JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Modification of IgE Binding during Heat Processing of the Cow's Milk Allergen β-Lactoglobulin

BRITT-MARIE EHN,*,†,‡ BO EKSTRAND,† ULF BENGTSSON,‡ AND STAFFAN AHLSTEDT§

SIK-The Swedish Institute for Food and Biotechnology, Göteborg, Sweden; Department of Respiratory Medicine and Allergology, The Sahlgrenska Academy at Göteborg University, Göteborg, Sweden; and Karolinska Institutet, Stockholm, Sweden

The effect of heat treatment on the IgE binding ability of β-lactoglobulin, as pure protein or in whole milk, was studied by inhibition of IgE antibody binding using FEIA-CAP inhibition. A slight but significant decreased IgE binding was seen between unheated and heat-treated β-lactoglobulin solution at 74 °C (IC_{50} = 2.03 and 3.59 μg/mL, respectively, $p = 0.032$). A more pronounced decrease was found at 90 °C with an IC_{50} of 8.45 μg/mL ($p = 0.014$). The inhibition of IgE binding of milk after heat treatment at 90 °C was also significantly decreased ($p = 0.007$). However, at all heat treatments, a similar total amount of IgE antibodies could be inhibited at a sufficiently high concentration of β-lactoglobulin. The inhibiting ability of β-lactoglobulin was significantly impaired in some fermented acidified milk products such as yogurt as compared to that in nonfermented milk ($p < 0.001$). There was only a small difference of IgE binding between the native forms of genetic variants A and B.

KEYWORDS: Milk; allergenicity; IgE antibody; IgE antibody inhibition; heat processing; β-lactoglobulin

INTRODUCTION

It is important to gain better knowledge of the influence of raw material composition and process design on the structure of food allergens and the allergenic potential of food products (*1*). It is frequently mentioned in the literature that heat treatment decreases the allergenicity of cow's milk (*2*−*5*). Previous experiments with guinea pigs showed promising results (*6*−*9*), but subsequent studies show that heat treatment is not sufficient to make whey protein or milk formulas hypoallergenic and that, for example, proteolytic degradation is also needed (*10*−*12*).

There is a connection between the level of specific IgE binding to an allergen and the patient's reaction. Thus, Sampson and Ho (*13*) found that a certain level of IgE could predict an allergic reaction with 95% certainty, but Osterballe and Bindslev-Jensen (*14*) could not link the IgE antibody levels to the amount of allergen required to evoke an allergic reaction. In egg-allergic children with low IgE concentration egg white was tolerated after cooking (*15*). Thus, it may be concluded that even if heat treatment does not make the allergen totally nonallergenic, it can still be of importance to quantify the degree of decreased IgE binding in a heat-treated allergen, such as cow's milk.

β-Lactoglobulin (BLG), as well as all of the major proteins in milk, are reported to be allergenic (*16*, *17*). It exists naturally as a dimer, each subunit having a molecular mass of 18 kDa (162 residues). The three-dimensional structure of native BLG, wich belongs to the lipocalin family, has been thoroughly studied (*18*−*20*). Heat effects on cow's milk are to a large extent caused by the behavior of BLG during denaturation and aggregation (*21*, *22*). Thus, pure BLG solutions were considered a good initial model system for this study.

There are a number of modifications at different structural levels during heat denaturation of BLG (*23*). Fast changes in the environment of aromatic amino acids occur and become irreversible at 65−70 °C, such as irreversible modification of the tertiary structure due to disulfide bond formation. At temperatures >40 °C, the free SH group (Cys 121) becomes accessible (*24*).

BLG exhibits a complex unfolding mechanism during denaturation, comprising at least two other species different from the native and completely denatured unfolded states (*25*). Dimer dissociation is a necessary step for a sequential polymerization, and cohesion of hydrophobic patches is the major driving force for aggregation (*26*). However, the denaturation process as shown in the calorimetric studies is dependent on concentration and heating rate (*27*). Heat-stable dimers, trimers, and tetramers of BLG have been observed at all stages of denaturation (*28*, *29*). These experiments prove the existence of oligomers and larger aggregates as necessary intermediate stages in heat-induced aggregation (*30*).

Six different genetic variants of bovine BLG have been identified (*31*). The most prevalent are variants A and B, which differ only at positions 64 (Asp → Gly) and 118 (Val → Ala).

* Address correspondence to this author at SIK-The Swedish Institute for Food and Biotechnology, P.O. Box 5401, S-402 29 Göteborg, Sweden (telephone +46-31-3355604; fax +46-31-833782; e-mail ben@sik.se).
† SIK-The Swedish Institute for Food and Biotechnology.
‡ Sahlgrenska Academy at Göteborg University.
§ Karolinska Institutet.

10.1021/jf0304371 CCC: $27.50
Published on Web 02/14/2004

These variants behave differently in heat-induced denaturation and aggregation processes (*19*, *22*). This might be expected to cause differences in allergenicity during heating.

The antigenic and allergenic epitopes of BLG have been extensively analyzed (*32*−*37*). Seló et al. (*35*) identified three major allergenic epitopes and also found a number of other epitopes scattered along the BLG sequence. They also pointed at the great variability and heterogeneity of the human IgE response against BLG. Thermal processing not only takes away epitope structures, it may also create new ones (*38*). A combination of heat treatment and proteolysis leads to digestion of transiently exposed regions. This leads to the destruction of most epitopic sites (*39*).

The denaturation of whey proteins can be followed by changes in antibody binding (*40*). In a recent study, Clement et al. (*41*) found two epitopes characteristic of the native BLG structure. By means of monoclonal antibodies Kaminogawa et al. (*42*) were able to monitor local conformational changes during denaturation. A set of murine monoclonal antibodies against BLG B could discriminate between a fresh solution and a solution that had been stored for 2 days at 4 °C and between BLG that had been purified by salt fractionation and by gel filtration (*43*). Negroni et al. (*44*) developed immunometric assays based on monoclonal antibodies that are specific to native and heat-denatured BLG.

The influence of individual epitopes on the clinical manifestation of allergy is still an open question (*45*), but there might be interesting differences between cases with transient and persistent allergy toward cow's milk connected with conformational and sequential epitopes (*46*).

The methodology first described as the radioallergosorbent test (RAST) (*47*, *48*) is well-established to determine IgE antibodies to allergens as an adjunct tool in allergy diagnosis. To further reveal the extent and precision of IgE antibody binding, an inhibition technique was established (*49*). Using such a procedure the relative IgE binding capability of a protein in relation to other allergenic proteins was characterized. The best established method using a solid phase with a very high capacity to bind antibodies (*50*) was chosen in this work because it offers the accuracy and reproducibility with high precision necessary to determine the effect on IgE binding in BLG of different heat treatments with minimal influence of other antibodies and because the results were easily interpretable as IgE concentration in units per milliliter.

We studied the effect of heating BLG solutions, both as the native mixture of BLG genetic variants and the separated forms A and B, on their ability to bind to IgE antibodies in patient sera. We also measured the IgE binding to BLG in heated whole milk samples and in some fermented dairy products such as yogurt and other types of acidified milk products.

MATERIALS AND METHODS

Reagents and Materials. BLG from bovine milk was purchased from Sigma Chemical Co. (St. Louis, MO). The genetic variants of BLG (A and B) were studied both as a mixture and isolated. Standard whole milk (3% fat), low-fat milk (0.5% fat), yogurt (3% fat), and sour milk (3% fat) (Arla Foods, Gothenburg, Sweden) were used. The buffer was a 0.01 M standard phosphate-buffered saline solution (PBS), pH 7.4. Specific IgE negative control was purchased from Pharmacia Diagnostics AB (Uppsala, Sweden).

Serum Samples. Serum samples from subjects sensitized to bovine milk were collected from Sahlgrenska University Hospital (Göteborg, Sweden). All individuals had elevated levels of IgE to cow's milk, >3.5 kU_A/L, determined by a specific IgE detection test (CAP System FEIA, Pharmacia Diagnostics).

Table 1. Specific IgE against Cow's Milk Proteins in Serum Pools

serum pool	specific IgE (kU/L)				
	caseins	β-lactoglobulin	α-lactalbumin	BSA	lactoferrin
1	22.1	8.0	5.7	0.8	0.4
2	13.2	4.6	4.2	2.9	2.8

The serum samples were pooled, in order to have consistent samples for repeated testing, and used in the subsequent antibody inhibition experiments. Serum pool 1: included samples from 25 patients, aged from 3 months to 42 years. Serum pool 2 comprised samples from 54 patients, aged from 3 months to 51 years. The single serum samples used in the pool were 0.1−2.5 mL in volume (total volumes of pools 1 and 2 were 23 and 35 mL, respectively). From their specific milk IgE antibody concentrations, no single patient was expected to dominate the final inhibition pattern of the pools. In addition, individual sera from patients having a clinical diagnosis of allergic reactions to cow's milk, 13 adults and 20 children, were collected. Three of the children had developed a tolerance toward milk when the specimens were taken. The specific IgE antibodies toward the major cow's milk allergens were determined both for pools of sera and for individual samples.

Processing Experiments. Solutions of BLG in aliquots of 2 mL were treated with heat, at different degrees and for different times, in a water bath (Lauda M3). After heat treatment, the samples were cooled to 25 °C. The protein concentrations were 0.2 and 1.6 mg/mL in experiments with serum pools 1 and 2, respectively.

IgE Binding Inhibition Studies. Inhibition of IgE binding was done mainly as described by Yman et al. (*48*) using the Pharmacia CAP system FEIA. The protein was dissolved in PBS, pH 7.4. The samples were divided into two parts, one that was left untreated and the other heat-treated. The protein solutions were then diluted further in PBS, 1/3 serial dilutions, with concentrations ranging from 1620 to 0.082 μg/mL. Sixty microliters of each protein solution was mixed with 60 μL of serum and incubated for 1 h at 25 °C. Serum pool mixed with PBS was used as a control of total anti-BLG IgE concentration, and IgE-negative serum was used as a control.

The concentration of unbound specific IgE antibody was plotted against allergen concentration. The values were given in kU_A/L (1 unit = 2.44 ng). The protein concentration needed for 50% inhibition (called the IC_{50}) was calculated from the inhibition curves.

The milk products were diluted, used in inhibition, and evaluated in the same way as described above. All experiments were done three times, and the mean values with standard deviation are used in the figures and tables. The coefficients of variation were found to range from 12 to 18% in the different serum pools.

The study was approved by the Research Ethics Committee, Göteborg University.

Statistics. Student's *t* test for paired data, one-sided, was used to compare IC_{50} values before and after heat treatment of the samples. For comparing the genetic variants of BLG, Student's *t* test for nonpaired data, two-sided, was used. When the commercially processed fermented and nonfermented samples were compared, Student's *t* test for nonpaired data, one-sided, was used.

RESULTS

IgE Spectra. The specific IgE spectra of the serum pools against the major milk proteins tested (casein, BLG, α-lactalbumin, bovine serum albumin, and lactoferrin) are given in **Table 1**. When the individual IgE spectra were determined against these proteins in serum samples from 33 other patients, not included in the serum pools, most of the adults had IgE antibodies against two or three of the proteins (11/13), whereas most of the children had IgE against three or four proteins (15/20). This indicated that the serum pools used were representative in demonstrating the multispecificity most often present in individual serum samples from cow's milk allergic patients (*16*).

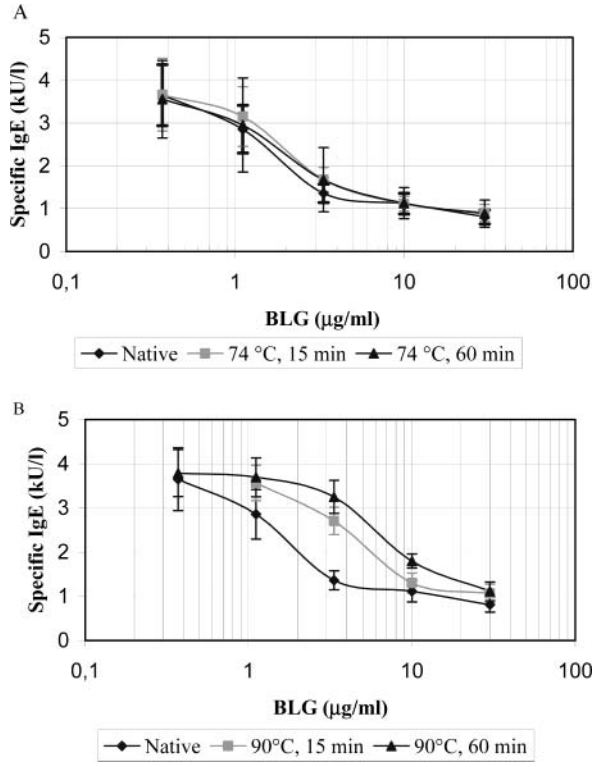

Figure 1. Inhibition of anti-β-lactoglobulin IgE in serum pool 1; comparison between native and heat-treated β-lactoglobulin: (**A**) 74 °C; (**B**) 90 °C. Results are presented as mean values ± 1 SD.

Table 2. β-Lactoglobulin Concentration at 50% Inhibition of IgE Binding (IC_{50}) in Experiments with Pure β-Lactoglobulin and Milk Products

β-lactoglobulin variant/milk product	serum pool	IC_{50} (μg/mL) MV[a]	1 SD[b]	significance[c]
β-lactoglobulin (A + B)	1	2.03	0.24	
β-lactoglobulin (A + B), 74 °C, 60 min	1	3.59	0.75	0.032 (*1–2*)
β-lactoglobulin (A + B), 90 °C, 60 min	1	8.45	2.10	0.014 (*1–3*)
				0.022 (*2–3*)
β-lactoglobulin (A + B)	2	2.40	0.42	
β-lactoglobulin A	2	5.10	0.38	
β-lactoglobulin B	2	2.56	0.12	0.004 (*5–6*)
β-lactoglobulin A, 90 °C, 60 min	2	49.7	19.7	
β-lactoglobulin B, 90 °C, 60 min	2	75.7	41.0	>0.20 (*7–8*)
low-fat milk	2	1.41	0.19	
low-fat milk, 90 °C, 60 min	2	245	51	0.007 (*9–10*)
low-fat milk, boiled for 15 min	2	109	31	0.014 (*9–11*)
whole milk	2	1.85	0.15	
fermented sour milk	2	37.3	9.3	0.01 (*12–13*)
yogurt	2	51.4	8.2	<0.001 (*12–14*)

[a] Mean values of three independent experiments. [b] Standard deviation. [c] Significance of differences between variants, *p*-values.

Inhibition Studies. The inhibition studies with native and heat-modified BLG solutions showed that, when BLG was heated for 60 min at 74 °C (**Figure 1A**), a higher concentration was needed for inhibition than in the case of the native control, which indicated impaired IgE antibody binding. For the BLG heated for 60 min at 90 °C (**Figure 1B**) the difference compared with the native was more pronounced, but using a higher concentration still gave pronounced inhibition. A comparison of the IC_{50} values (**Table 2**) showed that there was a significant difference for both of the heat-modified solutions, 60 min at 74 °C ($p = 0.032$) and 60 min at 90 °C ($p = 0.014$), in comparison with the native one. The difference between the heat-modified solutions was also significant ($p = 0.022$).

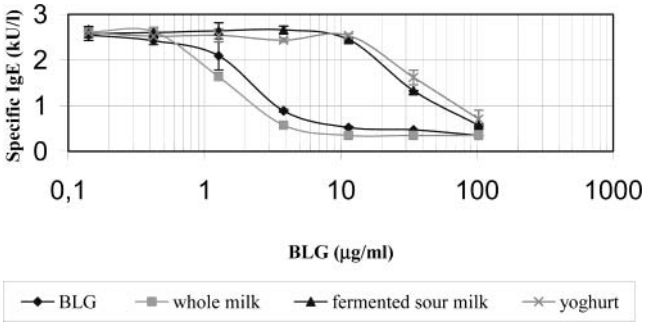

Figure 2. Inhibition of anti-β-lactoglobulin IgE in serum pool 2; comparison between whole milk, fermented sour milk, and yogurt. Results are presented as mean values ± 1 SD.

The genetic variants of BLG, A and B (**Table 2**), showed a relatively small but significant difference ($p = 0.004$) in IgE binding between the two in native form. After heat treatment, however, there was no significant difference between the genetic variants ($p > 0.20$).

The inhibition of the IgE binding after heat treatment (90 °C, 60 min) was also significantly decreased in low-fat milk ($p = 0.007$) (**Table 2**).

When low-fat milk was boiled at 100 °C for 15 min, there was still an inhibition of IgE binding but at a 100 times higher concentration than for untreated low-fat milk. There was a significant difference between the IC_{50} values (see **Table 2**; $p = 0.014$), although the maximum inhibition was still at a similar level as for native samples.

To study fully denatured BLG, milk was sterilized in an autoclave at 132 °C for 5 min (results not shown). Also, in this case there were some signs of inhibition, although starting at concentrations >100 μg/mL and with an IC_{50} > 925 μg/mL.

Complex products such as yogurt and fermented sour milk showed a low inhibition of IgE binding as compared to whole milk (**Figure 2**). A comparison of the IC_{50} values showed that the difference was significant (yogurt, $p < 0.001$; sour milk, $p = 0.01$) (**Table 2**).

DISCUSSION

The commonly used heat treatments for milk during processing, such as pasteurization, preheating, and sterilization, cause denaturation of the whey proteins. The denaturation temperature of BLG is measured to be 70 °C, with a residual structure unfolding near 130 °C (*21*, *51*). The reaction kinetics of the irreversible denaturation of BLG has been extensively studied (*52*). For irreversible denaturation, the time needed is dependent on temperature—at a lower temperature such as 70 °C, 30% denaturation requires >30 min; at 90 °C such a time gives >90% denaturation (*52*). The most extreme heat treatment in this study was sufficient to cause considerable denaturation of the protein structure; still there was some inhibition at concentrations >100 μg/mL.

Our results showed that there is impaired IgE binding to BLG after heat treatment, both in pure BLG solutions and in heated milk. However, it was not possible to totally eliminate IgE binding epitopes. There was always binding at some higher concentration, and the same inhibition as for untreated samples seems to be achieved for the heat-treated samples only at higher concentrations. One explanation might be that all of the different epitopes were still present but were available in lower concentrations or had been partly modified in such a way that although

they bound IgE antibodies, the binding was of lower affinity. Another explanation would be that the heating has destroyed some of the epitopes, as well as unmasked or created new ones.

Van Beresteijn et al. (*53*) reported no difference in IgE titer between the two genetic variants, A and B, in their native form. Our findings indicated a small but significant difference in IgE binding ability, although the clinical significance of this difference was not verified. One of the positions—64—of the two amino acids distinguishing between the A and B forms is situated outside the main antigenic and IgE-binding epitopes according to the literature (*35*, *37*).

Our experiments with BLG solutions were done at lower concentrations than those used in food systems, but they generally illustrated the high heat stability of the protein and its allergic epitopes. Heating milk to decrease its allergenicity is not a straightforward process; some data in the literature point at a high stability of allergenic properties of the whey proteins and great individual variation (*3*, *54*, *55*). There is no clear evidence in the literature for a significantly decreased clinical effect on allergy after heat treatment.

BLG plays an important role when heating whole milk, but the interaction with other milk proteins, especially the caseins, and the factors mentioned above result in the overall heat-stable profile of milk. In whole milk, BLG can bind to casein micelles (*51*) and coaggregate with α-lactalbumin, which has an extensive effect on surface availability (*56*).

In hypoallergenic formulas, both heat treatment and hydrolysis are used to decrease allergenicity. Despite this, the formulas may cause a reaction in some allergic children (*57*, *58*). In a recent investigation of 12 hydrolyzed milk formulas, residual antigenic BLG was found in all products by ELISA and immunoblotting (*59*).

The allergenic epitopes of BLG seemed to be extremely heat stable (90 °C, 60 min). This may suggest that linear epitopes are the most important epitopes in the patients studied here (*37*). It is also reported that heating BLG exposes so-called hidden epitopes (*38*, *60*), which may be one explanation for the IgE binding even after heat treatment.

One of the major IgE binding epitopes on BLG is situated in the region containing the free SH group in Cys 121 (*35*, *37*). This SH group is known to be exposed after moderate heat treatment (*24*) and oxidized at the high temperatures used in this study. However, in our experiments we observed IgE binding at a final level after heat treatment similar to that found in native BLG. This suggested that the allergenic epitope closest to this SH group is most probably still identifiable by the IgE antibodies.

The low inhibition capacity of fermented milk and yogurt, that is, the decrease in IgE binding ability, can be explained by a number of factors. Both the fermented sour milk and the yogurt are treated with high pasteurization, in contrast to whole milk, witch is only low pasteurized. The BLG in yogurt and fermented sour milk may be structurally modified by the starter culture enzyme activity in such a way that it affects the allergenic epitopes. Jedrychowski and Wróblewska (*12*) found that acid fermentation reduced the antigenicity of BLG considerably. The BLG might not be available for IgE binding in yogurt due to association with the protein gel structure or other aggregates.

This study made no comparison between the transient and the persistent forms of cow's milk allergy to identify whether the former had a higher degree of IgE binding to conformational epitopes, which might be inhibited by heat treatment. This can be explored in further studies. It would also be of interest to study the combined effects of heat treatment and proteolysis in fermented milk products.

ABBREVATIONS USED

BLG, β-lactoglobulin; FEIA, fluoro enzyme immunoassay; IC_{50}, protein concentration needed for 50% inhibition; PBS, phosphate-buffered saline solution.

ACKNOWLEDGMENT

We thank all the staff involved at the Clinic of Allergy, the Laboratory of Allergy and the Clinic of Paediatric Allergy at Sahlgrenska University Hospital for their help in finding cow's milk allergic patients and collecting material. We thank Anne-Li Karlsson and Göran Åkesson for their technical assistance.

Supporting Information Available: Specific IgE in serum samples from adults and children (table) and inhibition of anti-β-lactoglobulin IgE in serum pool 2 (figures). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Besler, M.; Steinhart, H.; Paschke, A. Stability of food allergens and allergenicity of processed foods. *J. Chromatogr. B* **2001**, *756*, 207−228.

(2) Kocho, K. Milk allergy with special reference to a histopathologico-anatomic study of its prevention. II. Prevention of intestinal hypersensitivity by means of digestive ferments and heating of milk. *Nippon Shonika Gakkai Zasshi* **1966**, *70*, 606−614.

(3) Baldo, B. Milk allergies. *Aust. J. Dairy Technol.* **1984**, *39*, 120−128.

(4) Gjesing, B.; Österballe, O.; Schwartz, B.; Wahn, U.; Löwenstein, H. Allergen-specific IgE antibodies against antigenic components in cow milk and milk substitutes. *Allergy* **1986**, *41*, 51−56.

(5) Nörgaard, A.; Bernard, H.; Wal, J. M.; Peltre, G.; Skov, P. S.; Poulsen, L. K.; Bindslev-Jensen, C. Allergenicity of individual cow milk proteins in DBPCFC-positive milk allergic adults. *J. Allergy Clin. Immunol.* **1996**, *97*, 237.

(6) Anderson, K. J.; McLaughlan, P.; Devey, M. E.; Coombs, R. R. Anaphylactic sensitivity of guinea-pigs drinking different preparation of cow's milk and infant formulae. *Clin. Exp. Immunol.* **1979**, *35*, 454−461.

(7) McLaughlan, P.; Anderson, K. J.; Widdowson, E. M.; Coombs, R. R. Effect of heat on the anaphylactic-sensitising capacity of cow's milk, goat's milk, and various infant formulae fed to guinea-pigs. *Arch. Dis. Child* **1981**, *56*, 165−171.

(8) Kilshaw, P. J.; Heppell, L. M.; Ford, J. E. Effects of heat treatment of cow's milk and whey on the nutritional quality and antigenic properties. *Arch. Dis. Child* **1982**, *57*, 842−847.

(9) Heppell, L. M.; Cant, A. J.; Kilshaw, P. J. Reduction in the antigenicity of whey proteins by heat treatment: a possible strategy for producing a hypoallergenic infant milk formula. *Br. J. Nutr.* **1984**, *51*, 29−36.

(10) Guesry, P. R.; Secretin, M. C.; Jost, R.; Pahud, J. J.; Monti, J. C. Milk formulas in the prevention of food allergy. *Allergy Proc.* **1991**, *12*, 221−226.

(11) Lee, Y. H. Food-processing approaches to altering allergenic potential of milk-based formula. *J. Pediatr.* **1992**, *121*, 47−50.

(12) Jedrychowski, L.; Wróblewska, B. Reduction of the antigenicity of whey proteins by lactic acid fermentation. *Food Agric. Immunol.* **1999**, *11*, 91−99.

(13) Sampson, H. A.; Ho, D. G. Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. *J. Allergy Clin. Immunol.* **1997**, *100*, 444−451.

(14) Osterballe, M.; Bindslev-Jensen, C. Threshold levels in food challenge and specific IgE in patients with egg allergy: Is there a relationship? *J. Allergy Clin. Immunol.* **2003**, *112*, 196−201.

(15) Boyano Martínez, T.; García-Ara, C.; Díaz-Pena, J. M.; Muños, F. M.; García Sánchez, G.; Esteban, M. M. Validity of specific IgE antibodies in children with egg allergy. *Clin. Exp. Allergy* **2001**, *31*, 1464−1469.
(16) Wal, J. M.; Bernard, H.; Créminon, C.; Hamberger, C.; David, B.; Peltre, G. Cow's milk allergy: the humoral immune response to eight purified allergens. In *Advances in Mucosal Immunology*; Mestecky, J., et al., Eds.; Plenum Press: New York, 1995; pp 879−881.
(17) Wal, J. M. Structure and function of milk allergens. *Allergy* **2001**, *56* (Suppl. 67), 35−38.
(18) Brownlow, S.; Marais Cabral, J. H.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C. T.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution−still an enigmatic lipocalin. *Structure* **1997**, *5*, 481−495.
(19) Sawyer, L.; Kontopidis, G.; Wu, S.-Y. β-Lactoglobulin−a three-dimensional perspective. *Int. J. Food Sci. Technol.* **1999**, *34*, 409−418.
(20) Sawyer, L.; Kontopidis, G. The core lipocalin, bovine β-lactoglobulin. *Biochim. Biophys. Acta* **2000**, *1482*, 136−148.
(21) de Wit, J. N. Structure and functional behaviour of whey proteins. *Neth. Milk Dairy J.* **1981**, *35*, 47−64.
(22) Elofsson, U. β-Lactoglobulins in solution and at the solid/liquid interface. Thesis, Lund University, Lund, Sweden, 1996.
(23) Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106−112.
(24) Ekstrand, B.; Mullan, W. M. A.; Waterhouse, A. Inhibition of the antibacterial lactoperoxidase-thiocyanate-hydrogen peroxide system by heat-treated milk. *J. Food Prot.* **1985**, *48*, 494−498.
(25) Garcia-Hernandez, E.; Hernandez-Arana, A.; Zubillaga, R. A.; Rojo-Dominguez, A. Spectroscopic and thermodynamic evidence for a complex denaturation mechanism of bovine β-lactoglobulin A. *Biochem. Mol. Biol. Int.* **1998**, *45*, 761−768.
(26) Cairoli, S.; Iametti, S.; Bonomi, F. Reversible and irreversible modifications of beta-lactoglobulin upon exposure to heat. *J. Protein Chem.* **1994**, *13*, 347−354.
(27) Qi, X. L.; Brownlow, S.; Holt, C.; Sellers, P. Thermal denaturation of β-lactoglobulin: effect of protein concentration at pH 6.75 and 8.05. *Biochim. Biophys. Acta* **1995**, *1248*, 43−49.
(28) Fang, Y.; Dalgleish, D. G. Conformation of β-lactoglobulin studied by FTIR: Effect of pH, temperature, and adsorption to the oil−water interface. *J. Colloid Interface Sci.* **1997**, *196*, 292−298.
(29) Bauer, R.; Carrotta, R.; Rischel, C.; Ogendal, L. Characterization and isolation of intermediates in β-lactoglobulin heat aggregation at high pH. *Biophys. J.* **2000**, *79*, 1030−1038.
(30) Carrotta, R.; Bauer, R.; Waninge, R.; Rischel, C. Conformational characterization of oligomeric intermediates and aggregates in β-lactoglobulin heat aggregation. *Protein Sci.* **2001**, *10*, 1312−1318.
(31) Hambling, S. G.; McAlpine, A. S.; Sawyer, L. β-lactoglobulin. In *Advances in Dairy Chemistry−I*; Fox, P. F., Ed.; Elsevier: Amsterdam, The Netherlands, 1992; pp 141−190.
(32) Ball, G.; Shelton, M. J.; Walsh, B. J.; Hill, D. J.; Hosking, C. S.; Howden, M. E. H. A major continuous allergenic epitope of bovine β-lactoglobulin recognized by human IgE binding. *Clin. Exp. Allergy* **1994**, *24*, 758−764.
(33) Williams, S. C.; Badley, R. A.; Davis, P. J.; Puijk, W. C.; Meloen, R. H. Identification of epitopes within beta lactoglobulin recognised by polyclonal antibodies using phage display and PEPSCAN. *J. Immunol. Methods* **1998**, *213*, 1−17.
(34) Sélo, I.; Négroni, L.; Créminon, C.; Yvon, M.; Peltre, G.; Wal, J.-M. Allergy to bovine β-lactoglobulin: Specificity of human IgE using cyanogen bromide-derived peptides. *Int. Arch. Allergy Immunol.* **1998**, *117*, 20−28.
(35) Sélo, I.; Clément, G.; Bernard, H.; Chatel, J.-M.; Créminon, C.; Peltre, G.; Wal, J.-M. Allergy to bovine β-lactoglobulin: Specificity of human IgE to tryptic peptides. *Clin. Exp. Allergy* **1999**, *29*, 1055−1063.
(36) Miller, K.; Meredith, C.; Selo, I.; Wal, J. M. Allergy to bovine beta-lactoglobulin: specificity of immunoglobulin E generated in the Brown Norway rat to tryptic and synthetic peptides. *Clin. Exp. Allergy* **1999**, *29*, 1696−1704.
(37) Järvinen, K. M.; Chatchatee, P.; Bardina, L.; Beyer, L.; Sampson, H. A. IgE and IgG binding epitopes on alpha-lactalbumin and β-lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.* **2001**, *126*, 111−118.
(38) Davis, P. J.; Williams, S. C. Protein modification by thermal processing. *Allergy* **1998**, *53* (Suppl. 46), 102−105.
(39) Iametti, S.; Rasmussen, P.; Frökiaer, H.; Ferranti, P.; Addeo, F.; Bonomi, F. Proteolysis of bovine β-lactoglobulin during thermal treatment in subdenaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. *Eur. J. Biochem.* **2002**, *269*, 1362−1372.
(40) Levieux, D. Heat denaturation of whey proteins. Comparative studies with physical and immunological methods. *Ann. Rech. Vet.* **1980**, *11*, 89−97.
(41) Clement, G.; Boquet, D.; Frobert, Y.; Bernard, H.; Negroni, L.; Chatel, J. M.; Adel-Patient, K.; Creminon, C.; Wal, J. M.; Grassi, J. Epitopic characterization of native beta-lactoglobulin. *J. Immunol. Methods* **2002**, *266*, 67−78.
(42) Kaminogawa, S.; Shimizu, M.; Ametani, A.; Hattori, M.; Ando, O.; Hachimura, S.; Nakamura, Y.; Totsuka, M.; Yamauchi, K. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. *Biochim. Biopohys. Acta* **1989**, *998*, 50−56.
(43) Venien, A.; Levieux, D.; Astier, C.; Briand, L.; Chobert, J. M.; Haertle, T. Production and epitopic characterization of monoclonal antibodies against bovine β-lactoglobulin. *J. Dairy Sci.* **1997**, *80*, 1977−1987.
(44) Negroni, L.; Bernard, H.; Clement, G.; Chatel, J. M.; Brune, P.; Frobert, Y.; Wal, J. M.; Grassi, J. Two-site enzyme immunometric assays for determination of native and denatured β-lactoglobulin. *J. Immunol. Methods* **1998**, *220*, 25−37.
(45) Heinzmann, A.; Blattman, S.; Spuergin, P.; Forster, J.; Deichman, K. A. The recognition pattern of sequential B cell epitopes of β-lactoglobulin does not vary with the clinical manifestations of cow's milk allergy. *Int. Arch. Allergy Immunol.* **1999**, *120*, 280−286.
(46) Järvinen, K. M.; Beyer, K.; Vila, L.; Chatchatee, P.; Busse, P. J.; Sampson, H. A. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J. Allergy Clin. Immunol.* **2002**, *110*, 293−297.
(47) Wide, L.; Bennich, H.; Johansson, S. G. Diagnosis of allergy by an in vitro test for allergen antibodies. *Lancet* **1967**, *2*, 1105−1107.
(48) Yman, L.; Ponterius, G.; Brandt, R. Rast-based allergen assay methods. *Dev. Biol. Stand.* **1975**, *29*, 151−165.
(49) Schröder, H.; Yman, L. Standardization of the RAST inhibition assay. *Allergy* **1980**, *35*, 234−236.
(50) Yman, L. Standardization of IgE antibody assay. *J. Int. Fed. Clin. Chem.* **1991**, *3*, 198−203.
(51) Walstra, P.; Jenness, R. *Dairy Chemistry and Physics*; Wiley: New York, 1984.
(52) Dannenberg, F.; Kessler, H.-G. Application of reaction kinetics to the denaturation of whey proteins in heated milk. *Milchwissenschaft* **1988**, *43*, 3−7.
(53) van Beresteijn, E. C. H.; Meijer, R. J. G. M.; Schmidt, D. G. Residual antigenicity of hypoallergenic infant formulas and the occurrence of milk-specific IgE antibodies in patients with clinical allergy. *J. Allergy Clin. Immunol.* **1995**, *96*, 365−374.
(54) Jost, R.; Fritsche, R.; Pahud, J. J. Reduction of milk protein allergenicity through processing. *Bibl. Nutr. Diet.* **1991**, *48*, 127−137.
(55) Jost, R.; Monti, J. C.; Pahud, J. J. Reduction of whey protein allergenicity by processing. *Adv. Exp. Med. Biol.* **1991**, *289*, 309−320.

(56) Bertrand-Harb, C.; Baday, A.; Dalgalarrondo, M.; Chobert, J. M.; Haertle, T. Thermal modification of structure and co-denaturation of α-lactalbumin and β-lactoglobulin induce changes of solubility and susceptibility to proteases. *Nahrung* **2002**, *46*, 283−289.

(57) Cantani, A.; Micera, M. Immunogenicity of hydrolysate formulas in children (part 1). Analysis of 202 reactions. *J. Invest. Allergol. Clin. Immunol.* **2000**, *10*, 261−276.

(58) Cantani, A.; Micera, M. Immunogenicity of hydrolysate formulas in children (part 2): 41 case reports. *J. Invest. Allergol. Clin. Immunol.* **2001**, *11*, 21−26.

(59) Rosendal, A.; Barkholt, V. Detection of potentially allergenic material in 12 hydrolyzed milk formulas. *J. Dairy Sci.* **2000**, *83*, 2200−2210.

(60) Haddad, Z. H.; Kalra, V.; Verma, S. IgE antibodies to peptic and peptic-tryptic digests of β-lactoglobulin: significance in food hypersensitivity. *Ann. Allergy* **1979**, *42*, 368−371.

Received for review June 12, 2003. Revised manuscript received December 3, 2003. Accepted December 30, 2003. We gratefully acknowledge the financial support of the KK Foundation and the VL Foundation R&D, Lidköping, Sweden, and the company AnalyCen Nordic AB.

JF0304371