Peptic and Tryptic Hydrolysis of Native and Heated Whey Protein to Reduce Its Antigenicity

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ABSTRACT

This study examined the effects of enzymes on the production and antigenicity of native and heated whey protein concentrate (WPC) hydrolysates. Native and heated (10 min at 100°C) WPC (2% protein solution) were incubated at 50°C for 30, 60, 90, and 120 min with 0.1, 0.5, and 1% pepsin and then with 0.1, 0.5, and 1% trypsin on a protein-equivalent basis. A greater degree of hydrolysis was achieved and greater nonprotein nitrogen concentrations were obtained in heated WPC than in native WPC at all incubation times. Hydrolysis of WPC was increased with an increasing level of enzymes and higher incubation times. The highest hydrolysis (25.23%) was observed in heated WPC incubated with 1% pepsin and then with 1% trypsin for 120 min. High molecular weight bands, such as BSA, were completely eliminated from sodium dodecyl sulfate-PAGE of both native and heated WPC hydrolysates produced with pepsin for the 30-min incubation. The α -lactalbumin in native WPC was slightly degraded when incubated with 0.1% pepsin and then with 0.1% trypsin; however, it was almost completely hydrolyzed within 60 min of incubation with 0.5% pepsin and then with 0.5% trypsin. Incubation of native WPC with 1% pepsin and then with 1% trypsin for 30 min completely removed the BSA and α -lactalbumin. The β -lactoglobulin in native WPC was not affected by the pepsin and trypsin treatments. The β -lactoglobulin in heated WPC was partially hydrolyzed by the 0.1 and 0.5% pepsin and trypsin treatments and was completely degraded by the 1% pepsin and trypsin treatment. Antigenicity reversibly mimicked the hydrolysis of WPC and the removal of β -lactoglobulin from hydrolysates. Antigenicity in heated and native WPC was reduced with an increasing level of enzymes. A low antigenic response was observed in heated WPC compared with native WPC. The lowest antigenicity was

observed when heated WPC was incubated with 1% pepsin and then with 1% trypsin. These results suggested that incubation of heated WPC with 1% pepsin and then with 1% trypsin was the most effective for producing low-antigenic hydrolysates by WPC hydrolysis and obtaining low molecular weight small peptides. Further research is warranted to identify the low molecular weight small peptides in the WPC hydrolysates produced by pepsin and trypsin, which may enhance the use of whey.

Key words: hydrolysis, antigenicity, whey, pepsin and trypsin

INTRODUCTION

Milk protein represents an important source of AA and peptides in the human diet. In recent years, whey has gained immense recognition as a protein source in functional foods, infant formulas, and bakery products (van Beresteijn et al., 1994; Clemente, 2000). Whey protein hydrolysates are extensively being prepared and used for nutritional support of human patients with various physiological insufficiencies and anomalies (Halken and Host, 1997).

Native whey proteins are not fully hydrolyzed by digestive enzymes because of the presence of disulfide bonds and other chemical barriers in their structures (Schmidt et al., 1995), which possess antigenic activities for humans (Kananen et al., 2000). In particular, β -LG can induce a milk antigenic response in human infants because of their underdeveloped gastrointestinal tracts (Zeiger et al., 1986) and immune systems (Pintado et al., 1999). Allergic reactions to cow's milk are favored by rapid absorption of incompletely digested milk proteins. This partial digestion can be explained by the low acidity of the gastrointestinal tract during infancy (Kananen et al., 2000) along with the high buffering capacity of milk, and by the low exocrine pancreatic functioning (Nakamura et al., 1993). Symptoms such as gastrointestinal alterations, urticaria, angioedema, atopic dermatitis, allergic rhinitis, asthma, or chronic cough have been described (Heyman, 1999). Because an allergy to cow's milk develops

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in 3% of the pediatric population (Hudson, 1995), milk manufacturers have had to face the problem of reducing the antigenicity of whey protein.

Several treatments have been suggested to reduce the antigenicity of whey proteins, including physical removal of β -LG through UF (Olsen et al., 2003), its denaturing through heat treatment (O'Connell and Fox, 2001), biological conversion through bacterial fermentation (Sütas et al., 1996), and enzymatic hydrolysis (Ragno et al., 1993). However, physical, thermal, and fermentation techniques usually produce bitter peptides (Heyman, 1999), diminish the other protein and peptide functions, and are expensive (Kananen et al., 2000).

Enzymatic hydrolysis of whey protein offers a practical way to reduce its antigenicity (Heyman, 1999). Furthermore, it can yield a variety of new peptides that may offer many physiological benefits for humans (Otte et al., 1997). However, use of a single *endo* and *exo* proteolytic enzyme of animal, plant, or microbial origin has shown no promise in minimizing the antigenicity of whey proteins (Svenning et al., 2000). Thus, this study was conducted to test the hypothesis that thermal treatment, along with hydrolysis of whey proteins by pepsin and trypsin, may reduce their antigenicity. The objectives of this study were to investigate the hydrolytic properties of whey protein concentrate (WPC) and the antigenicity of its hydrolysates by using thermal and enzymatic treatments.

MATERIALS AND METHODS

Substrates and Enzymes

The WPC used in this study was purchased from Hilmar (Hilmar 8000, Hilmar, CA). A 25-g quantity of WPC (80% protein) was dissolved in 1 L of double-distilled water to produce a 2% WPC solution on a protein-equivalent basis. The whey solution was defatted by ultracentrifugation (Supra 25K, Hanil Sci., Seoul, Korea) and demineralized in distilled water by dialysis sacks (Sigma, St. Louis, MO). The WPC solution was heated for 10 min at 100°C. The native WPC solution without heat treatment was used in the experiment as a control. Pepsin (porcine gastric mucosa, activity 0.8 to 2.5 units/g of protein) and trypsin (bovine pancreas, activity 3.3 Anson units/g of protein) were purchased from Sigma and Novo Nordisk (Bagsværd, Denmark), respectively.

Preparation of WPC Hydrolysates with Pepsin and Trypsin

Enzymatic hydrolysis of WPC was carried out by the method of Adamson and Reynolds (1996). Native and

heated WPC solutions were adjusted to pH 2 by addition of 0.5 N HCl for peptic hydrolysis. The native and heated WPC solutions were incubated at 50°C for 30, 60, 90, or 120 min with 0.1, 0.5, or 1% pepsin on a protein-equivalent basis. After peptic hydrolysis, the pepsin was inactivated by heating (at 90°C for 10 min) in a water bath. The native and heated WPC were incubated for 120 min with each level of pepsin and then used for tryptic hydrolysis. The WPC solutions were adjusted to pH 8 with 0.5 N NaOH and incubated with 0.1, 0.5, or 1% trypsin for 30, 60, 90, or 120 min. In these solutions, the trypsin was inactivated by heating (at 90°C for 10 min) in a water bath. The hydrolyzed solutions were centrifuged, and their supernatants were harvested as hydrolysates and stored at -20°C for further analyses. During incubation, the pH of the WPC solution was maintained with a pH-stat (Metrohm Ltd., Herisau, Switzerland) at pH 2 and 8 for peptic and tryptic hydrolysis by continuous addition of 0.5 N HCl and 0.5 N NaOH, respectively.

Degree of Hydrolysis and Nonprotein Nitrogen Estimation

The degree of hydrolysis (DH) of native and heated WPC by peptic and tryptic enzymes for the different incubation times was determined according to Adler-Nissen (1979), and the NPN concentration in WPC hydrolysates was estimated by the method of Lowry et al. (1951).

SDS-PAGE

Sodium dodecyl sulfate-PAGE was used to estimate the hydrolysis of BSA, α -LA, and β -LG in hydrolysates of native and heated WPC according to the method described by Laemmli (1970). The separating gel, 14% (wt/vol) acrylamide with 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) in an acetic acid:methanol:H₂O solution (1:1:5, by vol) and destained in an acetic acid:methanol:H₂O solution (1:3:6, by vol).

ELISA

Antigenicity was determined in whey protein (Sigma) and WPC hydrolysates by competitive ELISA (Schmidt et al., 1995). The microtiter plates were used as a solid support. Single wells of ELISA plates were coated with 100 μ L of whey protein (1 mg/mL in 0.05 mol of sodium bicarbonate buffer, pH 9.6). In 1.5-mL glass vials, 10⁻³, 10⁻², 10⁻¹, 10⁰, 10¹, 10², and 10³ μ g/mL of whey protein hydrolysates in PBS (0.15 mol of

Figure 1. Mean ($\pm$ SD) hydrolysis of native and heated whey protein concentrate (WPC). Native and heated (10 min at 100°C) WPC (2% protein solution) were incubated at 50°C for 30, 60, 90, and 120 min with 0.1, 0.5, and 1% of pepsin and then trypsin on a protein-equivalent basis. Legend: native WPC 0.1% (◆), native WPC 0.5% (■), native WPC 1% (▲), heated WPC 0.1% (◇), heated WPC 0.5% (□), and heated WPC 1% (△).

NaCl, 0.01 mol of phosphate buffer, pH 7.4) containing 0.05% Tween 20 (PBST) were incubated overnight at 4°C with a constant amount of antiwhey (antibodies against bovine whey proteins, rabbit, 1:1,000 in 0.05 mol of PBS; Sigma). Residual free binding sites were blocked with 250 μ L/well of 0.5% gelatin in PBST for 2 h at 37°C. After removing the solutions, the wells were washed 3 times with PBST. Plates were incubated at 37°C for 2 h. After washing, the antiwhey that reacted with the plate-bound antigens was determined with peroxidase-conjugated goat antirabbit IgG (1:1,000 in 0.05 M PBS; Sigma). A 100- μ L solution of *o*-phenylene-diamine dihydrochloride [FAST OPD peroxidase substrate tablet set (Sigma) in deionized water] was added to each well. After 30 min, the reaction was stopped by adding 100- μ L solutions of 2 N H₂SO₄. Absorption was measured at 490 nm on an ELISA plate reader (MR700 Microplate Reader, Dynatech Laboratories Inc., Alexandria, VA). Ten replications were carried out at each step of the experiment. Data on WPC hydrolysis, NPN contents, and antigenicity are presented as means $\pm$ standard deviations.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis of WPC

The DH of native and heated WPC with pepsin and trypsin enzymes is shown in Figure 1. Hydrolysis of

both native and heated WPC with pepsin was small in magnitude. The DH of WPC ranged from 4.71 to 25.23%, depending on the enzyme concentration and whether the substrate was heated or native. The WPC hydrolyzed slowly for 120 min of incubation with pepsin. Hydrolysis increased rapidly thereafter during 30 min of incubation with trypsin and then increased slowly with incubation time (Figure 1). Hydrolysis was greater in heated WPC than in native WPC at all incubation times. This result is in agreement with Mullally et al. (1997), who reported that the rate of hydrolysis in heated whey proteins was significantly higher compared with their native forms. Hydrolysis of WPC was increased with increasing levels of pepsin and trypsin and with increasing incubation time (Figure 1). The maximum hydrolysis (25.23%) was observed in heated WPC when it was incubated with 1% pepsin and then with 1% trypsin (Figure 1). The rate of proteolysis is known to depend on the conformation of proteins (Green and Neurath, 1954). Changes in 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 exposure of the hydrophobic areas (Brunner, 1977). Trypsin has specificity for bonds associated with the hydrophobic side chains of Phe, Tyr, and Trp at pH

Figure 2. Mean ($\pm$ SD) nonprotein nitrogen in enzymatic hydrolysates of native and heated whey protein concentrate (WPC). Native and heated (10 min at 100°C) WPC (2% protein solution) were incubated at 50°C for 30, 60, 90, and 120 min with 0.1, 0.5, and 1% of pepsin and then trypsin on a protein-equivalent basis. Legend: native WPC 0.1% (◆), native WPC 0.5% (■), native WPC 1% (▲), heated WPC 0.1% (◇), heated WPC 0.5% (□), and heated WPC 1% (△).

8. These results indicate that heated WPC underwent extensive conformational changes at pH 8, resulting in the exposure of hydrophobic groups to the solvent, thus allowing access of the enzyme to at least a few strategic peptide bonds. The specificity of Trp for Arg and Lys was also established because of its deep primary binding site at the bottom of a narrow pocket occupied by a negatively charged Asp at position 189. These cleavages may then induce further structural changes, eventually allowing cleavage at many points (Mullally et al., 1997). This explains the higher rate of tryptic proteolysis compared with peptic hydrolysis at all incubation times

Mean NPN concentrations in native and heated WPC after enzymatic hydrolysis are presented in Figure 2. The NPN concentrations in native and heated WPC increased rapidly during the first 60 min of incubation with pepsin, and thereafter, the concentrations increased steadily with increasing incubation time. A higher NPN concentration was observed in heated WPC compared with native WPC when incubated with pepsin. The NPN concentrations in both heated and native solution increased rapidly when incubated with trypsin for 30 min, and thereafter, the concentrations increased with increasing incubation time. A higher

NPN concentration was observed in WPC solution incubated with a higher level of pepsin and trypsin. The highest NPN concentration was observed in heated WPC when incubated with 1% pepsin and then with 1% trypsin. The NPN in WPC represents small peptides, AA, and soluble ammonia nitrogen (Leonil et al., 1997). The higher NPN concentration of heated WPC incubated with 1% pepsin and then with 1% trypsin indicated higher proteolytic activity, possibly yielding low molecular weight peptides and AA.

SDS-PAGE

The electrophoretic patterns of heated and native WPC hydrolysates produced with pepsin and trypsin enzymes at various incubation times are shown in Figures 3 and 4, respectively. When native WPC was hydrolyzed with pepsin, the high molecular weight bands, such as those of BSA (66 KDa), were no longer visible after 30 min of incubation (Figure 3A). The α -LA (14.4 KDa) in native WPC was slightly degraded when incubated with 0.1% pepsin and then with 0.1% trypsin (Figure 3A); however, it was almost completely hydrolyzed within 60 min of incubation with 0.5% pepsin and then with 0.5% trypsin (Figure 3B). Incubation

Figure 3. Sodium dodecyl sulfate-PAGE patterns of hydrolysates of native whey protein concentrate (WPC) produced by 0.1% (A), 0.5% (B) and 1% (C) pepsin and trypsin treatments. Native WPC (2% protein solution) was incubated at 50°C for 30, 60, 90, and 120 min with 0.1, 0.5, and 1% pepsin and then trypsin on a protein-equivalent basis. Lane A: standard broad-range marker (Bio-Rad, Hercules, CA): phosphorylase b (97.4 KDa), BSA (66.2 KDa), ovalbumin (45 KDa), carbonic anhydrase (31 KDa), trypsin inhibitor (21.5 KDa), and lysozyme (14.4 KDa). Lane B: native WPC. Lanes 1 to 4: native WPC hydrolysates produced by incubation with pepsin for 30, 60, 90, and 120 min, respectively. Lanes 5 to 6: native WPC hydrolysates produced by incubation with trypsin for 30, 60, 90, and 120 min, respectively.

of native WPC with 1% pepsin and then with 1% trypsin for 30 min completely removed the BSA and α -LA (Figure 3C). The β -LG in native WPC was not affected by the 0.1 and 0.5% enzyme concentrations and showed slight degradation when incubated with 1% pepsin and trypsin. These results indicate that native β -LG is quite resistant to proteolysis by pepsin at pH 2 (Figure 3). Kella and Kinsella (1988) suggested that acid stability of β -LG could result from the increased internal hydrogen bonding that arises either between 2 titrated carboxyl groups or between 1 amide and 1 carboxyl group. Thus, the resistance of β -LG to peptic degradability in native WPC hydrolysates may reflect its stable conformation at pH 2. Pepsin has specificity for Trp, Tyr, Phe, Leu, and Ile (Schmidt and van Markwijk, 1993). The resistance of native β -LG to peptic digestibility indicates that these groups were not ac-

Figure 4. Sodium dodecyl sulfate-PAGE patterns of hydrolysates of heated whey protein concentrate (WPC) produced by 0.1% (A), 0.5% (B), and 1% (C) pepsin and trypsin treatments. Heated WPC (2% protein solution) was incubated at 50°C for 30, 60, 90, and 120 min with 0.1, 0.5, and 1% of pepsin and then trypsin on a protein-equivalent basis. Lane A: standard broad-range marker (Bio-Rad, Hercules, CA): phosphorylase b (97.4 KDa), BSA (66.2 KDa), ovalbumin (45 KDa), carbonic anhydrase (31 KDa), trypsin inhibitor (21.5 KDa), and lysozyme (14.4 KDa). Lane B: heated WPC. Lanes 1 to 4: heated WPC hydrolysates produced by incubation with pepsin for 30, 60, 90, and 120 min, respectively. Lanes 5 to 6: heated WPC hydrolysates produced by incubation with trypsin for 30, 60, 90, and 120 min, respectively.

cessible to the enzyme. Intermolecular S-S bonds maintain the structural integrity and increase the stability of whey proteins (Cantor and Schimmel, 1980). Reduction of these bonds destabilizes the conformation of β -LG. The BSA and α -LA contents of heated WPC were extensively degraded within 30 min of incubation with 0.1% pepsin (Figure 4). The β -LG in heated WPC was partially hydrolyzed by the 0.5 and 1% pepsin treatments. The 0.1% pepsin and trypsin treatment did not affect the β -LG content of heated WPC. The β -LG in heated WPC was partially hydrolyzed by the 0.5% pepsin and trypsin treatment and completely degraded by the 1% pepsin and trypsin treatment. Because β -LG and α -LA are thermolabile (McKenzie, 1971), heat processing may have altered their hydrolytic characteristics. Heating whey at 90°C rapidly increased the hydrolysis of β -LG by cleavage of the S-S

Figure 5. Mean ($\pm$ SD) inhibition rate of binding between native and heated whey protein concentrate (WPC) hydrolysates and rabbit antbovine whey protein antiserum. Native and heated (10 min at 100°C) WPC (2% protein solution) were incubated at 50°C for 30, 60, 90, and 120 min with 0.1, 0.5, and 1% of pepsin and then trypsin on a protein-equivalent basis. Legend: whey protein (●), native WPC 0.1% (◆), native WPC 0.5% (■), native WPC 1% (▲), heated WPC 0.1% (◇), heated WPC 0.5% (□), and heated WPC 1% (△).

bonds (Høst et al., 1990). The present findings indicate that β -LG in heated WPC underwent substantial pH-induced conformational changes at pH 2 and then at pH 8. It may be suggested, on the basis of earlier reports, that β -LG (dimer) dissociates to a monomer and undergoes a reversible conformational change above pH 7, causing the Trp and tyrosyl residues to be exposed to the solvent (Reddy et al., 1988). β -Lactoglobulin contains 4 Trp groups per dimer. Two of them are "buried" and the other 2 are "exposed," or 4 of them are partly "buried" (McKenzie, 1971). Complete removal of β -LG from heated WPC with the pepsin and trypsin treatment may be attributed to dissociation of the dimer to a monomer at a higher pH 8 (McKenzie, 1971), followed by S-H oxidation and S-S interchange (Reddy et al., 1988).

Antigenicity of WPC Hydrolysates

The antigenicity of WPC hydrolysates harvested at 120 min of incubation with pepsin and trypsin were measured with rabbit antbovine whey antibody by

indirect ELISA inhibition (Figure 5). Antigenicity reversibly mimicked the hydrolytic patterns of WPC and removal of β -LG from its hydrolysates. Antigenicity in heated and native WPC was reduced with increasing levels of enzymes. A low antigenic response was observed in heated WPC compared with native WPC at all levels of enzymes. The lowest antigenicity was observed when heated WPC was incubated with 1% pepsin and then with 1% trypsin. The antigenic response in infants consuming formulas based on proteins from cow's milk has been attributed mainly to their β -LG and α -LA contents (Svenning et al., 2000). In the present study, the reduction in antigenic response of the heated WPC hydrolysates obtained by the pepsin and trypsin treatment seemed to correspond with the reduction of their β -LG and α -LA contents. This reduction may be ascribed to the splitting of the epitope sequence because of enzymatic hydrolysis (Lieske and Konrad, 1996). Ena et al. (1995) reported, consistent with the present findings, that the antigenicity of whey protein was also slightly reduced when it was incubated with fungal proteinases and pancreatic extracts.

The specificity of the enzymes used possibly influenced the degradation of epitopic areas, which are responsible for the immunological reactions (Svenning et al., 2000). These authors explained that neither the DH nor the molecular mass distribution of hydrolysates reflected the degree of reduced antigenicity of individual whey proteins. However, in the present study, as the DH of WPC increased, its antigenicity was reduced. The results of the present experiment indicate that hydrolysates obtained with heated WPC by the combined treatment of pepsin and trypsin could lower its antigenicity better than those achieved in previous experiments (Asselin et al., 1989; Ena et al., 1995; Svenning et al., 2000) with single proteolytic enzymes (trypsin, pepsin, or chymotrypsin). The present results are in contrast with the findings of Ena et al. (1995), who reported a partial reduction in the antigenicity of whey with enzymes (pepsin and corolase-7092) and suggested a UF step for the complete removal of α -LA and β -LG from whey before it is used in infant formulas.

CONCLUSIONS

Hydrolysis by pepsin and trypsin was greater in heated WPC than in native WPC. Hydrolysis in heated WPC was increased with an increasing level of pepsin and trypsin. Antigenicity reversibly mimicked the hydrolysis of WPC and removal of β -LG from its hydrolysates. Treatment with 1% pepsin and then 1% trypsin completely removed BSA, α -LA, and β -LG from the hydrolysates of WPC. Complete removal of α -LA and β -LG from the WPC hydrolysates possibly reduced their antigenicity. Further research is warranted to identify the low molecular weight small peptides in WPC hydrolysates produced by pepsin and trypsin, which may help enhance the value and use of whey in human foods.

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