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Rational Design of Enzyme Compositions for the Production of Functional Hydrolysates of Cow Milk Whey Proteins

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Abstract—The design of enzyme compositions for the preparation of functional hydrolysates of whey proteins is studied. Analysis of the protein profiles of the whey from hard, semihard, and soft cheeses showed that the whey contains 50–63% β-lactoglobulin, 19–20% α-lactalbumin, and up to 11% κ-casein. According to the protein profile, the whey from Circassian cheese contains 76% casein (α-, β-, and κ-casein), 12% β-lactoglobulin, and 12% α-lactalbumin. Based on the *in silico* analysis, the rational design of a multienzyme composition was carried out for the hydrolysis of whey with a known protein composition taking into account the content of amino acid descriptors of biological activity and bitter taste. For the hydrolysis of whey from hard (Montazio), soft (Mozzarella and Gorgonzola), and semihard (Caciotta) cheeses, the determined optimum ratio of the Protamex and Alcalase enzymes was 3.0 : 1.0% (90 min, 50°C). For soft pickled unripened cheese (Circassian), the optimum ratio of the Thermolysin and Alcalase enzymes was 2.0 : 1.0% (60°C, 120 min). The use of the bioinformatics approach made it possible to obtain hydrolysates with acceptable organoleptic properties and predetermined antioxidant (400–500 μM TE/g protein) and antihypertensive (IC_{50} 537–2500 mg protein/L) activities.

Keywords: cheese whey, protein hydrolysates, rational design of the enzyme composition, biologically active peptides, antioxidant activity, antihypertensive properties

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INTRODUCTION

The universal functionality of biologically active peptides defines their important role in the pharmaceutical and nutraceutical industries and in the area of functional nutrition. The market for biologically active peptides is currently growing, and the number of food products that contain hydrolysates of various protein sources (commercially available or under development) is increasing.

A modern global trend in the development of functional food products is the use of rapid testing technologies to determine a wide range of molecular parameters of the product, in particular, the entire set of peptides that are contained in a product or can be released during its hydrolysis. Foodomics is a modern discipline that includes estimation of the composition of food products and assessment of the consequences of biotechnological production processes and their impact on human health [1]. The addition of biologically active peptides to food products is one of the promising strategies for the engineering of functional products with antioxidant and antihypertensive properties aimed at combating diabetes, hypertension, and other major diseases. Thus it becomes important to identify suitable sources of biopeptides among protein-containing raw materials and to find selective methods of hydrolysis that will release biologically active fragments from protein chain.

Computational methods make it possible to predict the content of bioactive peptides in hydrolysates of proteins with known amino acid sequences. Databases containing hundreds of peptides with known biological functions allow for the prediction of new peptides with improved biological activity, identification of biological raw materials that contain such peptides, and the choice of enzymes for selective proteolysis [2].

Cheese whey is the most promising source of biopeptides because of its large-scale production, availability, and high content of biologically active fragments. The global dairy industry produces over 285 million tons of milk per annum [3]. Milk contains 85–95% whey, which accounts for 55% of milk nutrients. Whey is the main waste product in the production of cheeses. Whey nutrients are mainly represented

by lactose (4.5–5%) and soluble proteins (0.6–0.8%). About half of the total whey volume is processed and converted into various food products, whereas the other half is disposed of as waste. Around the world, the currently unused whey is considered a source of proteins and biologically active peptides.

The extract of β-lactoglobulin fragments from the BioZate whey protein hydrolysate (Davisco Foods International Inc., United States) and a mixture of casein fragments from the whey of Festivo fermented hard cheese (MTT Agrifood Research, Finland) have an antihypertensive effect. The hydrolysate of the Bio-PURE-GMP whey prevents tooth decay (according to the manufacturer's advertising), affects blood coagulability, and exhibits antimicrobial properties. The Capolac casein hydrolysates (Arla Foods Ingredients, Sweden) enhance mineral absorption, whereas PRODIET F200/Lactium dietary dairy products (Ingredia, France) reduce the symptoms caused by stress. The Immunel mixture of milk peptides (Wild Co., Germany) reduces inflammation and promotes regenerative processes in the gastrointestinal tract. Other functional properties of commercially available dairy hydrolysates and isolated peptides include regulation of the blood sugar level, reduction of psoriasis and acne symptoms, sedative effect, improvement of muscle activity and recovery, anticancer effect, support of the general immunity, and improvement of sleep and memory [4].

Although attempts to vary proteases and their combinations in a random manner in order to produce hydrolysates with diverse properties have been described in the literature, a bioinformatics approach is rarely applied to enable the rational use of protease specificity for the selection of an enzyme composition [5].

The goal of the work is to obtain functional hydrolysates of cow-milk whey proteins by the bioinformatics approach so that these hydrolysates have predetermined biological properties and satisfactory organoleptic characteristics.

EXPERIMENTAL

Materials. Whey samples from the Mozzarella, Gorgonzola, Caciotta, Montasio, and Circassian cheeses used in this study were provided by the Sfoggiatech cheese manufacturer (Tradizioni Italiane).

Whey proteins were precipitated by ammonium sulfate (70% saturation) to study the qualitative and quantitative composition. The precipitated proteins were filtered through a pleated paper filter. The precipitate from the filter was redissolved in a minimum volume of distilled water, after which the protein solution was dialyzed against distilled water. After dialysis, the solution was passed through a Chromafil CA/S membrane (Germany) with a pore size of 0.45 μm. Proteins were determined with a BCA Protein assay kit (Pierce, USA) according to the manufacturer's instructions. Denaturing PAGE electrophoresis was then performed [6]. All samples were applied in the same amount (10 μg protein per lane). The protein lanes were then identified by MALDI-TOF/TOF mass spectrometry on an UltrafleXtreme tandem MALDI-TOF/TOF mass spectrometer (Bruker, Germany). An Infinity 1000/26MX gel documentation system (VilberLourmat, France) was used to obtain protein maps. Analysis of the protein maps and the densitometric determination of the mass fraction of a given protein in the total content of identified proteins were performed with the Image-Master 2D Platinum, v.7, software (GE Healthcare, United States). Mass spectra were processed with the FlexAnalysis 3.3 software package (Bruker Daltonics, Germany). Proteins were identified using the Mascot software (www.matrixscience.com). Proteins with an NCBI score above 74 were considered to be reliably identified.

In silico design of a multienzyme composition for the hydrolysis of whey proteins obtained during the production of hard, semihard, soft, and pickled cheeses. The amino acid sequences of the major protein components identified in the studied whey samples were obtained from the NCBI database. The possibility of obtaining biologically active hydrolysates without a bitter taste through directed proteolysis was then studied. Proteases were selected for in silico digestion of protein components based on the enzyme specificities and the presence of the descriptors of biological activity and bitter taste in the predicted peptide profiles. In the selection of proteases, the goal was to minimize the number of residual fragments that contain bitter taste descriptors while maximally preserving the fragments that contain descriptors of biological activity.

Enzymatic hydrolysis of whey. Before hydrolysis, all whey samples were separated on an ESB-02-04 laboratory electric separator of milk (Russia), and whey protein concentrates were obtained. The latter step was required because the total protein content in whey was relatively low, ranging from 0.29% for Circassian cheese to 1.1% for Mozzarella cheese (Table 1). The concentrates were prepared on a pilot AL 362 ultrafiltration device equipped with roll-type membranes (Altair, Russia). The resulting concentrates of the whey samples from Mozzarella, Caciotta, Gorgonzola, and Montasio cheeses contained 8–11 wt % solids and 3.2–3.8% total protein. The whey from Circassian cheese was concentrated to ~7 wt % solids and 0.7–0.8% total protein.

The enzymatic hydrolysis of the whey from Montasio, Caciotta, Gorgonzola, and Mozzarella cheeses by the combination of Protamex (Novozymes A/S, Denmark) and Alcalase (Novozymes A/S, Denmark) enzyme preparations (**EP**s) was conducted under previously selected conditions: a reaction time of 90 min, a Protamex-to-Alcalase ratio of 3.0 : 1.0 (wt % of the

Table 1. Qualitative and quantitative composition of proteins in the studied whey samples

<table>
<tr><th rowspan="2">Protein</th><th colspan="5">Content in whey, %</th></tr>
<tr><th>Montazio</th><th>Caciotta</th><th>Gorgonzola</th><th>Mozzarella</th><th>Circassian</th></tr>
<tr><td>α-lactalbumin</td><td>19</td><td>20</td><td>20</td><td>20</td><td>12</td></tr>
<tr><td>β-lactoglobulin</td><td>56</td><td>50</td><td>63</td><td>56</td><td>12</td></tr>
<tr><td>BSA</td><td>6</td><td>8</td><td>6</td><td>5</td><td>~0</td></tr>
<tr><td>α-s1-casein</td><td rowspan="3">~0</td><td rowspan="3">~0</td><td rowspan="3">~0</td><td rowspan="3">~0</td><td rowspan="2">26</td></tr>
<tr><td>α-s2-casein</td></tr>
<tr><td>β-casein</td><td rowspan="2">50</td></tr>
<tr><td>κ-casein</td><td>11</td><td>9</td><td>5</td><td>4</td></tr>
<tr><td>Immunoglobulin G1, heavy chain</td><td>5</td><td>8</td><td>4</td><td>8</td><td rowspan="3">~0</td></tr>
<tr><td>Lactoperoxidase</td><td>2</td><td>3</td><td>1.4</td><td>5</td></tr>
<tr><td>Xanthine oxidase</td><td>1</td><td>2</td><td>0.6</td><td>2</td></tr>
</table>

substrate weight), a pH of 7.0, and a temperature of 50°C. It has been shown that the hydrolysis of rennet cheeses under these conditions leads to the production of biologically active hydrolysates with reduced allergenic potency [7].

The hydrolysis of whey proteins from Circassian cheese was carried out at a pH of 7.8 and a temperature of 60°C with Thermolysin (Sigma-Aldrich, United States) and Alcalase EPs. To determine the optimum Thermolysin-to-Alcalase ratio, the hydrolysis of four whey concentrate samples was performed at different concentrations of both enzymes. The degree of hydrolysis and the content of free amino acids were measured in the hydrolysates 90, 120, and 150 min after the start of the reaction.

Enzymatic hydrolysis was carried out in 250-mL flasks with ground stoppers on an IKA RT5 Power multiposition magnetic stirrer with a temperature control plate (IKA Werke, Germany) at 66 rpm. The reaction temperature was 50°C for the mixture of Protamex and Alcalase and 60°C for the mixture of Thermolysin and Alcalase. The reaction temperature was selected based on the temperature optima of the enzymes. The temperature accuracy (±0.1°C) during the enzymatic hydrolysis was monitored with an ETS-D5 contact thermometer (IKA Werke, Germany). The desired amount of concentrated (×100) EP solution was added to preheated cheese whey at a pH of 7.0 (the pH was adjusted by 5 M sodium hydroxide solution).

Statistical processing of the results was carried out using the Statistica 8.0 software package (StatSoft Inc., United States).

Determination of the functional properties of hydrolysates. The organoleptic characteristics of the obtained hydrolysates were individually evaluated by the presence or absence of bitter and salty tastes.

Degree of hydrolysis and the content of free amino acids (faas). The degree of hydrolysis and the FAA content in the hydrolysates were determined by spectrophotometry as described in [8, 9]. The optical density of the solutions was measured at wavelengths of 340 and 420 nm (for the hydrolysates and FAAs, respectively) on a Synergy 2 microplate photometer—fluorometer (BioTek, United States) with 92-well nonabsorbent UV-transparent flat-bottomed UV-Star plates (Greiner BioOne, Germany).

The in vitro antioxidant activity in the samples of cheese whey hydrolysates was determined on a Synergy 2 microplate photometer—fluorometer (BioTek, United States) by the ORAC (Oxygen Radical Absorbance Capacity) fluorescence method [10] with generation of peroxyl radicals in the reaction medium [11].

The in vitro hypotensive activity in the samples of cheese whey hydrolysates was determined by their ability to inhibit angiotensin I-converting enzyme (**ACE**). ACE is a key part of the renin—angiotensin system that regulates blood pressure in humans. The most sensitive method for determining the ACE-inhibitory activity of a substance is the method based on the use of ACE substrates with internal fluorescence quenching. o-Aminobenzoyl-Phe-Arg-Lys(dinitrophenyl)-Pro was used as such substrate. The measurements were carried out on a Synergy 2 microplate photometer— fluorometer (BioTek, United States).

Molecular weight distribution. Cheese whey hydrolysates were fractionated by gel filtration. The molecular weight distribution of the whey samples and their enzymatic hydrolysates was evaluated by exclusion chromatography. The chromatographic system comprised a Varian ProStar HPLC chromatograph (United

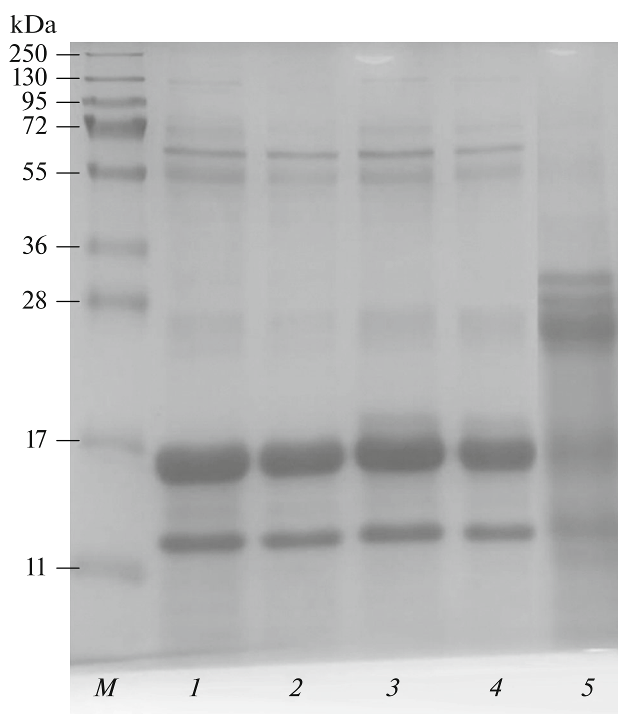

Fig. 1. PAAG electrophoresis of protein fractions of whey from Mozzarella (*1*), Gorgonzola (*2*), Cachotta (*3*), Montazio (*4*), and Circassian (*5*) cheeses. *M*—molecular weight markers.

States) with a PS210 SDM pump, a PS410 Autosampler, and a BioSep-SEC-S 2000 (7.8 × 300 mm) from Phenomenex, United States. Such columns are used to carry out the analytical separation of low molecular weight proteins and peptides by gel filtration. The column was calibrated by standard water-soluble proteins and peptides from GE Healthcare (United States), Serva (Germany) and Sigma (United States) with molecular weights ranging from 451 to 450000 Da. The optical density was detected in the range of 190–330 nm at the base wavelength of 214 nm on a photodiode array detector (Varian 335 PDA, United States). The eluent was 50 mM potassium phosphate buffer with a pH of 6.9. The elution rate was 1 mL/min, and the volume of the loaded sample was 20 μL. Samples were prefiltered through a 0.45-μm syringe filter.

Analysis of the peptide profile of the hydrolysates. The peptide profile of the whey hydrolysates was analyzed in a system comprised of an Agilent 1100 chromatograph (Agilent Technologies, United States) and an LTQ-FT Ultra mass spectrometer (Thermo, Germany). Reversed-phase gradient chromatography was used to separate peptides: for this purpose, a 1-μL sample was loaded on a column (75 μm × 12 cm, Reprosil-Pur Basic C18, 3 μm, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) [12]. The following solvents were used as a mobile phase: solvent A was H_2O–HCOOH (1000 : 1, vol/vol) and solvent B was CH_3CN–HCOOH (1000 : 1, vol/vol). The linear gradient ranged from 3 to 50% solvent B in 90 min. Mass spectrometric analysis of peptide fractions was carried out using the Xcalibur software (Thermo Electron, Germany) in a two-stage mode of automatic spectrum measurement. At the first stage, the exact masses of peptides were measured on a Fourier transform-ion cyclotron resonance-mass spectrometer (**ICR FT**) in the m/z range 100–1600 at the resolution R = 50 000 for m/z of 400. At the second stage, the three highest peaks were selected from the ICR mass spectrum, and the collision-induced fragmentation in a linear quadrupole ion trap was performed for those peaks (the number of ions was 3×10^4). Peptides were identified using the Mascot software, version 2.0.04 (Matrix Science, UK). The Uniprot Bostaurus database was used for protein identification. The automatic filtration and validation of proteins and peptides was carried out using the Scaffold 4.0 software (version Scaffold-01_07_00, ProteomeSoftware, Oregon, United States).

RESULTS AND DISCUSSION

Qualitative and quantitative composition of raw materials. The protein content of the whey from rennet cheeses differed from that of the whey from Circassian cheese (the latter belongs to the group of soft unripened cheeses). The main difference in the production technology of these cheeses is the coagulation method: Circassian cheese is produced by acidic coagulation and high-temperature treatment of casein clot without the use of bacterial cultures and rennet enzymes while hard, semihard, and soft cheeses are obtained by rennet coagulation with the addition of a lactic acid starter culture. Analysis of the protein profiles (Fig. 1) showed that the whey from hard, semihard, and soft cheeses contains 50–63% β-lactoglobulin, 19–20% α-lactalbumin, and up to 11% κ-casein. The protein profile of the whey from Circassian cheese comprises of 76% casein (including 50% β- and κ-casein and 26% α-casein), 12% β-lactoglobulin, and 12% α-lactalbumin (Table 1).

Rational design of multienzyme compositions. Mass fractions of casein and whey proteins were determined in the whey samples. As follows from Table 1, the main components of whey from rennet cheeses were α-lactalbumin (α-**LA**) and β-lactoglobulin (β-**LG**), whereas a mixture of β- and κ-caseins makes up for 76% of the whey from Circassian cheese.

The amino acid sequences of the major protein components of the studied whey samples were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/protein). The possibility of directed proteolysis yielding biologically active hydrolysates without the bitter taste was studied. For this purpose, the amino acid sequences were analyzed for the presence

of amino acid descriptors of bitter taste and biological activity.

Table 2 shows the total number of amino acids that are present in the studied proteins and define the bitter taste of potential products of proteolysis, as well as their antioxidant, antihypertensive, and antimicrobial activities. In general, the amino acid profiles of whey proteins are similar to the amino acid profile of the β- and κ-casein mixture. The only substantial difference is the content of proline, which is very low in casein.

According to the literature, high proline content is a descriptor of antimicrobial activity of peptides [13]. Thus, the use of proline-specific proteases should decrease the probability of the formation of such peptides during the hydrolysis. Analysis of the specificity of commercially available proteases and their mixtures (Table 3) [14, 15] showed that proline could inhibit the proteolytic activity of trypsin, chymotrypsin, and thermolysin, which limited the applicability of these enzymes for the proteolysis of α-LA and β-LG. Numerous matches between the descriptors of bitter taste and biological activity (especially antioxidant and antihypertensive activities) made it very difficult to obtain biologically active hydrolysates with positive organoleptic properties. Hydrophobicity is a common feature of most of those descriptors. However, alanine and isoleucine, while being present among the described descriptors of bitter taste, are almost never reported as descriptors of biological activity. According to the data on the specificity of proteases [14, 15], the use of a combination of Alcalase and Thermolysin makes it possible to cleave both N-terminal and C-terminal peptide bonds that hold alanine and isoleucine in peptides and thus to release these amino acids in the free form. This does not result in the loss of biological activity but decreases the probability of bitter peptides formation. This combination of enzymes can also hydrolyze C- and N-terminal peptide bonds of phenylalanine, leucine, and valine. This property is also typical of Protamex, an EP reported to effectively eliminate bitter taste via the specific cleavage of C- and N-terminal peptide bonds with hydrophobic amino acids.

Since the specificity of Protamex does not depend on the content of proline in proteins, it appears reasonable to use this enzyme preparation for the hydrolysis of whey proteins with high proline content. In order to hydrolyze caseins, this enzyme can be replaced by the Alcalase-Thermolysin mixture that is similar to Protamex in its specificity to the hydrolyzed peptide bonds.

Thus, an enzyme composition consisting of Alcalase and Thermolysin was chosen for the hydrolysis of whey from Circassian cheese, whereas the Protamex enzyme preparation combined with small amounts of Alcalase was chosen for the hydrolysis of whey from Mozzarella, Caciotta, Gorgonzola, and Montasio cheeses.

Table 2. Amount of biologically active amino acids in the sequences of whey proteins

α-lactalbumin, β-lactoglobulin		β-casein, κ-casein	
amino acid	amount	amino acid	amount
Bitter taste			
P	55	P	10
L	40	L	44
Y	13	Y	8
F	16	F	10
A	25	A	24
W	2	W	6
I	24	I	19
Antioxidant activity			
W	2	W	6
Y	13	Y	8
M	11	M	8
L	40	L	44
T	26	T	17
F	16	F	10
C	3	C	15
Hypotensive activity			
W	2	W	6
Y	13	Y	8
C	3	C	15
M	11	M	8
L	40	L	44
T	26	T	17
F	16	F	10
Antimicrobial activity			
H	9	H	6
R	9	R	4
P	55	P	10

Enzymatic hydrolysis of whey proteins. According to electrophoresis, the whey from Montasio, Caciotta, Gorgonzola, and Mozzarella cheeses primarily contained α-LA and β-LG. This was confirmed by analysis of the elution profiles of the whey samples. Since the initial protein composition of the studied whey samples was very similar, the same enzyme compositions were used for their hydrolysis. The elution profiles of the obtained hydrolysates also turned out to be similar. The predominant fraction of the whey from Circassian cheese consisted of casein proteins. The elution profiles of the Circassian cheese whey and its hydrolysate were significantly different from those of the other four samples studied (Fig. 2).

Table 3. Specificity of proteolytic enzymes and enzyme preparations to the hydrolized peptide bonds

Enzyme preparation	Origin	Specificity*
Proteinase K	–	P1: A, E, F, I, L, T, V, W, or Y
Trypsin	Pancreas of pigs or cattle	P1: R and K (not before P) P2: W and M
Chymotrypsin	Pancreas of pigs or cattle	P1: L, F, Y, W, and M (not before P)
Alcalase	*Bacillius licheniformis*	P1: large uncharged amino acids V, L, I, F, Y, and W
Neutrase	*Bacillius amyloliquefacience*	P'1: F, L, and V
Thermolysin	*Bacillius thermoproteolyticus*	P1': I, F, L, V, A, and M (not when P'' - P)
Protamex	*Bacillius subtilis*	P1: V, L, I, F, Y, and W P1': F, L, and V Low content of hydrophobic amino acids hydrolysates, compared with other enzymes
Pepsin (pH 1.3)	Gastric mucosa of pigs or cattle	P1': F, L, W, and Y P1: no R P2: no P P3: no H, K, and R P2': no P
Pepsin (pH 2)	Gastric mucosa of pigs or cattle	P1': F, L P1: no R P2: no P P3: no H, K, and R P2': no P

* Pn, Pn' is the C- or N-terminal location of an amino acid with respect to the hydrolyzed peptide bond; *n* is the remoteness from the hydrolyzed bond.

During hydrolysis, the high molecular weight fraction (>10 kDa) decreased significantly (from 68–78 to 11–23%), whereas the medium molecular weight (3–10 kDa) and low molecular weight (10 kDa) fractions increased (Figs. 2, 3). Medium and low molecular weight fractions account for about 40 and 55% of the obtained hydrolyzates, respectively. All hydrolysates obtained under optimal conditions had favorable organoleptic characteristics (without pronounced bitter or salty taste).

Under the selected conditions, the hydrolysis degree was 10–15% for the whey from Montasio, Caciotta, Gorgonzola, and Mozzarella cheeses, and the content of free amino acids ranged from 6 to 10%. In the case of Circassian cheese, the hydrolysis degree and the content of free amino acids increased with the duration of hydrolysis. In the latter case, the presence of Alcalase in the enzyme mixture significantly increased the degree of hydrolysis (from 16–17 to 19–23%) without a significant increase of the FAA content (Figs. 4a, 4b). The use of Thermolysin in combination with Alcalase led to a reduced (~3%) content of free amino acids in the hydrolysates of whey proteins from Circassian cheese. The optimum ratio that provided the maximum degree of hydrolysis and a moderate content of free amino acids was 2 : 1 (Figs. 4a, 4b).

Identification of biologically active peptides and in vitro verification of the biological activity of the studied whey hydrolysates. A total of 83 to 102 peptides with sizes of 8–26 amino acid residues were identified in the protein hydrolysates of whey from Caciotta, Mozzarella, Montasio, and Gorgonzola. 85 peptides with a length of 5–14 amino acid residues and an average molecular weight of 925 ± 65 Da were found in the hydrolysate of Circassian cheese whey. Approximately 61% of the total number of identified peptides originated from β- and κ-caseins. This data was in agreement with the predominance of casein fractions in the composition of the whey from Circassian cheese. A hydrolysis degree of 13–17% was achieved under the experimental conditions, and the content of free amino acids in the hydrolysates ranged from 6 to 11 mg/mL in glutamic acid equivalents. The antioxidant capacity against peroxyl radical was 450–550 μmol TE/g protein, with an exception of Circas-

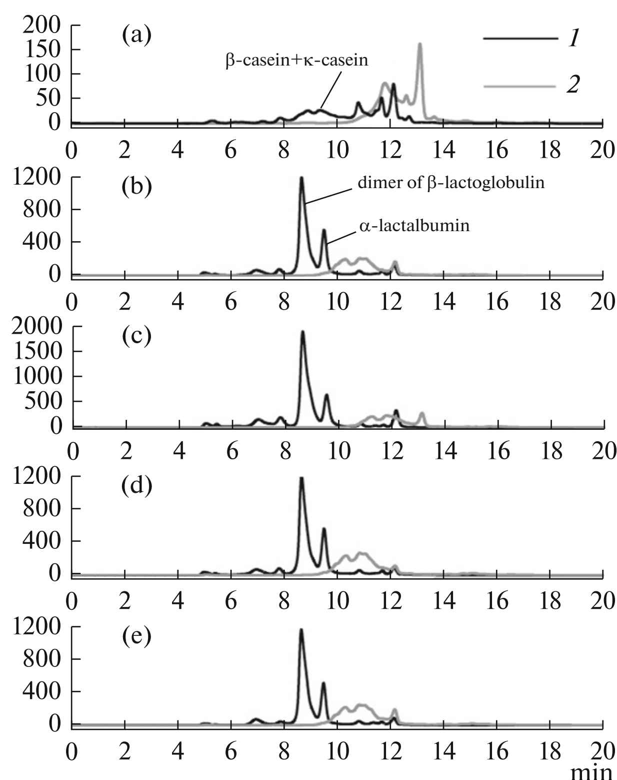

Fig. 2. Elution profiles of whey samples (*1*) and their hydrolysates (*2*): a—Circassian cheese, b—Montazio, c—Mozzarella, d—Gorgonzola, and e—Caciotta.

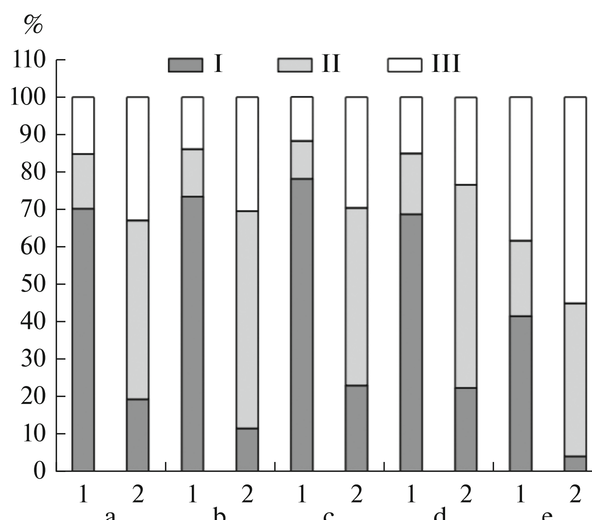

Fig. 3. Ratio of protein–peptide fractions (%) with different molecular weights in whey samples (*1*) and their enzymatic hydrolysates (*2*); a—Caciotta, b—Mozzarella, c—Montazio, d—Gorgonzola, and e—Circassian cheese. I: > 10 kDa, II: 3–10 kDa, III: < 3 kDa.

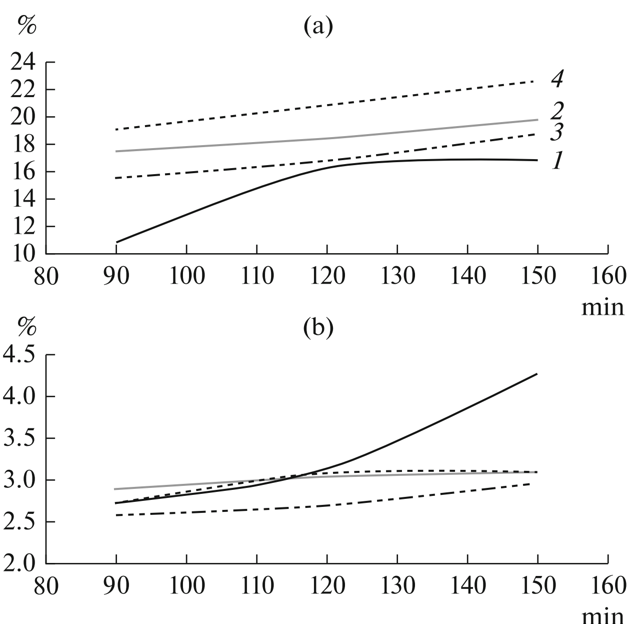

Fig. 4. Changes in the degree of hydrolysis (*a*, %) and the formation of free amino acids (*b*, %) with an increase in the duration of the hydrolysis of Circassian cheese whey proteins by Thermolysin and Alcalase at different ratios of the enzyme preparations: *1*—2.5 : 0; *2*—2.0 : 0.5; *3*—1.5 : 1.0; *4*—2.0 : 1.0.

sian cheese whey, in which a slightly higher degree of hydrolysis (21.55%) was compensated for by a decreased content of free amino acids (2.94 mg/mL in glutamic acid equivalents).

Sequence analysis of the identified peptides was performed with the BioPep database [16]. The peptides containing antioxidant, antihypertensive, and antimicrobial fragments with previously annotated activity were identified in all five hydrolysates. It should be noted that the identified peptides containing the VVPP fragment also carry a C-terminal phenylalanine (Table 4), which may significantly enhance the ACE-inhibitory activity of these peptides [17].

The LVRTPEVDDE, LVRTPEVDDEAL, LVRTPEVDDEALE, and LVRTPEVDDEALEK peptides containing the LVRT fragment (IC_{50} 1000 μM) [18] were found in almost all hydrolysates. However, since that fragment was located at the N-terminus of the peptides, its contribution in the ACE-inhibitory activity of those peptides would hardly be significant. This might also apply to the AIPPKKN, AIPPKKNQDKTEIPT, MAIPPKKNQDKTEIPT, MAIPPKKN, AIPPKKNQDKTEIPTINT, and MAIPPKKNQDKTEIPTINT peptides that contain the ACE-inhibitory fragment AIPP(IC_{50} 900 μM) [19] either at the N-terminus or in the middle of the sequence (Table 4). The ACE-inhibitory fragment VLDTDYK (IC_{50} 946 μM) [20] was also rather common. The greatest amount of fragments with annotated ACE-inhibitory activity was present in the hydrolysate of Circassian cheese whey. This whey sample was also characterized by the smallest size of the identified peptides (5–14 amino acid

Table 4. Hypotensive peptides identified in whey hydrolysates

Precursor protein	Identified peptide	Whey sample*	ACE-inhibitory peptide	IC_{50}, μM
α-LA	NNDSTE**YGLF**	1	**YGLF**	733 [19]
	IVQNNDSTE**YGLF**	2		
	AIVQNNDSTE**YGLF**	1, 2, 4		
β-LG	**LVRT**PEVDDE	1–4	**LVRT**	1000 [17]
	LVRTPEVDDEAL	1–4		
	LVRTPEVDDEALE	1–5		
	LVRTPEVDDEALEK	1–4		
	VLDTDYKK	2	**VLDTDYK**	946 [20]
	L**VLDTDYK**KY	1, 2, 4		
	VLDTDYKKY	6, 7, 8, 9, 10		
	VLDTDYKKYL	1–4		
	VLDTDYKKYLL	3		
	DTDYKK**YLLF**	4	**YLLF**	172 [16]
	DIQK**VAGTWY**S	4	**VAGTWY**	1682 [16]
	K**VAGTWY**SL	4		
κ-casein	**AIPP**KKN	5	**AIPP**	900 [19]
	AIPPKKNQDKTEIPT	2, 3, 5		
	M**AIPP**KKNQDKTEIPT	2, 3, 4		
	M**AIPP**KKN	1–4		
	AIPPKKNQDKTEIPTINT	1, 3		
	M**AIPP**KKNQDKTEIPTINT	3		
	SRYPS	5	**SRYPS**	[26]
	F**LPYPY**	5	**LPYPY**	28.90 [28]
	M**ARHPHP**H	5	**ARHPHP**	[26]
β-casein	**VVPP**F	5	**VVPP**	258.21 [29]
	TPV**VVPP**F	1–5		
	IPPLTQTPV**VVPP**F	2		
	NIPPLTQTPVVVPPF	1,2	**NIPPLTQTPV, VVPP**	173 [29]
	SLPQ**NIPPLTQTPVVVPP**F	1–4		
	NIPPLTQTPVVVPPFL	1		
	VYPFPGPIP	5	**VYPFPG**	221 [29]
	VYPFPGPI	4, 5	**VYPFPGPI**	500 [30]
	VYPFPGP	5	**YPFPGPIP**	500 [19]
	VYPFPGPIHN	1, 2, 4	**YPFPGPI**	500 [19]
	V**YPFPGPIP**NS	5		
	L**VYPFPGPI**HN	1, 2, 4		
	L**VYPFPGPIP**N	1, 5	**LVYP**	170 [31]
	LVYPFPGP	4, 5		
	QSEEQQQTEDELQ**DKIHPF**	4, 5	**DKIHPF**	257 [32]
	QQQTEDELQ**DKIHPF**	3		
	YQEPVLGPVRGPFP	4	**YQEPVL**	280 [19]
	YQEPVLGPVRGPFPI	4		
	EMPFPKYPVEPF	4	**EMPFPK**	423 [20]
	KVLPVPQK	4, 5	**KVLPVPQ**	1000 [33]
	AVPYPQ	5	**AVPYP**	80 [19]
	K**AVPYP**Q	5		
	AVPYPQRDMP	5	**AVPYPQR**	15 [34]
	LHLPLP	5	**LHLPLP**	2.90 [31]
	LHLPLPL	5	**LHLPLPL**	432.7 [31]

* Whey samples: 1—Caciotta, 2—Mozzarella, 3—Montazio, 4—Gorgonzola, 5—Circassian cheese.

Table 5. Biological activity of whey samples and their hydrolysates

Sample	Antioxidant activity, μM TE/g protein	ACE-inhibitory activity, IC_{50}, mg protein/L
Caciotta		
Whey	39.5 ± 13.0	33481 ± 112
Hydrolysate	417.2 ± 22.0	2563 ± 32
Mozzarella		
Whey	170.8 ± 14.0	16162 ± 98
Hydrolysate	517.8 ± 57.2	1572 ± 28
Montazio		
Whey	97.2 ± 7.9	25703 ± 96
Hydrolysate	511.4 ± 25.1	2242 ± 133
Gorgonzola		
Whey	124.2 ± 11.1	22599 ± 56
Hydrolysate	424.7 ± 14.3	1681 ± 34
Circassian cheese		
Whey	154.3 ± 7.4	3548 ± 113
Hydrolysate	497.1 ± 62.9	537 ± 25

residues) and the maximum ACE-inhibitory activity of the hydrolyzate (Table 5).

The largest amount of peptides containing the residues of redox-active amino acids (tyrosine, tryptophan, methionine, cysteine, histidine, and phenylalanine) [21] was found in the hydrolysate of Gorgonzola cheese whey; these peptides accounted for approximately 75% of the total number of identified peptides. In the hydrolysates of the Caciotta, Mozzarella, Montasio, and Circassian cheese whey samples, 58, 63, 70, and 66% peptides, respectively, contained redox-active amino acids. The most common antioxidant fragment in the composition of the identified peptides was YVEEL. The antioxidant activity of the YVEEL peptide obtained by the hydrolysis of β-lactoglobulin was 0.799 μM TE/μM peptide (against peroxyl radical) (Table 6) [22]. In general, the antioxidant capacity of the studied hydrolysates was slightly lower than that of β-lactoglobulin (700–2100 μM TE/g protein) and α-lactalbumin (1000–3000 μM TE/g protein) hydrolyzed by various proteases [22]. However, due to the low degree of hydrolysis, the organoleptic characteristics of the obtained hydrolysates were so favorable that those hydrolysates could be considered as functional food ingredients.

The following antioxidant fragments were identified in the peptides of the Gorgonzola hydrolysate: WYSL (4.51 μM TE/μM) [23] in the KVAGTWYSL

Table 6. Antioxidant peptides identified in whey protein–peptide hydrolysates

Protein precursor	Identified peptide	Whey number*	Antioxidant peptide	Antioxidant activity
β-LG	V**YVEEL**KPTPE	2, 3	**YVEEL**	0.799 μM TE/μM, ORAC [22]
	V**YVEEL**KPTPEGDLEIL	2, 3, 4		
	V**YVEEL**KPTPEGDLEILL	1		
	V**YVEEL**KPTPEGDLE	1–4		
	KVAGT**WYSL**	4	**WYSL**	4.51 μM TE/μM, ORAC [23]
κ-casein	**SRYPS**	5	**SRYPS**	– [25]
	M**ARHPHP**H	5	**ARHPHP**	– [25]
β-casein	**YQEPVLGP**VRGPFP	4	**YQEP** **YQEPVLGP**	0.102 ± 0.10 mM, TEAC [23]
	YQEPVLGPVRGPFPI	4		0.124 ± 0.11 mM, TEAC [24]
	KVLPVPQK	4	**KVLPVPQ** **VLPVPQ**	– [25]
	VLPVPQK	5		
	A**VPYPQ**	5	**VPYPQ**	2450 μM, ORAC [27]
	KA**VPYPQ**	5		
	AVPYPQRDMP	5	**AVPYPQR, VPYPQ**	– [25]

* Whey number: 1—Caciotta, 2—Mozzarella, 3—Montazio, 4—Gorgonzola, 5—Circassian cheese.

peptide; YQEP (0.102 ± 0.10 mM TE) and YQEPVLGP (0.124 ± 0.11 mM TE) [24] in the peptides YQEPVLGPVRGPFP and YQEPVLGPVRGPFPI; and KVLPVPQ, which can inhibit lipoxygenase activity [25], in the KVLPVPQK peptide of the casein origin. The antioxidant peptide SRYPS was identified in the hydrolysate of Circassian cheese whey. The activity of this peptide was confirmed in a system that involved ABTS-cation radical quenching [26]. The same hydrolysate contained peptides with the following antioxidant fragments: VLPVPQ [27] in VLPVPQK, ARHPHP [28] in MARHPHPH, and AVPYPQR [25] and VPYPQ (2450 μM TE/mM, ORAC) [27] in AVPYPQ, KAVPYPQ, and AVPYPQRDMP. Almost all the peptides identified in the hydrolysates of whey from Mozzarella and Montasio—with the exceptions of KFDKALKALPMH, NDECAQKK, ENDECAQKK (β-lactoglobulin), AIPPKKNQDKTEIPT, IPPKKNQDKTEIPT (κ-casein), KFGERALKAW (serum albumin), QQQTEDELQDKIHP, QQQTEDELQDKIHPF (β-casein), and LENTVKETIKY (Glycosylation-dependent cell adhesion molecule) — were also present in the Caciotta and Gorgonzola cheese whey hydrolysates with lower antioxidant capacity (Table 6). The relatively high antioxidant capacity of the Circassian cheese whey hydrolysate can be accounted for the presence of unique peptides containing hydrophobic and aromatic amino acid residues: SLPEW, VSLPEW, and GVSLPEW of the β-lactoglobulin origin; VRSPAQ, YQQKP, TVPAKS, SNTVPAK, and LSNTVPAK of the κ-casein origin; MHQPHQPLPPT, MHQPHQP, AMAPK, INKKI, LNVPGE, VLPVPQ, VMFPPQ, MPFPKYP, HKEMPFPKYP, MAPKHKEMPFPKYP, VMFPPQS, and FPGPIPN of the β-casein origin.

CONCLUSIONS

With the use of bioinformatics, hydrolysates with predetermined antioxidant and antihypertensive activities were obtained from whey samples with different protein compositions.

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