

Effect of Conjugation of Cow Milk Whey Protein with Polyethylene Glycol on Changes in their Immunoreactive and Allergic Properties

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Polyethylene glycol (PEG) was conjugated with milk proteins to determine the changes of the immunoreactive properties of α -lactalbumin (α -la) and β -lactoglobulin (β -lg). The competitive ELISA method was applied to determine the antigenicity of modified protein, using rabbit polyclonal antibodies. The immunoreactivity decreased to $0.90 \mu\text{g ml}^{-1}$ α -la (which corresponded with 0.05%) and $0.14 \mu\text{g ml}^{-1}$ β -lg (which corresponded with 0.08% of the immunoreactivity observed in raw milk). The determination of allergenicity of modified whey proteins was carried out on a group of 10 allergic patients and it was insignificantly weaker than that of a control sample of milk allergens.

Keywords: Cow's milk allergy, whey protein (α -la and β -lg), polyethylene glycol (PEG), ELISA

INTRODUCTION

Modified whey proteins (α -lactalbumin [α -la] and β -lactoglobulin [β -lg]) and casein are protein sources and components of baby formulas – an alternative dietetic solution for those who suffer cow milk intolerance. The immunoreactive properties of the particular milk components may be changed as a result of applying heat treatment, physical (microwaves, ultrasounds), chemical, enzymatic and biotechnological processes through lactic acid fermentation (Wróblewska, 1996).

Polyethylene glycol (PEG) is a non-toxic compound authorized in the USA by the Food and Drug Administration (FDA) for application in therapeutics for external and internal use. Adenine deaminase (ADA) was the first PEG conjugated compound introduced into medicine in 1991 to be administered to patients with the enzyme deficiency (Inada *et al.*,

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1995). Currently, over 40 proteins (including interferon γ , interleukin 2, ovalbumin, haemoglobin, immunoglobulin, myoglobin, ribonuclease, soybean trypsin inhibitor and alkaline phosphatase) have been modified with the use of either PEG or its derivatives: PEG₁ [2-0-methoxypolyethylene glycol)-4,6-dichloro-*s*-triazine], PEG₂ [2,4-bis(O-methoxy-polyethylene glycol)-6chloro-*s*-triazine]. In mild process conditions, the above compounds react with the lysine ϵ -amine groups or free amine groups of protein. PEG is soluble in water and many organic solvents. These properties are responsible for its excellent ability to cross-bond with other substances (e.g. proteins).

PEG has been widely applied. It was found that proteins combined with an active PEG derivative are able to maintain their biological activity. At the same time, such conjugates are responsible for a significantly lower, or even completely reduced, organism immunological response to native proteins. The application of m-PEG polymer chain in clinical medicine to a global camouflage of the foreign cell surface, mainly that of red blood cells, is crucial in blood transfusion and transplantations (Scott, 1998). Both the PEG-protein and PEG-liposome conjugates are applied in the pharmaceutical industry and medicine. PEG, placed superficially, is used in obtaining materials of neutral surface charge. PEG is also frequently used to form bonds both between the particles of the same compound and between a particle and a surface of a drug, for example. The particles conjugated with PEG are used to purify mixtures of biologically-active compounds. Biopolymers are synthesized on PEG carriers. PEG has additionally found application in controlling the solubility of different substances such as the organic solvent solubility of enzymes or the water solubility of enzyme substrates, dyes, chemotherapeutic substances as well as compounds contributing to food flavour aroma sensation (Harris, 1992).

The aim of the completed experiments was to determine the change of the immunoreactive properties of cow milk α -la and β -lg as a result of their conjugation with PEG.

MATERIALS AND METHODS

The experimental material was fat-free cow milk modified by bonding its immunoreactive whey proteins with PEG 8000. The milk was supplemented with PEG (Sigma, St Louis, MO, USA, cat. no. P 2139) in the following six amounts: 0.05; 0.1; 0.15; 0.5; 1.0; 1.5 g PEG per 10 ml of milk (Matsushima *et al.*, 1992). PEG was added in three portions, gently stirring the resulting suspension for 1 h at 4°C. The obtained samples were centrifuged at $5000 \times g$ for 15 min, then frozen and stored until the analyses were performed (Wróblewska & Jędrychowski, 1996).

Analysis of Protein Immunoreactive Properties

The ELISA method. The indirect and competitive ELISA methods with polyclonal rabbit antibodies towards α -la and β -lg, whose production was described earlier (Wróblewska & Jędrychowski, 1994), were applied in the experiments.

Antibodies were obtained from four New Zealand white rabbits applying the immunization protocol according to Durants *et al.* (1991). Immunogen prepared for the first immunization contained 0.5 ml of protein solution in 0.9% sodium chloride (2.0 mg ml^{-1}) emulsified with equivalent volume of Freund's complete adjuvant. The next three immunizations were made at monthly intervals in the presence of incomplete adjuvant, and the last two intramuscular (maintaining) immunizations were made without adjuvant with the same antigen volumes and concentrations as described previously. Production of antibodies and increases in their titre were controlled by the indirect ELISA method taking blood samples from the rabbit marginal vein 2–3 days prior to the subsequent scheduled immunization.

The days after the last immunization rabbits were bled out. Blood was incubated for 1 h at 30°C and left overnight at 4°C. Following centrifugation at $1500 \times g$ for 20 min, serum IgG antibodies were obtained at 20% saturation with sodium sulphate. After centrifugation at

1500 × g, 30 min, the residue was dissolved in phosphate buffer, pH 8.0, and dialysed for 15 h at 4°C with the phosphate buffer being changed four times. The IgG fraction obtained was lyophilized according to the Boehringer of Mannheim Biochemica (1991/1992).

Reagents

The following reagents were used in both ELISA methods: α -la (Sigma, cat. no. L-6010), β -lg (Sigma, cat. no. L-6879), a conjugate of goat anti-rabbit immunoglobulin with horseradish peroxidase (Sigma, cat. no. A-6154), a 50 mM-carbonate buffer solution for microplate coating, pH 9.8, a buffer for rinsing microplates – saline solution, buffered with 10 mM-phosphate solution supplemented with 0.5% Tween 20, 10 mM-phosphate buffer solution for diluting samples, serum and conjugate, pH 7.4 (Sigma, cat. no. P-4417), a substrate for colour effect in reading out absorbance values, o-PD (*o*-phenyldiamine dihydrochloride), (Sigma, cat. no. P-3804), a 9 mM-citrate buffer, pH 5.0, for o-PD.

The Indirect ELISA Method

The indirect ELISA method was used to estimate the concentration of the applied polyclonal antibodies (PABs) (Jędrychowski and Wróblewska, 1999). The 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-PD (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).

The Competitive ELISA Method

The competitive ELISA method was used for a quantitative immunoreactivity estimation of the samples obtained through PEG modification. Microplates were coated with the antigen in the concentrations determined earlier in the indirect ELISA method (1 μ g ml⁻¹ for β -lg and 5 μ g ml⁻¹ for α -la) in a 9 mM-carbonate buffer solution, pH 9.6, in the amount 100 μ l per well. The microplates with the antigen were incubated for 18 h at 4°C, then rinsed four times with 10 mM-phosphate-buffered saline, pH 7.4, containing 0.5% Tween-20. This procedure was repeated after each step of this method. The places of the microplates that were not filled by the antigen were filled with a 1.5% gelatin solution of 150 μ l per well and incubated for 30 min at 25°C. After rinsing the microplates, the wells were filled with both a sample containing the antigen and the polyclonal rabbit antibodies obtained for a given antigen (50 μ l of each solution of an adequate concentration per well). In order to blend the process components, the microplates were placed in the Janke and Kunkel (IKA-SCHÜTTLER MTSZ) shaker for 5 min and incubated for 1 h at 37°C. After rinsing the microplate, a substrate was added (o-PD solution in citrate buffer, pH 5.0) and after 30 min the process was stopped with 4 M-H₂SO₄ solution. The absorbance was determined by the ORGANON-TEKNIKA automatic Reader 510 at a wavelength of $\lambda = 492$ nm.

The obtained results were processed with the Immunofit™ EIA/RIA software by Beckman.

TABLE 1. Effect of cow milk protein modification with PEG on immunoreactive properties changes

Addition of PEG (g/10 ml of milk)	Residual immunoreactivity ^a			
	α-la		β-Ig	
	μg ml ⁻¹	%	μg ml ⁻¹	%
0.05	282.10	25.64	12.47	4.79
0.10	116.95	10.63	2.73	1.04
0.15	23.46	2.41	2.46	0.95
0.50	1.76	0.16	0.86	0.33
1.00	1.30	0.12	0.30	0.11
1.50	0.90	0.08	0.14	0.05

^aCalculated as % raw milk immunoreactivity.

The Allergenicity Analysis of Modified Whey Proteins

The allergenicity evaluation of PEG modified proteins samples was carried out on 10 patients of the Dr J. Biziel Provincial Hospital in Bydgoszcz, Poland. The skin tests were completed with cow milk as the positive control test. The organism allergenic response was expressed as a diameter of the resulting redness on the forearm in millimetres. The observed average diameter was qualified into one of the three positive reaction groups: <10 mm (+ +), 15–20 mm (+ + +) and >20 mm (+ + + +).

RESULTS

Modification of Allergenic Properties of Cow Milk Whey Proteins Through Conjugation With PEG 8000

Cow milk whey proteins were chemically modified through their combination with PEG added in six different amounts. The results describing the remaining post-process immunoreactivity were included in Table 1. It was found that the larger the amount of PEG, the lower the immunoreactivity was. The considerable decrease of residual immunoreactivity was noticed in the samples in which the amount of PEG added to 10 ml of milk was 0.15 g. The estimated amounts of residual immunoreactive protein were 2.14% and 0.95% for α-la and β-Ig, respectively, calculated as a per cent raw milk immunoreactivity.

The supplementation of 1.5 g PEG per 10ml of milk sample resulted in an immunoreactivity decrease to 0.90 μg ml⁻¹ of a sample for α-la (which corresponded with 0.05% of the immunoreactivity observed in control samples) and to 0.14 μg ml⁻¹ of a sample for β-Ig (which corresponded with 0.08% of the immunoreactivity observed in raw milk).

The Allergenity Analysis of the Modified Whey Proteins

Based on the results of clinical examinations, carried out in compliance with ethical norms on a group of 10 patients of the J. Biziel Provincial Hospital in Bydgoszcz, it was found that besides the quite advantageous results obtained *in vitro*, the α-la and β-Ig allergenic property decrease was still unsatisfactory (Table 2). The experiment showed that the PEG modified preparations of cow milk proteins, in the case of five patients, resulted in the same strong skin reactions as for milk. Four patients reacted insignificantly weaker and one responded with a weak immunological reaction. The immune reaction of 10 examined allergic patients was summarized on the 80% level in comparison to 100% positive control sample of milk. In conclusion, it was stated that the process of modification, despite the considerable decrease

TABLE 2. Assessment of allergenic properties of milk protein modified with PEG (provocative dermal test PRICK)

Patient	Sample		
	Milk allergen	Blank sample	Milk protein sample after modification with PEG
S.K.	+++	—	+++
A.J.	+++	—	++
B.G.	+++	—	+++
S.J.	+++	—	+++
W.R.	+++	—	++
K.L.	+++	—	+++
P.Z.	+++	—	++
R.U.	+++	—	+++
G.L.	+++	—	++
H.I.	+++	—	+

of immunoreactivity observed *in vitro* with the use of the ELISA method, still does not ensure a completely safe preparation free of allergenic properties.

DISCUSSION

The aim of this study was to estimate the immunoreactivity changes in cow milk allergic proteins (α -la and β -lg) as a consequence of their conjugation with PEG. The allergenicity of conjugates on allergic patients by skin test was also determined.

PEG is a non-toxic substance; when conjugated to proteins it shields them from interaction with other macromolecules. It also has an effect on the induction of immune tolerance through Th-cells. The mechanisms of this phenomenon however have not yet been explained (Saito *et al.*, 2000). It is known from the literature that protein chemical modification with PEG or its derivatives may be used to adjust protein products to particular applications or to eliminate their undesirable properties as well as modifying their physical, chemical or functional properties, mainly due to its non-toxicity (Okada *et al.*, 1985; Sasaki *et al.*, 1993).

These references provide us with the information that protein modification with PEG may also have a positive influence on enzyme stability, chemical denaturation and proteolysis (Inada *et al.*, 1995).

The results obtained by Matsushima *et al.* (1992), Sasaki *et al.* (1993) and Abuchowski *et al.* (1977) confirmed that the modification of L-asparaginase and bovine serum albumin (BSA) is possibly decreasing its immunoreactivity. It was found that the complete elimination of asparaginase immunoreactivity was obtained after modification of 34% of the amine groups with a PEG derivative of a molecular weight of 100 kDa (PM₁₀₀). Whereas when applying the derivative of molecular weight 13 kDa (PM₁₃) the same effect was achieved after modifying 50% of the amine groups (Sasaki *et al.*, 1993). To evaluate the immunoreactivity changes, the Kabat and Mayers method of 1961 was applied, however the currently available ELISA method allows for a more precise determination of the results.

Hellman and Carlsson (1996) proposed to use PEG polymer in immunotherapy through allergen cross-linking. Monomethoxy-polyethylene glycol coupling or immune complex formation may also be used to reduce the allergenicity of the antigen and to tolerize or

redirect the immune response to a T-helper 1 response (Hellman Carlsson, 1996). Immunotherapy is safe and widely used in the treatment allergic diseases, and the above proposition seems to be promising.

In 1999, So *et al.* prepared three proteins: hen egg lysozyme, ovalbumin and bovine gamma globulin as conjugates with monomethoxypolyethylene glycol (mPEG). They found that the ratio 1.5–2.0 the molecular weight of the conjugated total mPEG to protein is the most appropriate ratio of tolerance induction – specifically from T and B cell responses (So *et al.*, 1999).

In his study, Saito (2000) used transgenic mice with ovalbumin specific Th-cell receptor (TCR) genes and examined the *in vitro* and *in vivo* responses of OVA-specific Th-cells towards tolerogenic PEG-conjugate. When stimulated with PEG-OVA *in vitro*, transgenic ovalbumin (OVA)-specific Th-cells proliferated and produced interleukin 2. Conjugate PEG-OVA administered into the circulation of transgenic mice induced no responses in peripheral OVA-specific Th-cells. (Saito *et al.*, 2000).

The conjugation of a protein molecule with any bigger molecule of the carrier seems to be an increasingly popular way to reduce immunogenicity of different allergens. Kabayashi (Kabayashi *et al.*, 2001) estimated the immunogenicity of the preparations of β -lg conjugated with carboxymethyl dextran (CMD). Structural analysis by intrinsic fluorescence CD spectra and ELISA with monoclonal antibodies (MAbs) indicated that the surface of β -lg in each conjugate was covered by CMD without any great disruption of its native conformation. The antibody response to β -lg estimated in mice was reduced. The results of B-cell epitope scanning using overlapping synthesized peptide showed that the linear epitope profiles of the conjugates were similar to those of β -lg, whereas the antibody response to each epitope was reduced. It was concluded that conjugation with CMD of a higher molecular weight, is effective in reducing the immunogenicity of β -lg and that masking of epitopes by CMD is responsible for the reduced immunogenicity (Kabayashi *et al.*, 2001).

Similar observations were made by Caliceti (Caliceti *et al.*, 2001). They prepared four different polymer conjugates of uricase. There were no relevant differences in protein conformation among the native forms and the conjugates, but immunogenicity and antigenicity were altered by polymer conjugation. It was found that monomethoxypoly (ethylene glycol) 10 000 Da reduced the protein antigenicity and poly(N-vinylpyrrolidone) 6000 Da increased antigenicity in comparison to native uricase. All conjugates induced a lower production of mouse anti-native uricase antibodies, and induced anti polymer immunoresponse (Caliceti, 2001).

In our work we found a significant reduction of residual immunoreactivity α -la and β -lg when PEG was added to milk. The immunoreactivity of raw milk was estimated at 100%. The reaction between 0.05 g PEG and the whey proteins present in 10 ml of milk reduced immunoreactivity of α -la and β -lg to 25.64 and 4.79%, respectively. The addition of 0.10 g of PEG to 10 ml of milk reduced residual immunoreactivity more intensively for both α -la and β -lg proteins (to 10.63 and 1.04%, respectively). The addition of 0.15 g of PEG to milk further reduced immunoreactive properties of the proteins to 2.41 and 0.95% (α -la and β -lg). The further portions of PEG added to milk reduced immunoreactivity to a low degree. At the end of this experiment we obtained the lowest number of immunoreactivity after the addition of 1.50 g of PEG to 10 ml of milk. The residual immunoreactivity estimated for α -la was 0.08% and for β -lg 0.05%.

It is suspected that the decrease of protein immunoreactivity could have been caused by covering the epitope area, i.e. places on the protein surface that have the ability to bond antibodies of α -la and β -lg, respectively. The conditions of conjugation reaction were mild (4°C) and any conformation changes within the protein molecule were not expected. The reduction of immunoreactivity of residual α -la and β -lg depended on the number of polymer chains bound to the protein surface. These observations are similar to other research (Kabayashi *et al.*, 2001). The molecular weight of polymer conjugated to protein is a very important factor. Caliceti indicated that a polymer of 10 000 Da reduced antigenicity and that

of 6000 Da increased antigenicity of protein (Caliceti *et al.*, 2001). PEG used in our work has a molecular weight of 8000 Da. Therefore, immunoreactivity decreased in all samples after conjugation and the changes were connected primarily with the quantity of PEG.

The skin test results obtained after challenge in allergic patients were very important. Only one person indicated considerably lower allergenicity, and the results of five people remained the same when tested for milk allergen. However, Caliceti (2001) showed that his uricase conjugates elicited both anti-native protein and anti-polymer immunoresponses. It is possible that in our example the patients reacted to free α -la or β -lg epitopes not bound to PEG or a PEG chain. The production of specific antibodies should be done to prove this thesis.

Protein modification with PEG holds out hope of obtaining non-immunogenic and non-toxic compound forms for people with allergies. It is also necessary to examine the biological characteristics of such modified products. Immunoreactivity examination *in vitro* (the ELISA method) may not in this case be regarded as determining the clinical suitability of the modified proteins. It is crucial to carry out further examinations in such cases and we must verify the suitability of such a type of protein chemical modification in cases of hypoallergenic food products.

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