



## Peptide Analysis and the Bioactivity of Whey Protein Hydrolysates from Cheese Whey with Several Enzymes

Renda Kankanamge Chaturika Jeewanthi<sup>1</sup>, Myeong Hee Kim<sup>1</sup>, Na-Kyoung Lee<sup>1</sup>, Yoh Chang Yoon<sup>1</sup>, and Hyun-Dong Paik<sup>1,2\*</sup>

<sup>1</sup>Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea

<sup>2</sup>Bio/Molecular Informatics Center, Konkuk University, Seoul 05029, Korea

### Abstract

The aim of this study was identifying a suitable food grade enzymes to hydrolyze whey protein concentrates (WPCs), to give the highest bioactivity. WPCs from ultrafiltration retentate were adjusted to 35% protein (WPC-35) and hydrolyzed by enzymes, alcalase,  $\alpha$ -chymotrypsin, pepsin, protease M, protease S, and trypsin at different hydrolysis times (0, 0.5, 1, 2, 3, 4, and 5 h). These 36 types of hydrolysates were analyzed for their prominent peptides  $\beta$ -lactoglobulin ( $\beta$ -Lg) and  $\alpha$ -lactalbumin ( $\alpha$ -La), to identify the proteolytic activity of each enzyme. Protease S showed the highest proteolytic activity and angiotensin converting enzyme inhibitory activity of IC<sub>50</sub>, 0.099 mg/mL (91.55%) while trypsin showed the weakest effect. Anti-hypertensive and antioxidative peptides associated with  $\beta$ -Lg hydrolysates were identified in WPC-35 hydrolysates (WPH-35) that hydrolyzed by the enzymes, trypsin and protease S. WPH-35 treated with protease S in 0.5 h, responded positively to usage as a bioactive component in different applications of pharmaceutical or related industries.

**Keywords** whey protein hydrolysates, protease S, proteolytic activity, bioactive peptides

### Introduction

The whey industry has a short history but continues to innovate, focusing on finding new uses and new markets for value-added ingredients. Whey manufacturers are capable of producing numerous whey products by adjusting protein levels, minerals, lactose contents, functional properties, and other specifications. Besides the functional properties like water binding, forming gels, emulsifying, foaming, fat binding, color and texture enhancing in food products (Jeewanthi *et al.*, 2015a), whey proteins (WPs) have numerous nutritional properties which concern by food manufacturers. WPs contain a relatively high level of essential amino acids with a high nutritional value and are an important source of bioactive sequences (Korhonen, 2002; Silvestre *et al.*, 2012). There has been a growing interest in recent years in bioactive peptides derived from WPs. Such peptides have been found to express various bioactivities with protective functions such as reducing allergenicity, regulation of digestion, nutrient uptake, and metabolic or physiological regulation. These biologically active peptides are inactive within the sequence of parent protein and release during mechanical or chemical cleavage.

The term bioactivity refers to food components that can affect biological pro-

Received August 16, 2016  
Revised December 29, 2016  
Accepted December 31, 2016

#### Corresponding author

Hyun-Dong Paik  
Department of Food Science and  
Biotechnology of Animal Resources,  
Konkuk University, Seoul 05029, Korea  
Tel: +82-2-2049-6011  
Fax: +82-2-2455-3082  
E-mail: [hdpaik@konkuk.ac.kr](mailto:hdpaik@konkuk.ac.kr)

© This is an open access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

cesses or substrates and hence have an impact on body function or condition and health. Whey-based peptides have demonstrated activity that may reduce both hypertension and dyslipidemia. Literature has reported that bioactive whey peptides are involved in angiotensin-converting enzyme (ACE) inhibitory activity, opioid-like activity, antithrombotic activity, and cholesterol-reducing activity (Gerdes *et al.*, 2001). ACE-inhibitory activity is currently among the most documented bioactivity of whey peptides (Gauthier *et al.*, 2003). ACE is involved in hypertension, and its level is an important risk factor in the development of heart and vascular disease. In the human body, ACE converts the inactive peptide angiotensin I into the vasoconstrictive peptide angiotensin II and inactivates bradykinin (Meisel and Schlimme, 1996). The blockage of ACE and prevention of the amount of angiotensin II in the blood result in a decrease in blood pressure in hypertensive patients.  $\beta$ -lactoglobulin ( $\beta$ -Lg) is responsible for the majority of bioactive properties of WP products (Otte *et al.*, 1996). Bioactive peptides have been found in the enzymatic hydrolysates of  $\beta$ -Lg and  $\alpha$ -lactalbumin ( $\alpha$ -La).

Several studies have suggested the need for rapid and cost-effective methods for the production and isolation of bioactive peptides from whey and that can also be used on a larger industrial scale (Tavares and Malcata, 2013; Zambrowicz *et al.*, 2013). Bioactive peptides, including those that are inactive within the sequence of the parent protein, can be released in three ways: enzymatic hydrolysis, fermentation of milk with proteolytic starters, and proteolysis by microorganisms (Foegeding *et al.*, 2002). Researchers have shown that limited enzymatic hydrolysis of WPs can result in improved functionality and biological activities (Pouliot *et al.*, 2009; Spellman *et al.*, 2009). Food industry uses several enzymes for food production. However, using particular enzymes for an each food has a great influence for the food quality.

WPC-35 is a protein source which available in market at a cheaper cost compared with the products of other WPC percentages or isolates. Adjusting the protein content of 35% from fresh whey needs less processing. This concentration of WPs widely use in food industry for baby foods, dairy foods, bakery products, confectionaries, chocolates, nutritional supplements, tablets, and as a replacer of skim milk. This study was aimed at identifying an enzyme that encompassing bioactive peptides and ACE-inhibitory effect of WPC-35 hydrolysates, out of six different food-grade enzymes which are using currently in food manufacturing industry. Moreover, this study concerned

on identifying the most appropriate whey protein hydrolysates (WPHs) which can be added to food as long time hydrolyzed protein or partly purified peptides for formulation of a wide range of functional food products in health attributes.

## Materials and Methods

### WPC-35 preparations

WPC was produced using the method of Jeewanthi *et al.* (2014). Fresh mozzarella cheese whey remained after mozzarella cheese production at the lab was clarified and subjected to heat treatment at 70°C in a vat for 5 min after adjusting the pH to 7 from 6.32, followed by cooling to 50°C. Ultrafiltration (UF) was carried out with inlet and outlet pressures of 6 bar and 2 bar, respectively. The membrane module used was equipped with GR-60-PP flat sheet UF membranes (Danish Separation Systems AS, Denmark). The molecular weight cut-off range was 10 kDa. This experiment was carried out in a spiral wound-type module (DSS LabUnit M20, Alfa Laval Nakskov, Denmark), and the total effective surface area of the membranes was 0.036 m<sup>2</sup>. After filtration, the permeate was removed, and the retentate was subjected to spray drying using the B-191 Mini spray dryer (BUCHI, Switzerland) in order to obtain dried WPC at a protein content of approximately 35%. The volume reduction of whey was 75% to obtain WPC-35 (Jayaprakasha and Yoon, 2005). The following formula was used to calculate the volume reduction. The inlet and outlet air temperatures were 175°C and 75°C, respectively.

$$\text{Volume reduction (\%)} = \frac{\text{Permeated volume (mL)}}{\text{Original volume (mL)}} \times 100$$

### General component measurement of WPC-35

The protein content was measured by the Kjeldahl method (nitrogen factor 6.38), and fat by the Röse-Gottlieb method (AOAC, 2000). Moreover, total solid, ash, and lactose contents were determined according to the methods of AOAC (2000).

### Preparation of WPC-35 hydrolysates

WPC-35 hydrolysates (WPHs) were produced according to the method of Jeewanthi *et al.* (2014). The enzymes were added to a 10% WPC-35 solution at a ratio of 1:25 (w/v) and placed in a shaking incubator (VS-8480S, Vision Scientific Co., Ltd., Korea) at 37°C. Aliquots of each

enzyme solution were collected at 0.5, 1, 2, 3, 4, and 5 h while shaking at 180 rpm. The aliquots were then placed in a 95°C water bath for 10 min to inactivate the enzymes and cooled to room temperature. The mixture was centrifuged at 3,000 rpm (Combi-514R, Hanil Science Industrial Co., Ltd., Korea) for 30 min, and the upper solution was dried completely using a freeze dryer (FDU-1200, EYELA, Japan) for 24 h. The dried powder was then sealed and refrigerated.

### Hydrolysis enzymes

The commercial enzymes used were alcalase 2.4L (Novozymes, Denmark, EC 3.4.21.62), protease M (Amano Enzyme, Japan), protease S (Amano Enzyme, E.C. 3.4.24.28), trypsin (Novozymes, Denmark, EC 3.4.21.4),  $\alpha$ -chymotrypsin (Sigma-Aldrich Co., USA, EC 3.4.21.1), and pepsin (Wako Pure Chemical Industries Ltd., Japan, EC 3.4.23.1).

### Proteolytic activity of enzymes for WPC-35

Mini-PROTEAN tetra electrophoresis, which includes BIO-RAD gel electrophoresis system, was used to evaluate peptide formation of the hydrolysates. In brief, 10 mg of sample was dissolved in 1 mL of sample buffer (Sigma-Aldrich Co., USA) for 5 min at 100°C to minimize protein denaturing. Each sample was then stained with coomassie brilliant blue G (Sigma-Aldrich Co., USA). Distinct bands within the gel appeared after running WPH in a polyacrylamide gel. Seebue plus2®pre-stained standard was used as the reference material. Degree of hydrolysis (DH) was estimated using 2,4,6-trinitrobenzene sulfonic acid (Sigma-Aldrich Co., USA) (Jeewanthi *et al.*, 2015b).

### ACE-inhibitory activity

The ACE-inhibitory activity of WPH was measured using a modified method, which used by Lim *et al.* (2011). ACE solution was prepared by stirring 1 g of rabbit acetone lung powder (Sigma-Aldrich Co., USA) with 10 mL of 0.1 M sodium borate buffer containing 0.4 M sodium chloride at 4°C for 24 h. From this solution supernatant was used after centrifuge (Combi-514R, Hanil Science Industrial Co., Ltd., Korea) for 30 min at 12,000 rpm. WPHs 10% solution sample of 50  $\mu$ L was mixed with 100  $\mu$ L of 0.1 M sodium borate buffer (pH 8.3). Then 50  $\mu$ L of ACE solution was added and incubated at 37°C for 5 min. In addition, 50  $\mu$ L of Hip-His-Leu solution (Sigma-Aldrich Co., USA) was added and incubated for 30 min.

To stop the enzymatic reaction, 250  $\mu$ L of 0.5 N HCl was added. Produced hippuric acid was extracted by vortexing for 15 s after addition of 1.5 mL of ethyl acetate. One milliliter of supernatant was transferred in to a clean glass test tube after centrifuging for 2,500 rpm for 10 min. Then the tubes were placed in dry oven at 100°C for complete evaporation and remained brown ring was dissolved well using 3 mL of deionized water. Each sample's absorbance was measured using a 228 nm spectrophotometer (OPTIZEN 2120UV, Mecasys Co., Ltd., Korea). This experiment was performed in triplicate and the results are the average of three values. The ACE inhibitory activity was expressed in two ways, as a percentage of inhibition and IC50 value, which is defined as the concentration of WPC hydrolysates (mg/mL) necessary to reduce the activity of this enzyme at 50%.

The following formula was used to calculate the percentage of ACE-inhibitory activity:

$$\text{ACE-inhibitory activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

### Bioactive peptides

A Q-Star mass spectrometer (Applied Bio-systems/MDS SCIEX, Canada) was used for the analysis of bioactive peptides of 3 h-hydrolyzed WPH by trypsin and protease S. The method used for analysis was the offline electrospray-ionization-mass (ESI/MS), which was identified by Schober *et al.* (2012) from their previous studies, as a suitable method for analyze of WPHs. The aliquots were diluted with acetonitrile at a ratio of 1:1. Each prepared diluted sample of 10  $\mu$ L filled into nano ESI emitters (GC 120 F-10, DNU MS, Germany). The spray voltage was gradually increased to 1,600 volts, and the flow rate of the ionized molecules was analyzed at 2-200  $\mu$ L/min.

### Statistical analysis

Mean values and standard deviation were measured using the SAS software package. One-way analysis of variance (ANOVA) and Duncan's multiple range test ( $p < 0.05$ ) were used to establish the significance of the differences. The peptide probability was calculated according to mascot probability scores (<http://www.matrixscience.com>). It was calculated the scores as  $-10 \times \text{Log}(\text{Peak value})$ .

## Results and Discussion

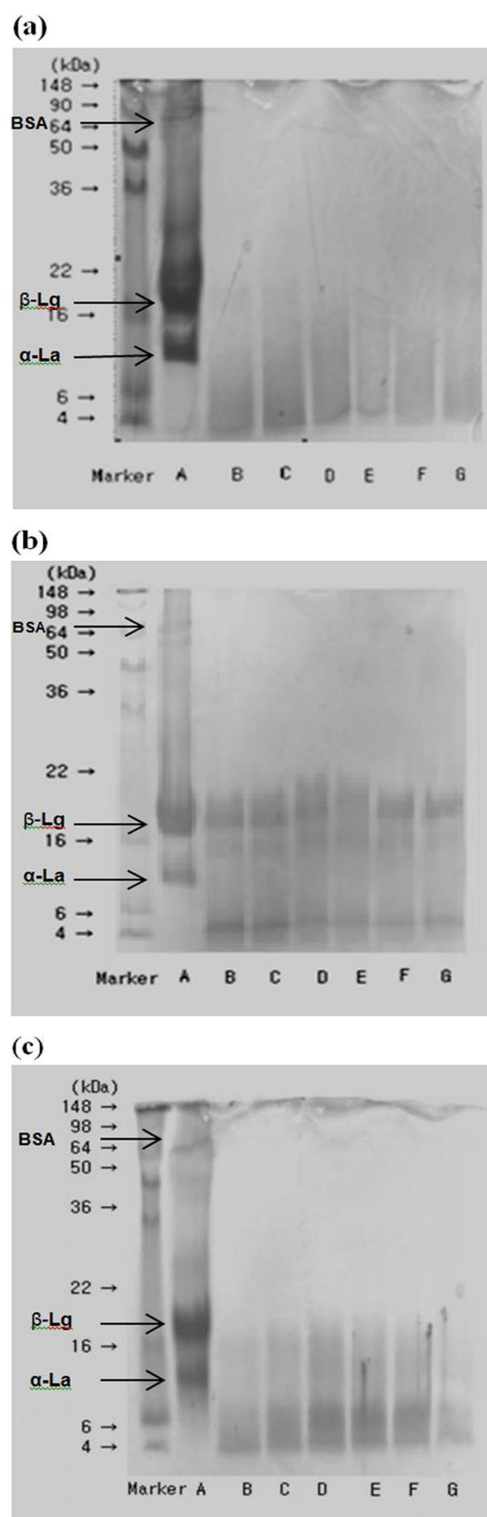
### General components of WPC-35

Usage of UF and spray drying methods to manufacture WPC-35 has a relatively easy scale-up, comparing to other membrane separation techniques or preparative chromatographic techniques. Short time heat treatment of this method provides an appropriate background to study the performance of WPH since the bioactive peptides remain intact and the WPs remain non-denatured manner. The protein concentration of 35% was able to achieve within a less processing time and inexpensive status due to minimal utilization of energy and time as well as its easy scale up, ease of membrane exchange, by contrast with other commercially available higher protein concentrations.

The following nutritional parameters were determined for the general compositions. The components of manufactured WPC-35 were 34.94% protein, 53.32% lactose, 3.50% fat, and 3.24% ash. The protein content of permeate was lesser than 0.1% along the UF process. The scientific study of Weigle *et al.* (2005) has been reported that an isocaloric high-protein diet with 30% protein, 20% fat, and 50% carbohydrate was markedly increased satiety and decreased energy intake, body weight, and body fat. Lactose is the major component of WPC-35 which considers as the least valuable component. The optimum results in high protein diets which aimed at weight reduction are achievable when intake is more balanced with carbohydrate (Luhovyy *et al.*, 2005). Relatively lactose has a low glycemic index compared to the other carbohydrates in food and it is hard to utilize. This reason upgrades the value of lactose on top of the other health benefits such as increase mineral absorption, contribute as a probiotic and a prolonged energy supplier. Calcium, magnesium, and phosphorous are the major minerals available in WPC and zinc (Jelen, 1992) is available in trace amounts.

### Proteolytic activity of enzymes for WPC-35

WPC is a complex blend of individual proteins. Compositional patterns were produced from the electrophoresis of WPHs, and the hydrolysate from protease S and pepsin can be seen in Fig. 1. In intact WPCs, the bands appeared in the range of 50-98 kDa, 16-22 kDa, and 6-16 kDa. It was shown that BSA (66.4 kDa),  $\beta$ -Lg (18 kDa), and  $\alpha$ -La (14 kDa) are the distinct proteins of intact WPCs. Damodaran (1996) has reported that  $\beta$ -Lg remains as a dimer with a molecular weight of 36 kDa between pH values 5.2-7.2. The pH values of all experimented WPHs



**Fig. 1. Proteolytic activity of WPC-35 hydrolysates treated by (a) protease S, (b) pepsin, and (c) alcalase at hydrolysis time.** Lane A-G: 0, 0.5, 1, 2, 3, 4, and 5 h of WPC-35 hydrolysates.

solutions were laid between these ranges except pepsin hydrolysates. The pH values of pepsin hydrolysates were

2.94-2.63 ( $p < 0.05$ ) at 0-5 h hydrolysis (data not shown).

$\alpha$ -La is the second most abundant protein in WP. It is a compact globular protein with a similar structure and composition to human milk proteins.  $\alpha$ -La considers as an important protein in WPH supplemented nutritional foods due to Cys, Ile, Leu, Trp, and Val residues. The structure of this protein is highly heterogeneous,  $\alpha$ -helix forms with weak hydrophobic interactions.

Depending on the type of enzyme, the protein bands were located in different locations; however, the 2 h hydrolysis samples of every enzyme showed a common pattern in bands, where the degraded peptides commonly appeared between 16-22 kDa and 6-16 kDa. Protease S showed the highest proteolytic activity of the selected enzymes of WPC-35 (Fig. 1(a)), while pepsin showing the least (Fig. 1(b)). Our previous experiments showed that the protease S had the second highest DH among these enzymes for WPC-35 as 9.96-11.2% (hydrolyzed for 0.5-5 h) (Jeewanthi *et al.*, 2014). In WPC-50 and WPC-60 (whey protein concentration with 50% and 60%), DH for protease S was reported in-between 26.4-28.77% (hydrolyzed for 0.5-5 h) and superior to enzymes named flavourzyme, protease A, protease M, and pepsin (Jeewanthi *et al.*, 2015b). The results indicate that pepsin is less capable of hydrolyzing  $\beta$ -Lg within 5 h and available at a higher purity. DH of pepsin was reported as 8.26-10.34% for 0.5-5 h hydrolysis of WPC-35. In trypsin hydrolysis samples, the  $\beta$ -Lg and  $\alpha$ -La contents decreased over time (data not shown). After 4 h,  $\beta$ -Lg was no longer detectable and much less  $\alpha$ -La was observed.  $\beta$ -Lg was more rapidly broken down by trypsin and less rapidly by pepsin compared to  $\alpha$ -La of whole whey (Pintado and Malcata, 2000). At the beginning of the reaction, trypsin partially hydrolyses  $\beta$ -Lg, although  $\alpha$ -La and BSA are more resistant to hydrolysis (Custodio *et al.*, 2005). All of the experimental results above agreed with the results. Trypsin was exhibited 8.78-11.23% of DH% (hydrolyzed for 0.5-5 h). Alca-

lase showed the second highest proteolytic activity, and peptide bands were found between 4-10 kDa (Fig. 1(c)). Chymotrypsin reacted with most of the  $\beta$ -Lg, visualized as increasingly weaker bands with time, and with little of the BSA (data not shown).

#### ACE-inhibitory activity

The preferred ACE inhibitors are those peptides that contain dicarboxylic amino acids in the N-terminal position and branched-chain amino acid residues, such as Val and Ile (Wu and Ding, 2002), while the C-terminal position is represented by hydrophobic amino acids such as Trp, Tyr, Phe or Pro (Yust *et al.*, 2003). However, ACE shows poorer affinity when dicarboxylic amino acid in final position or residue Pro comes before the last position (Tavares and Malcata, 2013). ACE inhibiting peptides which have antihypertensive properties have been released through an enzymatic treatment of bovine  $\beta$ -Lg and WPCs (Wang *et al.*, 2010). The calculated values for the ACE-inhibitory effect of different WPHs are shown in Table 1. The results showed 6.06% ( $IC_{50} = 0.972$  mg/mL) of ACE-inhibitory effect in intact WPC-35. Mullally *et al.* (1997) also reported 10% lesser ACE-inhibitory effect in whey protein isolates (WPIs). Just after the hydrolysis of WPC-35, the values were heavily increased by at least ten-fold of their initial value. Among the experimental enzymes, protease S and alcalase hydrolysates showed the strongest ACE-inhibitory effect, while trypsin hydrolysates showed the weakest effect. The experiments of Wang *et al.* (2010) reported similar results for alcalase followed with neutrase, by showing stronger ACE-inhibitory effect compared to trypsin in hydrolysates of WPIs 5% solution. According to our results, ACE-inhibitory activity can be accelerated by the higher proteolytic activities of the protease S after releasing many amino residues between 2-12 kDa those considering as the most responsible ACE-inhibitor peptides (Lopez-Fandino *et al.*, 2006). With the rea-

**Table 1. IC50 values of ACE-inhibitory activity in whey protein 35% hydrolysates of enzymes at different hydrolysis time**

Enzyme		Hydrolysis time (h)			
		0	2	3	4
Alcalase	$IC_{50}$	$0.972 \pm 0.001^a$	$0.107 \pm 0.003^a$	$0.126 \pm 0.032^{bc}$	$0.317 \pm 0.029^b$
Chymotrypsin	$IC_{50}$	$0.972 \pm 0.001^a$	$0.180 \pm 0.005^c$	$0.157 \pm 0.004^b$	$0.121 \pm 0.001^a$
Pepsin	$IC_{50}$	$0.972 \pm 0.001^a$	$0.179 \pm 0.012^b$	$0.177 \pm 0.006^a$	$0.186 \pm 0.008^{ba}$
Protease M	$IC_{50}$	$0.972 \pm 0.001^a$	$0.153 \pm 0.005^a$	$0.136 \pm 0.002^a$	$0.154 \pm 0.009^b$
Protease S	$IC_{50}$	$0.972 \pm 0.001^a$	$0.108 \pm 0.003^b$	$0.103 \pm 0.010^{ba}$	$0.099 \pm 0.006^{ba}$
Trypsin	$IC_{50}$	$0.972 \pm 0.001^a$	$0.258 \pm 0.012^b$	$0.196 \pm 0.009^a$	$0.243 \pm 0.010^b$

<sup>1</sup> $IC_{50}$ : Concentration of hydrolysates (mg/mL) necessary to the activity of this enzyme by 50%; <sup>a-c</sup>Means with the different letters in a same column are significantly different by Duncan's multiple range test ( $p < 0.05$ ).

son of protease S is a thermostable neutral protease, the WPH solution has given a favorable atmosphere to perform enzyme activity than the other acidic or base enzymes. Alcalase treated WPHs are the second highest score resulted for ACE-inhibitory activity and Fig. 1(c) revealed that those peptides are lies between 4-10 kDa.

The pH of WPHs in a 10% solution of every enzyme was ranged between in 6.03-6.22 except pepsin hydrolysates (data not shown). On the other hand, it delayed the activity of trypsin which favors a base environment. Protease M, pepsin, and trypsin showed maximal activity with 3 h hydrolysates. The bioactive peptides responsible for ACE-inhibitory degradation, seemed starts and dominate or inhibit the novel formation of peptides at this level (Meisel and Schlimme, 1996). These results show that the specificity of the enzyme had an obvious effect on the resulting ACE-inhibitory activity besides every enzyme hydrolysates has capability of increasing ACE-inhibitory effect.

### Bioactive peptides

The major protein in WPCs is  $\beta$ -Lg which has 162 amino acids. This protein remains its primary structure unchanged in gastric digestion due to resistance of enzymatic or acidic digestion along the passage through stomach (Barros *et al.*, 2001). Hydrolysis of intact structure of  $\beta$ -Lg activates the bioavailability of the peptides and recognition of those is important in food manufacturing industry since it is the major protein in WPHs.

Experimented WPH was selected for peptide analysis, looking at trypsin and protease S hydrolysates (3 h) showed the lowest and highest ACE-inhibitory activity respectively. Peptide analysis of  $\beta$ -Lg results are shown in Tables 2 and 3. These are the enzymes that showed ACE-inhibitory active peptides that fractioned by both ends from the parental proteins as LAMA and LDAQSAPLR within 3 h (least hydrolysis time).

They confirmed that protease S generated LDAQSAPLR (Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg) in fragment 32-40 and LVLDTDYK (Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys)

**Table 2. The peptide analysis of  $\beta$ -lactoglobulin after WPC-35 hydrolysis for 3 h by trypsin**

Sequence (Fragments)	PP <sup>1</sup>	Sequence (Fragments)	PP
ALKALPMH	34.70	LKPTPEGDLEILL	20.49
ALKALPMHIR	83.33	LSFNPTQLE	25.38
ALPMHIRLSFNPTQLEEQCHI	51.39	LSFNPTQLEE	20.69
AMAASDISLLDAQSAPLR <sup>3</sup>	68.67	LSFNPTQLEEQCHI	77.51
DIQKVAGTWYSL <sup>2,3</sup>	34.34	<sup>2,3</sup> LVLDTDYKKY	51.37
FNPTQLEEQCHI	75.42	LVRTPEVDDEA	67.53
GLDIQKVAGTWY <sup>2,3</sup>	41.89	LVRTPEVDDEALE	79.73
GLDIQKVAGTWYS <sup>2,3</sup>	78.11	LVRTPEVDDEALEK	143.31
GLDIQKVAGTWYSL <sup>2,3</sup>	50.71	MKGLDIQK	35.54
GLDIQKVAGTWYSLA	55.14	SFNPTQLEEQCHI	32.67
GLDIQKVAGTWYSLAMAASDISLLDAQSAPLR <sup>3</sup>	82.49	SLAMAASDISLLDAQSAPLR <sup>3</sup>	70.12
IDALNENKV	57.49	SLAMAASDISLLDAQSAPLRVY	56.04
IIAEKTKIPA	88.18	<b>TKIPAVFK<sup>2</sup></b>	31.34
IIAEKTKIPAV	71.87	TPEVDDEALEKFDK	84.27
IIAEKTKIPAVF	61.96	TPEVDDEALEKFDKALK	122.61
IIAEKTKIPAVFK <sup>2</sup>	96.36	<sup>2,3</sup> VAGTWYSLAMAASDISLLDAQSAPLR <sup>3</sup>	65.97
<b>LAMA<sup>3</sup></b>	62.54	VEELKPTPEGDLE	41.75
<b>LDAQSAPLR<sup>3</sup></b>	18.10	VEELKPTPEGDLEIL	39.20
LIVTQT	25.95	VEELKPTPEGDLEILLQ	66.79
LIVTQTMK	64.99	VEELKPTPEGDLEILLQK	50.18
LIVTQTMKG	79.06	VLDTDYKKY	67.88
LIVTQTMKGLDIQK	90.75	VRTPEVDDEA	60.49
LIVTQTMKGLDIQKVAGTWY <sup>2,3</sup>	45.73	VRTPEVDDEALEK	70.41
LIVTQTMKGLDIQKVAGTWYS <sup>2,3</sup>	109.54	VYVEELKPTPEGDLEILLQK	83.00
LIVTQTMKGLDIQKVAGTWYSL <sup>2,3</sup>	57.28	YVEELKPTPEGDLEIL	52.27
LKALPMH	22.02	YVEELKPTPEGDLEILL	54.56
LKPTPEGDLEIL	19.67		

<sup>1</sup>Peptide probability (PP), > 10, Peptide confidence: high; <sup>2</sup>peptide having antioxidant activity; <sup>3</sup>peptide having ACE-inhibitory activity.

**Table 3. The peptide analysis of  $\beta$ -lactoglobulin after WPC-35 hydrolysis for 3 h by protease S**

Sequence (Fragments)	PP <sup>1</sup>	Sequence (Fragments)	PP
AASDISL	18.46	LKPTPEGDLEILL	24.76
DALNENKVLVLDTDYKKYL	15.18	LKPTPEGDLEILLQK	29.16
FNPTQLEEQ	32.39	LKPTPEGDLEILLQKW	74.64
FNPTQLEEQCHI	53.99	<b>LLDAQSAPLRVYVEE</b>	81.24
IAEKTKIPA	52.39	LNENKVLVLDTDYKKY	121.29
IAEKTKIPAVFK <sup>2</sup>	36.31	LSFNPTQLEEQCHI	56.87
IDALNENKV	30.40	<sup>3</sup> <b>LVLDTDYKKY</b>	70.36
IDALNENKVL	19.07	<sup>3</sup> <b>LVLDTDYKKYL</b>	85.25
IDALNENKVLVLDTDYKK	96.05	LVRTPEVDDEA	73.10
IDALNENKVLVLDTDYKKY	120.15	LVRTPEVDDEAL	23.86
IIAEKTKIP	31.74	LVRTPEVDDEALEK	130.02
IIAEKTKIPA	55.18	SNPTQLEEQ	49.87
IIAEKTKIPAV	44.34	SNPTQLEEQCHI	50.75
IIAEKTKIPAVFK <sup>2</sup>	50.73	SLAMAASDISLLDAQSAPLRVYVEELK-	107.76
IIAEKTKIPAVFKI	21.08	PTPEGDLEILL	
<b>LDAQSAPLR<sup>3</sup></b>	41.53	VEELKPTPEGDLEIL	56.13
<sup>3</sup> <b>LDAQSAPLRVY</b>	87.27	VFKIDALNENKV	87.67
<sup>3</sup> <b>LDAQSAPLRVYVEE</b>	70.24	VLDTDYKKY	61.53
LDTDYKKY	45.80	VLDTDYKKYL	32.80
LIVTQT	19.47	<sup>2,3</sup> <b>VLVLDTDYKKY</b>	54.98
LIVTQTMKG	67.29	VRTPEVDDEA	57.61
LKALPMH	22.43	VRTPEVDDEALEK	72.48
LKPTPEGDLE	70.36	VYVEELKPTPEGDLEIL	36.23
LKPTPEGDLEIL	25.45	YVEELKPTPEGDLEILL	40.45

<sup>1</sup>Peptide probability (PP), > 10, Peptide confidence: high; <sup>2</sup>peptide having antioxidant activity; <sup>3</sup>peptide having ACE-inhibitory activity.

in fragment 92-100 from  $\beta$ -Lg with a higher confidence levels. Moreover, in  $\beta$ -Lg, the hydrolysates by trypsin generated LAMA (Leu-Ala-Met-Ala) with a high confidence value, VAGTWY (Val-Ala-Gly-Thr-Trp-Tyr) and LDAQSAPLR. The peptides LAMA, LDAQSAPLR, LVLDTDYK, and VAGTWY have been identified as ACE-inhibitory peptides (Pihlanto-Leppala *et al.*, 2000).

The presence of hydrophobic amino acids which Ala (A), Gly (G), Ile (I), Leu (L), Phe (F), Trp (W), Tyr (Y), and Val (V), and unique amino acids such as Pro (P) and Gly (G) in a peptide sequence increase the lipid inhibitory activity (Hermansen, 2000). The peptides LVLDTDYK (Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys), TKIPAVFK (The-Lys-Ile-Pro-Ala-Val-Phe-Lys), WYSL (Trp-Tyr-Ser-Leu), and VAGTWY has been reported as high radical scavenging active peptides (Conway *et al.*, 2013; Zhang *et al.*, 2013). VAGTWY, TKIPAVFK, and WYSL are shown in trypsin treated WPHs, while TKIPAVFK and LVLDTDYK in protease S treated hydrolysates. The peptides AASDISLLDAQSAPLR (Ala-Ala-Ser-Asp-Ile-Ser-Leu-Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg), PAVFK (Pro-Ala-Val-Phe-Lys), VLVLDTDYK (Val-Leu-Val-Leu-Asp-Thr-Asp-

Tyr-Lys), and VAGTWY have been reported to hold antibacterial effects (Exposito and Recio, 2006). Protease S treated hydrolysates of  $\beta$ -Lg consisted VLVLDTDYK peptides and did not appear in trypsin WPH. Both peptides AASDISLLDAQSAPLR and PAVFK observed in both hydrolysates, approves the presence in antibacterial effects of hydrolyzed WPHs of both samples. The more proteolytic active enzymes with WPC are able to release these peptides of interest, resulting in more bioactivity of the peptides. WPHs can be used as a complementary treatment in combination with lifestyle modifications, such as weight reduction, exercise, and a controlled diet, which have been reported to be some of the most important tools for the effective reduction of blood pressure (Host and Halken, 2004). Careful selection of enzymes and temperature for proteolysis produces the maximum biological activity.

## Conclusion

Based on the findings of this study, the hydrolysates of WPC-35 exhibited improved ACE-inhibitory activity by

releasing peptides that are inactive in intact WPs with the influence of enzymatic activity. It is known that  $\beta$ -Lg and  $\alpha$ -La are the prominent peptides in whey proteins and are available for bioactive functions like ACE-inhibitory effects after hydrolysis. Protease S had the highest proteolytic activity compared to the other enzymes for hydrolysis of WPCs. Protease S treated WPHs showed the highest ACE-inhibitory effect out of the other experimented enzymes, and second was alcalase. Protease S released ACE-inhibitory active peptide LDAQSAPLR and trypsin released LAMA, and LDAQSAPLR after 3 h hydrolysis. Among the experimented enzymes protease S had better performances in hydrolyzing WPC-35. WPCs had many other inactive bioactive peptides of antibacterial and antioxidant activity that are available to enhance by releasing with enzymatic hydrolysis. Based on the specific bioactive peptides of the hydrolysates, various applications and alterations could be carried out with proper selection of the enzymes.

## Acknowledgements

This study was supported by Korea Institute of Planning & Evaluation for Technology of Food, Agriculture, Forestry & Fisheries, Ministry for Food, Agriculture, Forestry and Fisheries Korea (#109136-3) and Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012-0006686).

## References

1. AOAC. (2000) Official Methods of Analysis. 17<sup>th</sup> ed, Association of Official Analytical Chemists, Gaithersburg, MD, USA, Chapter 33, pp. 4-53.
2. Barros, R. M., Ferreira, C. A., Silva, S. V., and Malcata, F. X. (2001) Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate. *Enzyme Microb. Tech.* **29**, 541-547.
3. Conway, V., Gauthier, S. F., and Pouliot, Y. (2013) Antioxidant activities of buttermilk proteins, whey proteins, and their enzymatic hydrolysates. *J. Agric. Food Chem.* **61**, 364-372.
4. Custodio, M. F., Goulart, A. J., Marques, D. P., Giordano, R. C., Giordano, R. L. C., and Monti, R. (2005) Hydrolysis of cheese whey proteins with trypsin, chymotrypsin and carboxypeptidase A. *Braz. J. Food Nutr.* **16**, 105-109.
5. Damodaran, S. (1996) Functional Properties. In: Food proteins, properties and characterization. Nakai, S. and Modler, H. W. (eds) VCH Publishers, New York, USA. pp. 167-234.
6. Exposito, I. L. and Recio, I. (2006) Antibacterial activity of peptides and holding variants from milk proteins. *Int. Dairy J.* **16**, 1294-1305.
7. Foegeding, E. A., Davis, J. P., Doucet, D., and McGuffey, K. (2002) Advances in modifying and understanding whey protein functionality. *Trends Food Sci. Tech.* **13**, 151-159.
8. Gauthier, S. F. and Stela, Y. P. (2003) Functional and biological properties of peptides obtained by enzymatic hydrolysis of whey proteins. *J. Dairy Sci.* **86**, 78-87.
9. Gerdes, S. K., Harper, W. J., and Miller, G. (2001) Bioactive components of whey and cardiovascular health. In: Application monograph cardiovascular health. U.S. Dairy Export Council, Arlington, USA. pp. VA1- VA 8.
10. Hermansen, K. (2000) Diet, blood pressure and hypertension. *Brit. J. Nutr.* **83**, S113-S119.
11. Host, A. and Halken, S. (2004) Hypoallergenic formulas when, to whom and how long: After more than 15 years we know the right indication. *Allergy* **59**, 45-52.
12. Jayaprakasha, H. M. and Yoon, Y. C. (2005) Production of functional whey protein concentrate by monitoring the process of ultrafiltration. *Asian-Aust. J. Anim. Sci.* **3**, 433-438.
13. Jeewanthi, R. K. C., Lee, N. K., and Paik, H. D. (2015a) Improved functional characteristics of whey protein hydrolysates in food industry. *Korean J. Food Sci. An.* **35**, 350-359.
14. Jeewanthi, R. K. C., Lee, N. K., Lee, S. K., Yoon, Y. C., and Paik, H. D. (2015b) Physicochemical characterization of hydrolysates of whey protein concentrates for their use in nutritional beverages. *Food Sci. Biotechnol.* **24**, 1335-1340.
15. Jeewanthi, R. K. C., Paik, H. D., Lee, N. K., Kim, M. H., Kim, S. Y., and Yoon, Y. C. (2014) Characteristics of whey protein hydrolysates from cheese whey, favors on various food applications. *Chem. Ind. Chem. Eng. Q.* **20**, 503-509.
16. Jelen, P. (1992) Whey cheese and beverages. In: Whey and lactose processing. Zadow, J. G. (ed) Elsevier, London and New York. pp. 157-194.
17. Korhonen, H. (2002) Technology options for new nutritional concepts. *Int. J. Dairy Technol.* **55**, 79-88.
18. Lim, S. M., Lee, N. K., Park, K. K., Yoon, Y. C., and Paik, H. D. (2011) ACE-inhibitory effect and physicochemical characteristics of yogurt beverage with whey protein hydrolysates. *Korean J. Food Sci. An.* **31**, 886-892.
19. Lopez-Fandino, R., Otte, J., and Van, C. J. (2006) Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *Int. Dairy J.* **16**, 1277-1293.
20. Luhovyy, B. L., Akhavan, T., and Anderson, G. H. (2007) Whey proteins in the regulation of food intake and safety. *J. Am. Coll. Nutr.* **6**, 704-712.
21. Meisel, H. and Schlimme, E. (1996) Bioactive peptides derived from milk proteins: Ingredients for functional foods. *Kieler Milchw. Forsch.* **48**, 343-357.
22. Mullally, M. M., Meisel, H., and Fitzgerald, R. J. (1997) ACE-I inhibitory activities of gastric and pancreatic proteinase digest of whey proteins. *Int. Dairy J.* **7**, 299-303.
23. Otte, J., Ju, Z. Y., Faergemand, M., Lomholt, S. B., and Qvist, K. B. (1996) Protease-induced aggregation and gelation of



- whey proteins. *J. Food Sci.* **61**, 911-915.
24. Pihlanto-Leppala, A., Koskinen, P., Piilola, K., Tupasela, T., and Korhonen, H. (2000) ACE-I Inhibitory properties of whey protein digests: Concentration & characterization of active peptides. *J. Dairy Res.* **67**, 53-64.
  25. Pintado, M. E. and Malcata, F. X. (2000) Hydrolysis of ovine, caprine and bovine whey proteins by trypsin and pepsin. *J. Bioprocess. Eng.* **23**, 275-282.
  26. Pouliot, Y., Guy, M. M., Tremblay, M., Gaonac'h, A. C., Chay Pak Ting, B. P., Gauthier, S. F., and Voyer, N. (2009) Isolation and characterization of an aggregating peptide from a tryptic hydrolysate of whey proteins. *J. Agr. Food Chem.* **57**, 3760-3764.
  27. Schober, Y., Yoo, S. H., Paik, H. D., Park, E. J., Spengler, B., Rompp, A., Jayaprakasha, H. M., and Yoon, Y. C. (2012) Characterization of bioactive peptides derived by enzymatic hydrolysis of whey protein concentrates. *Milchwissenschaft* **67**, 55-57.
  28. Silvestre, M. P. C., Silva, M. R., Silva, V. D. M., Souza, M. W. S., Lopes, J. R. C. O., and Afonso, W. O. (2012) Analysis of whey protein hydrolysates: Peptide profile and ACE inhibitory activity. *Braz. J. Pharm. Sci.* **48**, 747-757.
  29. Spellman, D., O'cuinn, G., and Fitzgerald, R. J. (2009) Bitterness in *Bacillus* proteinase hydrolysates of whey proteins. *Food Chem.* **114**, 440-446.
  30. Tavares, T. G. and Malcata, F. X. (2013) Whey Proteins as Source of Bioactive Peptides Against Hypertension. In: Bioactive food peptides in health and diseases. Hernandez-Ledesma, B., and Hsieh, C. C. (eds), Intech, Rijeka, Croatia. pp. 75-95.
  31. Wang, L., Mao, X., Cheng, X., Xiong, X., and Ren, F. (2010) Effect of enzyme type and hydrolysis conditions on the *in vitro* angiotensin I-converting enzyme inhibitory activity and as content of hydrolysed whey protein isolate. *Int. J. Food Sci. Tech.* **45**, 807-812.
  32. Weigle, D. S., Breen, P. A., Matthys, C. C., Callahan, H. S., Meeuws, K. E., Burden, V. R., and Purnell, J. Q. (2005) A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations. *Am. J. Clin. Nutr.* **82**, 41-48.
  33. Wu, J. and Ding, X. (2002) Characterization of inhibition and stability of soy-protein-derived ACE-I inhibitory peptides. *Food Res. Int.* **35**, 367-375.
  34. Yust, M. M., Pedroche, J., Girón-Calle, J., Alaiz, M., Millán, F., and Vioque, J. (2003) Production of ACE inhibitory peptides by digestion of chickpea legumin with alcalase. *Food Chem.* **80**, 1-7.
  35. Zambrowicz, A., Timmer, M., Polanowski, A., Lubec, G., and Trziszka, T. (2013) Manufacturing of peptides exhibiting biological activity. *Amino Acids.* **44**, 315-320.
  36. Zhang, Q. X., Wu, H., Ling, Y. F., and Lu, R. R. (2013) Isolation and identification of antioxidant peptides derived from whey protein enzymatic hydrolysate by consecutive chromatography and Q-TOF MS. *J. Dairy Res.* **80**, 367-373.