RESEARCH AND DEVELOPMENT OF A PEPTIDE COMPLEX TECHNOLOGY

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Abstract: Results of biotechnological research on controlled hydrolysis of casein and production of peptide complexes are summarized in the present article. Selection of processing parameters and optimization of the conditions of enzymatic hydrolysis of milk proteins allowed for the development of a resource-efficient technology for the production of peptide complexes. Enzymatic hydrolysis of peptide bonds R_1-CONH-R_2 + H_2O → R_1COOH + NH_2R_2 was considered using the example of casein and a range of proteolytic enzymes (trypsin and chymotrypsin) belonging to the hydrolase class. Enzyme solution (0.1% m/v) was added to each protein solution so that the final enzyme-substrate ratio was 1:25, 1:50, or 1:100. The enzyme-substrate ratio of 1:50 was shown to be optimal, and the recommended temperature and pH values were 50±1°C and 7.50±0.01, respectively. The degree of hydrolysis is one of the parameters characterizing the overall changes in the amino acid composition of proteins. Therefore, a time period of 12.00±0.05 h was chosen as the optimal duration of the hydrolysis process. Further research was focused on the analysis of peptide profiles using MALDI-TOF MS based on identification of peptide sequences. The studies have shown that casein hydrolysates are rich in biologically active peptide complexes. The detection of such complexes in hydrolysates of casein was the main result of the study. For example, the peptide β-casokinin (amino acid sequence Ala-Val-Pro-Tyr-Pro-Gln-Arg) is an inhibitor of angiotensin-converting enzyme.

Keywords: casein, enzyme preparations, peptides, prevention, hypertensive disease, chronic cardiac insufficiency

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INTRODUCTION

According to a report by the All-Russian Healthcare Organization, the mortality from cardiovascular disease in Russia is the highest in the world. Deaths caused by cardiovascular diseases account for 57% of total mortality in Russia, with 1.3 million people dying from these diseases every year. Coronary heart disease and hypertension remain the most common diseases of the cardiovascular system. High blood pressure remains unnoticed very often due to the lack of clinical manifestations, and therefore it is a risk factor for the development of coronary heart disease, congestive heart failure, stroke, and disturbances of the function of kidneys and other organs [1].

The development of technology for the manufacturing of therapeutics and functional foods intended for the prevention of cardiovascular disease is of especial importance. Functional foods of plant and animal origin play an important role in the prevention of disease. Functional foods are systematically used in the form of special diets capable both of preventing disturbances of physiological functions and metabolic processes in the body and of improving human health due to the presence of essential nutrients. These food products play a considerable role in the prevention of various diseases. Proper nutrition including nutriceuticals and functional foods enhances immunity and defense capacity of the body and activates anabolic processes, having a general positive effect on health [2].

The development and implementation of food for persons who are prone to or suffering from hypertension is currently in progress in Russia. Notably, foods enriched in peptides, individual amino acids, or fatty acids, as well as dairy products obtained using selected strains of lactic acid bacteria, and other products of this type are entering the market of functional foods and dietary supplements [4]. At this, the interest towards the structure and function of low molecular weight peptides produced by controlled enzymatic hydrolysis of high-quality proteins fulfilling a range of specific biological functions in the body is increasing.

A large number of biologically active peptides that are either naturally present in food or formed during enzymatic or chemical (acid) hydrolysis of the dietary proteins was identified during the past decade. Natural processing of dietary protein in the digestive tract (and preceding fermentation in the case of pre-fermented foods) enables the release of these peptides from the proteins and their functioning as independent regulatory units with hormone-like activity. A considerable number of such peptides possessing a wide range of biological functions has been identified already [3, 4].

Raw materials of animal origin, namely, milk and dairy products, are among the sources of biologically active peptides. However, such peptides can be isolated from a variety of foods such as egg, fish, shellfish, grains (rice, wheat, buckwheat, barley, and maize), soybeans, and radishes. Functional proteins from milk have an exceptionally broad range of biological activity. Bioactive peptides can be formed from both

casein (α_s, β, k, and κ-casein) and whey proteins (lactoferrin and immunoglobulin, α-lactalbumin, and β-lactog-lobulin) [5]. Use of dietary products enriched with peptides derived from milk proteins and capable of blocking the conversion of the inactive peptide angiotensin I into the active compound angiotensin II is an efficient and safe way of lowering blood pressure. Nitric oxide activates the enzyme guanylate cyclase and promotes the formation of cyclic guanosine monophosphate, which has vasodilatory activity due to its effect on smooth muscle cells [1]. Supplementation of food with functional whey casoplatelins possessing an antithrombotic effect reduces the risk of complications in patients suffering from hypertension and hypercholesterolemia [7].

Technology of peptide isolation from natural products is crucial. The structure and the biological properties of the peptides formed depend on physico-chemical parameters of the hydrolysis. Enzymatic hydrolysis is most often used for the processing of proteins in food industry.

Both casein and whey proteins from cow's milk can undergo hydrolysis. The biological value of whey proteins is higher than that of caseins due to the higher content of essential amino acids cysteine and tryptophan, and therefore whey hydrolysates are considered more physiological than casein hydrolysates. However, formation of small peptides during hydrolysis is among the advantages of casein making it more efficient in the dietary treatment of food allergy [8]. Casein hydrolysates are very often used as additional ingredients in functional foods and children's nutrition.

Various methods are used for casein hydrolysis. Four factors of the highest importance for the production of a casein hydrolysate with desired properties are the following: the degree of hydrolysis, which determines the formation of shorter peptides with lower allergenic capacity; low content of free amino acids, which is considered advantageous if the hydrolysate is added to foodstuffs; reduced bitterness; and a high total yield of peptides and amino acids [11]. Protein hydrolysates produced by enzymatic hydrolysis compensate for protein deficit and maintain the nitrogen balance in patients after gastrointestinal surgery, severe burns, etc. Development of technologies for the production of milk protein hydrolysates is of particular relevance due to the lack of protein preparations and protein hydrolysates of enhanced biological value; the supply of enteral nutrition products and specialized breast milk substitutes based on protein hydrolysates is also insufficient [9].

Peptides isolated from α-, β-, and κ-casein and lactoferrin (such as β-casokinins, casomorphins (caseomorphins), ecsorphin derived from α-casein, caseinophosphopeptides, and lactoferricin) exhibit antihypertensive properties, as well as antimicrobial and antithrombotic activity (Table 1) [10].

Table 1. Examples of biologically active peptides derived from casein hydrolyzates

System of the body	Physiological function	Peptide source	Fragment	Amino acid sequence of the peptides
Cardiovascular system	Antihypertensive	α_{s1}-casein	144–151	Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp
		α_{s1}-casein	129–134	Leu-Ala-Tyr-Phe-Tyr-Pro
		α_{s1}-casein	181–186	Thr-Thr-Met-Pro-Leu-Trp
	Antioxidant	κ-casein	117–127	Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met
	Antithrombotic	κ-casein	106–116	Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys
	Hypocholesterinemic	β-casein	99–101	Val-Pro-Pro
		κ-casein	129–131	Ile-Pro-Pro

In view of the above, the aim of the present work was the investigation and development of a technology for the production of biologically active peptide complexes intended to reduce blood pressure and prevent chronic heart failure.

OBJECTS AND METHODS OF RESEARCH

The milk protein casein (GOST 17626-81) was selected as the object of investigation, since it is the most easily accessible and biologically valuable source of protein.

Conventional, standard and original methods of research were used in the present work. Sampling and preparation of samples for the analysis were carried out in accordance with GOST 26809-86 «Milk and dairy products. Acceptance rules, methods for sampling and preparation of samples for analysis» and GOST 9225-84 «Milk and dairy products. Methods for microbiological analysis».

Enzymes capable of cleaving polypeptide chains into short peptides, namely, trypsin (activity 50-60 U/mg protein) and chymotrypsin (activity 40-60 U/mg protein) were chosen for the enzymatic hydrolysis; both enzymes were from Sigma (the United States).

Hydrolysis was performed with constant stirring in a thermostat at optimum temperature (50 ± 1 °C) and pH (7.5 ± 0.01, phosphate buffer) recommended by the manufacturers of the proteolytic enzymes used.

Total nitrogen/protein was determined according to the method of Dumas; the nitrogen formed was registered by the thermal conductivity detector of the «Rapid N cube» nitrogen analyzer. Amino nitrogen was determined spectrophotometrically using 2,4,6-trinit-

robenzene sulfonic acid (TNBS); the method is based on spectrophotometric detection of chromophores generated in the reaction of primary amines with TNBS. The amount of amino nitrogen was determined using a calibration curve constructed with standard dilutions of a known substance. The degree of hydrolysis of the protein was determined as the ratio of amino nitrogen to total nitrogen.

Qualitative and quantitative amino acid analysis was performed using an automatic amino acid analyzer Aracus PMA GmbH. The procedure of amino acid detection is based on separation of molecules on a cation exchanger with elution in a stepwise pH gradient and postcolumn derivatization with ninhydrin.

Molecular weight distribution of peptides and proteins in the obtained hydrolysates was evaluated using electrophoresis according to the procedure of Laemmli. Denaturing polyacrylamide gel (12% separating and 4% stacking gel) with 0.1% SDS-Na was used for the separation of proteins. Electrophoresis was performed at 15 mA in 1×electrode buffer supplemented with 0.1% SDS-Na. The gels were viewed on a UV transilluminator TCP-20M (Vilber Lourmat, the United States) at illumination wavelength of 312 nm. Gel documentation system Vitran-Photo was used for data processing.

Amino acid sequences of the peptides formed were determined by chromatography-mass spectrometry on an Agilent 5975 C system: the MALDI-TOF approach involving separation of ions by mass/charge ratio was used.

Identification of peptide sequences was accomplished by searching the NCBI (http://www.ncbi.nlm.nih.gov/) and SwissProt (http://web.expasy.org) databases.

RESULTS AND DISCUSSION

Development of technology for the production of peptide complexes possessing biological activity involves controlled hydrolysis of casein by enzyme preparations to achieve the predetermined degree of hydrolysis and subsequent separation of peptides. Selection of processing parameters and optimization of the conditions of the enzymatic hydrolysis of casein enables the development of a resource-efficient technology for the production of peptide complexes. The optimal enzyme - substrate ratio was chosen and the effect of fractional composition of the proteins on hydrolysis efficiency was investigated at the present stage of research. The final enzyme-substrate ratio after addition of 0.1 % solution of the enzyme to the protein solution was 1:25 in the first series of experiments, 1:50 in the second series of experiments, and 1:100 in the third series of experiments. The results of these experiments are shown in Fig. 1 and Table 2.

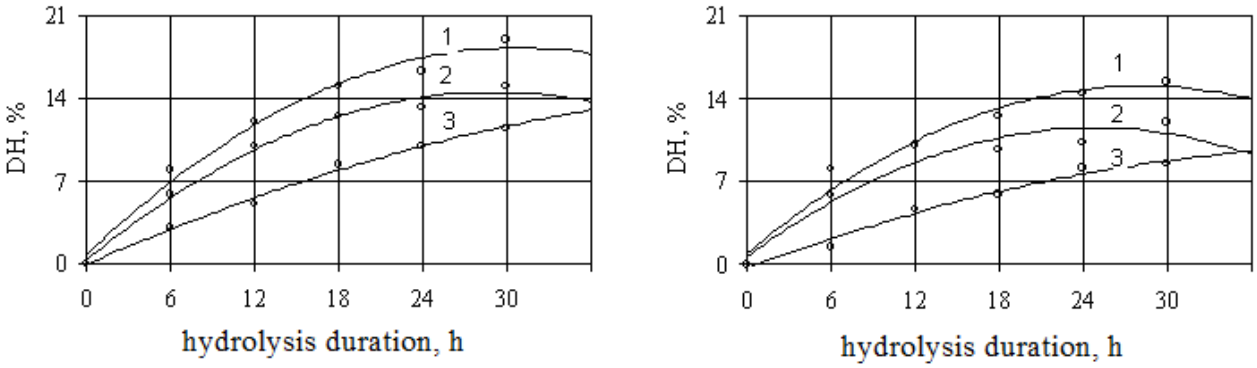

Fig. 1. Dependence of the degree of hydrolysis on process duration. Enzyme - substrate ratio: 1 - 1:25; 2 - 1:50; 3 - 1:100 ((a) trypsin treatment, (b) chymotrypsin treatment).

Table 2. Basic parameters of hydrolysates obtained by treatment with proteolytic enzymes

Parameter	Control	Enzyme-substrate ratio at hydrolysis duration, h								
		1:25			1:50			1:100		
		6.00± 0.05	12.00± 0.05	24.00± 0.05	6.00± 0.05	12.00± 0.05	24.00± 0.05	6.00± 0.05	12.00± 0.05	24.00± 0.05
Trypsin										
Degree of hydrolysis, %	0	3.00± 0.18	5.00± 0.30	10.10± 0.61	5.80± 0.45	10.00± 0.60	12.50± 0.75	8.00± 0.48	12.00± 0.72	16.30± 0.98
pH	7.50	7.40	7.25	7.18	7.38	7.36	7.29	7.42	7.35	7.24
Chymotrypsin										
Degree of hydrolysis, %	0	1.50± 0.09	4.70± 0.28	8.12± 0.79	5.82± 0.35	10.36± 0.62	11.22± 0.67	8.56± 0.51	10.17± 0.61	14.40± 0.86
pH	7.5	7.12	7.22	7.06	7.50	7.28	7.20	7.30	7.29	7.26

High efficiency of trypsin used at an enzyme - substrate ratio of 1:25 was demonstrated in the present study: the degree of hydrolysis exceeded 3% after 6.00 ± 0.05 h of treatment, and by twelve hours of treatment it was above 5%. When the enzyme-substrate ratio was 1:50, the degree of hydrolysis equaled 5.80 ± 0.45 and 10.0 ± 0.60%, respectively, for the two timepoints mentioned above. The degree of hydrolysis at an enzyme - substrate ratio of 1: 100 was significantly higher than in the other experiments and amounted to 12.0 ± 0.72% after six hours of fermentation. The active acidity of the mixture was found to change only slightly at different enzyme-substrate ratios; during the whole treatment process, it remained within a range of 7.0-8.0, which is optimal for the enzyme activity. A similar course of enzymatic hydrolysis was observed in experiments with chymotrypsin. The reaction rate was lower at enzyme - substrate ratios of 1:25 or 1:100, and therefore liberation of the peptides from the polypeptide chain was suppressed.

Changes in the degree of hydrolysis were correlated with changes in the molecular weight of the hydrolysate related to formation of low molecular weight peptides. An attempt to determine the molecular weight distribution of the peptides formed at different time points for each enzyme-substrate ratio was undertaken in order to fully understand the patterns of hydrolysis. Electrophoresis was used to study the kinetics of hydrolysis and assess the molecular weight distribution of the fragments found in the hydrolysates. Native casein with a molecular weight of 28 kDa was used as a control. The molecular weight distribution of the peptides produced by enzymatic hydrolysis of casein by trypsin and chymotrypsin are illustrated by Fig. 2 and Table 3; the molecular weight distribution was assessed using electrophoresis in polyacrylamide gel.

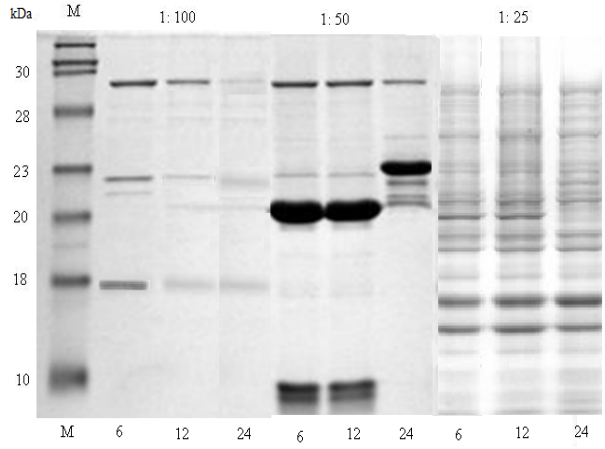

Fig. 2. Polyacrylamide gel electrophoresis of tryptic hydrolysates of casein (enzyme-substrate ratio 1:25, 1:50, or 1:100; treatment duration 6.00 ± 0.05, 12 ± 0.05, and 24 ± 0.05 h).

Redistribution of peptide fractions was shown to occur during hydrolysis by trypsin and chymotrypsin at all the enzyme-substrate ratios used. As the duration of the fermentation process increased, the amount of peptide structures with a molecular mass of 30 kDa decreased and that of low-molecular peptides and free amino acids increased. An additional estimate of the properties of the hydrolysate obtained was based on the analysis of the dynamics of free amino acid accumulation in the hydrolysate produced under various conditions. Amino acids constituted about 25.8% of the hydrolysate, the content of lysine, leucine, arginine, serine, and tyrosine being the highest.

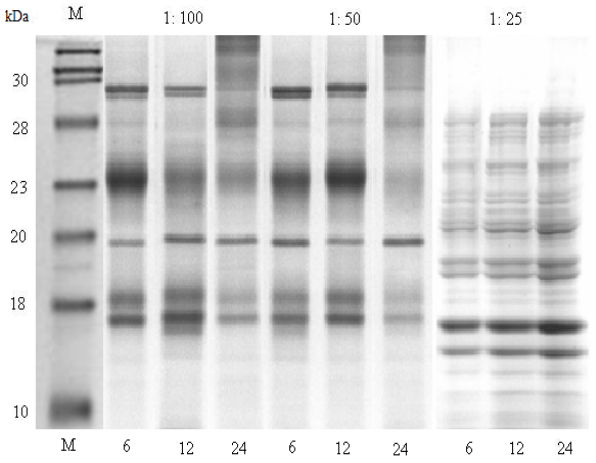

Fig. 3. Polyacrylamide gel electrophoresis of chymotryptic hydrolysates of casein (enzyme-substrate ratio 1:25, 1:50, or 1:100; treatment duration 6.00 ± 0.05, 12 ± 0.05, and 24 ± 0.05 h).

The results of the present study allow for the conclusion that accumulation of amino acids formed due to the action of proteolytic enzymes occurs along with the cleavage of intact substrate molecules resulting in formation of peptide products already present in the mixture. Despite the high specificity of the proteolytic enzymes used, they are capable of cleaving a polypeptide chain into low molecular weight peptides and free amino acids. The optimal parameters of the hydrolysis reaction were selected according to the results of the experiments described above. The optimal parameters for an enzyme system consisting of trypsin and chymotrypsin were the following: temperature 50 ± 1°C, enzyme-substrate ratio of 1:50, and process duration of 12.00 ± 0.05 hours.

Analysis of peptide profiles with MALDI-TOF MS-based peptide identification was the next step of the study. Peptides of many different types were detected in casein hydrolysates by MALDI-TOF mass spectrometry. Multicomponent composition of the samples studied was demonstrated using analysis of the mass spectra. The chromatogram of the tryptic hydrolysate of casein is shown in Fig. 4 and the peaks are listed in Table 3. The chromatogram of the hydrolysate formed after chymotrypsin treatment is shown in Fig. 5 and the peaks are listed in Table 4.

Analysis of the peptide profiles indicates that all investigated hydrolysate fractions contain both short and long peptides, which consist of two or more amino acid residues. Identification of peptide sequences obtained was performed by searching the NCBI (http://www.ncbi.nlm.nih.gov/) and SwissProt (http://web.expasy.org) databases. The results of the comparative studies are shown in Table 5.

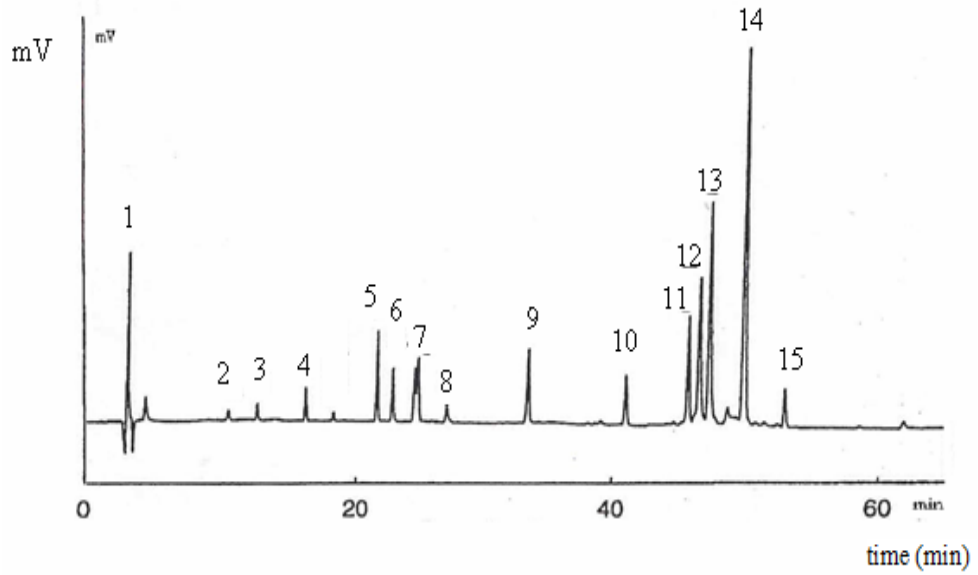

Fig. 4. Chromatogram of the tryptic hydrolyzate of casein (the location of the peptide fragments within the complete protein sequence is indicated by Latin letters).

Table 3. Characteristics of the peptide profiles generated by tryptic hydrolysis of casein

Peak number	Molecular weight, Da	Location in the polypeptide chain	Amino acid sequence
1	3803	1–25	RELEELNVPGEIVESLSSSEESITR
2	615	26–28	INK
3	98	29	K
4	390	30–32	IEK
5	1463	33–48	FQSEEQQTEDELQDK
6	4451	49–97	IHPFAQTQSLVYPFPGPIHNSLPQNIPPLTPVVVPPFLQPEVMGVSK
7	180	98–99	VK
8	664	100–105	EAMAPK
9	233	106–107	HK
10	690	108–113	EMPFPK
11	2107	184–202	DMPILYQEPVLGPVR
12	589	170–176	VLPVPQK
13	687	177–183	AVPYPQR
14	5172	114–169	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPWVMFP PQSVLSLSQSK
15	585	203–209	GPFPIIV

Note. A – alanine; D – aspartic acid; E – glutamic acid; G – glycine; H – histidine; I – isoleucine ; L – leucine; K – lysine; N – asparagine ; Q – glutamine ; P – proline; S – serine; T – threonine; W – tryptophan ; Y – tyrosine ; V – valine.

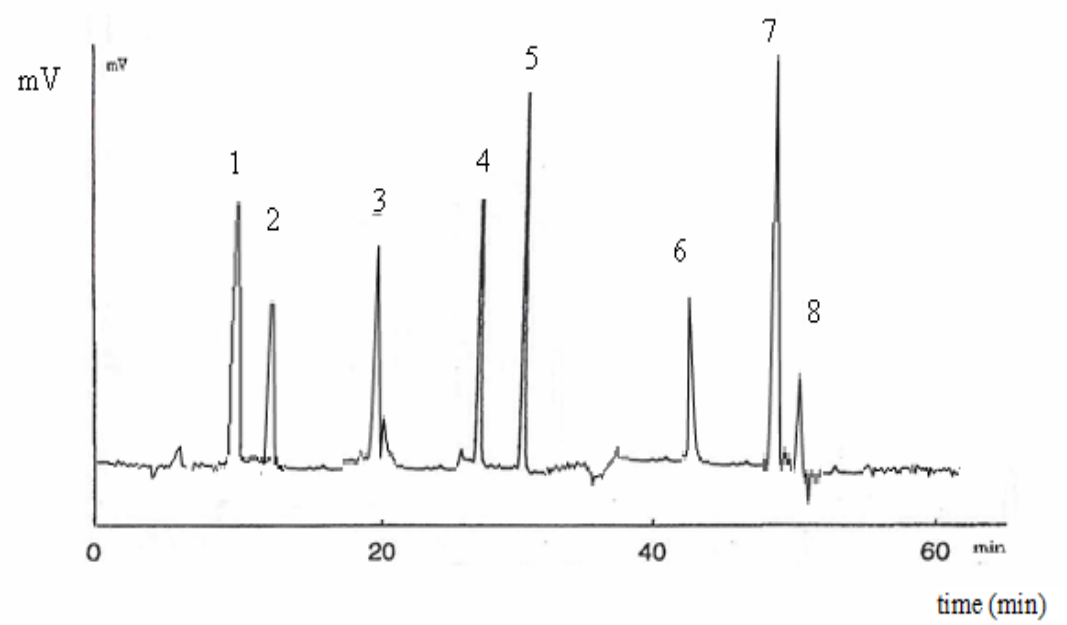

Fig. 5. Chromatogram of casein hydrolysate produced by chymotrypsin treatment (the location of peptide fragments within the complete protein sequence is indicated by Latin letters).

Table 4. Characteristics of the peptide profiles generated by hydrolysis of casein with chymotrypsin

Peak number	Molecular weight, Da	Location in the polypeptide chain	Amino acid sequence
1	3803	1–25	RELEELNVPGEIVESLSSSEESITR
2	1802	26–32	INKKIEKF
3	2329	33–51	QSEEQQQTEDELQDKIHPF
4	3754	52–86	AQTQSLVYPFPGPIPNSLPQ NIPPLTQTPVVVPPF
5	3668	87–118	LQPEVMGVSKVKEAMAPKHK EMPFPKYPVEPF
6	2734	119–142	TESQSLTLTDVENLHLPLPL LQSW
7	5286	143–189	MHQPHQPLPPTVMFPPQSVL SLSQSKVLPVPQKAVPYPQR DMPIQAF
8	1717	190–209	QEPVLGPVRGPFPIIV

Note. A – alanine; D – aspartic acid; E – glutamic acid; G – glycine; H – histidine; I – isoleucine ; L – leucine; K – lysine; N – asparagine ; Q – glutamine ; P – proline; S – serine; T – threonine; W – tripotofan ; Y – tyrosine ; V – valine.

Table 5. Peptides identified in the hydrolysates investigated

Fragment	Enzyme used	Amino acid sequence of the peptide	Name	Function
1–25	trypsin, chymotrypsin	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(P)-Leu-Ser(P)Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Thr-Arg	phosphopeptide	mineral absorption stimulant
177–183	trypsin	Ala-Val-Pro-Tyr-Pro-Gln-Arg	β-casokinin	inhibitor of angiotensin converting enzyme

CONCLUSIONS

Peptide complexes with different molecular weight and amino acid sequence were detected in casein hydrolysates during the present study. Investigation of the controlled hydrolysis enabled us to determine the optimal process parameters and the amounts of the enzymes added. Optimal parameters for enzymatic hydrolysis were the following: temperature 50 ± 1ºC, duration of 6.00 ± 0.5 h, pH 7.5 ± 0.1, and enzyme-substrate ratio of 1:50.

Analysis of peptide profiles with peptide identification by MALDI-TOF mass spectrometry showed that peptide complexes with different molecular weight were formed under optimal conditions of enzymatic hydrolysis. A peptide with the amino acid sequence Ala-Val-Pro-Tyr-Pro-Gln-Arg was reported to possess antihypertensive activity [12, 13]. The amino acid sequence of a component of a peptide complex obtained from β-casein by controlled hydrolysis was identical to that of the peptide mentioned above. Due to its sequence features, the peptide obtained is of interest for research related to its biological activity and its use for lowering blood pressure and prevention of chronic heart failure.

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