

REDUCTION OF ALLERGENICITY AND INCREASING THE BIOLOGICAL VALUE OF BUFFALO'S MILK PROTEINS BY ENZYMATIC MODIFICATION

S. HUSSEIN, É. GELENCSEER and GY. HAJÓS

Central Food Research Institute
H-1022 Budapest
Herman Ottó u. 15. Hungary

Received for Publication December 22, 1994

Accepted for Publication September 1, 1995

ABSTRACT

Buffalo milk proteins were covalently enriched with methionine (Met) during the enzymatic peptide modification (EPM) process. The maximal Met-enrichment occurred when the α -chymotryptic hydrolysate of buffalo's milk proteins was treated with methionine ethyl ester in the presence of α -chymotrypsin during the EPM. Methionine content of this product was more than three times (5.4%) higher than that of the original buffalo milk protein (1.5%). The electrophoretic patterns of the modified proteins showed that the enzymatic reaction was mainly characterized by transpeptidation. Molecular masses of the modified protein fractions were influenced by the concentration of L-methionine ethyl esters in the reaction mixture during the EPM. EPM products from the buffalo milk protein hydrolysates with α -chymotrypsin and trypsin or with pepsin and trypsin showed low allergenic activities compared with those when buffalo milk proteins were hydrolyzed with α -chymotrypsin or with pepsin alone.

INTRODUCTION

Buffalo milk is popular in Egypt, as well as in other parts of the world. However, the nutritional value of its proteins have not been fully investigated. The biological value of proteins is mainly determined by the content of essential amino acids. The biological utilization of mixtures of free amino acids is much less effective

¹Address for correspondence: Gy. Hajós, CFRI, Budapest H-1536 PoBox: 393.

Journal of Food Biochemistry 19 (1995) 239-252. All Rights Reserved.

© Copyright 1996 by Food & Nutrition Press, Inc., Trumbull, Connecticut.

and energy consuming than that of foods consisting of peptides and proteins of the same composition (Silk 1974; Matthews and Adibi 1976). The first procedure using enzyme catalysis for introducing appropriate essential amino acids and thus improving the nutritional value of food proteins, the plastein reaction, was proposed by Yamashita *et al.* (1979) and Aso *et al.* (1985).

Several investigators have evaluated the immunogenicity and/or allergenicity of enzymatically hydrolyzed casein (Takasa *et al.* 1979; Otani *et al.* 1990; Nakamura *et al.* 1993) and whey proteins (Asselin *et al.* 1988) by different techniques for potential use in infant formula. The allergenicity of milk was related to heterogeneity of protein constituents, and many authors considered β -lactoglobulin as being the major allergen because of its absence in human milk (Bahna and Heiner 1980).

Whitaker and Puigserver (1982) reported that enzymatic modification of proteins is a suitable process for designed amino acid incorporation and structure modification of the protein chains. The enzymatic process allows enrichment of the proteolysis products with essential amino acids which are terminally bound covalently to peptides (Hajós *et al.* 1988; 1989; 1990; Lorenzen and Schlimme 1992).

Recently an enzymatic method has been developed for covalent methionine enrichment of proteolytic hydrolysates of milk and plant proteins (Hussein *et al.* 1991; Hussein and Hajós 1992; Hajós *et al.* 1993).

The role of methionine is particularly important in nutrition (FAO/WHO 1973) because it is a limiting essential amino acid of buffalo and cow milk proteins. Because of its special hydrophobic character, the surface and the structure of the protein undergoes conformational modification during covalent Met-enrichment and consequently, its allergenic character may be altered.

A utilitarian motive of our present work is to know how the covalent methionine incorporation, together with the enzymatic modification (EPM) method, influence the biological value and the antigenic character of the buffalo milk proteins.

MATERIALS AND METHODS

Enzymes

Enzymes used included α -chymotrypsin (EC 3.4.21.1; Sigma); trypsin (EC 3.4.21.4; Serva); pepsin (EC 3.4.23.1; Calbiochem); carboxypeptidase A (EC 3.4.17.1; Serva); carboxypeptidase B (EC 3.4.17.2; Serva); and leucine aminopeptidase (EC 3.4.11.1; Serva).

Proteins

Raw bulk buffalo milk was obtained from the Faculty of Agriculture of the Menofia University herd in Egypt. The milk was skimmed twice using a cream separator and freeze dried.

TABLE 1.
METHIONINE CONCENTRATION IN THE REACTION MIXTURE

Sample No.	Methionine Added (g/g Hydrolysate)	Methionine Ethyl Ester Added (g/g Hydrolysate)
EPM 0	0.00	0.00
EPM 2	0.14	0.21
EPM 4	0.34	0.49
EPM 6	0.48	0.70
EPM 8	0.63	0.90
EPM 10	0.91	1.32

Hydrolysates of Buffalo Milk Proteins

In the present work we used four different hydrolysates of buffalo milk proteins as substrates of the EPM reactions. These substrates are the following: α -chymotryptic hydrolysate; peptic hydrolysate; α -chymotryptic and tryptic hydrolysate; and peptic and tryptic hydrolysate of buffalo milk proteins. Production of these hydrolysates were:

Buffalo milk proteins were hydrolyzed with α -chymotrypsin at pH 7.5 or with pepsin at pH 2.0. The incubation was carried out at 37C for 2 h. The reaction mixture was stirred during hydrolysis and after that freeze-dried.

Buffalo milk proteins were hydrolyzed with α -chymotrypsin at pH 7.5 under the same conditions for 1 h, then trypsin was added at pH 7.5 at the same conditions for 1 h, then freeze-dried.

Buffalo milk proteins were hydrolyzed with pepsin at pH 2.0 for 1 h and then trypsin was added at pH 7.5 under the same conditions as mentioned above.

EPM Products of Buffalo Milk Proteins

Tryptic, α -chymotryptic and/or peptic hydrolysates of buffalo milk protein were used to produce EPM-products in the presence of α -chymotrypsin (pH 6.0) or pepsin (pH 4.0) as catalysts. The concentration of substrate was 25% (w/v). The ratios of methionine ethyl ester added (L-methionine ethyl ester concentration ranged from 210 mg/g protein (EPM 2) to 132 mg/g protein (EPM 10)) to substrates are given in Table 1. The EPM 0 is a modified protein product produced without methionine ethyl ester addition to the reaction mixture; however, samples EPM 2, 4, 6, 8 and 10 refer to the ratio of methionine ethyl ester added to the reaction mixture as shown in Table 1. The enzyme:protein ratio was 1:100. The incubation was carried out at 37C; the incubation time was 16 h. After incubation the products were dialyzed through a cellophane membrane against distilled water. The nondialyzable fraction was freeze-dried.

SDS-PAGE

Molecular mass distribution of the protein fractions was investigated by SDS-PAGE according to Delincée and Hajós (1984). The protein zones were stained with Coomassie Brilliant Blue.

Amino Acid Content

Aliquots of the samples were hydrolyzed with 6 M HCl in a tube flushed with N₂ at 105°C for 24 h and the amino acids were subjected to thin layer ion-exchange chromatography (Dévényi 1974). In situ quantitative evaluation of the methionine spots was carried out by a Biotech-Fischer video densitometer (identified and compared to the control methionine).

Leucine Aminopeptidase (LAP) Treatment

A suspension of the EPM-product (5 mg) in 200 µl of 0.1 M NH₄HCO₃ buffer was incubated with LAP. Samples (10 µl) were investigated after incubating for 0, 10, 30 and 60 min, respectively, and the samples were immediately mixed with an equal volume of concentrated HCl to stop the enzyme reaction. Free amino acids were separated by thin layer ion-exchange chromatography.

Carboxypeptidase Treatment

The samples were incubated with a mixture of carboxypeptidase A and carboxypeptidase B. The treatment and the amino acid determination were the same as in the case of leucine aminopeptidase.

ELISA Test

The allergenic character of samples was measured *in vitro* by the competitive indirect ELISA test (Gelencsér *et al.* 1992). Inhibitor assays were performed with use of polystyrene plates (Sülysáp, Hungary). Buffalo milk protein antigen was coated on the assay wells by incubating 100 µl/well of protein at 5 µg/ml in carbonate buffer, at pH 9.4 for overnight at 4°C. Between each step, the plates were washed three times with phosphate-buffered saline (PBS), pH 7.4, 0.1 M containing 0.5% Tween 20. Free binding sites were blocked by incubation of wells with 200 µl/well of PBS buffer, pH 7.4 containing 0.5% gelatin. The wells then received 50 µl/well of primary antibodies (human sera containing specific IgG antibodies to milk protein antigens, purchased from the Gastroenterological Center of Madarász Children's Hospital, Hungary) at working dilution 1:160 in PBS.

buffer, pH 7.4, and by 50 μl /well of competitive antigens (EPM products) at concentrations of 1×10^{-3} $\mu\text{l}/\text{ml}$ to 5×10^{-3} $\mu\text{l}/\text{ml}$ in PBS buffer, pH 7.4, for 1 h at 37°C. The HRP-labeled goat-Ig-anti-human IgG conjugate (HUMAN, Hungary) was mixed by adding 100 μl /well of conjugate at working dilution (1:160) in PBS buffer, pH 7.4, and incubating for 1 h at 37°C. The HRP-activity was measured in 100 μl /well sodium citrate substrate solution, containing OPD and H_2O_2 . After incubation for 30 min with substrate solution at room temperature and inhibition with 50 μl /well of 4 M H_2SO_4 solution, the optical density was measured in a Titer-Tek Multiskan (Flow, UK) at 492 nm. Results were expressed in percent by reference to a standard serum.

Allergenic Character:

$$A(\%) = \left(1 - \frac{A_i - A_{bl}}{A_{st} - A_{bl}}\right) \times 100 \quad \text{where}$$

A = allergenic activity of samples

A_i = absorbance of the samples at 492 nm

A_{bl} = absorbance of the blank solution at 492 nm

A_{st} = absorbance of the standard serum at 492 nm

RESULTS AND DISCUSSION

Electrophoretic Patterns of the EPM Products

The electrophoretic patterns of protein fractions of the buffalo milk proteins, the α -chymotryptic hydrolysate and the EPM-products separated by SDS-PAGE are shown in Fig. 1. There are substantial differences between the peptides of the EPM-products and those of the buffalo milk protein hydrolysate. Molecular weights of the modified protein fractions were influenced by the concentration of L-methionine ethyl ester in the reaction mixture during enzymatic modification. Figure 2 shows the major zones of the peptic hydrolysate of buffalo milk proteins. The changes in the zones indicate that transpeptidation is the major process in the enzymatic modification.

Incorporation of L-Methionine into Buffalo Milk Protein Hydrolysate by Enzymatic Modification

Methionine content of the buffalo milk protein hydrolysate and EPM products are shown in Table 2. This comparison indicated that the methionine content in the EPM products with methionine incorporation were found to be higher than

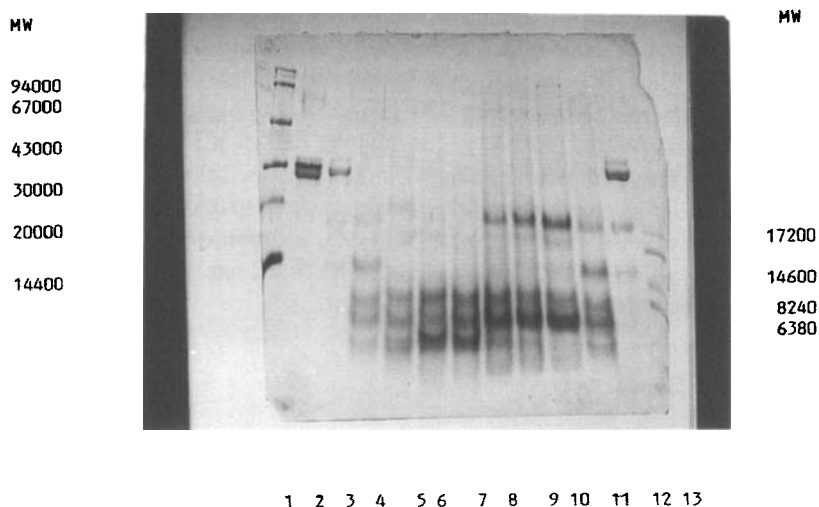


FIG. 1. SDS-PAGE PATTERNS OF BUFFALO MILK PROTEIN, ITS HYDROLYSATE AND ENZYMATICALLY MODIFIED PRODUCTS

1: LMW (low molecular weight controls); 2: Buffalo casein; 3, 12: Buffalo milk proteins; 4, 11: α -chymotryptic hydrolysate of buffalo milk proteins; 5: EPM 0 (EPM product without Met-enrichment); 6, 7, 8, 9, 10: EPM products with different Met-enrichment; 13: PMW (peptide molecular weight control)

that of the substrate protein. The optimum of L-methionine incorporation when α -chymotrypsin was used as a catalyst (EPM 4) was at a ratio of 34 g L-Met/100 g hydrolysate (Table 1). Methionine content of EPM 4 is greater by 3.8% than that of the sample without Met addition (EPM 0). The optimum of Met incorporation when pepsin was used as a catalyst (EPM 2) was at a ratio of 14 g L-Met/100 g hydrolysate in the reaction mixture (Table 1). Methionine content of EPM 2 is greater by 3.0% than that of the control sample (EPM 0).

The maximal enrichment of the covalently bound methionine in the products (produced in the case of α -chymotrypsin) was about four times as high as that of the original substrate; however, in the case of products produced using pepsin as catalyst. The Met-contents of the products were three times as high as that of the original substrate protein. The methionine level of these products satisfies the methionine requirement proposed by FAO/WHO (1973).

Methionine contents of the two EPM products, the EPM A (produced from α -chymotryptic and tryptic hydrolysate of the buffalo milk proteins, and catalyzed by α -chymotrypsin), and the EPM B (produced from peptic and tryptic hydrolysate of the buffalo milk proteins, and catalyzed by pepsin) are shown in

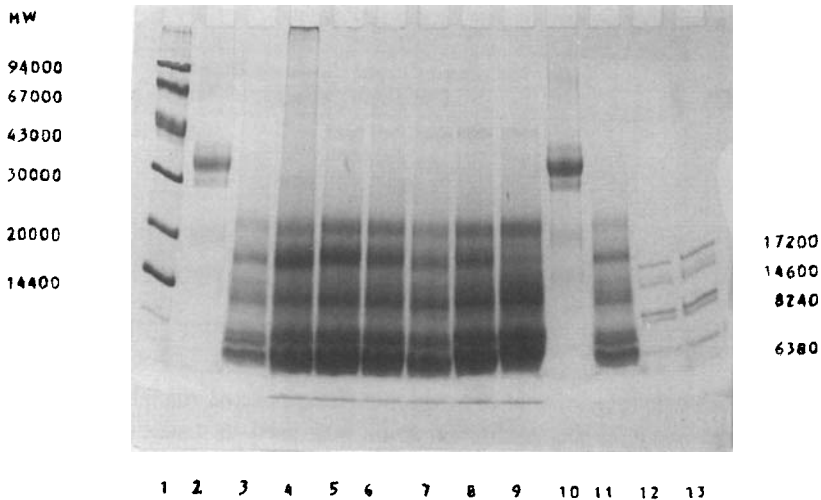


FIG. 2. SDS-PAGE PATTERNS OF BUFFALO'S MILK PROTEINS, ITS PEPTIC HYDROLYSATE AND ITS MODIFIED EPM PRODUCTS

1 : LMW; 2, 10: Buffalo's milk proteins; 3, 11: Buffalo's milk protein hydrolysate with pepsin; 4: EPM 0 (EPM product without Met-enrichment); 5, 6, 7, 8, 9: EPM products (produced in the presence of pepsin, as catalyst) with different Met-enrichment; 12, 13: PMW

TABLE 2.
METHIONINE CONTENT OF THE MODIFIED BUFFALO MILK PROTEINS

EPM	Methionine Content Covalently Bound (g Met/100 g Protein)	
	EPM A	EPM B
0	1.58	1.95
2	3.21	4.52
4	5.44	3.20
6	4.77	3.10
8	5.61	2.82
10	5.65	2.95

EPM A: EPM products from α -chymotryptic hydrolysates of the buffalo milk proteins, produced with α -chymotrypsin as catalyst.

EPM B: EPM products from peptic hydrolysates of the buffalo milk proteins, produced with pepsin as catalyst.

Table 3. Methionine contents in all EPM products with methionine incorporation were found to be higher than that of the original substrate proteins.

TABLE 3.
THE METHIONINE CONTENT OF THE MODIFIED BUFFALO MILK PROTEINS¹

EPM	Methionine Content Covalently Bound (g Met/100 g Protein)	
	EPM A	EPM B
0	1.68	1.69
2	2.83	3.51
4	4.82	2.56
6	4.37	2.56
8	3.75	3.06
10	3.56	3.00

¹After treatment with exopeptidases.

The results show that the amount of methionine incorporated changed with the type of enzyme used. When α -chymotrypsin was used as catalyst with α -chymotryptic and tryptic hydrolysate as substrate the methionine incorporation was higher compared with pepsin as catalyst with peptic and tryptic hydrolysate of buffalo milk proteins.

The effects of exopeptidases on samples are shown in Fig. 3. In samples taken at 0 min, where HCl was added together with exopeptidases to prevent digestion, no free amino acids were detectable. This finding proved that both the excess methionine ethyl ester and free methionine were removed from the reaction mixture during dialysis. In samples obtained after 10–60 min, increasing amounts of free amino acids were detected as a result of exopeptidase action.

Significant amount of Met was detectable in the EPM samples hydrolyzed by carboxypeptidase A + B, while Met was totally absent in the buffalo milk protein hydrolysate.

The amino acid content of the C-terminal residues of the α -chymotryptic hydrolysate and of the Met-enriched EPM product (EPM 4) (EPM A, Table 2) of buffalo milk proteins after densitometric evaluations are shown in Fig. 3. In Fig. 3, four intense spots can be seen corresponding to the amino acids removed by carboxypeptidases from the C-terminal of the α -chymotryptic hydrolysate of buffalo milk proteins. Interestingly, among these free amino acids, methionine was not found. The EPM sample shows five intense spots under the same circumstances. One of these intense spots corresponded to methionine.

These results suggest that enzymatic peptide modification is a suitable method for covalent Met-enrichment, that is, for increasing the nutritive value of buffalo milk protein by the incorporation of a given quantity of the limiting essential amino acids.

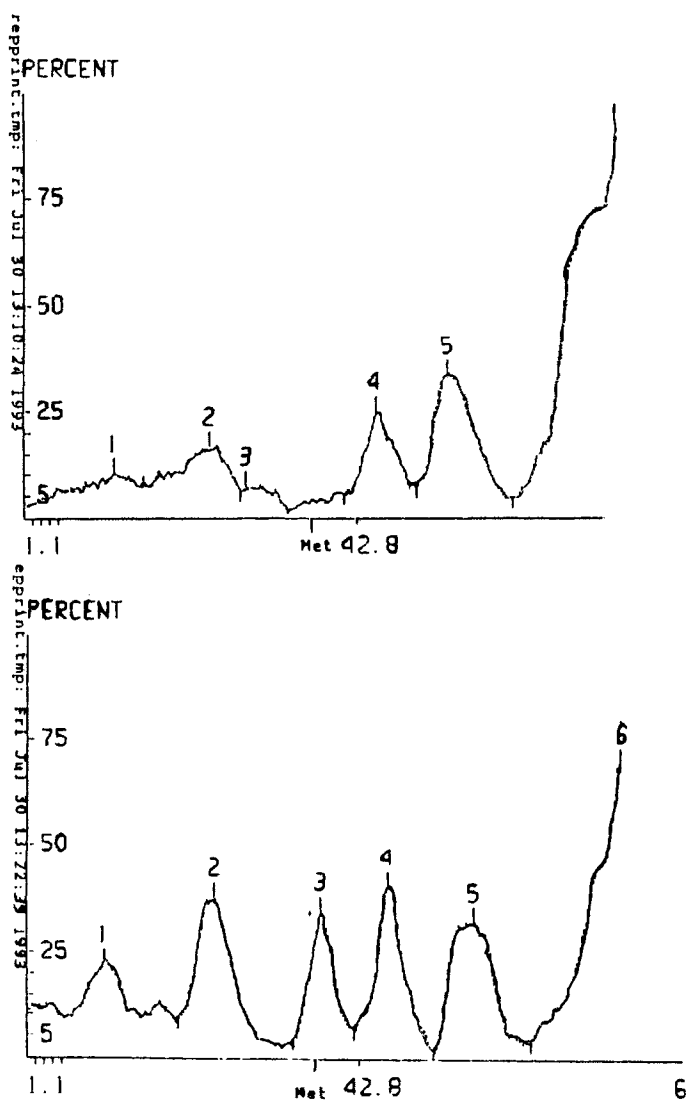


FIG. 3. DENSITOGAM OF THE LIBERATED AMINO ACIDS FROM THE 60 MIN SAMPLE OF THE CARBOXYPEPTIDASE TREATED α -CHYMOTRYPTIC HYDROLYSATE AND OF THE METHIONINE ENRICHMENT EPM-PRODUCT OF THE BUFFALO MILK PROTEIN

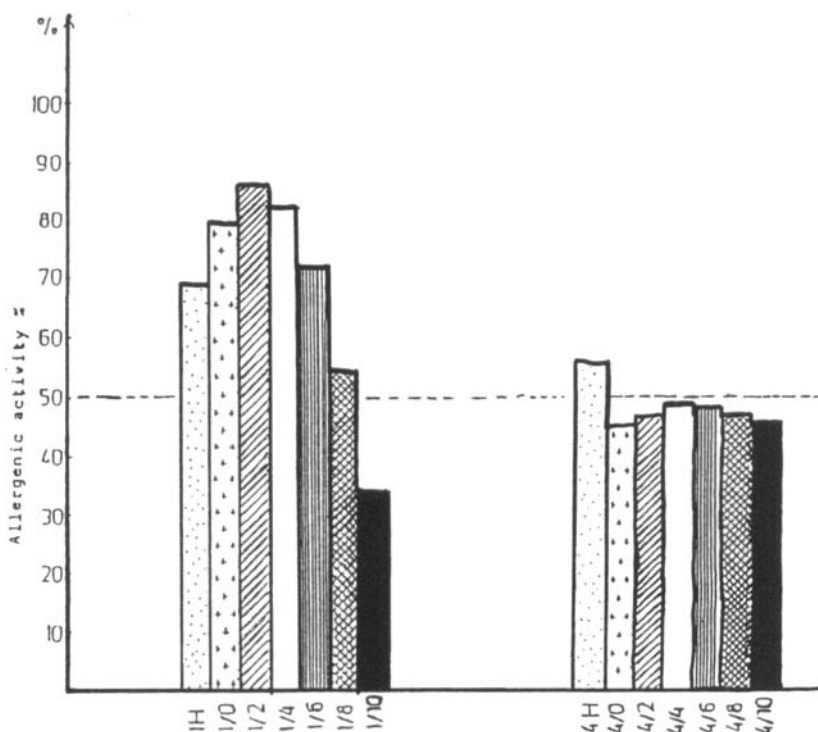


FIG. 4. ALLERGENIC CHARACTER OF THE ENZYMATICALLY MODIFIED BUFFALO'S MILK PROTEIN PRODUCTS

1H: α -chymotryptic hydrolysate of buffalo milk protein; 1/0: EPM product without Met-enrichment; 1/2, 1/4, 1/6, 1/8, 1/10: EPM products with different Met-enrichment in the presence of α -chymotrypsin; 4H: Peptic hydrolysate of buffalo milk protein; 4/0: EPM product without Met-enrichment; 4/2, 4/4, 4/6, 4/8, 4/10: EPM products with different Met-enrichment in the presence of pepsin.

Allergenic Character of the Buffalo Milk Protein Hydrolysate and EPM Products

Figures 4 and 6 show that the allergenicity of α -chymotryptic and peptic hydrolysates and EPM products was significantly reduced. The allergenic character of the EPM products produced in the presence of α -chymotrypsin was significantly reduced by the increase of the concentration of methionine ethyl ester in the reaction mixtures. The results also show that the allergenic character of EPM products produced in the presence of pepsin catalyst was not affected by the concentration of methionine ethyl ester in the reaction mixtures. In this case all EPM products showed low allergenic activity (under 50%).

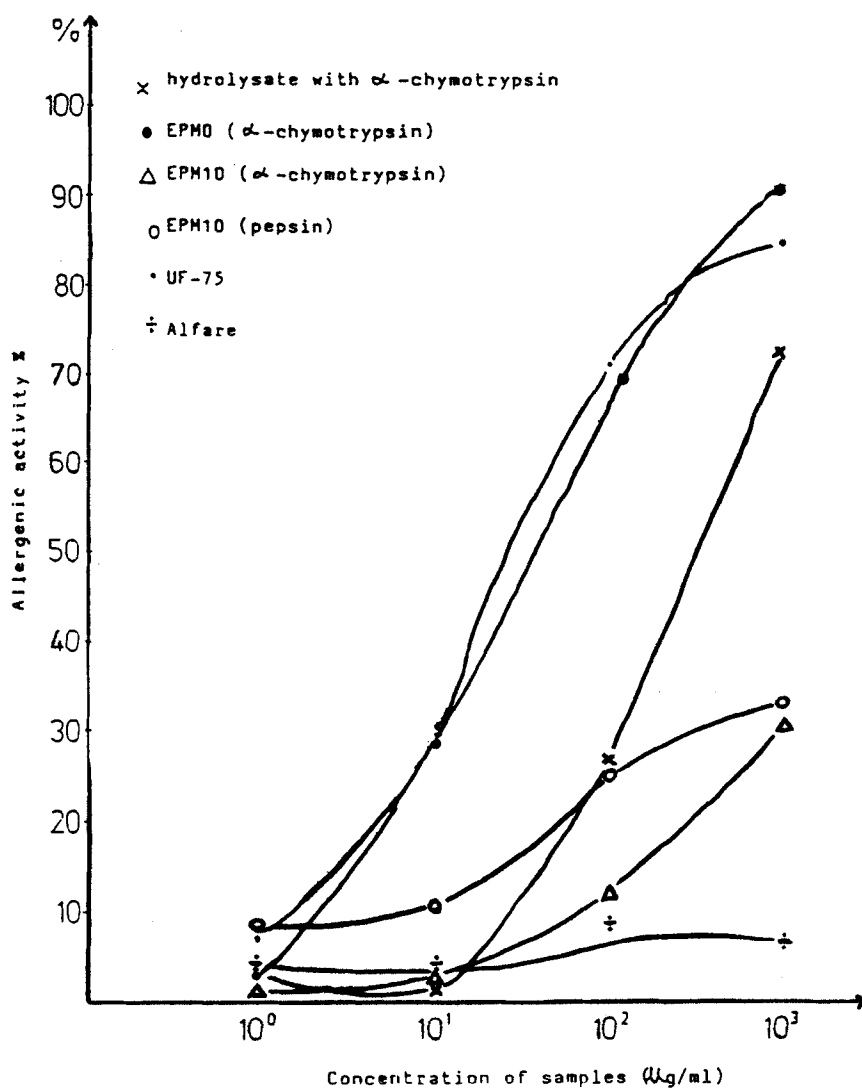


FIG. 5. POTENTIAL ALLERGENIC ACTIVITY OF MODIFIED BUFFALO MILK PROTEIN PRODUCTS BY COMPETITIVE INDIRECT ELISA

Figure 5 shows the potential allergenic activity of the modified buffalo milk protein products investigated by competitive indirect ELISA. At a concentration of $1 \times 10^3 \mu\text{g/ml}$, the samples of EPM 10, both in the case of α -chymotrypsin

and pepsin as catalyst, showed low allergenic activity in comparison with the hydrolysates.

The allergenic character of the different EPM products from buffalo's milk protein hydrolysates are shown in Fig. 6. Allergenic character of the EPM A samples decreased gradually from 89% (sample EPM 0) to 3% (EPM 10). Figure 6 also shows the allergenic character of EPM products from a peptic and tryptic hydrolysate of buffalo milk proteins when pepsin was used as a catalyst (EPM B). Allergenic character of the EPM B samples decreased from 64% (EPM 0) to 30% (EPM 10). The results showed that the allergenic character of EPM A was lower than that of the samples of EPM B. Allergenic character of modified buffalo milk proteins was significantly reduced by the increase of the concentration of methionine ethyl ester in the reaction mixture. The ratio of reduction of the allergenicity was very high when α -chymotrypsin was used as the catalyst compared with pepsin as the catalyst.

The potential allergenic activity significantly dropped in the case of buffalo milk protein hydrolysates because of the cleavage of a significant number of peptide bonds. The most important decrease in the allergenic properties was found in the EPM products because of modification of sequential and conformational

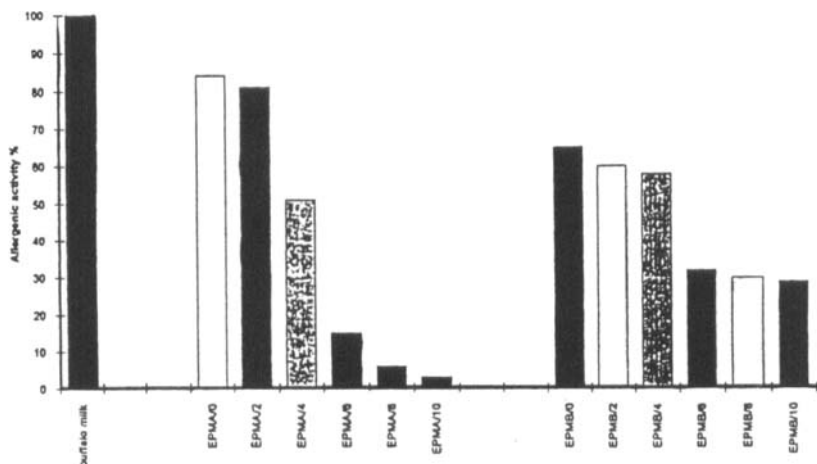


FIG. 6. ALLERGENIC CHARACTER OF THE ENZYMATICALLY MODIFIED BUFFALO'S MILK PROTEINS PRODUCTS

EPM A: EPM products from α -chymotryptic and tryptic hydrolysate of the buffalo's milk proteins, produced in the presence of α -chymotrypsin as catalyst; EPM B: EPM products from peptic and tryptic hydrolysate of the buffalo's milk proteins, produced in the presence of pepsin; EPM 0: EPM product without Met-enrichment; EPM 2–EPM 10: EPM products with different Met-enrichment (see Table 1)

determinants of the proteins structure by transpeptidation and covalent incorporation of Met.

Our results show that the allergenic character of buffalo milk proteins can be reduced by the enzymatic peptide modification process with methionine enrichment.

REFERENCES

- ASO, H., KIMURA, H., WATANABE, M. and ARAI, S. 1985. Chemical properties of enzymatically modified proteins produced from soy protein by covalent attachment of methionine. *Agric. Biol. Chem.* **49**, 1649-1654.
- ASSELIN, J., AMIOT, J., GAUTHIER, S.F., MOURAD, W. and HEBERT, J. 1988. Immunogenicity and allergenicity of whey protein hydrolysates. *J. Food Sci.* **53**, 1028-1039.
- BAHNA, S.L. and HEINER, D.C. 1980. *Allergies to Milk*, Grune and Stratton, New York.
- DELIN CÉE, H. and HAJÓS, GY. 1984. Investigation of plasteins by SDS polyacrylamide gel electrophoresis. *Acta Alimentaria* **13**, 309-319.
- DÉVÉNYI, T. 1974. Thin-layer ion-exchange chromatography. *Hungarian Scientific Instruments* **30**.
- FAO/WHO 1973. Energy and protein requirements. Report of a Joint FAO/WHO Ad Hoc Expert Committee, FAO Nutrition Meeting Report Series No. 52, Rome.
- GELENCSÉR, É., POLGÁR, M. and HAJÓS, GY. 1992. Screening of food protein allergy in childhood by an allergen-specific IgG-ELISA test. In *Biochemistry of Diseases*. 1st IUBMB Conference. June 1-6, Nagoya.
- HAJÓS, GY., ÉLIÁS, I. and HALÁSZ, A. 1988. Methionine enrichment of milk protein by enzymatic peptide modification. *J. Food Sci.* **53**, 739-742.
- HAJÓS, GY., HALÁSZ, A. and BÉKÉS, F. 1989. Designed protein modification by enzymatic technique. *Acta Alimentaria* **18**, 325-330.
- HAJÓS, GY., HUSSEIN, S. and GELENCSÉR, É. 1993. Enzymatic peptide modification of food proteins. In *Food Proteins: Structure and Functionality* (K.D. Schwenke and R. Mothes, eds.) pp. 82-86, VCH, Weinheim.
- HAJÓS, GY., SZARVAS, T. and VÁMOS-VIGYÁZÓ, L. 1990. Radioactive methionine incorporation into peptide chains by enzymatic modification. *J. Food Biochemistry* **14**, 381-394.
- HUSSEIN, S., GELENCSÉR, É and HAJÓS, GY. 1991. Separation of hypoallergenic fractions of modified buffalo milk protein. Abstract of the 3rd International conference on biochemical separation. pp. 115-116, Sopron, Hungary.

- HUSSEIN, S. and HAJÓS, GY. 1992. Reduction of the allergenicity of buffalo's milk proteins by enzymatic modification. 21st FEBS meeting, Dublin, Ireland.
- LORENZEN, P.C. and SCHLIMME, E. 1992. The plastein reaction: properties in comparison with sample proteolysis. *Milchwissenschaft* 47, 499-504.
- MATTHEWS, D.M. and ADIBI, S.A. 1976. Peptide absorption. *Gastroenterology* 71, 151-161.
- NAKAMURA, T., SYUKUNOBE, Y., SAKURAI, T. and IDOTA, T. 1993. Enzymatic production of hypoallergenic peptide from casein. *Milchwissenschaft* 48, 11-14.
- OTANI, H., DONG, X.Y. and HOSONE, A. 1990. Preparation of low immunogenic peptide fragments from cow milk casein. *Milchwissenschaft* 45, 217-220.
- SILK, D.B.A. 1974. Peptide absorption in man. *Gut* 15, 495-501.
- TAKASA, M., FUKUWATARI, Y., KAWASE, K., KIYOSAWA, I.L., OGASA, K., SUZUKI, S. and KUROMI, T. 1979. Antigenicity of casein enzymatic hydrolysate. *J. Dairy Sci.* 62, 1570-1576.
- WHITAKER, J.R. and PUIGSERVER, A.J. 1982. Fundamentals and applications of enzymatic modifications of proteins: an overview. *Adv. Chem. Ser.* 198, 57-87.
- YAMASHITA, M., ARAI, S., AMANO, Y. and FUJIMAKI, M. 1979. A novel one-step process for enzymatic incorporation of amino acids into proteins: application to soy protein and flour for enhancing their methionine levels. *Agric. Biol. Chem.* 43, 1065-1068.