

Reduction of the Antigenicity of Whey Proteins by Lactic Acid Fermentation

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Lactic acid bacteria (399 meso- and thermophilic strains or mixed cultures) were screened for their ability to reduce the antigenicity of bovine α -lactalbumin (α -la) and β -lactoglobulin (β -lg). Residual antigenicity of these proteins in whey from the fermented milks was determined by indirect ELISA and competitive ELISA for α -la and β -lg, using rabbit polyclonal antibodies. The antigenicity was related to allergenicity, as determined by skin-testing with selected samples of whey from the fermented milks. The antigenicity of whey proteins was found to be reduced by over 99% as compared to raw milk after the lactic acid fermentation of sterilized cow's milk. The allergenicity of α -la and β -lg was not, however, eliminated, being only slightly attenuated.

Keywords: cow's milk allergy, ELISA, lactic acid fermentation

INTRODUCTION

Sensitivities to certain foods or food ingredients affect both children and adults. The most serious sensitivities are those that affect children, particularly infants and babies because of influence on their growth. Milk, being the first food for newborns cannot be eaten by them. Some allergies can still develop in children on infant food formulas with modified bovine proteins. It is therefore necessary to eliminate the major milk allergens β -lactoglobulin (β -lg) and α -lactalbumin (α -la). To reduce milk allergenicity, heat treatment of milk (Heppel *et al.*, 1984) and enzymatic hydrolysis of milk proteins (Boza *et al.*, 1994, 1995; Ena *et al.*, 1995; Mutilangi *et al.*, 1995; Nakamura *et al.*, 1992; Pahud *et al.*, 1985; Schmidt & Poll, 1991) have been used. These approaches are not so far entirely satisfactory. Severe heating only reduces the milk allergenicity, yet adversely affects the nutritional and sensory properties of treated milk. On the other hand, enzymatic hydrolysis requires specific enzymes, degrading allergenicity-responsible epitopes and removal of bitter peptides. Both, heat treatment and enzymatic hydrolysis can increase the allergenicity of whey proteins by generating cryptotopes (Paraf, 1996).

Fermented milk products such as kefir or yogurt are well recognized for their health-promoting properties. The effect of yogurt on the immune system in mice was found to be dependent on the storage time (Perdigón *et al.*, 1995), suggesting the importance of the balance between particular cultures and, consequently, their immunogenic potential. Orally administered *Lactobacilli* were reported to increase the IgA release and phagocyte and lymphocyte activities in mice and humans (Perdigón *et al.*, 1986; 1988; 1990). The *Lactobacillus* strains used in yogurts show immunostimulating activity by influencing the production of γ -interferon and lymphokines (De Simone *et al.*, 1986).

The objective of the present work was to test the meso and thermophilic strains or cultures for their ability to reduce cow's milk protein antigenicity and allergenicity.

MATERIALS AND METHODS

Reagents

Reagent grade chemicals were from Sigma. Assay buffers: carbonate/bicarbonate coating buffer, pH 9.8, phosphate-buffered saline (PBS)-Tween wash buffer, pH 7.4. Block solution: 1.5% gelatin (cat. no. G-2500). Enzyme substrate: o-phenylene-diamine dihydrochloride (o-PD; cat. no. P-3804) in 0.1 M-citrate-phosphate buffer, pH 5.0. Second antibody: anti-rabbit IgG (whole molecule) peroxidase conjugate, antibody developed in goat (cat no. A-6154).

Whey Preparation

Raw cow's milk from one selected herd of infection-free cows was sterilized (110°C for 10 min), cooled and fermented by meso- or thermophilic lactic acid bacteria at 25 and 37°C, respectively, until the coagulum was formed. Samples were then mixed and centrifuged (6000 $\times$ g for 15 min) and the resulting whey was portioned (1 ml) and kept frozen until analysis. All strains and cultures of lactic acid bacteria, i.e. 228 mesophiles and 161 thermophiles (Table 1) were from the Biolacta-Texel company, Olsztyn, Poland. α -la (Sigma, cat. no. L 6010) and β -lg (Sigma, cat. no. L 6879) were used as antigens. Polyclonal rabbit antibodies against α -la and β -lg were obtained as previously described (Wróblewska, 1994). The serum was purified in the presence of Na₂SO₄ according to the method reported in the Boehringer Mannheim Biochemical (1991/1992) catalogue.

ELISA Methods

Indirect ELISA and competitive ELISA were used as previously described (Wróblewska, 1994, 1995). Indirect ELISA was used to determine the titre of polyclonal antibodies. The final procedure was as follows: the microtitre plate was coated with 100 μ l/well of antigen diluted in 50 mM-carbonate buffer, pH 9.8 and incubated for 12–18 h at 4°C. When α -la content was determined, the microplate was coated with α -la antigen diluted to 5 μ g ml⁻¹. For β -lg determination, the dilution of β -lg antigen employed was 1 μ g ml⁻¹. The plate was then washed four times with 10 mM-phosphate buffer, pH 7.4, containing 0.5% Tween-20. This washing system was used after every analytical step. Residual free binding sites were blocked with 150 μ l/well of 1.5% gelatin in coating buffer for 30 min at 25°C. The plate was washed, coated with 100 μ l/well of 10-fold-diluted antibody and incubated for 1 h at 37°C. After washing, the plate was incubated for 1 h at 37°C with 100 μ l/well of peroxidase-conjugated goat anti-rabbit immunoglobulin, followed by washing and addition of o-phenylene-diamine dihydrochloride (Sigma, cat. no. P-3804) in 9 mM-citrate buffer, pH 5.0. After incubating plates for 30 min, 100 μ l 4 mM-sulphuric acid was added to stop the reaction. Absorbance was read at 492 nm on an automated plate reader (Reader 510, Organon Teknika, Belgium). α -la and β -lg standard curves are shown in Figure 1.

Competitive ELISA was used to determine the residual antigenicity of α -la and β -lg in whey after lactic acid fermentation of cow's milk. When using the ELISA procedure described above, the solution of rabbit antibodies (50 μ l/well) and the whey sample examined

TABLE 1. Residual immunoreactivity of α -la and β -lg in sample analysed from cow milk (the best results from 228 mesophilic samples)

Sample analysed		Residual immunoreactivity ^a			
		α -la		β -lg	
		($\mu\text{g ml}^{-1}$)	(%)	($\mu\text{g ml}^{-1}$)	(%)
<i>Lactococcus lactis</i> ssp. <i>lactis</i>	1w14	1.95	0.17	22.72	8.74
	w83	1.98	0.18	3.56	1.37
	1w65	4.21	0.38	6.91	2.66
	358	2.06	0.18	15.15	5.83
	136	1.15	0.10	8.75	3.36
<i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i>	408	2.65	0.24	4.44	1.71
	412	3.17	0.28	2.85	1.09
	506	4.01	0.36	3.79	1.45
	3F7	7.81	0.71	5.32	2.05
	8F6	5.34	0.48	3.97	1.53
<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	32	0.04	0.004	1.83	0.70
	34	1.46	0.13	1.71	0.66
	3z16	0.84	0.07	1.29	0.49
	R6	1.07	0.09	1.24	0.47
	3z67	0.69	0.06	2.05	0.79
<i>Lactococcus lactis</i> ssp. <i>lactis</i> biovar. (<i>diacetylactis</i>)	23	0.09	0.008	1.91	0.74
	2306	4.78	0.43	6.45	2.48
	292	3.44	0.31	3.39	1.30
	196	24.64	2.24	9.69	3.72
	2T7	5.41	0.49	6.46	2.48
Mesophilic culture	14D	10.93	0.99	1.75	0.67
	13T	5.98	0.54	3.49	1.34
	21T	4.39	0.39	3.56	1.37
	IIJM	6.16	0.56	2.85	1.09
	2957 SMADL	0.76	0.06	23.28	8.95

^aCalculated as % raw milk immunoreactivity.

(50 μl /well) were added at the same time to the antigen-coated and gelatin-blocked well. The percentage of residual antigenicity of α -la and β -lg was calculated in relation to a raw milk, assumed 100%. The values from triplicates were computed using the ImmunofitTM EIA/RIA program (Beckman).

Analysis of Whey Allergenicity

Ten cow milk-allergic patients, children and newborns (interviews, skin tests) of the Chair and Clinic of Allergy and Internal Diseases at the Dr J. Biziel Hospital, Bydgoszcz, Poland, were evaluated by skin testing with the samples of whey the antigenicity of which was reduced the most (above 99%, Table 1) after the lactic acid fermentation. Whey samples were filtered through an 0.22 μm microbiological filter, transferred to sterile vials, freeze-dried and lyophilized. The lyophilized samples of whey were dissolved in 3.5 ml of the physiological sterile salt solution to prepare the solutions with the concentration of antigens (α -la and β -lg) corresponding to the initial concentration in whey. The skin testing was carried out by intradermal injecting of 0.1 ml of whey or standard cow milk allergen (positive control) and diluent (negative control) from Sevac, Czech Republic, into a palmar area of forearm. After 20 min, the diameter of the wheal was measured (in millimeters) for two perpendicular

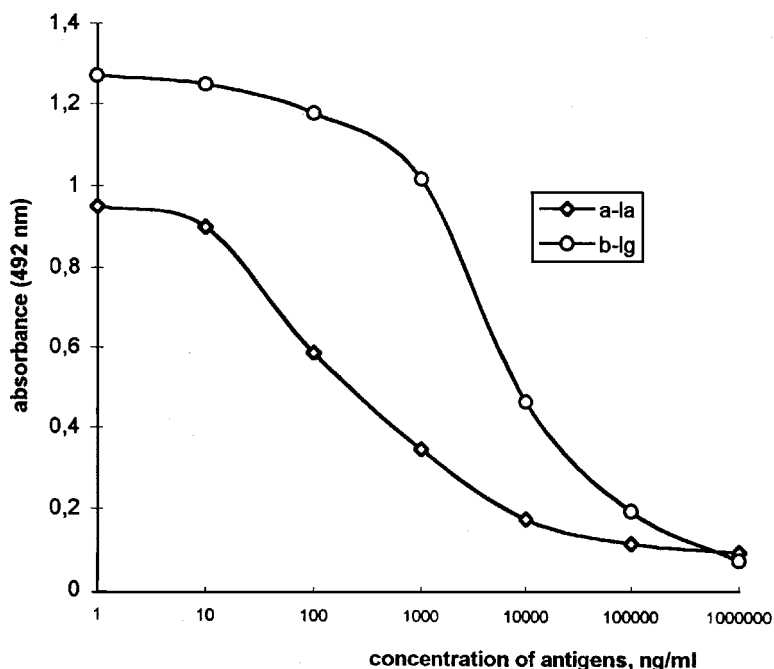


FIG. 1. Standard curves for α -la and β -lg.

dimensions. The test was positive when the average diameter was 10 mm (++) , 15 mm (+++) , 20 mm and above (++++).

RESULTS AND DISCUSSION

In our earlier work, we determined the effects of heating at 60–90°C for 15 s to 1 h on immunoreactivity of cow's milk (Wróblewska & Jedrychowski, 1994; Wróblewska, 1996). We found that with the severity of heat treatment the amount of immunodetectable α -la and β -lg decreased. In the most drastic conditions of the heating (90°C for 15 min), the levels of immunoreactive α -la and β -lg were 12.72 and 18.74%, respectively, as calculated in relation to raw milk (data not shown). Duranti *et al.* (1989;1991) reported that upon sterilization the α -la immunoreactivity decreased to 8% as compared to raw milk. To further reduce the residual immunoreactivity of whey proteins we suggest using the lactic acid fermentation. The antigenicity of whey proteins was markedly reduced during fermentation of milk with the majority of the bacteria examined. The proteolytic system of microorganisms used in the dairy industry for cheese ripening and fermented milks has been described by many authors (Thomas & Mills, 1981; Visser, 1981; Thomas & Pritchard, 1987; Sasaki *et al.*, 1995; Khalid & Marth, 1990; Juillard *et al.*, 1996; Tirelli *et al.*, 1997). These microorganisms are mostly thermophiles of *Lactobacillus* or *Streptococcus* species. Relatively fewer studies have been devoted to the recognition of the efficiency of microbial enzymes to reduce the immunoreactivity of whey proteins. The most effective mesophiles (chosen from 228) and thermophiles (chosen from 161) used in this work are listed in Tables 1 and 2, respectively.

Irrespective of mesophilic culture, the relative percentages of residual antigenicity for α -la and β -lg varied, generally being smaller and less differentiated for α -la than β -lg. The levels of reduction of whey protein antigenicities reported below include those being caused by sterilization. Fourteen strains out of the 20 *L. lactis* ssp. *lactis* examined and all examined

TABLE 2. Residual immunoreactivity of α -la and β -lg in fermented milk sample analysed from cow milk (the best results from 161 thermophilic samples)

Sample analysed		Residual immunoreactivity ^a			
		α -la		β -lg	
Species	Strain	($\mu\text{g mL}^{-1}$)	(%)	($\mu\text{g mL}^{-1}$)	(%)
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>	J7	7.09	0.64	5.74	2.20
	TB5	0.83	0.07	4.52	1.73
	b-5	12.09	1.09	2.46	0.95
	SL	4.45	0.40	1.06	0.41
	S11	0.14	0.01	8.59	3.30
<i>Lactobacillus helveticus</i>	IV	1.78	0.16	8.43	3.24
	Cu-1-18	1.73	0.16	7.70	2.96
	122	2.60	0.23	5.39	2.07
	2 Visby	7.37	0.67	3.04	1.17
	5V	3.29	0.29	0.96	0.37
<i>Streptococcus salivarius</i> ssp. <i>thermophilus</i>	JT3	2.20	0.20	2.76	1.06
	G-1	2.46	0.22	2.31	0.88
	A	3.94	0.36	4.61	1.77
	5ch	2.12	0.19	4.41	1.69
	G-4	1.15	0.10	6.11	2.35
<i>Lactobacillus casei</i>	137	124.10	11.28	3.00	1.15
	7BR	16.10	1.46	3.38	1.30
	II/77	70.98	6.45	1.13	0.43
	Cu-18	11.41	1.04	2.87	1.10
	2	6.20	0.56	5.69	2.18
<i>Lactobacillus delbrueckii</i> ssp. <i>lactis</i>	147	23.65	2.15	8.01	3.08
	168	12.43	1.13	25.52	9.81
	202V	35.85	3.26	19.88	7.64
	F-16	13.39	1.22	45.52	17.51
	4FD	11.35	1.03	48.12	18.51
<i>Lactobacillus acidophilus</i>	6T	18.13	1.65	3.73	1.43
	172	6.84	0.62	3.51	1.35
	1ndz	15.91	1.45	1.45	0.56
	2536	2.16	0.19	5.59	2.15
	67L	1.08	0.09	3.81	1.46
Yogurt strains	Jog1	39.70	3.61	0.49	0.19
	JogT	8.96	0.81	28.46	10.95
	JogZM	6.47	0.59	0.57	0.22
	Napoj AC	3.99	0.36	0.45	0.17
	Napoj Th	2.07	0.19	1.12	0.43

^aCalculated as % raw milk immunoreactivity.

strains of *L. mesenteroides* ssp. *cremoris* reduced the α -la antigenicity by over 99% and β -lg antigenicity within the range from 34 to 99%. The abilities of *L. lactis* ssp. *cremoris* strains were different. Nineteen of them reduced the α -la antigenicity by 56.97 to 99.99%. One strain (strain 266) increased the α -la antigenicity, suggesting that either cryptotopes were generated or cross-reactions affected the result (data not shown).

Twenty-two strains of *L. lactis* ssp. *lactis* (biovar. *diacetylactis*) decreased the amount of immunodetectable α -la by 90.91% to 99.99% and one strain (2w96) by 47.61% as compared to raw milk. These strains reduced the β -lg antigenicity by 56.50 to 99.26%. Strain 23 appeared to be the most effective factor reducing the antigenicity of both α -la (99.99%) and β -lg (99.26%).

The mesophilic cultures, composed of the strains described above, did not act synergetically when fermenting milk. Interdependence between residual immunoreactivity of α -la and β -lg after lactic acid fermentation with mesophilic cultures is shown in Figure 2. 'A' marks the mesophilic cultures, the enzymes of which caused reductions in the immunoreactivity of both α -la and β -lg. Cultures of the enzymes which reduced mostly β -lg immunoreactivity, with high immunoreactivity of α -la, are marked 'B'. 'C' marks the cultures that strongly reduced α -la immunoreactivity but not β -lg immunoreactivity. In spite of the fact that the statistical arrangement of data was random, most strains revealed reduced activity against both tested proteins. The most beneficial composition of strains had culture SMADL for reducing the α -la antigenicity (0.06% residual antigenicity) and culture 14D for reducing the β -lg antigenicity (0.67% residual antigenicity).

The changes in whey protein antigenicity with thermophilic bacteria are shown in Table 2. Among the strains of *L. delbrueckii* ssp. *bulgaricus*, the strains S₁₁ and SL reduced the α -la (99.99%) and β -lg (99.59%) antigenicities the most. These results are important because *L. delbrueckii* ssp. *bulgaricus* is a component strain of yogurt culture. It can then be appropriately selected for the most effective reduction of antigenicity of whey proteins in yogurts.

The *L. helveticus* strains decreased the α -la and β -lg antigenicities by 99.84 to 91.15% and 99.84 to 27.81%, respectively. The most effective strain among *S. salvarius* ssp. *thermophilus* species was strain G-4, which decreased the α -la antigenicity by 99.90% and strain G-1 which decreased the β -lg antigenicity by 99.12%.

The *L. casei* strains reduced the α -la antigenicity by 78.46 to 99.44% and β -lg antigenicity by 42.14 to 99.57%. The most effective strains for reduction of antigenicity were *L. casei* 2 (for α -la) and *L. casei* III/77 (for β -lg) (Table 2).

The *L. delbrueckii* ssp. *lactis* strains reduced the α -la antigenicity by 98.97% (strain 4FD) to 96.74% and β -lg antigenicity by 96.92% (strain 147) to 82.49%.

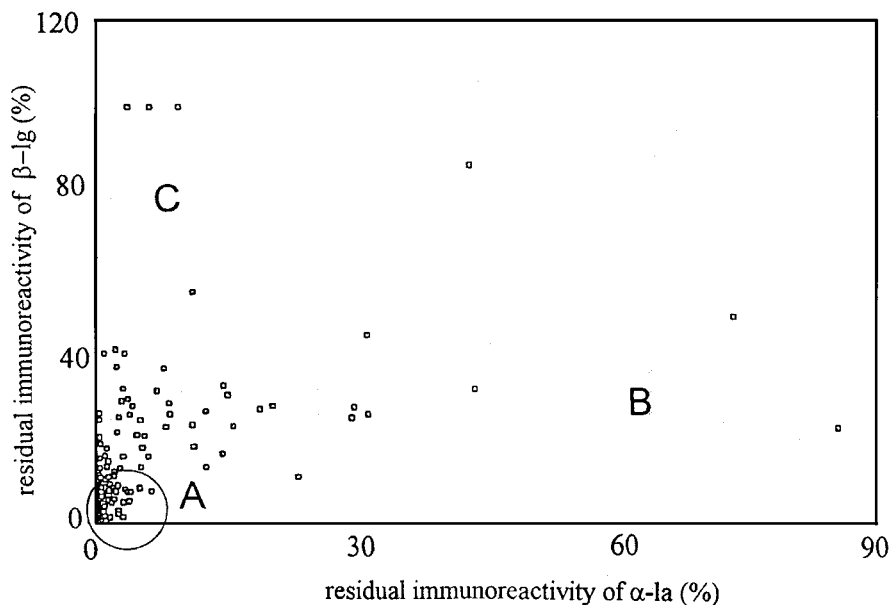


FIG. 2. Interdependence between residual immunoreactivity of α -la and β -lg after milk fermentation with application of mesophilic cultures: A - of low content of remnant α -la and β -lg with immunoreactive properties; B - of low content of remnant α -la with immunoreactive properties; C - of low content of remnant β -lg with immunoreactive properties.

TABLE 3. Assessment of allergenic properties of milk protein modified in milk fermentation process employing selected strains and cultures (provocative dermal test PRICK)

Patient	Milk allergen	Blank sample	Samples										
			Napoj Th	Napoj Ac	<i>L. lactis</i> ssp. <i>cremoris</i> 32	<i>S. Salivarius</i> ssp. <i>thermophilus</i> G-1	<i>L. lactis</i> ssp. <i>lactis</i> (biovar <i>diacetilactis</i>) 23	<i>L. acidophilus</i> Ind2	<i>L. delbrueckii</i> ssp. <i>bulgaricus</i> SL	<i>L. casei</i> 3/II	<i>L. helveticus</i> 5V	Mesophilic culture 14 D	
1. S. J.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
2. A. R.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
3. J. R.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
4. K. K.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
5. K. A.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
6. N. E.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
7. B. P.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
8. N. E.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
9. S. E.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
10. J. P.	++ +	—	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
Test result (mm) ^a	16	0	13	13	12.5	12.5	13.5	13.0	13.5	13.5	12.5	15.5	

^a Average diameter of wheal: ++ 10 mm, +++ 15 mm, ++++ 20 mm and more.

The *L. acidophilus* strains are used more frequently nowadays for fermented milk products such as yogurt due to their probiotic properties, i.e. immunologic system enhancement, adherence to intestine epithelial cells and balancing the intestine microflora (O'Sullivan, 1992). Some strains, e.g. NFCO 1748, decrease the amount of faecal enzymes, protect the intestine in drug-induced diarrhoea or radiotherapy-induced diarrhoea and are effective in constipation treatment (Lee & Sepo, 1995). Among 31 strains of *L. acidophilus* examined, 28 strains decreased the α -la and β -lg antigenicities with the 99.91% maximum reduction of α -la antigenicity (strain 67L). The residual β -lg antigenicity varied more broadly (9.41–227.15 $\mu\text{g l}^{-1}$), with one strain which caused the β -lg antigenicity to increase as compared to raw milk (data not shown). The residual α -la and β -lg antigenicities of yogurt cultures were low (Table 2).

The present study was aimed at determining the influence of enzymes originating from a wide range of microbiological cultures on milk immunoreactivity. There is a need to characterize, in particular, enzymes responsible for the changes of the immunoreactivity.

Despite the fermentation of milk causing important reduction in antigenicity of whey proteins, their *in vivo* allergenicity was not eliminated (Table 3). Only slight attenuation of the immediate reaction to the provocation test was observed.

CONCLUSIONS

Lactic acid fermentation was found to be suitable for decreasing milk immunoreactivity, which in sterilized milk was over 99%. None of the studied strains and cultures reduced the sterilized milk antigenicity by 100%. The assumed line of study seems to be interesting and promising. The product obtained retains good organoleptic properties (slightly acid taste and odour), important for potential consumers of milk products characterized by reduced immunoreactivity.

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