# THESIS APPROVAL

## GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Food Science)

**DEGREE**

Food Science | Food Science and Technology

**FIELD** | **DEPARTMENT**

**TITLE:** Peptic-Modified Whey Protein Concentrate and its Antibrowning Effect on Heated Glycine-Sugar and Whey Protein-Sugar Mixtures

**NAME:** Miss Narumol Rongsirikul

**THIS THESIS HAS BEEN ACCEPTED BY**

_____ **THESIS ADVISOR**

( Associate Professor Parichat Hongsprabhas, Ph.D. )

_____ **THESIS CO-ADVISOR**

( Assistant Professor Tanaboon Sajjaanantakul, Ph.D. )

_____ **THESIS CO-ADVISOR**

( Assistant Professor Wannee Jirapakkul, Ph.D. )

_____ **DEPARTMENT HEAD**

( Assistant Professor Wannee Jirapakkul, Ph.D. )

**APPROVED BY THE GRADUATE SCHOOL ON** _____

_____ **DEAN**

( Associate Professor Gunjana Theeragool, D.Agr. )

THESIS

# PEPTIC-MODIFIED WHEY PROTEIN CONCENTRATE AND ITS ANTIBROWNING EFFECT ON HEATED GLYCINE-SUGAR AND WHEY PROTEIN-SUGAR MIXTURES

NARUMOL RONGSIRIKUL

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Food Science)
Graduate School, Kasetsart University
2012

Narumol Rongsirikul 2012: Peptic-Modified Whey Protein Concentrate and its Antibrowning Effect on Heated Glycine-Sugar and Whey Protein-Sugar Mixtures. Master of Science (Food Science), Major Field: Food Science, Department of Food Science and Technology. Thesis Advisor:
Associate Professor Parichat Hongsprabhas, Ph.D. 93 pages.

Maillard reactions are non-enzymatic browning which generate advanced glycation end products (AGEs) and Maillard reaction products (MRPs). This research investigated the production and characteristics of the modified whey protein concentrate (MWPC) with antioxidant capacity if they had antibrowning effect on sterilized glycine-reducing sugar and whey protein-reducing sugar suspensions. The -modification processes were carried out by heating a 2% WPC suspension at 80 °C for 30 min at its natural pH of 6.6 or acidic pH of 2.0, followed by peptic treatment at 37 °C for 60 min. Acidic heating of WPC and peptic treatment reduced protein aggregation and slightly increased antioxidant capacity measured as oxygen radical absorbance capacity (ORAC) assay ($p \leq 0.05$). Spray-drying of MWPC further hydrolysed proteins, especially α-lactalbumin. The MWPC had increased antioxidant capacity measured as Trolox equivalent antioxidant capacity (TEAC), amino groups but lowered sulfhydryl groups and the exposed aromatic amino acids. The reconstituted MWPC was able to reduce the formation of AGEs in fructose-containing mixtures heated at 80 to 95 °C for 45-60 min. The addition of MWPC to reducing sugar-containing mixture; namely fructose, glucose and lactose, was also able to lower brown pigment formation of the mixed suspensions containing 0.1 M reducing sugar and 0.5-1.0% protein sterilized at 121 °C for 15 min ($p \leq 0.05$). Overall, the study showed that the MWPC had potential use as antibrowning agent, particularly against AGEs formation during sterilization process.

_____ _____ __ / __ / __

Student's signature Thesis Advisor's signature

# ACKNOWLEDGEMENTS

This thesis could not be successfully completed without help and all suggestion of many kind persons. I would like to express my gratitude to all who gave me the possibility to complete this thesis. First and foremost, I would like to gratefully thank my major advisor, Associate Professor Dr. Parichat Hongsprabhas, who gave valuable advice and guidance for research of this thesis along with appreciative suggestion, checked and corrected the fault of this thesis until the completion. I would like to give thank to Assistant Professor Dr. Tanaboon Sajjaanantakul and Assistant Professor Dr. Wannee Jirapakkul, my co-advisors, for all of comments and good suggestions.

I would like to express special thank Professor Dr. Tzou-Chi Huang for giving me a chance to learn the cell culture and cytotoxic techniques and also suggested me during being an exchange student at the Department of Food Science, National Pingtung University of Science and Technology (NPUST), Taiwan. I also would like to thank all members of cell line laboratory for warm welcome and helpful. This research was financially supported by the Center for Advanced Studies in Agriculture and Food (CASAF), National Research University Program, Kasetsart University. Therefore, I would like to take my sincere thanks in this opportunity.

It is a pleasure to thank those who made this thesis possible. I am deepest appreciate to my colleagues in 2413 laboratory room for support and suggestion to me and the gratefully thanks to my relation and my friends for their help and encouragement and for all friendship that we have had in time.

Finally, my graduation would not be achieved without best wish from my parents, Mrs. Supa and Mr. Ekkamol Rongsirikul so I am indebted and heartfelt thank for their help, encouragement, enthusiasm and financial support until I complete.

Narumol Rongsirikul

March 2012

# TABLE OF CONTENTS

**Page**

# LIST OF TABLES

# LIST OF TABLES (Continued)

# LIST OF FIGURES

# LIST OF FIGURES (Continued)

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AA | = | amino acid |
| AAPH | = | 2,2’-azobis(2-amidinopropane)dihydrochloride |
| ABTS | = | 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt |
| AGEs | = | advanced glycation end-products |
| ARP | = | Amadori rearrangement product |
| AUC | = | area under the fluorescence decay curve |
| BHA | = | butylated hydroxyanisole |
| BHT | = | butylated hydroxytoluene |
| BSA | = | bovine serum albumin |
| DTT | = | dithiothreitol |
| DTNB | = | 5,5'-dithiobis(2-nitrobenzoate) |
| DMSO | = | dimethyl sulfoxide |
| CD | = | circular dichroism |
| CT | = | cycle time (min) |
| Da | = | Dalton |
| DHAA | = | dehydroascorbic acid |
| DSC | = | differential scanning calorimetry |
| EC | = | Enzyme Commission (number) |
| FBS | = | fetal bovine serum |
| GI | = | gastro-intestinal |
| HCl | = | hydrochloric acid |
| HepG2 | = | human hepablastoma |
| HSA | = | human serum albumin |
| $IC_{50}$ | = | 50% inhibition concentration |
| β-ME | = | β-mercaptoethanol |
| µmol | = | micromole |
| µM | = | micromolar |
| MEM | = | Eagle’s minimum essential medium |

# LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| MRPs | = | Maillard reaction products |
| MTT | = | 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide |
| MW | = | molecular weight |
| MWPC | = | modified whey protein concentrate |
| OD | = | optical density |
| OPA | = | *o*-phthaldialdehyde |
| ORAC | = | oxygen radical absorbance-fluorescein |
| ORD | = | optical rotatory dispersion |
| pI | = | isoelectric pH |
| PBS | = | phosphate buffered saline |
| w/v | = | weight by volume |
| ROS | = | reactive oxygen species |
| SDS-PAGE | = | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SH | = | sulfhydryl group |
| TE | = | Trolox equivalent |
| TEAC | = | Trolox equivalent antioxidant capacity |
| TBARS | = | thiobarbituric acid-reactive substances |
| TEMET | = | N,N,N',N'-tetramethylethylenediamine |
| wb | = | wet basis |
| WPC | = | whey protein concentrate |
| WPI | = | whey protein isolate |

# PEPTIC-MODIFIED WHEY PROTEIN CONCENTRATE AND ITS ANTIBROWNING EFFECT ON HEATED GLYCINE-SUGAR AND WHEY PROTEIN-SUGAR MIXTURES

## INTRODUCTION

Maillard reactions are non-enzymatic browning that can occur in foods and biological systems. In foods, Maillard reactions result in the changes of colour, aroma and taste. Factors affecting the degree of Maillard reactions include temperature, time (Ames, 1990), pH (Kim and Lee, 2008), reactants (Sumaya-Martinez *et al.*, 2005), water activity, buffer type and concentration, the presence of oxygen, light, and metals (Narayan, 1998). The Maillard reaction is initiated by the glycation of amino groups by carbonyl group of sugar or dicarbonyl group from vitamin C or lipids. The aldose sugars gives rise to Amadori products; while ketose sugar results in Heyns products. Further reactions after Amadori/Heyns rearrangement involve many pathways, which give rise to various products called advanced glycation end-products (AGEs) during the intermediate stages of redox reactions. At the final stages of Maillard reactions, the Maillard reaction products (MRPs) are usually composed of the polymerized products having brown colour called melanoidins.

The presence of carbonyl in aldehyde or ketone is crucial for Maillard reactions to occur, both in foods and biological systems. In addition, the molecular weight (MW) of reducing sugar is also important. The pentose sugars (e.g. ribose, arabinose and xylose) are more reactive than the hexoses (e.g. glucose, fructose and galactose) and reducing disaccharides (e.g. maltose or lactose) (Narayan, 1998). Among hexoses, fructose was found to turn brown more quickly than did glucose (Brands *et. al.*, 2000).

The brown pigment formation in fructose-containing products could occur via both Maillard reactions and caramelization to the higher extent than those found in

glucose-containing products (Brands *et al*. 2000). Nowadays, fructose has been widely used in the form of high-fructose corn syrup, particularly in carbonated beverages and other sweetened drinks, baked goods, candies, canned fruit and so on. The consumption of fructose in the United States has increased dramatically since the past several decades (Gaby, 2005). Therefore, the better understanding in antibrowning activity of fructose-containing products requires investigation.

Maillard reaction products could be advantages and/or disadvantages in foods. Apart from being the sources of colours and flavours, they are known to be antioxidants, antimutagens and antibiotics (Friedman, 2005). However, there are a lot of evidence showed that the MRPs are carcinogens, allergens and mutagens (Friedman, 2005). The AGEs are also known to cause aging in human (Brownlee, 1995); produce reactive oxygen species (ROS) (Bigl *et al.*, 2008) and are involved in chronic diseases (Munch *et al.*, 1997). Melanoidins are also known as mutagenic compounds (Bigl *et al.*, 2008).

The classical methods in controlling Maillard reactions are: elimination of reactive substances, lowering of pH, chelating of trace minerals, limiting water content, maintaining low temperature and using of sulfite as inhibitor or bleaching substances (Peng *et al.*, 2008). However, the inhibition of AGEs and MRPs by inhibiting oxidation mechanisms using natural compounds has been recently investigated. For example, the use of polyphenolic compounds (Wu and Yen, 2005; Schamberger and Labuza, 2007), amino acids (Friedman and Molnar-Perl, 1990), peptides and proteins (Tong *et al.*, 2000; Peng *et al.*, 2008; Hongsprabhas *et al.*, 2011) in inhibiting the full inter-molecular crosslinking pathways in Maillard reactions.

Amino acids, especially sulfur-containing amino acid cysteine and methionine, aromatic amino acid (tryptophan, tyrosine and phenylalanine) and amidazole-containing amino acid (histidine) can interact with free radicals. The free amino acids that were not generally found in foods and the extensive proteolysis do not increase the antioxidant capacity (Chan *et al.*, 1994; Ostdal *et al.*, 1999; Zhou and

Decker 1999; Rival *et al.*, 2001). Nonetheless, some antioxidative food peptides can be obtained from natural sources; e.g. glutathaione (γ-Glu-Cys-Gly), carnosine (β-alanyl-L-1-methylhistidine) and anserine (β-alanyl-L-3-methylhistidine) (Samaranayaka and Li-Chan, 2011). In addition, the hydrolysis of food protein also generates the antioxidative food peptides; e.g. through food protein hydrolytic processing, microbial fermentation and also through gastro-intestinal (GI) digestion. Samaranayaka and Li-Chan (2011) have reviewed the molecular characteristics and possible mechanisms of antioxidative peptide from different food sources. They indicated that various antioxidative peptides with different characteristics were generated via different enzymes, recovering process and the raw materials used as the sources of peptides. It was concluded that the antioxidant potential is attributed to the amino acid composition, sequences, and size of peptide (Samaranayaka and Li-Chan, 2011).

Although the production of bioactive food peptides and amino acid sequences of antioxidative food peptides and hydrolysates have been investigated extensively as reviewed by Samaranayaka and Li-Chan (2011), little is known on the potential use of antioxidative food peptides and hydrolysates in inhibiting or delaying Maillard reactions in processed foods. Hydrolysis of food proteins could increase the $NH_2$−group, the main reactant for Maillard reactions. Yet, the protein hydrolysates, particularly whey protein hydrolysate obtained from tryptic hydrolysis (Hongsprabhas *et al.*, 2011), were reported to have both antioxidant capacity and antibrowning capacity in pasteurized and sterilized whey protein-lactose mixture.

The purposes of this study were to further explore the influences of enzymatic modification of whey proteins, emphasizing on pepsin due to its specificity in cleaving peptide bond linking aromatic amino acid residues to other residues, on the alterations in their antioxidant capacity and antibrowning activity when used in heated glycine-sugar and protein-sugar mixtures. The mechanisms of antibrowning reactions were also explored to get better understanding on the roles of reactive groups in modified whey proteins responsible for antibrowning activities.

# OBJECTIVES

The objectives of this research were:

1. To modify the commercial WPC by heat treatment to unfold whey proteins, followed by peptic hydrolysis and spray-drying,

2. To evaluate the alterations in exposed reactive groups of proteins and antioxidant capacity after modification and spray-drying,

3. To assess the abilities of reconstituted modified whey protein concentrate (MWPC) in preventing brown pigment formation, and

4. To investigate the antibrowning mechanisms of MWPC in heated glycine-sugar and protein-sugar mixture.

# LITERATURE REVIEW

Food protein plays an important role in nutritional and functional properties required in foods. From nutrition standpoints, protein is the nutrient used for growth and metabolic maintenance for homeostasis rather than the caloric sources although protein can provide 4 kcal/g. Protein comprises of amino acids linked together by peptide bonds. The essential amino acids, which cannot be synthesized in the body, can be obtained from proteineous foods, particularly from muscle foods, dairy, egg and legumes.

Biofunctional properties of proteins and amino acids include enzymatic activities for energy pathways, protein and amino synthesis, immunomodulation and regulatories of body functions. For examples, tryptophan is a precursor of serotonin neurotransmitter. Sleep-wake rhythm (Yogman *et al.*, 1982), the response to stress, and other physiological processes (Anisman and Zacharko, 1991) are regulated by this serotonin. Cysteine is a residue in tripeptide glutathione that is an important compound in antioxidative systems of the neonate (Heine *et al.*, 1991) and against intestinal tumors (Mcintosh *et al.*, 1995). Cysteine is also the precursor of taurine (Lo, 1996), which is essential for infant, young baby, including man when the synthesis is inadequate (Sturman *et al.*, 1976). Taurine plays an important role in brain development (Sturman and Hayes, 1980). Apparently proteins have an important role in maintaining good health. During the past decade, there have been extensive evidences of using food-derived proteins and peptides as functional food and nutraceuticals for health-promoting benefits.

Of all food proteins investigated for their roles in biofunctional properties, dairy proteins have received a lot of attention and some were manufactured at commercial scale, such as micellar caseins, caseinates and whey protein products. This thesis emphasized on further modifications of whey protein concentrate (WPC), which are currently imported to Thailand and used as skimmed milk replacer with reasonable cost in dairy product industries.

## 1. Whey proteins

Whey is a by-product from cheese and caseinate manufacturings. When casein is separated from milk during cheese production via activities of chymosin (rennet), pH adjustment and heating coagulated caseins, the by-product is called sweet whey. Whey is mainly composed of proteins, lactose and ash as shown in Table 1 (Zadow, 1994; Smithers *et al.*, 1996). Whey has been used to make more valuable products since the last 20 years by separating and purifying the protein fraction to whey protein concentrate (WPC) and whey protein isolate (WPI) rather than using whey as animal feed. The production of WPC is by ultrafiltration of whey, followed by drying process. The WPC can contain up to 80-85% protein (Tetra Pak, 1995). Whey proteins isolates (WPI) are composed of protein mainly above 90% and manufactured by ion exchange chromatographic technology after ultrafiltration.

**Table 1** Composition of bovine milk and whey

| Component | Content (% w/v) | |
|---|---|---|
| | Milk | Whey |
| Casein protein | 2.8 | <0.1 |
| Whey protein | 0.7 | 0.7 |
| Fat | 3.7 | 0.1 |
| Ash | 0.7 | 0.5 |
| Lactose | 4.9 | 4.9 |
| Total solids | 12.8 | 6.3 |

**Source:** Smithers (2008)

Whey protein is also the important source of essential amino acids. It is rich in branched-chain amino acid like leucine, isoleucine and valine (Smithers, 2008). The amino acids in whey proteins are shown in Table 2.

**Table 2** Amino acid composition in bovine milk protein, casein, and whey protein (g AA/100 g protein)

| Amino acid | Total protein | Casein | Whey protein |
|---|---|---|---|
| Alanine | 3.7 | 3.1 | 5.5 |
| Arginine | 3.6 | 4.1 | 3.3 |
| Aspartic acid | 8.2 | 7.0 | 11.0 |
| Cystine | 0.8 | 0.3 | 3.0 |
| Glutamic acid | 22.8 | 23.4 | 15.5 |
| Glycine | 2.2 | 2.1 | 3.5 |
| Histidine | 2.8 | 3.0 | 2.4 |
| Isoleucine | 6.2 | 5.7 | 7.0 |
| Leucine | 10.4 | 10.5 | 11.8 |
| Lysine | 8.3 | 8.2 | 9.6 |
| Methionine | 2.9 | 3.0 | 2.4 |
| Phenylalanine | 5.3 | 5.1 | 4.2 |
| Proline | 10.2 | 12.0 | 4.4 |
| Serine | 5.8 | 5.5 | 5.5 |
| Threonine | 4.8 | 4.4 | 8.5 |
| Tryptophan | 1.5 | 1.5 | 2.1 |
| Tyrosine | 5.4 | 6.1 | 4.2 |
| Valine | 6.8 | 7.0 | 7.5 |

**Source:** Belitz and Grosch (1999)

In addition, whey protein is rich in sulphur-containing amino acids like methionine and cysteine, compared to meat and casein as shown in Figure 1 (Smithers, 2008). Casein is rich in essential amino acid methionine while whey protein is rich in cysteine. Moreover, the biological value (BV) of whey protein is also 15% higher than that of BV of egg (Smithers, 2008). .

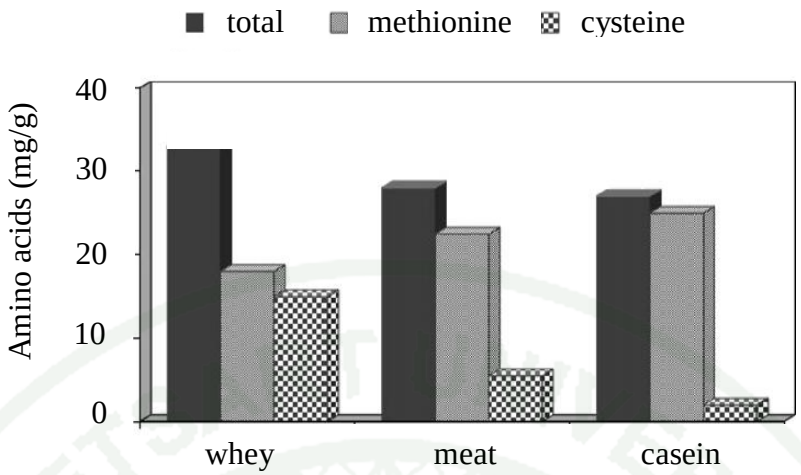



**Figure 1** The amount of sulphur amino acids in whey, meat and casein

**Source:** Smithers (2008)

Whey protein comprises of ~50% β-lactoglobulin, ~20% α-lactalbumin, ~6% serum albumin, ~10% immunoglobulins and ~12% other components (e.g. lactoferrin, lactoperoxidase, lysozyme and growth factors) (Smithers, 2008 ; Lam and Moughan 2011). The major whey proteins are reviewed as follows:

1.1 β-Lactoglobulin (β-lg)

β-Lactoglobulin is the major whey protein from milk of ruminants, monogastrics including human. The variety of ligand-like small hydrophobic molecule as fatty acid and vitamin A could bind to this bovine globulin. The biological function of β-lactoglobulin is not been clear (Sawyer and Kontopidis, 2000; Kontopidis *et al.*, 2004). However, it is probably involved in storage and transportation of the hydrophobic substances such as fatty acids and retinoids naturally present in bovine milk (Pérez and Calvo, 1995; Wang *et al.*, 1997; Kontopidis *et al.*, 2002).

The β-lactoglobulin is a small soluble globular protein, with the molecular weight (MW) of monomer is 18,350 Da (Brownlow *et al.*, 1997). The optical rotatory

dispersion (ORD) and circular dichroism (CD) of β-lactoglobulin showed that ruminant β-lactoglobulin at neutral pH is a dimer of two identical or nearly-identical monomers. When pH was below 2, β-lactoglobulin exists as a monomer. At pH above 9, β-lactoglobulin irreversibly aggregates (Groves *et al.*, 1951). The β-lactoglobulin A and B genetic variants are both consist of 162 amino acids as shown in Figure 2. The two intrachain disulfide bridges (Cys66-Cys160 and Cys106-Cys-119) and a free sulfhydryl group (SH) at Cys121 were shown in the primary sequence (McKenzie *et al*, 1972; Creamer *et al.*, 1983). This polypeptide folds into a compact globular conformation. The buried Cys121 in native molecule of β-lactoglobulin becomes exposed and is more reactive on dissociation of dimers and/or partial unfolding of the molecule; i.e. with increasing temperature (Sawyer, 1967; Qi *et al.*, 1995; Creamer and MacGibbon, 1996). The free SH group is important to heat-induced denaturation of the β-lactoglobulin (Sawyer, 1967) since it affects the stability of milk (Fox, 1995). Free SH group is also involved in thermally induced aggregation and gelation near and above neutral pH through sulfhydryl-disulfide interchange reactions (Roefs and Kruif, 1994; Sawyer *et al.*, 1994; Gezimati *et al.*, 1996). The stability of β-lactoglobulin also depends on the genetic variants. The differential scanning calorimetry (DSC) showed that of β-lactoglobulin A was less stable than was the B variant (Imafadon *et al.*, 1991; McSwiney, 1994).

The secondary structure of β-lactoglobulin is predomainly anti-parallel β-sheet and eight of nine stands form a hydrophobic barrel (β-barrel) bordered on one side of C-terminal by an α-helix (Papiz *et al.*, 1986) as shown in Figure 3. The globular structure of β-lactoglobulin protein is very stable to the acids and proteolytic enzymes present in the stomach (Papiz *et al.*, 1986).

**β-lg BOVINA A** $^{1}$**L** IVTQTMKGLDIQKVAGTW **Y**$^{20}$ SLAMAASDISLLDAQSAPLR$^{40}$
**β-lg BOVINA B** $^{1}$**L** IVTQTMKGLDIQKVAGTW **Y**$^{20}$ SLAMAASDISLLDAQSAPLR$^{40}$

**β-lg BOVINA A** VYVEELKPTPEG **D** LEILLQK$^{60}$ WEN **D** ECAQKKIIAEKTKIPA$^{80}$
**β-lg BOVINA B** VYVEELKPTPEG **D** LEILLQK$^{60}$ WEN **G** ECAQKKIIAEKTKIPA$^{80}$

**β-lg BOVINA A** VFKIDALNENKVLVLDTDYK$^{100}$ KYLLFCMENSAEPEQSL **V** CQ$^{120}$
**β-lg BOVINA B** VFKIDALNENKVLVLDTDYK$^{100}$ KYLLFCMENSAEPEQSL **A** CQ$^{120}$

**β-lg BOVINA A** CLVRTPEVD **D** EALEKFDKAL$^{140}$ KALPMHIRL **S** FNPTQLE
**β-lg BOVINA B** CLVRTPEVD **D** EALEKFDKAL$^{140}$ KALPMHIRL **S** FNPTQLE

**β-lg BOVINA A** **E** QC$^{160}$ H **I**$^{162}$
**β-lg BOVINA B** **E** QC$^{160}$ H **I**$^{162}$

**Figure 2** Amino acid sequences of β-lactoglobulin A and B variants

**Source:** Hernández-Ledesma *et al.* (2008)

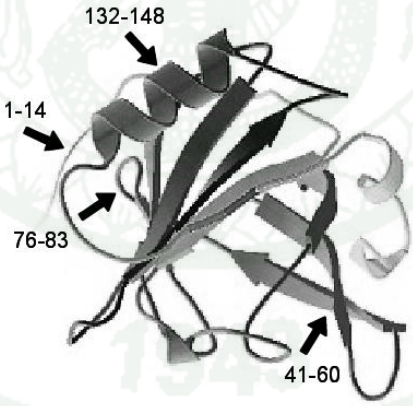



**Figure 3** Three-dimensional conformation of tertiary structure of β-lactoglobulin

**Source:** Girard *et al.* (2003)

Otte *et al.* (1997) investigated the hydrolysis of β-lactoglobulin by bromelain, pepsin, papain, trypsin and endoprotease Arg-C. Bromelain-catalysed hydrolysis was rapid and resulted in a large number of small peptides. Pepsin, however, hydrolysed only a fraction of the β-lactoglobulin, the few fragments were released and they were small peptides. Both papain and trypsin hydrolysed β-lactoglobulin to medium-sized peptides (1-5 kDa). Nonetheless, endoproteinase Arg-C did not hydrolyse native β-lactoglobulin.

However, the heat-treated β-lactoglobulin could be hydrolysed by endoproteinase Arg-C and six peptides with molecular masses around 2 kDa were identified (Otte *et al.* 1997). The fragments of protein hydrolysates from papain, trypsin and endoprotease Arg-C is shown in Figure 4. These results revealed the compact structure β-lactoglobulin, which buried the disulfide bond Cys66-Cys160; and the Cys106 and Cys-119 inside. As the result, β-lactoglobulin quite stable against hydrolysis, unless the protein was denatured.


**Figure 4** Primary structure of β-lactoglobulin (illustrated after the sequence reported by Eigel *et al.*, 1984). The fragments identifed are marked by arrows. Fragments were produced by hydrolysis with papain (full line), trypsin (broken line) and endoprotease Arg-C (dotted line)

**Source:** Otte *et al.* (1997)

1.2 α-Lactalbumin (α-la)

The α-lactalbumin is the second major whey protein. This globular protein has MW of 14,147 Da for genetic variant A and 14,175 Da for genetic B (Brew and Grobler, 1992). It is composed of the 123 amino acid residue and contains four intramolecular disulfides (located at Cys 60-120, Cys 8-111, Cys 61-77 and Cys 73-91) and no free SH group (Vanaman *et al.*, 1970; Fox, 1989; Figure 5). The isoelectric pH (pI) of α-lactalbumin is between 4-5. The α-lactalbumin is calcium metalloprotein with a single high affinity of $Ca^{2+}$ -binding site (Hiraoka *et al.*, 1980).

Three aspartyl residues participate in the $Ca^{2+}$-binding site in α-lactalbumin (Stuart *et al*., 1986). The α-lactalbumin was thus frequently used as a simple model of calcium-binding protein. This primary site can also bind to $Na^{+}$, $Mn^{2+}$ and $Mg^{2+}$, but with a much lower affinity (Kronman, 1989).

Native α-lactalbumin consists of two domains. They are α-helical domain and a β-sheet domain (Kuwajima, 1996). The α-domain is shown in residues 1-39 and residues 82-123. The β-domain is residues 40-81. There are four α-helices-A [residues 5-11], B[23-24], C[86-99] and D[105-109] and three $3_{10}$-helices[12-16, 101-104 and 115-119] in the α-helical domain and an anti-parallel β-sheet[40-50] and a $3_{10}$-helix[76-82] in the β-sheet domain. The native structure of human α-lactalbumin is also shown in Figure 6.

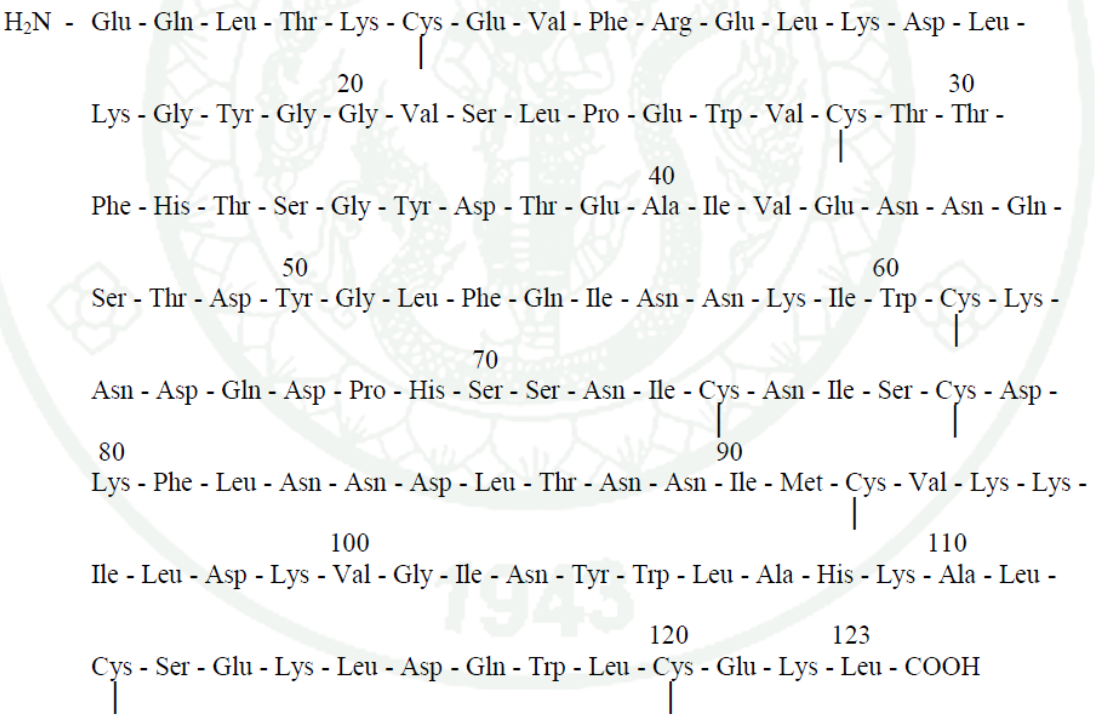



**Figure 5** The complete amino acid sequence of bovine α-lactalbumin

**Source:** Brew *et al*. (1970)

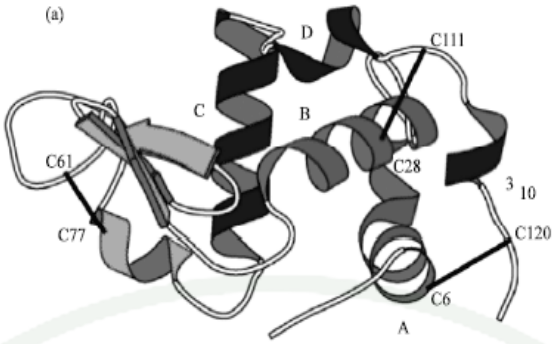



**Figure 6** The native structure of human α-lactalbumin

**Source:** Rösner and Redfield (2009)

The term molten globule (MG) state has been used to describe the partially-unfolded and non-native protein conformation (Hirose 1993; Kuwajima 1989). Molten globule state of α-lactalbumin is composed of denatured state (Shonle, 1996). In non-native state, two types of conformation were reported. The first was a highly denatured or fully unfolded state and the second is the compact intermediates, which have been observed under both transient and equilibrium conditions (Figure 7).

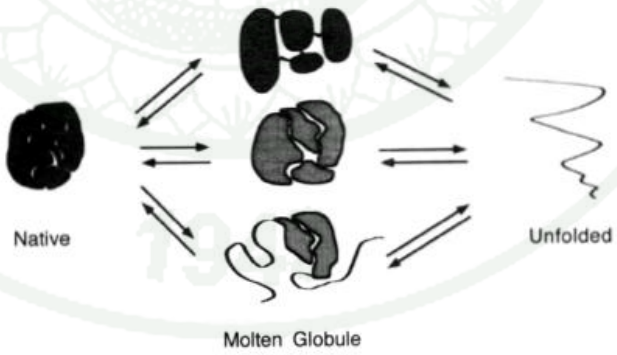



**Figure 7** Schematic of various type of molten globule from X-ray scattering

**Source:** Kataoka *et al*. (1997)

The transitions from one form to the other are quite possible because potential energy barrier was not high between the conformers (Palaniappan, 2001). Thus, the molten globule is defined as structural state that contains substantial secondary structure, with the absence of native-like tertiary structure, yet retains compact globular structure (Dolgikh *et al*., 1981). The molten globule state can also be induced without cooperative thermal denaturation (Ptitsyn, 1987). The molten globule state of α-lactalbumin can be formed by the moderate, strong denaturant concentration (e.g. guanidine hydrochloride or urea) or acid denaturation or removal of calcium at low salt concentration and neutral pH (Kuwajima, 1989).

The α-lactalbumin is considered thermostable because it can fully re-nature upon cooling (>90% thermoreversible) although it has the lowest denaturation temperature ($T_d$ ~ 61°C) (de Wit and Klarenbeek, 1984; Ruegg *et al.*, 1977). In the absence of $Ca^{2+}$, the $T_d$ of α-lactalbumin was decreased (Bernal and Jelen, 1984). This suggested that calcium bound intermediate maintained the intact hydrophobic core (Vanderheeren and Hanssens, 1994), causing the stable structure to α-lactalbumin. In addition, $Ca^{2+}$ bound, thermally-unfolded intermediate of α-lactalbumin has only been found when the temperature was up to 80 °C (Vanderheeren and Hanssens 1994; Vanderheeren *et al.*, 1996). Below pH 3.5, no thermal transition can be detected because a conformational transition (Kronman, 1965), caused by ionization of carboxyl and tyrosyl residues, resulted in the total loss of calcium binding ability (Kuwajima *et al.*, 1981).

### 1.3 Bovine serum albumin (BSA)

Albumins are soluble in water or dilute salt solutions and are coagulated by heat (Osborne, 1907). These proteins are able to bind variety of hydrophobic ligands such as fatty acids, lysolecithin, bilirubin, warfarin, tryptophan, steroids, anaesthetics and several dyes. They play an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood. It has been determined that serum albumin is chiefly responsible for the maintenance of blood pH (Figge *et al*., 1991). The primary structure is constituted of a single chain of

about 580 amino acid residues. The secondary structure of bovine serum albumin contained about 68%-50% α-helix of six turns and 16%-18% β-sheet and 17 disulfide bridges (Sjoholm and Ljungstedt, 1973; Reed *et al*., 1975; Foster, 1997). It is composed by three domains: I, II and III, as shown in Figure 8; which confer to the protein of a heart shaped form (Bos *et al*., 1989). Each domain is constituted by two subdomains, namely, IAB, IC, IIAB, IIC, IIIAB, IIIC, respectively. From the spectroscopic point of view, the main differences between BSA and human serum albumin (HSA) is that the BSA has two tryptophan residues at Trp134 and Trp212; while HSA has only one Trp214 (Peters, 1985).

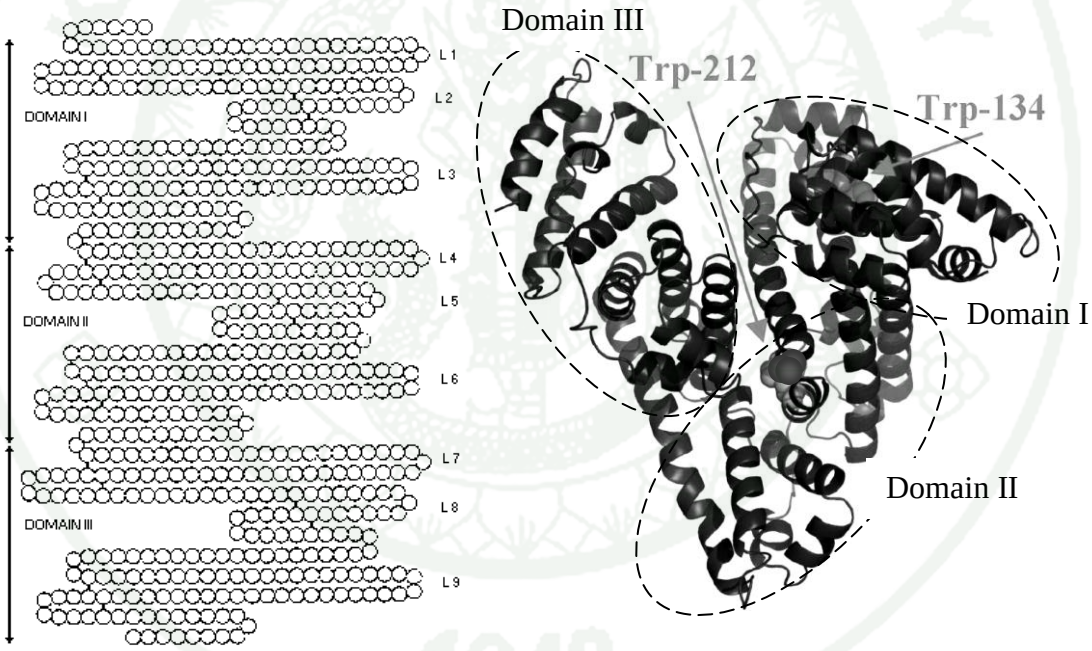



**Figure 8** The **structure** of BSA

**Source:** Peters (1985); Dubeau *et al*. (2010); Wu *et al*. (2011)

Figure 9 shows the amino acid composition of BSA. Albumins are characterized by a low content of tryptophan and methionine and a high content of cystine and the charged amino acids: aspartic and glutamic acids, lysine, and arginine.

The glycine and isoleucine content of BSA are lower than that in the averaged protein. BSA has molecular weight around 66,200 Da (Peters, 1985).

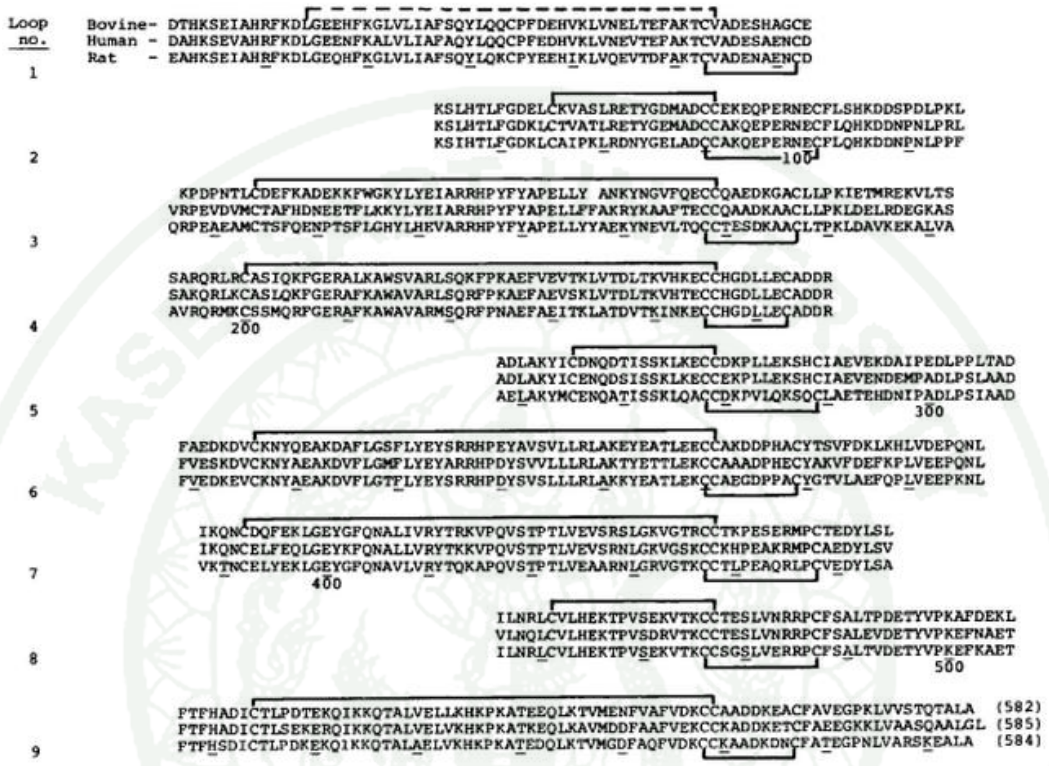



**Figure 9** The amino acid composition of BSA

**Source:** Peters (1985)

Harmsen and Braam (1969) showed that alkali or heat denaturation of BSA caused a partial loss of α-helical structure with no formation of β-sheet. However, β-sheet can be formed when BSA was heated above 72 °C. Heat treatment of serum albumin can result in two structural stages. The first stage is reversible whilst the second stage is irreversible. However, the structural changes do not necessarily result in a complete destruction of the ordered structure (Kuznetsow *et al*., 1975; Lin and Koenig, 1976). Heating of BSA up to 65°C can be regarded as the first stage, with subsequent heating above that can be described as the second stage

(Wetzel *et al*., 1980). The β-sheet formed was more pronounced upon cooling and was reported to be concentration-dependent (Wetzel *et al*., 1980).

**2. Bioactive whey proteins**

There are many researches on bioactivities associated with protein and peptides. Hernández-Ledesma *et al.* (2008) described peptides derived from β-lactoglobulin on its biological properties on antihypertensive, antimicrobial, immunostimulating peptides, opioid, hypocholesterolaemic and antioxidant capacity as shown in Table 3. The most prevalence activities of whey protein-derived peptides were antioxidant and antimicrobial activities, as well as hypocholesterolemic activity.

The use of synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in foods have limitations due to their adverse effects. The replacement of synthetic antioxidants by the natural ones from dietary sources has been proposed (Chen *et al.*, 1992). Whey proteins have been considered as the natural source to produce the natural antioxidative protein hydrolysates (Chatterton *et al.*, 2006). This is because protein hydrolysates were shown to inhibit deleterious changes of lipid oxidation. Whey proteins hydrolysed by different enzymes were shown to have capacity in decreasing the thiobarbituric acid-reactive substances (TBARS) formation in a liposome-oxidizing system (Peña-Ramos and Xiong, 2001, 2003, 2004). WPI hydrolysed by alcalase, protamex and flavourzyme was reported that although the amino acid composition of WPI and WPI hydrolysate mixture were similar, the antioxidation activity of WPI hydrolysate mixture was higher than those in WPI. The fraction of >45 kDa in WPI hydrolysate showed higher TBARS inhibition effect than the lower MW fractions and hydrolysate mixtures did. The most of lower MW fractions of the commercial WPI hydrolysates showed the higher inhibitory effect (Peña-Ramos and Xiong, 2004).

It has been shown that not only the MW of peptides, the antioxidant capacity also depends on the composition of amino acids and its concentration. Peña-Ramos and Xiong (2004) also studied the correlation between peptide size and amino acid

concentration on the antioxidation activity of TBARS inhibition and indicated that antioxidant activity of whey protein hydrolysates depended on the MWs of peptides. In addition, the antioxidant capacity was strong when the peptides contained high concentration of histridine and hydrophobic amino acids. In addition, Peng *et al*. (2009) fractionated the alcalase-hydrolysate from WPI and reported that the fractions containing small MW peptides of 0.1–2.8 kDa was effective in scavenging 1,1-diphenyl-2-picrylhydrazyl radicals ($DPPH^{\bullet}$) and the trapped hydroxyl ($^{\bullet}OH$) and superoxide ($O^{\bullet}_2$) radicals determined by electron spin resonance (ESR) than all other fractions or the mixed hydrolysates.

**Table 3** Bioactive peptides derived from β-lactoglobulin

| Fragment | Sequence | Origin | Activity | Reference |
|---|---|---|---|---|
| β-lg f(42-46) | YVEEL | Hydrolysis with thermolysin | Antioxidant | Hernández-Ledesma *et al.* (2005) |
| β-lg f(145-149) | MHIRL | Hydrolysis with thermolysin | Antioxidant | Hernández-Ledesma *et al.* (2005) |
| β-lg f(19-29) | WYSLAMAASDI | Hydrolysis with thermolysin | Antioxidant | Hernández-Ledesma *et al.* (2005) |
| β-lg f(15-20) | AGTWY | Hydrolysis with pepsin | Antimicrobial | Pellegrini *et al.* (2001) |
| β-lg f(25-40) | AASDISLLDAQSAPLR | Hydrolysis with pepsin | Antimicrobial | Pellegrini *et al.* (2001) |
| β-lg f(78-83) | IPAVFK | Hydrolysis with pepsin | Antimicrobial | Pellegrini *et al.* (2001) |
| β-lg f(92-100) | VLVLDTDYK | Hydrolysis with pepsin | Antimicrobial | Pellegrini *et al.* (2001) |
| β-lg f(102-105) | YLLF | Hydrolysis with pepsin+trypsin or pepsin + chymotrypsin | Opioid | Antila *et al.* (1991); Sipola *et al.* (2002) |
| β-lg f(146-149) | HIRL | Hydrolysis with chymotrypsin | Opioid; antihypertensive; Analgestic Antinociceptive | Yoshikawa *et al.* (1991) Yamauchi *et al.* (2003a) |
| | | | Hypocholesterolemic | Yamauchi *et al.* (2003b) |
| β-lg f(71-75) | HAEK | Hydrolysis with trypsin | Hypocholesterolemic | Nagaoka *et al.* (2001) |
| β-lg f(9-14) | GLDIQK | Hydrolysis with trypsin | Hypocholesterolemic | Nagaoka *et al.* (2001) |
| β-lg f(142-146) | ALPMH | Hydrolysis with trypsin | Hypocholesterolemic | Nagaoka *et al.* (2001) |
| β-lg f(41-60) | VYVEELKPTPEGDLEILLQK | Hydrolysis with trypsin | Hypocholesterolemic | Nagaoka *et al.* (2001) |

**Source:** Hernández-Ledesma *et al.* (2008)

Hernández-Ledesma *et al*. (2005) showed that corolase PP was the most appropriate enzyme to hydrolyse α-lactabumin and β-lactoglobulin, compared to the commercial proteases like pepsin, trypsin, chymotrypsin and thermolysin as shown in Table 4. The hydrolysates obtained had the highest antioxidant capacity measured by oxygen radical absorbance (ORAC) assay.

**Table 4** The radical scavenging activity of the hydrolysates and their corresponding permeates (F < 3 kDa) from α-lactalbumin and β-lactoglobulin by pepsin, trypsin, chymotrypsin, corolase PP, and thermolysin

| Enzyme | ORAC value (μmol of Trolox equivalence/mg protein) | | | |
|---|---|---|---|---|
| | α- lactalbumin | | β-lactoglobulin | |
| | hydrolysate | F < 3kDa | hydrolysate | F < 3kDa |
| Pepsin | 1.065±0.056 | 0.790±0.017 | 0.701±0.033 | 0.821±0.007 |
| Trypsin | 1.031±0.046 | 0.942±0.011 | 0.979±0.022 | 0.667±0.012 |
| Chymotrypsin | 2.528±0.078 | 1.755±0.051 | 1.378±0.032 | 1.508±0.039 |
| Corolase PP | 2.954±0.106 | 2.315±0.080 | 2.151±0.051 | 1.897±0.019 |
| Thermolysin | 2.039±0.018 | 1.365±0.030 | 1.657±0.038 | 1.519±0.005 |

**Source:** Hernández-Ledesma *et al*. (2005)

### 3. Non-enzymatic browning reactions in foods

Browning reactions in foods involve enzymatic and non-enzymatic reactions. Of all non-enzymatic reactions crucial in foods, the Maillard reaction is one of the most studied reactions. The rest of non-enzymatic browning reactions include the ascorbic acid browning, lipid browning and caramelization (Villamiel *et al*., 2006).

Ascorbic acid browning is defined as the thermal decomposition of ascorbic acid under both aerobic and anaerobic conditions by oxidative or non-oxidative mechanisms in the presence or absence of amino compounds (Wedzicha, 1984). When oxygen is present, ascorbic acid is degraded to dehydroascorbic acid (DHAA).

The DHAA is not stable and spontaneously converts to 2, 3-diketo-L-gulonic acid (Lee and Nagy 1996). Under anaerobic conditions, DHAA is not formed but undergone the generation of diketogulonic acid via its keto tautomer, followed by β-elimination at C-4 from this compound and decarboxylation to give 3-deoxypentosone, which is further degraded to furfural. While diketogulonic acid from aerobic conditions was decarboxylated to form xylosone and converted to reductones. These oxidation products: furfural, reductones and 3-deoxypentosone, may contribute to the browning of foods by means of a Maillard reaction (Fennema 1976; Beliz and Grosch 1997). These products have been detected in both model systems and foods containing ascorbic acid (Yin and Brunk 1991; Davies and Wedzicha 1994). This degradation causes the loss of commercial value in citrus product. The degradation of ascorbic acid followed by Maillard browning also of concern in non-citrus foods like asparagus, broccoli, cauliflower, peas, potatoes, spinach, apples, green beans, apricots, melons, strawberries, corn and dehydrated fruits (Belitz and Grosch, 1997).

Lipid peroxidation occurs by the action of oxygen and reactive oxygen species on the fatty acids, especially the unsaturated ones. Lipid oxidation produces the electrophilic carbonyl compounds and interaction with neucleophiles such as free amino group of amino acids, peptides or proteins. Therefore, the oxidized lipids like aldehydes and ketones can subsequently react with amino acids and form brown pigments via Maillard reaction (Hidalgo and Zamora, 2000). This reaction can also take place and produced the different end-products from pure lipid oxidation (Gillatt and Rossell, 1992). It can simultaneously occur of pure lipid and lipid-amino acid oxidations (Hidalgo and Zamora, 2000). It is possible that peroxidative products induce browning reaction of the Amadori compounds (Hermosin *et al.*, 1992).

## 3.1 Maillard reactions

The most common chemical reactions in complex food that can occur during food processing and storage are Maillard reactions. These reactions involve a complex of chemical reactions involvement of carbonylic intermediates. It occurs naturally during the processing, cooking, and storage of foods. French chemist Louis Camille Maillard is the first scientist who described this reaction but the first coherent scheme (Figure 10) was put forward by Hodge in 1953. Maillard reactions are the condensation of ε-amino groups of proteins and α-amino groups of terminal amino acids with reducing-end carbonyl group of acyclic sugars that give numerous compounds called Maillard reactions products or MRPs. These products occur via various stages of reaction and are dependent on pH, reactant, temperature and time.

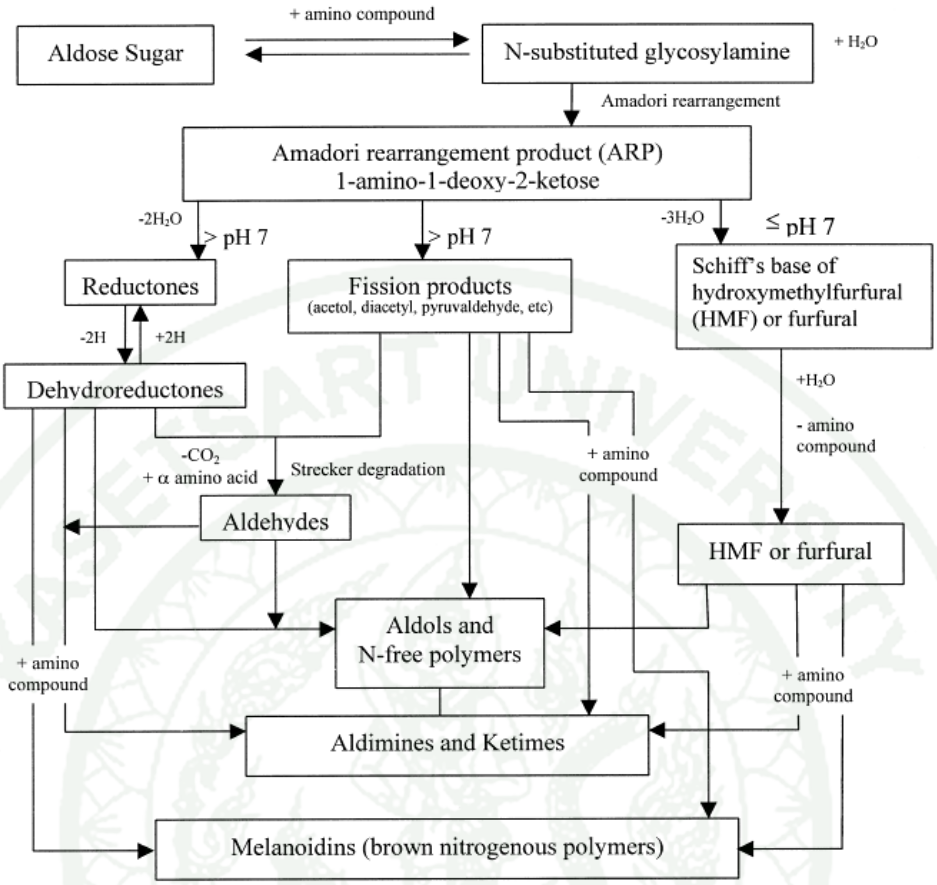



**Figure 10** Maillard reaction scheme

**Source:** Hodge (1953)

### 3.1.1 The initial reaction

The initial reaction or the early stage of Maillard reactions is the condensation of an amino acid with reducing sugar. The condensation of an amino group (nonionized amino group, ε-$NH_2$ of lysine or terminal $NH_2$-group) with a carbonyl group (carbon atom double-bonded to an oxygen atom; C=O) in reducing sugar would lose a molecule of water to form N-substituted aldosylamine or Schiff base (carbon-nitrogen double bond with the nitrogen atom connected to an aryl or alkyl group; C=N–R). This is unstable and undergoes the Amadori rearrangement to form 1-amino-1-deoxy-2-ketoses (ketosamines; Figure 11 a)), which can undergo subsequent dehydration, fission and polymerization reactions and formed complexes.

If the initial sugar reactant is a ketose, a glycosylamine is formed by the same mechanism as for aldoses, but it can then undergo a reverse-Amadori (Heyns) rearrangement to yield a 2-amino aldose (Figure 11 b)).

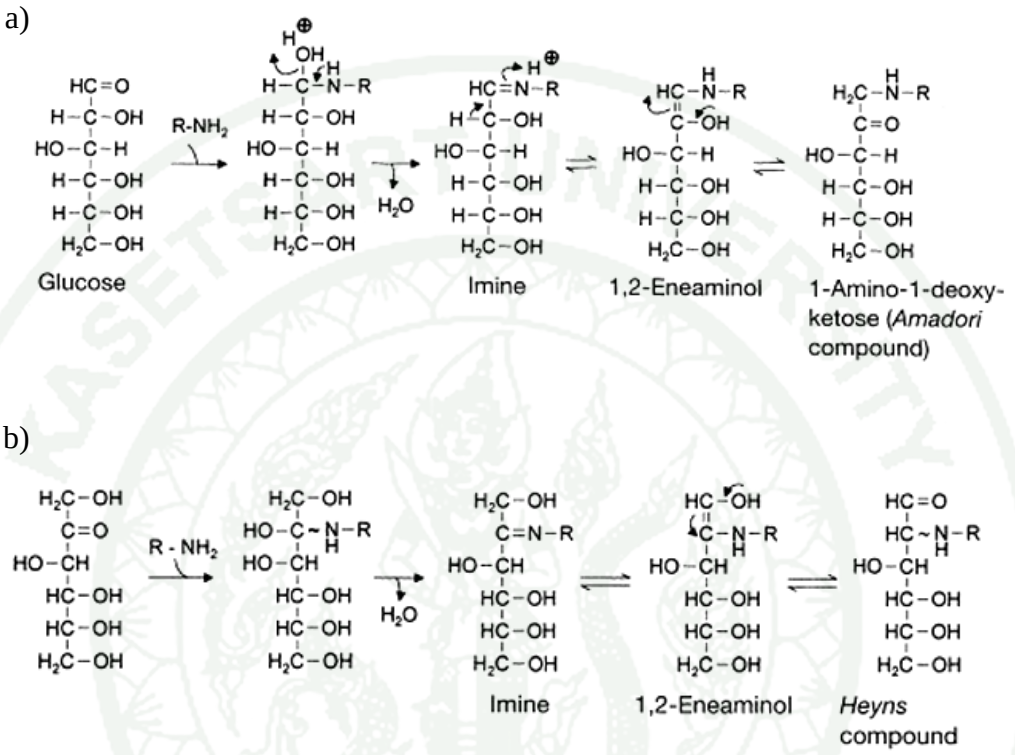



**Figure 11** Amadori products (1-amino-1-deoxy-ketoses; ketosamines) and Heyns products (2-amino-2-deoxy-aldoses; aldosamines)

**Source:** Belitz *et al.* (2004)

Amadori compounds are precursors of numerous flavor, aroma and brown polymer compounds. They are formed before the occurrence of sensory changes. Therefore, the determination provides the sensitive indicator for early detection of the changes caused by Maillard reactions. (Olano and Martínez-Castro, 1996)

The degradation of Amadori rearrangement product (ARP) can occur after the formation of ARP. The degradation of the ketosamine products of the

Amadori rearrangement product is dependent on the pH of the system. They can then react via three pathways; i.e. the formation of reductones, the formation of the highly reactive fission product and the Schiff's base of hydroxyl furfural and methylfurfural.

The reductones formation is the first pathway of ARP degradation. It occurs when pH is above 7. ARP simply further dehydrate by the loss of two water molecules into reductones and dehydro-reductones (α-dicarbonyl compound) (Martins *et al.*, 2001).

The formation of the highly reactive fission product is the product from the second degradation pathway of ARP. The formation of the highly reactive fission products takes part in further reaction at pH above 7. This pathway is the formation of short chain hydrolytic fission products such as diacetyl, acetol, pyruvaldehyde, which then undergo the Strecker degradation with amino acids to aldehydes and by condensation to aldols. Negative aromas like 2- and 3-methyl-butanal and other aldehydes are formed via this pathway (Martins *et al.*, 2001).

Schiff base of hydroxyl furfural and methylfurfural is the product occurs when the ARP is formed and pH $\leq$ 7. This pathway involves the loss of 3 water molecules, then a reaction with amino acids and water occurs, which undergo aldol condensation and polymerise further to true melanoids (Martins *et al.*, 2001).

### 3.1.2 Advanced stage of Maillard reactions

The advanced stage leads to the breakdown of Amadori products or other products related to the Schiff's base and the formation of degradation product, reactive intermediates and volatile compounds (Villamiel *et al.*, 2006). Reactive intermediate like 3-deoxyglucosone participates in cross-linking of proteins at a much faster rate than the glucose itself, and their further degradation leads to the formation of 5-hydroxymethyl-2-furaldehyde and pyraline (Feather *et al.*, 1995)

In the advanced stage, many reactions take place such as cyclisation, dehydration or further condensation. These reactions lead to continually formed highly UV-absorbing and colorless compounds. In the advanced phase of the reaction, Amadori products undergo further transformation to fluorescent, colored substances and cross-linked polymers. They are defined as advanced glycation end products (AGEs).

### 3.1.3 The final stage of Maillard reactions

In the final stage, the formation of brown nitrogenous polymers and co-polymer occurs. They are known as melanoidins (Badoud *et al*., 1995), which provide brown pigments to food products. Melanoidins have been described as low molecular weight colored substances that able to cross-link with protein via ε-amino groups of lysine or arginine to produce high molecular weight colored melanoidins. It has been also postulated that they are polymer consisting of repeating units of furans and/or pyrroles formed during advanced stages of Maillard linked by polycondensation reactions (Martins and van Boekel 2003).

## 3.2 Caramelization

Commercial caramel is divided into four classes (Institute of Medicine, 2003) based on the reactants. Class I is a plain caramel or caustic caramel, prepared by heating carbohydrates with or without acids or alkaline but no ammonium or sulfite compounds. Class II is a caustic sulfite caramel, prepared by heating carbohydrates with or without acids or alkaline in the presented of sulfite compound but no ammonium compound. Class III is an ammonia caramel, prepared by heating carbohydrates with or without acids or alkaline in the presence of ammonium compounds and no sulfite compound. Class IV is a sulfite ammonia caramel, prepared by heating carbohydrates, with or without acids or alkaline but in the presence of ammonium and sulfite compounds (Institute of Medicine, 2003). In addition, this reaction can also be divided by electrical charge into three groups: positive caramel, negative caramel and spirit caramel (Greenshields, 1973).

Caramelization of reducing carbohydrates starts with the opening of the hemiacetal ring followed by enolization, which proceeds via acid and base catalyzed mechanism, leading to the formation of isomeric carbohydrates. The interconversion of sugars through their enediols increases with increasing pH and is called the Lobry de Bruyn-Alberda van Ekenstein transformation (Kroh, 1994). In the first step of degradation, alkaline degradation could lead to the formation of pyruvaldehyde and lactic acid. In acidic degradation, enolisation occurs, followed by elimination of water molecules and cyclisation. This lead to the formation of three important intermediate 5-hydroxymethyl-furfural (5-HMF), 2(-2-hydroxyacétyl)-furans and maltol. These molecules are part of the volatile fraction responsible for the characteristic aroma of caramel. However, in acid condition, the formation of isomeric carbohydrates was low and dehydration is favored, leading to furaldehyde compounds: 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde. HMF is produced in higher yield in fructose than in glucose when unbuffered acids are used as catalysets. The enolization of glucose greatly increased in unbuffered acid solution (Fennema, 1976). In the second step of caramelization, the reactions of condensation and polymerization are more specific. The loss of two water molecules with cyclisation gives rise to the major constituent of the volatile fraction of caramel, fructose dianhydrides (DAF). This step leads to the formation of dark brown high MW compounds.

The caramelization reactions have been studied during heat treatment of sugar at high temperatures (Sugisawa and Edo, 1966). However, del Pilar Buera *et al.* (1987) reported the non-enzymatic browning in high water activity ($a_w$) and liquid system and revealed that the significant colour formation of caramelization can occur even at high $a_w$ (0.9) and also under mild temperature.

**4. Antibrowning agents**

In food, heat-induced changes in color usually occur especially in the presence of reducing sugar through Maillard reactions and caramelization. These reactions affect qualities of foods, both desirable and undesirable. Controlling Maillard reactions can be achieved by elimination of reactive substances, lowering of pH, chelating of trace minerals, limiting water content, maintaining low temperature and using of sulfite as inhibitor or bleaching substances (Peng *et al*, 2008).

Some reagents have been used to inhibit the non-enzymatic browning such as sulfiting agents (Iyengar and McEvily, 1992). Sulfiting agents commonly used in foods are sulfur dioxide, sulfurous acid, sodium (or potassium) sulfite, bisulfite, or metabisulfite. These reagents inhibit non-enzymatic browning by reacting with the carbonyl intermediates, thereby blocking pigment formation (Wedzicha, 1987). The products allowed to use sulfites include dehydrated fruits and vegetables, pre-peeled potatoes, fresh grapes, and wine (Taylor *et al*., 1986).

However, consumer awareness of the risks associated with sulfites and the revised regulations have created the needs for substitutes. Many of conventional alternatives to sulfite includes ascorbic acid–based formulations, cysteine, 4-hexylresorcinol have also been used in inhibiting enzymatic browning (Sapers *et al.*, 2002). Nevertheless, not many compounds have been used to inhibit Maillard reactions. Some of the inhibitors for Maillard reactions such as natural compounds have been recently investigated. For example, the use of polyphenolic compounds (Wu and Yen, 2005; Schamberger and Labuza, 2007), amino acids (Friedman and Molnar-Perl, 1990), peptides and proteins (Tong *et al.*, 2000; Peng *et al.*, 2008; Hongsprabhas *et al.*, 2011) in inhibiting the full inter-molecular crosslinking pathways during advanced stage of Maillard reactions.

**5. Antioxidative agents**

Oxidation-reduction reactions in biological systems involve the transfer of electrons between oxidant and reductant. These are essential reactions for cell survival such as the conversion of nutrient molecules to energy by oxidization in mitochondria called the electron transport system (ETS) and the cell signaling. Radicals are atoms, molecules, or ions with unpaired electrons on an open shell configuration (Leopoldini *et al.*, 2004). The reduction of oxygen to water in living bodies involve intermediate products, activated oxygen metabolites or prooxidants such as hydroxy radicals superoxide anion and $H_2O_2$. The lack of equilibrium between antioxidation and oxidation results in increasing oxidative damage and oxidative stress, which is accompanied by inflammation, aging and carcinogenesis (Weitzman and Gordon, 1990; Ames, 1993; Borek, 2001).

In foods, oxidation reaction occurs when electrons are transfered between chemicals that had different oxidation states. Oxidation reaction in food is a destructive process, causing the loss of nutritional values and changes in the qualities. Oxidation of fats and oils leads to rancidity. In fruits such as apples, it can result in the formation of compounds, which cause discoloration of fruit through enzymatic reactions. Antioxidants are usually added to food to slow the rate of oxidation and, if used properly, they can extend the shelf-life of foods.

Antioxidant is any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge, 1989). Antioxidants in food are of interest for at least four reasons. First, endogenous or added antioxidants may protect components of food itself against oxidative damage. Second is the effect from the absorbed dietary antioxidant. Third is the effect from unabsorbed food-derived antioxidant such as the ability of unabsorbed phenolic to chelate iron ions and scavenge reactive species in stomach and colon. The last is the extract for therapeutic. For endogenouse or added antioxidants, the antioxidants are of interest due to its ability to prevent the off-flavors, rancidity and similar phenomena in food

industry. Food scientists often equate antioxidants with inhibitors of lipid peroxidation and consequent food deterioration (Halliwel, 2002).

5.1 Synthetic antioxidants

Synthetic antioxidant compounds shown in Figure 12 required in food must be non-toxic and highly active at low concentration. Lipophilic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tocopherols and dodecylgallate are suitably used in o/w emulsions. More polar antioxidants such as tert-butylhydroquinone (TBHQ) and propylgallate are very active in fats and oils; thus they enrich at the surface of fat and contact with air. Utilization of antioxidants is often regulated by the governments through the controls on use of food additives. In North America, incorporation of antioxidants is

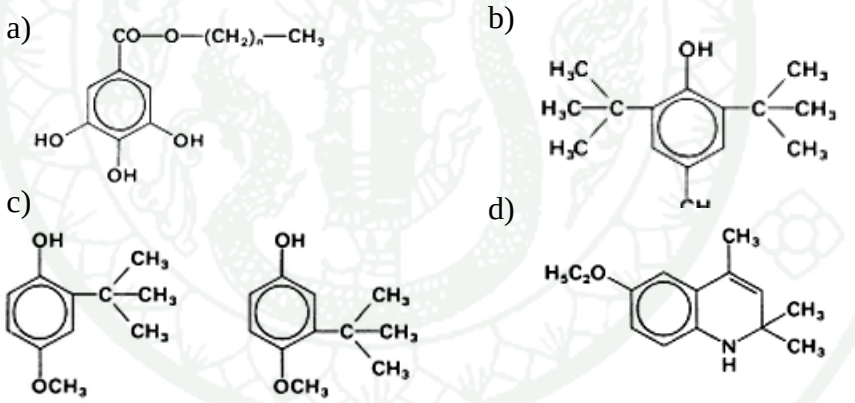



**Figure 12** Synthetic antioxidant compounds; a) Propyl (n=2); octyl (n=7) and dodecyl (n=11) gallate; b) 2, 6- Di- tert- butyl- p- hydroxytoluene (BHT); c) tret- butyl-4-hydroxyanisole (BHA) the two isomer of 2- and 3- tert- butyl-4-hydroxyanisole was show as mixture in commercial BHA.; d) 6- ethoxy-1, 2-dihydro-2, 2, 4- trimethylquinoline (ethoxyquin)

**Source:** Belitz *et al.* (2004)

permitted at maximum level of 0.01% for any one antioxidant, and maximum of 0.02% for any combination (Belitz *et al*., 2004).

5.2 Natural antioxidants

Plants and animals have the necessary antioxidants and of enzyme to defense oxidative stress. Dietary antioxidants, such as water-soluble vitamin C and phenolic compounds, as well as lipid-soluble vitamin E (tocopherol) and carotenoids present in vegetables, contribute antioxidation mechanisms against oxidative stress (Podsędek, 2007). Natural antioxidants from plant products may be more effective in reducing ROS levels compared to the single synthetic antioxidants due to the synergistic actions of a wide range of biomolecules such as vitamins C and E, phenolic compounds, carotenoids, terpenoids and phytomicronutrients (Gutteridge and Halliwell, 2010). In addition, the dietary intake of synthetic antioxidants could cause genotoxicity and carcinogenicity at high concentrations (de Oliveira *et al.*, 2009; Gutteridge and Halliwell, 2010).

Antioxidant potential from natural food and plant extract has been investigated in raw and cooked pork patties (McCarthy *et al*., 2001). When compared at the level of 0.25% aloe vera, 0.01% fenugreek, 0.25% ginseng, 0.10% mustard, 0.10% rosemary, 0.05% sage, 0.1% soya protein, 0.25% tea catechins and 4% WPC against 0.01% of synthetic antioxidant BHA or BHT, the TBARS values indicated that BHA/BHT are the most effective antioxidant, followed by tea catechins, rosemary, sage, WPC, mustard, aloe vera, fenugreek, soya protein and ginseng respectively (McCarthy *et al*., 2001).

The ability of protein to interact with free radical in food could also lead to the development of novel antioxidant technologies. The oxidative susceptibility of amino acid residue to free radical attack is dictated in large part by its functional R group or side chain. Twenty amino acids are potential oxidizable. However, the most reactive amino acid capable of antioxidation tends to be those containing either nucleophilic sulphur-containing side chain (cysteine and methionine) or aromatic side

chain (tryptophan, tyrosine and phenylalanine), of which the hydrogen is easily abstracted. Aromatic amino acid residues are also the targets for ROS attack (Elias *et al.*, 2008). Figure 13 shows the oxidation of phenylalanine residues to *ortho*- and *meta*-tyrosine derivatives (Stadtman and Levine, 2003)

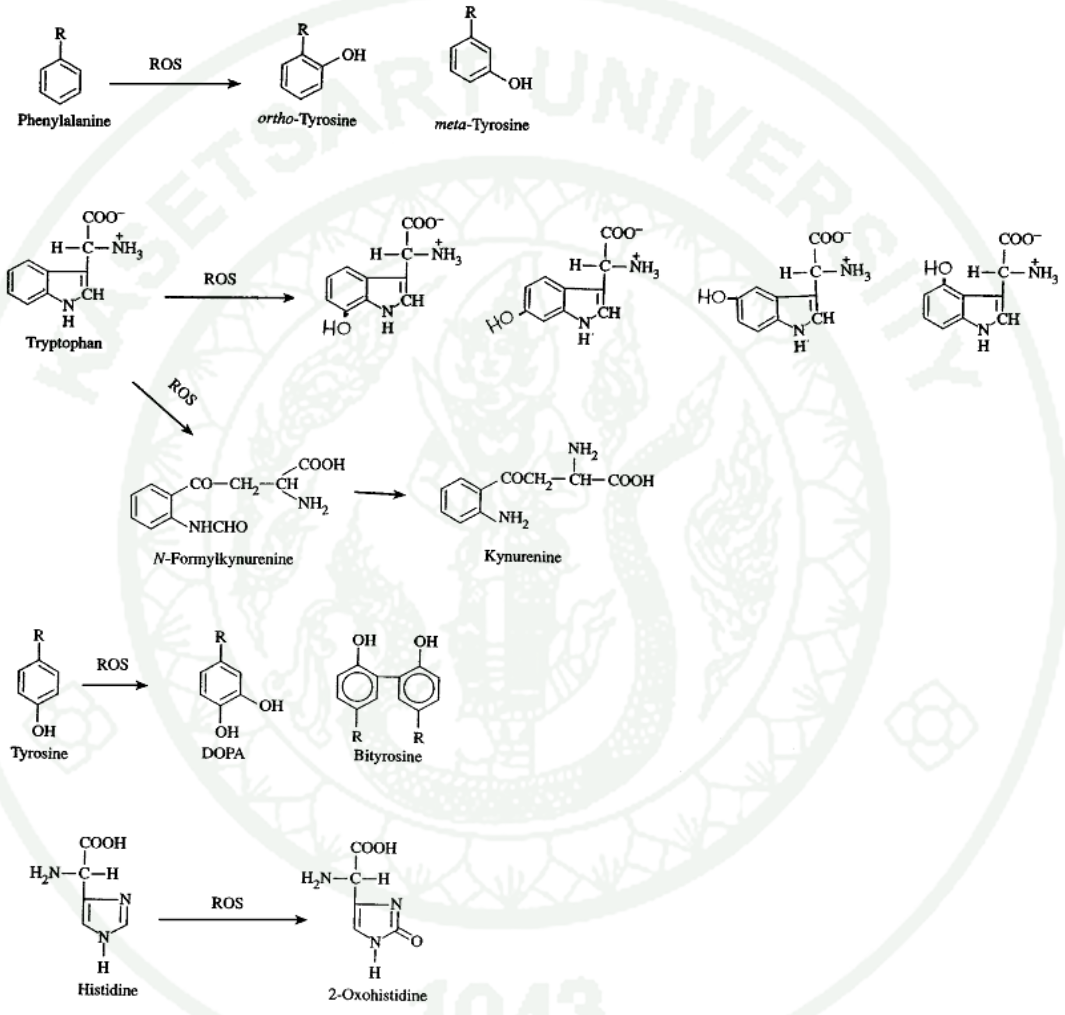



**Figure 13** Oxidation of aromatic amino acids

**Source:** Stadtman and Levine (2003)

### 5.3 Antioxidation measurement

The antioxidation evaluation of antioxidant compounds are hydrogen atom transfer (HAT) reaction-based assay and single electron transfer (ET) reaction-based assay. The HAT is the transfer of hydrogen atom from the antioxidant molecules to the radical. In HAT, the oxidized antioxidant contains radical ($ArO^{\bullet}$) that is more stable than the substrate ($R^{\bullet}$) shown in Eq. (1). Single electron transfer (SET), however, is the transfer of an electron to the radical and makes the radical become stable than the substrate molecule. The oxidized antioxidant becomes charged free radical as shown in Eq. (2) but is more stable than the substrate $R^{\bullet}$ (Leopoldini *et al.*, 2004).

$$R^{\bullet} + ArOH \Rightarrow RH + ArO^{\bullet} \qquad \text{Eq. (1)}$$

$$R^{\bullet} + ArOH \Rightarrow R^{-} + ArOH^{\bullet +} \qquad \text{Eq. (2)}$$

In HAT-based assays, these methods are composed of synthetic radical generator, oxidizable molecular probe and antioxidant. HAT-based methods are more relevant to radical chain-breaking antioxidant capacity (Huang *et al.*, 2005). For example, the oxygen radical absorbance capacity (ORAC) assay is composed of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) radical generator and fluorescein probe.

In contrast, SET-based assays involve one redox reaction, in which the oxidant is also the probe for monitoring the reaction (Ndhlala, 2010). Two components in the reaction are the antioxidant and oxidant probe. (Leopoldini *et al.*, 2004). When electron from antioxidant was removed, the color of oxidant would change in proportional to the concentration of antioxidants in the reaction mixture. The trolox equivalent antioxidant capacity (TEAC) assay uses 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical as oxidant and probe. This assay is based on the discolouration of dark blue solution ABTS by antioxidant compounds. The standard antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (Trolox) is used for comparison (Re *et al.*, 1999). The TEAC assay is

highly dependent on the time of incubation and the ratio of sample quantity to ABTS radical concentration (Roginsky and Lissi, 2005). However, it is stable over a wide pH range and can be used to study pH effects on antioxidant mechanisms (Lemanska *et al.*, 2001). TEAC assay can be used to measure the antioxidant capacity of hydrophilic and lipophilic compounds in test samples (Arno, 2000). Another method for SET-based assay is the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay. This method is based on the scavenging of DPPH by antioxidants. The deep purple color of DPPH becomes colorless and standard antioxidant such as BHT would be used for comparison. DPPH radical scavenging capacity assay is valid to quantify samples with hydrophilic or lipophilic antioxidants and it is one of the most extensively used (Ndhlala, 2010).

# MATERIALS AND METHODS

## Materials

### 1. Effect of peptic modification on the characteristics of whey protein concentrate (WPC)

A) Materials and equipments for the preparation of the modified WPC

1) Commercial WPC was imported and repacked by a local distributor in Thailand. It contained 75.83% protein (wet basis [wb]), 6.5% moisture content (wb), 2.71% ash (wb), 1.05% fat (wb) and 13.91% carbohydrate (wb) (AOAC, 2000).

2) Porcine gastric mucosa pepsin (EC 3.4.23.1; 367 $Umg^{-1}$) was purchased from Sigma Chemical Co. (St. Louis MO, USA).

3) pH meter (model Orion 2-star, Thermo Fisher Sciencetific Inc., Waltham, USA)

4) Water bath stirrer (EYELA PS-100, Tokyo Rikakikai Co., Ltd**.,** Tokyo, Japan)

B) Materials and equipments for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

1) Acetic acid (Mallinckrodt Chemical Works, St. Louis, MO, USA)
2) Acrylamide PAGE (GE Healthcare Bio-Sciences AB, Sweden)
3) Ammonium persulphate (APS; BioRad, München, Germany)
4) Coomassie Brilliant Blue (BioRad, München, Germany)
5) Glycerol (Fisher Scientific, Inc., Fair Lawn, NJ, USA)
6) Glycine (BioRad, München, Germany)
7) Methylenebis methylene-acrylamide (GE Healthcare Bio-Sciences AB, Sweden)
8) Methanol (Mallinckrodt Chemical Works, St. Louis, MO, USA)
9) β-mercaptoethanol (BioRad, München, Germany)

10) Sodium dodecyl sulfate (GE Healthcare Bio-Sciences AB, Sweden)

11) Tris(hydroxymethyl)-aminomethane (GE Healthcare Bio-Sciences AB, Sweden)

12) N,N,N',N'-tetramethylethylenediamine (TEMET; $C_6H_{16}N_2$; ultrapure, USB corporation, Cleveland, OH, USA)

13) Mini ProteanII equipment (BioRad, München, Germany)

14) Microcentrifuge (Labnet Spectrafuge 16M, Labnet International Inc., Woodbridge, NJ, USA)

C) Materials and equipments for the determination of brown pigment formation

1) UV-Visible spectrophotometer (Spectronic GENESYS 10; Thermo Fisher Scientific, Waltham MA, USA)

D) Materials and equipments for the determination of oxygen radical absorbance capacity (ORAC)

1) Fluorescein (Na salt) was purchased from Sigma Chemical Co. (St. Louis MO, USA)

2) The 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox; Aldrich Chemical Co., Milwaukee WI, USA)

3) The 2, 2'- azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany)

4) Phosphate buffer pH 8: di-Sodiumhydrogen ortho-phosphate dihydrate ($Na_2HPO_4.2H_2O$) and Sodiumdihydrogen ortho-phosphate ($NaH_2PO_4.2H_2O$) (Ajax Finechem, Sydney, Australia)

5) 96-well black microplates (Corning Inc., Corning NY, USA)

6) Fluorescence microplate reader (FLUOstar OPTIMA microplate reader, BMG Labtech, Offenburg, Germany)

**2. The effect of spray-drying on the properties of reconstituted modified whey protein concentrate**

A) Materials and equipments for the preparation of spray-dried modified WPC

1) Phosphorus pentoxide ($P_2O_5$; Aldrich Chemical Co., Milwaukee WI, USA)

2) Trehalose (kindly provided by the East Asiatic Company Ltd., Bangkok, Thailand) was used as carbohydrate excipient during spray-drying.

3) Mini Spray Dryer with two-fluid nozzle (B-190; Buchi, Flawil, Switzerland)

4) Karl Fischer titration (Metrohm 787 KF Titrino Karl Fisher**,** Herisau, Switzerland)

B) Chemical reagents for the determination of Trolox equivalent antioxidant capacity (TEAC)

1) 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Fluka Chemical Co, Buchs, Switzerland)

2) Phosphate buffered saline (PBS) from di-Sodiumhydrogen ortho-phosphate dihydrate ($Na_2HPO_4.2H_2O$), Sodiumdihydrogen ortho-phosphate ($NaH_2PO_4.2H_2O$) and Sodium chorine (NaCl) (Ajax Finechem, Sydney, Australia)

3) Potassium persulfate ($K_2S_2O_8$; Aldrich Chemical Co., Milwaukee, WI, USA)

4) Spectrophotometer Spectronic 20 (Spectronic Instruments, Inc, Rochester, NY, USA)

C) Materials and equipments for the determination the reactive amino groups (OPA method)

1) Deionized water
2) Dithiothreitol 99% (DTT; GE Healthcare Bio-Sciences AB, Sweden)
3) Ethanol 99% (Mallinckrodt Chemical Works, St. Louis, MO, USA)
4) *o*-phthaldialdehyde 97% (OPA; Sigma Chemical Co., St. Louis, MO, USA)
5) Sodium-dodecyl-sulfate (SDS; GE Healthcare Bio-Sciences AB, Sweden)
6) Sodium-tetraborate decahydrate (Ajax Finechem; Sydney, Australia)
7) Microplate reader (TECAN Infinite M 200 PRO, Männedorf, Switzerland)

D) Materials and equipments for the determination of free sulfhydryl groups

1) Distilled water
2) Urea Tris-Glycine were purchase from Ajax Finechem (Sydney, Australia)
3) Ellman's reagent ([5, 5'-dithiobis (2-nitrobenzoate)] (DTNB); Fluka Chemical Co, Buchs, Switzerland)
4) TECAN Infinite M200 PRO, Männedorf, Switzerland

E) Materials and equipments for size measurement

1) Double deionized water
2) Zetasizer Nano-ZS Instuments (ZEN 3600, Malvern Instrument Ltd., Worcestershire, UK)

F) Materials and equipments for cell culture

1) Eagle's minimum essential medium (MEM; Gibco®, Invitrogen, Carlsbad, CA, USA)

2) Fetal bovine serum (FBS; HyClone, Logan, UT, USA)

3) Flask and treat with 0.2 µm filtered (polystyrene filter, Corning, Corning, NY, USA)

4) Hep G2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

5) Potassium chloride cell culture tested (Sigma Chemical Co., St. Louis, MO, USA)

6) Potassium phosphate monobasic plant cell (Sigma Chemical Co., St. Louis, MO, USA)

7) Sodium bicarbonate cell culture tested (Sigma Chemical Co., St. Louis, MO, USA)

8) Sodium bicarbonate cell culture tested (Sigma Chemical Co., St. Louis, MO, USA)

9) Sodium chloride cell culture tested (Sigma Chemical Co., St. Louis, MO, USA)

10) Sodium phosphate cell culture tested (Sigma Chemical Co., St. Louis, MO, USA)

11) Syring Filter 25 mm. 0.2 um. (Corning Inc., *Corning*, NY, USA)

12) Trypan blue solution cell culture tested (Sigma Chemical Co., St. Louis, MO, USA)

13) Thioglycolate broth (Sigma Chemical Co., St. Louis, MO, USA)

14) Carbon dioxide incubator (CB 210 $CO_2$ Incubator, Binder,Tuttlingen, Germany)

G) Materials and equipments for cytotoxic activity tests

1) 96- well Polystyrene sterile plate (Corning Inc., Corning, NY, USA)

2) Dimethyl sulfoxide plant cell culture (DMSO; Sigma Chemical Co., St. Louis, MO, USA)

3) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, Carlsbad, CA, USA)

4) Trypsin from porcine pancreas cell; 1:250 (Sigma Chemical Co., St. Louis, MO, USA)

5) Carbon dioxide incubator (CB 210 $CO_2$ Incubator, Binder,Tuttlingen, Germany)

H) Efficacy of spray-dried modified whey protein concentrate (MWPC) in heated fructose-containing mixtures

1) Glycine and fructose were purchased from Ajax Finechem (Sydney, Australia)

2) Phosphate buffer pH 8: di-Sodiumhydrogen ortho-phosphate dihydrate ($Na_2HPO_4.2H_2O$) and Sodiumdihydrogen ortho-phosphate ($NaH_2PO_4.2H_2O$) (Ajax Finechem, Sydney, Australia)

3) Water bath stirrer (EYELA PS-100 Tokyo Rikakikai Co., Ltd., Tokyo, Japan)

I) Efficacy of spray-dried modified whey protein concentrate (MWPC) in sterilized reducing sugar-containing mixtures

1) Glycine, fructose, glucose and lactose were purchased from Ajax Finechem (Sydney, Australia)

2) Phosphate buffer pH 8: di-Sodiumhydrogen ortho-phosphate dihydrate ($Na_2HPO_4.2H_2O$) and Sodiumdihydrogen ortho-phosphate ($NaH_2PO_4.2H_2O$) (Ajax Finechem, Sydney, Australia)

3) Autoclave (Hirayama HA-300 MII, Japan)

## Methods

### 1. Effect of peptic modification on the characteristics of whey protein concentrate

A) The modification of whey protein concentrate

Commercial WPC was modified by pre-heat treatment followed by peptic treatment. Pre-heat treatment was performed by preparing 2% protein WPC solution in distilled water, heated in a water bath at 80±2 °C for 30 min at natural pH (~6.6) or at pH 2 (adjusted by 5.0 M HCl), cooled down to room temperature, and added with pepsin. The enzyme to substrate ratio of 1:17 was activated by 4 M urea and 3 M guanidine. Peptic treatment was carried out at pH 2.0 in a water bath at 37 °C for 1 h with constant stirring and inactivated by adjusting the pH to 8.0 by 2.0 M NaOH.

A 2% (w/v) protein of freshly modified WPC was reheated in a water bath at 80 °C for 30 min in the absence or presence of lactose at 0.6 and 1.2 mg/ml, cooled in an ice bath to room temperature. The molecular weight, brown pigment formation and antioxidant capacity was measured using methods described below.

B) The molecular weight profile of modified whey protein concentrate

Molecular weight profile of WPC was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970) using a Mini ProteanII equipment. Separating gel at 14% was used. WPC samples were prepared in eppendorf at the concentration of 1 mg $mL^{-1}$ in a sample buffer containing 1 mL 0.5 M Tris-HCl pH 6.8, 0.8 mL glycerol, 1.6 mL 10% SDS, 1% bromophenol blue in the presence or absence of β-mercaptoethanol (β-ME), then boiled in a water bath at 100 °C for 10 min. The centrifugation was done (5,000 rpm for 10 min) after cooling and 7 µL of protein samples were loaded into each well at the quantity of 0.007 mg protein per well. Gels were stained by Coomassie brilliant blue and destained by destaining solution.

C) Brown pigment formation in modified whey protein concentrate before and after reheating

UV-visible spectrophotometer was used in order to measure the brown pigment formation at 0.08% (w/v) protein by measuring the absorbance at 420 nm and 620 nm. Brown pigment formation was determined as $OD_{420}$ subtracted by $OD_{620}$ according to method described by Pan and Melton (2007).

D) The oxygen radical absorbance capacity assay (ORAC) before and after reheating

The oxygen radical absorbance capacity assay (ORAC) using fluorescein as the fluorescent probe as descrided by Ou *et al.* (2001) modified by Dávalos *et al.* (2004) was performed on WPC and its modified products before and after spray-drying. Briefly, 1.17 mM fluorescein stock solution was prepared by dissolving 0.0110 g fluorescein in 25 mL 75 mM phosphate buffer pH 7.4 (kept at 4 °C for less than 30 days). The working solution at 1.167 µM was prepared by using the 24.953 µL stock solution adjusted to 25 mL using 75 mM phosphate buffer pH 7.4. The 200 µM Trolox stock solution in a 75 mM phostphate buffer pH 7.4 was prepared and used as standard (0.5-8 µM). In a 96-well plate, 20 µL of 0.08 mg protein $mL^{-1}$ in a 75 mM phosphate buffer pH 7.4 was pipetted into each well. The working fluorescein 120 µL was added into the well to obtain the final concentration of 700 nM. The 40 mM AAPH was used as the radical and fed by a fluorescence microplate reader. The gain fluorescent intensity was read and the buffer was replaced for the sample and used as blank. The positive and negative controls of each sample were carried out by adding buffer to replace the radical and fluorescein respectively. After incubation at 37°C for 15 min, the fluorescence intensity was measured by FLUOstar at 485-P excitation and 520-P emission. The area under the fluorescence decay curve (AUC) was calculated as following equation (Prior *et al.*, 2003):

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 +, \ldots, + f_i/f_4) \times CT$$

Where CT was the cycle time (min), $f_i$ was fluorescence reading at cycle *i* and $f_4$ was the initial fluorescence reading at cycle 4. The average AUC was subtracted with the AUC of blank the obtained net AUC.

**2. The effect of spray-drying on the properties of reconstituted modified whey protein concentrate**

A) Preparation of spray-dried modified whey protein concentrate

The 6.25% (w/v) protein of the freshly modified WPC suspension was added with trehalose to obtain the final solid concentration of 20%. The protein-trehalose suspension was spray-dried in a spray-dryer by using two-fluid flow nozzle and co-current flow of B-190 Buchi mini spray dryer at feeding rate 4.0 ± 0.5 mL/min, inlet temperature 130 °C and outlet temperature 100 °C using the air flow rate of drying air around 600 normal L/h. The sample, designated as MWPC, was kept in a desiccator for 2 week at room temperature (~30°C) and measured for the moisture content by Karl Fischer titration. Spray-dried powder of MWPC was kept in an aluminium foil pouch in a dessicator containing $P_2O_5$ for about 15 days. After that, aluminum foil pouch was sealed and kept at -20 °C prior to reconstitution with 0.1 M phosphate buffer pH 8 and analysed using methods described below.

B) Characteristics of reconstituted modified whey protein concentrate

1) Trolox equivalent antioxidant capacity (TEAC)

Samples were measured for the radical scavenging capacity by the Trolox equivalent antioxidant capacity (TEAC) assay as described by Re *et al.*, 1999. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) and kept in the dark for 12-16 h. This $ABTS^{\bullet+}$ radical solution was diluted with 5 mM phosphate buffered saline (PBS) pH 7.4 to the absorbance at 734 nm of 0.7±0.02. The 30 µL of 0.25% protein was added into 3 mL of the $ABTS^{\bullet+}$ radical solution, then the reactions were allowed to proceed

at 30˚C for 4 min and the absorbance at 734 nm was read. The PBS was used as blank and 5 mM Trolox stock solution was used as standard. The Trolox equivalent antioxidant capacity was calculated as µmol Trolox equivalent (TE)/mg protein.

2) Determination of the exposed aromatic rings

The exposed aromatic rings were measured as UV absorbance at 280 nm of 0.03% protein solution in quartz cuvette (Aitken and Learmonth, 1996).

3) Determination of reactive amino groups

The reactive amino groups were determined by *o*-phtaldialdehyde (OPA) solution using method described by Nielsen *et.al.* (2001). OPA solution was composed of 200 mg SDS and 7.62 g sodium tetra-borate in 150 mL deionized water, followed by the addition of 97% OPA for 160 mg in 4 mL ethanol. The 176 mg of 99% dithiothreitol (DTT) was added and then adjust volume with deionized water to 200 mL. The 0.2 mL of 0.1% protein sample in distilled water was mixed with 2 mL OPA solution. Samples in quartz cuvette were measured for the absorbance at 340 nm after 2 minutes by TECAN micro plate reader (TECAN Infinite M200 PRO, Männedorf, Switzerland).

4) Determinations of free sulfhydryl groups

The free sulfhydryl group content was determined using method described by Beveridge *et.al.* (1974). The sample was diluted with distilled water to1% protein and added with 0.5 mL with 2.5 mL of 8 M urea Tris-glycine and 0.02 mL Ellman's reagent (5,5'-dithiobis(2-nitrobenzoate), DTNB). The solutions were then mixed for 2 min and measured for the absorbance at 420 nm. The absorbance was calculated as following equation (Beveridge *et al.*, 1974):

$$\mu M\ SH/g = (73.53 \times A_{412} \times D)/C$$

where $A_{412}$ = the absorbance at 412 nm; C = the sample concentration in mg solid/mL; D = the dilution factor; 73.53 is derived from $10^6/(1.36\times10^4)$; $1.36\times10^4$ is the molar absorptivity (Ellman, 1959) and $10^6$ is for conversions from the molar basis to the μM $mL^{-1}$ basis and from mg solid to g solid.

5) Particle size distribution

Particle size distribution of reconstituted 0.1% protein of WPC and MWPC reconstituted in double deionized water was measured by Zetasizer Nano using refractive index of 1.33 at room temperature.

6) Cell survival assay

The human hepablastoma HepG2 (ATTC HB-8065) cell was purchased from the American Type Culture Collection (ATCC, Rockville, MA, USA). The HepG2 were cultured in a flask and treated with 0.2 μm filtered (polystyrene filter, Corning, Corning, NY) Eagle's minimum essential medium (MEM; Gibco®, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and incubated under 37 °C, 5.0% $CO_2$ atmosphere conditions.

7) Cytotoxic activity tests

After incubation, the trypsinization was done by removing the medium and washed the cells with 3 mL phosphate buffered saline (PBS). After that, the PBS was removed and 3 mL trypsin solution was added. The HepG2 cells were incubated in trypsin at 37 °C for 5 min to digest the connecting tissue, giving rise to single cells. Total cell counting was done after staining with tryphan blue under microscope by using hemacytometer. Dead cells were stained with tryphan blue while survival cells were not. The density of dead and survival cells were calculated by average the amount of cells in the four corner squares of hemacytometer in the volume of a square. The $2\times10^4$ survived cells was seeded in each well of the sterile

96-well plate (Corning, Corning, NY) and incubated for 24 h. Sample was dissolved with 1% DMSO and adjusted the volume with MEM medium to reach the desired protein percentage. This sample medium solution was pipetted to into 24-hour-incubated cell after the old medium was removed. The 100 µL MWPC sample was added and incubated for 48 h. The sample was removed and washed with PBS. After that 100 µL MEM without phenol red and 10 µL MTT were filled in the dark and covered with foil. After incubation for 2 h, 100 µL SDS was added in the dark, cover with foil, and the sample was incubate again for 4 h to extract the formazan in cells. The dark purple was developed and measured by the absorbance at 570 nm. The non treated sample was used as the control of 100% survival.

C) Efficacy of spray-dried modified whey protein concentrate (MWPC) in heated fructose-containing mixtures

Spray-dried MWPC sample was reconstituted in 0.1 M phosphate buffer pH 8 containing 0.1 M fructose and in the absence or presence of glycine at the final protein concentration of 1% and 0.5%. The protein content of glycine to MWPC was 1:1. The fructose-containing mixture was heated in a water bath at 80, 85, 90 and 95 °C for 15, 30, 45 and 60 min. The samples were measured for brown pigment and AGEs formation using methods described below.

1) Brown pigment formation

The brown pigment formation of samples was measured by using 200 µL of sample and determined spectrophotometrically at 420 nm (Cämmerer, 1999) in a 96-well transparent flat bottom using microplate reader (TECAN Infinite M200 PRO, Männedorf, Switzerland).

2) Fluorescent AGEs formation

Fluorescent AGEs of samples were determined using 200 µL of sample in a 96-well transparent flat bottom by microplate reader (TECAN Infinite

M200 PRO) and using the excitation at 380 nm and emission at 465 nm The percentage of fluorescence unit was calculated by using $1gmL^{-1}$ in a 0.1 M $H_2SO_4$ as 100% (Morales and van Boekel, 1997).

D) Efficacy of spray-dried modified whey protein concentrate (MWPC) in sterilized reducing sugar-containing mixtures

The fructose-glycine, glucose-glycine and lactose-glycine, as well as fructose-WPC, glucose- WPC and lactose-WPC suspensions containing 0.1 M sugars were prepared in the absence or presence of 0, 0.25 and 0.50% MWPC. The sterilization (121 °C, 15 min) of sugar-protein suspension was carried out in 0.1 M phosphate buffer pH 8 using the concentration of N-source from glycine or WPC to MWPC as 0.50:0.50 and 0.25:0.25 percentage. The brown pigment were measured at the dilution of 25 times.

Statistical analysis

The experiments were carried out in two separate trials of spray-drying; each trial was run in triplicate. The data were analyzed by analysis of variance (ANOVA) with significance at $p \leq 0.05$. Significant differences among mean values were determined by Duncan's multiple range tests. All statistical analyses were performed using SPSS software Version 12.

# RESULTS AND DISCUSSION

## 1. Effect of peptic modification on the characteristics of whey protein concentrate

### 1.1 Effect of pre-heat treatment on the characteristics of whey protein concentrate

The major whey proteins shown in SDS-PAGE were β-lactoglobulin (MW~18 kDa) and α-lactalbumin (MW~14 kDa) (Figure 14). The commercial WPC used in this study also showed the bands having MWs of more than 65 kDa and between 45 to 55 kDa. Pre-heat treatment at 80 °C for 30 min (Figure 14, lane 2) resulted in the aggregation of proteins shown as sedimentation in the wells and the loss of the bands having MW between 45 – 50 kDa and that above 65 kDa. The presence of a new band of 32 kDa (Figure 14, lane 3) and the sedimentation may result from the aggregation of β-lactoglobulin and α-lactalbumin since the band intensity of β-lactoglobulin at 18 kDa and α-lactalbumin at 14 kDa was decreased.

The acidified and heated WPC showed disappearance of the 32 kDa band (Figure 14, lane 3). The bands of 50 kDa and above 65 kDa were present although they were not whey proteins originally found in milk. This showed that the acidified and heated WPC decreased the disulfide bond between 18 kDa β-lactoglobulin and 14 kDa α-lactalbumin and between the BSA fractions. Under reducing conditions, all aggregated proteins were dissociated. Nonetheless, the SDS-PAGE showed no apparent changes in the MWs of major whey proteins although the proteins were heated under acidification at pH of 2.0

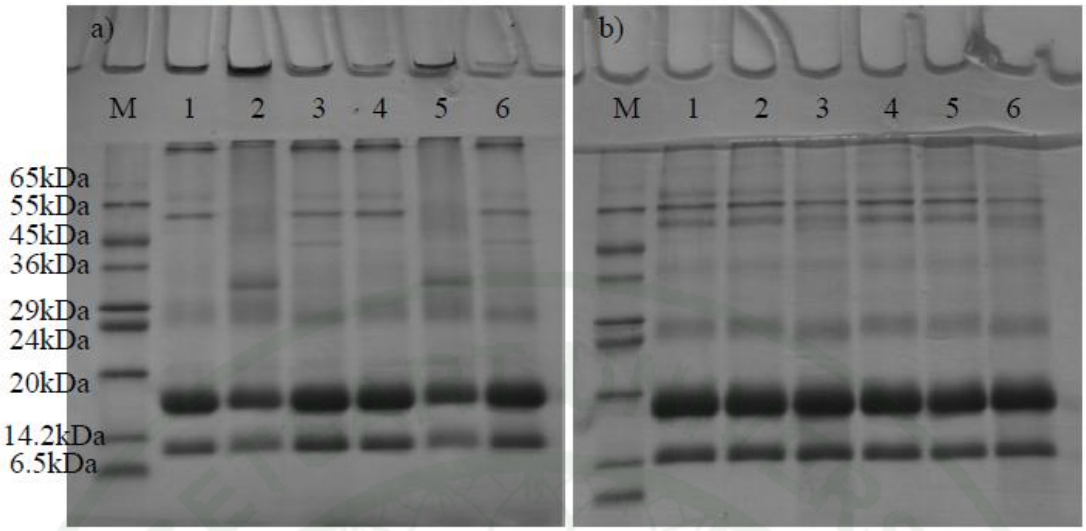



**Figure 14** Molecular weight profiles in SDS-PAGE of whey protein concentrate (WPC) and peptically modified whey protein concentrate obtained from different types of modification without β-mercaptoethanol (a) and with β-mercaptoethanol (b): lane M = low-range molecular weight marker; lane 1 = commercial WPC; lane 2 = heated WPC; lane 3 = acidified and heated WPC; lane 4 = peptically modified WPC; lane 5 = peptically modified WPC from heated WPC; and lane 6 = peptically modified WPC from acidified and heated WPC.

The acidified and heated WPC had the lowest brown pigment formation and the highest oxygen radical absorbance capacity (ORAC) as shown in Table 5. The acidic modification could retard the brown pigment formation during heating due to the effect of low pH that caused the $NH_2-$ group of side chain to protonate, therefore preventing the initial step of Maillard reactions to occur via covalent bond formation and the formation of Schiff base (Ajandouz and Puigserver, 1999). From the SDS-PAGE result, the decreasing aggregation between β-lactoglobulin and α-lactalbumin and between the BSA fractions via disulfide bond and the generation of the 45 kDa band were observed, which may lead to the increase in the ORAC of the acidified and heated WPC (Table 5). The increase of ORAC might be caused by the exposure of the reactive groups capable of hydrogen atom transfer (HAT).

**Table 5** Effect of pre-heat treatment on brown pigment formation and oxygen radical absorbance capacity of whey protein concentrate

| Types of WPC | Brown pigment formation of 0.08 % protein (w/v) (measured as $OD_{420}$) | Oxygen radical absorbance capacity (ORAC) (μmol TE/mg protein) |
|---|---|---|
| Commercial WPC | $0.111^{b} \pm 0.003$ | $0.196^{b} \pm 0.010$ |
| Heated WPC (80°C, 30 min) | $0.285^{a} \pm 0.047$ | $0.173^{b} \pm 0.030$ |
| Acidified and heated WPC (80°C, 30 min) | $0.062^{b} \pm 0.004$ | $0.319^{a} \pm 0.026$ |

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

1.2 Effect of peptic treatment on the characteristics of pre-heated whey protein products

There has been suggestion that whey proteins needed to be unfolded to become sensitive to the action of gut enzymes for a rapid and complete hydrolysis by pepsin and trypsin (Reddy *et al.*, 1988; Schmidt and van Markwijk, 1993). Evidently the hydrolysis of acidified and heated WPC by pepsin investigated in the present study did not alter the MWs of major whey proteins, particularly β-lactoglobulin and α-lactalbumin as shown in lanes 4 to 6 (Figure 14). The present study showed that the compact structure of whey proteins was quite stable to peptic digestion although the WPC was heated at 80 °C for 30 min at its natural pH or under acidic condition prior to peptic hydrolysis.

Even though the SDS-PAGE did not show apparent changes in the MWs of β-lactoglobulin and α-lactalbumin, peptic treatment further decreased brown pigment formation of commercial WPC, heated WPC and acidified and heated WPC

(Table 6). In addition, peptic modification greatly increased oxygen radical absorbance capacity of all whey protein products (Table 7). This result indicated that whey protein was not modified by peptic hydrolysis to generate the low molecular weight proteins/peptides. The increasing ORAC might be due to the alterations in the molecular structure of whey proteins, which resulted in the more capability in transferring hydrogen atom or subsequent radical dimerization from sulfur amino or attack the radicals by the exposed aromatic amino acid (Elias *et al.*, 2008).

**Table 6** Effect of peptic treatment on brown pigment formation of whey protein products

| Types of WPC | Brown pigment formation ($OD_{420}$) of 0.08% protein (w/v) | |
|---|---|---|
| | Before peptic treatment | After peptic treatment |
| Commercial WPC | $0.111^{b}$±0.003 | $0.047^{c}$±0.003 |
| Heated WPC (80°C, 30 min) | $0.285^{a}$±0.047 | $0.055^{c}$±0.011 |
| Acidified and heated WPC (80°C, 30 min) | $0.062^{c}$±0.004 | $0.048^{c}$±0.002 |

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

**Table 7** Effect of pre-heating and peptic treatment on oxygen radical absorbance capacity

| Types of WPC | Oxygen radical absorbance capacity (μmol TE/mg protein) | |
|---|---|---|
| | Before peptic treatment | After peptic treatment |
| Commercial WPC | $0.196^{c}$ ±0.010 | $0.549^{ab}$ ±0.027 |
| Heated WPC (80˚C, 30 min) | $0.173^{c}$ ±0.030 | $0.691^{a}$ ±0.156 |
| Acidified and heated WPC (80˚C, 30 min) | $0.319^{bc}$ ±0.026 | $0.751^{a}$ ±0.165 |

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

The modification of whey proteins by pepsin significantly increased ORAC of whey protein products ($p \leq 0.05$, Table 7). Nonetheless, the peptically hydrolysed whey proteins appeared to be different, depending on the pre-heat treatment employed to WPC prior to peptic hydrolysis. When the acidified and heated WPC was peptically hydrolysed and further re-heated at pH 8.0, 80 °C for 30 min in the presence of lactose, the brown pigment formation was prevented (Table 8). The peptically hydrolysed commercial WPC and peptically hydrolysed heated WPC, however, were more prone to brown pigment formation after being re-heated at 80 °C for 30 min compared to the peptically hydrolysate from acidified and heated WPC.

**Table 8** Effect of re-heating (80˚C, 30 min) and additional lactose concentration on brown pigment formation of peptically hydrolysed whey proteins prepared from different types of WPC

| Re-heat treatment | Added lactose concentration ($mg\ mL^{-1}$) | Brown pigment formation (measured as $OD_{420}$) of 0.08% protein (w/v) of peptically hydrolysed whey protein products from | | |
|---|---|---|---|---|
| | | Commmercial WPC | Heated WPC (80˚C, 30 min) | Acidified and heated WPC (80˚C, 30 min) |
| Before re-heating | 0 | $0.047^{d} \pm 0.003$ | $0.055^{cd} \pm 0.011$ | $0.048^{d} \pm 0.002$ |
| After re-heating | 0 | $0.099^{a} \pm 0.009$ | $0.073^{b} \pm 0.002$ | $0.053^{cd} \pm 0.003$ |
| | 0.6 | $0.090^{a} \pm 0.015$ | $0.074^{b} \pm 0.002$ | $0.050^{cd} \pm 0.002$ |
| | 1.2 | $0.097^{a} \pm 0.009$ | $0.065^{bc} \pm 0.009$ | $0.050^{cd} \pm 0.002$ |

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

Prior to re-heat treatment, the peptically hydrolysates from commercial WPC, heated WPC and acidified and heated WPC had fairly high ORAC of above 0.5 µmol TE/mg protein (Table 9). However, re-heat treatment, in the absence or in the presence of lactose, lowered the ORAC of the peptically hydrolysed whey protein products significantly ($p \leq 0.05$).

**Table 9** Effect of re-heating (80˚C, 30 min) and additional lactose concentration on oxygen radical absorbance capacity of peptically hydrolysed whey proteins prepared from different types of WPC

| Re-heat treatment | Added lactose concentration ($mg\ mL^{-1}$) | Oxygen radical absorbance capacity (μmol TE/mg protein) of peptically hydrolysed whey protein products from | | |
|---|---|---|---|---|
| | | Commercial WPC | Heated WPC (80˚C, 30 min) | Acidified and heated WPC (80˚C, 30 min) |
| Before re-heating | 0 | $0.549^{b}$ ±0.027 | $0.691^{ab}$ ±0.156 | $0.751^{a}$ ±0.165 |
| After re-heating | 0 | $0.293^{c}$ ±0.016 | $0.327^{c}$ ±0.006 | $0.272^{c}$ ±0.112 |
| | 0.6 | $0.267^{c}$ ±0.009 | $0.310^{c}$ ±0.009 | $0.282^{c}$ ±0.080 |
| | 1.2 | $0.282^{c}$ ±0.008 | $0.315^{c}$ ±0.007 | $0.274^{c}$ ±0.110 |

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

Although WPC was not hydrolysed to the small MW peptides, the modification by pepsin using acidified and heated WPC as the substrate was able to increase the ORAC. This modification also retarded brown pigment formation when the solution was re-heated at 80 °C, 30 min. It is possible that the alterations in ORAC and brown pigment formation were due to the alterations in the molecular structure of whey proteins, which exposed more reactive groups responsible for the oxygen radical scavenging, rather than due to the influence of hydrolysis of whey proteins to the small MW peptides.

**2. The effect of spray-drying on the properties of reconstituted modified whey protein concentrate**

2.1 Characteristics of reconstituted modified whey protein concentrate

Although modification of acidified and heated WPC by pepsin did not apparently change the MWs of whey proteins (Figure 15, lane 3), SDS-PAGE showed that spray-drying of modified whey proteins caused the hydrolysis of most proteins, particularly α-lactalbumin, to the smaller peptides of less than 6 kDa (Figure 15, lane 4). β-lactoglobulin (18 kDa) showed the least hydrolysis, observed as less intensed band after spray-drying, compared to that before spray-drying. This suggested that further hydrolysis occurred during spray-drying although the pepsin was inactivated by pH adjustment to pH of 8.0 prior to spray-drying. Nonetheless, it should be noted that α-lactalbumin, of which the tertiary structure was held by intramolecular disulfide bond and $Ca^{2+}$-crosslinking (Hiraoka *et al*., 1980; Vanaman *et al*., 1970) was prone to further hydrolysis during spray-drying and entirely hydrolysed. This was probably due to the loss of $Ca^{2+}$-ion during peptic hydrolysis at pH of 2.0

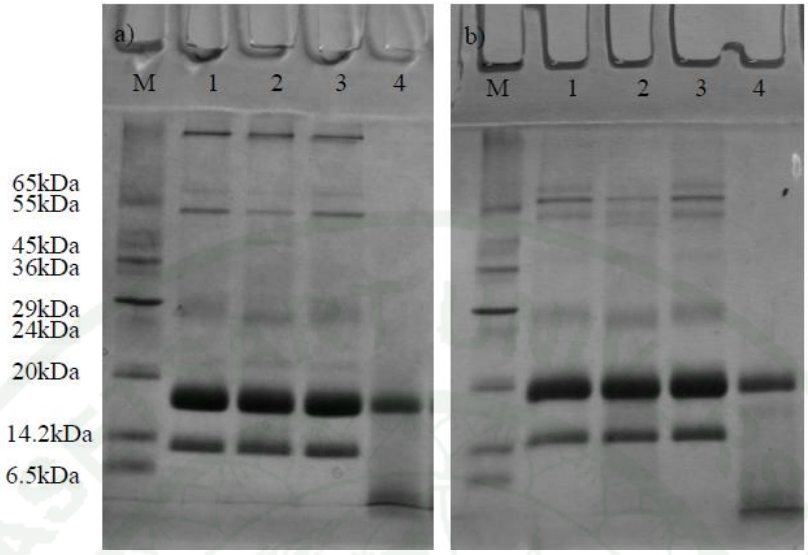



**Figure 15** Molecular weight profiles of spray-dried WPC and spray-dried modified WPC (acidified and heated WPC prior to peptic hydrolysis) without β-mercaptoethanol (a) and with β-mercaptoethanol (b): lane M = low-range molecular weight marker; lane 1 = commercial WPC; lane 2 = spray-dried WPC in trehalose excipient; lane 3 = freshly modified WPC; lane 4 = spray-dried modified WPC in trehalose excipient.

Nonetheless, the size distributions of reconstituted unmodified WPC (commercial WPC), WPC spray-dried with trehalose and MWPC (peptically hydrolysed heated and acidified WPC), before and after spray-drying, were quite similar in terms of the majority of the population (Figure 16). Most whey protein particles had the length-scale size of around 100 – 200 nm despite the hydrolysis of whey proteins shown in Figure 16. This was probably due to the aggregation of whey protein molecular at pH 7.45.

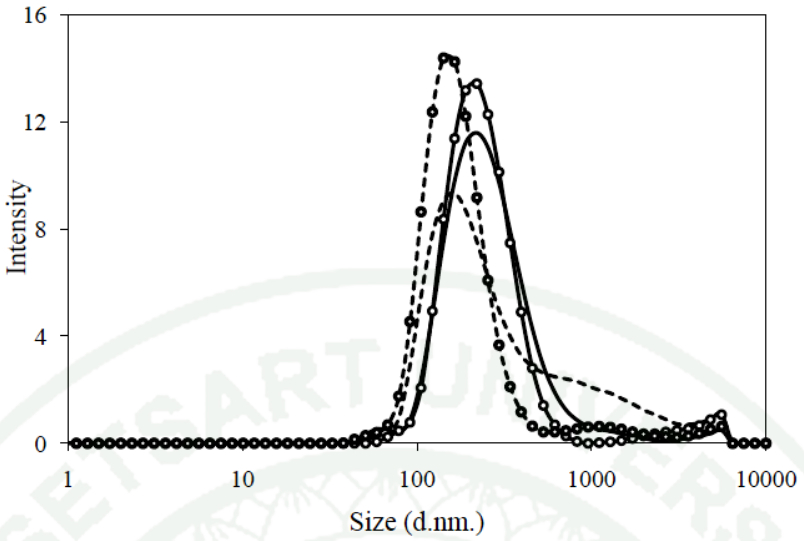



**Figure 16** Effect of modification and spray-drying on size distribution of whey protein products: ——— commercial WPC; – – – Reconstituted spray-dried WPC in trehalose excipient; —O— freshly modified WPC before spray-drying; – O – reconstituted MWPC spray-dried in trehalose excipient.

After spray-drying, the MWPC had lower exposed aromatic side chain and showed drastic decrease in sulfhydryl group content (Table 10). The reactive amino group was greatly increased after spray-drying (Table 10), which coincided with the lower MWs of whey proteins (Figure 15).

Pepsin hydrolysed protein by cleaving the peptide bond linking aromatic acid residues with the others. It is likely that the peptides self-associated though hydrophobic interactions among the terminal aromatic side chain. Therefore, the exposed aromatic amino acid was decreased. Despite the reduction in exposed aromatic side chain and sulfhydryl group in MWPC after spray-drying, the reconstituted MWPC had an increased $ABTS^{\bullet+}$ radical scavenging capacity, measured as Trolox equivalent antioxidant capacity (TEAC), for about two times compared to the commercial WPC before and after spray-drying and the MWPC before spray-drying. Trolox equivalent antioxidant capacity (TEAC) is base on electron transfer (ET) mechanism which reflected the ability of aromatic ring to scavenge free radical.

Results suggested that although the hydrolysates self-associated though aromatic ring, the ability of MWPC to transfer electron to $ABTS^{\bullet+}$ was increased after modification.

**Table 10** Effect of spray-drying on reactive groups of whey protein products

| Treatment | TEAC (µmol Trolox/mg protein) | Exposed aromatic amino acid ($OD_{280nm}$) | Reactive amino groups ($OD_{340nm}$) | Free SH groups (µM SH/g protein) |
|---|---|---|---|---|
| Commercial WPC | $0.144^{c}$±0.004 | $0.510^{b}$±0.004 | $0.437^{b}$±0.066 | $12.79^{a}$±1.40 |
| Commercial WPC spray-dried in trehalose excipient | $0.264^{b}$±0.027 | $0.540^{a}$±0.007 | $0.467^{b}$±0.075 | $14.45^{a}$±1.55 |
| Freshly prepared MWPC | $0.234^{b}$±0.015 | $0.470^{c}$±0.013 | $0.472^{b}$±0.064 | $12.94^{a}$±1.49 |
| MWPC spray-dried in trehalose excipient | $0.464^{a}$±0.007 | $0.396^{d}$±0.014 | $0.754^{a}$±0.136 | $6.17^{b}$±0.17 |

Means followed by different superscripts within column are significantly different ($p \leq 0.05$).

The small MWs of whey proteins after peptic modification and spray-drying decreased sulfhydryl group, which is essential precursor of glutathione synthesis in cell. Figure 17 illustrates the cytotoxic effect of reconstituted MWPC on hepablastoma Hep G2 by MTT test. It was found that the survival of hepatic cancer cells was reduced to 50%, the so-called 50% inhibition concentration ($IC_{50}$), when MWPC spray-dried in trehalose excipient was added in growing media above 1.8

mg/mL. Although Hongsprabhas *et al*. (2011) have demonstrated earlier that HepG2 was quite tolerant against MRPs cytotoxicity induced by heated WPC, it is possible that the modification of WPC investigated in this study, which caused the drastic loss of sulfhydryl group and the formation of MRPs, lowered the survival of HepG2.

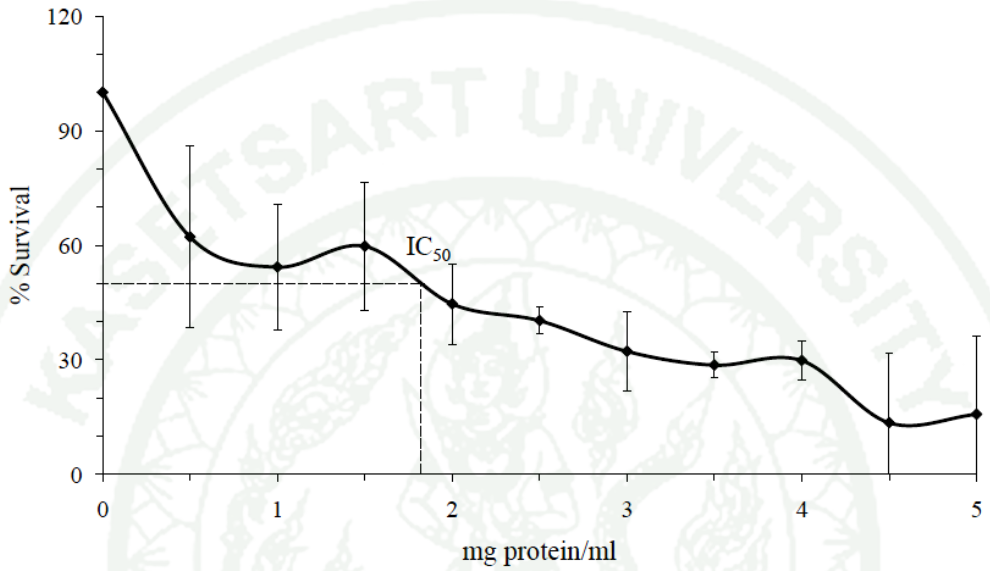



**Figure 17** Cytotoxicity of reconstituted MWPC on hepablastoma HepG2. Bars represent standard deviation.

Despite the loss in sulfhydryl group, which is the most probable reactive group responsible for hydrogen atom transfer in oxygen radical absorbance assay and the lowering of hepablastoma HepG2 survival, the MWPC was further investigated for its efficacy as antibrowning agent in thermally processed food systems as reported in the following sections. This is to explore the potential use of MWPC as antibrowning agent and further investigate the mechanisms involved in antibrowning activities by the MWPC prepared in the present study. However, the influences of reconstituted MWPC spray-dried in trehalose excipient on cytotoxicity in cancer and normal cells merit further investigation whether the MWPC could be used as antiglycation compound in the biological systems.

2.2 Efficacy of spray-dried modified whey protein concentrate (MWPC) in heated fructose-containing mixtures

The brown pigment formation of fructose (Figure 18) indicated the influence of browning by non-Maillard type of reaction. Del Pilar Buera *et al.* (1987) revealed that even though the water activity was high as 0.90 and the temperature was about 55 °C, the caramelization could occur. Therefore, brown pigment formation of heated fructose was likely due to both Maillard reactions and caramelization.

Modification of WPC by peptic hydrolysis of acidified and heated WPC and spray-drying increased the $NH_2-$ group, in combination with the loss of SH– group. Both of which are crucial reactants for Maillard reactions since they can form Schiff base during the early stage of Maillard reactions and rearrange to Amadori or Heyns products. Figure 18 illustrates that prolong heating of fructose, fructose-glycine, fructose-MWPC and fructose-glycine-MWPC in 0.1M phosphate buffer pH 8 increased AGEs formation (Figure 18 (a)) but not significantly altered brown pigment formation (Figure 18 (b)). The heated fructose-0.50%glycine mixture had the highest AGEs formation, followed by fructose-0.50%glycine-0.50%MWPC and fructose-0.25%glycine-0.25%MWPC after heating at 80 °C (Figure 18 (a)). During heating at 80 °C, all mixture turned brown due to the presence of fructose of 0.1 M, which favored Maillard reactions.

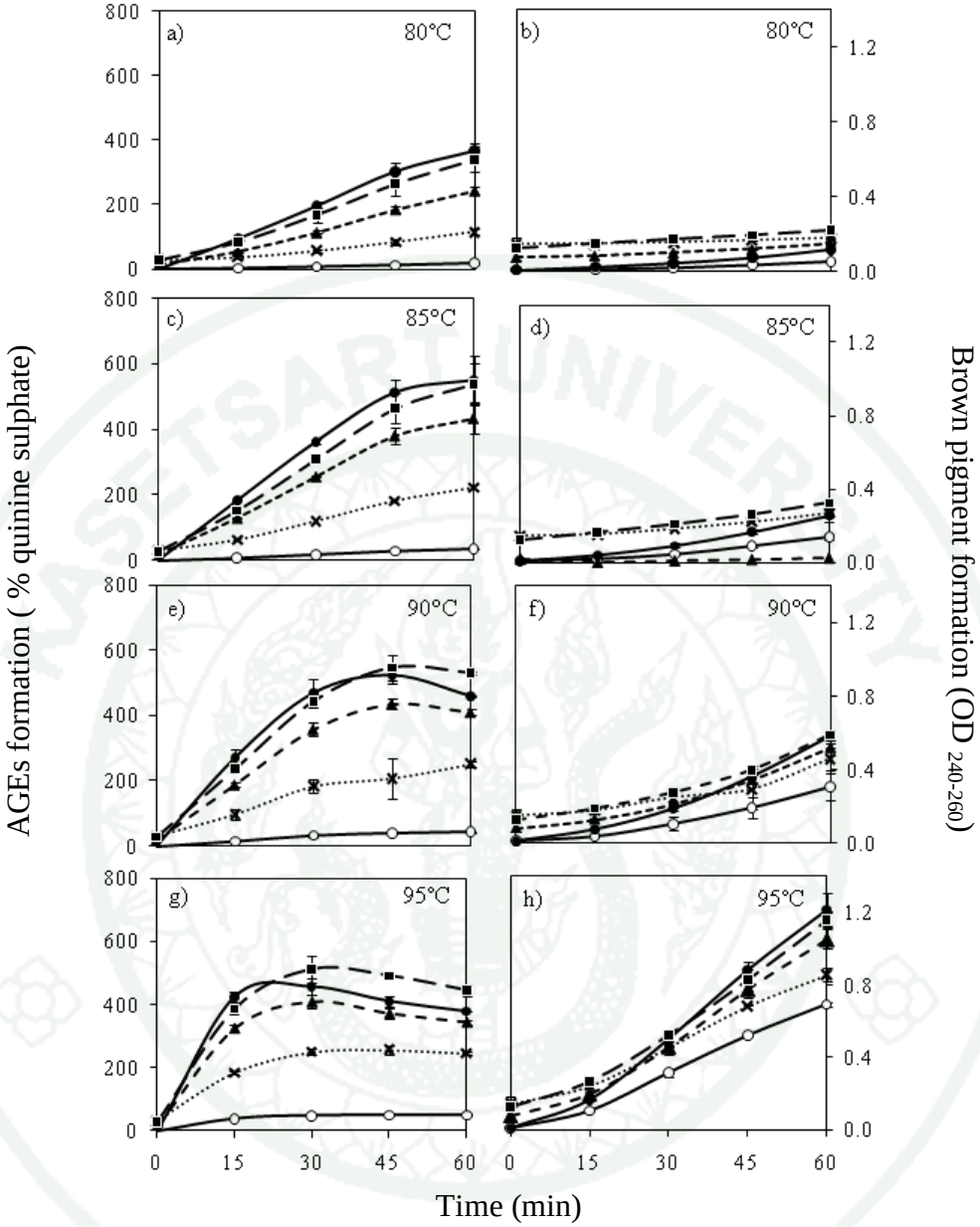



**Figure 18** Effect of glycine and MWPC addition on the formation of advanced glycation end-products (AGEs) (left) and brown pigment (right) after heating with fructose at 80 °C (a and b), 85°C(c and d), 90°C(e and f), and 95°C (g and h). —○— 0.1M fructose; —●— 0.1M fructose + 0.50% glycine; ···×··· 0.1M fructose + 0.50%MWPC; – ▲ – 0.1M fructose + 0.25%glycine + 0.25% MWPC; –■– 0.1M fructose + 0.50%glycine + 0.50% MWPC

The reaction rate of the AGEs and brown pigment formation were accelerated when the temperature was increased from 80 to 95 °C. AGEs formation of fructose-0.50%glycine at 80 °C reached 400% quinine sulphate in 60 min. In addition, the AGEs reached maximum between 400-600% faster at high temperatures. The AGEs formation of fructose-0.50%glycine mixture were constant and converted to brown pigment when AGEs content reached about 600% quinine equivalent after heating at 85 °C for 45 min (Figure 18 (c and d)). The AGEs were converted to brown pigment after being heated up to 45 min at 90 °C, observed as the the decline of AGEs and high content brown pigment (Figure 18 (e and f)). Fructose-0.50%MWPC turned to AGEs and brown pigment at slower rate than did fructose-0.50%glycine. The mixture of fructose-glycine-MWPC has mixed characters of glycine and MWPC.

When the mixtures were heated at 95 °C, the fructose-0.50%glycine mixture had the highest AGEs content, followed by fructose-glycine-MWPC (at both concentration) and fructose-MWPC (Figure 18 (g)). The highest AGEs contents were observed between 15 – 30 min of heating. After 30 min, the AGEs in fructose-0.50%glycine and fructose-glycine-MWPC started to decline. However, the AGEs in fructose-0.50%MWPC remained constant after 30 min of heating. The drastic increase in brown pigment formation of all mixtures was observed after 15 min heating. This suggested that the AGEs formed at 95 °C could convert to brown pigments after being heated for 15 min.

The low content of AGEs in the presence of MWPC may result from the inhibition of AGEs formation or the progress of AGEs to brown pigment formation. Figure 18 (g) indicated that the heated fructose-MWPC undergone the least brown pigment formation and the least accumulation of AGEs. This suggested that the MWPC inhibit the brown pigment formation most likely by inhibiting the formation of AGEs.

2.3 Efficacy of spray-dried modified whey protein concentrate (MWPC) in sterilized reducing sugar-containing mixtures

The effect of antibrowning activity of MWPC on the inhibition of brown pigment formation in sterilized mixtures containing different sugars was shown in Table 11. Of all sugars investigated, fructose was most effective sugar that formed brown pigment with glycine, followed by glucose and lactose. The presence of MWPC decreased the brown pigment formation of sterilized mixed suspension regardless of reducing sugars used. At 0.50% concentration of N-source, the sterilized glycine-sugar mixtures had the highest brown pigment formation. Substitution of glycine with 0.25% MWPC lowered the brown pigment formation.

**Table 11** Effect of glycine and MWPC addition on brown pigment formation of reducing sugar-containing mixture after sterilization (121 °C, 15 min)

| N-source | | MWPC | Browning pigment formation (measured as $OD_{420}$ of 25 time dilution) | | |
|---|---|---|---|---|---|
| Glycine | Commercial WPC | | 0.1M Fructose | 0.1M Glucose | 0.1M Lactose |
| None | None | None | $0.185^{e}\pm0.009$ | $0.172^{de}\pm0.001$ | $0.095^{d}\pm0.023$ |
| None | None | 0.50% | $0.248^{cd}\pm0.011$ | $0.189^{de}\pm0.002$ | $0.137^{d}\pm0.011$ |
| 0.50% | None | None | $0.448^{a}\pm0.027$ | $0.384^{a}\pm0.005$ | $0.295^{a}\pm0.030$ |
| None | 0.50% | None | $0.215^{de}\pm0.023$ | $0.171^{de}\pm0.005$ | $0.122^{d}\pm0.002$ |
| 0.25% | None | 0.25% | $0.286^{c}\pm0.000$ | $0.225^{c}\pm0.010$ | $0.181^{c}\pm0.017$ |
| None | 0.25% | 0.25% | $0.213^{de}\pm0.004$ | $0.160^{e}\pm0.000$ | $0.107^{d}\pm0.007$ |
| 0.50% | None | 0.50% | $0.364^{b}\pm0.002$ | $0.307^{b}\pm0.004$ | $0.234^{b}\pm0.010$ |
| None | 0.50% | 0.50% | $0.171^{e}\pm0.045$ | $0.131^{f}\pm0.025$ | $0.120^{d}\pm0.031$ |

Means within the same column followed by different superscripts are significantly different ($p\leq0.05$).

# CONCLUSIONS AND RECOMMENDATIONS

The oxygen radical absorbance capacity (ORAC) and ABTS• radical scavenging capacity (TEAC) in whey proteins, which indicates capacity of MWPC in transferring hydrogen atom and electron to free radicals, respectively, were improved by peptic treatment of acidified and heated WPC and spray-drying. The modification drastically decreased exposed sulfhydryl (SH) group and the exposed aromatic side chain; while increasing the amino group content although it did not entirely hydrolyse whey proteins to the small MW peptides.

The α-lactalbumin was selectively hydrolysed to the small MW peptides using peptic modification and spray-drying. The survival of HepG2 was decreased despite of an increase in oxygen radical absorbance capacity and ABTS• radical scavenging capacity. Less cell survived in the presence of MWPC was probably due to the loss of cysteine in MWPC, which is the precursor in the synthesis of glutathione, the natural antioxidant used at cellular level. However, the cell death mechanisms of HepG2 induced by MWPC spray-dried in trehalose excipient required further investigation for the influence of AGEs and MRPs.

When the reconstituted MWPC was added in the thermally processed glycine-reducing sugar mixtures, it was found that MWPC could retard brown pigment formation by inhibiting the formation of AGEs. Although an increase in $NH_2$– group after modification process increased the susceptibility of whey proteins to glycation compared to the commercial WPC by providing more reactive amino group for Maillard reactions to occur, the MWPC was effectively able to inhibit brown pigment formation even under sterilization scheme. Nonetheless, further investigations on the mechanisms in antibrowning ability of MWPC in thermally process foods containing different sugars are needed.

# LITERATURE CITED

Aitken, A. and M. Learmonth. 1996. Protein determination by UV absorption, pp 3-6. *In* J. M. Walker, ed. **Protein Protocols Handbook,** 2nd ed. Humana Press Totowa, N.J.

Ajandouz, E. H and A. Puigserver. 1999. Nonenzymatic browning reaction of essential amino acids: Effect of pH on caramelization and Maillard reaction kinetics. **J. Agric. Food Chem.** 1 (15): 928-943.

Ames, B. N., M. K. Shigenaga and T. M. Hage. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. **Proc. Nat. Acad. Sci. USA** 90: 7915–7922.

Ames, J. M. 1990. Control of the Maillard reaction in food systems. **Trends Food Sci. Tech.** 1: 150–154.

Anisman, H. and R. M. Zacharko. 1991. Multiple neurochemical and behavioral consequences of stressors: implications for depression, pp. 57–82. *In* S. E. File, ed. **Psychopharmacology of Anxiolytics and Antidepressants**. Pergamon, NY.

Antila, P., I. Paakari, A. Järvinen, M. J. Mattila, M. Laukkanen, A. Pihlanto–Leppälä, P. Mäntsälä and J. Hellman. 1991. Opioid peptides derived from in–vitro proteolysis of bovine whey proteins. **Int. Dairy J**. 1: 215–229.

AOAC. 2000. Official Methods of Analysis, 17th ed. **Association of Official Analytical Chemists**. Arlingtion, Virginia.

Arno, M. B. 2000. Some methodological problems in the determination of antioxidant acitivity using chromogen radicals: a practical case. **Trends Food Sci. Tech.** 11: 419–421.

Badoud, R., L. B. Fay, F. Hunston and G. Pratz. 1995. Periodate oxidative degradation of Amadori compounds: formation of N[epsilon]–carboxymethyllysine and N carboxymethylamino acids as markers of the early Maillard reaction, pp. 208–220. *In* TCh Lee, H. J. Kim, eds. **Chemical Markers for Processed and Stored Foods**. ACS Symposium Series 631, Chicago, Illinois, US.

Belitz, H. D. and W. Grosch. 1997. **Food Chemistry**, 2nd ed. Springer, Berlin, Germany.

_____and_____. 1999. *In* **Food Chemistry**, 2nd ed. Springer–Verlag, Berlin, Germany.

_____and_____ and P. Schieberle. 2004. **Food Chemistry**, 3 rd ed. Springer, Berlin, Germany.

Bernal, V. and P. Jelen. 1984. Effect of calcium binding on thermal denaturation of bovine α–lactalbumin. **J. Dairy Sci.** 67: 2452– 2454.

Beveridge, T., S. J. Toma and S. Nakai. 1974. Determination of SH and S–S groups in some food proteins using Ellman's reagent. **J. Food Sci.** 39: 49–51.

Bigl, K., F. Gaunitz, A. Schmitt, S. Rothemund, R. Schliebs, G. Munch and T. Arendt. 2008. Cytotoxicity of advanced glycation endproducts in human micro– and astroglial cell lines depends on the degree of protein glycation. **J. Neural Transmission.** 115: 1545–1556.

Borek, C. 2001. Antioxidant health effects of aged garlic extract. **J. Nutrition.** 131: 1010S–1015S.

Bos, O. J. M., J. F. A. Labro, M .J. E. Fischer, J. Witling and L. H. M. Janssen. 1989. The molecular mechanism of the neutral–to–base transition of human serum albumin. **J. Biol. Chem.** 264: 953–959.

Brands, C. M., G. M. Alink, M. A. van Boekel and W. M. Jongen. 2000. Mutagenicity of heated sugar–casein systems: effect of the Maillard reaction. **J. Agric Food Chem.** 48(6): 2271–5.

Brew, K. and J. A. Grobler. 1992. α–Lactalbumin, pp 191–229. *In* P. F. Fox, ed. **Adv. Dairy Chem.** Elsevier Appl. Sci., London, England.

_____, F. J. Castellino, T. C. Vanaman and R. L. Hill. 1970. The complete amino acid sequence of bovine α–lactalbumin. **J. Biol. Chem.** 10: 4570–4582.

Brownlow, S., J. H. Morais Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. North and L. Sawyer. 1997. Bovine beta–lactoglobulin at 1.8 Å resolution–still an enigmatic lipocalin. **Structure** 5: 481–495.

Cämmerer, B., B. L. Wedzicha and L. W. Kroh. 1999. Nonenzymatic browning reactions of retro–aldol degradation products of carbohydrates. **Eur. Food Res. Tech.** 209: 261–265.

Chan, K. M., E. A. Decker, J. B. Lee and D. A. Butterfield. 1994. EPR spin-trapping studies of the hydroxyl radical scavenging ability of carnosine and related dipeptides. **J. Agric Food Chem.** 42: 1407–1410.

Chatterton, D. E. W., G. Smithers, P. Roupas and A. Brodkorb. 2006. Bioactivity of β–lactoglobulin and α–lactalbumin–technological implications for processing. **Int. Dairy J**. 16: 1229–1240.

Chen, C. H., A. M. Pearson and J. L. Gray. 1992. Effect of synthetic antioxidants (BHA, BHT and PG) on the mutagenicity of IQ–like compounds. **Food Chem.** 43: 177–183.

Creamer, L. K. and A. K. H. MacGibbon. 1996. Some recent advances in the basic chemistry of milk proteins and lipids. **Int. Dairy J.** 6: 539–568.

Creamer, L. K., D. A. D. Parry and G. N. Malcolm. 1983. Secondary Structure of bovine β–lactoglobulin B. **Arc. Biochem. Biophys.** 227: 98–105.

Dávalos, A., C. Gómez–Cordovés and B. Bartolomé. 2004. Extending applicability of oxygen radical absorbance capacity (ORAC–Fluorescein) assay. **J. Agric. Food Chem. 52:** 48–54.

Davies, C. G. A. and B. L. Wedzicha. 1994. Ascorbic acid browning: The incorporation of C1 from ascorbic acid into melanoidins. **Food Chem.** 49: 165–167.

del Pilar Buera, M., J. Chirife, S. L. Resnik and R. D. Lozano. 1987. Nonenzymatic browning in liquid model systems of high water activity: Kinetics of color changes due to caramelization of various single sugars. **J. Food Sci.** 52(4): 1059–1062.

de Oliveira, O. C., I.B. Valentim, C. A. Silva, E. J. H. Bechara, M. P. De Barros, C. M. Mano and M. O. F Goulart. 2009. Total phenolic content and free radical scavenging activities of methanolic extract powders of tropical fruit residues. **Food Chem**. 115: 469–475.

de Wit, J. N. and G. Klarenbeek. 1984. Effects of various heat treatments on structure and solubility of whey proteins. **J. Dairy Sci.** 67: 2701–2710.

Dolgikh, D. A., R. I. Gilmanshin, E. V. Brazhnikov, V. E. Bychkova, G. V. S emisotnov, S. Y. Venyaminov and O. B. Ptitsyn. 1981. α– Lactalbumin: compact state with fluctuating tertiary structure? **FEBS Letters** 136: 311–315.

Dubeau, S., P. Bourassa, T. J. Thomas and H. A. Tajmir–Riahi. 2010. Biogenic and synthetic polyamines bind bovine serum albumin. **Biomacromolec.** 11(6): 1507–1515.

Elias, R. J., S. S. Kellerby and E. A. Decker. 2008. Antioxidant activity of proteins and peptides. **Crit. Rev. Food Sci. Nutrition. 48**: 430–441.

Ellman, G. D. 1959. Tissue sulfhydryl groups. **Arc. of Biochem. Biophys.** 82: 70–72.

Feather, M. S., T. G. Flynn, K. A. Munro, T. J. Kubiseski and D. J. Walton. 1995. Catalysis of reduction of carbohydrate 2–oxoaldehydes (osones) by mammalian aldose reductase and aldehyde reductase. **Biochim. Biophys. Acta** 1244: 10–16.

Fennema, O. R. 1976. **Food Chemistry**. 2nd ed. Marcel Dekker, NY.

Figge, J., T. H. Rossing and V. Fencl. 1991. The role of serum–proteins in acid–base equilibria. **J. Lab. Clin. Med.** 117: 453–467.

Foster, J. F. 1997. **Albumin, Structure, Function and Uses**. Academic Press Inc., USA.

Fox, P. F. 1989. The milk protein system, pp. 1–53. *In* P. F. Fox, ed. **Developments in Dairy Chemistry Vol. 4**. Elsevier Applied Science, NY.

Fox, P. F. 1995. **Heat Induced Changes in Milk**. 2nd ed. International Dairy Federation, Brussels, Belgium.

Friedman, M. 2005. Biological effects of Maillard browning products that may affect acrylamide safety in food. **Chemistry and Safety of Acrylamide in Food.** 561: 135–156.

Friedman, M. and I. Molnar–Perl. 1990. Inhibition of browning by sulfur amino Acids. 1. Heated amino acid–glucose systems. **J. Agric. Food Chem.** 38: 1642–1647.

Gaby, A. R. 2005. Adverse effects of dietary fructose. **Alternat. Med. Rev.** 10: 294–306.

Gezimati, J., H. Singh and L. K. Creamer. 1996. Heat–induced interactions and gelation of mixtures of bovine $\alpha$–lactoglobulin and serum albumin. **J. Agric. Food Chem.** 44: 804–810.

Gillatt, P. N., and J. B. Rossell. 1992. The interaction of oxidized lipids with proteins, pp. 65–118. *In* F.B. Padley, ed. **Advances in Applied Lipid Research.** JAI Press, London, UK.

Girard, M., S. L. Turgeon and S. F. Gauthier. 2003. Quantification of the interactions between beta lactoglobulin and pectin through capillary electrophoresis analysis. **J. Agric. Food Chem**. 51: 6043–6049.

Greenshields, R. N. 1973. Caramel—Part 2: Manufacture, composition and property. **Process Biochem**. 8(4): 17–20.

Groves, M. L., N. J. Hipp and T. L. McMeekin. 1951. Effect of pH on the denaturation of β–lactoglobulin and its dodecyl sulfate derivative. **J. Am. Chem. Soc.** 73: 2790.

Gutteridge, J. M. C. and B. Halliwell. 2010. Antioxidants: Molecules, medicines and myths. **Biochem. Biophys. Res. Commun.** 393: 561–564.

Halliwell, B. 2002. Food–derived antioxidants: How to evaluate their importance in food and in vivo. *In* E. Cadenasand L. Packer, eds. **Handbook of Antioxidants.** Taylor and Francis group, CRC press, Boca Raton, FL, USA.

Halliwell, B. and J. M. C Gutteridge. 1989. **Free Radicals in Biology and Medicine**, 2nd ed. Clarendon Press, Oxford, UK.

Harmsen, B. J. M. and W. J. M. Braam. 1969. On the conformation of bovine serum albumin after alkaline or thermal denaturation. **Int. J. Protein Research 1**: 225–233.

Heine, W. E., P. D. Klein and P. J. Reeds. 1991. The importance of α–lactalbumin in infant nutrition. **J. Nutrition** 121: 277–83.

Hermosín, I., F. Ledl, Gómez and A. Sánchez. 1992. lnfluence of oxidized lipids on the non–enzymatic browning reaction: interaction between linoleic acid and an Amadori compound. **Chem. Phys. Lipids** 63: 265–270.

Hernández–Ledesma, B., I. Recio and L. Amigo. 2008. β–lactoglobulin as source of bioactive peptides. **Amino Acids** 35: 257–265.

_____, A. Dávalos, B. Bartolome and L. Amigo. 2005. Preparation of antioxidant enzymatic hydrolyzates from α–lactalbumin and β–lactoglobulin. Identification of peptides by HPLC–MS=MS. **J. Agric. Food Chem.** 53: 588–593.

Hidalgo, F. J. and R. Zamora. 2000. The role of lipids in nonenzymatic browning. **Grasas Aceites** 51: 35–49.

Hiraoka, Y., T. Segawa, K. Kuwajima, S. Sugai and N. Murai. 1980. α–lactalbumin: A calcium metalloprotein. **Biochem. Biophys. Res. Comm.** 95: 1098–1104.

Hirose, M. 1993. Molten globule of food proteins. **Trends Food Sci. Tech.** 4: 48–52.

Hodge, J.E. 1953. Chemistry of browning reactions in model systems. **J. Agric. Food Chem.** 1: 928–943.

Hongsprabhas, P., P. Kerdchouay and P. Sakulsom. 2011. Lowering the Maillard reaction products (MRPs) in heated whey protein products and their cytotoxicity in human cell models by whey protein hydrolysate. **Food Res. Int.** 44(3): 748–754.

Huang, D., B. Ou and R. L. Prior. 2005. The chemistry behind antioxidant capacity assays. **J. Agric. Food Chem.** 53: 1841–1856.

Imafadon, G. I., K. F. Ng–Kwai–Hang, V. R. Harwalkar and C. Y. Ma. 1991. Differential scanning calorimetric study of different genetic variants of β–lactoglobulin. **J. Dairy Res.** 74: 2416–2422.

Institute of Medicine (U.S.). 2003. **Committee on Food Chemicals Codex**. The National Academies Press**,** Washington DC, USA.

Iyengar, R. and A. J. McEvily. 1992. Anti–browning agents: Alternatives to the use of sulfites in foods. **Trends Food Sci. Tech.** 3: 60–64.

Kataoka, M., K. Kuwajima, F. Tokunaga and Y. Goto. 1997. Structural characterization of the molten globule of α–lactalbumin by solution X–ray scattering. **Protein Sci.** 6: 422–430.

Kim, J. S. and Y. S. Lee. 2008. Effect of reaction pH on enolization and racemization reactions of glucose and fructose on heating with amino acid enantiomers and formation of melanoidins as result of the Maillard reaction. **Food Chem.** 108: 582–592.

Kontopidis, G., C. Holt and L. Sawyer. 2002. The ligand–binding site of bovine beta–lactoglobulin: evidence for a function? **J. Mol. Biol.** 318: 1043–1055.

Kontopidis, G., C. Holt and L. Sawyer. 2004. Invited review: β–lactoglobulin: binding properties, structure, and function. **J. Dairy Sci.** 87: 785–796.

Kroh, L. W. 1994. Caramelization in food and beverages. **Food Chem.** 51: 373–379.

Kronman, M. J. 1989. Metal–ion binding and the molecular conformational properties of α–lactalbumin. **Crit. Rev. Biochem. Molec. Biol.** 24 (6): 565–667.

_____, L. Cerankow and L. G. Holmes. 1965. Inter– and intramolecular interactions of alpha–lactalbumin. 3. Spectral Changes at Acid pH. **Biochem.** 4: 518–525.

Kuwajima, K. 1989. The molten globule state as a clue for understanding the folding and cooperativity of globular–protein structure. **Proteins** 6: 87–103.

_____. 1996. The molten globule state of alpha–lactalbumin. **FASEB Journal** 10: 102–109.

_____, Y. Ogawa and S. Sugai. 1981. Role of the interaction between ionizable groups in the folding of bovine alpha–lactalbumin. **J. Biochem.** 89: 759–770.

Kuznetsow, A. N., B. Ebert, G. Lassmann and A. B. Shapiro. 1975. Adsorption of small molecules to bovine serum albumin studied by the spin–probe method. **Biochim. Biophys. Acta** 379: 139–146.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** 227: 680–685.

Lam, S. M. S. C. C. and P. J. Moughan. Chapter 72-Whey protein and satiety: implication for diet and behavior, pp 1107-1126. *In* V. R. Preedy, R. R Watson and C. R. Martin, eds. **The International Handbook of Behavior, Diet and Nutrition**. 1st ed. Springer, UK.

Lee, H. S. and S.Nagy. 1996. Chemical degradation indicators to monitor the quality of processed and stored citrus products, pp. 86–91. *In* T. C. Lee and H. J. Kim, eds. **Chemical Markers for Processed and Stored Foods**. American Chemical Society, Washington DC.

Lemanska, K., H. Szymusiak, B. Tyrkowska, R. Zielinski, A. E. M. F. Soffer and I. M. C. M. Rietjens. 2001. The influence of pH on the antioxidant properties and the mechanisms of antioxidant action of hydroxyflavones. **Free Radical Biol. Med.** 31: 869–881.

Leopoldini, M., T. Marino, R. Nino and M. Toscano. 2004. Antioxidant properties of phenolic compounds: H–atom versus electron transfer mechanism. **J. Phys. Chem. A** 108: 4916–4922.

Lin, V. J. C. and J. L. Koenig. 1976. Raman studies of bovine serum albumin. **Biopolymers** 15: 203–218.

Lo, C. W. 1996. Human milk: nutritional properties, pp. 436–48. *In* W. A. Walker and J. B. Watkins, eds. **Nutrition in Pediatrics.** B. C. Decker, Hamilton, Canada.

Martins, S. I. F. S., W. M. F. Jongen and M. A. J. S. van Boekel. 2001. A review of Maillard reaction in food and implications to kinetic modelling. **Trends Food Sci. Tech.** 11: 364–373.

_____ and M. A. J. S. Van Boekel. 2003. Melanoidins extinction coefficient in the glucose–glycine Maillard reaction. **Food Chem.** 83: 135–142.

McCarthy, T. L., J. P. Kerry, J. F. Kerry, P. B. Lynch and D. J. Buckley. 2001. Assessment of the antioxidant potential of natural food and plant extracts in fresh and previously frozen pork patties. **Meat Sci.** 57: 177–184.

Mcintosh, G. H., G. D. Regester, R. K. Lelue, P. J. Royle and G. W. Smithers. 1995. Dairy proteins protect against dimethylhydrazine–induced intestinal cancers in rats. **J. Nutrition** 125: 809–816.

McKenzie, H. A., G. B. Ralston and D. C. Shaw. 1972. Location of sulphydryl and disulphide groups in bovine β–lactoglobulins and effects of urea. **Biochem.** 11: 4539–4547.

McSwiney, M., H. Singh, O. Campanella and L.K. Creamer. 1994. Thermal gelation and denaturation of bovine β–lactoglobulins A and B. **J. Dairy Res.** 61: 221–232.

Morales F. J. and van Boekel M. A. J. S. 1997. A Study on Advanced Maillard Reaction in Heated Casein/Sugar Solutions: Fluorescence Accumulation. **Int. Dairy J.** 7: 675–683.

Munch, G., J. Thome, P. Foley, R. Schinzel and P. Riederer. 1997. Advanced glycation endproducts in ageing and Alzheimer's disease. **Brain Res. Rev. 23**: 134–143.

Nagaoka, S., Y. Futamura, K. Miwa, T. Awano, K. Yamauchi, Y. Kanamaru, K. Tadashi and T. Kuwata. 2001. Identification of novel hypocholesterolaemic peptides derived from bovine milk β–lactoglobulin. **Biochem. Biophys. Res. Commun.** 281: 11–17.

Narayan, K. A. 1998. Biochemical aspects: nutritional bioavailability, pp. 125–174. *In* I. A. Taub and P. Singh, eds. **Food Storage Stability.** CRS press, NY.

Ndhlala, A. R., M. Moyo and J. Van. 2010. Staden Natural Antioxidants: Fascinating or Mythical Biomolecules? **Molecules** 15: 6905–6930.

Nielsen, P. M., D. Petersen and C. Dambmann. 2001. Improved method for determining food protein degree of hydrolysis. **J. Food Sci.** 66: 642–646.

Olano, A. and I. Martínez–Castro. 1996. Nonenzymatic browning, pp 1683–1721. *In* L.M.L. Nollet, ed. **Handbook of Food Analysis**. Marcel Dekker, NY

Osborne, T. B. 1907. **The Proteins of the Wheat Kernel**. Carnegie Institute, Washington DC, USA.

Ostdal, H., H. J. Andersen and M. J. Davies. 1999. Formation of long-lived radicals on proteins by radical transfer from heme enzymes-a common process? **Arch Biochem. Biophys.** 362: 105–112.

Otte, J., S. B. Lomholt, R. Ipsen, H. Stapelfeldt, J. T. Bukrinsky and K. B. Qvist. 1997. Aggregate formation during hydrolysis of β–lactoglobulin with a Glu and Asp specific protease from *Bacillus licheniformis*. **J. Agric. Food Chem.** 45: 4889–4896.

Ou, B., M. Hampsch–Woodill and R. L. Prior. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. **J. Agric. Food Chem. 49**: 4619–4626.

Pan, G. G. and L. D. Melton. 2007. Nonenzymatic browning of lactose and caseinate during dry heating at different relative humidities. **J. Agric. Food Chem** 55: 10036–10042.

Papiz, M. Z., L. Sawyer, E. E. Eliopoulos, A. C. T. North, J. B. C. Findlay, R. Sivaprasadarao, T. A. Jones, M. E. Newcomer and P. J. Kraulis. 1986. The structure of β–lactoglobulin and its similarity to plasma retinol binding protein. **Nature** 324: 383–385.

Palaniappan, L., 2001. Ultrasonic study of 2–butanol in cyclohexane with toluene. **Indian J. Phys.** 75: 515–518.

Pellegrini, A., C. Dettling, U. Thomas and P. Hunziker. 2001. Isolation and characterization of four bactericidal domains in the bovine β–lactoglobulin. **Biochimica et Biophysica Acta** 1526: 131–140.

Péna–Ramos, E. A. and Y. L. Xiong. 2001. Antioxidative activity of whey protein hydrolyzates in a liposomal system. **J. Dairy Sci.** 84: 2577–2583.

_____ and _____. 2003. Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. **Meat Sci**. 64: 259–263.

_____,_____, and G. E. Arteaga. 2004. Fractionation and characterization for antioxidant activity of hydrolysed whey protein. **J. Sci. Food Agric.** 84: 1908–1918.

Peng, X., Z. Zheng , K. W. Cheng , F. Shan , G. X. Ren , F. Chen and M. Wang . 2008. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. **Food Chem.** 106: 475–481.

_____, Y. L. Xiong, B. Kong. 2009. Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. **Food Chem.** 113: 196–201.

Pérez, M. D. and M. Calvo. 1995. Interaction of β–lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: a review. **J. Dairy Sci.** 78: 978–988.

Peters, T. 1985. Serum albumin. **Advances Protein Chem.** 37: 161–245.

Podsędek, A. 2007. Natural antioxidants and antioxidant activity of *Brassica* vegetables: A review. **LWT Food Sci. Tech.** 40: 1–11.

Prior, R. L., H. Hoang, L. Gu, X. Wu, M. Bacchiocca, L. Howard, M. Hampsch–Woodill, D. Huang, B. Ou and R. Jacob. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC)) of plasma and other biological and food samples. **J. Agric. Food Chem.** 51: 3273–3279.

Ptitsyn, O. B. 1987. Protein folding: Hypotheses and experiments**. J. Proein Chem.** 6: 273– 293.

Qi, X. L., S. Brownlow, C. Holt and P. Sellers. 1995. Thermal denaturation of β–lactoglobulin: Effect of protein concentration at pH 6.75 and 8.05. **Biochimica et Biophysica Acta** 1248: 43–49.

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice–Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biol. Med.** 26: 1231–1237.

Reed, R. G., R.C. Feldhoff, O. L. Clute and T. Peters Jr. 1975. Fragments of bovine serum albumin produced by limited proteolysis. Conformation and ligand binding. **Biochem.** 14: 4578–4573.

Reddy, I. M., N. K. D. Kella and J. E. Kinsella. 1988. Structural and conformational basis of the resistance of β-lactoglobulin to peptic and chymotryptic digestion. **J. Agric. Food Chem.** 36: 737–741.

Rival, S. G., S. Fornaroli, C. G. Boeriu and H. J. Wichers. 2001. Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. **J. Agric. Food Chem.** 49: 287–294.

Roefs, S. and K. Kruif. 1994. A Model for the denaturation and aggregation of β–lactoglobulin. **Eur. J. Biochem.** 226: 883–889.

Roginsky, V. and E. A. Lissi. 2005. Review of methods to determine chain–breaking antioxidant activity in food. **Food Chem.** 92: 235–254.

Rösner, H. I. and C. Redfield. 2009. The human alpha–lactalbumin molten globule*:* comparison of structural preferences at pH 2 and pH 7. **J. Mol. Biol**. 394(2): 351–362.

Ruegg, M., U. Moor and B. Blanc. 1977. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. **J. Dairy Res.** 44: 509– 520.

Samaranayaka, A. G. P. and E. C. Y. Li–Chan. 2011. Food–derived peptidic antioxidants: A review of their production, assessment, and potential applications. **J. Functional Foods** 3: 229–254.

Sapers, G. M., K. B. Hicks and R. L. Miller. 2002. Antibrowning agents, pp 543–61. *In* A. L. Branen, P. M. Davidson, S. Salminen and J. H. Thorngate, eds. **Food Additives,** 2nd ed. Marcel Dekker, NY.

Sawyer, W. H. 1967. Heat denaturation of bovine β–lactoglobulin and relevance of disulphide aggregation. **J. Dairy Sci**. 51: 323–329.

Sawyer, L. and G. Kontopidis. 2000. The core lipocalin, bovine betalactoglobulin. **Biochim. Biophys. Acta** 1482: 136–148.

_____, J. H. M. Cabral, and C. A. Batt. 1994. Protein engineering studies of β–lactoglobulin, pp. 114–126. *In* A. T. Andrews and J. Varley, eds. **Biochemistry of Milk Proteins**. The Royal Society of Chemistry, Cambridge, UK.

Schamberger, G. P. and T. P. Labuza. 2007. Effect of green tea flavonoids on Maillard browning in UHT milk. **LWT Food Sci. Tech.** 40: 1410–1417.

Schmidt, D. G. and van B. W. Markwijk. 1993. Enzymatic hydrolysis of whey proteins. Influence of heat treatment of α-lactalbumin and β-lactoglobulin on their proteolysis by pepsin and papain. **Netherlands Milk and Dairy Journal** 47: 15–22.

Shonle, D. 1996. The denatured state (the other half of the folding equation) and its role in protein stability. **FASEB J**. 10: 27–34.

Sipola, M., P. Finckenberg, H. Vapaatalo, A. Pihlanto–Leppälä, H. Korhonen, R. Korpela and M. L. Nurminen. 2002. α–Lactorphin and β–lactorphin improve arterial function in spontaneously hypertensive rats. **Life Sci**. 71: 1245–1253.

Sjoholm, I. and I. Ljungstedt. 1973. Studies on the tryptophan and drug–binding properties of human serum albumin fragments by affinity chromatography and circular dichroism measurements. **J. Biol. Chem.** 248: 8434–8441.

Smithers, G. W. 2008. Whey and whey proteins–From 'gutter–to–gold'. **Int. Dairy J.** 18: 695–704.

Smithers, G. W., F. J. Ballard, A. D. Copeland, K. J. De Silva, D. A. Dionysius and G. L. Francis. 1996. New opportunities from the isolation and utilization of whey proteins. **J. Dairy Sci.** 79: 1454–1459.

Stadtman, E. R. and R. L. Levine. 2003. Free radical–mediated oxidation of free amino acids and amino acid residues in proteins. **Amino Acids** 25: 207–218.

Stuart, D. I., K. R. Acharya, N. P. C. Walker, S. C. Smith, M. Lewis and D. C. Phillips. 1986. α–Lactalbumin possesses a novel calcium binding loop. **Nature** 324: 84–87.

Sturman, J. A. and K. C. Hayes. 1980. The biology of taurine in nutrition and development , pp. 231–299. *In* H. H. Draper, ed. **Adv. Nutr. Res. 3.** Plenum Press, NY.

_____, D. K. Rassin and G. E. Gaull. 1976. Taurine in development: Is it essential in the neonate? **Pediatric Res.** 10: 415–688.

Sugisawa, H. and H. Edo. 1966. The thermal degradation of sugars I. Thermal polymerization of glucose. **J. Food Sci.** 31(4): 561–565.

Sumaya–Martinez, M. T., S. Thomas, B. Linard, A. Binet and F. Guerard. 2005. Effect of Maillard reaction conditions on browning and antiradical activity of sugar–tuna stomach hydrolysate model system. **Food Res. Int.** 38: 1045–1050.

Taylor, S. L., N. A. Higley and R. K. Bush. 1986. Sulfites in foods: Uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity. **Adv. Food Research** 30: 1–76.

Tetra Pak. 1995. **Dairy Processing Handbook**. Tetra Pak Processing Systems AB, Lund, Sweden.

Tong, L. M., S. Sasaki, D. J. McClements and E. A. Decker. 2000. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. **J. Agric. Food Chem.** 48:1473–1478.

Vanaman, T. C., K. Brew and R. L. Hill. 1970. Disulfide bonds of bovine alpha–lactalbumin. **J. Biol. Chem.** 245: 4583–4590.

Vanderheeren, G. and I. Hanssens. 1994. Thermal unfolding of bovine α–lactalbumin. **J. Biol. Chem.** 269(10): 7090– 7094.

_____, I. Hanssens, W. Meijberg and A. Vanaerschot. 1996. Thermodynamic characterization of the partially unfolded state of $Ca^{2+}$–loaded bovine alpha–lactalbumin: Evidence that partial unfolding can precede $Ca^{2+}$ release. **Biochem.** 35: 16753–16759.

Villamiel, M., M. D. del Castillo and N. Corzo. 2006. 4. Browning Reactions, pp. 83–85. *In* Y. H. Hui, W. K. Nip, L. M. L. Nollet, G. Paliyath and B. K Simpson. **Food Biochemistry and Food Processing**. Wiley–Blackwell, US

Wang, Q., J. C. Allen and H. E. Swaisgood. 1997. Binding of vitamin D and cholesterol to beta–lactoglobulin**. J. Dairy Sci**. 80: 1054–1059.

Wedzicha, B. L. 1984. **Chemistry of sulfur dioxide in foods**. Elsevier Applied Science Pubishers, London, UK.

_____. 1987. Review: Chemistry of sulphur dioxide in vegetable dehydration. **Int. J. Food Sci. Tech.** 22: 433–450.

Weitzman S. A. and L. I. Gordon. 1990. Inflammation and cancer: role of phagocyte–generated oxidants in carcinogenesis. **Blood** 76: 655–663.

Wetzel, R., M. Becker, J. Behlke, H. Billwitz, S. Bohm, B. Ebert, H. Hamann, J. Krumbiegel and G. Lasmann. 1980. Temperature behaviour of human serum albumin. **Eur. J. Biochem.** 104: 469–478.

Wu, C. H. and G. C. Yen. 2005. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. **J. Agric. Food Chem.** 53: 3167–3173.

Wu, H., P. Wang, X. Hu, Z. Dai and X. Zou. 2011. Site–selective probe for investigating the asynchronous unfolding of domains in bovine serum albumin. **Talanta** 84: 881–886.

Yamauchi, R., K. Ohinata and M. Yoshikawa. 2003a. β–lactotensin and neurotensin rapidly reduce serum cholesterol via NT2 receptor. **Peptides** 24: 1955–1961.

_____, H. Usui, J. Yunden, Y. Takenaka, F. Tani and M. Yoshikawa. 2003b. Characterization of β–lactotensin, a bioactive peptide derived from bovine β–lactoglobulin, as a neurotensin agonist. **Biosci, Biotech, and Biochem.** 67(4): 940–943.

Yin, D. Z. and U. T. Brunk. 1991. Oxidized ascorbic acid and reaction products between ascorbic and amino acids might constitute part of age pigments. **Mechanisms of Ageing and Development** 61(1): 99–112.

Yoshikawa, M., R. Sasaki, H. Chiba and F. Tani. 1991**. Japan Kokai Tokkyo Koho**: 220197 (Sep. 27)

Yogman, M. W., S. H. Zeisel and C. Roberts. 1982. Assessing effects of serotonin precursors on newborn behavior. **J. Psychiatric Res**. 17: 123–33.

Zadow, J. G. 1994. Utilization of milk components: Whey, pp. 313–373. *In* R. K. Robinson, ed. **Modern dairy technology, advances in milk processing,** Vol. 1. Chapman and Hall UK, London.

Zhou, S. and E. A. Decker. 1999. Ability of amino acids, dipeptides, polyamines, and sulfhydryls to quench hexanal, a saturated aldehydic lipid oxidation product. **J. Agric. Food Chem.** 47: 1932–1936.

# APPENDIX

**1. The preparation of oxygen radical absorbance capacity (ORAC) assay**

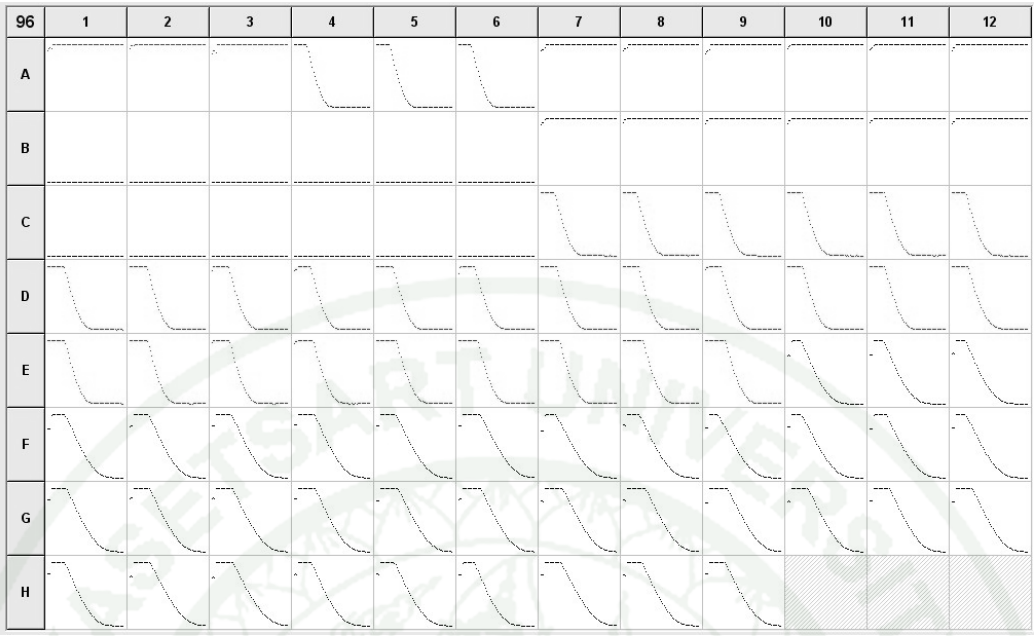
The oxygen radical absorbance capacity was determined as describe before and the reactants were react in 96 wells black plate The reaction was done follow these steps;

1.1 The PBS was added volume of 20 µL for blank, 60 µL for each positive control and 120 µL for each negative control.

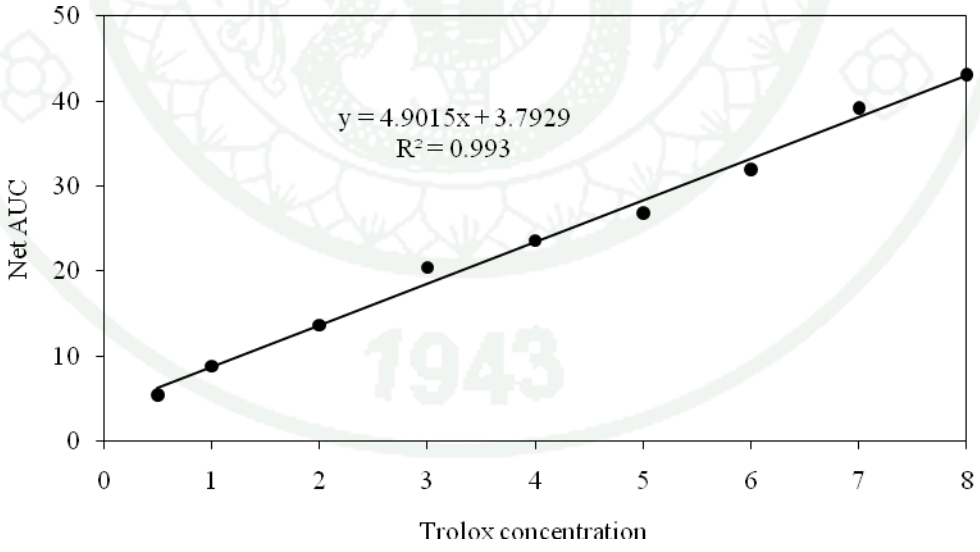
1.2 The 0.08 mg protein $mL^{-1}$ sample or standard Trolox (5-80 µM) was added volume of 20 µL.

1.3 The fluorescein was added volume of 120 µL and covered with black lid.

All tests were done triplicate by three wells were one replicate. After fluorescein was added, plate was rapidly placed in fluorescence microplate reader in order to incubate to 37 °C, 15 min. The AAPH solution was fed by machine and fluorescent intensity was read at fourth cycle. The data was record into graph as shown in Appendix Figure 1. The area under curve (AUC) of sample was compared the relation with those of Trolox standard by using $R^2$ equation of Trolox standard (Appendix Figure 2) to calculate the Trolox equivalent per mg protein.




**Appendix Figure 1** The fluorescein fluorescence decay curve induced by AAPH; in the presence of standard Trolox 0.5-8 µM (C7-E9); 0.08 mg protein $mL^{-1}$ modified whey protein (E10-H9).




**Appendix Figure 2** Linear relation between 0.5-8 µM standard Trolox and the net area under curve (AUC) for oxygen radical absorbance capacity (ORAC)

**2. The preparation of SDS-PAGE method**

2.1 Sample preparation

The samples were prepared at concentration of 0.35 mg protein in 350 ml buffer. The buffer compose of 3.8 ml distilled water, 1 ml 0.5 M Tris-HCl, pH 6.8, 0.8 ml glyceral, 1.6 ml 10% SDS, 0.4 ml β-mercaptoethanol, and 0.4 ml 1% bromophenol blue. The buffer of non β-mercaptoethanol was prepared by replace β-mercaptoethanol with distilled water. After mixed with buffer, samples were heating at 100 °C for 10 min and cool in water before added into the well.

2.2 Gel preparation

Polyacrylamide gel was prepared by sequentially mixing reagents (Appendix Table 1). After mixing, distilled water, 1.5 M Tris-HCl, pH 8.8, 0.5 M Tris-HCl, pH 6.8, 10% SDS and the stock acrylamide/bis (30%T and 2.67%C) the solution was degassed to avoid the air bubble in gel matrix that may lead to chipped gel. The freshly prepared 10% ammonium persulphate (APS) and TEMET was added to catalyze the gel formation therefore the solution was rapidly poured in to glass cast.

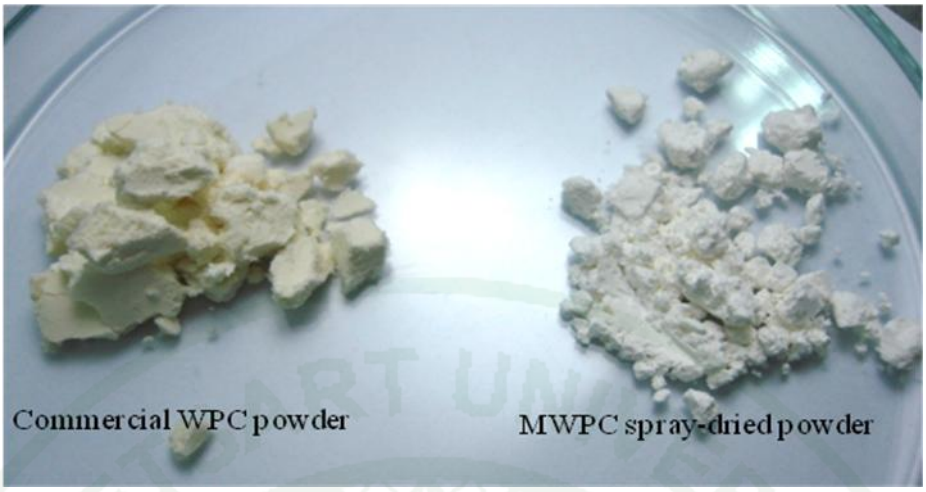
The 5X electrode (running) buffer pH 8.3 was prepared by solute 9 g tris base, 43.2 g glycine and 3 g SDS and adjust with distilled water to 600 ml and kept at 4°C. After the samples were loaded into the well, the electric was supplied at 150 V until sample was run to the bottom of gel before the edge about 0.5-1 cm. Then remove the gel and stain with staining solution. The staining solution was prepared by adding 0.1% coomassia blue in the solution of 40% methanol and 10% acetic acid and filter with filter paper. Methanol, distilled water and glacial acetic acid at ratio 100:825:75 were used to prepare 1L of destaining solution.

**Appendix Table 1** Gel preparation for SDS-PAGE

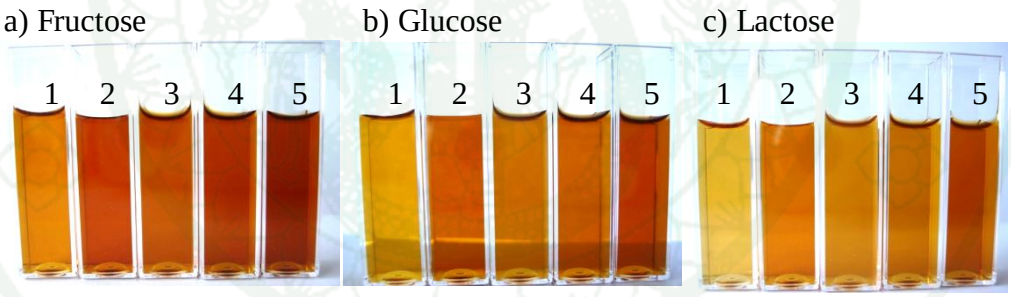
| Gel composition | Separating gel | | | | Stacking gel |
|---|---|---|---|---|---|
| | 7.5% | 12% | 14% | 15% | 4% |
| 1. Distilled water | 4.85 ml | 3.35 ml | 2.68 ml | 2.35 ml | 6.1 ml |
| 2. 1.5 M Tris-HCl, pH 8.8 | 2.5 ml | 2.5 ml | 2.5 ml | 2.5 ml | - |
| 3. 0.5 M Tris-HCl, pH 6.8 | - | - | - | - | 2.5 ml |
| 4. 10% SDS | 100 µl | 100 µl | 100µl | 100µl | 100 µl |
| 5. Acrylamide/bis (30%T, 2.67%C stock) Degas | 2.5 ml | 4 ml | 4.67 ml | 5 ml | 1.33 ml |
| 6. 10% Ammonium persulphate (APS) fresh daily | 50 µl | 50 µl | 50 µl | 50 µl | 50 µl |
| 7. TEMET | 5 µl | 5 µl | 5 µl | 5 µl | 10 µl |
| Total Monomer | 10 ml | 10ml | 10 ml | 10 ml | 10 ml |

**3. Efficacy of spray-dried modified whey protein concentrate (MWPC) in sterilized reducing sugar-containing mixtures: Brown color after sterilize at 121 °C, 15 min**

Appearance of commercial WPC powder and spray-dried MWPC after modified WPC was spray-dried to be the powder was shown in Appendix Figure 3. When heating at 121 °C, 15 min of the solution contain 0.1 M sugar (fructose, glucose, lactose) at pH 8 by using 0.1M phosphate buffer pH 8 in the present of 0.50% glycine, 0.50% MWPC or mix of 0.25% glycine and 0.25% MWPC or mix of 0.50% glycine and 0.50% MWPC the brown color was shown in Appendix Figure 4.




**Appendix Figure 3** Appearance of commercial WPC powder and spray-dried MWPC




**Appendix Figure 4** Brown pigment formation in sterilized reducing sugars, glycine, glycine-sugar and whey protein-sugar mixtures 1) 0.1M reducing sugar, 2) 0.1M reducing sugar+0.50% glycine, 3) 0.1M reducing sugar + 0.50% MWPC, 4) 0.1M reducing sugar + 0.25% glycine + 0.25% MWPC and 5) 0.1M reducing sugar + 0.50% glycine + 0.50% MWPC

# CURRICULUM VITAE

**NAME:** Ms. Narumol Rongsirikul

**BIRTH DATE:** May 12, 1985

**BIRTH PLACE:** Bangkok, Thailand

**EDUCATION:**

| YEAR | INSTITUTE | DEGREE/DIPLOMA |
|---|---|---|
| 2008 | Mahidol Univ. | B.Sc. (Food Tech.) |

**SCHOLARSHIP:** Center of Advanced Studies on Agriculture and Foods (CASAF), National Research University program (NRU), Kasetsart University [Special Research Unit: Controlled Release Delivery Systems for Bioactive and Functional Food Ingredients (CAFT1)] (2009)

**PUBLICATION:** Rongsirikul, N. and P. Hongsprabhas. 2011. Preparation of antioxidative whey protein concentrate with antibrowning activity against Maillard reactions, pp 621-629**.** *In* **Proceedings of the 49th Kasetsart University Annual Conference.** Kasetsart University, Thailand