Influence of Alcalase and Transglutaminase on Immunoreactivity of Cow Milk Whey Proteins

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Abstract

Wróblewska B., Jędrychowski L., Hajós G., Szabó E. (2008): **Influence of Alcalase and transglutaminase on immunoreactivity of cow milk whey proteins.** Czech J. Food Sci., **26**: 15–23

The aim of the research was to determine the changes in the immunoreactivity of whey protein concentrate (WPC) modified by two enzymes: proteinase, Alcalase 2.4L FG (Novo Nordisk), and cross-linked transglutaminase (EC 2.3.2.13, Activa^{TM} P, m-TG, Ajinomoto). The new products were characterised by 2D electrophoresis, immunoblotting, and ELISA methods. The WPC hydrolysate obtained with Alcalase contained proteins and peptides characterised mostly by low molecular weight peptides (MW < 14.4 kDa) in the pH range of 3–10. Immunoblotting showed strong immunoreactive properties of the hydrolysate with α-la and β-lg polyclonal rabbit antibodies. The 2D electrophoretic patterns of WPC and its modified product obtained with m-TG did no differ significantly. However, the immunoblot analysis demonstrated that WPC showed a stronger reactivity towards IgE of allergic patients as compared to WPC with m-TG. ELISA methods showed that two-step hydrolysis with Alcalase followed by m-TG significantly reduced the immunoreactive properties of whey proteins. No cross reactions were observed with α-la and only about 0.6% cross-reactivity with β-lg.

Keywords: whey proteins; immunoreactivity; Alcalase; transglutaminase

Properties of cow milk proteins and their hydrolysates enable to extend the production of therapeutic formulae due to new functions of bioactive peptides recently found, e.g. opioid agonist, hypertension agent, mineral binding agent, antioxidant, functional protein and antideppressioner. One of the most important problems is to develop the technology for the production of an entirely safe formula for cow milk allergic patients. So far, different hydrolysates with various degrees of protein hydrolysis which are available on the market do not satisfy the expectations of physicians and patients as they still contain proteins with allergenic properties. According to UE Directive 2003/89/EC of November 10, 2003 which enforces the necessity of labelling the foods containing allergens, increasing efforts are needed to lower the allergenicity of specific formulae.

The most popular modification of milk proteins is hydrolysis with trypsin (Lametti *et al.* 2002), microbial enzymes (Kleber *et al*. 2006; Bernasconi *et al.* 2006; Jędrychowski & Wróblewska 1999), and ultra-high pressure treatment and temperature (Kleber *et al*. 2007). Literature reports on benefits

Supported by the National Grant No. N31204231/2678.

of other types of enzyme such as transglutaminase, which catalyses the cross-linking reaction between protein molecules and is mainly used to improve the functional properties of food (Truong *et al.* 2004; Soeda *et al.* 2006; Jaros *et al.* 2006). It has a great potential to improve the firmness, elasticity, viscosity, heat stability, and water holding capacity of prepared foods (meat, fish, soybean, wheat products) through a mild enzyme reaction (Kuraishi *et al.* 2001), and was also reported to alter the characteristics of gelatine and collagen (Motoki & Seguro 1998; Kashiwagi *et al.* 2002). Transglutaminase catalyses the acyl-transfer reaction between γ-carboxyamide group of peptide-bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors) including the ε-amino group of lysine residues (Motoki & Seguro 1998). As a result of the cross-linking of the peptide-bound glutamine and lysine residues, ε-(γ-glutaminyl) lysine isopeptide bonds and high molecular weight polymers are formed. Two other reactions are also catalysed by TG: deamidation and amine incorporation (Özrenk 2006).

Transglutaminases are enzymes that occur in plants, animal tissues, body fluids, and microorganisms. Transglutaminase, Activa^{TM} P, (m-TG) preparation was succesfully isolated from a variant of *Streptoverticillium mobaraense* by Ajinomoto Inc. (Germany) by a simple, cost-efficient technology of production, and now m-TG is widely used on commercial scale for proteins cross-linking.

Milk proteins are a suitable substrate for m-TG as they are characterised by high contents of glutamine and lysine amino acid residues (Menéndez Aguirre 2006). Casein is a better substrate than whey proteins (e.g. β-lactoglobulin), nevertheless denaturation or chemical disruption of disulfide bonds increases its reactivity (Gerrard 2002). Milk products prepared with m-TG were shown to display an increased gel strength, less syneresis and more creamy consistency (Lorenzen *et al.* 2002). Cross-linking reduced mesh sizes of the milk protein network and distribution of proteins in the product was more regular. O' Sullivan *et al.* (2002) proved that m-TG rendered casein micelles extremely stable to a range of disruptive forces. Sharma *et al.* (2002) proved that industrial α-la was able to cross-react with TG even without the reduction of disulphide bonds. The precise modification of amino acids (lysine, and glutamine) in holo and apo-α-la by TG was identified at 30 and 50 degrees which offers the possibility of preparing products with various degrees of modification with different physical properties (Nieuwenhuizen 2003). Using m-TG in dairy products becomes of a great interest to food scientists (Stepaniak 2004; Özrenk 2006; Jaros *et al.* 2006). No literature data exist concerning immunoreactivity or allergenicity of dairy products prepared with m-TG. Allergic properties of microbial transglutaminase were evaluated according the 2001 FAO/WHO Decision Tree (Pedersen *et al.* 2004). They found homology between five amino acid fragments of m-TG and cod fish allergen identified earlier (Gad c 1). Homology of six amino acid fragments can indicate the allergenic potential of a new protein product. According to the Decision Tree, the most significant step in the allergic risk evaluation of a novel food is the determination of the reaction level between the new proteins and specific allergic patients sera. In the study mentioned, no reaction was observed between patient IgE and m-TG (Pedersen *et al.* 2004; Poulsen 2004).

The aim of this study was to determine the effect of enzymatic hydrolysis of WPC with Alcalase, cross-linked WPC with m-TG, and combined modification of cow milk whey proteins with Alcalase and m-TG on the immunoreactivity of proteins.

MATERIALS AND METHODS

Raw materials. A commercial, 65% whey protein concentrate (WPC) obtained from Laktopol Company, (Suwalki, Poland), was used as the substrate for the hydrolysis reaction in this study.

Enzymes. Protease, Alcalase 2.4 FG from *Bacillus licheniformis* (purchased from Novo Nordisk, Denmark) and transglutaminase MP-Activia (m-TG) (Ajinomoto, GmbH, Hamburg, Germany) were used.

WPC hydrolysis. A 35% solution of WPC proteins was prepared (the amount of proteins equivalent to raw milk), and then Alcalase was added (15 mAU enzyme/g proteins, enzyme dose determined previously, data not published). Hydrolysis was carried out (temperature of reaction 50°C, pH 8.0) for 140 min until a constant level of the degree of hydrolysis (DH) had been obtained. The sample was then heated at 90°C/5 min to inactivate the enzyme.

Degree of hydrolysis. The determination of the degree of hydrolysis (DH) was performed using

the pH-stat method (Adler-Nissen 1986). The total amount of peptide bonds was determined after the hydrolysis of proteins with 6 mol/l HCl at the temperature of 105°C (Hajós *et al*. 1988). TNBS method was used to determine free α-amino groups (Panasiuk *et al*. 1998).

Transglutaminase-cross-linking of whey protein. The addition of m-TG to 3.5% WPC protein solution was 0.105 g/100 ml. The solution was stirred for 1.5 h at the temperature of 37°C and the sample was then heated at 90°C/5 min to inactivate the enzyme.

Transglutaminase-cross-linking of whey protein hydrolysate. The addition of m-TG to 3.5% WPC hydrolysate protein solution prepared with Alcalase was 0.105 g/100 ml. The solution was stirred for 1.5 h at the temperature of 37°C and the sample was afterwards heated at 90°C/5 min to inactivate the enzyme.

Total protein concentration by Bradford assay. The determination of the total content of proteins was made in triplicate to estimate the optimal conditions for electrophoresis, 2D and ELISA (Bradford 1976).

Separation of proteins by SDS-PAGE. Defatted samples of WPC and its modified products (12–125 μg dry mass) were separated by SDS-PAGE (75 mm × 83 mm × 1.5 mm) using the buffer system of Laemmli (1970). The stacking gel contained 125mM Tris/HCl, 0.1% w/v SDS, pH 6.8, whereas the resolving gel (5–20% gradient) contained 340mM Tris/HCl, 0.1% w/v SDS, pH 8.8, and the electrophoretic buffer – 25mM Tris, 192mM glycine, 0.1% w/v SDS. The sample buffer contained 62mM Tris, 10% v/v glycerine, 3% w/v SDS, and 1% v/v β-mercapto-ethanol, pH 6.8. The separation was conducted at 200 V for approximately 70 min at room temperature.

Separation of proteins by two dimensional (2D) electrophoresis and confirmation of immunoreactivity by Western blots. The first dimension (Isoelectric Focusing, IEF) was run in the immobilised pH gradient (Immobilised pH Gradient, IPG) strip. Each sample was dissolved in rehydratation buffer (8 mol/l urea, 1% 3-(3-cholamidopropyl)dimethyloammonio-1-propane sulfonate (CHAPS), 20% mmol/l DTT). The protein extract (0.09–0.36 mg) was applied to 7 cm IPG strips for rehydratation and focusing with a linear voltage slope up to 24 000 volt-hours in the pHaser isoelectric focusing unit Bio-Rad Protean IEF Cell (Philadelphia, PA, USA). Before running the SDS-PAGE, IPG strips were equilibrated with urea (6 mol/l), SDS (2%), and glycerol (20%) containing Tris-HCl buffer in the presence of 130 mmol/l DTT for 15 min followed by 135 mmol/l jodoacetamide for 15 minutes. The strips were then loaded on the home-casted vertical SDS-PAGE in Bio-Rad Protean 3 Cell. For the molecular weight determination, Prestained SDS-PAGE Standard (BIO-RAD) was used.

After electrophoresis, proteins were either stained using Coomassie Blue (Reanal, Budapest, Hungary) or subsequently transferred onto a 0.45 μm supported nitrocellulose membrane (Bio-Rad) by semidry blotting for 60 min at 0.8 mA/cm^2. Prior to blotting, the gel and the membrane were equilibrated in 25mM Tris, 192mM glycerine, and 20% v/v methanol. Imaging of the gels and blots was carried out with a Bio-Rad Gel Doc 2000 system.

Human sera were obtained from cow milk allergic children, remaining under medical care of the Children Gastrointestinal Centre, Madarasz Children's Hospital in Budapest (Hungary).

Immunological assay. After the transfer onto nitrocellulose membrane, free binding sites were blocked by incubation in Tris-buffered saline (TBS) supplemented with 0.05% v/v Tween 20. The identification of immunoreactive proteins was performed overnight using undiluted individual sera obtained from milk allergic patients. After washing, the bound antibodies were detected with peroxidase-conjugated goat anti-human IgE (Sigma, Germany) diluted 1:120. The binding patterns were visualised using a substrate solution consisting of 4-chloronaphtol (Sigma, Germany), H_2O_2 and ethanol in 16mM phosphate buffered saline solution.

Immunoreactivity of modified WPC products determined by competitive ELISA. Microplates (Nunc®, Roskilde, Denmark) were coated with the antigen in triplicate (1 μg/ml for β-lg and 5 μg/ml for α-la) in a 9mM carbonate buffer solution, pH 9.6, in the amount of 100 μl/well. The microplates with the antigen were incubated for 18 h at 4°C, then rinsed 4 times with 10mM phosphate-buffered saline, pH 7.4, containing 0.5% Tween 20. This procedure was repeated after each step of this method. The residual free binding sites were blocked with 150 μl/well of 1.5% gelatine solution and incubated for 30 min at 25°C. Then the wells were filled with both samples containing the antigen and the polyclonal rabbit antibodies,

obtained in our own laboratory against the given antigens, α-la and β-lg (50 µl of each solution of an adequate concentration per well), and incubated for 1.5 h at 37°C. After rinsing the microplate, the substrate 3,3',5,5'-tetramethylbenzidine (TMB-solution in citrate buffer, pH 5.0) was added and after 30 min the process was terminated with 50 µl 2M H_2SO_4 solution. The absorbance was read at 492 nm using the Sunrise, Tecan (Austria). The results obtained were processed by means of Immunofit^{TM} EIA/RIA software by Beckman.

RESULTS AND DISCUSSION

In the reported study two enzymes were used for the milk whey protein modification, i.e. serine endopeptidase – Alcalase 2.4.FG, and transglutaminase (m-TG). Alcalase is an enzyme hydrolysing peptide bonds it the vicinity of serine, glycine, and aromatic amino acids residues. The final DH obtained during hydrolysis of WPC using Alcalase was 14.5%. This was similar to the findings of other authors (Spellman *et al.* 2003; Severin & Xia 2006). In the previous study (Wróblewska *et al.* 2004), the authors concluded that WPC hydrolysis with Alcalase caused a significant decrease in the immunoreactivity of cow milk whey proteins, but the hydrolysate was still allergenic, although most peptides had molecular weights within the range of 369–1046 Da.

The cross-linking reaction is more complex. The catalytic mechanism of m-TG was desribed by Kashiwagi *et al.* (2002) as based on a ping pong reaction of guinea pig liver transglutaminase which was postulated by Folk and Cole (1965) to consist of three phases: (1) a glutamine substrate binds to catalytic cysteine residue of the enzyme in a binary complex as thioester, (2) ammonia dissociates with the formation of an acyl enzyme intermediate, (3) the acyl enzyme intermediate reacts with the second substrate, either through an acyl acceptor, which can be almost any primary amine, to form γ-glutamyl-amine product or via water to form glutamic acid product (Menéndez Aguirre 2006). During the cross reaction, it was difficult to maintain constant pH which required adjusting acidity by the addition of 0.5M NaOH or 0.5M HCl. It was difficult to determine in which phase of the chemical interaction the cross reaction proceeded. Therefore, the authors applied various conditions of m-TG reaction (Jaros *et al.* 2006).

Most studies described that denaturation of globular proteins prior to the cross-linking reaction was necessary and made the access of m-TG to proteins easier (Sharma *et al.* 2001), and that e.g. dithiotheritol was required for cross-linking α-la (Aboumahmoud & Savello 1990; Wilcox & Swaisgood; 2002; Eissa & Khan 2006). Nieuwenhuizen *et al.* (2003) concluded that modification of lysine and glutamine residues in two forms of α-la (apo and holo) depended on the reaction conditions. At 30°C, no lysine or glutamine was modified in holo α-la, whereas in apo α-la lysines 13, 16, 108, and 114, and glutamines 39 and 43,

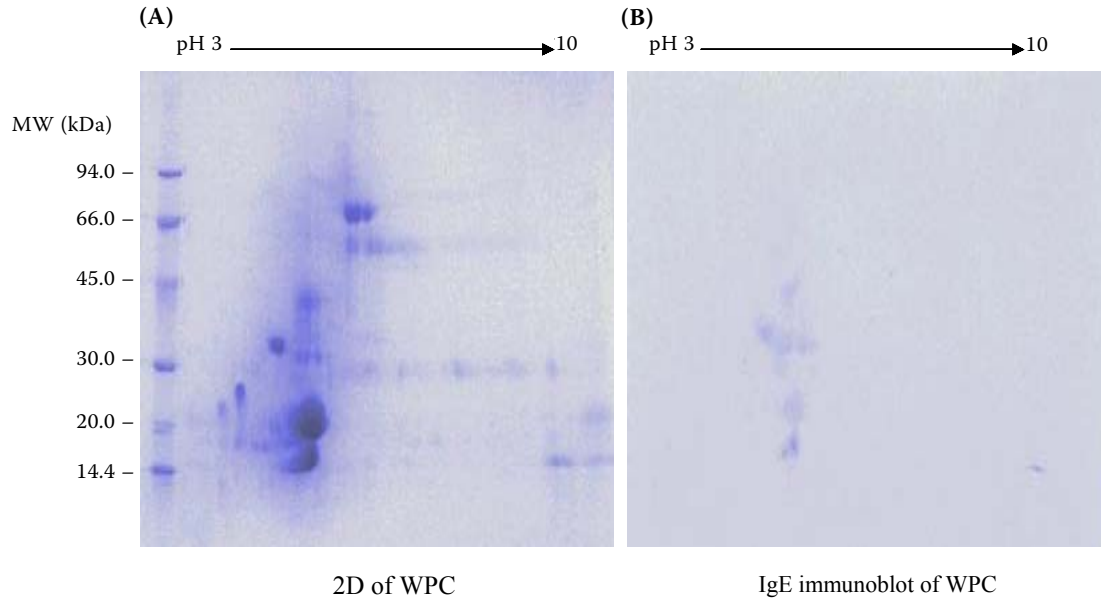

Figure 1. (A) 2D electrophoresis pattern of WPC; (B) Immunoblot of WPC proteins with human IgE

were subject to modification. At 50°C, lysines 13, 16, 108, and 114, but no glutamine, were modified in holo α-la, whereas in apo α-la lysines 5, 13, 16, 108, and 114, and glutamines 39, 43, 54, 65, and 117, underwent modification. The aim of this study was to modify whey proteins (WPC) with m-TG without previous denaturation and to determine the influence of m-TG on proteins immunoreactivity. The first study with m-TG, used to modify the native whey protein (β-lg), was published by Nieuwenhuizen *et al.* (2004). They showed that both isoforms, β-lg(A) and β-lg(B), can be successfully modified with m-TG under non-reducing and non-denaturing conditions.

To determine the changes within the structure of WPC proteins after the enzymatic modification, some analytical methods, including immunologic ones, were applied.

Two dimensional electrophoresis (2D) indicated the most distinct whey milk protein spots in the pH range of 4.5–5.5 and at pH 10.0 (Figure 1A). Their location corresponded to major milk allergens: α-la (14,2 kDa), β-lg (18.6 kDa), and casein fractions (28–32 kDa). There was also a distinct spot of bovine serum albumin, BSA (66 kDa). Allergenicity of WPC was determined applying 2D electrophoresis immunoblot with sera from cows' milk allergic patients. The immunoreactive identified spots corresponded to α-la, β-lg, and casein fractions (pH 4–5), and one unidentified immunoreactive spot to protein of 14.2 kDa and 10 pH (Figure 1B).

Figure 2A and B depict typical spots responsible for all major milk whey proteins, 14.2 kDa (α-la), 18.6 kDa (β-lg) and slightly stained spots in the range of 28–32 kDa, probably as a technological contamination with casein fractions. Very intensive hydrolysis of WPC with Alcalase showed a peptide mixture observed below 14.2 kDa (Figure 2A). Probably, hydrolysis of WPC with Alcalase resulted in the reduction of the main whey allergic proteins (α-la and β-lg). Due to the fact that no difference was observed in SDS-PAGE electrophoresis between WPC and WPC modified with m-TG, it can be concluded that WPC was not modified with m-TG (Figure 2B). The small difference between 2D immunoblot pictures of WPC (Figure 1B) and WPC with m-TG (Figure 4B) showed that the reaction of milk whey protein with transglutaminase can reduce allergenicity without previous denaturation. However, De Jong and Koppelman (2002) pointed out that some proteins, e.g. β-lg, needed a pre-treatment to open the protein structure to enhance cross-linking and also to enhance acyl-transfer. When m-TG was added to untreated milk, only casein was able to cross-react, and whey protein remained intact. They pointed out that, probably, the concentration of transglutaminase higher than that of transglutaminase inhibitor in cow milk can evoke cross-linking reaction of casein (De Jong *et al.* 2003).

After WPC hydrolysis with Alcalase 91, spots were revealed which could belong to the immunoreactive proteins and peptides (Figure 3A). Most of

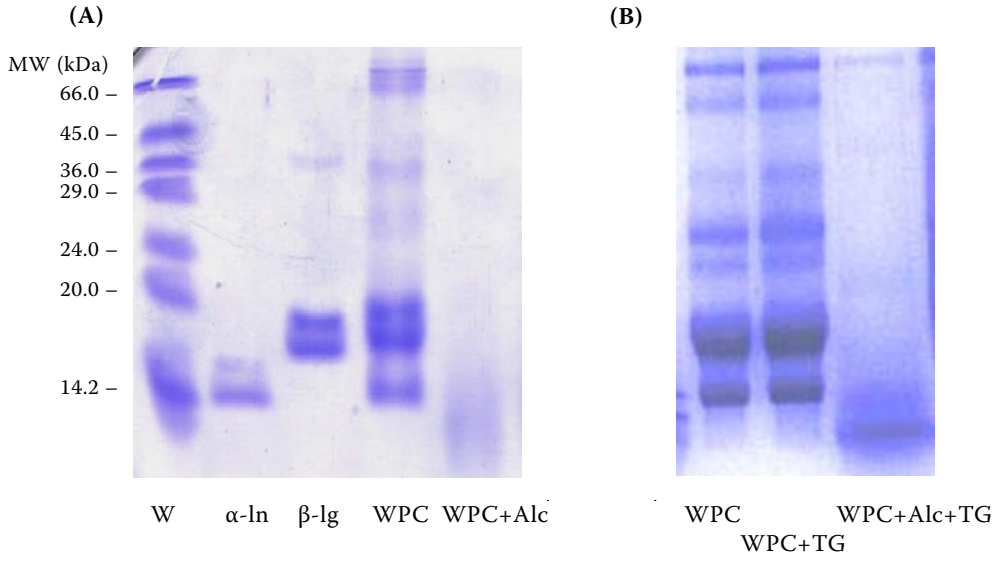

Figure 2. SDS-PAGE of WPC and its hydrolysates: (A) molecular standards (W – weight standard, α-la, β-lg), WPC – whey protein concentrate, WPC+Alc – WPC hydrolysate with Alcalase; (B) WPC – whey protein concentrate, WPC+TG – WPC with transglutaminase, WPC+Alc+TG – WPC with Alcalase and transglutaminase

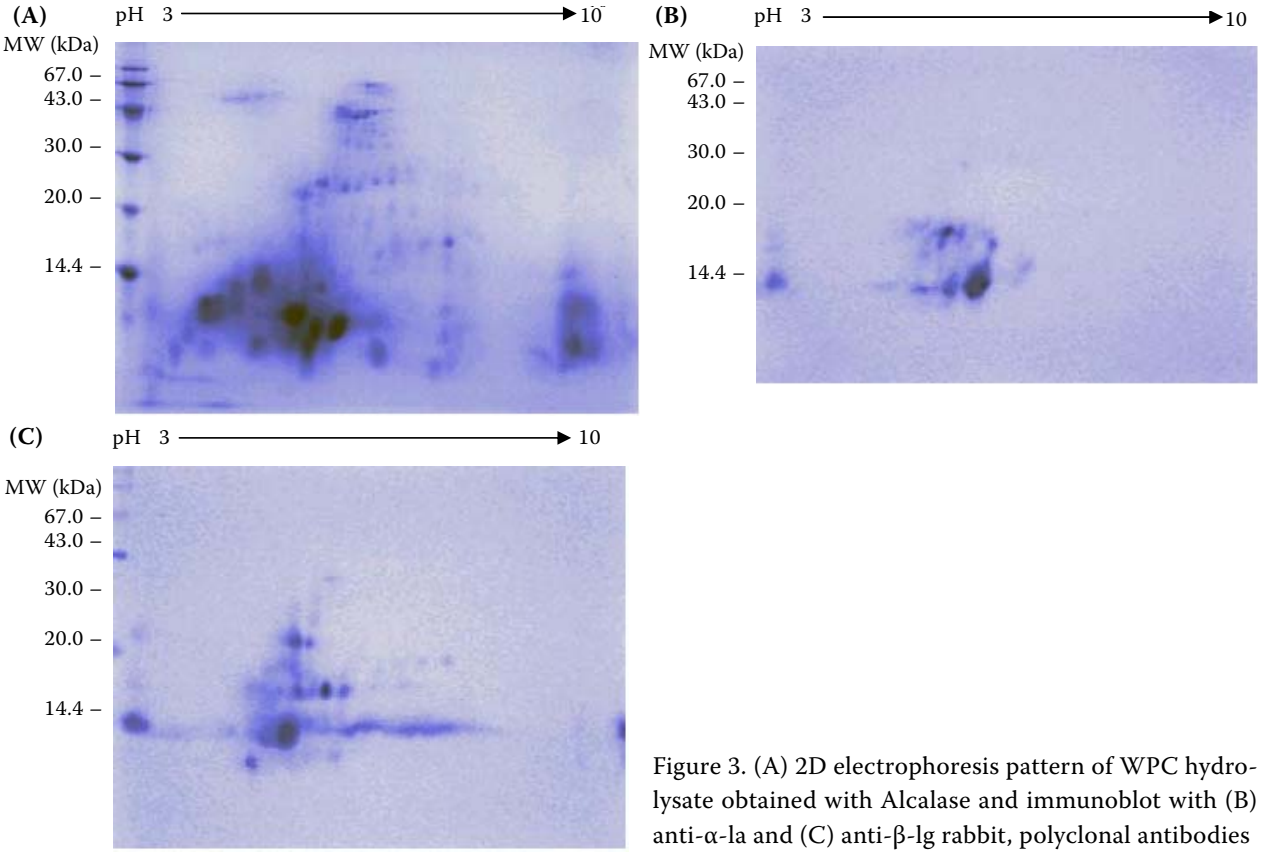

Figure 3. (A) 2D electrophoresis pattern of WPC hydrolysate obtained with Alcalase and immunoblot with (B) anti-α-la and (C) anti-β-lg rabbit, polyclonal antibodies

them were stained in the range of the peptide area. Immunoblot of WPC hydrolysate produced with Alcalase as prepared with anti-α-la and anti-β-lg rabbit polyclonal antibodies, revealed its immunoreactive character (Figure 3B and C).

Figure 4 depicts WPC proteins modified with m-TG. Some spots occur in the pH range corresponding to the electrophoretical positions of major milk proteins: α-la (14.2 kDa), β-lg (18.6 kDa) casein fractions (29–32 kDa), and BSA (66 kDa). Yet on

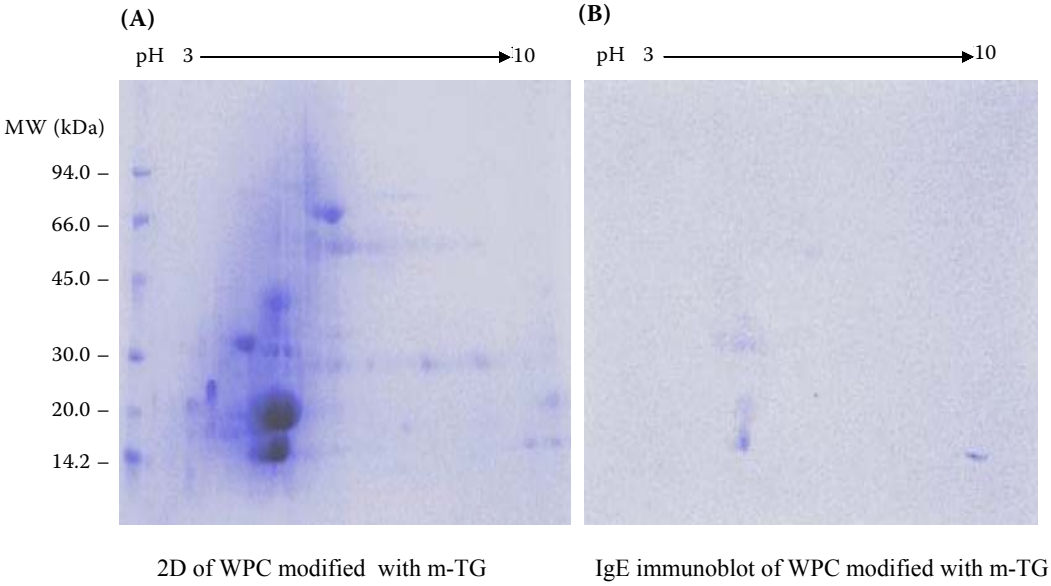

Figure 4. (A) 2D electrophoresis pattern of WPC modified with m-TG, (B) Immunoblot of WPC proteins modified with m-TG with human IgE immunoblot

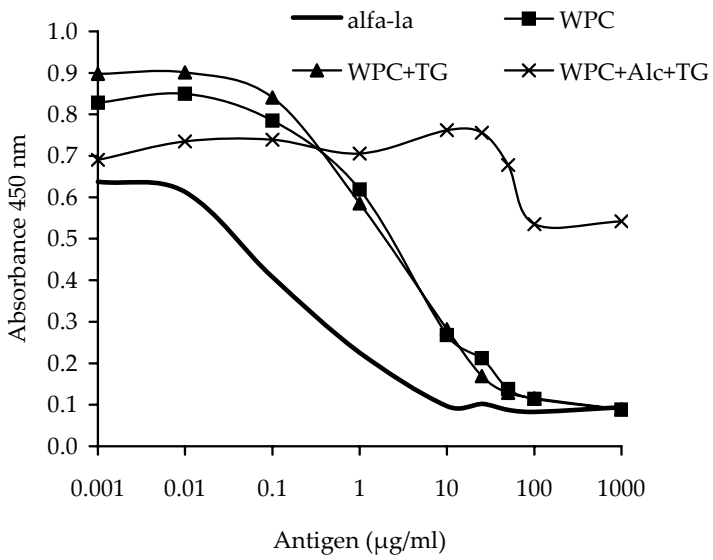

Figure 5. Results of competitive ELISA: reaction of WPC and its modified products with rabbit polyclonal anti-α-la antibodies

the immunoblott (Figure 4B) only two week spots are noticeable corresponding to IgE patient sera reaction with α-la (one at pH 4–5 and the other near pH 10), and a very week IgE answer to β-lg. This fact may prove that cross-linking with m-TG is a more advantageous process to achieve a low immunoreactivity of milk protein than hydrolysis with Alcalase (Figure 1B).

ELISA method was used to determine the immunoreactivity of WPC enzymatic modification products after the reaction with rabbit polyclonal antibodies directed to main whey allergens α-la (Figure 5) and β-lg (Figure 6). In both figures, the curves of WPC and WCP cross-linked with TG have a similar shape and immunoreactive range, which may indicate insignificant changes within the epitope structures. Hydrolysis of WPC with Alcalase changed the immunoreactive reaction to a high extent and resulted in a partial reduction of immunoreactivity. However, the addition of m-TG to WPC hydrolysate prepared with Alcalase considerably changed the shape of the curve, which indicated a reduction of WPC product immunoreactivity after two-step modification (with Alcalase and subsequently m-TG). No reaction occurred between the modified product (WPC Alcalase hydrolysate after incubation with m-TG) and α-la antibodies (Figure 5), but the epitopes responsible for the reaction with β-lg antibodies (0.6 %) were still present (Figure 6). It appears that cross-linking, acyl-transfer, and deamidation of WPC hydrolysate produced with Alcalase could change the structure of proteins mainly in the epitope area responsible for the allergic reaction with IgE. The reactions of hydrolysis and subsequent conjugation by cross reaction may

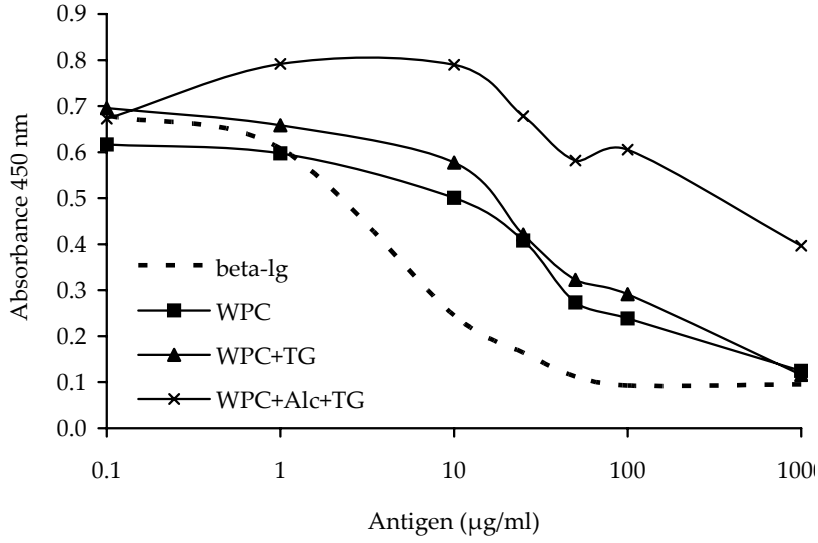

Figure 6. Results of competitive ELISA: reaction of WPC and its modified product with rabbit polyclonal anti-β-lg antibodies

be a new method for reducing the immunoreactivity and allergenicity of protein formulae.

CONCLUSIONS

Hydrolysis of whey proteins did not guarantee obtaining quite a safe hypoallergenic product. During proteolytical reaction, the conformational epitopes are probably destroyed but the linear epitopes are still present. Apart from that, whey proteins can contain casein particles as a technological contamination and between milk proteins, especially β-lg and casein, cross reactions proceed. All the findings mentioned can indicate that hypoallergenic formulas were characterised by residual antigenicity, and cross-linking seems to be a more advantageous solution which can be proposed to prepare milk protein as a base for hypoallergenic formula.

A new technological proposal – the application of two-step enzymatic modification of WPC with proteinase (Alcalase) and cross-linked enzyme (m-TG), is presented in this study.

The competitive ELISA methods showed that this modification reduced the immunoreactive properties of α-la (no cross-reaction was observed) and significantly lowered the immunoreactivity of β-lg (about 0.6% cross-reactivity). It seems necessary to determine the allergenicity of such a product in the future.

Acknowledgements. The authors thank the Ajinomoto company for transglutaminase, (m-TG) Activa^{TM} P, and Marianne Polgar from Children Gastrointestinal Centre, Madarasz Children's Hospital in Budapest (Hungary) for human sera.

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Received for publication September 25, 2006
Accepted after corrections November 22, 2007

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