Digestibility of Bovine Milk Whey Protein and β-Lactoglobulin in Vitro and in Vivo

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Bovine milk whey proteins were treated with porcine stomach pepsin, bovine chymosin, human uropepsin, and gastric juice from rat stomach at various pH values. Although α-lactalbumin and bovine serum albumin were hydrolyzed by these proteases, β-lactoglobulin (β-LG) was not hydrolyzed by any pepsin samples at any pH in vitro. Heat-denatured whey proteins, including heated β-LG, were easily hydrolyzed by pepsins. Native β-LG injected into the stomach of a rat was not digested in stomach in vivo, whereas heat-denatured β-LG was digested in the stomach of a rat. Native β-LG as well as the heated sample was hydrolyzed by both commercially available porcine pancreatin and the rat pancreatic juice collected from rat intestine in vitro. Native β-LG injected into the stomach was digested in the intestine in vivo as well as heat-denatured β-LG. The body weights of rats fed heated β-LG increased more rapidly than those of rats fed native β-LG.

Keywords: *β-Lactoglobulin; milk whey protein; pepsin; pancreatic enzymes; digestibility*

INTRODUCTION

Mammals provide nutrients to offspring through milk. Milk components, including whey proteins, vary from species to species. β-Lactoglobulin (β-LG) is a major protein in bovine milk but appears in small amounts in human, rat, and pig milk (Conti et al., 1980; Hambling et al., 1992; Bell et al., 1981). The milk of one mammal species is not always nourishing for other species, for example, β-LG sometimes behaves as an allergenic protein to human infants (Bahna, 1978). In general, however, bovine milk has important nutritional properties beneficial to the health and growth of infants. Thus, various improvements have been sought in preparing formulated milk for infants (Asselin et al., 1989).

Milk protein is usually digested in the stomach by pepsin, further hydrolyzed in the intestine, and then absorbed through the intestinal wall. Schmidt and Van Marwisk (1993) reported that bovine β-LG is not easily hydrolyzed by pepsin and the effect of heat treatment on the digestibility of whey proteins including β-LG was investigated. Their study was based on a hydrolysis experiment in vitro using porcine pepsin, which is commercially available. This resistance of β-LG against pepsin supports the hypothesis that β-LG functions as a retinol-binding protein (Papiz et al., 1986), because the β-LG molecule holding a retinol molecule can transport it to the intestine through the stomach without hydrolysis of the β-LG molecule. On the other hand, it was reported that the protein efficiency ratio (PER) value of bovine milk whey protein was higher than that of casein or other proteins (Smietana et al., 1986; Forsum et al., 1974; Renner, 1983), which was assessed using rats as the experimental animal. There may be a possibility that the pepsin in the stomach of a rat can digest the native β-LG molecule.

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The objective of the present study is to clarify whether bovine milk whey proteins and bovine β-LG can be digested in the stomach and intestine by porcine, human, bovine, and rat pepsin and pancreatin using both the in vitro and the in vivo system. Heat-denatured soluble whey protein (PWP) and heat-denatured soluble β-LG were examined to clarify whether they are hydrolyzed in the stomach and intestine. We also investigated a difference in the nutritive effects of unheated and heated β-LG by animal experiment using rats.

MATERIALS AND METHODS

Protein, Enzyme Samples, and Other Reagents. Acid whey protein concentrate was obtained from Danmark Protein A/S (Videbaek, Denmark). Protein compositions of whey protein concentrates and the details of the preparation of whey protein isolates (WPI) from whey protein concentrate are described elsewhere (Kinekawa and Kitabatake, 1995; Kitabatake et al., 1994). Briefly, casein in the whey protein concentrate fraction was removed by acid precipitation at pH 4.5 and protein in the supernatant; whey protein isolate (WPI) was collected by ammonium sulfate saturation (75%). WPI was usually kept as 75% ammonium sulfate precipitation. When used, the precipitate was dissolved and dialyzed against distilled and deionized water. PWP was prepared as follows: WPI was exhaustively dialyzed against distilled and deionized water, and the pH of the dialyzed WPI was adjusted to 7.0 by addition of NaOH. The clear WPI solution was heated at 80 °C for 1 h. After cooling, heat-treated WPI showed a clear liquid, which is named PWP (Kinekawa and Kitabatake, 1995).

Both β-LG A (variant A; L-7880) and β-LG (containing variants A and B; L-0130) were obtained from Sigma Chemical Co. (St. Louis, MO), as were α-lactalbumin (α-LA), bovine hemoglobin (H-2500), chymosin (R-7751, 20 units/mg), and porcine stomach pepsin (activity = 3200−4500 units/mg of protein, essentially salt-free; P-6887). Human uropepsinogen (>1700 units/mg) was a gift from Japan Chemical Research Co. Ltd. (Kobe, Japan). Pepstatin-A was obtained from Peptide Institute (Osaka, Japan). Pancreatin was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals were of reagent grade and obtained from Nacalai Tesque and Wako Pure Chemical Industries Ltd. (Osaka, Japan).

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Protein Concentration. Protein concentration of whey protein was determined using an absorbance at 280 nm based on the value of $E(1\%, 1\ cm) = 11.7$, which was calculated from the composition of whey protein (Leonard et al., 1963). Protein concentrations of β-LG A, β-LG, and porcine pepsin were measured using an absorbance of 280 nm, an extinction coefficient at 1% (w/v), and 1 cm light paths of 9.6, 9.6, and 13.2, respectively (Townend et al., 1960; Leonard et al., 1963; Kronman and Andreotti, 1964). The extinction coefficients at 278 nm at 1%(w/v) and 1 cm light path of human pepsin, chymosin, and pancreatin were 17.3, 14.3, and 13.6, respectively (Fasman, 1976; Ryle, 1970).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was carried out with a 5−20% gradient gel (SPG-520L; Atto Co., Tokyo, Japan) according to the instruction manual of the indicated manufacturer. A protein sample (6.6 μg) was loaded in each lane, and the bands were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% methanol.

Size Exclusion Chromatography. Size-exclusion chromatography of the solutions was performed by HPLC (L-6000; Hitachi Ltd., Tokyo, Japan) using a G 2000 SWL column (Tosoh Co., Tokyo, Japan). The column was equilibrated and the sample was eluted with 20 mM sodium phosphate buffer, pH 7.0, containing 0.5 M sodium sulfate at room temperature. The flow rate was set at 1.0 mL/min, and absorbance was measured at 280 nm.

Digestion of Whey Proteins and Hemoglobin in Vitro. WPI, PWP, β-LG A, β-LG with and without heating, and hemoglobin were treated with commercially available various pepsins and pancreatin, and gastric juice and pancreatic juice collected from rat stomach and pancreas, respectively.

The pH of substrate proteins was adjusted to 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, or 7.0 by addition of 1 N HCl. Porcine pepsin treatment, described elsewhere (Kinekawa and Kitabatake, 1996), briefly was as follows: The protein concentration of each substrate solution was adjusted to 70 mg/mL by the addition of distilled water. Fifty microliters of pepsin solution (10 mg/mL 0.01 N HCl) was added to 1.5 mL of substrate solution at pH 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, or 7.0 and incubated for 1 h at 37 °C. An aliquot of each solution was applied to SDS−PAGE or size exclusion HPLC.

Human uropepsinogen (>1700 units/mg) was dissolved in 50 μL of distilled water, and 2.5 μL of 0.2 N HCl was added. The solution reached 0.63 mg/mL 0.01 N HCl and was incubated for 10 min at 37 °C to convert human uropepsinogen to human uropepsin. Ten microliters of human uropepsin was added to 64 μL of substrate solution (10 mg/mL), the pH of which was previously adjusted to 2.0 by the addition of HCl, and then incubated at 37 °C for 1 h.

Chymosin was dissolved once in 50 mM sodium acetate buffer, pH 3.5, at 0.2 mg/mL, and then 5 μL of the chymosin solution was added to 100 μL of substrate solution (70 mg/mL) in which substrate protein was dissolved in 50 mM sodium phosphate buffer, pH 6.3. The reaction was allowed to continue at 37 °C for 1 h.

Pancreatin was dissolved in distilled water, and then 11.7 μL of pancreatin solution (25 mg/mL) was added to 250 μL of the substrate solution containing 10 mg of β-LG, 5 mM NaCl, and 20 mM $CaCl_2$ in 0.1 M borate buffer, pH 7.5. The reaction was allowed to continue at 37 °C for 1 h.

Rat gastric juice was collected using the stomach injection method as follows: 1 mL of carbonated water containing sucrose was injected into the stomach of infant rats (Wister, male, 3 weeks), which were previously starved for 12 h, using a stomach sonde (small type for mouse). After 1 min, the sonde was put into the stomach again, and ~100 μL of gastric juice was collected. The gastric juice obtained was kept on ice-cold water and used for the experiment on the same day. Ten microliters of gastric juice (A_{280nm} = 12.2−13.1, pH 2.0) was added to 50 μL of substrate solution (10 mg/mL, β-LG). After reaction at pH 2.0 and 37 °C for 1 h, samples were applied on SDS−PAGE.

Rat pancreatic juice was collected from pancreatic duct of rats (Wister, male, 20 weeks, ~700 g) by cannula insertion. After the rats were anesthetized with nembutal injections (1 μL/g of body weight), the abdominal region (~2 cm) of a rat was exposed, and liver and duodenum were pulled out through the incision region. The bile duct was then ligated and a cannula (Silastic medical tubing; i.d. = 0.02 in., o.d. = 0.037 in., Dow Corning Co., MI) was inserted into the pancreatic duct and fixed with adhesives (Aron Alpha, Toagosei Co., Ltd., Tokyo, Japan). The liver and duodenum were restored in the body, and the cannula were drawn outside through another incision region on the dorsum of the rat. After the incision region was stuck with adhesives, the rat was maintained in a ballman cage and the liquid exuded from cannula was collected. Yellow fluid was first secreted, and then clear juice was obtained. This clear fluid was considered to be pancreatic juice. At least 10 mL of pancreatic juice could be collected from each rat by this method. The secreted pancreatic juice was kept in ice-cold water and used on the same day. Pancreatic juice (2.5 μL, A_{278nm} = 61.6, pH 7.5) collected from rats was added to 250 μL of substrate solution (10 mg of β-LG, 5 mM NaCl, and 20 mM $CaCl_2$ in 0.1 M borate buffer, pH 7.5). After reaction at pH 7.5 and 37 °C for 1 h, samples were submitted to size exclusion chromatography.

Digestion of β-LG in Vivo. Heated (80 °C, 1 h) and unheated β-LG were used as substrates. Ten milliliters of either β-LG (20 mg/mL) or NaCl solution (0.9%, for control test) was injected into the stomach of a rat (Wister, male, 7 weeks, starved for 12 h) using a stomach sonde (small type for mouse). After 40 min, the rat was put in the ether atmosphere for 1 min and nembutal was injected to the heart. The abdominal region of the rat was then exposed, and the stomach and three consecutive parts of the intestine (15, 15−30, and 30−60 mm from the stomach) were clicked by forceps and excised. The stomach and intestine parts were immediately frozen with liquid nitrogen and kept at −80 °C. The frozen stomach and intestine parts were thawed and cut off longitudinally. The content was collected by washing with distilled water and then lyophilized. The powdered sample was dissolved in SDS solution and analyzed by SDS−PAGE and Western blotting using a rabbit anti-β-LG antibody (preparation described below).

Preparation of Rabbit Anti-β-LG Antiserum and Western Blotting. A rabbit was immunized with β-LG A that had been purified by Sephadex G-75 chromatography to remove the contaminated protein. First, 2 mg of purified β-LG A was emulsified with Freund's complete adjuvant (Nacalai Tesque) and injected into the rabbit. At both the second and fourth weeks, 2 mg of β-LG A with incomplete adjuvant (Nacalai Tesque) was injected as a boost. Blood was then drawn from the ear, and serum was separated by centrifugation. The serum was used as β-LG A antiserum for Western blotting.

Western Blotting of β-LG. After SDS−PAGE, the protein on the gel was transferred to a poly(vinylidene difluoride) membrane (Clear blot P sheet, Atto Co. Ltd., Tokyo, Japan) using Horize blot AE-6675-P (Atto Co., Ltd.). The membrane was washed with Tris-buffered saline (20 mM Tris, 137 mM NaCl, 2.6 mM KCl, pH 7.5) and blocked with ovalbumin (2%, diluted with Tris-buffered saline). The membrane was incubated with β-LG antiserum (500-fold dilution with saline) and was treated with alkaline phosphatase-linked anti-immunoglobulin G antibody. Color development was carried out by incubation with nitroblue tetrazolium (Promega Co., Madison, WI) and 5-bromo-4-chloro-3-indolylphosphate (Promega Co.).

Purification of β-LG from Whey Protein with Pepsin for Animal Experiments. A large amount of β-LG (>100 g) was prepared from WPI using the "pepsin treatment method" (Kinekawa and Kitabatake, 1996), which was carried out as follows: The pH of the whey protein solution was adjusted to 2.0 by addition of 2 N HCl. Protein concentration was adjusted to 70 mg/mL, and the solution was preincubated at 37 °C for 10 min. The enzymatic reaction was carried out at 37 °C for a given period of time with a protein-to-enzyme ratio of 200:1 (w/w). The protein fraction was collected by ammonium sulfate precipitation (75%) and either dialyzed against distilled water (20-kDa pore size of the membrane) or filtered and washed twice with distilled water by ultrafiltration (30-kDa pore size

of the membrane). Although β-LG is 18-kDa, β-LG molecules are associated under these conditions. Therefore, β-LG did not pass through the membrane having 20- or 30-kDa pore size and remained on the membrane filter under these conditions. The dialysate and the filtrate were centrifuged at 13 000 rpm (20800*g*) for 30 min (SCR20-B, Hitachi, Ltd., rotor RPR20-2). β-LG in the supernatant fraction was precipitated by addition of ammonium sulfate (75%) and kept at 4 °C until use.

Animal Experiments. Purified β-LG described above was exhaustively dialyzed against distilled and deionized water. After dialysis, the protein concentration and pH of the β-LG solution were adjusted to 30 mg/mL and 7.0, respectively. Half of the β-LG solution was heated at 80 °C for 1 h. The heated β-LG yielded a transparent liquid. The rats (Wister, male, 3 weeks) were divided into three groups, each consisting of eight rats. Heated or unheated purified β-LG was fed to the rats ad libitum, and the volume of β-LG ingestion was recorded every day. The rats of the first group were given unheated β-LG solution and a specially prepared protein-free feed: modified-AIN assorted feed containing 55.3% cornstarch, 13.3% gelatinized potato starch, 10.7% cellulose powder, 8.0% edible oil, 4.7% minerals (AIN-76), 6.7% sucrose, and 1.3% vitamins (AIN-76 and choline tartrate). Heated β-LG solution were given to the rats of the second group, and the protein-free feed ad libitum and distilled water instead of protein solution and modified-AIN assorted feed were given to those of the third group. The rats were reared individually in a cage in an environmentally controlled animal room maintained at 22 °C and 60% relative humidity. The animal room was lighted from 6:00 a.m. to 6:00 p.m. The volume of liquid intake and body weight were measured for each rat between 9:00 and 10:00 a.m. every day.

Protein efficiency ratio (PER) and net protein ratio (NPR) were calculated every week and every 10 days, respectively, using the following equations.

$$\text{PER} = \text{wt gain (g)/protein consumed (g)}$$

$$\text{NPR} = [\text{wt gain (g)} - \text{wt of protein-free group (g)}]/\text{protein ingested (g)}$$

RESULTS AND DISCUSSION

Digestion of Heated and Unheated Whey Proteins by Porcine Pepsin, Human Pepsin, Chymosin, and Gastric Juice in Vitro. The hydrolytic pattern of unheated whey protein digestion by porcine pepsin was observed using SDS−PAGE (Figure 1A). The unheated standard whey protein sample gave several dense bands corresponding to β-LG (18.4 kDa), α-LA (14.4 kDa), and serum albumin [bovine serum albumin (BSA), 67 kDa]. After treatment with porcine pepsin, the distribution of these bands in SDS−PAGE was similar to that of the unheated whey proteins when treated at pH 4.0 and above, indicating that porcine pepsin could not hydrolyze these proteins above pH 4.0. At pH 3.5, the bands corresponding to α-LA and serum albumin weakened, and some bands with a molecular weight of 20−35 kDa disappeared, while the bands corresponding to β-LG stayed the same. Below pH 3.5 the band corresponding to α-LA and other bands having lower molecular weights disappeared completely. However, β-LG was not hydrolyzed by porcine pepsin at any pH. When heated whey protein PWP (the whey protein heated under the salt-free condition) was used as a substrate, the hydrolytic pattern was different from that of unheated whey protein (Figure 1B). Even above pH 3.5, β-LG, α-LA, and BSA were changed and appeared to be hydrolyzed. At pH 2.0 and 1.5, which are in the optimum pH region for pepsin, the proteins, including β-LG, were hydrolyzed to smaller molecular weight

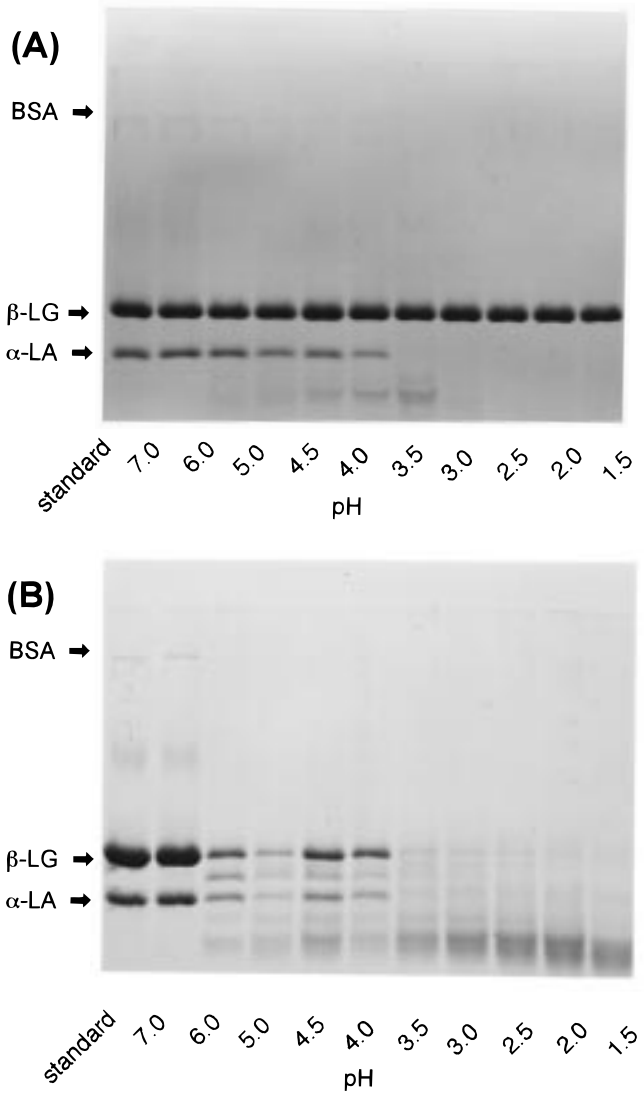

Figure 1. SDS−PAGE of WPI and processed whey protein treated with porcine pepsin. pH values of acid WPI solution (A) and processed whey protein solution (B; acid whey protein preheated at 80 °C for 1 h under salt-free condition) were adjusted to indicated values by addition of 1 N HCl. Protein concentration of each solution was adjusted to 70 mg/mL by addition of distilled water. Fifty microliters of pepsin solution (10 mg/mL 0.01 N HCl) was added to 1.5 mL of acid WPI solution of various pH values and incubated for 1 h at 37 °C. An aliquot of each solution was applied to SDS−PAGE. Standard, the WPI without pepsin.

peptides, indicating that heat treatment of whey proteins makes these globular proteins susceptible to porcine pepsin action at an acid pH.

In the case of unheated whey protein, prolonged incubation and/or further addition of porcine pepsin to the reaction mixture did not result in the hydrolysis of β-LG in whey protein. These results indicate that β-LG in the whey protein sample was not hydrolyzed by porcine pepsin at any pH if not heated as shown previously (Kinekawa and Kitabatake, 1996).

Next, to determine whether human pepsin can hydrolyze β-LG, uropepsinogen, which is found in urine and has enzyme properties, including substrate specificity, the same as those of the pepsin in stomach (Sun et al., 1985), was used. The hydrolytic pattern by uropepsin was similar to that of porcine pepsin (Figure 2); that is, human pepsin is also able to hydrolyze heated β-LG but not unheated β-LG. As a control, unheated hemo-

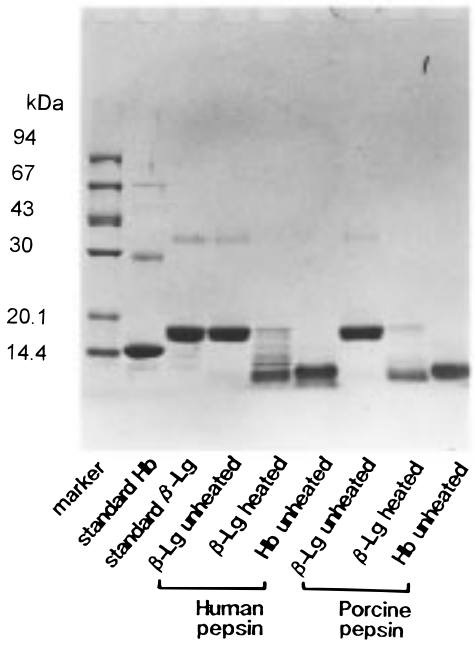

Figure 2. SDS−PAGE of heated (80 °C, 1 h) and unheated β-LG A and hemoglobin treated with human and porcine pepsin. Human uropepsinogen (0.63 mg/mL) was prepared with 0.2 N HCl and converted to human uropepsin. Ten microliters of human pepsin (0.63 mg/mL) was added to 64 μL of β-LG A (β-LG) solution (10 mg/mL) or hemoglobin solution (Hb; 10 mg/mL), which were previously dissolved in 0.2 N HCl. After reaction at pH 2.0 and 37 °C for 1 h, samples were submitted to SDS−PAGE. Molecular marker kit contained α-LA (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa), and phosphorylase B (94 kDa).

globin was treated by both human and porcine pepsin and was hydrolyzed by both.

Calf has chymosin instead of pepsin, and for this reason β-LG, whey protein, and PWP were treated with chymosin. Unheated β-LG and the β-LG fraction of whey protein were not hydrolyzed, but those of PWP were (data not shown).

Rat is the standard animal used in experiments to estimate the nutritive value of some protein. Therefore, it is important to know whether rat pepsin can hydrolyze the β-LG or not. Gastric juice was collected directly from the stomach of a rat with a stomach sonde. To facilitate the process, we used a carbonated sucrose solution. Injection of carbonated sucrose solution expands the stomach with carbonic gas following injection, and sucrose promotes the excretion of gastric juice, which made it possible to collect gastric juice easily, using the sonde. Figure 3 shows that unheated β-LG was not hydrolyzed and heated β-LG was hydrolyzed by gastric juice, which was completely inhibited by the addition of pepstatin-A, indicating that the hydrolysis of heated β-LG by gastric juice is due to rat pepsin contained in the gastric juice.

Digestion of Heated and Unheated β-LG by Porcine Pancreatin and Rat Pancreatic Juice (in Vitro). Unheated β-LG is not digested in the stomach. We examined whether whey protein and β-LG can be digested by pancreatic enzyme in vitro. First, the effects of commercially available pancreatic enzymes (pancreatin) on heated and unheated β-LG were examined. Figure 4 shows the hydrolytic patterns of both types of β-LG. Size exclusion HPLC analysis of unheated β-LG

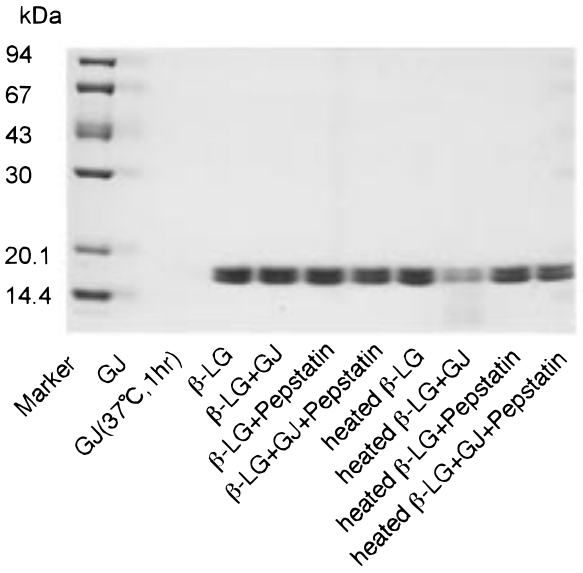

Figure 3. SDS−PAGE of heated (80 °C, 1 h) and unheated β-LG A that were treated with gastric juice collected from rat stomach. Reaction mixture contained 10 μL of gastric juice (A_{280nm} = 12.2−13.1, pH 2.0) and 50 μL of substrate solution (10 mg/mL, β-LG A). After reaction at pH 2.0 and 37 °C for 1 h, samples were submitted to SDS−PAGE. Marker proteins are the same as in Figure 2. GJ, gastric juice.

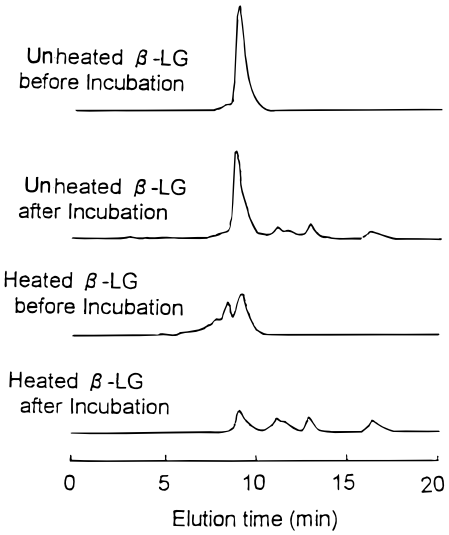

Figure 4. Size exclusion chromatography patterns of hydrolysis of heated (80 °C, 1 h) and nonheated β-LG A (β-LG) treated with porcine pancreatin. Reaction mixture contained 11.7 μL of porcine pancreatin solution (A_{278nm} = 13.6, pH 7.5) and 250 μL of substrate solution (10 mg of β-lactoglobulin A, 5 mM NaCl, and 20 mM $CaCl_2$ in 0.1 M borate buffer, pH 7.5). After reaction at pH 7.5 and 37 °C for 1 h, samples were submitted to size exclusion chromatography.

gave a single peak. After pancreatin treatment, several peaks having lower molecular size appeared, indicating that unheated β-LG can be hydrolyzed by pancreatin. HPLC analysis of the heated β-LG showed several peaks with short retention time, indicating formation of aggregates of heat-denatured β-LG molecules. After treatment with pancreatin, such molecule species having higher molecular size disappeared and several peaks with low molecular size appeared. Compared to unheated β-LG, heated β-LG seems to be degraded more rapidly. Preincubation of β-LG with porcine pepsin had no effect on hydrolysis by pancreatin as pepsin has no effect on unheated β-LG.

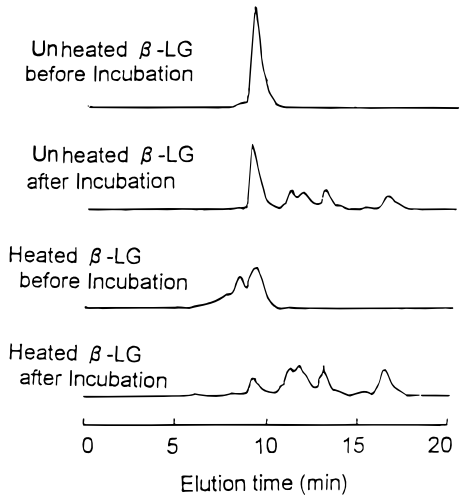

Figure 5. Size exclusion chromatography patterns of hydrolysis of heated (80 °C, 1 h) and unheated β-LG A (β-LG) treated with rat pancreatic juice collected. Reaction mixture contained 2.5 μL of pancreatic juice collected from rats (A_{278nm} = 61.6, pH 7.5) and 250 μL of substrate solution (10 mg of β-LG A, 5 mM NaCl, and 20 mM $CaCl_2$ in 0.1 M borate buffer, pH 7.5). After reaction at pH 7.5 and 37 °C for 1 h, samples were submitted to size exclusion chromatography.

Instead of commercially available pancreatic enzymes, rat pancreatic juice was collected, and the effects on heated and unheated β-LG were examined (Figure 5). Both types of β-LG were degraded by incubation with rat gastric juice. Heated β-LG was hydrolyzed more rapidly than unheated β-LG. However, unheated β-LG was also hydrolyzed well by pancreatic juice.

Digestion of Heated and Unheated β-LG in Vivo. In the in vitro experiment described above, unheated β-LG could not be hydrolyzed by pepsin but was hydrolyzed by pancreatin and pancreatic juice. To confirm resistance to hydrolysis of unheated β-LG in the stomach and its digestibility in the intestine, we carried out an in vivo experiment using rat. A given amount of β-LG with or without heating was directly injected into the stomach of a rat using a stomach sonde. After 40 min, the stomach and three consecutive parts of the intestine (15, 15−30, and 30−60 mm from the stomach) were removed and their contents analyzed by SDS−PAGE and Western blotting using an anti-β-LG antibody (Figures 6 and 7).

The band corresponding to β-LG was found on the SDS−PAGE of the stomach contents of the rat injected with unheated β-LG, whereas the stomach contents of the rat injected with heated β-LG showed no band with the same R_f value as that of standard β-LG and bands having lower molecular size appeared (Figure 6). This means that heated β-LG was hydrolyzed in the stomach, but unheated β-LG was not in vivo. On the SDS−PAGE of the content of the intestine, the band corresponding to β-LG was not observed in any case. Unheated as well as heated β-LG was hydrolyzed in the intestine in vivo. Large spots or bands were found in the intestinal fractions in both rats fed unheated β-LG and those fed heated β-LG. Western blotting shows whether these spots included β-LG or the hydrolyzed compounds of β-LG (Figure 7). Unheated β-LG was not hydrolyzed and was detected in the stomach as a large hollow band, which is similar to that of standard β-LG in Figure 7, confirming unheated β-LG was not hydrolyzed and stayed in the rat stomach. Some unhydrolyzed heated β-LG was also detected in the stomach. This amount,

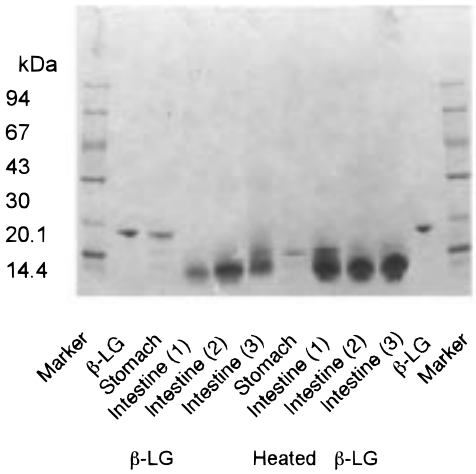

Figure 6. SDS−PAGE of heated (80 °C, 1 h) and unheated β-LG A and B (β-LG) that were digested in rat stomach and intestine in vivo. Ten milliliters of both β-LG A and B (20 mg/mL) and NaCl solution (0.9%, for control test) were injected into stomach of rats (Wister, male, 7 weeks, starved for 12 h) using a stomach sonde (small type for mouse). After 40 min, stomach and three consecutive parts (1, 2, 3) of intestine (15, 15−30, and 30−60 mm from stomach) were removed and their contents extracted and lyophilized. Contents were then dissolved in SDS solution and analyzed by SDS−PAGE. Marker proteins are the same as in Figure 2.

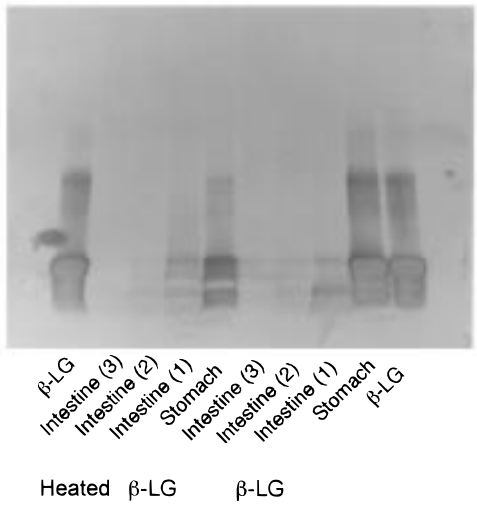

Figure 7. Western blotting patterns using rabbit anti β-LG antibody of heated (80 °C, 1 h) and unheated β-LG A and B (β-LG) that were digested in rat stomach and intestine in vivo. After SDS−PAGE, proteins on gel were transferred to poly(vinylidene difluoride) membrane. Membrane was washed with Tris-buffered saline and blocked with ovalbumin. Membrane was incubated with β-LG antiserum (500-fold dilution with saline) and was treated with alkaline phosphatase-linked anti-immunoglobulin G antibody. Color development was carried out by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Details are shown in Figure 6.

however, was much less than that found in the stomach of the rat fed heated β-LG. From the contents of the intestines, several bands including a weak β-LG band were found in both cases, indicating that hydrolyzed β-LG could be recognized by the antibody or that some of the contents in the intestine was nonspecifically detected. Unheated β-LG is not observed in the content of the intestine (Figure 7), suggesting unheated β-LG as well as heated β-LG can be hydrolyzed in the intestine under the condition of this study, which is in agreement with the result of the in vitro experiments shown in Figures 4 and 5.

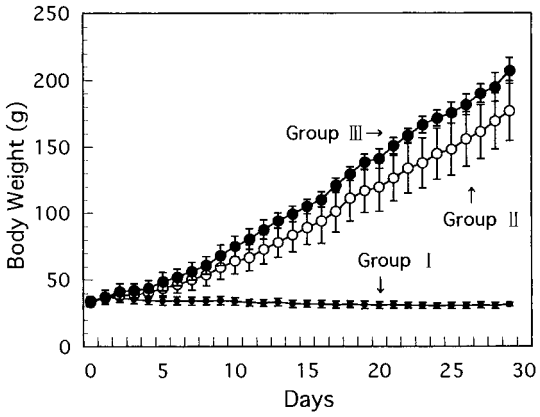

Figure 8. Change in body weight of rats (Wister, male, 3 weeks, 8 rats × 3 groups) fed heated (80 °C, 1 h) and unheated β-LG solution: group I, protein-free feed, modified-AIN assorted feed and water; group II, modified-AIN assorted feed and nonheated β-LG solution (3% protein, pH 7.0); group III, modified-AIN assorted feed and heated β-LG solution (3% protein, pH 7.0).

Table 1. Feed Intake, Body Weight Gain, PER, and NPR of Rats (Wister, Male, 3 Weeks, 8 Rats × 3 Groups) Fed Heated (80 °C, 1 h) and Unheated β-LG Solution

	unheated β-LG	heated β-LG	p value
feed intake (g)	34.40 ± 9.47	44.43 ± 4.45	0.000
body weight gain (g)	143.98 ± 23.66	172.88 ± 11.70	0.000
PER			
first week	6.44 ± 0.92	5.98 ± 0.73	0.312
second week	4.90 ± 0.81	4.95 ± 0.38	0.086
third week	4.50 ± 0.82	4.29 ± 0.40	0.135
fourth week	4.11 ± 0.74	3.83 ± 0.46	0.228
NPR			
10 days	5.36 ± 0.97	5.57 ± 0.74	0.282
20 days	4.62 ± 0.85	4.39 ± 0.38	0.108
10 days	4.13 ± 0.78	3.97 ± 0.41	0.159

This study shows that unheated β-LG was not hydrolyzed well in the stomach, whereas heated β-LG was hydrolyzed well in stomach, and both heated and unheated β-LG were hydrolyzed well in the intestine. It is generally known that β-LG is well digested and absorbed, which might be because unheated β-LG is degraded in the intestine.

Nutritional Evaluation of Heated and Unheated β-LG. To examine the nutritional effect of heating β-LG on the growth of rats, a large amount of β-LG (>100 g) was purified from acid whey protein according to the pepsin method (Kinekawa and Kitabatake, 1996), and unheated or heated β-LG was fed to rats (3 weeks) for 29 days as their only protein source. Change in body weight is shown in Figure 8. The rate of body weight increase in rats fed heated β-LG was higher than that of rats fed unheated β-LG. However, the PER and the NPR did not show significant difference ($p < 0.05$) between heated and unheated β-LG (Table 1). This may be because heated β-LG is more easily digested and more quickly absorbed than unheated β-LG, or it may be because rats preferred heated β-LG as it has a faint yet distinctive smell as a result of the heat treatment.

β-LG is the major protein component of whey protein and is commonly ingested by humans through bovine milk and as a food ingredient. Although whey protein is also used as a base for infant formula as it is highly nutritious, bovine β-LG resists digestion by not only porcine pepsin (Schmidt and Van Marwisk, 1993) but also calf, human, and rat pepsin, as shown in this study. This discrepancy, that bovine β-LG is highly nutritive yet nondigestive, can be explained by the fact that unheated β-LG can be digested and absorbed in the upper part of intestine. However, the absorption rate of unheated β-LG should be lower than that of heated β-LG; this might be reflected in the results of the nutrition experiment shown in Figure 8.

Unheated β-LG differs from heated β-LG with respect to not only digestion rate but also degradation pattern (the peptide species and amounts of peptides that occurred in vivo are shown in Figure 5). This means that the physiological activity of peptides, which heated β-LG produces after hydrolysis in vivo, and the allergenic effects of β-LG or these peptides might differ from those of unheated β-LG. This subject demands further investigation, particularly as whey protein is widely used in infant formulas. The physiological and structural properties of soluble heated β-LG and processed whey protein are under investigation.

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