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Modification of IgE Binding to β-Lactoglobulin by Fermentation and Proteolysis of Cow's Milk

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The effect of fermentation by *Lactobacilli* and of proteolytic hydrolysis of whole milk on the IgE binding ability of β-lactoglobulin was studied using an ELISA inhibition assay. Sera from nine adult milk allergic patients were tested. The individual sera showed a similar inhibition pattern in the changes during fermentation and proteolysis. The degradation of β-lactoglobulin was studied with liquid chromatography. In general, fermentation with *Lactobacilli* gave little effect on IgE binding, even though chromatography data showed a gradual degradation of β-lactoglobulin. Proteolysis with trypsin, however, gave extensive degradation of β-lactoglobulin and strongly decreased IgE binding. In addition, we measured the inhibition pattern of β-lactoglobulin in various selected commercially available fermented milk products. These showed an IgE binding capacity similar to that of nonfermented high pasteurized milk.

KEYWORDS: **Milk; allergenicity; IgE antibody; IgE antibody inhibition; fermentation; lactic acid bacteria; trypsin; proteolysis; β-lactoglobulin**

INTRODUCTION

It is important to gain better knowledge of the influence of food processing on the structure of food allergens and the allergenic potential of food products (*1*). It has long been known that food processing can affect the clinical allergenic potential of food (*2*). The allergenic potential of a protein can be interpreted as either its ability to sensitize (induce IgE antibodies), its ability to cause symptoms in an already sensitized subject, or simply its capacity to bind IgE antibodies (*3*). This study investigated IgE binding to β-lactoglobulin in treated milk products.

β-Lactoglobulin (BLG), as all of the major proteins in milk, has an allergenic potential (*4−7*). Our previously published results (*8*) show that heat treatment significantly decreases but does not eliminate the IgE binding ability of BLG.

A certain cutoff level of milk-specific IgE has been shown to predict an allergic reaction with a certainty of 90−95% (*9*, *10*). However, the severity of a patient's reaction has no correlation with specific IgE levels (*9*). Boyano Martinez et al. (*11*) demonstrated that some egg allergic children tolerated cooked egg white but not raw and that those children had lower levels of specific IgE than children that reacted to both forms of egg white. In allergic patients with low levels of specific IgE or patients evolving tolerance, a reduction in the allergenicity of the food might allow some consumption. However, it must be emphasized that any reintroduction of an allergenic food must be approached very carefully and under physician's supervision.

A number of studies have been published on the effects of proteolysis in combination with heat treatment on the immunoreactivity of milk proteins (*12−14*). Bonomi et al. (*15*) noted a relation between proteolytic degradation during heat treatment and other physical treatments such as high pressure and decreased immunoreactivity of BLG. However, several of the commercially available hydrolyzed milk formulas still contain immunoreactive material (*16−19*). We therefore wanted to quantify the IgE binding of BLG during proteolytic treatment of milk.

We also wished to evaluate how fermentation processes affect the IgE binding ability of BLG in milk. In a previous paper, we reported some preliminary data on IgE binding to BLG in some fermented dairy products such as yogurt and other types of acidified milk products (*8*). This prompted the question of the structural changes in fermented milk products in general. Jedrychowski and Wroblewska (*20*) studied the effect of lactic acid fermentation on antigenicity and found a reduced binding ability to rabbit polyclonal antibodies. When milk allergic

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patients were skin tested with whey samples from fermented whole milk, however, the reaction was only slightly attenuated. One of the *Lactobacillus* strains that gave a reduction in the skin test was *L. helveticus*.

The proteolytic activity of lactic acid bacteria has been reported to consist of an extracellularly located system that releases a fraction of oligopeptides in the range of 4−8 amino acids, which are transported into the cells and further degraded by peptidases (*21*, *22*). However, there is a great diversity in the specificity of the proteinases of the starter cultures (*23*).

The antigenic and allergenic epitopes of BLG have been extensively analyzed (*24*−*29*). Sélo et al. (*30*) identified three major allergenic epitopes and also found a number of other epitopes scattered along the BLG sequence. They furthermore indicated the great variability and heterogeneity of the human IgE response against BLG.

In this work, we analyzed IgE positive sera from individual cow's milk allergic patients and their ability to bind to BLG after the fermentation of milk with lactic acid bacteria and after proteolysis with trypsin. The protein degradation was analyzed with liquid chromatography to relate the chemical degree of proteolysis to the loss in IgE binding ability.

The IgE binding ability of BLG in several commercial fermented milk products was also investigated.

MATERIALS AND METHODS

Serum Samples. Individual serum samples from 19 adult patients with a clinical diagnosis of cow's milk allergy were collected from Sahlgrenska University Hospital (Göteborg, Sweden). All patients had specific IgE antibodies toward milk ≥0.99 kU_A/L (1 unit = 2.44 ng) determined by the FEIA-CAP System (Pharmacia Diagnostics, Uppsala, Sweden). The presence of specific IgE antibodies toward the major cow's milk allergens was determined in each serum. Fourteen individuals had elevated levels of specific IgE to BLG of >0.35 kU_A/L. Nine of these sera with a BLG-specific IgE level exceeding 5 kU_A/L were used in this study for ELISA inhibition. The nine sera had specific IgE antibodies toward milk ≥11.8 kU_A/L, a IgE level exceeding the cutoff value of 5.8 kU_A/L suggested by Sampson and Ho (*9*). Serum from one nonallergic person was used as a negative control.

Processing Cow's Milk. Milk from the Kungsängen experimental herd at the Swedish Agricultural University was collected fresh after morning milking. The milk samples were thereafter incubated at 45 °C and defatted to a fat concentration of <0.1%. This was followed by heating at 72 °C for 30 s in a plate heat exchanger to simulate the industrial process of pasteurization to avoid undesirable microbial growth. Some milk samples were heated at 90 °C for 4 min, similar to the industrial process followed in making fermented milk products. Immediately after heat treatment, milk was rapidly cooled to the storage temperature of 8 °C. After 2 h of cold storage, the skim milk was incubated at 30 °C for 60 min and thereafter subjected to hydrolysis or fermentation. Three different lines of trials were carried out: proteolysis with 10 g/L trypsin (Merck Labs, Darmsdorf, Germany), and fermentation with *Lactobacillus helveticus* 174 (8 g/L) and with *L. helveticus* 192 (8 g/L; both *Lactobacilli* strains from Danisco AB, Norrköping, Sweden). The experiments were conducted at 30 °C for 212 h. Samples were taken from the substrate with certain hours intervals, ultracentrifuged (100 000*g* × 30 min), and immediately frozen and stored at −77 °C.

Commercial Milk Samples. Seven commercially available fermented milk products were purchased in food stores and were defatted and ultracentrifuged before they were used in the experiments.

Liquid Chromatography. Reversed-phase high performance liquid chromatography (RP-HPLC) was used to monitor the degradation of milk proteins during hydrolysis. The following experimental system was used: LaChrom Elite HPLC D-7000 system MerckHitachi (Hitachi Ltd., Tokyo, Japan) connected to a Hewlett-Packard Kayak XM-600 PC using a Thermo-Hypersil (Thermo Electron Corp., Waltham, MA) C18 RP-HPLC column. The method used included water−acetonitrile (90/10) as base buffer (buffer A) and acetonitrile as eluant (buffer B). Both A and B buffers contained 0.1% trifluoroacetic acid for pH adjustment (the final pH of both buffers was 2.5). The gradient used was 0% buffer B up to 10 min and 0−100% buffer B in a linear fashion between 10 and 60 min. Each run was followed by a double washing step (100% B followed by 100% A) to ensure proper equilibration of the column. Eluted peaks on the chromatogram were detected at 214 nm and integrated using D-7000 HPLC System Manager version NT 4.1 software from Merck Hitachi (Hitachi HiTech, USA). The samples for HPLC were prepared as follows: skim milk was mixed 1:1 with reduction buffer containing 8 M urea and 20 mM dithiothreitol (DTT) and incubated for 60 min at 30 °C. Prior to injecting 100 μL of sample onto the RP column, the reduced sample was diluted 3-fold in buffer A containing 6 M urea.

IgE Binding Inhibition and ELISA. All incubations were carried out at room temp. Microtiter plates, polystyrene MaxiSorp 96 U-well from NUNC (Roskilde, Denmark), were coated with 120 μL/well of BLG (L3908) from Sigma Chemical Co. (St. Louis, MO) at a concentration of 5 μg/mL in a 0.01 M standard phosphate buffered saline solution (PBS), pH 7.4, and incubated overnight. The milk samples from the experiments with *L. helveticus* and trypsin were reduced with DTT as described above. All milk samples were diluted in PBS, 1/3 serial dilutions. The milk samples hydrolyzed with trypsin were first mixed 1:1 with 5 g/L trypsin inhibitor (from soybean, type I-S, SIGMA T9003) in PBS before the serial dilutions. Patient serum was diluted between 1/2−1/7 in PBS to give an appropriate maximal absorbance. 60 μL of milk solution was mixed with 60 μL of serum sample and incubated for 1 h at 25 °C. The BLG-coated wells were washed three times with PBS with 0.05% Tween 20 (PBST). This washing step was followed after four subsequent incubations. 50 μL of serum samples, preincubated with milk solutions, was added to the wells and incubated for 1 h. 50 μL of monoclonal mouse anti-human IgE (SIGMA I 6510, clone GE-1) diluted 1/10 000 in PBST was added per well and incubated for 1 h. 50 μL of goat anti-mouse IgG conjugated with biotin (SIGMA B9904) diluted 1/50 000 in PBST was added per well and incubated for 1 h. 50 μL of streptavidin complexed with biotinylated horseradish peroxidase (K0377) from DakoCytomation (Glostrup, Denmark) diluted 1/2500 in PBST was added per well, and the plates were incubated for 30 min. After a final washing step, 50 μL of 3,3′,5,5′-tetramethylbenzidine liquid substrate system for ELISA (SIGMA) was added. The reaction was stopped after an appropriate time, 40−60 min, by adding 100 μL per well of 1 M HCl, and the absorbance was read at 450 nm with an ELISA-reader SPECTRAMAX 340 (Molecular Device Corp., Menlo Park, CA). Uninhibited serum sample was used as a control for the maximal absorbance of each serum. Serum from a nonallergic person was used as a negative control.

The absorbance of unbound specific IgE antibody was plotted against the original, estimated BLG concentration in the milk sample used. The protein concentration needed for 50% inhibition (IC_{50}) was calculated from the graph.

All samples were done in duplicates. The coefficients of variation of the IC_{50} values were 20%.

Statistics. The Wilcoxon signed rank test for paired data was used to compare the IC_{50} values before and after different treatments. A p value of <0.05 was considered statistically significant. The statistical analysis was performed with SPSS version 12.0.1 for Windows (SPSS, Chicago, IL).

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

RESULTS

Specific IgE. The specific IgE reactivities of the nine individual sera against milk and five milk proteins (casein, BLG, α-lactalbumin, bovine serum albumin, and lactoferrin) are given in **Table 1**. All of the sera had specific IgE against three or more of the milk proteins, which is in agreement with results of earlier studies (*4*−*6*).

Liquid Chromatography. RP-HPLC data for the samples taken from the fermentation and proteolysis experiments are

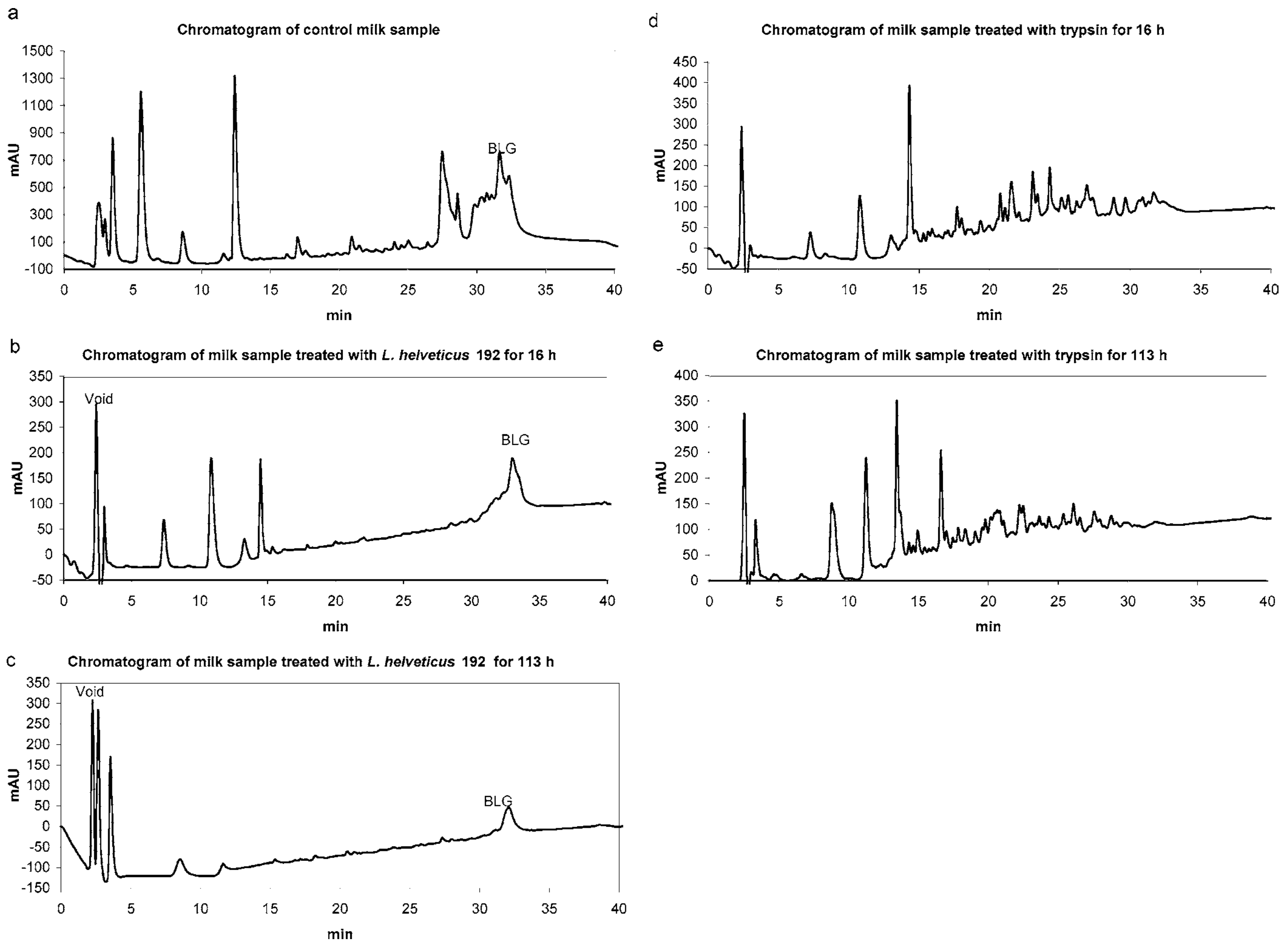

Figure 1. (**a**–**e**) RP-HPLC of samples from fermentation and proteolysis experiments. All samples were heat treated at 72 °C for 30 s before fermentation or proteolysis with trypsin.

Table 1. Specific IgE for Serum Samples Used in Inhibition Experiments

patient	age		kU/L					
$n = 9$	year	sex	milk	caseins	BLG	α-lactalbumin	BSA	lactoferrin
A4	47	F	>100	>100	16.5	44.3	<0.35	2.52
A7	36	F	45.8	49.4	15.6	<0.35	<0.35	<0.35
A11	29	F	14.5	16.0	5.75	<0.35	<0.35	<0.35
A12	26	F	28.1	21.2	21.3	19.5	<0.35	<0.35
A15	52	M	>100	25.6	19.9	<0.35	<0.35	<0.35
A17	24	M	11.8	11.7	14.6	<0.35	<0.35	<0.35
A18	22	M	>100	>100	32.4	24.5	0.47	<0.35
A19	29	M	15.6	11.7	5.3	3.5	<0.35	12.8
A21	19	F	53.8	55.0	8.61	34.2	<0.35	2.78

shown in **Figure 1a**−**e**. A change in the peptide pattern after fermentation and proteolysis can readily be observed. The amount and number of peptide peaks clearly increases with tryptic digestion; see **Figure 1d**,**e**. The BLG peak gradually decreases in the fermented sample (**Figure 1b**,**c**). The amount of intact BLG in relation to an untreated milk sample is given in **Table 2**. There is a gradual degradation in fermented samples, leaving 60% BLG intact after 16 h and 18% after 212 h. The tryptic digestion shows a rapid decline of intact BLG where only 12% intact BLG is left after 16 h of proteolysis with trypsin and trace amounts after 23 h.

Heat Treatment. The heat treatment at 72 °C for 30 s did not seem to have any effect on IgE binding as judged by the IC_{50} for one of the different sera. The IC_{50} for fresh milk was

Table 2. Chromatographic Evaluation of Intact BLG Content in Samples from Fermentation and Proteolysis Experiments[a]

	processing time (h)					
milk sample	16	23	40	113	137	212
L. helveticus 174	60	48	44	34	24	
L. helveticus 192	60	52	46	34	28	18
Trypsin	12	4	0	6	0	0

[a] Amount of intact BLG (%) in relation to a control sample. All milk samples were treated at 72 °C for 30 s.

0.20 and 0.22 μg/mL for the milk treated at 72 °C for 30 s. Heating at 90 °C for 4 min had a clear effect, reducing IgE binding and thereby increasing the IC_{50} to 4.96 μg/mL.

Reduction with DTT decreased the IgE binding capacity of BLG in the milk samples heat treated at 72 °C for 30 s, giving a significant increase of the IC_{50} concentration ($p < 0.01$) (**Tables 3** and **4**).

Fermentation Experiments. The fermented samples (*L. helveticus* 192) taken at different times of treatment were very similar in their IgE binding capacity of BLG (**Figure 2**), and there was no significant difference from control samples after 16 ($p = 0.11$) and 113 ($p > 0.20$) h of fermentation (**Table 3**). Surprisingly, there was no gradual decrease in IgE binding with time, as could be expected from the chromatography data (**Figure 1b**,**c**, **Table 2**).

Milk samples fermented with *L. helveticus* 174 showed results similar to those for *L. helveticus* 192 (data not shown).

Table 3. IC_{50} for Milk Samples Treated with *L. helveticus* 192[a]

patient $n = 9$	IC_{50}, μg/mL			
	not reduced and no fermentation	reduced		
		no fermentation	*L. helveticus* 8 mg/mL	
			16 h	113 h
A4	0.16	4.09	3.59	2.78
A7	0.42	2.82	3.03	2.82
A11	0.11	1.37	2.39	1.68
A12	0.57	4.86	5.92	4.67
A15	1.45	6.43	15.33	8.79
A17	0.16	1.44	3.25	3.0
A18	0.34	10.11	8.59	6.76
A19	0.22	2.55	3.54	3.15
A21	0.47	5.24	6.58	4.88

[a] All milk samples were treated at 72 °C for 30 s.

Table 4. IC_{50} for Milk Samples Treated with Trypsin[a]

patient $n = 9$	IC_{50}, μg/mL			
	not reduced and no trypsin	reduced		
		no trypsin	trypsin 10 mg/mL	
			16 h	113 h
A4	0.08	0.84	>400	>400
A7	0.48	4.60	9.20	6.00
A11	0.24	0.67	>400	>400
A12	0.26	2.04	392	292
A15	0.55	3.43	>400	>400
A17	0.09	0.88	>400	>400
A18	0.11	2.23	>400	>400
A19	0.22	2.08	>400	>400
A21	0.84	8.02	>400	>400

[a] All milk samples were treated at 72 °C for 30 s.

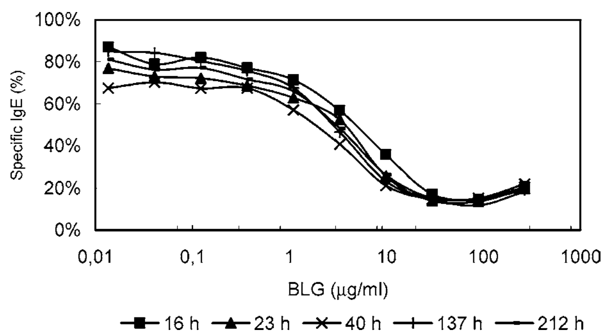

Figure 2. Inhibition of anti-β-lactoglobulin IgE in serum A15. A comparison between milk samples fermented with *L. helveticus* 192 for different periods of time.

Commercial Fermented Milk Products. Seven commercial fermented milk products were analyzed for their IgE binding capacity in one of the sera (**Figure 3**). They were all very similar, and no decrease in the IgE binding was observed as compared to a control heated at 90 °C for 4 min (high pasteurization), which is the standard heat treatment in commercial fermented milk products.

Proteolysis Experiments. Milk samples treated with trypsin were analyzed for IgE inhibition (**Figure 4**). Very little IgE binding ability remained after only 16 h, and the typical sinusoidal inhibition curve was no longer observable. A measurable IC_{50} was not achieved at a BLG concentration of 400 μg/mL for most of the sera tested; see **Table 4**. There was a significant difference as compared to the control, with a p-value

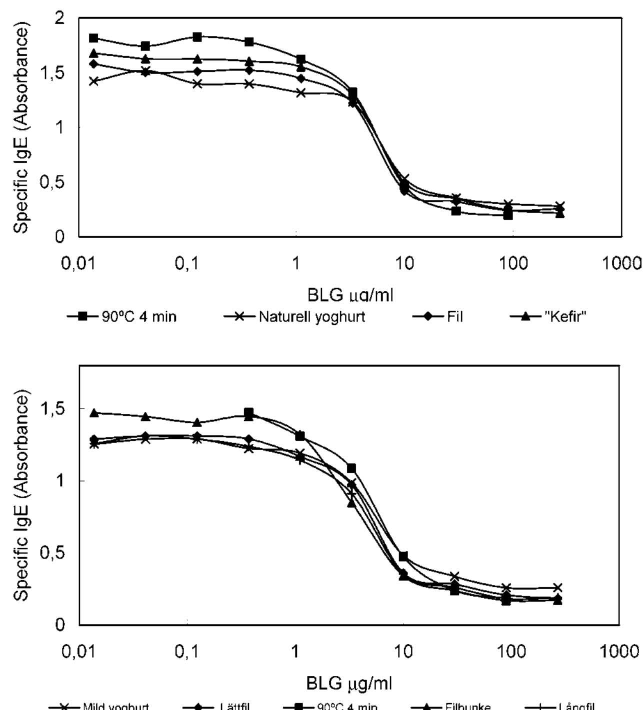

Figure 3. Inhibition of anti-β-lactoglobulin IgE in serum A15. A comparison between commercially available fermented milk products.

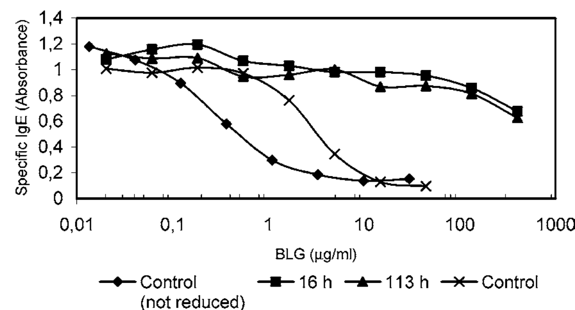

Figure 4. Inhibition of anti-β-lactoglobulin IgE in serum A12. A comparison between milk samples digested with trypsin for different periods of time. The milk samples were treated at 72 °C for 30 s.

of <0.01 for the milk samples treated with tryptic digestion for 16 and 113 h.

DISCUSSION

The extracellular proteolytic degradation during fermentation with *Lactobacilli* is clearly visible in the chromatographic analyses in the reduction of the BLG peak, but this did not affect the epitope recognition by IgE as judged by the unchanged inhibition pattern after this treatment. This suggests that the extracellular proteolytic activity in the fermentation process did not extensively degrade the IgE epitopes. Even though chromatography data show that only 18% intact BLG remains after 212 h of fermentation, the degradation might only be partial, leaving peptides long enough to bind the antibodies. It is also possible that there is a greater access to some buried epitopes.

Several reports demonstrate that lactic acid bacteria have effects in the GI tract on the immune reactions connected to hypersensitivity toward foods (for reviews, see refs *31*, *32*). In these cases, the lactobacilli often have to be viable (*33*). The direct effect on the allergenic proteins has not been well investigated, but there are some reports, for example, on proteolytic degradation of proteins during fermentation (*20*)

leading to decreased antigenicity. Lactic acid bacteria used in the fermentation of sour dough have been shown to produce proteolytic enzymes that hydrolyzed a gliadin peptide that is toxic to celiac patients (*34*).

As can be seen from the RP-HPLC chromatograms (**Figure 1d**,**e**) and **Table 2**, the decrease in the native BLG peak in the milk samples with tryptic proteolysis is approximately 88% after 16 h, and, after 113 h, 6% of the BLG remained unhydrolyzed. It can be noticed that the formation of peptides is relatively proportional. The peptide pattern in the chromatograms between 5 and 35 min contains peptides with a wide range of hydrophobicity, the principle of separation in a reversed-phase system. This indicates that tryptic proteolysis has hydrolyzed the milk into a large number of different peptides, all containing hydrophobic amino acid residues. This does not seem to be the case for hydrolysis during fermentation with *Lactobacilli*. In those chromatograms (**Figure 1b**,**c**), while the BLG peak gradually decreases, we cannot observe any peptide patterns similar to those in the chromatograms of tryptic hydrolysis (**Figure 1d**,**e**). It can be suggested that the peptides from fermentation with *Lactobacilli* are larger, have a significantly lower hydrophobicity, and thus might elute in the void peak. There is also the possibility that the components of the fermented milk, a very complex product, may interfere with the separation on RP-HPLC, thus making the results difficult to evaluate (*35*).

The proteolysis with trypsin in this study apparently degraded all of the intact BLG. Very little IgE binding capacity remains in the BLG after 16 h of proteolysis of milk, suggesting that most of the important IgE epitopes have been destroyed.

In general, proteolysis of milk proteins considerably reduces their allergenicity (*36*−*38*). Enzymatic proteolysis is used together with other processes when producing hypoallergenic milk formulas (*13*). Many milk allergic children tolerate extensively hydrolyzed formulas and have been successfully treated with them (*39*−*41*). However, there are some reports of reactions toward both casein and whey hydrolysate formulas (*18*). The specific enzymatic hydrolysis of trypsin will certainly impair many active epitopes, which has been found to give a much less potent allergenic mixture, in tests on mice (*42*). Yet some of the allergenic epitopes of BLG have been shown to survive the hydrolysis (*16*, *30*).

One of the major IgE binding epitopes on BLG is situated in the region containing the free thiol (−SH) group in Cys 121 (*29*, *30*). The free SH group is known to be exposed after moderate heat treatment (*43*) and oxidized at high temperatures. Samples reduced with DTT showed a decrease in IgE binding ability. The reduction caused by DTT leads to the breaking of disulfide bridges. A change of this kind would probably destroy some conformational epitopes. However, in our experiments, we observed the IgE binding to be at a similar final level after reduction with DTT. Thioredoxin treatment has been reported to reduce the allergenicity of milk in an animal model (*44*).

In conclusion, fermentation with strains of lactic acid bacteria did not give a significant decrease in the IgE binding ability of BLG. Proteolytic degradation with trypsin gave a significant decrease in IgE binding, and, although no sinusoidal inhibition curve could be seen after extensive hydrolysis, a partial inhibition was still present at high concentrations.

The IgE binding ability of BLG in proteolyzed milk was reduced with all individual sera studied, suggesting that the reduced binding seen is a general phenomenon. In our earlier paper (8), we used pooled sera and a fluoro enzyme immuno assay (FEIA-CAP) inhibition technique based on the methodology first described as the radioallergosorbent test (RAST) (*45*, *46*) to determine the IgE antibody binding capacity of BLG. In the present work, a new ELISA method was developed to study IgE binding that made it possible to screen a greater number of samples and use small volumes of individual patient sera. Thus, we could avoid the risk of one or a few sera masking the reactions of others. The results were comparable to those obtained with the FEIA-CAP method.

It would be desirable to achieve in parallel a good monitoring of protein degradation and of changes in the IgE binding capacity of the protein. A decrease in IgE binding ability should be correlated to the clinical manifestation of allergy in patients. There is a possibility that it will have clinical consequences in patients with low specific IgE levels or who are evolving tolerance. There is an obvious advantage in eliminating the IgE binding capacity of milk products while still preserving peptides that are long enough to allow T-cell tolerance to evolve in milk allergic patients. Controlled processing of milk products may provide such a product.

ABBREVIATIONS USED

BLG, β-lactoglobulin; DTT, dithiothreitol; FEIA, fluoro enzyme immuno assay; IC_{50}, the protein concentration needed for 50% inhibition; PBS; phosphate buffered saline solution; PBST; phosphate buffered saline solution with Tween 20; RP-HPLC, reversed phase high-pressure liquid chromatography.

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