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ENZYMATIC HYDROLYSIS OF COW'S WHEY MILK PROTEINS IN THE ASPECT OF THEIR UTILIZATION FOR THE PRODUCTION OF HYPOALLERGENIC FORMULAS

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Key words: immunoreactivity, whey proteins, enzymatic hydrolysis, ELISA, sensory evaluation

The aim of the study was to lower the immunoreactivity of milk whey protein (WPC), α-lactalbumin (α-la) and β-lactoglobulin (β-lg) by means of one-step enzymatic hydrolysis with two different doses of Alcalase (A and 2A) against WPC substrate, Lactozyme and two-step enzymatic hydrolysis (Alcalase with pepsin, Alcalase and papain, and Alcalase and Lactozyme). The highest degree of hydrolysis (DH) was noticed for WPC hydrolysis with 2A. Electrophoresis was used to determine changes in WPC mass proteins obtained after hydrolyses, whereas competitive ELISA to determine their immunoreactivity, and sensory evaluation to estimate the desirability of particular products. The lowest WPC immunoreactivity was obtained after hydrolysis with Alcalase.

Analytical sensory evaluation (QDA) and hedonic rating proved that the sensory quality of the hydrolysates was mainly dependent on the bitterness and dairy taste. These negative attributes were partly reduced by using papain and Alcalase. The addition of Lactozyme increased the amount of glucose in hydrolysates eight times, but did not influence their general palatability. The results obtained with the QDA method were confirmed by the Principal Component Analysis (PCA).

INTRODUCTION

Food allergy to milk is usually the first reaction of hypersensitivity occurring already in infancy and playing a significant role in inducing different types of allergies with different clinical symptoms. Studies have shown that over 80% of children with allergy to milk develop asthma or other symptoms of the respiratory tract [Exl & Fritsché, 2001]. The most important whey protein allergens are α-lactalbumin (α-la) and β-lactoglobulin (β-lg). Both these proteins are likely to provoke allergic responses. Within β-lg structure, there were distinguished multiple significant IgE-dependent amino acid fragments: 1–8, 9–14, 25–40 78–83, 84–91, 92–100, 123–135 [Wal, 2002], 17–58, 59–93, 99–108, 109–123 [Maynard *et al.*, 1997], 21–40, 41–60, 107–117, 148–168 [Miller *et al.*, 1999], 47–56, 67–76, 75–84, 127–136, 141–150 [Järvinen *et al.*, 2002] as well as the IgE-dependent ones: 1–24, 67–77, 82–92, 85–95, 117–127 [Miller *et al.*, 1999], 10–18, 60–80, 91–94, 105–117 [Maynard *et al.*, 1997]. α-La contains in its structure immunodominating epitopes in 6–10:S-S:115–123, 59–94 [Wal, 2002], 13–22 [Järvinen *et al.*, 2002], 17–58, 99–108, 109–123 [Maynard *et al.*, 1996], 42–49 [Senocq *et al.*, 2001]. Whey protein hydrolysates were practically used in the production of formulas for infants with atopy and adsorption and digestion disorders with immunological background. A technology which would ensure obtaining a completely safe and universal formula still remains a problem. A mixture of peptides obtained as a result of casein enzymatic hydrolysis with molecular weight of below 500 Da still displays allergenic properties [Calvo & Gomez, 2002]. Only amino acid mixtures are completely non-allergenic products [Høst & Halken, 2004]; other hypoallergenic products available on the market contain proteins potent to induce allergic mechanisms.

New products can be qualified as therapeutic milk-replacing preparations only if they are well tolerated by 90% of the population with confirmed hypersensitivity to protein being the basis of the hydrolysate studied.

Milk allergy is often accompanied by lactose intolerance classified as non-allergic enzymopathy [Czernecki & Targoński, 2002]. Lactose intolerance was demonstrated in 4% of the children with allergy to cow's milk protein [Woś, 2000]. The cause of intolerance is a lack or deficiency of lactase (β-galactosidase) in the brush border of the small intestine mucosa. In such cases, saccharide not absorbed in the small intestine causes osmotic fluid transfer to the intestine lumen. Moreover, non-digested lactose passes to the large intestine, where it is fermented by *Coli* bacteria. In this process, gases are produced as a by-product and the monosaccharides formed cannot be absorbed in the large intestine, which deepens the osmotic effect being the result of fluid transfer to the intestine lumen [Swagerty, 2002]. In patients with very low lactase level, the time of symptom onset is not always correlated with the time of lactose consumption. The symptoms of lactose intolerance include flatulence, abdomen aches and diarrhoea, but they are not specific. In addition, the large intestine's hypersensitivity syndrome is likely to occur [Turnbull, 2000].

Apart from the immunoreactive properties of hydrolysed products also their sensory evaluation is highly signifi-

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cant. The most advantageous would be an individual selection of food preparation based on interview data, degree of risk to develop allergy and tolerance to the preparation by child-patient [Kaczmarski *et al.*, 2000]. So far attempts to produce a hypoallergenic formula completely free from allergenic and immunogenic properties and satisfactorily tasty have been unsuccessful.

The taste and smell of formulas consumed in early infancy have a strong influence on later food preferences. This has been confirmed in a study on a group of 53 two-week-old infants fed Nutramigen or Enfamil in the Flavor Programming During Infancy project [Menella *et al.*, 2004].

The aim of this work was to examine the influence of one- and two-step enzymatic WPC hydrolysis on the immunoreactive properties and sensory quality of hydrolysates and to explain which of their sensory attributes are the most important for consumers.

MATERIALS

In this study, commercial whey protein isolates (WPC), obtained from Laktopol Company, Suwałki, Poland, were applied as a substrate of hydrolysis reaction. Protease from *Sublilisina carlsberg* as Alcalase 2.4 FG, Lactozyme^{TM} 3000 L HPG (purchased from Novo Nordisk), papain – EC 3.4.22.2 (Sigma cat. No. P-3375), pepsin – EC 3.4.23.1 (Sigma cat. No. P-7000), were used as enzymes.

The proteolytic activity of enzymes was determined with the Anson's method with the use of haemoglobin as a substrate according to Mejbaum-Katzenellenbogen & Mochnacka [1969]. The values obtained reached: 1.83 AU/g protein for Alcalase, 3.15 AU/g for papain and 4.63 AU/g for pepsin.

METHODS

Treatment of milk with enzymes. The detailed process of hydrolysis was described earlier [Wróblewska *et al.*, 2004]. We used the following enzymes: Alcalase, an increased dose of Alcalase against the substrate, (in this study described as A2), Lactozyme, Alcalase and pepsin, Alcalase and papain, Alcalase and Lactozyme. Briefly, the conditions for the one-step hydrolysis of WPC with Alcalase were as follows: the amount of substrate – 500 mL (3.5% of protein), temp. 50°C, pH 8.0, the addition of enzyme 15 mAU/g of protein (data estimated according to the earlier study [Wróblewska *et al.*, 2004]), or 30 mAU/g of protein (2A). The main question was to determine the influence of an enzyme dose on changes in the hydrolysate's immunoreactivity. The hydrolysis was continued for 120 min. The sample was heated at 90°C/5 min to inactivate the enzyme, then cooled immediately and lyophylised. During the "two-step" hydrolysis, the second enzyme (15 mAU/g) was added after 100 min of the process. The conditions for each enzyme were as follows: for pepsin – pH 2 and temp. 37°C, and for papain – pH 8.0 and temp. 50°C. Lactozyme was added as a 2 mL/L protein solution, according to Application Sheet of Novozymes [Special Food/2001-09443-03.pdf]. The hydrolysis was carried out for 12 h at 4°C, then the sample was heated at 90°C/5 min to inactivate the enzyme, cooled immediately and lyophylised. The determination of a degree of hydrolysis (DH) was performed with the pH-stat method [Adler-Nissen, 1986]. The total amount of peptide bonds was determined after hydrolysis of proteins with 6 mol/L HCl at a temperature of 105°C [Hajós, 1988]. To determine free α-amino groups, use was made of the TNBS method [Panasiuk *et al.*, 1998].

Electrophoretic method. Electrophoretic separations of WPC protein and its hydrolysates were performed with MINIPOL – Kucharczyk TE (Poland) using 15% polyacrylamide gel (SDS-PAGE) [Laemmli, 1970]. Before electrophoresis, protein samples and hydrolysates were heated by boiling for 3 min with SDS (3% w/v) and 2-mercaptoethanol (0.1% v/v). Low molecular markers (Sigma) ranging from 66000 to 6500 Da were used as a standard. The gels were run in a Tris-glycine buffer, pH 8.3, and total protein in gels was stained with Coomassi Brilliant Blue R-250. The conditions of electrophoresis were as follows: 25 mA, time 1.5 h.

The competitive ELISA method. Microplates were coated with the antigen at concentrations determined earlier in the indirect ELISA method (1 μg/mL for β-lg and 5 μg/mL for α-la) in a 9 mmol/L carbonate buffer solution, 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 mmol/L 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 with 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 a 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 a citrate buffer, pH 5.0) and after 30 min the process was stopped with 4 mol/L H_2SO_4 solution. The absorbance was read with an ORGANON-TEKNIKA automatic Reader 510 at a wave length of λ=492 nm.

The results obtained were processed with the ImmunofitT^{M} EIA/RIA software by Beckman.

Glucose concentration. In the study, use was made of a diagnostic kit for the determination of an increase in glucose concentration after hydrolysis (P.Z. Cormay, Poland). Briefly, WPC and its Lactozyme hydrolysates (100 μL) were mixed with trichloroacid (TCA), (1 mL of 3% solution). The sample was centrifuged (5 min/2000 g). Then 100 μL of supernatant was added to 1 mL of 2-Reagent (GOD+POD+4-Aminoantipirine) and incubated for 5 min at a temp. 37°C. The absorbance was measured at a wave length of 520 nm (Spectrophotometer DU® 7500, Beckman, USA). The concentration of glucose was calculated from a standard curve.

Sensory evaluation method. The sensory quality of the hydrolysates was evaluated with the Quantitative Descrip-

tion Analysis (QDA) [Stone & Sidel, 1993]. The QDA procedure used in the study was in agreement with requirements of the international standard [ISO/DIS 13299:1998]. Eight attributes (developed by the sensory panel in a preliminary procedure) describing odour and taste have been used to asses the sensory quality of hydrolysates. Definitions of these attributes are presented in Table 1. The intensity of each attribute was evaluated on a 10-cm linear scale anchored on both sides for odour and taste intensity as "none" (0) and "very intensive" (10). The overall quality test for hydrolysates was conducted using the same type of scale anchored on both ends: unlinking (0) – extremely liking (10).

Sensory panel. The panel of sensory assessors was trained and monitored according to ISO standards [8586-1:1993]. The sensory evaluation of the samples was performed by 8 panellists, all with at least one year of experience in discrimination and descriptive tests on different food products.

Prior to their participation in the experiments, the subjects were trained to rate the perceived intensity of the following different sensations: sourness, bitterness and astringency, using aqueous solutions of different concentrations of citric acid, quinine sulphate, caffeine and tannic acid.

Preparation of samples and testing conditions. The 2 g samples were suspended in 40 mL of boiled and cooled water (20°C) The solution was magnetically stirred for 15 min, transferred to a 200-mL volumetric flask and adjusted with water. For testing, 10 mL volumes of individual samples were prepared from each solution, coded and presented in random to each panel member to avoid carry-over effect. One-minute break was taken between samples, during which the panellists were asked to eat unsalted biscuit and rinse their mouth thoroughly with spring water. Each sample of hydrolysate was evaluated in two replications; hence 16 individual results were the basis for presented mean values and for further statistical data processing. The analysis was performed in a standardised test room provided with 6 test booths [ISO 8589:1998] and with computerised system ANALSENS for data collection and processing [Baryłko-Pikielna, 1992].

Statistical analysis. Principal component analysis (PCA) was applied for general assessment of similarity-dissimilarity of the evaluated samples and for description of their attributes. The statistical analysis was carried out with Senstat for Windows (Deltaworks software, 1996, The Netherlands).

RESULTS AND DISCUSSION

On using enzymatic hydrolysis for protein modification it is possible to intentionally design the final product in respect of the degree of hydrolysis. Hydrolysates obtained in the studies presented were characterised by a hydrolysis degree (DH) ranging from 14.5% (with Alcalase) to 18% (with 2A or two-step hydrolysis with Alcalase and pepsin), (Table 1). Spellman *et al.* [2003] obtained a similar hydrolysis degree of WPC, *i.e.* 14%, using Alcalase 22.4L; in their case hydrolysis proceeded most effectively for the first 160 min and reached DH of 13%. For the next 200 min DH was growing only slightly to reach 15%.

TABLE 1. Degree of hydrolysis (DH) of the WPC hydrolysates obtained with proteolytic enzymes.

Enzyme	DH (%)
Alcalase	14.5
Alcalase (2A)	18.0
Alcalase + pepsin	17.5
Alcalase + papain	15.9

The application of Lactozyme caused about 80% increase in glucose level during hydrolysis, from 100 mg/mL in WPC to 984 mg/mL in WPC hydrolysates prepared with Lactozyme. Simultaneously, this means that the lactose level was lower compared to substrate. The increase in glucose level was proposed as the improvement of hydrolysate sweet taste. No taste improvement was, however, observed. The amount of lactose that remained in hydrolysates was inessential, because every dose can cause intolerance. Roy & Gupta [2002] used Lactozyme to hydrolyse whey protein, and obtained a high level of glucose from lactose, up to 90%.

SDS-PAGE electrophoresis practically does not provide the possibility to identify the molecular mass of particular peptides but it is clearly seen that the obtained protein products underwent proteolytic degradation. The SDS-PAGE of WPC protein and its hydrolysates is presented in Figure 1. The analysis of WPC substrate electrophoregram shows two distinct bands corresponding to the presence of α-la and β-lg. The substrate contains also clear bands in the molecular weight range of 66 and 36 kDa, which indicates the presence of BSA and a number of casein fractions.

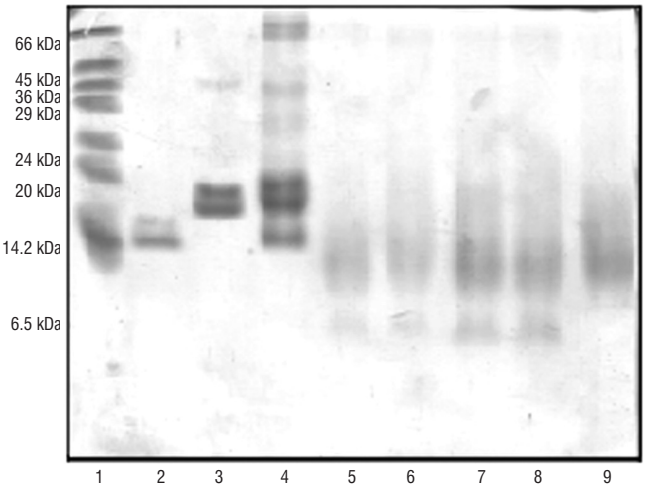

FIGURE 1. SDS-PAGE eletrophoregram of: (1) marker of MW, (2) α-la, (3) β-lg, (4) WPC; hydrolysates: (5) WPC+Alcalase, (6) WPC+Alcalase (2A), (7) WPC+Alcalase and pepsin, (8) WPC+Alcalase+Lactozyme, and (9) WPC+Alcalase and papain.

However, the electrophoregrams of hydrolysate obtained with proteolytic enzymes show a high similarity of their protein-peptide profiles. There is a broad band of intense colour within peptide fractions below 14 kDa. In the

case of hydrolysis with Alcalase and Lactozyme, a decrease in the amount of proteins with high molecular weight is also visible, which can be seen in the broad peptide band below 14 kDa. Lactozyme, which is an amylolytic enzyme, was added only to change the chemical composition of saccharides and, potentially, the sensory properties of the product.

Smyth & Fitzgerald [1998] hydrolysed a WPC preparation applying Alcalase 0.6L. After 30 min of the process they observed the presence of protein fractions of molecular weight below 30 kDa. Further hydrolysis increased the percentage of low-molecular peptides even more. Electrophoregrams of hydrolysate proteins obtained after 8-h hydrolysis revealed only the presence of fractions below 14 kDa. The application of Neutrase, a neutral proteinase from *Bacillus subtilis*, caused similar results.

Ena *et al.* [1995] studied conformation changes of proteins in WPC preparations during hydrolysis with Corolase 7092™ (peptidases from *Aspergillus strains*) and with pepsin and Corolase PP™ (a mixture of pancreatic enzymes). Electrophoregrams of hydrolysates obtained with Corolase 7029™ showed a significant decrease in the amount of high-molecular protein fractions after 75 min of hydrolysis. After another 85 min, particular fractions were practically undetectable with the SDS-PAGE method. Hydrolysis of α-la standard proceeded more slowly. Even after 180 min a clear fraction of non-hydrolysed α-la was observed. The β-lg standard turned out more vulnerable to the enzyme. Electrophoregram made after 60 min of hydrolysis using Corolase 7029 did not reveal the presence of non-hydrolysed protein. The next stage was a two-step hydrolysis with pepsin and Corolase PP. After 190 min of hydrolysis, there were observed small fractions containing proteins with molecular weight of over 16 kDa [Ena *et al.*, 1995].

The capability of the obtained hydrolysates to react with anti-α-la and anti-β-lg antibodies was assessed using competitive ELISA (Figures 2 and 3). In the case of WPC hydrolysates, their allergenicity was clearly reduced compared to the reaction with allergen as pure as α-la.

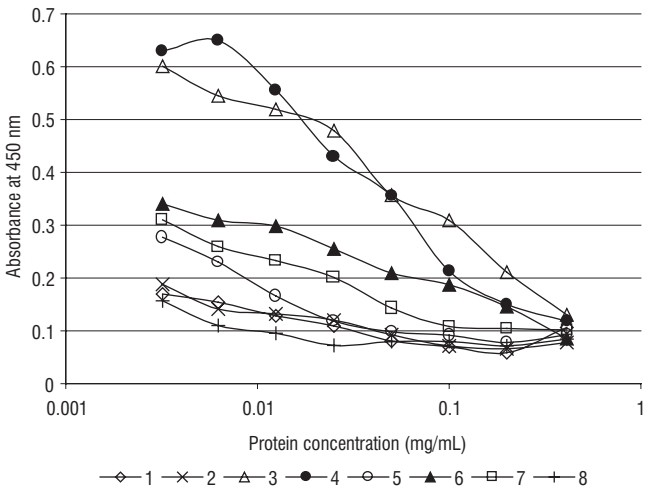

FIGURE 2. Results of ELISA assay of WPC hydrolysates with rabbit anti-α-la: (1) α-la standard, (2) WPC, hydrolysates: (3) WPC+Alcalase, (4) WPC+Alcalase (2A), (5) WPC+Alcalase and pepsin, (6) WPC+Alcalase+Lactozyme, (7) WPC+Alcalase+papain, and (8) WPC+Lactozyme.

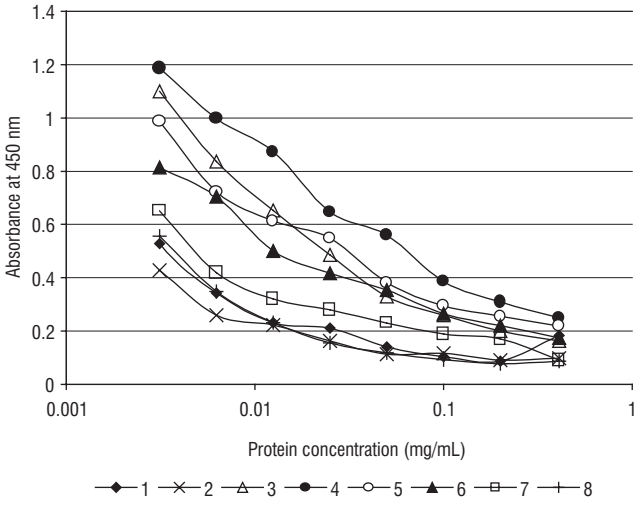

FIGURE 3. Results of ELISA assay of WPC hydrolysates with rabbit anti-β-lg: (1) β-lg standard, (2) WPC, hydrolysates: (3) WPC+Alcalase, (4) WPC+Alcalase (2A), (5) WPC+Alcalase and pepsin, (6) WPC+Alcalase+Lactozyme, (7) WPC+Alcalase+papain, and (8) WPC+Lactozyme.

The lowest immunoreactivity to anti-α-la antibodies was found for WPC hydrolysates obtained using Alcalase and its double dose. A high increase in the immunoreactivity was observed for the preparation obtained using Alcalase and pepsin compared to the hydrolysate obtained with Alcalase. During hydrolysis, the epitopes of proteins were likely to be exposed or plastein reaction proceeded leading to the formation of protein aggregations of various physical and chemical properties which were not observed in the substrate [Stevenson *et al.*, 1999]. Preparations obtained using Alcalase and Alcalase and Lactozyme also had diversified immunoreactivity. In this case, however, Lactozyme acted differently than with the hydrolysate obtained using pepsin. An increase in the immunoreactive properties of the preparation was also observed as a result of enzymatic hydrolysis.

Kananen *et al.* [2000] subjected whey proteins to enzymatic modification using pepsin and trypsin. Three-hour hydrolysis of a preparation with pepsin reduced its allergenicity by 50% (ELISA test), whereas 30-min incubation with trypsin resulted in lowering its antigenicity to next to zero. This confirms weak hydrolytic properties of pepsin towards protein fragments with epitope-like character. Bhatty [1983], comparing vulnerability of different proteins to pepsin action, observed that milk proteins were little vulnerable to hydrolysis. Even following long, several-hour hydrolysis, the hydrolysates were found to contain protein fractions with high molecular weight.

The WPC preparation with Alcalase and the WPC hydrolysate with Alacalase and Lactozyme had similar immunoreactivity because – as an amylolytic enzyme – Lactozyme did not reveal proteolytic action to α-la. Neither did it have a significant influence on the immunoreactivity of protein formerly not subjected to enzymatic hydrolysis with proteolytic enzymes.

The most effective lowering of β-lactoglobulin allergenicity was observed for WPC hydrolysate obtained with a double amount of Alcalase. Preparations hydrolysed in arrangements with pepsin revealed antigenic properties

similar to those of the preparation hydrolysed with Alcalase.

The immunoreactivity of hydrolysate with Alcalase slightly increased following additional use of Lactozyme. It can be supposed that Lactozyme is an enzyme which may bring about changes in glycoprotein structure in the case of reaction with lactose bound into α-la complexes.

Other authors reported that enzymes may affect changes within glycoproteins in the case of α-lactoalbumin. Shida *et al.* [1994] reported that hydrolysis products of glycoproteins consisting of lactose and α-la lose their capability to re-form complexes following hydrolysis. The effect is accounted for by β-galactosidase capability to remove terminal galactoside residues from glycocomplexes. The differences between the enzyme influence on α-la and β-lg result most probably from an increased accessibility of lactose for β-galactosidase when it is bound to α-lactalbumin.

β-Lactoglobulin is a protein which due to a high lysine content is capable of forming complexes with lactose. Morgan *et al.* [1999] studied the vulnerability of lactose β-lactoglobulin present in a commercial preparation of β-lactoglobulin to the action of β-galactosidase isolated from *Kluyveromyces lactis* yeast. No hydrolytic action of the enzyme on lactose bound to complexes with proteins was observed even following long (6-h) hydrolysis. However, the initial incubation of the product with trypsin, and next with β-galactosidase, caused a further decrease in lactose level by 50%. Although the application of β-galactosidase considerably lowered the level of lactose in preparations, it did not improve significantly the antigenic properties of proteins.

Considering the reduction of immunoreactivity, the most advantageous appears to be WPC hydrolysis using only Alcalase or its double amount against the WPC. Thus obtained preparations were characterised by the lowest degree of reaction to both anti-α-la and anti-β-lg.

Cow's milk proteins in a native form, or thermally treated, do not display negative sensory properties. They are neither bitter, nor pungent. It is the use of enzymatic hydrolysis that brings about the formation of peptides responsible for the bitter taste in the ready formula.

The overall quality of WPC hydrolysates was shown in Figure 4. The Alcalase and papain WPC hydrolysate was estimated as the most desirable (4.1 arbitrary units).

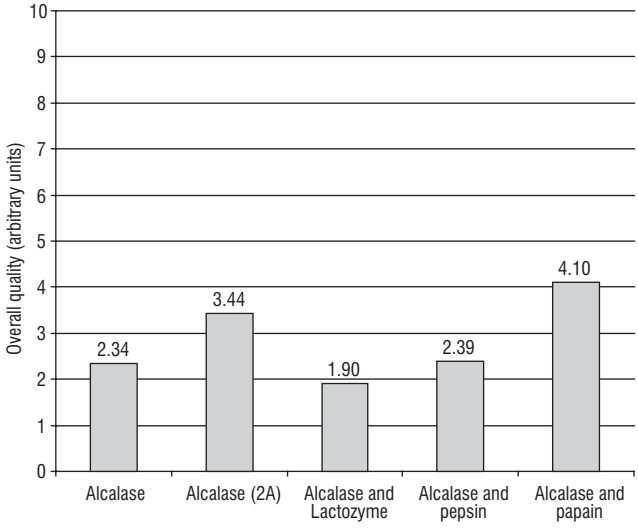

FIGURE 4. Hedonic scores of investigated hydrolysates

Sensory profiles of the hydrolysates in relation to the enzymes used for the hydrolysis of WPC were displayed as spider diagrams in Figure 5a – 5e. At the first glance one could see that the profiles of the samples were differentiated. General eight attributes were selected as the factors influencing the odour and taste of hydrolysates (Table 2). Four of them, *i.e.* dairy (an attribute connected with pungency), bitter, soap-like, rancid fat, which can be regarded as negative for the quality were present in all the products. However, bitter and dairy taste described most of the sensory variations of the samples.

TABLE 2. Attributes (descriptors) used in a sensory analysis of hydrolysates and their definitions.

Attribute	Definition
Odour attributes	
Dairy odour	specific note associated with some dairy product (like pungent)
Creamy odour	odour typical of full fat cream (35% fat)
Taste attributes	
Bitter taste	basic taste typical of caffeine in water (0.5%)
Soap-like taste	taste note associated to NaOH diluted in water (0.1%)
Dairy taste	specific note reminding of some dairy products (like pungent)
Rancid fat taste	taste illustrated by iso-butyric acid (0.1%)
Salty taste	basic taste associated to NaCl diluted in water (0.8%)
Sweet taste	basic taste illustrated by sucrose diluted in water (3%)

The results of the overall quality evaluation of hydrolysates were as follows: Alcalase and papain > Alcalase (2A)> Alcalase and pepsin = Alcalase > Alcalase and Lactozyme. The relation between the overall quality and intensity of selected attributes is demonstrated in Figure 6. These results indicate that the general liking of the hydrolysates was dependent on dairy and bitter taste (which seemed to be closely related to one another). For example, the hydrolysate treated with Alcalase and then Lactozyme proved a high intensity of these attributes and the lowest degree of general liking. In contrast, the hydrolysate treated with Alcalase and then papain demonstrated the lowest intensity of dairy and bitter taste and the highest degree of general liking. It should be emphasized that the sensory profiles of the hydrolysates revealed also positive attributes properties affecting their quality, *i.e.* sweet taste and creamy odour. Their intensity was the highest in the hydrolysate treated with Alcalase and then papain which had the highest overall quality scores.

Data obtained from the profile analysis was subjected to PCA employing statistical software. Two principal components, PC1 and PC2, were extracted which accounted for 72% of the total data variance (Figure 7). The projection of data points ascribed to the samples and to input attributes on the plane reflects, in a comprehensive graphical manner, the similarities and dissimilarities between them. It can be

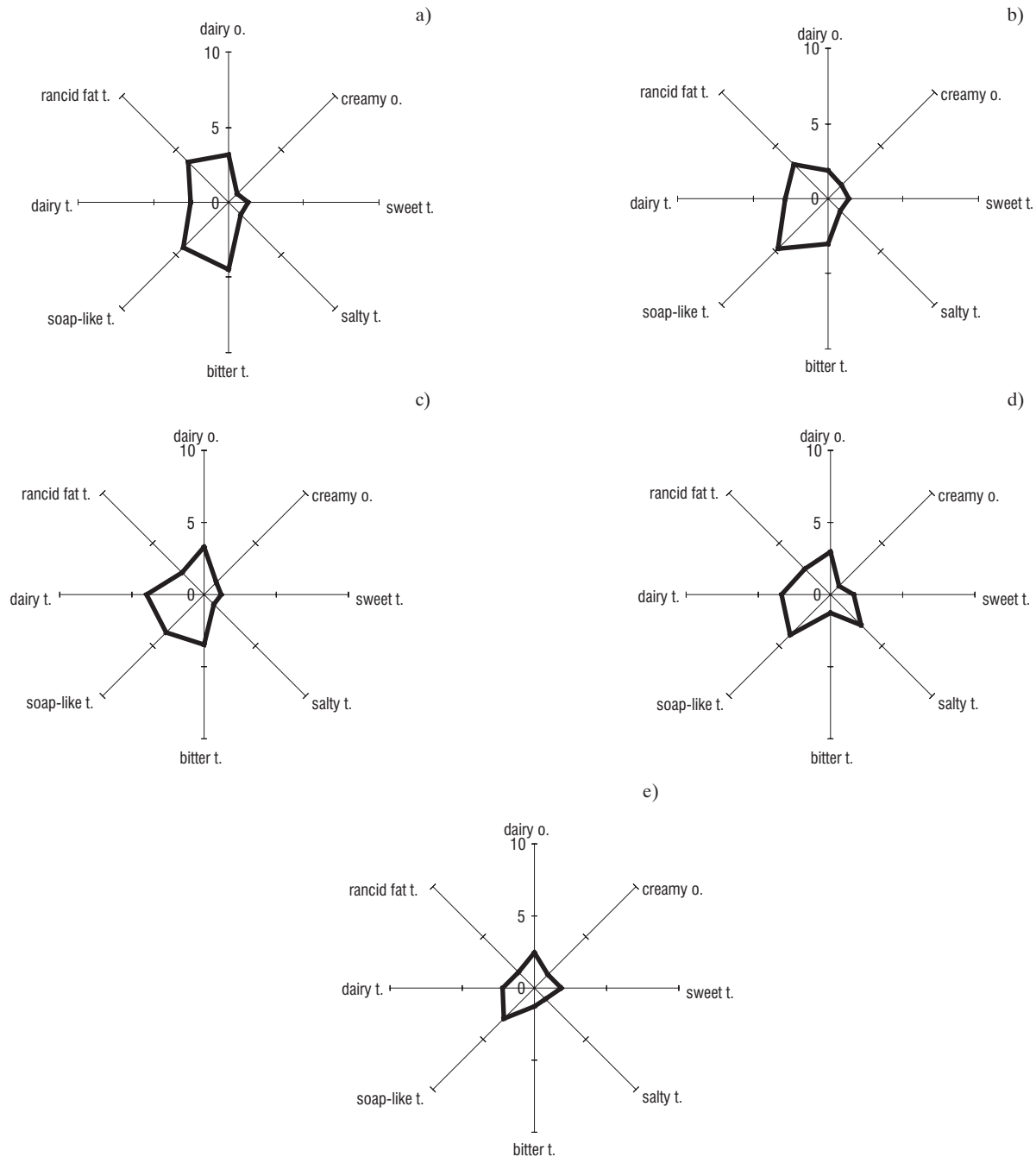

FIGURE 5. Spider diagrams of sensory profiling of hydrolysates: a – Alcalase, b – Alcalase (2A), c – Alcalase and Lactozyme, d – Alcalase and pepsin, e – Alcalase and papain.

seen that in the diagram two samples: Alcalase and papain and Alcalase (2A), were located opposite to the other hydrolysates. They were characterised by more intensive sweet taste and creamy odour than the others, which caused their higher overall quality. The remaining three hydrolysates (Alcalase and pepsin, Alcalase and Lactozyme and Alcalase) were mainly characterised by negative attributes and were located opposite to the overall quality.

Enzymatic hydrolysis of proteins frequently results in bitter taste, which is due to the formation of low molecular mass peptides composed of mainly hydrophobic amino acids [Kirimura *et al.*, 1969; Saha & Hayashi, 2001]. The formation of bitter peptide is the most serious problem in the practical use of food protein hydrolysates. Bitter taste is caused by the release of peptides containing hydrophobic amino acids, such leucine, isoleucine, valine, phenyloalanine, tyrosine and tryptophan, during the process [Pedersen, 1994]. Matoba *et al.* [1970] isolated three bitter peptides with the structure of Gly-Pro-Phe-Pro-Val-Ile, Phe-Phe-Val-Alα-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys and Phe-Alα-Leu-Pro-Gln-Tyr-Leu-Lys from trypsin casein hydrolysates. The peptides are built mostly of amino acids containing hydrophobic groups. This thesis, however, has not been confirmed by Lee & Warthesen's [1996] who demonstrated that in none of the so-called "bitter peptide" studied in cheddar cheese there occurred tryptophane or

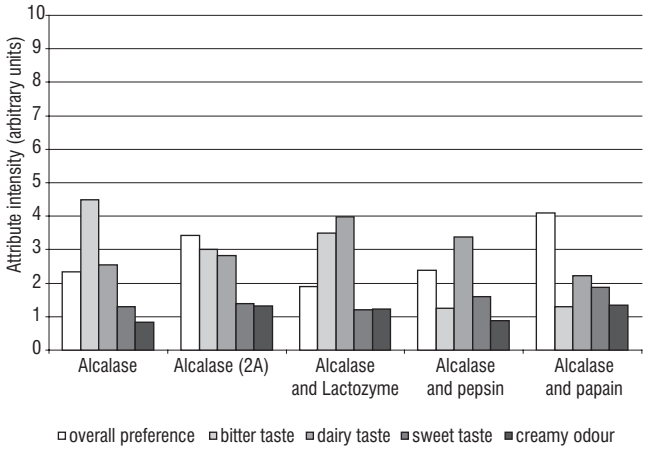

FIGURE 6. Relatonship between intensity of taste and overall quality of hydrolysates.

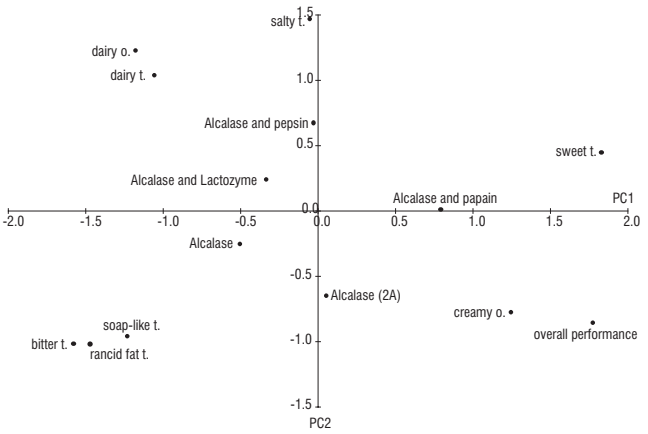

FIGURE 7. PCA plot of sensory profiling results (odour and flavour) of hydrolysate samples (variability converted by PC1 – 46%, by PC2 – 26%).

phenylalanine. Saha & Hayashi [2001] pointed out that also some hydrophylic amino acids, such as serine or glutamate, could strengthen the undesirable effect. The bitter taste is observed mainly in the preparations containing peptides with molecular weight below 10 kDa; bitterness intensity is proportional to the intensity of hydrolysis and the activity of the protease used. Bumburger & Belitz [1993], having carried out studies with trypsin-hydrolysed casein, postulated that in that case, apart from hydrolysis degree, an important role was also played by the change in the protein structure of the product formed.

In our study, the application of the "two-step" hydrolysis with Alcalase and then papain improved the sensory quality of the product. However, using Alcalase and then Lactozyme did not improve the quality of the hydrolysate as it was expected.

During the production of hydrolysates, the total solids are a very important item. The sensory evaluation of hydrolysates prepared with Debitrase™ HYW20 showed that hydrolysates with an equivalent of DH were less bitter when generated at 300 g TS/L than those generated at 50 g TS/L Hydrophobic bitter peptide peak present at a higher concentration in hydrolysates generated at low TS was identified as β-lg (43-57) [Spellman *et al.*, 2005].

CONCLUSIONS

The lowest WPC immunoreactivity was obtained after hydrolysis with Alcalase. However, increasing the dose of this enzyme against substrate did not result in considerable changes in the reaction with anti-α-la and anti-β-lg; hence it is not necessary to increase the enzyme dose during hydrolysate production. The use of Lactozyme following Alcalase led to obtaining a product with lowered protein immunoreactivity and lactose level. Studies on the possibility of suggesting it as a special diet should be continued. Good hypoallergenic formula for patients suffering simultaneously from allergy and lactose intolerance is still under construction.

We found that the hydrolysates showed four undesirable notes among which bitterness and dairy taste influenced the unpleasant taste of these products to the greatest extent. The most neutral, regarding bitterness and dairy taste, was the hydrolysate treated with Alcalase and then papain. In hedonic rating this sample received the highest note.

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ENZYMATYCZNA HYDROLIZA BIAŁEK SERWATKOWYCH MLEKA KROWIEGO W ASPEKCIE WYKORZYSTANIA ICH DO PRODUKCJI HYPOALERGICZNYCH ODŻYWEK

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Celem przedstawionych badań było obniżenie immunoreaktywności białek serwatkowych mleka (WPC)) tj. α-laktoalbuminy (α-la) i β-laktoglobuliny (β-lg) w wyniku jednostopniowej hydrolizy enzymatycznej z zastosowaniem dwóch różnych dawek enzymu Alkalazy (A i 2A) w stosunku do substratu, Laktozymu i dwustopniowej hydrolizy enzymatycznej, z wykorzystaniem układów enzymowych: Alkalaza i pepsyna, Alkalaza i papaina, Alkalaza i Laktozym. Najwyższy stopień hydrolizy (DH=18%) zanotowano dla hydrolizatu 2A (tab. 1). Spośród metod analitycznych zastosowano elektroforezę do przedstawienia zmian w obrębie białek WPC poddanych hydrolizie (rys. 1.), natomiast metodę konkurencyjną ELISA do oznaczenia immunoreaktywności uzyskanych prób (rys. 2 i 3). Ocena sensoryczna pozwoliła na określenie pożądalności poszczególnych produktów (rys. 4). Najniższą immunoreaktywność stwierdzono dla hydrolizatu uzyskanego przy zastosowaniu Alkalazy. Dodatek Laktozymu spowodował ośmiokrotny wzrost ilości glukozy, ale nie miał wpływu na podniesienie pożądalności produktu (rys. 5 i 6). Wyniki uzyskane metodą QDA zostały potwierdzone analizą PCA (rys. 7).