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IMMUNOMETRIC METHODS OF ANALYSIS AS A TOOL FOR DETERMINING THE ANTIGENIC PROPERTIES OF COW MILK PROTEINS AND THEIR HYDROLYSATES

Barbara Wróblewska, Magdalena Karamać, Agata Szymkiewicz*

Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

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Milk is a common and inexpensive source of proteins which are the basis of the production of modified formulas for human nutrition of patients suffering from allergies, phenylketonuria, liver diseases and as a protein supplement in energetic drinks, geriatric products, sport nutrition and weight-control diets. The paradox is that almost all milk proteins are allergenic, so their structure should be modified by technological treatment. The degree of modification is usually determined by immunometric methods. In this paper, three of these methods: dot-immunobinding, immunoblotting, and ELISA are described.

INTRODUCTION

Food is one of the factors which has the ability to induce an allergic response in the human immunological system. Allergies can be seen from two different points of view. The first is from patients' suffering from varying symptoms of food allergies, *e.g.* gastrointestinal abnormalities, vomiting, diarrhea and skin urticaria. They are seeking the best medicine to provide immediate relief. The second, scientific point of view, is the search for the best theoretical solution connected with the underlying causes and mechanisms of allergies, the different routes of enzymes in human body and the reaction to foreign antigens by the human immune system. Patients and scientists are both seeking solutions to producing non-allergenic food, as the prevention of allergies is always easier than their treatment. To date, there is no common structure that can predict whether an antigen is a strong food allergen or not. The allergens are divided into four common groups depending on their structures: (1) alpha-helical proteins; (2) largely beta-sheet proteins with a prominent helix in close contact (β-lactoglobulin); (3) alpha + beta-structures (α-lactalbumin); and (4) serpins [Aalberse, 2000].

All structures are unique and their identification requires immunological detection methods without excessive manipulation - which can change their epitopes. Such methods of analysis are based on the reactions between an antigen and a specific antibody. Generally, food allergens are characterized by the following attributes: a compact structure which is stabilized by disulfide bonds, the molecules are heat stable proteins, resistant to hydrolysis by digestive enzymes and they are glycosylated. Epitopes (linear/conformational) are hydrophylic structures exposed at the surface of the native protein, where amino acid sequence homology corresponds with human proteins at low levels.

Cow milk is the most common food allergen during infancy and early childhood. The treatment of choice for cow milk allergy is the avoidance of cow milk; substitutes such as soy formulas, hydrolysed cow milk casein and whey formulas and elemental formulas are usually recommended [Plebani *et al.*, 1997].

There are several possible modifications of cow milk protein, such as thermal processing (pasteurization, ultrasounds, microwaves), enzymatic hydrolysis (trypsin, α-chymotrypsin, rennin, pepsin, Alcalase, papain *etc.*), chemical reaction: (succinilation, acetylation, phosphorylation, conjugation with polyethylene glycol and bovine serum albumin with the use of glutaraldehyde) and lactic acid fermentation with meso- and thermophilic biological strains and cultures [Wróblewska, 1996]. All of these approaches decrease the antigenic properties of cow milk proteins, but none of them reduce them completely.

Cow milk is a specific source of allergen which offers the opportunity to investigate three different situations: β-lg which has no homologue with native human milk, α-la which has a homologue with very restricted expression and serum albumin whose homologue is ubiquitous in the human immune system [Aalberse & Stapel, 2001].

Milk is a very popular model of a mixture of different allergens with some well characterized epitopes. Chatchatee *et al.* [2001] found 6 major and 3 minor IgE-binding, as well as 5 major and 1 minor IgG-binding regions on αs1-casein. In addition, they identified 2 unique epitopes (AA 69-78 and 173-194) that are recognized only by IgE antibodies from patients with persistent milk allergy. Epitopic characterization of native β-lg with two selected monoclonal antibodies from a panel of 52 mAbs were found to be suitable for monitoring of native β-lg in food product and manufacturing processes [Clement *et al.*, 2002].

The use of polyclonal antibodies in analytical methods permits the identification of the type of heat treatment that

*Author's address for correspondence: Barbara Wróblewska, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, ul. Tuwima 10, 10-747 Olsztyn, Poland; tel. (48 89) 523 46 88; fax: (48 89) 524 01 24; e-mail: basia@pan.olsztyn.pl

the milk had undergone. Fukal *et al.* [2002] applied the combination of the two antibodies (against native α-la and β-lg) to the categorization of raw, pasteurized, UHT and bath-sterilized types of milk. During pasteurization, denaturation of α-la probably uncovered epitopes, while more severe heat processing damaged immunodominant epitopes and sterilization almost completely destroyed them. Milk immunoreactivity measured with anti-β-lg antibodies increased in pasteurized and UHT milk. In sterilized milk there were only minor changes. Slight denaturation caused the unfolding of the β-lg and the appearance of new epitopes which were damaged during sterilization.

Polyclonal antibodies are a mixture of antibodies directed against multiple antigenic determinants on a molecule and they are useful for control of residual antigenic activity in hydrolysed formulas [Plebani *et al.*, 1997].

This paper presents analytical tools to identify and quantify milk protein allergens which can be determined with the application of antibodies.

MATERIALS

In this study, commercial whey protein isolate (WPC) and sodium caseinate (obtained from Laktopol Company, Suwałki, Poland) were applied as a substrate of hydrolysis reaction. Protease *Sublilisina carlsberg* - Alcalase 2.4 FG (purchased from Novo Nordisk), pronase from *Streptomyces griseus*, and papain E C 3.4.22.2 (Sigma) were used as enzymes.

METHODS

The effect of hydrolysis modification on milk protein was determined with immunometric methods. Usually this group of analytical methods detects the presence of residual antigenic and allergenic components in a native or processed state [Docena *et al.*, 2002].

Electrophoretic and immunoblotting methods. Electrophoretic separations of protein extracts were performed with 12% polyacrylamide gel (SDS-PAGE) according to Laemmli under denaturing conditions [Laemmli, 1970]. Before electrophoresis, all protein samples were boiled for 3 min in the presence of SDS (3% w/v) and 2-mercaptoethanol (0.1% v/v). The gels were run in a Tris--glycine buffer, pH 8.3 and the total protein in gels was stained with Coomassi Brilliant Blue R-250.

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane in the apparatus for the socalled "wet" electrotransfer using a Tris-glycine buffer with methanol, pH 8.3 (192 mmol/L glicyne, 25 mmol/L Tris and 20 % v/v methanol).

In order to detect antigenic fractions, the membrane was incubated overnight at 4°C in a serum solution containing rabbit polyclonal antibodies against selected milk proteins. Antigen-antibody complexes were visualised on the membrane by incubation in the solution of goat anti-rabbit IgG labelled with peroxidase. The reaction of enzyme with substrate (H_2O_2/4-chloro-1--naphtol) produced navy blue bands at the site of conjugated antibodies.

Dot-immunobinding. The amount of 3 μL of the hydrolysate sample (concentration of protein 1 mg·mL^{-1}) were applied directly to a sheet of nitrocellulose and allowed to dry. The OVA was used as a negative control and the WPC as a positive control. The membrane was blocked with a blocking buffer (TRIS-HCl buffer, 150 mM NaCl, 0.2% Tween, pH 7.5) for 10 min. Rabbit antibodies against α-la, β-lg, and casein fractions (α-, β-, κ-) diluted 1:500 in the same buffer were applied overnight as a further step of analysis at room temperature. The final reaction was visualized with H_2O_2/4-chloro-1-naftol. This method was an alternative method to immunoblotting, but in non--denaturing conditions.

Enzyme-linked immunosorbent assay (ELISA)

Reagents. The following reagents were used in both ELISA methods: α-lactalbumin (Sigma, cat. no. L-6010), β-lactoglobulin (Sigma, cat. no. L-6879), a conjugate of goat anti-rabbit immunoglobulin with 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 a 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 color effect in reading out absorbance values, o-PD (ophenylediamine 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. The procedure was as follows: the microtitre plate was coated with 100 mL/well of antigen diluted in a 50 mM carbonate buffer, pH 9.8 and incubated for 12-18 h at 4°C. When the α-la content was determined, the microplate was coated with α-la antigen diluted to 5 μg·mL^{-1}. For β-lg determination, the dilution of β-lg antigen employed was 1 μg·mL^{-1}. The plate was then washed four times with a 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 μL/well of 1.5% gelatine in a coating buffer for 30 min at 25°C. The plate was washed, coated with 100 μL/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 μL/well of peroxidase-conjugated goat anti-rabbit immunoglobin, followed by washing and the addition of ophenylenediamine dihydrochloride (Sigma, cat. No. P-3804) in 9 mM citrate buffer, pH 5.0. After incubating the plates for 30 min, 100 μM of 4 M 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. Microplates were coated with the antigen in the concentrations determined earlier in the indirect ELISA method (1 μg·mL^{-1} for β-lg and 5 μg·mL^{-1} for α-la) in a 9 mM/L carbonate buffer solution at pH 9.6 in the amount of 100 μL per well. The microplates with the antigen were incubated for 18 h at 4°C, then rinsed 4 times with 10 mM phosphate-buffered saline at 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% gelatine solution of 150 μL 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 μL of each solution of an adequate concentration per well). In order to blend the process components, the microplates were placed in the Janke&Kunkel (IKA-SCHUTTLER 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 a 4 M H_2SO_4 solution. The absorbance was determined by the ORGANON-TEKNIKA automatic Reader 510 at a wave length of λ=492 nm.

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

RESULTS AND DISCUSSION

Dot-immunobinding

Dot-immunobinding is a simple, useful method for tentative estimation of immunoreactivity of a sample. It can be an alternative to immunoblotting. The samples are applied directly onto nitrocellulose membranes. Usually, the final estimation concerning the allergenicity of antigen molecule is a clear "yes" or "no" answer.

WPC hydrolysates prepared with different enzymes such as Alcalase, papain or protease incubated with anti β-lg and α-la antibodies were immunoreactive, but more interesting was which part of these hydrolysates was the most and the less immunoreactive. So, the hydrolysed WPC were separated by the chromatography system using a Sephadex column 75 HP 10/30 and some fractions were isolated. The results are summarized in Table 1. Generally, the first and second fraction contained unhydrolysed protein such as α-la and β-lg, and it was visualized by distinct dots which indicated the reaction between the protein epitopes and the antibodies directed towards them. The latter fraction consists of peptides and the sensitivity of the applied type of assay did not indicate a reaction between the WPC protein and the antibodies.

The very useful example to use dot-blot analysis was shown by a study of an American group of researchers. They prepared 25 decapeptides of α_{s1}-cas, α_{s2}-cas, κ-cas α-la and β-lg, according to the known IgE-binding regions of cow milk proteins, comprising the core epitopes, for synthesis on a cellulose-derived membrane. Sera from 10 patients with persistent CMA and 10 patients who had outgrown their milk allergy were used to investigate the differences in epitope recognition. The patients with persistent CMA showed binding to more numerous epitopes in the caseins than patients who had outgrown their allergy. Five of these IgE-binding epitopes (two of α_{s1}-cas , one of α_{s2}-cas, two of κ-cas) showed no binding by any of the patients with transient allergies. These epitopes were classified as the informative epitopes [Jarvinen *et al.*, 2002]. The linear epitopes from α_{s1}-cas and β-casein which reacted with IgE antibodies are characteristic for children who have achieved tolerance [Vila *et al.*, 2001].

Immunoblotting

Immunoblotting is an analytical method in which the proteins are separated under denaturing conditions. The process of denaturation can usually easily destroy conformational epitopes which are dependent on a 3-D structure. In practice, these epitopes are mostly reactive and renaturate on the nitrocellulose membrane, and X-ray crystallography of the allergen-antibody-complex is needed for epitope identification [Becker & Reese, 2001]. Immunoblotting is inefficient for some "important" allergens and overefficient for some "unimportant" allergens and can therefore be deceptive [Aalberse *et al.*, 1998].

Immunoblotting identified a very distinct reaction between α-la (14.2 kDa) present in WPC and in papain hydrolysate using anti-α-la antibodies. WPC reacted with anti-β-lg antibodies. But there was also observed a trace of a protein with a higher molecular mass (about 33 kDa). A protein with such a molecular mass can indicate the presence of processed and/or unprocessed traces of casein or BSA and unsatisfactory technological separation casein from whey protein. This situation was also reported by other authors [Docena *et al.*, 2002]. Only papain WPC hydrolysate reacted with anti-β-lg antibodies. There were no reactions between epitopes of sodium caseinate hydrolysates and related anti-α-, β- or κ-casein antibodies.

Immunoblotting demonstrated the cross-reactivity between mammalian proteins. The Italian group of researchers, while researching an alternative solution for children allergic to cow milk protein, noted the reaction between milk protein from different animals and anti-β monoclonal antibodies. Antibodies can recognize β-lg from cow, goat, buffalo and ewe milk, while weak cross--reactivity was observed with mare's, mule's and donkey's milk protein. Camel milk did not react with antibodies, so it seems that it could be an interesting alternative protein source of protein for allergic people [Restani *et al.*, 2002].

TABLE 1. The results of dot-blot of WPC hydrolysates produced with different enzymes.

Hydrolysates of WPC produced with enzymes	Dot-blot of hydrolysates	Dot-blot of fraction of hydrolysates			
		I	II	III	IV
Incubated with anti-α-la:					
Papain	++++	++++	++	+	-
Alcalase	++++	++++	++	+	-
Pronase	++++	++++	++	+	-
Incubated with ant-β-lg:					
Papain	++++	++++	+++	++	-
Alcalase	++++	++++	++	+	-
Pronase	++++	++++	++	+	-

Currently, commercially available gels are recommended, because it is difficult to achieve reproducible results by using homemade gels for peptide electrophoresis. Immunoblotting produced the first indications that conformational epitopes may be destroyed under denaturing analysis conditions [Becker & Reese, 2001].

ELISA

ELISA is a useful type of analysis to determine the quantity of antigens. There are some formats of analysis: direct, indirect, with avidin-biotin complex and the "sandwich" capture assay.

The indirect ELISA method can estimate the level of titre of specific rabbit polyclonal antibodies against milk proteins, which varied from 1:2 000 to 1:50 000. The competitive ELISA method found immunological similarities within the casein fractions and no cross-reactions between α-la and β-lg (Figure 1). The reaction between epitopes of papain hydrolysate and β-lg antibodies was about 6% and with α-la antibodies was below 1%. The immunoreactivity of protease and Alcalase hydrolysates was below 1%. The ELISA method proved that all fractions selected by chromatography were immunoreactive. Together with a decrease in the molecular mass of protein presence in the next fraction, the immunoreactivity of the determined proteins also decreased, but none of them was completely free from antigenicity.

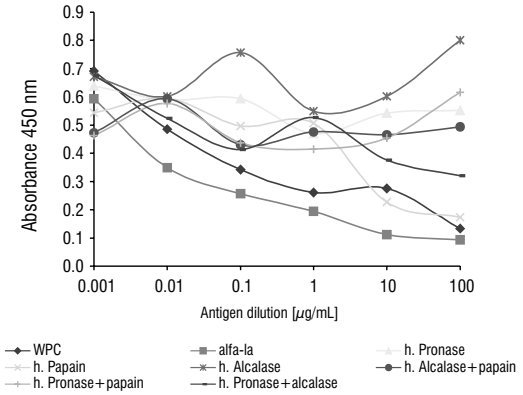

FIGURE 1. ELISA results of cross-reactivity of WPC hydrolysates after incubation with α-la antibodies.

Generally, it is claimed that monoclonal antibodies are more useful in the ELISA method because of their uniqueness. But in some cases, polyclonal antibodies are also very suitable. Danish researchers compared the standard curves of three indirect competitive ELISA assays for β-lg, prepared for use with mouse monoclonal antibodies, rabbit polyclonal antibodies and commercial Cortecs kit with sheep antibodies. The detection limit with Mabs, where only one epitope was reactive, was not satisfactory compared with Pabs, which were superior [Mariager *et al.*, 1994]. Polyclonal antibodies often prove to be even more useful, particularly in an exhibition test in combination with purified (recombinant) allergens [Aalberse *et al.*, 1998]. Allergen--specific monoclonal antibodies can be used to develop quantitative assays in allergen standardization or to measure "hidden" allergens in foods [Becker & Reese, 2001].

An interesting application of the ELISA method was shown by a group of French scientists, concerning the epitopic characterization of native bovine β-lg. This kind of research demanded high-tech methods of analysis such as a surface plasmon resonance biosensor, circular dichroism and MALDI-TOF mass spectrometry. Finally, two monoclonal antibodies suitable for monitoring native β-lg in food products and manufacturing processes were chosen [Clement *et al.*, 2002].

CONCLUSIONS

Immunometric methods are very useful in determination of protein allergenicity, but it is obvious that no single epitope is responsible for the entire protein allergenicity, most parts of the protein molecules contain fragments which bind antibodies. Dot-blot and immunoblotting are simple methods and provide the basic data on possible reactions between allergen and antibodies. ELISA can determine the quantity of residual immunoreactivity of protein, but for a more detailed epitopic characterization it should be amplified with: surface plasmon resonance (SPR - BIACORE 2000), circular dichroism (CD), and MALDI-TOF mass spectrometry.

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