ENZYMATIC MODIFICATION OF BUFFALO'S AND COW'S MILK PROTEINS

Candidate (Ph.D) Dissertation in Food Science
by

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CONTENTS

1. INTRODUCTION

The world's annual supply of around 500 million tons of milk comes mainly from 3 billion cows. Buffalo's are the second largest source of milk supply. Some 130 million buffalo's in the world provide over 30 million tons of milk annually.

Buffalo's milk is very popular in Egypt because of its nutritional and technological characteristics, however, buffalo's milk proteins have not been fully investigated in point of view of food safety.

Milk protein allergy in new-born babies and young children is a serious concern. The allergenicity of milk was related to heterogenicity of protein constituents and many authors considered ß-lactoglobulin as being the major allergen because of its absence in human milk (Bahna and Heiner 1980).

Food protein allergy has a raising trend in the world. People suffering from food allergy must constantly avoid offending foods. They need hypoallergenic proteins.

Plant proteins and some animal proteins are usually deficient or contain low amount of one or more of the essential amino acids. For example, the limiting essential amino acid of legume seed protein is methionine while cereal grain's is lysine. A deficiency in a limiting essential amino acid results in a lowered protein efficiency ratio (PER value). For example, the PER value for most common beans is in the range of 0.9-1.0 while the addition of methionine to 3% (of dry bean weight) increased the value to about 2.6 (Antunes et.al., 1979).There are several potential and real solutions to improve the PER value of proteins. These include:

(a) Fortification of the protein source, however, there are a number of disadvantages. These include:

(1) possible losses of the added amino acid during food preparation and processing;

(2) side reactions of the amino acid occurred during the processing;

(3) undesirable effect on sensory properties of the food

(4) possible decreased biological utilization of free amino acids. Free amino acids may come to the blood stream ahead of those released by proteolysis of proteins in the gut, thereby not being available simultaneously with the other essential amino acids for the synthesis of the required proteins and enzymes.

(b) Mixing of proteins with complementary amino acids, if they are available, is an economical and appropriate way of meeting the daily nutritional needs of the essential amino acids.

(c) Covalent attachment of essential amino acids to proteins.

Some of the purposes and advantages of covalent incorporation of limiting essential amino acids into high protein foods include:

(1) improvement of nutritional quality;

(2) prevention of loss of added amino acid;

(3) alleviation of undesirable flavors of the free amino acid (or products of the amino acid);

(4) possible changes in functional properties of the modified proteins;

(5) to prevent deteriorate reactions during processing and storage of the food.

Limiting essential amino acids can be attached efficiently to food proteins in the laboratory by enzymatic modification with improvement of the nutritional value of the protein, proportional to the amount of essential amino acid incorporated.

Proteins are fundamentally important as nutrients, but now they seem to be much more. Through the versatility of their properties, they can become key ingredients that determine many parameters of food quality. The covalent attachment of a highly hydrophobic amino acid to a hydrophylic protein with the aid of protease action could be a method to fulfill the purpose.

Earlier an enzymatic method has been elaborated in our laboratory for covalent methionine enrichment of proteolytic hydrolysates of milk and plant proteins. As a continuation of these efforts I decided now to deal with the incorporation of methionine into different proteolysates of buffalo's milk and cow's milk proteins. The role of methionine is particularly important in the nutrition because:

- it is a limiting essential amino acid of the buffalo's and cow's milk proteins.
- of its special hydrophobic character, the surface and the structure of the protein undergoes modification and consequently, its allergenic character may be altered.

Aims of the research work

The objectives of this study are as follows:

1. Increasing the biological value of buffalo's milk proteins by covalent methionine-incorporation during enzymatic peptide modification.
2. Reducing allergenic character of buffalo's milk proteins by enzymatic peptide modification.
3. Production of enzymatically modified buffalo and cow caseins with reduced antigenic character
4. To study covalent attachment of methionine to κ-casein by enzymatic peptide modification.
5. Producting proteins of modified amino acid content for particular nutritional purposes.

2. LITERATURE

2.1 Enzymatic peptide modification of proteins

One of the basic questions of the food science is how to improve the quality of food proteins, i.e. how to modify their structures.

The enzymatic modification is a protease-catalyzed process which leads to efficient production of a plastein from a protein hydrolyzate. The ability of proteinases to catalyze peptide bond synthesis has a historical background. In 1886, Danilewsky(1886) discovered that an insoluble protein-like substance was formed if a small amount of pepsin or pancreatin was added to a concentrated solution of peptone or to a neutralized proteolytic digest of ovalbumin or any kind of other proteins. Van't Hoff (1898) suggested that trypsin might posses capacity to catalyze the synthesis of proteins from degradation products originally generated by its own proteolytic action. The term plastein was first used by Sawjalov (1901) to designate the precipitate resulting from the addition of rennin to a partial-hydrolysate of fibrin.

Determann and co-workers (1965 a and b), Determann and Köhler (1965) reported molecular weight distribution of enzymatic condensation products, the specificity of pepsin in the enzymatic modification of prolin-containing peptides. Experiments in which the pentapeptide L-tyrosyl-L-leucyl-glycyl-L-glutamyl-L-phenylalanin was the substrate and pepsin was the catalyst, the dimer, tetramer and pentamer of the original peptide could be detected in the plastein product.

Efficiency of the pepsin-catalyzed peptide bond formation is highly influenced by the character of C-terminal amino acid. Similarly to the pepsin-catalyzed hydrolysis also in the plastein reaction the C-terminal phenylalanine and tyrosine give the highest synthesis rate while alanine at the C-terminal prevents the condensation in the plastein reaction.

Pepsin has a significant stereospecificity in peptide bond cleavage, and the same was conformed to plastein formation. Determann et al. (1965) found a more pronounced specificity of pepsin in peptide condensation than in peptide-bond hydrolysis.

Appropriate chain length of the substrate is also important for peptide bond synthesis, Determann and Köhler (1965) stated that monomers have to consist of at least four amino acids to act as substrate in the plastein reaction.

Horowitz and Haurowitz (1959) studied the α–chymotrypsin catalyzed synthesis of the enzymatic modification and concluded that it proceeded through transpeptidation. Yamashita et al. (1970) observed that both transpeptidation and condensation were involved in the efficient formation of a plastein reaction with a 10 % TCA-insoluble substance from a soy protein hydrolysate.

The enzymatic peptide modification requires different conditions from those that are necessary for the enzymatic hydrolysis of proteins:

1. Substrate has to be a low molecular weight peptide or peptide mixture. Tsai et al. (1974) found that the fractions having average molecular weights of 1043 and 635 respectively, produced plasteins much more effectively than the lower and higher molecular weight fractions. Hofsten and Lalasidis (1976) reported that a whey protein

hydrolysate fraction with an average molecular weight of less than 1000 can form a plastein product with a molecular weight of 2000-3000. Therefore, the molecular size of the substrate is one of the most important factors that have influence on plastein formation.

2. Extremely high substrate concentration (20 to 50 %) can serve as a driving force for this reverse reaction (Fujimaki et al. 1971, 1977; Eriksen and Fagerson 1976; Fruton 1982; Sukan and Andrews 1982 a,b; Jakubka 1987; Kullmann 1987).

3. The reaction system should be kept between pH 4 and 7, irrespective of the optimal pH values of proteinases in their usual proteolysis reaction (Yamashita et al. 1971). The optimum pH for most proteases, that form plastein these measurements were carried out with soy globulin hydrolysate was found to be within the narrow range of 4-7 (Table 1). For example pepsin is an acid protease, and one of its characteristics is a high hydrolase activity at low pH. Even at pH 1, it is active and is able to hydrolyze proteins. As Yamashita et al. (1971) demonstrated that no appreciable amount of plastein was formed at pH 1-2, even if all the other conditions were favourable. The optimum for pepsin to produce plastein is around pH 4.5. On the other hand, α–chymotrypsin, a serine proteinase, displays its maximum rate of protein hydrolysis close to pH 8. However, effective plastein formation from soy globulin hydrolysate using α–chymotripsin occurred at pH 5.3.

Table 1.: ***Optimum pH values for hydrolysis of soy proteins with proteinases and of plastein synthesis from a peptic hydrolysate of soy protein with the same proteinases.***

Enzyme	Optimum pH values	
	Hydrolysis	Synthesis
pepsin	1.6	4.5
α–chymotrypsin	7-8	5.0-6.0
trypsin	8.0	5.5-6.5
papain	5-6	5.0-5.0

A new peptide bond can be formed by either transpeptidation or condensation. Yamashita and co-workers (1973) proposed that plastein reaction is mainly a polycondensation reaction based on the fact that they demonstrated an actual peptide-enzyme intermediate. If plastein reaction is merely a formation of new peptide bonds a decrease in the total number of free amino groups should take place. As above mentioned, Horowitz and Haurowitz (1959)were unable to find any decrease of free amino groups during the plastein formation. A similar observation was made by V. Hofsten (1974), V. Hofsten and Lalasidis (1976) and Monti and Jost (1979), who could not detect significant molecular weight increase during plastein formation. Hajós and Halász (1982) investigated changes in molecular weight distribution of these enzymatic products in comparison to their substrates. They stated that during plastein reaction transpeptidation occurs which results in lower and higher molecular weight products than those of the substrate. In the case of a papain-catalyzed reaction a relatively greater amount of high

molecular weight (30KDa) product could be detected which hint polycondensation.

Edwards and Shipe (1978); Sukan and Andrews (1982) belive that the aggregates produced during plastein reaction are not the results of new covalent bonds but hydrophobic and ionic bonds. Results of V. Hofsten (1974) on gel formation of whey-based plastein catalyzed by esterase showed that the amount of low molecular peptides increased indicating that the gel formation is independent of the increase in peptide chain length. Furthermore, gels produced under optimal conditions were soluble in 8M urea or 6M guanidine at neutral pH which indicate that the gels are not held together by covalent bonds.

Aso and his co-workers (1974) concluded that hydrophobic forces are major factors in plastein chain assembly. They found that compared with the substrate, the water-insoluble product contained smaller amount of hydrophilic and larger amount of hydrophobic amino acid residues.

Plastein formation is characterized, according to Arai et al. (1975), by plastein productivity and insolubility values. It was shown that the hydrophilic substrates where values are lower than 0.4 and hydrophopic substrates where is higher than 0.6 produced plastein in low yields. Substrates which can be characterized by approximately 0.5 value produce plastein much more efficiently.

In the presence of NaCl an increased amount of plastein yield was achieved by Tanimoto et al. (1975). At lower concentration (0.1M) it was considered to be a result of enzyme activation and at higher concentration (0.8M) a salting out effect of products occurred. The favorable effect of salt addition was also stated by V.

Hofsten (1974) and it corresponds to findings related to enzyme-catalyzed peptide synthesis.

In several aspects plasteins react like proteins. They form colored compounds with ninhydrin and give complex compounds with Cu^{2+} can be precipitated with trichloracetic acid and hydrolysed by various proteolytic enzymes. Belikov and Gololobov (1986) suggest that plasteins consist of conglomerates of intertwinned polypeptide chains with different lengths and structures.

Andrews and Alichanidis (1990) observed that no appreciable amounts of new peptides were formed. They ruled out the hypothesis of covalent bond formation and transpeptidation as reaction mechanism. They therefore concluded that the plastein synthesis reaction is a purely entropy-driven physical aggregation process.

Lorenzen and Schlimme (1993) reported that plasteins obtained with pancreatin as physiological enzyme system differ markedly in their composition and behaviour from serine proteinase plasteins, which reflects the influence of the peptidases on plastein formation. High tyrosine contents (approximately 35 mol%) indicate that strongly hydrophobic, proteolysis-resistant, tyrosine-containing peptides are concentrated in the pancreatin plasteins.

Transpeptidation in the course of enzymatic modification was also verified by Hajós et al. (1990); Hussein and Hajós (1993). Our results showed that transpeptidation plays an essential role in the EPM reaction. Also, the modified proteins obtained when α-chymotrypsin was used as catalyst differ markedly in their molecular weight and methionine content from those, in which pepsin was used as catalyst.

2.2 Covalent amino acids enrichment of peptides for nutritional purposes

During the last two decades the utilization of non-conventional protein sources for human and animal nutrition became one of the most important problems of research on food technology and food science. The biological value of proteins is mainly determined by the presence of essential amino acids. The biological utilization of a mixture of free amino acids is much less effective and energy consuming than that of foods consisting of peptide and proteins of the same composition (Silk 1974; Matthews and Adibi 1976).

The results that amino acid esters and not free amino acids are incorporated into plastein seems to confirm that plastein is formed essentially by transpeptidation, i.e. by the transfer of amino acyl residues from donor peptides to acceptor peptides or amino acids.

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.

Plasteins from combined protein hydrolysates with complementary essential amino acid profiles seem to be a more economic way of nutritional value improvement than incorporation of pure amino acid esters.

Yamashita et al. (1970) first studied the production of combined plastein with soy protein hydrolysate and hydrolysed wool keratin with hydrolysed soy protein and hydrolysed ovalbumin. Wool keratin and ovalbumin are rich in sulfur amino acids which is the first limiting essential amino acid of soy protein. Plastein reaction can be catalyzed by soluble or immobilized proteinases. Pallavicini et al. (1983) compared the effect of soluble α–

chymotrypsin with α–chymotrypsin immobilized on chitin on plastein formation from low molecular weight peptides of a peptic digest of soybean protein. The results indicated that the plasteins from soluble α–chymotrypsin were richer in glycine, valine, leucine and serine than the plastein prepared with immobilized α–chymotrypsin. Plasteins prepared by immobilized α–chymotrypsin catalyzed were rich in glutamic acid, lysine, alanine and threonine, suggesting a much more hydrophilic plastein than prepared from the soluble form of the enzyme.

Enzyme catalyzed peptide bond synthesis may result in a oligomerization process of the incorporated amino acid. As the investigations of Gaertner and Puigserver (1982) stated the polymethionyl-casein type peptide has a good in vitro digestibility. The plastein reaction proved to be a convenient means for the elimination of the bitter taste from enzymatic protein hydrolysates. It has been found that, when the bitter hydrolysates were used as substrates of the plastein reaction, the resulting products are tasteless.

Noar and Shipe (1984) concluded that the incorporation of methionine by a one step enzymatic process (Yamashita et al. 1979) was due to the formation of hydrophobic bonds.

Aso et al. (1979) investigated a model system in which, instead of a protein hydrolysate, N-benzoylglycine ethyl ester (Bz-Gly-OET) was used as substrate. The substrate was first incubated with papain at pH 6.0 and 37C. After 15 minutes an amino acid ester was added and incubated. Analysis of the reaction and its products indicated that the following reaction occurred.

Bz-Gly-OET + AA-OR → Bz-Gly-AA-OR + ETOH

where AA-OR refers to the amino acid ester.

The results of Sukan and Andrews (1982) showed that hydrophobic amino acids such as phenylalanine, leucine, isoleucine, tyrosine, valine and proline were preferentially incorporated into plastein at the expense of hydrophilic amino acids. In some other researches a trend of a prefential incorporation of hydrophobic amino acids in the protein product in the enzyme-catalysed reactions has been reported (Horowitz and Haurowitz, 1959; Eriksen and Fagerson, 1976; Fujimaki et al., 1977; Nötzold et al., 1983; Winkler et al., 1988).

Aso et al. (1974) incorporated individually the three essential amino acids, lysine, threonine and tryptophan into zein in which they were deficient. They found that the lysine, thereonine and tryptophan content of the respective plasteins were 2.14, 9.23 and 9.71 %, where those in the zein used as a starting material were 0.20, 2.40 and 0.38 % respectively.

Fujimaki (1978) studied the effect of various amounts of L-lysine ethyl ester by adding them to gluten hydrolysates and found that the lysine content of the plastein depended on the amount of L-lysine ethyl ester used. There was a sigmoidal relationship between the amount of lysine ethyl ester used and the amount of lysine incorporated. The curve reached a plateau when one part of the ester was mixed with two parts of the gluten hydrolysate.

In most cases the resulting protein hydrolysates, though free from non-protein impurities, were accompanied by another problem: development of a bitter taste. The synthesis reaction leading to plastein formation has been found very effective for the debittering

of protein hydrolysates (Fujimaki et al. 1971; Fujimaki et al. 1977). It was also possible to incorporate essential amino acids during the plastein reaction with formation of a plastein whose amino acid composition has already been altered. Thus, a process for the improvement of food protein is suggested, in which a combination of enzymatic protein hydrolysis and plastein synthesis takes place (Fig. 1.).

Arai et al. (1979) found that in the papain-catalyzed incorporation of methionine into soy protein, self-polymerization of methionine ethyl ester occurred depending on the initial concentrations of methionine ethyl ester and soy protein. Polymethionine can hardly be digested in the gastrointestinal tracts of animals. (Arai et al., 1988).

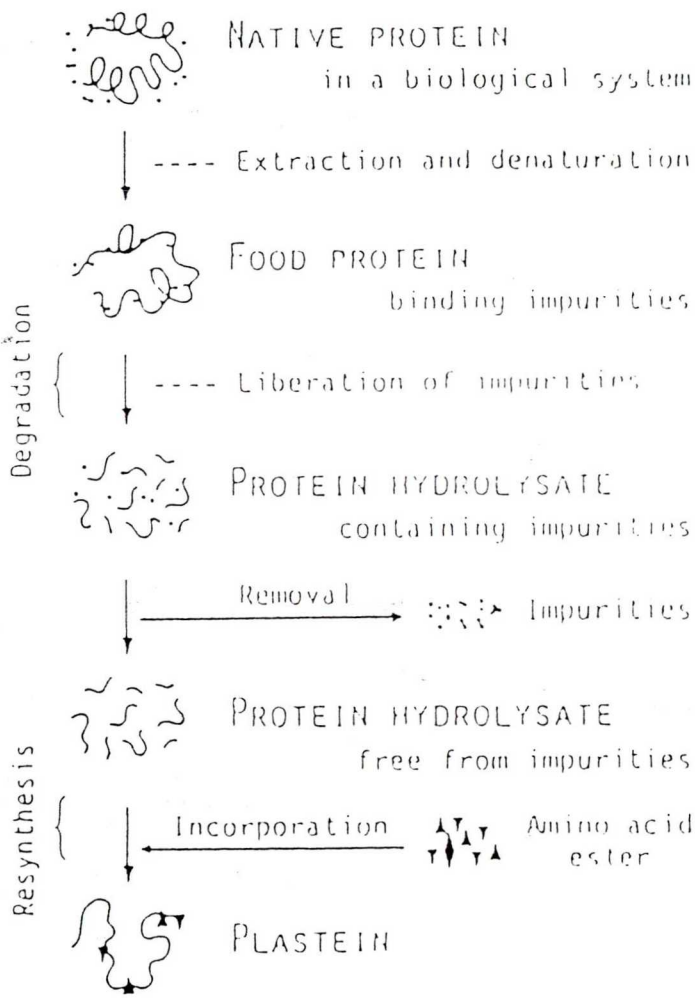
The enzymatic process allows enrichment of the proteolysis products with essential amino acids which are terminally bound covalently to peptides (Hajós et al., 1988; Hajós et al. 1989; Lorenzen and Schlimme, 1992; Demeczky et al. 1994.).

In this laboratory we (Hussein and Hajós, 1993) stated that enzymatic peptide modification is a suitable method for increasing the nutritive value of also buffalo's milk proteins by the incorporation of a given quantity of the limiting essential amino acids.

Lorenzen and Schlimme (1993) reported that serine as L-serine ethyl ester can be incorporated during normal proteolysis into proteolysis products. The increase in the serine content is about half, compared with plastein products obtained by the plastein reaction.

The nutritional quality of plasteins is of great importance. In vitro digestibility of plastein showed comparable digestibility for

Fig. 1.: ***Schematic representation of a combined process of enzymatic protein hydrolysis and resynthesis for producing a plastein with an improved acceptability and an improved amino acid composition, Fujimaki et at. (1977)***



the original protein and the resulted plastein (Yamashita et al., 1970). Rat studies showed an average in vivo digestibility of 90.3 %, with was similar to that of soy protein isolates. Results of Noack and Hajós (1984) and Hajós et al. (1989) confirmed that the casein-based enzymatic modified product digested under physiological conditions showed no impairment in digestibility in comparison to casein. Enzymatic protein modification by plastein reaction was proposed by Yamashita et al. (1976) to prepare a product containing less phenylalanine and more tyrosine in order to satisfy the special requirements of patients who suffer of phenylketonuria. Fish protein concentrate and soy protein isolates were used as protein sources. Each substrate component was hydrolysed subsequently by pepsin and pronase. Aromatic amino acids were removed by absorption to Sephadex G-15. The aromatic acid free hydrolysates were the substrates of the plastein reaction where tyrosine ethyl ester and tryptophan ethyl ester were incorporated. The resulting plasteins contained no free amino acids and were almost tasteless. Low phenylalanine (0.25%) and high tyrosine (7.6%) content of the plasteins made them acceptable as food materials for patients with phenylketonuria.

These different suggestions on how the amino acids are incorporated have given rise to polimics in the literature continuing to the present day, since the reaction mechanism of the process (enzymatic modification of proteins) has not been fully clarified as yet. That is why Hajós (1986) preferred to use the term enzymatic peptide modification (EPM) instead of the generally adapted term of plastein reaction.

2.3 Milk allergy

Cow's milk is an adequate source of high-quality protein. It is ingested daily and in quantities that exceed those of any other single food in infants. Therefore, cow's milk allergy in newborn babies and young children is a serious concern.

Hippocrates (460-370 B.C.) was the first to record that cow's milk could cause gastric upset and urticaria, Chabot, (1951). Later, Galen (131-210) described a case of allergy to goat's milk. (O'Keefe, 1963). Although ruminant milk has probably been used as a food to some degree since man began to record his activities, in early times human breast milk was the major food available infants. When the mother was unable to nurse her baby, a wet nurse was almost the only alternative.

Bahna (1978) estimated that 0.3 to 7.5 % babies-representing about 100,000 babies each year in USA- and from 3.0 to 5.0 % of one year old infants in Japan are allergic to cow's milk protein.

The allergenic effect of cow's milk was related to heterogenicity of protein constituents and many authors considered β-lactoglobulin as being the major allergen because of its absence in human milk (Moneret-Vautrin et al., 1982; Bahna and Heiner, 1980). However, the absence of β-lactoglobulin in human milk is under controversy (Conti et al., 1980; Brignon et al. 1985). Goldman et al. (1963) found that 37 of milk allergic patients challenged orally with β-lactoglobulin and 23 of them showed symptoms. Haddad et al. (1979) have also detected specific antibodies immunoglobulin E (IgE) against β-lactoglobulin in patients.

Gjesing et al. (1986) have shown that milk allergic children had mainly IgE against α-lactalbumin, β-lactoglobulin, albumin and immunoglobulin. These are the four major proteins of bovine whey, as well as IgE against the three casein components.

Jeness et al. (1967) noted that antibody of bovine β-lactoglobulin could react with ß-lactoglobulin from milk of cow, water buffalo, goat, and sheep.

2.4 Reducing the allergenicity of milk proteins

A number of methods have been applied in order to decrease or eliminate the allergenicity of this allergen. Heat constitutes the main method of processing of milk. Heating causes certain changes in milk proteins, including unfolding of polypeptide chains and rupture of disulfide linkages. Ratner et al. (1958) showed that heating of whey proteins reduced antigenicity but no change was observed with caseins. Hanson and Mansson (1961) found that casein resisted a temperature of 120°C for 15 minutes, β-lactoglobulin and α-lactalbumin resisted heating up to 100°C, but bovine serum proteins were denatured between 70° and 80°C.

Bleumink and Young (1968) demonstrated that heat treatment of milk induced associations between ß-lactoglobulin and lactose by Maillard reaction and that the allergenic capacity of the product was a hundred times bigger than that of native β-lactoglobulin. The Maillard reaction was also in close connection with loss of 24 % of available lysine (Heppell et al. 1983).

McLaughlan et al. (1981) observed that the milk with severe heat treatment during manufacturing of commercial baby milk

formula sensitized guinea pigs less efficiently than mildly heated preparations. Kilshaw et al. (1982) found that severe heat treatment on 121°C for 20 minutes of diafiltered whey completely abolished the sensitizing capacity of β-lactoglobulin.

Goat milk proteins showed crossed immunologic reactions with bovine proteins, especially with caseins, ß-lactoglobulin and α-lactalbumin (Bahna and Heiner, 1980).

Similarly, mixed results have been obtained with enzymatic hydrolysis. Protein hydrolysates have been used since the 1940s for the nutritional management of individuals who cannot digest intact protein (Cuthbertson, 1950). The most prevalent use of such hydrolysates however has been for feeding infants suffering from food (particularly cow's milk) hypersensitivity. Formulae designed for allergic infants have used primarily hydrolysates of whey proteins or casein (Takasa et al., 1979; Pahud and Schwarz, 1984; Knights, 1985; Cordle et al., 1991).

Haddad et al. (1979) demonstrated that the treatment of ß-lactoglobulin with pepsin or pepsin and trypsin in vitro resulted in the formation of a breakdown product with the ability of eliciting the formation of antibodies. However, hydrolysates from enzyme fragmentation of ß-lactoglobulin by trypsin, α–chymotrypsin and pepsin had no antigenic activity (Kurisaki et al., 1985). The difference in the results may be attributed to the fact that native ß-lactoglobulin was removed from the hydrolysate by gel filtration by the latter workers. Otani (1981) also recognised no antigenic activity of any peptide with molecular weights of 10,000 or less liberated from ß-lactoglobulin by the pepsin, trypsin and α–chymotrypsin.

Dietary products made with casein-hydrolysates are also available for children allergic to these proteins (Moneret-Vautrin et al. 1982).

Enzymatic hydrolysis of whey proteins seems to be an efficient method for the production of hypoallergenic infant food formula and a good alternative for children allergic to whey proteins (Asselin et al., 1988, 1989).

Otani et al., (1990) indicated that the α–chymotrypsin digest casein with molecular weight smaller than 3500 is less immunogenic when it is injected intradermally.

Cordle et al., (1991) showed that the protein hydrolysates (casein and soy protein) had substantially lower immunogenicity than the parent proteins. Both of the protein hydrolysates seem to be promising candidates for use in hypoallergenic infant feeding systems.

Schmidt et al. (1990) reported that a whey protein hydrolysate (with a degree of hydrolysis of 8-15%) free from antigenicity may be prepared by use of a mixture of an enzyme with high activity against α-lactalbumin, and an enzyme with high activity against ß-lactoglobulin.

Nakamura et al. (1991) found that casein hydrolysate showed hardly any antigenicity. ELISA showed that antigenicity of casein hydrolysate was approx. 10000 times lower than that of the intact bovine casein. Casein hydrolysate could be used in many kinds of food and in development of hypoallergenic infant formulas.

Mahmoud et al. (1992) found that extensive antigenicity loss occurred during the first 10 % of hydrolysis time and, subsequently relatively small changes occurred.

Nakamura et al. (1993) showed that hydrolysis with Rhizopus proteinase followed by A. oryzae proteinase was the most efficient combination of enzymes to reduce antigenicity of casein. Excess hydrolysis caused unpalatability, bitterness off-flavour and loss of nutritional value.

Hajós et. al. (1993) studied the effect of various amount of L-methionine ethyl ester on allergenic character of modified casein by adding them to buffalo's casein hydrolysate with α-chymotrypsin and found that the change of the allergenic character of modified buffalo's casein showed fairly curious results. The more amount of L-methionine ethyl ester was added to the reaction mixtur, the lower level of the allergenic activity was observed.

The enzymatic modification process is a suitable method for reduction the allergenicity and for improvement of biological value by incorporation of essential amino acids into the milk and soy protein chains (Hussein et al., 1991; Hussein and Hajós, 1992; Hajós et al., 1993 a and b).

3. MATERIALS AND METHODS

3.1 Materials

Enzymes

Endopeptidases:

α–chymotrypsin (5U/mg)	(Sigma)	EC 3.4.21.1
Trypsin (6U/mg)	(Serva)	EC 3.4.21.4
Pepsin (20mAnson/mg)	(Calbiochem)	EC 3.4.23.1

Exopeptidases:

Carboxypeptidase A (50U/mg)	(Serva)	EC 3.4.17.1
Carboxypeptidase B (50U/mg)	(Serva)	EC 3.4.17.2
Leucine aminopeptidase	(Serva)	EC 3.4.11.1

Proteins

Bovine casein (Reanal)

Buffalo's milk protein

Buffalo casein

κ-casein

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

3.2 Methods

3.2.1 *Preparation of whole cow and buffaloe casein*

Casein was precipitated from unpasteurized cow's or buffalo's skim milk by acidification to pH 4.6 through the slow addition of IN

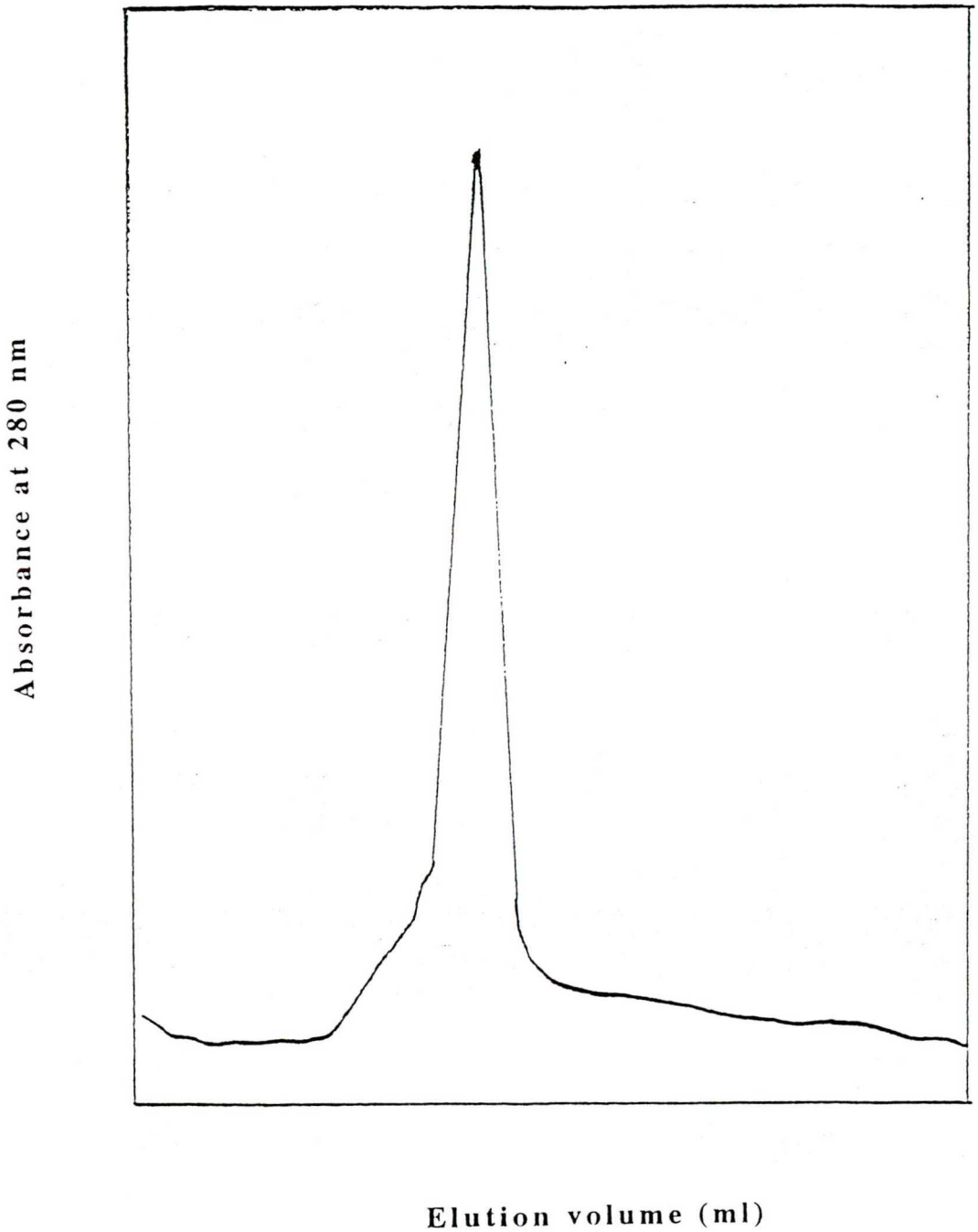
HCl. The precipitate was separated by filtering the solution through cheesecloth. After being washed with distilled water, the precipitate was redissolved by slowly adding IN NaOH to a final solution pH of 7.0. This precipitation, washing, and neutralization were repeated three times, and the solution of casein (pH 7.0) was lyophilized and stored at -20C until used.

3.2.2 *Isolation of κ-casein*

Whole casein was dissolved in 6.6M urea to a 10 % solution and pH was lowered to 1.5 by slow addition of 3.5M H_2SO_4 (Zittle and Custer, 1963). The solution was diluted with H_2O to 2.2 M urea. After 2h the protein was precipitated by centrifugation at 3000 rpm for 20 minutes at room temperature. The κ-casein in the supernatant was precipited by the addition of (132 g) 1M ammonium sulfate to each liter of supernatant. The precipitate is collected, suspended in water, and dissolved by the addition of IN NaOH to a final pH of 7.5. The solution was dialyzed and freeze-dried. These preparations were freed of contaminants by ethanol precipitation (McKenzie and Wake, 1961). This was done as follows: One volume of a 1% pH 7.o solution of κ-casein prepared by the sulfuric acid method was mixed with two volumes of ethanol. One molal ammonium acetate in 75 % ethanol was added until the typical sticky precipitate was obtained. The precipitate was dissolved in water with addition of NaOH to bring the pH to 7.5, dialyzed, and freeze-dried.

The purity of κ-casein was investigated by FPLC (Fig. 2.)

Fig. 2.: *FPLC chromatogram of the κ-casein prepared by the sulfuric acid method after further purification with ethanol*



3.2.3 *Preparation of protein hydrolysate*

3.2.3.1 *Hydrolysates of buffalo's milk proteins*

- Buffalo's milk proteins were hydrolysed with α-chymotrypsin at pH 7.5 or with pepsin at pH 2.0. The incubation was carried out at 37°C for 2h. The reaction mixture was stirred during hydrolysis and after that freeze-dried.
- Buffalo's milk proteins were hydrolysed with α-chymotrypsin at pH: 7,5 under the same conditions for 1 hour, then trypsin was added at pH: 7,5 at the same conditions for 1 hour then freeze-dried.
- Buffalo's milk proteins were hydrolysed with pepsin at pH:2.0 for 1 hour, then the pH was adjusted to pH:7,5 and trypsin was added for 1 hour under the same conditions as mentioned above.

3.2.3.2 *Hydrolysates of buffalo casein*

- Buffalo's casein was hydrolysed with α-chymotrypsin at pH:7,5 or with pepsin at pH:2,0 under the same conditions as mentioned above.
- Buffalo's casein was hydrolysed with a mixture of α-chymotrypsin and trypsin at pH: 7,5 for one hour, then the pH was adjusted to pH: 2,0 and pepsin was added and the reaction mixture was incubated at 37°C for 1 hour.

3.2.3.3 *Hydrolysates of the cow's casein*

Cow's casein was hydrolysed with α–chymotrypsin at pH: 7,5 or with pepsin at pH: 2,0. .

3.2.3.4 *Hydrolysate of κ–casein*

κ–casein was hydrolysed with α–chymotrypsin at pH: 7,5 under the same conditions as mentioned above.

3.2.4 ***Enzymatic peptide modification (EPM)***

The enzymatic peptide modification was carried out with enzyme used in the same protein hydrolysate.

3.2.4.1 *EPM products of buffalo's milk proteins*

- Buffalo's milk protein hydrolysates with α–chymotrypsin or pepsin 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 the substrate 25 % w/v. The ratios of methionine ethyl ester added to substrate can be seen on Table 2. The EPM0 is a modified protein produced without methionine ethyl ester in the reaction mixture but the samples EPM2, 4, 6, 8, 10 refer to the ratio of methionine ethyl ester added to the reaction mixture. The enzyme-protein ratio was 1:100. The incubation was carried out at 37°C; incubation time 16h.

The EPM products were, after incubation, simultaneously dialyzed for 48h through a cellophane membrane against distilled water. The nondialyzable fractions were freeze-dried.

- Buffalo's milk protein hydrolysates with α-chymotrypsin and trypsin one by one were used to produce two types of EPM products, the first with α-chymotrypsin at pH:6.0 and second with trypsin at pH:6.0. The reaction mixture was incubated at 37°C for 1 hour.
- Buffalo's milk proteins hydrolysates with pepsin and trypsin were used to produce two EPM products.

3.2.4.2 *EPM products of the buffalo casein*

- Buffalo's casein hydrolysates with α-chymotrypsin or pepsin were used at the concentration of 25% to produce EPM products.
- Buffalo's casein hydrolysate with α-chymotrypsin and trypsin followed with pepsin were used to produce three different EPM products: the first with α-chymotrypsin, (pH:6.0) ,the second with trypsin (pH:6.0)and the third with pepsin (pH:4.0).

3.2.4.3 *EPM products of the cow's casein*

Cow's casein hydrolysates with α-chymotrypsin or pepsin were used to produce EPM products in the presence of α-chymotrypsin(pH:6.0) or pepsin (pH:4.0) as catalysts.

3.2.4.4 *EPM products of the κ-casein*

κ-casein hydrolysate with α-chymotrypsin was mixed at pH:6.0 with α-chymotrypsin without (EPM0) or with addition (EPM6) of L- methionine ethyl ester (69gL-Met ethyl ester /100g hydrolysate). The incubation of the reaction mixture was carried out at 37°C for 16 hour.

Table 2: ***Methionine concentration in the reaction mixture***

Number of the samples	Methionine added (g/1g hydrolyzate)	Methionine ethyl ester added (g/1g hydrolysate)
EPM 0	0.00	0.000
EPM 2	0.14	0.210
EPM 4	0.34	0.487
EPM 6	0.48	0.697
EPM 8	0.63	0.902
EPM 10	0.91	1.317

3.2.5 ***Determination of the degree of hydrolysis (DH)***

The trinitrobenzene sulfonic acid (TNBS) method (Adler-Nissen, 1979) was used to determine the free amino groups in the reaction mixture. Photometric determination of free amino acid was carried out in solution with sodium dodecyl sulfate (SDS). The degree of hydrolysis (DH) is defined (Adler-Nissen, 1982) as the percentage of peptide bonds cleaved:

$$DH\% = \frac{\text{number of free amino groups}}{\text{total number of amino acid residues}}$$

The total number of amino acid residues was experimentaly determined from the hydrochloric acid hydrolysates of the samples.

3.2.6 *SDS-PAGE*

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was carried out with small modification by the method of Weber and Osborn (1975) using 10 % polyacrylamide gel (bisacrylamide-acrylamide, 1:37) containing 0.1 M sodium phosphate buffer (pH 7.2), 0.1 % SDS and 6 M urea. The electrophoresis buffer consisted of 0.1 M sodium phosphate. 0.1 % SDS, pH 7.2 and the sample buffer was 0.01 M sodium phosphate, 1 % SDS, 2 % β-mercapto ethanol, pH 7.2. Samples were heated in sample buffer for 2 minutes at 100°C, then it was cooled and 6 M urea was added. Electrophoresis conditions were 50V for 1h, followed by 100V for about 4-5h, until the dye front of added bromophenol blue had migrated about 9 cm. Protein staining with Coomassie Brilliant Blue R-250, involving the use of formaldhyde fixation to avoid losses of low molecular weight peptides. A more sensitive staining was achieved by the silver staining procedure of Merril et al. (1982).

Control protein mixtures to the SDS-PAGE

LMW (low MW proteins)

Phosphorylase b	M.W.	94000
Bovine serum albumin		67000
Ovalbumin		43000
Carbonic anhydrase		30000
Soy bean trypsin inhibitor		20000

α-lactalbumin	14400
PMW (peptides)	
Myoglobin	17200
Myoglobin I+II	14600
Myoglobin I	8240
Myoglobin II	6380
Myoglobin III	2560

Densitometry

Densitometry of the gel slab were carried out by a Shimadzu corporation chart type 200-91527.

3.2.7 *Isoelectric focusing*

a. in Sephadex: isoelectric focusing of EPM products and substrates were performed on a glass slab (size 15x20 cm and 0.7 mm layer of 1.5 M urea and 8.0 % Sephades containing 2 % carrier ampholyte (Symolyte) pH 3.5-10.0). The prints obtained from the separations on Sephadex were stained for protein with Coomassie Brillant Blue G 250. (Radola, 1973).

b. PAGIF:(Addeo et.al 1990)

1. Reagents for production of the urea containing polyacrylamide gels

1.1 Stock gel solution

4.85 g acrylamide

0.15 g N,N-methylene-bis-acrylamide (B1S)

48.05 g urea

12.22 g glycerol (87% w/v)

in water and filled up to 100 ml and store in a brown glass bottle in the refrigerator.

1.2 Catalyst solutions

40 % w/v ammonium persulfate (PER)

N,N,N,N-tetramethylethylene diamine (TEMED)

2. Anode solution

5.77 g phosphoric acid (85 w/v) was dissolved in water and diluted to 100 ml.

3. Cathode solution

2.00 g sodium hydroxide was dissolved in water and diluted to 100 ml with water.

4. Protein dissolving buffer

5.75 g glycerol (87% w/v)

24.03 g urea

250 µl ß-mercaptoethanol

in water and filled up to 50 ml.

5. Fixative

150 g of trichloroacetic acid was dissolved in water and was filled up to 1000 ml.

6. Destaning solution

500 ml of methanol and 200 ml glacial acetic acid were diluted to 2000 ml with distilled water.

7. Staining solutions

7.1 Staining solution (stock solution 1)

3.0 g Comassie Brillant Blue G 250 was dissolved in 1000 ml 90 % (v/v)methanol using magnetic stirrer and filtered through two medium-speed folded filters.

7.2 Staining solution (stock solution 2)

5.0 g copper sulphate pentahydrate was dissolved in 1000 ml 20 % (v/v) acetic acid.

7.3 Staining solution (working solution)

125 ml of each of the stock solutions (7.1, 7.2) were mixed together immediately prior to stain.

Preparation of the urea containing acrylamide gels

Gel solution was prepared by mixing additives and ampholyts with stock gel solution (1.1)

9.0 ml stock solution

24 mg β-alanine

500 µl ampholyte pH 3.0-10.0

The gel solution was mixed and de-gassed for two minutes in vacuo.

10 µl Temed and PER was added to the solutions.

Table 3.: ***Conditions of isoelectric focusing***

Step	Time (minutes)	Voltage (V)	Current (mA)	Power (w)
1. Prefocusing	30	2500	15	constant 4
2. Sample focusing	60	2500	15	constant 4
3. Final focusing	60	2500	5	maximum 20

Protein staining

- Protein fixation

The gel was put immediately into a staining dish filled with 200 ml fixative, left for 15 minutes, shaked occasionally.

- Washing and staining the gel plate

The fixative was drained off and washed the gel plate twice for 30 seconds each time with 100 ml destaining solution.

The destaining solution was poured off and filled the dish with 250 ml staining solution; and was allowed to stain for 45 minutes with gentle shaking.

- Destaining the gel plate

The staining solution was poured off and the, gel plate was washed twice with 100 ml destaining solution each time, then shaked for at least 2x15 minutes with 200 ml destaining solution until the back ground became clear and uncoloured. Then the gel plate was rinsed with distilled water (2x2 minutes).

3.2.8 *Preparative Isoelectric Focusing*

A Bio-Rad Rotofor cell was assembled for use according to the manufactures instructions (BIO-RAD 1990).

The Rotofor cell, (Bio Rad, Richmond, CR), separates up to 50 ml of protein-ampholyte solution into 20 fractions in approximately 4 h. Each fraction has about 2-3 ml of native protein solution and may contain several miligrams of proteins. This electrofocusing chamber makes narrow bands of focused protein by rotation at 1 rpm around the focusing axis to minimize gravity-induced convection and electroosmosis destabilization of the gradient. This unit has been used to purify several proteins including enzymes and antibodies (Egen et al 1988; Evans et al. 1989; Wynne and Yada 1991; Petrash et al. 1991). One of the examples of its use with foods is the separation of wheat gluten proteins (Ng et al. 1989; Curioni et al. 1990).

The fractions obtained from the preparative scale IEF allows the second dimension of analysis to be of a varied nature.

Each of the 20 fractions obtained from the Rotofor device was then analyzed by SDS-polyacrylamide gel electrophoresis.

Fifty mg of EPM product was dissolved in 1 ml of 4 M urea after dissolved added 49 ml distilled water and 1.25 ml ampholyte (Symolyte) (pH range 3-10). Prefocusing was performed for 1h at 12 W constant power. The EPM solution was then loaded into the Rotofor cell for focusing and IEF was conducted at 12W constant power for 4h at 5°C. The initial conditions were 496V and 24mA, and at equilibrium the values were 815V and 15mA, respectively.

3.2.9 *Amino Acid content*

An aliquot of the samples was hydrolysed with 6 M HCl in a tube flushed with N_2 at 105°C for 24h and the amino acids were subjected to thin layer ion exchange chromatography. The amino acids were developed with an acetonic ninhydrin reagent with warming to 70°C for 10 minutes (Dévényi, 1976; Hajós and Sajgó, 1972).

In situ quantitative evaluation of the methionine was carried out by Biotech. Fischer densitometer (identified and compared to the control methionine).

3.2.10 *N-terminal amino acids of the peptides*

Leucine aminopeptidase (LAP) treatment

A suspension of the EPM-product (5mg) was incubated with LAP in 100 µl 0.1 M NH_4HCO_3 buffer. Samples (10 µl) were investigated after incubating for 0, 10, 30 and 60 minutes, respectively, at which times each sample was immediately mixed with an equal volume of conc. HCl to stop the enzyme reaction.

Free amino acids were separated by thin-layer ion exchange chromatography (Dévényi, 1976; Hajós and Sajgó, 1972).

3.2.11 *C-terminal amino acids*

Carboxypeptidase treatment

The samples were incubated with a mixture (1:1) of carboxypeptidase A (50U/mg) and carboxypeptidase B (50U/mg). The treatment and the amino acid determination were the same as in the leucine aminopeptidase treatment.

3.2.12 *Chromatographic separation*

The apparatus used was the Pharmacia fast protein liquid chromatography (FPLC) system with UV-M monitor and OH-850 recorder (Radelkis). For analytical scale a prepacked anion-exchange MONO Q column (HR 5/5 Pharmacia)was applied. The buffer of low strength contained 0.02 mol tris and 3.5 M urea at pH 7.5. The buffer of high strength was the same composition with the addition of 1 mol NaCl. The buffers were filtered through a 0.22 μm Millipore filter (GVWP 04700). A sample volume of 200 μl (300 μg) was injected, the eluent flow rate was 1ml/min. and the absorbance was measured at 280 nm.

Preparative separation was carried out on a Q-Sepharose HR 10/30 column (Pharmacia), eluent flow rate 4 cm^3min^{-1}, and the sample load was 200 mg/run. Other conditions were the same as discribed above.

3.2.13 *ELISA test*

The allergenic character of samples was measured in vitro by competitive indirect ELISA test (Hajós, et. al. 1991; Gelencsér, et.al. 1992). Inhibitor assays were performed with use of polystyrene plates (Sülysáp, Hungary). Buffalo's milk protein or buffalo casein, or cow casein antigen was coated on the assay wells by incubating 100 µl/well of protein at 5 g/ml in carbonate buffer, pH 9.4 for overnight at 4C. Between each step, the plates were washed three times with phostate-buffered saline (PBS), pH 7.4, 0.1 mol 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. Free binding sites were blocked by incubation of wells with 200 µl/well of PBS buffer, pH 7.4 containing 0,5 % (w/v) gelatin. The wells were then incubated by 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. 10^{-3} µl/ml to -10^{3} µl/ml in PBS buffer pH 7.4 for 1 hour at 37C. The HRP-labeled goat - Ig-anti-human IgG conjugate (HUMAN, Hungary) by incubating 100 µl/well of conjugate at working dilution (1:160) in PBS buffer pH 7.4 for 1 hour at 37C. The HRP-activity was measured in 100 µl/well sodium citrate substrate solution, contaning OPD and H_2O_2. After incubation for 30 min. with substrate solution at room temperature and inhibition with 50 µl/well of 4N H_2SO_4 solution the optical density was

measured in a titer-tek multiskan (Flow, UK) at 492 nm. Results were expressed in percentage by the reference to a standard serum.

Allergenic character:

$$A(\%) = \left(1\frac{A_i - A_{bl}}{A_{st} - A_{bl}}\right)x100$$ where:

A = allergenic activity of samples involved in the inhibition assays

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

4. RESULTS AND DISCUSSION

4.1 Enzymatic modification of buffalo's milk proteins:

Buffalo's milk proteins were submitted to hydrolysis with α–chymotrypsin or pepsin or α–chymotrypsin and trypsin and by pepsin followed by trypsin. Enzymatic peptide modification was carried out using L-methionine ethyl ester in the reaction mixture, and α–chymotrypsin or pepsin were the catalysts of the reactions.

4.1.1 *Electrophoretic patterns of the buffalo's milk proteins*

SDS-PAGE

The electrophoretic patterns and the densitograms of protein fractions of the buffalo's milk protein, hydrolysate with α–chymotrypsin and EPM products separated by SDS-PAGE are shown in Figs 3 and 4.

These results illustrate that zones of small molecular mass α–chymotryptic hydrolysate of buffalo's milk protein (in the range of 6000-20000 molecular mass) increased, whereas the yield in zones of high molecular mass decreased during the EPM reaction. The separation revealed little differences between EPM products (0,2 and 10), showed the greatest intensity on staining in the molecular range of about 5000-8000 molecular mass.

Minor zones were detected at positions corresponding to molecular mass about 14000 and 22000 molecular mass. Also, the results revealed that the increase in the zones of high molecular mass and intensity became predominant above 20000 molecular mass of the EPM products (6, 8 and 10). These EPM products (6, 8, 10) showed definite zones ranged from 6000 to 20000 molecular mass,

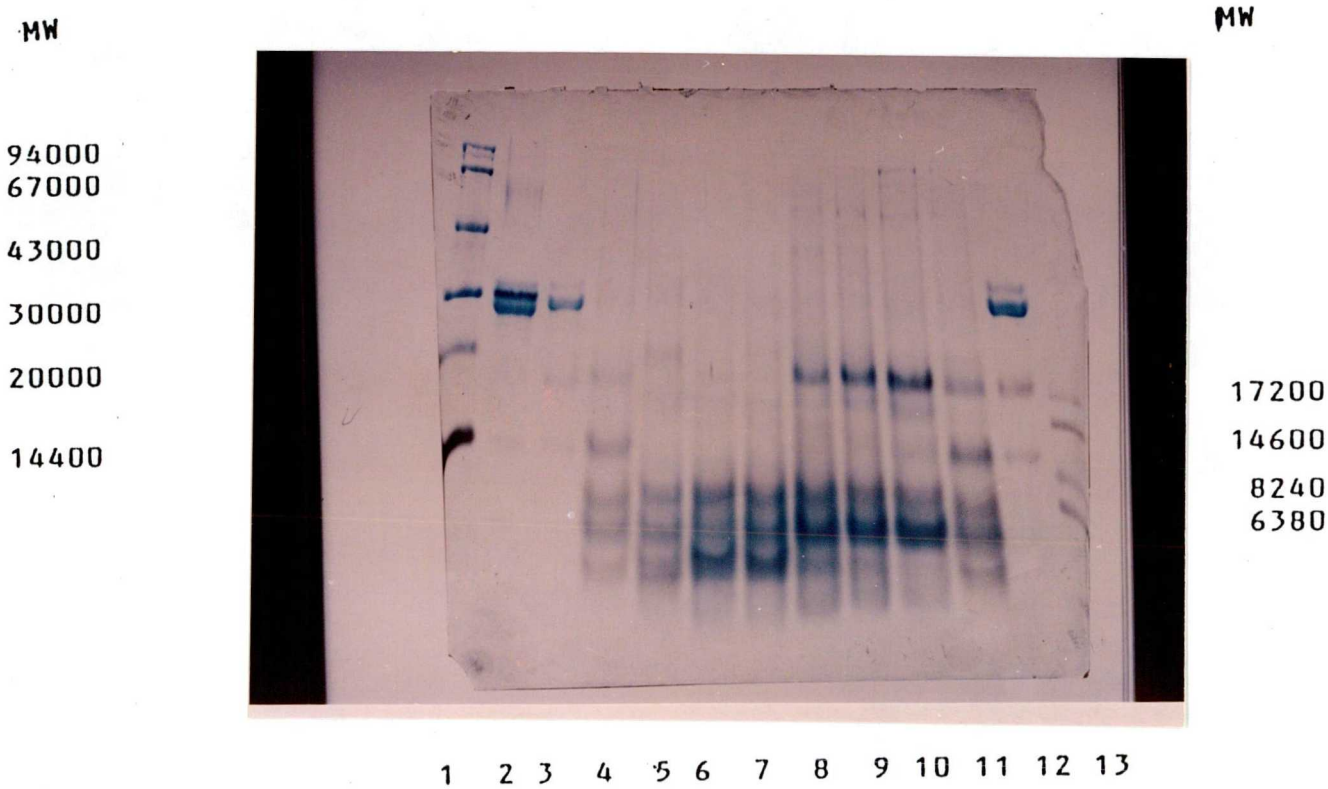
these changes of the molecular mass and intensity are essentially influenced by the concentration of L-methionine ethyl ester in the reaction mixture.

The patterns of peptides produced by a peptic-hydrolysis was of course completely different (Fig.5), the pepsin caused a complete loss of α- and β-caseins. The substrate for enzymatic modification (samples 3 and 11) showed the greatest intensity on staining in the molecular range of about 17200-20000 Dalton. Minor zones were detected at the positions corresponding to molecular weights of about 6000, 8000, 14000, 17000 and 30000. From Fig. 5., it can be seen that the molecular weights of EPM products were higher than those of the hydrolysate. It can also be seen that the numbers of fractions of EPM products are lower than hydrolysate. It can also be seen that, zones intensity of EPM products (EPM0 to 10) increased by increasing the concentration of L-methionine ethyl ester in the reaction mixture.

The changes in the zones indicate that transpeptidation is the major process in the enzymatic modification.

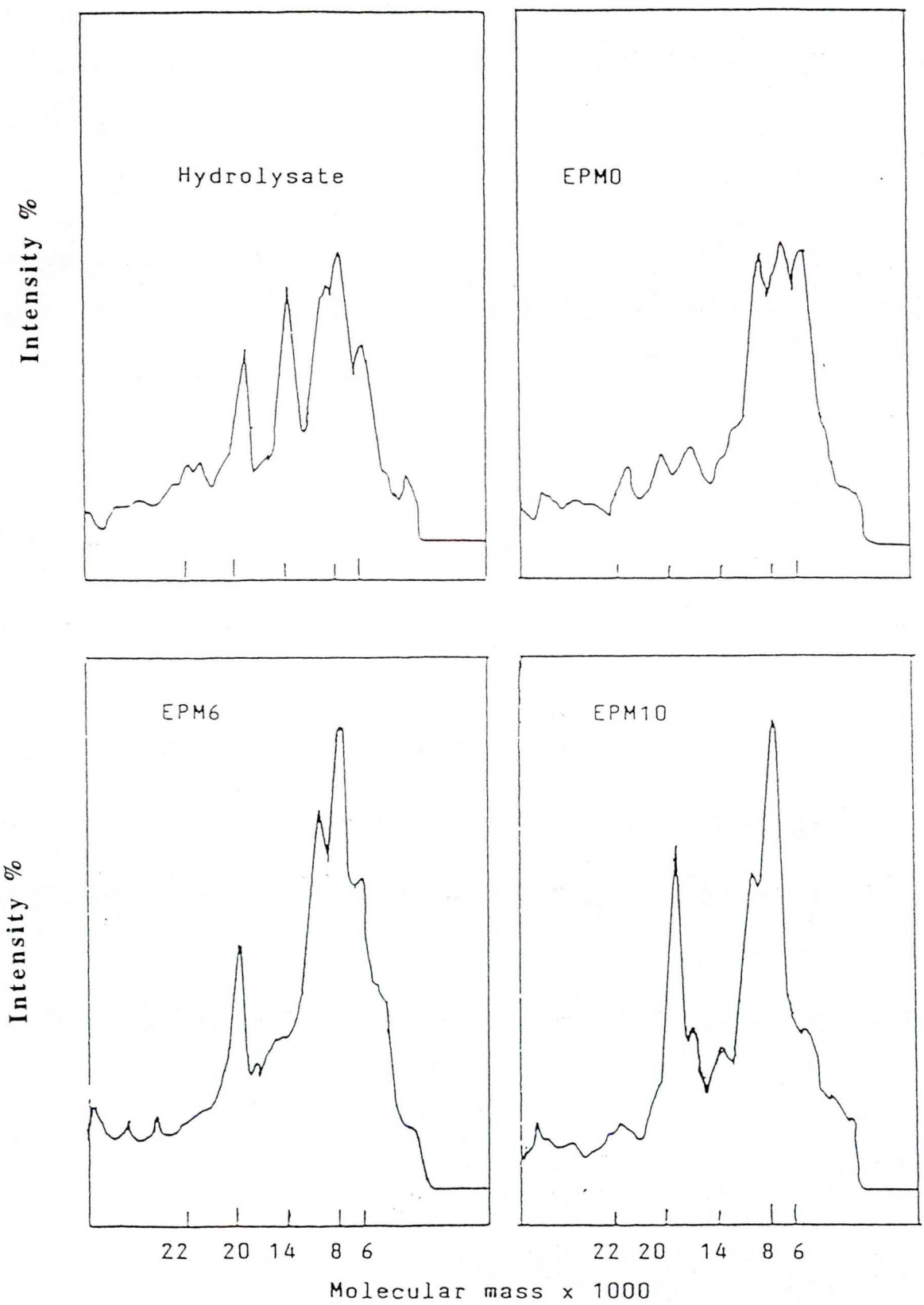
The electrophoretic patterns of protein fractions of the buffalo's milk protein, hydrolysate with α–chymotrypsin and trypsin, hydrolysate with pepsin followed by trypsin, and EPM products are shown in Figs. 6 and 7. These results indicate that both hydrolysates are very low molecular mass comparison with the α–chymotryptic or peptic hydrolysates α–chymotrypsin hydrolysis the bonds involving aromatic amino acids and to a much less extent leucine and methionine (Neil et al., 1966), while trypsin is more specific for basic amino acids. Pepsin exhibits specificities for peptide bonds C-terminal to aromatic residues, leucine, methionine

Fig. 3.1 ***SDS-PAGE patterns of the buffalo's milk protein, its enzymatically modified products***



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

Fig. 4.: ***Densitograms of protein fractions of the buffalo's milk protein hydrolysate and EPM products separated by SDS-PAGE***



and glutamic acid (Tang, 1963). The combination of proteases gave increase in hydrolysis when compared with that of hydrolysates obtained by any protease enzyme alone. Fig.6. shows that there are considerable differences between hydrolysate and EPM products by α–chymotrypsin as catalyste. Clearly that when the amount of methionine ethyl ester increased in the reaction mixture, the differences increased. The separation revealed that little differences between EPM0 and EPM2, both EPMs contain five fractions ranged from mol. mass 14000 to 30000. The intensity of fraction 30000 decreased gradually, by increasing the methionine ethyl ester in the reaction mixture, but intensity of zones mol. mass 20000 and 14000 increased gradually.

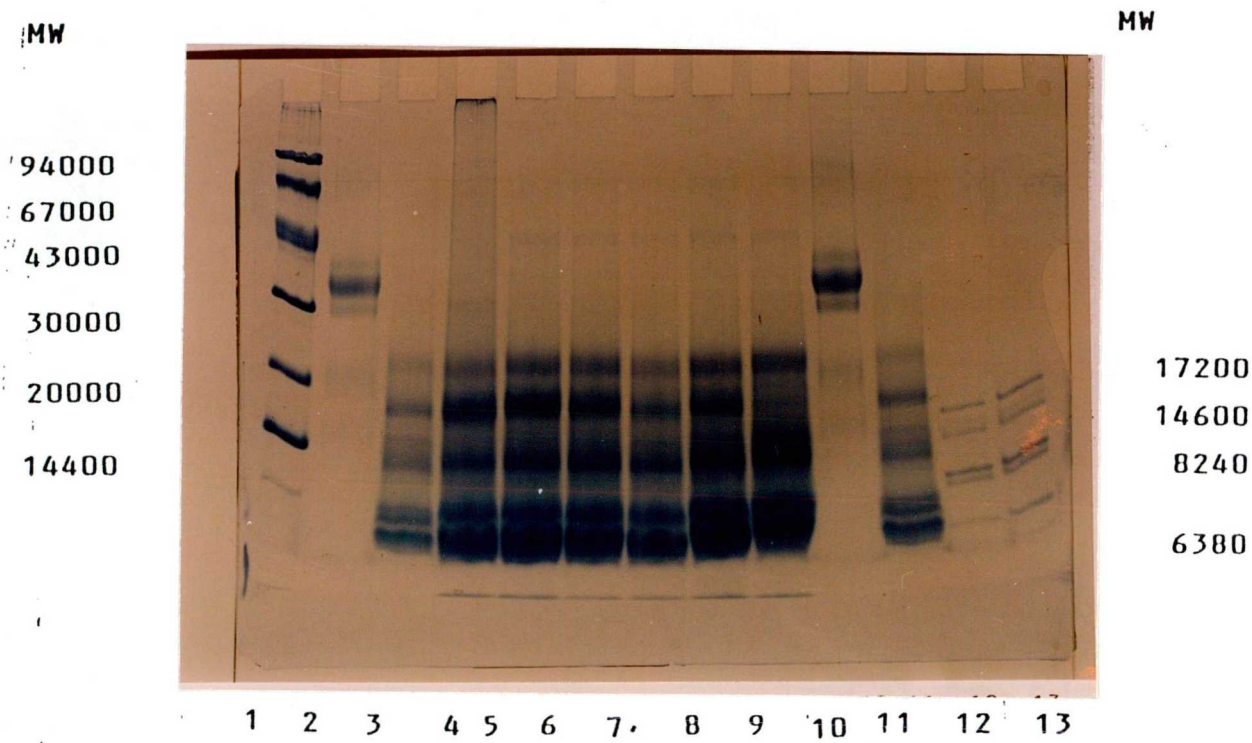
Fig. (7.) Show the differences between buffalo's milk proteins hydrolysates with pepsin followed with trypsin and EPM products in the presence of pepsin as catalyst. It can be seen that the molecular weight of EPM products increased by increasing the concentration of L-methionine ethyl ester in the reaction mixture. Also showed that little differences between EPM6, EPM8 and EPM 10.

Isoelectric focusing

Changes of the isoelectric points of the peptides and proteins during EPM processes were detected by isoelectric focusing Sephadex, or PAGIF are shown in Figs. 8, 9, 10, 11

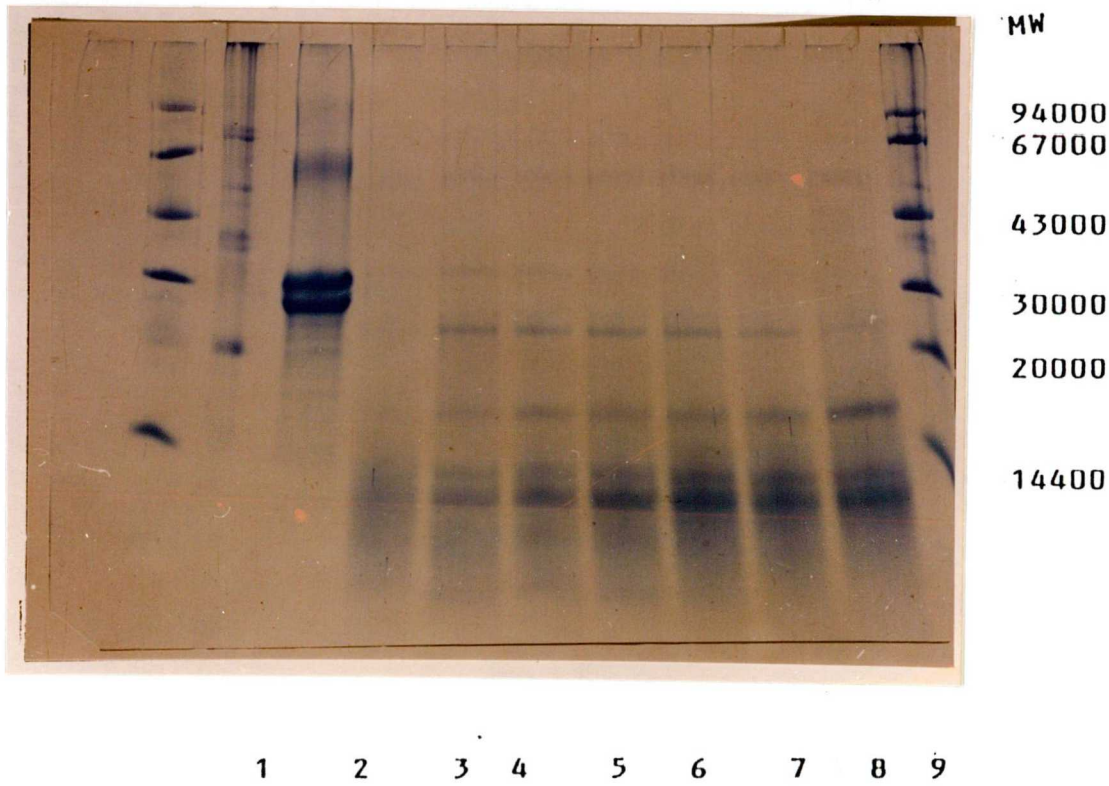
Fig.8. shows the fractions obtained by isoelectric focusing on Sephadex of the EPM products and buffalo's milk hydrolysate with α–chymotrypsin. It can be seen that considerable differences can be found between the peptides of the EPM products and those of the buffalo's milk protein hydrolysate, and great differences between

Fig. 5.: ***SDS-PAGE patterns of buffalo's milk proteins, its peptic hydrolysate and its modified EPM products***



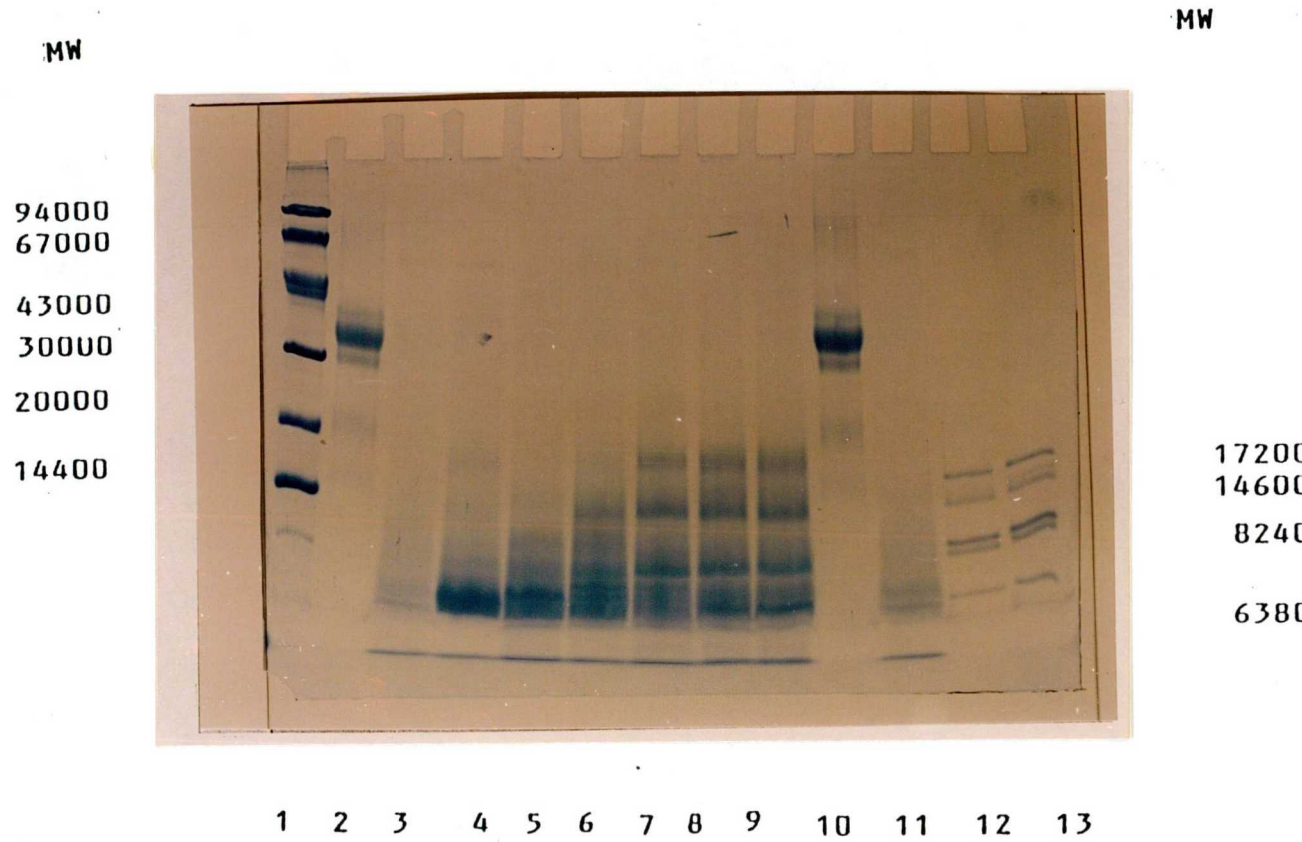
1	LMW
2, 10	Buffalo's milk proteins
3, 11	Buffalo's milk proteins hydrolysate with pepsin
4	EPM 0 (EPM product without Met-enrichment)
5, 6, 7, 8, 9	EPM products (produced in the presence of pepsin, as catalyst)
12, 13	PMW

Fig. 6.: ***SDS-PAGE patterns of the buffalo's milk proteins, its hydrolysate with α-chymotrypsin and trypsin, and its modified EPM products catalyst with α-chymotrypsin***



1	Buffalo's milk proteins
2	Buffalo's milk proteins hydrolysate with α-chymotrypsin and trypsin
3	EPM 0 (EPM product without Met-enrichment)
4, 5, 6, 7, 8	EPM products (produced in the presence of α-chymotrypsin as catalyst)
9	LMW

Fig. 7.: ***SDS-PAGE patterns of the buffalo's milk proteins, its hydrolysate with pepsin followed by trypsin, and modified buffalo's milk proteins catalyst with pepsin***



1	LMW
2, 10	Buffalo's milk proteins
3, 11	Buffalo's milk proteins hydrolysate with pepsin followed by trypsin
4	EPM 0 (EPM product without Met-enrichment)
5, 6, 7, 8, 9	EPM products with different Met-enrichment
12, 13	PMW

all EPM products, this difference may be dependent on the concentration of L-methionine ethyl ester in the reaction mixture. It is clear the difference between EPM6 and both EPM 0 and EPM 10, EPM 6 contain fraction at IF. 5.0 at high intensity.

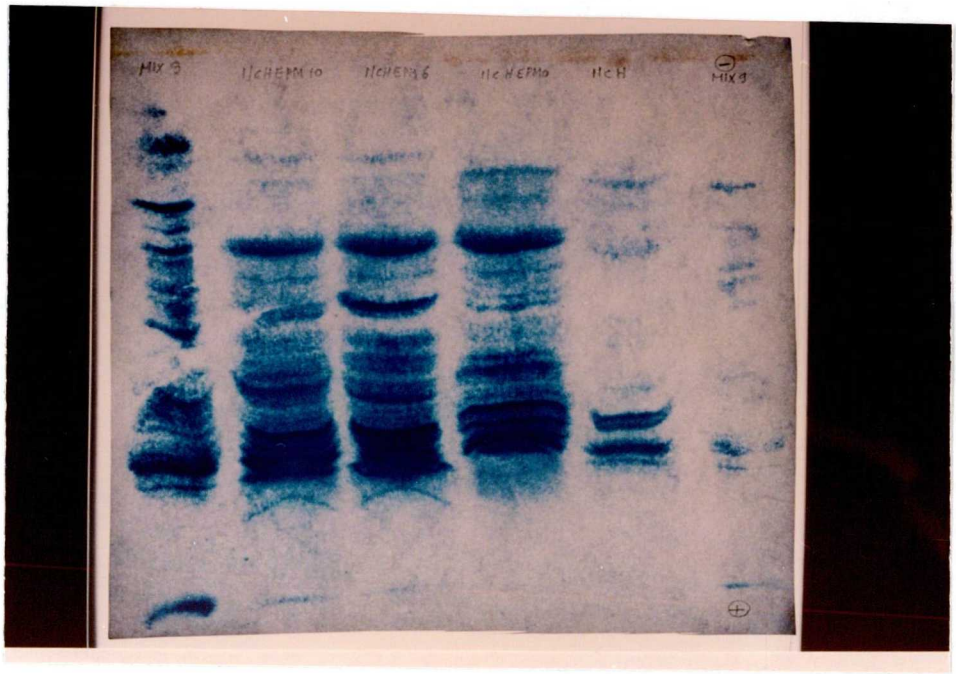
The fractions obtained by PAGIF of the EPM products and buffalo's milk protein hydrolysate with pepsin are shown in Fig. 9. It can be seen that differences between hydrolysate and EPM products, the intensity of EPM fractions is higher compared with hydrolysate fractions. The changes in the zones indicate that transpeptidation is the major process in the enzymatic modification.

Fig. 10. shows that the differences between hydrolysate with pepsin followed by trypsin, the hydrolysate were related to bands of IF near 4.0. Also results revealed that higher differences between EPM 0 and EPM 6 and 10.

Fig. 11. show that the differences between hydrolysate with α–chymotrypsin and trypsin, and EPM products when α–chymotrypsin was used as catalyst. Also results revealed that significant differences between EPM product without methionine ethyl ester and with methionine ethyl ester in the reaction mixture, and the differences increase by increasing amount of methionine ethyl ester in the reaction mixture.

The separation by net charge of protein fractions of both EPM products was found to be significantly different from that of the substrate. Results indicate that a great number of peptide bonds were cleaved and formed due to a considerable transpeptidation. It is interesting to note that, in this respect, the samples both with and without methionine addition behaved in an analogous way. This suggests that transpeptidation takes place in all EPM reactions.

Fig. 8.: ***Fractions obtained by isoelectric focusing on Sephadex of the buffalo's milk proteins hydrolysate with α-chymotrypsin and EPM products, catalyst with α-chymotrypsin***



1 2 3 4 5 6

1, 6 Mix 9 (Protein Test Mixture 9, pI-Marker Proteins, SERVA)

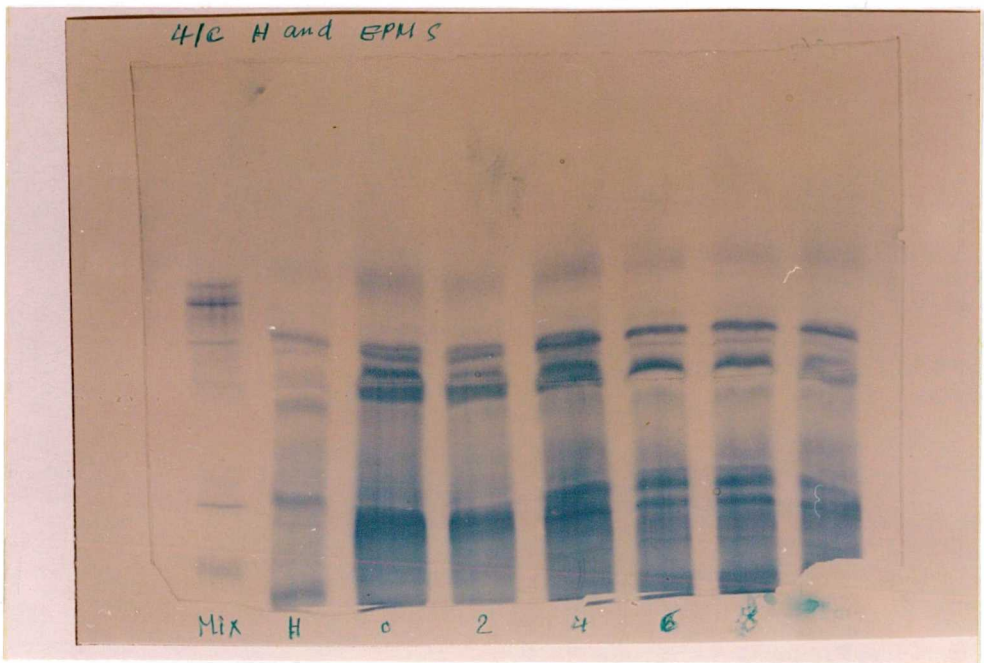
2 EPM 0 (EPM product without methionine enrichment)

3 EPM 6 (EPM products with different Met-enrichment)

4 EPM 10 (EPM products with different Met-enrichment

5 Buffalo's milk proteins hydrolysate with α-chymotrypsin

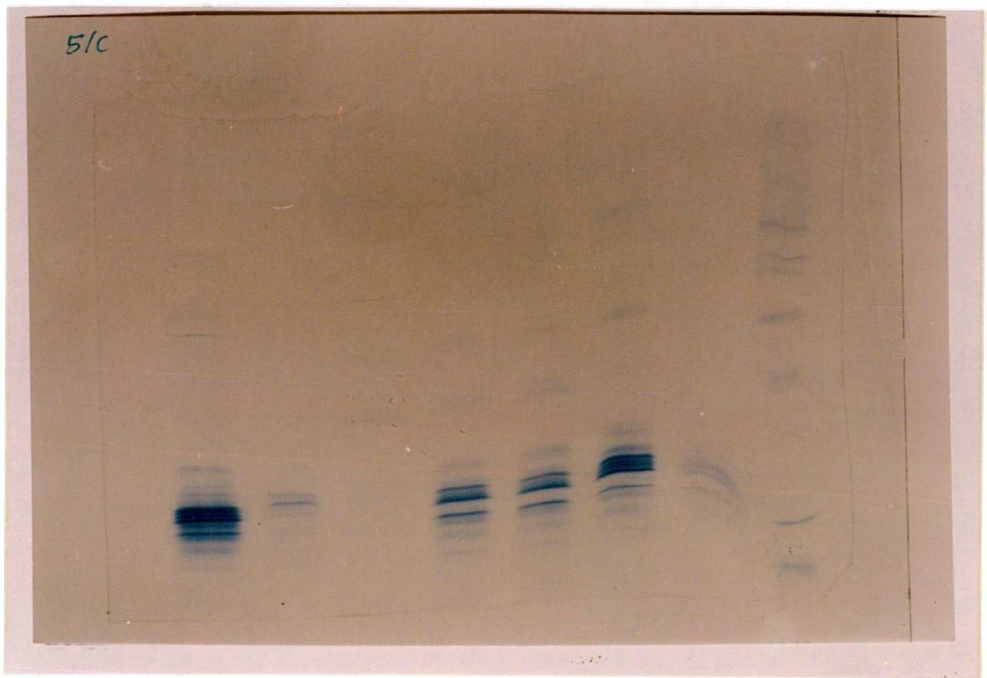
Fig. 9.: ***Fractions obtained by PAGE-IEF of the buffalo's milk proteins hydrolysate with pepsin and EPM products, catalyst with pepsin***



1 2 3 4 5 6 7 8

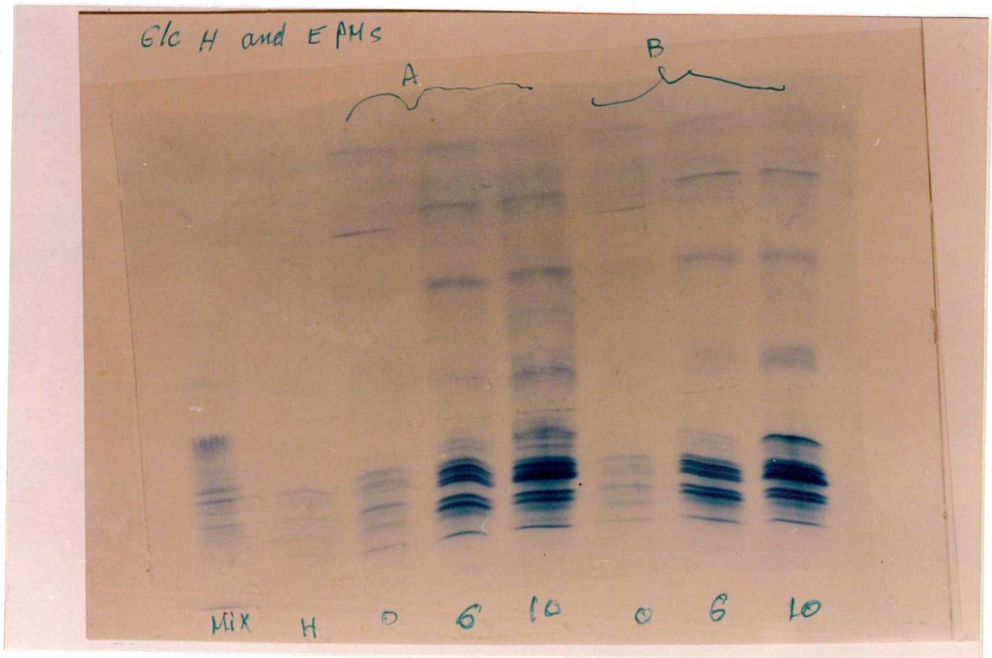
1	Mix 9 (pI-Marker Proteins, SERVA)
2	Buffalo's milk protein hydrolysate with pepsin
3	EPM 0 (EPM product without Met-enrichment)
4, 5, 6, 7, 8	EPM 2 - EPM 10 (EPM products with different Met-enrichment)

Fig. 10.: ***PAGE-IEF electrophoresis patterns of the buffalo's milk proteins hydrolysate with pepsin followed by trypsin and EPM products catalystes with pepsin (EPM A) and trypsin (EPM B)***



Mix 9	Protein test mixture 9 (pI Marker proteins, SERVA)
H	Hydrolysis with pepsin followed by trypsin
A/0	EPM 0 catalyst with pepsin (without methionine enrichment)
A/6, A/10	EPM 6 and EPM 10 catalyst with pepsin (with different Met-enrichment)
B/0	EPM 0 catalyst with trypsin (without Met-enrichment)
B/6, B/10	EPM 6 and EPM 10 catalyst with trypsin (with different Met-enrichment)

Fig. 11.: ***Fractions obtained by PAGE-IEF of the buffalo's milk proteins hydrolysate with α-chymotrypsin and trypsin, and EPM products catalyst with α-chymotrypsin (EPM A) and trypsin (EPM B)***



1 Mix 9 protein test mixture 9 (pI-Marker proteins, SERVA)

2 Buffalo's milk protein hydrolysate with α-chymotrypsin and trypsin

3 EPM A/O ┐ EPM products with and without Met-

4 EPM A/6 │ enrichment

5 EPM A/10 ┘ (α-chymotrypsin used as catalyst)

6 EPM B/0 ┐ EPM products with and without Met-

7 EPM B/6 │ enrichment

8 EPM B/10 ┘ (trypsin used as catalyst)

4.1.2 *Incorporation of L-methionine into buffalo's milk protein hydrolysate by enzymatic modification*

Methionine content of the buffalo's milk protein hydrolysate and EPM products are shown in Table 4. This comparison shows methionine content in the EPM products with methionine incorporation were found to be higher than that of the substrate protein. The optimum of L-methionine incorporation when α–chymotrypsin used as catalyst (EPM4) was found at a ratio of 34 g L-Met/100 g hydrolysate (48 g L-Met ethyl ester/100 g hydrolysate). Methionine content of EPM4 is greater by 3.8 % than that EPM0. The optimum when pepsin was used as catalyst (EPM2) was found at a ratio of 14 g L-Met/100 g hydrolysate (21 g L-Met ethyl ester/100 g hydrolysate). Methionine content of EPM2 is greater by 3.0% than that of EPM 0.

Table 4.: *Methionine content of the modified buffalo's milk proteins.*

EPM	Methonine 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

Methionine content of buffalo's milk proteins is 1.5 g/100 g protein

EPM A: EPM products from α-chymotryptic hydrolysate of the buffalo's milk proteins, produced in the presence of α-chymotrypsin as catalyst.

EPM B: EPM products from peptic hydrolysate of the buffalo's milk proteins, produced in the presence of pepsin as catalyst.

The results showed that the maximal enrichment of the methionine in the products was in the case of α–chymotrypsin catalyst covalently bound, about four times and in the case of pepsin was three times as high as that of the substrate protein. The methionine level of the products satisfies the methionine demand proposed by FAO/WHO (1973).

Methionine content of EPM products, when buffalo's milk proteins hydrolysate with α–chymotrypsin and trypsin or with pepsin followed by trypsin, and α–chymotrypsin or pepsin were used as catalysts respectively, are shown in Table 5., Methionine contents in the EPM products with methionine incorporation were found to be higher than that of the substrate protein.

Table 5.: ***The Methionine content of the modified buffalo's milk proteins.***

EPM	Methionine content covalently bound (g Met/100g protein)	
	EPMA	EPMB
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

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 as catalyst.

The optimum of L-methionine incorporation was found to be a ratio of 34 g L-Met/100 g hydrolysate (EPM4) when α–chymotrypsin was used as catalyst. The optimal incorporation of L-methionine in the case of pepsin catalyst was 14 g L-Met/100 g hydrolysate (EPM2). The results revaled that the amount of methionine incorporated changed with the type of the enzyme applied. When α–chymotrypsin was used as catalyst in the case of α-chymotryptic hydrolysate of buffalo's milk proteins the amount of methionine incorporated was higher compared with the cases when pepsin was used as catalyst in the case of peptic hydrolysate of buffalo's milk proteins.

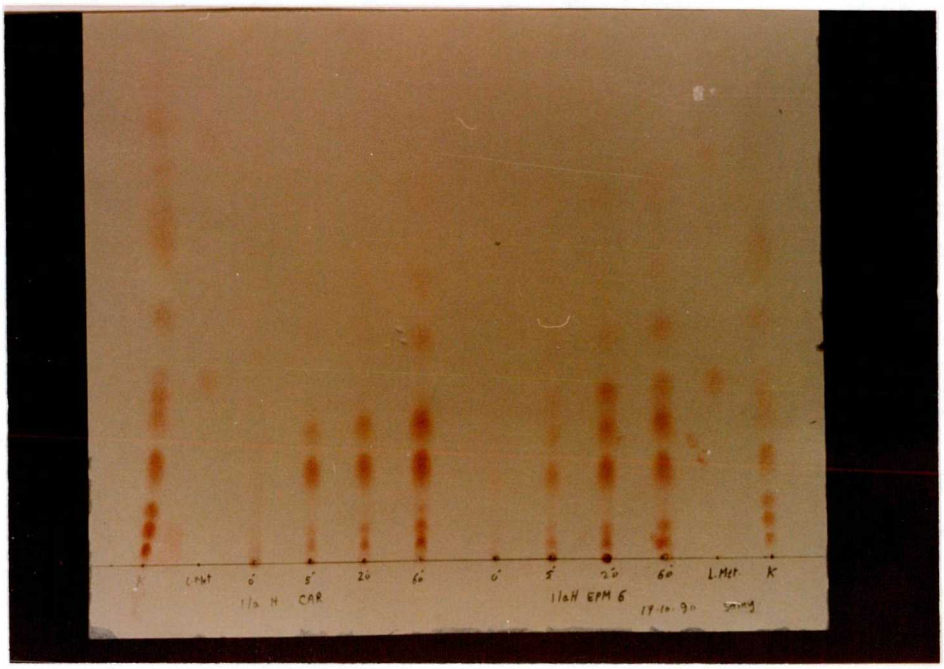
The effects of exopeptidases on samples are shown in (Fig. 12.). In samples taken at 0 min., where HCl was added together with exopeptidase to prevent digestion no free amino acid was present. This finding proved that both the excess methionine ethyl ester and methionine were totally removed from the reaction mixture during dialysis. In samples obtained after 10-60 min., increasing amounts of free amino acids could be detected as results of exopeptidase action.

In Fig. 12. can be seen that methionine was in the EPM product, while it was totally absent in the buffalo's milk protein hydrolysate.

The C-terminal residues of α-chymotryptic hydrolysate of buffalo's milk proteins and EPM products catalyst with α–chymotrypsin are shown in Fig. 13. and 14. .On the Fig. 13. after 60 min., four intense spots can be seen; these correspond to the amino acids removed by carboxypeptidase from the C-terminal. Interestingly, among these free amino acids, methionine could not be found comparison to reference samples. At the Fig. 14. EPM shows five intense spots. One of the intense spots corresponded to methionine. There were no difference between EPM product with α–chymotrypsin and pepsin in the C-terminal.

This results suggest that enzymatic peptide modification is a suitable method for increasing the nutritive value of buffalo's milk protein by the incorporation of a given quality of the limiting essential amino acids according to the FAO/WHO recommendation (1973).

Fig. 12.: ***Liberation of amino acids during treatment of buffalo's milk protein hydrolysate (H), and EPM product with mixture from carboxypeptidase A and B***



H: α-chymotryptic hydrolysate of the buffalo milk proteins

EPM: modified product in the presence of Met-enrichment

Fig. 13.: ***Densitogram of amino acids of the 60 minutes carboxypeptidase (A+B) hydrolysate of the buffalo milk proteins hydrolysate with α-chymotrypsin***

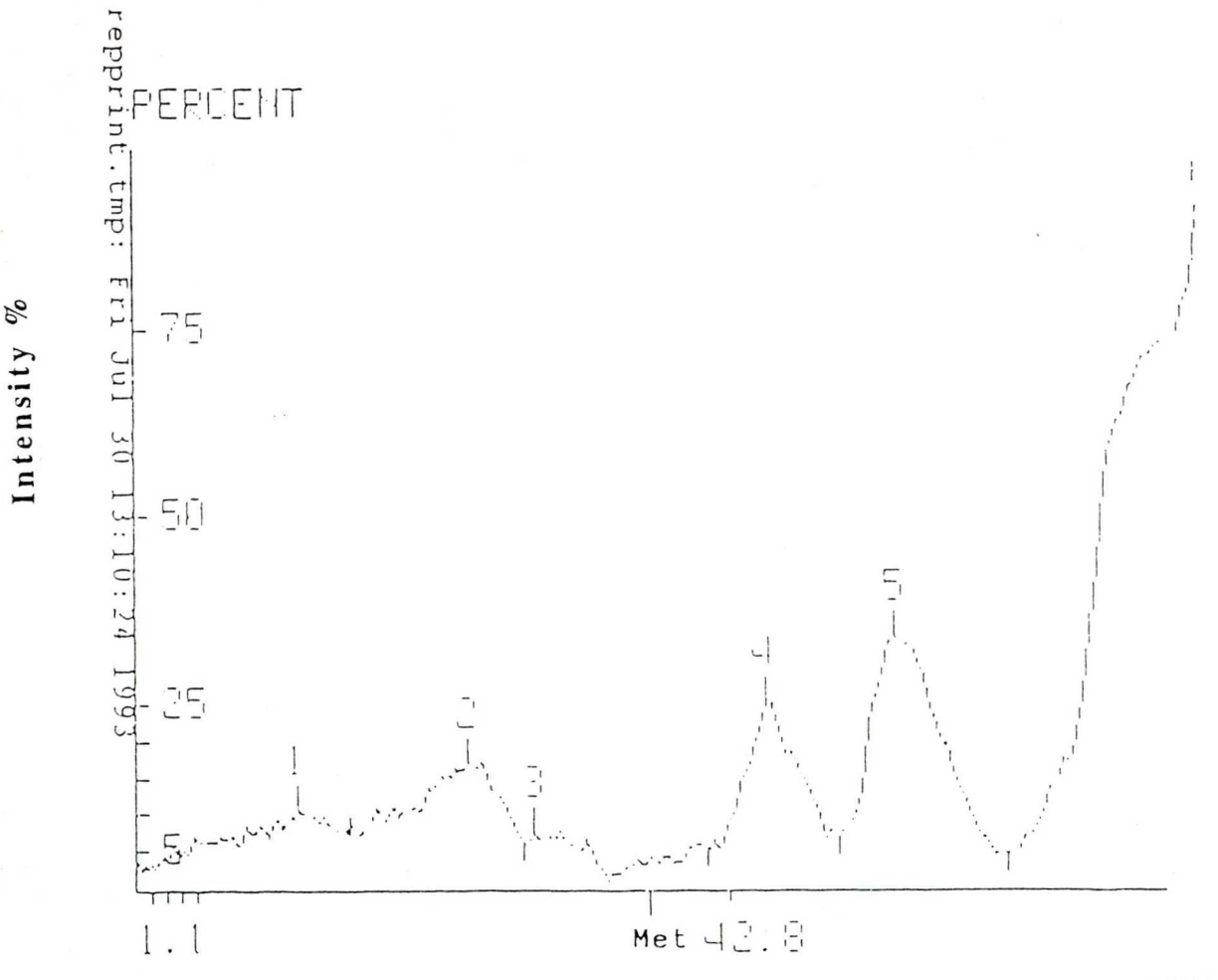
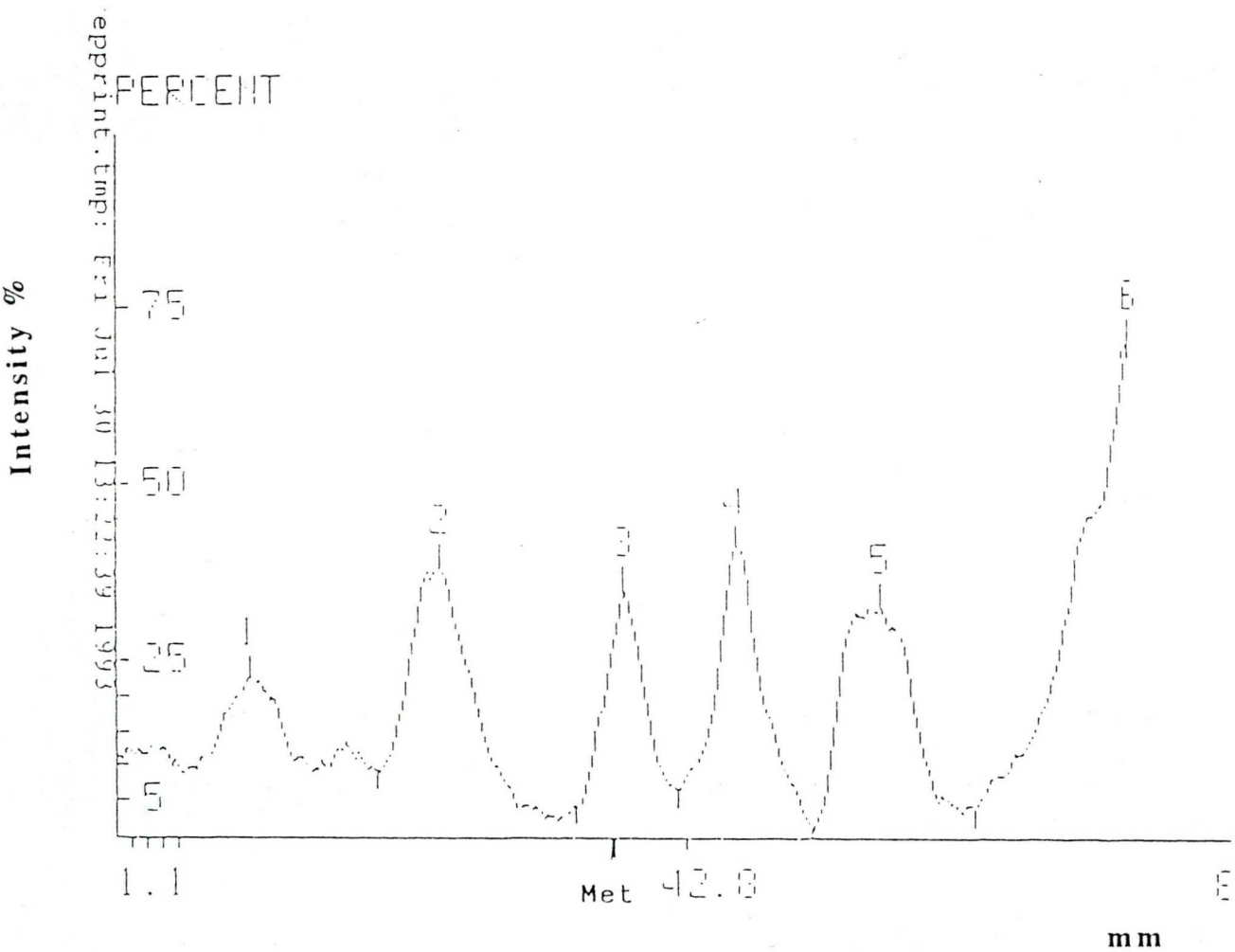


Fig. 14.: ***Densitogram of amino acids in the sample taken of the 60 minutes carboxypeptidase (A+B) hydrolysate of the methionine enriched EPM product***



4.1.3 *Allergenic character of the buffalo's milk protein hydrolysate and EPM products*

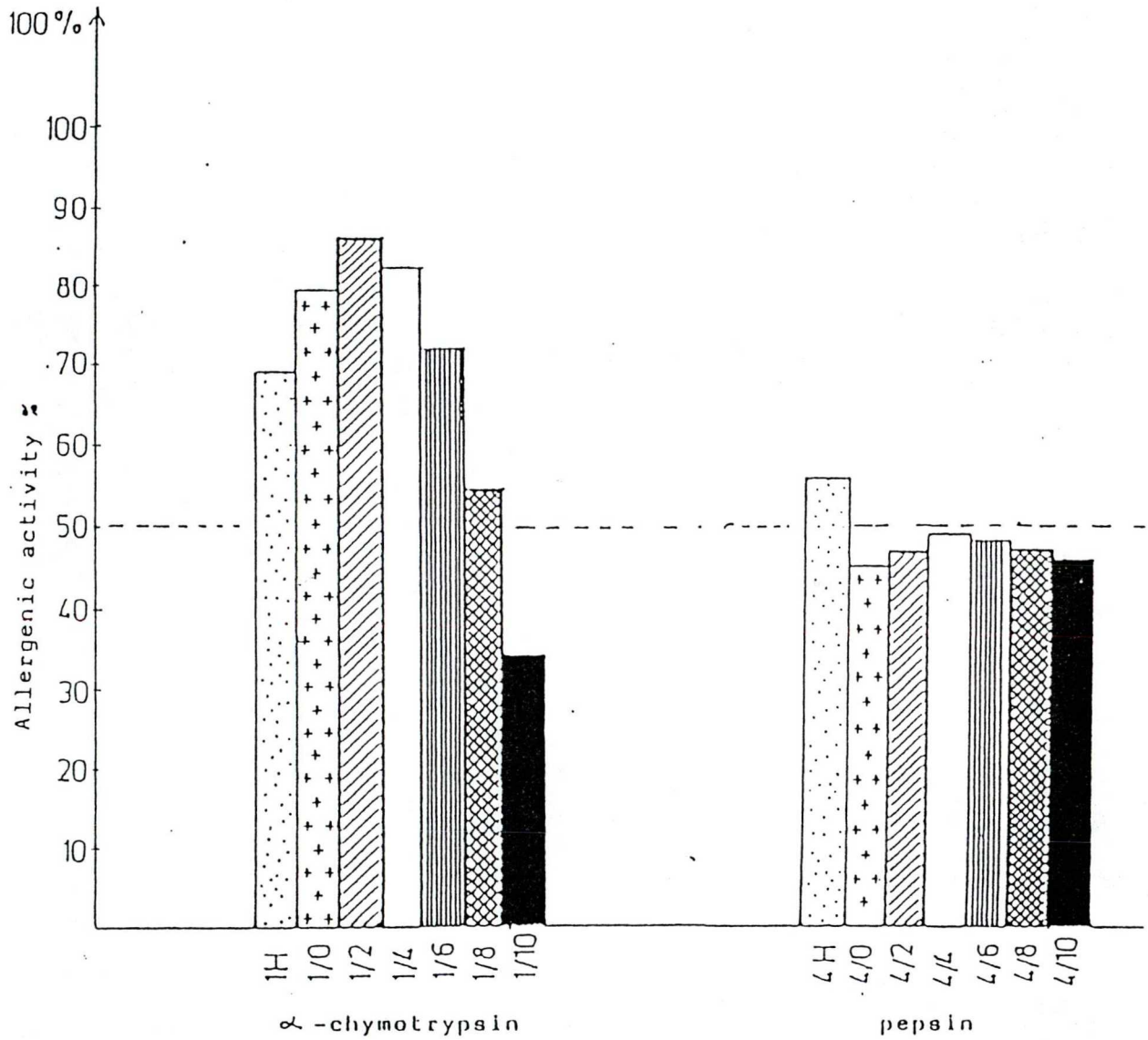
The results of the enzyme-linked immunosorbent assay (ELISA) are presented in Fig. 15., 16., 17.

Fig. 15. shows that the allergenicity of α-chymotryptic and peptic hydrolysate were significantly reduced, peptic hydrolysate is very highly reduced in allerginecity (56%) compared with α-chymotryptic (69%). It also can be seen that the reduction of the allergenic character of the modified buffalo's milk protein α-chymotrypsin catalyst was significantly improved by the increase of the concentration of methionine ethyl ester in the reaction mixture. EPM products (0, 2, 4, 6) appeared high allergenic character comparison to hydrolysate, whilst EPM8 and 10 exhibited low allergenic character 54% and 34%, respectively. The results showed also that the allergenic character of EPM products, where pepsin was used as catalyst were not effected by the concentration of methionine ethyl ester in the reaction mixture. All EPM products charactriezed by low allergenic activity under 50%, ranged from 49% (EPM4) to 45% EPM0. It can also be seen that EPM10 product with α-chymotrypsin of 34% was very low compared with EPM10 product with pepsin of 46%.

Fig. 16. shows that allergenic activity increased by increasing the concentration of samples. At the concentration of 10^3µg/ml, EPM 10 in both α-chymotrypsin or pepsin was used as catalyst appeared low allergenic activity comparison with both hydrolysates.

Fig. 17. shows the allergenic character of EPM products from buffalo's milk protein hydrolysate with α-chymotrypsin and trypsin, α-chymotrypsin was used as catalyste (EPMA). Allergenic

Fig. 15.: ***Allergenic character of the enzymatically modified buffalo's milk protein products***



1H	α-chymotrypsin hydrolysate
1/0	EPM product without Met-enrichment
1/2, 1/4, 1/6, 1/8, 1/10	EPM products with different Met-enrichment
4H	Peptic hydrolysate
4/0	EPM product without Met-enrichment
4/2, 4/4, 4/6, 4/8, 4/10	EPM products with different Met-enrichment

Fig. 16.: ***Potential allergenic activity of modified buffalo's milk protein products by competitive indirect ELISA***

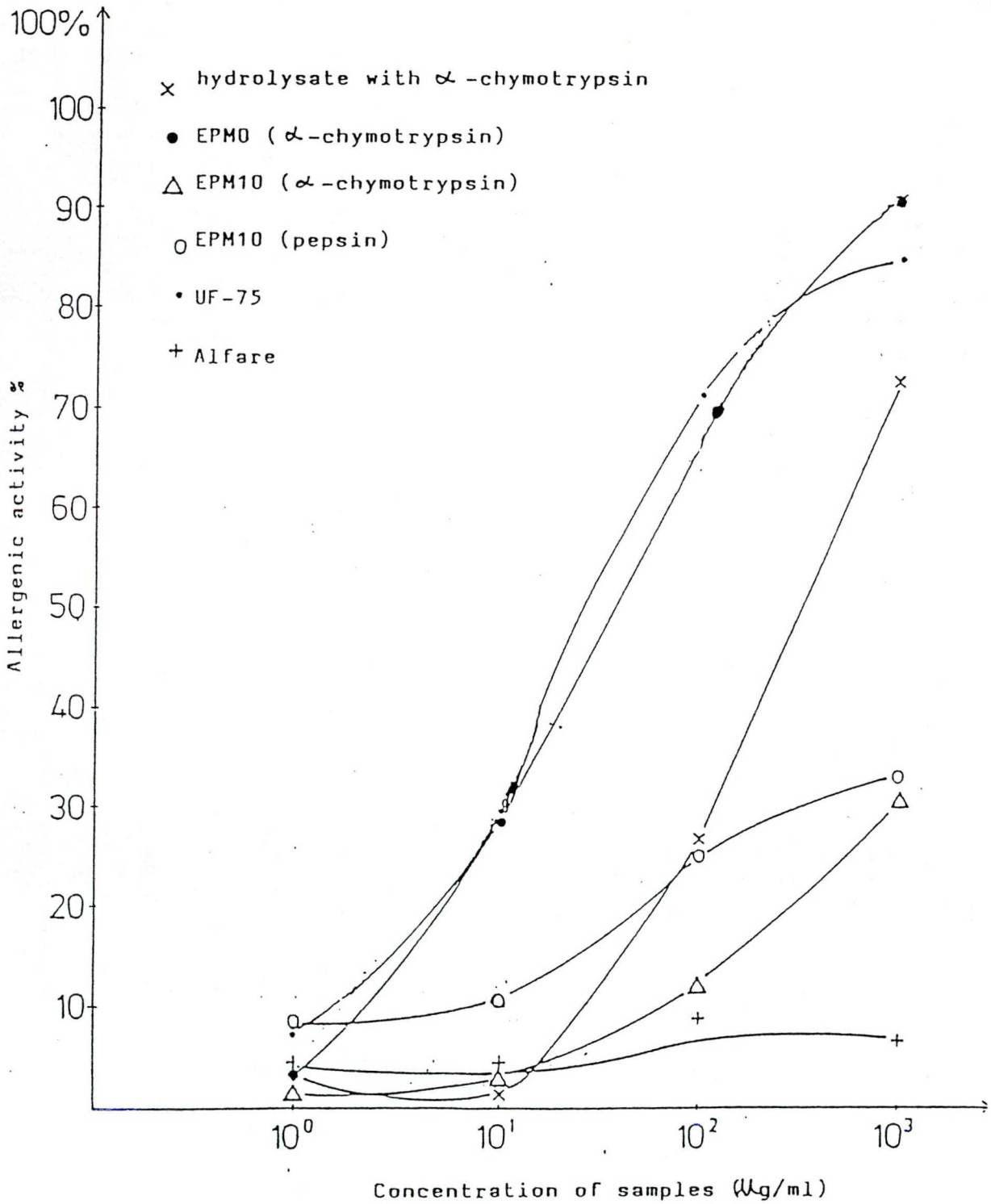
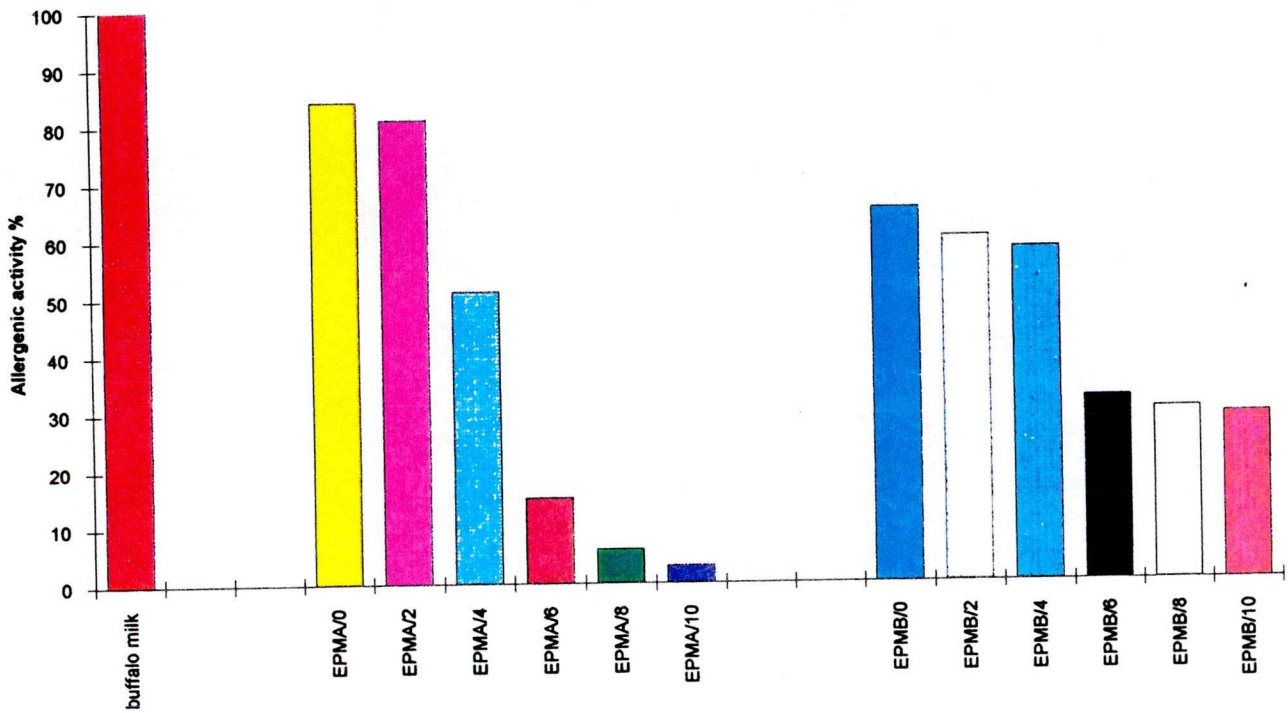


Fig. 17.: ***Allergenic character of the enzymatically modified buffalo's milk proteins products***



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 0: EPM products with different Met-enrichment (see Table 2.)

character decreased gradually until EPM4 from 89% EPM0 to 50% EPM4, and then decreased sharply until EPM10, 3%. The same Fig. 17. show that allergenic character of EPM products from buffalo's milk protein hydrolysate with pepsin and trypsin, pepsin was used as catalyste (EPMB). Allergenic character decreased from 64% EPM0 to 30% EPM10. The results showed also that the allergenic character of EPMA was lower than that from EPMB. The reduction of the allergenic character of modified buffalo's milk proteins is significantly improved 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 catalyst comparison with pepsin as catalyst.

The potential allergenic activity significantly dropped in case of buffalo's milk protein hydrolysates because of the cleavage of a great number of peptide bonds. The most essential decrease in the allergenic properties was measured in EPM products because of the modification of the structure of sequential and conformation determinants by transpeptidation and covalent incorporation of special amino acids.

This study was indicated that among EPM products from:

1. buffalo's milk protein hydrolysate with α–chymotrypsin and α–chymotrypsin used as catalyst

2. buffalo's milk protein hydrolysate with pepsin and pepsin used as catalyst

3. buffalo's milk protein hydrolysate with α–chymotrypsin and trypsin, and α–chymotrypsin used as catalyst.

4. and buffalo's milk protein hydrolysate with pepsin followed by trypsin, pepsin was used as catalyst were the most efficient

enzymes to reduce the allergenicity and improve the biological value of buffalo's milk proteins.

4.2 Enzymatic peptide modification of buffalo casein and cow casein

The main difference between cow and buffalo caseins is that the latter contains 2α_{S1}-casein fractions differing only by a phosphate group. Another difference lies in the relative proportion of α_{S1}-, α_{S2}, β-, and κ-caseins in the whole casein. In the buffalo, these proportions are approximately 30, 18, 34 and 15% as compared to 39, 8, 34 and 15%, respectively, in the cow (Addeo et al. 1977). The sum $\alpha_{S1}+\alpha_{S2}$ was approximately the same in both species as were the proportions of κ- and β-caseins, but that of αS_2-casein in buffalo whole casein was higher.

Addeo et al. (1977) found that cow and buffalo κ-casein were very similar in some respects. Fraction κ_1, the main buffalo κ-casein fraction, which represents approximately 40% of total κ-casein, was devoid of carbohydrates and it was thus similar to its bovine counterpart. However the κ_1 fraction in cow's milk represents only 25% of total κ-casein and this explains why the total carbohydrate content of whole buffalo κ-casein was lower than that of its bovine counterpart.

Addoe et al (1977) established that buffalo β-casein was similar to its bovine counterpart. The amino acid compositions of both proteins were very similar, but buffalo β-casein seemed to contain only 4 phosphate groups instead of 5 in the cow portein.

Two α_{S1}-casein fractions were present in buffalo whole casein. Their amino acid compositions, N- and C-terminal sequences were identical but their phosphate contents are 7 and 8 phosphate

groups/mol respectively. The amino acid compositions of buffalo and cow α_{S1} casein were similar.

Similarly, 2 α_{S2}-casein fractions exist in buffalo whole casein. Their amino acid compositions and N- and C-terminal end are identically, but they have 10 and 11 phosphate groups/mol, respectively. The amino composition and N- and C-terminal sequences of buffalo α_{S2}-casein clearly establish its homology with bovine α_{S2}-casein.

Buffalo's casein and cow's casein were submitted to hydrolysis with α–chymotrypsin, pepsin, α–chymotrypsin and trypsin, pepsin and trypsin or with α–chymotrypsin + trypsin followed by pepsin. Enzymatic peptide modification (EPM) was carried out using L-methionine ethyl ester in the reaction mixture and α–chymotrypsin or pepsin were the catalysts of the reactions.

EPM products were analyzed with SDS-PAGE, Iso electeric focusing, methionine content, C and N terminals and allergenic activity.

4.2.1 *Electrophoretic patterns of the EPM products of buffalo and cow caseins*

The electrophoretic patterns and the densitograms of protein fractions of the buffalo casein, buffalo casein hydrolysate with α–chymotrypsin and EPM products separated with SDS-PAGE are shown in Figs 18., 19., 20., 21. These results illustrate that buffalo's casein contain four zones ranged from 30000 to 80000 mol. mass. Hydrolysis of buffalo casein with α–chymotrypsin degradated the major casein fractions (30000 and 17000 mol. mass)

and produced four new fractions ranged from 14000 to 2500 mol. mass.

The principal protein zones in EPM products were similar in number and relative mobilities of the zones of casein hydrolysate.

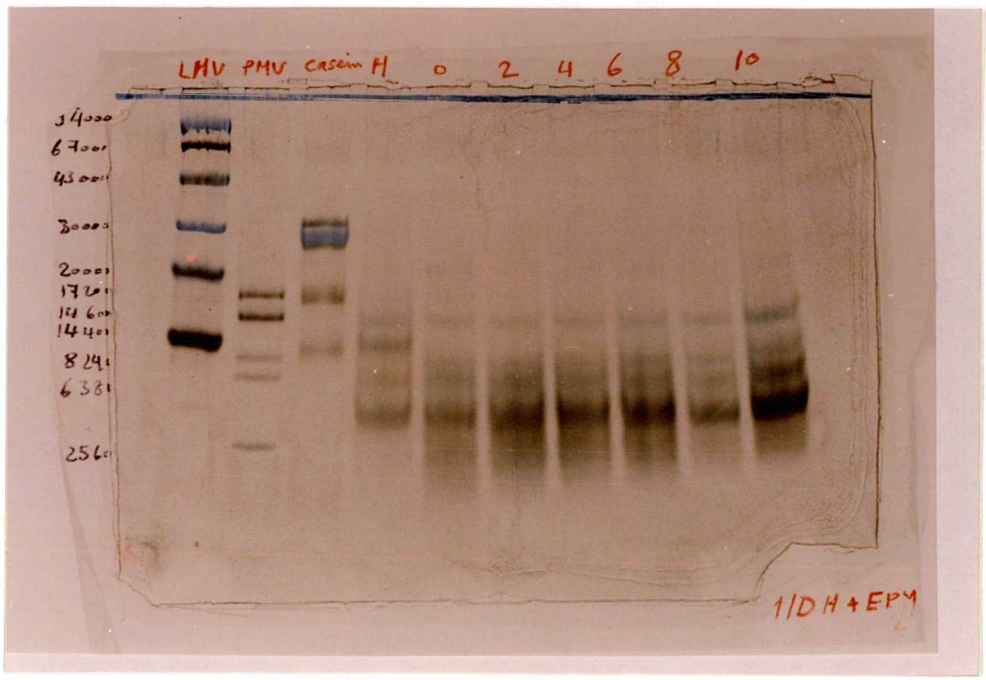
From electrophoretograms presented in Fig 18. and 20., it can be seen that the difference between EPMO and EPM4, where the intensity of fraction 6000 mol. mass increased. Fig 18. and 21. show that the differences between EPM6 and EPM10 showed the greates intensity on staining in the mol. mass about 6000. Generally, these changes of the molecular mass and intensity were essentially influenced by the concentration of L-methionine ethyl ester in the reaction mixture.

The electrophoretic patterns of protein fractions of the cow's milk protein, hydrolysate with α–chymotrypsin and EPM products separated by SDS-PAGE are shown in Fig. 22. The α–chymotrypsin caused a complete loss of α- and β-caseins. The substrate for enzymatic modification (Sample 4) showed 4 fractions at lowest intensity in the molecular range of about 17000 to as small as 2500 mol-mass. From Fig. 22., it can be seen that the molecular weights and intensity of EPM products were higher than those of the hydrolysate. It can also be seen that zones intensity of EPM products increased by increasing the concentration of L-methionine ethyl ester in the reaction mixture.

The changes in the zones indicate that transpeptidation was the major process in the enzymatic modification.

Fig. 23. and 24. show the effect of pepsin on buffalo casein and cow casein to hydrolysis and catalyst the modified protein products. There was no difference between both hydrolysates products with pepsin. The pepsin caused a complete loss of α- and β-caseins.

Fig. 18.1 ***SDS-PAGE patterns of the buffalo casein, its hydrolysate with α-chymotrypsin, and enzymatically modified products***



1 2 3 4 5 6 7 8 9 10

1	LMW
2	PMW
3	Buffalo casein
4	α-chymotryptic hydrolysate of the buffalo milk proteins
5	EPM 0 (EPM product without Met-entrichment)
6, 7, 8, 9, 10	EPM products with different Met-enrichment

Fig. 19.: ***Densitograms of protein fractions of the buffalo milk casein and buffalo casein hydrolysate with α-chymotrypsin separated by SDS-PAGE***

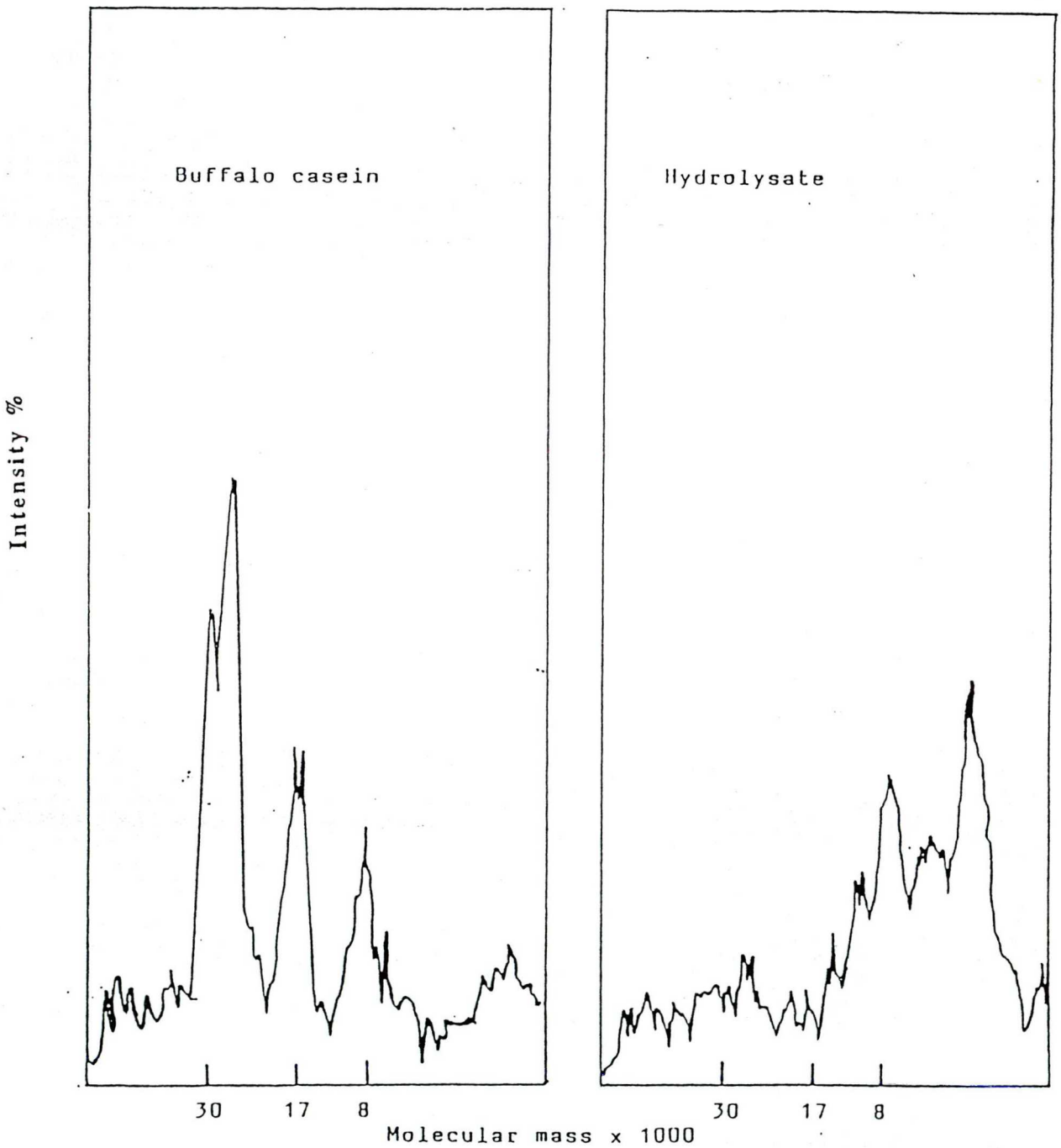


Fig. 20.: ***Densitograms of protein fractions of the modified buffalo casein (EPM 0 and EPM 4) separated by SDS-PAGE***

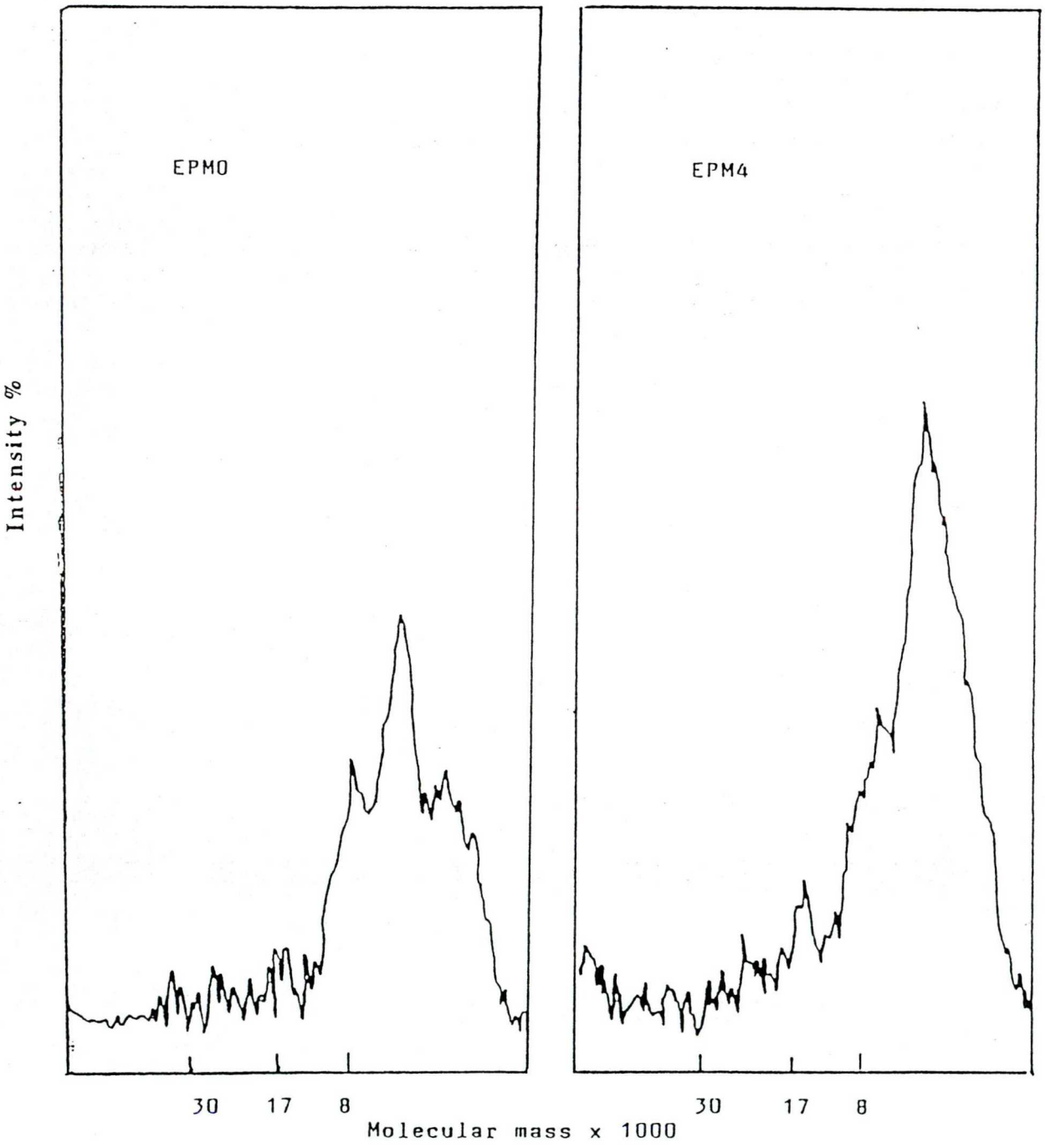


Fig. 21.: ***Densitograms of protein fractions of the modified buffalo casein (EPM 6 and EPM 10) separated by SDS-PAGE***

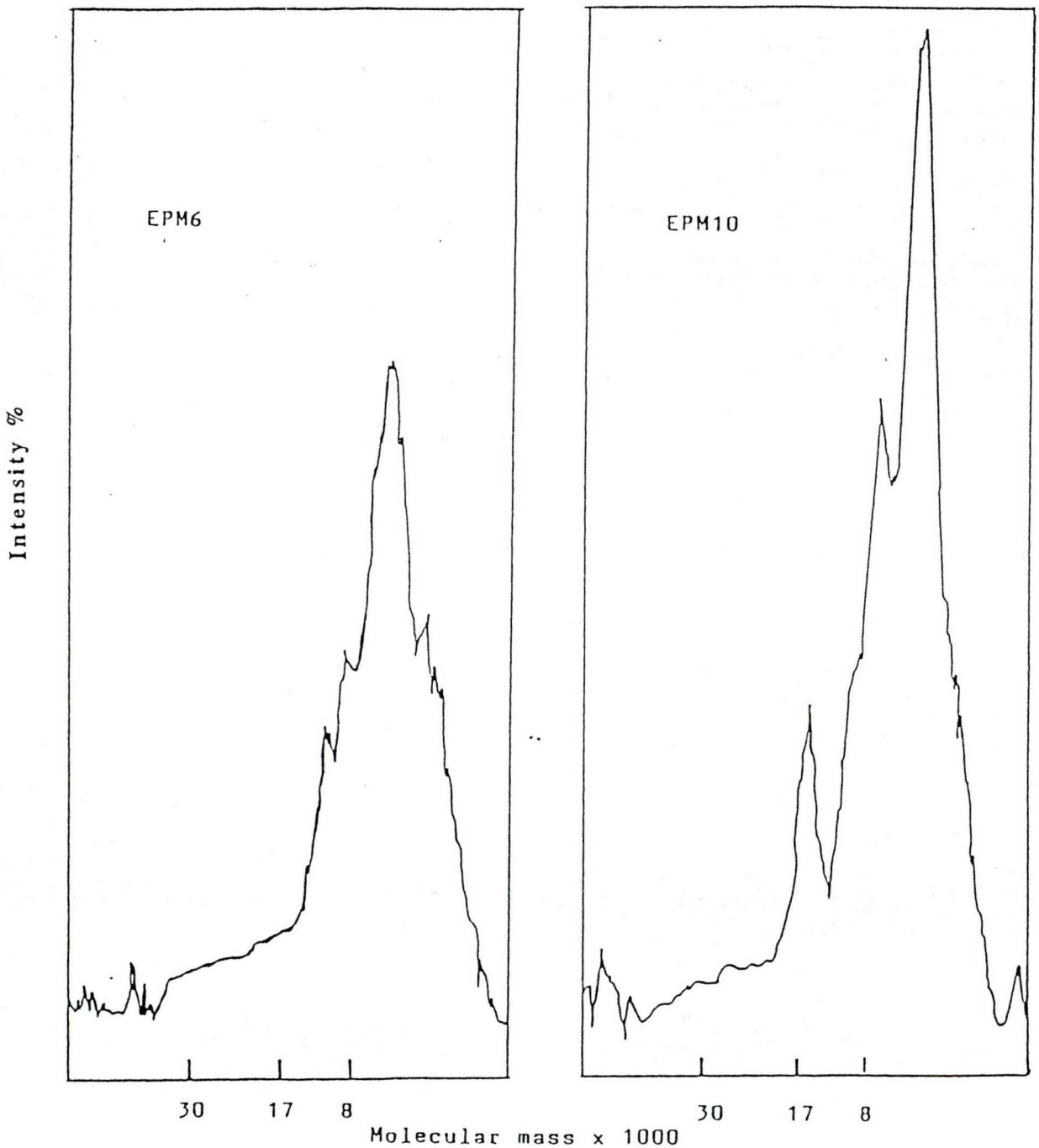
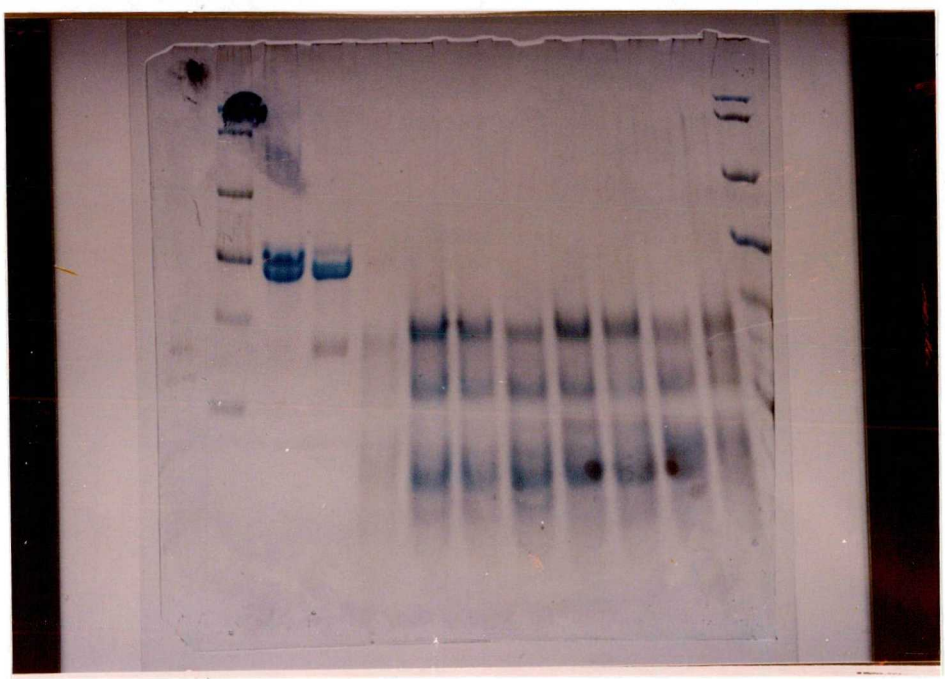
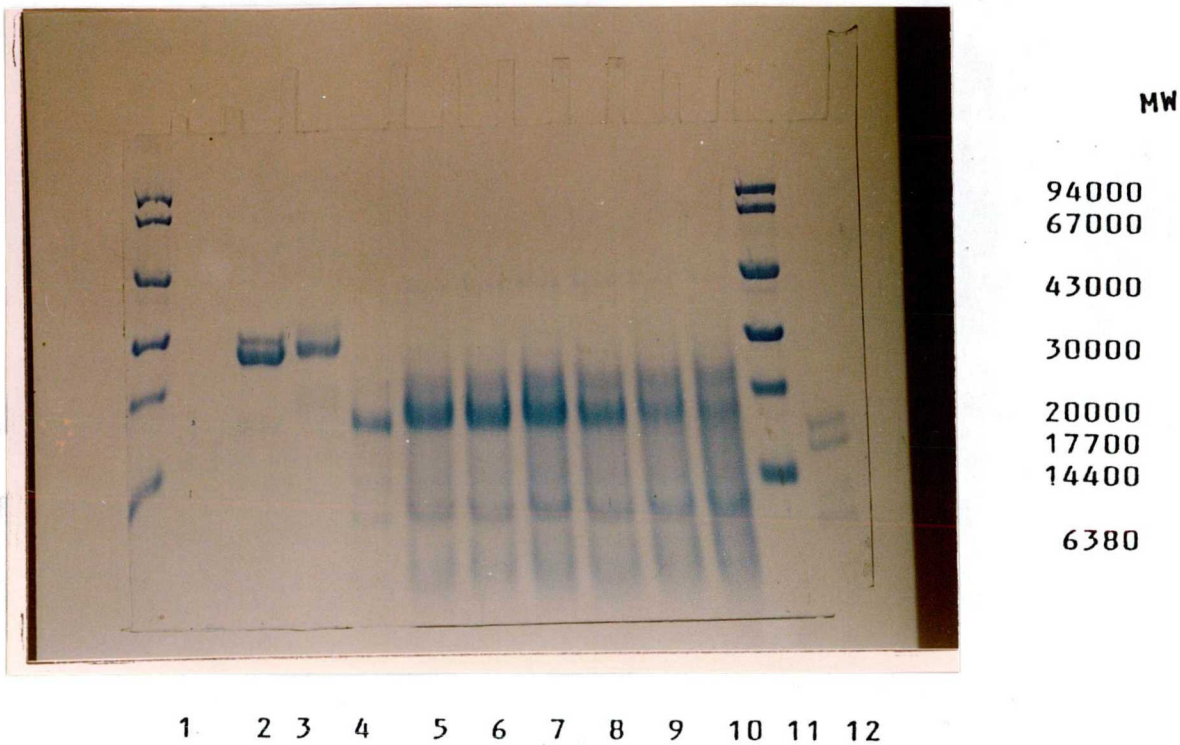


Fig. 22.: ***SDS-PAGE patterns of the cow casin, its hydrolysate with α-chymotrypsin and modified cow casein***



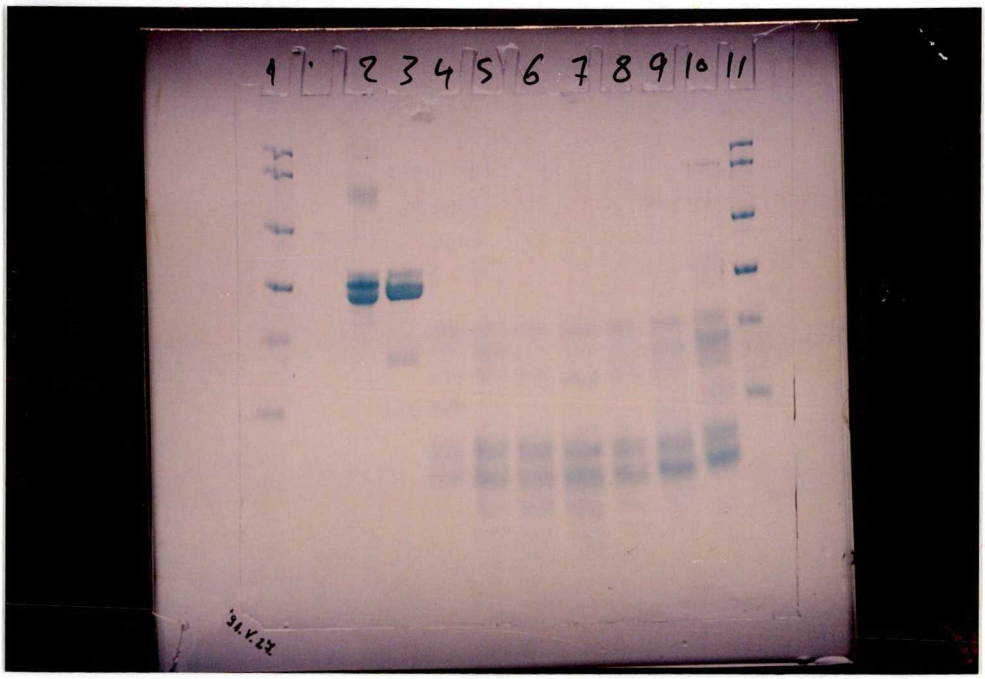
1, 11	LMW
2, 3	Cow casein
4	Cow casein hydrolysate with α-chymotrypsin
5	EPM 0 (EPM product without Met-enrichement)
6, 7, 8, 9, 10	EPM products with different Met-enrichment

Fig. 23.: ***SDS-PAGE patterns of the buffalo casein, its peptic hydrolysate and its modified EPM products***



1, 11	LMW
2, 3	Buffalo casein
4	Buffalo casein hydrolysate with pepsin
5	EPM 0
6, 7, 8, 9, 10	EPM products (produced in the presence of pepsin, as catalyst)
12	PMW

Fig. 24.: ***SDS-PAGE patterns of the cow casein, its peptic hydrolysate and its modified EPM products***



1, 11	LMW
2	Buffalo casein
3	Cow casein
4	Peptic hydrolysate of cow's casein
5	EPM 0 (EPM product without Met-enrichment)
6, 7, 8, 9, 10	EPM products with different Met-enrichment produced in the presence of pepsin, as catalyt

From Fig. 24., it can be seen that EPM0 hydrolysate during enzymatic process. Also can be seen that molecular weights of EPM products increased by increasing the concentration of methionin ethyl ester in the reaction mixture.

Fig. 24.show that the intensity of zones in the range of 6000-30000 mol. mass of EPM products gradualy increased by increasing the concentration of methionine ethyl ester in the reaction mixture.

4.2.2 *Degree of hydrolysis (DH)*

Hydrolysis and synthesis of peptide bonds as consequences of enzymatic peptide modification have been studied by determination of the degree of hydrolysis using the TNBS-method.

The degree of hydrolysis of buffalo's milk protein and casein hydrolysate with α–chymotrypsin and EPM products in the precense of methionine ethyl ester in the reaction mixture are shown in Table 6.

Table 6.:. *Determination of degree of hydrolysis (DH)*

Sample	Buffalo's milk protein		Casein	
	hydrolysate	EPM	hydrolysate	EPM
DH	11.2	11.8	16.7	11.2

The degree of hydrolysis of casein hydrolysate was about 1.5 times higher than the degree of hydrolysis of buffalo's milk proteins hydrolysate at the same conditions. This may be due to the effect of α–chymotrypsin. The effect on pure casein was higher than the effect on whole buffalo proteins (casein and whey protein). In the case of EPM producte from buffalo proteins hydrolysate the degree of hydrolysis increased from 11.2% (hydrolysate) to 11.8% (EPM producte). This may be due to bond cleavage occured during enzymatic process. Lozano and Combes (1992) concluded that in the plastein reaction catalyzed by α–chymotrypsin, all possible biocatalytic pathway: hydrolysis, synthetic-transpeptidation, and synthetic-condensation could occur simultaneously.

In the case of EPM producte from casein hydrolysate, the degree of hydrolysis decreased from 16.7% for casein hydrolysate to 11.2% for EPM producte. These due to the lose of low molecular weight peptide during dialysis and were not able to dye by protein staining low molecular weight peptides The determination of the degree of hydrolysis revealed that transpeptidation was the major process during enyzmatic modification. This was supported by the lack of change in the average molecular mass of peptide.

4.2.3 *Incorporation of L-methionine into buffalo and cow casein hydrolysates by enzymatic modification*

Table 7. shows the methionine content of buffalo and cow caseins hydrolysates and EPM products when α–chymotrypsin used as catalyste. This comparison shows methionine content in the EPM products with methionine incorporation were found to be higher than that of the substrate protein. The results showed that

methionine content of EPM products from cow casein is higher than that of the EPM products from buffalo casein.

Table 7.: ***Methionine content of the modified buffalo's and cow's casein, α–chymotrypsin used as catalyst***

Samples	Methionine covalently bound (g Met/100g protein)	
	buffalo casein	cow casein
EPM 0	1.3	1.6
EPM 2	2.8	4.3
EPM 4	2.1	3.0
EPM 6	1.9	3.45
EPM 8	1.6	3.75
EPM 10	1.3	2.7

The optimum of L-methionine incorporation in the case of cow casein and buffalo casein (EPM2) was found at a ratio of 14 g Met/100g hydrolysate in this enzymatic process. Methionine content of EPM2 in the case of cow casein is greater by 2.7% than that EPM0 but in the case of buffalo casein is greater by 1.5%. Also methionine content of EPM2 in the case of cow casein is greater by 1.5% than that of EPM2 in the case of buffalo casein.

Methionine content of buffalo and cow caseins hydrolysates and EPM products catalyst with pepsin are shown in Table 8. The optimum of L-methionine incorporation when pepsin used as catalyst in the case of cow casein (EPM4) was found at a ratio of 34

g L.Met/100g hydrolysate but in the case of buffalo casein (EPM2) at a ratio of 14 g L.Met/100g hydrolysate was found .

The results revaled that the amount of methionine incorporated changed with the type of the casein and enzyme applied.

Table 8.: Methionine content of the modified buffalo and cow casein, pepsin used as catalyst

EPM	Methionine covalently bound (g Met/100g protein)	
	buffalo casein	cow casein
0	1.5	1.4
2	2.6	2.5
4	2.2	3.0
6	1.8	1.9
8	1.6	1.7
10	1.3	1.7

In the case of buffalo casein, the bigger amount of methionine ethyl ester was added to the reaction mixture, the less amount of covalently bound methionine was found in the products. A possible interpretation of this surprising contradiction is that the changes in the ratio between methionine ethyl ester and protein hydrolysate influence the degree of the amino acid incorporation and thereby the primary structure of the products and, consequently, the functionality of the proteins.

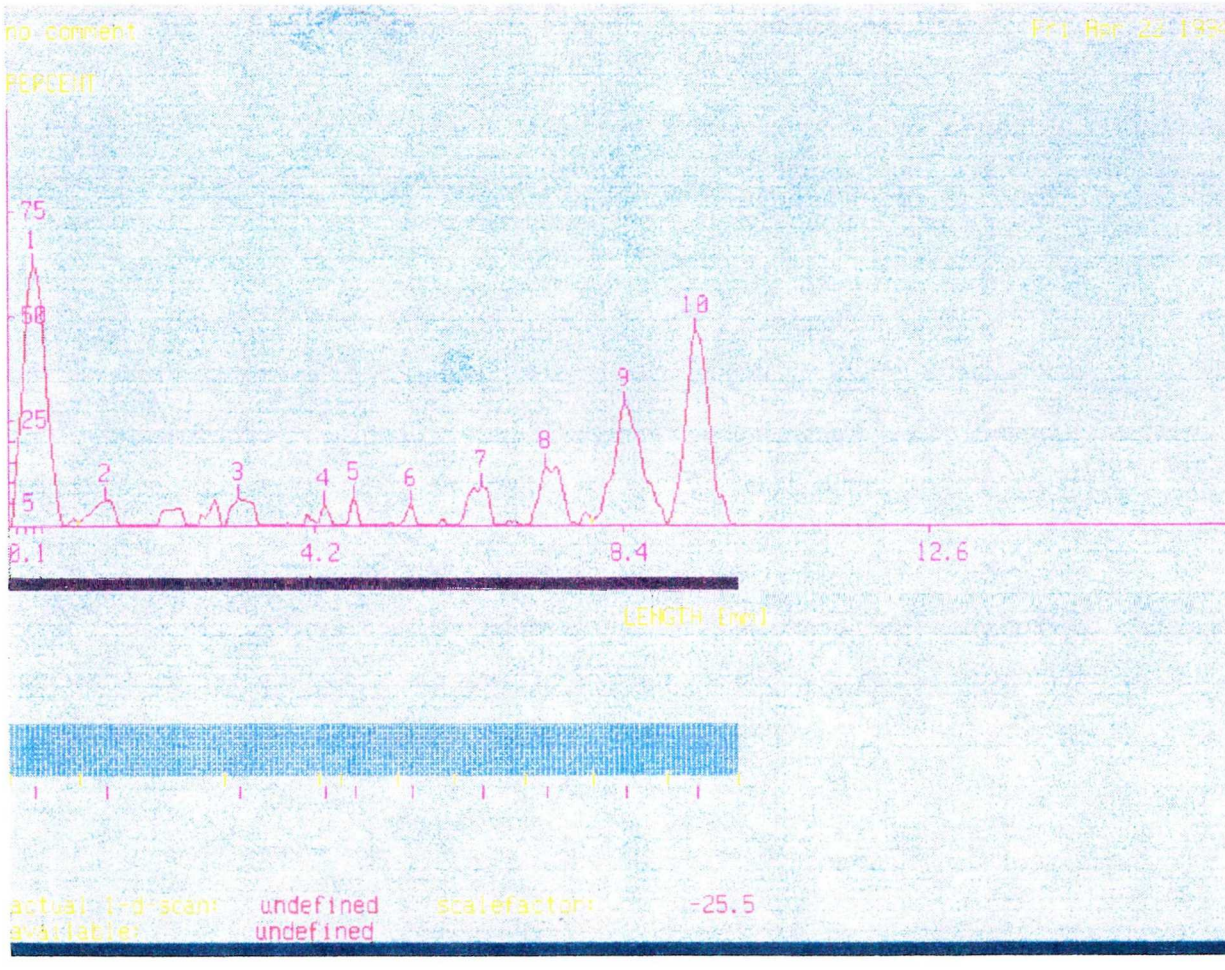
Generally, when α–chymotrypsin was used as catalyst the amount of methionine incorporated is higher compared with pepsin. The results also revealed that the amount of methionine incorporated in the case of cow casein is higher than in the case of buffalo casein.

The effects of exopeptidases on buffalo casein hydrolysate and EPM product are shown in Fig. 25. In samples taken at zero time, where HCl was added together with exopeptidase to prevent digestion, no free amino acid was present. In Fig. 25. it can be seen that methionine was in the EPM product, while it was absent in the buffalo casein hydrolysate.

In the case of EPM product, samples obtained after 10 min. to 60 min., increasing amount of L-methionine could be detected as a results of exopeptidase action. Fig. 25. and Table 9. show that the amount of free L-methionine after 60 min. was five times the amount of free L-methionine after 10 min. As methionine is preferently bound to the C-terminal end of the peptide carboxypeptidase could be an effecient catalyzer for amino acid incorporation by enzymatic process. On the other hand, with LAP treatment no significant amount of methionine existed at N-terminals of the EPM molecules.

These results suggest that enzymatic peptide modification is a suitable method for increasing the nutritive value of buffalo and cow caseins by the incorporation of a given quantity of a paticular limiting essential amino acid.

Fig. 25.: ***L-Methionine hydrolysed by carboxypeptidase (A and B)***



α-chymotryptic hydrolysate of buffalo casein and EPM product treated with carboxypeptidase (A+B), invesitgated after incubating for 0, 10, 30 and 60 minutes, respecitvely.

Hydrolysate: 2, 3, 4, 5 samples obtained after 0, 10, 30 and 60 minutes respectively

EPM product: samples 6, 7, 8, 9 obtained after 0, 10, 30 and 60 minutes respectively

1, 10 control Met

Table 9.: ***Samy-quantitative evaluation of methionine-content of the samples separated by thin layer ion exchange chromatography***

number	position	area (pixel)	height (%)	% area (%)	absolute val.
1	0.3044	3917	100.0	36.5	0.00
2	3.3489	150	6.3	1.4	0.00
3	4.0515	170	7.8	1.6	0.00
4	5.4098	140	6.3	1.3	0.00
5	6.4637	186	11.8	1.7	0.00
6	7.0023	318	11.8	3.0	0.00
7	7.4707	692	18.8	6.5	0.00
8	8.4309	1794	39.2	16.7	0.00
9	9.4614	3358	76.1	31.3	0.00
		10725		100.0	0.00

Buffalo casein hydrolysate and EPM product treated with carboxypeptidase (A+B), investigated after incubating for 0, 10, 30 and 60 minutes, respectively.

1	control
2, 3, 4, 5	samples obtained after 0, 10, 30 and 60 minutes, respectively
6, 7, 8, 9	samples obtained after 0, 10, 30 and 60 minutes, respectively

4.2.4 *Separation of a methionine-enriched fraction from the enzymatically modified cow casein by fast protein liquid chromatography*

The applied analytical and preparative methods were carried out according to Juillerat and co-workers (1989) for phosphopeptides from whole casein by fast protein liquid chromatography using MONO Q strongly basic anion-exchange resin both for analytical and preparative separations. The tryptic peptides were dissolved in Tris buffer (20 mmol, pH 8.0) and eluted by linear salt gradient.

In case of α-chymotryptic hydrolysate of casein and its methione-enriched product segmented gradient (Haanoot et al., 1986, Barrefors et al., 1985) was applied on the MONO Q HR 5/5 column. The preparative separation was performed on Q-Sepharose resin at low pressure and fast flow rate.

Figures 26. a, b, and c show the chromatograms of the hydrolysate and the modified products on MONO Q HR 5/5 anion-exchanger at different pH values: pH 8.0, pH 8.5, pH 8.8, respectively. The difference between the chromatograms of the hydrolysate and the EPM-product is better revealed at higher pH value (Fig. 26c).

On the Q-Sepharose HR 10/30 preparative column the modified product was separated with the same gradient, which was developed on MONO Q. By this way the EPM-product was separated to seven fractions (Fig. 27). The incorporated methionine content of fractions was investigated after the digestion with exopeptidases to compare with that of hydrolysate. The second fraction which was

eluted at the start of salt gradient, contains remarkably high percent of methionine bound at the end of the peptide chains.

The densitometric evaluation shows (Fig. 28, Table 10.) that the methionine content of the EPM-product was three times higher than that of the hydrolysate.

Table 10.: ***Quantitative evaluation of methionine-content of the samples separated by cation exchange thin-layer chromatography***

No.	Position (mm)	Area	Area (%)
1	7.3394	810	4.5
2.	20.9174	2292	12.8
3	36.6972	2640	15.8
4	53.5780	2126	11.9
5	69.3578	1569	8.8
6	85.5046	49	0.3
7	100.5505	3612	20.2
8	115.9633	1225	6.8
9	133.5780	735	4.1
10	148.9908	746	4.2
11	159.2661	420	2.3
12	179.0826	99	0.6
13	193.0275	1567	8.8
		17.890	100.0

The anion-exchange separation was suitable for the analytical separation of α-chymotryptic casein hydrolysate and the methionine-enriched EPM-product.

This fact can be explained, that the covalently incorporated methionine modifies the net charge of certain molecules in the

Fig. 26.a.: *Separation of the α-chymotryptic casein hydrolisate (A) and the methionine-enriched EPM-product (B) on MONO Q resin, column HR 5/5, AU 0.01, pH8.0*

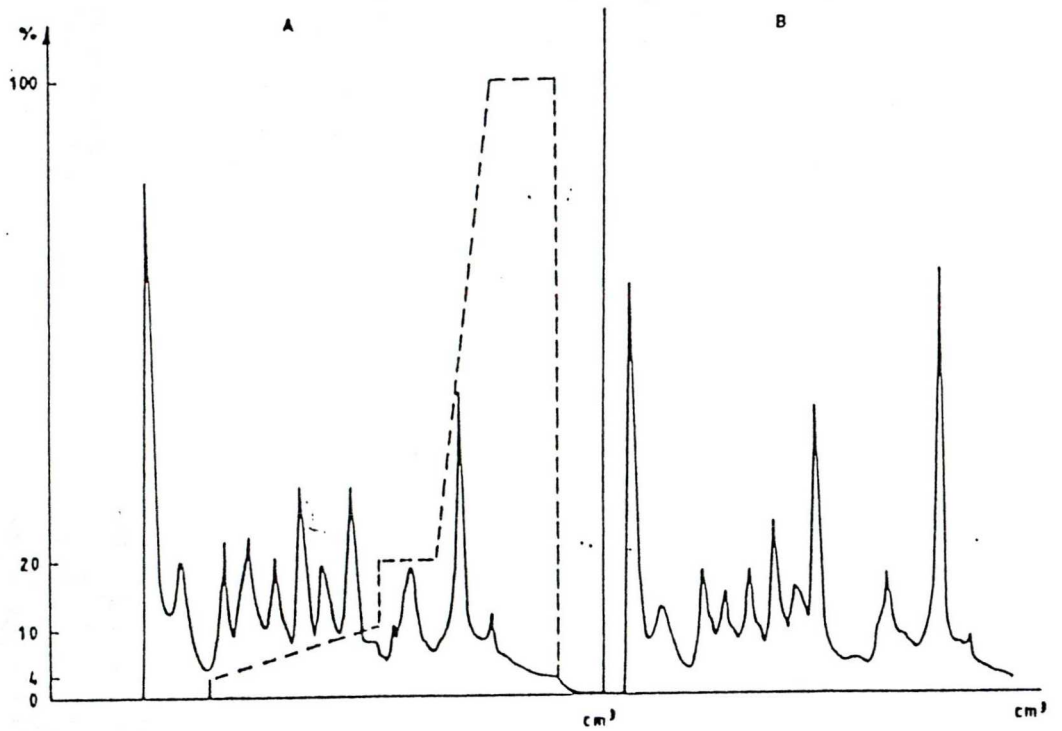


Fig. 26.b.: *Separation of the α-chymotryptic hydrolysate (A) and the methionine-enriched EPM-product (B) on MONO Q resin, column HR 5/5, AU 0.01, pH 8.5*

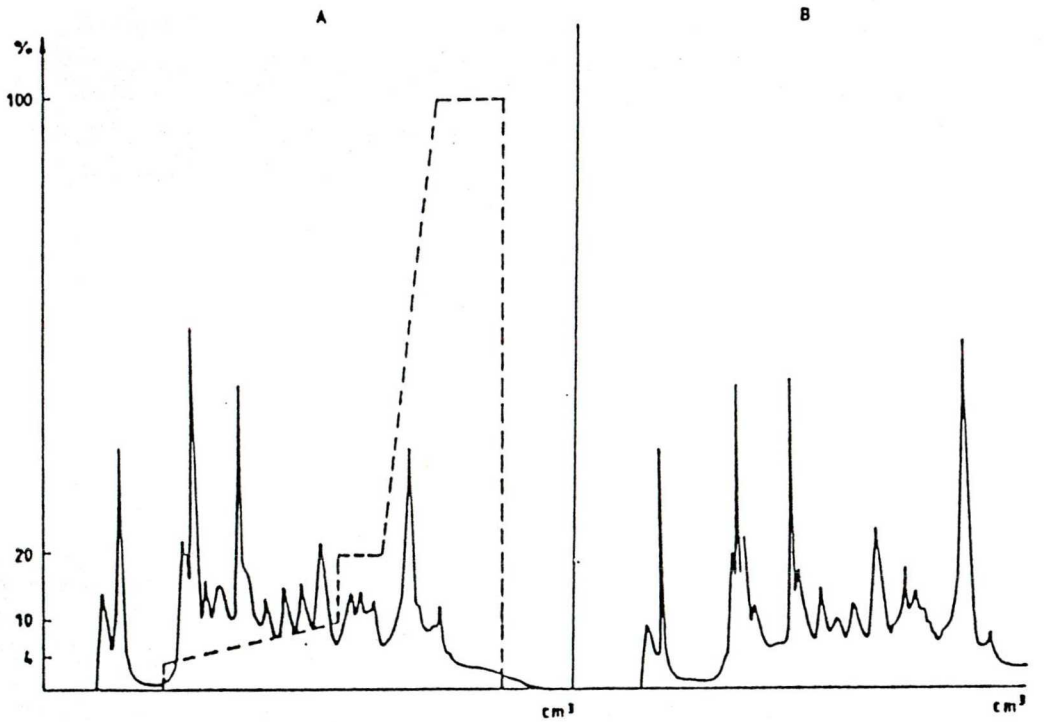


Fig. 26.c.: *Separation of the α-chymotryptic hydrolysate (A) and the methionine-enriched EPM-product (B) on MONO Q resin, column HR 5/5, AU 0.01, pH 8.5*

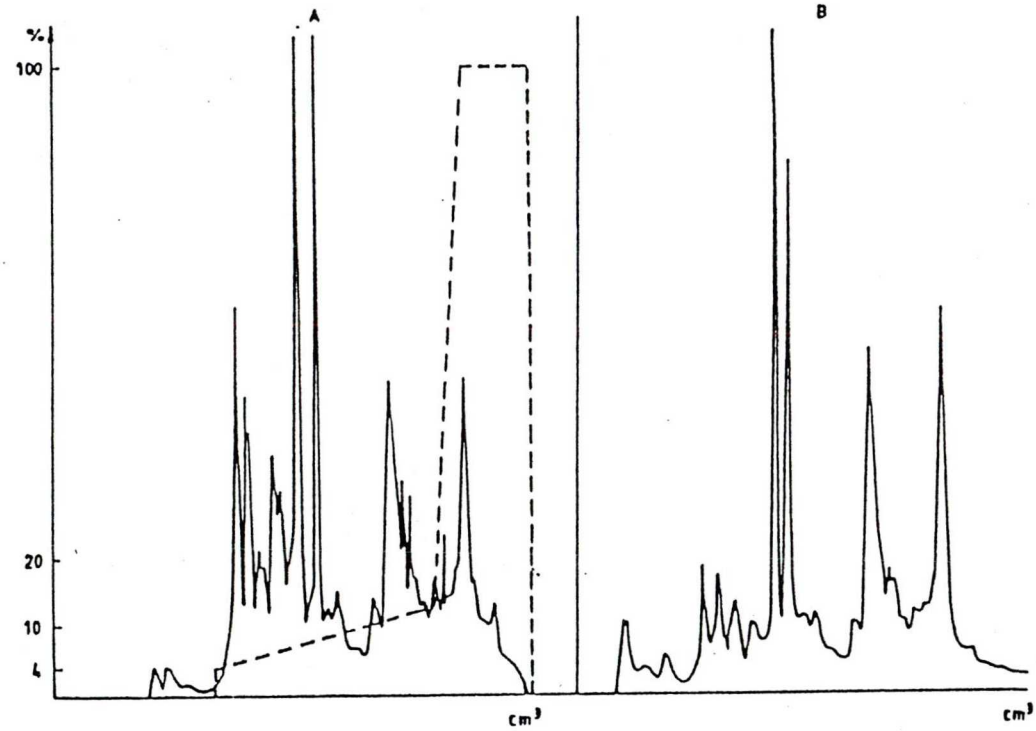


Fig. 27.: ***Separation of the methionine-enriched EPM-product on Q Sepharose resin, column HR 10/30, AU 2.0, pH 8.0***

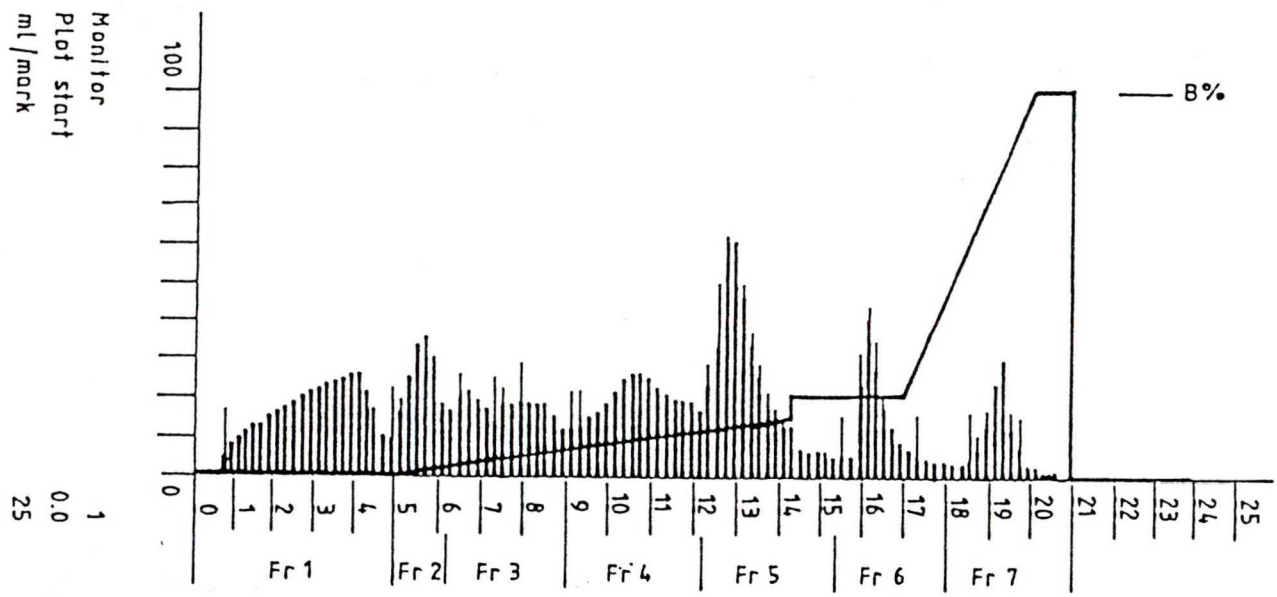
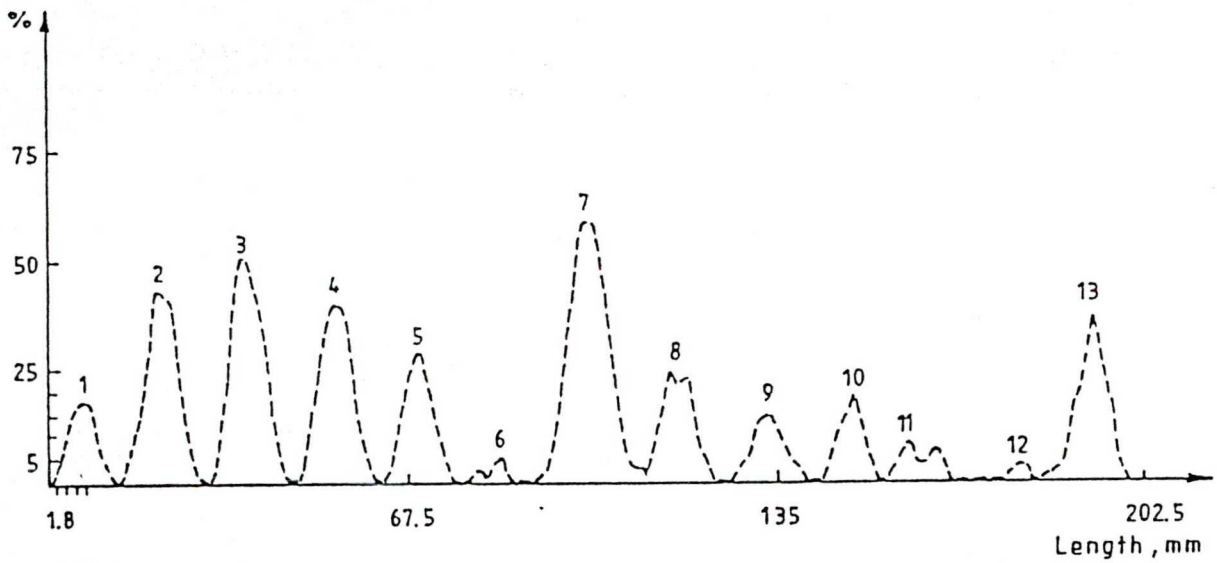


Fig. 28.: *Densitometric evaluation of the methionine spots on the chromatoplate. 1 and 13: amino acid standard, 2: methionine, 3-6: samples from reaction mixture (2nd fraction), 7: methionine, 8-11: samples from reaction mixture (hydrolysate), 12: methionine*



peptide mixture and that modifies their binding properties to the ion-exchange matrix.

The preparative separation of the methionine-enirched EPM-product resulted in a fraction of high percent of covalenly bound methionine.

By the use of ion exchange columns Mono Q and Q-Sepharose developed for FPLC it was possible to find a suitable separation method for a proteolysate and a methionine-enriched EPM product of casein.

4.2.5 *Methionine-enriched peptides in EPM products of buffalo casein*

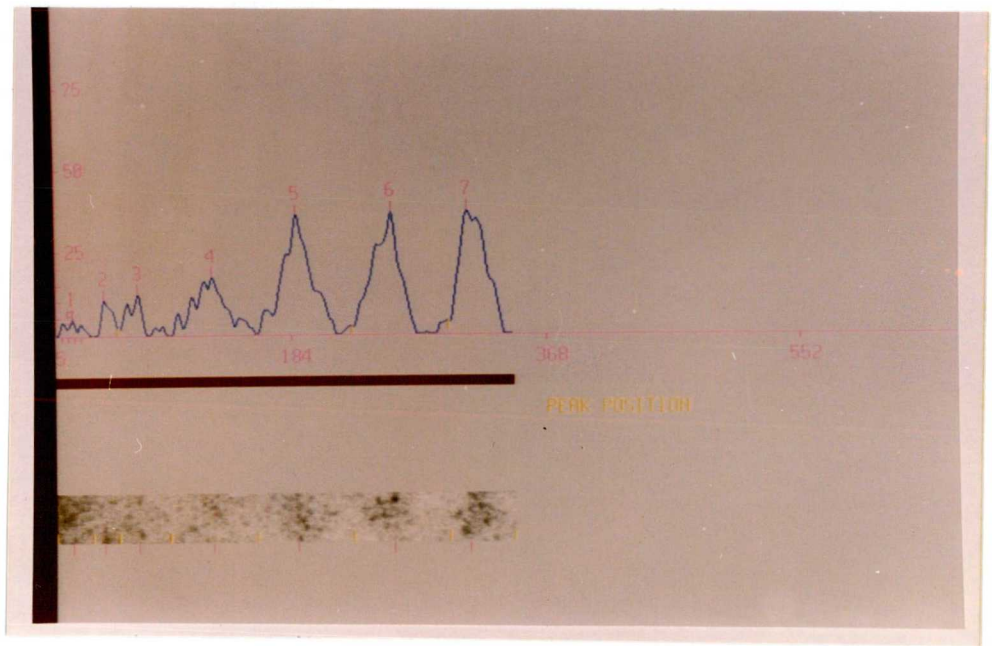
The incorporated methionine content of some fractions obtained by separation of hydrolysate and EPM-product was investigated by Sephadex isoelectric focusing (IEF). The hydrolysate was made of buffalo casein. The catalyst of the hydrolysis was α–chymotrypsin. The same enzyme for the EPM reaction was used. Then protein fractions according to their isoelectric points by isoelectric focusing on a 0,7 mm thick Sephadex (IEF) thin layer were separated. After the separation three new zones appeared in the IEF pattern of the EPM product compared to the IEF pattern of the appropriate hydrolysate. The new zones were cut out from the paper print and the protein content of these three fractions was investigated after eluting with distilled water and freeze drying .

The incorporated methionine content of the fractions was investigated after hydrolysis with 6M HCl and the amino acids were subjected to thin layer ion exchange chromatography.

Fig. 29. shows that the methionine content of the fractions obtained from EPM product had remarkably higher methionine content comparison with that of the hydrolysate fractions.

The above mentioned results prove that all three new IEF-zones of the EPM product (that didn't appear in the IEF pattern of the hydrolysate) contain peptides that have covalently incorporated methionine.

Fig. 29.: ***Methionine content of two fractions from buffalo casein hydrolysate with α-chymotrypsin and three fractions from methionine-enriched buffalo casein separated by Sephadex IEF***



2, 3, 4 Met-content of fractions obtained from buffalo casein hydrolysate with α-chymotrypsin

5, 6, 7 Met-content of fractions obtained from Met-enrichment buffalo casein

4.2.6 *Two-dimensional analysis of EPM proteins using preparative isoelectric focusing followed by SDS-PAGE*

SDS-PAGE for modified buffalo's milk proteins separated from preparative IEF are shown in Fig.30. Most proteins were focused within pH 4.0-5.0. No pretreatment of the EPM sample was necesarry. The EPM product contained five fractions (A, B, C, D, E) ranged from 20000 to 2500 mol. mass.

Fig. 30. shows well-resolved typical bands of EPM fractions on SDS-PAGE, they were identified with both LMW and PMW standard. This gel was used to verify the apparent molecular weights of EPM sample. Most of A (20000 mol. mass) fraction were concentrated in the fractions 6, 7, 8, B (14400 mol. mass) fraction was in fractions 6, 7, 8, and the others were mostly in fractions 1 to 5.

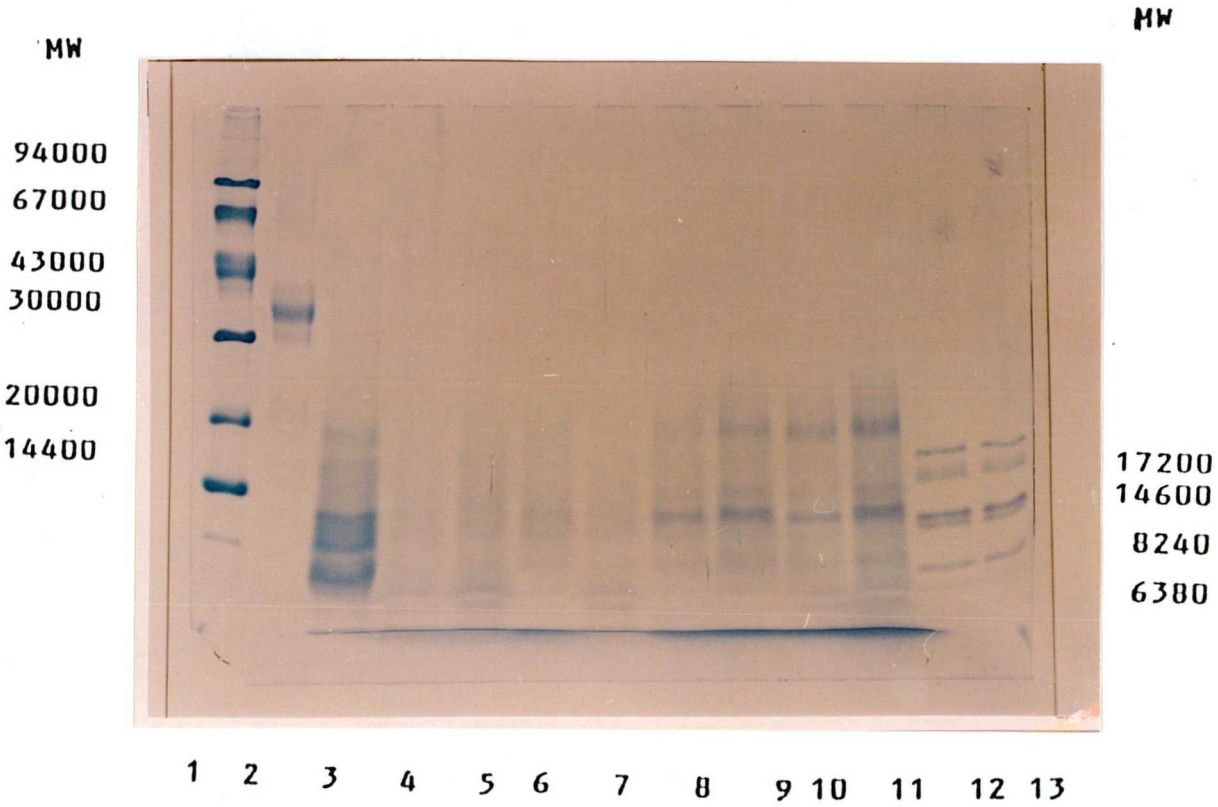
Preparative IEF followed by SDS-PAGE was found to be very useful for the analysis of all the proteins in EPM sample.

4.2.7 *Reduction of the allergenicity of buffalo casein and cow casein by enzymatic modification*

Allergenic character of cow's and buffalo's milk casein, and EPM modified casein, were investigated. Allergenic character of the Met-enriched, EPM-modified buffalo casein samples were compared with that of the untreated casein and buffalo casein hydrolysate with α–chymotrypsin (Fig 31.).

The samples of this EPM series contained different methionine concentration (in the form of Met ethyl ester) in the reaction mixture. Fig 31. shows that the reduction of the allergenic character of the modified buffalo casein is significantly improved by the

Fig. 30.1 ***SDS-PAGE for modified protein fractions (EPM) separated from preparative IEF***



1	LMW
2	Buffalo milk protein
3	EPM (Modified buffalo's milk proteins of with Met-enrichment)
4-11	Fractions are obtained from EPM with Met-enrichment
12, 13	PMW

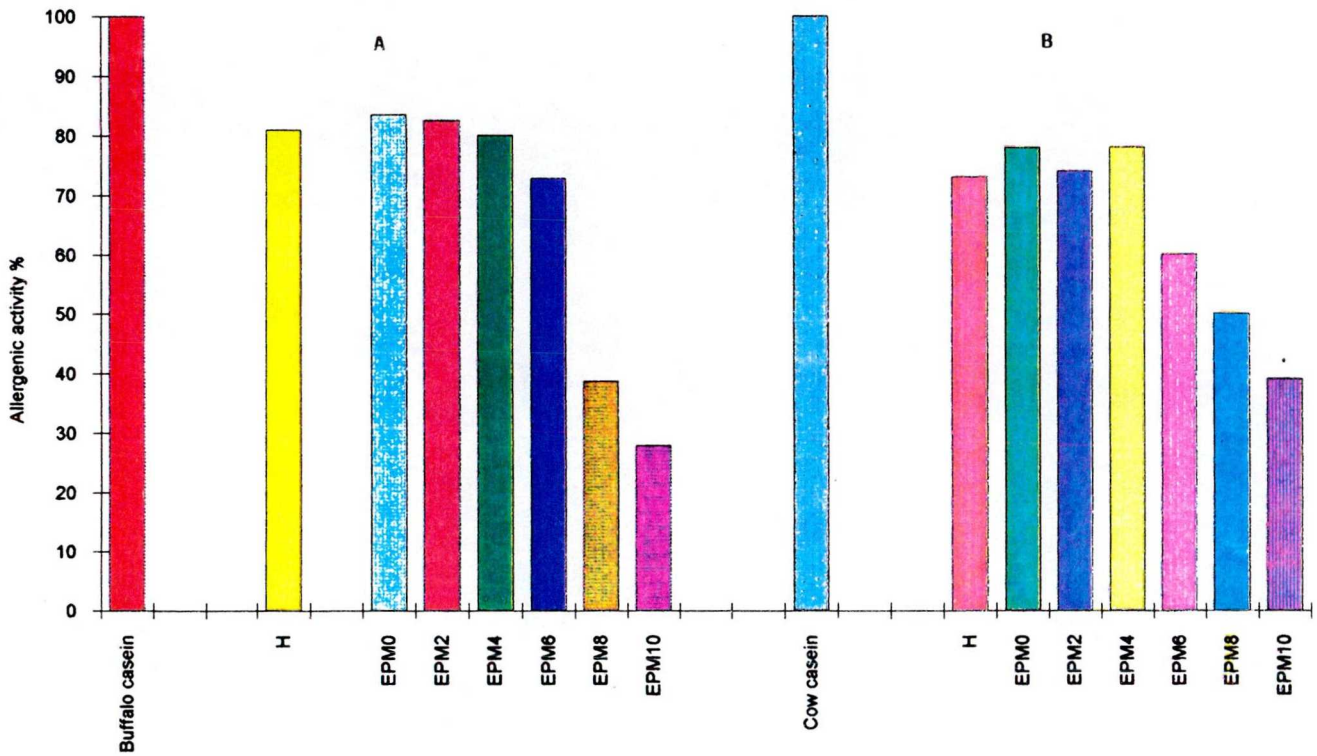
increase of the concentration of Met ester in the reaction mixtures. The hydrolysis of buffalo casein with α–chymotrypsin eliminated about 15% of total allergenic activity. There were little differences between casein hydrolysate and the sample EPM0 (without methionine), and samples of EPM2, 4, 6 where the Met concentration was(82.3%, 80%, 72.8%) in the reaction mixtures respectively. The biggest difference, however between was the samples EPM8, EPM10 and the casein hydrolysate.

The allergenic character of cow's milk casein, cow's milk casein hydrolysate, its EPM products with and without methionine enrichment were also investigated (Fig. 31.). Little differences between both cow casein and buffalo casein hydrolysates with α–chymotrypsin, and revealed the same trend of decrease in allergenic activity. The decreased allergenic activity of EPM products may be due to transpeptidation processes in the course of EPM.

Fig. 32. shows the allergenic activity of cow casein and buffalo casein hydrolysates with pepsin and EPM products in the presence of different methionine ethyl ester concentration in the reaction mixture. The results showed that both hydrolysates were characterized by low allergenic activity, but cow casein hydrolysate was very low comparison with buffalo casein hydrolysate, 30% and 16%, respectively. The results showed also that the allergenic activity of both EPM products were very low and was not affected by the concentration of methionine ethyl ester in the reaction mixture.

Allergenic character of buffalo casein, buffalo casein hydrolysate with α–chymotrypsin and trypsin were followed by pepsin and EPM products when α–chymotrypsin (EPMA), trypsin (EPMB) or pepsin (EPMC) were used as catalysts in the presence of

Fig. 31.: ***Allergenic character of the enzymatically modified buffalo and cow casein products***



A: α-chymotryptic hydrolysate of buffalo casein and EPM products in the presence of α-chymotrypsin as catalyst

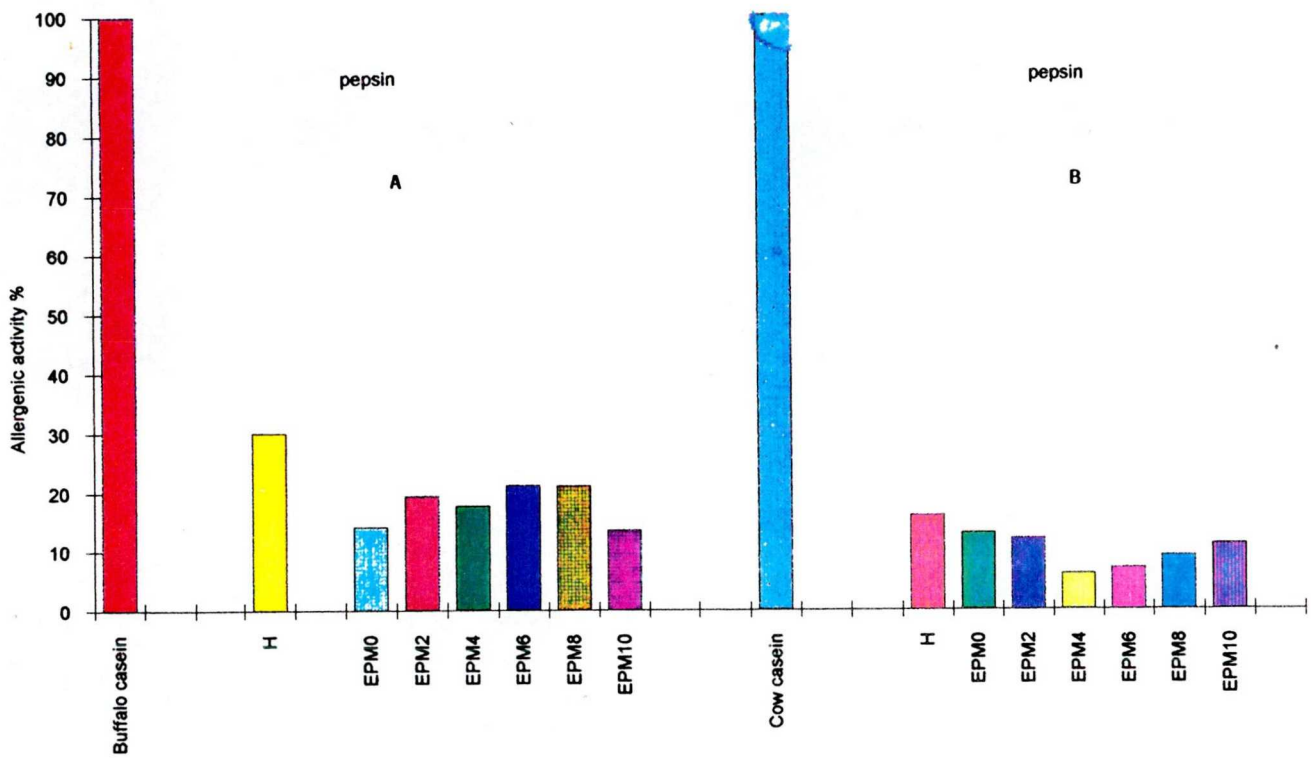
B: α-chymotryptic hydrolysate of cow casein and EPM products in the presence of α-chymotrypsin as catalyst

EPM O: EPM product without Met-enrichment

EPM 2-EPM 10: EPM products with Met-enrichment

(see Table 2.)

Fig. 32.: ***Allergenic character of the enzymatically modified buffalo and cow caseins products***



A: peptic hydrolysate of buffalo casein and EPM products in the presence of pepsin as catalyst

B: peptic hydrolysate of cow casein and EPM products in the presence of pepsin as catalyst

EPM O: EPM product without Met-enrichment

EPM 2-EPM 10: EPM products with Met-enrichment

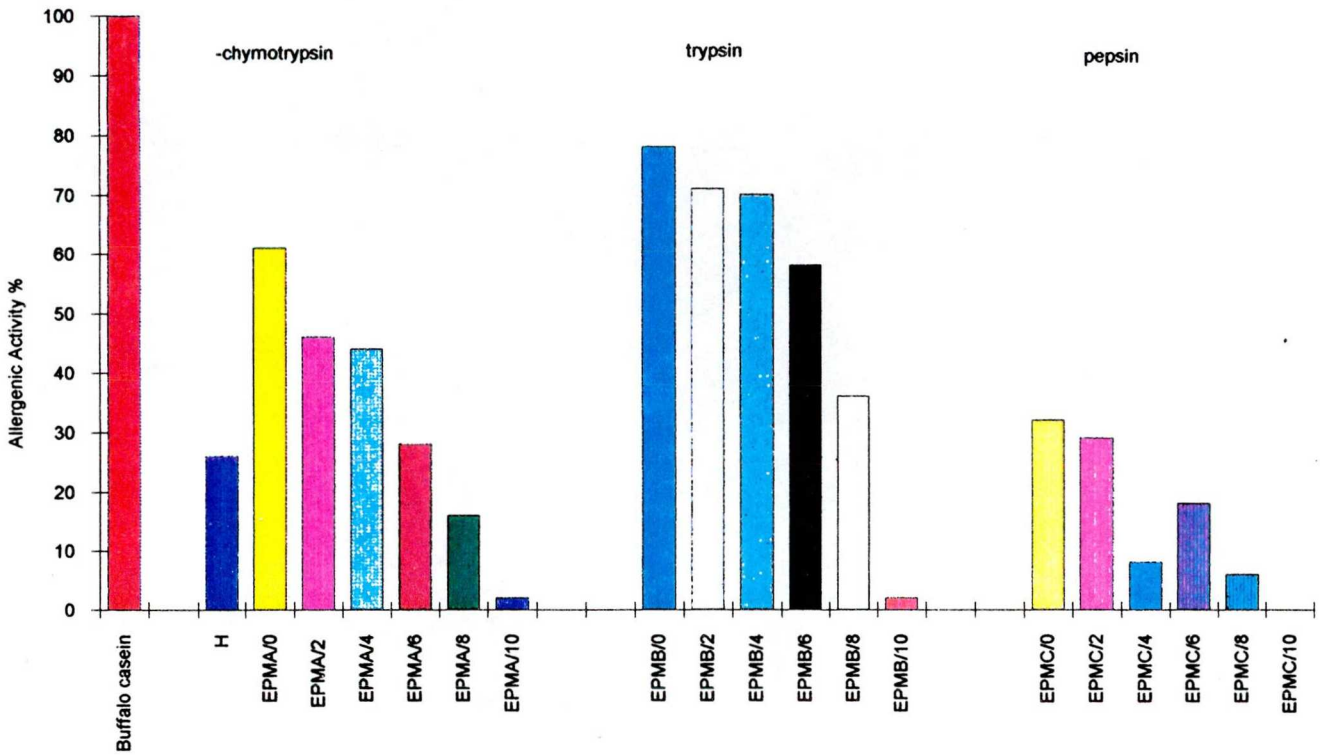
(see Table 2.)

different concentration of methionine ethyl ester in the reaction mixtures are shown in Fig. 33.

Fig. 33. show that the allergenicity of hydrolysate was significantly reduced to 26%. It also can be seen that the reduction of the allergenicity of the modified buffalo casein hydrolysate with three enzymes, α–chymotrypsin catalyst is significantly improved by the increase of the concentration of methionine ethyl ester in the reaction mixture. All EPM products in the presence of methionine ethyl ester exhibited low allergenic character, below 50%. Allergenic character decreased from 61% (EPMO) to 2% (EPM10). The ratio of reduction the allergenicity in the case of EPMA is very high compared with allergenicity of buffalo casein hydrolysate with α–chymotrypsin as catalyst (see Fig. 31.), these may be due to the molecular mass of EPM products. Fig.33. show that EPMB, where trypsin was used as catalyst only EPM8 and EPM10 appeared low allergenic activity, 36% and 2%, respectively. It also can be seen that EPMC, when pepsin was used as catalyst, all EPM products showed very low allergenic activity compared with EPMA, and were not effected by the concentration of methionine ethyl ester in the reaction mixture. Generally, buffalo casein and cow casein when hydrolyzed with pepsin alone, or with pepsin followed by trypsin or by α–chymotrypsin and trypsin followed by pepsin and pepsin was used as catalyst in all cases, EPM products revealed low allergenic activity and not effected by the amount of methionine ethyl ester in the reaction mixture.

The change of the allergenic character of the EPM samples of buffalo and cow caseins showed fairly curious results. The more

Fig. 33.: ***Allergenic character of the enzymatically modified buffalo casein produced from buffalo casein hydrolysate with mixture of (α-chymotrypsin and trypsin) followed by pepsin***



A: EPM products in the presence of α-chymotrypsin

B: EPM products in the presence of trypsin

C: EPM products in the presence of pepsin

EPM A/O, EPM B/0, EPM C/0: EPM products without Met-enrichment

EPM A/2-10
EPM B/2-10
EPM C/2-10
} EPM products with different Met-enrichment (see Table 2.)

amount of methionine ethyl ester was added to the reaction mixture, the lower level of the allergenic character could be detected.

The allergenicity of the enzymatically hydrolyzed products significantly decreased in comparison to the untreated caseins, presumably because of the cleavage of a great number of peptide bonds. The fact that the allergenic character of proteins could be decreased by this enzyme-catalyzed method was due, very probably, to two different factors: the transpeptidation and the amino acid enrichment of the modified peptides.

4.3 Enzymatic modification of κ-casein

κ-casein is part of the casein complex which constitutes about 80% of the proteins in cow and buffalo's milk. It is associated with the other casein components into micelles and it protects the latter against flocculation by calcium ions (Wheelock and Knight 1969). Milk coagulation begins when milk-clotting enzymes cleave κ-casein. This limited proteolysis destabilizes the casein micelles and triggers their aggregation (McMahon and Brown 1984). Limited hydrolysis of κ-casein by chymosin to release κ-casein macropeptide (κ-MP; residues 106 to 169) initiates the coagulation of paracasein micelles that eventually form curds, which are processed to make cheese. Para-κ-casein (residues 1 to 105 of κ-casein) remains as part of the casein micelles.

El-Negoumy (1968) detected at least 11 breakdown products of κ-casein, most of which were negatively charged. Moreover, κ-casein was the only component of whole casein attacked within 50 min. by chymosin, and it yielded several slow and fast moving components. All milk-clotting enzymes had the same specific proteolytic action on κ-casein at the beginning of the reaction;

however, each enzyme ultimately produced different Para-κ-casein (Lawrence and Creamer 1969).

Tam and Whitaker (1972) studied hydrolysis of several caseins by bovine pepsin, α–chymosin, Cryphonectria parasitica and M. pusillus rennet's. Initial hydrolysis of κ-casein was greatest at pH 6. Cryphonectria parasitica rennet was the most proteolytic toward all casein substrates and displayed high proteolytic activity on whole caseins and α_S-casein and κ-casein components. In contrast, Mucor miehei rennet had more moderate activity, similar to that of chymosin, however, its activity was not specific for κ-casein.

Shammet et.al. (1992) found that chymosin was the only enzyme capable of transforming κ-casein solely to macropeptide and Para-κ-casein. The microbial enzymes were not specific and hydrolysed para κ-casein to smaller peptide fragments.

The main purpose of this research work was to study the enzymatic modification of κ-casein.

κ-casein was submitted to hydrolysis with α–chymotrypsin. Enzymatic peptide modification products with and without L-methionine ethyl ester enrichment were produced from this proteolytic hydrolysates of κ-casein.

4.3.1 *Incorporation of L-methionine into κ-casein hydrolysate by enzymatic modification*

Methionine content of κ-casein and EPM products (EPM0 and EPM6) are shown in Table 11. The results show that methionine content in the EPM product with methionine incorporation were found to be higher than that of both κ-casein and hydrolysate. Methionine content of EPM is greater by 4.6% than that of κ-casein.

Table 11.: ***Methionine content of the κ-casein, the sample of EPM 0 and one of the methionine-enriched EPM products***

Samples	Methionine %
κ-casein	1.7
EPM product from	
κ-casein without Met-enrichment	1.5
EPM-product from κ-casein	
with Met-enrichment	6.3

The quantitative evaluation was carried out by Videodensitometer.

The results showed that the covalently bound methionine content of the product in the case of α–chymotrypsin catalyst was four times as high as of substrate.

The effect of exopeptidases on modified κ-casein are shown in Fig. 34 and Table 12.There were no amino acid present in sample, that was taken at zero time, where HCl was added together with exopeptidase to prevent digestion .

In the case of the modified κ-casein the samples were obtained in every 10 minutes for an hour. The amount of the libertated L-methionine didn't incrase after the first 10 minutes.

Fig. 34.: ***L-methionine liberated during treatment with carboxypeptidase A and B from modified κ-casein. Where: 1, 2, 3, 4, are samples obtained after 0, 10, 30 and 60 minutes of exopeptidase treatment, 5: control Met***

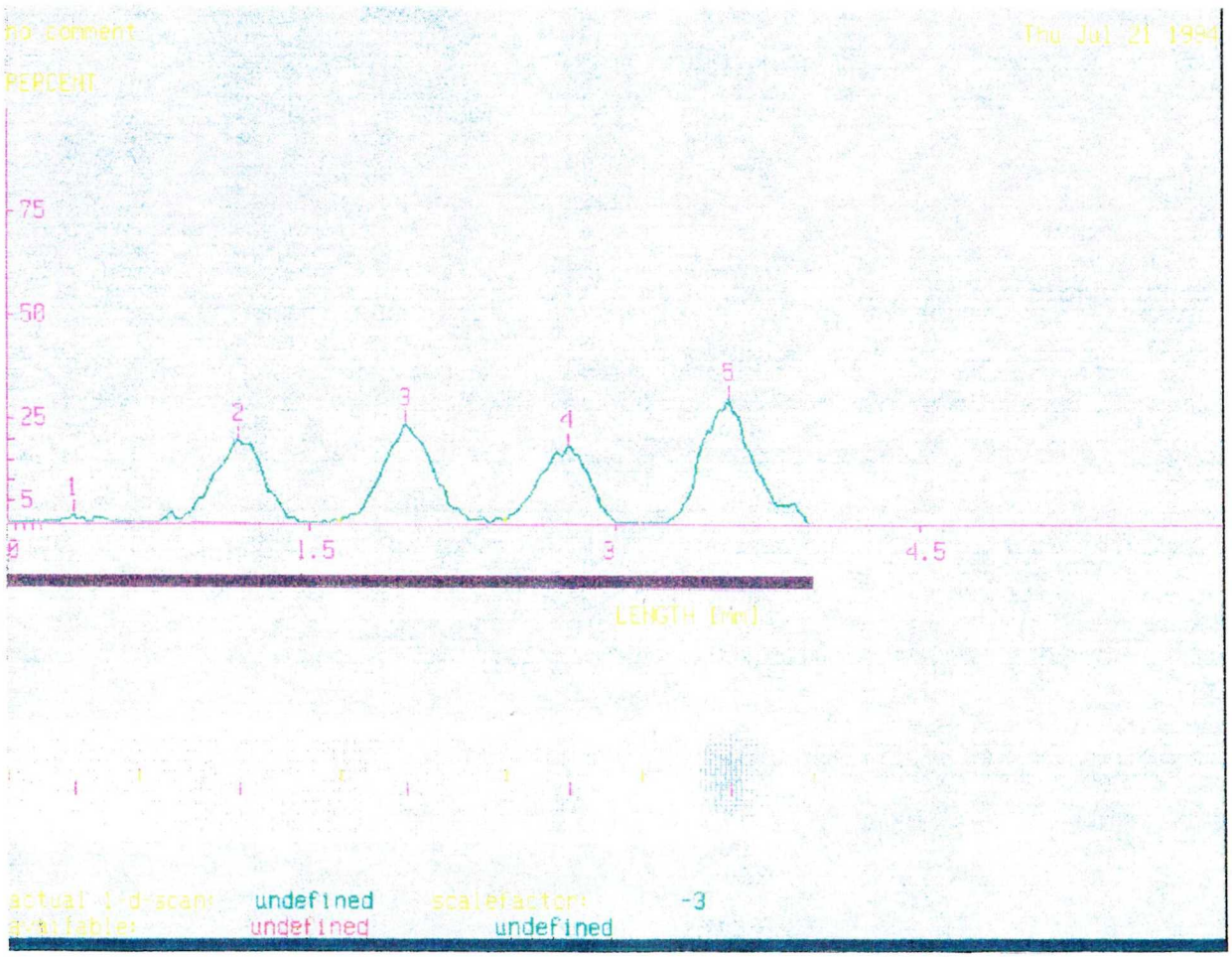


Table 12.: ***Quantitative evaluation of methionine content of the samples separated by thin layer ion exchange chromatography***

number	position (mm)	area (pixel)	height (%)	% area (%)
1	0.5369	378	5.9	1.9
2	1.5996	4644	52.5	23.3
3	2.6510	5046	63.9	25.3
4	3.6689	4544	54.9	22.8
5	4.6868	5310	70.6	26.7
		19922		100.0

1, 2, 3, 4: Samples obtained after 0, 10, 30 and 60 minutes, of exopeptidase treatment

5: Control Met

5. SUMMARY

A special enzymatic technique called enzymatic peptide modification(EPM) has been elaborated for designed modification of milk proteins. I proved that by the use of enzymatic process the biological value of milk proteins can be improved and the allergenic character of proteins can be reduced.

The achieved results could be summarized as follows:

I. Enzymatic modification of buffalo's milk proteins.

1. Molecular mass of modified protein fractions was influenced by the concentration of L-methionine ethyl ester in the reaction mixture when α–chymotrypsin was used as catalyst.

2. The fractions of the modified buffalo's milk proteins obtained by isoelectric focusing indicate that transpeptidation is the major process in the enzymatic reaction.

3. The results showed that the maximal covalent enrichment of the methionine in the EPM-products occurred when α–chymotryptic or α–chymotryptic and tryptic hydrolysates of buffalo's milk protein were used as substrates. When α–chymotrypsin was used as catalyst during both EPM reactions, the maximal covalently bound methionine content was four times and three times higher than that of the substrates, respectively. The maximal enrichment of the methionine in those EPM products produced from the peptic or peptic and tryptic hydrolysates of buffalo's milk proteins and catalysed by pepsin, was twice and three times higher than that of the substrates, respectively.

The fact that the improvement of the methionine content was due to the covalently bound methionine was confirmed by the exopeptidase treatments. All results show that the covalently bound

methionine was bound mainly to the C-terminal of the modified peptides.

4.EPM-products from the buffalo's milk protein hydrolysates with α–chymotrypsin and trypsin or with pepsin and trypsin showed low allergenic activites compared with those when the buffalo's milk proteins was hydrolyzed with α–chymotrypsin or with pepsin alone.

The allergenic character of the α–chymotrypic modified buffalo's milk proteins was significantly reduced by the increase of the concentration of methionine ethyl ester in the reaction mixture.

II. Enzymatic modification of buffalo and cow caseins:

1.Our results from electrophoretic patterns of protein fractions of the modified buffalo and cow caseins showed that the changes in the zones indicate that transpeptidation is the major process in the enzymatic modification.

2. According to our results the product: EPM 2 showed the maximal enrichment of methionine in the modified buffalo's and cow's caseins in the presence of α-chymotrypsin as catalyst. The methionine content of the sample of EPM 2 in the case of cow's casein is 4.3%, however, it is 2.8% in the case of the buffalo's casein sample of EPM 2.

The optimum of the L-methionine incorporation was in the presence of pepsin catalyst in the case of cow casein at the sample EPM 4 (3%) and in the case of buffalo casein at EPM 2 (2.6%).

Generally, when α-chymotrypsin was used as catalyst, the amount of methionine incorporated was higher compared with the case when pepsin was present as catalyst in the reaction mixture.

3.The incorporated methionine content of some fractions obtained by Sephadex IEF separation of buffalo casein hydrolysate and EPM product was also investigated.

The results proved that all the three new IEF-zones of the EPM product contained peptides of covalently incorporated methionine.

4. The results showed that the degree of hydrolysis of casein hydrolysate was 1.5 times as high as that of the buffalo's milk proteins hydrolysate at the same conditions. The degree of hydrolysis and of EPM product of buffalo milk proteins was 11%. In the case of EPM product from casein hydrolysate, the degree of hydrolysis decreased from 16.7% to 11.2%.

5. Our results proved that the allergenic character of EPM-products of both cow's and buffalo's caseins can be reduced during the enzymatic process with methionine enrichment.

5.1. The reduction of the allergenic character of the modified buffalo casein was significantly modified by the increase of the concentration of methionine ethyl ester in the reaction mixture when α–chymotrypsin was used as catalyst. In the case of modified cow casein the same trend was observed.

5.2. The allergenic activity of EPM products from buffalo or cow casein in the case of pepsin catalyst was very low and was not affected by the concentration of methionine ethyl ester in the reaction mixture.

5.3.Our results reveled that allergenic activity of all the EPM products from hydrolysates of buffalo casein (with a mixture of α–chymotrypsin and trypsin followed by pepsin) showed low allergenic activity.

III. Enzymatic modification of κ-casein

Methionine content of the modified κ-casein was 6.3%, however, the methionine content of the unmodified κ-casein was 1.7%. Our results showed that the methionine enrichment was successful even in the case of the purified κ-casein.

Our work proved that the enzymatic peptide modification is a suitable method for the covalent methionine enrichment and for the reduction of the allergenic character of buffalo's and cow's milk proteins.

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