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Nutritional evaluation of whey protein hydrolysate: chemical composition, peptide profile, and osmolarity

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Abstract

The expediency of processing whey is confirmed by the value of its composition, amount of raw materials, and the possibility of obtaining a wide range of food products and ingredients based on it. The most appropriate technological method for reducing the antigenicity of whey proteins in the food industry is enzymatic hydrolysis. The work aims to study the chemical composition and properties of hydrolysis products of cheese whey UF concentrate with enzyme preparations Promod 439L and Flavorpro 766MDP to determine processing areas of the obtained hydrolysate. At the same time, 65% and 40% of peptide bonds in the antigenic epitopes of β-lactoglobulin and α-lactoalbumin, respectively, were broken without a significant increase in the osmolarity of the food system (291±2 mOsm/dm^3 in the hydrolysate). Among the products of proteolysis, short-chain peptides predominate. The resulting product indicates reduced allergenicity and high content of essential amino acids. The resulting hydrolysate can be used in the technology of various dairy products to replace skimmed milk in the preparation of a normalized mixture, manufacture of products for preventive nutrition of people allergic to cow's milk proteins, and as the main ingredient in beverages for sports nutrition.

Keywords: ultrafiltration concentrate of cheese whey; enzymatic hydrolysis; molecular weight distribution; peptide profile; chemical composition; physicochemical properties.

Practical Application: The results can be used to replace skimmed milk in normalized mixture and as main beverage ingredient.

Introduction

The Food Security Doctrine of the Russian Federation for the period until 2030 aims at providing the population with high-quality and safe food products for an active and healthy lifestyle. At the same time, resource conservation, compliance with environmental safety, rational use of natural resources, and environmental protection are current priority areas in the course for sustainable development of the state. The main task of food enterprises in the Russian Federation is to reduce the negative impact of their economic activities on the environment by introducing the best available technologies. They (concerning the dairy industry) focus on the processing of secondary raw materials (whey) with an annually growing volume (Trindade et al., 2019; Khramtsov, 2019). In Russia, the total volume of whey in 2020 was about 10 million tons. In terms of absolutely dry matter, it amounts to a few tens of tons.

The expediency of processing whey is confirmed by its valuable protein-carbohydrate-mineral composition, availability of many raw materials (1/5 of the volume of milk processed in the Russian Federation), and opportunities for obtaining a wide range of food products and ingredients with a high added value on its basis. That is possible due to the fractionation of whey components by the size of their molecules. Micro-, ultra-, infiltration, reverse osmosis, and electrodialysis are utilized. At the same time, whey protein concentrates are the highest in demand because of their ability to exhibit important physicochemical properties, such as fat- and moisture-retaining, emulsifying, and performing some technological functions in food systems (Guralnick et al., 2021). In addition, they do not have the status of food additives and "E-index" therefore provide a "clean" label of the finished food product (Nastaj et al., 2019). One of the main disadvantages of whey is the allergenicity of protein components, especially β-lactoglobulin, the content of which is up to 50% of all whey proteins (Agarkova et al., 2020). A β-lactoglobulin molecule contains some linear IgE-binding epitopes, some of which are potentially allergenic since their size is no more than 5 amino acid residues.Reduction of the whey protein complex antigenicity is possible due to the removal or cleavage of high-molecular allergenic components, including β-lactoglobulin (Knol et al., 2019). Since native whey proteins show their immunoreactivity only when the molecule retains the tertiary structure, various technological methods (heat treatment, high-pressure application, enzymatic hydrolysis, etc.) can change their allergenic potential (Wal, 2004). As a result, conformational transitions occur in protein molecules. That leads to changes in the structure and digestibility of denatured proteins, as well as to destruction or limited access to antigenic epitopes. Prolonged heating of dairy raw materials reduces its nutritional value and can result in lower solubility and insufficient digestibility of the product, which is a significant disadvantage for reducing allergenicity by thermal denaturation.

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The main thermodynamic approach to modifications caused by high hydrostatic pressure is based on the compressibility of molecules and changes in their volume (ΔV) (Lullien-Pellerin & Balny, 2002). Such a physical impact leads to an equilibrium shift in favor of the smallest total volume. The pressure has a destructive effect on the quaternary (200 MPa) structure of most globular proteins but has a relatively little impact on the secondary structure (>300 – 700 MPa). Protein denaturation includes dissociation of oligomeric proteins, unfolding, and aggregation. The covalent bonds of the protein remain intact. These changes depend on the structure and concentration of the protein, pressure, temperature, pH, ionic strength, and the composition of the solvent. Denaturation under pressure is an easily controlled process, and it causes less significant rearrangements in the protein globule than temperature or chemical exposure (Mazri et al., 2012).

Reducing the allergenic potential of whey proteins is possible due to the removal of lactoglobulins from milk using selective specific sorbents. The greatest interest is the precipitation of protein substances using such a specific sorbent as chitosan, which can selectively interact with various milk proteins, forming an insoluble complex. It should be noted that chitosan is a low-toxic and biodegradable polymer (Chen et al., 2015; Evdokimov et al., 2015).

In the food industry, enzymatic hydrolysis of whey proteins is the most appropriate technique, which allows obtaining hydrolysates with given molecular weight distribution and residual antigenicity (Eberhardt et al., 2019; Brandelli et al., 2015; Martorell-Aragonés et al., 2015). The choice of enzymes is justified by their specificity concerning the type of peptide bond, which determines the existence of products with different degrees of protein hydrolysis. An important advantage of using protein proteolysis is the possibility of obtaining easily digestible short-chain peptides that can have a certain physiological effect on the human body (Brandelli et al., 2015; Tavares et al., 2011; Prosekov et al., 2016; Bamdad et al., 2017).

Hydrolysates are a mixture of peptides and free amino acids, some of which can give products of hydrolysis a bitter taste or aftertaste. This is due to the cleavage of polypeptide bonds between amino acids containing hydrophobic lateral chains in their structure. Hydrophobic lateral chains in the original proteins and polypeptides are not available for binding to the tongue receptors and subsequent taste recognition, and therefore, they are not characterized by a bitter taste. In the process of proteolysis, peptides of a shorter length are formed. This results in the release of submerged hydrophobic areas onto the surface of the molecule, so they become accessible to the language receptors, and the bitter taste of hydrolysate increases. According to the literature, peptides having average hydrophobicity of amino acid side residues less than 1300 kcal/mol have no bitter taste (Acquah et al., 2018; Iwaniak et al., 2020).

The ability of peptides to develop bitterness also depends on the molecular weight. The stereochemical selectivity concerning receptor sites located in each receptor cavity is associated with the factors of molecular volume (Fennema et al., 2017). Peptides with a molecular weight of less than 6kDa can be potentially bitter since larger peptides cannot penetrate the receptor sites.

To obtain hydrolysates with suitable organoleptic properties, it is advisable to use combinations of proteinases and peptidases for regulated protein hydrolysis (Song et al., 2020).Concerning the presented relevance, the purpose of the research was to study the chemical composition and properties of hydrolysis products of cheese whey UF concentrate with enzyme preparations Promod 439L and Flavorpro 766MDP to determine processing areas of the obtained hydrolysate.

Promod 439L is a serine protease (from *Bacillus licheniformis*), the substrate for which is both β-lactoglobulin and α-lactoalbumin. Flavorpro766 MDP is a mix of amino- and carboxypeptidases (from *Aspergillus spp.*). It is an enzyme that can exhibit the properties of both exo- and endopeptidase, cleaving off amino acids with high hydrophobicity and thus reducing the bitterness of the resulting peptides (Biocatalysts, 2021).To achieve this goal, the following tasks have been defined:

— To identify free amino acids and establish an amino acid sequence of peptides formed as a result of hydrolysis in the UF concentrate of cheese whey with enzyme preparations, Promod 439L and Flavorpro766 MDP;

— To predict a decrease in the allergenicity of the hydrolysate based on the analysis of molecular mass distribution, the peptides formed, their length, and changes in electric charge;

— To evaluate the quality of the obtained hydrolysate and ascertain the advisability of using it in the technology of specific food product line groups.

2 Materials and methods

2.1 Objects and methods of the research

The objects of the research were: cheese whey, ultrafiltration concentrate (UF concentrate) of cheese whey, and its hydrolysate obtained using enzymes Promod 439L and Flavorpro 766MDP (Biocatalysts Limited, Cardiff, Wales, United Kingdom). All raw materials used in the research, according to organoleptic, physical, chemical, and microbiological parameters, met the requirements of TR CU 021/2011 and 033/2013 (Table 1).

A sampling of research objects and their preparation for analysis were carried out following GOST 26809.1-2014 "Milk and Dairy Products. Acceptance Rules, Sampling Methods and Sample Preparation for Analysis. Part 1. Milk, Dairy products, Dairy Components and Milk-containing Products (as amended)". To study physical and chemical parameters, as well as to identify the chemical composition of raw materials and experimental samples, standard arbitration, and commonly accepted research methods, described in various regulatory documents of the Russian Federation, as well as modified, improved, and special techniques involving application of modern devices and information technologies were used.

Table 1. Chemical composition and properties of cheese whey and its UF concentrate.

Name of the indicator	Cheese whey	Cheese whey UF-concentrate with concentration factor 3.9
pH	6.61 ± 0.04	6.53 ± 0.04
Milk in dry matter, %	6.31 ± 0.50	8.60 ± 0.50
Fat in dry matter, %	0.05 ± 0.05	0.10 ± 0.05
Total protein in dry matter, %	0.82 ± 0.06	3.19 ± 0.06
Lactose in dry matter, %	4.91 ± 0.70	4.58 ± 0.70
Ash in dry matter, %	0.52 ± 0.15	0.61 ± 0.15
Coliform bacteria	not detected	not detected
MATOMO, CFU/dm^3	$3 \cdot 10^3$	$8 \cdot 10^3$

2.2 Production of UF concentrate and hydrolysate of whey proteins

The ultrafiltration of whey was carried out at PJSC Voronezh Dairy Plant using MMS (Kalacheevsky cheese factory, Kalach town, Voronezh region, Russia) Swissflow UF installation with ceramic membranes. The protein concentration factor was 3.8 – 3.9, total mass fraction of protein in the concentrate was not less than 3.0% (Melnikova & Bogdanova, 2021).

The resulting UF concentrate of cheese whey was hydrolyzed using the enzymes Promod 439L and Flavorpro 766MDP in the amount of 1.5% and 3.0% of the total protein weight in the UF concentrate, respectively, for (6 ± 0.5) hours at a temperature of (52 ± 2) °C (Melnikova & Bogdanova, 2021). The enzyme Promod 439L cleaves the peptide bonds formed by the carboxyl group of glutamic acid, and Flavorpro 766MDP provides additional fragmentation of large peptides (Biocatalysts, 2021).The enzyme was inactivated by heating the mixture to a temperature of (80 ± 2)°C and holding it for 5 minutes. The hydrolyzed mixture was filtered and cooled to t = (4 ± 2)°C.

2.3 Analysis of the amino acid sequence of proteins and peptides

The analysis of an amino acid sequence of proteins and peptideswas conducted in the Research Equipment Sharing Center "Structural and functional studies of proteins and RNA" of the Institute of Protein Research, Russian Academy of Sciences using the chromato-mass spectrometric method. The Mini-PROTEAN Tetra System (BIO-RAD, CA, USA) was used for the electrophoretic separation of the sample proteins, and the nanoflow chromatograph Easy-nLC 1000 (Thermo Fisher Scientific, MA, USA) was used for mass chromatographic analysis of the resulted proteins. The detection was carried out using a high-resolution mass spectrometer Orbitrap Elite ETD (Thermo Fisher Scientific, MA, USA). The separation was made on a capillary column 150 mm long with a diameter of 75 microns filled with the phase Aeris 1.7 microns PEPTIDE XB-C18 (Phenomenex, CA, USA) under laboratory conditions. The analysis of mass spectrometric data was performed using the commercial program PeaksStudio 7.5 (Bioinformatics Solutions Inc. (BSI), Waterloo, Canada). *De novo* sequencing was used to identify the results of mass spectrometry.

2.4 Free amino acid content and total content of certain amino acids

The amino acid composition of whey protein hydrolysate was evaluated in the Research Equipment Sharing Center "Control and Management of Energy-efficient Projects" at Voronezh State University of Engineering Technologies by ion-exchange chromatography with post-column derivatization of ninhydrin using liquid chromatograph Shimadzu LC-20 Prominence (Shimadzu Europa GmbH, Germany) according to GOST 32195-2013 and GOST 32201-2013.

2.5 Microstructure analysis

Microscopic examination of the samples was carried out using the preparation "crushed drop", the Altami Bio 1 microscope (Altami Ltd., Saint Petersburg), and the Canon camera adaptor at a magnification of 600/0.85 times.

2.6 Osmolarity

It was calculated by multiplying the osmolality of the solution by its density. Osmolality was determined according to GOST R 55578-2013 using an automatic cryoscopicosmometer OSMOMAT 030 D (Gonotec GmbH, Berlin, Germany) in the Moloko testing laboratory of the All-Russian Research Institute of the Dairy Industry.

2.7 Statistical analysis of the results obtained

Experimental studies of each sample were replicated 5-10 times in a three-time sequence. Calculations, charting and their description were carried out by mathematical statistics using Microsoft Office 19 applications for Windows (Microsoft Corporation, WA, USA). The results are presented taking into account the errors found by the least-squares method. The confidence interval is $P > 0.95$.

3 Results

Since the degree and the rate of proteolysis significantly depend on the position of hydrolyzable bond in protein carbon chain and the chemical nature of side groups of neighboring amino acid residues, an increase in the ratio between the substrate and the enzyme by ultrafiltration, as well as the duration of incubation up to (6.0 ± 0.5) hours is provided.

In the course of experimental studies, the efficiency of proteolysis in cheese whey UF concentrate was evaluated, the amino acid sequence of peptides present in the hydrolysate, as well as their length, molecular weight, charge, were identified, the average hydrophobicity was calculated. To obtain objective data, similar indicators were determined in a cheese whey UF concentrate sample. The chemical composition and physicochemical properties of whey proteins hydrolysate were identified (Table 2).

The amino acid sequence is represented in a single-letter code. The sections determined during mass spectrometric analysis are highlighted in gray (i.e., at least one set of peptides was found that covers, sequentially or overlapping, the marked part of this sequence). Examples of such peptides are marked in blue under the corresponding sections of the sequence (Figure 1). Individual sections of the protein sequence with the most representative peptides are clearly visible.

Table 2. Chemical composition and properties of whey proteins hydrolysate

Name of the indicator	Value
Moisture, %	91.42 ± 0.50
Dry matter, % including:	8.58 ± 0.50
Total protein, %	3.18 ± 0.06
Total nitrogen, %	0.508 ± 0.01
Nonprotein nitrogen, %	0.321 ± 0.01
True protein, %	1.17 ± 0.06
Whey proteins, %, including:	0.88 ± 0.06
β-lactoglobulin, mg/cm^3	0.76 ± 0.06
α-lactoalbumin, mg/cm^3	4.45 ± 0.06
Lactoferrin, mg/cm^3	0.034 ± 0.006
Lactose, %	4.51 ± 0.70
Fat, %	0.10 ± 0.05
Ash, %	0.62 ± 0.15
pH	6.45 ± 0.04
Osmolarity, mOsm/dm^3	291 ± 2

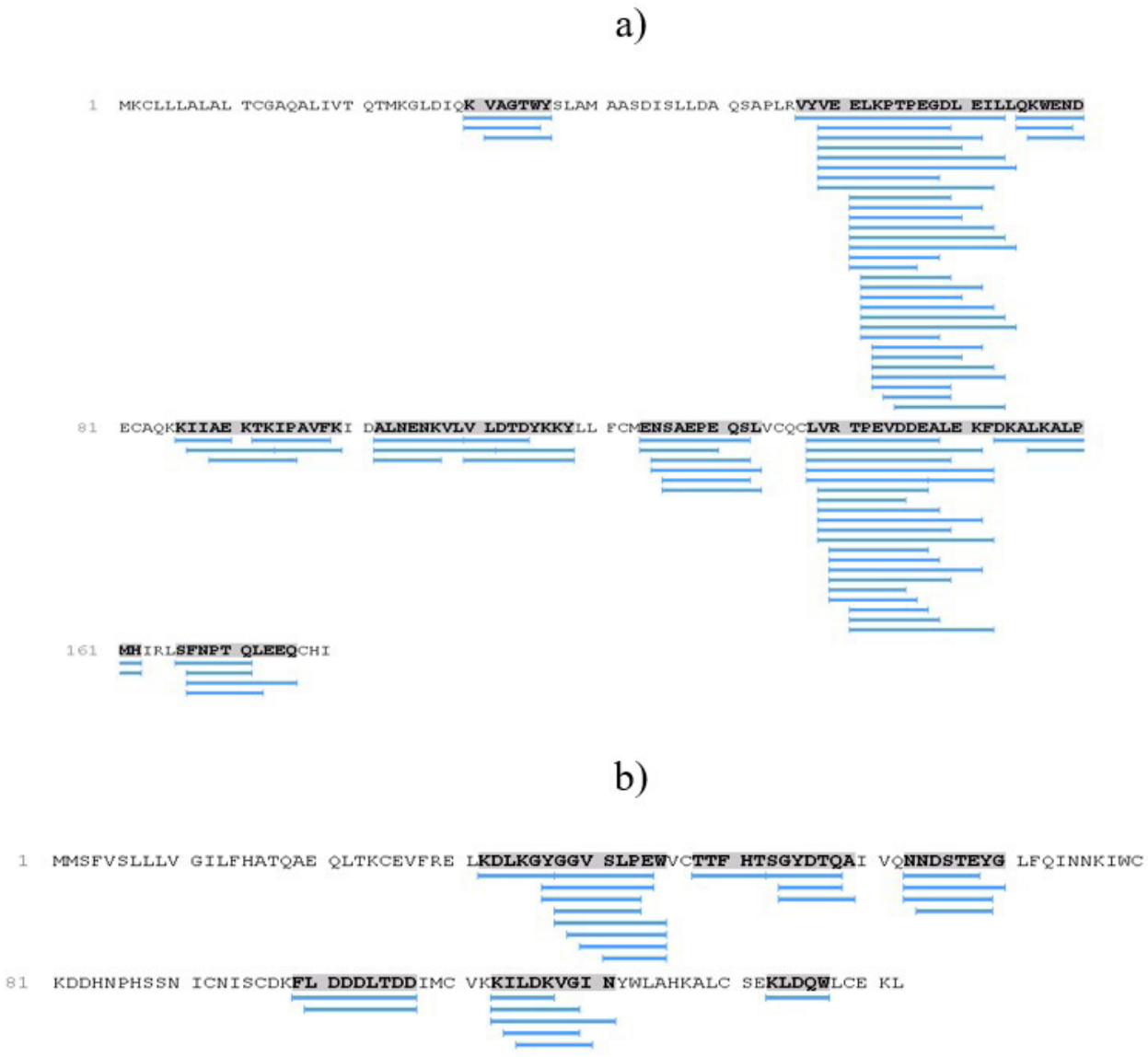

Figure 1. Peptides of whey protein hydrolysate—derivatives of: (a) β-lactoglobulin; (b) α-lactoalbumin.

The experimental sample contains β-lactoglobulin derivatives containing from 5 to 17 amino acid residues with a molecular weight of 561 to 1943 Da, as well as α-lactoalbumin derivatives containing from 5 to 9 amino acid residues with a molecular weight of 528 to 1067 Da (Figure 2). The length of the peptides-derivatives of β-lactoglobulin and α-lactoalbumin present in cheese whey UF concentrate was between 5 and 25 amino acid residues with a molecular weight of 624 to 2817 Da.

Proteolysis contributed to the change in peptides charge due to the cleavage of some amino acids and their redistribution on the surface, which resulted in a decrease in the self-association of peptides and overall hydrophobicity. At the same time, the solubility of the nitrogen-containing components of the hydrolysate dry matter increased as a result of the destruction of the double electric layer of protein molecules. This led to an increase in their resistance to precipitation during high-temperature pasteurization (Figure 3) (Krameret al., 2012).

4 Discussion

According to the results of the conducted studies, it has been found that β-lactoglobulin and α-lactoalbumin were subjected to hydrolysis to a greater extent. Moreover, 65% and 40% of the

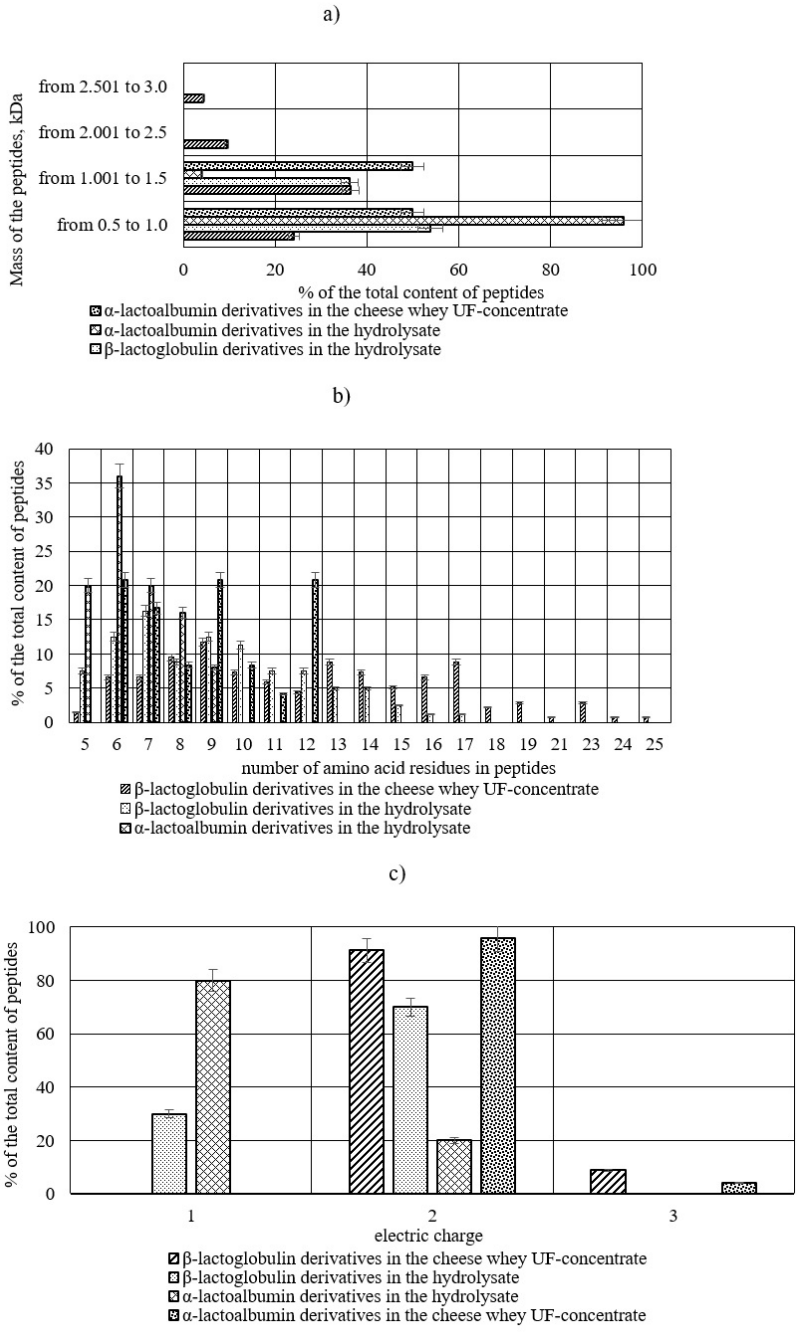

Figure 2. Distribution of peptides identified in experimental samples: (a) by molecular weight; (b) by length; (c) by charge.

peptide bonds in the antigenic epitopes of β-lactoglobulin and α-lactoalbumin, respectively, were broken without a significant increase in the osmolarity of the food system.Among the products of proteolysis, short-chain peptides predominate. The content of free amino acids that cause the bitter taste of hydrolysate (phenylalanine, tryptophan, tyrosine, leucine) changed slightly in comparison with the initial UF concentrate (Figure 4). Therefore, the resulting whey protein hydrolysate is characterized by reduced allergenicity, high content of essential amino acids as well asbranched chain, andabsence of bitter taste. It can quickly replenish the loss of fluid and energy in the body without causing an osmotic imbalance in the gastrointestinal tract.

According to the literature (Tavares et al., 2011; Prosekov et al., 2016; Bamdad et al., 2017), some short-chain peptides can exhibit antioxidant properties due to the presence of certain amino acids in them. The binding of free radicals by peptides occurs by hydrophobic terminal amino acids, such as *Ala* (A), *Pro* (P), *Val* (V), *Ile* (I), *Leu* (L), *Phe* (F), *Trp* (W), *Tyr* (Y) and *Met* (M). In addition, amino acids with aromatic residues (histidine (H) and proline (P)) can donate electrons to charged radicals. Since these amino acids are present as the terminal residue of peptides formed as a result of proteolysis (Figure 1), whey protein hydrolysate can be recommended as an effective tool to deal with oxidative stress.

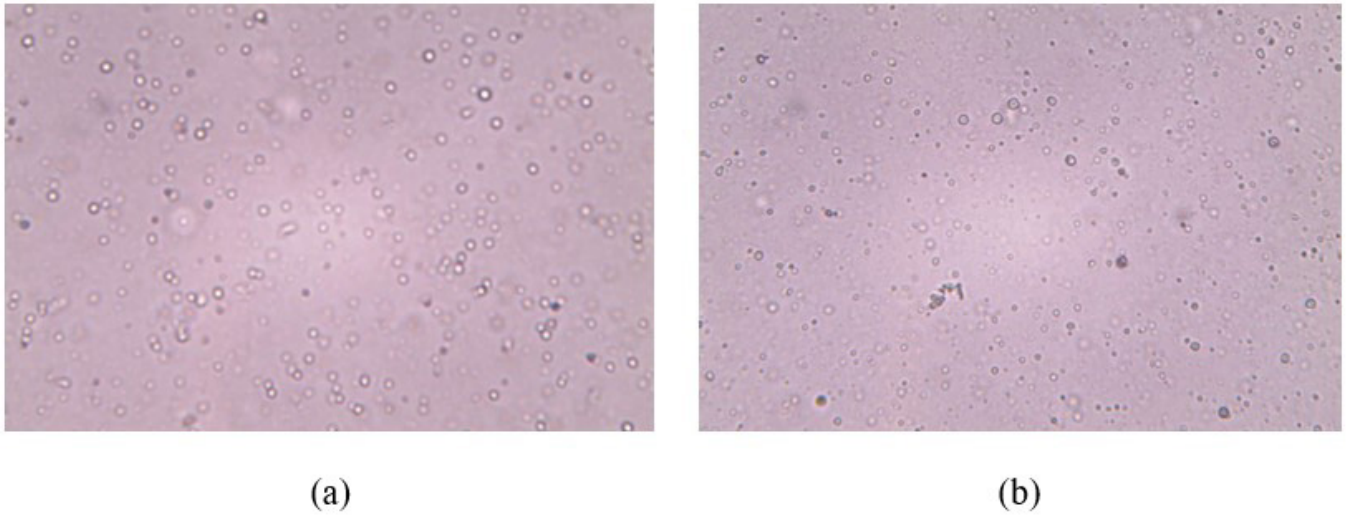

Figure 3. Microstructure of the studied samples after pasteurization at t = (80 ± 2) °C, τ = 15 sec (magnification 600/0. 85): (a) cheese whey UF concentrate; (b) hydrolysate of whey proteins.

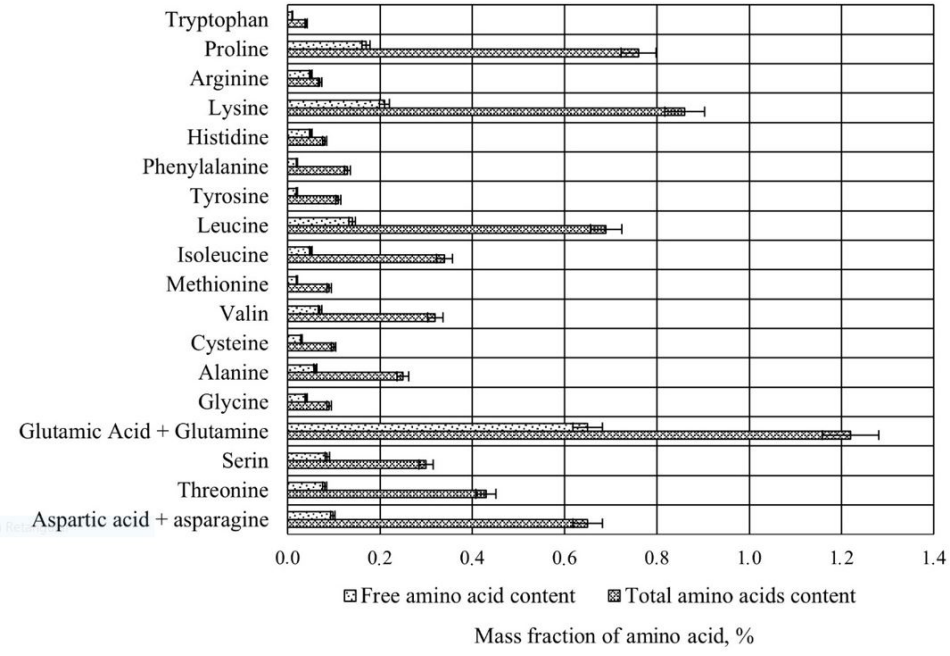

Figure 4. Total content and content of free amino acids in whey protein hydrolysate.

The limitations of the conducted experimental studies are the difficulties associated with the complete inactivation and separation of enzyme preparations from hydrolysate, which affect the reliability and interpretation of the results obtained.

5 Conclusion

Based on experimental data, the effectiveness of hydrolysis of proteins in cheese whey UF concentrate to obtain low osmolarity short-chain peptides with suitable organoleptic properties using enzymes Promod 439L and Flavorpro 766MDP has been proved. Further research will focus on the storage ability and determination of quality and safety parameters of whey protein hydrolysate. The resulting hydrolysate can be used in the technology of various dairy products to replace skimmed milk when making a normalized mixture and also as a main ingredient of beverages for sports nutrition taking into account sensory profiling of the products (Tirloni et al., 2019). This allows expanding the assortment line of domestic products for preventive nutrition of people suffering from allergies to cow's milk proteins, as well as ensuring import substitution in the segment of functional food products.

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