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THESIS

INFLUENCES OF FOOD MATRIX ON PROTEIN DIGESTIBILITY AND OIL RELEASE OF HIGH-CALORIC EMULSION UNDER *IN VITRO* GASTROINTESTINAL CONDITIONS

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This study investigated the effects of heat treatment sequence, calcium lactate concentration (0, 25, and 100 mM), and soy residue (okara) addition on the fabrication of protein matrix at the oil-water interface of the liquid food emulsion. The modeled pasteurized oil-in-water liquid emulsion contained 3.75% (w/v) protein from sodium caseinate and soy protein isolate, 2.0 % (w/v) cassava starch, 11.75 % (w/v) cassava maltodextrin and 3.33 % (v/v) rice bran oil. The MW of proteins and peptides, % oil released and microstructure of liquid emulsion subjected to peptic and tryptic digestion over time, in the absence or presence of bile acid, were investigated. The *two-stage heating process* applied during emulsion preparation led to the slower release of free fat under stomach condition compared with the *one-stage heating process* ($p<0.05$) due to the longer lag phase prior to oil release. Calcium lactate-induced protein aggregation led to the formation of large MW (>250 kDa) at the interface. Increasing calcium lactate concentration did not have significant effect on free oil released after peptic digestion ($p\geq0.05$). Nevertheless, at 100 mM calcium lactate, the proteins at the interface was increased, compared with those at the lower calcium lactate and there was no oil separated after 30 min tryptic digestion, particularly when bile acid was also present. The presence of pectinase-hydrolyzed okara in the emulsion subjected to the *two-stage heating process* prolonged the lag phase required before the release of oil. However, the oil released to the high extent after tryptic digestion for 30 min. This study has demonstrated that the protein matrix at the interface and in the bulk phase influenced the release of oil by prolonging the lag phase or shortening the lag phase during peptic digestion. From industrial standpoints, the production of healthy foods with controlled digestion and nutrient releases can be facilitated by controlling the preferential adsorption of protein types at the oil-water interface, induction of protein or peptide re-adsorption at the interface and/or changing the matrix composition at the oil-water interface. These approaches can be manipulated by protein choices and emulsification procedure.

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INTRODUCTION

Many people have risks on non-communicable chronic diseases such as diabetes, coronary heart diseases, high blood pressure and cancer. Too much fat diet is the main cause of their disease development. Many food manufacturers are trying to identify the method for reducing fat content in food products. In the case of high-caloric diet, controlling scheme for the amount and rate of lipid released from food during digestion in gastrointestinal tract (GI) received limited investigation.

The ideal oil-in-water liquid emulsion for drinks and beverages need to have low viscosity, and be stable in the presence of electrolytes after pasteurization, sterilization and storage for consumption. The liquid food formulae containing only soy proteins are deficient in essential amino acid methionine. Caseinate, the milk proteins, can be added to the formulae. However, caseinate is sensitive to ionic calcium, which could lead to heavy aggregation and possible gelation in the presence of complete electrolytes in the formulae.

This study attempted to understand the roles of mixed proteins; i.e., soy protein isolate (SPI) and sodium caseinate (SCN) composite at the interface of the emulsion. They were used as protein source in the model liquid food formulae providing 1.0 kcal/mL. The formulae contained caloric distribution from carbohydrate:protein:lipid of 55:15:30. The fabrication of different protein matrix characteristics at the oil-water interface was used as a tool to understand how to control lipid release from the emulsion under *in vitro* GI studies. The insights on the nature of protein matrix at the interface generated during food processing and its fate caused by pH shifting, peptic digestion, tryptic digestion and the presence of bile acid

in the GI tract may help improving the food process that could control the release of macronutrients during digestion in the GI tract.

OBJECTIVES

1. To study the physical and chemical properties of protein composites between sodium caseinate (SCN) and soy protein isolate (SPI),

2. To fabricate protein matrix at oil-water interface and in bulk aqueous phase of the model liquid emulsion through heat treatment sequence, calcium lactate concentration and addition of soy residue, and

3. To determine the protein pattern at the oil-water interface and in the bulk aqueous phase, protein digestibility and oil release of the model liquid food under stomach and duodenum conditions (*in vitro* study).

LITERATURE REVIEW

1. Protein-stabilized oil-in-water emulsion

Proteins in plants and animals are found in many forms, including metabolic proteins (e.g., enzymatic, hormonal and structural proteins) and storage proteins (e.g., egg albumin, seed proteins and caseins). Storage proteins are used as the main constituent of various food products because they have high nutritional value and provide desirable functional properties, including water holding capacity, foaming and gelation to food products (Damodaran and Paraf, 1997).

Food emulsions (e.g., milk, cream, ice cream, butter, cheese, dressing and mayonnaise) represent a heterogeneous mixture of liquid. They are mixtures of oil and water, which are thermodynamically unstable. Oil and water tend to separate during long-term storage. Proteins are surface-active molecules with hydrophobic side chains of proteins close to the oil surface and hydrophilic residues favored in the aqueous phase (Dalgleish, 1997a). Thereby they are widely used as emulsifiers in food industry. Emulsifying properties of each protein depend on size, shape, charge, and hydrophobicity of proteins (Zayas, 1997).

1.1 Soy proteins

A polypeptide chain that forms the spherical shape is referred as globular protein. The folding to the globular shape is mainly due to a large number of hydrophobic residues, which are thermodynamically unfavorable with water. A polypeptide chain folds and the internal of the molecules are composed mainly of the hydrophobic amino acid residues; while the polar and/or charged amino acid are mainly present on the surface (Damodaran, 1996).

Soy proteins are often employed as emulsifier in food products. The majority of soy proteins are the storage proteins, which consist mostly of globulins. The globulins are soluble in salt and the two major globulins present in soy proteins

are *β*-conglycinin and glycinin. The soy storage proteins can be fractionated into four main groups on the basic of sedimentation velocity as 2S, 7S, 11S and 15S (Naismith, 1955). *β*-conglycinin is the main constitute of the 7S fraction, whereas glycinin is the main component of the 11S fraction. *β*-conglycinin (7S globulin) with molecular mass of 62-200 kDa, is a trimer which consists of three subunits as α′, α and β. The molecular weights of each subunit are 58, 57 and 42 kDa, respectively (Petruccelli and Añón, 1995). Glycinin (11S globulin) is a hexamer with a molecular mass of 300-380 kDa. Each subunit is composed of acidic (35 kDa) and basic (18-20 kDa) polypeptides. Both acidic and basic polypeptides were linked together by disulfide bond (Staswick *et al*., 1984). Five subunits were identified in glycinin as $A_{1a}B_{1b}$ (53.6 kDa), A_2B_{1a} (52.4 kDa), $A_{1b}B_2$ (52.2 kDa), A_3B_4 (55.4 kDa) and $A_5A_4B_3$ (61.2 kDa) (Nielsen, 1985). Due to the large number of sulfhydryl (SH) groups in the glycinin, sulfhydryl-disulfide (SH-SS) interchange within a molecule (Table 1) resulted in the formation of glycinin gel with harder texture than that of *β*-conglycinin gel at 95˚C (Renkema *et al*., 2001). However, *β*-conglycinin provided stronger emulsifying ability than did glycinin (Utsumi *et al*., 1997).

Table 1 Numbers of cysteine and cystine in each subunit of *β*-conglycinin and glycinin

	Subunit	Cysteine (SH)	Cystine (SS)
β-Conglycinin (7S)	α′	1	0
	α	1	0
	β	0	0
Glycinin (11S)	$A_{1a}B_{1b}$	2	3
	A_2B_{1a}	2	3
	$A_{1b}B_2$	2	3
	A_3B_4	2	2
	$A_5A_4B_3$	2	2

Source: Utsumi *et al*. (1997)

The commercial soy proteins have been used in the form of soy protein concentrate (SPC) and soy protein isolate (SPI). SPI is manufactured by grinding the defatted beans in an alkali solution (pH~8.0) to separate the soluble fraction. After that, the protein is precipitated by adjusting the pH to 4.5 (Puppo and Añón, 1998). Generally, the isoelectric pH (pI) of soy protein is around 4.8 to 6.4 (Liu, 1997). However, soy protein is not an ideal protein, because it is deficient in the essential amino acid methionine. Soy contains methionine for only 1.39 g/16 g of N; while the recommendation by the Food and Agricultural Organization (FAO) is 3.5 g/16 g of N (Friedman *et al*., 1991; Friedman and Brandon, 2001).

Heat treatment is the most common physical means for improving the emulsifying properties of globular proteins. Heat treatment induces denaturation or unfolding of protein that leads to the exposure of hydrophobic groups and increases surface hydrophobicity (Kato *et al*., 1983). Heat treatment after the emulsion formation further exposes hydrophobic groups or other functional groups (i.e., sulfhydryl group) of the adsorbed protein on the interface and altered the interfacial protein (McClements, 2004). The soy protein-stabilized emulsions are prone to flocculation or aggregation and creating a highly viscous emulsion during heating (Roesch and Corredig, 2002). This is the limit to drinks or beverage application.

Flexible phosphoproteins (e.g., α-casein, β-casein, ovotransferrin, etc.) can interact on the surfaces of unfolding globular proteins and show the polyanion characters on the surface, a so called “chaperone properties” (Matsudomi *et al*., 2004). Using protein composites between globular proteins and flexible phosphoproteins in the emulsion may alter the aggregation characteristics of the protein-stabilized emulsion droplets during heat treatment. Thus, this study attempted to use protein composites for both nutrition and surface modification purposes.

1.2 Caseins and caseinates

Flexible phosphoproteins like caseins are widely used as ingredient in food products because they possess high nutritive value and are excellent emulsifier.

Caseins have flexible structure and hold no tertiary structure and little secondary structure. Thereby they are easily unfolded at the oil-water interface (Zayas, 1997). Caseins are contained for approximately 80% of total protein in bovine milk. Four main types of casein; namely κ-casein, α_{s1}-casein, β-casein and α_{s2}-casein are present in a mass ratio of about 1:4:4:1 with molecular weights of 19, 22, 24 and 25 kDa, respectively (Lee *et al*., 1992; Fox and McSweebey, 1998). Generally, α_{s1}-casein, β-casein and α_{s2}-casein have serine residues, which conjugate with phosphate to form phosphoserine (SerP). The amount of phosphoserine residues in α_{s1}-casein and β-casein is 8 and 5 residues per molecule of casein, respectively (Agboola and Dalgleish, 1995). In the presence of calcium, this casein strongly aggregates with calcium phosphate as casein micelles (30-300 nm) (Figure 1) (Walstra and Jenness, 1984). The micelle can be stabilized in solution because κ-casein with hydrophilic moiety is present on the surface (Dalgleish, 1990).

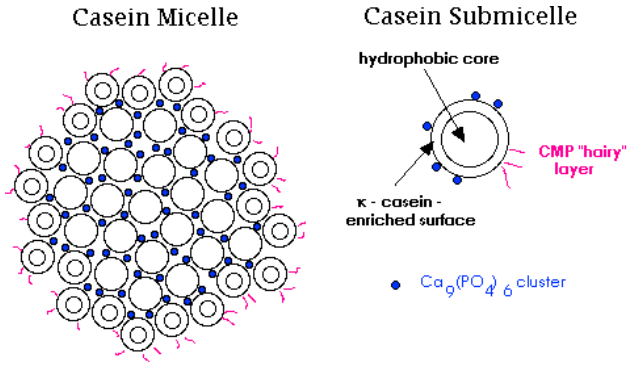

Figure 1 Schematic of casein micelle

Source: Walstra and Jenness (1984)

Commercial caseins are in the form of sodium-, calcium- and potassium-caseinates. The micellar structure of casein is destroyed during the production of caseinate due to the removal of calcium within the structure (Kinsella, 1984). Sodium casinate (SCN) exhibits higher solubility than other caseinates (Konstance and Strange, 1991). They are widely used as an ingredient in foods such as ice-cream, coffee whitener and whipped toppings (Doxastakis, 1989). SCN is manufactured by

precipitating the casein from milk by lowering the pH to 4.6, which is isoelectric pH of casein micelle. The casein curd is then washed with water, re-neutralization to pH~7.0 with NaOH before drying. Thus, SCN contains a mixture of casein monomers, especially phosphorylated forms and possesses the character of casein nanoparticles (10-20 nm) (Lucey *et al*., 2000). The pI of SCN was thus lower to around 3.75 to 4.0 (Jahaniaval *et al*., 2000).

The SCN-stabilized emulsion is stable over a wide range of temperature (Kinsella, 1984; Hunt and Dalgleish, 1995). Addition of divalent electrolytes, i.e. calcium ions, resulted in flocculation and aggregation of the emulsion droplets. This makes the SCN-stabilized emulsion prone to creaming and an increase in viscosity (Agboola and Dalgleish, 1996; Dalgleish, 1997b; Dickinson and Golding, 1998; Dickinson and Davies, 1999). Flocculation of the emulsion induced by ionic calcium may be due to calcium-induced bridging between adsorbed casein molecules on different droplets or the decreasing of charge and thickness of the adsorbed layers at the interface (Dickinson *et al*., 1992; Agboola and Dalgleish, 1995). Calcium can bind with negatively charged phosphoserine residues on both α_{s1}-casein and β-casein. This reduces the surface charge on the protein and change the conformation of the adsorbed layer around the emulsion to collapse that lead to a decrease in thickness of protein layers (Dalgleish, 1997b).

1.3 Formation of interfacial film at the interface for emulsion stabilization

Proteins can stabilize emulsion by both steric and charge mechanisms. Steric mechanism arises from proteins that form the film surrounding the oil droplets (Figure 2). As a result, the proteins prevent immediate flocculation or coalescence of the oil droplets (McClements, 2004).

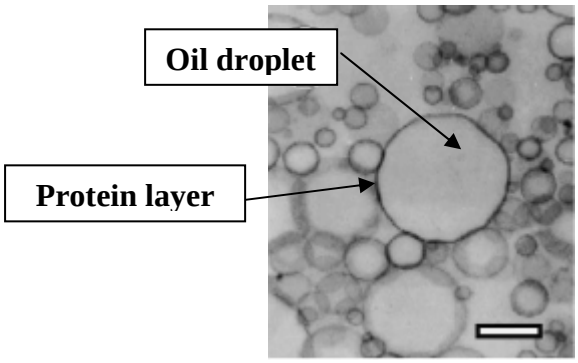

Figure 2 Transmission electron micrograph of an emulsion of soya oil stabilized by sodium caseinate. The surface is covered by monolayer of protein. Bar scale= 300 nm.

Source: Dalgleish (2006)

The formation of the interfacial film is three-step processes (Zayas, 1997) including: (1) diffusion of proteins to the interface, which depends on molecular weight of protein. Caseinates have small molecular weight size. Therefore, they may diffuse quickly to the interface compared to soy proteins (Tornberg *et al*., 1982). The molecular weight of soy proteins is around 180 to 380 kDa; while casein monomer is 25 to 30 kDa (Cheftel *et al*., 1985; Kilara and Harwalkar, 1996; Manski *et al*., 2007) (2) protein adsorbed at the interface. The stability of the emulsion is increased with increasing protein concentration at the interface. The adsorption of protein is enhanced by salt addition before homogenization and resulted in a decrease in surface repulsion. Salt also induces excess protein in the aqueous phase move to the interface during homogenization (Mulvihill and Murphy, 1991; Radford *et al*., 2004). The increasing of protein concentration (up to 2.0% w/v) is the method for increasing protein concentration at the interface of the emulsion containing high salt concentration. Salt can induce precipitation of unadsorbed proteins from the serum to the oil surface (Schokker and Dalgleish, 2000) and (3) changes of protein conformation resulted from unfolding of protein molecules. The adsorbed proteins can also blend with other neighboring molecules and aggregate to form a thicker layer

surrounding the oil droplets. This is because the unfolded proteins expose the reactive polar groups into solution.

Hence, the mechanical properties of the interfacial film depend on the structure of the adsorbed protein and the strength of the interactions between them. Of the latter, the thickness and shape of interfacial film are important on emulsion stability and may influence protein digestibility and oil release from food structure during digestion in gastrointestinal tract.

2. Physico-chemical changes of oil-in-water emulsion during digestion

2.1 Lipid digestion and absorption in gastrointestinal tract

Gastrointestinal tract (GI) is the main part of digestive system where digestion and absorption of nutrients occur. It involves mouth, esophagus, stomach, small intestine, large intestine and anus (Figure 3). Lipid digestion and absorption are series of events in the small intestine. Initially, ingested food is mixed with saliva and reduced to small size by chewing to form a bolus. Bolus passes the esophagus into the stomach and mixed with gastric enzyme (i.e. pepsin and lipase) (Tso and Crissinger, 2000; Grosvenor and Smolin, 2002).

In the case of lipid embedded within food matrices consisting of proteins, carbohydrates and other compositions (Figure 4), its digestion depends on how fast gastric enzyme can breakdown the matrix surrounding them. The rate of matrix disintegration depends on altering lipid droplet size, interfacial properties, and manipulating food matrix structure and composition (Pasquier *et al*., 1996; Beysseriat *et al*., 2006; Mun *et al*., 2007; McClements *et al*., 2008).

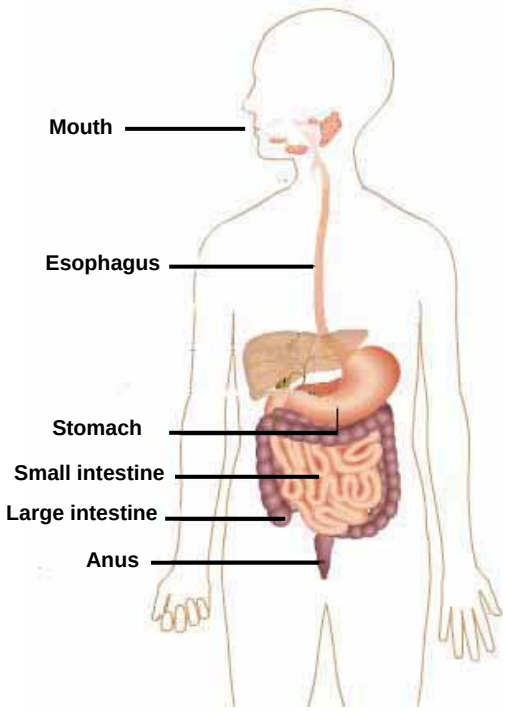

Figure 3 Schematic of gastrointestinal tract

Source: Whitney and Rolfes (1993)

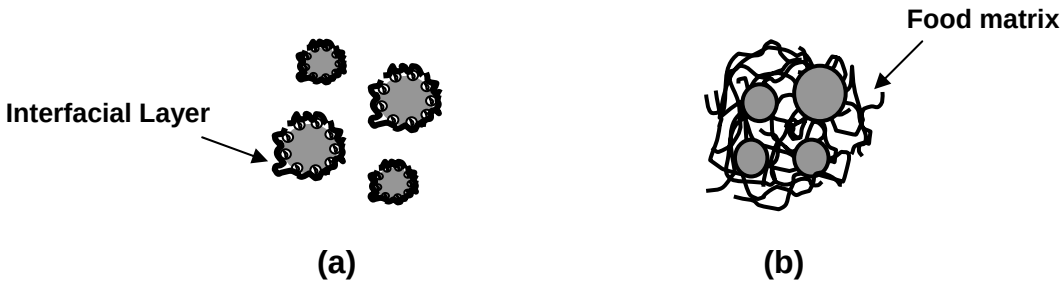

Figure 4 Schematic of the different matrices to alter lipid bioavailability (a) matrix at the interface (b) the oil droplet entrapped within food matrix

Source: McClements *et al*. (2008)

In the stomach, lipid released from food matrix is converted into the larger droplet size due to droplet coalescence. Due to the high acidic condition in stomach (pH 1-2), lipases produced in the stomach are not reactive. However short- or medium-chain fatty acid can be hydrolyzed (Grosvenor and Smolin, 2002). After the stomach digesta entered the small intestine (pH 5.4-7.0), the lipid droplet is covered by bile acid (anionic surfactant) and broken down into small droplets. Lipase secreted from the pancreas binds to the surface of oil droplets and hydrolyzes triacylglycerol to diacylglycerols, monoacylglycerols, and free fatty acids (Tso and Crissinger, 2000). The lipid digestive products form smaller droplets and are coated with bile acid, known as "micelles" move close to the villi, these micelle can diffuse into the mucosal cell of the small intestine. The bile acids in the micelles are reabsorbed and returned to the liver to be reused (Hofmann, 1977).

It is recommended that the increased consumption of the dietary fiber can prevent lipid digestion and absorption in the GI tract, particularly soluble dietary fiber. This is due to the increase in viscosity of bolus or digesta in the stomach and small intestine. Dietary fiber binds bile acid, interacts with lipase, thereby reducing the enzyme activity (Pasquier *et al*., 1996; Thongngam and McClements, 2005; Drzikova *et al*., 2005).

Controlling of lipid release from food structure during digestion in the GI tract is important for improving health of humans (McClements *et al*., 2008). Human who has low lipid digestion or adsorption requires an increase of lipid release from food structure; while individuals who are risk of non-communicable chronic disease such as diabetes mellitus, coronary heart diseases, high blood pressure and cancer require a decrease of lipid release (Grosvenor and Smolin, 2002). Thus, understanding of the factors affecting lipid released from food structures during digestion in the GI tract would help fabricating foods for specific nutritional performance (McClements *et al*., 2008).

2.2 Enteral or tube feeding

Enteral formulae are nutritious liquid emulsions widely used in hospitals as complete nutrient products administered into the GI tract for patients who cannot swallow. They contain carbohydrates, proteins, lipids, vitamins, minerals and water that meet the requirements when provided in adequate volume. The standard formulae for individual with normal GI function usually contain 1.0 kcal/mL and caloric distribution from carbohydrate:protein:lipid of 37-55:15-25:30-45 if fluid intake needs not to be restricted. The calorie sources are usually in the form of biopolymers in the case of polymeric formulation. This is to lower the osmolality of formulae that could cause osmotic diarrhea if the osmolality is much higher than the isotonic osmolality of 300 mOsmol/kg (Pfeiffer *et al*., 1998). Carbohydrates used may be in the form of glucose syrup, maltodextrin or other forms of complex carbohydrate (Hongsprabhas and Hongsprabhas, 2000). Protein sources in the commercial products include intact SCN and/or SPI. Hydrolyzed casein, caseinate and whey proteins, as well as free amino acids, also find their uses in the oligomeric or elemental formulae, which have higher osmolality than the polymeric ones. The formulae also contain corn oil, or soybean oil, to provide both energy source and essential fatty acids.

Apart from the caloric dense characteristics from the above mentioned carbohydrate and protein sources, the pathogen-free formulae need to have low viscosity (ca. 100-150 mPa.s) to flow through the tubing of 3.3-5.7 mm. (10-16 French unit; Fr) in diameter to enter the GI tract. The presence of electrolytes to meet requirement is also challenging for the manufacturers to optimize the process for such high caloric emulsions with various mono-, di- and tri-valent ions at high ionic strength (Nanovic, 2005) to avoid flocculation, coalescence or even gelation during pasteurization, or sterilization, and storage.

2.3 Alterations of protein characteristics at interface

2.3.1 The interactions of adsorbed proteins at the interface

Generally, globular proteins are sensitive to heat treatment. On heating, protein molecules undergo conformational changes characterized by a partial unfolding of the globular structure and expose reactive groups such as hydrophobic regions and SH groups. The unfolded molecules aggregate, leading to the formation of insoluble aggregates with higher MW (Kinsella and Whitehead, 1989). The degree of polymerization of soluble aggregates to insoluble aggregates can be regulated by altering the number of reactive groups. Disulfide bond formation is a prerequisite for globular protein polymerization during heating (Zhu and Damodaran, 1994). Altering the number of reactive groups could be done by adding the thiol reagents such as cysteine, *N*-ethylmaleimide and *β*-mercaptoethanol. At low protein concentration, soluble or insoluble aggregates occur. However, further aggregation of these aggregates can be induced by addition ion or ionic strength (Barbut and Foegeding, 1993; Hongsprabhas and Barbut, 1997a; Ja and Kilara, 1998; Marangoni *et al*., 2000; Alting *et al*., 2003; Maltais *et al*., 2005). When such aggregate system was used in the emulsification, disulfide linkages appear to form between adsorbed proteins in the emulsion. The adsorbed proteins are responsible for the stability of the adsorbed layer at the oil-water interface, which is extremely difficult to be displaced by other protein molecules or surfactants (Roth *et al*., 2000; Dalgleish *et al*., 2002).

2.3.2 Competitive adsorption among proteins at the interface

In emulsion stabilized by mixed proteins (e.g., subunits of casein or globular proteins), competitive absorption at the oil-water interface could occur among these proteins. For example, α_{s1}-casein at the oil-water interface of the emulsions was reported that it could be displaced by β-casein (Dickinson *et al*., 1988). The β-casein added to the whey-stabilized emulsion could also displace β-lactoglobulin at the interface, but the displaced proteins were re-adsorbed during subsequent heat treatment (Dickinson, 1994; Brun and Dalgleish, 1999).

2.3.3 Carbohydrate-induced flocculation of oil-in-water emulsion

Addition of soluble polysaccharide to a protein-stabilized oil-in-water emulsion may induce two types of flocculation (i.e., depletion- or bridging-flocculation) (Dickinson and Euston, 1991). It depends on the type and concentration of polysaccharide, molecular size, pH and ionic strength (Beysseriat *et al*., 2006). Depletion flocculation occurred in the presence of non-ionic polysaccharide (i.e., guar gum, locust bean gum) or sugar (Bourriot *et al*., 1999; Schorsch *et al*., 1999). The carbohydrates increased the attractive force between the droplets due to the osmotic effect associated with the exclusion of the biopolymers from a narrow region surrounding each droplet (McClements, 2000). At neutral pH and high concentration of anionic polysaccharide (i.e., high methoxyl pectin (HMP), pectin and carrageenan), the depletion flocculation could be induced due to the non-adsorbed polysaccharide (Surh *et al*., 2006). At low pH, protein shows positively charged, the negatively charged polysaccharides were adsorbed onto the oil droplets via positively charged interfacial protein, causing bridging of the emulsion (Moreau *et al*., 2003; Surh *et al*., 2006). When polysaccharide was present on the surface at sufficient amount, the aggregation of the emulsion droplets stabilized by caseinate during acidification could be prevented (Liu *et al*., 2008).

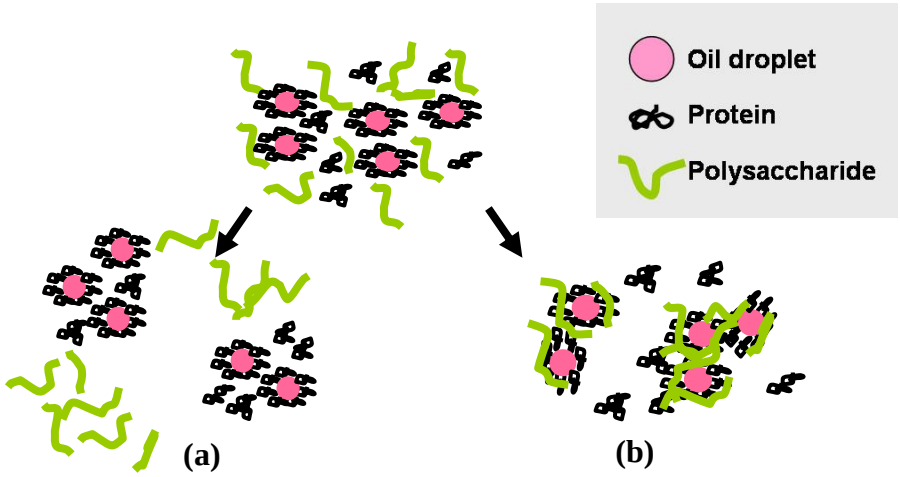

Figure 5 Schematic of soluble polysaccharide induced flocculation of the protein-based emulsion droplets with different mechanisms (a) depletion (b) bridging.

3. Soy residue from soymilk production (okara)

Soy residue or okara is a by-product of soymilk and tofu preparation collected after filtration. Dried okara contained 25.4-28.4% protein, 9.3-10.9% oil, 40.2-43.6% insoluble fiber, 12.6-14.6% soluble fiber, and 3.8-5.3% soluble carbohydrates (Van der Riet *et al*., 1989). Therefore, okara is a rich source of dietary fiber, especially pectin-like polysaccharides. Okara also contains a high quality protein (Wang and Cavins, 1989; Ma *et al*., 1997). It is used as an ingredient in many food products such as pickle, tempeh, salads, sauces, baked goods and desserts (O'Toole, 1999). Extractions of protein or soluble polysaccharides from okara have been reported (Yamaguchi *et al*., 1996; Yoshii *et al*., 1996; Ma *et al*., 1997; Fischer *et al*., 2001). Due to the highly structured fiber and high water holding capacity of okara, it promotes highly viscous product. Application of okara in liquid food is still limited. Commercial pectinase is able to digest both primary and secondary cell wall of okara; it is therefore used as the solubility improvement of okara (Kasai *et al*., 2004). However, the ability of pectinase on improving the flow ability of okara depends on moisture content. At intermediate moisture content, acidic hydrolysis of okara alone could reduce the consistency of okara more effectively than using acid and pectinase co-hydrolysis (Tudthong *et al*., 2007).

Using SPI-containing enteral formulae did not only increase the viscosity of the emulsion, but also the protein quality and digestibility were slightly lower than that of SCN-based one (Castillo *et al*., 2002). However, SCN-containing enteral formulae are sensitive to the ionic calcium and may be prone to creaming and viscosity increase when the ionic calcium is added. Despite the importance of maintaining nutritional values and physical characteristics of the enteral formulae, limited data exist on the processing and storage effects on the protein functions at the oil-water interface and in bulk phase when the proteins are present at high concentration. To acquire both nutritional adequacy and practical handling of colloidal stability, this study attempted to use SCN and SPI composite as protein source. The modeled enteral formulae with 1.0 kcal/mL and caloric distribution from carbohydrate:protein:lipid of 55:15:30 were prepared by fabricating the protein matrix

with different characteristics such as using different heat treatment sequences, calcium lactate addition at different concentrations and addition of soy residue or okara with different degrees of pectinase hydrolysis. The carbohydrate sources providing the calories were cassava starch and cassava maltodextrin. The understandings on the fate of proteins at the interface during peptic and tryptic digestion may help designing interfacial matrix of the oil-in-water emulsion possessing different characters for a controlled release of nutrients.

MATERIALS AND METHODS

Materials

1. Raw Materials

1.1 Soy protein isolate (PROFAM 974, Archer Daniels Midland, Decatur, USA) containing 6.58% moisture, 82.14% protein (Nx6.25) and 0.51% fat based on dry basis (AOAC, 2000) (Appendix A).

1.2 Sodium caseinate (High viscous, Gansu Hualing Milk Products Group, Gansu, China) containing 6.92% moisture, 80.62% protein (Nx6.25) and 0.43% fat based on dry basis (AOAC, 2000) (Appendix A).

1.3 Food grade cassava starch (Jade leaf, Bangkok Interfood Co., Bangkok, Thailand) containing 11.24% moisture, 0.17% protein (Nx6.25) and 88.59% carbohydrate based on dry basis (AOAC, 2000) (Appendix A).

1.4 Cassava maltodextrin (Neo-Maldex®, Neotech Food, Bangkok, Thailand) containing 6.00% moisture, 94.00% carbohydrate based on dry basis.

1.5 Refined rice bran oil (King, Thai Edible oil, Bangkok, Thailand)

1.6 Soy residue (okara) from soy milk production was kindly supported by Lactasoy (Thailand) Inc.

2. Reagents

2.1 Reagents for adjusting pH of protein solution

2.1.1 Lactic acid ($CH_3CHOHCOOH$; Lactic acid 85%; Analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.1.2 Hydrochloric acid (HCl; Analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.1.3 Sodium hydroxide (NaOH; Analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.2 Reagents for inducting the protein composite aggregates

2.2.1 Calcium-L-lactate Pentahydrate ($C_6H_{10}CaO_6.5H_2O$; Analytical grade, Fluka, Sigma-Aldrich Chemic GmGH, Zwijndreoht, Netherlands)

2.2.2 DL-lactic acid sodium salt solution ($C_3H_5NaO_3$; Analytical grade, Fluka Chemika, Fluka Chemic AG., Buchs, Swizerlands)

2.3 Reagents for evaluating the sulfhydryl-disulfide (SH-SS) interchange

2.3.1 5, 5′-Dithiobis (2-nitrobenzoic acid) (3, 3′-6) (Ellman's reagent solution: $C_{14}H_8N_2O_8S_2$; Analytical grade, Fluka Biochemica, Fluka Chemie GmbH, Steinheim, Germany)

2.3.2 EDTA (Analytical grade, Fluka Chemika, Fluka Chemic AG., Buchs, Switzerlands)

2.3.3 *N*-ethylmaleimide ($C_6H_7NO_2$; Analytical grade, Fluka, Sigma-Aldrich, Steinheim, Germany)

2.4 Reagents for reconstitution of pepsin, trypsin and bile acid

2.4.1 Pepsin (E.C. 3.4.23.1; 1:10,000, Product code No.P 7000, Sigma Chemical, St. Louis, USA)

2.4.2 Trypsin (E.C. 3.4.21.4; Product code No.T8003, Sigma Chemical, St. Louis, USA)

2.4.3 Bile extract from porcine (Sigma no. B8631, Sigma Chemical, St. Louis, USA)

2.4.4 Urea (CH_4N_2O; Analytical grade, Fluka, Fluka Chemie GmbH, Vienna, Austria)

2.4.5 Guanidine Hydrochloride ($CH_5N_3.HCl$; Analytical grade, Fluka, Fluka Chemie GmbH, Steinheim, Germany)

2.4.6 Monosodium phosphate dihydrate ($NaH_2PO_4.2H_2O$; Analytical grade, MERCK, Merck KBaA, Darmstadt, Germany)

2.4.7 Disodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$; Analytical grade, MERCK, Merck KBaA, Darmstadt, Germany)

2.5 Reagents for determination the change of the emulsion during peptic or tryptic digestion

2.5.1 Oil Red "O" (product No.75087; Fluka Chemika, St. Louis, USA)

2.5.2 Rhodamine B (hexyl ester) perchlorate (Invitrogen™, Invitrogen, Oregon, USA)

2.5.3 Trichroloacetic acid ($C_2HCl_3O_2$; POCH; Analytical grade, POCH SA, Sowińskiego, Poland)

2.6 Reagents for modification the soy milk residue

2.6.1 Lactic acid (L-LAC FG 88; food grade, Shanxi Leda Biochemical, Taiyuan, China)

2.6.2 Pectinase (Pectinex® Ultra SP-L, novozymes, Novozymes A/S, Dittingen, Switzerland)

2.7 Reagents for protein analysis (Kjeldahl method)

2.7.1 Boric acid (H_3BO_3; Analytical grade, MERCK, Meck KGaA, Darmstadt, Germany)

2.7.2 Bromocresol green ($C_{21}H_{14}Br_4O_5S$; Analytical grade, Labchem, Ajax Finechem, Auckland, New Zealand)

2.7.3 Copper (II) sulfate ($CuSO_4.5H_2O$; Analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.7.4 Methyl red ($C_{15}H_{15}N_3O_2$; Panreac, PANREAC QUIMICA SA, España, Spain)

2.7.5 Potassium sulfate (K_2SO_4; Analytical grade, MERCK, Merck KBaA, Darmstadt, Germany)

2.7.6 Sodium hydroxide (NaOH; commercial grade, Thasco chemical CO., Bangkok, Thailand)

2.7.7 Sulfuric acid (H_2SO_4; Analytical grade, MERCK, Meck KGaA, Darmstadt, Germany)

2.8 Reagents for protein analysis (Lowry's method)

2.8.1 Albumin from bovine serum (Fluka BioChemika, Fluka Chemic GmbH, Buchs, Switzerland)

2.8.2 Copper (II) sulfate ($CuSO_4.5H_2O$; Analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.8.3 Folin-Ciocalteau's phenol reagent (Fluka BioChemika, Sigma-Aldrich Chemic BmbH, Buchs, Switzerland)

2.8.4 Potassium sodium (+)-Tartrate ($KNaC_4H_4O_6.H_2O$; Analytical grade, UVIVAR, Ajax Finechem, Seven Hills, Australia)

2.8.5 Sodium carbonate anhydrous (Na_2CO_3; Analytical grade, UNIVAR, Ajax Finchem, Seven Hills, Australia)

2.9 Reagent for fat analysis

2.9.1 Petroleum ether (35˚C-60˚C, Analytical grade, Mallinckrodt CHEMICALS, Mallinckrodt baker, Phillipsburg, USA)

2.10 Reagents for fiber analysis

2.10.1 Ethanol (C_2H_5OH; Analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.10.2 Octan-1-ol ($CH_3(CH_2)_7OH$; UNILAB, Asia Pacific Specialty Chemicals Limited, Seven Hills, Australia)

2.10.3 Sodium hydroxide (NaOH; Analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.10.4 Sulfuric acid (H_2SO_4; Analytical grade, MERCK, Meck KGaA, Darmstadt, Germany)

2.11 Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophorisis (SDS-PAGE)

2.11.1 Acrylamide PAGE (CH_2:$CHCONH_2$; PlusOne, Amersham Biosciences AB, Uppsala, Sweden)

2.11.2 Ammonium Persulfate (BIO-RAD, Bio-Rad Laboratories, Hercules, USA)

2.11.3 Brilliant Blue R (Coomassie^{TM} Brilliant blue R-250, Usb, USB corperation, Cleveland, USA)

2.11.4 Bromophenol blue sodium salt ($C_{27}H_{28}Br_2O_5S$; Labchem, Ajax Finechem Pty Ltd, Taren Point, Australia)

2.11.5 Glacial Acetic acid (CH_3COOH; Baker Analyzed®, Mallinckrodt Baker, Phillipsburg, USA)

2.11.6 Glycerol ($CH_2OHCHOHCH_2$ OH, CARLO ERBA, Cario Erba Reagenti SpA, Rodano, Italy)

2.11.7 Glycine (BIO-RAD, Bio-Rad Laboratories, Hercules, USA)

2.11.8 Hydrochloric acid (HCl; Analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.11.9 Methanol (CH_3OH; Carlo ERBA, Carlo Erba Reagenti SpA, Rodano, Italy)

2.11.10 N, N´ Methylenebisacrylamide (CH_2:$CHCONH)_2CH_2$; PlusOne, GE Healthcare Bio-Science AB, Uppsala, Sweden)

2.11.11 2-Mercaptoethanol ($HSCH_2CH_2OH$; BIO-RAD, Bio-Rad Laboratories, Hercules, USA)

2.11.12 Sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$; Usb, USB corporation, Cleveland, USA)

2.11.13 N,N,N´, N´-tetramethylethylenediamine (Usb, Amersham International, Buckinghamshire, England)

2.11.14 Tris (hydroxyl methyl) aminomethane ($NH_2C(CH_2OH)_3$; Usb, USB corporation, Cleveland, USA)

2.12 Reagent for reducing sugar content

2.12.1 Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2$; Analytical grade, UNIVAR, Ajax Finchem, Seven Hills, Australia)

2.12.2 Benzoic acid (C_6H_5COOH; UNIVAR, Analytical grade, Ajax Finchem, Seven Hills, Australia)

2.12.3 Copper (II) sulfate ($CuSO_4.5H_2O$; Analytical grade, Fisher Chemicals, Fisher Scientific UK Limited, Loughborough, UK)

2.12.4 D-galacturonic acid ($C_6H_{10}O_7.H_2O$; Fluka, Sigma-Aldrich, Bratislava, Slovakia)

2.12.5 Di-sodium hydrogen arsenate heptahydrate ($AsHNa_2O_4.5H_2O$; Fluka, Fluka Chemie GmbH, Madrid, Spain)

2.12.6 Sodium acetate hydrated ($CH_3COONa.3H_2O$; Analytical grade, UNIVAR, Ajax Finchem, Seven Hills, Australia)

2.12.7 Sodium choride (NaCl; Analytical grade, UNIVAR, Ajax Finchem, Seven Hills, Australia)

2.12.8 Sodium sulphate (Na_2SO_4; Analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.12.9 Sulfuric acid (H_2SO_4; Analytical grade, Baker analyzed®, Mallinckrodt Baker, Phillipsburg, USA)

Methods

1. Characteristics of heated sodium caseinate and soy protein isolate composites

1.1 Effect of protein composite ratios, pH of solvent and ionic strength on the appearance of protein composites between sodium caseinate (SCN) and soy protein isolate (SPI)

Ten milliliters of protein composite suspensions (15% protein w/v) were prepared in water pH 3.0 or 5.4 using 1.0 M lactic acid in a 25 mL beaker using the protein ratios between SCN and SPI of 1:0, 0.7:0.3, 0.5:0.5, 0.3:0.7 and 0:1 (w/w). The protein suspensions were heated at 80˚C for 30 min and cooled to room temperature (30˚C). Calcium lactate or sodium lactate was added to the suspensions. The final concentrations of calcium lactate were 0, 10 and 50 mM; while the final concentrations of sodium lactate were 0, 100 and 200 mM. The reaction was allowed to proceed for 30 min at 30˚C. The composite suspensions were re-heated at 80˚C for 30 min and cooled down to 30˚C. The appearance of the suspensions was observed.

1.2. Effect of heat treatment on physicochemical properties of protein composites

1.2 1 Preparation the heated protein composites between sodium caseinate (SCN) and soy protein isolate (SPI)

Protein composite suspensions (10% protein w/v) were prepared in distilled water adjusted to pH 3.0, 4.5 or 5.4 using 1.0 M lactic acid. The composite suspensions were prepared to contain protein from SCN and SPI at the ratios of 1:0, 0.7:0.3, 0.5:0.5, 0.3:0.7 and 0:1 (w/w). Then they were analyzed for apparent viscosity, SDS-PAGE, accessible sulfhydryl content and microstructure before and after heating at 80˚C for 30 min.

1.2.2 Apparent viscosity

Fifty milliliters of composite suspensions (10% protein w/v) were prepared in a 100 mL beaker, stirred at 25˚C using a magnetic stirrer for 30 min and measured for apparent viscosity at different temperatures using a digital Brookfield Rheometer model RVDV III (Brookfield Engineering Laboratories Inc., Stoughton, USA) equipped with the UL-adapter at a constant shear rate at 12.2 s^{-1}. The samples were heated from 25˚C to 80˚C at a heating rate of 1˚C/min and recorded for apparent viscosity.

1.2.3 Accessible sulfhydryl (SH) content of composite aggregates

The accessible SH group content was determined and calculated by the method described by Shimada and Cheftel (1989). Five milliliters of the unheated or heated (at 80˚C 30 min) protein composite suspensions containing 10% protein (w/v) in a 25 mL beaker were prepared and diluted to 2% (protein w/v) using water pH 3.0 or pH 5.4 adjusted by 1.0 M lactic acid. One milliliter of the diluted protein suspension was further diluted with 8 mL Tris-glycine buffer (10.4 g Tris, 6.9 g glycine and 1.2 g EDTA per litre), pH 8.0 and 1 mL of Ellman's reagent solution (4 mg per mL DTNB in Tris-glycine buffer, pH 8.0). The reaction was allowed to proceed for 30 min at room temperature and filtered through the Millipore filter (Millipore Corporation, Bedford, MA, USA) with the pore size of 0.22 μm. The absorbance was measured by the spectrophotometer at 412 nm (Spectronic 20^{+}, Spectronic Instruments, Inc., Rochester, USA) and a blank was 9 mL Tris-glycine buffer and 1 mL of Ellman's reagent solution. The accessible SH content was calculated as followed:

$$\mu\text{M } SH\ g^{-1}\ protein = \frac{73.53 \times Absorbance\ at\ 412\ nm}{mg\ protein} \quad (1)$$

1.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The MW profiles of proteins of the unheated and the heated (at 80˚C 30 min) were carried out using the SDS-PAGE (Laemmli, 1970) in a 4% stacking gel and 15% separating gels using a Bio-Rad mini-Protein II cell (Bio-Rad Laboratories, Hercules, USA). The separation was performed at a constant voltage (150 V) for 50 min with a stacking gel containing 15% acrylamide and running gel containing 4% acrylamide concentration. A continuous buffer system, containing 0.375 M Tris–HCl, pH 8.8, and 10% SDS for the separating gel and 0.124 M Tris, 0.959 M glycine, and 0.1% SDS, pH 8.3, for the running buffer, were used. Protein samples were mixed with the dissociating sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, with or without 5% β-mercaptoethanol and 1% (w/v) bromophenol blue) to give a concentration of 4 mg protein/mL and the solutions were heated at 100˚C for 3 min and centrifuged at 5000 rpm (Labnet Spectrafuge 16M, Labnet International Inc., Woodbridge, USA) for 5 min to remove insoluble material. To each well, 5 μL sample solution was loaded. Gel slabs were fixed and stained simultaneously using the Bio-Rad Coomassie blue R-250 stain solution (40% methanol, 10% acetic acid, 0.1% Coomassie blue R-250) for 30 min and destained by Bio-Rad Coomassie blue R-250 destain solution for 5 hr with 2–3 changes of destain solution. Molecular weight of each band was determined by using full-range rainbow molecular weight markers, MW~10–250 kDa (RPN 8000, Amersham Biosciences UK Limited, Buckinghamshire, UK) as the MW standards. Duplicate runs were carried out.

1.2.5 Microstructure

The unheated and heated (at 80˚C 30 min) protein composite suspensions (5 mL) were prepared as described in section 1.2.1 in a 30 mL glass vial. Rhodamine B (0.01% in 95% ethanol) was added to protein suspensions. After incubation for 5 min, each sample was loaded onto a well slide and observed for a location of fluorescent-labeled protein using the Confocal Laser Scanning Microscopy or CLSM (Axio Imager MI, Carl Zeiss PTe Ltd, Jena, Thüringen, Germany). A

He/Ne laser was used as a laser source at the excitation wavelength of 543 nm. The micrographs were acquired at 1024x1024 pixels using the LSM 5 PAS-CAL program.

1.3 Effect of salt addition on aggregation of pre-heated protein composites

1.3.1 Turbidity

Ten milliliters of protein composite suspensions (10% protein w/v) were prepared in water pH 3.0 or 5.4 in a 25 mL beaker using the protein ratios between SCN and SPI of 0.7:0.3, 0.5:0.5 and 0.3:0.7 (w/w), respectively. The protein suspensions were heated at 80˚C for 30 min and cooled to room temperature (30˚C). A stock solution of 0.167 M of *N*-ethylmaleimide (NEM), a sulfhydryl-blocking agent, was added to the pre-heated composite suspensions to obtain the final concentration of NEM at 20 mM in the solvent have corresponding pH prior to the calcium lactate addition. The composite suspensions, both in the absence and presence of NEM, were diluted to 0.04% (protein w/v) with a stock solution of calcium lactate or sodium lactate in water adjusted to pH 3.0 or 5.4. The final concentrations of calcium lactate were 0, 5, 15, 25, 35, 45 and 60 mM; while the final concentrations of sodium lactate were 0, 15, 45, 75, 105, 135, 165, 210 and 250 mM. The reaction was allowed to proceed for 30 min at 30˚C and the optical density was measured as OD_{500} (Spectronic 20^{+}, Spectronic Instruments Inc., Rochester, USA) using the method described by Hongsprabhas and Barbut (1997a) and calculated turbidity (Pearce and Kinsella, 1978) as followed:

$$Turbidity = \frac{2.303 * OD_{500}}{pathlength\ (cm)} \quad (2)$$

1.3.2 Microstructure

Five milliliters of protein composite suspensions (10% protein w/v) were prepared as described in section 1.2.1 in a 30 mL glass vial. The protein suspensions were heated at 80˚C for 30 min and cooled to room temperature (30˚C).

Calcium lactate was added. The reaction was allowed to proceed for 30 min at 30˚C. The final concentrations of calcium lactate were 0 and 25 mM. Rhodamine B (0.01% in 95% ethanol) was added to protein suspensions. After incubation for 5 min, each sample was loaded onto a well slide and observed for a location of fluorescent-labeled protein using the Confocal Laser Scanning Microscopy or CLSM (Axio Imager MI, Carl Zeiss PTe Ltd, Jena, Thüringen, Germany). A He/Ne laser was used as a laser source at the excitation wavelength of 543 nm. The micrographs were acquired at 1024x1024 pixels using the LSM 5 PAS-CAL program.

1.3.3 Determination of peptide released from salt-induced protein composite aggregates during peptic digestion

One hundred milliliters or ninety two milliliters of the protein composite suspension (4% protein w/v) were prepared in water pH 3.0 in a 150 mL beaker using the protein ratios between SCN and SPI of 1:0, 0.7:0.3 and 0:1 (w/w), respectively. The protein suspensions were heated at 80˚C for 30 min and cooled to room temperature (30˚C). Eight milliliters of a stock solution 0.25 M of *N*-ethylmalemide (NEM) was added to 92 mL pre-heated composites to obtain the final concentration of NEM at 20 mM. Calcium lactate was added to all samples. The final concentrations of calcium lactate were 0 and 25 mM. The reaction was allowed to proceed for 30 min at 30˚C. The protein composite suspensions were adjusted to pH 2.0 by adding 5M HCl. The samples were hydrolyzed by pepsin with enzyme to substrate ratio of 1:17 at 37˚C for 0, 15, 30, 45, and 60 min. The hydrolyzed protein composites were pipetted into the Eppendorf tubes. The serum phase was collected by centrifugation at 14000 rpm for 10 min by a microcentrifuge (Labnet Spectrafuge 16M, Labnet International, Inc., Woodbridge, USA) and removed with syringe. The peptide released in the serum phase was obtained by mixing those sera with trichroloacetic acid (TCA) at the final concentration of 10% (w/v) TCA. The solution was allowed to stand for 10 min at room temperature. The insoluble protein was removed by centrifugation at 14000 rpm for 10 min. Supernatants were collected and analyzed for the protein content (Nx6.25) by Kjeldahl method (AOAC, 2000) (Appendix A2).

2. Effect of heat treatment sequences on *in vitro* protein digestibility and characteristics of liquid oil-in-water emulsion containing 25 mM calcium lactate

2.1 Preparation of liquid oil-in-water (o/w) emulsion powder

A total of 3.5 L liquid o/w emulsions to provide the calories of 1.0 kcal/mL was prepared with the solid material about 21% (w/v), containing 3.75%(w/v) protein from SCN and SPI at the ratio of 0.7:0.3, cassava starch 2.0%(w/v), maltodextrin 11.75%(w/v) dispersed in 3.34 L of water pH 3.0 (adjusted by 1 M lactic acid). The suspensions was pre-heated at 80˚C for 30 min, cooled to room temperature (27˚C), and emulsified with 3.33 %(v/v) rice bran oil using a high speed colloid mill (2F-colloid mill, APV Gaulin Inc., Wilmington, USA) for 3 min at 24000 rpm. The 107.91g of calcium lactate was added to the emulsion at 38˚C-40˚C to obtain the final concentration of calcium lactate of 25 mM. This method for preparing the liquid emulsion described was designated as "*one-stage heating process*".

In the "*two-stage heating process*", the emulsions were further re-heated at 80˚C for 30 min to generate the salt-induced protein aggregates with compact particle and loosening network (Hongsprabhas and Barbut, 1998). The emulsions prepared by "*one-stage heating process*" and "*two-stage heating process*" were spray-dried by spray dryer (GEA Niro A/S, Niro A/S, Soeborg, Denmark). The inlet air was 160˚C and the outlet air was 85˚C with the flow of drying air of 1 m^3/min and the liquid feed was 16 mL/min. The emulsion powder was stored at -20˚C prior to analysis.

2.2 *In vitro* digestion of protein in the reconstituted liquid o/w emulsion

The method for protein digestion *in vitro* was as described by Glahn *et al*. (1998). Preparation of the liquid o/w emulsions to provide 1.0 kcal/mL of calories was by dispersed the emulsion powder 2.083 g in 10 ml of hot distilled water (75˚C) in a 25 ml beaker. Samples were stirred at room temperature (25˚C) for 10 min and

adjusted to pH 2.0 by adding 5M HCl. The samples were first hydrolyzed by pepsin with enzyme to substrate ratio of 1:17 at 37˚C for 0, 15, 45, 60, 75, 90, 105, 120, 135 and 150 min. The pepsin-digested hydrolyzate was adjusted to pH 7.0 by 2 N NaOH. Trypsin with enzyme to substrate ratio of 1:100, with or without 0.02 g bile extract in 200 µL of a 0.1 M phosphate buffer, pH 7.0, was added to obtain final concentration of bile acid in the emulsion was 0.72 µmol/mL. The samples were incubated in water bath at 37˚C for 30 min.

2.3 Emulsion stability

The method for evaluating emulsions stability was carried out using method described by McDermott *et al*. (1981). Before digestion by pepsin and trypsin, Oil Red "O" was added to the emulsion to indicate the oil phase. The hydrolyzed emulsion was centrifuged at 12,000 rpm for 10 min by a micro-hematocrit centrifuge (KHT-400, Gemmy Industrial Corp., Taipei, Taiwan). After centrifugation, the hydrolyzed emulsion was separated into four layers with a red free fat layer on the top, opaque cream layer, translucent serum layer and opaque sediment. The free fat height and total height were measured with a vernier caliper and the results were reported as % free fat height compared with the total height.

2.4 Separation of protein or peptide at the oil-water interface and in the bulk phase

One milliliter of the hydrolyzed emulsion was pipetted into an Eppendorf tube and centrifuged at 14000 rpm for 10 min by a microcentrifuge (Labnet Spectrafuge 16M, Labnet International Inc., Woodbridge, USA) to separate the cream phase (top) from the serum phase (bottom). The serum phase was removed by syringe. The cream was separated and dried on filter paper (No.1, Whatman, Whatman International Ltd., Maidstone, England) and then resuspended in 0.5 mL of the extraction buffer containing 0.5 M Tris-HCL (pH 6.8), 10% glycerol and 10% (w/v) SDS (Manion and Corredig, 2006). The concentration of extracted proteins

from the cream phase and the serum phase were determined by Lowry's method (Lowry *et al*., 1951).

2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The MW profiles of protein in the serum phase and at the interface of the hydrolyzed emulsion were evaluated. The aliquots of the serum were added to dissociating buffer (0.5 M Tris-HCl, pH 6.8, glycerol, with or without 5% β-mercaptoethanol, 1% (w/v) bromophenol blue). Aliquots of the re-suspended cream were added with 20 μL of 1% (w/v) bromophenol blue. The SDS-PAGE was carried out using a method described in section 1.1.4. An aliquot of the sample solution containing 0.03 mg protein was loaded into each well. The electrophoresis was run at a constant current of 25 mA for serum phase and at a constant voltage of 150 V for cream phase.

2.6 Determination of peptide released from the emulsion during digestion

Twenty five milliliters of the liquid o/w emulsion was prepared in a 50 mL beaker and was digested by pepsin and trypsin as described in section 2.2. The hydrolyzed emulsion was pipetted into the Eppendorf tubes. The serum phase was collected by centrifugation at 14000 rpm for 10 min by a microcentrifuge (Labnet Spectrafuge 16M, Labnet International, Inc., Woodbridge, USA) and removed with syringe. The peptide released in the serum phase was obtained by mixing those sera with trichroloacetic acid (TCA) at the final concentration of 10% (w/v) TCA. The solution was allowed to stand for 10 min at room temperature. The insoluble protein was removed by centrifugation at 14000 rpm for 10 min. Supernatants were collected and analyzed for the protein content (Nx6.25) by Kjeldahl method (AOAC, 2000) (Appendix A2)

2.7 Microstructure

Rhodamine B (0.01% in 95% ethanol) was added to the hydrolyzed emulsion. After incubation for 5 min, each sample was loaded onto a well slide and observed for a location of fluorescent-labelled protein using the Confocal Laser Scanning Microscopy or CLSM (Axio Imager MI, Carl Zeiss PTe Ltd, Jena, Thüringen, Germany). A He/Ne laser was used as a laser source at the excitation wavelength of 543 nm. The micrographs were acquired at 512x512 pixels using the LSM 5 PAS-CAL program.

3. Effect of calcium lactate concentration on *in vitro* protein digestibility and characteristics of emulsion prepared by *two-stage heating process*

3.1 Preparation of *two-stage heated emulsion* powder containing different calcium lactate concentrations

A total of 3.5 L liquid o/w emulsions to provide the calories of 1.0 kcal/mL was prepared with the solid material about 21% (w/v), containing 3.75%(w/v) protein from SCN and SPI at the ratio of 0.7:0.3, cassava starch 2.0%(w/v), maltodextrin 11.75%(w/v) dispersed in 3.34 L of water pH 3.0 (adjusted by 1 M lactic acid). The suspensions was pre-heated at 80˚C for 30 min, cooled to room temperature (27˚C), and emulsified with 3.33 %(v/v) rice bran oil using a high speed colloid mill (2F-colloid mill, APV Gaulin Inc., Wilmington, USA) for 3 min at 24000 rpm. The 0, 26.98 and 107.91g of calcium lactate were added to the emulsion at 38˚C-40˚C to obtain the final concentration of calcium lactate of 0, 25, and 100 mM, respectively. The emulsions were re-heated at 80˚C for 30 min. The *two-stage heated emulsion* containing different calcium lactate concentrations were spray-dried by a spray dryer as described in section 2.1. Then they were analyzed for *in vitro* protein digestibility, emulsion stability, SDS-PAGE, determination of peptide released from the emulsion during digestion, and microstructure as described in section 2.2-2.7.

3.2 Determination of calcium content in the bulk aqueous phase

Twenty milliliters of the hydrolyzed emulsion was pipetted into the Eppendorf tubes. The serum phase was collected by centrifugation at 14000 rpm for 10 min by a microcentrifuge and removed with syringe. The peptide released in the serum phase was obtained by mixing those sera with trichroloacetic acid (TCA) at the final concentration of 10% (w/v) TCA. The solution was allowed to stand for 10 min at room temperature. The insoluble protein was removed by centrifugation at 14000 rpm for 10 min. Supernatants were collected and analyzed for the soluble calcium content with the Atomic Absorption Spectrometry using an air-acetylene flame.

4. Effect of soy residue (okara) addition on *in vitro* protein digestibility and characteristics of emulsion prepared by *two-stage heating process*

4.1 Soy residue and modified soy residue powder

Fresh soy residue was hydrolyzed by pectinase (1% w/w of total solid soy residue) and incubated at room temperature (30˚C) for 20 hr Okara cake was separated by cheese cloth. Mungbean starch, as a sorbent material, was added to fresh okara or pectinase-hydrolyzed okara (PHO) at the ratio of starch:total solid of okara as 1:1. The samples were dried at 65˚C in a jet spouted bed at King Mongkut's University of Technology Thonburi. The dried samples were milled and passed through 100 mesh sieve. Powders were kept in plastic bag and stored at -20˚C prior to analysis for chemical composition (AOAC, 2000), reducing sugar content (Milner and Avigad, 1967) (Appendix A5) and colour value in CIE system using spectrophotometer (model CM-3500d, Konica Minolta Ltd., Osaka, Japan). Okara powder contained 12.68% moisture, 8.52% protein (Nx5.52), 4.6% fat, 3.01% crude fiber and 0.054% ash based on dry basis (AOAC, 2000). PHO contained 8.79% moisture, 12.65% protein (Nx5.52), 10.14% fat, 6.89% crude fiber and 0.361% ash based on dry basis. They were used as protein and crude fiber source in reconstituted emulsion containing 0 mM added calcium lactate.

4.2 *In vitro* digestion of protein in the reconstituted liquid o/w emulsion

Okara or PHO powder was dissolved in 10 mL distilled water in a 25 mL beaker to obtain 0.08% crude fiber (w/v) in the suspension. The suspension was stirred at 80˚C for 30 min and cooled to 75˚C, then added with 2.083 g of the *two-stage heated emulsion* powder and cooled to room temperature (25˚C). The suspension containing 0.08% crude fiber (w/v) with 1 kcal/mL and caloric distribution from carbonydrate:protein:lipid of 55:15:30 was digested by pepsin and trypsin as described in section 2.2 and determined for apparent viscosity, emulsion stability, SDS-PAGE, % peptide released from the emulsion, and microstructure as describe in section 1.2.2 and 2.3-2.7.

6. Statistical analysis

The high-caloric liquid emulsions and their spray-dried products were prepared in two separated trials. Each trial was run and evaluated in duplicates. The data were analyzed by Analysis of Variance (ANOVA) with significance at $p<0.05$. Significant difference among mean values was determined by Duncan's Multiple Range Test. All statistical analyses were performed using the SPSS Software Version 12.

RESULTS AND DISCUSSION

Results

1. Characteristics of heated sodium caseinate and soy protein isolate composites

1.1 Effects of protein composite ratios, pH of solvent and ionic strength on appearance of protein composites between sodium caseinate and soy protein isolate

At high protein concentration, the heated SPI formed gel and SCN was viscous fluid. The presence of 10 mM calcium lactate and re-heat treatment at 80˚C for 30 min resulted in the formation of large SCN aggregate to form a clump. When the protein was used in the form of composites between SCN and SPI, the ability to form three-dimensional structure of *two-stage heated protein* in the absence or presence of salt was altered. Figure 6 indicated that the ability to form three-dimensional structure of heated protein composites depended on the ratio of SCN:SPI, pH of solvent, different types of salt (monovalent vs. divalent ion), and ionic strength.

The increase of the SCN fraction from 0.3 to 0.5 and 0.7 and/or using water pH 3.0 as solvent could reduce the ability to form three-dimensional structure of *two-stage heated protein* in the presence of 10 mM calcium lactate. It should be noted that using mixed protein between phosphorylated caseins and soy proteins together with water possessing different pHs could alter network formation ability of protein suspensions.

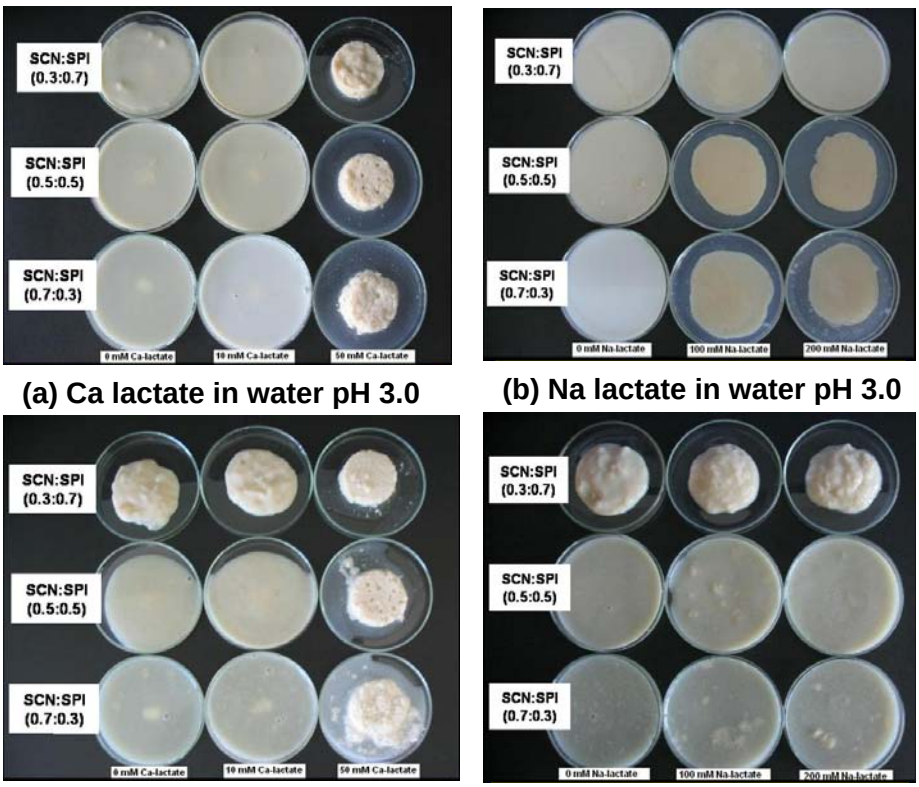

Figure 6 Appearance of 15% protein (w/v) suspensions/gels prepared by heating at 80˚C for 30 min, cooled down to 30˚C, added with different concentrations of (a, c) calcium lactate (b, d) sodium lactate and re-heated at 80˚C for 30 min, a so-called "*two-stage heating process*".

1.2 Effect of heat treatment on physicochemical properties of protein composite

Figure 7 illustrates the apparent viscosity of the unheated and heated protein suspensions containing 10% protein (w/v) in water pH 3.0, 4.5 and 5.4. The unheated SPI suspensions were highly viscous and the viscosity could not be recorded by Brookfield Rheometer equipped with a UL-adapter. Thus, viscosity of this fraction (SCN:SPI=0:1) was not reported. Before heating, the composite suspensions containing SCN to SPI of 0.3:0.7 had the highest viscosity, followed by SCN

suspensions. The increase of SCN ratio from 0.3 to 0.5 and 0.7 in the unheated composite decreased the viscosity of suspensions (Figure 7a).

Upon heating to 80˚C, the viscosity of the composite containing SCN to SPI of 0.3:0.7 and SCN suspensions increased (Figure 7b). However, the heated composite suspensions containing high ratio of SCN had lower viscosity than the unheated ones. The composite containing SCN:SPI at the ratio of 0.7:0.3 showed the lowest viscosity, which was below 100 mPa.s. Furthermore, using water with different initial pHs (adjusted by 1M lactic acid) resulted in different responses on the viscosity of the protein suspensions both before and after heating, although the final pH of all composite suspensions was around 6.38 to 7.22, which was above the pI of both protein sources.

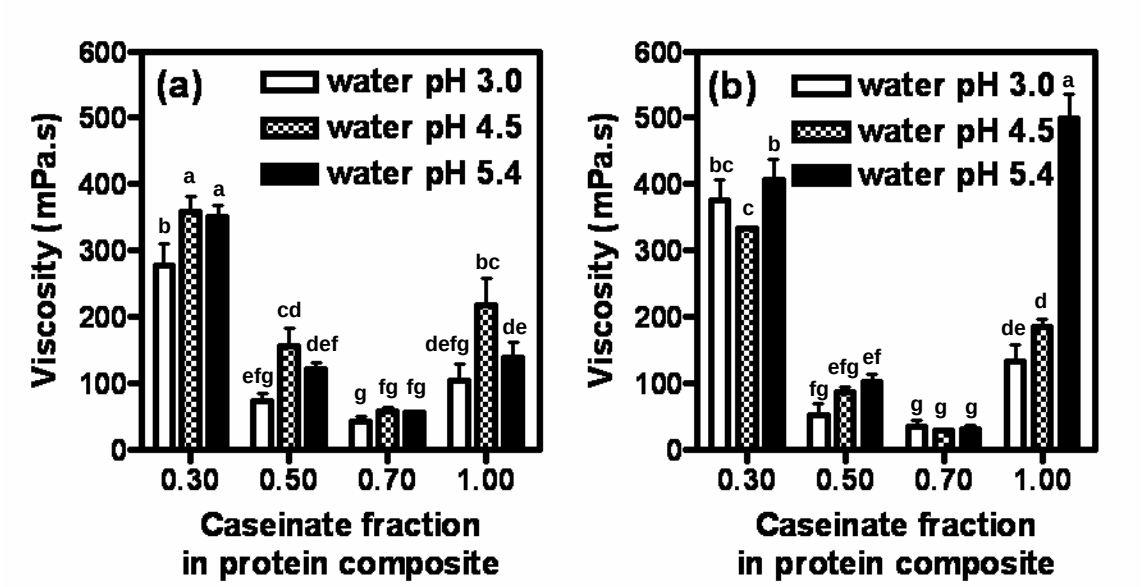

Figure 7 Effect of caseinate fraction on apparent viscosity of protein composite suspensions (10% protein w/v) dispersed in water at different pHs (a) before and (b) after heating at 80˚C. Bars represent standard deviation.

Figure 8 illustrates the microstructure of SPI, SCN-SPI composites (at the ratio of 0.7:0.3) and SCN. Before heating in water pH 3.0, the SPI aggregates showed spherical structure with the particle size in the range of 30 to 100 μm (Figure 8a). In contrast, the signal of red fluorescence in SCN (Figure 8c) was different from that of

SPI and SCN-SPI composites although all micrographs were recorded by using the re-used condition. The microstructure of the protein aggregates remained unchanged (Figure 8d, 8e, 8f) at the corresponding protein ratios after heat treatment.

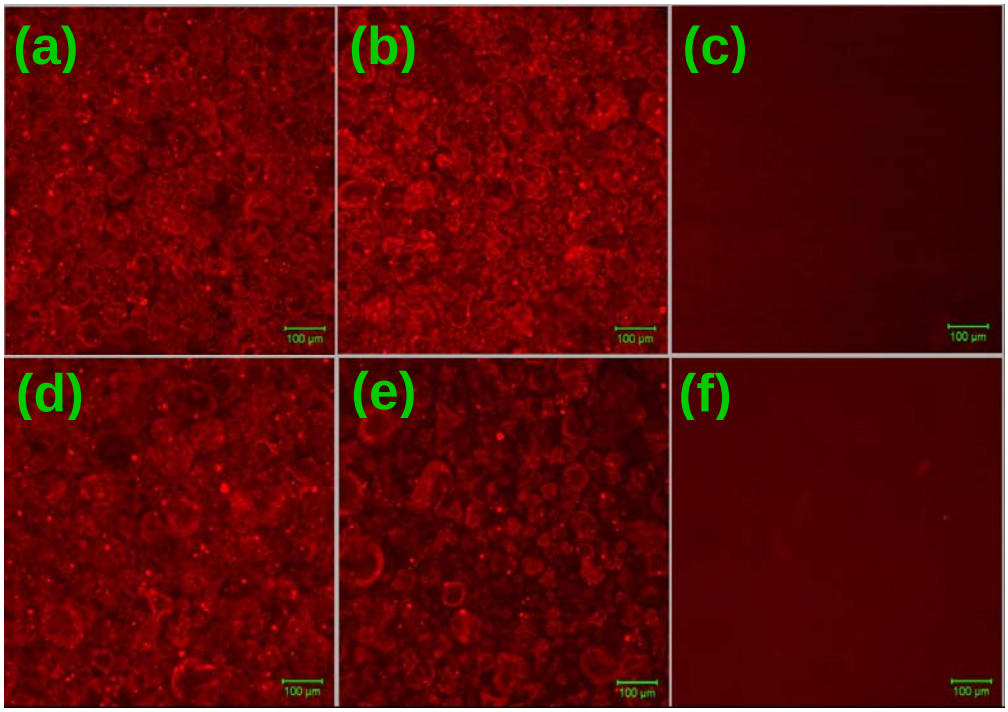

Figure 8 Confocal laser scanning micrographs of soy protein isolate (SPI), SCN-SPI composite (SCN:SPI = 0.7:0.3 protein w/w) and sodium caseinate (SCN) suspension (10% protein w/v) before and after heat treatment at 80˚C for 30 min in water pH 3.0; (a) unheated SPI; (b) unheated SCN-SPI composite; (c) unheated SCN; (d) heated SPI; (e) heated SCN-SPI composite; (f) heated SCN. Proteins were stained with Rhodamine B and are shown by red fluorescence. Bar scale= 100 µm.

Thus, the decrease in the viscosity of protein composites containing high SCN fraction may be due to the dilution of spherical structure from the decrease in SPI ratio or chaperone properties of phosphorylated casein. Phosphorylated casein (i.e. α- and β-casein) can interact on the surfaces of unfolding globular proteins and resulted in the repulsion of there aggregates (Matsudomi *et al*., 2004).

The MW profiles of SCN and SCN:SPI composite were analyzed by SDS-PAGE electrophoresis to determine changes in the polypeptide composition after heating (Figure 9). The unheated SCN contained large MW aggregates with MW more than 250 kDa that did not enter the stacking gel and deposited in the well. The major proteins in the unheated SCN suspension had the MW between 25 and 30 kDa observed as the high intensity bands (Figure 9; lane 2), which are higher than the native casein monomers; namely κ-casein, α_{s1}-casein, β-casein and α_{s2}-casein have the MW of 19, 22, 24 and 25 kDa, respectively. There were protein bands between 50-250 kDa present in lane 2, which is SCN before heating. This was probably due to the polymerization of casein monomer during commercial operations (Guo *et al*., 1999).

After heating at 80˚C for 30 min, the heated SCN retained the bands between 25 and 30 kDa and the higher MW of more than 250 kDa (Figure 9; lane 4) as in the unheated ones. The presence of reducing agent, *β*-mercaptoethanol, which reduced the disulfide bond in protein, showed that the bands between 25 and 30 were retained. However, there was the disappearance of the protein in the well, as well as the appearance of two bands between 15 and 25 kDa (Figure 9; lane 6). These bands were probably κ-casein (MW~19 kDa) and α_{s2}-casein (MW~25 kDa). The intermolecular disulfide bond may involve in the formation of SCN aggregates prior to the heat treatment used in this study. The main bovine casein which formed disulfide bond could be κ-casein and α_{s2}-casein, of which each contained two cysteinyl residues per molecule (Dalgleish, 1997a).

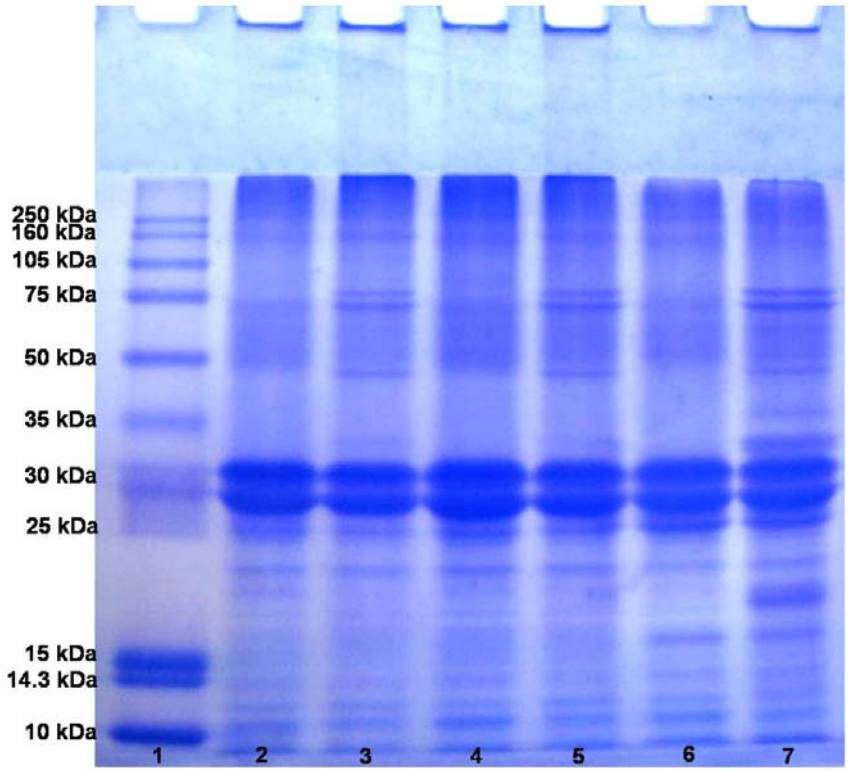

Figure 9 SDS-PAGE of sodium caseinate (SCN) and SCN-soy protein isolate (SPI) composite suspensions (SCN:SPI=0.7:0.3 protein w/w). Lane 1= wide range MW standards; lane 2= unheated SCN; lane 3= unheated SCN-SPI composite; lane 4= heated SCN at 80˚C for 30 min; lane 5= heated SCN-SPI composite at 80˚C for 30 min; lane 6= heated SCN in the presence of *β*-mercaptoethanol; land 7= heated SCN-SPI composite in the presence of *β*-mercaptoethanol.

In samples containing SPI at the ratio of SCN:SPI as 0.7:0.3, the unheated SCN-SPI composite showed the bands between 35 and 250 kDa (Figure 9; lane 3), which were not observed in the SCN. Thus, these bands were part of protein profiles of SPI, particularly 7S soy polypeptide (MW~62-200 kDa). Because soy protein also contained 11S soy polypeptide (MW~350 kDa) (Cheftel *et al*., 1985; Kilara and Harwalkar, 1996), not all of the soy proteins passed through the stacking gel, observed as the high intensity band with MW larger than 250 kDa. After heating of SCN-SPI composite, all bands still remained (Figure 9; lane 5). It indicated that heat

treatment did not alter the MW of protein bands in SCN-SPI composite at the ratio of 0.7:0.3. Under reducing condition (Figure 9; lane 7); there were additional protein bands between 15 and 25 kDa, 30-35 kDa and 35 kDa intensely visible. These bands were likely κ-casein, basic subunit of 11S, α_{s2}-casein, and acidic subunit of 11S. A band appeared between 30 and 35 kDa, may be SCN-SPI or SPI-SPI aggregates, which may be involved aggregation during heat treatment.

The soluble and insoluble aggregates of protein still contained accessible sulfhydryl (SH)-groups shown in Table 2. Heat treatment used in this study did not affect SH content of either SCN or SPI ($p \geq 0.05$). In water pH 3.0, which is close to pI of phosphorelated casein, the unheated SCN had lower accessible SH content than the unheated SPI. However, when both proteins existed together in the composite, heat treatment level off the accessible contents of the composite to those in between the range found in unheated SCN and SPI. Nevertheless, heat treatment employed to SPI in water pH 5.4, which was close to the pI of soy proteins, drastically increased the SH content of the SPI suspension compared to that of SPI suspension subjected to heat treatment in water pH 3.0.

Table 2 Effect of caseinate fraction on accessible sulfhydryl (SH) content of sodium caseinate (SCN) and soy protein isolate (SPI) composites (10% protein w/v) dispersed in water with different pHs

SCN : SPI	Accessible SH content (μM SH g^{-1} protein)	
	Water pH = 3.0	Water pH = 5.4
Unheated		
0:1	27.57 ^{abA}	29.41 ^{abA}
1:0	12.87 ^{cA}	16.54 ^{abA}
Heated		
0:1	20.83 ^{abcA}	39.22 ^{abA}
0.3:0.7	29.41 ^{aA}	28.19 ^{abA}
0.5:0.5	18.38 ^{abcA}	20.83 ^{abA}
0.7:0.3	13.48 ^{bcA}	8.58 ^{bA}
1:0	15.93 ^{abcB}	35.54 ^{aA}

Means in the same column followed by different lower case superscripts are significantly different ($p<0.05$). Means in the same row followed by different upper case superscripts are significantly different ($p<0.05$).

1.3 Effect of salt addition on aggregation of pre-heated protein composites

The calcium lactate-mediated aggregation of the pre-heated protein composite suspension was monitored by turbidity (Figure 10). In the presence of sulfhydryl-blocking agent NEM, the increase in calcium lactate concentration slightly increased the turbidity of the pre-heated protein suspension. However, in the absence of NEM, the increase in calcium lactate concentration significantly raised the turbidity of pre-heated protein suspension of all ratios when water pH was 5.4 (Figure 10c, 10f, 10i). In addition, at the ratio of SCN:SPI of 0.7:0.3 in water pH 3.0, the absence of NEM drastically increased the turbidity (Figure 10g). This suggested that sulfhydryl disulfide (SH-SS) interchange was induced by calcium-induced aggregation of mixed protein, though their aggregates had low accessible SH contents.

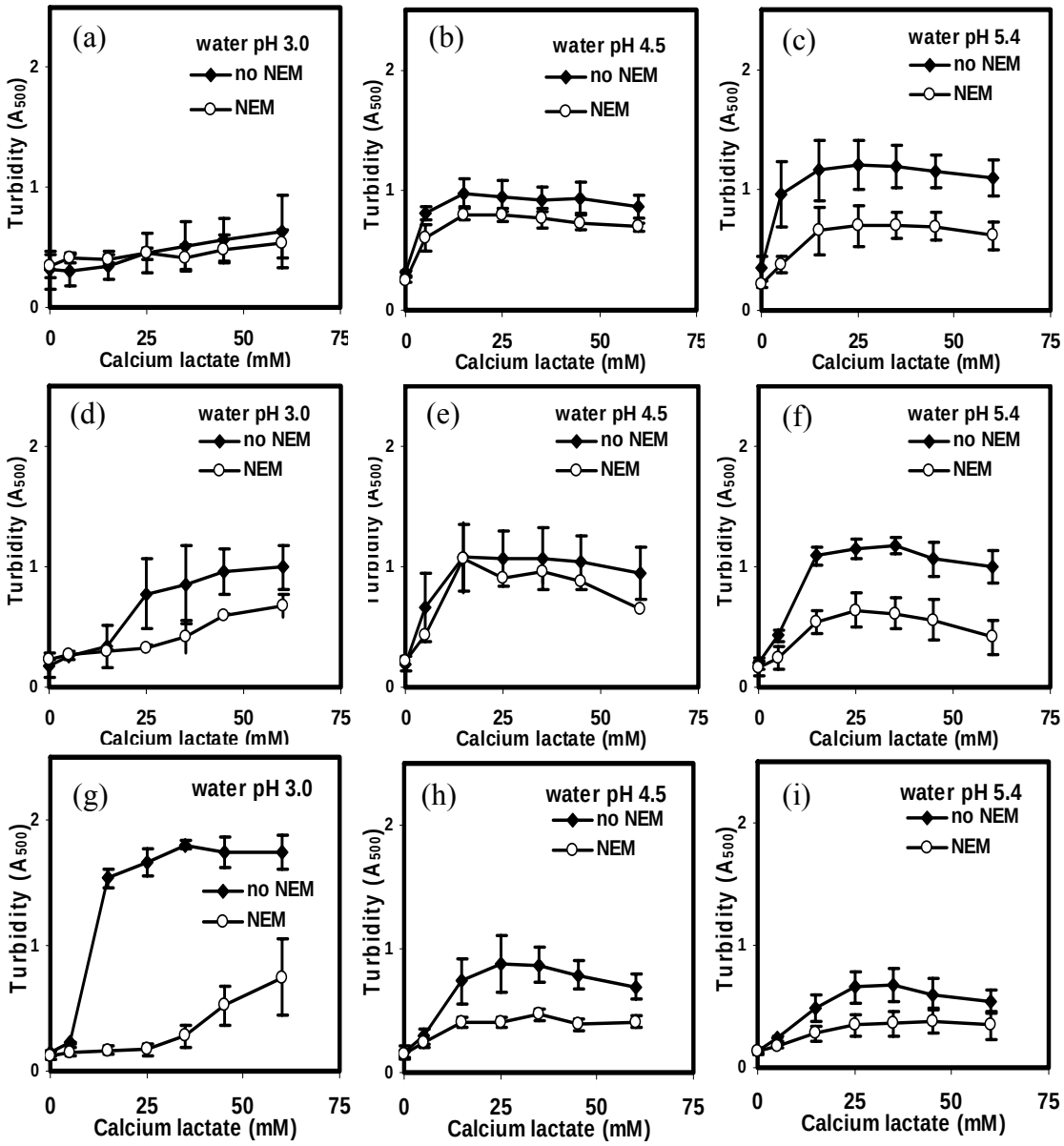

Figure 10 Effect of calcium lactate concentration on turbidity of heated protein composite suspensions containing different ratios of sodium caseinate (SCN) and soy protein isolate (SPI) in the absence and presence of sulfhydryl-blocking agent, *N*-ethylmaleimide (NEM), in water at different pHs. (a), (b), (c) SCN:SPI=0.3:0.7; (d), (e), (f) SCN:SPI=0.5:0.5; (g), (h), (i) SCN:SPI=0.7:0.3. Bars represent standard deviation.

The spherical aggregates of SCN:SPI composites at the ratio of 0.7:0.3 before and after calcium lactate addition in water pH 3.0 are illustrated in Figure 11. The CLSM micrographs showed that the salt-induced aggregates of protein composite remained hollow structure, resembling to the SPI aggregates. Although the size of the aggregates was not altered in the presence of calcium lactate, the aggregates appeared to be denser in terms of the signal of the fluoresced proteins.

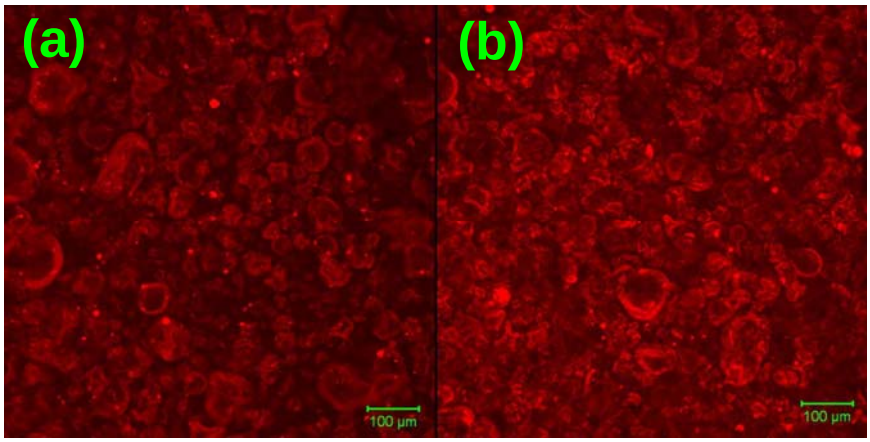

Figure 11 Confocal laser scanning micrographs of heated composite protein aggregates containing sodium caseinate (SCN) and soy protein isolate (SPI) at the ratio of 0.7:0.3 (protein w/w) in water pH 3.0; (a) no calcium lactate addition; (b) with calcium lactate addition at the concentration of 25 mM. Proteins were stained with Rhodamine B and are shown by red fluorescence. Bar scale= 100 µm.

The influence of sodium lactate-mediated aggregation of heated protein composites is shown in Figure 12. The increase in sodium lactate concentration only increased the turbidity of protein composite dispersed at water pH 3.0 ($p<0.05$) (Figure 12a, 12d, 12g). The SH-SS interchange was involved in the sodium lactate-mediated aggregation of SCN to SPI of 0.7:0.3 at pH 3.0.

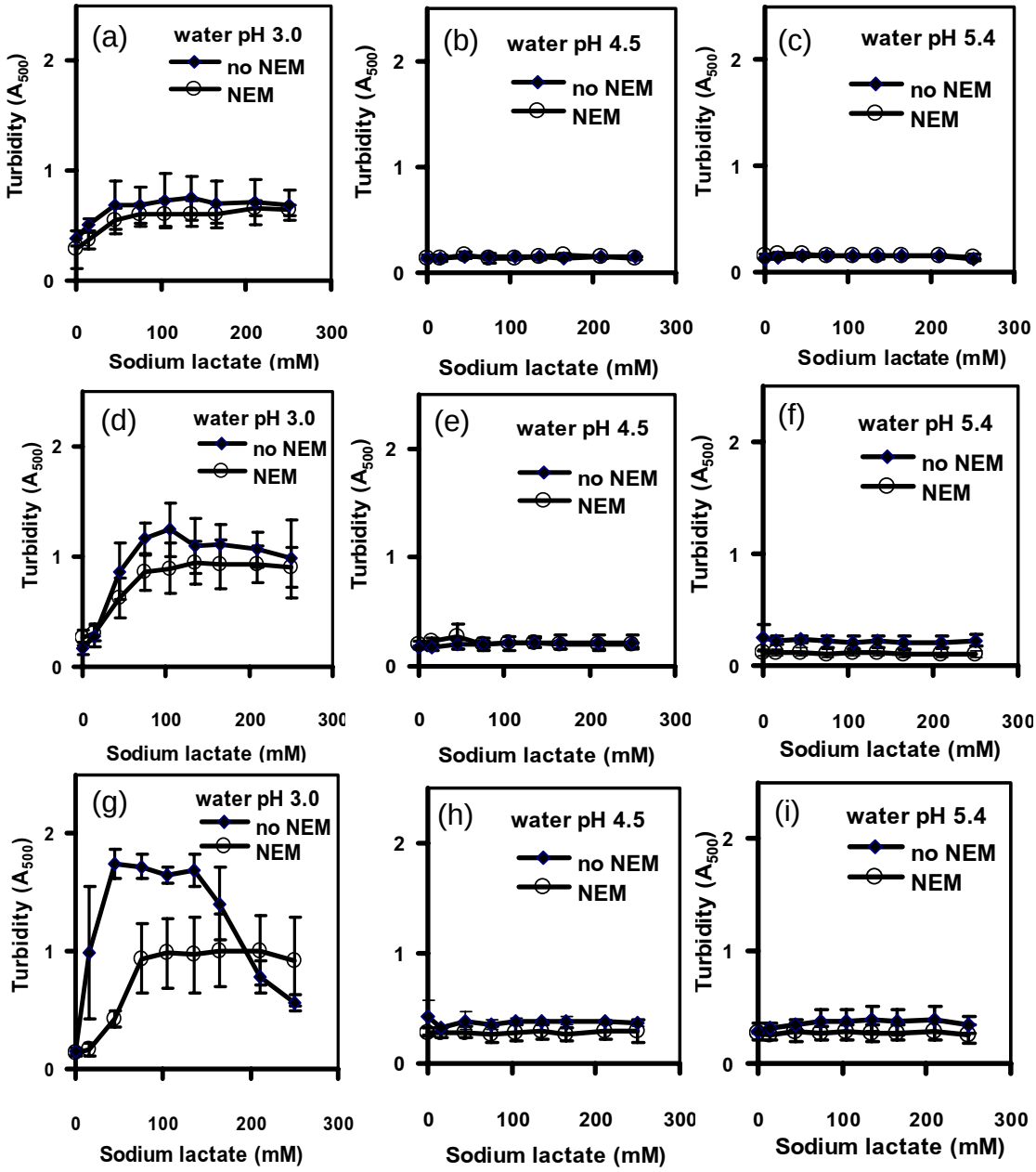

Figure 12 Effect of sodium lactate concentration on turbidity of heated protein composite suspensions containing different ratios of sodium caseinate (SCN) and soy protein isolate (SPI) in the absence and presence of sulfhydryl-blocking agent, *N*-ethylmaleimide (NEM), in water at different pHs. (a), (b), (c) SCN:SPI=0.3:0.7; (d), (e), (f) SCN:SPI=0.5:0.5; (g), (h), (i) SCN:SPI=0.7:0.3. Bars represent standard deviation.

Table 3 showed the TCA-soluble protein in the serum phase of SPI, SCN-SPI composite (at the ratio of 0.7:0.3) and SCN suspension in the presence of 0 mM and 25 mM calcium lactate during peptic digestion at pH 2.0. In the presence of NEM, the initial protein content (at time 0 min) in serum phase was higher than those in the absence of NEM despite the types of protein. Prolonging digestion time increased the soluble protein contents in the serum phase regardless of calcium lactate concentration. However, the SPI suspension had the highest TCA-soluble protein in the serum phase compared with other suspensions after digestion ($p<0.05$). This suggested that the SH-SS interchange did not affect the protein digestibility in bulk aqueous phase, but the protein digestibility of these aggregates depended on the type and ratio of proteins.

Table 3 Effect of protein type, calcium lactate concentration and the sulfhydryl-blocking agent (NEM) on the TCA-soluble protein content in the serum phase of protein suspension (4% protein w/v) during peptic digestion at pH 2.0

Treatment	Protein content (%)				
	Peptic digestion time (min)				
	0	15	30	45	60
SPI					
0 mM Ca-lactate	0.04 ^{aB}	1.80 ^{aA}	2.10 ^{aA}	2.39 ^{aA}	2.25 ^{abA}
0 mM Ca-lactate with NEM	0.18 ^{bB}	1.71 ^{abA}	2.15 ^{aA}	2.39 ^{aA}	2.35 ^{aA}
25 mM Ca-lactate	0.05 ^{aC}	1.53 ^{abcB}	1.64 ^{abAB}	2.16 ^{abA}	2.19 ^{abcA}
25 mM Ca-lactate with NEM	0.17 ^{bC}	1.68 ^{abB}	1.70 ^{abB}	2.48 ^{aA}	2.38 ^{aA}
SCN:SPI (0.7:0.3)					
0 mM Ca-lactate	0.04 ^{aB}	1.27 ^{abcA}	1.43 ^{bA}	1.53 ^{bcA}	1.38 ^{deA}
0 mM Ca-lactate with NEM	0.17 ^{bB}	1.33 ^{abcA}	1.23 ^{bA}	1.56 ^{bcA}	1.64 ^{cdeA}
25 mM Ca-lactate	0.04 ^{aC}	1.21 ^{bcB}	1.32 ^{bB}	1.49 ^{cAB}	1.74 ^{bcdA}
25 mM Ca-lactate with NEM	0.16 ^{bC}	1.21 ^{bcB}	1.49 ^{abA}	1.62 ^{bcA}	1.68 ^{bcdeA}
SCN					
0 mM Ca-lactate	0.04 ^{aC}	1.12 ^{cB}	1.34 ^{bAB}	1.38 ^{cA}	1.49 ^{deA}
0 mM Ca-lactate with NEM	0.16 ^{bB}	1.42 ^{abcA}	1.32 ^{bA}	1.71 ^{bcA}	1.39 ^{deA}
25 mM Ca-lactate	0.04 ^{aB}	1.08 ^{cA}	1.14 ^{bA}	1.32 ^{cA}	1.10 ^{eA}
25 mM Ca-lactate with NEM	0.17 ^{bB}	1.35 ^{abcA}	1.33 ^{bA}	1.42 ^{cA}	1.21 ^{deA}

Means in the same column followed by different lower case superscripts are significantly different ($p<0.05$). Means in the same row followed by different upper case superscripts are significantly different ($p<0.05$).

2. Effect of heat treatment sequences on *in vitro* protein digestibility and characteristics of liquid oil-in-water emulsion

The protein composite at the ratio SCN:SPI of 0.7:0.3 in water pH 3.0 had the lowest viscosity (~100 mPa.s.) was used as a protein source in our high-caloric emulsion. The emulsions contained 25 mM added calcium lactate addition prepared by *one-stage heating process* was more homogeneous than the *two-stage heating process* ones (Figure 13). This suggested that the aggregates in the emulsion could be modified after the salt-induced aggregates process by a re-heating step, a so-called "*two-stage heating process*".

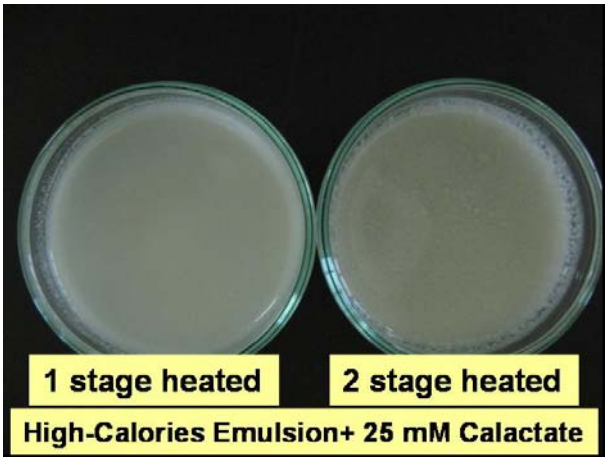

Figure 13 Reconstituted of liquid o/w emulsion (provided 1 kcal/mL) from the powder contained 13.75% carbohydrate, 3.75 % protein from sodium caseinate and soy protein isolate (SCN:SPI=0.7:0.3), and 3.33% rice bran oil in the presence of 25 mM calcium lactate prepared by different heat sequences. Each emulsion had the pH around 5.12 to 5.30.

The reconstituted emulsion prepared by *one-stage heating process* had the protein content of 0.70% in the cream phase, which was lowered to 0.52% during the first 60 min of digestion by pepsin at pH 2.0 (Figure 14). On the other hand, the protein content in the cream phase of the emulsion prepared by *two-stage heating process* was raised from 0.30% to 1.06% during the first 60 min of digestion (Figure

14a). This increase in the protein content in the cream phase was likely to be responsible for the delay time of oil to release from the emulsion prepared by *two-stage heating process* during peptic digestion (Figure 14b). The lowering of the protein content in cream phase of the emulsion prepared by *one-stage heating process* was likely be responsible for the short delay time of 15 min before the oil was released and the higher extent of oil released from the emulsion during 150 min of peptic digestion.

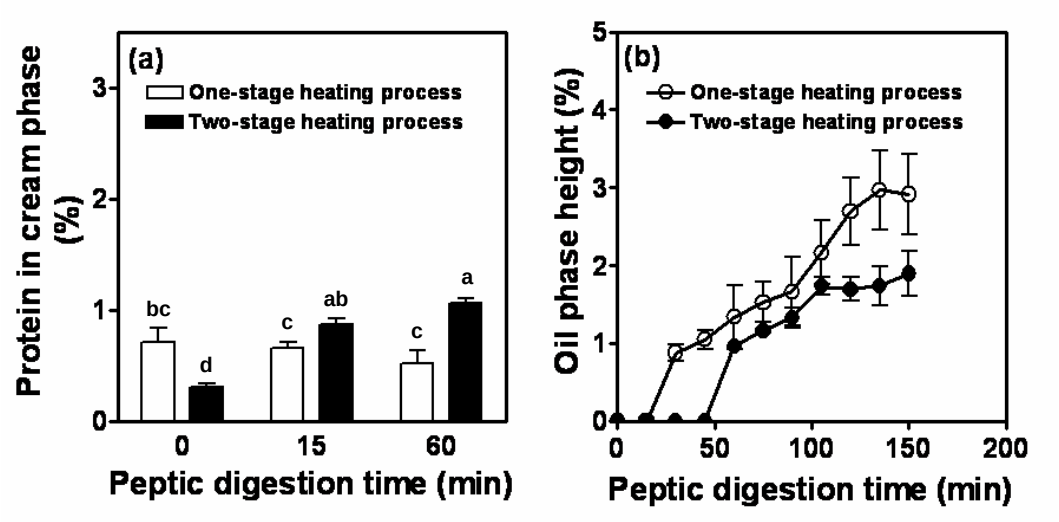

Figure 14 Effect of heating sequence on (a) protein concentration in cream phase and (b) oil released from the emulsion containing 25 mM calcium lactate (1 kcal/mL) during peptic digestion at pH 2.0. Bars represent standard deviation.

Table 4 shows that the TCA-soluble protein content in serum phase of the reconstituted emulsion prepared by *one-stage heating process* increased from 0.09% to 1.18% during the first 15 minute of digestion and remained plateau after prolonged digestion. Likewise, the TCA-soluble protein content in the serum phase of the reconstituted emulsion prepared by *two-stage heating process* increased in the similar manner as to that of the emulsion prepared by *one-stage heating process* during the first 15 min of digestion.

Table 4 Effect of heating sequence on the TCA-soluble protein content in the serum phase of the emulsion containing 25 mM calcium lactate (1 kcal/mL) during peptic digestion at pH 2.0

Treatment	Protein content (%)				
	Peptic digestion time (min)				
	0	15	30	45	60
One-stage heating process	0.09 ^{c}	1.18 ^{a}	1.20 ^{a}	1.28 ^{a}	1.45 ^{a}
Two-stage heating process	0.09 ^{c}	0.98 ^{bc}	1.08 ^{ab}	1.29 ^{a}	1.29 ^{a}

Means followed by different superscripts are significantly different ($p<0.05$).

Figure 15 illustrates the CLSM of the emulsion prepared by different heating schemes before and after peptic digestion. The reconstituted emulsion prepared by *one-stage heating process* (Figure 15a) had much larger oil droplet size, i.e. mostly 20 μm than did the one from *two-stage heating process*, which was mostly less than 5 μm (Figure 15e). Upon acidification of the reconstituted emulsion from pH around 5.3-5.5 to pH of 2.0, the oil droplets flocculated. The microstructure of the protein at the interface of emulsion prepared by *one-stage* and *two-stage heating processes* showed aggregation in different manners. In emulsion prepared by *one-stage heating process*, the oil droplets with thin coated protein flocculated heavily when the pH was adjusted to 2.0 and the globular shape of the droplet was retained (Figure 15b). On the contrary, the oil droplets in the emulsion prepared by *two-stage heating process* aggregated greatly and entrapped the oil droplets within the matrix (Figure 15f). Thicker interfacial protein layer was observed in the case of emulsion prepared by *one-stage heating process* after the addition of pepsin and allowing the digestion to proceed for 15 min and the emulsion contained various oil droplet size (Figure 15c).

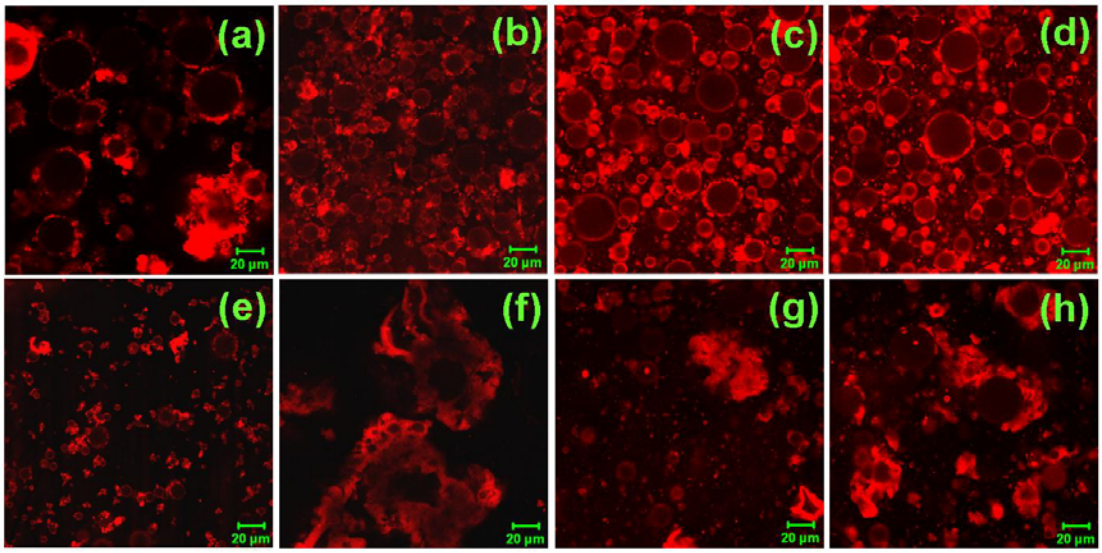

Figure 15 Confocal laser scanning micrographs of o/w emulsion containing 25 mM calcium lactate (1 kcal/mL) prepared by (a-d) *one-stage heating process* and (e-h) *two-stage-heating process*. (a, e) reconstituted emulsion; (b, f) reconstituted emulsion adjusted to pH 2.0, digestion time was 0 min; (c, g) reconstituted emulsion added with pepsin, digestion time was 15 min at pH 2.0; and (d, h) reconstituted emulsion added with pepsin, digestion time was 60 min at pH 2.0. Protein was fluoresced in red by Rhodamine B. Bar scale= 20 µm.

This phenomenon was not evident in the peptic-hydrolyzed emulsion prepared by *two-stage heating process*. Its oil droplets had thin interfacial protein although the emulsion showed disintegration of the protein matrix and the existence of individual oil droplets with small size (Figure 15g). Over 60 min of digestion, the microstructure of the interfacial protein film in the emulsion and the oil droplet size prepared by *one-stage heating process* remained unchanged (Figure 15d). However, the oil droplets in the emulsion prepared by *two-stage heating process* showed larger size and they were bridged by the protein matrix as a clump (Figure 15h).

Different heating schemes employed during emulsion preparation resulted in preferential adsorption of proteins at the oil-water interface (Figure 16). The majority of the protein at the interface or in cream phase had MW between 25-30 kDa (Figures 16a, 16b; lane 1) before digestion by pepsin. This was because the protein ratio between SCN to SPI of 0.7:0.3 was used in the formulae. The protein profile in SDS-PAGE suggested that most of the proteins at the interface were phosphorylated caseins. Prior to digestion, the cream phase in the emulsion prepared by *one-stage heating process* incorporated proteins of the MW above 50 kDa, most of which were 7S soy polypeptides. The high intensity bands (Figure 16a; lane 1) at that MW range, compared with those in emulsion prepared by *two-stage heating process* (Figure 16b; lane 1), suggested different partitioning of polypeptides at the interface induced by second heating scheme. It appeared that soy polypeptides were present in fewer amounts than casein at the interface when *two-stage heating process* was employed.

Further digestion by pepsin hydrolyzed most of the polypeptides to the MW of less than 35 kDa during the first 60 min of digestion. Nonetheless, the second heating scheme employed in *two-stage heating process* not only resulted in the low distribution of soy polypeptides at the interface, but also generated the large MW above 250 kDa precipitated in the well (Figure 16b; lanes 2 and 3). This fraction was slowly digested and still present even after digestion by pepsin for 60 min. However, further digestion by trypsin hydrolyzed most of the polypeptides at the interface to the MW of lower than 15 kDa.

Heating schemes resulted in slight difference in polypeptides in the serum phase (Figures 16c, 16d) compared to those in the cream phase (Figures 16a, 16b). The polypeptides with MW slightly below 25 kDa and between 10 and 14.3 kDa were clearly observed in the serum phase as a consequence of preferential adsorption in the cream phase (Figures 16c, 16d; lane 1). Similar to the protein in the cream phase, the polypeptides in the serum phase were hydrolyzed to the MW of less than 35 kDa after peptic digestion for 60 min and to less than 15 kDa by tryptic digestion.

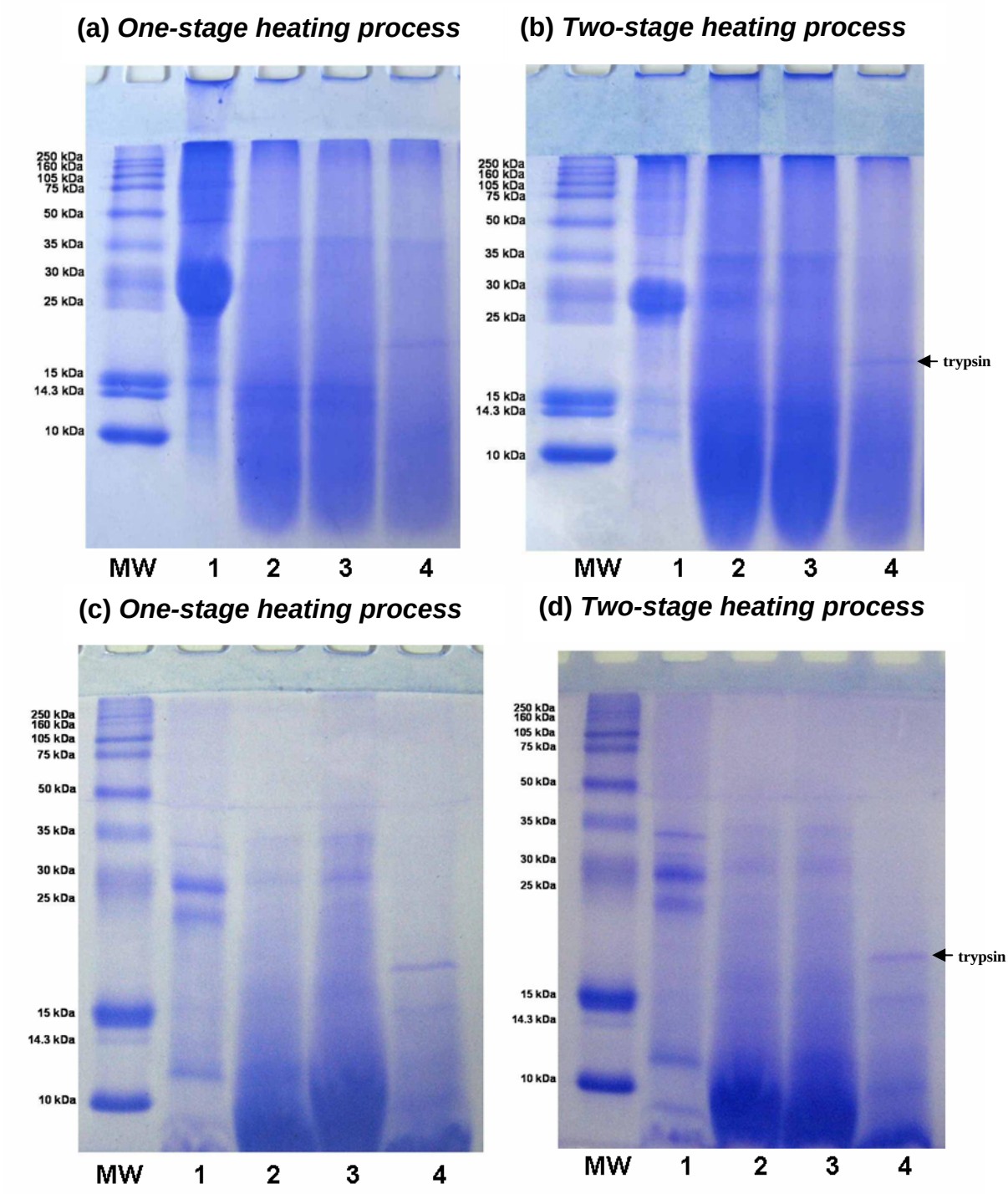

Figure 16 Effect of heating sequence on the MW characteristics of protein in (a,b) cream phase and (c,d) serum phase of reconstituted emulsion containing 25 mM calcium lactate (1 kcal/mL). Lane 1=peptic digestion for 0 min; lane 2=peptic digestion for 15 min; lane 3=peptic digestion for 60 min; and lane 4=peptic digestion for 15 min followed by tryptic digestion for 30 min.

In contrast with the protein digestibility shown in Table 3, this study showed that the protein digestibility relied upon the partitioning of proteins whether they are at the interface or in bulk aqueous phase. Therefore, it is likely that the digestion characters of protein at the interface are different from those in bulk aqueous phase.

Further hydrolysis by trypsin released the oil from the emulsions (Table 5). Nevertheless, the presence of bile acid did not alter the oil release characteristics although the emulsions were hydrolyzed for 60 min. The tryptic-hydrolyzed emulsion prepared by *two-stage heating process* showed less oil released than did the one prepared by *one-stage heating.*

Table 5 Effect of heating sequence and bile acid on oil released from the emulsion containing 25 mM calcium lactate (1 kcal/mL) after being digested by pepsin for 15 min followed by trypsin for 30 min or 60 min at pH 7.0

Digestion	Oil phase height (%)	
	One-stage heating process	*Two-stage heating process*
15 min pepsin+30 min trypsin	1.856 ^{abc}	1.582 ^{c}
15 min pepsin+30 min trypsin+bile acid	2.156 ^{ab}	1.919 ^{abc}
15 min pepsin+60 min trypsin	2.092 ^{ab}	1.729 ^{bc}
15 min pepsin+60 min trypsin+bile acid	2.283 ^{a}	1.845 ^{abc}

Means followed by different superscripts are significantly different ($p<0.05$).

This study showed that although heating scheme influenced preferential adsorption of casein over soy protein at the interface, the overall release of oil fraction was not affected by heating schemes.

3. Effect of calcium lactate concentration on *in vitro* protein digestibility and characteristics of emulsion prepared by *two-stage heating process*

The appearance of reconstituted of liquid o/w emulsions prepared by *two-stage heating process* in the presence of different calcium lactate concentrations are shown in Figure 17. At high concentration of calcium lactate, the large particle separated in the suspension was evident.

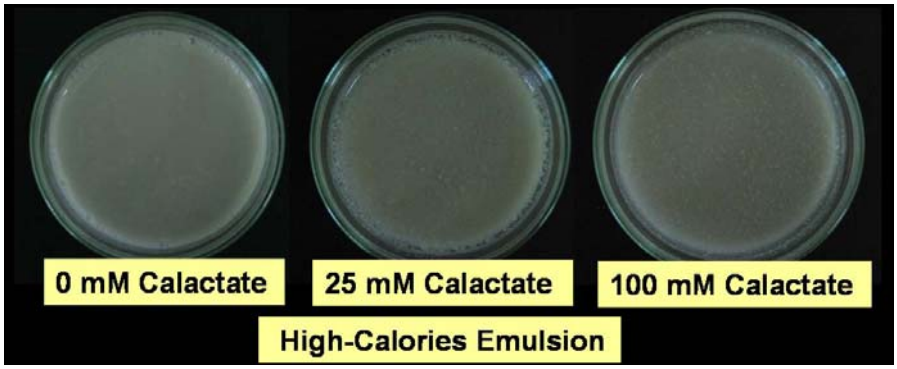

Figure 17 Reconstituted of liquid o/w emulsion (providing 1 kcal/mL) from the powder contained 13.75% carbohydrate, 3.75 % protein from sodium caseinate and soy protein isolate (SCN:SPI=0.7:0.3), and 3.33% rice bran oil in the presence of different calcium lactate concentrations prepared by *two-stage heating process*. Each emulsion had the pH around 5.30 to 5.46.

Prior to the digestion by pepsin, raising the concentration of added calcium lactate from 0 mM to 100 mM lowered the protein content in the cream phase (Figure 18a) of reconstituted emulsion prepared by *two-stage heating process*. In the absence of calcium lactate, the protein content in the cream phase remained unchanged during 60 min peptic digestion of the emulsion at pH 2.0. However, the addition of calcium lactate resulted in an increase in protein contents in the cream phase during the first 60 min of peptic digestion. In the presence of 25 mM calcium lactate, the cream phase of the emulsion had the highest protein content. Despite the increase in protein content in the cream phase in the emulsion containing added calcium lactate, the oil release characteristics of all emulsions during peptic digestion remained unaltered

over the digestion time of 150 min by pepsin (Figure 18b). This suggested that the second heating step alone resulted in the interfacial characteristics that can prolong the release of oil phase regardless of calcium lactate addition.

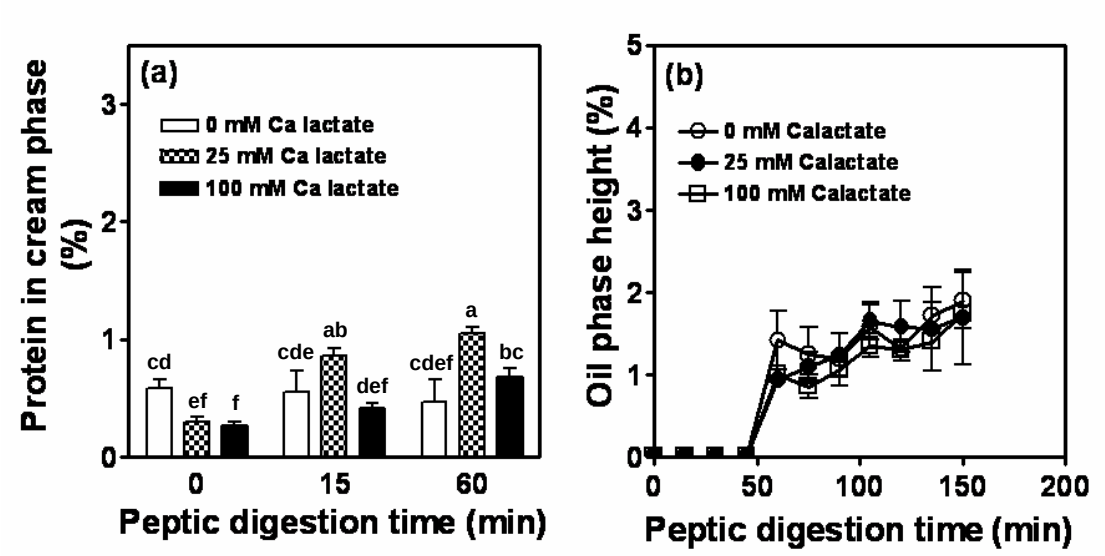

Figure 18 Effect of calcium lactate concentration on (a) protein content in cream phase and (b) oil released from the emulsion prepared by *two-stage heating process* during peptic digestion at pH 2.0. Bars represent standard deviation.

Nevertheless, Figure 19 illustrates the CLSM of the emulsion before and after peptic digestion. The emulsions were prepared by adding different concentrations of calcium lactate. The reconstituted emulsion prepared in the absence of calcium lactate had large oil droplet size with coated protein entity to the higher extent than did the emulsions containing 25 and 100 mM added calcium lactate (Figures 19a, 19e, 19i). Upon acidification of the reconstituted emulsion to the pH of 2.0, there was the clumping of the protein fraction and the flocculation of the oil droplets, particularly in the emulsion containing 25 mM of added calcium lactate (Figure 19f). Disintegration of the flocculated protein matrix and the oil droplets, as well as the increase in the oil droplet size, became evident after the emulsion was digested for 15 min by pepsin (Figures 19c, 19g, 19k). After 60 min of peptic digestion, the protein fraction in the

emulsion with added calcium lactate of 100 mM re-aggregated to large particles dispersed more densely than the others (Figure 19l).

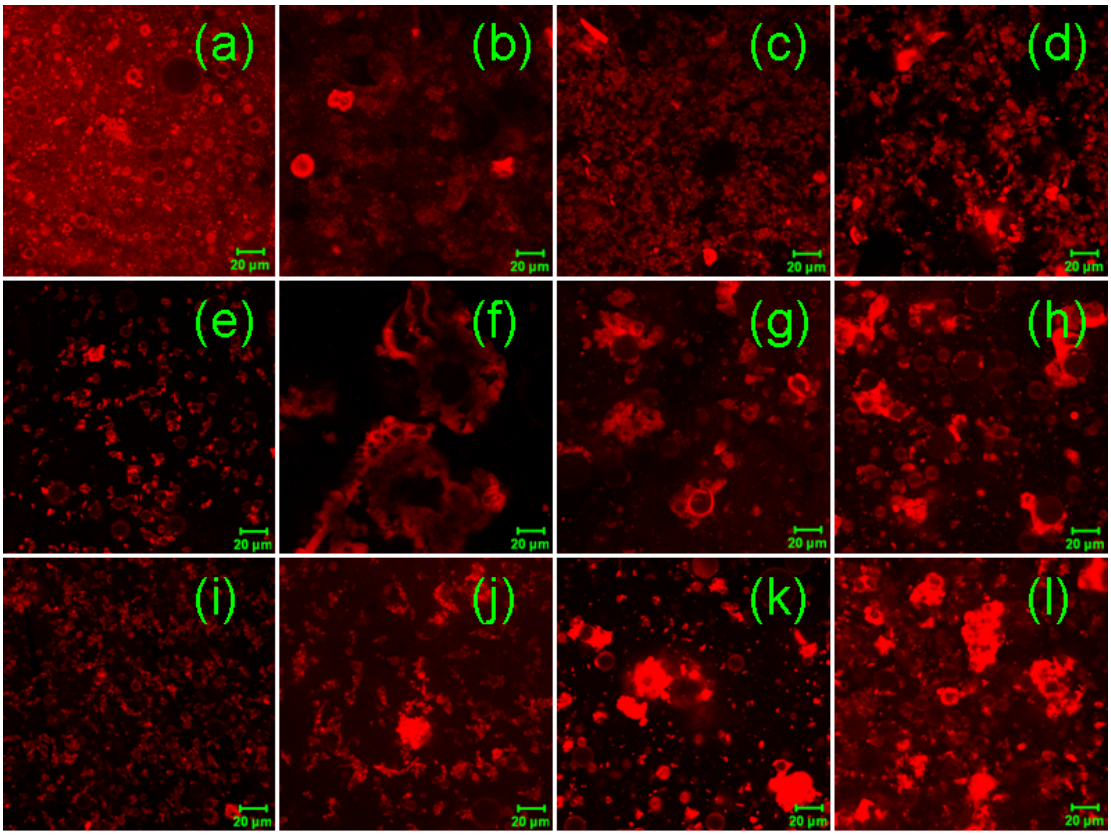

Figure 19 Confocal laser scanning micrographs of o/w emulsion prepared by *two-stage heating process* (1 kcal/mL) and contained (a-d) 0 mM added calcium lactate, (e-f) 25 mM added calcium lactate and (i-l) 100 mM added calcium lactate. (a, e, i) reconstituted emulsion; (b, f, j) reconstituted emulsion adjusted to pH 2.0; (c, g, k) reconstituted emulsion added with pepsin, digestion time was 15 min at pH 2.0; and (d, h, l) reconstituted emulsion added with pepsin, digestion time was 60 min at pH 2.0. Protein was fluoresced in red by Rhodamine B. Bar scale= 20 µm.

Table 6 shows that calcium was present mostly in the serum phase. After peptic and tryptic digestion, the amount of calcium was not significantly different from those of indigested ones ($p \geq 0.05$). The serum phase still contained high amount of calcium, although the high MW of protein or peptide was precipitated by TCA. It

should be noted that calcium in the serum phase may be in the form of ionic calcium and/or calcium bound to proteins or peptides that had MW of less than 14.3 kDa. The soluble protein fragments with the highest calcium binding capacity had MW about 14.4 or 8 to 9 kDa (Bao *et al*., 2008). The excess calcium in the serum phase may be important in the induction of protein or peptide to re-adsorb at interface during digestion.

Table 6 Effect of calcium lactate concentration on the soluble calcium content in the serum phase of the emulsion prepared by *two-stage heating process* (1 kcal/mL) during peptic and tryptic digestion at the final pH 7.0

Treatment	Calcium content in solution (calculation) (mg/ 100g sample)	Soluble calcium in serum phase (mg/ 100g sample)	
		Without TCA	With TCA
0 mM Ca-lactate			
0 min pepsin	No added calcium	9.07 ^{dA}	12.58 ^{cA}
15 min pepsin		15.89 ^{dA}	15.53 ^{cA}
15 min pepsin+30 min tryp		15.25 ^{dA}	14.18 ^{cA}
25 mM Ca-lactate			
0 min pepsin	497	249.71 ^{cA}	177.10 ^{bA}
15 min pepsin		228.17 ^{cA}	181.93 ^{bA}
15 min pepsin+30 min tryp		246.14 ^{cA}	212.85 ^{bA}
100 mM Ca-lactate			
0 min pepsin	1784	1098.79 ^{aA}	837.02 ^{aA}
15 min pepsin		931.04 ^{bA}	730.60 ^{aB}
15 min pepsin+30 min tryp		825.76 ^{bA}	826.90 ^{aA}

Means in the same column followed by different lower case superscripts are significantly different ($p<0.05$). Means in the same row followed by different upper case superscripts are significantly different ($p<0.05$).

Preferential adsorption of soy and phosphorylated proteins at the interface and serum phase were also observed as the calcium lactate concentration increased (Figure 20). In the cream phase of emulsion added with 100 mM calcium lactate, there was an absence of the polypeptides with MW above 50 kDa and between 25-30 kDa, which were mainly soy proteins and phosphorylated caseins, respectively (Figure 20c). The polypeptides with a much lower MW, i.e. less than 15 kDa, were present in the cream phase of such an emulsion. These polypeptides were not adsorbed at the oil-water interface of the emulsions prepared in the absence of calcium lactate and the one containing 25 mM calcium lactate (Figures 20a, 20b, respectively).

Upon peptic and tryptic digestion, the protein bands present in the cream phase of the emulsion containing 0 mM of added calcium lactate had very low intensity (Figure 20a; lanes 2,3,4), compared to those in the cream phase of the emulsions containing 25 and 100 mM of added calcium lactate. Further digestion of the latter two emulsions by trypsin hydrolyzed most of the polypeptides at the interface to the MW of lower than 15 kDa.

Calcium lactate concentration also resulted in the difference in polypeptides distribution in the serum phase (Figures 20d, 20e, 20f) compared to those in cream phase (Figures 20a, 20b, 20c). The polypeptides with the MW above 30 kDa were observed only in the serum phase of the emulsion prepared in the absence of calcium lactate. The polypeptides with MW slightly below 25 kDa and between 10 and 14.3 kDa were observed in the serum phase of emulsions containing 0 and 25 mM calcium lactate and with less intensity in the serum phase of the emulsion containing 100 mM calcium lactate. The increase of calcium lactate to 100 mM may reduce the solubility of the phosphorylated caseins. It is likely that most of them were precipitated in the sediment and not participated in the emulsion stabilization.

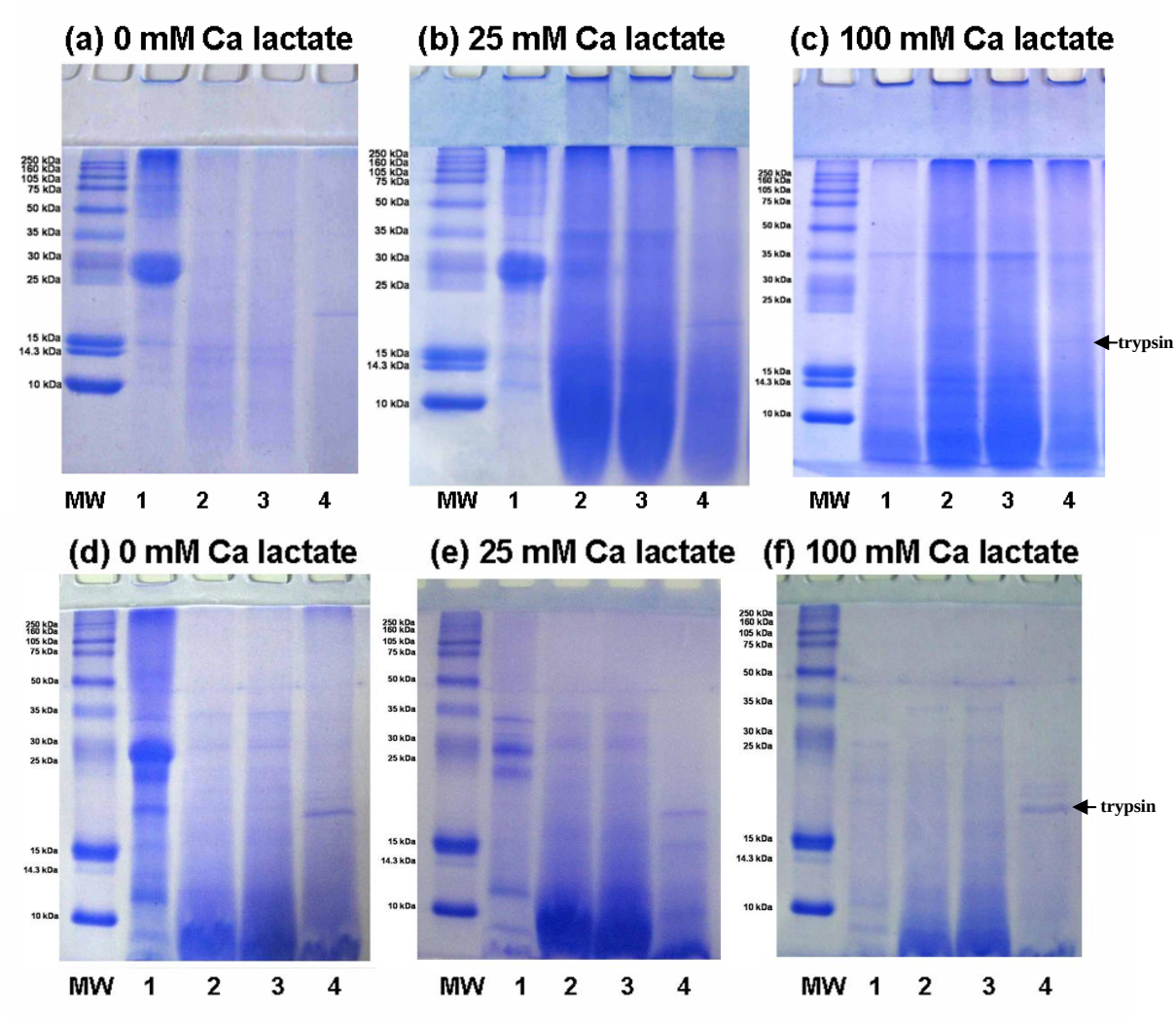

Figure 20 Effect of calcium lactate concentration on the MW characteristics of protein in (a-c) cream phase and (d-f) serum phase of reconstituted emulsion prepared by *two-stage heating process* (1 kcal/mL). Lane 1=peptic digestion for 0 min; lane 2=peptic digestion for 15 min; lane 3=peptic digestion for 60 min; and lane 4=peptic digestion for 15 min followed by tryptic digestion for 30 min.

The tryptic-hydrolyzed emulsion lost their stability the most in the presence of 25 mM calcium lactate (Table 7). After trypsin digestion for 30 min, the emulsion containing 100 mM of added calcium lactate was able to withstand both the presence of bile acid and protein digestion. No detectable oil separation after centrifugation was observed in this case.

Table 7 Effect of calcium lactate concentration and bile acid on oil released from the emulsion prepared by *two-stage heating process* (1 kcal/mL) after being digested by pepsin for 15 min followed by trypsin for 30 min or 60 min at pH 7.0

Digestion	Oil phase height (%)		
	0 mM Ca lactate	25 mM Ca lactate	100 mM Ca lactate
15 min pepsin+30 min trypsin	1.013 ^{b}	1.582 ^{a}	0.947 ^{b}
15 min pepsin+30 min trypsin+bile acid	1.157 ^{b}	1.919 ^{a}	0.000 ^{c}
15 min pepsin+ 60 min trypsin	1.110 ^{b}	1.729 ^{a}	1.018 ^{b}
15 min pepsin+60 min trypsin+bile acid	1.135 ^{b}	1.845 ^{a}	1.025 ^{b}

Means followed by different superscripts are significantly different ($p<0.05$).

This study indicated that the preferential adsorption of proteins at the interface can be further manipulated by increasing calcium lactate concentration although calcium ion did not directly present in the bound forms with proteins or peptides.

4. Effect of soy residue (okara) addition on *in vitro* protein digestibility and characteristics of emulsion prepared by *two-stage heating process*

In this study, soy residue (okara) or pectinase-hydrolyzed okara (PHO) was used as protein and fiber source in the liquid food formulae. Table 8 show that pectinase hydrolysis significantly altered the reducing sugar content of the powder ($p<0.05$). The increase in reducing sugar of polysaccharide, indicated that the polysaccharide could be hydrolyzed into small MW carbohydrate (Milner and Avigad, 1967).

Table 8 The reducing sugar content of okara and pectinase-hydrolyzed okara powder

Treatment	Reducing sugar (mg/mL) of 10% w/v suspension
Okara powder	0.191 ± 0.074^{b}
Pectinase-hydrolyzed okara (PHO)	0.363 ± 0.059^{a}

Means in the same column followed by different superscripts are significantly different ($p<0.05$).

However, PHO powder had brownish colour to the higher extent than okara (Table 9). It should be noted that some reducing sugar in PHO may be involved in Maillard reaction during drying process.

Table 9 The colour value of okara and pectinase-hydrolyzed okara powder

Treatment		CIE system of powder		
		L*	a*	b*
Okara powder		89.16 [a]	-0.6 [a]	11.21 [a]
Pectinase-hydrolyzed okara (PHO)		85.00 [b]	0.27 [b]	16.07 [b]

Means in the same column followed by different superscripts are significantly different ($p<0.05$).

When okara or PHO was present in the reconstituted emulsion, the microstructure and the apparent viscosity of the emulsion were altered (Figure 21 and Figure 22). In the absence of okara, the reconstituted emulsion contained individual oil droplets with particle size around 2-10 μm with protein at the interface separated in the protein-rich aqueous phase (Figure 21a). The CLSM showed aggregation of protein-stabilized oil droplets appeared in the presence of PHO (Figure 21c). The oil droplets with particle size around 30 μm (dark oil droplet surrounded by red protein). However, these droplet aggregates did not affect the apparent viscosity of emulsion. The emulsion had low viscosity, which was below 150 mPa.s (Figure 22). In contrast, the presence of okara resulted in the oil droplets with average size around 13 μm. However, they were more connected in this matrix (Figure 21b). The apparent viscosity of the reconstituted emulsion containing okara drastically increased to 350 mPa.s ($p<0.05$).

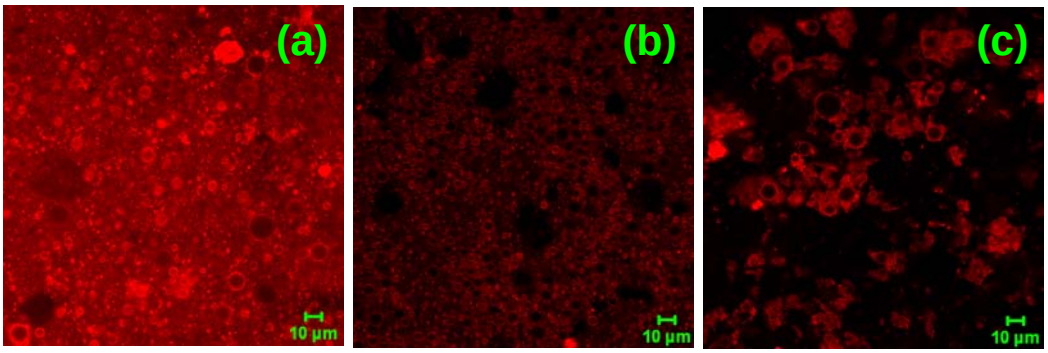

Figure 21 Confocal laser scanning micrographs of the reconstituted *two-stage heated emulsions* in the absence of calcium lactate (1 kcal/mL) containing okara and modified okara (a) the emulsion; (b) the emulsion with okara and (c) the emulsion with pectinase-hydrolyzed okara (PHO). Each emulsion had the pH around 5.12 to 5.30. Proteins were stained with Rhodamine B and are shown by red fluorescence. Bar scale= 10 µm.

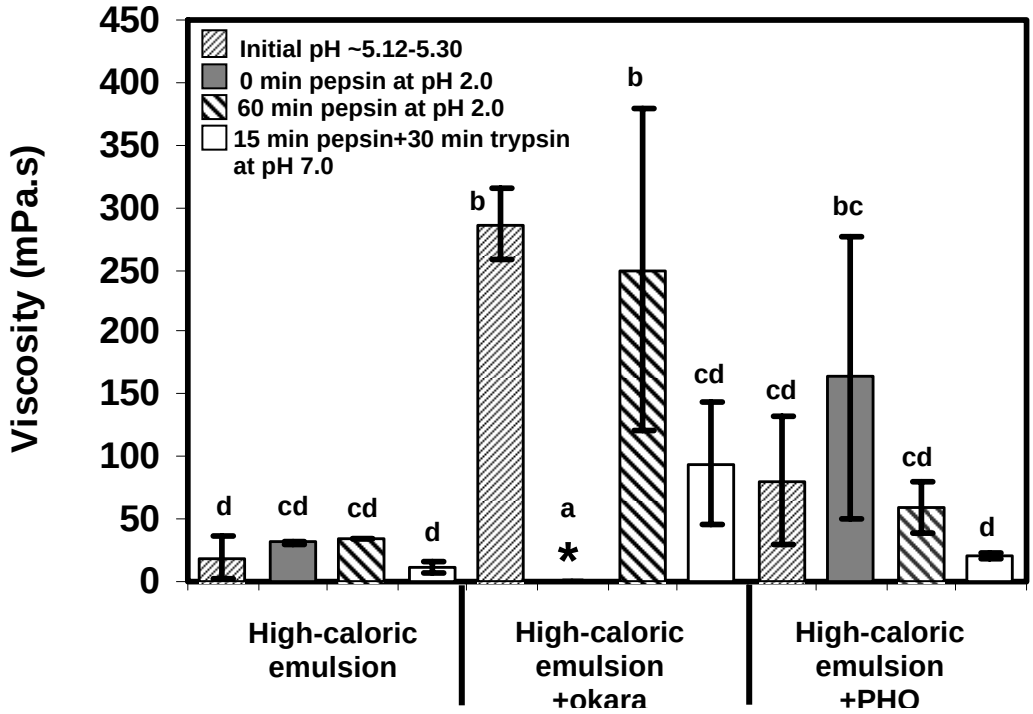

Figure 22 Apparent viscosity of the reconstituted *two-stage heated emulsions* (in the absence of calcium lactate) containing no okara, okara, and pectinase-hydrolyzed okara (PHO) before and after peptic and/or tryptic digestion. Measurements were carried out at 25˚C.

Upon acidification of the reconstituted emulsion containing okara or PHO from pH around 5.12-5.30 to pH of 2.0, both the apparent viscosity and the microstructure were altered. In the presence of okara, the reconstituted emulsion developed thick suspension that could not be recorded by Brookfield Rheometer. The high viscosity resulted in the clumping of the protein fraction into matrix observed in Figure 21. This led to the entrapment of the oil droplets within the matrix. The aggregates in the emulsion were large in size (Figure 23a). In contrast, the presence of PHO did not alter the three-dimensional network formation (Figure 23d). Thus, the emulsion contained PHO still had low viscosity.

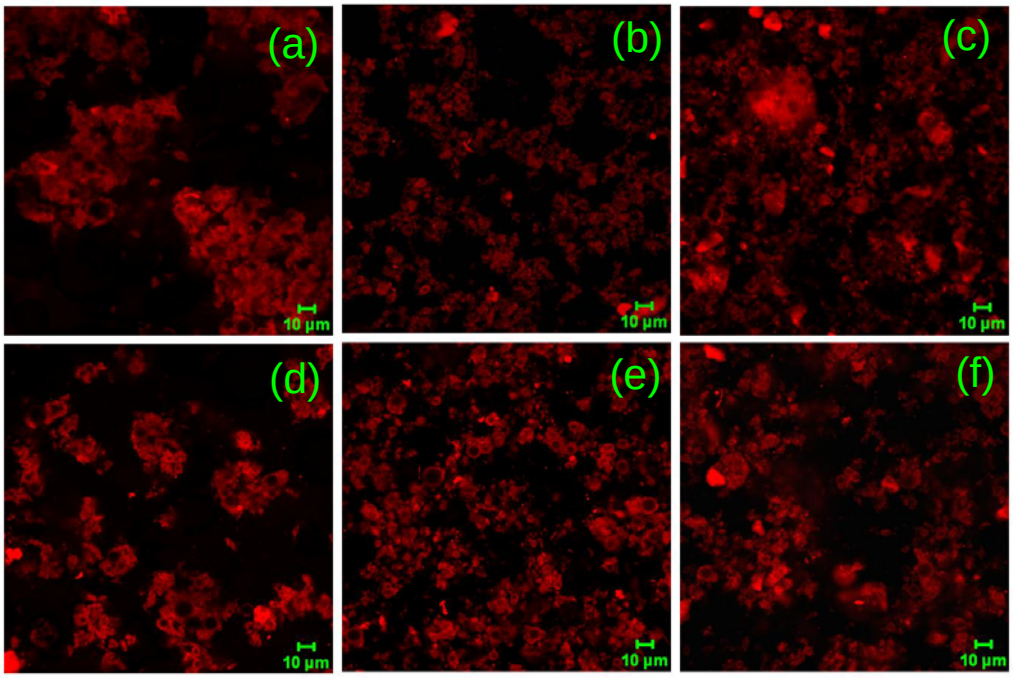

Figure 23 Confocal laser scanning micrographs of the reconstituted *two-stage heated liquid o/w emulsions* in the absence of added calcium lactate (1 kcal/mL) with okara and modified okara (a-c) the emulsion with okara and (d-f) the emulsion with pectinase-hydrolyzed okara (PHO). (a, d) reconstituted emulsion adjusted to pH 2.0; (b, e) reconstituted emulsion added with pepsin, digestion time was 15 min at pH 2.0; and (c, f) reconstituted emulsion added with pepsin, digestion time was 60 min at pH 2.0. Protein was fluoresced in red by Rhodamine B. Bar scale= 10 µm.

After 15 min of peptic digestion, the flocculation of protein matrix and the oil droplets were disintegrated. However, bridging of the oil droplets was still present (Figure 23b, 23e). Thicker layer at the interface was clearly observed in the emulsion containing PHO. In the emulsion containing okara, the oil droplets with small size had thinner protein layer at the interface and the increase in oil droplets size after peptic digestion for 60 min (Figure 23c) was observed. The oil droplets in the emulsion containing PHO showed larger size than the one added with okara and they were present as a clump of the small oil droplets with thinner protein film (Figure 23f).

The protein content in the cream phase of the emulsion is shown in Figure 24a. In the absence of okara, the protein content in the cream phase remained unchanged during 60 min peptic digestion of the emulsion at pH 2.0. The addition of okara resulted in an increase of protein content in the cream phase during the first 60 min of peptic digestion ($p<0.05$). Although the protein content in the cream phase in the emulsion containing okara increased, oil was separated during first 15 min of peptic digestion. In contrast, the emulsion containing PHO had the long delay time of 135 min before the oil was released (Figure 24b). This suggested that a clump of the small oil droplets at the oil-water interface of the emulsion was likely to be more responsible for the long delay time than the protein content at the interface.

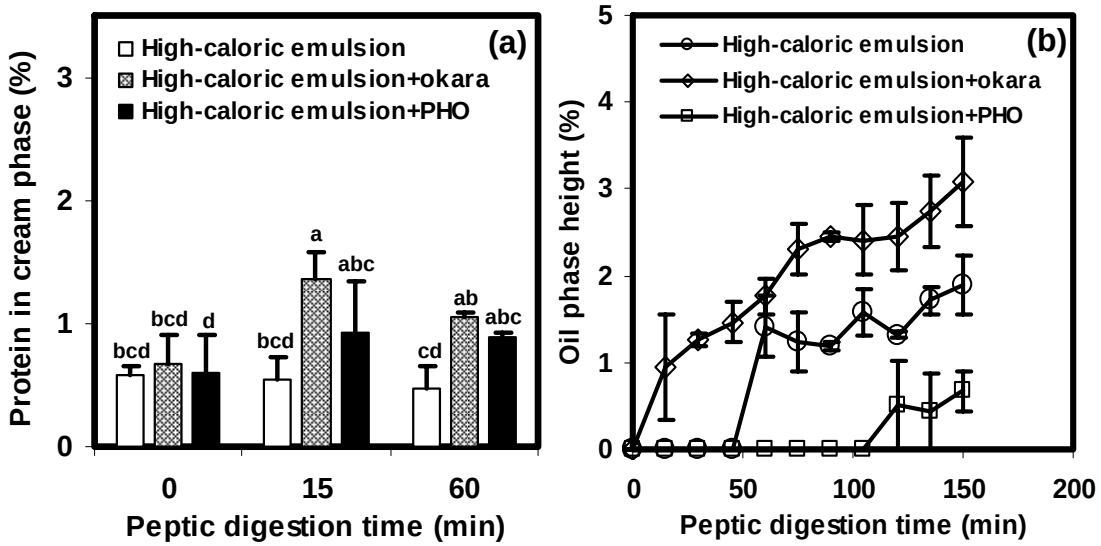

Figure 24 Effect of okara and pectinase-hydrolyzed okara (PHO) addition on (a) protein content in cream phase (b) oil released from the reconstituted *two-stage heated emulsion* in the absence of calcium lactate (1 kcal/mL) during peptic digestion at pH 2.0. Bars represent standard deviation.

The use of PHO altered the polypeptide profiles (Figure 25). PHO and okara resulted in preferential adsorption of protein at the interface. The majority of protein at the interface of all emulsions was the MW between 24-31 kDa, which were phosphorylated caseins. However, the cream phase of the emulsion containing PHO exhibited less intensity band than the others (Figure 25c; lane1). In the absence of okara and PHO, the cream phase in the emulsion incorporated proteins with the MW above 52 kDa, most of which were 7S soy polypeptides (Figure 25a; lane1). These polypeptides were still present and showed an increase in band intensity when okara was added to the emulsion (Figure 25b; lane1). In addition, the cream phase of this emulsion had polypeptides incorporated at the interface, i.e. the bands referred to acidic (18-20 kDa) and basic (31-38 kDa) of 11S soy polypeptides which were not observed before. In the cream phase of emulsion containing PHO, there was an absence of the polypeptides with MW above 52 kDa. However, 11S soy polypeptide, particularly basic polypeptides slightly adsorbed at the interface. Moreover, the polypeptides with MW less than 12 kDa were also incorporated at the interface

(Figure 25c; lane1). These polypeptides were less adsorbed at the oil-water interface of other emulsions. Further digestion by pepsin hydrolyzed most of the polypeptides to the MW of less than 35 kDa during the first 60 min of digestion and to less than 15 kDa by tryptic digestion.

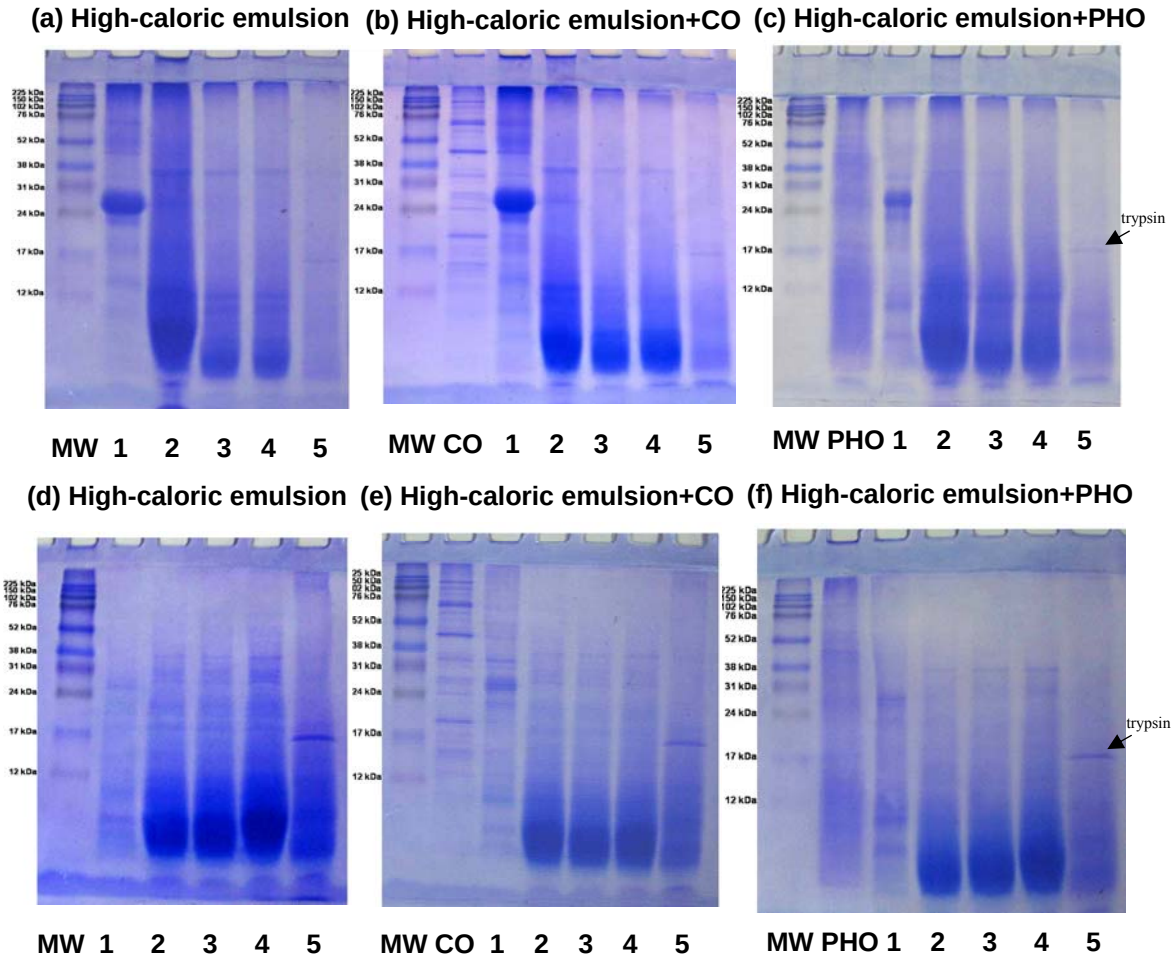

Figure 25 Effect of okara and pectinase-hydrolyzed okara (PHO) addition on the MW characteristics of protein in (a-c) cream phase and (d-f) serum phase of reconstituted *two-stage heated emulsion* containing 0 mM calcium lactate. Lane 1=peptic digestion for 0 min; lane 2=peptic digestion for 15 min; lane 3=peptic digestion for 30 min; lane 4=peptic digestion for 60 min; and lane 5=peptic digestion for 15 min followed by tryptic digestion for 30 min.

Okara present in the emulsion resulted in a slight difference in polypeptide profiles in the serum phase (Figures 25e) compared to those in cream phase (Figures 25b). The polypeptides with MW above 52 kDa were clearly observed in the serum phase as a consequence of preferential adsorption in the cream phase (Figures 25e, lane 1). Similar to the protein in the cream phase, the polypeptides in the serum phase were hydrolyzed to the MW of less than 35 kDa after peptic digestion for 60 min and to less than 15 kDa by tryptic digestion.

Although the emulsion containing PHO did not release the oil after peptic digestion, the tryptic-hydrolyzed emulsion lost their stability and the oil could be released in the presence of bile acid (Table 10). This indicated that the matrix at the oil-water interface of the emulsion could be altered by the presence of modified okara. The resulting digesta may be further modified by bile acid and the shift of pH from 2.0 to 7.0.

Table 10 Effect of bile acid on oil released from the *two-stage heated emulsion* containing 0 mM calcium lactate with pectinase-hydrolyzed okara (PHO) addition after being digested by pepsin for 15 min followed by trypsin for 30 min or 60 min at pH 7.0

Digestion	Oil released (%)
15 min pepsin+30 min trypsin	0.663 ^{b}
15 min pepsin+30 min trypsin+bile acid	0.974 ^{ab}
15 min pepsin+60 min trypsin	0.862 ^{ab}
15 min pepsin+60 min trypsin+bile acid	1.325 ^{a}

Means followed by different superscripts are significantly different ($p<0.05$).

Discussion

This study showed that mixed protein between SCN:SPI could be used as protein source in the liquid food formulae. The final viscosity of the model liquid oil-in-water emulsion provided caloric distribution from carbohydrate:protein:lipid of 55:15:30 was below 50 mPa.s, which was suitable for drinks. The first heat treatment at 80˚C for 30 min induced proteins to form soluble or insoluble aggregates. After homogenization, these aggregates adsorbed at the oil-water interface and formed matrix surrounding the oil droplets. The new findings of this study were that the characteristics of protein matrix could be further fabricated via re-heating step or *the second heating process* after the emulsion was formed, calcium lactate concentration and soy residue addition. Moreover, the protein matrix at the interface generated during food processing was altered again due to the shift of pH in the GI tract. The fabrication of the adsorbed protein matrix on the oil-water interface influenced the amount of oil and time required to release oil from emulsion during digestion in the GI tract. The increase or decrease of the oil release depended on two factors; i.e. (1) the preferential adsorption of phosphorylated caseins and soy proteins due to heat treatment and (2) re-adsorption of protein or peptide on the oil-water interface during digestion due to the increase in calcium lactate concentration. The summary of these results was conceptualized in Figure 26.

Because phosphorylated caseins were the main constitutes of the protein matrix at the oil-water interface of the emulsion prepared by *two-stage heating process*. The phosphorylated caseins were prone to further alteration by acidification to pH 2.0 under the stomach condition and resulted in the flocculation of the oil droplets entrapped in the protein matrix. The *two-stage heating process* resulted in the emulsion with slower release of oil under *in vitro* stomach and duodenal conditions, compared to the one prepared by *one-stage heating process*. The delay time of oil release was about 30 min longer during peptic digestion of the emulsion prepared by *two-stage heating process*. In addition, the amount of oil release from the emulsion prepared by *two-stage heating process* under stomach condition was lower than the one prepared by *one-stage heating process*.

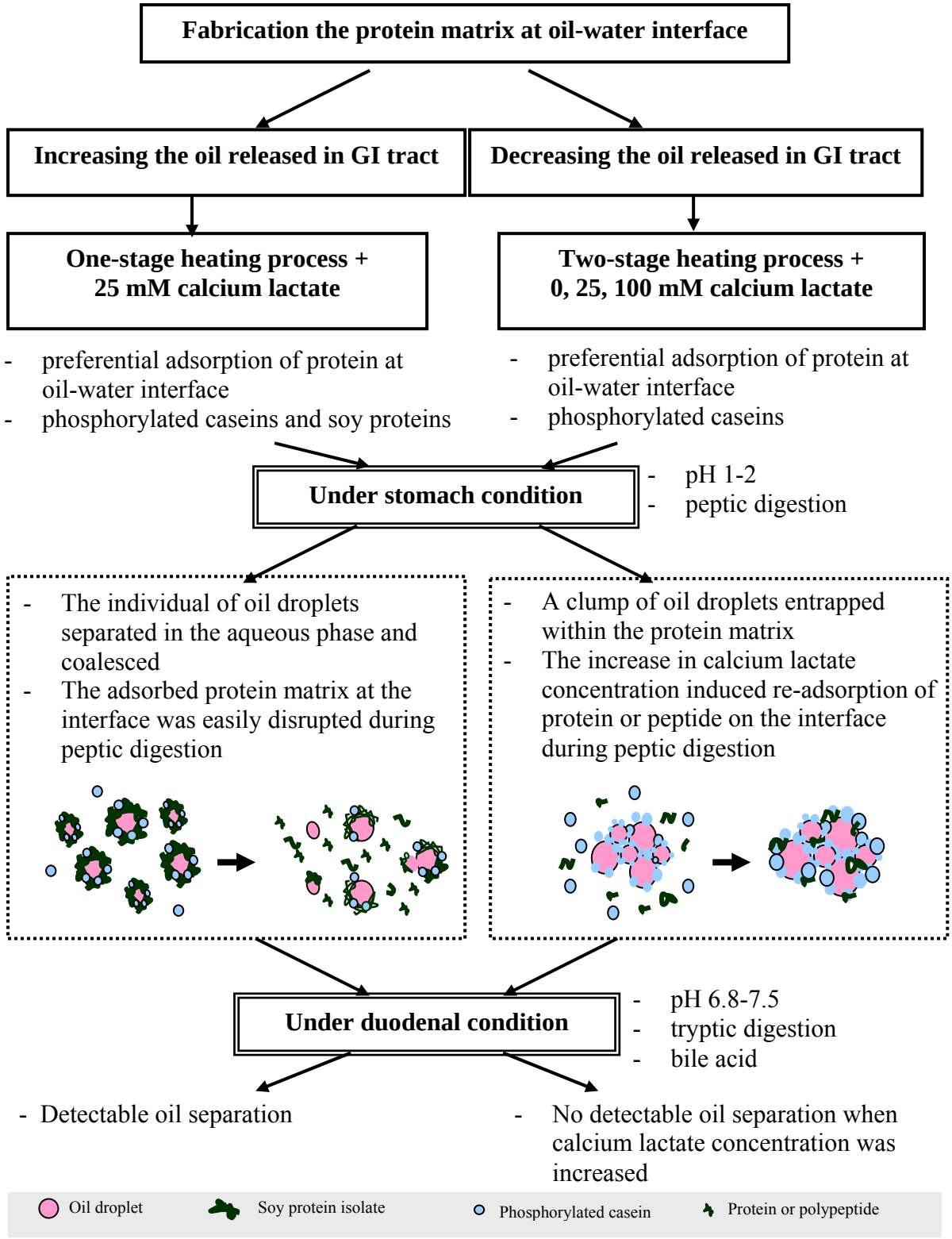

Figure 26 Schematic diagram summarizing the influences of food processing and calcium lactate concentration on oil release from the emulsion under GI tract condition.

Preferential adsorption of caseins at the oil-water interface in the emulsions prepared by *two-stage heating process* can be further manipulated by calcium lactate concentration. High calcium lactate could induce the binding of calcium ions between anionic phosphoseryl residues on the major individual caseins (Agboola and Dalgleish, 1995). In addition, calcium lactate-mediated aggregates involved SH-SS interchange, leading to the formation of SS-linked aggregates. However, SS-linkages of the adsorbed proteins did not affect protein digestion under the GI condition. The formation of such aggregates at 100 mM of added calcium lactate could lead to the non-adsorbable protein conformation at the interface that precipitated during centrifugation. However, the excess calcium content in the serum phase may induce the re-adsorption of protein or peptide on the oil-water interface during digestion. Thereby, the protein contents at the interface of the reconstituted emulsion increased after digestion.

The addition of soy residue (okara) to the emulsion could promote either prolong or reduce the time of oil to release under *in vitro* stomach condition, depending on the modified composition of okara (i.e. polysaccharide chain, protein) (Figure 27). The different MW of polysaccharide, due to pectinase hydrolyzed, may need further clarification. The modified okara promoted phase separation of the emulsion at pH around 5.12-5.24, leading to the oil droplets flocculation as small particle. Phosphorylated caseins were the main constitutes of the protein matrix at the oil-water interface, while soy polypeptide (soy protein isolate and okara) and phosphorylated casein were the main constitutes of the protein matrix at the oil-water interface of the emulsion containing okara. The fact that soy protein was more easily hydrolyzed by pepsin than caseinate, the reconstituted emulsion containing okara was shortened the time of oil to release under *in vitro* stomach, compared with those containing PHO. The reconstituted emulsion containing PHO delayed time before the oil release to about 135 min during peptic digestion. This emulsion had low in viscosity, the main course of stabilized the emulsion during peptic digestion may be due to a clump of the small droplets aggregates at the interface.

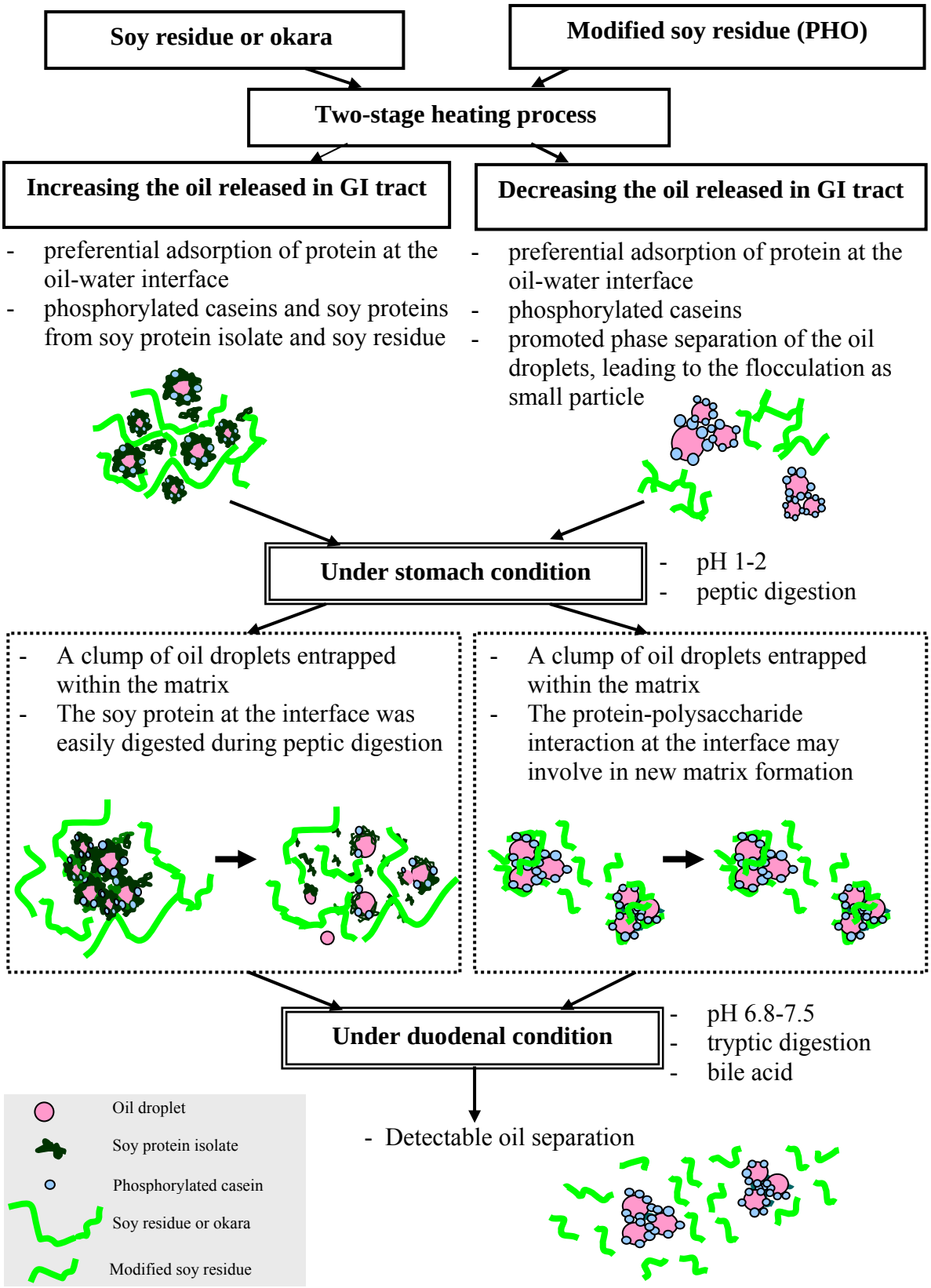

Figure 27 Schematic diagram summarizing the effect of soy residue addition on oil release from the emulsion under GI tract condition.

The data showed that the emulsion containing PHO released the oil under duodenal condition to the lower extent than that in the absence of PHO (Table 10 vs. Table 7, respectively). It should be noted that a clump of the small oil droplets, may arise from polysaccharide interacted with the adsorbed protein on the oil droplets, causing bridging flocculation of the emulsion at pH 2.0.

CONCLUSION AND RECOMMENDATION

Conclusion

This study demonstrated that the protein matrix at the interface and in the bulk aqueous phase of liquid oil-in-water emulsion can be fabricated by heat treatment sequence, calcium lactate concentration and addition of the soy residue from soymilk production. Such matrix influenced the release of oil by prolonging the lag phase or shortening the lag phase during peptic digestion and tryptic digestion. This study also introduced a so called "*two-stage heating process*" in the preparation of high-caloric emulsions containing cassava starch as part of carbohydrate source that can be used in the preparation of the basal formula, in a powdered form, for fortification of minerals, vitamins and dietary fiber to meet individual's requirement.

Recommendation

This study found that the factors affecting the fabrication of protein matrix at the interface to control the amount and time for oil to release. However, these new findings were observed in the lab scale. Thus, these factors should be studied at pilot scale and evaluated for the lipid digestibility. In addition, this study still had some section that needed further clarification, including (1) the mechanisms governing protein and peptide species at the interface of the emulsions prepared by different heating schemes to have different aggregation mechanisms (2) the investigation influence on the MW of polysaccharide in okara after pectinase hydrolysis and (3) the understanding of charge of protein at interface and pectinase-hydrolyzed okara (PHO) at highly acidic stomach pH to slightly alkali pH in duodenum with and without bile acid.

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APPENDICES

APPENDIX A

Chemical analysis

Method of Analysis

A1 Determination of moisture content (AOAC, 2000 Method 925.10)

1. Principle

The principle of this method is the heating and drying of a sample at high temperature, using hot air oven. Generally, the temperature is mostly the boiling point of water, which is 100˚C. The free water is removed from a sample. In oven drying methods, the sample is heated under specified conditions, and the loss of weight is used to calculate the moisture content of the sample. The time may be from a few minutes to over 24 hr. depending on type of sample and temperature of drying.

2. Apparatus

2.1 Analytical balance
2.2 Hot air oven
2.3 Moisture can and lid
2.4 Desiccator

3. Procedure

The moisture cans and lids are dried in a hot air over at 130˚C for 3 hr. and stored dry moisture cans and lids in desiccator before use. The sample weighing about 1.0-1.5 g is placed in an aluminum pan with known weight and dried in hot air oven at 130˚C until constant weight. After that the moisture cans are cooled in desiccator and weighed.

4. Calculation

$$\%Moisture = \frac{wt\ of\ wet\ sample - wt\ of\ dry\ sample}{wt\ of\ wet\ sample} \times 100$$

A2 Determination of protein content (Kjeldahl Method)

1. Principle

This method determines protein content in food products by using sulfuric acid in the presence of catalysts digest proteins and other organic components. The total organic nitrogen is converted to ammonium sulfate. The digesta is neutralized with alkali. The ammonia is distilled into a boric acid solution. The borate anions formed are titrated with standardized acid, which is converted to nitrogen in the sample. This nitrogen comes from both proteins and nonprotein components. The nitrogen content can convert to percent crude protein with multiply a conversion factor (i.e 6.25 for egg or meat proteins, 6.38 for milk proteins and 5.52 for soy proteins).

2. Apparatus

2.1 Analytical balance
2.2 Digested Kjeldahl tube
2.3 Digestion Unit (model K-435, BÜCHI)
2.4 Scrubber (model B-414, BÜCHI)
2.5 Distrillation Unit (model B-324, BÜCHI)
2.6 Flask

3. Reagents

3.1 2% (w/v) Boric acid
3.2 Bromocresol green
3.3 Copper (II) sulfate
3.4 Methyl red
3.5 Potassium sulfate
3.6 40% (w/v) Sodium hydroxide
3.7 Sulfuric acid

3.8 0.1 N Sulfuric acid solution

4. Procedure

4.1 The solid sample weighing about 0.5-1.0 g. or 10 mL of liquid sample is placed in the digested Kjeldahl tube.

4.2 Catalysts (mixed 10 g potassium sulfate and 0.5 g copper (II) sulfate) and a few the glass beads are added.

4.3 Twenty milliliters of reagent grade sulfuric acid are added. The tubes are carried to Digestion Unit where protein nitrogen is liberated to form ammonium ions, sulfuric acid oxidizes organic matter and combines with ammonium formed, carbon and hydrogen are converted to carbon dioxide and water.

4.4 The tubes are taken from Digestion Unit when the sample changed to clear solution and cooled at room temperature (30˚C).

4.5 A tube is connected with Distrillation Unit. Sixty milliliters of distilled water and 60 mL of 40% (w/v) sodium hydroxide are added.

4.6 Distillation is continued at moderate rate until all ammonia has passed into the absorbing acid or 60 mL of 2% (w/v) boric acid containing the indicators bromocresol green and methyl red in flask.

4.7 The flask is remove and titrated with 0.1 N sulfuric acid solution. A reagent blank should be run.

4. Calculation

$$\%Nitrogen = \frac{(V_1 - V_2) \times N \times f \times 0.014 \times 100}{Wt\ of\ dry\ sample\ (mg)}$$

$$\%\,\mathrm{Pr}\,otein = \%\,Nitrogen \times conversion\ factor$$

In which,

V_1= sample titer (mL of 0.1 N sulfuric acid solution)
V_2= blank titer (mL of 0.1 N sulfuric acid solution)
N = Normality of sulfuric acid solution
f = factor of sulfuric acid solution:

$$f = \frac{E}{121.14 \times N \times V}$$

In which,

N = Normality of the acid
V = consumption of acid in mL
E = the Tris buffer weighing 120 mg

A3 Determination of fat content (AOAC, 2000 Method 920.39)

1. Principle

This method determines the fat content in food products by organic solvent extraction. The commonly organic solvent is hexane or petroleum ether, which is nonpolar solvents. Because of moisture reduce efficiency of solvent penetration, the sample should be dried and reduced particle size before analyze.

2. Apparatus

2.1 Soxhlex
2.2 Extraction cups
2.3 Thimbles
2.4 Analytical balance
2.5 Hot air oven
2.6 Desiccator

3. Reagent

Petroleum ether (35˚C-60˚C)

4. Procedure

4.1 The predried sample weighing about 0.5 g. (W_1) in No.1 Whatman is placed in a thimber.

4.2 Weigh a dried extraction cup (W_2) and add 50-75 mL of petroleum ether in the cup.

4.3 The thimbers and the extraction cups are carried to Extraction Unit.

4.4 The extraction in Extraction Unit is divided into two steps, including boiling with solvent for 20 min and rinsing with solvent for 45-60 min.

4.5 The extraction cups with extracted fat are dried in a hot air oven at 100˚C for 30 min and cooled in desiccator.

4.6 Weigh the dried extraction cups with extracted fat (W_3).

4. Calculation

$$\%\,Fat = \frac{(W_3 - W_2)}{W_1} \times 100$$

In which,

W_1= weight of the sample (g)

W_2= weight of a dried extraction cup (g)

W_3= weight of a dried extraction cup with extracted fat (g)

A4 Determination of crude fiber content (AOAC, 2000 Method 962.09)

1. Principle

This method determines the crude fiber content by sequential extraction of the sample with mild acidic and basic solution. After that insoluble residue is collected by filtration and the residue is dried. Thus, crude fiber refers to the amounts of cellulose and lignin in the sample. Hemicelluloses, pectin and hydrocolloid gums dissolve in hot water or alkali solution.

2. Apparatus

2.1 Fibertec
2.2 Crucible
2.3 Hot air oven
2.4 Muffle furnace
2.5 Desiccator
2.6 Analytical balance

3. Reagents

3.1 95 % Ethanol
3.2 Octan-1-ol (Anti-foaming)
3.3 0.313 N Sodium hydroxide solution
3.4 0.255 N Sulfuric acid solution

4. Procedure

4.1 The sample weighing about 0.5-3 g. (W_1) is placed in a crucible and carried the crucible to Fibertec Hot Extraction Unit.

4.2 Add 100-200 mL of 0.255 N sulfuric acid solution to column and a little Anti-foaming is also added.

4.3 Extract for 30 min and drain the sulfuric acid solution.

4.4 Rinse with hot distilled water and add 100-200 mL of 0.313 N sodium hydroxide solution to column.

4.5 Extract for 30 min and drain the sodium hydroxide solution.

4.6 95% Ethyl alcohol is added. The crucible with extracted residues is dried in a hot air oven at 105-110˚C for 2 hr. and cooled in desiccator.

4.6 Weigh the crucible with dried residue (W_2)

4.7 The crucible with dried residue is burned in a Muffle furnace at 550±2˚C and cooled in desiccator.

4.8 Weigh the crucible with ash (W_3).

5. Calculation

$$\%\,Crude\ fiber = \frac{W_2 - W_3}{W_1} \times 100$$

In which,

W_1= weight of the sample (g)

W_2= weight of a crucible with dried residue (g)

W_3= weight of a crucible with ash (g)

A5 Determination of ash content (AOAC, 2000 Method 923.03)

1. Principle

The principle of this method is the heating of a sample at high temperature, using a Muffle furnace. Generally, the temperature is 500-600˚C. At this condition, water and volatiles are vaporized, and organic substances are burned. Thus, ash content refers to the inorganic residue.

2. Apparatus

2.1 Porcelain crucible

2.2 Muffle furnace

2.3 Desiccator

2.4 Analytical balance

2.5 Bunsen

3. Procedure

3.1 The porcelain crucibles are burned in a Muffle furnace at 550±2 ˚C for 3 hr and stored dry crucibles in desiccator before use.

3.2 The sample weighing about 1 g (W_1) is placed in a porcelain crucible that known weight (W).

3.3 The crucible is preheated by Bunsen and placed in a cool Muffle furnace. Use tongs and gloves.

3.4 Burn the crucibles for 16 hr and cool in desiccator.

3.5 Weigh the crucible with ash (W_2)

4. Calculation

$$\%\,ash = \frac{W_2 - W}{W_1 - W} \times 100$$

In which,

W= weight of a porcelain crucible (g)

W_1= weight of the sample (g)

W_2= weight of a crucible with ash (g)

A6 Determination of reducing sugar content (Milner and Avigad, 1967)

1. Principle

The principle of this method is the reduction of Cu^{2+} ions in an arsenomolybdate complexes, which are prepared by reacting ammonium molybdate and sodium arsenate in sulfuric acid to Cu^{+} ions by reducing sugars. The reactions result in a strong blue color.

2. Apparatus

2.1 Spectrophotometer
2.2 Water bath
2.3 Vortex

3. Reagents

3.1 Copper solution (*A*) is prepared by dissolving 280 g of anhydrous sodium sulphate and 40 g of sodium chloride in 720 mL of distilled water. Complete dissolution will occur when 100 mL of 2 M acetate buffer (pH 5.1) and 65 mL of 0.32 M cupric sulphate is added to a salt solution at about 60˚C. The final pH of solution is 4.8 (using 10 N hydrochloric acid or sodium hydroxide). The volume is then adjusted with water to one liter. The reagent is stored at 37˚C in a brown bottle, is stable for at least two months.

3.2 Arsenomolybdate reagent (*B*) is prepared by dissolving 25 g of ammonium molybdate in 450 mL of distilled water. Twenty-one milliliters of reagent grade sulfuric acid and 3 g of di-sodium hydrogen arsenate heptahydrate in 25 mL of distilled water are added to ammonium molybdate solution. The reagent is stored at 37˚C for 24 to 48 hr before use.

3.3 Standard sugar solution is a 0.1 M stock solution of galacturonic acid in prepared in 0.05% benzoic acid and kept at 4˚C.

4. Procedure

4.1 A 0.5 mL of sample or standard is placed in test-tubes and 1.5 mL of reagent A is added.

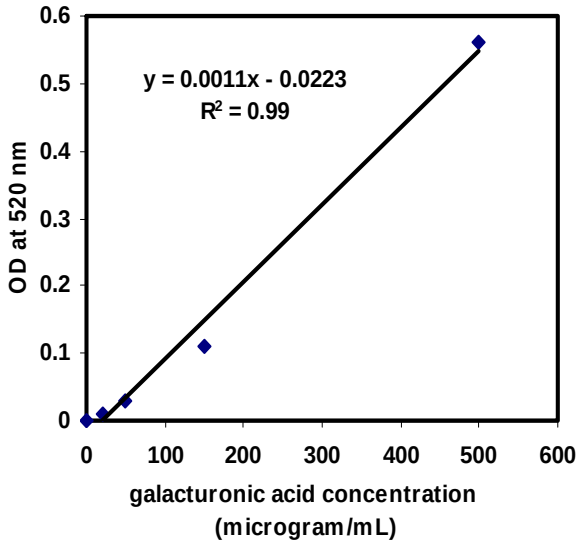
4.2 Mix and cover with glass marbles. The solution is immersed in a boiling water bath for 10 min and cooled in an ice bath without shaking.

4.3 Add 1.0 mL of reagent *B*. Shake gently and add 2.0 mL of water.

4.4 Read the absorbance at 520 nm and a reagent blank should be run.

5. Calculation

The reducing sugar content of unknown solutions is determined by comparing the color of standard solution.



Appendix Figure A1 Standard absorption curves of galacturonic acid

A7 Determination of protein content (Lowry's method, Lowry *et al*. 1951)

1. Principle

This method is very sensitive due to combination between the Biuret reaction and using Folin-Ciocalteau phenol reagent. The peptide bonds of proteins react with copper under alkaline condition to produced Cu^{+} and give a deep blue color. In addition, Folin-Ciocalteau chemistry, in which a complex mixture of inorganic salts (phosphomolybdic-phosphotungstic acid) react with tyrosine and tryptophan residues to give a blue green color.

2. Apparatus

2.1 Analytical balance
2.2 Spectrophotometer
2.3 Water bath
2.4 Cuvette
2.5 Vortex

3. Reagents

3.1 Solution *A* is prepared by dissolving 2% (w/v) of sodium carbonate in distilled water.

3.2 Solution *B* is prepared by dissolving 1% (w/v) of copper sulfate in distilled water

3.3 Solution *C* is prepared by dissolving 2% (w/v) of sodium potassium tartrate in distilled water

3.4 Alkaline copper reagent (prepare fresh, within 1 hr of use) is prepared by mixing 1 mL of solution *B* and 1 mL of solution *C* with 98 mL of solution *A*.

3.5 Folin-Ciocalteau's phenol reagent

3.6 Standards bovine serum albumin solution is a stock bovine serum albumin solution containing 2 mg/mL protein in distilled water.

3.7 2 N Sodium hydroxide

4. Procedure

4.1 A 0.2 mL of sample or standard is placed in test-tubes and 0.2 N of sodium hydroxide.

4.2 Hydrolyze at 100˚C for 10 min in a boiling water bath and cool to room temperature.

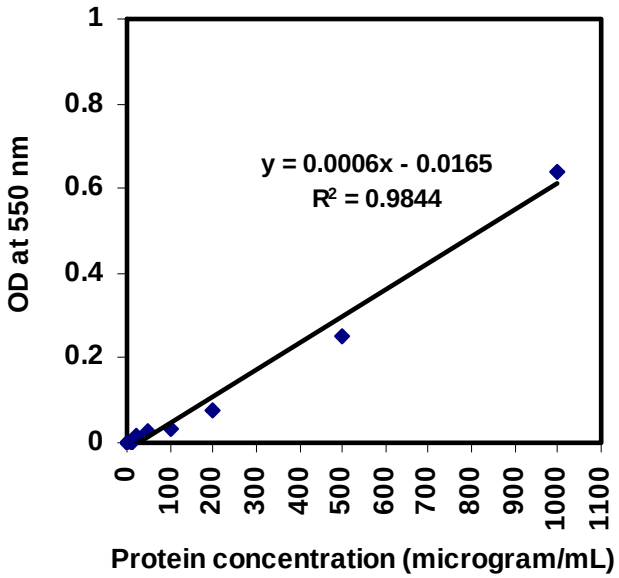
4.3 Add 2 mL of freshly alkaline copper reagent and is allowed to proceed for 10 min at room temperature.

4.4 Add 0.2 mL of Folin reagent and let the mixture stand at room temperature for 30-60 min (do not exceed 60 min)

4.5 Read the absorbance at 550 nm and a blank reagent should be run.

5. Calculation

A standard curve for Lowry's assay using know amounts of bovine serum albumin (BSA) and determine the protein content of unknown solutions by comparing the color of standard solution.



Appendix Figure A2 Standard absorption curves of bovine serum albumin.

APPENDIX B
Statistical analysis

Appendix Table B1 Statistical analysis of the apparent viscosity of unheated protein composite suspensions (10% w/v protein) at SCN fraction was 0.3, 0.5, 0.7 and 1.0

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Fraction of SCN	3	99388.97	33129.657	22.605	0.000
Error	8	11724.77	1465.596		
Total	11	111113.7			
Water pH 4.5					
Fraction of SCN	3	144677.8	48225.930	21.945	0.000
Error	8	17580.32	2197.541		
Total	11	162258.1			
Water pH 5.4					
Fraction of SCN	3	146760.7	48920.225	61.903	0.000
Error	8	6322.221	790.278		
Total	11	153082.9			

Appendix Table B2 Statistical analysis of the apparent viscosity of heated protein composite suspensions (10% w/v protein) at SCN fraction was 0.3, 0.5, 0.7 and 1.0

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Fraction of SCN	3	223499.6	74499.864	54.698	0.000
Error	8	10896.17	1362.021		
Total	11	234395.8			
Water pH 4.5					
Fraction of SCN	3	158973.6	52991.215	312.518	0.000
Error	8	1356.497	169.562		
Total	11	160330.1			
Water pH 5.4					
Fraction of SCN	3	471782.6	157260.875	86.192	0.000
Error	8	14596.39	1824.548		
Total	11	486379.0			

Appendix Table B3 Statistical analysis of the apparent viscosity of heated protein composite suspensions (10% w/v protein) in water pH 3.0, 4.5 and 5.4

Source	df	SS	MS	F	Sig.
SCN fraction=0.3					
Water pH	2	8447.167	4223.583	2.252	0.186
Error	6	11253.33	1875.556		
Total	8	19700.50			
SCN fraction=0.5					
Water pH	2	3956.334	1978.167	4.205	0.072
Error	6	2822.478	470.413		
Total	8	6778.812			
SCN fraction=0.7					
Water pH	2	61.562	30.781	0.272	0.771
Error	6	678.335	113.056		
Total	8	739.897			
SCN fraction=1.0					
Water pH	2	236538.3	118269.134	58.671	0.000
Error	6	12094.91	2015.818		
Total	8	248633.2			

Appendix Table B4 Statistical analysis of the apparent viscosity of protein composite suspensions (10% w/v protein) after heat treatment at 80˚C for 30 min

Source		df	SD	MD	t-test	Sig. (2-tailed)	95%	
							Lower	Upper
Water pH 3.0								
SCN fraction=0.3	Equal variances assumed	4	45.299	-99.39	-2.194	0.093	-225.162	26.382
SCN fraction=0.5	Equal variances assumed	4	19.850	21.656	1.091	0.337	-33.456	76.769
SCN fraction=0.7	Equal variances assumed	4	10.570	7.220	0.683	0.532	-22.128	36.568
SCN fraction=1.0	Equal variances assumed	4	34.818	-29.786	-0.855	0.440	-126.458	66.885

Appendix Table B4 (Continued)

Source		df	SD	MD	t-test	Sig. (2-tailed)	95%	
							Lower	Upper
Water pH 4.5								
SCN fraction=0.3	Equal variances assumed	4	23.109	27.123	1.174	0.306	-37.038	91.285
SCN fraction=0.5	Equal variances assumed	4	28.632	70.300	2.455	0.070	-9.1978	149.798
SCN fraction=0.7	Equal variances assumed	4	7.576	27.517	3.632	0.022	6.4907	48.553
SCN fraction=1.0	Equal variances assumed	4	41.771	32.957	0.789	0.474	-83.019	148.932
Water pH 5.4								
SCN fraction=0.3	Equal variances assumed	4	35.986	-57.333	-1.593	0.186	-157.248	42.581
SCN fraction=0.5	Equal variances assumed	4	14.711	19.583	1.331	0.254	-21.261	60.428
SCN fraction=0.7	Equal variances assumed	4	6.736	25.440	3.776	0.019	6.736	44.144
SCN fraction=1.0	Equal variances assumed	4	43.927	-362.330	-8.248	0.001	-484.295	-240.371

Appendix Table B5 Statistical analysis of the accessible sulfhydryl content of heated Protein composite at the ratio of SCN:SPI of 1:0, 0.7:0.3, 0.5:0.5, 0.3:0.7, and 0:1 in water pH 3.0 and 5.4

Source		df	SD	MD	t-test	Sig. (2-tailed)	95%	
							Lower	Upper
SCN	Equal variances assumed	2	9.193	-1.840	-0.200	0.860	-41.394	37.714
SPI	Equal variances assumed	2	2.5986	-3.675	-1.414	0.293	-14.856	7.506
HSCN	Equal variances assumed	4	9.1711	-18.383	-2.004	0.116	-43.846	7.079
HSCN7	Equal variances assumed	4	10.6814	1.2267	0.115	0.914	-28.429	30.883
HSCN5	Equal variances assumed	4	8.5781	-2.450	-0.286	0.789	-26.266	21.366
HSCN3	Equal variances assumed	4	3.4672	4.9033	1.414	0.230	-4.723	14.529

Appendix Table B5 (Continued)

Source		df	SD	MD	t-test	Sig. (2-tailed)	95%	
							Lower	Upper
HSPI	Equal variances assumed	4	6.2483	-19.610	-3.138	0.035	-36.958	-2.262

Appendix Table B6 Statistical analysis of the accessible sulfhydryl content of heated soy protein isolate (SPI) and sodium caseinate (SCN) after heat treatment at 80˚C for 30 min

Source		df	SD	MD	t-test	Sig. (2-tailed)	95%	
							Lower	Upper
Water pH 3.0								
SPI	Equal variances assumed	3	7.029	6.741	0.959	0.408	-15.630	29.113
SCN	Equal variances assumed	3	4.405	-3.063	-0.695	0.537	-17.081	10.954
Water pH 5.4								
SPI	Equal variances assumed	3	11.735	-9.801	-0.835	0.465	-47.148	27.545
SCN	Equal variances assumed	3	7.030	-18.998	-2.703	0.074	-41.370	3.373

Appendix Table B7 Statistical analysis of the accessible sulfhydryl content of heated protein composite at the ratio of SCN:SPI of 1:0, 0.7:0.3, 0.5:0.5, 0.3:0.7, and 0:1

Source	df	SS	MS	F	Sig.
Water pH 3.0					
The ratios of proteins	4	450.604	112.651	2.194	0.143
Error	10	513.508	51.351		
Total	14	964.112			
Water pH 5.4					
The ratios of proteins	4	1799.080	449.770	3.159	0.064
Error	10	1423.573	142.357		
Total	14	3222.652			

Appendix Table B8 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.3:0.7 after the increase in calcium lactate concentration

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Calcium lactate Conc.	6	0.308	5.127E-02	1.449	0.265
Error	14	0.495	3.538E-02		
Total	20	0.803			
Water pH 4.5					
Calcium lactate Conc.	6	0.973	0.162	16.369	0.000
Error	14	0.139	9.909E-03		
Total	20	1.112			
Water pH 5.4					
Calcium lactate Conc.	6	1.691	0.282	7.634	0.001
Error	14	0.517	3.691E-02		
Total	20	2.207			

Appendix Table B9 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.5:0.5 after the increase in calcium lactate concentration

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Calcium lactate Conc.	6	2.202	0.367	8.509	0.001
Error	14	0.604	4.314E-02		
Total	20	2.806			
Water pH 4.5					
Calcium lactate Conc.	6	1.949	0.325	6.017	0.003
Error	14	0.756	5.398E-02		
Total	20	2.704			
Water pH 5.4					
Calcium lactate Conc.	6	2.749	0.458	53.190	0.000
Error	14	0.121	8.612E-03		
Total	20	2.869			

Appendix Table B10 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.7:0.3 after the increase in calcium lactate concentration

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Calcium lactate Conc.	6	9.984	1.664	230.594	0.000
Error	14	0.101	7.216E-03		
Total	20	10.085			
Water pH 4.5					
Calcium lactate Conc.	6	1.482	0.247	12.769	0.000
Error	14	0.271	1.935E-02		
Total	20	1.753			
Water pH 5.4					
Calcium lactate Conc.	6	0.797	0.133	12.547	0.000
Error	14	0.148	1.059E-02		
Total	20	0.945			

Appendix Table B11 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.3:0.7 after the increase in calcium lactate concentration in the presence of NEM

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Calcium lactate Conc.	6	6.827E-02	1.138E-02	1.514	0.244
Error	14	0.105	7.514E-03		
Total	20	0.173			
Water pH 4.5					
Calcium lactate Conc.	6	0.670	0.112	27.218	0.000
Error	14	5.744E-02	4.103E-03		
Total	20	0.727			
Water pH 5.4					
Calcium lactate Conc.	6	0.662	0.110	7.030	0.001
Error	14	0.220	1.568E-02		
Total	20	0.881			

Appendix Table B12 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.5:0.5 after the increase in calcium lactate concentration in the presence of NEM

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Calcium lactate Conc.	6	0.538	8.963E-02	20.999	0.000
Error	14	5.976E-02	4.268E-03		
Total	20	0.598			
Water pH 4.5					
Calcium lactate Conc.	6	1.731	0.288	20.349	0.000
Error	14	0.198	1.418E-02		
Total	20	1.929			
Water pH 5.4					
Calcium lactate Conc.	6	0.627	0.105	7.023	0.001
Error	14	0.208	1.488E-02		
Total	20	0.836			

Appendix Table B13 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.7:0.3 after the increase in calcium lactate concentration in the presence of NEM

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Calcium lactate Conc.	6	0.998	0.166	9.003	0.000
Error	14	0.259	1.848E-02		
Total	20	1.257			
Water pH 4.5					
Calcium lactate Conc.	6	0.229	3.810E-02	23.109	0.000
Error	14	2.308E-02	1.649E-03		
Total	20	0.252			
Water pH 5.4					
Calcium lactate Conc.	6	0.174	2.901E-02	4.435	0.010
Error	14	9.157E-02	6.541E-03		
Total	20	0.266			

Appendix Table B14 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.3:0.7 after the increase in sodium lactate concentration

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Sodium lactate Conc.	8	0.359	4.484E-02	1.395	0.264
Error	18	0.579	3.215E-02		
Total	26	0.937			
Water pH 4.5					
Sodium lactate Conc.	8	1.844E-02	2.305E-03	0.573	0.787
Error	18	7.244E-02	4.025E-03		
Total	26	9.088E-02			
Water pH 5.4					
Sodium lactate Conc.	8	3.430E-02	4.287E-03	0.509	0.834
Error	18	0.151	8.415E-03		
Total	26	0.186			

Appendix Table B15 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.5:0.5 after the increase in sodium lactate concentration

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Sodium lactate Conc.	8	3.718	0.465	10.389	0.000
Error	18	0.805	4.473E-02		
Total	26	4.523			
Water pH 4.5					
Sodium lactate Conc.	8	8.946E-03	1.118E-03	0.394	0.909
Error	18	5.104E-02	2.835E-03		
Total	26	5.998E-02			
Water pH 5.4					
Sodium lactate Conc.	8	3.899E-03	4.874E-04	0.140	0.996
Error	18	6.284E-02	3.491E-03		
Total	26	6.674E-02			

Appendix Table B16 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.7:0.3 after the increase in sodium lactate concentration

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Sodium lactate Conc.	8	8.34	1.042	19.394	0.000
Error	18	0.968	5.375E-02		
Total	26	9.308			
Water pH 4.5					
Sodium lactate Conc.	8	9.800E-04	1.225E-04	0.854	0.570
Error	18	2.581E-03	1.434E-04		
Total	26	3.561E-03			
Water pH 5.4					
Sodium lactate Conc.	8	2.622E-03	3.278E-04	2.204	0.078
Error	18	2.677E-03	1.487E-04		
Total	26	5.299E-03			

Appendix Table B17 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.3:0.7 after the increase in sodium lactate concentration in the presence of NEM

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Sodium lactate Conc.	8	0.411	5.132E-02	4.433	0.004
Error	18	0.208	1.158E-02		
Total	26	0.619			
Water pH 4.5					
Sodium lactate Conc.	8	2.043E-03	2.554E-04	0.057	1.000
Error	18	8.011E-02	4.450E-03		
Total	26	8.215E-02			
Water pH 5.4					
Sodium lactate Conc.	8	2.279E-03	2.849E-04	0.064	1.000
Error	18	8.072E-02	4.485E-03		
Total	26	8.300E-02			

Appendix Table B18 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.5:0.5 after the increase in sodium lactate concentration in the presence of NEM

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Sodium lactate Conc.	8	1.807	0.226	7.631	0.000
Error	18	0.533	2.961E-02		
Total	26	2.340			
Water pH 4.5					
Sodium lactate Conc.	8	1.615E-02	2.019E-03	0.906	0.533
Error	18	4.012E-02	2.229E-03		
Total	26	5.628E-02			
Water pH 5.4					
Sodium lactate Conc.	8	1.892E-03	2.365E-04	0.189	0.989
Error	18	2.250E-02	1.250E-03		
Total	26	2.439E-02			

Appendix Table B19 Statistical analysis of turbidity of heated protein composites at the ratio of SCN:SPI of 0.7:0.3 after the increase in sodium lactate concentration in the presence of NEM

Source	df	SS	MS	F	Sig.
Water pH 3.0					
Sodium lactate Conc.	8	3.359	0.420	6.11	0.001
Error	18	1.237	6.872E-02		
Total	26	4.596			
Water pH 4.5					
Sodium lactate Conc.	8	5.112E-03	6.390E-04	1.025	0.453
Error	18	1.122E-02	6.234E-04		
Total	26	1.633E-02			
Water pH 5.4					
Sodium lactate Conc.	8	1.773E-03	2.216E-04	0.61	0.758
Error	18	6.541E-03	3.634E-04		
Total	26	8.314E-03			

Appendix Table B20 Statistical analysis of TCA-soluble protein of SPI suspension containing 0 mM calcium lactate after peptic digestion about 0, 15, 30, 45, and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
No NEM					
Digestion time	4	7.416	1.854	30.723	0.001
Error	5	.302	6.035E-02		
Total	9	7.718			
With NEM					
Digestion time	4	6.806	1.701	19.312	.003
Error	5	.441	8.810E-02		
Total	9	7.246			

Appendix Table B21 Statistical analysis of TCA-soluble protein of SPI suspension containing 25 mM calcium lactate after peptic digestion about 0, 15, 30, 45, and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
No NEM					
Digestion time	4	6.126	1.531	33.843	.001
Error	5	.226	4.525E-02		
Total	9	6.356			
With NEM					
Digestion time	4	6.853	1.713	45.195	.000
Error	5	.190	3.791E-02		
Total	9	7.043			

Appendix Table B22 Statistical analysis of TCA-soluble protein of SCN suspension containing 0 mM calcium lactate after peptic digestion about 0, 15, 30, 45, and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
No NEM					
Digestion time	4	2.823	.706	76.292	.000
Error	5	4.625E-02	9.250E-03		
Total	9	2.869			

Appendix Table B22 (Continued)

Source	df	SS	MS	F	Sig.
With NEM					
Digestion time	4	2.883	.721	10.978	.011
Error	5	.328	6.566E-02		
Total	9	3.212			

Appendix Table B23 Statistical analysis of TCA-soluble protein of SCN suspension containing 25 mM calcium lactate after peptic digestion about 0, 15, 30, 45, and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
No NEM					
Digestion time	4	2.084	.521	5.715	.042
Error	5	.456	9.114E-02		
Total	9	2.539			
With NEM					
Digestion time	4	2.167	.542	7.533	.024
Error	5	.360	7.192E-02		
Total	9	2.527			

Appendix Table B24 Statistical analysis of TCA-soluble protein of SCN-SPI suspension containing 0 mM calcium lactate after peptic digestion about 0, 15, 30, 45, and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
No NEM					
Digestion time	4	3.032	.758	8.315	.020
Error	5	.456	9.115E-02		
Total	9	3.487			
With NEM					
Digestion time	4	2.813	.703	16.031	.005
Error	5	.219	4.387E-02		
Total	9	3.032			

Appendix Table B25 Statistical analysis of TCA-soluble protein of SCN-SPI suspension containing 25 mM calcium lactate after peptic digestion about 0, 15, 30, 45, and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
No NEM					
Digestion time	4	3.463	.866	63.846	.000
Error	5	6.780E-02	1.356E-02		
Total	9	3.531			
With NEM					
Digestion time	4	3.122	.781	97.201	.000
Error	5	4.015E-02	8.030E-03		
Total	9	3.162			

Appendix Table B26 Statistical analysis of TCA-soluble protein of SPI, SCN-SPI , and SCN suspension containing 0 and 25 mM calcium lactate with and without NEM after pectic digestion about 0, 15, 30, 45 and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
Time 0 min					
Treatment	11	.101	9.150E-03	37.219	.000
Error	12	2.950E-03	2.458E-04		
Total	13	.104			
Time 15 min					
Treatment	11	1.247	.113	2.246	.090
Error	12	.606	5.049E-02		
Total	13	1.853			
Time 30 min					
Treatment	11	2.335	.212	2.707	.051
Error	12	.941	7.842E-02		
Total	13	3.276			
Time 45 min					
Treatment	11	4.260	.387	5.242	.004
Error	12	.886	7.387E-02		
Total	13	5.146			

Appendix Table B26 (Continued)

Source	df	SS	MS	F	Sig.
Time 60 min					
Treatment	11	4.592	.417	6.670	.001
Error	12	.751	6.259E-02		
Total	13	5.343			

Appendix Table B27 Statistical analysis of protein content in cream phase of the emulsion containing 25 mM calcium lactate prepared by *one-stage heating process* and *two-stage heating process* during peptic digestion at pH 2.0

Source		df	SD	MD	t-test	Sig. (2-tailed)	95% Lower	95% Upper
0 min digestion	Equal variances assumed	2	0.9656	4.005	4.147	0.054	-0.1498	8.1598
15 min digestion	Equal variances assumed	2	0.562	-2.128	-3.785	0.063	-4.547	0.2908
60 min digestion	Equal variances assumed	2	0.864	-5.403	-6.254	0.025	-9.1208	-1.6861

Appendix Table B28 Statistical analysis of TCA-soluble protein content in the serum phase of the emulsion containing 25 mM calcium lactate prepared by *one-stage heating process* and *two-stage heating process* during peptic digestion for 0, 15, 30, 45, and 60 min at pH 2.0

Source	df	SS	MS	F	Sig.
One-stage heating process					
Digestion time	4	1.970	0.493	17.944	0.004
Error	5	0.137	2.745E-02		
Total	9	2.107			
Two-stage heating process					
Digestion time	4	2.339	0.585	220.626	0.000
Error	5	1.325E-02	2.650E-03		
Total	9	2.352			

Appendix Table B29 Statistical analysis of oil release from the emulsion containing 25 mM calcium lactate prepared by *one-stage heating process* and *two-stage heating process* after tryptic digestion in the absence and presence of bile acid

Source	df	SS	MS	F	Sig.
Treatment	7	.752	.107	3.274	.059
Error	8	.262	3.281E-02		
Total	15	1.014			

Appendix Table B30 Statistical analysis of protein content in cream phase of the emulsion prepared by *two-stage heating process* containing 0, 25, and 100 mM calcium lactate during peptic digestion at pH 2.0

Source	df	SS	MS	F	Sig.
Calcium lactate Conc.	8	105.947	13.243	12.204	.001
Error	9	9.766	1.085		
Total	17	115.714			

Appendix Table B31 Statistical analysis of soluble calcium in the serum phase of the emulsion prepared by *two-stage heating process* containing 0, 25, and 100 mM calcium lactate after peptic and tryptic digestion

Source	df	SS	MS	F	Sig.
Treatment	8	2951439	368929.866	115.975	.000
Error	9	28630.10	3181.122		
Total	17	2980067			

Appendix Table B32 Statistical analysis of TCA-soluble calcium in the serum phase of the emulsion prepared by *two-stage heating process* containing 0, 25, and 100 mM calcium lactate after peptic and tryptic digestion

Source	df	SS	MS	F	Sig.
Treatment	8	2045445	255680.672	59.836	.000
Error	9	39787.34	4420.815		
Total	17	2085233			

Appendix Table B33 Statistical analysis of soluble calcium in the serum phase of the emulsion prepared by *two-stage heating process* containing 0, 25, and 100 mM calcium lactate after peptic and tryptic digestion in the absence and presence of TCA

Source		df	SD	MD	t-test	Sig. (2-tailed)	95%	
							Lower	Upper
0 mM Ca-lactate								
0 min peptic digestion	Equal variances assumed	2	5.5708	-3.501	-0.628	0.594	-27.470	20.468
15 min peptic digestion	Equal variances assumed	2	1.8483	0.361	0.195	0.863	-7.591	8.314
Peptic+tryptic digestion	Equal variances assumed	2	3.3389	1.077	0.323	0.778	-13.288	15.444
25 mM Ca-lactate								
0 min peptic digestion	Equal variances assumed	2	22.000	72.6105	3.300	0.081	-22.050	167.271
15 min peptic digestion	Equal variances assumed	2	12.994	46.238	3.558	0.071	-9.670	102.148
Peptic+tryptic digestion	Equal variances assumed	2	37.861	33.291	0.879	0.472	-129.611	196.194
100 mM Ca-lactate								
0 min peptic digestion	Equal variances assumed	2	116.577	261.766	2.245	0.154	-239.825	763.358
15 min peptic digestion	Equal variances assumed	2	41.099	200.442	4.877	0.04	23.605	377.279
Peptic+tryptic digestion	Equal variances assumed	2	129.604	-1.144	-0.009	0.994	-558.787	556.499

Appendix Table B34 Statistical analysis of oil release from the emulsion prepared by *two-stage heating process* containing 0, 25, and 100 mM calcium lactate after tryptic digestion in the absence and presence of bile acid

Source	df	SS	MS	F	Sig.
Calcium lactate Conc.	11	5.953	.541	19.314	.000
Error	12	.336	2.802E-02		
Total	23	6.289			

Appendix Table B35 Statistical analysis of colour value (L*, a*, b*) of okara and pectinase-hydrolyzed okara (PHO)

Source		df	SD	MD	t-test	Sig. (2-tailed)	95% Lower	95% Upper
L*	Equal variances assumed	2	0.1275	4.160	32.634	0.001	3.611	4.708
a*	Equal variances assumed	2	2.062E-02	-0.865	-41.959	0.001	-0.954	-0.776
b*	Equal variances assumed	2	0.163	-4.860	-45.719	0.000	-5.317	-4.403

Appendix B Table 36 Statistical analysis of the apparent viscosity of the *two-stage heated emulsion* containing 0 mM calcium lactate with no okara, okara or pectinase-hydrolyzed okara (PHO) after peptic and tryptic digestion

Source	df	SS	MS	F	Sig.
Treatment	11	1685888	153362.518	51.387	0.000
Error	12	35789.99	2982.499		
Total	23	1721678			

Appendix Table B37 Statistical analysis of protein content in cream phase of the *two-stage heated emulsion* containing 0 mM calcium lactate with no okara, okara or pectinase-hydrolyzed okara (PHO) addition during peptic digestion at pH 2.0

Source	df	SS	MS	F	Sig.
Okara addition	8	1.860	0.233	5.334	0.011
Error	9	0.392	4.360E-02		
Total	17	2.253			

Appendix Table B38 Statistical analysis of oil release from the *two-stage heated Emulsion* containing 0 mM calcium lactate with pectinase-hydrolyzed okara (PHO) addition after tryptic digestion in the absence and presence of bile acid

Source	df	SS	MS	F	Sig.
Okara addition	3	0.461	0.154	3.592	0.124
Error	4	0.171	4.279E-02		
Total	7	0.632			

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