Enzymatic Hydrolysis of Heated Whey: Iron-Binding Ability of Peptides and Antigenic Protein Fractions

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ABSTRACT

This study evaluated the influence of various enzymes on the hydrolysis of whey protein concentrate (WPC) to reduce its antigenic fractions and to quantify the peptides having iron-binding ability in its hydrolysates. Heated (for 10 min at 100°C) WPC (2% protein solution) was incubated with 2% each of Alcalase, Flavourzyme, papain, and trypsin for 30, 60, 90, 120, 150, 180, and 240 min at 50°C. The highest hydrolysis of WPC was observed after 240 min of incubation with Alcalase (12.4%), followed by Flavourzyme (12.0%), trypsin (10.4%), and papain (8.53%). The nonprotein nitrogen contents of WPC hydrolysate followed the hydrolytic pattern of whey. The major antigenic fractions (β -lactoglobulin) in WPC were degraded within 60 min of its incubation with Alcalase, Flavourzyme, or papain. Chromatograms of enzymatic hydrolysates of heated WPC also indicated complete degradation of β -lactoglobulin, α -lactalbumin, and BSA. The highest iron solubility was noticed in hydrolysates derived with Alcalase (95%), followed by those produced with trypsin (90%), papain (87%), and Flavourzyme (81%). Eluted fraction 1 (F-1) and fraction 2 (F-2) were the respective peaks for the 0.25 and 0.5 M NaCl chromatographic step gradient for analysis of hydrolysates. Iron-binding ability was noticeably higher in F-1 than in F-2 of all hydrolysates of WPC. The highest iron contents in F-1 were observed in WPC hydrolysates derived with Alcalase (0.2 mg/kg), followed by hydrolysates derived with Flavourzyme (0.14 mg/kg), trypsin (0.14 mg/kg), and papain (0.08 mg/kg). Iron concentrations in the F-2 fraction of all enzymatic hydrolysates of WPC were low and ranged from 0.03 to 0.05 mg/kg. Fraction 1 may describe a new class of iron chelates based on the reaction of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ with a mixture of peptides obtained by the enzymatic hydrolysis of WPC. The chromatogram of

Alcalase F-1 indicated numerous small peaks of shorter wavelengths, which probably indicated a variety of new peptides with greater ability to bind with iron. Alcalase F-1 had higher Ala (18.38%), Lys (17.97%), and Phe (16.58%) concentrations, whereas the presence of Pro, Gly, and Tyr was not detected. Alcalase was more effective than other enzymes at producing a hydrolysate for the separation of iron-binding peptides derived from WPC.

Key words: whey, hydrolysis, iron-binding ability

INTRODUCTION

Whey proteins represent nearly 20% of the total bovine milk proteins (Fox, 1989). The known major fractions of whey proteins are β -LG, α -LA, Ig, and BSA (Walzem et al., 2002). In recent years, whey has gained immense recognition as a protein source in functional foods and infant formulas (Clemente, 2000). However, β -LG can induce milk allergy in human infants because of the underdeveloped infantile gastrointestinal tract (Zeiger et al., 1986) and immune system (Pintado et al., 1999). Because cow's milk allergy develops in 3% of the pediatric population (Hudson, 1995), infant formula manufacturers have had to face the problem of reducing whey protein antigenicity. Enzymatic hydrolysis of whey offers a practical way to reduce its antigenic protein fractions (Heyman, 1999). Furthermore, hydrolysis can yield a variety of new peptides that may offer many physiological benefits for humans (Otte et al., 1997).

Whey protein hydrolysates are being extensively prepared and used to nutritionally support human patients with various physiological insufficiencies and anomalies (Halken and Host, 1997). Several peptides with considerable biological roles have been identified in the enzymatic hydrolysates of whey, for example, opioid peptide (Meisel and FitzGerald, 2000), angiotensin I-converting enzyme-inhibitory peptide (Gobbetti et al., 2004), antithrombotic peptide (Chabance et al., 1995), immunomodulatory peptide (Mercier et al., 2004), anticarcinogenic peptide (Marshall, 2004), and mineral carrier peptide (Kim and Lim, 2004).

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Recently, lactoferricin, a peptide derived through peptic hydrolysis of whey, was shown to have iron-binding capacity (Wakabayashi et al., 2003). Such peptides can help in the prevention of anemia, a widespread nutritional deficiency particularly common in children and women (WHO, 2001). Whey protein has considerable binding ability for divalent and trivalent cations (Chaud et al., 2002), an attribute that could be used together with protein hydrolysis to improve iron bioavailability to anemic subjects (Kim et al., 2007).

Proteolytic enzymes extracted from the gastric gland (pepsin) of microbial origin (Alcalase) or from vegetable juices (papain) have been used for the hydrolysis of milk proteins (Kim et al., 2007). However, enzymes from different origins may vary in their capacity (specificity or hydrolytic action) to hydrolyze whey proteins, and thus may influence the physicochemical characteristics of the hydrolysates (Bertrand-Harb et al., 2002), their biological role (FitzGerald and Meisel, 1999), and the iron-binding ability of derived peptides. This study examined the influence of various enzymes (Alcalase, Flavourzyme, papain, and trypsin) on whey protein hydrolysis, with particular emphasis on reducing its antigenic fractions and quantifying the peptides having iron-binding ability in its hydrolysates.

MATERIALS AND METHODS

Whey Protein, Enzymes, and Reagents

The whey protein concentrate (WPC) used in this study was purchased from Hilmar Inc. (Hilmar 8000, Hilmar Inc., Hilmar, CA). A 2% (wt/vol) WPC solution on a protein-equivalent basis was prepared by dissolving 25 g of WPC (80% protein) in 1 L of deionized water. This WPC solution was defatted by ultracentrifugation at $12,000 \times g$ for 20 min (Supra 25K, Hanil Sci., Incheon, Korea). Dialysis tubing of 33×21 mm size (Sigma-Aldrich, St. Louis, MO) was used to demineralize the WPC solution. Alcalase [from *Bacillus globigii* (*Bacillus licheniformis*), activity 2.4 units/g of protein] and Flavourzyme (from *Aspergillus oryzae*, activity 500 units/g of protein) were procured from Sigma-Aldrich. Papain (from *Carica papaya* L., activity 400,000 units/g of protein) was purchased from Amano Enzymes Co. (Nagaya, Japan). Trypsin (from bovine pancreas, activity 3.3 Anson units/g of protein) was purchased from Novo Nordisk (Bagsvaerd, Denmark). Analytical grade BSA, trinitrobenzenesulfonic acid, trifluoroacetic acid (TFA; HPLC grade), and all other reagents were purchased from Sigma-Aldrich.

Preparation of Hydrolysates and Degree of Hydrolysis

The WPC hydrolysates were prepared by thermal (Guo et al., 1995) and enzymatic (Adamson and Reyn-

olds, 1996) hydrolysis. The pH of WPC solutions were initially adjusted to 8 using 0.5 M NaOH to avoid any coagulation during thermal hydrolysis of protein, and the solution was then heated for 10 min at 100°C. To study the enzymatic hydrolysis of protein by each enzyme, 100 mL of WPC solution was used after heat treatment. The pH of the each WPC solution was adjusted to 8 by using 0.5 M NaOH for all enzymes. Enzymes were dissolved in deionized water to prepare a 1% solution on a protein-equivalent basis. Enzyme solutions were added to the WPC reaction mixture at the ratio of 1:50 (enzyme:substrate, vol/vol, protein basis). The pH of each reaction mixture was maintained at a constant level, as described earlier, by using a pH-stat technique (Metrohm Ltd., Herisau, Switzerland). The temperature was maintained at 50°C throughout protein hydrolysis. During hydrolysis, samples were withdrawn after 30, 60, 90, 120, 150, 180, and 240 min and the enzymes were inactivated by heating the reaction mixture for 10 min at 90°C. The supernatants were taken as WPC hydrolysates and the precipitates were discarded. The WPC hydrolysates were stored at -20°C for subsequent estimation of degree of hydrolysis (DH), peptide identification, and iron-binding capacity. The DH of WPC by various enzymes for the different incubation times were determined according to Adler-Nissen (1979), and the NPN concentrations in WPC hydrolysates were estimated by using the method of Lowry et al. (1951).

SDS-PAGE

Sodium dodecyl sulfate-PAGE was used to estimate the DH of WPC incubated with various enzyme combinations for different time durations and their α -LA, β -LG, and BSA concentrations, as described by Laemmli (1970). The separating gel 14% (wt/vol) acrylamide, having pH 8.8, and the stacking gel 3% (wt/vol) acrylamide, with pH 6.8, were used. Gels were stained with 0.2% (wt/vol) Coomassie Brilliant Blue R-250 (Sigma Co., St. Louis, MO) in an acetic acid:methanol:H₂O solution (1:1:5, by vol) and were destained in an acetic acid:methanol:H₂O solution (1:3:6, by vol).

Reversed-Phase HPLC

Protein-peptides in enzymatic hydrolysates and fraction 1 (F-1; eluted fraction is the peak for the 0.25 M NaCl chromatographic step gradient) of Alcalase hydrolysates were analyzed by reversed-phase HPLC (RP-HPLC) on a Zorbax 300SB (Agilent Technologies Inc., Palo Alto, CA) C₁₈ column (4.6×250 mm) equilibrated with solvent A (0.1% TFA in H₂O) and eluted with a linear gradient of solvent B (0.1% TFA in acetoni-

Figure 1. Mean ($\pm$ SD) hydrolysis of heated whey protein concentrate (WPC) by different enzymes. Heated (for 10 min at 100°C) WPC (2% protein solution) was incubated at 50°C for 30, 60, 90, 120, 150, 180, and 240 min with 2% (on a protein-equivalent basis) Alcalase (♦), Flavourzyme (■), trypsin (▲), and papain (●)

trile) for 40 min. Runs were conducted at room temperature on an HPLC system (Agilent 1100, Agilent Technologies) at a flow rate of 1 mL/min, and the absorbance of the column eluate was monitored at 214 nm. The injection volume was generally 10 μ L and the concentration of protein material applied was approximately equivalent to 0.5 mg/mL of protein. All samples were filtered through 0.2- μ m syringe filters prior to application to the C₁₈ column.

Determination of the Iron-Binding Ability of WPC and Its Hydrolysates

Suspensions of the WPC and of its hydrolysates (supernatants) were used to prepare 1% protein (wt/vol) solutions in deionized water, with pH adjusted to 5. In these solutions, the iron-binding capacity of the protein-peptides was determined after addition of 0.1% (wt/vol) FeSO₄ (Sigma-Aldrich) and incubation at 37°C for 1 h.

The iron solubility of WPC and its hydrolysates was determined (as an indicator of the iron-binding capacity of protein-peptides) by inductively coupled plasma (ICP) spectroscopy (ICPS 7510, Shimadzu, Kyoto, Japan). Iron solubility was expressed as a percentage of the total iron-ion contents, and was calculated by equation [1]:

$$[\text{iron solubility}]_{\%} = \frac{[\text{iron-ion}]_{\text{supernatant}}}{[\text{iron-ion}]_{\text{total}}} \times 100, \quad [1]$$

where $[\text{iron-ion}]_{\text{supernatant}}$ is the supernatant iron-ion concentration from the suspension and $[\text{iron-ion}]_{\text{total}}$ is the total iron-ion concentration of the suspension.

Separation and Ability of Iron-Binding Protein

Separation of the iron-binding peptide from the WPC hydrolysate was conducted by the method of Rose et al. (1969). Diethylaminoethylcellulose (DEAE; Whatman DE 52, Whatman, Brentford, Middlesex, UK) was equilibrated in 500 mL of 20 mM Tris-HCl buffer at pH 7.8. A slurry of equilibrated DEAE was packed in a glass column (20 $\times$ 2.5 cm). Enzymatic hydrolysates were dissolved in the same buffer (pH 7.8) and loaded onto the column, then eluted by a step gradient with the same buffer containing 0.25, 0.5, and 0.75 M NaCl. The flow rate was 3 mL/min, the fraction volume was 15 mL per tube, and elutions were monitored at 280 nm. The 40-mL injection volume contained approximately 200 mg of protein. Samples were filtered through 0.5- μ m syringe filters prior to application to the column.

Iron-binding abilities of peptides in all WPC hydrolysates were determined by ICP spectroscopy at a radio frequency power of 1.2 kW, coolant gas of 14 μ L/min, plasma gas of 1.2 μ L/min, and carrier gas of 0.7 μ L/min. The ICP standard iron (AnApex Co. Ltd., Daejeon, Korea), having a wavelength of 259.94 nm, was used. All samples were filtered through 0.2- μ m syringe filters prior to their application to the ICP instrument.

Figure 2. Mean ($\pm$ SD) NPN fractions in enzymatic hydrolysates of heated whey protein concentrate (WPC). Heated (for 10 min at 100°C) WPC (2% protein solution) was incubated at 50°C for 30, 60, 90, 120, 150, 180, and 240 min with 2% (on protein-equivalent basis) Alcalase (◆), Flavourzyme (■), trypsin (▲), and papain (●)

Figure 3. Sodium dodecyl sulfate-PAGE patterns of enzymatic hydrolysates of heated whey protein concentrate (WPC). Heated WPC (2% protein solution) was incubated at 50°C for 30, 60, 90, 120, 150, 180, and 240 min with 2% (on protein-equivalent basis) Alcalase (a), Flavourzyme (b), trypsin (c), and papain (d). A = standard broad-range marker (Bio-Rad, Hercules, CA): myosin (209 kDa), β -galactosidase (124 kDa), BSA (80 kDa), ovalbumin (49.1 kDa), carbonic anhydrase (34.8 kDa), soybean trypsin inhibitor (28.9 kDa), lysozyme (20.6 kDa), and aprotinin (7.1 kDa); B = WPC; C = heated WPC; lanes 1 to 7 = heated WPC hydrolysates produced at 30, 60, 90, 120, 150, 180, and 240 min of incubation, respectively, with different enzymes.

Figure 4. Reversed-phase HPLC chromatograms of enzymatic hydrolysates of whey protein concentrate (WPC). Heated WPC (2% protein solution) was incubated at 50°C for 240 min with 2% (on a protein-equivalent basis) Alcalase (A), Flavourzyme (B), trypsin (C), and papain (D). Hydrolysates were applied to the column. The columns were equilibrated with solvent A (0.1% trifluoroacetic acid in H₂O) and eluted with a linear gradient of solvent B (0.1% trifluoroacetic acid in acetonitrile). The flow rate was 1 mL/min, injection volume was 10 μ L, and detection was at 214 nm.

AA Analysis and Protein Determination

Amino acid analysis of the iron-binding protein-peptide fraction of the Alcalase hydrolysate was performed by the method of Moore et al. (1958). Iron-binding protein-peptides (exactly 1 mg of protein) were exhaustively dialyzed against distilled water and lyophilized, then hydrolyzed in 1 mL of 6 M HCl in evacuated tubes at 110°C for 24 h. After speed-vacuum concentration (Maxi-Dry Plus, Heto-Holten A/S, Allerød, Denmark), the sample was dissolved in 0.2 M sodium citrate loading buffer (pH 2.2), and then filtered through 0.2- μ m syringe filters. Amino acid determination was carried out on an AA analyzer (Biochem 20, Pharmacia, Uppsala, Sweden). Protein concentrations in the enzyme preparations, hydrolysates, and fractions were determined by the dye-binding method of Bradford (1976). Bovine serum albumin (Sigma-Aldrich) was used as the standard.

Ten replications were carried out at each step of the experiment. Data on whey hydrolysis, iron solubility in enzymatic hydrolysates, and iron contents of the Alcalase hydrolysate fractions are presented as mean $\pm$ SD.

RESULTS AND DISCUSSION

Protein Hydrolysis

Heated WPC rapidly degraded within 30 min of incubation with different enzymes, and its hydrolysis slowly increased thereafter (Figure 1). More extensive hydrolysis of WPC was observed with Alcalase and Flavourzyme than with trypsin and papain at all incubation times. Extensive hydrolysis of whey protein with Alcalase was demonstrated previously by Smyth and Fitzgerald (1998) and Kim et al. (2007) by SDS-PAGE and HPLC analysis, respectively. The higher hydrolysis of WPC with Alcalase and Flavourzyme may be attributed

Figure 5. Diethylaminoethylcellulose ion-exchange column chromatogram of heated whey protein concentrate hydrolysates derived from Alcalase (A), Flavourzyme (B), trypsin (C), and papain (D) treatments, respectively. Enzymatic hydrolysate dissolved in 20 mM Tris-HCl buffer (pH 7.8) was applied to the column. The column, packed with diethylaminoethylcellulose, was washed with the same buffer and then eluted with a step gradient of NaCl as indicated. The flow rate was 3 mL/min, fraction volume was 15 mL per tube, and elution was monitored at 280 nm.

to their broader specificities in cleaving various peptide bonds. The greater hydrolysis of WPC by Alcalase may be ascribed to its hydrolyzing ability on peptide bonds with adjacent aromatic AA residues (Kim et al., 2007). Alcalase has been used extensively to prepare soluble hydrolysates of soy protein (Fox, 1989) and fish protein (Rebeca et al., 1991). The highest hydrolysis of WPC was observed after 240 min of incubation with Alcalase (12.4%), followed by Flavourzyme (12.0%), trypsin (10.4%), and papain (8.53%). Alcalase contains several different proteinases, each with different specificities, because it is a relatively crude bacterial extract of *B. licheniformis* (Sukan and Andrews, 1982). This property of Alcalase resulted in a significantly higher DH of whey fractions compared with other enzymes.

The NPN contents of WPC hydrolysates followed the hydrolytic pattern of whey. The NPN concentrations in WPC hydrolysates were also increased within 30 min of incubation with various enzymes, and thereafter NPN steadily increased with increasing incubation times (Figure 2). The highest NPN concentration was noticed after 240 min of incubation with Alcalase, followed by Flavourzyme, trypsin and papain. Similar results were reported previously by Kim et al. (2007). During enzymatic hydrolysis, proteins are cleaved to smaller molecules, namely, smaller peptides and free AA. Nonprotein nitrogen in WPC represents the small peptides, AA, and soluble ammonia nitrogen (Leonil et al., 1997). The higher NPN concentrations of heated WPC with Alcalase and Flavourzyme indicated their extensive proteolytic activity to yield possibly low molecular weight peptides and AA. The rate of proteolysis depends on the conformation of proteins (Green and Neurath, 1954). Changes in the conformation, which alter the number of accessible peptide bonds, alter the rate of proteolysis (Kella and Kinsella, 1988). Heated WPC undergoes temperature-dependent thermodenaturation and conformational changes, resulting in the exposure of hydrophobic areas (Brunner, 1977). The DH achieved during a hydrolysis reaction is expected to be related to the activity and specificity of the enzyme. For example, the high DH attained during WPC hydrolysis with Flavourzyme may, in part, be attributed to the presence of high levels of aminopeptidase activity (Smyth and FitzGerald, 1998).

Antigenic Fractions

Electrophoretic patterns indicated that β -LG (the major antigenic whey fraction) in WPC was not affected by the heat treatment (Figure 3). Sodium dodecyl sulfate-PAGE revealed complete removal of β -LG, α -LA, and BSA in all enzymatic hydrolysates of WPC at 240 min of incubation. The major antigenic fraction in WPC de-

graded within 60 min of its incubation with Alcalase, Flavourzyme, and trypsin (Figure 3). Electrophoretic patterns of papain WPC hydrolysates indicated a few remaining traces of both β -LG and α -LA even after 240 min of incubation. The relation between antigenicity (the ability of a substance to bind to specific antibodies) and allergenicity (its ability to elicit an allergic reaction and an immune response) implies that the antigenic peptides of β -LG can also be allergenic. Although, in the current study, antigenic and allergenic responses of enzymatic hydrolysates of WPC were not estimated, previous studies (van Beresteijn et al., 1994; Kananen et al., 2000) have indicated a positive relation between β -LG concentration and whey antigenicity. Complete degradation of this fraction to low molecular weight peptides with Alcalase, Flavourzyme, or trypsin could lower the antigenicity and allergenicity of whey.

The chromatograms of enzymatic hydrolysates of heated WPC also indicated complete degradation of β -LG, α -LA, and BSA (Figure 4). Because β -LG and α -LA are thermolabile (McKenzie, 1971), heat processing might have altered only their hydrolytic characteristics in the current study. Heating whey at 90°C rapidly increased the hydrolysis of β -LG by cleavage of the sulfur-sulfur bond (Høst et al., 1990). Furthermore, β -LG (a dimer) dissociates to a monomer and undergoes a reversible conformational change above pH 7, causing the exposure of Trp and tyrosyl residues to solvent (Reddy et al., 1988). Many new peaks of shorter wavelengths appeared on the chromatograms of enzymatic hydrolysates of heated WPC, which is an indication of whey degradation to new peptides of shorter chain lengths. Heated WPC hydrolysates derived with Alcalase, Flavourzyme, and trypsin produced shorter peaks in greater numbers on the chromatograms compared with heated WPC hydrolysates derived with papain.

Separation of Iron-Binding Protein

Iron solubility in WPC hydrolysates ranged from 81 to 90% and was higher than that noticed with WPC (Table 1). The highest iron solubility was observed in heated WPC hydrolysates derived with Alcalase (95%), followed by those produced with trypsin (90%), papain (87%), and Flavourzyme (81%). Eluted F-1 and fraction 2 (**F-2**) are the respective peaks for the 0.25 and 0.5 M NaCl chromatographic step gradient for analysis of enzymatic hydrolysates (Figure 5). Iron-binding ability was noticeably higher in F-1 compared with F-2 of all enzymatic hydrolysates of WPC (Figure 6). The highest iron contents in F-1 were observed in WPC hydrolysates derived with Alcalase (0.2 mg/kg), followed by hydrolysates derived with Flavourzyme (0.14 mg/kg), trypsin (0.14 mg/kg), and papain (0.08 mg/kg). Iron concentra-

Table 1. Iron solubility of whey protein concentrate (WPC) and its hydrolysates

Item	Iron content (mg/kg)		Iron solubility (%)
	Fraction 1	Fraction 2	
WPC	0.89 ± 0.02	0.62 ± 0.01	70 ± 0.3
WPC hydrolysate			
Alcalase	0.88 ± 0.03	0.84 ± 0.02	95 ± 0.5
Flavourzyme	0.86 ± 0.01	0.70 ± 0.03	81 ± 0.4
Trypsin	0.88 ± 0.02	0.79 ± 0.02	90 ± 0.2
Papain	0.87 ± 0.01	0.76 ± 0.01	87 ± 0.1

tions in F-2 of all enzymatic hydrolysates of WPC were low and ranged between 0.03 and 0.05 mg/kg. The F-1 may describe a new class of iron chelates based on the reaction of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ with a mixture of peptides obtained by the enzymatic hydrolysis of WPC. These results are similar to those of other researchers (Friedlander and Norman, 1980; Kim et al., 2007) who have reported separation of diatomic mineral-binding protein with a 0.22 to 0.25 *M* NaCl step gradient. The differences in the type of AA (Doucet et al., 2003), net charge, and length of peptides (Chaud et al., 2002) seemed to influence the extent of complex formation with iron in the present study. The exact mechanism of iron chelation by the Alcalase hydrolysate protein-peptides is not clear and is beyond the scope of this study. However, previous works have suggested that peptide iron chelation is mainly a function of the net charge on the AA (Reddy and Mahoney, 1995) and peptides (Chaud et al., 2002). Peptides have shown greater affinity to bind with iron at pH 5 (Chaud et al., 2002; Kim et al., 2007). Those authors explained that AA (except for L-Tyr) appear to form complexes with iron through carboxyl oxygen, al-

though the ϵ -amino nitrogen of Lys, the guanidino nitrogen of Arg, and the imidazole nitrogen of His may also have been involved in the iron ligand binding. However, α -amino nitrogen in the AA appeared to lack affinity for iron, and L-Tyr appeared to form complexes with iron through phenolate oxygen (Chaud et al., 2002). At neutral pH, L-Cys reduces iron(III) and complexes with iron(II) through sulfur bonds (Hamed and Silver, 1983). The F-1 fraction, which has shown greater affinity to bind with iron, was ultrafiltered through a 3-kDa molecular mass cutoff membrane by using a concentrator (Membrane 3 kDa MWCO PES, Sartorius, Edgewood, NY) to remove large peptides from the sample. Ultrafiltration was carried out to have small peptides of 3 kDa, the size below which physiological activity is evident. To identify the peptide pattern of the F-1 iron-enriched fraction derived from Alcalase hydrolysate, the RP-HPLC chromatogram was measured (Figure 7). This chromatogram of Alcalase F-1 indicated numerous small peaks of shorter wavelengths, which probably indicate a variety of new peptides with greater ability to bind with iron.

AA Composition

The AA profile of the WPC and the F-1 eluted by ion-exchange chromatography of the Alcalase hydrolysate are presented in Table 2. The WPC had the highest Glu (17.80%) concentration and the lowest amount of Cys (0.68%). Alcalase F-1 had higher Ala (18.38%), Lys (17.97%), and Phe (16.58%) concentrations, whereas the presence of Pro, Gly, and Tyr were not detected. Previously, Ward (1983) reported that the peptides on the carboxyl side of Phe, Tyr, and Trp were hydrolyzed with

Figure 6. Mean iron contents (mg/kg) of fraction 1 and fraction 2 of enzymatic hydrolysates of heated whey protein concentrate.

Figure 7. Reversed-phase HPLC chromatogram of fraction 1 of Alcalase hydrolysates (Figure 5). Heated whey protein concentrate (2% protein solution) was incubated at 50°C for 240 min with 2% (on a protein-equivalent basis) Alcalase. Fraction 1 was applied to the column. The columns were equilibrated with solvent A (0.1% trifluoroacetic acid in H₂O) and eluted with a linear gradient of solvent B (0.1% trifluoroacetic acid in acetonitrile). The flow rate was 1 mL/min, injection volume was 10 μ L, and detection was at 214 nm.

Alcalase (Doucet et al., 2003). Adamson and Reynolds (1996) explained that peptide bonds on the carboxyl side of Glu, Met, Leu, Tyr, Lys, and Gln residues were cleaved when Alcalase was used to prepare CN phosphopeptides. The specificity for the peptide bond adjacent to the sulfur-containing residue Met was also observed when the cleavage frequency of whey protein with Alcalase was evaluated by Doucet et al. (2003) and Kim et al. (2007). The current findings are similar to the reports of Pintado et al. (1999), Kim and Lim (2004), and Kim et al. (2007) on the AA compositions of enzymatic hydrolysates of whey. The AA composition of Alcalase hydrolysate F-1 also mimicked the N-terminal AA composition of iron-binding peptides derived previously through CN hydrolysis with Alcalase (Choi et al., 1998).

CONCLUSIONS

More extensive hydrolysis of WPC and higher NPN concentrations were observed with Alcalase and Flavourzyme compared with trypsin and papain. Sodium dodecyl sulfate-PAGE and RP-HPLC revealed complete degradation of β -LG, α -LA, and BSA within 60 min of its incubation with Alcalase, Flavourzyme, and trypsin. The highest iron solubility was noticed in hydrolysates derived with Alcalase (95%), followed by those produced with trypsin (90%), papain (87%), and Flavourzyme (81%). Iron-binding ability was higher in F-1 compared with F-2 of all enzymatic hydrolysates. The highest iron

Table 2. Amino acid composition of whey protein concentrate (WPC) and peptide fraction (F-1)¹ eluted from ion-exchange chromatography of Alcalase hydrolysate²

AA	WPC (%)	Alcalase F-1 (%)
Asp	7.55	4.13
Thr	8.49	2.27
Ser	6.53	2.45
Glu	17.80	6.81
Pro	8.27	ND ³
Gly	3.87	ND
Ala	9.07	18.38
Val	4.90	3.19
Cys	0.68	7.02
Met	1.66	2.56
Ile	4.93	3.23
Leu	10.36	7.18
Tyr	1.73	ND
Phe	2.81	16.58
Lys	8.29	17.97
His	1.42	5.88
Arg	1.63	2.35
Total molar ratio	100.00	100.00

¹Peptides less than 3 kDa.

²The WPC hydrolysate was produced by thermal denaturation of the WPC (100°C for 10 min), followed by treatment with Alcalase (1% on a protein basis) for 240 min.

³Not determined.

contents in F-1 were noticed in WPC hydrolysates derived with Alcalase (0.2 mg/kg). Alcalase F-1 indicated numerous small peaks of shorter wavelengths, which indicated a variety of new peptides with greater ability to bind with iron. Alcalase F-1 had higher Ala (18.38%), Lys (17.97%), and Phe (16.58%) concentrations, whereas the presence of Pro, Gly, and Tyr were not detected.

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