ปัจจัยที่มีผลต่อคุณสมบัติและความคงตัวของเวย์ไฮโดรไลเซต

นางสาววิทิตดา อวัยวานนท์

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FACTORS AFFECTING PROPERTIES AND STABILITY OF WHEY HYDROLYSATES

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วิทิตดา อวัยวานนท์ : ปัจจัยที่มีผลต่อคุณสมบัติและความคงตัวของเวย์ไฮโดรไลเซต . (FACTORS AFFECTING PROPERTIES AND STABILITY OF WHEY HYDROLYSATES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.อังคณา ตันติธุวานนท์ , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.วราภรณ์ สุวกูล, 103 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของสภาวะในการไฮโดรไลซิสด้วยเอนไซม์ที่มีต่อแบบแผนโปรตีน ค่าการไฮโดรไลซิส และความสามารถในการกำจัดอนุพันธ์ออกซิเจนที่ว่องไวและศึกษาความคงตัวของเวย์ไฮโดรไลเซต สภาวะในการไฮโดรไลซิสด้วยเอนไซม์ของเวย์เกิดขึ้นโดยใช้ชนิดของเอนไซม์ (ทริปซิน ไคโมทริปซินและปาเปน) อัตราส่วนเอนไซม์ต่อสารตั้งต้น (1/1000, 1/200 และ 1/100) และระยะเวลาในการไฮโดรไลซิส (1, 3 และ 5 ชั่วโมง) ที่แตกต่างกัน จากแบบแผนโปรตีนของเวย์ไฮโดรไลเซต พบว่าความสามารถในการย่อยโปรตีนที่เป็นส่วนประกอบของเวย์โดยทริปซินและไคโมทริปซินเพิ่มขึ้นเมื่ออัตราส่วนเอนไซม์ต่อสารตั้งต้นและระยะเวลาในการไฮโดรไลซิสเพิ่มขึ้น ในขณะที่ความสามารถในการย่อยโปรตีนโดยปาเปนเพิ่มขึ้นตามอัตราส่วนเอนไซม์ต่อสารตั้งต้น การเพิ่มอัตราส่วนเอนไซม์ต่อสารตั้งต้นและระยะเวลาในการไฮโดรไลซิสส่งผลให้ค่าการไฮโดรไลซิสของเวย์ไฮโดรไลเซตเพิ่มขึ้น ปาเปนมีความสามารถในการย่อยโปรตีนที่เป็นส่วนประกอบของเวย์สูงที่สุดตามด้วยทริปซินและไคโมทริปซิน การศึกษาความสามารถในการกำจัดอนุพันธ์ออกซิเจนที่ว่องไวของเวย์ไฮโดรไลเซตในเซลล์เคราติโนไซต์ของมนุษย์พบว่าชนิดของเอนไซม์มีผลต่อความสามารถในการกำจัดอนุพันธ์ออกซิเจนที่ว่องไวของเวย์ไฮโดรไลเซตมากที่สุด เวย์ไฮโดรไลเซตที่เตรียมจากปาเปนมีความสามารถในการกำจัดอนุพันธ์ออกซิเจนที่ว่องไวสูงที่สุด ตามด้วยเวย์ไฮโดรไลเซตที่เตรียมจากทริปซินและเวย์ไฮโดรไลเซตที่เตรียมจากไคโมทริปซิน แม้ว่าเวย์ไฮโดรไลเซตประกอบด้วยซีสเตอีนที่เป็นสารตั้งต้นในการสังเคราะห์กลูตาไธโอนในปริมาณสูง แต่ความสามารถในการกำจัดอนุพันธ์ออกซิเจนที่ว่องไวของเวย์ไฮโดรไลเซตไม่ได้เกิดจากการกระตุ้นการสร้างกลูตาไธโอนในเซลล์ การศึกษาความคงตัวของผงไลโอฟิไลส์เวย์ไฮโดรไลเซตในสภาวะเร่งเป็นเวลา 3 เดือน พบว่าเปปไทด์ที่เป็นส่วนประกอบของเวย์ไฮโดรไลเซตเกิดความไม่คงตัว ส่งผลให้ความสามารถในการกำจัดอนุพันธ์ออกซิเจนที่ว่องไวของเวย์ไฮโดรไลเซตลดลง

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VITITDA AWAIWANONT: FACTORS AFFECTING PROPERTIES AND STABILITY OF WHEY HYDROLYSATES. THESIS ADVISOR: ASST. PROF. ANGKANA TANTITUVANONT, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. WARAPORN SUWAKUL, Ph.D., 103 pp.

The objectives of this study were to investigate the effect of the enzymatic hydrolysis conditions on the properties of whey hydrolysates as protein patterns, degree of hydrolysis (DH) and reactive oxygen species (ROS) scavenging activity and to investigate the stability of whey hydrolysates. Enzymatic hydrolysis conditions of whey were performed at various types of enzyme (trypsin, chymotrypsin and papain), enzyme to substrate ratio (E/S: 1/1000, 1/200 and 1/100) and hydrolysis time (1, 3 and 5 hr). From the protein patterns of whey hydrolysates, increased E/S and hydrolysis time improved the digestion of whey by trypsin and chymotrypsin. While the digestion of whey by papain was increased by the E/S. Increased E/S and hydrolysis time resulted in increasing the DH of whey hydrolysates. Papain had the greatest digestive ability to hydrolyze whey followed by trypsin and chymotrypsin. The ROS scavenging activity of whey hydrolysates was investigated in human keratinocyte cells (HaCaT cells). The types of enzyme had the greatest effect on the activity. Whey hydrolyzed with papain showed the highest activity followed by whey hydrolyzed with trypsin and chymotrypsin. Although whey hydrolysates were rich in cysteine, a glutathione precursor, our study showed that the ROS scavenging activity of whey hydrolysates was not mediated by the stimulation of glutathione synthesis. The stability of lyophilized whey hydrolysate powders was investigated under accelerated condition. After 3 month's storage, the peptides in whey hydrolysate powders were unstable resulting in decreased ROS scavenging activity.

Department : Pharmaceutics and Industrial Phamacy Student's Signature .....
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LIST OF ABBREVIATIONS

α-La	=	α-lactalbumin
ANOVA	=	analysis of variance
β-Lg	=	β-lactoglobulin
BSA	=	bovine serum albumin
BSO	=	buthionine sulfoximine
°C	=	degree Celsius
cm^2	=	square centrimeter
conc	=	concentration
DCF	=	2,7-dichlorofluorescein
DCFH	=	2,7-dichlorodihydrofluorescein
DCFH-DA	=	2,7-dichlorodihydrofluorescein diacetate
df	=	degree of freedom
DH	=	degree of hydrolysis
DMA	=	9, 10 -dimethylanthracene
DMEM	=	Dulbecco's modified Eagle medium
DMNQ	=	2, 3-dimethoxy-1,4-napthoquinone
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
DPPH	=	1,1-diphenyl-2-picryl hydrazyl
E/S	=	enzyme to substrate
ESR	=	electron spin resonance
et al.,	=	*et alii*, 'and others'
FBS	=	fetal bovine serum
FRAP	=	ferric reducing antioxidant power
g	=	gram
γ-GCS	=	γ-glutamylcysteine synthetase
GPx	=	glutathione peroxidase
GSH	=	reduced glutathione
GSSG	=	oxidized glutathione

HaCaT	=	human keratinocyte cell line
HCl	=	hydrochloric acid
HE	=	dihydroethidium
hr	=	hour
k	=	release rate constant
kDa	=	kilodalton
L	=	liter
MCB	=	monochlorobimane
mg	=	milligram
min	=	minute
ml	=	milliliter
MTT	=	thiazolyl blue tetrazolium bromide
MW	=	molecular weight
mM	=	millimolar
n	=	sample size
N	=	normality
NAC	=	N-acetyl-cysteine
NADPH	=	nicotinamide adenine dinucleotide phosphate
NaOH	=	sodium hydroxide
nm	=	nanaometer
PBS	=	phosphate buffered saline
Prx	=	peroxiredoxin
pH	=	the negative logarithm of the hydrogen ion concentration
RH	=	relative humidity
ROS	=	reactive oxygen species
sec	=	second
SD	=	standard deviation
SDS	=	sodium dodecyl sulfate
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	=	superoxide dismutase

TEMED	=	N, N, N, N-tetramethyl-ethylenediamine
TNBS	=	trinitrobenzene sulfonic acid
µg	=	microgram
µl	=	microliter
UV	=	ultraviolet
V	=	volt
v/v	=	volume/volume
WPC	=	whey protein concentrate
WPI	=	whey protein isolate
w/v	=	weight by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Nowadays, impact of aging on the function and appearance of skin is receiving a growing interest. Various cosmetic products are used for protecting skin aging (Rabe et al., 2006). A group of the products that has grown in decade is peptides. Cosmetic peptides can improve signs of skin aging by reducing wrinkle depth, improving skin firmness and inhibiting collagen degradation (Lupo and Cole, 2007). An interesting source of the cosmetic peptides is whey proteins.

Whey is complex proteins from milk. It is considered as a dairy waste from cheese and casein production. The protein components in whey are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins, lactoferrin, lactoperoxidase, bovine serum albumin (BSA) and glycomacropeptide. They have several biological properties as muscle anabolism, immunomodulation, anticancer and antioxidant (Smithers, 2008). Since whey contains various bioactive proteins, the utilization of whey as nutritional supplement has been reported. In recent years, the cosmetic properties of whey have been discovered. The properties include enhancing skin and hair hydration (Tomita et al., 1994), inhibiting melanogenesis (Nakajima et al., 1997) and promoting collagen synthesis (Collons, Mammone, and Marenus, 2001). These making whey become an interesting cosmetic ingredient. For protection of skin aging, various antioxidants are recommended to reduce oxidative damage of skin. Whey also acts as an antioxidant. It might be beneficial to prevent skin aging. The antioxidant activity of whey is presented by increasing glutathione synthesis. Whey is rich in cysteine, a glutathione precursor. Supplementation of whey improved synthesis of glutathione (Micke et al., 2001; Sukkar et al., 2008). Micke et al. (2001) reported that an oral administration of whey with a daily dose of 45 g for 2 weeks could increase the level of plasma glutathione by 50%. However, the topical antioxidant effect of whey to protect skin aging is limited. Since whey contains the high molecular weight (MW) proteins, the protein components in whey can not penetrate into the skin for protecting oxidative damage. Enzymatic hydrolysis is a common technique to

decrease size of whey proteins resulting in increased skin and cellular penetration. Additionally, this technique was effective in improving the antioxidant activity of whey. The previous studies reported that the bioactive peptides which were produced by the enzymatic hydrolysis of whey showed the ROS scavenging (Hernandez-ledesma et al., 2005; Peng, Xiong, and Kong, 2009) and reducing property (Peng et al., 2009). These properties contribute to the antioxidant activity of whey hydrolysates. The activities of the bioactive peptides depend on the specific peptide sequences which are buried in the compact structure of native whey. Modification of the protein structure by the enzymatic hydrolysis results in opening the bioactive sequences. Alteration in the hydrolysis conditions as the enzyme to substrate ratio (E/S), hydrolysis time and types of enzyme can affect the properties of the peptides in whey hydrolysates (Amiot et al., 2004; Hernandez-ledesma et al., 2005). Even the antioxidant activity of whey hydrolysates has been reported, the study on the ability of whey hydrolysates to scavenge ROS in aging skin is not available. The study was focused on development of whey hydrolysate powder serving antioxidant activity for topical cosmetic products. For this reason, the effect of the hydrolysis conditions on the properties of whey hydrolysates particularly the scavenging activity against ROS in human keratinocyte cells was investigated in this study.

Stability is an important factor that has to consider before the product releasing to marketplaces. For whey hydrolysate, instability of the bioactive peptides might be accelerated by water resulting in decreased bioactivities (Lai and Topp, 1999). To protect the bioactivities of the peptides, a dehydration technique as freeze drying is used to improve the stability of whey hydrolysate. Although whey hydrolysate is removal of water by freeze drying, instability of the hydrolysate can be mediated by chemical and physical reactions. For this reason, the stability of whey hydrolysate powder is investigated in this study.

This study was aimed to investigate the effect of the hydrolysis conditions: enzyme to substrate ratio (E/S), hydrolysis time and types of enzyme on the properties of whey hydrolysates as the protein patterns, degree of hydrolysis (DH) and ROS scavenging activity and evaluate the stability of selected freeze dried whey hydrolysates under accelerated conditions.

The purposes of this study were as follows:

1. To investigate the effect of the hydrolysis conditions as the E/S, hydrolysis time and types of enzyme on the properties of whey hydrolysates
2. To investigate the stability of selected whey hydrolysates and freeze dried whey hydrolysates

CHAPTER II

LITERATURE REVIEW

Whey

Milk possesses two major groups of proteins: caseins and whey proteins. In cheese production, casein curd is coagulated by acid or enzyme to make cheese, the remaining liquid calls whey. In the past, whey was discharged into environment as waste product causing high pollution. Several countries have regulated disposal of the dairy wastes by encourage for researching in treatment of the wastes. Such legislations have taken the lead in studying information and utilizations of whey (Smithers, 2008). Nowadays whey becomes a valuable product which is a functional food. Various products of whey proteins are available in marketplaces which contain a range of compositions: protein, fat, lactose and mineral content (Table 1).

Table 1 Types of commercially available of whey proteins (Marshall, 2004)

Types of whey proteins	Protein concentration	Fat, lactose and mineral content
Undenatured whey proteins	25-89%	• Some fat, lactose and minerals • Fat, lactose and mineral content decrease as protein concentration increases.
Hydrolyzed whey protein	Variable	• Varies with protein concentration
Whey protein contentrate (WPC)	25-89%	• Some fat, lactose and minerals • Fat, lactose and mineral content decrease as protein concentration increases.
Whey protein isolate (WPI)	90-95%	• Little if any

The biological components of whey are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins, lactoferrin, lactoperoxidase, bovine serum albumin (BSA) and glycomacropeptide (Table 2). They contribute to a variety of biological activities as muscle anabolism (Tipton et al., 2007), bone formation (Takada, Aoe and Kumegawa, 1996), anticancer (Bounous, 2000), antimicrobial (Early et al., 2001), antihypertensive (Ven et al., 2002), immunostimulating (Low et al., 2003) and antioxidant activity (Hernandez-ledesma et al., 2005; Liu, Chen and Mao, 2007; Pena-Ramos and Xiong, 2001; Peng et al., 2009; Tseng et al., 2006; Tong et al., 2000). The antioxidant activity of whey is contributed by increasing glutathione synthesis. Whey is rich in cysteine, a glutathione precursor. An oral administration of whey improved the synthesis of glutathione (Micke et al., 2001; Sukkar et al., 2008). In addition, in vitro cell culture demonstrated an increased intracellular glutathione when supplementation of whey resulting in the protection of ROS-induced cell damage (Kent et al, 2003; Tseng et al., 2006). Lactoferrin and BSA, the minor whey components, were metal-ion chelators (Tong et al., 2000). These proteins were able to chelate metal ion that catalyzed Fenton reaction consequently the ROS generation was decreased. Additionally, the antioxidant activity of whey is improved by enzymatic hydrolysis technique. Peptides obtained from whey hydrolysates played antioxidant activity via ferric reducing antioxidant activity and radical scavenging activity as 1,1-diphenyl-2-picryl hydrazyl (DPPH), superoxide anion and hydroxyl radical scavenging activity (Peng et al., 2009). Protein hydrolysates from the major components of whey, β-Lg and α-La, also exhibited the antioxidant activity. These hydrolysates played peroxyl radical scavenging activity (Hernandez-ledesma et al., 2005).

Table 2 Protein components in whey (Marshall, 2004; Madureira et al., 2007)

Whey components	Molecular weight (kDa)	% of whey protein
β-lactogobulin	18,277	50-55
α-lactalbumin	14,175	20-25
Immunoglobulins	25,000 (light chain) + 50,000-70,000 (heavy chain)	10-15
Lactoferrin	80,000	1-2
Lactoperoxidase	70,000	0.5
Bovine serum albumin	66,267	5-10
Glycomacropeptide	6,700	10-15

Moreover, the cosmetic properties of whey hydrolysates have been reported. Collons et al. (2001) reported that whey can improve the effect of vitamin C on boosting collagen synthesis. Co-treatment of whey with vitamin C increased the collagen synthesis by 30% compared with treatment of vitamin C alone. Inhibition of melanogenesis by β-Lg, a major protein component in whey, was found in human melanocyte cells. This protein suppressed tyrosinase activity of the cells as a consequence of decreased pigmentation (Nakajima et al., 1997).

Enzymatic Hydrolysis

Enzymatic hydrolysis is often used technique to modify nutritional and functional properties of protein. During hydrolysis process, the protein is hydrolyzed by the enzyme to produce bioactive sequence peptides. The hydrolyzed protein is normally called protein hydrolysate. The protein hydrolysates are rich in low molecular weight peptides which present bioactive properties. The bioactive properties of whey are also improved by this technique. Several studies have been reported the bioactive properties of whey hydrolysates as cell proliferation (Amiot et al., 2004), immunomodulation (Mercier et al., 2004), antihypertension (Ven et al., 2002) and antioxidant (Pena-Ramos and Xiong, 2001). Functionality of the peptide in the hydrolysates is depending on the hydrolysis conditions: pH, hydrolysis

temperature, hydrolysis time, enzyme to substrate ratio (E/S), and especially types of enzyme.

1. pH

pH is an important factor to be controlled for enzymatic hydrolysis. It affects ability of enzyme to hydrolyze protein substrate. Most enzymes are effective in narrow pH range. A change in pH either higher or lower range might alter structure of enzymes resulting in loss of enzyme activity. The pH for enzymatic hydrolysis should represent the physiological conditions of selected enzyme. For instance, papain is a cysteine protease presenting in papaya. The optimum pH for papain is approximately 6-8 (Kong and Xiong; Pena-Ramos and Xiong, 2001; Qian, Jung and Kim, 2008). Trypsin and chymorypsin are proteolytic enzymes from small intestine. The optimal operating pH of these enzymes is about 8 that is environmental condition of small intestine. Decreased pH below 5 caused inactivation of the enzymes (Outzen et al., 1996). Pepsin is digestive protease in gastric juice. The optimal pH is approximately 2. Increased pH attenuates its activity. If pH is up to 8, the enzyme is irreversibly inactivated (Outzen et al., 1996; Pande et al., 2009; Pena-Ramos and Xiong, 2001). There is only a study regarding the effect of pH on enzymatic hydrolysis of whey. In this study, whey was hydrolyzed by trypsin at 37^{o}C under different pH: 8 and 9 (Mota et al., 2006). The peptides in whey hydrolysates obtained from pH 8 were similar to the peptides obtained from pH 9. This finding indicated that increased pH did not affect the obtained hydrolysates. However, no pH effect observing in the study might be due to the narrow range of pH. The enzyme activity of trypsin was relatively comparable under the studied pH (Outzen et al., 1996).

2. Hydrolysis Temperature

Temperature can affect the mobility between enzyme and substrate molecule. Increasing temperature enhances the mobility and promotes enzyme-substrate reaction (Palmer, 1995). Enzymatic hydrolysis rate of soy protein and whey is improved by increasing hydrolysis temperature (Kong et al., 2008; Mota et al., 2006). Additionally, increase in hydrolysis temperature resulted in unfolding of protein structure (Havea, Singh, Creamer, 2000; Hong and Creamer, 2002; Tong et al., 2000). This facilitated

susceptibility between enzyme and substrate as a consequence of improved protein digestion. However, high hydrolysis temperature can alter conformation of enzyme leading to loss of enzyme activity. Therefore, preheat treatment of protein before enzymatic hydrolysis is of interest to improve digestion of protein substrate (Pena-Ramos and Xiong, 2001; Peng et al., 2009).

In general, the optimal temperature for hydrolyzing protein depends on the types of enzyme as 37^{o}C for trypsin, chymotrypsin and papain (Je et al., 2007; Kent et al., 2003; Kong and Xiong, 2006; Pena-Ramos and Xiong, 2001), 45^{o}C for corolase PP (Ven et al., 2002) and 50-65^{o}C for alcalase (Dong et al., 2008; Kong and Xiong, 2006; Peng et al., 2009).

3. Hydrolysis Time

Hydrolysis time is a period of protein hydrolysis. Increased hydrolysis time leads to further hydrolysis of peptide bonds that affects bioactivity of the protein hydrolysate (Palmer, 1995). Most studies selected long hydrolysis time to produce protein hydrolysates because they expected that complete hydrolysis of the proteins will produce protein hydrolysates which showed high bioactivities (Hernandez-ledesma et al., 2005; Je et al., 2007; Peng et al., 2009; Rajapakse et al., 2005). Peng et al. (2009) found that the ferric reducing antioxidant power (FRAP) of whey hydrolysates was improved with the increase of hydrolysis time. At the hydrolysis time of 5 hr providing a maximum degree of hydrolysis (DH), whey hydrolysate showed the highest activity.

However, a maximum bioactivities of protein hydrolysates was not necessary to obtain from a maximum hydrolysis time. Whey hydrolyzed with papain did not show the maximum antioxidant activity at the maximum hydrolysis time (Pena-Ramos and Xiong, 2001). The antioxidant activity of the hydrolysates was not improved by enzymatic hydrolysis. As increased hydrolysis time from 0.5 to 6 hr, the hydrolysates were not able to inhibit lipid oxidation in a liposome system. This was in agreement with the study of Pena-Ramos and Xiong (2003) reporting that the antioxidant activity of whey hydrolyzed with flavourzyme for 1 hr was not different from native whey. The antioxidant activity of zein hydrolyzed with papain was the highest at the hydrolysis time of 4 hr (Kong and Xiong, 2006). Increased hydrolysis

time over 4 hr decreased the activity of the hydrolysates. The hydroxyl radical scavenging activity of carp hydrolyzed with alcalase did not increase with the hydrolysis time (Dong et al., 2008). The hydrolysate providing the maximum activity was produced from the hydrolysis time of 2 hr. These indicated that bioactive properties of protein hydrolysates had no direct relationship with the hydrolysis time. The bioactive properties of protein hydrolysates may depend upon specific structure of peptides (Hernandez-ledesma et al., 2005; Je et al., 2008; Kong and Xiong, 2006).

4. Enzyme to Substrate Ratio (E/S)

The amount of enzyme used in the hydrolysis process is typically presented as the content of available enzyme reacted with the substrate. High E/S attributes high hydrolysis of peptide bonds. It also takes short hydrolysis time to reach maximum hydrolysis (Amoit et al., 2004). Kong et al. (2008) found that required hydrolysis time for obtaining pre-defined DH of soy protein was inverse-proportional to the E/S. The time needed for hydrolyzing the soy protein to reach 4% DH was 300 min for the E/S of 1/100 and 100 min for the E/S of 1/25. This indicated that high E/S was able to hydrolyze the protein at higher rate than low E/S. Additionally, low E/S might limit capacity of the enzyme to hydrolyze protein results in partial hydrolysis of the protein substrate. Amiot et al. (2004) found that a maximum DH of milk protein hydrolysates increased when the E/S increased. The maximum DH of whey hydrolyzed with trypsin at the E/S of 1/1000 was 6.8% while the maximum DH of the hydrolysate obtained from the E/S of 1/100 was 9.5%. Low E/S limits the digestive ability of enzyme where high E/S is costly. The amount of enzyme used for protein hydrolysis should be optimum. However, the studies on the effect of the E/S on enzymatic hydrolysis of whey protein are limited.

5. Types of Enzyme

A type of enzyme is a crucial factor for enzymatic hydrolysis. Different types of enzyme cleave peptide bonds of different amino acids producing various protein hydrolysates. For instance, chymotrypsin is a serine protease enzyme. The enzyme is susceptible to various amino acids. The specific amino acids for chymotrypsin are aromatic and hydrophobic amino acids as tyrosine, phenylalanine, tryptophan and

methionine. Subsequently, the peptides produced by chymotrypsin have aromatic or hydrophobic amino acids at the end of the fragments (Kruat., 1977; Whitford, 2005). These peptides are essential for promoting cell growth. Milk hydrolyzed with chymotrypsin showed growth promoting activity on the human keratinocyte cells (Amiot et al., 2004). The bioactive peptides were approximately 800 kDa. They contained hydrophobic amino acids which important for cell growth, at the end of their sequences. Trypsin is widely used enzyme for enzymatic hydrolysis. This enzyme has higher specificity to amino acids compared with chymotrypsin. The specific amino acids for trypsin are lysine and arginine. Thus the peptides in protein hydrolysates obtained from trypsin actually contain lysine or arginine at the end of the fragments. Papain is the cysteine protease which contains cysteine residue at the active site. This enzyme preferably cleaves basic amino acids, leucine and glycine including ester and amide bonds. It is considered as a broad specific enzyme.

The digestive ability of enzyme is contributed by enzyme specificity. Enzyme which has high specificity is low ability to digest protein. Since the specific amino acids for trypsin are only two amino acids, this enzyme has low ability to digest protein substrate. The digestive ability of chymotrypsin is higher than that of trypsin because chymotrypsin has high extent of the specific amino acids than trypsin. However, the digestive ability of enzyme to substrate also depends on the amino acid compositions of protein substate. In case of chymotrypsin, if the protein substrate has low amount of aromatic and hydrophobic amino acids, the digestive ability of chymotrypsin might be lower than that of trypsin. Hernandez-ledesma et al. (2005) reported that the ability of chymotrypsin to digest β-Lg was lower than that of trypsin. This can be explained that the amount of aromatic and hydrophobic amino acids in β-Lg was lower than the amount of lysine and arginine resulting in limiting digestive ability of chymotrypsin.

Several studies found that the bioactivity of protein hydrolysates associated with the digestive ability of enzyme used (Hernandez-ledesma et al., 2005; Peng et al., 2009). Increased digestive ability of enzyme by increased hydrolysis time improved the antioxidant acitivity of whey hydrolysates (Peng et al., 2009). Moreover, enzyme that had high digestive ability was more effective in improving the ROS scavenging activity of protein hydrolysates than low digestive ability enzyme

(Hernandez-ledesma et al., 2005). Chymotrypsin had high ability to digest α-La compared with trypsin. The antioxidant activity of α-La hydrolyzed with chymotrypsin was also higher than the activity of β-Lg hydrolyzed with trypsin. However, Pena-Ramos and Xiong (2001) reported whey hydrolyzed with trypsin, chymotrypsin and papain were not able to inhibit the lipid oxidation in a liposomal system. Even though papain had the greatest digestive ability compared with trypsin and chymotrypsin, whey hydrolyzed with papain was ineffecitve in inhibiting lipid oxidation.

Bioactive Properties of Whey and Whey Hydrolysates

Whey proteins are well known for high nutritional value. The proteins contain a number of bioactive peptides and amino acids which are beneficial for health. The biological properties of whey as muscle anabolism, immunomodulation, increasing glutathione synthesis and anticancer have been extensively reported (Bounous 2000; Cribb et al., 2007; Low et al., 2003; Madureira et al., 2007; Marshall, 2004; Mercier et al., 2004; Tseng et al., 2006). Whey proteins are considered to be fast proteins. They are rapidly emptied from the stomach and fast absorbed in the small intestine resulting in transient peak in plasmatic amino acids (Sukkar and Bounous, 2004). This enhances the biological properties of whey. The previous studies reported that high concentrations of branched-chain amino acids in whey can promote protein synthesis and improve muscle strength (Marshall, 2004; Sukkar and Bounous, 2004). Thus whey is recommended as nutritional support for athletes. Supplementation of whey with resistance training provided improvements in strength and muscle hypertrophy (Cribb et al., 2007). Additionally, the ingestion of whey improved muscle protein balance in elderly persons (Katsanos et al., 2008)

Whey contains various bioactive proteins that affect the immune system (Low et al., 2003; Madureira et al., 2007; Marshall, 2004). In vitro study demonstrated whey protein isolate (WPI) significantly enhanced the proliferation of murine spleen lymphocytes (Mercier et al., 2004). The effect of whey on the immune system is also mediated by enhancement of the glutathione synthesis. Glutathione is important in improvement of immune and antioxidant system. Whey is a source of cysteine, a

glutathione precursor. Ingestion of whey increases the production of glutathione. An oral administration of whey proteins increased the glutathione levels of HIV-infected patients (Micke, Beeh and Buhl, 2002; Micke et al., 2001). In cell culture studies, the glutathione level of Jurkat T cells was enhanced by whey (Middleton et al., 2003). Supplementation of 10 mg/ml whey successfully improved the glutathione level and glutathione reductase activity of pheochromocytoma cells (Tseng et al., 2006). However, the improvement of the glutahione synthesis was dependent on sources of whey (Middleton et al., 2003).

The impact of whey on cancer prevention is contributed by anticancer and anticarcinogenic activity (Bounous 2000; Madureira et al., 2007; Sukkar and Bounous, 2004). Whey is capable of depleting tumor cells and inhibiting tumor proliferation via increasing glutathione synthesis. When glutathione level increases, the detoxification of carcinogens is improved. Increase in glutathione synthesis by supplementation of whey also promotes the antioxidant activity (Tseng et al., 2006). In vitro studies demonstrated whey exhibited the antioxidant activity. The protein components in whey can inhibit lipid oxidation in the cooked pork patties (Pena-Ramos and Xiong, 2003). The high MW components in whey can scavenge the peroxyl radical and inhibit the lipid oxidation (Tong et al., 2000). BSA and lactoferrin had the metal ion chelating activity.

Modification of the bioactive properties of whey by enzymatic hydrolysis has been studied. The commonly used enzymes for the enzymatic hydrolysis of whey are trypsin, chymotrypsin, pepsin, papain, alcalase, corolase PP and flavourzyme (Hernandez-ledesma et al., 2005; Mercier et al., 2004; Pena-Ramos and Xiong, 2001; Ven et al., 2002). The bioactive properties of whey hydrolysates are antihypertensive, immunomodulating and antioxidative activity. The antihypertensive activity of whey hydrolysates relates to the inhibition of the angiotensin converting enzyme (ACE). This enzyme promotes vasoconstriction resulting increased blood pressure. Whey hydrolyzed with corolase PP, a pancreatic enzyme mixture, exhibited the ACE inhibitory activity (Ven et al., 2002). The ACE inhibitory activity of whey hydrolyzed with PTN 3.0S increased with the DH (Mullally, Meisel and Fitzgerald, 1997). This activity was contributed by the peptides released from β-Lg and α-La.

The immunomodulating activity of whey hydrolysates was reported in the previous study. Whey hydrolyzed with the combination of trypsin and chymotrypsin increased the lymphocytes proliferation by releasing the immunomodulating peptides (Mercier et al., 2004). The bioactive peptides contributing the proliferative effect were smaller than 3 kDa. They were derived from β-Lg, α-La and glycomacropeptide. Since β-Lg and α-La are capable of inducing immune response, these proteins cause milk allergy in human infant. Enzymatic hydrolysis of β-Lg and α-La is a practical way to reduce milk allergy. The proteolytic enzymes that effectively minimize the antigenicity were alcalase, flavourzyme and trypsin (Kim et al., 2007; Zheng et al., 2008).

For the antioxidant activity, whey hydrolysates show the ROS scavenging and reducing activity. Enzymatic hydrolysis of β-Lg and α-La improved the peroxyl radical scavenging activity (Hernandez-ledesma et al., 2005). Increase in digestion of the proteins improved the scavenging activity. Among the enzymes studied, corolase PP was the most effective enzyme in improving the scavenging activity of β-Lg and α-La. Whey hydrolyzed with alcalase possessed the ferric reducing antioxidant and radical scavenging activity (Peng et al., 2009). The bioactive peptides in the hydrolysates efficiently scavenged the DPPH, superoxide anion and hydroxyl radical as presented by the reduction of electron spin resonance (ESR) signal intensity. Additionally, the antioxidant activity of whey hydrolysates is mediated by increasing glutathione synthesis (Marshall, 2004). Whey hydrolyzed with the mixture of trypsin, chymotrypsin and protease promoted the glutathione synthesis and protected oxidant–induced cell death in human prostate cells (Kent et al., 2003).

Characterization of Protein Hydrolysates

1. Degree of Hydrolysis (DH)

DH is a number of peptide bonds cleaved during protein hydrolysis. This parameter is used to control quality of protein hydrolysates. Common methods are employed to determine the DH of protein hydrolysates are the pH stat (Amiot et al., 2004; Guerard, 2007; Kong and Xiong, 2006; Peng et al., 2009) and trinitrobenzene sulfonic acid (TNBS) method (Pena-Ramos and Xiong, 2001; Ven et al., 2002).

The pH stat method is based on the titration of the protons released from peptides in protein hydrolysates. When enzymatic hydrolysis carries out at neutral or alkaline conditions, the protons are dissociated from the free amino groups of the peptides leading to decreasing pH of the reaction mixture. Alkaline reagents as KOH and NaOH are used for titration of the protons to maintain a constant pH (Dong et al., 2008; Mercier et al., 2004; Peng et al., 2009). The consumption of the reagents is proportional to the peptide bonds cleaved in protein hydrolysates. This method is simple, non-denaturing and real-time monitoring (Guerard et al., 2007; Mercier et al., 2004; Silvestre, 1997). However it is not suitable for protein hydrolysates obtained from extensive hydrolysis. Under extensive hydrolysis condition, protein substrate may be digested to tripeptides, dipeptides and free amino acids which have high pKa as a result of underestimating DH (Spellman et al., 2003).

The TNBS method is based on the reaction between the TNBS reagent and primary amino groups of the peptides in protein hydrolysates forming a chromophore (Figure 1). The effect of high pKa of the small peptides and free amino acids from extensive hydrolysis does not interfere in the action of TNBS reagent (Spellman et al., 2003). Therefore the DH obtained by the TNBS method would be more accurate than the DH obtained by the pH stat method. The TNBS method is effective in determining DH of protein hydrolysates. The disadvantage of the method is time-consuming because the process takes a long time for the reaction to be complete (Adler-Nissen, 1979; Spellman et al., 2003; Ven et al., 2002).

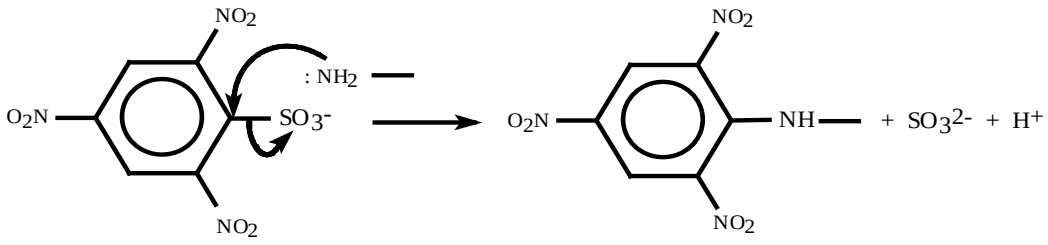


Figure 1 Scheme of the TNBS reaction with amino groups

Bioactivities of protein hydrolysates depend on characteristics of the hydrolysates including the DH (Amoit et al., 2004; Peng et al., 2009). Amiot et al.,

(2004) found that milk protein hydrolysate obtained from 6% DH contained higher extent of the small peptides than milk protein hydrolysate obtained from 4.5% DH. Milk protein hydrolysate presenting 6% DH effectively improved the growth of human keratinocyte cells. Peng et al., (2009) reported that (ferric reducing antioxidant power) FRAP value of whey hydrolyzed with alcalase was increased with increase of DH. When hydrolysis of whey reached a plateau, whey hydrolysate also showed the maximum FRAP value.

However, the antioxidant activity of protein hydrolysates is not necessary to be improved with the DH. The antioxidant activity of zein hydrolyzed with alcalase was the highest at 18% DH. Increase in the DH by increasing hydrolysis time decreased the activity of the hydrolysates (Kong and Xiong, 2006). Pena-Ramos and Xiong (2001) reported that the maximum inhibition of lipid oxidation was exhibited by whey hydrolyzed with protease F at the DH of 20%. As the DH increased to 35%, the activity of the hydrolysate was attenuated. Protein hydrolysates contained both antioxidative and prooxidative components. The prooxidative components in the hydrolysates were some peptides and amino acids. The effect of these components might overcome the antioxidant effect resulting in decreased antioxidant activity.

2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a gel electrophoresis technique used to demonstrate protein patterns of protein mixtures. The principle of this technique is based on the separation of the protein mixtures according to their molecular weight (MW). The protein mixtures are forced through a polyacrylamide gel in an electric field. The mobility of the proteins is depended on their MW, charge and conformation. In order to overcome the effect of charge and conformation, sample preparation is necessary for SDS-PAGE. The protein samples are treated with SDS and mercaptoethanol for disrupting secondary, tertiary and quaternary structure and reducing disulfide bonds of the proteins, respectively. Treated proteins are turned into linear structure with a series of negative charged SDS molecules (Figure 2). Thus the mobility of the proteins is inversely proportional to MW. Low MW protein presents high mobility. It moves

longer distance comparing high MW protein as shown in Figure 3 (Walker, 2005; Whitford, 2005).

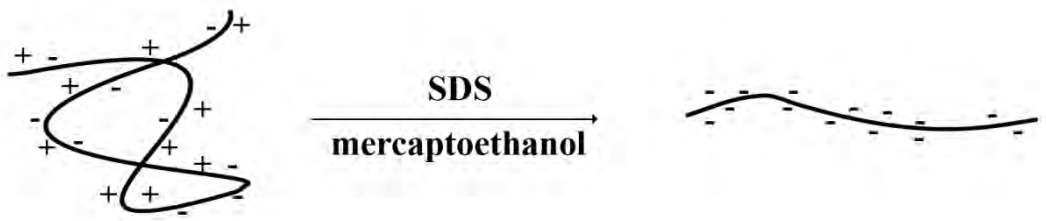


Figure 2 Diagram of protein transformation

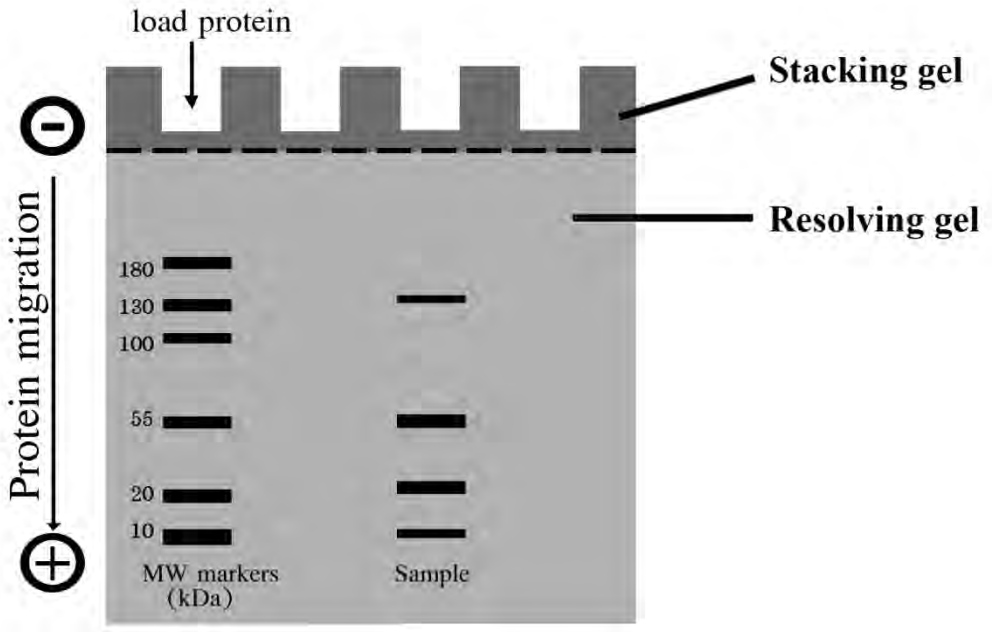


Figure 3 The protein pattern from SDS-PAGE technique

This technique can demonstrate the change in the protein compositions in the protein mixture. It is useful to determine the digestion of the protein components in protein hydrolysates (Alarcon et al., 2007; Goviddaraju and Srinivas, 2006; Pena-Ramos and Xiong, 2001) and determine the stability of protein mixture (Kehoe, Morris, Brodkorb, 2007; Havea et al., 2000). Kong and Xiong (2006) studied the digestion of zein by alcalase from the protein patterns of zein hydrolysates compared with native zein (Figure 4A). The reduction of the band intensities of zein components

showed that zein was digested by alcalase. α-zein was completely digested at the hydrolysis time of 3 hr, as indicated by disappearence of α-zein band. Moreover, this technique was able to demonstrate the difference in whey hydrolysates that obtained from different enzymes. At the 16% DH, the protein patterns of whey hydrolyzed with trypsin had the reduction of α-La band intensity while the protein patterns of whey hydrolyzed with pepsin showed disappearance of α-La band (Figure 4B and C). It indicated that at the same digestive ability of the enzymes, α-La was partially digested by trypsin and completely digested by pepsin (Pena-Ramos and Xiong, 2001).

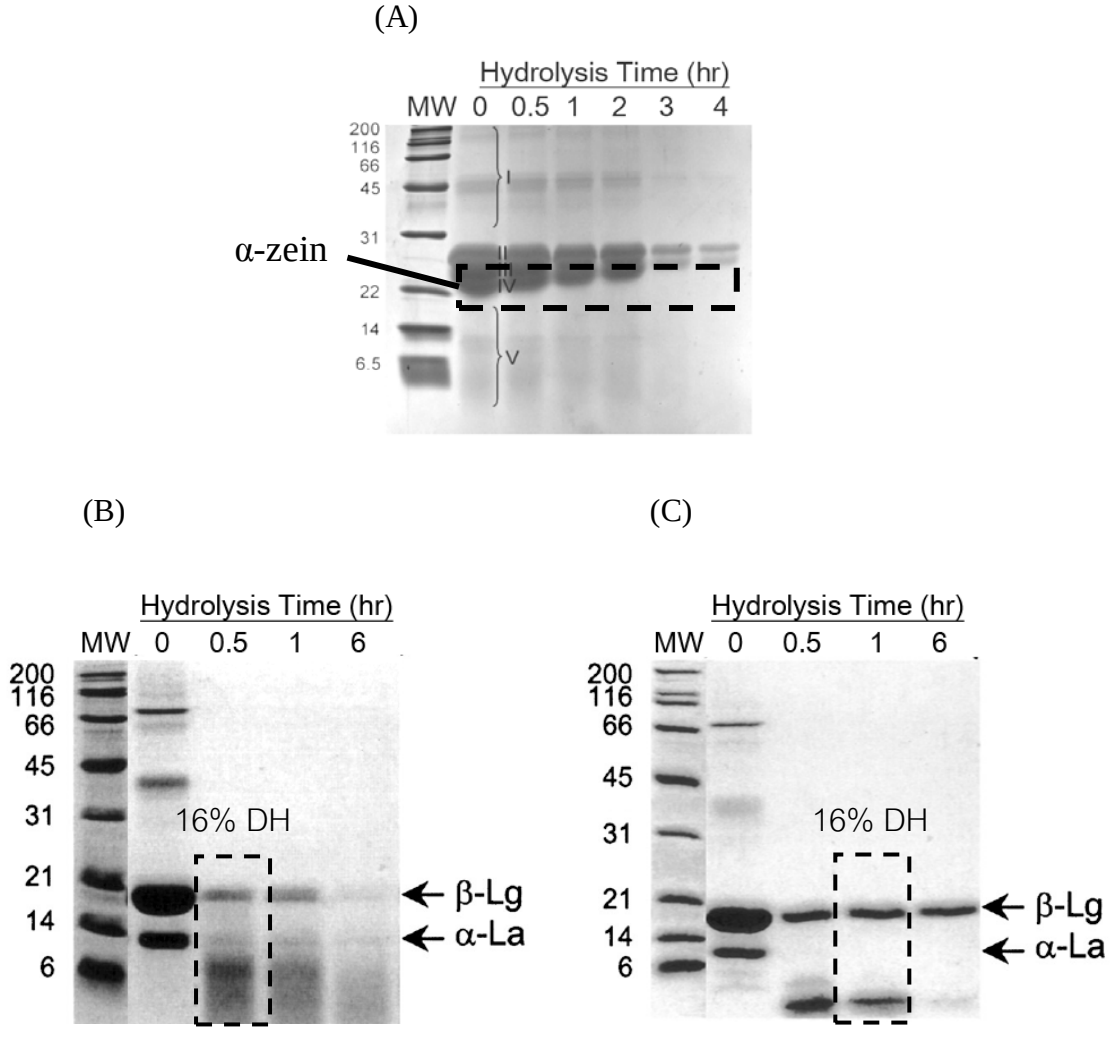


Figure 4 The protein patterns of zein hydrolyzed with alcalase (A) (Kong and Xiong, 2006) and whey hydrolyzed with trypsin (B) and pepsin (C) (Pena-Ramos and Xiong, 2001)

Stability of protein mixture is also determined by the SDS-PAGE technique. A change in size of the protein is demonstrated in the protein pattern. Havea et al. (2000) reported that after heating at 75°C, the appearance of additional protein bands was observed in the protein patterns of α-La and BSA mixture (Figure 5). This observation was found with the reduction of the band intensities of α-La and BSA. It suggested that α-La and BSA interacted with each other forming heat-induced aggregates.

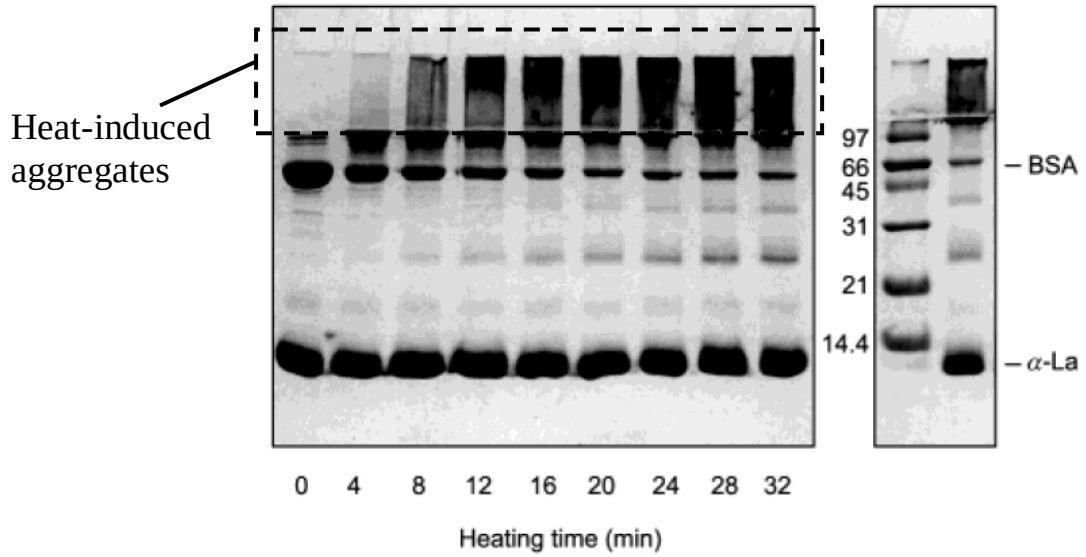


Figure 5 The protein patterns of α-La and BSA mixture under 75°C at various time (Havea et al., 2000)

Reactive Oxygen Species (ROS)

ROS are oxygen-derived molecules containing unpaired electron in atomic or molecular orbitals. They are essential to maintain homeostasis as host defense mechanisms and cellular signaling systems. The production of ROS mainly occurs in mitochondria. Electrons that leak from electron transport chain will combine molecular oxygen forming superoxide anion radical. This radical is depleted to less-reactive molecule, hydrogen peroxide via superoxide dismutase (SOD)-catalysed reaction. Hydrogen peroxide is enzymatically metabolized to molecular oxygen and water by hydrogen peroxide-removing enzymes, catalase, glutathione peroxidase

(GPx) and peroxiredoxin (Prx). Additionally, it is converted to hydroxyl radical via Fenton reaction. The radical is extremely reactive. It reacts close to its site of formation causing cell damage (Figure 6) (Nordberg and Arner, 2001). Under oxidative stress, the intracellular ROS are overproduced leading to deplete antioxidants and damage cellular components.

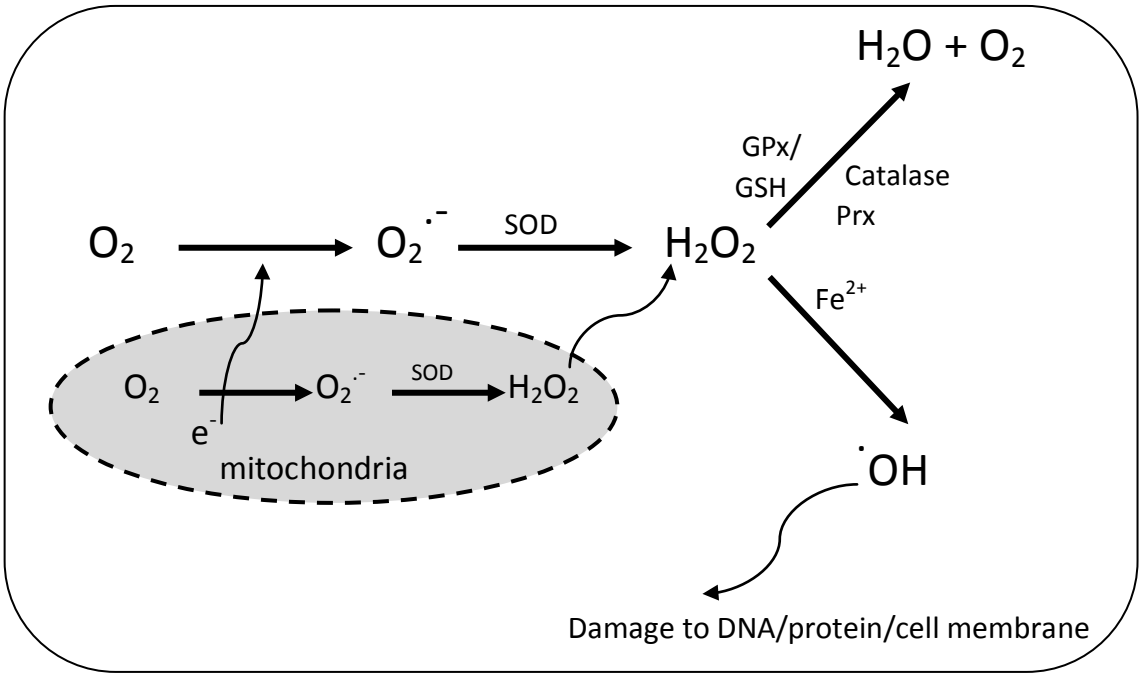


Figure 6 Fenton reaction in cells (modified from Nordberg and Arner (2001) and Valko et al. (2007))

In the study on the effect of oxidative stress in the cells, the substances that are commonly used to induce oxidative stress are quinones especially 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) (Heusler and Boehmer, 2004; Ishihara, Shiba, and Shimamoto, 2006; Pirev et al., 2008). The mechanism of the substance is similar to the ROS production in the physiological condition. DMNQ is reduced to semiquinone radical and undergoes autooxidation forming superoxide anion. The superoxide anion is converted to hydrogen peroxide and hydroxyl radical via Fenton reaction (Pinto and Castro, 2009).

A methodology for detecting ROS is usually based on fluorescence property of specific probes. The fluorescence probes are high sensitivity, specificity, simplicity

in data collection and high resolution in microscopic imaging techniques. The response of the fluorescence probes can be measured by various instruments as a microplate reader, flow cytometer and microscope. The selection of the probes is depending on types of ROS. For instance, 9, 10-dimethylanthracene (DMA) is specific to singlet oxygen. Dihydroethidium (HE) is specific to superoxide anion radical. 2,7-dichlorodihydrofluorescein (DCFH) is specific to hydrogen peroxide and hydroxyl radical. The fluorescence probe for investigating overall oxidative status of the cells should be specific to physiological ROS as DCFH (Myhre et al., 2003). This probe is used for detecting UV-generated ROS (Tobi, Paul and McMillan., 2000), arsenic-generated ROS (Shi et al., 2004) and overall intracellular ROS (Lai and Lee, 2006; Shim et al., 2008).

In general, DCFH is combined with diacetate forming 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). This probe diffuses passively through cell membrane and is de-esterified to non-fluorescent 2,7-dichlorodihydrofluorescein (DCFH) by intracellular esterases (Figure 7). In the presence of ROS, DCFH is oxidized to 2,7-dichlorofluorescein (DCF), highly fluorescent dichlorofluorescein which has excitation/emission wavelength at 498/522 nm (Figure 7). The emitted fluorescence response is directly proportional to concentration of intracellular ROS. Measurement of emitted fluorescence response is performed using flow cytometer (Gomes, Fernandes and Lima, 2005).

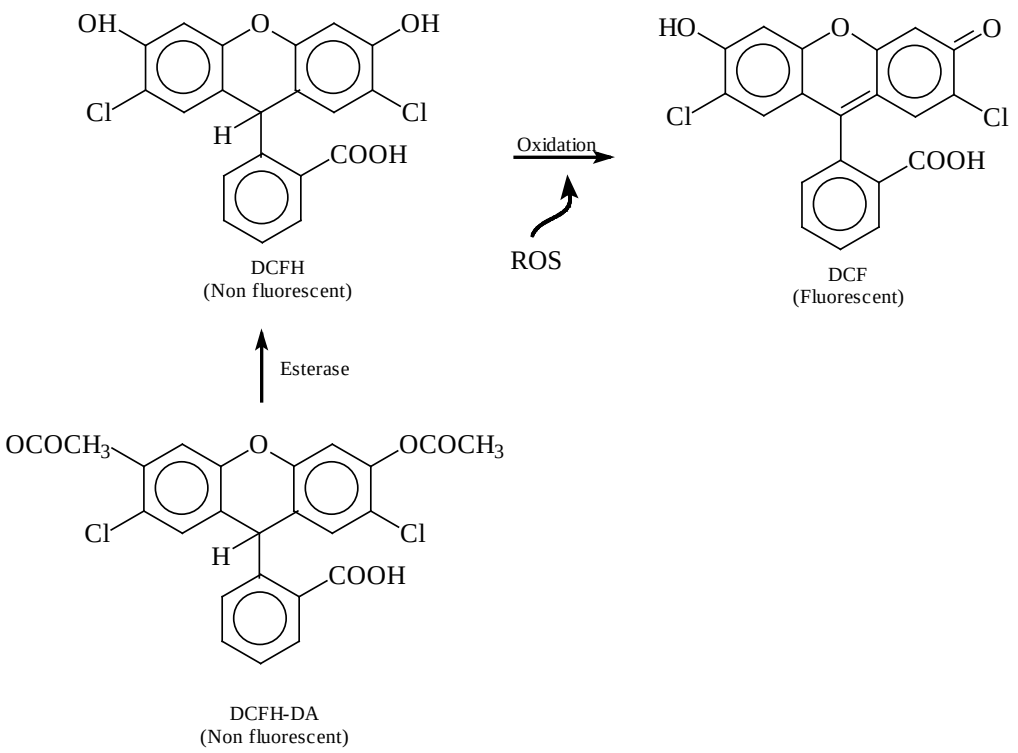


Figure 7 Mechanism of DCFH-DA de-esterification to DCFH and further oxidation to DCF (modified from Gomes et al. (2005))

Glutathione

Glutathione is sulfur-containing tripeptide which consists of glutamate, cysteine and glycine. The tripeptide is ubiquitous in mammalian and other living cells. It has two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). GSH plays an important role in cellular defenses against oxidative stress via direct reaction with ROS, enzymatic conjugation with electrophilic compounds and regeneration of vitamin C and E to active form (Valko et al, 2007). After these actions, GSH forms GSSG and regenerates to GSH by glutathione reductase with NADPH (Dickinson and Forman, 2002). Synthesis of glutathione requires two enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (Figure 8). The first step, glutamic acid reacts with cysteine to form γ-glutamyl-cysteine by γ-GCS. Following the reaction, γ-glutamyl-cysteine combines with glycine by the catalysis of glutathione synthetase producing glutathione (Marshall, 2004). Since availability of intracellular

cysteine is limited, rate-limiting step of glutathione synthesis is the first step (Dickinson and Forman, 2002; and Sen, 1997).

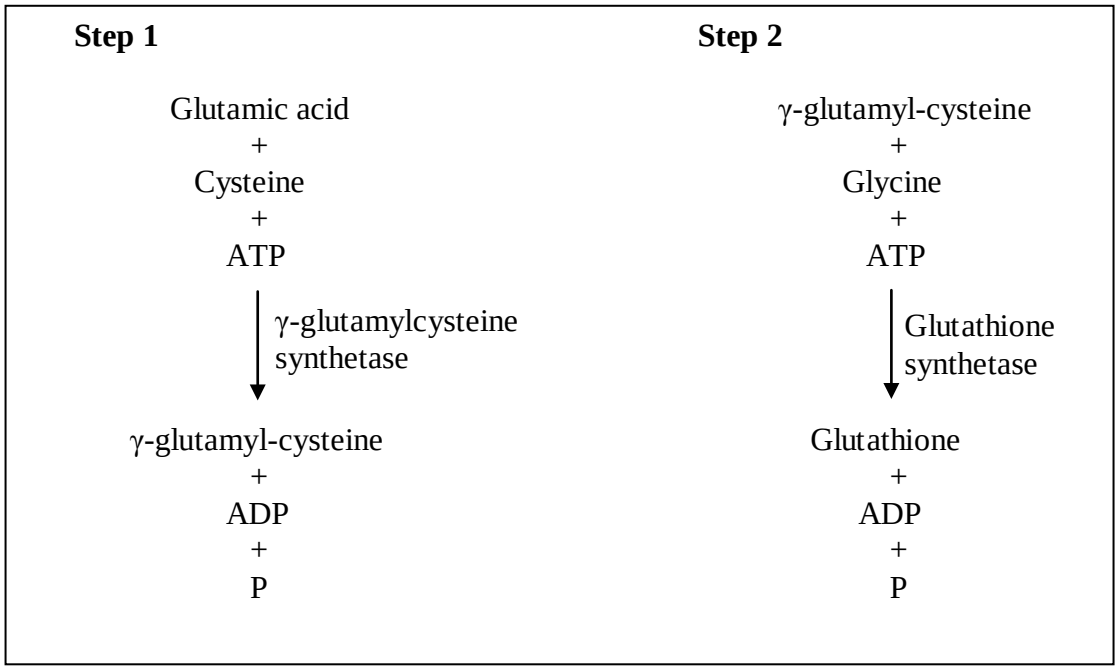


Figure 8 Synthesis of glutathione (modified from Marshall (2004))

Strategy to improve intracellular glutathione is increased availability of cysteine, a glutathione precursor. Administration of cysteine may improve glutathione synthesis. Since cysteine is easily oxidized and toxic, an administration of cysteine is not recommended. Delivery of cysteine-containing substances is received attention. Glutathione is also a cysteine-containing substance. However, increase in intracellular glutathione is not effective by glutathione administration because extracellular glutathione is mostly degraded by membrane-bound γ-glutamyl transpeptidase in extracellular compartment (Sen, 1997 and Sies, 1999). The other cysteine-containing substances are NAC, N-acetyl cysteine (NAC), oxothiazalidine carboxylate and glutathione analogs as glutathione monoester and glutathione diester (Anderson, 1998; Sen, 1997). These substances successfully increase intracellular glutathione (Figure 9). Additionally, a latest cysteine-containing substance that effectively improves intracellular glutathione is whey protein (Bounous, 2000). The previous

studies reported that supplementation of whey was effective in improved the glutathione levels in human prostate epithelial and pheochromatocytoma cells (Kent et al., 2003; Tseng et al., 2006). An oral administration of whey for three weeks can increase the glutathione level in Sprague-Dawley rats (Sukkar et al., 2008). Oral supplementation of whey protein with a daily dose of 45 g for 2 weeks increased the level of plasma glutathione by 50% in HIV-infected patients (Micke et al., 2001).

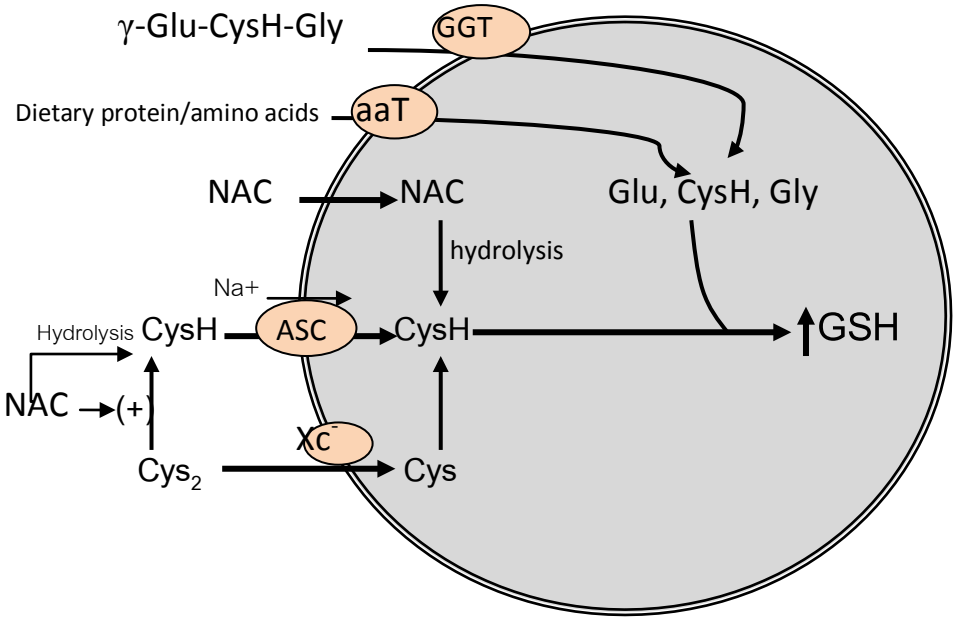


Figure 9 Mechanism of action of pro-glutathione (modified from Sen (1997))

A commonly used method for determining glutathione level in the living cells was monochlorobimane (MCB) conjugation. MCB is a non-fluorescent bimane. It freely penetrates across cell membrane to conjugate with GSH via catalysis of glutathione-s-transferase (GST) (Figure 10). GS-MCB conjugate is fluorescent at excitation/emission wavelengths of 390/460 nm. The emitted fluorescence is directly proportional to the glutathione level in the cells. The determination of intracellular glutathione using MCB was reported in rat astrocyte, human neurons, human neuroblastoma, human liver hepatocyte, human cervix carcinoma, human endometrium and Chinese hamster ovary cells (Schoonen et al., 2005; Sebastia` et al., 2003 and Waak and Dringen, 2006).

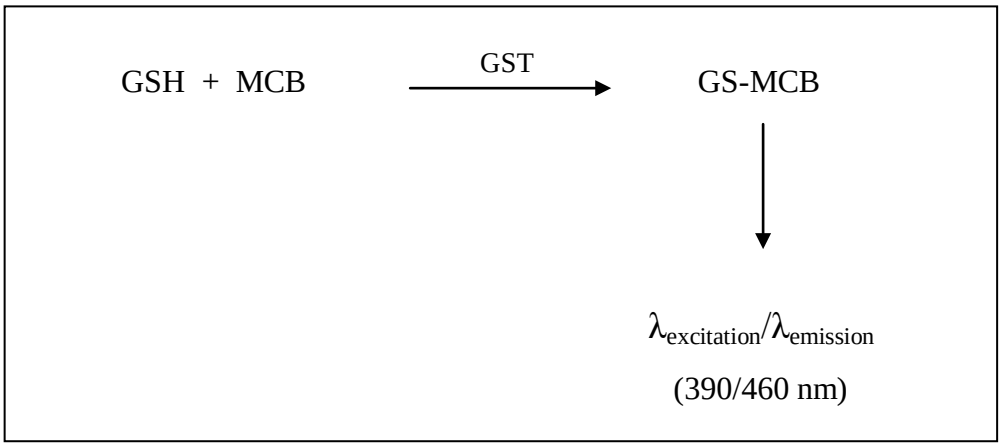


Figure10 The formation of GS-MCB conjugate

CHAPTER III

MATERIALS AND METHODS

Cell Culture

1. Human keratinocyte cell line, HaCaT cells (Cell Lines Service, Heidelberg, Germany, Lot no. 300493-144)

Chemicals

1. 40% Acrylamide/bis solution (Biorad, Hercules, CA, USA, Lot no. 1610144)
2. Ammonium persulfate (Biorad, Hercules, CA, USA, Lot no. 1610700)
3. Antibiotic Antimycotic Solution 100X (10,000 units penicillin, 10 mg Streptomycin and 25 µg amphotericin B per ml) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 028K2402)
4. β-mercaptoethanol (Biorad, Hercules, CA, USA, Lot no. 1610710)
5. Bio-Safe Coomassie (Biorad, Hercules, CA, USA, Lot no. BS081107)
6. Bromophenol blue (Biorad, Hercules, CA, USA, Lot no. 1610404)
7. Buthionine sulfoximine (BSO) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 1343040)
8. Chymotrypsin (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 086K7695)
9. 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 1000542555)
10. 2, 3-dimethoxy-1,4-napthoquinone (DMNQ) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 019K44098)
11. Dimethylsulfoxide (DMSO) Analytical Grade (Labscan Asia, Bangkok, Thailand, Lot no.07030033)
12. Disodium hydrogen phosphate (Ajax, Seven hills, Australia, Lot no. F2F136)
13. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA, Lot no. 562977)
14. Fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA, Lot no. 210080K)

15. Glycerol (Srichand United Dispensary Co., Ltd., Bangkok, Thailand)
16. Glycine (Biorad, Hercules, CA, USA, Lot no. 1610718)
17. Hydrochloric acid (Labscan Asia, Bangkok, Thailand, Lot no. G10W65)
18. L-glutamine (GlutaMAX) (Invitrogen, Carlsbad, CA, USA, Lot no. 654459)
19. L-leucine (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 085K5304)
20. Monochlorobimane (MCB) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 1393526)
21. N-acetyl-cysteine (NAC) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 1000542555)
22. N, N, N, N-tetramethyl-ethylenediamine (TEMED) (Biorad, Hercules, CA, USA, Lot no. 1610800)
23. PageRuler prestained protein ladder (Fermentas, Burlington, ON, Canada Lot no. 00036958)
24. Papain (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 118K1465)
25. Phosphate buffered saline (PBS)10X pH 7.4 (Invitrogen, Carlsbad, CA, USA, Lot no. 438129)
26. Sodium hydroxide (Merck, Darmstadt, Germany, Lot no. B0119798 726)
27. Sodium dihydrogen phosphate (Merck, Darmstadt, Germany, Lot no. KK220501045 530)
28. Sodium dodecyl sulphate (SDS) (Ajax Finechem, Auckland, New Zealand, Lot no. 0802175)
29. Sodium dodecyl sulphate (Biorad, Hercules, CA, USA, Lot no. 1610301)
30. Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 23396AJ)
31. Trinitrobenzene sulfonic acid (TNBS), 5% (w/v) (Sigma-Aldrich, St. Louis, MO, USA, Lot no. 068K5001)
32. Tris (Biorad, Hercules, CA, USA, Lot no. 1610719)
33. Trypan blue stain 0.4% (Invitrogen, Carlsbad, CA, USA, Lot no. 1368311)
34. Trypsin (Invitrogen, Sao Paulo, Brazil, Lot no.1256246)
35. 0.25% Trypsin-EDTA (Invitrogen, Burlington, ON, Canada, Lot no. 690176)
36. Whey protein isolated (HMS 90) (IMMUNOTHAI Co., Ltd., Bangkok, Thailand, Lot no. A074178HP)

Accessories

1. 25 cm^2 cell culture flask, canted neck with 0.2 µm vent cap (Corning Incorporated, New York, NY, USA)
2. 96-well black clear bottom cell cultured plate (Corning Incorporated, New York, NY, USA)
3. 96-well cell cultured plate (Corning Incorporated, New York, NY, USA)
4. Centrifuge tube 15 ml, plug seal cap (Corning Incorporated, New York, NY, USA)
5. Centrifuge tube 50 ml, plug seal cap (Corning Incorporated, New York, NY, USA)
6. Cryogenic vial (Corning Incorporated, New York, NY, USA)
7. Disposable sterile pipette 5 ml (Corning Incorporated, New York, NY, USA)
8. Disposable sterile pipette 10 ml (Corning Incorporated, New York, NY, USA)
9. Microcentrifuge tube 1.5 ml (Corning Incorporated, New York, NY, USA)
10. Pipette tips, 1-10 µl universal fit pipette tips (Corning Incorporated, New York, NY, USA)
11. Pipette tips, 20-200 µl universal fit pipette tips (Corning Incorporated, New York, NY, USA)
12. Pipette tips, 100-1000 µl universal fit pipette tips (Corning Incorporated, New York, NY, USA)

Equipment

1. Analytical balance (Model AX105 Delta Range, Mettler Toledo, Greifensee, Switzerland)
2. CO_2 incubator (Model 311, Thermo Elecrtron Corp., Marietta, OH, USA)
3. Centrifuge (Model Universal 320R, Hettich, Tuttlingen, Germany)
4. Flow cytometer (Model BD FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA)
5. Incubator (Model BED 115, WTC binder, Tuttlingen, Germany)
6. Laminar hood biosafety cabinet class II (Model ABS1200CL32MK2, ASTEC Microflow, Hants, UK)

7. Magnetic stirrer (Model RCT basic, KIKA works, Guaunghou, China)
8. Manifold freeze dryer (Model FD-6-85DMPO, Dura-dry, Warminster, PA, USA)
9. Micropipette (Biohit, Helsinki, Finland)
10. Microplate reader (Model VICTOR^3, Perkin Elmer, Beaconsfield, UK)
11. PowerPac basic power supply (Bio-rad, Hercules, CA, USA)
12. Orbital shaker (Model SO5, Stuart Scientific, Staffordshire, UK)
13. Peristaltic pump (Model ISM 829, ISMATEC, Glattbrugg, Switzerland)
14. pH meter (Model 420A, Orion Research Inc., Jacksonville, FL , USA)
15. Reversed microscope (Model CKX41, Olympus, Tubingen, Germany)
16. UV-Vis spectrophotometer (Model UV-1601, Shimadzu, Kyoto, Japan)
17. Vortex mixer (Model GENIE-2, Scientific Industries, Bohemia, NY, USA)
18. Water bath (Thermo NESLAB, Waltham, MA, USA)
19. Water bath (Grant Instruments, Cambridge, UK)

Methods

1. Preparation of Whey Hydrolysates

Conditions for preparation of whey hydrolysates were designed based on the 3^3 factorial designs. A total of 27 experimental trials were examined. The independent variables were hydrolysis time (x_1), enzymes to substrate ratio (E/S, x_2) and types of enzyme (x_3). Three levels of each independent variable were 1, 3 and 5 hr for hydrolysis time, 1/1000, 1/200 and 1/100 (w/w) for E/S, and chymotrypsin, trypsin and papain for types of enzyme were investigated. The dependent variables were categorized into 2 types: categorical variable and continuous variables. The categorical variable was protein patterns (y_1). The continuous variables were % DH (y_2) and relative DCF fluorescence (y_3). The relative DCF fluorescence was used to represent ROS scavenging activity. The design formulations were listed in Table 3. Each experiment was performed three times for each condition tested.

Whey hydrolysate was prepared according to Pena-Ramos, and Xiong (2001). Briefly, 20 mg of whey was mixed with 990 µl of 0.01 M sodium phosphate buffer pH 8.0. The solution was preheated at 90^{o}C for 5 min and then cooled down for 5 min at ambient temperature. After that, 10 µl of tested enzyme was added and incubated for appropriate time at 37^{o}C to hydrolyze the peptide bonds. The hydrolyzed sample was heated at 85^{o}C for 10 min to inactivate the enzyme. The hydrolysate was stored at 4^{o}C and used within 24 hr.

Table 3 Design formulations

Formulation	Type of enzyme	E/S (w/w)	Hydrolysis time (hr)
100-Ch1	Chymotrypsin	1/100	1
100-Ch3	Chymotrypsin	1/100	3
100-Ch5	Chymotrypsin	1/100	5
200-Ch1	Chymotrypsin	1/200	1
200-Ch3	Chymotrypsin	1/200	3
200-Ch5	Chymotrypsin	1/200	5
1000-Ch1	Chymotrypsin	1/1000	1
1000-Ch3	Chymotrypsin	1/1000	3
1000-Ch5	Chymotrypsin	1/1000	5
100-T1	Trypsin	1/100	1
100-T3	Trypsin	1/100	3
100-T5	Trypsin	1/100	5
200-T1	Trypsin	1/200	1
200-T3	Trypsin	1/200	3
200-T5	Trypsin	1/200	5
1000-T1	Trypsin	1/1000	1
1000-T3	Trypsin	1/1000	3
1000-T5	Trypsin	1/1000	5
100-Pa1	Papain	1/100	1
100-Pa3	Papain	1/100	3
100-Pa5	Papain	1/100	5
200-Pa1	Papain	1/200	1
200-Pa3	Papain	1/200	3
200-Pa5	Papain	1/200	5
1000-Pa1	Papain	1/1000	1
1000-Pa3	Papain	1/1000	3
1000-Pa5	Papain	1/1000	5

2. Characterization of Whey Hydrolysates

Whey hydrolysates were characterized as protein patterns, degree of hydrolysis (DH) and ROS scavenging activity.

2.1 Determination of Protein Patterns of Whey Hydrolysates by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein patterns of whey hydrolysates were determined by SDS-PAGE technique using a 15% acrylamide resolving gel and a 3% acrylamide stacking gel (Pena-Ramos, and Xiong, 2001). Briefly, resolving gel was applied into gel plates and left the gel to polymerize for 20 min. After 20 min, stacking gel was loaded over the resolving gel and allowed to polymerize for 40 min. The polymerized gels were placed in chamber containing running buffer. Approximately, 4 µl of protein ladder and 10 µl of tested substances were loaded in separate well of stacking gel. The gel was run for 2 hr at 85 V by PowerPac basic power supply. After that, the gel was removed from the chamber and washed three times with water. Then, the gel was stained with 10 ml of Biosafe Coomassie staining solution for 1 hr using orbital shaker. Stained gel was placed in 300 ml of water for 48 hr to remove the staining solution residue. Protein patterns of whey hydrolysates were compared with those of whey and the PageRuler prestained protein ladder (10-100 kDa). Each experiment was performed two times for each condition tested.

2.2 Determination of Degree of Hydrolysis (DH) of Whey Hydrolysates

The DH of whey hydrolysates was determined by measuring the amount of primary amino groups using the trinitrobenzene sulfonic acid (TNBS) method (Adler-Nissen, 1979). Whey and whey hydrolysates were dispersed in 1% w/v sodium dodecyl sulfate (SDS). A tested sample of 125 µl was mixed with 1 ml of 0.2125 M sodium phosphate buffer pH 8.2. After that, 2 ml of 0.1% w/v TNBS solution was added to form a chromophore. The sample solution was kept in the dark at 50^{o}C for 60 min. At the end of incubation, 2 ml of 0.1 N HCl was added to terminate the reaction. The solution was allowed to cool for 30 min at ambient temperature prior to

analysis of amino nitrogen content using UV-Vis spectrophotometer. The absorbance was read at 340 nm and transformed to the amount of amino nitrogen using a leucine standard curve. % DH was calculated by subtracting amino nitrogen of whey from those of whey hydrolysate and dividing by amino nitrogen content of peptide bonds in whey as in equation 1. The concentrations of leucine were 0.8, 1, 1.4, 1.6, 2 and 2.4 mM. The blank solution was 1% w/v SDS. Each experiment was performed three times for each condition tested.

$$\% \text{ DH} = \frac{(AN_2 - AN_1) \times 100}{\text{Npb}} \qquad \text{(equation 1)}$$

AN_1 = amino nitrogen content of whey

AN_2 = amino nitrogen content of whey hydrolysate

Npb = amino nitrogen content of peptide bonds in whey (123.3 mg/g of protein (Spellman et al., 2003))

2.3 Scavenging Activity of Whey and Whey Hydrolysates

Human keratinocyte cells line (HaCaT cells) were obtained from Cell Lines Service (Heidelberg, Germany). HaCaT cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% of 200 mM L-glutamine, 1% of 1,000,000 units/ml penicillin, 1,000 mg/ml streptomycin and 2.5 mg amphotericin. The cells were grown and maintained in 25 cm^2 cell culture flasks at 37°C in 5% CO_2 humidified atmosphere (Sanchez et al., 2006). Cells were used for experiment between passages 50 and 70.

2.3.1 Determination of Optimal Substances Concentration

Dose dependent studies were performed using thiazolyl blue tetrazolium bromide (MTT) assay to determine the effect of each tested substances on the cells viability and to find the optimal concentration of each tested substances needed for ROS scavenging activity study. The substances tested were 2, 3-dimethoxy-1,4-

napthoquinone (DMNQ, a ROS generator), N-acetyl-cysteine (NAC, a positive control), buthionine sulfoximine (BSO, a specific glutathione inhibitor), whey and whey hydrolysates. DMNQ concentrations tested were 10, 20 and 30 µM. NAC concentrations tested were 0.1, 1, 5 and 10 mM. BSO concentrations tested were 0.01, 0.1, 0.5 and 1 mM. Whey and whey hydrolysates concentrations tested were 0.1, 0.5, 1 and 2 mg/ml.

HaCaT cells were seeded in 96-well cell cultured plate at a cell density of 1×10^4 cell/well. At 70-80% confluence, the medium was removed and replaced with 180 µl complete medium. Approximately, 20 µl of tested substances were added gently to the cells in each well. Cells were incubated for 24 hr at 37°C under 5% CO_2 humidified atmosphere. After incubation for 24 hr, the cells were washed twice with 200µl of phosphate buffer saline (PBS) and further incubated with 50 µl of 0.45 mg/ml MTT reagent at 37°C for 4 hr. After 4 hr, the MTT solution was removed and 100µl of DMSO was added to dissolve the formazan crystal. The intensity of the MTT product was measured at 570 nm using microplate reader (Fotakis and Timbrell, 2006; Je et al., 2007). The relative percentage of cell viability was calculated by dividing the absorbance of treated cells by that of the control in each experiment (equation 2). Cultured cells incubated without the test compounds were served as untreated cells (control).

$$\% \text{ Cell viability} = \frac{A_{treatment} * 100}{A_{control}} \qquad \text{(equation 2)}$$

$A_{treatment}$ = absorbance of treated cells

$A_{control}$ = absorbance of untreated cells

Each experiment was performed three times in triplicates for each condition tested. DMNQ concentration causing 20% inhibition of cell viability (IC_{20}) was selected for stimulating the ROS production. Non cytotoxic concentrations of NAC, BSO, whey and whey hydrolysates were selected for ROS scavenging activity study.

2.3.2 Determination of Scavenging Activity of Whey and Whey Hydrolysates against DMNQ-generated ROS

The scavenging activity of whey and whey hydrolysates against DMNQ-induced ROS generation in HaCaT cells was investigated. The amount of intracellular ROS was determined by flow cytometer using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), a specific probe for ROS (Shim et al., 2008). The optimal concentrations of each tested substances were obtained from section 2.3.1.

HaCaT cells were cultured in 25-cm^2 cell cultured flask. At 90% confluence, the medium was removed and washed twice with 1 ml of PBS. The cells were incubated with 1 ml of 15 µM DCFH-DA in PBS for 30 min at 4°C. After 30 min incubation, the cells were washed 3 times with 1 ml of PBS followed by incubation with 200 µl of 0.25% trypsin-EDTA solution for 30 sec. The solution was then removed and the cells were gently washed with 1 ml of complete medium. After that, approximately 2 ml of serum-free medium was added. The cells were placed on ice and were scrapped using cell scrapper followed by trituration. And then, 2 ml of serum-free medium containing 30 µM DCFH-DA was added to give final concentration of 15 µM DCFH-DA. The cell suspension was divided to microcentrifuge tube for 360 µl/tube. The cells were pretreated with 40 µl of tested substances and incubated for 1 hr at 37°C under 5% CO_2 humidified atmosphere. After incubation for 1 hr, the cells were treated with 4 µl of 1 mM DMNQ to give final concentration of 10 µM DMNQ and incubated for 4 hr at 37°C under 5% CO_2 humidified atmosphere to induce intracellular ROS production. At the end of incubation, the intracellular ROS were expressed as DCF fluorescence measuring at excitation/emission wavelengths: 498/522 nm (Gomes et al., 2005) by flow cytometer using CellQuest Pro software. The DCF fluorescenc response was collected from 10,000 events for each sample. Relative DCF fluorescence was calculated by dividing the DCF fluorescence response of treated cells by that of the control in each experiment (equation 3). Cultured cells incubated without the test substances were served as untreated cells (control).

$$\text{Relative DCF fluorescence} = \frac{F_{treatment}}{F_{control}} \quad \text{(equation 3)}$$

$F_{treatment}$ = fluorescence response of treated cells

$F_{control}$ = fluorescence response of untreated cells

Each experiment was performed three times for each condition tested. Whey hydrolysate given the highest ROS scavenging activity was selected for investigating its ability to stimulate glutathione synthesis and for determining stability of whey hydrolysate powder.

3. Determination of Glutathione Synthesis

The effect of whey hydrolysates on glutathione synthesis was studied under the comparable condition of section 2.3.2 without DMNQ treatment using monochlorobimane (MCB), specific probe for glutathione (Sebastia` et al., 2003). The tested substances were BSO, a specific glutathione inhibitor, NAC, a positive control, and whey hydrolysate given the highest ROS scavenging activity chosen from section 2.3.2.

HaCaT cells were seeded in 96-well black clear bottom cell cultured plate at 2×10^4 cell/well. At 90% confluence, the medium was removed and replaced with 90 µl serum-free medium. Approximately, 10 µl of tested substances were added gently to the cells in each well. The cells were incubated for 5 hr at 37°C under 5% CO_2 humidified atmosphere. After incubation for 5 hr, the cells were washed twice with 200 µl of PBS and further incubated with 100 µl of 40 µM MCB reagent at 37°C for 2 hr. After 2 hr incubation, the fluorescence intensity of the conjugated product was read at the excitation/emission wavelengths: 390/460 nm by microplate reader using Wallac software. The relative percentage of glutathione was calculated by subtracting the fluorescence intensity of the blank from those of treated cells and dividing by that of the control in each experiment (equation 4). Blank was the cells in PBS before incubating with MCB. Cultured cell incubated without the tested substances were

served as untreated cells (control). Each experiment was performed three times in triplicates for each condition tested.

$$\% \text{ Glutathione} = \frac{(F_{treatment}-F_{treatment\ blank}) * 100}{(F_{control}-F_{control\ blank})} \quad \text{(equation 4)}$$

$F_{treatment}$ = fluorescence response of treated cells

$F_{treatment\ blank}$ = fluorescence response of treated cells blank

$F_{control}$ = fluorescence response of untreated cells

$F_{control\ blank}$ = fluorescence response of untreated cells blank

4. Evaluation of Stability of Whey Hydrolysates

4.1 Effect of Freeze Drying Process on Stability of Whey Hydrolysate Powder

In order to investigate the effect of freeze drying process on the stability of whey hydrolysate powders, whey hydrolysate given the highest ROS scavenging activity selected from section 2.3.2 was freshly prepared as described in section 1. The solution was freeze dried for 48 hr using manifold freeze dryer to obtain whey hydrolysate powder. The hydrolysate powder was characterized for the protein patterns, DH and ROS scavenging activity as described in section 2 within 24 hr. The properties of whey hydrolysate powder were compared with those of whey hydrolysate solutions. An experiment was repeated five times for each condition tested.

4.2 Stability of Whey Hydrolysate Powder under Accelerated Condition

Stability of whey hydrolysate powder was investigated under ICH guideline condition: 40^{o}C 75% relative humidity (RH) for 3 months (ICH Topic Q1A (R_2), 2003). Whey hydrolysate powder was stored in glass vial with rubber cap placing at

40^{o}C in 75%RH. The hydrolysate powder was withdrawn from the storage at intervals of 1 month for 3 months to determine the change in the protein patterns, DH and ROS scavenging activity as described in section 2. RH of 75% was obtained using saturated sodium chloride solution (Hong et al., 2005). The experiments were performed in triplicates for each condition tested.

5. Statistical Analysis

The percentage of DH and cell viability and relative DCF fluorescence were expressed as mean ± standard deviation (SD). Statistical analysis of these data was performed using analysis of variance (ANOVA) and Student's *t*-test. *p*-value less than 0.05 was considered significant difference. Multiple comparisons for analyzing significant difference among tested groups were Tukey and Dunnett's T3 for equal and unequal variances, respectively.

The effect of hydrolysis conditions on the DH values and ROS scavenging activity of whey hydrolysates was analyzed using three-way ANOVA. The effect of substances on the HaCaT cells viability was analyzed using one-way ANOVA and the multiple comparisons. The effect of substances on the relative DCF fluorescence of DMNQ-treated cells and the change in the DH values and relative DCF fluorescence in the stability study were analyzed using Student's *t*-test.

CHAPTER IV

RESULTS AND DISCUSSION

Effect of Hydrolysis Conditions on Protein Patterns of Whey Hydrolysates

The protein components in whey found in this study was similar to other reports (Dewit and Klarenbeek, 1984; Madureira et al., 2007; Pena-Ramos and Xiong, 2001). SDS-PAGE showed that whey composed of α-La (14 kDa), β-Lg (18 kDa), BSA (66 kDa), lactoperoxidase (70 kDa) and lactoferrin (80 kDa) (Figure 11).

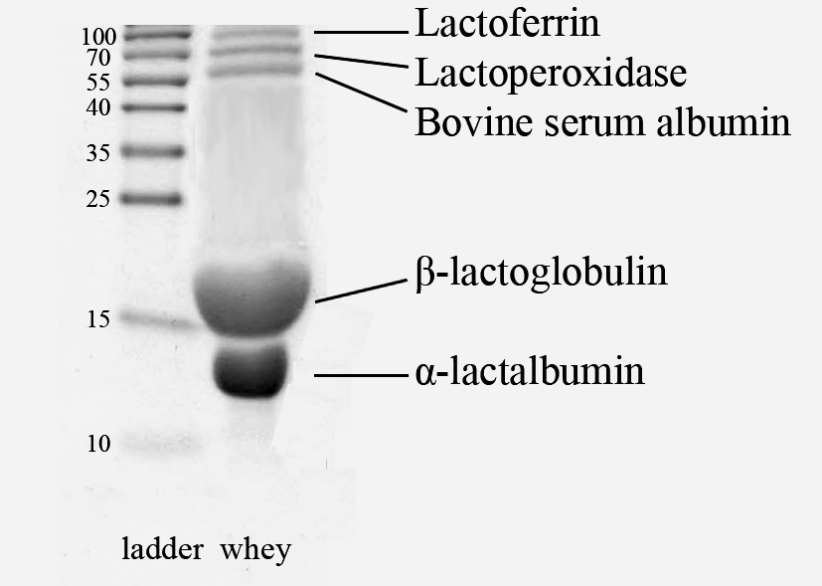


Figure 11 The protein pattern of whey

The protein patterns of whey hydrolysates were shown in Figures 12-14. Characterization studies of whey hydrolysates found that the protein patterns of whey hydrolysates depended on the E/S, hydrolysis time and types of enzyme.

The protein patterns of whey hydrolyzed with chymotrypsin was shown in Figure 12. When whey was hydrolyzed by chymotrypsin, the bands of high MW (>60 kDa) components, BSA, lactoperoxidase and lactoferrin, can not be detected in the gel within 1 hr at all E/S used in this study. For β-Lg and α-La, the major components in

whey, increased E/S and hydrolysis time increased the digestion of these proteins, as observed in the reduction of the protein bands. Increased E/S provided more available enzyme to hydrolyze whey while increased hydrolysis time extended the duration of reaction between enzyme and substrate, consequently the ability of enzyme to hydrolyze protein was improved (Guerard et al., 2007; Palmer, 1995). However, the appearance of β-Lg and α-La at the E/S of 1/100 under the hydrolysis time of 5 hr suggested that whey was not completely digested by chymotrypsin. This finding was consistent with the previous study by Pena-Ramos and Xiong (2001). The authors reported that the digestion of preheated whey by chymotrypsin at the E/S of 1/100 increased with the hydrolysis time. It is of interest that as the hydrolysis time increased up to 6 hr, the protein components in whey including β-Lg and α-La were completely digested by the enzyme. Thus, the complete digestion of β-Lg and α-La might be observed in this study if the hydrolysis time was extended to 6 hr.

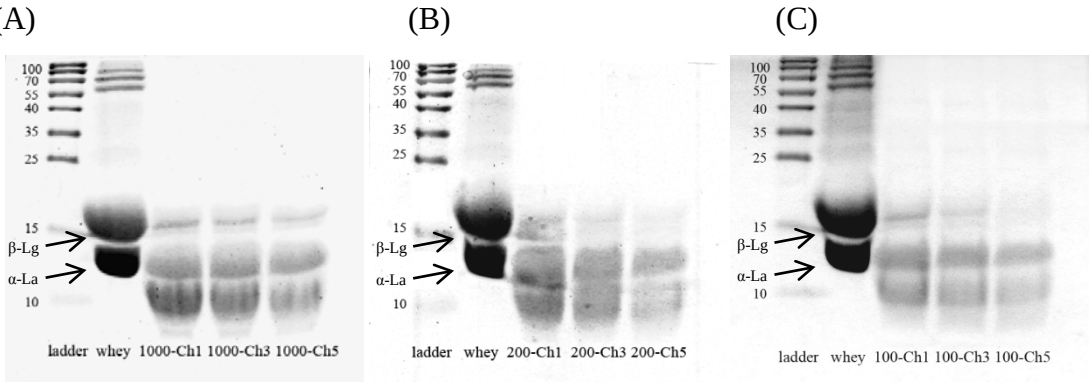


Figure 12 The protein patterns of whey hydrolyzed with chymotrypsin at the E/S of 1/1000 (A), 1/200 (B) and 1/100 (C) under various hydrolysis time: 1, 3 and 5 hr

For trypsin, the high MW components in whey were completely hydrolyzed within 1 hr at all E/S studied (Figure 13), as indicated by the disappearance of these protein bands in the gel. Similar to chymotrypsin, when the E/S and hydrolysis time increased, the digestion of β-Lg and α-La was improved. At the E/S of 1/100, β-Lg was completely hydrolyzed by trypsin within 1 hr, while α-La was partially hydrolyzed by this enzyme (Figure 13C). This result agreed with the previous study that β-Lg was more susceptible to trypsin than α-La (Hernandez-ledesma et al., 2005).

Moreover, the complete digestion of β-Lg by trypsin indicated that trypsin was more effective in hydrolyzing whey than chymotrypsin.

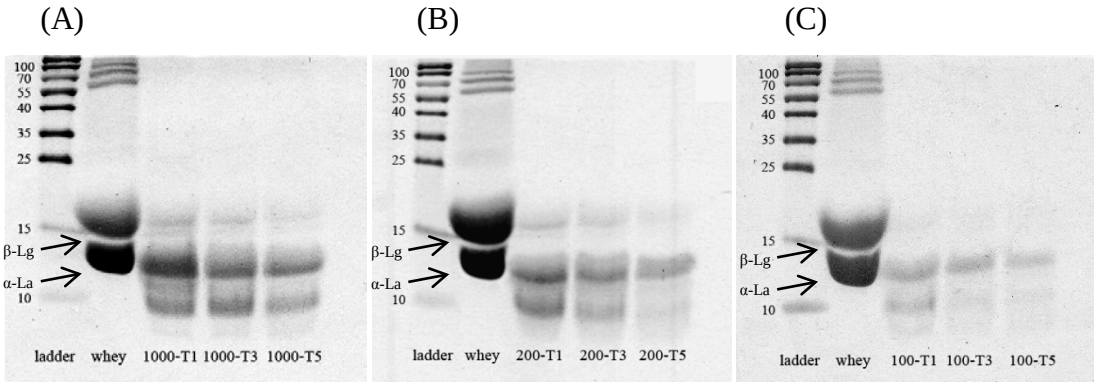


Figure 13 The protein patterns of whey hydrolyzed with trypsin at the E/S of 1/1000 (A), 1/200 (B) and 1/100 (C) under various hydrolysis time: 1, 3 and 5 hr

High susceptibility of trypsin to whey compared with chymotrypsin was associated with the ability of enzyme to digest protein substrate. Trypsin is a specific protease. The binding pocket of this enzyme is deep and narrow. In order for this enzyme to cleave the peptide bonds, the amino acids which possess a long side chain and positive charge needs to fit into its pocket to form an ionic bond with negative charged aspartate at the bottom of the pocket (Oleson, Ong, and Mann, 2004; Whitford, 2005). The specific amino acids for trypsin are only lysine and arginine thus the digestive ability of trypsin is quite limited. However, trypsin was effective in hydrolyzing whey in this study. The enzyme can completely digest most protein components in whey except α-La. This was explained that even the specific sites of trypsin were only two amino acids, these amino acids were abundant in whey leading to improved digestion of whey by the enzyme.

In contrast, chymotrypsin is broad specific than trypsin. The binding pocket of chymotrypsin is large and contains many hydrophobic groups as a result the specific amino acids for this enzyme are amino acids which possess hydrophobic and aromatic groups in their structure as phenylalanine, tyrosine, tryptophan and methionine (Amoit et al., 2004; Whitford, 2005). Although the number of the specific amino acids for chymotrypsin was higher than the specific amino acids for trypsin, the extent

of these amino acids was lower in whey. Thus the digestive ability of chymotrypsin was less than the ability of trypsin as indicated by the partial digestion of β-Lg (Figure 13). High digestive ability of trypsin compared with chymotrypsin was demonstrated in the enzymatic hydrolysis of β-Lg (Hernandez-ledesma et al., 2005). It suggested that high extent of the susceptible amino acids for trypsin in β-Lg contributed the digestive ability of the enzyme.

Similar to trypsin and chymotrypsin, the high MW components in whey were completely hydrolyzed by papain within 1 hr at all E/S studied (Figure 14). The digestive ability of papain on β-Lg and α-La improved with the increased E/S. The effect of the hydrolysis time on the digestive ability of papain was not observed in the protein patterns. This can be explained that the digestion of whey by papain was fast. The extensive digestion of whey in the first hour considerably decreased the concentration of the peptide bonds available for the enzyme. Thus increased hydrolysis time over 1 hr did not markedly improve the digestion of whey by papain. This result was supported by the study of Pena-Ramos and Xiong (2001) which demonstrated that papain was able to completly hydrolyze whey within 30 min. Moreover, papain was found to be the most effective enzyme in hydrolyzing whey compared to trypsin and chymotrypsin. This enzyme was able to completely hydrolyze β-Lg and α-La within 1 hr at the E/S of 1/200 and 1/100 (Figure 14B and C). The degradation products observed in the gels had MW lower than 15 kDa. However, the peptides had MW lower than 1 kDa could not be detected in the gel. They were removed from the gel during washing and staining process.

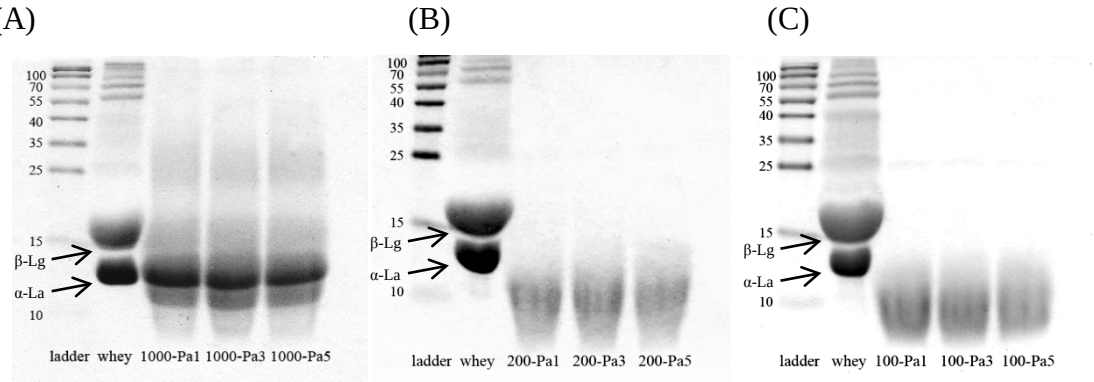


Figure 14 The protein patterns of whey hydrolyzed with papain at the E/S of 1/1000 (A), 1/200 (B) and 1/100 (C) under various hydrolysis time: 1, 3 and 5 hr

High digestive ability of papain was contributed by enzyme specificity. Papain is a broad specific enzyme that preferably cleaves the peptide bonds of leucine, glycine, histidine, lysine and arginine including ester and amide bonds (Sangeetha and Abraham, 2006). Therefore the amount of the specific sites for papain was higher than trypsin and chymotrypsin causing extensive hydrolysis of the protein components in whey particularly β-Lg and α-La. This finding was similar to the study of Pena-Ramos and Xiong (2001) indicating that papain was the most effective to hydrolyze whey compared with trypsin and chymotrypsin. At the E/S of 1/100, papain can completely hydrolyze all protein components in whey. However, the result from this study demonstrated that the E/S of 1/200 was sufficient to completely digest all whey components (Figure 14B). Increase in the amount of enzyme increased cost of production. Thus the most suitable condition for complete hydrolyzed whey was the E/S of 1/200 at the hydrolysis time of 1 hr using papain. This condition provided a complete hydrolysis of the protein components in whey with the lowest E/S and hydrolysis time.

Effect of Hydrolysis Conditions on Degree of Hydrolysis (DH) of Whey Hydrolysates

The DH of whey hydrolysates depended on the E/S, hydrolysis time and types of enzyme. The significant of the main effects on the DH was shown in Table 4 (p-value < 0.001). The E/S had the greatest effect on the DH followed by the hydrolysis time and types of enzyme.

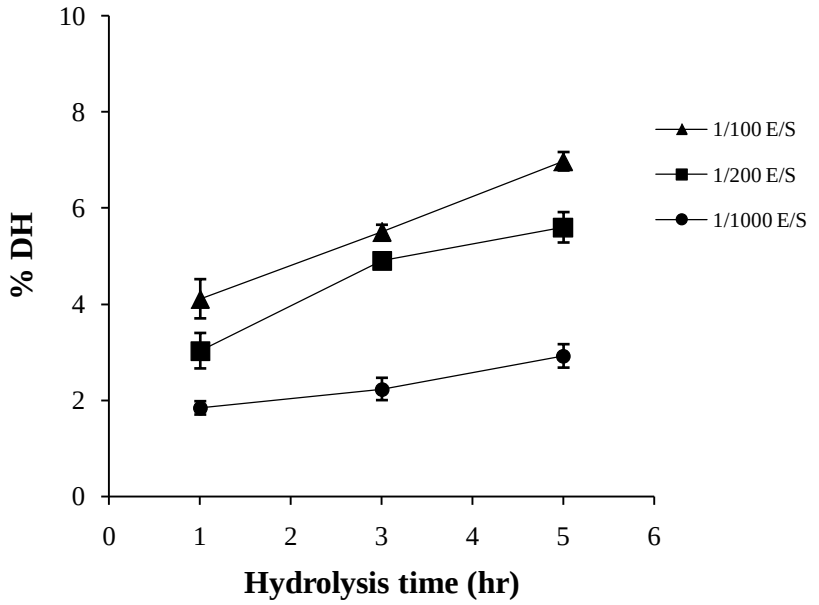
Table 4 ANOVA results: the effect of the hydrolysis conditions on DH values of whey hydrolysates

Source of variation	Sum of squares	df	Mean square	F	*p*-value
Total	2463.20	81			
Types[a]	25.06	2	12.532	182.744	< 0.001
E/S	377.22	2	188.609	2.750E3	< 0.001
Time[b]	49.27	2	24.635	359.231	< 0.001
Types × E/S	44.56	4	11.139	162.426	< 0.001
Types × time	4.08	4	1.021	14.884	< 0.001
E/S × time	1.99	4	.498	7.255	< 0.001
Types × E/S × time	2.20	8	.275	4.009	0.001
Error	3.70	54	.069		

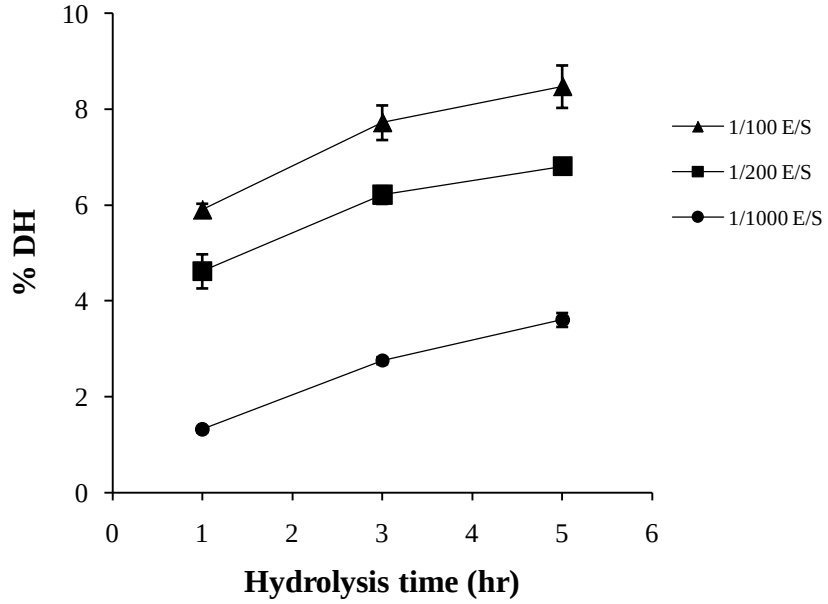
R^2= .993, [a] Types of enzyme, [b] Hydrolysis time

Regarding the E/S, the effect of E/S on the DH was concentration dependent in all enzymes and hydrolysis time studied (Figure 15). At the hydrolysis time of 5 hr, given the highest DH value, the DH value increased from 2.93 ± 0.24 to 6.97 ± 0.19% for chymotrypsin, 3.61 ± 0.15 to 8.48 ± 0.44% for trypsin and 1.42 ± 0.15 to 9.22 ± 0.44% for papain, as the E/S increased from 1/1000 to 1/100. As the E/S increased, the amount of free enzyme available for hydrolysis of whey increased leading to a higher DH value (Guerard et al., 2007).

(A)


10
8
6
4
2
0
% DH
0
1
2
3
4
5
6
Hydrolysis time (hr)
1/100 E/S
1/200 E/S
1/1000 E/S

(B)


10
8
6
4
2
0
% DH
0
1
2
3
4
5
6
Hydrolysis time (hr)
1/100 E/S
1/200 E/S
1/1000 E/S

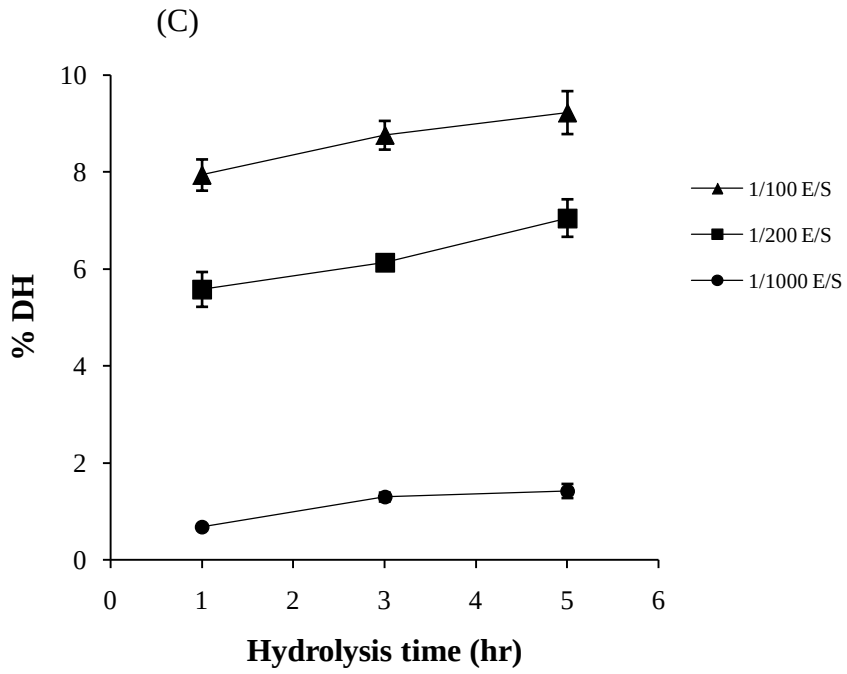


Figure 15 The DH profiles of whey hydrolysates at the E/S of 1/1000, 1/200 and 1/100 under various hydrolysis time: 1, 3 and 5 hr (A) whey hydrolyzed with chymotrypsin, (B) whey hydrolyzed with trypsin and (C) whey hydrolyzed with papain. Plots were mean ± SD (n = 3).

In addition, when the E/S increased from 1/1000 to 1/100, the difference in magnitude of the DH under the same hydrolysis time ranged between 2.27-4.05 for chymotrypsin, 4.58-4.97 for trypsin and 7.26-7.80 for papain (Table 5). It indicated that the effect of the E/S on the DH was influenced by the types of enzyme (*p*-value < 0.001) (Table 4). Whey hydrolyzed with papain had the greater effect of the E/S on the DH compared to whey hydrolyzed with chymotrypsin and trypsin. This finding was confirmed by the interaction plot between the types of enzyme and E/S (Figure 16A).

Table 5 The difference in magnitude of the DH (mean values) of whey hydrolysates using difference enzymes under the same hydrolysis time as the E/S increased from 1/1000 to 1/100

Enzyme	Hydrolysis time (hr)		
	1	3	5
Chymotrypsin	2.27	3.27	4.05
Trypsin	4.58	4.97	4.87
Papain	7.26	7.46	7.80

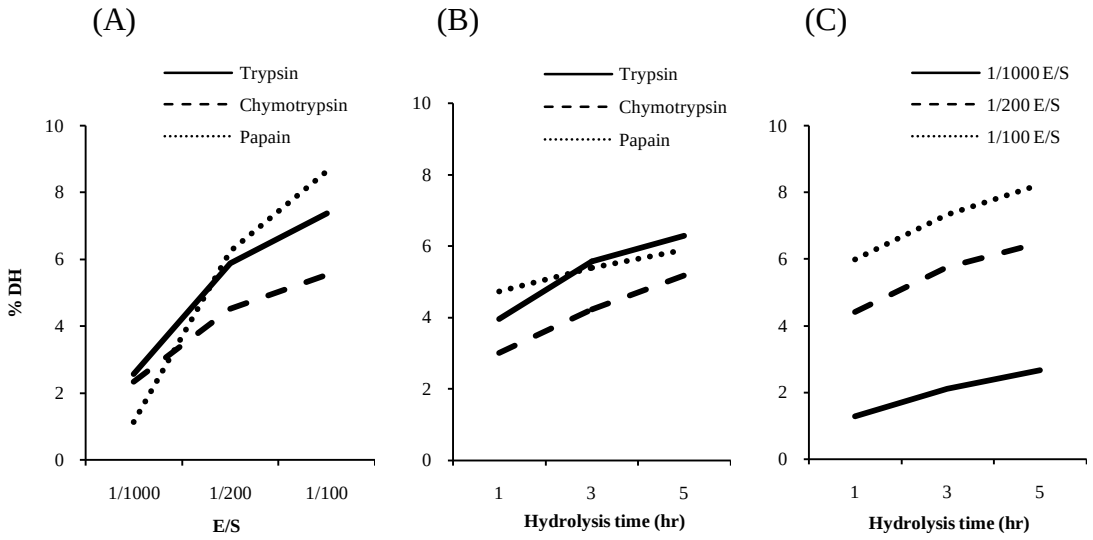


Figure 16 The effect on DH of: (A) types of enzyme-E/S interaction, (B) types of enzyme-hydrolysis time interaction and (C) E/S-hydrolysis time interaction

Regarding the hydrolysis time, the effect of the hydrolysis time on the DH was time dependent in all enzymes and E/S studied (Figure 15). At the E/S of 1/100, given the highest DH value, the DH value increased from 4.11 ± 0.41 to 6.97 ± 0.19% for chymotrypsin, 5.90 ± 0.13 to 8.48 ± 0.44% for trypsin and 7.94 ± 0.32 to 9.22 ± 0.44% for papain, as the hydrolysis time increased from 1 to 5 hr. When hydrolysis time increased, the duration allowed enzyme to interact with whey was prolonged resulting in a higher DH value (Palmer, 1995).

Additionally, when the hydrolysis time increased from 1 to 5 hr, the difference in magnitude of the DH under the same E/S ranged between 1.08-2.86 for chymotrypsin, 2.18-2.57 for trypsin and 0.75-1.47 for papain (Table 6). It indicated that the effect of the hydrolysis time on the DH was influenced by the types of enzyme (*p*-value < 0.001) (Table 4). Whey hydrolyzed with papain had the less effect of the hydrolysis time on the DH compared to whey hydrolyzed with chymotrypsin and trypsin. This finding was confirmed by the interaction plot between the types of enzyme and hydrolysis time (Figure 16B).

Table 6 The difference in magnitude of the DH (mean values) of whey hydrolysates using difference enzymes under the same E/S as the hydrolysis time increased from 1 to 5 hr

Enzyme	E/S		
	1/1000	1/200	1/100
Chymotrypsin	1.08	2.57	2.86
Trypsin	2.29	2.18	2.57
Papain	0.75	1.47	1.29

Moreover, the effect of the hydrolysis time on the DH also depended on the E/S (*p*-value < 0.001) (Table 4). For whey hydrolyzed with chymotrypsin, when the hydrolysis time increased from 1 to 5 hr, the difference in magnitude of the DH values was 1.08 for the E/S of 1/1000, 2.57 for the E/S of 1/200 and 2.86 for the E/S of 1/100 (Table 6). The hydrolysis time had less effect on the E/S of 1/1000 than the E/S of 1/200 and 1/100 indicating that the effect of the hydrolysis time on the DH of whey hydrolyzed with chymotrypsin depended on the E/S as presented by the interaction between the E/S and hydrolysis time (*p*-value < 0.001) (Table 7 and Figure 15A). For whey hydrolyzed with trypsin and papain, when the hydrolysis time increased from 1 to 5 hr, the difference in magnitude of the DH values was slightly different in all E/S studied (Table 6). This demonstrated that the effect of the hydrolysis time on the DH of whey hydrolyzed with trypsin and papain was independent on the E/S. Therefore, the DH of whey hydrolyzed with trypsin and papain was not affected by the interaction between the E/S and hydrolysis time (Tables 8 and 9).

Table 7 ANOVA results: the effect of the hydrolysis conditions on DH values of whey hydrolyzed with chymotrypsin

Source of variation	Sum of squares	df	Mean square	F	*p*-value
Total	533.81	27			
E/S	48.01	2	24.003	356.228	< 0.001
Time[a]	21.28	2	10.639	157.897	< 0.001
E/S × time	3.36	4	.839	12.449	< 0.001
Error	1.21	18	.067		

R^2= .984, [a] Hydrolysis time

Table 8 ANOVA results: the effect of the hydrolysis conditions on DH values of whey hydrolyzed with trypsin

Source of variation	Sum of squares	df	Mean square	F	*p*-value
Total	885.80	27			
E/S	108.99	2	54.493	919.450	< 0.001
Time[a]	25.90	2	12.951	218.515	< 0.001
E/S × time	.19	4	.048	.809	0.536
Error	1.07	18	.059		

R^2= .992, [a] Hydrolysis time

Table 9 ANOVA results: the effect of the hydrolysis conditions on DH values of whey hydrolyzed with papain

Source of variaton	Sum of squares	df	Mean square	F	*p*-value
Total	1043.58	27			
E/S	264.78	2	132.391	1.674E3	< 0.001
Time[a]	6.17	2	3.087	39.031	< 0.001
E/S × time	.64	4	.161	2.031	0.133
Error	1.42	18	.079		

R^2= .995, [a] Hydrolysis time

It is of interest that the DH of whey hydrolysates was dependent on the interaction between the E/S, hydrolysis time and types of enzyme. The DH of whey hydrolyzed with chymotrypsin was influenced by the interaction between the E/S and

hydrolysis time while the DH of whey hydrolyzed with trypsin and papain was not affected by the interaction (Tables 7-9). Thus the effect of the interaction between the E/S and hydrolysis time on the DH of whey hydrolysates also depended on the types of enzyme as presented by the three factors interaction (*p*-value = 0.001) (Table 4). Since the interaction between the E/S and hydrolysis time was only found in whey hydrolyzed with chymotrypsin, the overall interaction among three enzymes studied was not notably observed in the interaction plot between the E/S and hydrolysis time (Figure 16C).

The highest DH was obtained from the E/S of 1/100 with the hydrolysis time of 5 hr (Figure 15). Under this condition, whey hydrolyzed with trypsin (8.48 ± 0.44%) and papain (9.22 ± 0.44%) showed the highest DH followed by whey hydrolyzed with chymotrypsin (6.97 ± 0.19%). The digestive ability of trypsin and chymotrypsin was improved by the E/S and hydrolysis time while the digestive ability of papain was enhanced by the E/S. The hydrolysis time slightly affected the digestive ability of papain. It suggested that the digestion of whey by papain was faster than trypsin and chymotrypsin. This reaction was nearly complete within 1 hr. Over 1 hr of hydrolysis, the available peptide bonds for papain was extensively decreased leading to lower digestive rate. The digestion of whey by trypsin and chymotrypsin was slower than papain. After 1 hr of digestion, the susceptible peptide bonds for these enzymes were more available than the peptide bonds for papain. Thus increased hydrolysis time markedly improved the digestive ability of trypsin and chymotrypsin. These findings agreed with the previous study (Pena-Ramos and Xiong, 2001). In addition, at the comparable hydrolysis time, whey hydrolyzed with chymotrypsin had lower DH than whey hydrolyzed with trypsin (Figure 15). The similar result was found by Amoit et al. (2004) and Pena-Ramos and Xiong (2001).

The result from the protein patterns and DH studies indicated that papain had the highest ability to hydrolyze whey followed by trypsin and chymotrypsin (Figures 12-15). This enzyme was able to completely digest all protein components in whey and provided high DH. Although whey hydrolyzed with trypsin also showed the high DH, the enzyme can not completely digest α-La in whey. Chymotrypsin had the lowest digestive ability. It was not capable of completing digestion of whey and provided low DH. High ability of papain to digest whey might produce high amount

of bioactive peptides resulting in improving ROS scavenging acitivity of whey hydrolysates.

Scavenging Activity of Whey and Whey Hydrolysates

1. Determination of Optimal Substances Concentration

1.1 Determination of Optimal DMNQ Concentration

The effect of DMNQ on HaCaT cells viability was shown in Figure 17. DMNQ caused dose dependent toxicity in HaCaT cells. The percentage of cell viability of DMNQ-treated cells decreased from 81 ± 7 to 15 ± 4% as the concentrations increased from 10 to 30 µM. The result indicated that 10 µM of DMNQ caused 20% of inhibition of cell viability (IC_{20}). This concentration was chosen to induce ROS generation in further study. The mechanism of DMNQ toxicity was mediated by its ability to generate ROS. The accumulation of ROS in the cells would lead to oxidative stress. An electron from the electron transport chain in mitochondria reacted with DMNQ producing superoxide anion radical. The radical was transformed to hydrogen peroxide and hydroxyl radical via Fenton reaction resulted in cellular damage (Ishihara et al., 2006; Pinto and Castro, 2009).

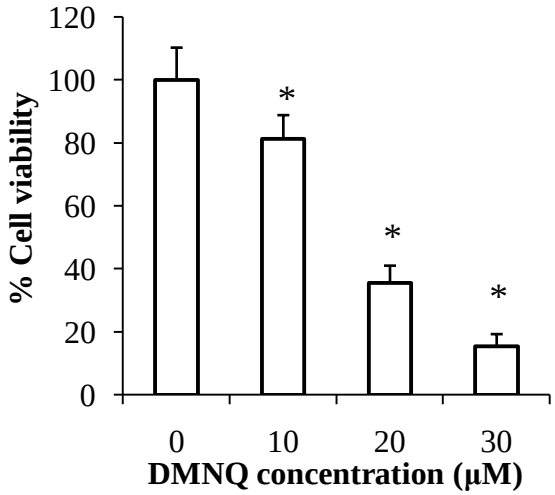


Figure 17 The effect of DMNQ on HaCaT cells viability. Plots were mean ± SD (n = 9). *p-value < 0.05 versus untreated cells.

1.2 Determination of Optimal NAC Concentration

The effect of NAC on HaCaT cells viability was shown in Figure 18. All concentrations of NAC did not cause any toxicity to HaCaT cells. Increased NAC concentration from 0.1 to 10 mM showed no significant difference in the percentage of the cell viability. The percentage of the cell viability of NAC-treated cells ranged from 103 ± 10 to 112 ± 10%. Previous studies reported that 5 mM of NAC effectively scavenged ROS in HaCaT cells (Tobi et al., 2000; Ledirac et al., 2005). Therefore this concentration was chosen for ROS scavenging activity study.

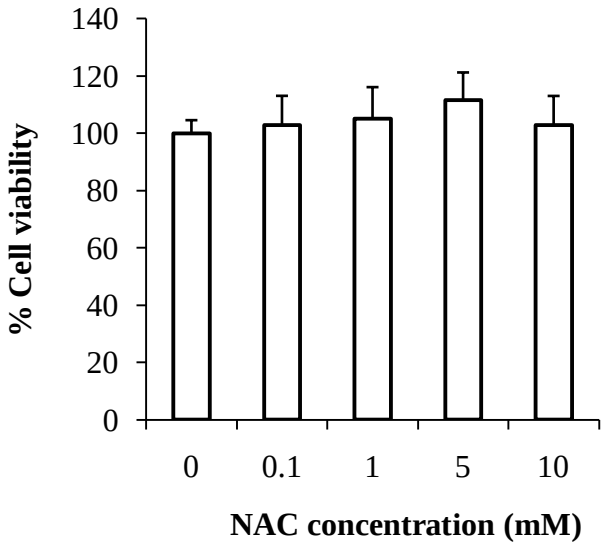


Figure 18 The effect of NAC on HaCaT cells viability. Plots were mean ± SD (n = 9).

1.3 Determination of Optimal BSO Concentration

The effect of BSO on HaCaT cells viability was shown in Figure 19. BSO was non-toxic to HaCaT cells at the tested concentration range. Increased BSO concentration showed no significant difference in the percentage of the cell viability. The percentage of the cell viability of BSO-treated cells ranged from 90 ± 11 to 99 ± 10% as the concentration increased from 0.01 to 1 mM. The previous studies reported that 1 mM of BSO was successful in depleting glutathione in HaCaT cells by 60% after incubation for 24 hr (Savini, Duflot and Avigliabo, 2000). Therefore this concentration was chosen for stimulation of glutathione synthesis study.

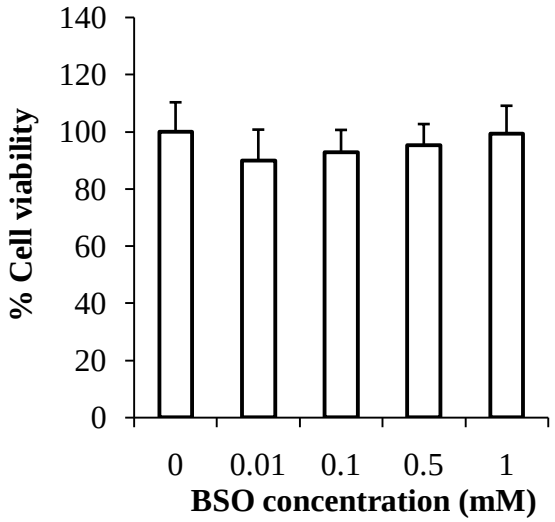


Figure 19 The effect of BSO on HaCaT cells viability. Plots were mean ± SD (n = 9).

1.4 Determination of Optimal Whey Concentration

The effect of whey on HaCaT cells viability was shown in Figure 20. Whey was non-toxic to HaCaT cells at the tested concentration range. Increased whey concentration from 0.1 to 2 mg/ml showed no significant difference in the percentage of the cell viability. The percentage of the cell viability of HaCaT cells treated with whey (0.1-2 mg/ml) ranged from 97 ± 5 to 108 ± 5%. Whey concentration of 2 mg/ml was the maximum concentration that could be prepared into solution. Thus, this concentration was chosen for ROS scavenging activity study.

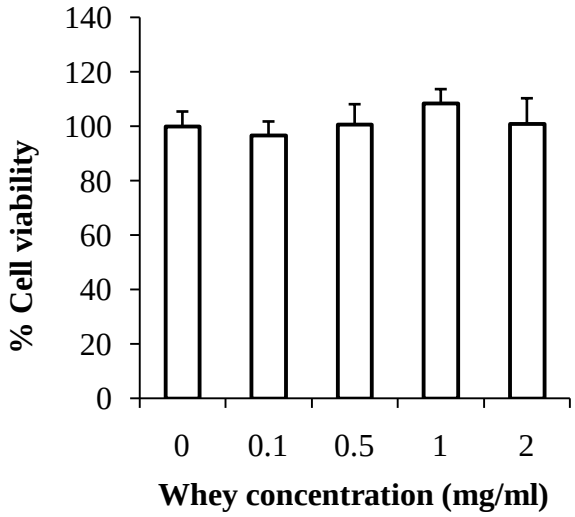


Figure 20 The effect of whey on HaCaT cells viability. Plots were mean ± SD (n = 9).

1.5 Determination of Optimal Whey Hydrolysates Concentration

The effect of whey hydrolysates on the vitality of HaCaT cells was shown in Figures 21-29. All concentrations of whey hydrolysates did not cause any toxicity to HaCaT cells. Whey hydrolysates concentration of 2 mg/ml was the maximum concentration could be prepared from whey solution. Therefore, 2 mg/ml of whey hydrolysates were chosen for ROS scavenging activity study.

In contrast, at the concentrations of 2 mg/ml, most formulations of whey hydrolysates especially whey hydrolyzed with chymotryspin significantly enhanced the percentage of the cell viability compared with that of untreated cells (p-value < 0.05). These results suggested that whey hydrolysates possibly had proliferative effect on HaCaT cells. The significant increase in the cell viability of whey hydrolysates was observed with nine formulations of whey hydrolyzed with chymotrypsin (p-value < 0.05, Figures 21-23), seven formulations of whey hydrolyzed with trypsin (p-value < 0.05, Figures 24-26) and three formulations of whey hydrolyzed with papain (p-value < 0.05, Figures 27-29). These formulations were 1000-Ch1, 1000-Ch3, 1000-Ch5, 200-Ch1, 200-Ch3, 200-Ch5, 100-Ch1, 100-Ch3, 100-Ch5, 1000-T1, 1000-T3, 1000-T5, 200-T1, 200-T3, 100-T1, 100-T3, 1000-Pa1, 1000-Pa3 and 1000-Pa5. The cell viability of HaCaT cells treated with 2 mg/ml of these hydrolysates ranged from

114 ± 9 to 144 ± 15%, for chymotrypsin, 113 ± 12 to 148 ± 13% for trypsin and 115 ± 8 to 144 ± 12% for papain (Table 10).

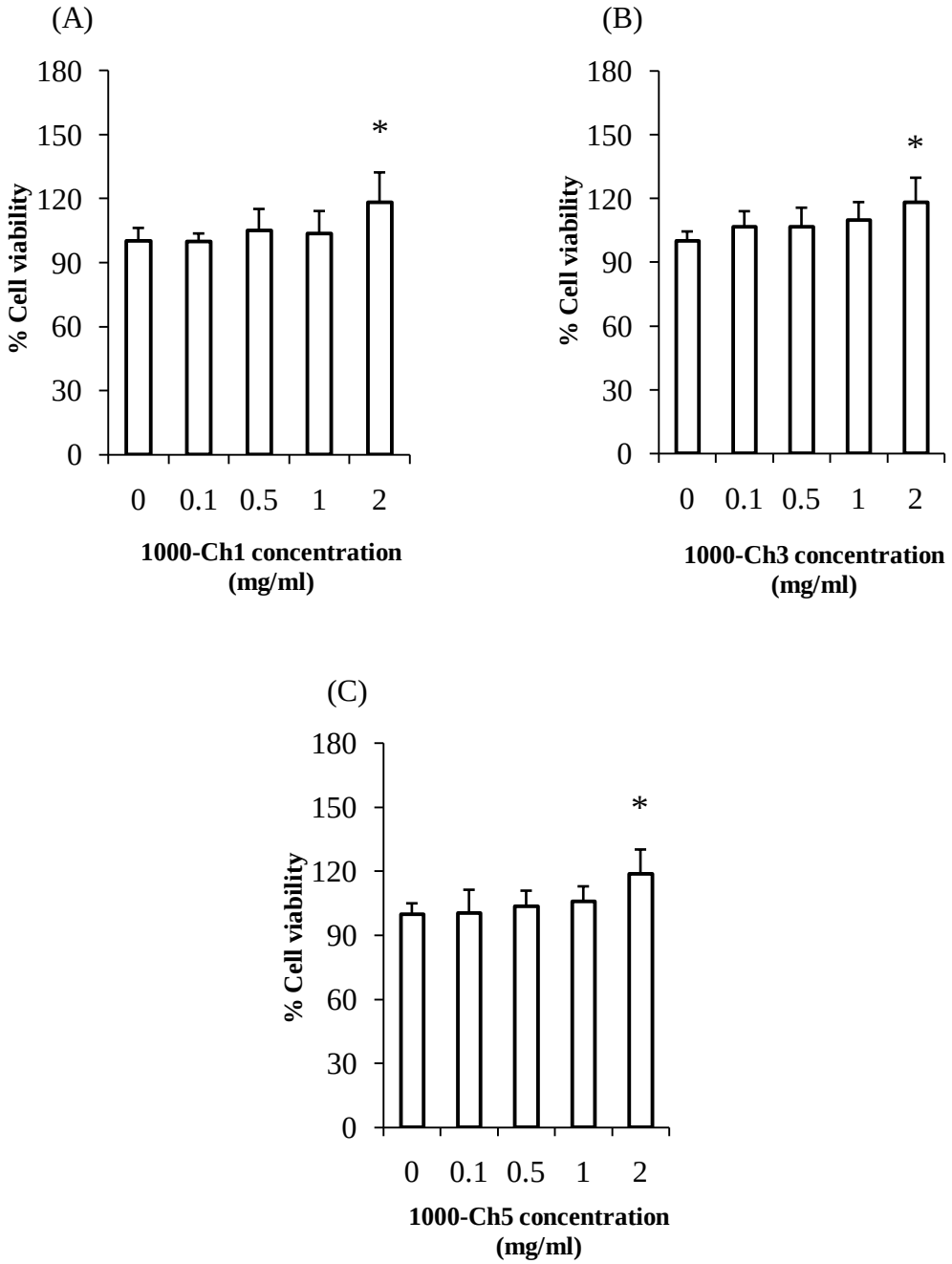


Figure 21 The effect of The effect of whey hydrolyzed with chymotrypsin at the E/S of 1/1000 on HaCaT cells viability: 1000-Ch1 (A), 1000-Ch3 (B) and 1000-Ch5 (C). Plots were mean ± SD (n = 9). *p-value < 0.05 versus untreated cells.

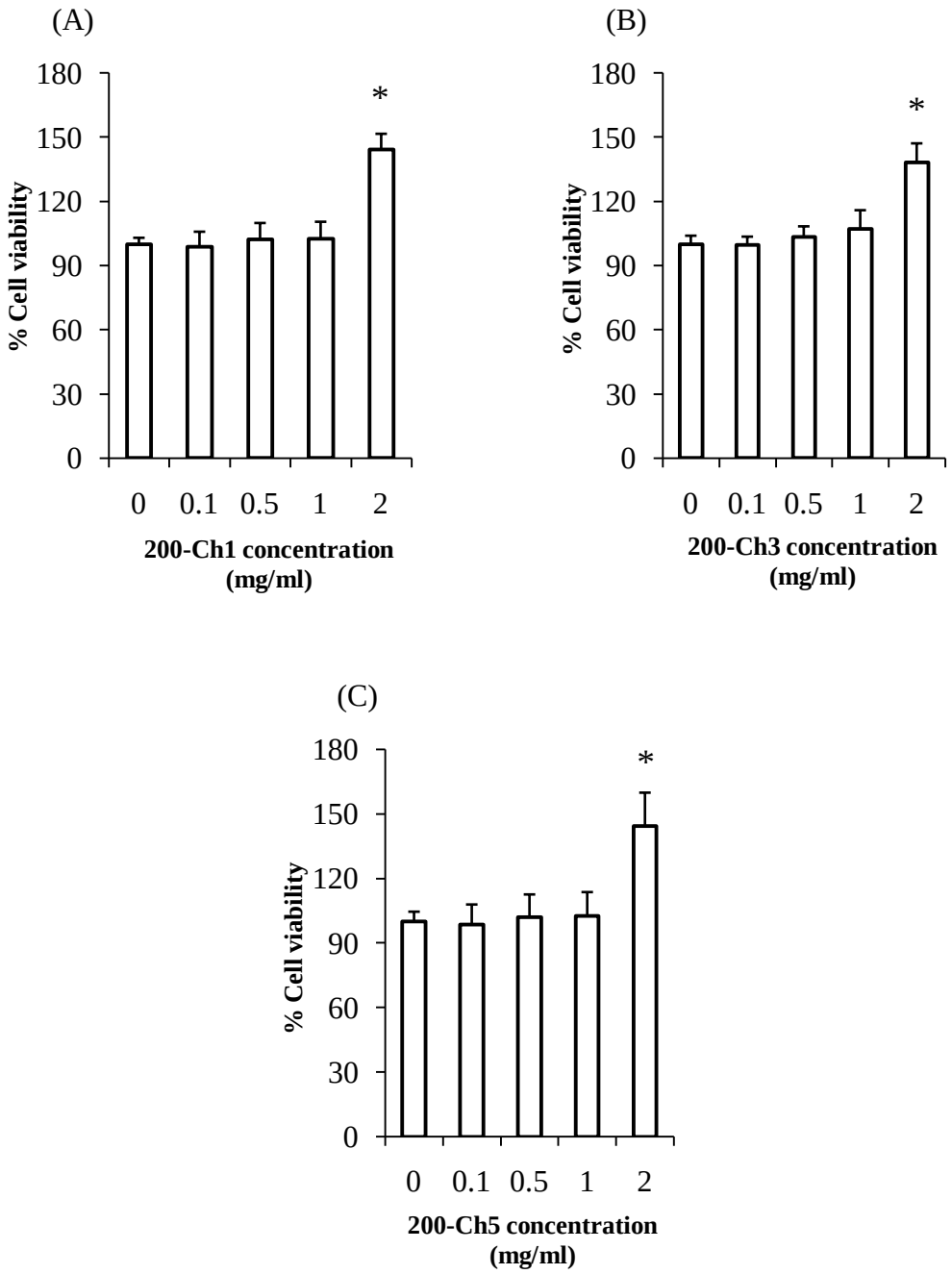


Figure 22 The effect of whey hydrolyzed with chymotrypsin at the E/S of 1/200 on HaCaT cells viability: 200-Ch1 (A), 200-Ch3 (B) and 200-Ch5 (C). Plots were mean ± SD (n = 9). **p*-value < 0.05 versus untreated cells.

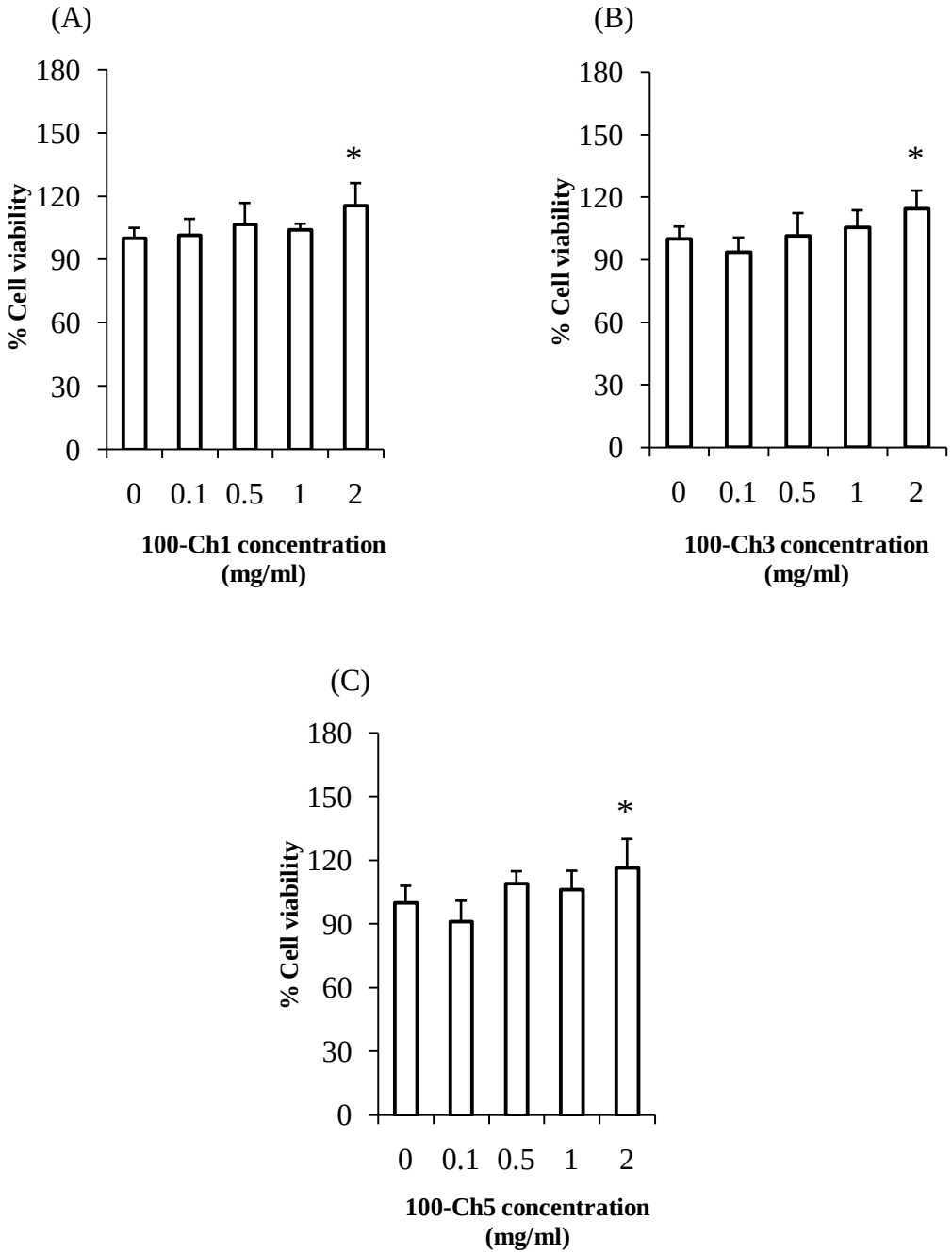


Figure 23 The effect of whey hydrolyzed with chymotrypsin at the E/S of 1/100 on HaCaT cells viability: 100-Ch1 (A), 100-Ch3 (B) and 100-Ch5 (C). Plots were mean ± SD (n = 9). **p*-value < 0.05 versus untreated cells.

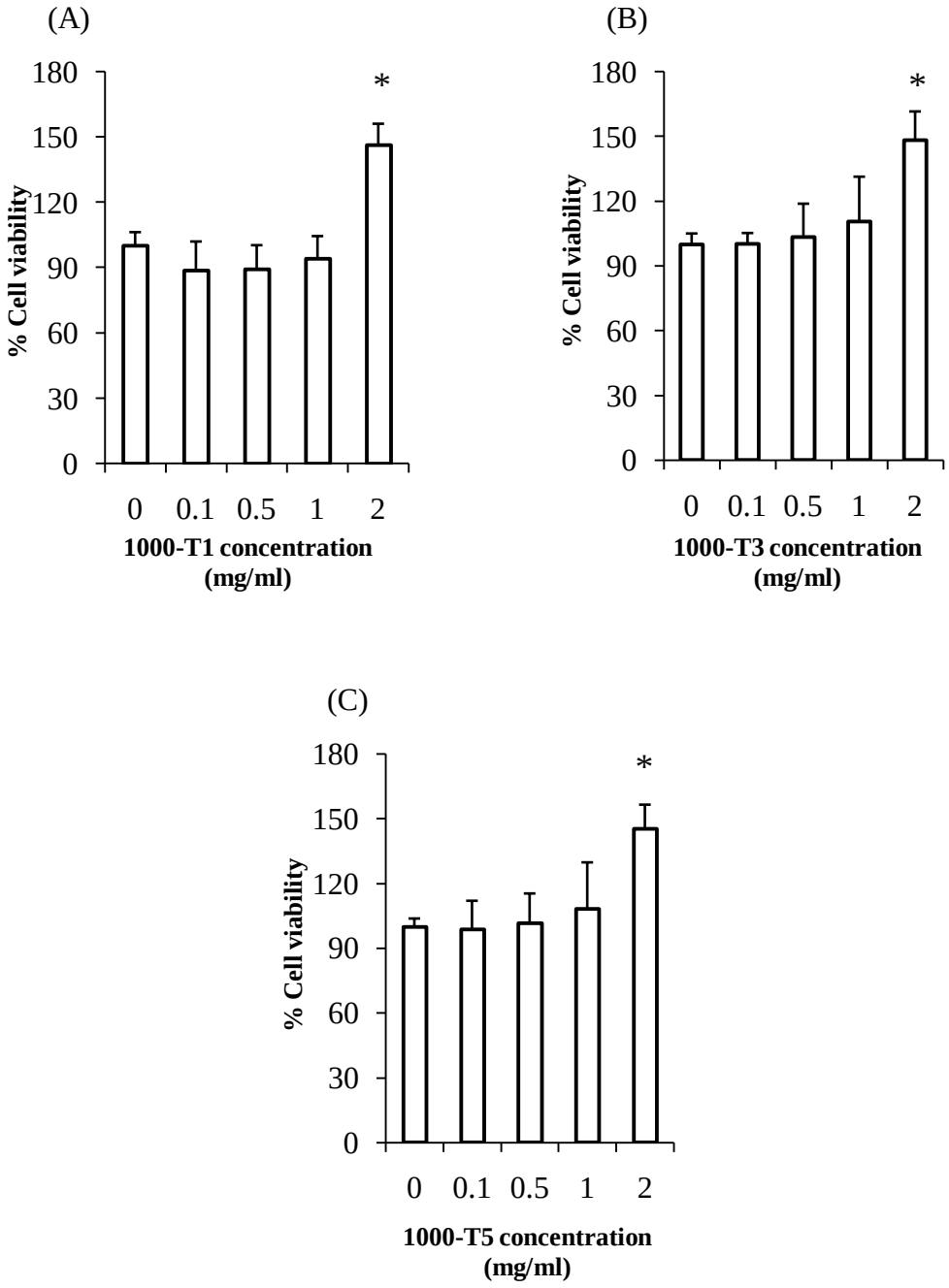


Figure 24 The effect of whey hydrolyzed with trypsin at the E/S of 1/1000 on HaCaT cells viability: 1000-T1 (A), 1000-T3 (B) and 1000-T5 (C). Plots were mean ± SD (n = 9). *p-value < 0.05 versus untreated cells.

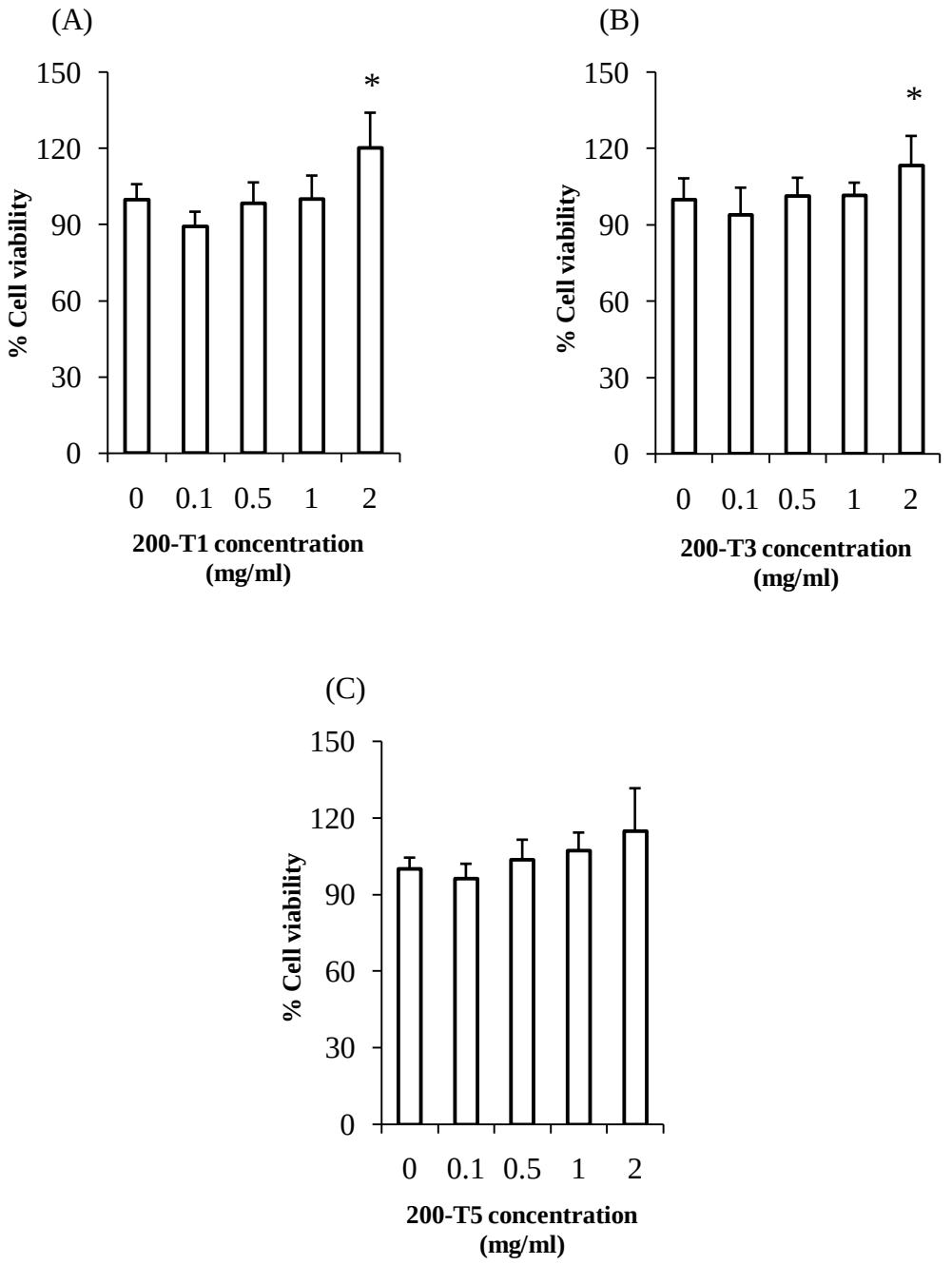


Figure 25 The effect of whey hydrolyzed with trypsin at the E/S of 1/200 on HaCaT cells viability: 200-T1 (A), 200-T3 (B) and 200-T5 (C). Plots were mean ± SD (n = 9). *p-value < 0.05 versus untreated cells.

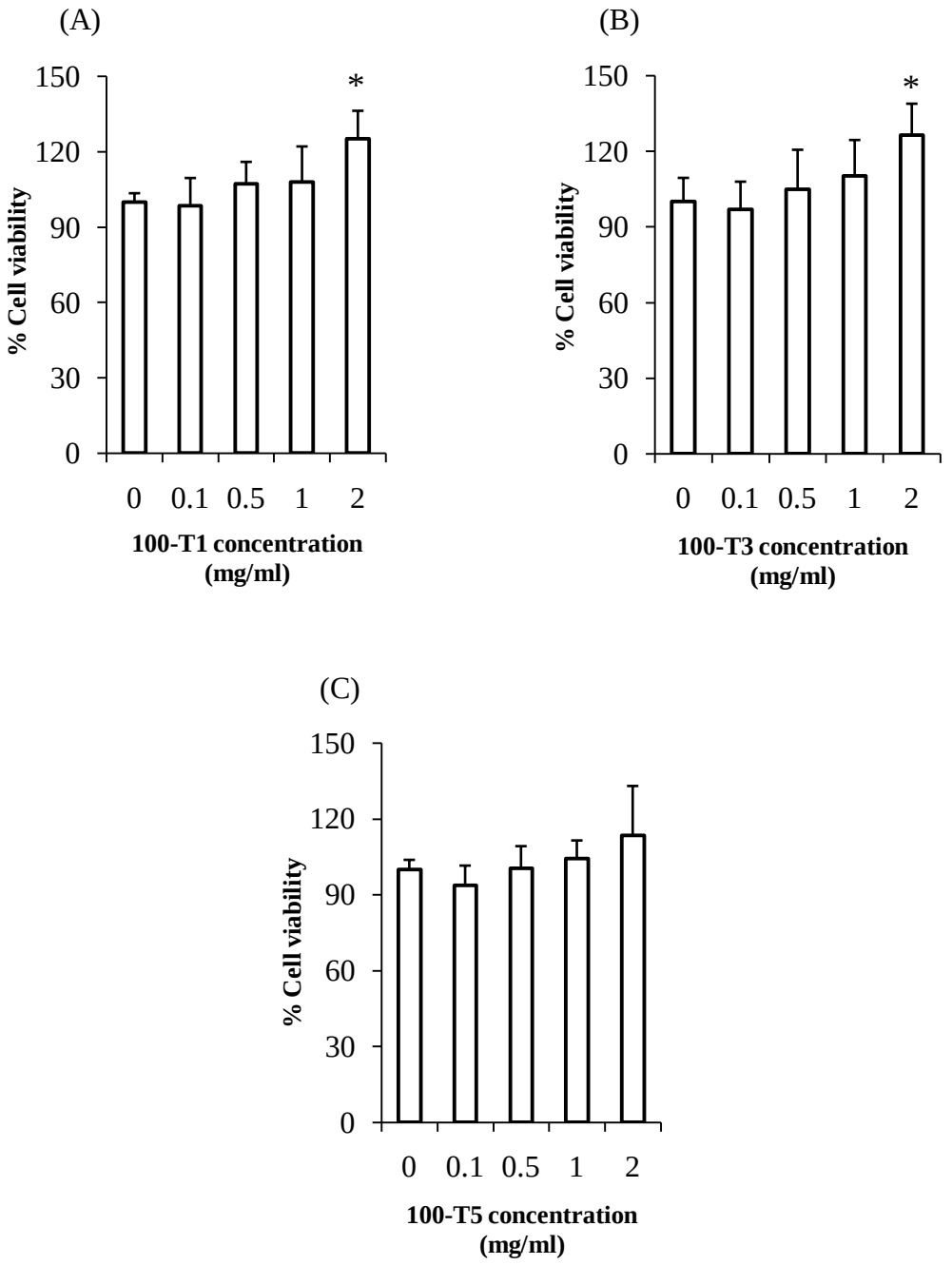


Figure 26 The effect of whey hydrolyzed with trypsin at the E/S of 1/100 on HaCaT cells viability: 100-T1 (A), 100-T3 (B) and 100-T5 (C). Plots were mean ± SD (n = 9). **p*-value < 0.05 versus untreated cells.

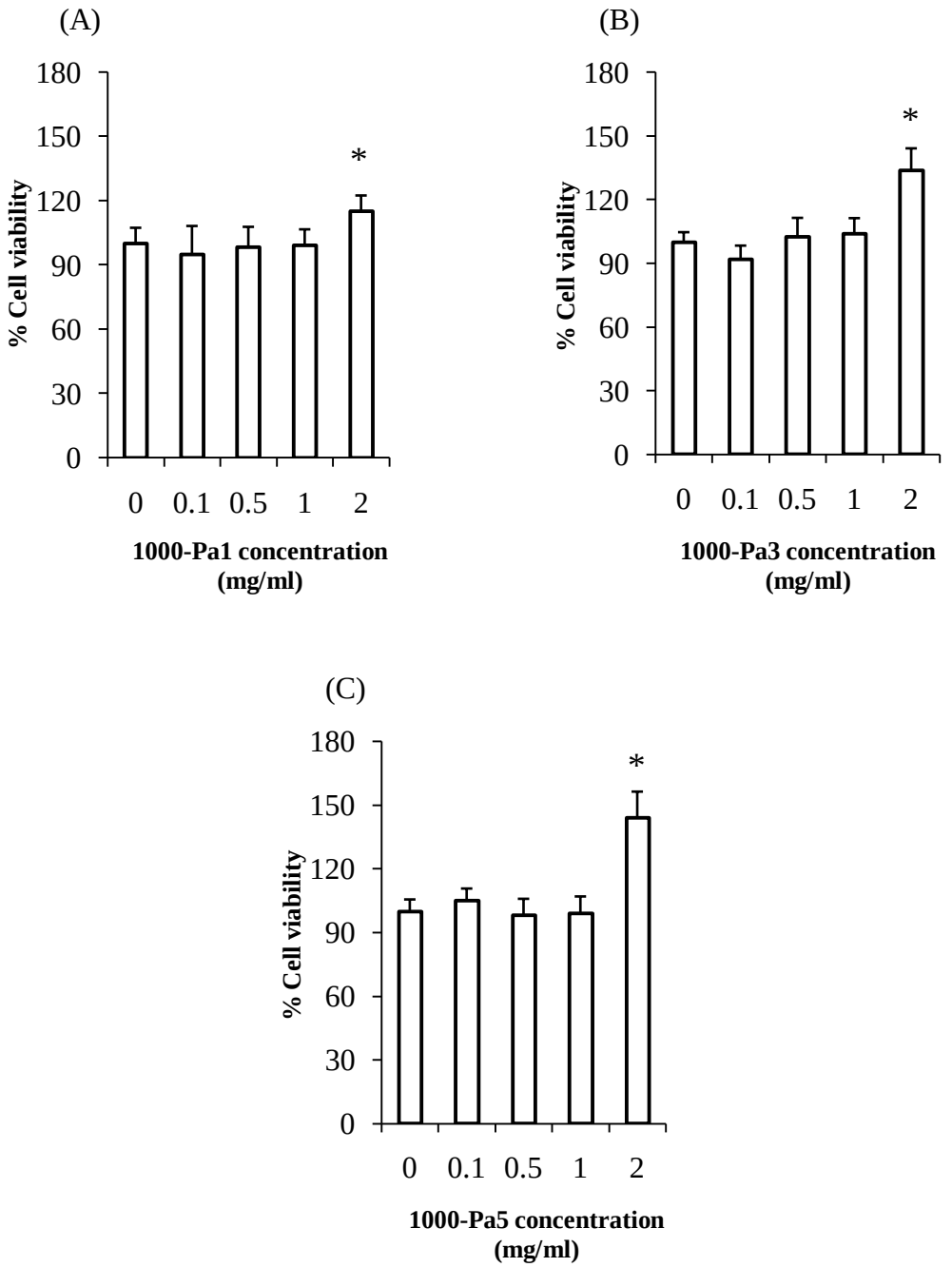


Figure 27 The effect of whey hydrolyzed with papain at the E/S of 1/1000 on HaCaT cells viability: 1000-Pa1 (A), 1000-Pa3 (B) and 1000-Pa5 (C). Plots were mean ± SD (n = 9). *p-value < 0.05 versus untreated cells.

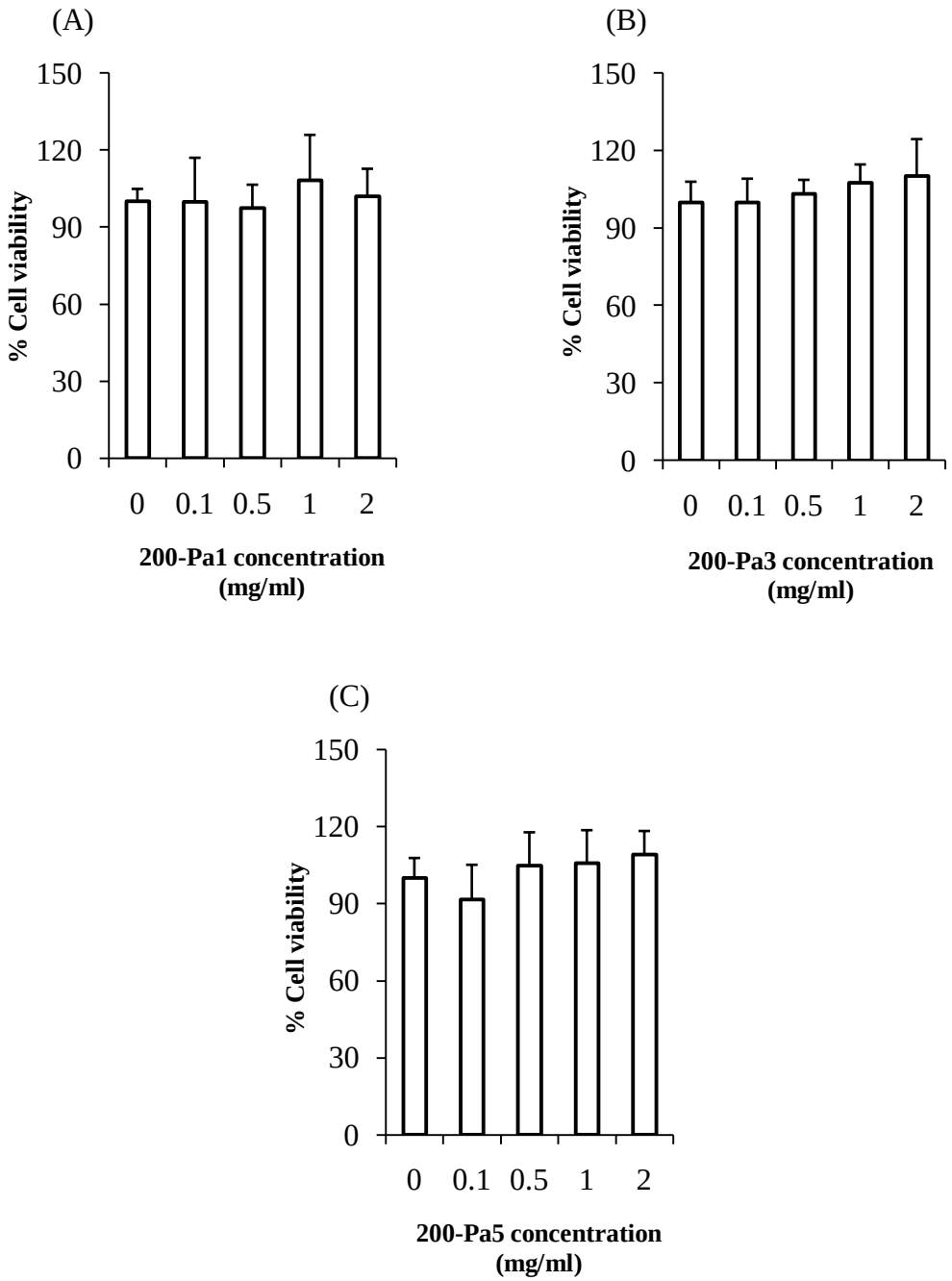


Figure 28 The effect of whey hydrolyzed with papain at the E/S of 1/200 on HaCaT cells viability: 200-Pa1 (A), 200-Pa3 (B) and 200-Pa5 (C). Plots were mean ± SD (n = 9).

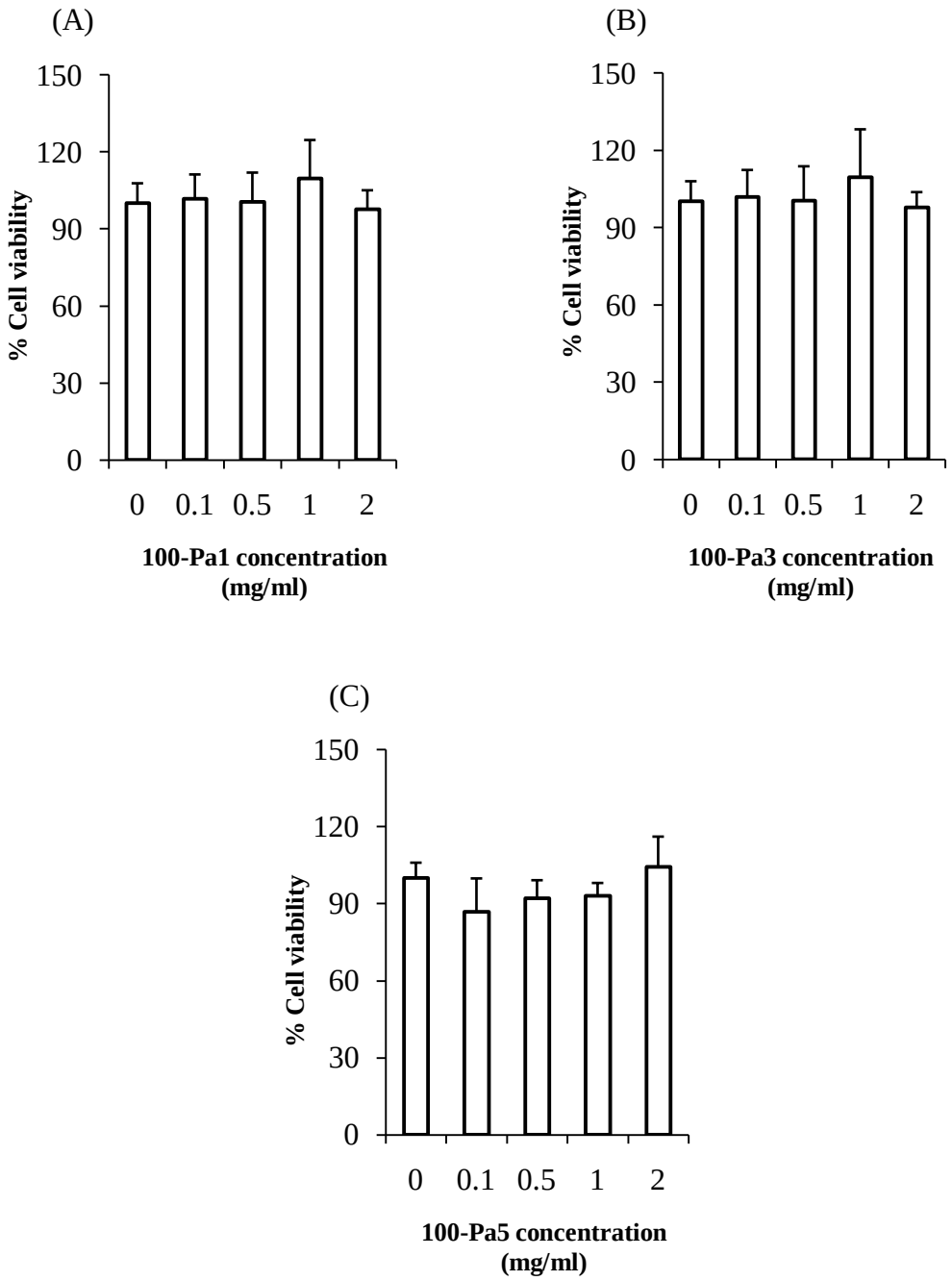


Figure 29 The effect of whey hydrolyzed with papain at the E/S of 1/100 on HaCaT cells viability: 100-Pa1 (A), 100-Pa3 (B) and 100-Pa5 (C). Plots were mean ± SD (n = 9).

Table 10 The effect of 2 mg/ml whey hydrolysates on HaCaT cells viability. Data were mean ± SD (n = 9).

Enzyme	E/S	Hydrolysis time (hr)		
		1	3	5
Trypsin	1/1000	146* ± 10	148* ± 13	146* ± 11
	1/200	120* ± 14	113* ± 12	115 ± 17
	1/100	125* ± 11	126* ± 13	113 ± 19
Chymotrypsin	1/1000	118* ± 14	118* ± 12	119* ± 11
	1/200	144* ± 7	138* ± 9	144* ± 15
	1/100	116* ± 11	114* ± 9	117* ± 13
Papain	1/1000	115* ± 8	134* ± 10	144* ± 12
	1/200	102 ± 11	110 ± 14	109 ± 9
	1/100	98 ± 7	96 ± 6	104 ± 12

*p-value < 0.05 versus untreated cells

The effect of whey on the cell viability was improved by enzymatic hydrolysis using trypsin, chymotrypsin and papain. HaCaT cells treated with native whey did not increase the cell viability compared to the untreated cells (Figure 20). The percentage of the cell viability of whey-treated cells at 2 mg/ml was 101 ± 9%. While treatment of 2 mg/ml whey hydrolysates showed the significant increase in the cell viability (Table 10). The maximum percentage of the cell viability of HaCaT cells treated with 2 mg/ml whey hydrolysates was nearly 150%.

Among all whey hydrolysates studied, whey hydrolyzed with chymotrypsin was the most capable in improving viability of HaCaT cells (Table 10). All formulations of whey hydrolyzed with chymotrypsin showed the significant increase in the cell viability. This might be due to the specificity of chymotrypsin itself. Chymotrypsin is an endopeptidase enzyme which is specific to the C-terminal of aromatic and hydrophobic amino acids (Oleson et al., 2004). The degradation products obtained from the enzyme actually contained aromatic or hydrophobic amino acids at the end of fragments. Most hydrophobic amino acids were essential for growth promoting effect in keratinocyte cells (Amoit et al., 2004). Therefore, the

hydrolysis of whey by chymotrypsin could increase the accessibility of the hydrophobic amino acids to the cells leading to an improvement of HaCaT cells viability. Although trypsin and papain were able to produce whey hydrolysates which had the growth promoting activity (Table 10), these enzymes did not directly cleave the peptide bonds of the hydrophobic amino acids that were important for keratinocyte growth. Thus the ability of whey hydrolyzed with trypsin and papain to improve the cell viability was less than the ability of whey hydrolyzed with chymotrypsin. The growth promoting activity was found in some formulation of these hydrolysates.

Additionally, extensive hydrolysis of whey did not effectively improve the growth promoting activity. In all enzymes studied, whey hydrolysates obtained from the E/S of 1/100 was less effective in increasing cell viability even though they had the highest DH (Figure 15 and Table 10). Under the extensive hydrolysis conditions, the bioactive peptides in whey hydrolysates were digested to amino acids resulting in decreased the amount of bioactive peptides in the hydrolysates. Therefore, the ability of whey hydrolysates to improve the cell viability was reduced. This result was supported by the previous study. Chabanon et al. (2008) reported that mixture of amino acids were no effect on promoting cell growth while the peptide containing such amino acids showed growth promoting activity. This indicated that bioactive sequence peptides were more effective in the activity than the constituent amino acids.

However, whey hydrolysates contained various peptides which had antioxidant activity (Hernandez-ledesma et al., 2005). These peptides might reduce MTT reagent to formazan crystal and subsequently increased the absorbance at 570 nm leading to overestimation of the cell viability. In order to confirm the proliferative effect of whey hydrolysates, the other methods for evaluating cell proliferation as determination of the total protein content and incorporation of [3H]-thymidine should be used.

2. Scavenging Activity of Whey and Whey Hydrolysates against DMNQ-generated ROS in HaCaT cells

Ten µM DMNQ was used to generate ROS in HaCaT cells and 5 mM NAC was used as a positive control. The results from this study showed that the relative DCF fluorescence response of DMNQ-treated cells was 1.42-fold compared to the untreated cells (baseline) (*p*-value = 0.012) (Figure 30). This increase in DCF fluorescence response of DMNQ-treated cells indicated that 10 µM DMNQ successfully generated the ROS in HaCaT cells. DMNQ undergoes electron reduction forming an unstable compound, semiquinone. This semiquinone can further donate an electron to molecular oxygen producing superoxide anion radical which can convert to hydrogen peroxide and hydroxyl radical via Fenton reaction (Brunmark and Cadenas, 1989; Ishihara et al., 2006; Valko et al., 2007).

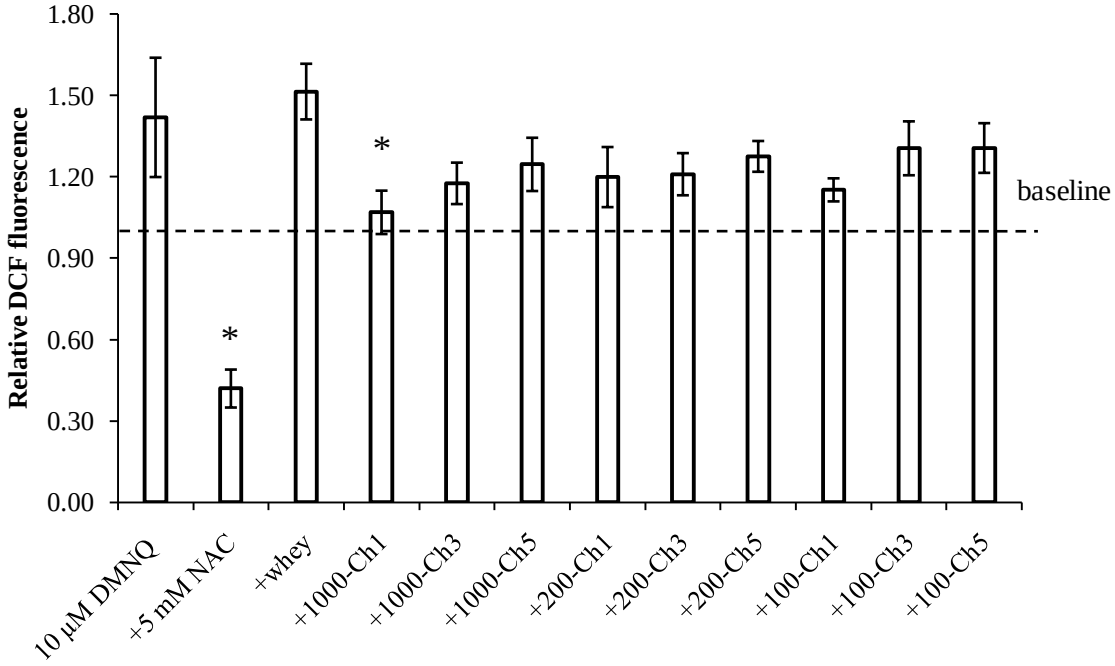


Figure 30 The effect of 2 mg/ml whey and whey hydrolyzed with chymotrypsin on DMNQ-induced ROS generation. Plots were mean ± SD (n = 3). **p*-value < 0.05 versus DMNQ-treated cells.

Flow cytometric analysis also showed that the relative DCF fluorescence response of DMNQ-treated cells with 5 mM NAC pretreatment was 0.42 ± 0.07 (Figure 30). This value was lower than the relative DCF fluorescence response of DMNQ-treated cells without NAC pretreatment indicating that NAC can scavenge the ROS generated by DMNQ. The ROS scavenging activity of NAC was contributed by its ability to directly scavenge the radical and indirectly stimulate glutathione synthesis (Koren et al., 2008: Tobi et al., 2000). Glutathione is known as an intracellular antioxidant. Increase in glutathione synthesis can enhance ROS scavenging activity of cells.

The scavenging activity of whey against DMNQ-generated ROS in HaCaT cells was shown in Figure 30. The result from flow cytometric analysis indicated that 2 mg/ml whey was not able to scavenge the ROS generated by DMNQ. The relative DCF fluorescence response of DMNQ-treated cells with 2 mg/ml whey pretreatment was 1.52 ± 0.10. This value was not significantly different from the relative DCF fluorescence response of DMNQ-treated cells without whey pretreatment (1.42 ± 0.22). Lacking ROS scavenging activity of whey might be due to the high MW protein components in whey. These proteins can not well penetrate into the cell thus inhibiting its ability to scavenge ROS (Amoit et al., 2004). Additionally, the active peptides and amino acids in whey were buried in the native structure. They were not able to expose to the ROS (Kong and Xiong, 2006). Therefore, the ROS scavenging activity of whey was not observed. This finding was in agreement with the study of Pena-Ramos and Xiong (2001) which found that native whey had no antioxidant activity. It was not able to reduce the oxidation of lipid compared with control. However, the previous studies demonstrated that the protein components in whey as lactoferrin, BSA and β-Lg had antioxidant activity (Tong et al., 2000). They were capable in inhibiting lipid oxidation and chelating metal ion. Moreover, whey is abundance of antioxidative amino acids as histidine, tryptophan, tyrosine, methionine, glycine and cysteine (Hernandez-ledesma et al., 2005: Pazos, Andersen, and Skibsted, 2006: Qian et al., 2008). These amino acids had hydrogen donating and metal ion chelating activity. Lacking antioxidative effect of whey that was observed in the study was due to inaccessibility of these bioactive proteins and amino acids to intracellular ROS.

In contrast, 2 mg/ml whey hydrolysates were able to inhibit DMNQ-generated ROS in HaCaT cells (Figures 30-32). The significant reduction of the relative DCF fluorescence response in HaCaT cells was observed with one formulation of whey hydrolyzed with chymotrypsin (p-value < 0.05, Figure 30), five formulations of whey hydrolyzed with trypsin (p-value < 0.05, Figure 31) and six formulations of whey hydrolyzed with papain (p-value < 0.01, Figure 32). These formulations were 1000-Ch1, 200-T1, 200-T3, 100-T1, 100-T3 100-T5, 200-Pa1, 200-Pa3, 200-Pa5, 100-Pa1, 100-Pa3 and 100-Pa5. The relative DCF fluorescence response of HaCaT cells pretreated with 2 mg/ml these hydrolysates were 1.07 ± 0.08 for chymotrypsin, 1.02 ± 0.09 to 1.07 ± 0.07 for trypsin and 0.67 ± 0.05 to 0.87 ± 0.13 for papain. These results suggested that whey hydrolyzed with papain had the greatest ROS scavenging activity compared with whey hydrolyzed with chymotrypsin and trypsin. The maximum ROS scavenging activity of whey hydrolyzed with papain was observed at the E/S of 1/100 under the hydrolysis time of 1 hr. This hydrolysate was able to reduce the relative DCF fluorescence response from 1.42 ± 0.22 to 0.67 ± 0.05.

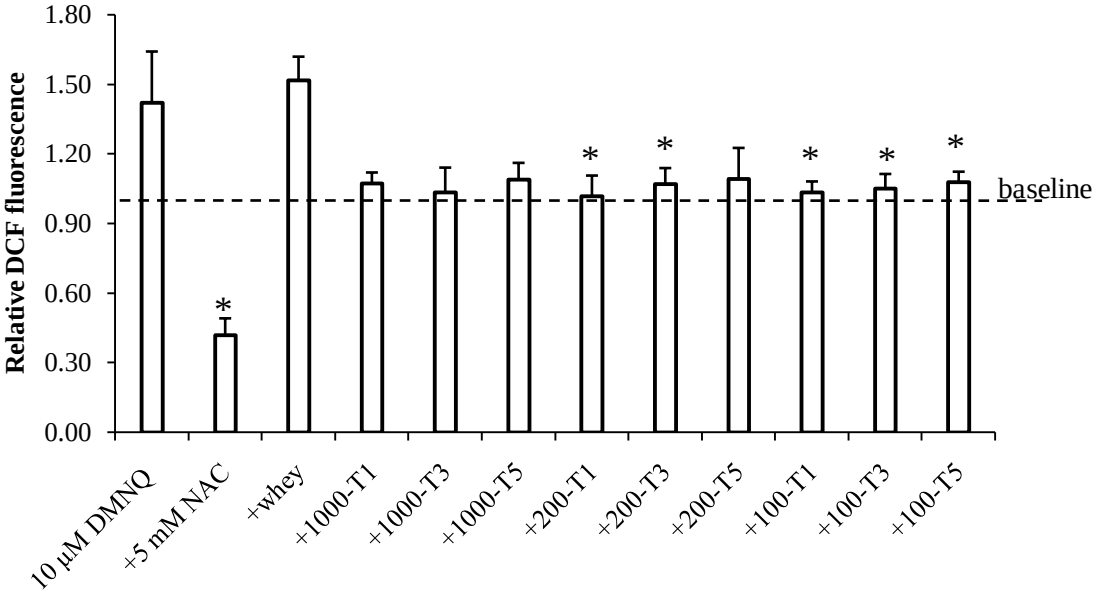


Figure 31 The effect of 2 mg/ml whey and whey hydrolyzed with trypsin on DMNQ-induced ROS generation. Plots were mean ± SD (n = 3). *p-value < 0.05 versus DMNQ-treated cells.

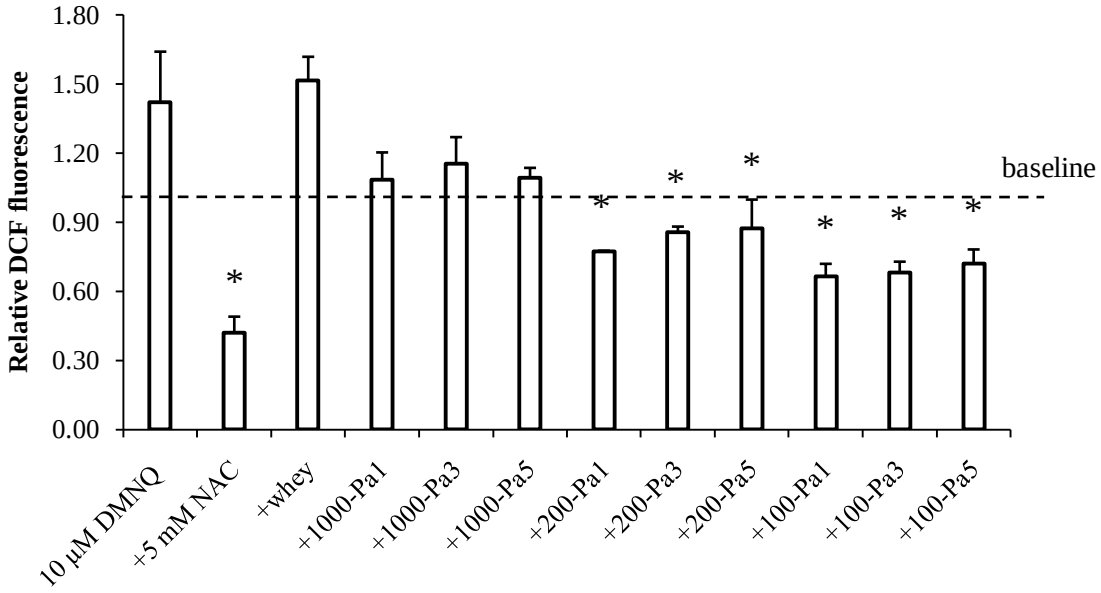


Figure 32 The effect of 2 mg/ml whey and whey hydrolyzed with papain on DMNQ-induced ROS generation. Plots were mean ± SD (n = 3). **p*-value < 0.05 versus DMNQ-treated cells.

The ROS scavenging activity of whey hydrolysates was contributed by the bioactive peptides which were buried in native whey. When whey was hydrolyzed by enzyme, the bioactive peptides were released from the native structure to scavenge ROS (Hernandez-ledesma et al., 2005; Pazos et al., 2006; Qian et al., 2008). Peng et al. (2009) reported that whey hydrolyzed with alcalase had the superoxide anion and hydroxyl radical scavenging activity. In the present study, these ROS were generated by DMNQ. Thus the ROS scavenging activity of whey hydrolyzed with trypsin, chymotrypsin and papain was possibly mediated by the scavenging of superoxide anion and hydroxyl radical. The mechanism of ROS scavenging activity of whey hydrolysates could be contributed by hydrogen donating, metal ion chelating and reducing activity (Dong et al., 2008; Kong and Xiong, 2006; Pazos et al., 2006; Peng et al, 2009; Tong et al., 2000.).

Kong and Xiong (2006) reported that enzymatic hydrolysis improved the hydrogen donating activity by increasing the N-terminal and C-terminal of branched-chain amino acids in whey. These terminals were able to donate hydrogen atoms to the ROS. When the ROS received the hydrogen atoms from the N-terminal and C-

terminal, they were transformed to more stable forms. Therefore, the oxidation of DCFH to DCF by the ROS was decreased as indicated in the reduction of the relative DCF fluorescence response.

The metal ion chelating activity of whey hydrolysates was contributed by the C-terminal of peptides (Dong et al., 2008). When the digestion of whey increased, the C-terminal of peptides increased. Consequently the metal ion which catalyzed Fenton reaction was chelate by the C-terminal leading to decrease in the ROS. Moreover, lactoferrin and BSA also showed the metal ion chelating activity (Pazos et al., 2006; Tong et al., 2000). These indicated that the ROS scavenging activity of whey hydrolysates probably mediated by the metal ion chelation.

Additionally, the ROS scavenging of whey hydrolysates might be attributed by the reducing activity. Peng et al. (2009) found that the reducing activity of whey hydrolysates was increased with the DH. At the highest DH, whey hydrolysates showed the highest reducing activity. It suggested that the peptides in the hydrolysates can terminate ROS by donation of electrons.

However, the ROS scavenging activity of whey hydrolysates can be mediated by increasing glutathione synthesis in the cells. Whey hydrolysates were rich in cysteine, a glutathione precursor. After treated HaCaT cells with whey hydrolysates, the glutathione level in the cells might increase resulting in improvement of the ability of the cells to scavenge ROS. Kent et al. (2003) reported that whey hydrolyzed with the mixture of trypsin, chymotrypsin and peptidase effectively increased the glutathione level of human prostate cells.

The results from the study demonstrated that the ROS scavenging activity of whey can be improved by the enzymatic hydrolysis. This activity of whey hydrolysates was possibly mediated by stimulation of glutathione synthesis and direct scavenging of the ROS via hydrogen donating, metal ion chelating and reducing activity. The protein components in whey that were important to scavenge the ROS might be β-Lg and α-La (Hernandez-ledesma et al., 2005). Enzymatic hydrolysis of these proteins produced the antioxidative peptides which were able to scavenge radical. However, the scavenging activity was depended on the hydrolysis conditions as the E/S, hydrolysis time and types of enyme.

3. Effect of Hydrolysis Conditions on Scavenging Activity of Whey Hydrolysates against DMNQ-generated ROS in HaCaT cells

The scavenging activity of whey hydrolysates against DMNQ-generated ROS in HaCaT cells depended on the E/S, hydrolysis time and types of enzyme. The significant of the main effects on the ROS scavenging activity of whey hydrolysates was shown in Table 11. The types of enzyme had the greatest effect on the ROS scavenging activity followed by the E/S and hydrolysis time.

Table 11 ANOVA results: the effect of hydrolysis conditions on scavenging activity of whey hydrolysates against DMNQ-generated ROS

Source of variation	Sum of squares	df	Mean square	F	*p*-value
Total	92.37	81			
Types[a]	1.55	2	.777	119.759	<.001
E/S	.18	2	.088	13.566	<.001
Time[b]	.09	2	.044	6.841	.002
Types × E/S	.69	4	.172	26.531	<.001
Types × time	.03	4	.007	1.074	.378
E/S × time	.00	4	.000	.058	.994
Types × E/S × time	.04	8	.005	.698	.692
Error	.35	54	.006		

R^2= .880, [a] Types of enzyme, [b] Hydrolysis time

For whey hydrolyzed with papain, the effect of the E/S on the ROS scavenging activity of the hydrolysates was concentration dependent (*p*-value < 0.001) (Figure 32 and Table 12). As the E/S increased from 1/1000 to 1/100, the relative DCF fluorescence response of whey hydrolyzed with papain decreased from 1.08 ± 0.12 to 0.67 ± 0.05, 1.15 ± 0.12 to 0.68 ± 0.05 and 1.09 ± 0.04 to 0.72 ± 0.06 for the hydrolysis time of 1, 3 and 5 hr, respectively. The hydrolysis time showed no significant effect on the ROS scavenging activity of whey hydrolyzed with papain.

Table 12 ANOVA results: the effect of the hydrolysis conditions on scavenging activity of whey hydrolyzed with papain against DMNQ-generated ROS.

Source of variation	Sum of squares	df	Mean square	F	*p*-value
Total	21.75	27			
E/S	.82	2	.412	66.669	<.001
Time[a]	.02	2	.010	1.622	.225
E/S × time	.01	4	.003	.484	.747
Error	.11	18	.006		

R^2= .885, [a] Hydrolysis time

For whey hydrolyzed with chymotrypsin, the effect of the hydrolysis time was inverse-proportion to the ROS scavenging activity (*p*-value = 0.009) (Figure 30 and Table 13). The E/S had no significant effect on the ROS scavenging activity of the hydrolysates. Increased hydrolysis time decreased the scavenging activity of whey hydrolyzed with chymotrypsin as observed by the increase in DCF fluorescence response. When the hydrolysis time increased from 1 to 5 hr, the relative DCF fluorescence response of whey hydrolyzed with chymotrypsin increased from 1.07 ± 0.08 to 1.25 ± 0.10, 1.20 ± 0.11 to 1.28 ± 0.06 and 1.15 ± 0.04 to 1.31 ± 0.09 at the E/S of 1/1000, 1/200 and 1/100, respectively.

Table 13 ANOVA results: the effect of the hydrolysis conditions on scavenging activity of whey hydrolyzed with chymotrypsin against DMNQ-generated ROS.

Source of variation	Sum of squares	df	Mean square	F	*p*-value
Total	40.21	27			
E/S	.04	2	.020	2.848	.084
Time[a]	.09	2	.043	6.177	.009
E/S × time	.02	4	.005	.695	.605
Error	.13	18	.007		

R^2= .536, [a] Hydrolysis time

For whey hydrolyzed with trypsin, the ROS scavenging activity was not influenced by the E/S (Figure 31 and Table 14). When the E/S increased from 1/1000 to 1/100, the relative DCF fluorescence response of whey hydrolyzed with trypsin

ranged from 1.07 ± 0.05 to 1.03 ± 0.05, 1.03 ± 0.11 to 1.05 ± 0.06 and 1.09 ± 0.07 to 1.08 ± 0.04 for the hydrolysis time of 1, 3 and 5 hr, respectively. In addition, the ROS scavenging activity of whey hydrolyzed with trypsin was not affected by the hydrolysis time. When the hydrolysis time increased from 1 to 5 hr, the relative DCF fluorescence response of whey hydrolyzed with trypsin ranged from 1.07 ± 0.05 to 1.09 ± 0.07, 1.02 ± 0.09 to 1.09 ± 0.13 and 1.03 ± 0.05 to 1.08 ± 0.04 at the E/S of 1/1000, 1/200 amd 1/100, respectively.

Table 14 ANOVA results: the effect of the hydrolysis conditions on scavenging activity of whey hydrolyzed with trypsin against DMNQ-generated ROS

Source of variation	Sum of squares	df	Mean square	F	*p*-value
Total	30.41	27			
E/S	.00	2	.000	.023	.977
Time[a]	.01	2	.005	.857	.441
E/S × time	.01	4	.002	.254	.903
Error	.11	18	.006		

R^2= .134, [a] Hydrolysis time

The effect of the E/S on the ROS scavenging activity of whey hydrolysates was dependent on the types of enzyme (Table 11 and Figure 33). When the E/S increased from 1/1000 to 1/100, the difference in magnitude of the relative DCF fluorescence response under the same hydrolysis time ranged between 0.37- 0.47 for papain, 0.06- 0.13 for chymotrypsin and 0.01-0.04 for trypsin (Table 15). It indicated that the effect of the E/S on the ROS scavenging activity was influenced by the types of enzyme. Whey hydrolyzed with papain had the greater effect of the E/S on the ROS scavenging activity compared to whey hydrolyzed with chymotrypsin and trypsin. This finding was confirmed by the interaction plot between the types of enzyme and E/S (Figure 33).

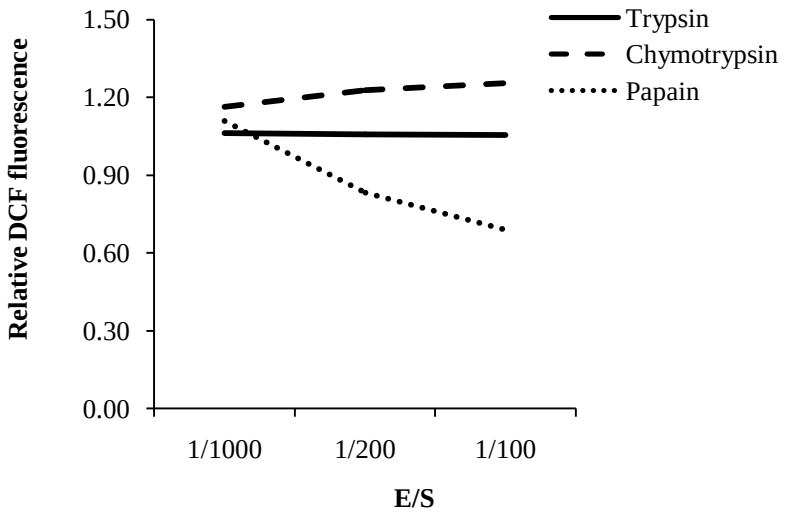


Figure 33 The effect of the types of enzyme-E/S interaction on scavenging activity of whey hydrolysates against DMNQ-generated ROS.

Table 15 The difference in magnitude of the relative DCF fluorescence response (mean values) of whey hydrolysates using difference enzymes under the same hydrolysis time as the E/S increased from 1/1000 to 1/100

Enzyme	Hydrolysis time (hr)		
	1	3	5
Papain	0.41	0.47	0.37
Chymotrypsin	0.08	0.13	0.06
Trypsin	0.04	0.02	0.01

The effect of the types of enzyme on the ROS scavenging activity of whey hydrolysates was reported in the previous study. Hernandez-ledesma et al. (2005) demonstrated that enzymes which had high ability to digest β-Lg and α-La, the major whey components, were effective to produce the antioxidative hydrolysates. In the present study, papain had the greatest digestive ability among three enzymes studied so whey hydrolyzed with papain showed the highest ROS scavenging activity compared with whey hydrolyzed with trypsin and chymotrypsin. Papain was a broad specific enzyme that preferably cleaved peptide bonds of several amino acids. This enzyme had high ability to digest β-Lg and α-La, the proteins containing antioxidative

peptides (Hernandez-ledesma et al., 2005; Pena-Ramos and Xiong, 2001). The digestive ability of papain was indicated in the DH and protein patterns studies (Figures 14 and 15). The result from the DH study showed that among three enzymes studied, whey hydrolyzed with papain at the E/S of 1/100 under the hydrolysis time of 5 hr gave the highest DH value (9.22 ± 0.44%). In addition, the protein patterns of whey hydrolyzed with papain indicated that papain was able to completely hydrolyzed all protein components in whey particularly β-Lg and α-La within 1 hr. At the E/S of 1/200 and 1/100, given complete hydrolysis of β-Lg and α-La, whey hydrolyzed with papain was significantly decreased DMNQ-generated ROS (Figure 32). Thus, the ROS scavenging activity of the hydrolysates might associate with the ability of papain to digest β-Lg and α-La.

For whey hydrolyzed with trypsin, the ROS scavenging activity of whey hydrolyzed with trypsin was lower than the activity of whey hydrolyzed with papain. Although the highest DH value of whey hydrolyzed with trypsin (8.48 ± 0.44%) was similar to that of whey hydrolyzed with papain (9.22 ± 0.44%), trypsin was not able to completely digest α-La that contained the antioxidative peptides (Figure 13). Incomplete hydrolysis of α-La might limit the ROS scavenging activity of whey hydrolyzed with trypsin (Hernandez-ledesma et al., 2005).

For whey hydrolyzed with chymotrypsin, the ROS scavenging activity of whey hydrolyzed with chymotrypsin was the less than those of whey hydrolyzed with trypsin and papain. This was corresponding to the digestive ability of chymotrypsin. Chymotrypsin had low ability to digest the protein components in whey as indicated in the DH and protein patterns studies (Figures 12 and 15). At the E/S of 1/100, given the highest DH value, the DH of whey hydrolyzed with chymotrypsin (6.97 ± 0.19%) was the lowest compared with those of whey hydrolyzed with trypsin (8.48 ± 0.44%) and papain (9.22 ± 0.44). Moreover, this enzyme was not able to completely hydrolyze the protein components in whey as indicated by the presence of β-Lg and α-La bands in the protein patterns of the hydrolysates. Low digestive ability of chymotrypsin limited the ability of the enzyme to produce bioactive peptides resulting in low ROS scavenging activity of these hydrolysates. The result confirmed that the ROS scavenging activity of whey hydrolysates correlated with the digestive ability of

enzyme. Moreover, the complete hydrolysis of β-Lg and α-La was also important to the scavenging activity of whey hydrolysates.

However, the factors affecting the ROS scavenging activity of whey hydrolysates was not only the types of enzyme. The results from this study found that the E/S and hydrolysis time also influenced the ROS scavenging activity of whey hydrolysates. The effect of the E/S and hydrolysis time on the scavenging activity depended on the types of enzyme.

For whey hydrolyzed with papain, the ROS scavenging activity of the hydrolysates increased when the digestive ability of papain increased (Figure 32). The results from the DH and protein patterns studies indicated that the digestive ability of papain was increased as the E/S increased (Figures 14 and 15). When the E/S increased, the bioactive peptides releasing from whey were increased. Thus, the ROS scavenging activity was also improved. Since the digestive ability of papain was not markedly improved by the hydrolysis time, increased hydrolysis time had no significant effect on the ROS scavenging activity of whey hydrolyzed with papain (Table 12).

In contrast to whey hydrolyzed with papain, the ROS scavenging activity of whey hydrolyzed with chymotryspin and trypsin under the hydrolysis conditions studied did not associate with the digestive ability of the enzymes. Increase in the E/S and hydrolysis time that improved the digestive ability of the enzyme as presented in the DH (Figure 15) and protein patterns studies (Figures 12 and 13) did not increase the ROS scavenging activity of whey hydrolyzed with chymotrypsin and trypsin (Figures 30 and 31). The E/S had no effect on the ROS scavenging activity of these hydrolysates (Tables 13 and 14). Increasing hydrolysis time resulted in decreased ROS scavenging activity of whey hydrolyzed with chymotrypsin while the activity of whey hydrolyzed with trypsin did not alter by the hydrolysis time. When the digestive ability of the enzyme increased, the production of the bioactive peptides was increased leading to improved ROS scavenging activity. However, the enzyme might further digest the bioactive peptides to inactive forms. Consequently, the ROS scavenging activity was decreased (Chen et al., 1996). Whey hydrolysates contained both bioactive peptides and inactive peptides. The observed ROS scavenging activity was the overall scavenging activity of both peptides. In case of whey hydrolyzed with

chymotrypsin, when hydrolysis time increased, the further digestion of the bioactive peptides might dominate thus decreasing their ROS scavenging activity. For whey hydrolyzed with trypsin, the production of the bioactive peptides was possibly equivalent to the digestion of the bioactive peptides resulting in unchanged overall ROS scavenging activity. Additionally low ability of chymotrypsin and trypsin to digest β-Lg and α-La might limit the ROS scavenging activity of whey hydrolyzed with chymotrypsin and trypsin.

The results from this study found that whey hydrolyzed with papain had the greatest ROS scavenging activity compared to whey hydrolyzed with trypsin and chymotrypsin. Six formulations of whey hydrolyzed with papain significantly decreased DMNQ-generated ROS (Figure 32). However, the statistically difference among the ROS scavenging activity of these hydrolysates was not observed. The hydrolysis time had no significant effect on the activity of whey hydrolyzed with papain (Table 12). Thus the appropriate time for hydrolyzing whey by papain was 1 hr, the shortest hydrolysis duration. Whey hydrolyzed with papain at the E/S of 1/200 and 1/100 under the hydrolysis time of 1 hr were selected to investigate their ability to stimulate glutathione synthesis and the stability of whey hydrolysate powders under accelerated condition in the further study.

Determination of Glutathione Synthesis

The ROS scavenging activity of whey hydrolysates could be mediated by the direct scavenging of ROS and stimulation of glutathione synthesis. To investigate the mechanism of the ROS scavenging activity of whey hydrolysates found in this study, whey hydrolysates given the highest ROS scavenging activity, 100-Pa1 and 200-Pa1, were examined their ability to stimulate glutathione synthesis under the condition used in the ROS scavenging activity study. BSO at the concentration of 1 mM was used as the negative control in this study. After treated HaCaT cells with 1 mM BSO, a specific glutathione inhibitor, the glutathione level in the cells decreased to 60 ± 6% compared with that of the untreated cells (p-value < 0.001, Figure 34). The reduction of the glutathione level by BSO was also reported in the previous studies (Sebastia` et al., 2003 and Tobi et al., 2000). The ability of BSO to reduce intracellular glutathione

was mediated by the inhibition of glutathione synthesis. BSO can irreversibly inhibit γ-glutamylcysteine synthetase (γ-GCS) that catalyzes the rate-limiting step of the glutathione synthesis (Griffith, 1999; Marshall, 2004; Sebastia` et al., 2003; Tobi et al., 2000). Consequently, the synthesis of glutathione was decreased as presented by the reduction of the glutathione level. This result indicated that the method used in the study was sufficient to determine the glutathione level in HaCaT cells.

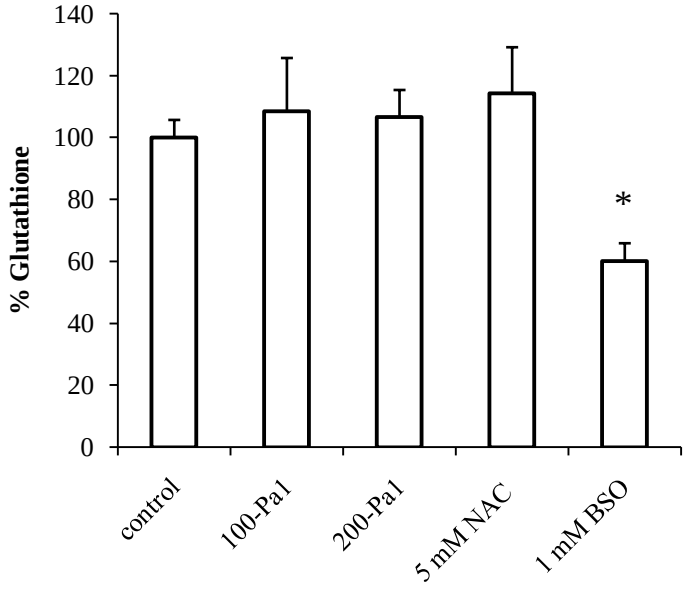


Figure 34 The effect of 2 mg/ml 100-Pa1 and 200-Pa1 on the glutathione level of HaCaT cells. Plots were mean ± SD (n = 9). **p*-value < 0.05 versus untreated cells.

NAC at the concentration of 5 mM was used as a positive control in the study. The effect of NAC on the glutathione synthesis was presented in Figure 34. The glutathione level of HaCaT cells treated with 5 mM NAC for 5 hr was 114 ± 15%. It was no significant difference from the glutathione level of the untreated cells (*p*-value = 0.144). This result indicated that 5 mM NAC was not able to stimulate the glutathione synthesis under studied condition. NAC is a known antioxidant which contains cysteine. It freely penetrates into the cells serving as a glutathione precursor. Another way, NAC is hydrolyzed to free cysteine and oxidized cysteine, cystine, undergoes intracellular transport by ASC and X_c^- transporter, respectively (Bannai and

Tateishi, 1986; Sen, 1997). Supplementation of NAC should improve the glutathione level of the cells. Lacking effect of NAC on the glutathione synthesis was unclear in this study. It might due to tight regulation of intracellular glutathione in HaCaT cells (Griffith, 1999; Sen, 1997). Under normal condition, the steady-state level of intracellular glutathione was maintained by γ-GCS. This enzyme catalyzes the rate-limiting step of glutathione synthesis. When the intracellular glutathione decreases the expression of γ-GCS subsequently improves as a consequence of increasing glutathione synthesis. At the steady-state level of intracellular glutathione, the synthesis of glutathione is limited by the decrease in the γ-GCS expression. Moreover, the activity of γ-GCS is also regulated via feedback inhibition by glutathione itself. High level of glutathione causes structural alteration in the enzyme leading to decreased glutathione synthesis. Even the incubation time and NAC concentration were increased up to 24 hr and 10 mM, respectively, the glutathione level of HaCaT cells was not improved. The glutathione level of HaCaT cells treated with 5 mM NAC for 24 hr was 92 ± 9% and the glutathione level of HaCaT cells treated with 10 mM NAC for 12 hr was 100 ± 11% (data from preliminary study). However, the increase in the glutathione level can be observed under nutrient-starved condition. Under this condition, the glutathione level of HaCaT cells treated with NAC for 12 hr was increased to 128 ± 10% for 5 mM NAC and 151 ± 21% for 10 mM NAC compared with that of the untreated cells (data from preliminary study). This indicated that the synthesis of glutathione was increased to compensate for the depleted glutathione level in the starved cells. Since the regulation of intracellular glutathione was impaired, the increasing intracellular glutathione was fluctuated under the nutrient-starved condition.

The effect of whey hydrolysates on the glutathione synthesis was presented in Figure 34. After treated HaCaT cells with 2 mg/ml of whey hydrolysates, the glutathione levels in the treated cells were 109 ± 17% for 100-Pa1 and 107 ± 9% for 200-Pa1, respectively. These were no significant difference from the glutathione level of the untreated cells, indicating that 100-Pa1 and 200-Pa1 were incapable of stimulating glutathione synthesis under studied condition. Thus the mechanism of the ROS scavenging activity of the whey hydrolysates found in the present study was not mediated by stimulation of glutathione synthesis. The protein components in whey as

β-Lg, α-La and BSA were cysteine-rich proteins (Kent et al., 2003). Digestion of whey by papain decreased size of these proteins to small peptides leading to increased cellular uptake. Supplementation of the hydrolysates should enhance the glutathione synthesis by increase in available cysteine, a glutathione precursor. However, the result showed no improvement of the glutathione synthesis by 100-Pa1 and 200-Pa1. Lacking effect of 100-Pa1 and 200-Pa1 on the stimulation of glutathione synthesis might be due to tight regulation of the glutathione level in the cells. Therefore the ROS scavenging activity of whey hydrolysates found in this study was possibly mediated by the direct scavenging of ROS as superoxide anion and hydroxyl radical (Peng et al., 2009).

Stability of Whey Hydrolysates

1. Effect of Freeze Drying Process on Stability of Whey Hydrolysate Powders

Whey hydrolysates given the highest ROS scavenging activity, 100-Pa1 and 200-Pa1, were freshly prepared and freeze dried for 48 hr. The effect of freeze drying process on the properties of whey hydrolysate powders as protein patterns, DH and ROS scavenging activity was investigated.

The protein patterns of whey hydrolysate powders were compared with those of whey hydrolysate solutions as presented in Figure 35. The protein patterns of 100-Pa1 and 200-Pa1 powder were similar to the protein patterns of their solutions. The peptide components in whey hydrolysate powders were lower than 14 kDa for 100-Pa1 and 15 kDa for 200-Pa1.

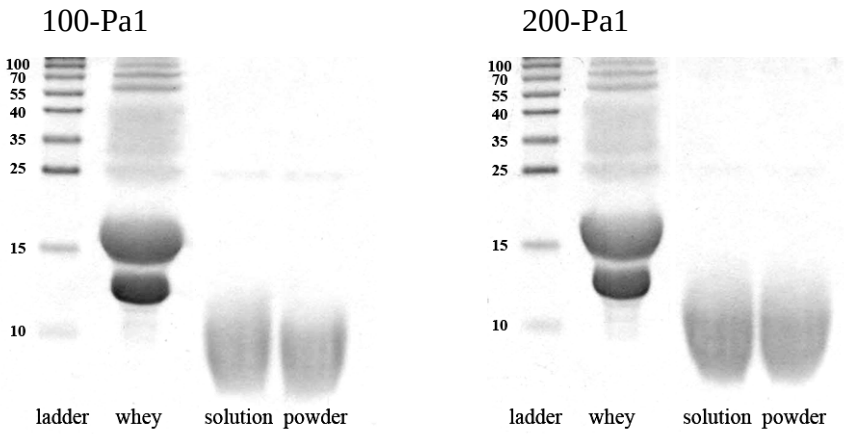


Figure 35 The protein patterns of whey hydrolysate solutions and powders

The DH values of whey hydrolysate powders showed no significant difference from those of whey hydrolysate solutions (Figure 36). The DH values of 100-Pa1 solution and powder were 8.28 ± 0.26 and 8.28 ± 0.61%, respectively. While the DH values of 200-Pa1 solution and powder were 5.81 ± 0.31 and 5.42 ± 0.23%, respectively. This result was in agreement with the protein patterns of whey hydrolysates (Figure 35). It suggested that freeze drying process did not affect the size and amount of the peptide components in whey hydrolysates.

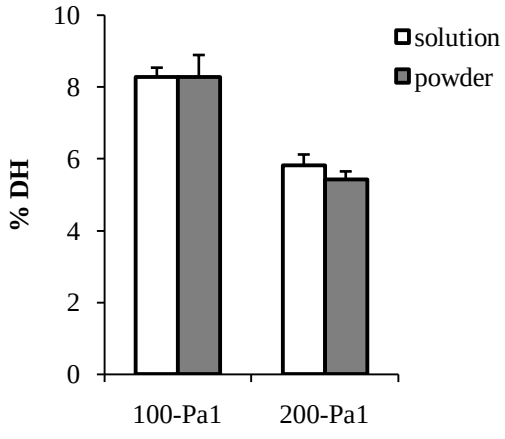


Figure 36 The DH of whey hydrolysate solutions and powders. Plots were mean ± SD (n = 5). *p-value < 0.05 versus the DH of their solutions.

The relative DCF fluorescence response of whey hydrolysate powders showed no significant difference from those of whey hydrolysate solutions (Figure 37). The

relative DCF fluorescence response of 100-Pa1 solution and powder were 0.73 ± 0.10 and 0.75 ± 0.05, respectively. While the relative DCF fluorescence response of 200-Pa1 solution and powder was 0.86 ± 0.04 and 0.88 ± 0.05, respectively. These results confirmed that freeze drying process did not influence the properties of whey hydrolysates as presented by no alteration in the protein patterns, DH and ROS scavenging activity of whey hydrolysates after freeze drying process.

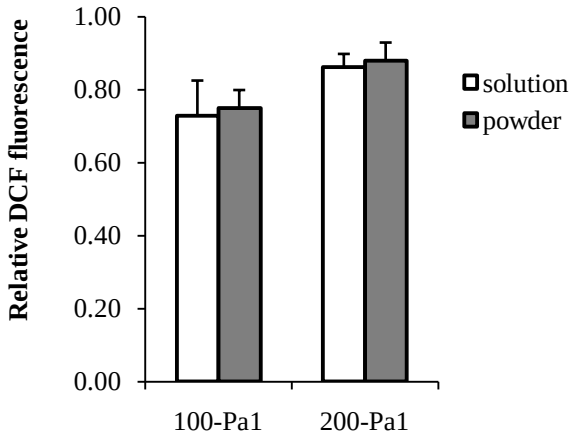


Figure 37 The ROS scavenging activity of whey hydrolysate solutions and powders. Plots were mean ± SD (n = 5). **p*-value < 0.05 versus the ROS scavenging activity of their solutions

2. Stability of Whey Hydrolysate Powders under Accelerated Condition

The stability of whey hydrolysate powders, 100-Pa1 and 200-Pa1, was studied under accelerated condition: 40^{o}C 75% RH (ICH Topic Q1A (R_2), 2003) for 3 months. The change in the properties of whey hydrolysate powders as protein patterns, DH and ROS scavenging activity was investigated.

The protein patterns of whey hydrolysate powders under accelerated condition were shown in Figure 38. After 3 month storage, the protein patterns of whey hydrolysate powders were not obviously different from the protein patterns of the hydrolysates powder before storage under the accelerated conditions.

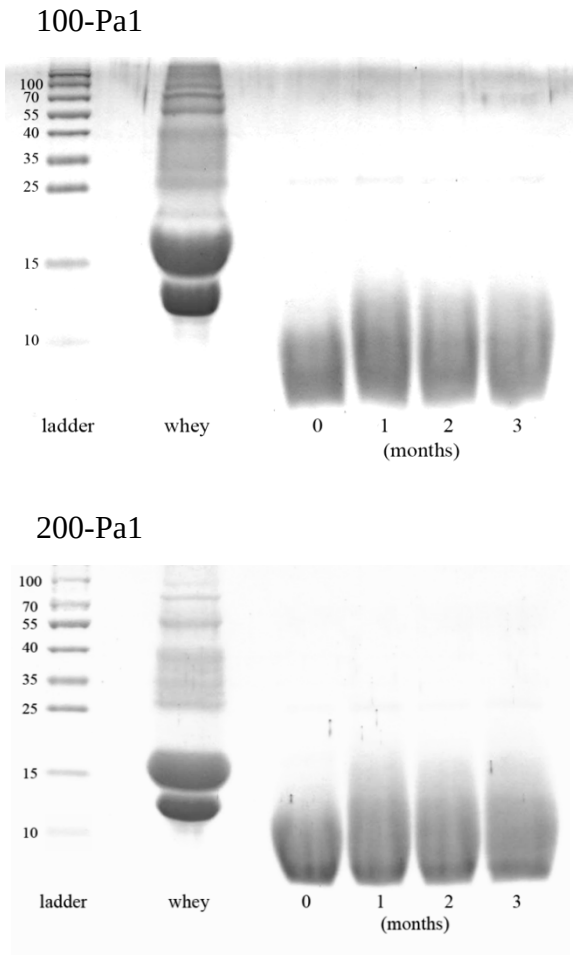


Figure 38 The protein patterns of whey hydroysate powders under accelerated condition

The DH of whey hydrolysate powders under accelerated condition was shown in Figure 39. The DH of whey hydrolysate powders were significantly decreased from 8.61 ± 0.24 to 6.88 ± 0.22% for 100-Pa1 (*p*-value = 0.001) and from 5.40 ± 0.32 to 4.52 ± 0.21% for 200-Pa1 (*p*-value = 0.016) over the storage periods.

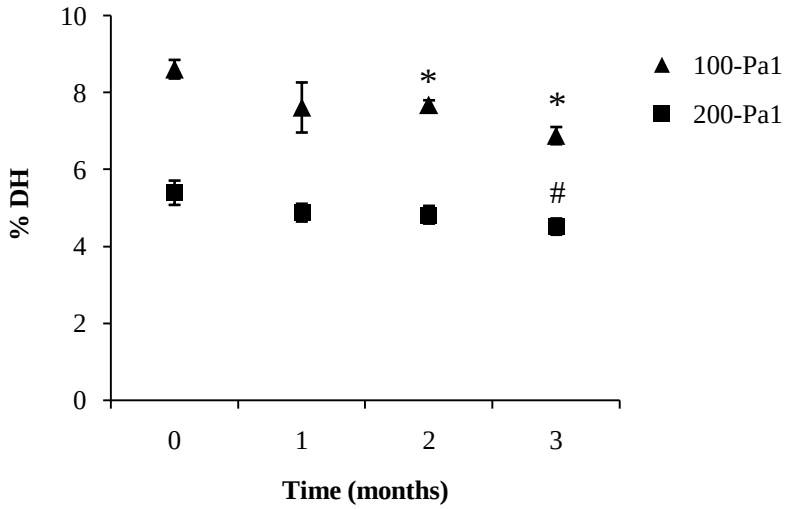


Figure 39 The DH of whey hydrolysate powders under accelerated condition. Plots were mean ± SD (n = 3). *p-value < 0.05 versus the DH of 100-Pa1 powder before storage, #p-value < 0.05 versus the DH of 200-Pa1 powder before storage.

The ROS scavenging activity of whey hydrolysate powders was shown in Figure 40. The relative DCF fluorescence response of whey hydrolysate powders was increased from 0.69 ± 0.02 to 0.94 ± 0.02 for 100-Pa1 (p-value = 0.001) and 0.83 ± 0.05 to 0.91 ± 0.03 for 200-Pa1 (p-value = 0.075) after 3 months storage. The result indicated that the ROS scavenging activity of whey hydrolysate powders was decreased by 36% for 100-Pa1 and 9.6% for 200-Pa1 compared with those of whey hydrolysate powders before storage. This implied that 200-Pa1 powder was more stable than 100-Pa1 powder.

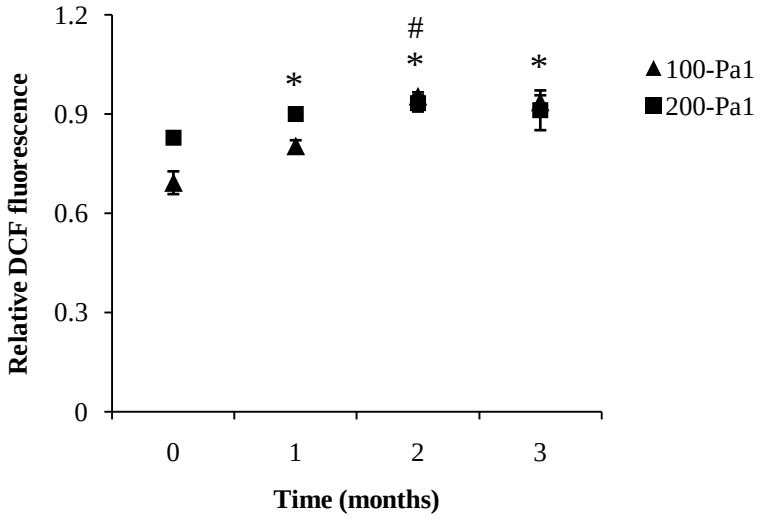


Figure 40 The ROS scavenging activity of whey hydrolysate powders under accelerated condition. Plots were mean ± SD (n = 3). **p*-value < 0.05 versus the relative DCF fluorescence response of 100-Pa1 powder before storage, $^{\#}$*p*-value < 0.05 versus the relative DCF fluorescence response of 200-Pa1 powder before storage.

The reduction of the ROS scavenging activity was contributed by alteration in the peptides components in whey hydrolysate powders. Over the storage period, the decrease in amount of these peptides was observed in the DH study (Figure 39). This finding indicated that the peptides in whey hydrolysate powders might interact with each other leading to aggregate formation. The aggregation caused the change in compositions and sequences of the bioactive peptides in whey hydrolysate powders. Consequently, the ability of whey hydrolysate powders to scavenge the ROS was decreased (Figure 40) (Chen et al., 1996; Liu, Chen and Mao, 2007). However, the aggregation of whey hydrolysate powders was not observed in the protein patterns study (Figure 38). It might be due to the SDS-PAGE condition used in this study was low resolution to determine the change in the peptides in whey hydrolysate powders. In order to confirm the aggregation of the peptides in whey hydrolysates, the resolution of SDS-PAGE technique should be improved by decreasing amount of loaded samples, increasing percentage of acrylamide resolving gel or decreasing duration for gel running. Additionally, the protein petterns of whey hydrolysate

powders should be determined by SDS-PAGE and native-PAGE technique to confirm aggregate formation.

CHAPTER V

CONCLUSIONS

The purposes of this study were to investigate the effect of the hydrolysis conditions: E/S, hydrolysis time and types of enzyme on the properties of whey hydrolysates as the protein patterns, DH and ROS scavenging activity and evaluate the stability of whey hydrolysate. The results of this study could be concluded as follow:

The digestion of whey by enzymes was investigated by the protein patterns. The effect of the hydrolysis conditions on the protein patterns of whey hydrolysates was depended on the E/S, hydrolysis time and types of enzyme. The digestive ability of chymotrypsin and trypsin was increased with the E/S and hydrolysis time while the digestive ability of papain was markedly improved by the E/S. The complete digestion of the protein components in whey was performed by papain at the E/S of 1/200 and 1/100 within 1 hr. It suggested that papain had the greatest susceptible to the protein components compared with trypsin and chymotrypsin.

The effect of the hydrolysis conditions on the DH of whey hydrolysates was depending on the E/S, hydrolysis time and types of enzyme. The effect of the E/S on the DH was concentration dependent and depended on the types of enzyme. The effect of the hydrolysis time on the DH was time dependent and depended on the E/S and types of enzyme. In addition, the interaction between the E/S and hydrolysis time on the DH was influenced by the types of enzyme. This interaction was only observed in whey hydrolyzed with chymotrypsin. The highest DH was obtained from the E/S of 1/100 with the hydrolysis time of 5 hr. Under this condition, whey hydrolyzed with papain and trypsin showed the highest DH compared to whey hydrolyzed with chymotrypsin.

For the ROS scavenging activity, whey hydrolyzed with chymotrypsin, trypsin and papain had the scavenging activity against DMNQ-generated ROS in HaCaT cells. The study of the effect of the hydrolysis conditions on the ROS scavenging

activity indicated that the types of enzyme had the greatest effect on the activity followed by the E/S and hydrolysis time. The ROS scavenging activity of whey hydrolyzed with papain increased with the E/S. While the ROS scavenging activity of whey hydrolyzed with chymotrypsin decreased as the hydrolysis time increased. The E/S and hydrolysis time was not significantly influence on the ROS scavenging activity of whey hydrolyzed with trypsin. Moreover, the interaction between the E/S and types of enzyme on the ROS scavenging activity was observed. Whey hydrolyzed with papain had the greater effect of the E/S on the ROS scavenging activity compared to whey hydrolyzed with chymotrypsin and trypsin. The highest ROS scavenging activity was found in whey hydrolyzed with papain at the E/S of 1/100 and 1/200. It suggested that high ROS scavenging activity of these hydrolysates was contributed by the complete digestion of the protein components in whey particularly β-Lg and α-La. Even whey hydrolysates were rich in cysteine-containing peptides. The ROS scavenging activity of whey hydrolysates was not mediated by the stimulation of the glutathione synthesis. Two formulations of whey hydrolyzed with papain showing the highest ROS scavenging activity, 100-Pa1 and 200-Pa1, were selected to evaluate the stability of whey hydrolysates.

The stability of whey hydrolysate powdes were investigated under accelerated condition for 3 months. After 3 month storage, the peptides in whey hydrolysate powders might be aggregation leading to decreased amount of peptide components. Consequently, the ROS scavenging activity of whey hydrolysate powders were decreased.

The results showed that the ROS scavenging activity of whey hydrolysates was improved by the enzymatic hydrolysis. Whey hydrolyzed with papain had the highest ROS scavenging activity compared with whey hydrolyzed with trypsin and chymotrypsin. The powders of whey hydrolyzed with papain were unstable under accelerated conditions for 3 months storage. The further study should identify specific bioactive peptide in whey hydrolysate and investigate instability mechanism of these in powder form in order to develop the formulation as an anti-aging product for topical used.

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APPENDICES

APPENDIX A

Preparation of SDS-PAGE Reagent

Preparation of SDS-PAGE Reagent

1. Preparation of Buffer

1.1 Preparation of Running Buffer

The mixture of 30 g of tris, 144 g of glycine and 10 g of SDS was dissolved in 900 ml of water and stirred for 30 min. The solution was adjusted volume to 1 L in volumetric flask giving 10-fold stock solution. 100 ml of stock solution was diluted with water to 1 L before use.

1.2 Preparation of Loading Buffer

0.2 g of SDS was dissolved in 2 ml of glycerol and 1.67 ml of 1.5 M tris-HCl (pH 6.8). 1 mg of bromophenol blue was dissolved in the solution followed by adding 1 ml of β-mercaptoethanol. The solution was stored in -20^{o}C.

2. Preparation of Samples

75 µl of 20 mg/ml whey or whey hydrolysate solution was mixed with 45 µl of loading buffer. The solution was heated at 95^{o}C for 10 min and cooled at room temperature before used.

3. Preparation of Acrylamide Gel

3.1 Preparation of 15% Acrylamide Resolving Gel

15% acrylamide resolving gel was prepared to characterize the protein patterns of whey hydrolysates. Briefly, 3.88 ml of water was mixed with 2.5 ml of 1.5 M tris-HCl (pH 8.8) and 3.92 ml of 40% acrylamide/bis solution followed by adding 100 µl 10% (w/v) SDS. The solution was immediately mixed with 60 µl of 1 0 %(w/v)

ammonium persulfate and 8 µl of N, N, N, N-tetramethyl-ethylenediamine (TEMED) prior use.

3.2 Preparation of 3% Acrylamide Stacking Gel

3% acrylamide stacking gel was used to concentrate the protein sample before entering the resolving gel. Briefly, 3.74 ml of water was mixed with 1.25 ml of 0.5 M tris-HCl (pH 6.8) and 410 µl of 40% acrylamide/bis solution followed by adding 50 µl of 10% (w/v) SDS. The solution was immediately mixed with 25 µl of 10% (w/v) ammonium persulfate and 6 µl of TEMED prior use.

APPENDIX B

Amino Acid Profile in Whey Protein Isolate (HMS 90)

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IMMUNOCAL®/HMS 90®
AMINO ACID PROFILE

AMINO ACID	mg per 10g protein	Percent
Tryptophan	182	1.82%
Threonine	754	7.54%
Isoleucine	618	6.18%
Leucine	990	9.90%
Lysine	863	8.63%
Methionine	227	2.27%
Cystine	227	2.27%
Phenylalanine	282	2.82%
Tyrosine	282	2.82%
Valine	572	5.72%
Arginine	154	1.54%
Histidine	163	1.63%
Alanine	509	5.09%
Aspartic acid	1153	11.53%
Glutamic acid	1789	17.89%
Glycine	182	1.82%
Proline	572	5.72%
Serine	481	4.81%

Jacques J. Cohen
Vice-President, International Operations
August 2008 This certificate is not valid if it is altered in any way whatsoever.

VITA

Miss Vititda Awaiwanont was born on April 25, 1984 in Bangkok, Thailand. She received her Bachelor Degree in Pharmacy from the Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2006. She continued the enrollment to the Master degree program in Pharmacy at Chulalongkorn University in the same year.