kenyon, 1984). They showed nodular episcleritis, peripheral corneal ulceration and wound necrosis with dehiscence. In 2003, Panilaitis et al. showed that soluble sericin proteins extracted from native silk fibres did not induce significant macrophage activation (Panilaitis et al., 2003). Later it was found that silk sericin increased the amounts of inflammatory mediators and proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) that are involved in the modulation of skin growth, repair and scarring during inflammation (Aramwit et al., 2009). However, the maximum levels of TNF-α and IL-1β released from monocytes and macrophage cells after silk sericin induction were 500 and 350 $pg\,mL^{-1}$, respectively. These levels of cytokines would not be sufficient to cause an inflammatory response or prevent cellular proliferation (Cosgrove et al., 2008; Khan et al., 2006; Wilusz et al., 2008), and these levels significantly decreased during wound healing. However, the effects will vary due to individual responses from different recipients.

Concluding remarks

The silk sericin protein, an environmentally unfriendly waste product derived from the manufacture of silk fibroin, has been proven to have great potential for many biomedical and biological applications. However, limitations on devising specific applications are caused by its ability to exist in many forms that depend on its method of extraction and purification, etc. Each specific application requires a particular form so it will be necessary to devise and understand how to prepare consistent products suitable for each. Areas that seem worthy for immediate exploration include its ability to replace serum for cell culture, facilitate wound healing and promote collagen synthesis. Its ability to form crosslink or blends with other polymers to produce more effective films that can be used for new drug delivery methods with reduced immunogenicity and increased drug stability or even new food packaging materials also should be further investigated. The future looks very promising but there is a need for a concerted coordinated effort to first understand its myriad

structures and how to control them. One purpose of this review is to try to activate such a process.

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Article

Comparative Clinical Study of Bactigras and Telfa AMD for Skin Graft Donor-Site Dressing

Pornprom Muangman [1], Sooksan Nitimonton [1] and Pornanong Aramwit [2,*]

[1] Burn Unit, Trauma Division, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand; E-Mails: pmuangman@yahoo.com (P.M.); ss79ntmt@yahoo.com (S.N.)

[2] Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

* Author to whom correspondence should be addressed; E-Mail: aramwit@gmail.com; Tel.: +66-089-921-7255; Fax: +66-02-218-8403.

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Abstract: The Bactigras® paraffin tulle coated with chlorhexidine is normally used for the treatment of donor-site wounds in burn patients who received split-thickness skin grafts in several centers. It has some disadvantages, such as adhesion to wound surfaces and pain from the irritation caused by this dressing. The Telfa AMD®, a non-adherent wound dressing which consists of absorbent cotton fibers impregnated with polyhexamethylene biguanide enclosed in a sleeve of thermoplastic polymers, is a new option for donor-site wound care which causes less adherence to the wound. The purpose of this study was to compare clinical efficacy of these two dressings for the management of donor-site wounds. Thirty-two patients who received split-thickness skin grafts by donor site harvesting from the thigh were enrolled in this study and randomized into two groups receiving either the Bactigras® or the Telfa AMD® wound treatment. Re-epithelialization, pain, infection and cost-effectiveness analyses were compared between both groups. The results showed that there was no significant difference in age, area of donor sites or length of hospital stays between the groups ($p > 0.05$). However, the day of re-epithelialization (≥90%) was significantly shorter in patients treated with the Telfa AMD® compared to the Bactigras® group (14.00 ± 3.05 *vs.* 9.25 ± 1.88 days for Bactigras® and Telfa AMD® groups, respectively, $p < 0.001$). The average pain score was also significantly lower in the Telfa AMD® group (1.57 ± 0.55 *vs.* 4.70 ± 1.16, $p < 0.001$). There was no difference in the cost

of treatment between the groups (4.64 ± 1.97 *vs.* 5.72 ± 2.54 USD, p = 0.19). This study indicated that the Telfa AMD® was an effective dressing for the treatment of donor-site wounds.

Keywords: Bactigras; burn; chlorhexidine; donor site wound; polyhexamethylene biguanide; Telfa AMD

1. Introduction

Split-thickness skin grafting is the most frequently used procedure in plastic surgery for the replacement of damaged or missing skin. The success of the procedure depends on the complete integration of the graft with the recipient bed and on the re-epithelialization of the skin graft donor site [1–3]. Treatment of the split-thickness autograft donor sites has been studied over the years but there is no standard treatment for managing these sites. The treatment protocol involves a variety of techniques and dressing materials, and all of them aim for a fast, spontaneous re-epithelialization of the donor sites [4]. Adequate wound treatment aims to prevent or reduce the risk of associated complications and to facilitate the healing process whilst considering the patients' physical and mental well-being during the treatment process [5]. The ideal treatment method protects the wounds from dehydration and mechanical trauma, prevents infection and reduces re-epithelialization time, and provides maximum comfort for the patient [6].

In general, the methods of treating donor wounds are categorized as open, semi-open, and closed [7]. The open method refers to the method where the wound remains exposed and it is allowed to heal without a dressing. The semi-open method means the wound bed is covered with dressing just once and then the wound is allowed to heal by the open method while in the closed method, the wound dressing is left intact for two to seven days. The most common approach is to make multiple dressing changes until the wound is completely healed. Among these techniques, the closed methods meet these requirements for adequate wound treatment to a large extent and have become the most attractive technique over the last decade [8,9]. Paraffin gauze dressing is recognized as a standard treatment for split-thickness skin graft donor sites [10]. It is considered to be non-adherent; nevertheless, it usually sticks to the wound surface while it absorbs exudate. Early removal of the dressing may lead to skin maceration or wound infection and wound epithelialization may slough off, accompanied by local pain aggravation and wound deepening [11].

Chlorhexidine is an antibacterial agent which is effective against a wide range of Gram-positive and Gram-negative bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA). Traditionally, mesh paraffin gauze with chlorhexidine is normally used for the treatment of donor-site wounds. Chlorhexidine can bind to bacterial cell walls at low concentrations, causing an alteration of the bacterial cell osmotic equilibrium and leakage. One of the disadvantages of this traditional gauze includes adherence to wounds, which can cause trauma to epithelial cells when removed. The Telfa AMD®, a non-adherent wound dressing, consists of a thin layer of absorbent cotton fibers impregnated with polyhexamethylene biguanide (PHMB), enclosed in a sleeve of poly (ethylene terephthalate), a thermoplastic polymer, that is perforated in a regular pattern and sealed along two edges [12,13].

Polyhexamethylene biguanide is a polymeric biguanide with a broad antimicrobial spectrum against both Gram-positive and Gram-negative bacteria, fungi and yeasts [14,15]. It has been used for several years as an antiseptic agent in medicine [16]. Polyhexamethylene biguanide binds to the surfaces of organisms causing instability and extensive disruption of their cytoplasmic membranes. It has a low systemic toxicity and poor absorption through skin. Since infection is another factor which retards wound healing, a broad spectrum antimicrobial agent may be beneficial for wound treatment as well as for split-thickness skin graft donor sites.

The purpose of this study was to compare the clinical efficacy of the PHMB-containing wound dressing in the thermoplastic polymer with the paraffin tulle dressing coated with chlorhexidine, the standard treatment for skin graft donor sites in the management of donor-site wounds.

2. Result and Discussion

The alternative dressing materials used for split-thickness skin graft donor sites present differences in healing times, infection and patient comfort. Since all of the dressings possess some unique properties, no ideal dressing is available on the market [17]. The challenge in managing donor site wounds is to promote healing as quickly as possible while minimizing adverse effects and complications [10]. If a complication such as infection occurs, the split-thickness defect may convert into a full-thickness loss, analogous to a third-degree burn [10]. Because of this, material which contains antibacterial agents should be applied; however, it should not hinder wound healing and, in addition, it should preferably have a promoting effect on epidermal healing [18]. In contrast to the great number of studies in which different techniques for the dressing of donor sites were evaluated, our present study compared the efficacy of two occlusive dressings, the Bactigras® dressing and the Telfa AMD®. The Bactigras® dressing is commonly used as a standard protocol of donor site wound dressing at our institute, which is similar to many burn centers using paraffin tulles coated with chlorhexidine [10]. The Telfa AMD® is commercially available as a non-adherence dressing with antimicrobial agents, which may have advantages in terms of patient comfort.

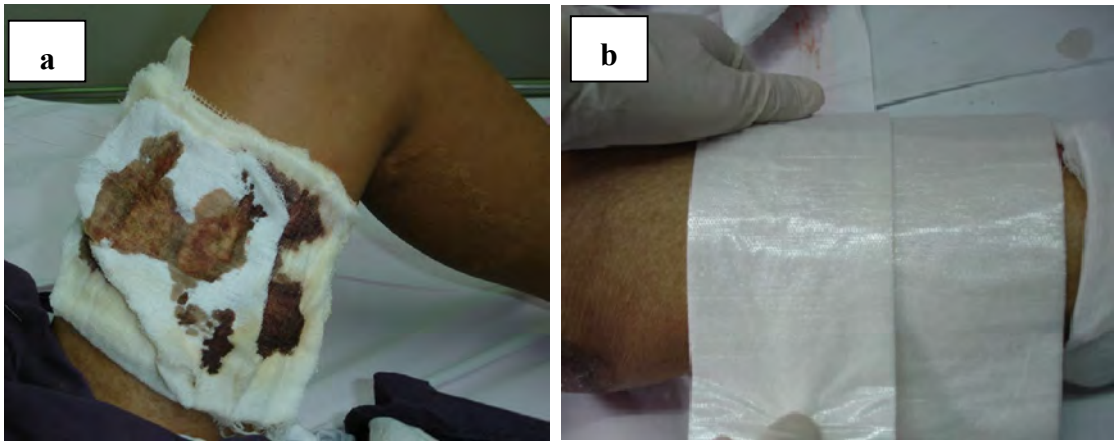
Thirty-two patients were enrolled in this study and all completed the follow-up period. Before the operation, all subjects were randomized into the two treatment arms. There were no significant differences in demographic parameters between the two groups at baseline including age, area of donor site and length of hospital stay. However, there was a significant difference in gender between both groups (Table 1). During the study period, clinical observations showed that both dressings were easily applied and did not require special supplies. The adhesion of the Telfa AMD® was lower than that of the Bactigras® dressing, and after moisturizing the Telfa AMD® was easily removed without damaging the newly formed epithelium. However, the Bactigras® dressing had a greater level of adhesion to the wound surfaces, and there was a risk of damaging the delicate epithelium. Figure 1 shows the new donor sites to be treated with the Bactigras® (Figure 1a) and Telfa AMD® (Figure 1b) dressings.

Table 1. Demographics data of patients in each group.

Demographics Data	Paraffin Tulle + Chlorhexidine Dressing (Bactigras®) (Range)	Cotton Fiber + PHMB (Telfa AMD®) (Range)	*p*-Value
Gender (male:female)	14:2	8:8	0.02 *
Age (years)	36.19 ± 19.81 (16–78)	29.13 ± 12.55 (17–50)	0.24
Area of donor sites (cm^2)	1,016.38 ± 498.56 (336–2,340)	935.83 ± 436.47 (270–2,052)	0.63
Length of hospital stays (days)	53.63 ± 36.22 (9–126)	47.69 ± 31.30 (13–124)	0.62

* indicates significant difference ($p < 0.05$).

Figure 1. New donor sites treated with Bactigras® (**a**) and Telfa AMD® (**b**), respectively.



The donor sites treated with the Telfa AMD® had a shorter re-epithelialization time and the length of time taken for more than 90% re-epithelialization was significantly different when compared to that of the patients in the Bactigras® group (Table 2).

Table 2. Efficacy of Bactigras® and Telfa AMD® in donor site wounds.

	Paraffin Tulle + Chlorhexidine Dressing (Bactigras®) (Range)	Cotton Fiber + PHMB (Telfa AMD®) (Range)	*p*-Value
Day of reepithelization (≥90%)	14.00 ± 3.05 (9–21)	9.25 ± 1.88 (7–13)	<0.001 *
Pain score	4.70 ± 1.16 (2.20–6.64)	1.57 ± 0.55 (0.57–2.57)	<0.001 *
Number of infection site	1	0	–
Cost of treatment (USD)	4.64 ± 1.97 (2.12–9.55)	5.72 ± 2.54 (1.73–12.09)	0.19

* indicates significant difference ($p < 0.05$).

The pain assessment also showed a statistically significant difference when comparing the applications of Telfa AMD® and the Bactigras® dressing. The average grade of pain in the Telfa AMD® applications was much lower on all evaluation days (Table 3). However, the average grades of pain in both groups were within the ranges of discomfort and very slight pain, not within the range of real pain, which requires analgesia.

Table 3. Average grade for the assessment of the pain in treatment with Bactigras® and Telfa AMD®.

Pain Score	Paraffin Tulle + Chlorhexidine Dressing (Bactigras®) (Range)	Cotton Fiber + PHMB (Telfa AMD®) (Range)	*p*-Value
First day	6.81 ± 1.17 (5–9)	2.56 ± 1.41 (0–5)	<0.001 *
Third day	6.38 ± 1.45 (4–9)	1.88 ± 1.20 (0–4)	<0.001 *
Seventh day	5.13 ± 2.03 (0–8)	1.13 ± 1.15 (0–4)	<0.001 *
14^{th}–21^{st} day	1.88 ± 2.33 (0–7)	0 0	<0.001 *

* indicates significant difference ($p < 0.05$).

Our results indicated that overall wound healing, as measured by the percentage of epithelialized dermis, was faster with the Telfa AMD® than with the Bactigras® dressing. The faster re-epithelialization rate observed with the Telfa AMD® can partially be explained by its physical properties since it contains poly (ethylene terephthalate) polymers. Since the Telfa AMD® has lower adhesion properties, it not only prevents trauma to the new and delicate epithelium during dressing removal, but it also provides a good moist environment, which is preferred for epithelial cell proliferation and migration [19]. This concept was well supported by evidence from many previous studies which showed faster re-epithelialization rates when moist-environment dressings were compared with traditional dry dressings [7,19–21].

On the other hand, the Bactigras® dressing has a greater absorptive effect, which resulted in a greater amount of adhesion. During dressing removal or patient movement, it is possible to damage the delicate epithelial cells which can slow wound re-epithelialization as well as increase patient discomfort. Therefore, the physical difference between these two dressings may be the reasons for our results. Moreover, a case of *Pseudomonas aeruginosa* infection, a bacterium which is of especial concern in patients with burns, was found in a patient treated with the Bactigras® dressing, which indicated that PHMB might be more beneficial in infection control than chlorhexidine. However, no mortality or any side effects from either of the dressings occurred in this study. With respect to bacterial growth, one patient in the Bactigras® group was found to have a local infection on the tenth day with *P. aeruginosa* (10^2), and no local infection was observed in the Telfa AMD® group. However, the microbial numbers were below the critical values. After receiving a standard systemic antibiotic, no isolated bacteria were found on the next day.

Pain is the main cause of patient discomfort which challenges burn treatment protocols. Our results indicated that the Telfa AMD® results in statistically less pain at the donor site from the first day of treatment, and the pain score was significantly reduced after three days. Even though the pain score has completely subjective characteristics, it is a reflection of how comfortable patients feel during treatment and it has been widely used in similar studies.

The results from the comparative cost-effective analysis showed that the cost difference between both dressings was insignificant. The total cost of patients treated with the Telfa AMD® was slightly higher than the Bactigras® dressing, which may have been due to the cost of the dressing itself.

3. Experimental Section

This was a prospective, randomized control study comprising 32 patients treated at the Siriraj Burn Unit, Thailand during December 2008–February 2010. It was designed to be open-labeled and observer blinded. The study was approved by Institutional Review Board Committee of the hospital, and written informed consent was obtained from each patient who enrolled in the study.

Twenty-two of the patients were men and ten were women, aged 16–78 years old. All monitored patients had similar burns with regard to the burn area and the depth of donor sites. Patients who need skin graft operation were randomized by computer and placed into two groups: 16 donor sites were treated with the Bactigras® paraffin tulle dressing coated with chlorhexidine (Smith & Nephew Healthcare Limited, Hull, UK), and 16 donor sites were treated with the Telfa AMD® cotton fiber impregnated with PHMB (Covidien, Mansfield, MA, USA). The demographic were collected from each subject in both group including age, gender, area of donor site (cm^2), operative time and length of hospital stay.

The donor site was at the proximal thigh area. The patients were excluded if they were allergic to paraffin, chlorhexidine, poly (ethylene terephthalate) or PHMB. They were also excluded if there were lesions on both thighs, if they had psychiatric problems or multiple injuries (more than two systems involved), if they were immunocompromised, such as with renal failure, cirrhosis or malnutrition, and if they were receiving radiation or chemotherapy for malignancy. Patients with diabetes mellitus, systemic lupus erythematosus or other connective tissue diseases, and patients who had donor sites in areas other than the thigh were also excluded. Patients who did not comply with the study protocol, or who had a skin graft which had previously been harvested from the same donor site area, could not be involved in this study either.

All of the skin grafts (0.010 inches thickness) were taken from the thigh using a Zimmer® Air Dermatome Skin Grafting System (Zimmer, Ltd., Swindon, UK). The area of donor site (cm^2) was calculated using Image J Java-based image processing program developed by the National Institutes of Heath. Immediately after harvest, the donor site was covered with a saline-soaked gauze for hemostasis until surgery was completed. The Bactigras® or Telfa AMD® dressing were applied to the donor wound covering about 1 cm of intact skin. The dressing was secured by a sterile gauze. The donor site wounds were inspected every day after operation. None of the dressings were changed until the wounds were completely dry and the dressings fell off.

A donor site follow-up chart was used to conduct the clinical follow-up of the healing process. The information gathered in the chart included the percentage of re-epithelialization of each donor site area,

the state of healthy skin on the periphery of each donor site and local signs of infection. Moreover, local pain was also followed-up using a visual analogue pain scale from 0 (no pain) to 10 (maximal severe pain) which was recorded at 30 minutes after the open wound, then at days 3, 7, 14 and 21 or when the dressing fell off. Normal saline solution was used to moisturize the dressing prior to removal and it is considered as neutral solution, no interaction has been found between normal saline and all dressing materials. The patients and the observer were blinded to the type of dressing in each donor site.

Infection was also evaluated by swab cultures for a microbiological analysis which was performed routinely once a week on Tuesday. A cost-effectiveness analysis was also compared between these treatment groups. The costs of the dressings, supplies and nursing labor were used to calculate the treatment cost.

Comparative analyses of the patients in both groups were performed using two-tailed unpaired student's *t*-test with SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as mean (±SD). A $p < 0.05$ was considered as statistically significant.

4. Conclusions

Both the Bactigras® dressing and the Telfa AMD® are easy to apply in clinical practice. They can both protect wounds against mechanical trauma and provide comfort for the patients. However, the Telfa AMD® provides a shorter re-epithelialization time, prevents infection and generates lower pain level in comparison with the Bactigras® dressing. The treatment cost difference between these two dressings is negligible.

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The Effect of Sterilization Methods on the Physical Properties of Silk Sericin Scaffolds

Tippawan Siritientong, Teerapol Srichana & Pornanong Aramwit

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Research Article

Theme: Sterile Products: Advances and Challenges in Formulation, Manufacturing, Devices and Regulatory Aspects
Guest Editors: Lavinia Lewis, Jim Agalloco, Bill Lambert, Russell Madsen, and Mark Staples

The Effect of Sterilization Methods on the Physical Properties of Silk Sericin Scaffolds

Tippawan Siritientong,[1] Teerapol Srichana,[2] and Pornanong Aramwit[1,3]

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Abstract. Protein-based biomaterials respond differently to sterilization methods. Since protein is a complex structure, heat, or irradiation may result in the loss of its physical or biological properties. Recent investigations have shown that sericin, a degumming silk protein, can be successfully formed into a 3-D scaffolds after mixing with other polymers which can be applied in skin tissue engineering. The objective of this study was to investigate the effectiveness of ethanol, ethylene oxide (EtO) and gamma irradiation on the sterilization of sericin scaffolds. The influence of these sterilization methods on the physical properties such as pore size, scaffold dimensions, swelling and mechanical properties, as well as the amount of sericin released from sericin/polyvinyl alcohol/glycerin scaffolds, were also investigated. Ethanol treatment was ineffective for sericin scaffold sterilization whereas gamma irradiation was the most effective technique for scaffold sterilization. Moreover, ethanol also caused significant changes in pore size resulting from shrinkage of the scaffold. Gamma-irradiated samples exhibited the highest swelling property, but they also lost the greatest amount of weight after immersion for 24 h compared with scaffolds obtained from other sterilization methods. The results of the maximum stress test and Young's modulus showed that gamma-irradiated and ethanol-treated scaffolds are more flexible than the EtO-treated and untreated scaffolds. The amount of sericin released, which was related to its collagen promoting effect, was highest from the gamma-irradiated scaffold. The results of this study indicate that gamma irradiation should have the greatest potential for sterilizing sericin scaffolds for skin tissue engineering.

KEY WORDS: ethanol; ethylene oxide; gamma irradiation; scaffold; sericin.

INTRODUCTION

Tissue engineering is widely anticipated to replace traditional graft procedures for treatment of tissue and organ defects. In tissue engineering, temporary implants are used to regenerate new tissue. A porous polymer matrix is often used as a guide for the cells to adhere, proliferate, and grow on (1). In order to obtain successful tissue generation *in vitro*, a scaffold with a highly specialized properties such as their topography, surface chemistry, mechanical properties, and degradation rates is required (2,3). These factors are critical and influence on the ability of cells to colonize a scaffold and eventually form an organized tissue construct. Implantation *in vivo* requires the scaffold to be biocompatible and to integrate within the surrounding natural tissue, and also to be completely eliminated from the host via biodegradation over a favorable time scale (4).

The demands on the scaffold materials are explicit for each specific application for which they are intended, giving rise to the need for a broad array of material properties. Protein-based materials such as silk protein, fibroin (fibrous protein), and sericin (degumming protein) have generated much interest in the biomedical and biotechnological fields due to their unique properties (5–8). Many researchers have successfully formed fibroin scaffolds for vascular tissue, connective tissue, and bone regeneration (9–11) whereas sericin scaffolds have been applied in skin substitution (12). Sericin is considered to be a waste material in textile manufacturing. However, its characteristics include high biocompatibility and biodegradability, low toxicity, and high hydrophilicity, and its low cost has also increased interest in the use of this compound in tissue engineering. Its potential and existing applications are extensive in medical, pharmaceutical, and cosmetic sectors. The effects of their production methods and the sterilization process used are often overlooked, even though they might have significant effects on the physical and biological properties of sericin scaffolds.

[1] Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Payathai Road, Bangkok, 10330, Thailand.
[2] Drug Delivery System Excellence Center and Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla, Thailand.
[3] To whom correspondence should be addressed. (e-mail: aramwit@gmail.com)

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A sterilization process is essential for every material or device for clinical use and the efficacy of sterilization techniques must be confirmed. Biomaterials with a complex architectures and hydrolytic degradation mechanisms from scaffolds may be easily damaged by harsh sterilization processes. Since sterilization treatments may adversely affect the material properties, any changes must be fully characterized and accounted for in the manufacturing process. These alterations may be detrimental or beneficial changes at the cellular level with respect to cell–surface interactions (13). Nevertheless, the challenge remains to discover an efficient and non-destructive sterilization protocol for biomaterial scaffolds which preserves their 3-D structure and ability to facilitate repair.

Biomedical devices prepared from biodegradable polymers are usually sterilized by ethylene oxide (EtO) because other sterilization procedures such as heat, steam or acid can cause extensive deformation of the devices and accelerate polymer degradation (14,15), whereas very little degradation occurs when EtO is used (16). However, in some polymers, EtO sterilization may lead to changes in the dimensions of scaffolds through shrinkage (16). Disinfection by ethanol is often used *in vitro* and is shown to produce no morphological or chemical damages to polyester scaffolds (16). Ethanol is considered as a strong immediate bactericidal activity (17) and virucidal activity at high concentration (ca. 95%) (18). It also has broad activity against most fungi-including yeasts and dermatophytes but virtually has no sporicidal activity (19). However, no study on ethanol as sterilize agent has been performed on protein-based biomaterials. Gamma irradiation is a common technique for sterilizing polymeric implants (20). Since scaffolds usually have a porous structure, a sterilization method is required that can penetrate such materials without leaving residues. Gamma irradiation is highly penetrative, although it causes a decrease in the tensile strength of hydrophobic polyurethanes (21). In some cases, the properties and performance can be negatively affected because of material degradation or induced cross-linking (22). Again, no study has been performed on the effect of gamma irradiation on protein-based biomaterial scaffolds.

Therefore, it is clear that each method will have advantages and limitations, the choice of a particular method must be carefully considered. The purpose of this study was to investigate changes in the physical properties of silk sericin scaffolds after sterilization by EtO, gamma irradiation, and ethanol treatment. Since the different sterilization processes may have different effects on various types of materials and different scaffold processing techniques, the release of sericin from scaffolds which may be beneficial for collagen production in wounds was also investigated.

MATERIALS AND METHODS

Preparation of a Three-Dimensional Silk Sericin and Polyvinyl Alcohol Scaffolds

The fresh, white-shell cocoons of *Bombyx mori* were kindly supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). Silkworm cocoons were produced in a controlled environment. After cutting the cocoons into pieces (about 5 mm^2), silk sericin was extracted using a high temperature and pressure degumming technique by mixing the silkworm cocoons with purified water (1 g of dry silk cocoon/30 mL of water) and the samples were autoclaved (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120°C for 60 min. After filtration through a membrane to remove fibroin, the sericin solution was concentrated until the desired concentration (approximately 7% *w*/*v* as measured by the BCA Protein Assay Reagent, Pierce, Rockford, IL, USA) was achieved.

Polyvinyl alcohol (PVA, Ajax Finechem, New South Wales, Australia, molecular weight 77,000–82,000) was dissolved at 80°C with constant stirring for about 4 h until it was completely dissolved to a concentration of 6% *w*/*v*. Glycerin, added as a plasticizer and to improve the scaffolds functional properties, was blended together with sericin and the PVA solution at room temperature for at least 30 min to make a final wet composition of 3% *w*/*v* sericin, 2% *w*/*v* PVA, and 1% *w*/*v* glycerin, which was then poured into a petri dish and frozen at −20°C, followed by lyophilization (Heto LL 3 000 lyophilizer, Allerod, Denmark) for 72 h.

EtO Sterilization and Gamma Irradiation

Scaffolds for EtO sterilization and gamma irradiation were packed in self-sealing sterilization pouches (saf-T-seal®, MD Industries, Maharashtra, India). Ethylene oxide sterilization was achieved in a 100% ethylene oxide atmosphere at 55°C for 3 h. The samples were then exposed to air for 12 h. Gamma irradiation sterilization was achieved at a dose of 25 kGray ^{60}Co at 55°C.

To validate ethylene oxide cycles used in the sterilization, overkill method has been performed using *Bacillus atrophaeus* (3M™ Attest™ 1294 rapid readout biological indicator for ethylene oxide, 3M Technologies Pte Ltd, Singapore). The overkill method is based on demonstrating that the sterilization of a microbial challenge (biological indicator) exceeds the challenge posed by the bioburden of the product. In our case, exposure of ethylene oxide for 3 h shows negative result of biological indicator which indicated that the ethylene oxide cycles are valid.

Ethanol Sterilization

The ethanol sterilization procedure was performed using a modified method from Karp *et al*. (23). A 200-mL 70% (*v*/*v*) ethanol solution (diluted from 96% *v*/*v* ethanol (Fisher Scientific UK Ltd, Loughborough, UK) with distilled water) was added to the scaffold. The samples were treated for 5 min and were subsequently rinsed three times with 100 mL deionized water.

Sterility Testing

All scaffolds were tested for sterility immediately following sterilization using a previously described procedure (24). Briefly, the samples were immersed in a Nutrient Agar Broth (Himedia Laboratories, Mumbai, India) to cultivate fastidious microorganisms and maintained under agitation at 25°C for 48 h. An untreated scaffold was used as the negative control while Allevyn (a commercial scaffold-like product, Smith & Nephew Medical Limited, London, UK) was used as the

positive control. Clouding of the broth after 48 h indicated contamination and inefficient sterilization, while a clear, uncontaminated broth indicated efficient sterilization, producing a sterile product. All experiments were performed in triplicate.

Bioburden Challenge Test

Test organisms that included Methicillin-resistant *Staphylococcus aureus* (MRSA; DMST 20645 lot no. 3273, DMST Culture Collection, The National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand) and *Bacillus subtilis* (ATCC 6633 DMST 15896 lot no. 3479, DMST Culture Collection, The National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand) were suspended in tryptic soy broth (TSB) to provide a final concentration of 10^2 and 10^4 colony forming unit (cfu)/mL. The inoculation of the test carrier was performed by using micropipette to place 20 μL of the test suspension on the surface of scaffolds and left to dry in the incubator for 18 h at 37°C (25). The scaffolds were then sterilized by ethanol, EtO, and gamma irradiation as previously mentioned protocol. After sterilization, the scaffolds were then transferred to test tubes containing TSB which were incubated at 25°C for 7 days. The turbidity of TSB was measured every day using UV spectrophotometer at wavelength 625 nm and using McFarland Standards as a reference to estimate the number of colonies. Unsterilized scaffolds with test suspension was used as positive control while sterilized scaffold without test suspension was identified as negative control, respectively. All experiments were performed in triplicate.

Changes in Morphology and dimensions

Scanning electron micrographs were taken on a JSM-5 800LV (JEOL JSM-5410LV, Tokyo, Japan) scanning electron microscope (SEM) at an acceleration voltage of 15 keV after cutting the scaffold into pieces, mounting onto aluminum stubs and sputter coating with gold at a 10–20 nm thickness. Sterilized and degraded scaffolds were checked for changes in gross (i.e., outer) scaffold dimensions (n=3 for each group), and the percentage of volume changes was calculated. The outer dimensions of the scaffolds were measured using a Micrometers Series 169-Non-Rotating Spindle Type (Mitutoyo Corporations, Kawasaki, Japan). All experiments were performed in triplicate.

Pore Size Measurement of Scaffolds

Pore size was measured by following a modified method from Kang *et al.* (26). The major and minor diameters of each scaffold were measured using a stereomicroscope equipped with an optical micrometer. The pore size was calculated as the geometric mean of the major and minor diameters, respectively. At least 100 pores were assessed, and the values presented indicate the mean ± standard deviation.

Fourier Transforms Infrared Spectroscopy

The FT-IR spectra of the compounds were recorded on a Perkin Elmer spectrometer by the KBr pellet technique. The sericin patch was ground into small pieces in mortar. For a verification of the compound structure, a KBr pellet was prepared by grinding of about 2 mg sericin samples with 200 mg KBr. The peak areas were calculated by the baseline technique. The standardization procedure was based on the preparation of a calibration curve between the integrated areas and the concentration of the salt in the KBr pellet. Infrared spectrum was recorded between 4,000 and 400 cm^{-1}.

Circular Dichroism

Circular dichroism (CD) was performed on a JASCO J-715 spectropolarimeter (Jasco Inc., Tokyo, Japan) using 0.1 cm path length quartz cell at temperature 20°C. The sample was analyzed in DDW. The UV spectra were measured at a protein concentration of 1 mg/mL from 250 to 190 nm with speed scan at 50 nm/min, response time constant was 1 s, spectral bandwidth was 2.0 nm, step resolution was 0.5 nm, and sensitivity was 10 mdeg. Triplicate scans of the CD spectra were averaged to reduce error and noise, and the baseline correction was subtracted by DDW background spectra. The percentage of α-helix, β-sheet, turns, and random coil were determined using analysis function built into the Jasco-715 spectropolarimeter software.

Swelling Properties

Swelling studies were carried out according to Mandal *et al.* (27) with slight modifications. Briefly, the lyophilized scaffolds were accurately weighed in the dry state and then immersed in 10 mL of purified water. At 24 h, the scaffolds were carefully removed, and the amount of water contained in the scaffolds was precisely determined by weighing them in the swollen state. The experiments were performed in triplicate under the same conditions. The percentage of swelling of the scaffolds at equilibrium was calculated using the following equation:

$$\% \text{ swelling} = [(W_t - W_0)/W_0] \times 100$$

where W_0 is the weight of the dried test sample and W_t is the weight of the swollen test sample.

Degradation Study

Newly processed scaffolds were prepared with a 3-D rectangular geometry, allowing for an accurate measure of their initial mass. The scaffolds were then divided into four groups and each group treated either by EtO, gamma irradiation, or ethanol, respectively, while a non-sterilized scaffold was used as the control. Each treated sample was placed in a perforated Eppendorf tube and immersed in phosphate buffer solution (pH 7.4) at 37°C for 24 h. The ratio of the sample mass to buffer volume was 1:1,000 (w/v), and the pH of the buffer was routinely checked. After 24 h, all samples were removed from the buffer, washed repeatedly

with deionized water, and dried under a vacuum (P= 0.01 mmHg, 24 h at room temperature). The dried samples were evaluated for changes in mass by accurately weighing them and the percentage weight loss was calculated using the following equation:

$$\% \text{ weight lost after 24 h immersion} = [(W_0 - W_t)/W_0] \times 100$$

where W_0 is the weight of the dried scaffold at the beginning (before immersion) and W_t is the weight of the dried sample after 24 h immersion. All experiments were performed in triplicate.

Mechanical Properties of the Scaffolds

Tensile strength tests using the Universal Testing Machine (Hounsfield H10KM, London, UK) were performed on dumb-bell-shaped samples of 25 mm length and 6 mm width at a cross-head rate of 5 mm/min (ASTM D412-06a). The maximum stress and Young's modulus were obtained from the stress and strain tensile curve. All experiments were performed in triplicate.

The Release of Sericin from Sericin Scaffolds

The release profile of sericin from the scaffolds was plotted after placing the scaffold samples (diameter 35 mm) into PBS (pH 7.4) at room temperature with continuous stirring in a closed-container. Samples (1.5 mL) were removed at different time points 0, 1, 15, 30 min, 1, 2, and 3 days, and the amount of sericin was measured immediately after sampling using a BCA protein assay kit (Pierce, Rockford, IL, USA). Briefly, the leached protein samples were collected, mixed with BCA reagents, and vortexed. The absorbance was measured at 562 nm and the amount of protein released was compared with a bovine serum albumin standard curve. All experiments were performed in triplicate.

Statistical Analysis

Data was expressed as the mean ± SD. The statistical significance was determined by paired and unpaired Student's t tests together with ANOVA. A value of $p<0.05$ was considered to be significant.

RESULTS

Effectiveness of Sterilization Methods and Scaffold Structure

After 48 h incubation, the untreated controls all produced signs of growth during sterility test whereas the Allevyn remained free of growth throughout the same time period. However, the broth in the tubes containing the ethanol-treated scaffold showed opacity after 24 h while the gamma-irradiated and EtO-treated scaffolds showed successful sterilization throughout the treatment durations. These results indicate the

Table I. Amounts of Microorganism Growth on Sericin Scaffolds

	Day						
	1	2	3	4	5	6	7
Positive control							
MRSA 10^2 cfu/mL	−	−	−	+	++++	++++	++++
MRSA 10^4 cfu/mL	−	−	++	++++	++++	++++	++++
B. subtilis 10^2 cfu/mL	−	++	++++	++++	++++	++++	++++
B. subtilis 10^4 cfu/mL	−	+++	++++	++++	++++	++++	++++
Negative control							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	++++	++++	++++	++++	++++	++++
MRSA 10^2 cfu/mL							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	−	−	−	−	−	−
MRSA 10^4 cfu/mL							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	−	−	−	+	++++	++++
B. subtilis 10^2 cfu/mL							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	−	+	++++	++++	++++	++++
B. subtilis 10^4 cfu/mL							
EtO	−	−	−	+	++++	++++	++++
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	+++	++++	++++	++++	++++	++++

− no growth, + amount of microorganism below 1.5×10^8 cfu/mL, ++ amount of microorganism between 1.5 and 6×10^8 cfu/mL, +++ amount of microorganism between 6 and 12×10^8 cfu/mL, ++++ amount of microorganism more than 12×10^8 cfu/mL

suitability of the sterilization techniques tested to these particular scaffolds. Table I shows the result of bioburden test which indicated that ethanol treatment is the least effective in sterilizing scaffold, similar to the result during sterility test, while gamma irradiation is the most effective method as shown by clear broth media after challenging with high bacterial suspension even after 7 days incubation.

Figure 1 shows the SEM cross-section images of scaffolds both before and after the sterilization treatment. Figure 1a is an image of the untreated control sample, which shows homogeneity throughout the sample, while Fig. 1b and c represent EtO- and gamma-irradiated scaffolds, respectively. The pore size in both the EtO- and gamma-irradiated scaffolds appears more irregular compared with the untreated sample and were more fragile under SEM; magnifications above ×2,500 resulted in damage to the samples within seconds. Ethanol treatment had a large impact on the scaffolds when compared to the controls. The samples sterilized in ethanol shows reduced porosity and increased of surface wrinkles (Fig. 1d).

Comparing Sterilization Techniques for Changes in Dimension and Pore Size

In all cases, the structure of the scaffolds changed during treatment. The samples sterilized by EtO, gamma irradiation, and ethanol were compared to untreated samples in regard to changes in dimension, shown as volume and pore size changes, as summarized in Fig. 2 and Table II, respectively. Ethylene oxide and gamma irradiation slightly altered the dimensions of the sericin scaffold whereas ethanol-treated samples shrank significantly compared to the other samples. After ethanol sterilization, there was a loss of approximately 80% of their initial volume. The ethanol-sterilized samples also looked softer than all of the other sterilized samples. Table II provides details of the pore size of sericin scaffolds both before and after sterilization treatment, indicating that ethanol-treated samples have a significantly smaller pore size compared with the control and EtO- and gamma-irradiated samples. Ethylene oxide and gamma irradiation slightly reduced the pore size but not to a significant level.

FT-IR Results

The FT-IR spectra of the sericin samples are shown in Fig. 3. These compounds exhibited the amide absorption bands of protein at ca. 3,286, 2,941, 1,664, 1,535, 1,415, and 1,046 cm^{-1}. The peak at 3,286 cm^{-1} corresponds to N–H stretching vibrations, and this may be caused by amide structure (only one stretching of secondary amine). The amide absorption primarily attributes to the C=O group peaks which can be seen

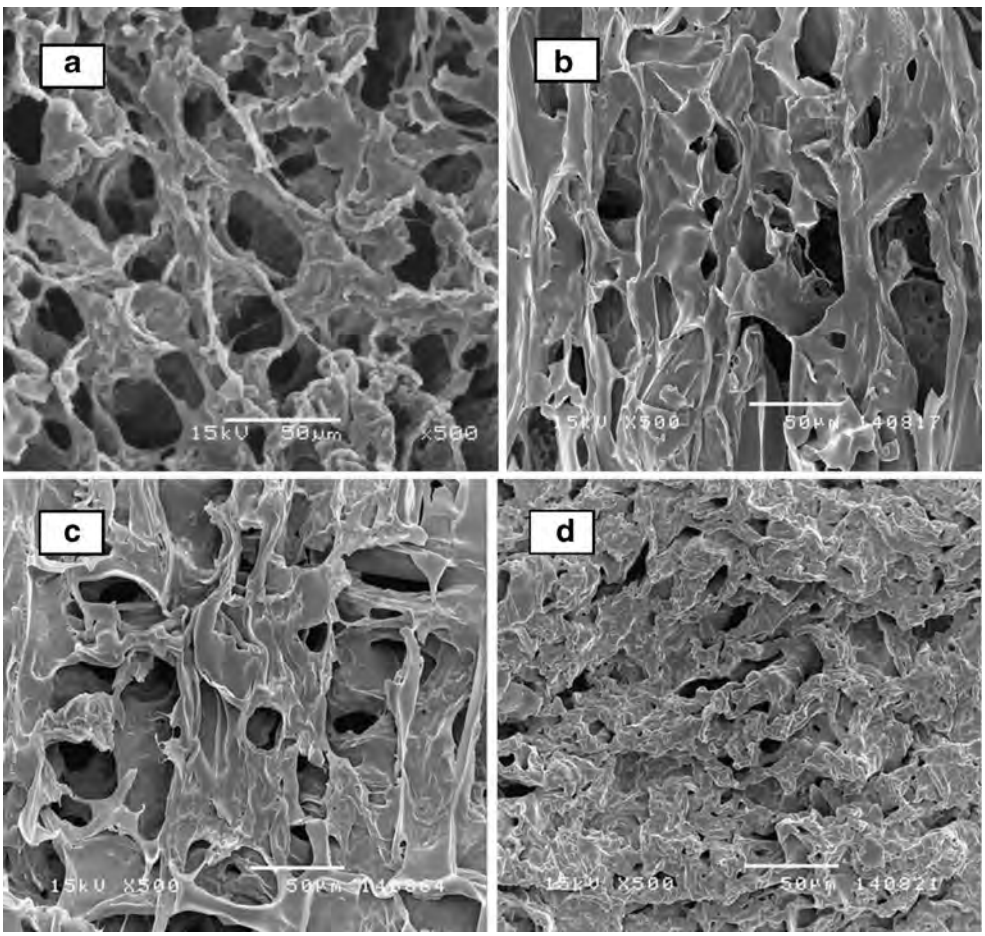

Fig. 1. SEM cross-section images of scaffolds. **a** sericin scaffold (unsterile), **b** ethylene oxide-treated sericin scaffold, **c** gamma irradiation-treated sericin scaffold, **d** ethanol-treated sericin scaffold

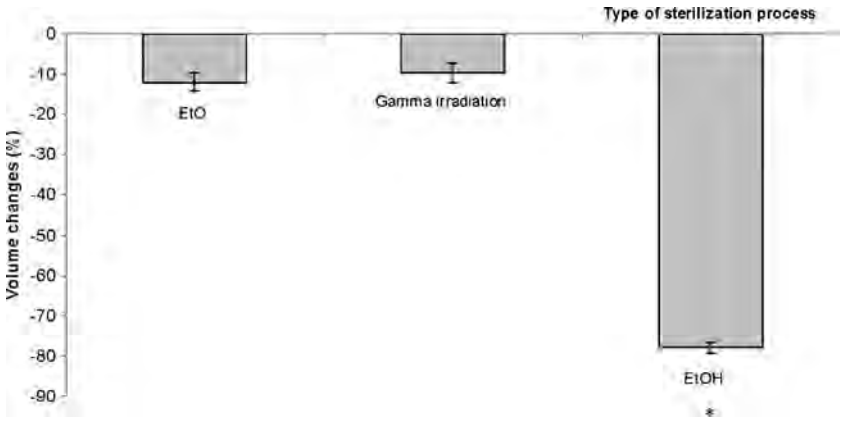

Fig. 2. Changes in the dimensions (shown as percentage of volume changes) of sericin scaffolds before and after sterilization treatment. *$p<0.05$ indicates significant differences

at 1,664 cm^{-1}. N–H bending and C–N groups exhibit characteristic band at 1,535 cm^{-1}. The band at 1,415 cm^{-1} is attributed to C–H and O–H bending, and the band between 1,099 and 1,046 cm^{-1} reveals to be C–OH vibrations stretching. The spectra of each sample are very similar to one another. It is postulated that the chemical structure of sericin scaffold did not change dramatically during treatments with ethanol, EtO, or gamma irradiation.

CD Result

The far-UV CD spectra of sericin scaffolds are shown in Fig. 4. They revealed strong negative peaks at 196 nm of untreated (3S2P1G) and ethanol-treated scaffolds suggesting a random coiled structure. However, in EtO-treated and gamma-irradiated scaffolds, the negative peaks shifted to 195 and 192 nm, respectively. A negative band at 220 nm suggested the presence of β-sheets in all samples. Absence of any positive bands around 190 nm excluded the presence of any α-helix in any sericin scaffolds in this study. The percentages of different secondary structures in the sericin scaffolds indicated 36–38% of β-sheet, 12–18% β-turn, and 45–49% random coil in untreated and ethanol-treated scaffolds. Whereas in EtO-treated and gamma-irradiated scaffolds, the percentage of β-sheet decreased to 28–29%, the percentage of random coil slightly decreased and percentage of β-turn slightly increased. Overall, the structures of sericin in this study were random coil, β-sheet, and β-turn. The treated samples did not change their conformation from the untreated scaffolds.

Table II. Pore Size of Sericin Scaffolds Before and After Sterilization Treatment

Sample	Pore size (μm)
Untreated (control)	43.81±18.62
Ethylene oxide	40.75±15.15
Gamma irradiation	40.30±13.22
Ethanol treatment	24.29±11.88*

*$p<0.05$, significant differences compared with other scaffolds

Swelling Properties

The swelling property of the sericin scaffolds before and after the sterilization treatment is shown in Fig. 5. All sericin scaffolds can absorb moisture efficiently, as shown by the high water holding capacity, including the untreated scaffold. However, the untreated scaffold became too fragile to be handled and broke into pieces while the ethanol-treated scaffold exhibited the highest intact structure after immersion for 24 h. The swelling property of the ethanol-treated scaffold was similar to that of the control, whereas gamma irradiation significantly increased the swelling property of the sericin scaffold. There was an approximately twofold swelling of the gamma-irradiated sample compared to the control after 24 h immersion. Ethylene oxide also increased the swelling property of the sericin scaffold compared to the untreated sample, but not to a significant level.

Degradation Study

Figure 6 represents the percentage of weight loss of the sericin scaffolds after 24 h immersion in water. The control lost approximately 90% of its weight after immersion which was significantly difference compared to the other samples, and as mentioned above, the control samples also had the least intact structure. All sterilization treatments reduced the loss of the scaffolds structure during immersion for 24 h, especially the ethanol-treated scaffold, which showed the smallest weight loss after immersion. The scaffolds treated with EtO and gamma irradiation lost approximately 60% and 70% of their initial weights, respectively, while the ethanol-treated scaffold only lost 40% compared to its initial weight.

Mechanical Properties of the Scaffolds

Figure 7 shows the mean maximum stress resistance of the sericin scaffolds before and after the sterilization treatments. The untreated scaffold exhibited the highest maximum stress resistance and was similar to the maximum stress resistance produced by the EtO-treated scaffold. Treatment by gamma irradiation and ethanol significantly reduced the maximum stress resistance of the sericin scaffolds. The

Effect of Sterilization on Sericin Scaffolds

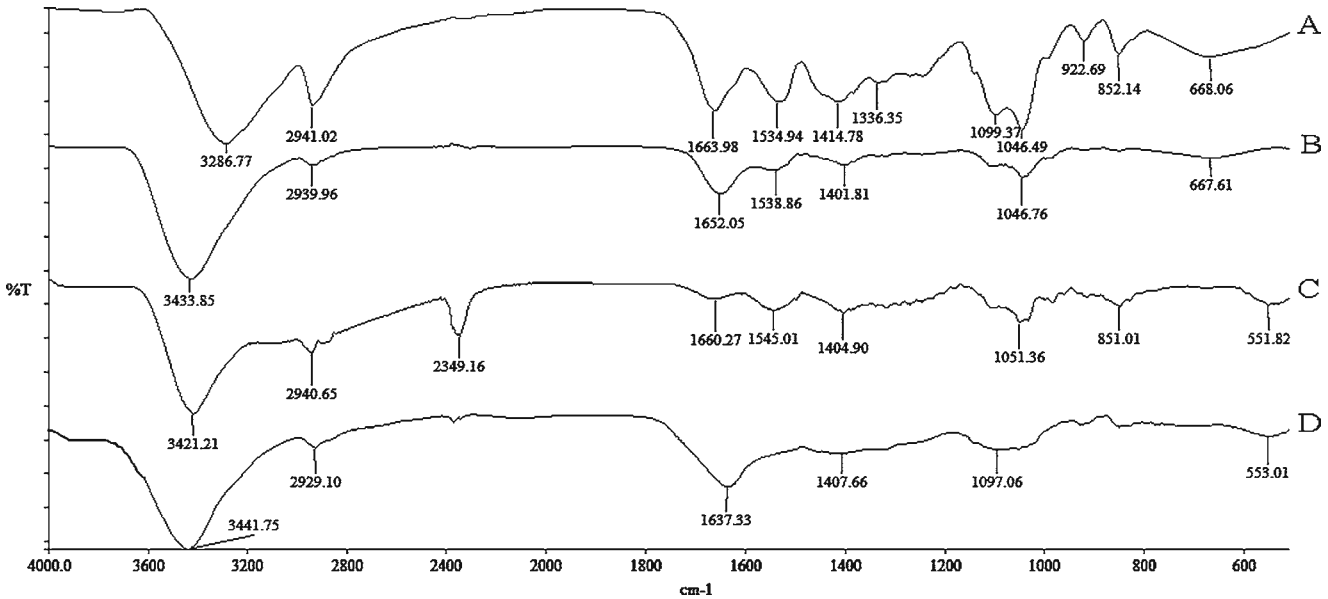

Fig. 3. FT-IR spectra of sericin scaffold before sterilization (*A*) and after sterilization with 70% ethanol (dry; *B*), ethylene oxide (*C*), and gamma radiation (*D*)

maximum stress resistance of the ethanol-treated scaffold was significantly lower when compared to the untreated and EtO-treated samples, which indicated that the untreated and EtO-treated scaffolds possessed relatively higher tensile strengths when compared to the gamma irradiation and ethanol-treated scaffolds.

Young's modulus of the sericin scaffolds before and after sterilization treatment are shown in Fig. 8. Gamma irradiation, similar to the ethanol-treated sericin scaffolds, had a lower Young's modulus compared with the untreated and EtO-treated scaffolds. The Young's modulus of the untreated and EtO-treated samples showed significant differences compared to the gamma-irradiated samples. These data indicated that the control and EtO-treated scaffolds were stiffer, whereas the gamma-irradiated and ethanol-treated scaffolds had more flexibility.

The Release of Sericin from the Sericin Scaffolds

Figure 9 shows the amount of sericin released from the scaffolds before and after sterilization. The gamma-irradiated scaffold released the highest amount of sericin, whereas the ethanol-treated scaffold released the lowest amount of protein, which was significantly different compared to the other scaffolds. Moreover, the amount of sericin released from the gamma-irradiated scaffold was significantly different from the amount of sericin released from the EtO-treated scaffold. The maximum amount of protein leaching from all

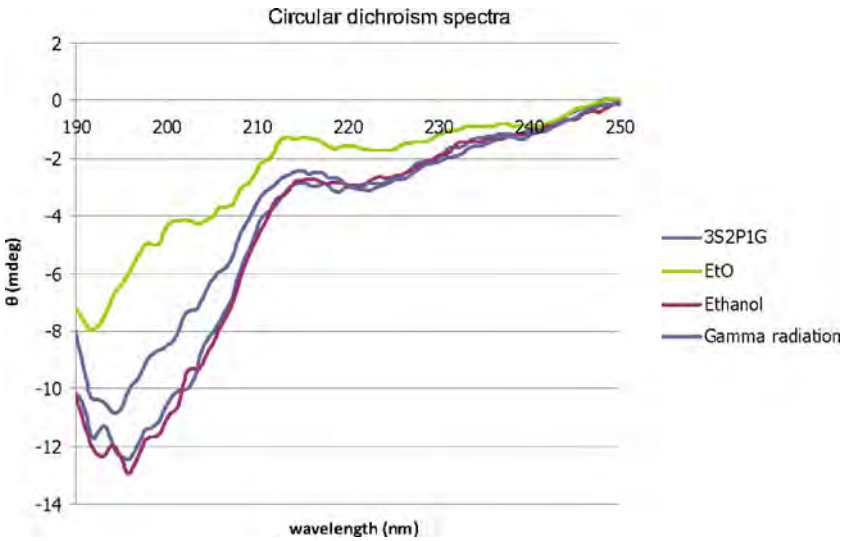

Fig. 4. The far-UV circular dichroism spectra of sericin scaffolds

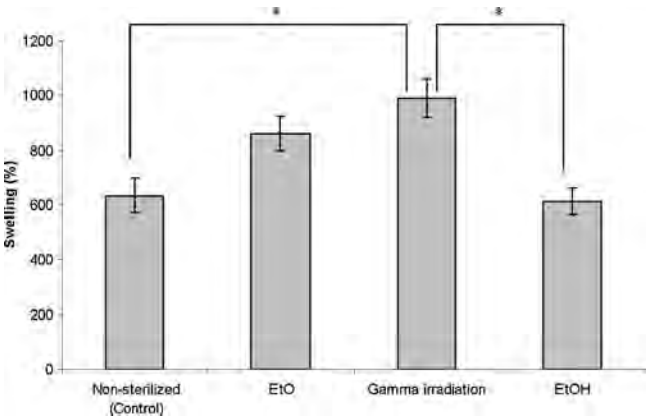

Fig. 5. The swelling property of sericin scaffolds before and after sterilization treatment. *$p<0.05$ significant differences

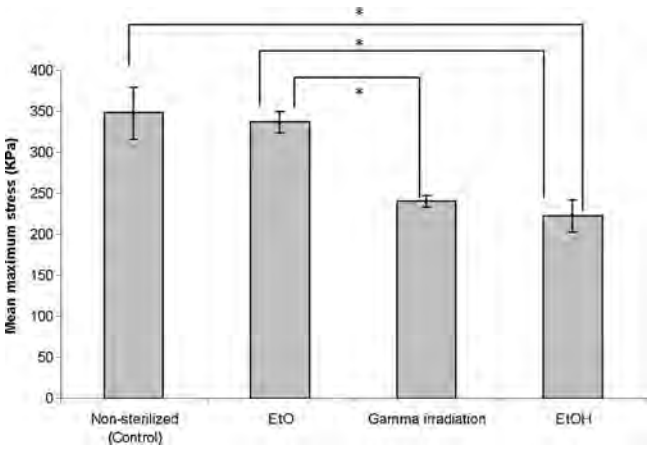

Fig. 7. The mean maximum stress resistance of the sericin scaffolds before and after sterilization treatment. *$p<0.05$ significant differences

the scaffolds was observed within 12 h. The fractions of protein released from the untreated, gamma-irradiated and EtO-treated scaffolds were approximately 3.15%, 3.44%, and 2.69%, respectively, while only 1.58% of sericin was released from the ethanol-treated scaffold.

DISCUSSION

Sericin, a silk protein, can be successfully formed 3-D scaffolds after mixing with PVA and glycerin, which can be used in tissue engineering. However, all biomaterials have to be efficiently sterilized before use. The sterilization method must be carefully selected since it can induce undesirable changes to the characteristics of the biomaterial that may affect cell–material interactions. Since sericin is mainly protein, heat, and radiation can alter its tertiary structure, which may result in its characteristics being changed. To the best of our knowledge, the published literature contains no reports on the sterilization of protein-based scaffolds.

Our results indicated the significant effects of the sterilization process on sericin scaffolds. Sterilization effectiveness was determined qualitatively by the absence of signs of growth after a 48-h incubation period. Growth was indicated by a change in opacity of the culture medium. Ethanol treatment showed the least effective sterilization method for the sericin scaffold. This result may have been due to the fact that ethanol is not actually a sterilizing agent, although it is a good disinfectant (due to its dehydration action and protein coagulation effect, which destroys membranes and denatures proteins); this solvent does not act on the endospores of many bacteria (28). Because of this, ethanol should only have a limited use as a surface-sterilizing agent. EtO and gamma irradiation indicated a successful sterilization was achieved. However, gamma irradiation is superior to EtO during challenge test.

Dimensional changes, expressed as percentage changes in volume, were observed for all samples. Gamma irradiation and EtO treatment decreased their initial volume by approximately 10%, whereas the ethanol-treated scaffolds decreased by approximately 80% of their initial volume. The effect of ethanol and EtO treatment on the dimension of the scaffolds are similar to results reported by others (16). While the overall dimensional change of all of the sterilized samples decreased, their degradation profiles were distinct: as shown in the results, the ethanol-treated scaffolds shrank considerably during the sterilization process, but remained at about 60% of their initial volume for the entire duration of the degradation study. The gamma-irradiated samples did not change their dimensions significantly during the sterilization

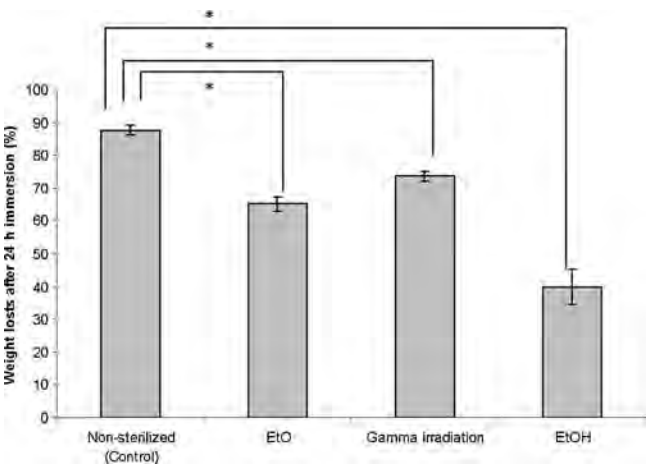

Fig. 6. Percentage of weight loss of sericin scaffolds before and after sterilization treatment, after 24 h immersion. *$p<0.05$ significant differences

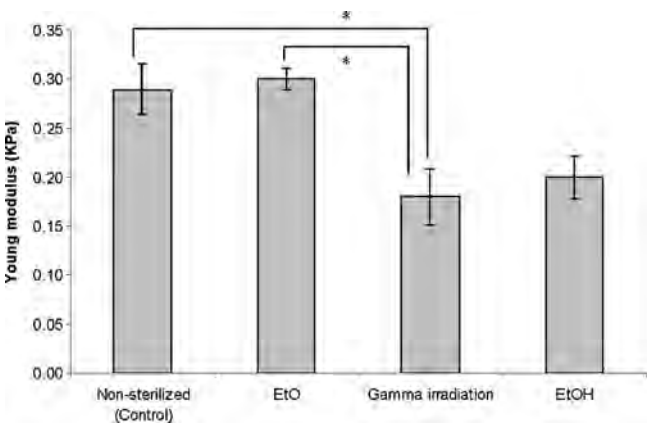

Fig. 8. Young's modulus of the sericin scaffolds before and after sterilization treatment. *$p<0.05$ indicates significant differences

Effect of Sterilization on Sericin Scaffolds

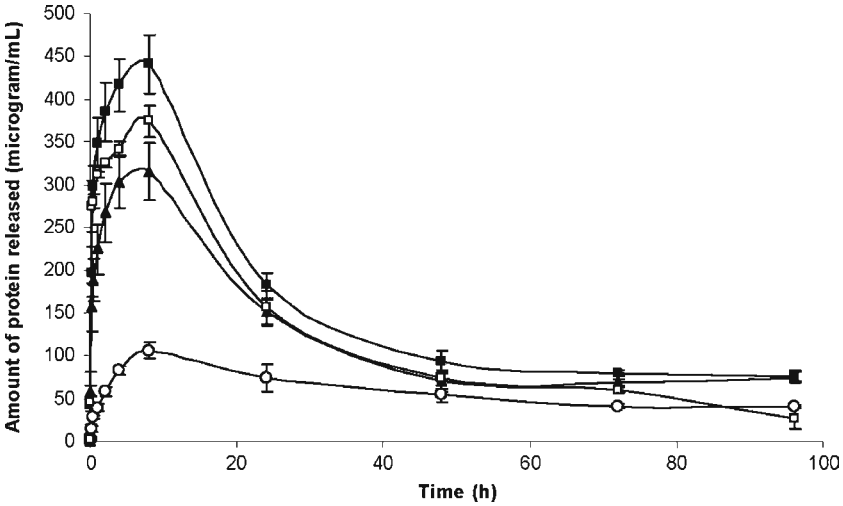

Fig. 9. The amount of sericin released from the scaffolds before and after sterilization: *unfilled squares* represents the untreated scaffold (control), *filled triangles* represents the EtO-treated scaffold, *filled squares* represents the gamma-irradiated scaffold, and *unfilled circles* represents the ethanol-treated scaffold

process, but they only remained at about 30% of their initial volume during the degradation study. Even though EtO and gamma irradiation were effective in the sterilization of the sericin scaffolds, the gamma-irradiated scaffolds are suitable only in low moisture environments in order to avoid its high degradation in the presence of water.

After being sterilized with EtO, gamma irradiation, and ethanol, the SEM images revealed that the sericin scaffolds responded to EtO and gamma irradiation in a similar manner, whereas the ethanol treatment produced significant effects on the scaffold. However, the physical appearance of the ethanol-treated scaffold did not show any imperfections, only a few surface wrinkles. After exposure to gamma irradiation and EtO, no significant differences were observed from before sterilization. The SEM and pore size measurements indicate that the ethanol treatment significantly reduced the pore size of the sericin scaffolds. However, the pore sizes of all of the sericin scaffolds in this study still remained within the range of 20–50 μm, which is suitable for the proliferation of keratinocytes (29).

The swelling property of a scaffold is essential for effective wound healing since it confers high exudates absorption capacity. However, the scaffolds should be stable and intact after moisture absorption. The reduced percentage weight loss of all of the treated samples indicated that the EtO, gamma irradiation, and ethanol also have an effect on the cross-linking or structure of sericin and results in more intact structures compared to the untreated samples. Even though most of the scaffolds showed a considerable weight loss after immersion for 24 h, in clinical applications, the moisture contained in wounds will not be a significant amount and the sericin scaffold should remain intact.

The mechanical properties of the scaffolds were influenced by the tested sterilization methods. Tensile strength was, in all cases, reduced after sterilization. The maximum stress resistance of all samples showed statistically significant losses when compared to the untreated scaffolds. The mechanical properties of the gamma-irradiated and ethanol-treated samples were quite similar: both scaffolds should be more flexible than the EtO-treated and untreated samples, but at the same time, they might break easily after receiving a certain amount of stretch. The results found here differ from those reported by others, which indicated that gamma irradiation reduced chitin-glycerol membrane elasticity significantly (28). The data of the present study confirmed that the effect of sterilization methods on physical properties, especially mechanical strength, may be different in various materials.

From our results, the sericin scaffold is highly elastic and flexible, which should be useful in wound care. Nevertheless, the low Young's modulus of the sericin scaffold may limit its use in tissue engineering: this similar mechanical property was found in other scaffolds such as the fibroin/hyaluronic acid composite and the chitosan scaffold, which can be used for neural tissue engineering and dermal substitution (30,31), but they are not suitable for bone tissue engineering.

Sericin can promote wound healing via activating collagen production: as the low levels of sericin released from the scaffold can be beneficial but at the same time, the matrix should also be stable. In all cases, protein release from scaffolds occurred simultaneously with mass loss and all of the scaffolds released sericin in a time-dependent manner. The release of PVA also occurred simultaneously with sericin as shown in previous study (12). The gamma-irradiated scaffold released the highest amount of sericin, which was even higher than that released from the untreated sample. This may have been due to a decrease in the molecular weight of the sericin after exposure to gamma irradiation, which is similar to results found in bovine serum albumin (32). The hydrolysate or smaller molecular weight of sericin can also support cellular viability especially in the cellular-damaged condition (33) and still promote collagen production as shown in heat-degraded sericin (34). The decrease in molecular weight, or less aggregation, allows protein to become released easily, which is another advantage of the gamma irradiation method for the sterilization of sericin scaffolds.

In contrast, ethanol released the smallest amount of sericin from the scaffold and exhibited a more intact

structure. The shrinkage and smaller pore size in the ethanol-treated scaffold indicated that there is a high entrapment of sericin itself and, with other components such as PVA in the scaffold, these result in less sericin being available for release.

Knowledge of the effects of sterilization allows the incorporation of these effects into the scaffold design. Some sterilization methods may show advantages or disadvantages compared to others. However, due to the delicate nature of sericin and the required degradability of tissue-engineered scaffolds, all factors related to their physical and biological properties after the sterilization process need to be considered.

CONCLUSIONS

Our results have indicated that sterilization processes have significant effects on the sericin scaffolds. Both gamma irradiation and EtO successfully reduced the chances of the sample becoming infected, but gamma irradiation is superior to EtO since it can sterilize scaffold for longer period of time especially in highly contaminated condition, whereas the ethanol treatment was not suitable for sterilizing this protein-based scaffold. Gamma irradiation and EtO did not cause any difference in the pore size or dimensions of the scaffold, whereas ethanol significantly reduced both pore size and the dimensions, as shown by the volume changes. With regard to the swelling property, the gamma-irradiated scaffold had the highest ability to absorb moisture while the ethanol-treated scaffold absorbed the least, but it remained the most intact scaffold after immersion in water for 24 h. The mechanical properties of gamma-irradiated and ethanol-treated scaffolds seem to be appropriate for further applications since they showed more flexibility. The highest amount of sericin released from a scaffold was found in the gamma-irradiated samples: this will be beneficial for activating collagen formation in wounds. While the results showed that none of the sterilization methods are ideal in that all methods caused some changes to the structural and physical properties, gamma irradiation should be the most appropriate procedure for sterilizing the sericin scaffolds for use in the tissue-engineering field.

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Formulation and characterization of silk sericin–PVA scaffold crosslinked with genipin

Pornanong Aramwit[a,*], Tippawan Siritientong[a], Sorada Kanokpanont[b], Teerapol Srichana[c]

[a] Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Payathai Road, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand
[c] Drug Delivery System Excellence Center and Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla, Thailand

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ABSTRACT

A porous-three-dimensional scaffold shows several advantages in terms of tissue engineering since it can provide a framework for cells to attach, proliferate and form an extracellular matrix. Sericin, a by-product from the silk industry, can form a three-dimensional scaffold with PVA after freeze-drying but has a fragile structure. Glycerin (as a plasticizer) and genipin (a crosslinking agent) are necessary to make a strong and stable matrix. Our objective was to investigate the properties of a three-dimensional silk sericin and PVA scaffold with and without glycerin and genipin at various concentrations. SEM showed that adding glycerin into scaffold gave better uniformity and porosity. Smaller pore sizes and better uniformity were found as the concentration of genipin in the scaffold increased. The results of FTIR indicated that glycerin retained a high moisture content and had a major effect at 3286 cm^{-1}, indicating the presence of water molecule in the matrix structure. Adding genipin into the scaffold resulted in a higher degree of crosslinking or fewer free ϵ-amino groups, as shown by the decrease in the stretching (=C–H) peak and absorption peaks around 1370–1650 cm^{-1}, respectively. The sericin/PVA scaffold had a low water sorption capacity, but adding glycerin significantly increased this property. Genipin further enhanced the moisture absorption capacity of the scaffold and extended the time taken to reach equilibrium. After immersing the sericin/PVA scaffold into purified water, the scaffold completely dissolved within an hour, whereas the scaffolds containing glycerin or glycerin with 0.1% genipin swelled 8 and 11 times, respectively, compared with the initial stage after 6 h of immersion. In terms of mechanical properties, the sericin/PVA/glycerin scaffold exhibited a similar compressive strength to the scaffold with a high genipin concentration, whereas a low concentration of genipin softened and reduced the compressive strength of the scaffold. A small amount of sericin was released from the scaffold and a higher concentration of genipin, resulting in less protein leaching compared to non-crosslinked sericin/PVA. The fraction of protein released from the sericin/PVA/glycerin scaffold was about 4%, with values of about 1 and 0.04% in the case of scaffolds with 0.01 and 0.1% genipin, respectively. All results indicated that the composition of the scaffolds had a significant effect on their physical properties, and that can easily be tuned to obtain scaffolds suitable for biological applications.

1. Introduction

Accidental damage to the epidermis by ulcers, burns or other traumatic incidents may result in a series of morbid consequences that restrict epidermal regeneration. In the case of wounds that extend entirely through the dermis, skin substitutes such as xenografts, allografts and autografts need to be employed for wound healing. The challenge of designing substrates that will allow specific biological interactions is demanding, particularly in the case of tissue engineered skin substitutes. Natural biomaterials such as collagen, silk and chitosan have received increasing attention in the field of biomedical engineering due to their unique properties, including non-toxicity, biodegradability and biocompatibility [1,2]. Porous-three-dimensional scaffolds that can provide a framework for cells to attach, proliferate and form their extracellular matrix play an important role in manipulating cell functions in this approach [3]. Since a suitable scaffold should possess the specific structure of the tissue it replaces and must be capable in turn of being replaced in time via the ingress of new cells [4], the choice of material is of prime concern [5]. However, natural biomaterials themselves are normally unable to meet all the requirements of their applications. Polymer blending is a useful technique for modifying the properties of a single polymer.

* Corresponding author. Tel.: +66 089 921 7255; fax: +66 02 218 8403.
E-mail address: aramwit@gmail.com (P. Aramwit).

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We have previously reported that silk sericin, a natural hydrophilic polymer extracted from silk cocoons during the degumming process, is non-toxic to fibroblast cells [6] and enhances wound healing by promoting collagen production in wounds [7]. Sericin is mainly comprised of serine and aspartic acid with strong polar side chains, thus enabling easy copolymerization and blending with other polymers to produce biocompatible materials with improved properties [8,9]. Earlier reports claimed that sericin was responsible for an immune response; however, few studies have investigated the use of sericin in the tissue engineering field. The immune response of sericin was subsequently proven to be dependent on the physical association with fibroin-silk fibers [10], but sericin itself generates very low immune responses [11]. Sericin forms fragile materials that are not suitable for use in medical applications, but Mandal et al. demonstrated that after blending with gelatin, silk sericin can form a scaffold and be a good candidate for tissue engineering applications [5]. In this study, polyvinyl alcohol (PVA) (a synthetic polymer with good biocompatibility, low toxicity and good mechanical properties) was blended with sericin. A crosslinking process is also believed to improve the permeability as well as the mechanical properties of proteins [12]. For this purpose, naturally occurring genipin was used to crosslink sericin. Genipin is found in traditional Chinese medicine and is extracted from gardenia fruit [13]. It is an effective naturally occurring crosslinking agent that can react with amino acids or proteins containing residues with primary amine groups such as lysine, hydroxylysine or arginine [14,15]. Sung et al. investigated the cytotoxicity, feasibility and biocompatibility of genipin for tissue fixation and found that genipin was 10,000 times less cytotoxic than the commonly used glutaraldehyde [16]. In addition, the treatment of animal wounds by genipin-crosslinked glue induced significantly lower inflammatory responses and more rapid recovery than those treated by aldehyde-crosslinked glues [16,17].

Glycerin, a commonly used plasticizer, has previously been mixed to improve silk film properties [18] and also helps to reduce phase separation between silk and PVA in the blend [19]. Glycerin content in blend films is important for the control of silk secondary structural transitions and influencing the mechanical properties of the films [20]. After mixing with silk, glycerin molecules interact with silk chains via intermolecular forces, most likely hydrogen bonds between hydroxyl groups of glycerin and amide groups of silk [19].

The purpose of this study was to investigate the properties of three-dimensional silk sericin and PVA scaffolds crosslinked with genipin at various concentrations that will have potential in tissue engineering due to their low toxicity.

2. Materials and methods

2.1. Preparation of three-dimensional silk sericin and PVA scaffold

The fresh, white-shell cocoons of *Bombyx mori* were kindly supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). Silkworm cocoons were produced in a controlled environment. After cutting the cocoons into pieces (about 5 mm^2), silk sericin was extracted using a high temperature and pressure degumming technique [21]. In brief, the silkworm cocoons were mixed with purified water (1 g of dry silk cocoon: 30 mL of water) and the samples were autoclaved (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120 °C for 60 min. After filtration through a membrane to remove fibroin, the sericin solution was concentrated until the desired concentration (approximately 7% (w/v)) measured by BCA Protein Assay Reagent, Pierce, Rockford, IL, USA) was achieved.

PVA (Ajax Finechem, New South Wales, Australia, molecular weight 77,000–82,000) was dissolved at 80 °C with constant stirring for about 4 h until it was completely dissolved to a concentration of 6% (w/v). Genipin was dissolved in ethyl alcohol to give a solution at a concentration of 20% (w/v). Sericin solution and PVA solution with and without glycerin were blended together at room temperature for at least 30 min to make a final wet composition of 3% (w/v) sericin, 2% (w/v) PVA and 1% (w/v) glycerin. Genipin solution at different concentrations (0.01–0.1% (w/v)) was added to the mixed solution of sericin, PVA and glycerin and stirred for 5 min, which was then poured into a petri-dish and frozen at −20 °C, followed by lyophilization (Heto LL 3000 lyophilizer, Allerod, Denmark) for 72 h. After drying, the scaffolds were accurately weighed to calculate the percentage of each component in dry weight basis.

2.2. Amino acid analysis

The amino acid composition of SS was measured by an amino acid analyzer (Hitachi L-8500A, Tokyo, Japan). Samples for amino acid analysis were hydrolyzed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) at 100 °C for 24 h under vacuum. All experiments were done in triplicate.

2.3. Scanning electron microscopy (SEM) of scaffolds

Samples of silk sericin/PVA and silk sericin/PVA with glycerin and genipin at various concentrations were cut into pieces and mounted onto aluminum stubs and sputter coated with gold at 10–20 nm thickness. Observations were performed with a JSM-5800LV scanning electron microscope (JEOL, Tokyo, Japan) at 15 keV.

2.4. Pore size measurement of scaffolds

Pore size was measured using a modified method from Kang et al. [22]. The major and minor diameters of each scaffold were measured using a stereo microscope equipped with an optical micrometer. The pore size was calculated as the geometric mean of the major and minor diameters, respectively. At least 100 pores were assessed and the values presented indicate the mean ± standard deviation.

2.5. Fourier transform infrared spectroscopy (FTIR) of scaffolds

FTIR spectra were obtained using a Spectrum One (Perkin–Elmer, MA, USA) spectrophotometer. The sample was ground into appropriate size particles and a small amount of sample was sealed into a KBr pellet (thickness less than 0.5 mm) by a hydraulic press prior to measurement at ambient temperature. Spectra were obtained at 4 cm^{-1} resolution, under a dry air purge, with the accumulation of 16 scans. The IR spectra of all scaffolds were recorded within the range of 4000–400 cm^{-1}. The spectrum of the KBr disc was subtracted from each sample spectrum.

2.6. Degree of crosslinking

The degree of crosslinking was determined using a modified method from Bubnis et al. [23]. The free amino groups of sericin were reacted with 2,4,6-trinitrobenzene sulfonic acid (TNBS). The absorbance of the product from the TNBS reaction was detected by UV spectroscopy. The relative degree of crosslinking was obtained from the differences between the absorbance values of non-crosslinked and crosslinked scaffolds. Briefly, about 5 mg of the crosslinked scaffolds were weighed into a test tube into which 1 ml of 0.5% TNBS solution and 1 ml of 4% sodium hydrogen carbonate ($NaHCO_3$, pH 8.5) were added. This was then heated in a water bath maintained at 40 °C for 2 h. The non-crosslinked primary amino

groups in the scaffolds reacted with TNBS and formed a soluble complex. This solution was further treated with 2 ml of 12.24N HCl at 70 °C for 2 h. The absorbance of the solutions was determined at 415 nm after suitable dilution spectrophotometrically. The degree of crosslinking was then calculated by the following equation, in comparison with the non-crosslinked samples:

$$\text{Degree of crosslinking}\,(\%) = \frac{1 - \text{Abs of crosslinked scaffold}}{\text{Abs of non-crosslinked scaffold}} \times 100$$

The experiment was performed in triplicate and values were expressed as the mean ± standard deviation.

2.7. Moisture absorption

The moisture absorption capacity of the silk sericin and PVA scaffolds was determined by placing the scaffolds into desiccators in which the relative humidity was controlled by salt solutions. Potassium chloride solution was used to obtain a relative humidity of 81.47 ± 1.48% at 25 °C [24]. Samples were removed from the desiccators after 0.5–120 h and carefully weighed. The moisture absorption capacity was calculated as the percentage of weight change compared to the initial weight. All experiments were performed in triplicate.

2.8. Swelling properties

Swelling studies were carried out according to Mandal et al. [5] with slight modifications. Briefly, the lyophilized scaffolds were accurately weighed in the dry state and then immersed in 10 mL of purified water. At various time intervals, the scaffolds were carefully removed and the amount of water contained in the scaffolds was determined precisely by weighing them in the swollen state. The experiments were performed in triplicate under the same conditions. The percentage swelling of the scaffolds at equilibrium was calculated using the following equation:

$$\%\,\text{swelling} = \frac{W_t - W_0}{W_0} \times 100$$

where W_0 is the weight of the dried test sample and W_t is the weight of the swollen test sample.

2.9. Mechanical properties of scaffolds

Compression tests were performed on flat samples of diameter 15 mm and thickness 8 mm with an Instron model 4301 instrument (Instron, Canton, MA), at a cross-head rate of 1 mm/min. Tangent modulus (E), stress and strain at the yield point (σ^* and ε^*), and the stress at 10% strain ($\sigma_{10\%}$) were obtained from the σ/ε compression curve. All experiments were performed in triplicate.

2.10. The release of sericin from sericin scaffolds

The release profile of sericin from scaffolds was plotted after placing the scaffold samples (diameter 35 mm) into PBS (pH 7.4) at room temperature with continuous stirring in a closed-container. The samples (1.5 mL) were taken out at different time points 0, 1, 15, 30 min, 1, 2 and 3 days, and the amount of sericin was measured using a BCA protein assay kit (Pierce, Rockford, IL). Briefly, the leached protein samples were collected, mixed with BCA reagents and vortexed. The absorbance was measured at 562 nm and the amount of protein released was compared with a bovine serum albumin standard curve. All experiments were performed in triplicate.

Table 1
Amino acid compositions of silk sericin.

Amino acid	Molar percent ± SD
Asp	15.64 ± 1.62
Thr	8.16 ± 0.62
Ser	33.63 ± 2.23
Glu	4.61 ± 0.35
Gly	15.03 ± 1.44
Ala	4.10 ± 0.22
Cys	0.44 ± 0.12
Val	2.88 ± 0.98
Met	3.39 ± 0.64
Ile	0.56 ± 0.18
Leu	1.00 ± 0.48
Tyr	3.45 ± 0.39
Phe	0.28 ± 0.09
Lys	2.35 ± 0.89
His	1.06 ± 0.21
Arg	2.87 ± 0.66
Pro	0.54 ± 0.11

Values are means by triplicate analysis.

2.11. Statistical analysis

Data was expressed as the mean ± SD. The statistical significance was determined by paired and unpaired Student's *t*-tests together with ANOVA. A value of $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Amino acid composition of silk sericin

Table 1 indicates the amino acid compositions of silk sericin. Serine, aspartic acid and glycine count for more than 50% of the whole amino acid compositions, similar to the results reported by others [25].

3.2. Morphology of silk sericin and PVA scaffold

Mixing sericin and PVA aqueous solution with or without glycerin can obtain a homogeneous mixture. It is expected that PVA only physically incorporated with sericin similar to the previous results of gelatin and PVA [26]. Liu et al. reported that gelatin had no significant effect on the thermal behavior of PVA, which indicated that no substantial change occurred in the PVA crystallite due to the presence of gelatin [26]. Genipin did not cause gel formation or significant increase in viscosity of sericin/PVA and glycerin solution (the viscosity of sericin/PVA/glycerin and sericin/PVA/glycerin with genipin solution were <0.3 dPa s). Scaffold composed of various concentrations of sericin or PVA, both ranging from 1 to 5% (w/v), were observed for their physical properties. The most suitable concentration of sericin and PVA which gave homogenous and stable matrix was sericin, PVA and glycerin at concentration 3, 2 and 1% (w/v) of wet weight basis, respectively (data not shown). It can easily form a scaffold after freeze-drying and appears as a smooth and homogenous material. After freeze-drying, final weight of the scaffold did not show significant difference compared with theoretical weight. From that result, various scaffolds composed of sericin (3% (w/v))/PVA (2% (w/v))/glycerin (1% (w/v)) and genipin at different concentrations were obtained. Without genipin, both sericin/PVA and sericin/PVA/glycerin scaffolds appeared off-white in color, which is the natural color of the silk cocoon. Genipin changed the color of the scaffold to pale blue (at a low concentration, 0.01%) and dark blue (at a high concentration, 0.1%) due to its own natural color. The sericin/PVA scaffold was rigid and less flexible compared to the scaffold composed of glycerin and genipin. Fig. 1A shows SEM images of a cross section of the sericin/PVA scaffold; the pores were not highly interconnected, both open and closed pore structures

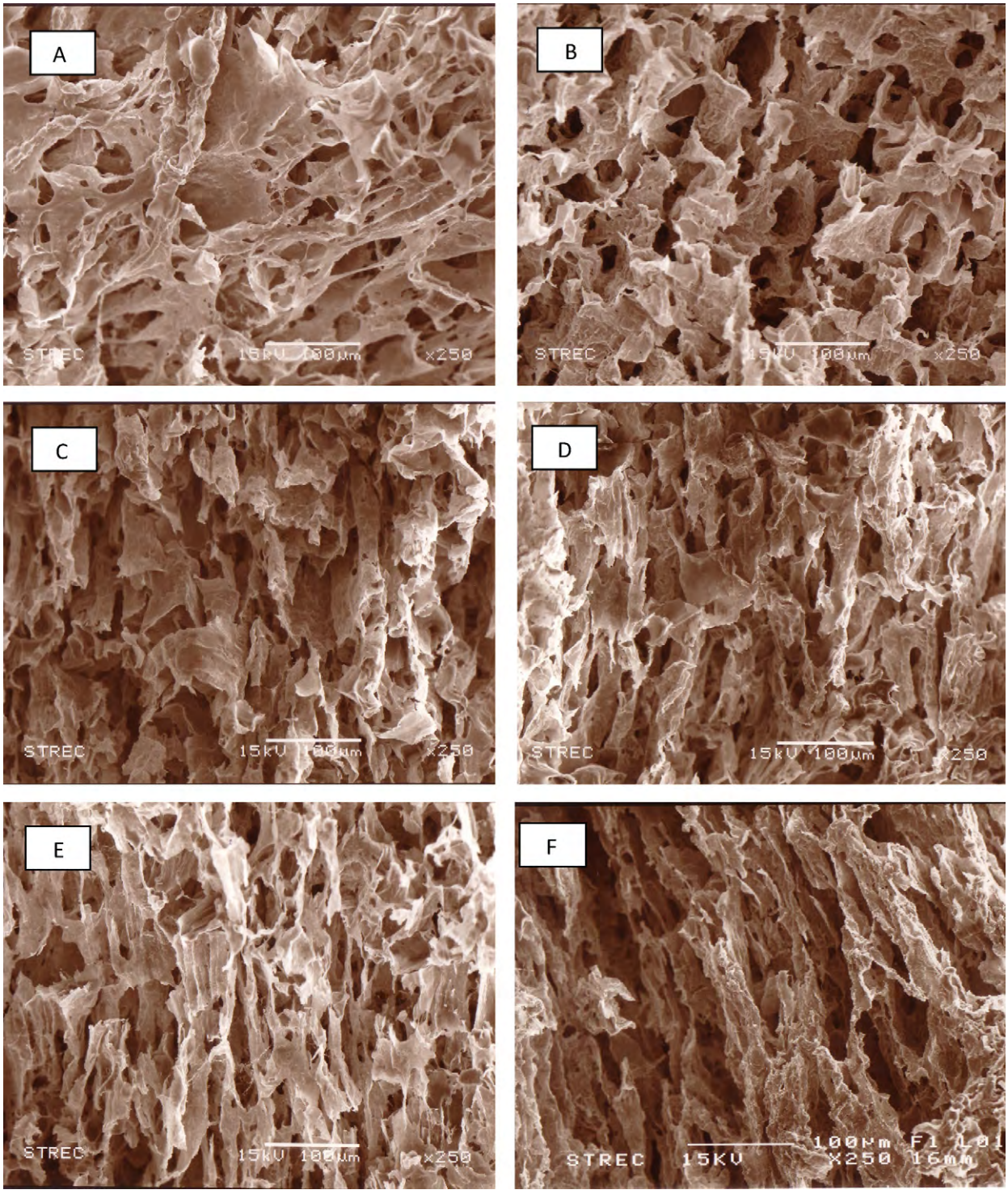

Fig. 1. SEM images of cross-section scaffold. A = sericin/PVA scaffold, B = sericin/PVA/glycerin scaffold, C = sericin/PVA/glycerin with 0.01% genipin scaffold, D = sericin/PVA/glycerin with 0.025% genipin scaffold, E = sericin/PVA/glycerin with 0.075% genipin scaffold, F = sericin/PVA/glycerin with 0.1% genipin scaffold.

were observed and the pore distribution was heterogeneous. However, better uniformity and porosity were observed in freeze-dried silk sericin/PVA/glycerin blended scaffolds (Fig. 1B). Different blend compositions ranging from 0.01 to 0.1% genipin were examined, with scaffolds showing smaller pore sizes and better uniformity as the genipin concentration increased (Fig. 1C–F). Table 2 exhibits the pore size distribution of sericin scaffolds. The sericin/PVA scaffold had a high pore size variation compared with the other types of scaffold while the sericin/PVA/glycerin scaffold exhibited smaller pore sizes and better uniformity compared with the sericin/PVA

Table 2
Pore size of sericin scaffolds ($n = 100$).

Scaffold compositions	Mean pore size ± SD (μm)
Sericin/PVA	39.13 ± 23.73
Sericin/PVA/glycerin	29.40 ± 12.60
Sericin/PVA/glycerin/0.01% genipin	48.19 ± 17.89
Sericin/PVA/glycerin/0.025% genipin	45.69 ± 15.38
Sericin/PVA/glycerin/0.05% genipin	40.87 ± 13.95
Sericin/PVA/glycerin/0.075% genipin	34.69 ± 13.87
Sericin/PVA/glycerin/0.1% genipin	33.63 ± 12.88

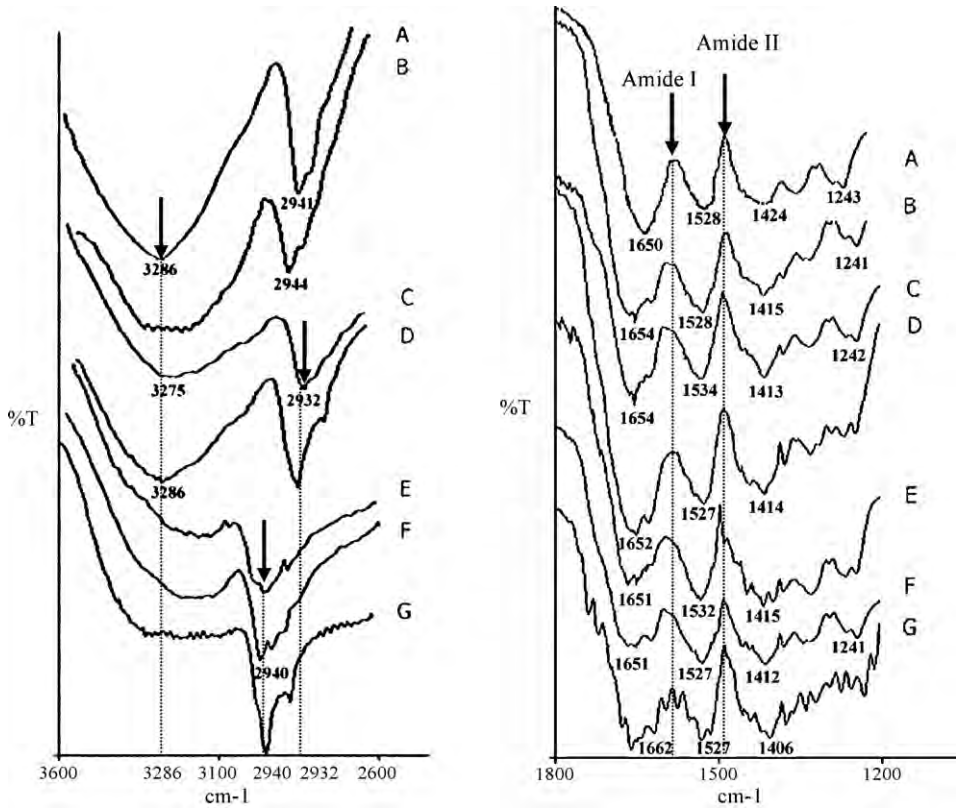

Fig. 2. FTIR spectra of sericin/PVA scaffold with and without glycerin and different concentrations of genipin. A represents sericin/PVA scaffold, B represents sericin/PVA/glycerin scaffold, C–G represent sericin/PVA/glycerin scaffold with 0.01, 0.025, 0.05, 0.075, 0.1% genipin, respectively.

scaffold. Adding genipin into the scaffolds resulted in an increase in the mean pore size which is an unexpected result. However, the size of the porous diameter decreased and uniformity increased following an increase in genipin concentration similar to the results of collagen crosslinking reported by Castaneda et al. [27]. All scaffolds were highly porous, which is quite suitable in terms of their use as tissue engineering material [3,28].

3.3. FTIR spectra

FTIR was used to confirm the secondary structural transition of dry sericin films. Fig. 2 represents the FTIR spectra of sericin/PVA scaffold with or without glycerin and various concentrations of genipin. The protein conformation was determined by identifying the peak positions of amide I, II and III corresponding to C=O, N–H and C–N stretching, respectively. As shown in Fig. 2, all scaffolds exhibited amide I and II peaks at around 1630 and 1520 cm^{-1}, and an amide III peak at 1230 cm^{-1}. The amide II band in Fig. 2 (B–G) in samples containing glycerin has a reduced intensity compared with that of the sericin/PVA scaffold, similar to the results reported by Gillgren et al. [29]. Generally, the amide II band responds to differences in the hydrogen bonding environment [30]. Thus, glycerin presumably has an effect on the hydrogen bonding of the amide groups such that amide–amide interactions are reduced by an increase in amide–plasticizer interactions.

The changes in the amide I band were similar in size to those of the amide II band. In the presence of glycerin, the intensity of the amide I band decreased. These results indicate that glycerin enhances the formation of α helical forms [29]. Furthermore, it can be assumed that the effect of glycerin is equally strong on the amide I band structure and the amide II band, similar to the results obtained with glycerin and zein, a cereal storage protein [29]. When 1% glycerin was added to the sericin/PVA scaffold as a plasticizer, the main change was the appearance of a broad peak at 3286 cm^{-1}, indicating that water was present in the sample. This was due to the moisture sorption properties of the glycerin plasticizer.

After adding different concentrations of genipin as a crosslinking agent to the sericin/PVA scaffold in the presence of glycerin, it was evident that the stretching (=C–H) peak and absorption peaks around 1370–1650 cm^{-1} diminished accordingly. This may be explained by the crosslinking effect to double bond and amides. These data indicated that adding the plasticizer and crosslinking agent drastically changed the molecular structures of sericin, providing it with its scaffold properties.

3.4. Degree of crosslinking

TNBS has been used as a UV chromophore in various procedures to determine primary amino groups in peptides and proteins [23]. Fig. 3 shows the percentage of crosslinks in the sericin/PVA/glycerin scaffolds with various compositions of genipin from 0.01 to 0.1% compared with that of the sericin/PVA and sericin/PVA/glycerin scaffolds. Higher concentrations of genipin in the scaffold resulted in a higher degree of crosslinking or fewer free ∈-amino groups. The addition of 0.1% genipin to the scaffold increased the degree of crosslinking by approximately 30% compared with the sericin/PVA/glycerin scaffold, and up to 80% when compared with the sericin/PVA scaffold alone. Genipin at 0.01% concentration showed significant difference in degree of crosslinking when compared with the scaffold composed of 0.075 and 0.1% genipin. The crosslinking mechanism of genipin and sericin containing amine is not well understood. It is expected that the reaction occurs with amino acid lysine, hydroxylysine and arginine which contain in the primary amine side chain of sericin [14]. Touyama et al. proposed mechanism of a genipin with a methylamine [31]. The reaction occurred through a nucleophilic attack of the primary

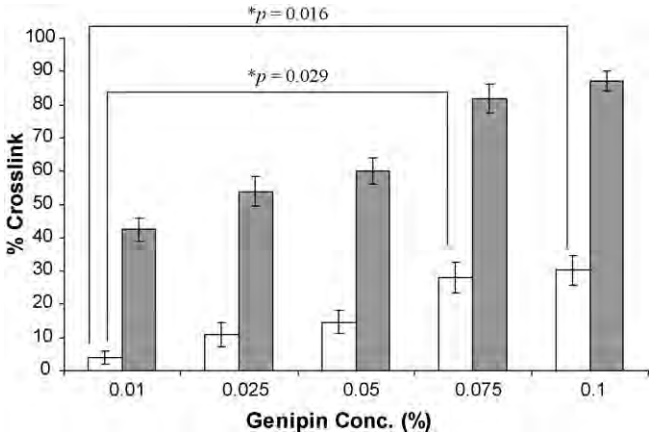

Fig. 3. Percentage of crosslinks in sericin/PVA/glycerin scaffold with various concentrations of genipin from 0.01 to 0.1% compared with crosslink that of the sericin/PVA and sericin/PVA/glycerin scaffolds, respectively. (□) indicates difference of percentage of crosslinks of sericin/PVA/glycerin + genipin with sericin/PVA/glycerin scaffold, (■) indicates difference of percentage of crosslinks of sericin/PVA/glycerin + genipin with sericin/PVA scaffold. * indicates significant differences at $p < 0.05$.

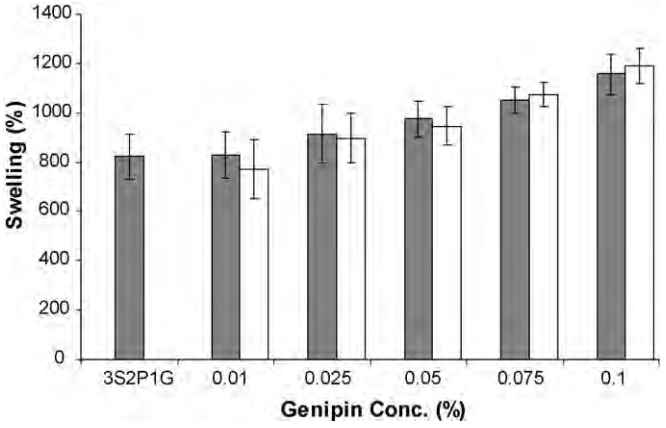

Fig. 5. The swelling of sericin/PVA scaffold with and without glycerin and various concentrations of genipin after immersion in water for 6 and 24 h. 3S2P1G = sericin/PVA/glycerin scaffold, 0.01–0.1% = sericin/PVA/glycerin with 0.01–0.1% genipin scaffold, respectively. (■) indicates percentage of swelling after 6 h of immersion, (□) indicates percentage of swelling after 24 h of immersion.

amine on the C_3 carbon of genipin. This caused an opening of the dihydropyran ring. An attack on the resulting aldehyde group by the secondary amine then followed. The final step in the formation of crosslinking is believed to be the dimerization produced by radical reactions. This indicates that genipin can form both intramolecular and intermolecular crosslinks. Glycerin can enhance the crosslinking in the sericin/PVA scaffold, similar to the result reported by Brault et al., which indicated that plasticizers such as glycerin can significantly enhance the formation of crosslinks within caseinates (milk proteins chains) [32]. This effect was explained by the preferential binding concept described by Gekko and Timasheff [33]. Similar behaviors were observed with other plasticizer such as propylene glycol and triethylene glycol [34]. Our results further indicated that genipin can effectively crosslink sericin, as in other proteins such as collagen, gelatin and casein [35–37].

3.5. Moisture absorption

Fig. 4 shows the percentage weight change of the scaffolds after placing them in a high humidity environment. The results indicated that the sericin/PVA scaffold had the lowest ability to absorb moisture, but that adding glycerin significantly increased this ability. This may partly be due to the moisture absorption capacity of glycerin itself. After 24 h, sericin/PVA scaffold absorbed moisture significantly less compared with scaffolds composed of genipin ($p = 0.003$, 0.002, 0.002, 0.022 and 0.000 for the case of 0.01–0.1% genipin, respectively). Genipin also enhanced the moisture absorption capacity of the sericin scaffold and extended the time taken to reach equilibrium. The time required to attain equilibrium swelling was higher for the sericin/PVA/glycerin scaffold with genipin at a concentration between 0.01 and 0.075% compared with the sericin/PVA scaffold with and without glycerin. Without genipin, the moisture absorption capacity of the sericin/PVA and sericin/PVA/glycerin scaffold reached the equilibrium within 3 days while those containing genipin had not reached equilibrium even after 5 days. Genipin concentration of the scaffolds between 0.01 and 0.1% produced an approximately 10% difference in weight change from moisture absorption.

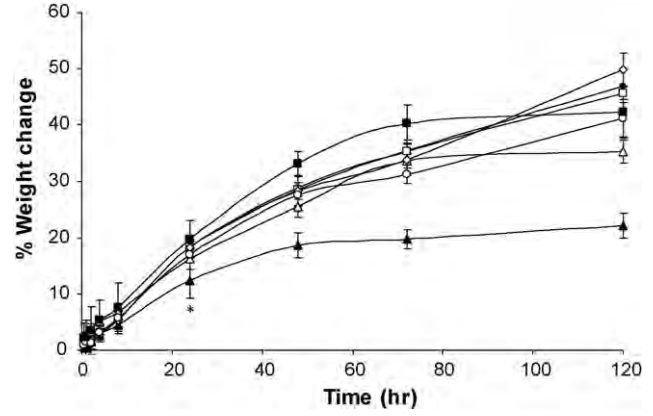

Fig. 4. The percentage weight change of sericin/PVA scaffold with and without glycerin and different concentrations of genipin after placing into high humidity (~80%) environment. (▲) represents sericin/PVA scaffold, (△) represents sericin/PVA/glycerin scaffold, (●)(□)(◊)(○) and (■) represent sericin/PVA/glycerin with 0.01, 0.025, 0.05, 0.075, 0.1% genipin scaffold, respectively. * indicates significant differences compared with sericin/PVA scaffold at $p < 0.05$.

Our results regarding the moisture absorption capacity of the sericin/PVA/glycerin scaffold with genipin as a crosslinking agent are similar to previously reported results [38]. Liu et al. found that increasing the degree of crosslinking by using genipin decreased the hydrophilicity of chitosan films which indicated that our scaffold composed of sericin/PVA/glycerin with 0.1% genipin (the highest degree of crosslink) should exhibit the lowest hydrophilicity. Since scaffold contained 0.1% genipin absorbed the highest amount of moisture (Fig. 4), it can be expected that the degree of hydrophilicity of the sericin scaffolds decreased with increasing moisture sorption capacity.

3.6. Swelling properties

The swelling of the sericin/PVA scaffold with and without glycerin and various concentrations of genipin after immersion in water for 6 and 24 h is shown in Fig. 5. The sericin/PVA scaffold was completely dissolved within 1 h. There was an 8-fold swelling of the sericin/PVA/glycerin scaffold compared with the initial weight after 6 h immersion and this scaffold was completely dissolved within 24 h. The swelling of sericin/PVA/glycerin with genipin increased over a period of time and was directly related to the percentage weight of genipin added to the scaffold base. At 0.1% genipin, the swelling after 6 and 24 h immersion was about 11 and 12 times that of the initial stage, respectively. However, no significant difference in swelling properties of sericin/PVA and sericin/PVA/glycerin scaf-

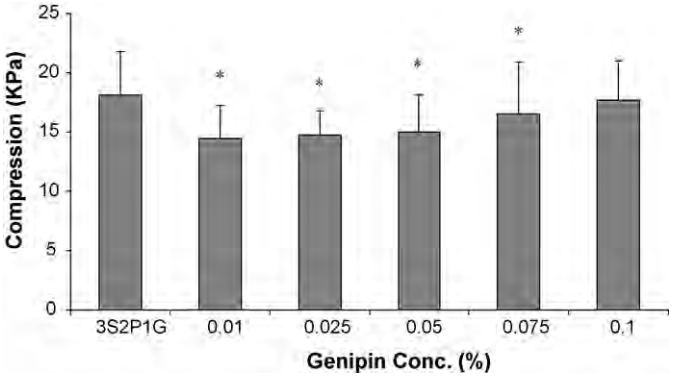

Fig. 6. The mechanical integrity of sericin/PVA/glycerin scaffold with different concentrations of genipin. Data represents mean ± SD (n = 3). 3S2P1G = sericin/PVA/glycerin scaffold, 0.01–0.1% = sericin/PVA/glycerin with 0.01–0.1% genipin scaffold, respectively. * indicates significant differences compared with 3S2P1G at $p < 0.05$.

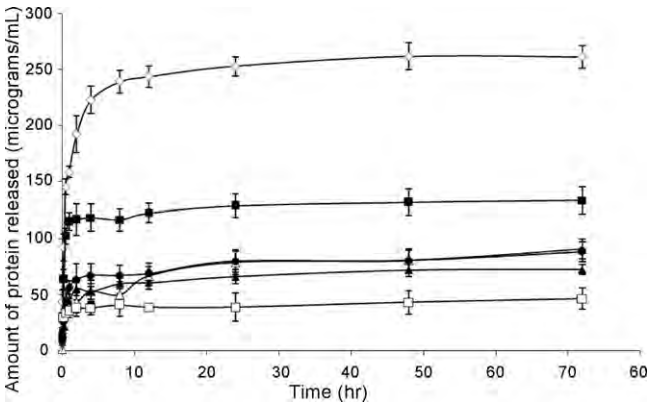

Fig. 7. The amount of protein released from the scaffolds. (◊) represents sericin/PVA/glycerin scaffold, (■)(△)(●)(▲)(□) represent sericin/PVA/glycerin with 0.01, 0.025, 0.05, 0.075 and 0.1% genipin scaffolds, respectively.

fold compared with scaffolds composed of genipin. Our finding is similar to the result reported by Chiono et al., which indicated that increasing the concentration of genipin led to greater swelling and degrees of dissolution of chitosan films [39]. A higher degree of genipin oligomerization resulted in a porous network with higher swelling properties [40]. The longer equilibrated moisture absorption time (Fig. 4) resulted in the higher swelling ratio (Fig. 5). This may be due to the flexible structure of the scaffold containing genipin, which was characterized by slow water sorption but a high water holding capacity, similar to that observed for mucin discs by Builders et al. [41]. The swelling properties at 6 and 24 h were not significantly different, because the three-dimensional scaffold allows its total surface area to interact with the water molecules during the initial swelling.

Our swelling results indicated that adding glycerin alone to the sericin/PVA scaffold is not enough to make scaffolds that are stable in an aqueous solution for 24 h. Genipin or other crosslinking agents are necessary in order to provide solid material suitable for biological applications.

3.7. Mechanical properties of scaffolds

Fig. 6 demonstrates the mechanical integrity of the sericin/PVA/glycerin scaffold with different concentrations of genipin. Without genipin, the scaffold exhibited greater compressive strength, similar to the scaffold with a high genipin concentration (0.1%). At a low concentration, genipin may soften and reduce the compressive strength of the scaffold but not to a significant level, while higher concentrations of genipin enhanced mechanical strength. Since silk sericin is amorphous in nature, the sericin scaffold normally has low mechanical strength. However, a plasticizer such as glycerin can increase the moisture content in the scaffold, it enhances the formation of crosslinks as also shown in other biopolymers [42]. Genipin can also increase the compressive strength of the scaffold due to its impact and hard structures. Our results showed that both plasticizer and crosslinking agent greatly improved the physical properties of the sericin/PVA scaffold. The concentration of genipin had a significant effect on the scaffold's characteristics. With regards to the physical appearance, glycerin greatly improved the flexibility of the sericin/PVA scaffold. Sericin/PVA/glycerin scaffold showed significant greater compressive strength compared with the scaffolds composed of 0.01–0.075% genipin (p = 0.003, 0.012, 0.016 and 0.012 for the case of 0.01–0.075% genipin, respectively). However, no significant difference in compressive strength of sericin/PVA/glycerin scaffold compared with the scaffolds composed of 0.1% genipin. Our findings suggested that glycerin or other plasticizers as well as the optimum concentration of genipin are required to provide the integrity and mechanical strength for the use of sericin in future applications.

According to the extremely high swelling property and very low compressive modulus of sericin/PVA/glycerin with genipin scaffold in the dry state, it indicates that this scaffold has high porosity with low density. This type of material is highly elastic and flexible which should be useful in fabrication of wound dressing. However, in this case the compressive modulus may still be even lower in the wet state, similar to the results found in others [43,44]. The low compressive modulus of sericin/PVA/glycerin with genipin scaffold may limit its use in tissue engineering, this similar mechanical property has been found in other scaffolds such as fibroin/hyaluronic acid composite or chitosan scaffold which could be used for neural tissue engineering or dermal substitution [45,46], but not suitable for bone tissue engineering.

3.8. Release of sericin from scaffolds

Fig. 7 demonstrates the amount of protein released from the scaffolds. The sericin/PVA scaffold completely dissolved and released all sericin in less than 30 min (data not shown). Fig. 7 shows that the sericin/PVA/glycerin scaffold without genipin released the highest amount of sericin, while higher genipin concentration led to the release of a lower amount of protein. Maximum protein leaching from all scaffolds was observed within 48 h. The fraction of protein released from the sericin/PVA/glycerin scaffold was approximately 4%, with values of about 1.03 and 0.04% in the case of scaffolds with 0.01 and 0.1% genipin, respectively.

As sericin can activate collagen production in wounds, low levels of sericin released from the scaffold will be beneficial and, at the same time, the matrix should also be stable. Even though the sericin/PVA scaffold released large amounts of sericin, the structure was completely degraded after immersion for a few hours. Since free sericin molecules that remain non-crosslinked contribute to the leached-out protein fraction, the sericin/PVA/glycerin scaffold that had the lowest degree of crosslinking compared to the scaffold with genipin exhibited higher sericin release, resulting in structural collapsed, which makes it unsuitable for further application. Adding genipin to the scaffold led to lower sericin release and a more intact structure. However, the fraction of protein released from the scaffold was quite low, with a maximum of about 4% in the scaffold without the crosslinking agent, while scaffolds with genipin released an even smaller amount of protein. Mandal et al. reported that these scaffolds can easily be tuned by varying their compositions to obtain the desired level of sericin release, which may be

significant in terms of wound healing and tissue engineering [5]. Due to its hydrophilic structure, PVA was also released from scaffolds, as monitored by thin layer chromatography–densitometric method after immersion for 24 and 48 h, respectively. Briefly, silica gel was used as a stationary phase and methanol–water mixture (50%) was used as a mobile phase. The R_f value of 0.8 was a spot of PVA and detected by densitometric method. The result indicated that 66–70% of PVA (mean $68.3 \pm 1.5\%$, $n = 3$) was released from sericin/PVA/glycerin with 0.05% genipin scaffold after 24 h water immersion. The amount of PVA released was constant even monitoring at a longer period of immersion (48 h) which indicates that the scaffold cannot release more PVA. While lower amount of PVA, approximately 33–40% (mean $36.7 \pm 2.6\%$, $n = 3$), was released from sericin/PVA/glycerin with 0.10% genipin scaffold under the same condition. The significant lower amount of PVA released from scaffold containing high concentration of genipin (higher degree of crosslink) may be due to the higher entrapment of PVA between sericin chain, resulting in less available amount of this polymer to be released ($p < 0.01$). Taking into account the high swelling and the amount of protein as well as PVA released, erosion might be the degradation behavior of sericin/PVA/glycerin scaffolds. Since small amount of sericin and some portions of PVA were released from scaffold, part of the scaffold structure still maintained and stable even after 48 h immersion.

4. Conclusion

Porous-three-dimensional sericin and PVA scaffolds can be formed but are fragile. Adding a plasticizer such as glycerin along with genipin as a crosslinking agent, markedly improved their properties. Uniform pore distribution, stable structures with good compressive strength, high swellability and the desired level of sericin release can be achieved by varying the concentration of the crosslinking agent. Sericin, which is considered as a waste product from the silk industry, can thus be modified to form a good candidate for biomaterial products. In term of tissue engineering application, sericin/PVA/glycerin scaffold with 0.1% genipin seems to be a good candidate since it shows good moisture absorption, high swelling degree and good mechanical strength. However, the amount of sericin released from scaffold with 0.1% genipin is much lower than others, biological tests needs to be performed in order to confirm its beneficial in tissue engineering applications.

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Article

In Vitro Evaluation of the Antimicrobial Effectiveness and Moisture Binding Properties of Wound Dressings

Pornanong Aramwit [1,*], Pornprom Muangman [2], Nantaporn Namviriyachote [1] and Teerapol Srichana [3]

[1] Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand; E-Mail: nantaporn.nam@gmail.com

[2] Burn Unit, Trauma Division, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand; E-Mail: pmuangman@yahoo.com

[3] Department of Pharmaceutical Technology and Drug Delivery System Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla 90110, Thailand; E-Mail: teerapol.s@psu.ac.th

* Author to whom correspondence should be addressed; E-Mail: aramwit@gmail.com; Tel.: +66-089-921-7255; Fax: +66-02-218-8403.

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Abstract: A variety of silver-coated dressings and some impregnated with other chemicals are now available in the market; however, there have been few studies analyzing their comparative efficacies as antimicrobial agents. Moreover, their properties for retaining an appropriate level of moisture that is critical for effective wound healing have never been reported. Five commercially available silver-containing and chlorhexidine dressings, Urgotul SSD®, Bactigras®, Acticoat®, Askina Calgitrol Ag® and Aquacel Ag®, were tested to determine their comparative antimicrobial effectiveness *in vitro* against five common wound pathogens, namely methicillin-sensitive and -resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Mepitel®, a flexible polyamide net coated with soft silicone, was used as a control. The zones of inhibition and both the rapidity and the extent of killing of these pathogens were evaluated. All five antimicrobial dressings investigated exerted some bactericidal activity, particularly against *E. coli*. The spectrum and rapidity of action ranged widely for the different dressings. Acticoat® had a broad spectrum of action against both Gram-positive and -negative bacteria. Other dressings demonstrated a narrower range of bactericidal activities.

Regarding the absorption and release of moisture, Askina Calgitrol Ag® absorbed and released the most moisture from the environment. Aquacel Ag® also exhibited good moisture absorption and moisture release, but to a lower degree. The other tested dressings absorbed or released very little moisture. Askina Calgitrol Ag® and Aquacel Ag® are good alternative dressings for treating wounds with high exudates and pus. An understanding of the characteristics of these dressings will be useful for utilizing them for specific requirements under specified conditions.

Keywords: antimicrobial; moisture absorption; wound dressing

1. Introduction

The skin is the largest human organ and acts as an extremely effective biological barrier. Cutaneous wounds are normally open to the environment and wound beds are favorable environments for bacterial growth. Burn wounds are open wounds and present a critical threat to burns victims, especially those with large areas of burns. This is the primary reason for dehydration, systemic infection and other complications suffered by burns victims [1-3]. One key factor for the effective treatment of burns patients is to close the wound as soon as possible [4,5]. Infections, either by bacteria or fungi, can lead to deterioration of the wound healing process [6] and severe systemic complications. The use of antibacterial agents locally and/or systemically can contribute to wound healing, especially for burn wounds. Their use inhibits microbial growth on or around the wounds and provides a suitable microenvironment for healing [1,2,4].

Wound management can be facilitated with dressings designed both to act as a temporary barrier and to promote wound healing [7]. Several topical antimicrobial agents are widely applied to wound dressings. Silver, in particular, has been a preferred additive to medicated wound dressings because it has broad antimicrobial and antifungal activity [8]. However, there are various forms and formulations of silver dressings available on the market, and little is known about their comparative effectiveness as antimicrobial agents and the spectrum of microbial killing that each provides. Other antimicrobials used in dressings are chlorhexidine, polyhexamethylene biguanide (PHMB) and iodine, but none of these have provided any evidence for promoting resistance. Silver has the advantage of having broad antimicrobial activities against Gram-negative and Gram-positive bacteria and there is also minimal development of bacterial resistance. However, there have been several reports that silver-impregnated dressings used on burns patients can induce hepatotoxicity and argyria-like symptoms [9,10]. It might soon be possible to meet the specific requirements for any particular circumstance, such as dressings that can adequately inhibit microbial growth, yet exhibit minimal silver toxicity and still enhance wound healing. Moreover, an appropriate dressing should be applied to a sensitive patient with wounds infected by pathogens.

Besides antimicrobial properties, the ability to absorb moisture is also an important factor for healing. Bolton *et al*. suggested that the use of more moisture-retentive dressings generally supports faster healing compared with less moisture-retentive dressings [11]. However, comparisons of

moisture absorption by dressings have never been studied even though the presence of moisture is known to accelerate the healing response compared with wounds that have been allowed to dry [12].

The objective of this study is to evaluate the antimicrobial effectiveness of five commercially available antimicrobial dressings *in vitro*. The moisture penetration of each dressing will also be investigated.

2. Results and Discussion

Table 1 shows the compositions of the studied coated dressings. All dressings contain either silver or chlorhexidine as an antimicrobial agent except for Mepitel® (which has been used as a control).

Table 1. Compositions of the studied coated dressings [13-15].

Dressing	Compositions of the dressing materials	Formulation compositions of the coated
Urgotul SSD®	Lipido-colloid dressing made of a polyester mesh impregnated with hydrocolloid particles (carboxymethylcellulose), vaseline particles and silver sulfadiazine	Silver sulfadiazine content 3.75%
Bactigras®	Chlorhexidine acetate 0.5% in white soft paraffin tulle dressing	Chlorhexidine acetate BP 0.5%
Acticoat®	Nanocrystalline silver being applied to high-density polyethylene mesh which is covered to either side of a rayon-polyester core	Nanocrystalline silver 105 mg/100 cm^2
Askina Calgitrol Ag®	Silver alginate wound dressing consists of an absorbent foam sheet. one surface of which is coated with an alginate matrix containing ionic silver together with a cleanser, moisturizer and a superabsorbent starch co-polymer	Silver 141 mg/100 cm^2
Aquacel Ag®	Sodium carboxymethylcellulose fibers containing 1.2% ionic silver. In the presence of exudate, the dressing absorbs liquid to form a gel, binding sodium ions and releasing silver ions	Silver 8.3 mg/100 cm^2
Mepitel®	Porous, semi-transparent, low-adherent wound contact layer, consisting of a flexible polyamide net coated with soft silicone	None

2.1. Corrected Zone of Inhibition Test

The result for the zones of inhibition generated by antimicrobial agents and dressings are presented in Table 2. The data show that all products generated an inhibitory zone against most individual microorganisms. All dressings inhibited bacterial populations to some extent except Bactigras®, which had no activity against *P. aeruginosa*. Acticoat® and Askina Calgitrol Ag® produced the largest zones of inhibition, which may due to the high concentration of silvercontained in these dressings (105 mg/100 cm^2 and 141 mg/100 cm^2, respectively) compared with 3.75% of silver sulfadiazine in

Urgotul SSD® and 0.5% chlorhexidine in Bactigras®. It is clear that different organisms produced differently sized zones of inhibition against the same dressing. Methicillin resistant *Staphylococcus aureus* (MRSA) and *B. subtilis* were less sensitive to the tested antimicrobial dressings, as shown by a smaller zone of inhibition compared with other organisms.

Table 2. Corrected zone of inhibitions (mm) generated by topical antimicrobial dressings.

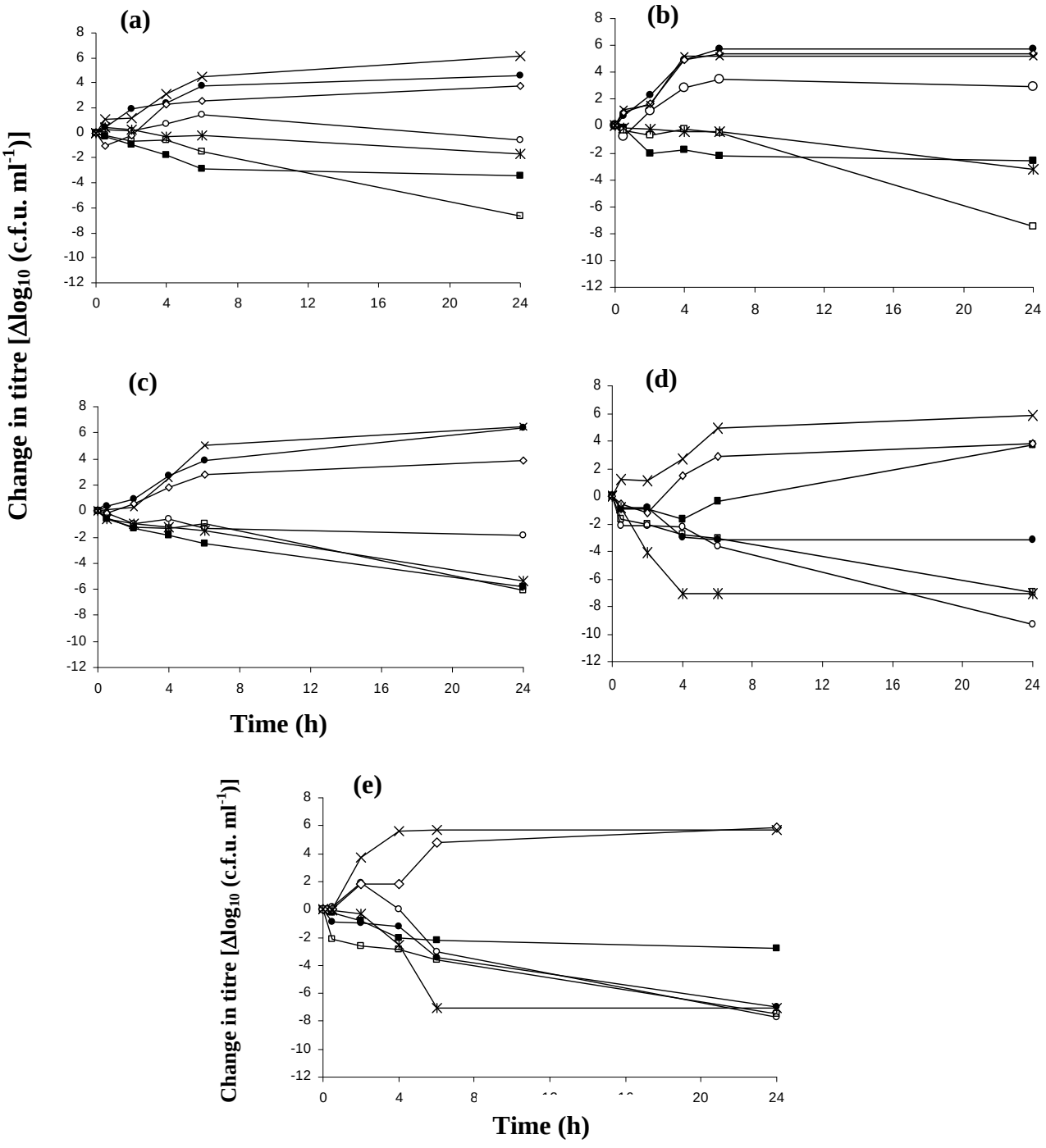
Microorganism	Urgotul SSD®	Bactigras®	Acticoat®	Askina Calgitrol Ag®	Aquacel Ag®	Mepitel®
S. aureus	1.41 ± 0.86	1.13 ± 0.42	13.30 ± 0.78	24.33 ± 3.12	12.97 ± 0.85	0.00
MRSA	0.19 ± 0.11	0.36 ± 0.33	6.69 ± 0.14	8.11 ± 4.33	1.84 ± 0.95	0.00
B. subtilis	2.39 ± 2.11	7.12 ± 1.24	10.98 ± 0.49	5.62 ± 1.48	6.69 ± 1.39	0.00
P. aeruginosa	9.05 ± 3.34	0	17.62 ± 4.82	21.08 ± 0.89	22.56 ± 1.77	0.00
E. coli	6.44 ± 1.22	0.78 ± 0.16	15.98 ± 0.84	12.42 ± 0.69	10.58 ± 0.47	0.00

Infection is a significant cause of delayed or prolonged wound healing, and high bacteria levels interfere with the progression of wound healing [16,17]. The broad antibacterial properties of silver and its derivatives have made it a good candidate and practical choice for creating silver dressings for wound care [13,14]. The results of this study show that all tested dressings investigated exerted some bactericidal activity, particularly on *E. coli*.

2.2. Bactericidal Activities of Antimicrobial Dressings

The spectrum and onset of action ranged widely for the various dressings. The bactericidal activities of the antimicrobial dressings against the five microorganisms are shown in Figure 1. Bactericidal activity was indicated by a reduction in bacterial counts presented as $\log_{10}$c.f.u. (colony forming units) mL^{-1} over time. These curves also indicated the rate of bacterial killing and provided an additional index of efficacy against the described isolate [18]. The normal growth rate of each organism was represented by the growth control and that of the Mepitel® dressing, which contained no antimicrobials. Overall, Acticoat® seemed to be the most effective dressing against these five tested organisms, especially with Gram-positive bacteria, whereas Urgotul SSD® and Bactigras® seemed to have a lower antimicrobial effect compared with the other dressings. For the Gram-positive bacteria, *S. aureus* (Figure 1a,b) and *B. subtilis* (Figure 1c), the Acticoat® dressing exerted maximal bactericidal activity, achieving more than a 4 log reduction of bacterial growth after 24 h. The killing patterns of *S. aureus* and *B. subtilis* by silver dressings were similar to MRSA, except for Aquacel Ag®, which slightly reduced both *S. aureus* and *B. subtilis* counts but had no effect on MRSA. With *P. aeruginosa* (Figure 1d), Acticoat®, Askina Calgitrol Ag® and Aquacel Ag® exhibited a good bactericidal effect. The maximal killing of *P. aeruginosa* was achieved at 4 h with Askina Calgitrol Ag® and the reduction in bacterial counts was sustained. The killing pattern for *E. coli* (Figure 1e) by Askina Calgitrol Ag® was similar to that for *P. aeruginosa* except for the maximal killing, which was found at 6 h. All dressings exhibited bactericidal activity and achieved more than a 4 log reduction of *E. coli* (Figure 1e) except for Bactigras®, which had a less pronounced effect.

Figure 1. The bactericidal activities of the antimicrobial dressings against five microorganisms. Values are the means of three experiments performed in triplicate. $\Delta\log_{10}$ c.f.u. ml^{-1} is the difference in $\Delta\log_{10}$ c.f.u. ml^{-1} at the time of bacterial inoculation, starting from $t = 0$. Strains: (**a**) Methicillin-sensitive *Staphylococcus aureus* (ATCC 6338P); (**b**) Methicillin-resistance *Staphylococcus aureus* (ATCC 25923); (**c**) *Bacillus subtilis* (ATCC 6633); (**d**) *Pseudomonas aeruginosa* (ATCC 27853); (**e**) *Escherichia coli* (ATCC 25922) and □ represents Acticoat®; ○ represents Aquacel Ag®; * represents Askina Calgitrol Ag®; ■ represents Bactigras®; ● represents Urgotul SSD®; ◊ represents Mepitel® and × represents growth control.

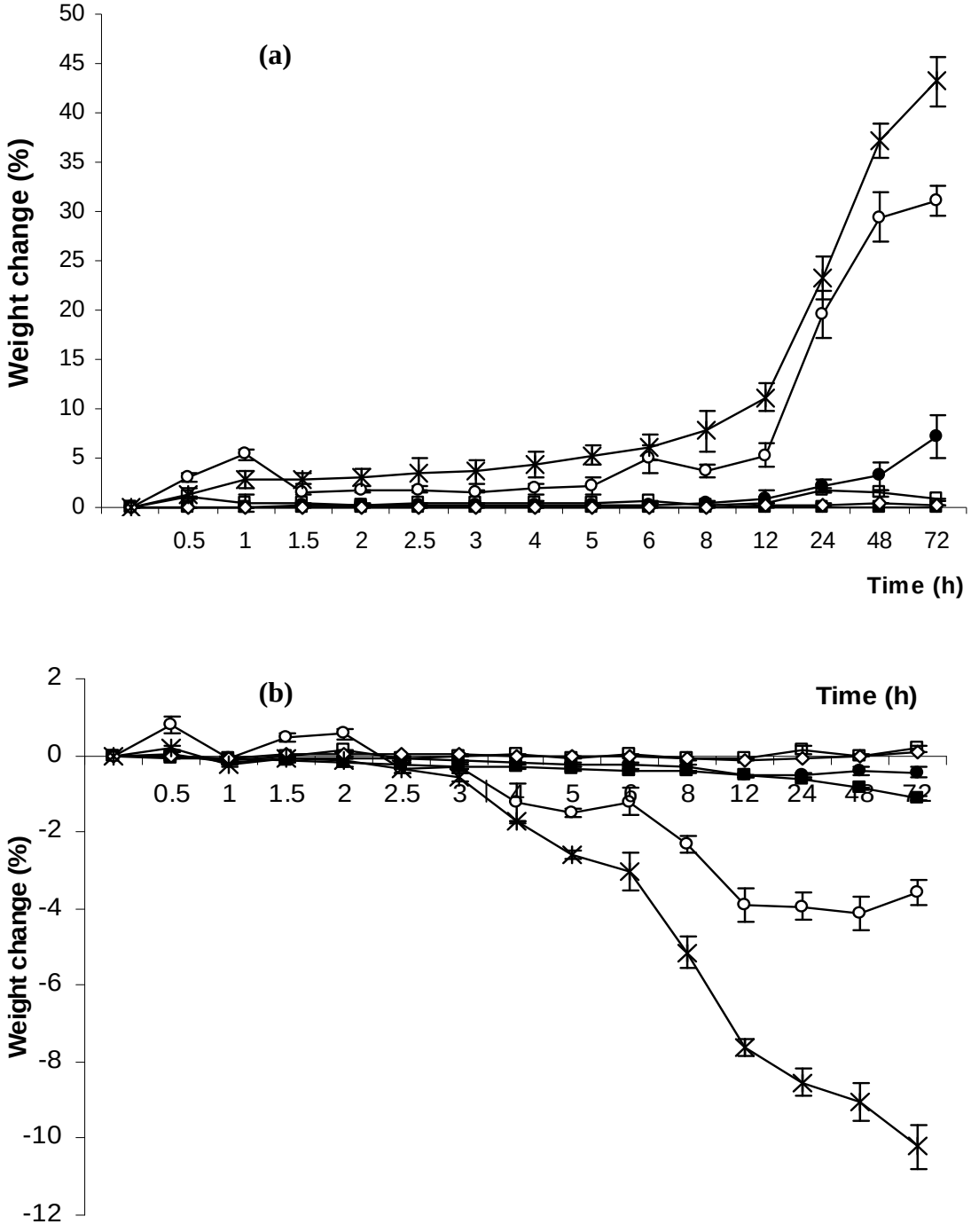


Acticoat® was effective and showed a broad spectrum of bactericidal activities on the bacteria tested with a long duration of action; these results are similar to those reported by Castellano *et al.* [19]. On the other hand, Askina Calgitrol Ag®, which contains the highest silver concentration compared with all tested dressings [14], had the most equivalent efficacy to Acticoat®. Since the silver form in Acticoat® is nanocrystalline, the molecular size and concentration of silver are higher than those in other dressings [20,21]. Because of its nanocrystalline form, Acticoat® also exhibits sustained-release of silver molecule resulting in longer duration of action. Urgotul SSD® showed bactericidal effects only with Gram-negative bacteria, similar to the results reported by Ip *et al.* [18]. The antimicrobial activity of Urgotul SSD® only generated from silver sulfadiazine, which shows the main activity on Gram-negative bacteria. Most dressings showed their bactericidal activity after the first hour of testing and the activity generally lasted for at least 24 h. One advantage of this rapid antibacterial action is that it allows wound healing to proceed without bacterial interference and reduces the likelihood that resistance will develop [18]. Since some dressings did not show their maximal bactericidal activity after 24 h, the limitation of this study is that it should have been extended for longer than 24 h, since some of the dressings might have a sustained effect for many days. Our study confirmed the effectiveness of silver and chlorhexidine dressings against a broad range of bacterial pathogens. With the enhanced bacterial killing effects, clinicians should be concerned that a too high level of silver could be delivered into the tissues and cause an adverse effect such as keratinocytes and fibroblasts toxicity that might affect on the recovery of wounds [18,22]. Suitable dressings for each use should be considered from the patient's sensitivity and the possible side effects from the level of silver in the dressings.

2.3. Wound Dressing Water Vapor Absorption

At steady state, potassium sulfate and potassium acetate in desiccators provided a relative humidity of 96.1 ± 1.5% and 22.4 ± 1.3% at 30 °C, respectively. Figure 2 shows the percentage weight change of each dressing after being placed into the desiccators at a relative humidity of 96.1% (Figure 2a) and 22.4% (Figure 2b), respectively for 0.5 to 72 h. Acticoat®, Bactigras®, Mepitel® and Urgotul SSD® absorbed or released very little moisture from the dressing at any humidity, whereas Askina Calgitrol Ag® absorbed and released the most moisture in humid conditions. After being placed in high humidity, Askina Calgitrol Ag® started to absorb moisture within 30 min and showed a significant weight change after 12 h. It also absorbed moisture close to 50% of its initial weight after being placed in a high humidity environment for 72 h and still did not reach a saturated condition. Nevertheless, it started to release moisture after placing it in a low humidity environment for 3 h and released approximately 10% of its weight after 72 h. In addition to Askina Calgitrol Ag®, Aquacel Ag® also showed a good absorption of moisture and had moisture release properties but to lower degree. Aquacel Ag® absorbed moisture up to 30% of its weight after 72 h in a high humidity environment after which it started to reach its saturated condition, whereas it showed approximately 4% moisture release after placing it in a low humidity environment for 72 h.

Figure 2. The percentage weight change of each dressing after placing into the desiccators with relative humidity at (**a**) 96.1% and (**b**) 22.4% for 0.5 to 72 h. □ represents Acticoat®; ○ represents Aquacel Ag®; * represents Askina Calgitrol Ag®; ■ represents Bactigras®; ● represents Urgotul SSD® and ◊ represents Mepitel®.



Since moisture is also an important factor for wound healing, the presence of moisture avoids the delays in healing response, which occur when wounds are allowed to dry out [12]. Excessive fluid retention at the wound surface, however, can result in poor healing and the maceration of the surrounding tissue [23]. Our results indicated that Askina Calgitrol Ag® absorbed and released the most moisture of the dressings tested. This was most evident with the foam dressings since there are

great variations in the location of the surface moisture [24]. The greatest percentage weight change occurred with Askina Calgitrol Ag® at high humidity since the foam dressings have a tendency to expand or shrink easily. Moreover, it contains superabsorbent starch co-polymer, which also increases the absorption capacity. These data indicated that Askina Calgitrol Ag® is a good alternative for treating wounds with high exudates and infection. Our result is the first report on the moisture absorption property of various antimicrobial dressings that will be beneficial for clinical application.

3. Experimental Section

3.1. Corrected Zone of Inhibition Test

The antimicrobial effect of each dressing, Urgotul SSD®, Bactigras®, Acticoat®, Askina Calgitrol Ag®, Aquacel Ag® and Mepitel®, was tested using corrected zone of inhibition method. This test was performed according to the method reported by Gallant-Behm with some modifications [25]. Briefly, the bacterial isolates were grown in broth for 4 to 6 h, and the broth was used to inoculate Muller-Hinton agar plates to form a confluent lawn. The various wound dressings (about 1 cm^2) were applied to the center of each lawn, and all plates were incubated for 24 h at 37 °C. The inhibition zone surrounding the tested dressing was then determined. No plate dehydration was observed around the dressings and all tests were performed in triplicate with results expressed as a mean with standard deviations.

3.2. Bactericidal Activities of Antimicrobial Dressings

In order to determine the onset and duration of antimicrobial activity of each dressing, bactericidal activities at different time points were determined by bacterial broth culture method which was adopted from Fraser *et al*. with some modifications [26]. Dressings (about 1 cm^2) were prepared in an aseptic manner. Each square was placed in a sterile vial and the dressing subjected to a pretreatment with 800 μL of distilled water for 10 min (according to a previously established protocol for an absorbancy test for the volume required and duration required for pretreatment). Tryptone soy broth (2.2 mL) was then added to each vial to make up to a total volume of 3 mL.

A suspension of each organism was prepared in broth from fresh colonies after overnight incubation and the turbidity was adjusted to the 0.5 McFarland standard (~1×10^8 c.f.u./mL). An aliquot (10 μL) of the bacterial suspension was added to each vial containing the dressing. Control broths with and without bacterial inoculation were also included. The vials were then incubated with agitation at 35 °C in a water bath. Aliquots of 10 μL of the bacterial broth were sampled from each vial at specific time intervals (0, 30 min and 2, 4, 6 and 24 h) and serial 10-fold dilutions for each aliquot were prepared in broth. Duplicate aliquots (25 μL) of each of the serially diluted samples were spread on plates. The plates were then incubated overnight at 35 °C and colonies counted (c.f.u./mL). The dilutions that allowed quantification (10-150 colonies) were counted and the mean counts calculated. Nine vials, containing the five antimicrobial dressings as well as the control dressing (Mepitel®) together with the culture and the broth controls, were included in each experiment for each organism. Plate counts were measured in triplicate and each experiment was repeated three times to obtain a mean value of c.f.u. counts.

3.3. Wound Dressing Water Vapor Absorption

Dressings (about 9 inch^2) were prepared in an aseptic manner and precisely weighed. Each dressing was placed in a desiccator pre-equilibrated with salts to make the relative humidity a desired value. Potassium sulfate or potassium acetate powder was placed in a desiccator to achieve a percentage relative humidity of about 90% and 20% at 30 °C, respectively, as reported by Greenspan [27]. After 30 min, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 24, 48 and 72 h, each dressing was taken from the desiccator using sterile forceps and again precisely weighed. The equilibrium moisture absorption was determined by the percentage weight change [28]. The experiments were performed in triplicate.

4. Conclusions

From five-tested antimicrobial dressings, Acticoat® was the most effective dressing against the tested organisms, whereas Urgotul SSD® and Bactigras® showed a lower antimicrobial effect compared with other dressings. Regarding the water vapor absorption activity, Askina Calgitrol Ag® absorbed and released the most moisture in humid conditions. Aquacel Ag® also showed good moisture absorption and release properties but to lower degree, while the other dressings hardly absorbed or released any moisture.

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Article

The Effect of Sericin from Various Extraction Methods on Cell Viability and Collagen Production

Pornanong Aramwit [1,*], Sorada Kanokpanont [2], Titpawan Nakpheng [3] and Teerapol Srichana [3]

[1] Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[2] Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand; E-Mail: sorada.k@chula.ac.th
[3] Department of Pharmaceutical Technology and Drug Delivery System Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla 90110, Thailand; E-Mails: titpawan@gmail.com (T.N.); teerapol.s@psu.ac.th (T.S.)

* Author to whom correspondence should be addressed; E-Mail: aramwit@gmail.com; Tel.: +66-089-921-7255; Fax: +66-02-218-8403.

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Abstract: Silk sericin (SS) can accelerate cell proliferation and attachment; however, SS can be extracted by various methods, which result in SS exhibiting different physical and biological properties. We found that SS produced from various extraction methods has different molecular weights**,** zeta potential, particle size and amino acid content. The MTT assay indicated that SS from all extraction methods had no toxicity to mouse fibroblast cells at concentrations up to 40 μg/mL after 24 h incubation, but SS obtained from some extraction methods can be toxic at higher concentrations. Heat-degraded SS was the least toxic to cells and activated the highest collagen production, while urea-extracted SS showed the lowest cell viability and collagen production. SS from urea extraction was severely harmful to cells at concentrations higher than 100 μg/mL. SS from all extraction methods could still promote collagen production in a concentration-dependent manner, even at high concentrations that are toxic to cells.

Keywords: sericin; cell viability; collagen; extraction; concentration

1. Introduction

Extracellular matrix proteins such as collagen, fibronectin, and gelatin are known to play important roles in the attachment and growth of mammalian cells. We recently showed that silk sericin (SS), a high molecular weight granular protein with adhesive and gelatin-like characteristics, can promote growth of the mouse fibroblast cell line L929, as well as activation of collagen production both *in vitro* and *in vivo* [1,2]. Many studies have also demonstrated that SS can accelerate the proliferation and attachment of several mammalian cell lines [3-5], and insect cell culture was also reported to be improved by SS [6]. Moreover, SS added to freezing media as an alternative to fetal bovine serum improved the survival of various cell lines during cryopreservation [7]. However, Terada *et al*. reported that culture supplemented with 1.0% SS resulted in no viable cells, which indicates that the presence of 1.0% SS is harmful to cells [3]. These data demonstrate that the concentration of SS supplemented to culture medium is also a significant factor for cell viability. Nevertheless, the optimal concentration of SS for promoting cell viability has never been reported.

SS can be extracted by various methods, such as high pressure and high temperature techniques, acid or alkaline solutions, or enzyme extraction. The method of extraction significantly affects the biochemical activities of silk proteins. Kurioka *et al*. reported that acid-degraded, alkali-degraded, and hot water-degraded SS powders exhibit different trypsin inhibitory activities and have different isoelectric points [8]. Furthermore, different SS extraction methods alter its amino acid composition, which may influence its cell-growth and collagen secretion in cells.

The objective of this study was to investigate the chemical properties of SS extracted from Thai silk strains via various extraction methods, which have never yet been investigated. In addition, we determined the effect of various concentrations of SS obtained from the different extraction methods on fibroblast cell viability and collagen production. These data yield important fundamental information for further development of SS as a serum-free medium supplement.

2. Results and Discussion

It has long been known that SS can accelerate the proliferation of several cell lines, including hybridoma cells [3,5,6,9]. Tsubouchi *et al*. also reported that SS can enhance the attachment of cultured human skin fibroblasts [4]. The attachment and subsequent proliferation of fibroblast cells are considered to play important roles in the healing process of skin lesions. In this study, the L929 mouse fibroblast cell line has been used as a model to investigate the roles of SS from various extraction methods on cell viability and collagen production.

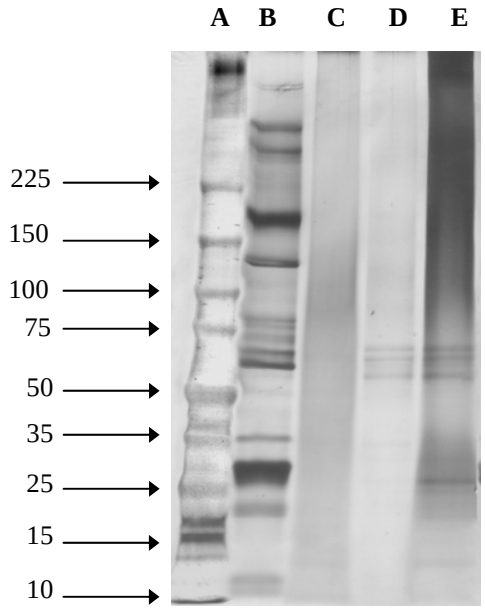
Recently, SS has been shown to have a protective effect against several toxicities, such as alcohol-induced gastric injury, in animal models [10]. However, the SS used in most previous reports [3,5,6,9,10] was extracted by heat or alkaline solution, even though SS can be extracted by various methods, which affect its physical and biological properties, as shown here. This is the first study to compare the enhancement of cell viability by SS at different concentrations derived by extraction procedures. We found that SS does not have only positive effects on cell viability, as at certain concentrations it starts to induce toxicity. This may be an explanation for the previously reported detrimental effects of SS in clinical uses [11,12]. Moreover, extraction methods also play an important role in SS activity. Many studies have used SS prepared by heat or alkaline extraction and

have reported the advantageous effects of this protein on cells. This is in agreement with our 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) result, which showed that SS extracted by heat, acid and alkaline solution is rarely toxic to cells at concentrations up to 100 μg/mL. Nevertheless, the extraction method has not previously been emphasized in relation to the use of SS, which may lead to confusion, as we have proven that SS derived by urea extraction method was severely toxic to cells. Since SS has been widely investigated for its use in biomedical applications, this point should be clarified in order to avoid misleading interpretation of results.

2.1. Molecular Weight of SS

SS extracted by various methods has different molecular weights, as shown in Figure 1. Different extraction methods provided different molecular weight SS, which may result in different chemical and biological properties. SS extracted with urea showed clear bands with molecular weights ranging from 10 to >225 kDa. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of acid-degraded and alkali-degraded SS showed distinguishable bands within the range of 50-150 kDa and 15-75 kDa, respectively. However, the number of bands from acid-degraded and alkali-degraded SS were much lower than that of SS extracted with urea. SDS-PAGE of SS prepared by the high temperature and high pressure degumming technique showed broad bands, with molecular weights ranging from 25-150 kDa. Our findings are in agreement with those reported by Sprague, which indicates that SS is a mixture of at least 15 different polypeptide chains, ranging in size from 20 to 220 kDa [13].

Figure 1. Molecular weight of SS extracted by various methods. (**A**) Marker (**B**) Urea-extracted sericin (**C**) Heat-degraded sericin (**D**) Acid-degraded sericin (**E**) Alkali-degraded sericin.



According to these studies, SS from heat, acid, alkaline and urea extraction methods show different molecular weights. This may seem insignificant, but it may in fact reflect biological properties of SS, such as its antimicrobial activity. Other researchers have reported that the antimicrobial property of SS

is derived from a low molecular weight protein, seroin, from *B. mori* [14,15]. Seroin is not involved in silk fiber construction and may play a role in protecting silk against microbial degradation [15]. Zurovec *et al*. reported that seroin polypeptides are present in silk as 22.5 and 23 kDa molecules, and that these polypeptides are liberated from other proteins when silk components are dissolved [14]. The implication of these findings is that urea extraction should be the only method that would provide SS with antimicrobial activity.

2.2. Particle Size and Zeta Potential Measurement

Table 1 shows the zeta potential and particle size of SS from different extraction methods. SS from all extraction methods exhibited negative zeta potential values. Zeta potential of SS from urea extraction yielded the highest negative charge, followed by acid-degraded SS, heat-degraded SS and finally alkali-degraded SS. Alkali-degraded SS had the largest particle size, followed by heat-degraded and acid-degraded SS, while SS extracted by urea solution had the smallest particle size. Since urea-extracted SS is present as a very small-sized compound in water, it may be in soluble form, while SS extracted by other methods may be present as hydrocolloids.

Table 1. Zeta potential and particle size of SS from different extraction methods.

Extraction method	Zeta potential (mV)	Mean size (nm)
Heat	-20.69 ± 2.14	110.42 ± 35.07
Acid	-32.12 ± 5.26	23.80 ± 16.07
Alkaline	-15.87 ± 2.89	824.42 ± 86.67
Urea	-68.36 ± 5.67	4.62 ± 2.44

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The magnitude of the zeta potential gives an indication of the potential stability of the system, where high zeta potentials (either negative or positive) indicates electrically stabilized particles, while colloids with low zeta potentials tend to coagulate or flocculate [16]. From our results, SS obtained from the alkaline extraction method had more of a tendency to coagulate in this solution, which corresponds to the largest particle size. However, SS obtained by heat, acid and urea extraction were stably dispersed with a lower degree of coagulation compared to alkali-degraded SS. Moreover, SS obtained from urea extraction was the most electrically stable and had the least tendency to coagulate, which was confirmed by the smallest mean particle size.

2.3. Amino Acid Analysis

Amino acid content in SS extracted from various methods is shown in Table 2. There were slight variations in the amino acid percentage in SS extracted by different methods; however, the main amino acid component in SS was still the same. Serine was the dominant amino acid in SS (~30%), while aspartic acid and glycine composed about 10-20%. The amount of methionine found in heat-degraded SS was significantly higher than in SS extracted by other methods, while the amount of tyrosine found in urea-extracted SS was significantly lower than in SS extracted by other methods. Moreover, heat-extracted SS, which contains the highest amount of methionine and cysteine, sulfur-containing

amino acids that can generate double-helical structures, can induce the highest levels of collagen production. This result corresponded with our previous report, which showed that methionine in SS relates to its collagen production activity [1].

Table 2. Amino acid composition of SS extracted using various methods (in mole%).

Amino acid	Extraction method of SS			
	Heat	**Urea**	**Acid**	**Alkaline**
Asp	15.64	18.31	15.93	19.88
Ser	33.63	31.27	31.86	30.01
Glu	4.61	5.27	5.75	5.93
Gly	15.03	11.23	10.49	11.01
His	1.06	3.26	2.47	1.72
Arg	2.87	5.41	4.92	4.92
Thr	8.16	8.36	8.51	6.49
Ala	4.10	4.33	3.72	4.21
Pro	0.54	1.46	0.78	1.24
Cys	0.54	0.39	0.53	0.23
Tyr	3.45	0.36	5.56	5.24
Val	2.88	2.96	2.95	2.94
Met	3.39	0.12	0.06	0.15
Lys	2.35	3.14	3.48	2.89
Ile	0.56	0.96	0.87	0.75
Leu	1.00	1.58	1.43	1.56
Phe	0.28	0.60	0.71	0.81

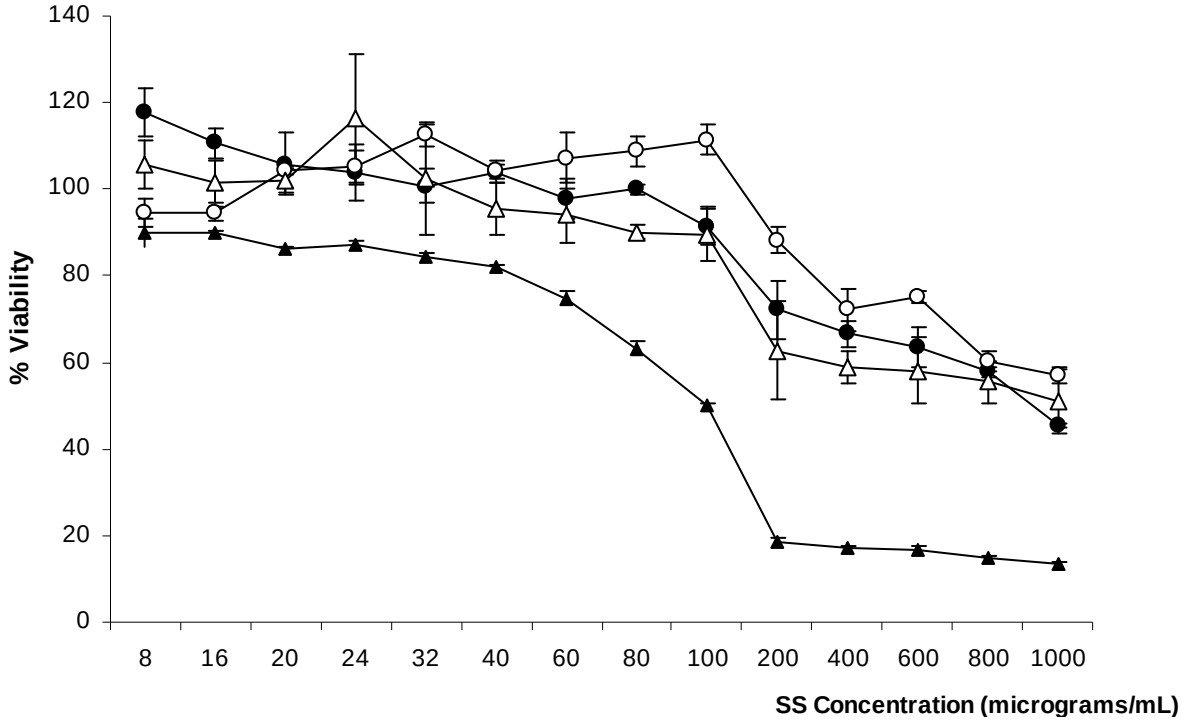
2.4. Cytotoxicity of SS Solution

The MTT assay indicated that SS solutions from all extraction methods had no toxicity to mouse fibroblast cells at concentrations up to 40 μg/mL after 24 h incubation (Figure 2). It also indicated that heat-degraded and alkali-degraded SS exhibit the least cell toxicity. SS derived from all extraction methods except urea extraction could promote cell viability at low concentrations. SS from urea extraction showed slight toxicity at concentration as low as 60 μg/mL and its toxicity became significant at concentrations higher than 100 μg/mL, while SS from other extraction methods showed toxicity to a lesser extent than urea-extracted SS, as shown by the percent viability of fibroblasts. These data indicate that extraction method and SS concentration have significant effects on growth and viability of fibroblast cells.

Heat-degraded SS showed the least toxicity to L929 cells at concentrations up to 100 μg/mL, while acid-degraded and alkali-degraded SS showed similar results, but at lower levels of activation, which is consistent with results of other reports [3,5,9]. However, at concentrations higher than 100 μg/mL, viability of L929 cells decreased. Similarly, Terada *et al*. reported that SS from alkaline extraction at low concentrations increased the population in HeLa (human epithelial cell) cultures, while higher concentration of SS (0.3%) did not [3]. This study also reported that SS at 1.0% was severely harmful

to the murine hybridoma (2E3-O) cell line [3]. From these data, we can conclude that concentration and the extraction method of SS, as well as the particular cell line, can affect the cell viability.

Figure 2. Viability study of L929 cells incubated with SS solutions via the MTT assay after incubation for 24 h. Error bars represent the standard error of the mean (n = 3). (Δ) Acid-degraded sericin, (○) Alkali-degraded sericin, (●) Heat-degraded sericin, (▲) Urea-extracted sericin.

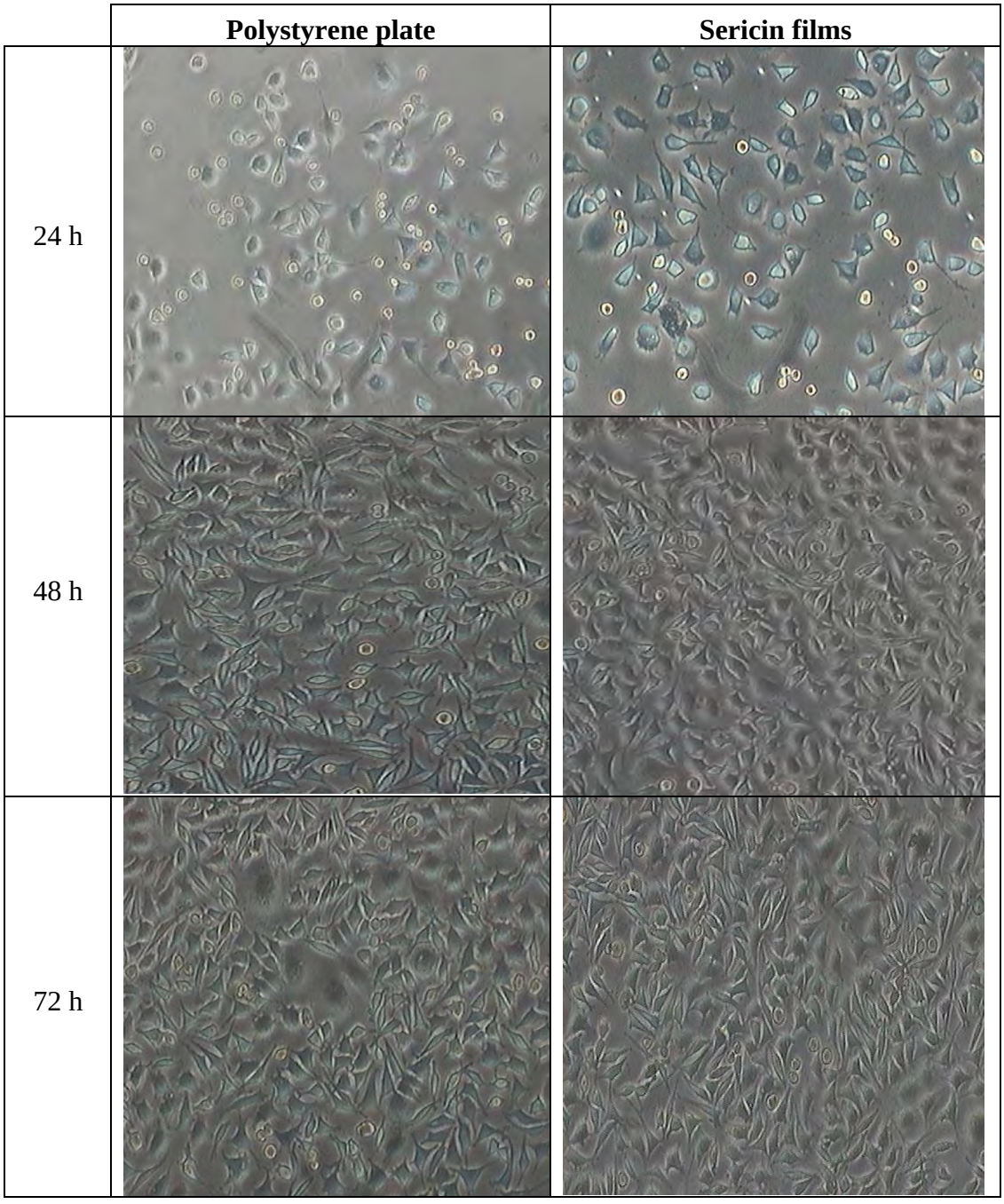


According to our results, the optimum concentration of SS for promoting cell viability depends on the SS extraction method. SS obtained by urea extraction should not be used as a supplement for serum-free medium, since it is toxic to cells. Heat-degraded and alkali-degraded SS are the most suitable for cell culture with the optimum concentration at 100 μg/mL. Low concentrations of acid-degraded SS were beneficial to cells. Concentrations as low as 8 μg/mL lead to the greatest cell viability.

2.5. Adherence of L929 Mouse Fibroblast Cell Line to SS Films

Since heat-degraded SS significantly promoted cell growth compared to other extraction techniques, and because of its chemical-free property, heat-degraded SS was used to study the adherence of fibroblast cells on SS films. The morphology of L929 mouse fibroblasts cultured on SS heat-extracted films observed at 24, 48, and 72 h are shown in Figure 3. Cells started attaching to SS films and began proliferating after 24 h similar to cells on styrene culture plates, which were used as a positive control. Approximately 70% of cells on both control and SS plate attached to the surface at 48 h and became confluent after 72 h. After 72 h, cells fully proliferated and formed complete pseudopodia like structures on styrene culture as well as SS plate. Moreover, the number of cells attached on SS plate at 72 h was slightly higher than number of cells on styrene culture plate.

Figure 3. Morphology of L929 mouse fibroblasts cultured on polystyrene culture plates and on SS films at 20X at 24 h, 48 h and 72 h after cell seeding at 20,000 cells/1,257 mm^2.

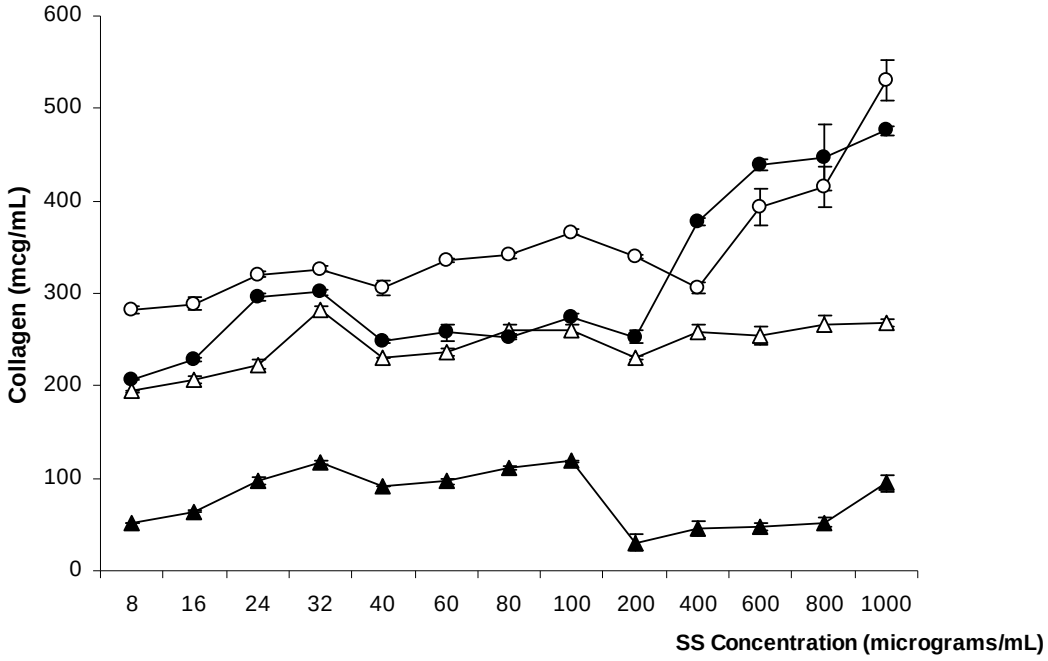


2.6. Determination of Soluble Collagen Production Induced by SS

SS extracted by all methods can induce collagen type 1 production from the fibroblast cell line L929 (Figure 4), while the negative control (fibroblast cells without SS as supplement in culture medium) did not produce any collagen (data not shown). However, urea-extracted SS induced the lowest amount of collagen production, compared to SS extracted by the other methods at all concentrations. Heat-degraded SS induced the highest collagen type 1 production at concentrations from 8-200 μg/mL. At concentrations higher than 200 μg/mL, alkali-degraded SS could activate the

highest collagen production. However, alkali-degraded and heat-degraded SS at concentrations higher than 200 μg/mL induced significant levels of collagen type 1, but resulted in fewer viable cells. These data indicate that SS at concentrations higher than 200 μg/mL can induce collagen production, even though it is toxic to cells.

Figure 4. Collagen type 1 production in fibroblast cell line L929 when various SS concentrations were added into the culture medium for 24 h to make the final concentration of SS in each well 8-1,000 μg/mL, respectively. Error bars represent the standard error of the mean (n = 3). (Δ) Acid-degrade sericin, (○) Alkali-degraded sericin, (●) Heat-degraded sericin, (▲) Urea-extracted sericin.



Enhancement of fibroblast collagen production in cells is normally related to transforming growth factor (TGF)-β [17], which is generally released only from surviving fibroblast cells 2 h after cells are activated by chemicals or trauma, and reaching peak levels after 12 h [18]. This supports our result that collagen content in cell culture still increased at SS concentrations higher than 100 μg/mL, even though the percentage of cell viability decreased. Collagen production may be generated from fibroblast cells, which are activated by silk protein at an early stage when most cells are still viable before SS becomes toxic to cells.

3. Experimental Section

3.1. Materials

3.1.1. Silkworm Cocoons

Fresh *Bombyx mori* cocoons were kindly supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). Native Thai silkworms, white cocoons, were produced in a controlled environment.

3.1.2. Fibroblast Cell Culture

The mouse fibroblast cell line L929 (Chinese Academy of Preventive Medical Sciences, Beijing, China) was cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U penicillin and 100 U streptomycin per mL) under 5% CO_2 at 37 °C. The medium was changed every 2 days. When cells reached confluence, they were harvested using 0.25% trypsin-EDTA (Gibco®, California, USA), followed by addition of fresh culture medium to create a new single cell suspension for further incubation.

3.1.3. Preparation of SS Powder Using a High Temperature and High Pressure Degumming Technique (Heat-Degraded SS Powder)

Cocoons of *B. mori* silkworms were cut into square pieces and extracted with purified water by autoclaving (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120 °C for 60 min. The aqueous solution obtained from autoclaving silk cocoons was collected and called heat-degraded SS. The aqueous solution was then filtered to remove insoluble material, which is fibroin. After that, the filtrate was frozen and lyophilized using a Heto LL 3000 lyophilizer (Allerod, Denmark) to obtain SS powder. The SS molecular weight from all strains was estimated by SDS-PAGE.

3.1.4. Preparation of SS by Citric Acid and Sodium Carbonate Solution (Acid-Degraded and Alkali-Degraded SS Powders)

Acid-degraded and alkali-degraded SS powders were extracted using a previously described method by Kurioka *et al*. with some modifications [8]. For acid-degraded SS powder preparations, cocoons were cut and added to a 1.25% citric acid solution, then boiled for 30 min. After removing insoluble fibers by paper filtration, the clear filtrate was immediately dialyzed in distilled water for three days using cellulose tubing (Cellusep T2, MWCO 6,000-8,000, Sequin, Texas, USA) and distilled water was changed regularly. The pH of the final solution was measured to verify complete removal of citric acid. The SS solution was then frozen and lyophilized. Alkali-degraded SS powder was prepared similarly, using 0.5% sodium carbonate solution instead of citric acid.

3.1.5. Preparation of SS by Urea Solution

SS extracted by urea solution was prepared using a previously described method with some modifications [4]. Freshly cut cocoon shells were soaked into 8 M urea aqueous solution for 30 min and then refluxed at 85 °C for 30 min. Centrifugation and filtration were performed to remove all insoluble residues. The solution was thoroughly dialyzed in distilled water using cellulose tubing (Cellusep T2, MWCO 6,000-8,000, Sequin, Texas, USA) for three days and distilled water was changed regularly. The pH of final solution was measured to verify complete removal of urea solution. The SS solution was frozen and then lyophilized.

3.2. Methods

3.2.1. Molecular Weight Determination

To determine the molecular weight of SS, polyacrylamide gel electrophoresis was performed as previously described with some modifications [19]. Briefly, sample solutions for SDS-PAGE were prepared by adding an equal volume of sample buffer (0.25 M Tris-HCl, pH 7.0 containing 4% SDS, 10% sucrose, 10% 2-mercaptoethanol, and 0.025% bromophenol blue) to each protein solution. Each sample solution was then incubated at 98 °C for 2-3 min and loaded onto a 5%-20% gradient gel (Atto Corporation, Tokyo, Japan). Electrophoresis was performed in 125 mM Tris base with 0.96 M glycine and 0.5% SDS, polypeptide bands were detected by silver staining.

3.2.2. Particle Size and Zeta Potential Measurement

The size of self-aggregates was measured by a dynamic light scattering method based on the particle size option in a Zetasizer Nano-ZS (ZEN 3600, Malvern Instruments Ltd., Worcestershire, UK). The scattered intensity was registered at a scattering angle of 90° at 25 °C. Zeta potentials were measured by a Zetasizer Nano-ZS instrument with palladium-coated electrodes. All samples were adjusted to pH 7.0 prior to particle size and zeta potential measurement. The zeta potential presented is the average value of analyses in triplicate.

3.2.3. Amino Acid Analysis

SS amino acid compositions were measured with an amino acid analyzer (Hitachi L-8500A, Tokyo, Japan). Samples were hydrolyzed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) at 100 °C for 24 h under vacuum prior to amino acid analysis. All experiments were performed in triplicate.

3.2.4. Cytotoxicity of SS Solution

L929 mouse fibroblast cells at an initial concentration of 2×10^4 cells/well were seeded in a 96-well plate in DMEM containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium. SS solutions of various concentrations in purified water were filter sterilized by 0.22 μm membrane filter (Sartorius Ltd., Epsom, UK) prior to adding to the culture medium to give final SS concentrations in each well at 8.0-1000 μg/mL. Cells without SS solution served as negative controls. Melittin, a peptide from bee venom toxin, from 0.125 to 1.0 mg/mL, was used as a positive control. After incubation for 24 h, MTT assay was performed to evaluate cell activity [20]. The absorbance was determined by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 570 nm. The percentage of viable cells was calculated and compared to the negative control. All experiments were done in triplicate.

3.2.5. Adherence of L929 Mouse Fibroblast Cell Line on SS Films

Heat-degraded SS films were cast from SS solution (0.1% w/v, in water pH 5) in polystyrene 12-wells cell culture plates (well diameter 20 mm). After air-drying, the films were crosslinked by

ultraviolet (UV) irradiation for 60 min and sterilized with 70% ethanol followed by phosphate-buffered saline (PBS, pH 7.5) before seeding cells. L929 mouse fibroblast cells were seeded onto the sterilized films (2×10^4 cells/well). Cells were cultured in DMEM containing 10% FBS and antibiotics (100 U penicillin and 100 U streptomycin per mL) under 5% CO_2 at 37 °C. Cells were harvested at 24, 48 and 72 h, and the morphology of cells on culture plates (control) and on films coated on culture plates were observed by light microscopy (Nikon, TS100, Melville, New York, USA). All experiments were done in triplicate.

3.2.6. Determination of Soluble Collagen Production Induced by SS

L929 mouse fibroblast cells were cultured at the same cell content and method as for the cytotoxicity study of SS solution. Cells without SS solution served as a negative control. Supernatants were collected after cell incubation for 24 h. The total amount of soluble collagen type 1 was assayed using the Sircol® collagen assay kit (Biocolor Ltd., Northern Ireland, UK). The results were determined by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 500 nm. All experiments were done in triplicate. The amount of collagen was calculated based on a standard curve of soluble collagen (standard bovine collagen type 1, produced from USA disease free animals).

4. Conclusions

SS can promote cell viability at certain concentrations, but it can be toxic to cells at higher concentrations. The method of extraction of SS also has significant effects on cell viability. Urea-extracted SS showed the lowest cell viability compared to SS extracted by heat, acid and alkaline methods. Urea-extracted SS was severely harmful to cells at concentrations higher than 100 μg/mL. Heat-degraded SS activated the highest collagen production, while urea-extracted SS showed the lowest level of collagen activation. SS from all extraction methods could still promote collagen production in a concentration-dependent manner, even at high concentrations that are toxic to cells, which indicate that collagen was generated before fibroblast cells departed.

Acknowledgements

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Properties and antityrosinase activity of sericin from various extraction methods

Pornanong Aramwit*[1], Siriporn Damrongsakkul†, Sorada Kanokpanont† and Teerapol Srichana‡

**Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand, †Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand, and ‡Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla 90110, Thailand*

The present study investigated the chemical properties and antityrosinase activities of SS (silk sericin) extracted from different Thai silk strains via various extraction methods. Different silk strains contain distinct SS with various amino acid compositions, which are significantly influenced by the extraction method used. Urea extraction of SS was the only method that provided clearly distinguishable bands and had the most significant impact on SS conformation as illustrated by FTIR (Fourier-transform infrared) spectra. The use of urea or either acidic or alkaline chemicals in the extraction process also influenced SS thermal behaviour. With regard to biological activity, SS extracted using urea exhibited the highest antityrosinase activity, whereas alkali-degraded SS showed no inhibition of mushroom tyrosinase. Pigments, primarily flavonoids and carotenoids from silk cocoons, were also found to enhance tyrosinase inhibition of SS.

Introduction

The silkworm, *Bombyx mori*, synthesizes and secretes two classes of silk proteins, SF (silk fibroin) and SS (silk sericin). SF is a fibrous protein that has been widely investigated for biomedical purposes owing to its physiochemical properties and relatively inert immune response [1]. SS, on the other hand, was long considered to simply be a waste product in the silk industry until it was found to have important biochemical functions such as antioxidant activity [2,3], antityrosinase activity [3] and effects on tumour progression [4–6] and can be used as serum-free freezing medium for mammalian cells [7]. Silks differ widely in composition, structure and biochemical properties depending on the specific source and strain. Thai silk, particularly the yellow Nangnoi silk, contains a significant amount of pigments, which are primarily associated with carotenoids and flavonoids [8,9]. These polyphenolic compounds are most commonly known for their antioxidant properties and other diverse biochemical functions, such as antityrosinase, antiallergy or anti-inflammatory activities [10,11].

Normally, pigments coexist and accumulate in the layers of cocoon sericin [12]. The components that give colour to silk cocoons are associated with phenolic compounds in mulberry leaves, the sole food for *B. mori* larvae, and the content of cocoon colour components varies depending on the silkworm strain [12]. There are several native strains of silkworm in Thailand with various colours of cocoon shells. The most common native Thai silk is Nangnoi (yellow cocoon shell), which has been found to contain several flavonoids such as c-prolinylquercetins, while other Thai silk cocoons have white and yellow–green shells. Hayashiya et al. [13] found that green cocoon shells contain at least nine fluorescent yellow compounds, five of which have been identified as flavonoid-related compounds. These flavonoid compounds, in addition to SS, are also responsible for the antioxidant properties of *B. mori* cocoons [3,12]. Although the types and amount of flavonoids in silk cocoons have been found to differ genetically, little is currently known about the antityrosinase activity generated from flavonoids or SS from different silkworm strains.

Tyrosinases are copper-containing enzymes that catalyse the ortho-hydroxylation of monophenols to catechols and their subsequent oxidation to ortho-quinones [14]. Tyrosinases are thought to play roles in cancer and neurodegenerative diseases such as Parkinson's disease [15]. In addition, tyrosinases represent a significant target in the fields of agriculture, food and medicine, which has led to widespread screening for compounds with potent antityrosinase activity.

Key words: amino acid, antityrosinase activity, extraction, pigment, sericin, silk strain.

Abbreviations used: DSC, differential scanning calorimetry; FTIR, Fourier-transform infrared; MWCO, molecular-mass cut-off; SF, silk fibroin; SS, silk sericin.

[1] To whom correspondence should be addressed (email aramwit@gmail.com).

Extraction methods significantly affect the biochemical activities of silk proteins. Kurioka et al. [16] reported that acid-, alkali- and hot water-degraded SS powders exhibit different trypsin inhibitory activities and pI values. Furthermore, different SS extraction methods may alter amino acid compositions, which are directly related to antityrosinase activity. Tyrosine-containing peptides are substrates for the tyrosinase enzyme and possibly contribute to its activation, whereas cysteine-containing peptides are known to effectively inhibit tyrosinase activity [14,17].

The objectives of the present study were to investigate the chemical properties of SS extracted from different Thai silk strains via various extraction methods. In addition, we determined the antityrosinase activity of SS and the effects of flavonoids from silkworms on tyrosinase inhibition.

Materials and methods

Silkworm cocoons

Fresh *B. mori* cocoons were kindly supplied by Chul Thai Silk (Petchaboon Province, Thailand). Silkworm cocoons were produced in a controlled environment from three Thai native silk strains [Chul 1/1 (bivoltine, white shell), Chul 3/2 (bivoltine, greenish shell) and Chul 4/2 (bivoltine, yellow shell)].

Preparation of SS powder using a high–temperature and high-pressure degumming technique (heat-degraded SS powder)

Cocoons of *B. mori* silkworms were cut into square pieces (approx. 5 mm^2). Coloured silkworm cocoons were extracted three times in 70% ethanol (1 g of silk cocoon and 30 ml of ethanol) for 24 h at room temperature (25 °C) to remove all flavonoids and carotenoids. After drying the remaining cocoon shells (~97% from initial cocoon weight), SS was extracted with purified water (1 g of dry silk cocoon and 30 ml of water) by autoclaving (SS-320; Tomy Seiko, Tokyo, Japan) at 120 °C and 15 lbf/in^2 (1 $lbf/in^2 = 6.9$ kPa) for 60 min. The aqueous solution obtained from autoclaving of silk cocoon was collected and called heat-degraded SS. The aqueous solution was then filtered to remove insoluble material, which is fibroin. After that, the filtrate was frozen and freeze-dried using a Heto LL 3000 lyophilizer (Heto-Holten A/S, Allerod, Denmark) to obtain SS powder. The SS molecular mass from all strains was estimated by SDS/PAGE.

Preparation of SS by citric acid and sodium carbonate solution (acid- and alkali-degraded SS powders)

Acid- and alkali-degraded SS powders were extracted by the same method as that described previously by Kurioka et al. [16] but with some modifications. For acid-degraded SS powder preparations, cocoons were cut and colours were extracted using the high-temperature and high-pressure degumming technique described above. The remaining cocoon shells were added into a 1.25% citric acid solution (1 g of dry silk cocoon and 18 ml of citric acid solution) and boiled for 30 min. After removing insoluble fibres by paper filtration, the clear filtrate was immediately dialysed in distilled water for 3 days using cellulose tubing [Cellusep T2; MWCO (molecular-mass cut-off) = 6000–8000; Sequin, TX, U.S.A.]. The SS solution was then frozen and freeze-dried.

Alkali-degraded SS powder was similarly prepared using 0.5% sodium carbonate solution instead of citric acid.

Preparation of SS by urea solution

Freshly cut cocoon shells (6 g) were soaked into aq. 8 M urea (150 ml) for 30 min and then refluxed at 85 °C for 30 min. Centrifugation and filtration were performed to remove all insoluble residues. The solution was thoroughly dialysed in distilled water using cellulose tubing (Cellusep T2; MWCO = 6000–8000; Sequin) for 3 days. The SS solution was frozen and freeze-dried using a Heto LL 3000 lyophilizer.

Measurement of SS powder colour

SS powder colour values were measured using a spectrocolorimeter tristimulus colour analyser (Model JS555; Color Techno System Corporation, Tokyo, Japan) calibrated with a white porcelain reference plate. Visible reflectance spectra (380–770 nm) were obtained using a silicone photocell and pulsed xenon lamp (illuminant D65, 0° view angle, illumination area diameter 8 nm). Colour parameters from spectra were calculated by the spectrocolorimeter. The colour coordinates of the uniform colour space CIELAB (CIE L*a*b* colour scale of the Commission Internationale d'Eclairage) were determined by reflectance (L^*) and chromaticity (a^* and b^*). The L^* value indicates brightness ranging from black ($L^* = 0$) to white ($L^* = 100$). The a^* value ranged from –60 (green) to 60 (red) and the b^* value ranged from –60 (blue) to 60 (yellow). All experiments were performed in triplicate.

Molecular mass determination

To determine the molecular mass of SS, PAGE was performed as previously described with some modifications [18]. Pigments from silk cocoons (Chul 3/2 and 4/2) were removed prior to SS extraction to confirm that carotenoids or flavonoids would not interfere with molecular mass determination. Briefly, sample solutions for SDS/PAGE were prepared by adding an equal volume of sample buffer [0.25 M Tris/HCl, pH 7.0, containing 4% (w/v) SDS, 10% sucrose, 10% (v/v) 2-mercaptoethanol and 0.025% Bromophenol Blue] to each protein solution. Each sample solution was then incubated at 98 °C for 2–3 min and loaded on to

a 5–20% gradient gel (Atto Corporation, Tokyo, Japan). Electrophoresis was performed in 125 mM Tris base with 0.96 M glycine and 0.5% SDS; polypeptide bands were detected by silver staining.

Amino acid analysis

SS amino acid compositions were measured with an amino acid analyser (Hitachi L-8500A; Hitachi, Tokyo, Japan). Samples for analysis were hydrolysed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Tokyo, Japan) at 100 °C for 24 h under vacuum. All experiments were performed in triplicate.

DSC (differential scanning calorimetry)

The thermal properties of SS powders obtained from various extraction processes were examined using a differential scanning calorimeter (DSC 204 F1 Phoenix®; Netzsch, Selb, Germany). Measurements were performed at a heating rate of 10 °C/min and a nitrogen flow rate of 60 ml/min from room temperature to 400 °C.

FTIR (Fourier-transform infrared) measurements

FTIR spectra of SS powders were obtained with an FTIR spectroscope (PerkinElmer) using KBr pellets. For all measurements, the thickness of the specimen was fixed at 2 mm.

Measurement of antityrosinase activity

Pigments from silk cocoons (Chul 3/2 and 4/2) were removed prior to SS extraction to confirm that antityrosinase activities were purely generated from SS, and carotenoids or flavonoids had no effect on the measurements. Assays were performed as previously described with minor modifications [19]. Tyrosinase (1000 units/ml; Sigma, St Louis, MO, U.S.A.) from a mushroom solution was prepared at a concentration of 100 units/ml in 0.2 M phosphate buffer solution (pH 6.5). Tyrosinase mushroom solution (150 μl) and phosphate buffer solution at pH 6.5 (300 μl) were mixed with or without the SS sample (0.8 mg). The mixture was then pre-incubated at 25 °C for 5 min before adding 300 μl of 1.25 mM dopa (3,4-dihydroxyphenylalanine; Sigma) solution, and the reaction was monitored at 475 nm. The percentage of inhibition of tyrosinase activity was calculated as

$$\text{Inhibition, (\%)} = [(A - B)/A] \times 100$$

where A represents the difference in the absorbance of the control sample between incubation time periods of 0.5 and 1.0 min, and B represents the difference in the absorbance of the test sample between the same incubation times.

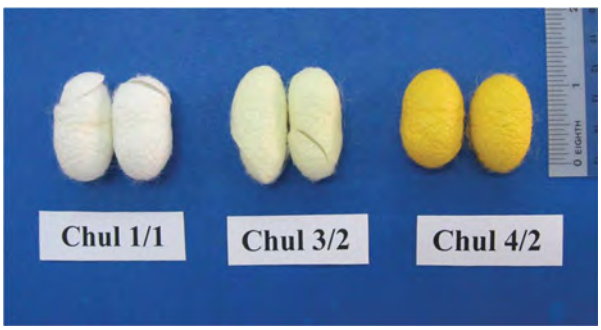

Figure 1 Physical appearance of Thai silk cocoons from three different strains: Chul 1/1 (white shell), Chul 3/2 (greenish shell) and Chul 4/2 (yellow shell)

Table 1 Percentage yield of SS extracted by different methods

Silk strain	Extraction method	Yield (%)
Chul 1/1	Heat	21.27 ± 3.83
	Urea	18.60 ± 4.08
	Acid	15.19 ± 2.36
	Base	12.18 ± 1.11
Chul 3/2	Heat	18.36 ± 0.29
	Urea	21.09 ± 7.19
	Acid	8.41 ± 1.14
	Base	6.93 ± 1.23
Chul 3/2	Heat[a]	17.00 ± 3.14
	Urea[a]	23.10 ± 4.12
	Acid[a]	8.33 ± 2.36
	Base[a]	5.93 ± 1.26
Chul 4/2	Heat	21.47 ± 0.62
	Urea	20.43 ± 1.06
	Acid	13.60 ± 2.13
	Base	12.69 ± 0.65
Chul 4/2	Heat[a]	19.58 ± 1.39
	Urea[a]	20.33 ± 3.26
	Acid[a]	11.86 ± 1.89
	Base[a]	11.60 ± 1.67

[a]SS yield from coloured silk cocoon after removal of pigment.

Each result is presented as the mean from three concurrent readings. Kojic acid was used as a positive control.

Statistical analyses

Results are expressed as means ± S.D. Statistical significance was determined by paired and unpaired Student's t tests together with ANOVA. $P < 0.05$ was considered statistically significant.

Results and discussion

Figure 1 displays the physical appearance of Thai silk cocoons from the three different Chul 1/1, Chul 3/2 and Chul 4/2 strains. SS yield using the various extraction methods are listed in Table 1. For all silk strains, SS extracted by the

Table 2 Colour values (reflectance measurements: L^*, a^* and b^*) in SS powder extracted by urea solution from Chul 3/2 and Chul 4/2 strains

Strain	L^* (mean ± S.D.)	a^* (mean ± S.D.)	b^* (mean ± S.D.)
Chul 3/2	93.41 ± 3.61	–4.02 ± 1.62[a]	15.53 ± 1.65[a]
Chul 3/2[b]	94.00 ± 4.56	–0.81 ± 0.22	6.29 ± 2.01
Chul 4/2	84.83 ± 3.89	–0.28 ± 0.14[a]	53.6 ± 2.23[a]
Chul 4/2[b]	94.43 ± 5.68	0.41 ± 0.29	3.34 ± 0.79

[a]Significant differences compared with same strain.
[b]Colour-extracted SS.

high-temperature and high-pressure method and by urea solution had higher yields compared with that extracted with citric acid and sodium carbonate solutions. Although ethanol can remove a significant amount of carotenoids and flavonoids from cocoon shells, minor amounts of both compounds were still present, as shown by chromaticity. SS extracted with urea appeared to give the most clearly distinguishable protein bands. SS yield in all silk strains was not affected by colour extraction from silk cocoons with ethanol, which indicated that no proteins had been dissolved by ethanol extraction.

Colour values (L^*, a^* and b^*) of SS powders from the Chul 3/2 and Chul 4/2 cocoons are shown in Table 2. SS powder from the Chul 3/2 strain was bright yellow and green in colour, as shown by an elevated negative value of a^* and high positive value of b^* respectively. However, SS powder generated from the Chul 3/2 cocoons after colour extraction showed much reduced a^* and b^* values, indicating that the green and yellow colour intensities decreased significantly, possibly from lower amounts of carotenoids and flavonoids in the cocoon shells. According to a^* and b^* values, the colour extraction process can remove approx. 80% of coloured pigments from the silk strain Chul 3/2. A similar result was also found in SS from the Chul 4/2 cocoons. Since silk cocoons from the Chul 4/2 strain have an intense yellow colour indicating higher pigment composition compared with the Chul 3/2 strain, the colour extraction process can successfully remove the pigment, as shown by the fact that b^* values were reduced by approx. 90%. The L^* and b^* values were used to determine the amount of coloured-pigment removal and we found a linear relationship with good correlation between changes in L^* and b^* values with the amount of coloured-pigment removal ($r^2 = 0.93$ for L^* and $r^2 = 0.94$ for b^*). It was also aligned with the studies by Peterson et al. [20]. From this correlation, more than 80% of pigments were removed from the Chul 3/2 and Chul 4/2 cocoons. These results show that carotenoids and flavonoids, along with other colour substances, are mostly removed from SS powders. Thus our molecular mass, amino acid content and antityrosinase activity results could be attributed to the effect of SS alone.

SS molecular masses from different strains and extraction methods are shown in Figure 2. SS extracted with

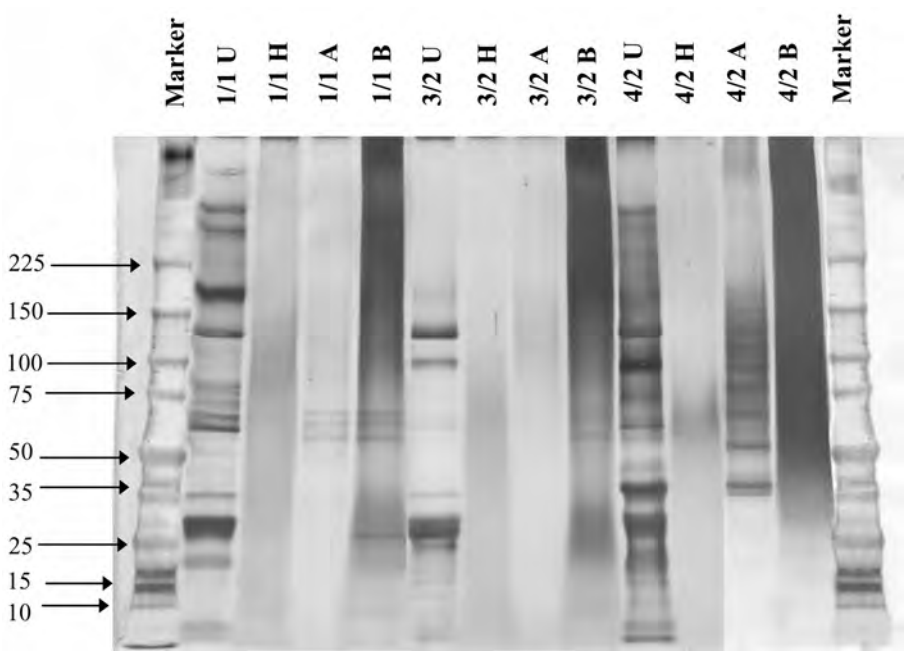

Figure 2 SDS/PAGE of SS extracted from Chul 1/1, Chul 3/2 and Chul 4/2 strains using the following methods: urea solution (U), high temperature and high pressure (H), citric acid solution (A) and sodium carbonate solution (B)

Different silk strains with various extraction methods show different molecular mass SS ranging from 10 to >225 kDa.

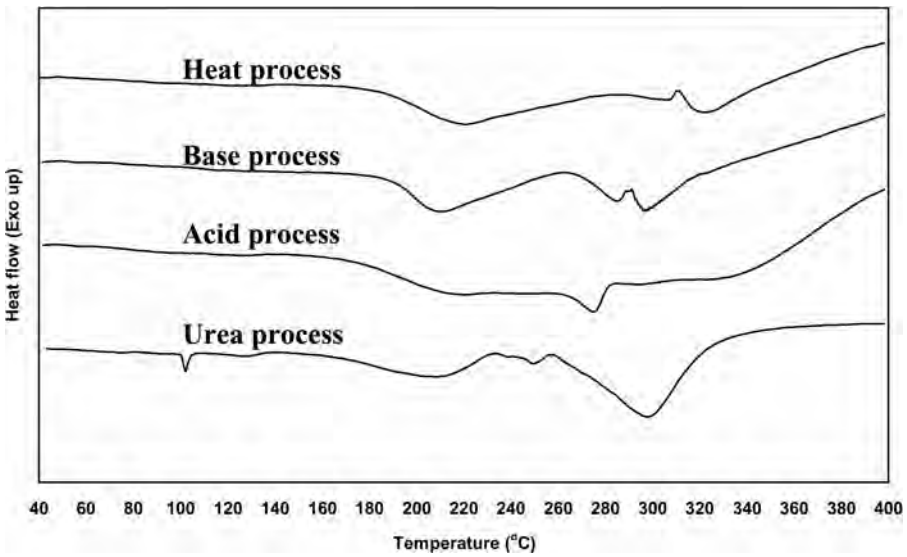

Figure 3 DSC of SS obtained by different extraction methods (acid, alkali, urea and heat processes), which showed an endothermic degradation peak at 210°C

Higher endothermic degradation temperatures of SS powder at 220°C were observed only from heat process SS.

urea from the Chul 1/1 and Chul 4/2 strains showed clear bands with molecular masses ranging from 10 to >225 kDa, whereas SS from the Chul 3/2 strain had a molecular mass in the range between 10 and 150 kDa. This result indicates that different silk strains contain distinct SS and other outer proteins, which may result in varying physical and biological activities. SDS/PAGE of acid- and alkali-degraded SS from all strains also displayed distinguishable bands within the range of 35–150 and 15–75 kDa respectively. However, the number of bands from acid- and alkali-degraded SS was much less than the number of bands from SS extracted with urea. SDS/PAGE of SS prepared by the high-temperature and high-pressure degumming technique showed continuous bands with molecular masses ranging from 50 to 150, 35 to 100 and 35 to 75 kDa for Chul 1/1, Chul 3/2 and Chul 4/2 respectively. Our findings are in agreement with those reported by Sprague [21], which indicated that SS is a mixture of at least 15 different polypeptide chains, ranging in size from 20 to 220 kDa.

The thermal behaviours of SS powders extracted from various processes are presented in Figure 3. We found that SS powder obtained from extraction methods using urea, acidic and alkaline solutions showed an endothermic degradation peak at 210°C. However, the endothermic degradation temperatures of SS powder at 220°C obtained from high-temperature and high-pressure degumming techniques were higher than those obtained from other processes. The observed degradation temperature corresponded to the 221°C reported by Lamoolphak et al. [22], implying that the use of chemicals during the extraction process influences the thermal stability of SS.

FTIR spectra of SS powders obtained from different extraction methods (heat, acid, alkali and urea) are shown in Figure 4. The peak positions of amide I (C=O stretching), amide II (N-H deformation and C-N stretching) and amide III (C-N stretching and N-H deformation) of SS powders derived from heat and alkali processes were located at 1650, 1530 and 1238 cm^{-1} respectively. These amide bands contribute to the primary random coil structure of SS [23,24]. Sericin powder obtained from acid extraction showed similar peak patterns except for the amide I characteristic peaks, which appeared at 1650 and 1624 cm^{-1}, indicating the presence of a random coil and β-sheet conformation respectively [25]. The SS powder prepared by urea extraction exhibited different characteristic peaks than those from SS extracted by other methods. The specific amide II peak was not clearly observed, while amide I occurred at 1650 and 1621 cm^{-1}, corresponding to a random coil and β-sheet respectively. In addition, the amide III characteristic peak shifted to 1225 cm^{-1} and a shoulder at 1250 cm^{-1}, indicating the presence of a β-sheet. Moreover, peaks at 1460 and 1150 cm^{-1} were found to correspond to urea in SS extracted using urea solutions [26]. However, the urea molecules presented in SS obtained by the urea extraction method might be incorporated as a part of SS molecules that cannot be removed by dialysis. This was supported by stable pH at a slightly acidic range after being thoroughly dialysed. The FTIR results revealed that the extraction process of SS could affect the chemical structure of SS. Among the four extraction methods used here, extraction with urea had the most significant impact on SS conformation.

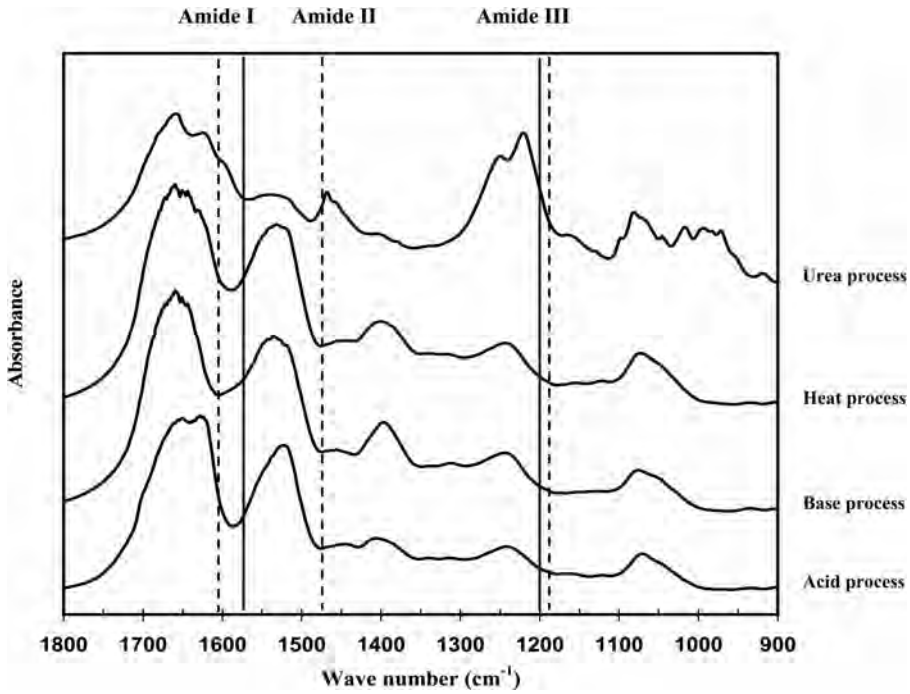

Figure 4 FTIR spectra of SS obtained from various extraction methods: acid, alkali, urea and heat processes

The solid vertical line indicates a β-sheet; the broken vertical line indicates a random coil. The peak positions of amide I (C=O stretching), amide II (N-H deformation and C-N stretching) and amide III (C-N stretching and N-H deformation) of SS from heat and alkali extractions were located at 1650, 1530 and 1238 cm^{-1} respectively. SS from acid extraction showed similar peak patterns except for the amide I characteristic peaks, which appeared at 1650 and 1624 cm^{-1}. The SS powder prepared by urea extraction exhibited different characteristic peaks than those from SS extracted by other methods, indicating that urea had the most significant impact on SS conformation.

Amino acid compositions of SS extracted by different methods are listed in Table 3 for all three strains. The results indicated that the Chul 1/1 strain contained the highest amount of methionine and cysteine residues. Whereas the methionine content in Chul 1/1 showed a significant difference ($P < 0.05$) when compared with both the Chul 3/2 and Chul 4/2 strains, the cysteine content in Chul 1/1 was significantly different ($P < 0.05$) when compared with the Chul 4/2 strain only. Moreover, the SS amino acid compositions from the same strain varied according to the method of SS extraction.

All SS had high amounts of serine and glycine with no significant differences between strains or extraction methods ($P > 0.05$). However, all strains did show a significant difference in aspartic acid content among the urea, heat and alkaline extraction methods, although heat and acid extraction showed no significant difference in aspartic acid levels. For all strains, alkaline extraction provided SS with the highest levels of aspartic acid. This may be due to the fact that aspartic acid normally has a negative charge at physiological pH, as the carboxylic side chain can easily be deprotonated in alkaline solutions to produce water-soluble $COO^{-}NH_4^{+}$ species. All extraction methods employed water-based solvents, thereby generating high amounts of water-soluble serine and glycine, whereas very few hydrophobic amino acids such as proline, leucine, isoleucine and phenylalanine were found. High arginine content in SS extracted from urea may be because of hydrogen-bonding between the guanidine group on arginine and the amino group on urea. We found the highest tyrosine content by acid extraction, which may due to protonation of tyrosine side chains.

We assessed direct inhibitory effects of SS on semi-purified tyrosinase and listed the results from our tyrosinase assays in Figure 5. Kojic acid was used as a positive control, as it significantly inhibits the catalytic activity of mushroom tyrosinase. SS, particularly from the Chul 3/2 strain, was found to inhibit mushroom tyrosinase. Overall, SS obtained from different extraction methods exhibited different degrees of tyrosinase inhibition, even within the same silk strain. SS from the Chul 1/1 strain showed significant differences in antityrosinase activity when the various extraction methods were compared. Our results indicate that SS extracted with urea had the highest degree of antityrosinase activity when compared with other extraction methods for all strains, whereas alkali-degraded SS showed no inhibition of mushroom tyrosinase. Only acid-degraded SS from coloured silk cocoons exhibited

Table 3 Composition of amino acids of SS from Chul 1/1, Chul 3/2 and Chul 4/2 strains extracted by different methods

Values are the means from triplicate analysis. H, heat; U, urea; A, acid; B, alkali.

Amino acid	Chul 1/1				Chul 3/2				Chul 4/2			
	H	U	A	B	H	U	A	B	H	U	A	B
Asp	15.64	18.31	15.93	19.88	15.62	17.93	16.00	21.58	15.97	17.69	16.61	19.92
Ser	33.63	31.27	31.86	30.01	34.50	32.24	32.01	28.41	33.84	30.69	31.95	27.59
Glu	4.61	5.27	5.75	5.93	4.76	6.02	5.40	7.66	4.86	5.97	5.88	7.03
Gly	15.03	11.23	10.49	11.01	15.09	10.75	10.38	11.16	15.14	10.96	10.69	12.58
His	1.06	3.26	2.47	1.72	1.22	2.82	2.83	2.38	1.37	2.50	2.29	2.15
Arg	2.87	5.41	4.92	4.92	2.95	5.21	4.87	3.79	3.09	5.71	5.24	4.83
Thr	8.16	8.36	8.51	6.49	8.43	8.78	8.78	6.09	8.34	9.04	8.30	5.56
Ala	4.10	4.33	3.72	4.21	4.45	3.80	3.57	3.96	4.98	4.63	3.56	4.40
Pro	0.54	1.46	0.78	1.24	0.62	0.79	0.73	0.92	0.71	1.16	0.79	1.01
Cys	0.44	0.39	0.53	0.23	0.43	0.33	0.50	0.19	0.27	0.42	0.52	0.16
Tyr	3.45	0.36	5.56	5.24	3.64	1.24	5.81	4.92	3.47	2.67	5.59	4.90
Val	2.88	2.96	2.95	2.94	3.04	3.28	3.03	3.03	2.92	2.98	2.76	2.99
Met	3.39	0.12	0.06	0.15	0.57	0.08	0.06	0.13	0.18	0.06	0.05	0.15
Lys	2.35	3.14	3.48	2.89	2.51	3.55	3.03	2.71	2.78	2.50	3.16	3.08
Ile	0.56	0.96	0.87	0.75	0.65	0.95	0.90	0.87	0.61	0.74	0.66	1.03
Leu	1.00	1.58	1.43	1.56	1.15	1.58	1.44	1.51	1.11	1.63	1.37	1.81
Phe	0.28	0.60	0.71	0.81	0.39	0.66	0.67	0.72	0.36	0.63	0.57	0.81

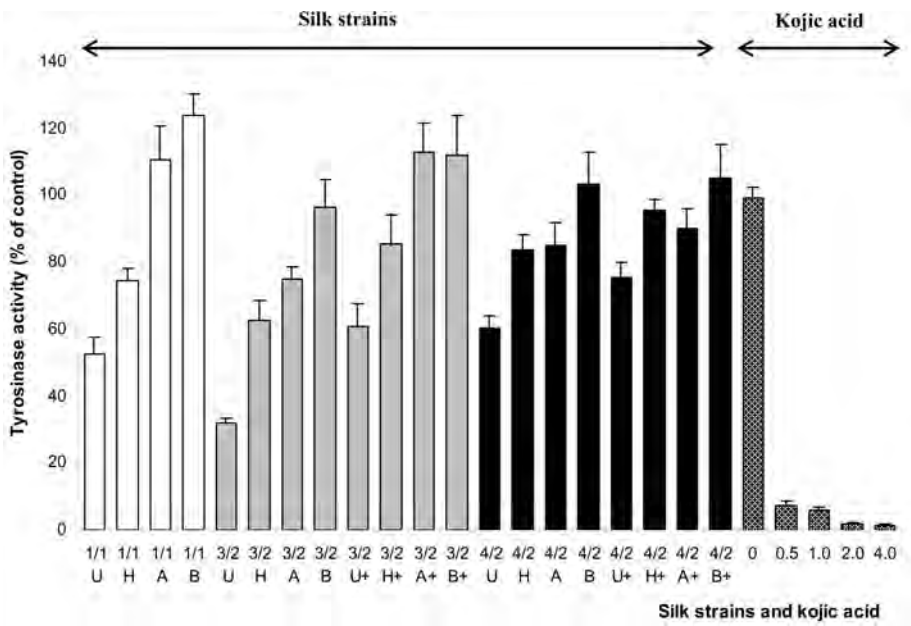

Figure 5 Effect of SS on mushroom tyrosinase activity compared with that of kojic acid

SS obtained by different extraction methods exhibited different degrees of tyrosinase inhibition, even within the same silk strain. SS, particularly from the Chul 3/2 strain, was found to inhibit mushroom tyrosinase. The '+' sign indicates SS from coloured silk cocoons after the removal of pigment. H, heat; U, urea; A, acid; B, alkali.

antityrosinase activity, but at a very low degree of inhibition. As shown in Table 2, the pigment extraction process was sufficiently effective so that it is reasonable to conclude from Figure 5 that SS itself has a significant antityrosinase activity. Pigments from silk cocoons were shown to also affect antityrosinase activity, as shown by the stronger tyrosinase inhibition of SS from coloured silk cocoons with or without pigment extraction. Comparing all strains and extraction methods, SS extracted from the Chul 3/2 strain by urea without pigment removal showed the highest degree of tyrosinase inhibition.

With regard to individual amino acids, Kahn [17] reported that cysteine appeared to be the best tyrosinase inhibitor. However, the observed inhibition was due to conjugation of cysteine with the enzymatically produced quinine rather than from direct enzyme inhibition [27]. This may also explain our result that acid-degraded SS showed the highest amount of cysteine but had less

antityrosinase activity than SS extracted with urea. As peptides that interact with tyrosinase can act as potential inhibitors, arginine-containing peptides are considered to be the most tyrosinase-binding peptides especially from the shorter peptides, whereas valine-containing peptide is one of the most tyrosinase-inhibiting peptides [14]. Sericin extracted with urea from all strains showed significantly high amounts of arginine and valine compared with SS extracted by other methods. Tyrosine-containing peptides are substrates for tyrosinases and thus may contribute to tyrosinase activation. Acid-degraded SS from all strains exhibited high tyrosine levels with a rather low degree of tyrosinase inhibition. Furthermore, Kahn [17] also indicated that peptides containing a negatively charged aspartic acid or glutamic acid residue are highly unfavourable for the tyrosinase–peptide interaction and therefore show lower antityrosinase activity [17]. This correlates to our alkali-degraded SS, which exhibited significantly high levels of aspartic acid and glutamic acid and low antityrosinase activity. However, all of the results presented here were from *in vitro* studies, and further *in vivo* studies are needed to determine biologically relevant antityrosinase activities.

In conclusion, we demonstrated that silk strains and extraction methods affect both physical and biological properties of SS. The urea extraction method appears to be the most efficient way to obtain clearly distinguishable bands with a broad range of molecular masses. Different extraction methods significantly affected the amino acid content of SS from all strains. With regard to the biological effects of SS, urea-extracted SS showed the highest antityrosinase activity, while alkali-degraded SS exhibited the lowest tyrosinase inhibition when compared with other extraction methods. Pigments from coloured cocoons also exhibited an antityrosinase effect, as shown by a lower degree of tyrosinase inhibition after pigment removal.

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