In Vitro Generation and Stability of the Lactokinin β -Lactoglobulin Fragment (142-148)

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ABSTRACT

The objectives of this study were to investigate the generation of β -lactoglobulin fragment (142-148) (β -LG f(142-148)) during the hydrolysis of whey proteins, and the in vitro stability of this fragment upon incubation with gastrointestinal and serum proteinases and peptidases. An enzyme immunoassay (EIA) protocol was developed for the quantification of β -LG f(142-148) in whey protein hydrolysates and in human blood serum. The minimum detection limit was 3 ng/mL. The level of the peptide in whey protein hydrolysates was influenced by the degree of hydrolysis (DH). As expected, highest levels of this peptide were found in hydrolysates generated with trypsin. Sequential incubation of hydrolysates at different DH values with pepsin and Corolase PP, to simulate gastrointestinal digestion, generally resulted in the degradation of β -LG f(142-148) as determined by EIA. Reversed-phase HPLC and angiotensin-I-converting enzyme (ACE) activity assays demonstrated that synthetic β -LG f(142-148) was rapidly degraded upon incubation with human serum. Furthermore, β -LG f(142-148) could not be detected by EIA in the sera of 2 human volunteers following its oral ingestion or in sera from these volunteers subsequently spiked with β -LG f(142-148). These in vitro results indicate that β -LG f(142-148) is probably not sufficiently stable to gastrointestinal and serum proteinases and peptidases to act as an hypotensive agent in humans following oral ingestion. The in vitro methodology described herein has general application in evaluating the hypotensive potential of food protein-derived ACE inhibitory peptides.

(Key words: lactokinin, whey protein, angiotensin-I-converting enzyme, hypertension)

Abbreviation key: ACE = angiotensin-I-converting enzyme, B/B₀ (%) = bound activity in the presence or absence of competitor, respectively, β -LG = β -lactoglobulin, BLGWP = β -lactoglobulin-enriched whey protein fraction, β -LG f(142-148) = peptide fragment corresponding to residues 142 to 148 of bovine β -lactoglobulin, DH = degree of hydrolysis, EIA = enzyme immunoassay, FAPGG = furanacryloyl-L-phenylalanylglycylglycine, IC₅₀ = concentration of inhibitory substance which reduces ACE activity by 50%, SGID = simulated gastrointestinal digestion, TPCK = L-1-tosylamide-2-phenylethyl chloromethyl ketone, WPC75 = whey protein concentrate 75.

INTRODUCTION

Angiotensin-I-converting enzyme (ACE) is a critical enzyme in the regulation of peripheral blood pressure. Angiotensin-I-converting enzyme, a dipeptide-liberating carboxypeptidase (peptidyl dipeptide hydrolase, EC 3.4.15.1), converts angiotensin I to angiotensin II, a highly potent vasoconstrictor molecule (Skeggs et al., 1956). However, ACE interacts with many different endogenous competitive inhibitors and substrates such as substance P, enkephalins, β -endorphin, and the vasodilatory peptide, bradykinin (Wyvratt and Patchett, 1985; FitzGerald et al., 2004). Inhibition of ACE exerts an antihypertensive effect by decreasing the level of angiotensin II and by maintaining a high level of the vasodilatory peptide, bradykinin (Erdös, 1975). Exogenous inhibitors of ACE displaying an antihypertensive effect were initially reported from snake venom (Ondetti et al., 1977). Several orally active pharmacological compounds, such as Captopril, were subsequently developed which exhibited potent hypotensive effects in vivo (Koike et al., 1980). Many food proteins such as fish (Ariyoshi, 1993), gelatin (Oshima et al., 1979), and milk (FitzGerald and Meisel, 2000; Meisel et al., 2004; Walsh and FitzGerald, 2004) are a source of ACE-inhibitory peptides. Milk protein-derived ACE inhibitory peptides

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are latent or encrypted within the primary sequences of the proteins (Meisel, 1998). Proteolysis of milk proteins (as occurs during processing with commercial enzyme preparations) may be used to generate a mixture of ACE inhibitory peptides in vitro (FitzGerald and Meisel, 1999; Pihlanto-Leppälä, 2001; Walsh and FitzGerald, 2004). Angiotensin-I-converting enzyme inhibitory peptides may also be produced during the generation of fermented dairy products (Takano, 1998; Gobbetti et al., 2002; Seppo et al., 2003). Human studies have linked the oral ingestion of fermented dairy products containing the potent casein-derived ACE inhibitory peptides, Ile-Pro-Pro and Val-Pro-Pro, with a hypotensive effect (Hata et al., 1996; Seppo et al., 2003). Ile-Pro-Pro and Val-Pro-Pro were found in the aortal fractions of spontaneously hypertensive rats following ingestion of Calpis sour milk (Masuda et al., 1996). These results demonstrate that Ile-Pro-Pro and Val-Pro-Pro were able to cross the intestinal barrier and reach their target organ. However, the general fate of in vitro produced ACE inhibitory peptides following oral ingestion does not appear to have been extensively studied. Following oral ingestion, biologically active peptides may be susceptible to further degradation by gastrointestinal, brush-border, and serum proteinases and peptidases (FitzGerald and Meisel, 2003). For example, α_{S1} -casein f(23-27) is a potent ACE inhibitory peptide in vitro, however, it exhibited no hypotensive effect in spontaneously hypertensive rats, presumably due to further degradation to inactive fragments following oral ingestion (Maruyama et al., 1987). The appearance of Ile-Pro-Pro and Val-Pro-Pro in aortal fractions may be linked to the fact that peptides containing proline residues are thought to be resistant to further hydrolysis (Kim et al., 1972; Yoshimoto et al., 1978).

Mullally et al. (1997a) reported that the tryptic peptide Ala-Leu-Pro-Met-His-Ile-Arg, corresponding to residues 142 to 148 of bovine β -lactoglobulin (β -LG), i.e., β -LG f(142-148), was a potent inhibitor of ACE activity in vitro. However, the potential of this peptide to act as a hypotensive agent has not been determined. The objectives of this study were: 1) to develop an enzyme immunoassay (EIA) protocol to quantify β -LG f(142-148); 2) to quantify the release of β -LG f(142-148) during hydrolysis of a β -lactoglobulin-enriched whey protein fraction (BLGWP) and whey protein concentrate 75 (WPC75) with different commercially available proteinase preparations; 3) to determine the stability of β -LG f(142-148) in hydrolysates to further digestion by gastrointestinal and serum proteinases/peptidases; and 4) to determine if β -LG f(142-148) can be detected in the serum of normotensive human volunteers following oral administration of β -LG f(142-148).

MATERIALS AND METHODS

Whey protein concentrate 75 (74.0% wt/wt protein) was obtained from a commercial supplier. The concentrate contained 0.59% Ca, 0.45% K, 0.45% P, 0.04% Cl^- , 0.057% Mg, and 0.22% Na (all on a wt/wt basis). A bovine whey protein fraction (88.7% protein wt/wt) enriched in β -lactoglobulin (BLGWP) was obtained from Glanbia Ingredients Ltd., (Ballyragget, Ireland). Proteinases received as gifts from manufacturers were: Protamex (a *Bacillus* proteinase complex) from Novo Nordisk A/S (Bagsvaerd, Denmark), Corolase PP (a porcine pancreatic enzyme preparation), Corolase 7089 (a *Bacillus subtilis* endoproteinase preparation), and Corolase PNL (an *Aspergillus sojae* preparation containing endo- and exopeptidase activities) from Röhm GmbH (Darmstadt, Germany). Porcine L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (13,700 N- α -benzoyl-L-arginine ethyl ester units/mg protein), ACE (rabbit lung acetone powder), EDTA, and N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) were obtained from Sigma-Aldrich, (Dublin, Ireland). Porcine pepsin (3200 to 4500 units/mg of protein) was obtained from Fluka (Sigma-Aldrich). All other chemicals were of analytical grade.

The β -LG f(142-148) peptide used for oral ingestion studies was synthesized by solid-phase peptide chemistry and Fmoc protection by Primm Peptidi (Milan, Italy). Smaller quantities of this peptide were synthesized using a similar protocol at the Institute Nationale de la Recherche Agronomique (Gif Sur Yvette, France) for immunochemical studies. Both peptide preparations were shown to be greater than 99% pure by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry. A single peak with $m/z = 838.7$ was observed, corresponding to the expected mass of the fragment.

Generation of Whey Protein Hydrolysates

Solutions (500 mL) of BLGWP and WPC75 (8.0% protein wt/wt) were allowed to hydrate while stirring at room temperature for at least 1 h before adjusting to pH 7.0 with 1 N HCl/NaOH. The enzyme-substrate ratio used varied for each enzyme-substrate combination (Table 1). During hydrolysis at 50°C, a constant pH was maintained by addition of 2 M NaOH using a pH-stat (Metrohm Ltd., Herisau, Switzerland). The degree of hydrolysis (DH, %), was calculated from the volume and molarity of NaOH used to maintain constant pH (Adler-Nissen, 1986). Hydrolysis reactions were allowed to proceed until no further increase in DH was evident. Throughout the duration of the hydrolysis reaction, samples were removed at defined DH values.

Table 1. Enzyme to substrate ratio, and final degree of hydrolysis (DH%) reached during the hydrolysis of a β -lactoglobulin-enriched whey protein fraction (BLGWP) and whey protein concentrate (WPC75) with a range of commercially available proteinase preparations.

Proteinase	BLGWP		WPC75	
	E:S ¹ (%)	DH ² (%)	E:S (%)	DH (%)
Corolase PNL	2.00	10.0	5.00	15.0
Corolase PP	0.70	16.1	2.00	22.0
Corolase 7089	1.20	10.0	ND ³	ND
Protamex	1.50	10.0	ND	ND
TPCK-trypsin ⁴	0.08	6.7	0.50	8.0

¹E:S = Enzyme to substrate ratio.

²DH = Maximum degree of hydrolysis reached when the substrate (8.0% protein) was hydrolyzed with the stated enzyme preparation at a specific E:S ratio at pH 7.0 and 50°C.

³ND = Not determined.

⁴TPCK = Tosyl-L-phenylalanyl-chloromethane.

To avoid gelation, WPC75 hydrolysates were diluted to 4.0% (wt/wt) protein before heat inactivation. Samples were heated at 80°C for 20 min to inactivate proteolytic and peptidolytic activity. Hydrolysates were cooled and stored at -20°C. As an enzyme control, samples of distilled water (500 mL) containing the different enzyme preparations were subjected to an identical heat treatment regimen as hydrolysate samples. All glassware was acid-washed before use.

Simulated Gastrointestinal Digestion

Hydrolysate samples were subjected to a 2-stage simulated gastrointestinal digestion (SGID) process. Hydrolysates were diluted to 2.0% (wt/wt) protein and the pH reduced to 2.0 using 1 N HCl. Following preincubation (37°C, 30 min), pepsin (1:40 wt/wt enzyme:substrate) was added to 20 mL of gently stirring hydrolysate and the reaction was incubated at 37°C. After 90 min, the pH was adjusted to 7.5 by adding 20 mL of 0.4 M Na₂HPO₄/NaH₂PO₄ buffer, pH 7.5. Corolase PP (1:100 wt/wt, enzyme:substrate) was then added and the sample was further incubated at 37°C while stirring. After 150 min, the hydrolysate was heated at 80°C for 20 min to terminate enzyme activity, cooled, and then stored at -20°C. Control hydrolysate samples without pepsin and Corolase PP (i.e., nonSGID) were subjected to identical treatments as test samples.

Protein Determination

Protein content was determined using a macro-Kjeldahl procedure (IDF, 1993).

Quantification of ACE Inhibitory Activity in Hydrolysates

Angiotensin-I-converting enzyme inhibitory activity was quantified using FAPGG as substrate (Murray et al., 2004). Inhibitory index values were obtained for hydrolysate samples by incorporating 50 μ L of a 7 mg/mL solution of hydrolysate in the assay (175 U/L ACE, 0.8 mM FAPGG) and quantifying the percentage inhibition of ACE vs. a control ACE assay without hydrolysate. The inhibitory potency (IC₅₀) of hydrolysates was determined from plots of percentage inhibition vs. log₁₀ [hydrolysate] (mg/L). Angiotensin-I-converting enzyme IC₅₀ values, that is, the amount of hydrolysate that mediated a 50% inhibition of ACE activity, were determined for selected hydrolysates. Captopril was used as a positive control inhibitory substance. The inhibitory potency (IC₅₀) of synthetic β -LG f(142-148) was determined to be 46.7 μ M. Triplicate analysis of independent duplicates was performed in all cases. Inhibition values quoted are means $\pm$ standard deviation.

Development of Enzyme Immunoassay for β -LG f(142-148)

Immunogen preparation. Synthetic β -LG f(142-148) was coupled to keyhole limpet hemocyanin via its amino groups using glutaraldehyde. Briefly, peptide (5 mg) and glutaraldehyde (12 μ L) were successively added to keyhole limpet hemocyanin (20 mg) dissolved in 6.6 mL of 0.1 M phosphate buffer, pH 7.4. After stirring for 18 h at 4°C, the mixture was divided into 1-mL aliquots and stored at -20°C.

Immunization. Anti- β -LG f(142-148) antisera was obtained by immunizing white 'Blanc-de Bouscat' adult male rabbits as follows: 2 mL of diluted immunogen (1/4 dilution) in water was emulsified with 2 mL of Freund's complete adjuvant and injected subcutaneously into 3 rabbits. Booster injections were given 6 wk later and every month thereafter. Rabbits were bled 10 d after each immunization. Sera were stored at 4°C after addition of sodium azide (0.01% wt/vol final). The experiments with rabbits were conducted in a manner that avoided unnecessary discomfort by following proper management and laboratory techniques.

Enzymatic tracer preparation. Enzymatic tracer was prepared by covalent linkage of peptide to the tetrameric form of acetylcholinesterase. This involved the reaction of thiol groups introduced into the peptide by N-succinimidyl-S-acetyl-thioacetate with maleimido groups previously incorporated into acetylcholinesterase by N-succinimidyl-4-(maleido-methyl)-cyclohexane-1-carboxylate, as previously described (Caruelle et al., 1988).

Competitive EIA. All dilutions were performed in EIA buffer consisting of 0.1 M potassium phosphate pH 7.4, containing 0.15 M NaCl, 0.1% (wt/vol) BSA, and 0.01% (wt/vol) sodium azide. Competitive assays were performed using 96-well microtiter plates coated with mouse monoclonal anti-rabbit IgG (10 μ g/mL in 0.05 M phosphate buffer pH 7.4 for 18 h at 4°C before saturating with EIA buffer), to ensure separation of bound and free moieties of the enzymatic tracer during the immunological reaction. The total reaction volume was 150 μ L, each component (enzymatic tracer, rabbit polyclonal antisera, and standard or sample) being added in 50- μ L aliquots. The enzymatic tracers were used at a concentration of 2 Ellman units/mL (Ellman et al., 1961), and the working dilutions for the corresponding rabbit antisera were previously determined by serial dilution experiments. After a 4-h incubation at 20°C, the plates were washed with 0.01 M phosphate buffer pH 7.4 containing 0.05% (wt/vol) Tween 20. The enzyme activity of the bound immunological complex was then quantified by the addition of 200 μ L/well of Ellman's reagent (mixture of 7.5×10^{-4} M substrate, acetylthiocholine iodide, and 5×10^{-4} M chromogen, 5,5'-dithiobis(2-nitrobenzoic acid)). After 1 to 2 h of gentle shaking in the dark at room temperature, the absorbance (414 nm) of each well was measured using an automatic plate reader (Titertek, Labsystem, Helsinki, Finland). Results were expressed in terms of the bound activity in the presence/absence of competitor (B/B_0 , respectively, %) as a function of the dose (logarithmic scale). All measurements for standards or samples were made in duplicate, and in quadruplicate for B_0 values. In all cases, variation in duplicate values was less than 10% of the mean value reported. A linear log-logit transformation was used to fit the standard curve (Meisel et al., 2003). The assay sensitivity was characterized by the dose of standard inducing a 50% lowering of the binding observed in the absence of competitor (B/B_0 50%). The minimum detectable concentration corresponded to an 80% lowering of the binding (B/B_0 80%).

Stability of β -LG f(142-148) in Human Sera

Synthetic β -LG f(142-148) (1.7 mg) was incubated with 1 mL of human blood serum at 37°C. After 0, 5, 15, 30, 45, and 60 min incubation, an aliquot (20 μ L) of the sulfosalicylic acid (5.0% wt/wt) soluble fraction was applied to a LiChroCART Superspher 100 C₁₈ end-capped reversed-phase HPLC column (125 $\times$ 4 mm, Merck, Darmstadt, Germany) fitted with a LiChrospher 100 C₁₈ guard column (4.0 $\times$ 4.0 mm, Merck). Solvents used were: solvent A (0.1% (vol/vol) trifluoroacetic acid in water) and solvent B (0.1% (vol/vol) trifluoroacetic acid in 50.0% (vol/vol) aqueous acetonitrile). Fraction-

ation was achieved by applying a linear gradient of 5 to 90.0% solvent B over a period of 85 min. The flow rate was 1 mL/min and eluting peptides were detected at 215 nm. In addition, the ACE inhibitory index values of HPLC fractions of β -LG f(142-148) from different stages of incubation with serum were determined. In this instance, ACE-inhibitory activity was quantified by measuring the amount of hippuric acid liberated by ACE from hippuryl-L-histidyl-L-leucine (Cushman and Cheung, 1971; Doig and Smiley, 1993). The synthetic hippuryl-L-histidyl-L-leucine substrate (0.25 μ M) and ACE (8.3 mU) were incubated for 30 min at 37°C with the freeze-dried (Speed Vac concentrator, Savant, Holbrook, NY) HPLC fractions solubilized in 0.15 mL. All components of the 0.25-mL assay mixtures were dissolved in 0.1 M sodium borate buffer, containing 0.3 M NaCl, pH 8.3.

Amino acid analysis. Freeze-dried samples were hydrolyzed with 6 mol/L HCl/1% vol/vol phenol in evacuated tubes at 110°C for 24 h. Amino acid analyses were carried out with an analyzer, type 4151 Alpha Plus (Pharmacia Biotech, Freiburg, Germany) using the ion-exchange resin Ultrapac 8 (Pharmacia Biotech 80 to 202845) and sodium citrate buffer system (Pharmacia Biotech). Calibration was achieved by running a mixture of standard amino acids (Sigma AA-S18, Taufkirchen, Germany).

Preliminary Clinical Investigation

Two healthy, fasted, normotensive male volunteers were studied in a single-blind, placebo-controlled crossover study. The subjects were orally administered capsules containing β -LG f(142-148) or lactose (as a placebo). Exclusion criteria for volunteer selection were history of atopy (asthma, eczema, or hayfever), allergy to cow's milk, lactose intolerance, and epilepsy. Both subjects initially received 4 mg of β -LG f(142-148) and had their blood pressure and serum ACE activity measured every 30 min for 6 h. Blood pressure was measured in the sitting position with a semiautomatic Omron sphygmomanometer (Omron Matsusaka, Japan). Three blood pressure readings were recorded and the mean value used. On a separate day, the subjects received placebo and their blood pressure and serum ACE activities were similarly quantified. This sequence was repeated for increasing doses (10, 25, 40, and 80 mg) of β -LG f(142-148). Subjects then received 80 mg of β -LG f(142-148) or placebo in a crossover fashion for 5 consecutive days and blood pressure and serum ACE activity after a 20-min rest in a semirecumbent position was recorded. All measurements were performed by the same doctor at 2 h postdose. Serum ACE activity was assayed by monitoring the change in absorbance at 340

nm of the hydrolysis of FAPGG to N-[3-(2-furyl)acryloyl]-L-phenylalanine (FAP) and glycyl-glycine (GG) on a Roche MIRA autoanalyzer (Roche Diagnostic Systems, Welwyn Garden City, UK). The study was approved by the Tayside Research Ethics Committee and both volunteers provided written, informed consent.

Statistical Analyses

One-way ANOVA was performed on all ACE inhibition data, comparing values from triplicate analysis at an equivalent degree of hydrolysis before and after SGID at a confidence level of 95% using SPSS, Version 11.0. A significant difference in results infers a significant difference at $P < 0.05$.

RESULTS AND DISCUSSION

Generation of Whey Protein Hydrolysates

The substrates WPC75 and BLGWP were hydrolyzed with a range of proteolytic activities. Hydrolysis reactions were allowed to proceed until a plateau was obtained, that is, no additional base was required to maintain constant pH. The DH% plateau attained varied for each enzyme and substrate combination (Table 1). Variations in the extent of hydrolysis between different enzyme and substrate combinations were attributed to the specificity of the different enzyme preparations. Commercial proteolytic preparations have previously been shown to possess several different enzyme activities (Mullally et al., 1997b; Smyth and FitzGerald, 1998).

ACE Inhibitory Activity of Hydrolysates

Unhydrolyzed BLGWP and WPC75 possessed little ACE inhibitory activity (~10% inhibition). These results are in agreement with previous reports (Mullally et al., 1997a). Hydrolysis of BLGWP and WPC75 resulted in the release of ACE inhibitory activity (Figures 1 and 2). The extent of release of ACE inhibitory peptides varied with the substrate and enzyme used for hydrolysis, and with DH (Figures 1 and 2). For example, the hydrolysis of BLGWP with TPCK-trypsin (Figure 1a) and Corolase PP (Figure 1d) resulted in the gradual release of ACE inhibitory activity, whereas hydrolysis with Protamex (Figure 1b), Corolase PNL (Figure 1c), and Corolase 7089 (Figure 1e) resulted in the relatively rapid release of ACE inhibitory peptides.

Digestion of intact BLGWP and WPC with pepsin and Corolase PP also resulted in the release of ACE inhibitory activity. In general, SGID treatment of BLGWP and WPC75 hydrolysates at different DH val-

ues resulted in further increases in the ACE inhibition indices (Figures 1 and 2).

The inhibitory potency of TPCK-trypsin hydrolysates of BLGWP was dependent on the extent of hydrolysis. The IC_{50} value was shown to decrease with increasing DH, e.g., hydrolysates at 4% DH had an IC_{50} of 260 mg/L, whereas the 6.7% DH hydrolysate had an IC_{50} of 206 mg/L ($P < 0.05$) (Figure 3). It was not possible to determine an IC_{50} value for hydrolysates at DH values less than 3% because hydrolysates at low DH were not potent inhibitors of ACE (IC_{50} values in excess of 350 mg/L were beyond the range of the assay). In agreement with Mullally et al., (1997a), no further hydrolysis of BLGWP was evident with TPCK-trypsin beyond a DH of 6.7%.

Application of SGID to intact BLGWP yielded a more potent ($P < 0.05$) hydrolysate with an IC_{50} of 180.6 mg/L. Simulated gastrointestinal digestion of TPCK-trypsin hydrolysates at different DH had varying effects on ACE inhibitory potency (Figure 3). At low DH (< 3%), SGID-treated hydrolysates were more potent than the nonSGID-hydrolysates. However, at 5 and 6% DH, SGID resulted in a decrease in the mean inhibitory potency of the hydrolysates, whereas SGID had no major effect on the inhibitory potency of the 6.7% DH hydrolysate. To date no reports appear to have detailed the effect of digestion with gastrointestinal proteinases on the ACE inhibitory potency of milk protein hydrolysates at different DH.

Immunochemical Detection of β -LG f(142-148)

Assays were performed to detect β -LG f(142-148) immunoreactivity in various hydrolysates (Table 2 and Figure 4). This test allowed determination of β -LG f(142-148) to a detection limit of 3 ng/mL. Control hydrolysate samples did not inhibit the binding of rabbit specific antibodies to β -LG f(142-148)-acetylcholinesterase, indicating no influence of the conditions of hydrolysis on the EIA.

Detection of β -LG f(142-148) in Whey Protein Hydrolysates

To validate the assay, the ability of the EIA system to detect β -LG f(142-148) in hydrolysates was determined. A WPC75 hydrolysate (WPC75 subjected only to SGID) was separated by reversed-phase HPLC and the fractions were analyzed for the presence of β -LG f(142-148). The main immunoreactive peak in the HPLC profile of the hydrolysate directly corresponded with the retention time of β -LG f(142-148), (Figure 5a, 5b). These results confirmed the specificity of the EIA and the existence of β -LG f(142-148) in the test hydroly-

Figure 1. Angiotensin-I-converting enzyme (ACE) inhibition indices (%) of a β -lactoglobulin-enriched whey protein fraction on digestion with (a) TPCK-trypsin, (b) Protamex, (c) Corolase PNL, (d) Corolase PP, and (e) Corolase 7089. Angiotensin-I-converting enzyme inhibition indices are plotted for hydrolysate samples (■) and for hydrolysates subjected to simulated gastrointestinal digestion (□). Data points are means of triplicate analysis $\pm$ standard deviation. *Indicates that values for samples at this degree of hydrolysis were significantly ($P < 0.05$) different.

sate. The assay did not detect β -LG f(142-148) in control samples containing water, pepsin, Corolase PP, and phosphate buffer (data not shown). No cross-immunoreactivity with whole β -LG under native and denatured forms was observed and as a consequence the EIA system detected low levels (0.01 to 0.1 μ g/mL) of β -LG f(142-148) in intact BLGWP and WPC75.

The amount of β -LG f(142-148) found in the hydrolysates was related to the substrate, the proteinase, and the hydrolysis conditions used (Figure 4 and Table 2). The peptide was present in TPCK-trypsin, Protamex,

Corolase PNL, Corolase PP, and Corolase 7089 hydrolysates of BLGWP (Figure 4). The amount of peptide within the hydrolysate was dependent on hydrolysis DH. In all cases, the amount of β -LG f(142-148) in the hydrolysates increased to a maximum level before decreasing with a further increase in DH. This was presumably due to the degradation of peptides containing the β -LG f(142-148) sequence at higher DH values. Interestingly, the TPCK-trypsin BLGWP hydrolysates at DH values of 2.0 and 3.0% contained higher concentrations of β -LG f(142-148) than the hydrolysates between

Figure 2. Angiotensin-I-converting enzyme (ACE) inhibition indices (%) of whey protein concentrate on digestion with (a) TPCK-Trypsin, (b) Corolase PNL, and (c) Corolase PP. Angiotensin-I-converting enzyme inhibition indices are plotted for hydrolysate samples (■) and for hydrolysates subjected to simulated gastrointestinal digestion (□). Data points are means of triplicate analysis $\pm$ standard deviation. *Indicates that values for samples at this degree of hydrolysis were significantly ($P < 0.05$) different.

Figure 3. Effect of TPCK-trypsin hydrolysis of a β -lactoglobulin-enriched whey protein fraction on angiotensin-I-converting enzyme (ACE) inhibitory potency (IC_{50} , mg/L protein). Angiotensin-I-converting enzyme inhibition indices are plotted for hydrolysate samples (■) and for hydrolysates subjected to simulated gastrointestinal digestion (□). The broken line (---) represents an IC_{50} value >350 mg/L, that is, beyond the quantifiable range of the assay. Data points are means of triplicate analysis $\pm$ standard deviation. *Indicates that values for samples at this degree of hydrolysis were significantly ($P < 0.05$) different.

5.0 and 6.7% DH (Figure 4a), with the latter hydrolysates being more potent inhibitors of ACE (Figure 3).

As expected, highest levels of β -LG f(142-148) were detected in the tryptic hydrolysates of BLGWP with the 3% DH hydrolysate having a mean value of $680 \mu\text{g}$ of peptide/mL. This corresponded to a theoretical yield of 26.6% (Figure 4a).

Simulated gastrointestinal digestion of all the BLGWP hydrolysates generated at different DH values resulted in a large decrease in the level of β -LG f(142-148) detected. For example, the 3% DH tryptic hydrolysate of BLGWP had $15 \mu\text{g/mL}$ of β -LG f(142-148) after SGID, compared with $680 \mu\text{g}$ peptide before SGID (Figure 4a). These results demonstrate that the SGID process, that is, incubation with pepsin and/or Corolase PP, leads to the degradation of β -LG f(142-148) in hydrolysates. Interestingly, SGID of intact β -LG resulted in the release of low levels of β -LG f(142-148), i.e., 8 to $36 \mu\text{g/mL}$ (Figure 4a to 4e).

The trypsin, Corolase PNL, and Corolase PP hydrolysates of WPC75 displayed similar trends to those of the BLGWP hydrolysates in terms of the effect of DH and SGID on the level of β -LG f(142-148), (Figures 4a to 4e and Table 2). Interestingly, the TPCK-trypsin hydrolysates of WPC75 appeared to contain higher levels of β -LG f(142-148) than the corresponding tryptic hydrolysates of BLGWP at certain DH values (Figure 4a and Table 2). At 2% DH, the tryptic hydrolysate of WPC75 contained $1160 \mu\text{g}$ of peptide/mL whereas the corres-

Table 2. Concentration ($\mu\text{g/mL}$) and theoretical yield of fragment (142-148) from β -lactoglobulin during the hydrolysis of whey protein concentrate (WPC75) to different degrees of hydrolysis (DH%) with different proteinase preparations. Hydrolysates at different degrees of hydrolysis values were subjected to simulated gastrointestinal digestion (SGID). Values for [β -LG f(142-148)] are the means of duplicate analysis (variation between duplicate values was <10% of mean).

Proteinase activity	DH ¹ after initial hydrolysis	NonSGID ² β -LG f(142-148) ($\mu\text{g/mL}$)	Yield ³ β -LG f(142-148) (%)	SGID ⁴ β -LG f(142-148) ($\mu\text{g/mL}$)
TPCK-trypsin ⁵	0	0.0	0.0	32.0
	1.0	720.0	39.5	6.0
	2.0	1160.0	63.6	5.0
	3.0	ND ⁶	ND	9.0
	4.0	950.0	52.1	6.0
	5.0	600.0	32.9	7.0
	6.0	ND	ND	9.0
	8.0	520.0	27.9	11.0
Corolase PNL	0	0.1	0.0	28.0
	3.0	46.0	2.5	2.0
	5.0	54.0	3.0	1.6
	7.0	37.0	2.0	2.5
	10.0	18.0	1.0	1.0
	12.0	9.0	0.5	1.0
	15.0	4.0	0.2	0.5
Corolase PP	0	0.0	0.0	12.0
	3.0	95.0	5.2	1.5
	5.0	75.0	4.1	1.0
	7.0	33.0	1.8	1.0
	10.0	20.0	1.1	1.0
	12.0	16.0	0.9	1.0
	14.0	9.0	0.5	0.5
	16.0	4.0	0.2	0.6
	18.0	0.6	0.0	0.4
	22.0	1.2	0.1	0.3

¹DH = Degree of hydrolysis.

²NonSGID = Samples were not exposed to proteinases of simulated gastrointestinal digestion but were exposed to the same buffer and temperature conditions of the SGID process.

³Yield = The yield of β -LG f(142-148) is expressed as a percentage of maximum theoretical concentration of β -LG f(142-148) which may be generated from β -lactoglobulin present in WPC75.

⁴SGID = Simulated gastrointestinal digestion.

⁵TPCK-trypsin = Tosyl-L-phenylalanyl-chloromethane-treated trypsin.

⁶ND = Not determined.

ponding BLGWP hydrolysate had 500 μg of peptide/mL. Furthermore, the calculated yield was higher in WPC75 than BLGWP hydrolysates, with an estimated 63.6% recovery of β -LG f(142-148) in the WPC75 hydrolysate. The reason for this difference in yield is unclear. It is possible that f(142-148) or a peptide containing f(142-148) may be more susceptible to further degradation during hydrolysis of a substrate enriched in β -LG.

Breakdown of β -LG f(142-148) may occur at several stages during the SGID process. β -Lg f(142-148) contains several sites potentially susceptible to pepsin cleavage. However, Mullally et al., (1997a) demonstrated that this peptide was essentially resistant to further degradation by pepsin at pH 2.0. β -Lactoglobulin f(142-148) also contains sites which are theoretically susceptible to hydrolysis by chymotrypsin (Hanekamp and Thorsness, 1998). However, Mullally et al. (1997a) observed very limited hydrolysis of this peptide on incu-

bation with excess TLCK-chymotrypsin activity. During the SGID process in the present study, Corolase PP was used as a source of trypsin and chymotrypsin activities. Mullally et al., (1994) demonstrated that Corolase PP also contained elastase activity. The observed degradation of β -LG f(142-148) during SGID in the present study may, in part, be due to its hydrolysis by elastase. Hydrolysis of WPC with elastase activity was shown to yield hydrolysates with low ACE inhibitory index values (Mullally et al., 1997b).

Stability of β -LG f(142-148) During Incubation with Human Sera

The stability of β -LG f(142-148) during incubation with human serum was determined by reversed-phase HPLC analysis and by monitoring changes in ACE inhibitory index. The HPLC profiles showed rapid degra-

Figure 4. Concentration ($\mu\text{g/mL}$) (■, □) and theoretical yield (○) of fragment (142-148) from β -lactoglobulin during the hydrolysis of a β -lactoglobulin-enriched whey protein fraction with different proteinase preparations. a) TPCk-trypsin, b) Protamex, c) Corolase PNL, d) Corolase PP, and e) Corolase 7089. Hydrolysates (■) at different degrees of hydrolysis values were subjected to simulated gastrointestinal digestion (□). Values for [β -LG f(142-148)] are the mean of duplicate analysis (variation between duplicate values was <10% of mean).

dition of β -LG f(142-148) during a 60-min incubation in serum (Figure 6). Furthermore, a decrease in the ACE inhibitory index value from 77.1 to 20.5% in the HPLC fractions corresponding to the retention time of β -LG f(142-148) (i.e., 40.6 min) was observed during the 60-min incubation. No ACE inhibitory activity was observed in the other fractions eluting from the C_{18} column. Approximately 10 inactive degradation products of β -LG f(142-148) were generated during incubation with human serum. The main degradation product, which appeared after 5 min incubation in serum, was

β -LG f(143-148), as determined by amino acid analysis (Figure 6).

Preliminary Clinical Investigation

The effects of oral ingestion of β -LG f(142-148) on 2 healthy, normotensive human volunteers was investigated. The results obtained following 5 d of ingestion of 80 mg of the peptide are presented in Table 3. Given that the investigation consisted of 2 volunteers, it is not possible to draw definitive conclusions about the

Figure 5. Reversed-phase HPLC profiles of (a) β -lactoglobulin f(142-148), where the line represents the gradient of solvent B (0.1% trifluoroacetic acid in acetonitrile), and (b) whey protein concentrate following simulated gastrointestinal digestion. The shaded histograms represent immunoactivity (% inhibition) to β -LG f(142-148) as a function of elution time.

Figure 6. Reversed-phase HPLC profiles [absorbance at 215 nm (mAU) as a function of elution time (min)] at different times after incubation at 37°C of β -lactoglobulin f(142-148) at a concentration of 1.7 mg/mL with the 5% sulfosalicylic acid soluble fraction of human blood serum. Angiotensin-I-converting enzyme inhibition indices (AI, %) of the heptapeptide (ALPMHIR) fraction (retention time = 40.6 min) were determined throughout the course of the incubation.

Table 3. Systolic and diastolic blood pressure, heart rate (in beats per min), and serum angiotensin-I-converting enzyme (ACE) activity in 2 human volunteers following oral administration of 80 mg of β -lactoglobulin f(142-148) daily for 5 consecutive days. Lactose was used as a placebo.

Subject	Placebo				Peptide			
	SBP ¹ (mm Hg)	DBP ² (mm Hg)	Heart rate	Serum ACE (U/L)	SBP (mm Hg)	DBP (mm Hg)	Heart rate (bpm)	Serum ACE (U/L)
1	113	60	58	13	112	71	72	12
2	112	69	68	37	121	79	69	37

¹SBP = Systolic blood pressure.

²DBP = Diastolic blood pressure.

results of this experiment. However, no adverse side effects were observed during or after administration of the peptide. Furthermore, β -LG f(142-148) or β -LG f(142-148)-like immunoreactivity could not be detected by EIA (minimum detection limit 3 ng/mL) in serum samples from the volunteers following oral ingestion of the peptide (data not shown). In addition, β -LG f(142-148) could not be detected, using the EIA test described herein, in sera from the volunteers when spiked with the peptide.

Overall, these results indicate that a peptide (β -LG f(142-148)), which is a potent inhibitor of ACE in vitro, may not necessarily be a potent antihypertensive peptide in vivo. The antihypertensive potential of peptides is dependent on their ability to be absorbed through the small intestine and to reach target organs without being degraded or inactivated by gastrointestinal or plasma proteinases and peptidases (FitzGerald and Meisel, 2003). Masuda et al. (1996) showed that the potent ACE-inhibitory casein-derived tripeptides, Ile-Pro-Pro and Val-Pro-Pro, were present in the solubilized aortic fraction of spontaneously hypertensive rats fed Calpis sour milk. On the other hand, it was previously shown that α _{S1}-casein f(23-27), a potent ACE inhibitor in vitro, had no hypotensive effect in spontaneously hypertensive rats (Maruyama et al., 1987). Our results confirm that only some peptides are resistant to degradation by intestinal proteinases and peptidases, are absorbed from the digesta, and are resistant to degradation by circulatory proteinases/peptidases. It is interesting to note that, even if it was resistant to degradation during gastrointestinal passage, β -LG f(142-148) was only transported at very low levels across Caco-2b monolayers (Vermeirssen et al., 2002). This data is further indication that the peptide probably does not act as a hypotensive agent in humans.

CONCLUSIONS

This study shows that β -LG f(142-148) and peptides containing a similar amino acid sequence are generated

during the hydrolysis of β -LG-enriched whey protein fraction and WPC75, with enzyme preparations having different specificities. β -Lactoglobulin f(142-148) was susceptible to degradation on incubation with gastrointestinal and human blood serum proteinase/peptidase activities in vitro. Given its susceptibility to such degradation, it would appear that β -LG f(142-148) probably does not have the potential to elicit a hypotensive response in humans.

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REFERENCES

- Adler-Nissen, J. 1986. A review of food protein hydrolysis – specific areas. Pages 57–109 in *Enzymatic Hydrolysis of Food Proteins*. Elsevier Applied Science, New York, NY.
- Ariyoshi, Y. 1993. Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends Food Sci. Technol.* 4:139–144.
- Caruelle, D., J. Grassi, J. Courty, B. Groux-Muscattelli, P. Pradelles, D. Barritault, and J. P. Caruelle. 1988. Development and testing of radio and enzyme immunoassays for acidic fibroblast growth factor. *Anal. Biochem.* 173:328–339.
- Cushman, D. W., and H. S. Cheung. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* 20:1637–1648.
- Doig, M. T., and J. W. Smiley. 1993. Direct injection assay of angiotensin-converting enzyme by high-performance liquid chromatography using a shielded hydrophobic phase column. *J. Chromatogr.* 613:145–149.
- Ellman, G. L., K. D. Courtney, V. Andres, and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetyl cholinesterase activity. *Biochem. Pharmacol.* 7:88–95.
- Erdős, E. G. 1975. Angiotensin-I-converting enzyme. *Circ. Res.* 36:247–255.
- FitzGerald, R. J., and H. Meisel. 1999. Lactokinins: Whey protein-derived ACE inhibitory peptides. *Nahrung* 43:165–167.

- FitzGerald, R. J., and H. Meisel. 2000. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr.* 84:S33–S37.
- FitzGerald, R. J., and H. Meisel. 2003. Milk protein hydrolysates and bioactive peptides. Pages 675–698 in *Advanced Dairy Chemistry – 1: Proteins*, 3rd edition, Part B. P. F. Fox and P. L. H. McSweeney, ed. Kluwer Academic/Plenum Publishers, New York, NY.
- FitzGerald, R. J., B. A. Murray, and D. J. Walsh. 2004. Hypotensive peptides from milk proteins. *J. Nutr.* 134:980–9885.
- Gobbetti, M., L. Stepaniak, M. De Angelis, A. Corsetti, and R. Di Cagno. 2002. Latent Bioactive Peptides in Milk Proteins: Proteolytic Activation and Significance in Dairy Processing. *Crit. Rev. Food Sci. Nutr.* 42:223–239.
- Hanekamp, T., and P. E. Thorsness. 1998. Chymotrypsin. Pages 1504–1507 in *Handbook of Proteolytic Enzymes*. A. J. Barrett, N. D. Rawlings, and J. F. Woessner ed. Academic Press, London, UK.
- Hata, Y., M. Yamamoto, M. Ohni, K. Nakajima, Y. Nakamura, and T. Takano. 1996. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* 64:767–771.
- IDF. (International Dairy Federation). 1993. Milk: Determination of nitrogen content. 28B: International Dairy Federation, Brussels, Belgium.
- Kim, Y. S., W. Bertwhistle, and Y. W. Kim. 1972. Peptide hydrolysis in the brush border and soluble fractions of small intestinal mucosa of rat and man. *J. Clin. Invest.* 51:1419–1430.
- Koike, H., K. Ito, B. S. Miyamoto, and H. Nishino. 1980. Effects of long-term blockade of angiotensin converting enzyme with captopril (SQ 14225) on hemodynamics and circulating blood volume in SHR. *Hypertension* 2:299–303.
- Maruyama, S., H. Mitachi, J. Awaya, M. Kurono, N. Tomizika, and H. Suzuki. 1987. Angiotensin I converting enzyme inhibitory activity of the C-terminal hexapeptide of α s1-casein. *Agric. Biol. Chem.* 51:2557–2561.
- Masuda, O., Y. Nakamura, and T. Takano. 1996. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J. Nutr.* 126:3063–3068.
- Meisel, H. 1998. Overview on milk protein-derived peptides. *Int. Dairy J.* 8:363–373.
- Meisel, H., H. Bernard, S. Fairweather-Tait, R. J. FitzGerald, R. Hartmann, C. N. Lane, D. McDonagh, B. Teucher, and J. M. Wal. 2003. Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjects. *Br. J. Nutr.* 89:351–358.
- Meisel, H., D. J. Walsh, B. A. Murray, and R. J. FitzGerald. 2004. Factors on body's regulation system. In *Nutraceutical proteins and peptides in health and disease*. Y. Mine, ed. Marcel Dekker, New York, NY.
- Mullally, M. M., D. M. O'Callaghan, R. J. FitzGerald, W. J. Donnelly, and J. P. Dalton. 1994. Proteolytic and peptidolytic activities in commercial pancreatic protease preparations and their relationship to some whey protein hydrolysate characteristics. *J. Agric. Food Chem.* 42:2973–2981.
- Mullally, M. M., H. Meisel, and R. J. FitzGerald. 1997a. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine β -lactoglobulin. *FEBS Lett.* 402:99–101.
- Mullally, M. M., H. Meisel, and R. J. FitzGerald. 1997b. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Int. Dairy J.* 7:299–303.
- Murray, B. A., D. J. Walsh, and R. J. FitzGerald. 2004. Modification of the furanacryloyl-L-phenylalanyl-glycylglycine assay for determination of angiotensin-I-converting enzyme inhibitory activity. *J. Biochem. Biophys. Methods* 59:127–137.
- Ondetti, M. A., B. Rubin, and D. W. Cushman. 1977. Design of specific inhibitors of angiotensin converting enzyme: New class of orally active antihypertensive agents. *Science* 196:441–444.
- Oshima, G., H. Shimabukuro, and K. Nagasawa. 1979. Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. *Biochim. Biophys. Acta* 566:128–137.
- Pihlanto-Leppälä, A. 2001. Bioactive peptides derived from bovine whey proteins: Opioid and ace-inhibitory peptides. *Trends Food Sci. Technol.* 11:347–356.
- Seppo, L., T. Jauhainen, T. Poussa, and R. Korpela. 2003. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. *Am. J. Clin. Nutr.* 77:326–330.
- Skeggs, L. T., J. E. Kahn, and N. P. Shumway. 1956. The preparation and function of the angiotensin-converting enzyme. *J. Exp. Med.* 103:295–299.
- Smyth, M., and R. J. FitzGerald. 1998. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. *Int. Dairy J.* 8:819–827.
- Takano, T. 1998. Milk derived peptides and hypertension reduction. *Int. Dairy J.* 8:375–381.
- Vermeirssen, V., B. Deplancke, K. A. Tappenden, J. Van Camp, H. R. Gaskins, and W. Verstraete. 2002. Intestinal transport of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer. *J. Pept. Sci.* 8:95–100.
- Walsh, D. J., and R. J. FitzGerald. 2004. Health-related functional value of dairy proteins and peptides. Pages 559–606 in *Proteins in Food Processing*. R. Yada, ed. Woodhead Publishing, Cambridge, UK.
- Wyvratt, M. J., and A. A. Patchett. 1985. Recent developments in the design of angiotensin-converting enzyme inhibitors. *Med. Res. Rev.* 5:483–531.
- Yoshimoto, T., M. Fischl, R. C. Orlowski, and R. Walter. 1978. Post-proline cleaving enzyme and post-proline dipeptidyl amino-peptidase comparison of two peptidases with high specificity for proline residues. *J. Biol. Chem.* 253:3708–3716.