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# Bioinformatic Modeling (In Silico) of Obtaining Bioactive Peptides from the Protein Matrix of Various Types of Milk Whey

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**Abstract:** Whey is a by-product of the production of various types of cottage cheese and cheese, casein, and coprecipitates. Conditions of milk coagulation directly affect the physico-chemical properties of whey and the formation of its protein profile. This fact makes it difficult to standardize the protein profile of milk whey for its further processing. Whey proteins have a great potential to release a wide range of bioactive peptides (BAP), capable of reducing the risk of a number of chronic food-related diseases. Computer modeling of an enzymatic hydrolysis of proteins is one of the ways to increase the efficiency of BAP release studies and to reduce the number of labor consuming experiments. This research is aimed at generating a digital model of the peptide complex of different whey types with predicted bioactivity, safety, and sensory properties using bioinformatic modeling approaches. The study was performed with the use of the proteomic databases tools according to the algorithm of hybrid strategy of bioinformatic modeling developed earlier. As a result of the study, taking into account the ranking of the proteins ratio in the protein profile, the hydrolysis by the protease complex chymotrypsin C-subtilisin was characterized as the maximum efficacy method to release peptides with both antioxidant and ACE-inhibitory activity. It was also observed that the bioactive peptides obtained as a result of in silico hydrolysis after GI digestion simulation can be considered safe in terms of allergic reactions and toxicological effects.

**Keywords:** whey; protein; bioactive peptides; in silico; antioxidative; ACE-inhibitory; DPP-IV-inhibitory; allergenicity; toxicity



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## 1. Introduction

Whey is a by-product of the production of various types of cottage cheese and cheese, casein, and coprecipitates. The yield of whey can be up to 85–90% of the raw milk. Today, the worldwide production of whey is about 190 million tons per year [1]. However, only around 50% of this whey is returned to the production chain. The remaining volume of whey is often disposed of with the wastewater without purification or processed for animal feed [2]. Whey is an organic contaminator with high BOD (40–60 g/L) and COD (50–80 g/L) values. In spite of this fact, there is a tendency to dispose of whey with wastewater. This is caused by high technical and material costs to process whey for food purposes [3]. Additionally, whey type determines the way of its processing due to specific properties of each of them. This also makes it difficult to introduce the idea of valorization on a large scale at milk processing plants [4,5]. In turn, whey type is determined by the type of used coagulation (heat and acid, rennet, acid and rennet, or heat in the presence of $Ca^{2+}$ coagulation), which helps separate the milk phase into a clot and a whey. Conditions of milk coagulation directly influence the formation of whey protein profile. This is caused by different isoelectric points of various casein fractions and whey proteins. The range of isoelectric points is from 3.3 (for proteose-peptone fraction) to 8.8 (for lactoferrin) [6,7].

Therefore, research into standardization of the whey composition and the development of new approaches to its processing are still relevant.

The development of modern analytical methods in the field of food peptidomics is a cause of interest in whey proteins. Whey proteins have a great potential to release a wide range of bioactive peptides (BAP), capable of reducing the risk of a number of chronic food-related diseases [8–10]. In particular, it was proven that enzymatic digestion of whey proteins in the GI tract, directional hydrolysis with commercial enzymatic preparations, or starter cultures' enzymes can lead to the release of BAP. These BAP show ACE and DPP-IV-inhibitory activity, as well as antioxidant, antimicrobial, immunomodulatory, and other properties [11,12]. At the same time, the study [13] reports that the enzymatic method, in terms of the safety, is preferable to hydrolysis with chemical reagents (alkalis or acids). According to [14], hydrolysis with commercial enzymatic preparations, compared to starter cultures' proteases, is more direct and effective in terms of enzyme stability to the environmental conditions and reproducibility of the results. Due to the functional properties of BAP, their use in the technology of dairy products with therapeutic and preventive effects is prospective.

Computer modeling of the enzymatic hydrolysis of whey proteins in silico is one of the ways to increase the efficiency of research on the release, identification, bioactive action assessment, allergenicity, and toxicity of BAP and reduce the number of expensive and labor-intensive experiments. The bioinformatic analysis in silico is reasonable to use in the preliminary stage of investigation of protein conversion on models of "digital twins" of whey. The authors of the study [15] performed a preliminary assessment of the bioactive potential of whey protein hydrolysates by using in silico. Hydrolysates were obtained by directional hydrolysis with enzymes produced by *E. faecalis* 2/28. Whey protein hydrolysates coincided in specificity with the commercial variants proposed in the BIOPEP database. The authors confirmed the results of in silico evaluation with in vitro assays. In turn, Ref. [16] noted that the in silico approach makes it possible to determine the appropriate methods for the purification of hydrolysates and evaluation of the sensory properties of the peptides and their allergenicity. Some other important parameters of the hydrolysis, e.g., the mechanism of amino acid action contribution into the bioactivity of certain peptides, are also possible to determine by in silico approach in order to protect key areas. There are a lot of bioinformatic tools and ways to use them. However, in our opinion, the optimal algorithm to use in silico is represented in the hybrid strategy of bioinformatic modeling we developed earlier [17]. The strategy of bioinformatic modeling allows to obtain the model of enzymatic protein digestion (taking into account their genetic polymorphism) by specific enzymes (one or several). The model presents a map of BAP with their potential bioactivity, predicted toxicity, allergenicity, sensory properties, stability for digestion in the gastrointestinal tract, and physicochemical and technological properties. It is worth noting that combining prognostic and technological approaches is an essential factor to modernize and improve the efficiency of whey processing methods. According to this, taking into account the variety of whey types and regular differences in its protein profile, our work was aimed at forming a digital model of a peptide complex of different whey types with predicted bioactivity, safety, and sensory properties.

## 2. Materials and Methods

### *2.1. Whey Samples*

Acid whey was obtained from the production of cottage cheese according to GOST 31453-2013 in the research-and-development plant of "All-Russian Dairy Research Institute" (Russia). Milk coagulation was induced by acid (CTW (AC)) and acid-rennet treatment (CTW (A/RC)). The whey obtained from the production of high-calcium coprecipitates (CPW (TCC)) was also produced in the research-and-development plant according to TU 49720-80. Sweet whey obtained from the production of semi-hard cheese (CHW (RC)) "Mantova" (TU 9225-002-09929631-14) and "Russian" (GOST 32260-2013) was provided by "Italian Traditions" (Russia) and Uva-Moloko LLC (Russia), respectively. Sweet whey

obtained from the production of soft stretch cheeses such as "Mozzarella" according to TU 10.51.40-004-09929631-17 (CHW (A/RC)) was provided by "Italian Traditions" (Russia). Whey obtained from the production of cheese "Adygeisky" (GOST 32263-2013) as a result of heat and acid induced coagulation of milk proteins (CHW (TAC)) was provided by CJSC "Adygeisky Molkombinat" (Russia). The experiment was carried out according to the plan presented in Figure 1.

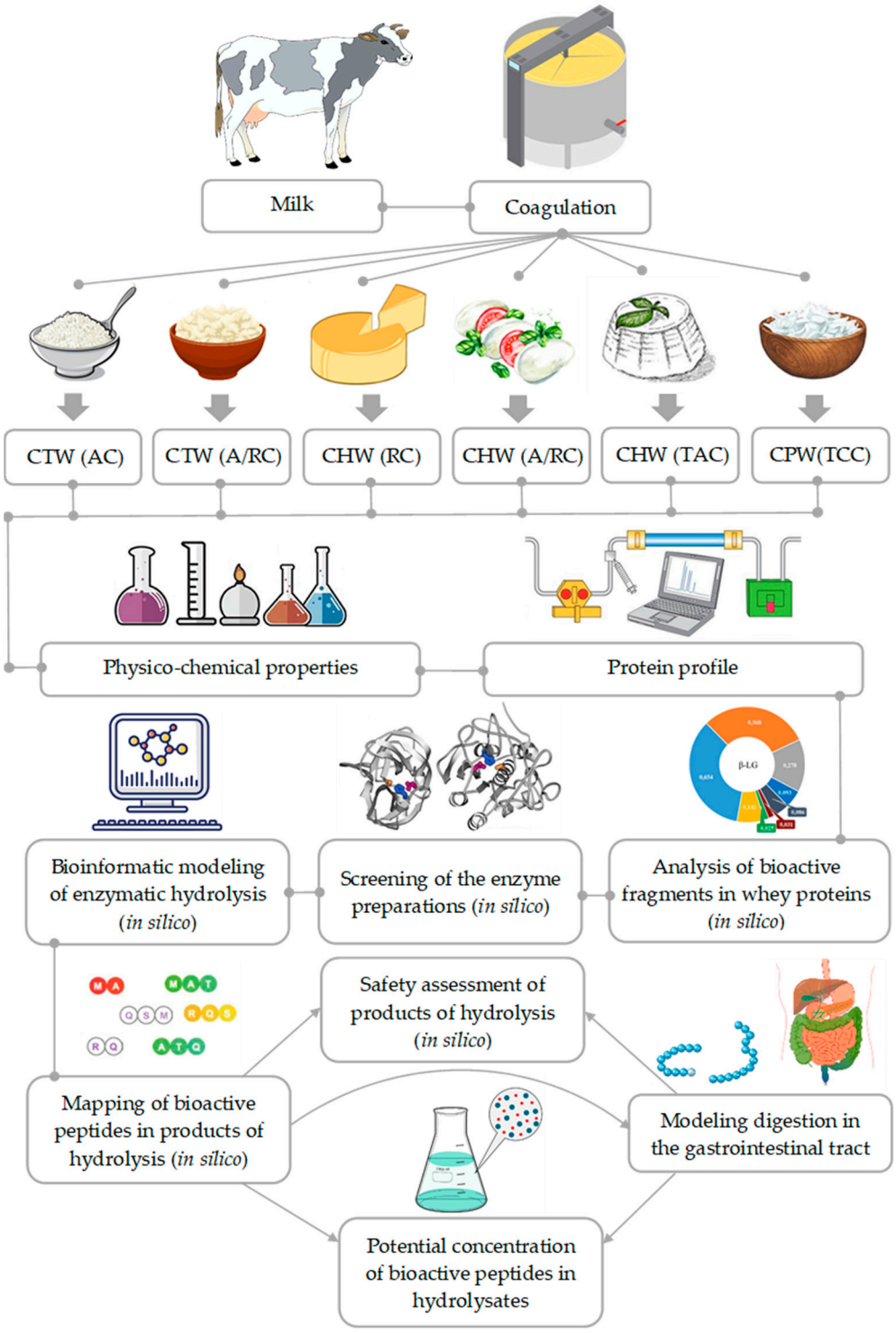


**Figure 1.** The experimental design.

### *2.2. Determination of Physicochemical Properties of Whey*

Standard methods were used to research the physico-chemical properties of the whey. Fat content was determined by Gerber acid method according to ISO 2446:2008. Total protein content was determined by Kjeldahl method with determination of total nitrogen content according to ISO 1871:2009, ISO 8968-1:2014 on Kjeltec-2400 Auto Analyzer (Foss Electric, Hilleroed, Denmark) with conversion factor 6.38. Casein and whey protein content were determined according to ISO 17997-1:2004. Milk protein fractions were determined by reversed-phase HPLC on an Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) according to the method in [18]. Lactose content was determined by enzymatic method according to ISO 26462:2010. pH was measured by potentiometric method with stationary pH-meter Aquasearcher AB33PH with electrode ST 320 (Ohaus, Parsippany, NJ, USA). Titratable acidity was determined by titration of the sample with 0.1 N NaOH solution in the presence of 1% alcoholic solution of phenolphthalein indicator and expressed in degrees Turner (°T). Total mineral content was determined by combustion of dried samples at 550 °C in a muffle electric furnace (MP-2UM, Utena, Lithuania) according to the method in [19]. Calcium content was determined by titrimetric method according to ISO 12081:2010.

### *2.3. Method of Bioinformatic Analysis*

Studies on the in silico release of bioactive peptides from a complex protein complex of milk whey were performed according to the algorithm of a hybrid strategy of bioinformatic modeling that we developed earlier [17]. The strategy algorithm was based on obtaining analytical data on the protein profile of raw materials and the amino acid sequence of proteins, followed by screening of bioactive amino acid sites, selection of optimal enzyme preparations, and modeling of hydrolysis, which was followed by evaluation of the bioactivity of peptides using proteomic databases. The main emphasis was focused on the safety of hydrolysis products to exclude the formation of peptides that could have negative effects on human organs' functions and health. The resistance of bioactive peptides to degradation in the gastrointestinal tract was another main emphasis.

### *2.4. Screening of Bioactive Sites in the Protein Structure and Release of Bioactive Peptides*

Screening of bioactive sites in the protein structure and the release of bioactive peptides was evaluated using a few parameters described previously in [20]:

1. Theoretical degree of hydrolysis (DH)

$$DH = d/D \times 100, \quad (1)$$

d—the number of hydrolyzed peptide bonds in the protein–peptide chain; D—total number of peptide bonds in the protein–peptide chain.

2. Frequency of bioactive fragments in the protein sequence (A)

$$A = a/N, \quad (2)$$

a—the number of fragments with a target activity; N—the number of amino acid residues.

3. Potential biological activity of protein fragments (B)

$$B = [\Sigma(a_i/IC_{50i})]/N, \quad (3)$$

$a_i$—the number of repeats of the $_i$-th bioactive fragment in the protein sequence; $IC_{50i}$—the concentration of the $_i$-th bioactive peptide corresponding to half-maximum inhibition [μM]; N—the number of amino acid residues.

4. Frequency of release of fragments with a target activity by selected enzymes ($A_E$)

$$A_E = d/N, \quad (4)$$

d—the number of peptides with a target activity (e.g., ACE inhibitors) released by selected enzymes; N—the number of amino acid residues in the protein.

5. Relative frequency of release of fragments with a target activity by selected enzymes (W)

$$W = A_E/A, \quad (5)$$

$A_E$—the frequency of release of fragments with a target activity by selected enzymes; A—the frequency of occurrence of bioactive fragments in the protein sequence.

6. Activity of fragments potentially released by proteolytic enzyme(s) ($B_E$)

$$B_E = [\Sigma(d_j/IC_{50j})]/N, \quad (6)$$

$d_j$—the number of repeats of the j-th bioactive fragment released by selected enzyme(s) from the protein sequence; $EC_{50j}$—the concentration of the j-th bioactive peptide corresponding to its half-maximal activity [μM]; $IC_{50j}$—the concentration of the j-th bioactive peptide corresponding to its half-maximal inhibition [μM]; N—the number of amino acid residues in the protein chain

7. Relative activity of fragments potentially released by proteolytic enzyme(s) (V)

$$V = B_E/B, \quad (7)$$

$B_E$—the activity of the fragments potentially released by the proteolytic enzyme(s); B—the potential biological activity of the protein fragments

8. The concentration of bioactive peptides was determined by the formula

$$M_{pep}^{A} = 10^3 \times \sum_{i=1}^{n} \times \frac{W_{pi} \sum_{\in peps_i^A} N_j M_j}{M_{pi}} \quad (8)$$

A—the bioactive function of peptides; $M_{pep}^{A}$—the concentration of bioactive peptides with function A (mg/100 g); *n*—the number of proteins; $pi - i$ protein, $peps_i^A$—bioactive peptides of the *i*-th protein with function A; $N_j$—the number of concrete peptide; $M_j$ —the molecular mass of the concrete peptide.

9. The content of free amino acids was determined by calculating individual amino acids in the obtained digestive models by a single enzyme or a combination of enzymes using Expasy Peptide Cutter tools (http://expasy.org (accessed on 16 March 2022)).

### *2.5. Simulations of Digestion in the Gastrointestinal Tract*

Prediction of bioactive peptide stability in the gastrointestinal tract was performed using the methodology described in [21,22]. These methods allow to simulate in silico digestion in the gastrointestinal tract by a complex of enzymes (pepsin, pancreatic elastase, and chymotrypsin A) with data processing in Expasy Peptide Cutter tools (http://expasy.org (accessed on 27 March 2022)).

### *2.6. Statistics*

Statistical analysis of the data was performed using the Statistica 2010 software package. All measurements were performed in 3 independent replicates. The results are presented as mean (±) standard deviation (SD). Statistical analysis was performed by means of a single-group-factor analysis of variance (ANOVA) at a significance value of $p < 0.05$.

## 3. Results

### *3.1. Protein Profile and Physico-Chemical Composition of Milk Whey*

The variability of protein profile and physico-chemical composition of milk whey depends mainly on the composition of raw milk (or concentrate), technological modes of its pretreatment, type and concentration of coagulating agent (calcium chloride, starter

cultures, rennet), and the modes of clot processing (pH, temperature during clot cutting, and further processing) [23]. The aim of this stage was to obtain the data on the protein profile and physico-chemical composition of milk whey obtained from the production of cottage cheeses, cheeses, and coprecipitates. Technologically reasonable methods were used for milk coagulation.

The presented data show that technological modes of milk coagulation influence the protein profile and physico-chemical composition of obtained milk whey (Table 1). Whey produced by acid and acid-rennet coagulation was characterized by low fat (0.12–0.14%) and total protein (0.47–0.55%) content. Lactose content was at 3.71–4.04%. This is caused by the sequence of biochemical reactions occurring in the process of milk fermentation by lactic acid microorganisms and the transformation of lactose into lactic acid. Due to an increase in lactic acid content, the system acidity increases too. Calcium content depended on the intensity of acid formation during acid and acid-rennet coagulation. Calcium content was 90.98 and 78.15 mg/100 g in whey produced by acid and acid-rennet coagulation, appropriately. A similar effect of calcium content (85.36 mg/100 g) was observed for CHW whey (TAC). Acidification of the system was reached by adding organic acids or acid whey. Minimal protein content (0.24–0.30%) and high lactose content (5.21–5.28%) were determined in the whey produced by heat-induced treatment of milk proteins under reduced acidity conditions (CHW (TAC) or the presence of calcium ions CPW (TCC)). The highest fat content (0.74–0.87%) and protein content (0.83–0.92%) were determined in the CHW (RC) and CHW (A/RC) whey. At the same time, the lactose and calcium, as well as acidity, were at different levels due to the presence of starter cultures in the CHW (A/RC) sample. Evaluation of the protein profile of whey from cottage cheese and cheese produced by acid, rennet, and acid-rennet coagulation of milk showed that it was represented by 48–54% β-LG, 20–22% α-LA, 2.9–7.3% BSA. The protein profile in these types of whey was also represented by 1.8–6.6% of the κ-CN fraction. In the CHW (RC) and CTW (AC) types of whey, β-CN (3.6–4.3%) and αS1-CN (1.0–1.8%) were also found. At the same time, the presence of αS1- and κ-CN in the whey was inconstant. This fact might be caused by their accidental transition into the casein dust during clot processing. The protein composition of whey produced by heat-acid and heat coagulation in the presence of $Ca^{2+}$ from milk was significantly different from other types of milk whey. The protein profile of CHW (TAC) and CPW (TAC) was characterized by 50–54% whey proteins (17–21% β-LG; 20–29% α-LA; 2.1–2.3% BSA) and 46–50% casein fractions (16.7% αS1-CN; 12.5–20% β-CN; 13.3–16.7% κ-CN).

**Table 1.** Protein profile and physico-chemical properties of milk whey.

| Name of Parameter | CTW (AC) | CTW (A/RC) | CHW (RC) | CHW (A/RC) | CHW (TAC) | CPW (TCC) |
|---|---|---|---|---|---|---|
| Fat, % | 0.12 ± 0.03 | 0.14 ± 0.05 | 0.87 ± 0.05 | 0.74 ± 0.14 | 0.53 ± 0.17 | 0.11 ± 0.06 |
| Protein, % | 0.55 ± 0.05 | 0.47 ± 0.05 | 0.92 ± 0.07 | 0.83 ± 0.09 | 0.30 ± 0.06 | 0.24 ± 0.02 |
| Casein, % | 0.04 ± 0.01 | 0.03 ± 0.02 | 0.08 ± 0.02 | 0.05 ± 0.01 | 0.15 ± 0.04 | 0.11 ± 0.03 |
| αS1-CN | 0.01 ± 0.01 | 0 | 0.01 ± 0.01 | 0 | 0.05 ± 0.03 | 0.04 ± 0.02 |
| αS2-CN | 0 | 0 | 0 | 0 | 0 | 0 |
| β-CN | 0.02 ± 0.01 | 0 | 0.04 ± 0.01 | 0 | 0.06 ± 0.03 | 0.03 ± 0.01 |
| κ-CN | 0.01 ± 0.01 | 0.03 ± 0.02 | 0.03 ± 0.02 | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.04 ± 0.01 |
| Whey protein, % | 0.51 ± 0.07 | 0.42 ± 0.04 | 0.84 ± 0.04 | 0.78 ± 0.06 | 0.15 ± 0.04 | 0.13 ± 0.02 |
| β-LG | 0.30 ± 0.02 | 0.24 ± 0.05 | 0.43 ± 0.04 | 0.45 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.02 |
| α-LA | 0.12 ± 0.01 | 0.10 ± 0.03 | 0.19 ± 0.02 | 0.17 ± 0.02 | 0.06 ± 0.02 | 0.07 ± 0.03 |
| BSA | 0.021 ± 0.003 | 0.033 ± 0.005 | 0.027 ± 0.004 | 0.038 ± 0.005 | 0.007 ± 0.002 | 0.005 ± 0.001 |
| LF | 0.0026 ± 0.0002 | 0.0027 ± 0.0006 | 0.0016 ± 0.0002 | 0.0028 ± 0.0003 | 0.0010 ± 0.0005 | 0.0009 ± 0.0004 |

**Table 1.** *Cont.*

| Name of Parameter | CTW (AC) | CTW (A/RC) | CHW (RC) | CHW (A/RC) | CHW (TAC) | CPW (TCC) |
|---|---|---|---|---|---|---|
| Lactose, % | 3.71 ± 0.19 | 4.04 ± 0.18 | 5.03 ± 0.17 | 4.46 ± 0.15 | 5.21 ± 0.18 | 5.28 ± 0.12 |
| Ash, % | 0.67 ± 0.04 | 0.61 ± 0.02 | 0.51 ± 0.02 | 0.53 ± 0.04 | 0.64 ± 0.04 | 0.57 ± 0.05 |
| Calcium, mg/100 g | 90.98 ± 2.72 | 78.15 ± 2.92 | 54.32 ± 3.44 | 66.31 ± 2.36 | 85.36 ± 3.31 | 63.05 ± 2.91 |
| Total solids, % | 6.13 ± 0.16 | 6.19 ± 0.21 | 7.34 ± 0.15 | 6.69 ± 0.19 | 7.04 ± 0.22 | 6.65 ± 0.21 |
| Titratable acidity, °T | 66 ± 3 | 63 ± 2 | 17.1 ± 0.2 | 29 ± 2 | 47 ± 3 | 15 ± 2 |
| pH | 4.55 ± 0.05 | 5.14 ± 0.05 | 6.39 ± 0.05 | 5.81 ± 0.02 | 5.45 ± 0.06 | 6.82 ± 0.04 |

CTW (AC)—whey from cottage cheese, acid coagulation; CTW (A/RC)—whey from cottage cheese, acid, and rennet-induced coagulation; CHW (RC)—whey from cheese, rennet coagulation; CHW (A/RC)—whey from cheese, acid, and rennet coagulation; CHW (TAC)—whey from cheese, temperature (heat), and acid-induced coagulation; CPW(TCC)—whey from coprecipitate, temperature (heat), and calcium-induced coagulation.

### *3.2. Screening Bioactive Sites in the Structure of Milk Whey Protein*

At this stage, we obtained data about amino acid sequences of major milk whey proteins (without signal peptide) taking into account protein gene polymorphism (dominant in cows in European countries). We used the following bioinformatic databases and associated tools: NCBI (www.ncbi.nlm.nih.gov (accessed on 11 March 2022)), Uniprot (www.uniprot.org (accessed on 11 March 2022)), and BIOPEP-UWM (www.biochemia.uwm.edu.pl (accessed on 11 March 2022)). Screening of bioactive peptides within the amino acid structure of the protein was carried out using bioinformatic database tools MBPDB (www.mbpdb.nws.oregonstate.edu (accessed on 11 March 2022)) and BIOPEP-UWM. Screening was performed to identify the most frequent bioactive fragments in the main milk whey proteins. As a result, all possible potentially released peptides were identified, and their bioactive potential was assessed by counting the annotated function references expressed as the frequency of occurrence of bioactive properties (Figure 2).

We found that DPP-4-inhibitory (A = 0.654 and 0.659), ACE-inhibitory (A = 0.568 and 0.431), and antioxidant (A = 0.278 and 0.073) were the most frequent bioactive fragments for β-LG and α-LA. A similar trend of DPP-4-inhibitory (A = 0.588 and 0.635), ACE-inhibitory (A = 0.408 and 0.473), and antioxidant (A = 0.088 and 0.065) activities was observed for BSA and LF. The highest frequency (in descending order) of DPP-4- and ACE-inhibitory peptides as well as antioxidant activity was also found in casein fractions (k-CN, β-CN, αS1-CN, αS2-CN), entering the whey mainly in the form of casein dust.

At the same time, potential ACE-inhibitory activity (Figure 3) was observed in higher values than DPP-4-inhibitory activity for all whey proteins. The potential ACE-inhibitory activity of β-LG was on average 5 times higher than that of the other whey proteins, 1.7 times higher than that of k-CN, β-CN, and 3.3–3.8 times higher than that of αS1-CN, αS2-CN. Thus, the next aim of this study was to release peptides with antioxidant and ACE-inhibitory activity from the protein complex of whey as the most optimal combinatorial biofunctionality.

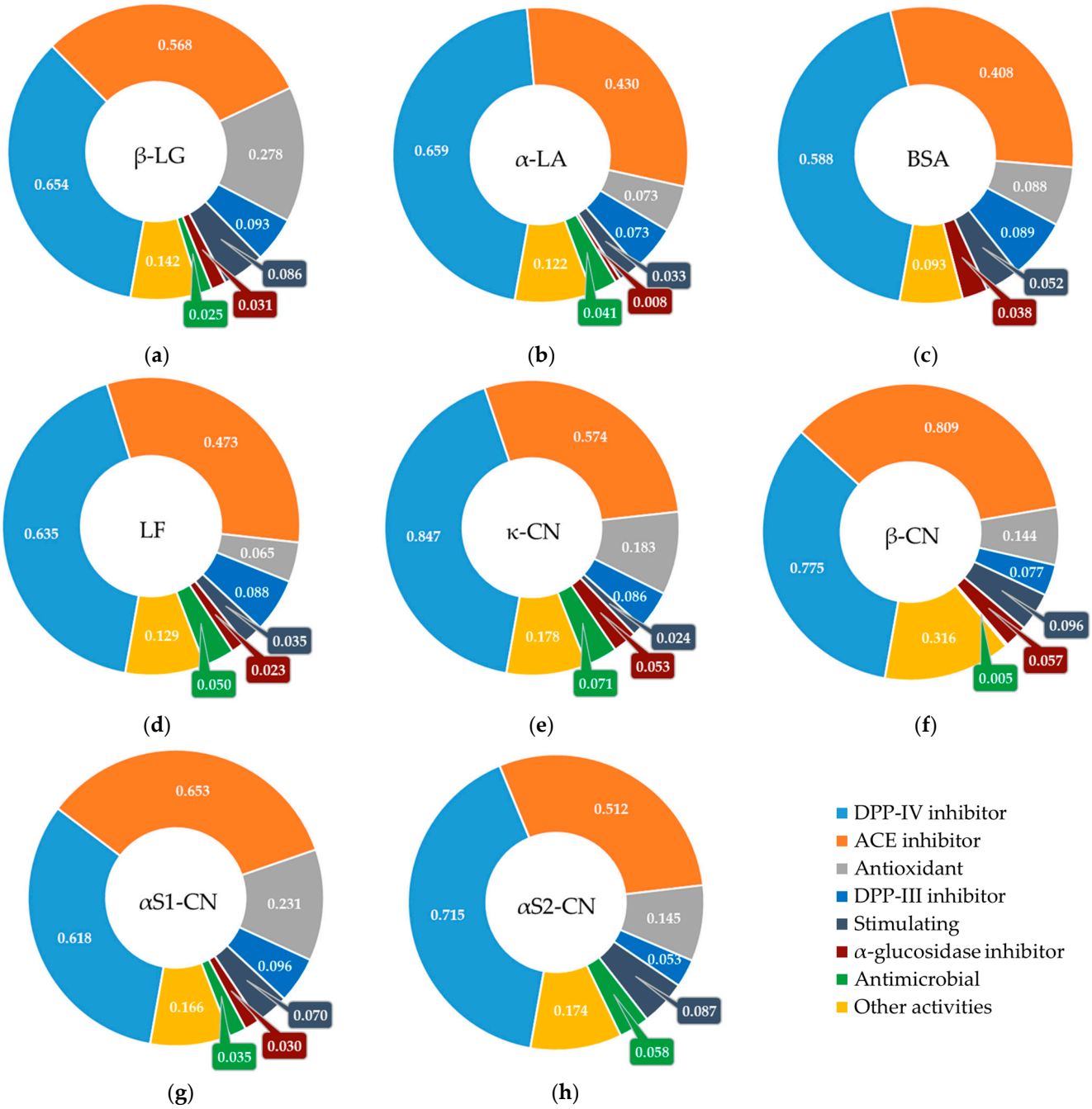


**Figure 2.** Frequency of bioactive fragments (A) in major whey proteins: (**a**) β-LG, (**b**) α-LA, (**c**) BSA, (**d**) LF, (**e**) κ-CN, (**f**) β-CN, (**g**) αS1-CN, (**h**) αS2-CN.

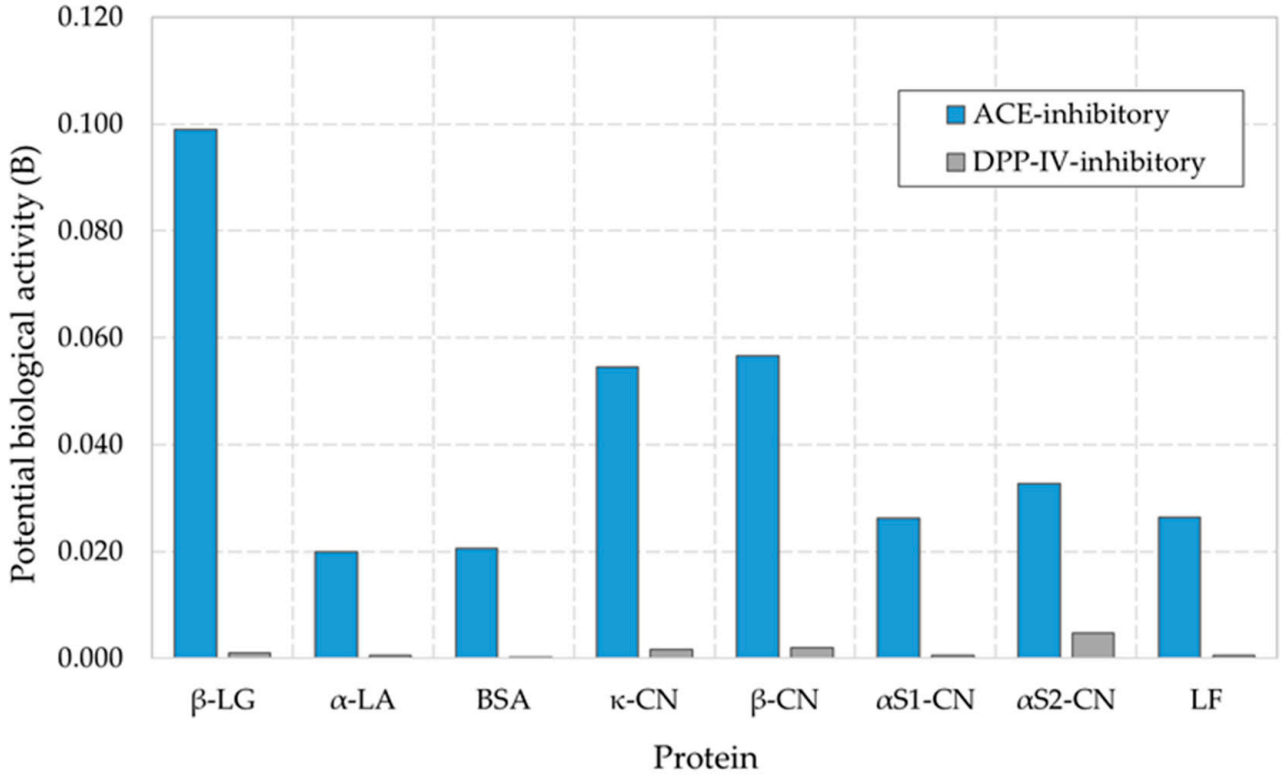


**Figure 3.** Potential biological activity (B) in major milk whey proteins.

### *3.3. Bioinformatic Modeling of Enzymatic Hydrolysis of Milk Whey Proteins by Enzymatic Preparations*

Computer modeling of substrate bioconversion with the release of peptides with antioxidant and ACE-inhibitory activities was performed. It was based on analytical data on the protein profile of the raw milk. Analysis of amino acid sequences of milk proteins using Expasy Peptide Cutter tools (http://expasy.org (accessed on 23 March 2022)) showed the specificity of 23 enzymes with respect to β-LG, BSA, LF, and caseins, specific enzymes for α-LA—20. A group of commercially available enzymes was selected for the study: pepsin, pancreatic elastase, proteinase P1, ficine, stem bromelain, chymotrypsin A and C, proteinase K, thermolysin, papain, subtilisin, cocolysin, chimaza, V-8 protease, pancreatic elastase II, and trypsin. Bioconversion was modeled by selected enzymes for each protein fraction. The results of bioinformatic modeling are presented in Tables 2 and 3.

Analysis of the data in Table 3 showed that the highest frequency of peptide release (in silico) with antioxidant and ACE-inhibitory activity from β-LG was observed with pepsin, pancreatic elastase, chymotrypsin C, and proteinase K. The highest relative activity of fragments potentially released by the enzyme was observed with pepsin, ficine, and subtilisin hydrolysis. It should be noted that the substrate specificity of pancreatic elastase II, V-8 protease, and trypsin revealed that these enzymes were not appropriate to release peptides with antioxidant activity from the β-LG structure. A high degree of hydrolysis and the release of a significant amount of free amino acids were observed for pepsin compared to other enzyme preparations (Table 2). As a result of α-LA hydrolysis, it was found that the highest frequency of release of peptides with antioxidant and ACE-inhibitory activity was noted with the enzymes ficaine, thermolysin, and papain. The highest relative ACE-inhibitory activity of the fragments was observed with the proteolytic enzymes pepsin, pancreatic elastase. The impossibility of releasing peptides with antioxidant activity was revealed by computer modeling of hydrolysis for the most enzymes (pepsin, pancreatic elastase, proteinase P1, chymotrypsin C, proteinase K, subtilisin, chymase, V-8 protease, pancreatic elastase II, trypsin). Evaluation of the hydrolysis (in silico) of BSA showed that

pepsin and ficaine provided a greater degree of release of peptides with ACE-inhibitory activity. The frequency of release of antioxidant peptides was significantly higher for bromelain stim. Hydrolysis of BSA by these enzymes was followed by the release of a huge amount of free amino acids (Table 2). This fact could adversely affect the sensory properties of the hydrolysate. Papain and proteinase K were the optimal enzymes in terms of both the frequency of release of peptides with antioxidant and ACE-inhibitory activity from the BSA structure and their predicted activity. Pepsin, chymotrypsin C, and subtilisin showed the highest cumulative frequency of peptide release with maximum antioxidant and ACE-inhibitory activity in the hydrolysis of LF. The free amino acid content of LF hydrolysis by chymotrypsin C and subtilisin is 3.4 and 5.5 times lower than that by pepsin.

**Table 2.** Evaluation of the predicted degree of hydrolysis of milk whey proteins and the release of free amino acids during the hydrolysis modeling by different proteases.

| Whey | β-LG | | α-LA | | BSA | | LF | | κ-CN | | β-CN | | αS1-CN | | αS2-CN | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DHt | FAA | DHt | FAA | DHt | FAA | DHt | FAA | DHt | FAA | DHt | FAA | DHt | FAA | DHt | FAA |
| Pepsin (pH > 2) EC 3.4.23.1 | 73.3 | 86 | 68.3 | 64 | 68.5 | 288 | 66.9 | 229 | 61.2 | 66 | 63.1 | 81 | 67.7 | 96 | 71.2 | 110 |
| Proteinase P1 EC 3.4.21.96 | 49.1 | 51 | 39.7 | 31 | 41.7 | 157 | 43.8 | 135 | 54.1 | 68 | 67.3 | 116 | 50.0 | 67 | 45.8 | 58 |
| Chymotrypsin EC 3.4.21.2 | 42.4 | 33 | 34.9 | 20 | 34.7 | 74 | 32.9 | 67 | 43.6 | 33 | 52.3 | 55 | 47.6 | 50 | 44.3 | 50 |
| Pancreatic elastase EC 3.4.21.36 | 47.3 | 38 | 42.1 | 26 | 42.3 | 130 | 49.7 | 135 | 48.3 | 46 | 41.6 | 35 | 43.1 | 38 | 42.9 | 45 |
| Ficine EC 3.4.22.3 | 37.0 | 25 | 38.9 | 23 | 41.8 | 119 | 43.8 | 110 | 30.8 | 22 | 35.5 | 28 | 41.7 | 43 | 38.7 | 43 |
| Stem bromelain EC 3.4.22.32 | 43.6 | 36 | 36.5 | 21 | 45.0 | 143 | 52.5 | 148 | 41.3 | 39 | 41.1 | 38 | 39.7 | 36 | 37.7 | 33 |
| Proteinase K EC 3.4.21.67 | 37.6 | 23 | 32.5 | 13 | 32.0 | 68 | 32.7 | 63 | 38.4 | 26 | 49.1 | 63 | 39.2 | 37 | 33.5 | 27 |
| Thermolysin EC 3.4.24.27 | 38.2 | 28 | 30.2 | 9 | 34.2 | 82 | 34.1 | 70 | 33.7 | 24 | 32.2 | 22 | 32.3 | 23 | 30.2 | 24 |
| Papain EC 3.4.22.2 | 36.4 | 16 | 28.6 | 13 | 34.8 | 66 | 38.5 | 64 | 34.9 | 20 | 31.3 | 21 | 31.4 | 29 | 29.7 | 15 |
| Subtilisin EC 3.4.21.62 | 27.3 | 16 | 28.6 | 8 | 26.8 | 46 | 30.2 | 41 | 26.2 | 13 | 32.2 | 25 | 29.4 | 18 | 29.3 | 15 |
| Chymotrypsin EC 3.4.21.1 | 26.1 | 12 | 28.6 | 10 | 24.2 | 35 | 22.7 | 31 | 20.1 | 9 | 24.3 | 13 | 26.5 | 16 | 25.0 | 15 |
| Cocolysin EC 3.4.24.30 | 32.7 | 18 | 25.4 | 7 | 28.2 | 56 | 27.2 | 44 | 27.3 | 14 | 23.4 | 11 | 27.0 | 16 | 23.6 | 15 |
| Chimaza EC 3.4.21.39 | 19.4 | 8 | 19.1 | 6 | 18.3 | 23 | 17.4 | 18 | 12.8 | 4 | 16.8 | 6 | 17.6 | 13 | 15.1 | 7 |
| Trypsin EC 3.4.21.4 | 10.9 | 2 | 10.32 | 2 | 13.7 | 7 | 13.4 | 6 | 8.1 | 1 | 7.2 | 2 | 9.8 | 3 | 14.2 | 6 |
| V-8 protease (pH = 7.8) EC 3.4.21.19 | 15.8 | 4 | 15.9 | 6 | 16.5 | 20 | 9.9 | 4 | 9.3 | 2 | 10.7 | 6 | 15.7 | 7 | 13.2 | 9 |
| Pancreatic elastase II EC 3.4.21.71 | 18.2 | 5 | 13.5 | 3 | 15.3 | 13 | 13.0 | 9 | 8.1 | 3 | 17.3 | 6 | 14.7 | 6 | 10.4 | 1 |

**Table 3.** Evaluation of the relative frequency (W) and activity of peptides (V) released from milk whey proteins when modeling hydrolysis by different proteases.

| Whey | Bioactivity | β-LG | | α-LA | | BSA | | LF | | κ-CN | | β-CN | | αS1-CN | | αS2-CN | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | W | V | W | V | W | V | W | V | W | V | W | V | W | V | W | V |
| Pepsin (pH > 2) | ACE-inhibitory | 0.111 | 0.019 | 0.170 | 0.164 | 0.172 | 0.094 | 0.106 | 0.020 | 0.102 | 0.089 | 0.144 | 0.159 | 0.148 | 0.136 | 0.094 | 0.163 |
| EC 3.4.23.1 | Antioxidative | 0.047 | - | - | - | - | - | 0.131 | - | 0.047 | - | 0.154 | - | 0.059 | - | 0.097 | - |
| Proteinase P1 | ACE-inhibitory | 0.067 | 0.002 | - | - | 0.043 | 0.027 | 0.036 | 0.009 | 0.070 | 0.010 | 0.077 | 0.087 | 0.055 | 0.114 | 0.062 | 0.006 |
| EC 3.4.21.96 | Antioxidative | 0.024 | - | 0.111 | - | 0.036 | - | - | - | 0.023 | - | 0.077 | - | 0.039 | - | 0.032 | - |
| Chymotrypsin | ACE-inhibitory | 0.089 | 0.005 | 0.076 | 0.020 | 0.108 | 0.013 | 0.170 | 0.026 | 0.109 | 0.047 | 0.077 | 0.029 | 0.098 | 0.024 | 0.094 | 0.105 |
| EC 3.4.21.2 | Antioxidative | 0.143 | - | - | - | 0.072 | - | 0.131 | - | 0.070 | - | 0.116 | - | 0.059 | - | 0.129 | - |
| Pancreatic elastase | ACE-inhibitory | 0.089 | 0.004 | 0.076 | 0.140 | 0.065 | 0.022 | 0.057 | 0.001 | 0.055 | 0.008 | 0.048 | 0.018 | 0.093 | 0.094 | 0.098 | 0.038 |
| EC 3.4.21.36 | Antioxidative | 0.072 | - | - | - | 0.036 | - | 0.044 | - | 0.070 | - | 0.039 | - | 0.079 | - | 0.097 | - |
| Ficine | ACE-inhibitory | 0.067 | 0.012 | 0.094 | 0.014 | 0.043 | 0.010 | 0.036 | 0.020 | 0.047 | 0.011 | 0.096 | 0.026 | 0.114 | 0.230 | 0.067 | 0.055 |
| EC 3.4.22.3 | Antioxidative | 0.047 | - | 0.221 | - | - | - | 0.087 | - | 0.047 | - | - | - | 0.020 | - | 0.064 | - |
| Stem bromelain | ACE-inhibitory | 0.056 | 0.003 | 0.038 | 0.00032 | 0.043 | 0.001 | 0.050 | 0.003 | 0.047 | 0.004 | 0.067 | 0.008 | 0.098 | 0.056 | 0.107 | 0.047 |
| EC 3.4.22.32 | Antioxidative | 0.047 | - | 0.111 | - | - | - | 0.087 | - | 0.047 | - | - | - | 0.118 | - | 0.032 | - |
| Proteinase K | ACE-inhibitory | 0.078 | 0.004 | 0.076 | 0.010 | 0.097 | 0.038 | 0.064 | 0.008 | 0.086 | 0.016 | 0.067 | 0.071 | 0.093 | 0.150 | 0.080 | 0.011 |
| EC 3.4.21.67 | Antioxidative | 0.047 | - | - | - | 0.036 | - | 0.044 | - | 0.093 | - | 0.077 | - | 0.079 | - | 0.161 | - |
| Thermolysin | ACE-inhibitory | 0.067 | 0.002 | 0.094 | 0.038 | 0.075 | 0.002 | 0.099 | 0.157 | 0.070 | 0.027 | 0.096 | 0.016 | 0.059 | 0.019 | 0.072 | 0.170 |
| EC 3.4.24.27 | Antioxidative | 0.047 | - | 0.111 | - | - | - | 0.087 | - | 0.047 | - | 0.116 | - | 0.039 | - | 0.032 | - |
| Papain | ACE-inhibitory | 0.044 | 0.003 | 0.057 | 0.00036 | 0.075 | 0.056 | 0.050 | 0.001 | 0.023 | 0.003 | 0.087 | 0.068 | 0.098 | 0.131 | 0.130 | 0.047 |
| EC 3.4.22.2 | Antioxidative | 0.024 | - | 0.221 | - | 0.036 | - | 0.087 | - | 0.023 | - | 0.039 | - | 0.059 | - | 0.064 | - |
| Subtilisin | ACE-inhibitory | 0.033 | 0.016 | 0.076 | 0.037 | 0.032 | 0.014 | 0.036 | 0.014 | 0.055 | 0.034 | 0.058 | 0.050 | 0.076 | 0.064 | 0.076 | 0.145 |
| EC 3.4.21.62 | Antioxidative | 0.047 | - | - | - | 0.036 | - | 0.087 | - | 0.093 | - | 0.039 | - | 0.020 | - | 0.193 | - |
| Chymotrypsin | ACE-inhibitory | 0.022 | 0.001 | 0.057 | 0.00017 | 0.054 | 0.008 | 0.021 | 0.013 | 0.047 | 0.014 | 0.019 | 0.00006 | 0.047 | 0.052 | 0.040 | 0.067 |
| EC 3.4.21.1 | Antioxidative | 0.024 | - | 0.111 | - | - | - | 0.044 | - | 0.116 | - | 0.039 | - | 0.039 | - | 0.161 | - |
| Cocolysin | ACE-inhibitory | 0.067 | 0.001 | 0.057 | 0.038 | 0.042 | 0.120 | 0.043 | 0.021 | 0.055 | 0.027 | 0.077 | 0.002 | 0.042 | 0.120 | 0.072 | 0.086 |
| EC 3.4.24.30 | Antioxidative | 0.047 | - | 0.111 | - | - | - | 0.044 | - | 0.047 | - | 0.116 | - | 0.039 | - | 0.032 | - |
| Chimaza | ACE-inhibitory | 0.022 | 0.001 | 0.038 | 0.004 | 0.043 | 0.011 | 0.028 | 0.013 | 0.039 | 0.013 | 0.019 | 0.003 | 0.034 | 0.044 | 0.031 | 0.058 |
| EC 3.4.21.39 | Antioxidative | 0.024 | - | - | - | - | - | 0.087 | - | 0.093 | - | - | - | - | - | 0.161 | - |
| Trypsin | ACE-inhibitory | 0.056 | 0.003 | 0.019 | 0.001 | 0.011 | 0.007 | 0.028 | 0.007 | 0.031 | 0.023 | 0.039 | 0.004 | 0.017 | 0.006 | 0.022 | 0.002 |
| EC 3.4.21.4 | Antioxidative | - | - | - | - | - | - | 0.044 | - | 0.023 | - | 0.039 | - | 0.059 | - | - | - |
| V-8 protease (pH = 7.8) | ACE-inhibitory | 0.011 | 0.000007 | 0.019 | 0.007 | - | - | - | - | - | - | 0.010 | 0.003 | 0.017 | 0.00013 | 0.004 | 0.000015 |
| EC 3.4.21.19 | Antioxidative | - | - | - | - | 0.036 | - | 0.036 | - | 0.023 | - | - | - | 0.039 | - | - | - |
| Pancreatic elastase II | ACE-inhibitory | 0.022 | 0.001 | 0.019 | 0.001 | 0.011 | 0.001 | 0.028 | 0.012 | 0.023 | 0.003 | 0.010 | 0.003 | 0.026 | 0.013 | 0.022 | 0.011 |
| EC 3.4.21.71 | Antioxidative | - | - | - | - | - | - | 0.044 | - | 0.047 | - | - | - | - | - | 0.032 | - |

Analysis of the obtained in silico data allowed us to identify enzymes (pancreatic elastase, chymotrypsin C, proteinase K, papain) in varying degrees, providing the highest frequency of release of antioxidant and ACE-inhibitory protein fragments with maximum activity from the κ-CN structure. Pepsin showed the highest frequency of release of ACE-inhibitory peptides with maximum predicted activity from the κ-CN structure, but does not have substrate specificity for the release of peptides possessing antioxidant activity. A similar pattern was observed during in silico hydrolysis of κ-CN with the enzymes stim bromelain, thermolysin, cocolysin, chymotrypsin A, chymase, trypsin, and pancreatic elastase II. Peptides with ACE-inhibitory activity were not released with V-8 protease. As a result of the evaluation of the efficiency of peptide release from β-CN, pepsin, chymotrypsin C, and thermolysin were the most optimal monoenzyme preparations to release peptides with maximum antioxidant and ACE-inhibitory activity. Lower frequency of release of bioactive peptides from the protein molecule and a lower activity of the ACE-inhibitory fragments were observed for other enzymes. Table 3 showed a similar trend for αS1-CN and β-CN hydrolysis. The highest tendency for the release of antioxidant and ACE-inhibitory peptides with maximum activity was observed when αS1-CN was hydrolyzed by pepsin, chymotrypsin C, and subtilisin. Hydrolysis of αS1-CN, β-CN, and κ-CN by V-8 protease showed inert kinetics to the release of ACE-inhibitory peptides from the protein structure. Pepsin, proteinase P1, chymotrypsin C, and proteinase K showed promising results for hydrolysis of αS2-CN. This was caused by their frequency of release of peptides with antioxidant and ACE-inhibitory activity. Hydrolysis of αS2-CN by pepsin was followed by formation of large amounts of free amino acids. Protein hydrolysis by chymotrypsin C resulted in a high frequency of release of antioxidant peptides and an average frequency of release and activity with ACE-inhibitory peptides.

At the next stage, in silico hydrolysis was performed using enzyme compositions in pairs and crosswise for each protein in the proteomic complex of milk whey using the software "Selection of Optimal Enzymes in β-lactoglobulin Proteolysis" (https://observablehq.com/@semipyatniy/optiferments (accessed on 25 March 2022)) synchronized with Expasy Peptide Cutter. The aim of this stage was to improve the efficiency of release of biopeptides with antioxidant and ACE-inhibitory activity. It was observed that using the bi-enzyme composition chymotrypsin C—subtilisin contributes to releasing the maximum total of ACE-inhibitory and antioxidant peptides. The modeling has taken into account the ranking of the ratio of proteins in the proteomic complex. Generalized data of bioinformatic modeling of hydrolysis of the protein complex of milk whey by the enzyme composition chymotrypsin C—subtilisin are shown in Table 4.

**Table 4.** Results of bioinformatic modeling of the hydrolysis of milk whey proteins by the enzyme complex chymotrypsin (EC 3.4.21.2)—subtilisin (EC 3.4.21.62).

| Protein | DHt | FAA | Activity | AE | W | BE | V |
|---|---|---|---|---|---|---|---|
| β-LG | 52.12 | 47 | ACE-inhibitory | 0.072 | 0.133 | 0.0022 | 0.023 |
| | | | Antioxidative | 0.036 | 0.143 | - | - |
| α-LA | 46.83 | 25 | ACE-inhibitory | 0.063 | 0.151 | 0.0035 | 0.146 |
| | | | Antioxidative | - | - | - | - |
| BSA | 46.83 | 125 | ACE-inhibitory | 0.072 | 0.182 | 0.0029 | 0.143 |
| | | | Antioxidative | 0.007 | 0.079 | - | - |
| LF | 47.26 | 113 | ACE-inhibitory | 0.071 | 0.156 | 0.0049 | 0.194 |
| | | | Antioxidative | 0.014 | 0.226 | - | - |
| κ-CN | 56.98 | 55 | ACE-inhibitory | 0.087 | 0.161 | 0.0011 | 0.021 |
| | | | Antioxidative | 0.017 | 0.107 | - | - |
| β-CN | 68.69 | 110 | ACE-inhibitory | 0.126 | 0.192 | 0.0016 | 0.031 |
| | | | Antioxidative | 0.009 | 0.087 | - | - |
| αS1-CN | 62.25 | 81 | ACE-inhibitory | 0.102 | 0.164 | 0.0039 | 0.151 |
| | | | Antioxidative | 0.015 | 0.070 | - | - |
| αS2-CN | 58.96 | 74 | ACE-inhibitory | 0.066 | 0.135 | 0.0025 | 0.080 |
| | | | Antioxidative | 0.019 | 0.154 | - | - |

ACE-inhibitory and antioxidative peptides with high frequency were released from all protein components of the complex, except α-LA (for peptides with antioxidant activity), during hydrolysis of milk whey with the selected enzyme composition.

### *3.4. Bioinformatic Analysis of Products of Enzymatic Hydrolysis of Milk Whey Proteins*

After the in silico hydrolysis of the complex protein matrix of milk whey with the selected enzymes, all reaction products were evaluated for biofunctionality using the tools of MBPDB (www.mbpdb.nws.oregonstate.edu (accessed on 26 March 2022)), AHTPDB (https://webs.iiitd.edu.in/raghava/ahtpdb (accessed on 26 March 2022)), and BIOPEP databases. In addition, we carried out prediction of the residual antigenicity of the obtained peptides using databases of IUIS (www.iuis.org (accessed on 26 March 2022)), BIOPEP, and AlgPred2 (https://webs.iiitd.edu.in/raghava/algpred2 (accessed on 26 March 2022)). Toxicity of the peptides was assessed using the ToxinPred database (https://webs.iiitd.edu.in/raghava/toxinpred/algo.php (accessed on 26 March 2022)). The flavor profile was predicted using the BIOPEP database and BitterDB (https://bitterdb.agri.huji.ac.il/dbbitter.php (accessed on 26 March 2022)). Peptide mapping of hydrolysis results (in silico) is presented in Table 5.

**Table 5.** Mapping of bioactive peptides in products of hydrolysis (in silico) of milk whey proteins.

| Peptide | Bioactivity | Protein Precursor | Location | Bitterness |
|---|---|---|---|---|
| AA | ACE inhibitor; DPP-IV inhibitor | k-CN | 67–68 | - |
| ACQ | Antioxidative | β-LG | 120–122 | - |
| | | BSA | 174–175; 319–320 | - |
| | | LF | 424–425 | - |
| AE | ACE inhibitor; DPP-IV inhibitor | αS1-CN | 64–65; 118–119 | - |
| | | β-LG | 113–114 | - |
| | | αS2-CN | 64–65 | - |
| AF | ACE inhibitor; DPP-IV inhibitor | β-CN | 195–196 | - |
| AI | ACE inhibitor | α-LA | 40–41 | - |
| AIP | ACE inhibitor | k-CN | 109–111 | - |
| | | BSA | 417–418; 505–506 | - |
| AL | DPP-IV inhibitor | LF | 42–43; 314–315; 327–328 | - |
| | | β-LG | 136–137 | - |
| | | αS2-CN | 179–180 | - |
| | | BSA | 154–155 | - |
| AP | ACE inhibitor; DPP-IV inhibitor | LF | 1–2 | - |
| | | β-CN | 105–106 | - |
| | | β-LG | 37–38 | - |
| AS | DPP-IV inhibitor | BSA | 196–197 | - |
| | | LF | 159–160 | - |
| AY | Antioxidative; ACE inhibitor; DPP-IV inhibitor | LF | 169–170 | - |
| | | αS1-CN | 147–148 | - |
| CAQ | Antioxidative | β-LG | 68–70 | - |
| CF | ACE inhibitor | BSA | 103–104; 502–503 | - |
| CHI | Antioxidative | β-LG | 164–166 | - |
| DKIHP | ACE inhibitor | β-CN | 47–51 | - |
| DP | DPP-IV inhibitor | BSA | 120–121 | - |
| | | LF | 109–110 | - |
| DQ | ACE inhibitor; DPP-IV inhibitor | α-LA | 118–119 | - |
| | | αS1-CN | 51–52 | - |
| | | αS2-CN | 112–113 | - |

**Table 5.** *Cont.*

| Peptide | Bioactivity | Protein Precursor | Location | Bitterness |
|---|---|---|---|---|
| DY | ACE inhibitor | BSA | 464–465 | bitter |
| GA | ACE inhibitor; DPP-IV inhibitor | LF | 151–152 | - |
| GE | ACE inhibitor; DPP-IV inhibitor | BSA | 15–16; 212–213; 410–411 | bitter |
| | | k-CN | 132–133 | bitter |
| | | LF | 181–182 | bitter |
| | | β-CN | 10–11 | bitter |
| | | β-LG | 66–67 | bitter |
| GF | ACE inhibitor; DPP-IV inhibitor | BSA | 413–414 | bitter |
| | | LF | 316–317 | bitter |
| GG | ACE inhibitor; DPP-IV inhibitor | α-LA | 19–20 | - |
| GK | ACE inhibitor | BSA | 444–445 | - |
| GL | ACE inhibitor; DPP-IV inhibitor | k-CN | 39–40 | bitter |
| | | LF | 459–460; 486–487 | bitter |
| | | α-LA | 51–52 | bitter |
| | | αS1-CN | 10–11 | bitter |
| GP | ACE inhibitor; DPP-IV inhibitor | BSA | 589–590 | bitter |
| | | β-CN | 66–67; 205–206 | bitter |
| | | αS2-CN | 104–105 | bitter |
| GQ | ACE inhibitor | LF | 302–303; 378–379 | - |
| GS | ACE inhibitor | BSA | 337–338 | - |
| | | LF | 298–299; 331–332 | - |
| GY | ACE inhibitor; DPP-IV inhibitor | LF | 197–198; 446–447 | bitter |
| | | α-LA | 35–36 | bitter |
| | | αS1-CN | 95–96 | bitter |
| HA | DPP-IV inhibitor | LF | 261–262 | - |
| HE | DPP-IV inhibitor | BSA | 477–478 | - |
| HF | DPP-IV inhibitor | BSA | 18–19 | - |
| HIRL | ACE inhibitor | β-LG | 150–153 | - |
| HL | Antioxidative; ACE inhibitor; DPP-IV inhibitor | k-CN | 104–105 | - |
| | | β-CN | 138–139 | - |
| HP | ACE inhibitor; DPP-IV inhibitor | k-CN | 102–103 | - |
| HS | DPP-IV inhibitor | α-LA | 70–71 | - |
| IAF | ACE inhibitor | BSA | 25–27 | - |
| IG | DPP-IV inhibitor | αS1-CN | 140–141 | - |
| II | DPP-IV inhibitor | β-CN | 213–214 | - |
| IL | ACE inhibitor; DPP-IV inhibitor | BSA | 469–470 | bitter |
| | | k-CN | 75–76 | bitter |
| | | β-LG | 56–57 | bitter |
| IN | DPP-IV inhibitor | k-CN | 51–52; 163–164 | bitter |
| | | α-LA | 55–56 | bitter |
| | | αS2-CN | 87–88 | - |
| IP | ACE inhibitor; DPP-IV inhibitor | k-CN | 26–27; 121–122 | bitter |
| | | LF | 483–484 | bitter |
| | | β-CN | 68–69; 76–77 | bitter |
| IQ | DPP-IV inhibitor | BSA | 208–209 | bitter |
| | | k-CN | 28–29 | bitter |
| | | β-CN | 193–194 | bitter |
| | | αS2-CN | 200–201 | - |
| IW | ACE inhibitor; DPP-IV inhibitor | LF | 275–276 | - |
| IY | Antioxidative; ACE inhibitor | LF | 83–84; 411–412 | - |
| KA | ACE inhibitor; DPP-IV inhibitor | β-CN | 180–181 | - |
| KCL | Antioxidative | LF | 203–205 | - |

**Table 5.** *Cont.*

| Peptide | Bioactivity | Protein Precursor | Location | Bitterness |
|---|---|---|---|---|
| KE | ACE inhibitor; DPP-IV inhibitor | BSA | 283–284 | - |
| | | LF | 251–252 | - |
| | | αS1-CN | 85–86; 128–129; 136–138 | - |
| | | αS2-CN | 32–33 | - |
| KF | ACE inhibitor; DPP-IV inhibitor | BSA | 210–211; 227–228 | bitter |
| | | LF | 285–286 | bitter |
| | | β-CN | 32–33 | bitter |
| | | β-LG | 139–140 | bitter |
| | | αS2-CN | 93–94; 177–178 | bitter |
| KKY | Antioxidative | β-LG | 102–104 | - |
| KL | ACE inhibitor | BSA | 116–117; 281–282; 408–409; 520–521; 591–592 | - |
| KL | ACE inhibitor | LF | 75–76 | - |
| | | α-LA | 116–117; 126–127 | - |
| KP | Antioxidative; ACE inhibitor; DPP-IV inhibitor | BSA | 118–119 | bitter |
| | | k-CN | 46–47 | bitter |
| | | β-LG | 47–48 | bitter |
| | | αS2-CN | 197–198 | bitter |
| KS | DPP-IV inhibitor | BSA | 66–67; 293–294 | - |
| | | LF | 290–291 | - |
| KT | DPP-IV inhibitor | BSA | 562–563 | - |
| KY | ACE inhibitor; DPP-IV inhibitor | BSA | 163–164 | - |
| | | β-CN | 115–116 | - |
| RF | ACE inhibitor; DPP-IV inhibitor | k-CN | 16–17 | - |
| | | αS1-CN | 22–23 | - |
| RL | ACE inhibitor; DPP-IV inhibitor | BSA | 202–203; 357–358; 472–473 | bitter |
| | | αS1-CN | 102–103; 121–123 | bitter |
| | | αS2-CN | 164–165 | bitter |
| RM | DPP-IV inhibitor | BSA | 458–459 | - |
| | | LF | 25–26 | - |
| RN | DPP-IV inhibitor | BSA | 100–101 | - |
| RP | ACE inhibitor; DPP-IV inhibitor | LF | 77–78; 137–138; 442–443 | - |
| | | αS1-CN | 1–2 | - |
| | | k-CN | 34–35 | - |
| RY | Antioxidative; ACE inhibitor; DPP-IV inhibitor | LF | 333–334 | - |
| | | αS1-CN | 92–93 | - |
| | | αS2-CN | 174–175 | - |
| TA | DPP-IV inhibitor | k-CN | 171–172 | - |
| TD | DPP-IV inhibitor | β-CN | 132–133 | - |
| TE | ACE inhibitor; DPP-IV inhibitor | BSA | 47–48 | - |
| | | LF | 143–144; 444–445 | - |
| | | α-LA | 48–49 | - |
| | | αS1-CN | 49–50 | - |
| | | β-CN | 41–42 | - |
| | | αS2-CN | 148–149; 158–159 | - |
| TF | ACE inhibitor; DPP-IV inhibitor | BSA | 523–524 | - |
| | | αS2-CN | 38–39 | - |

**Table 5.** *Cont.*

| Peptide | Bioactivity | Protein Precursor | Location | Bitterness |
|---|---|---|---|---|
| TK | DPP-IV inhibitor | BSA | 244–245 | - |
| TL | DPP-IV inhibitor | β-CN | 130–131 | - |
| | | αS2-CN | 126–127 | - |
| TM | DPP-IV inhibitor | BSA | 189–190 | - |
| | | β-LG | 6–7 | - |
| | | αS2-CN | 3–4 | - |
| TP | ACE inhibitor; DPP-IV inhibitor | BSA | 431–432; 507–508 | - |
| | | k-CN | 137–138 | - |
| | | β-CN | 82–83 | - |
| | | β-LG | 49–50 | - |
| TQ | ACE inhibitor; DPP-IV inhibitor | BSA | 596–597 | - |
| | | β-CN | 55–56; 80–81 | - |
| | | β-LG | 158–159 | - |
| TR | DPP-IV inhibitor | LF | 353–354 | - |
| TS | DPP-IV inhibitor | k-CN | 135–136 | - |
| | | αS2-CN | 134–135 | - |
| TT | DPP-IV inhibitor | LF | 218–219 | - |
| TW | Antioxidative; DPP-IV inhibitor | LF | 461–462 | - |
| TY | Antioxidative; DPP-IV inhibitor | BSA | 85–86; 511–512 | - |
| | | αS2-CN | 19–20 | - |
| VA | DPP-IV inhibitor | LF | 97–98; 450–451 | - |
| VAF | ACE inhibitor | BSA | 569–571 | - |
| | | LF | 212–214 | - |
| VAGTW | ACE inhibitor | β-LG | 15–19 | - |
| VAP | DPP-IV inhibitor | αS1-CN | 25–27 | - |
| VE | ACE inhibitor; DPP-IV inhibitor | BSA | 234–235; 300–301; 437–438; 545–546; 587–588 | bitter |
| | | k-CN | 143–144 | bitter |
| | | αS1-CN | 78–79 | bitter |
| | | β-CN | 13–14; 118–119; 134–135 | bitter |
| | | β-LG | 43–44 | bitter |
| VF | ACE inhibitor; DPP-IV inhibitor | BSA | 167–168; 384–385 | bitter |
| | | LF | 66–67; 220–221 | bitter |
| | | α-LA | 8–9 | bitter |
| | | αS1-CN | 31–32 | bitter |
| | | β-LG | 83–84 | bitter |
| | | αS2-CN | 150–151 | bitter |
| VGP | ACE inhibitor | LF | 360–362 | - |
| VKL | Antioxidative | BSA | 40–42 | - |
| VL | ACE inhibitor; DPP-IV inhibitor | BSA | 23–24; 194–195; 354–355; 475–476 | bitter |
| | | k-CN | 31–32; 80–81 | bitter |
| | | LF | 394–395; 396–397; 422–423; 440–441 | bitter |
| | | αS1-CN | 15–16 | bitter |
| | | β-CN | 166–167; 174–175; 203–204 | bitter |
| | | β-LG | 94–95; 96–97 | bitter |
| | | αS2-CN | 107–108 | bitter |

**Table 5.** *Cont.*

| Peptide | Bioactivity | Protein Precursor | Location | Bitterness |
|---|---|---|---|---|
| VM | ACE inhibitor; DPP-IV inhibitor | BSA | 564–565 | - |
| | | β-CN | 94–95; 159–160 | - |
| VN | ACE inhibitor; DPP-IV inhibitor | BSA | 43–44; 497–498 | - |
| | | LF | 489–490 | - |
| | | αS1-CN | 37–38 142–143 | - |
| VP | ACE inhibitor; DPP-IV inhibitor | BSA | 426–427; 513–514 | - |
| | | k-CN | 85–86 | - |
| | | LF | 162–163; 258–259; 420–421 | - |
| | | αS1-CN | 74–75; 88–89; 108–109; 114–115; 171–172 | - |
| | | β-CN | 8–9; 86–87; 177–178; 182–183 | - |
| | | αS2-CN | 119–120 | - |
| VQ | DPP-IV inhibitor | k-CN | 166–167 | - |
| | | α-LA | 42–43 | - |
| VRGP | ACE inhibitor | β-CN | 207–210 | - |
| VRY | ACE inhibitor | BSA | 420–422 | - |
| | | αS2-CN | 210–212 | - |
| VS | DPP-IV inhibitor | BSA | 352–353; 429–430; 439–440; 482–483; 594–595 | - |
| | | α-LA | 21–22 | - |
| | | β-CN | 97–98 | - |
| | | αS2-CN | 7–8 | - |
| VW | Antioxidative; ACE inhibitor; DPP-IV inhibitor | LF | 356–357 | - |
| VY | Antioxidative; ACE inhibitor; DPP-IV inhibitor | β-CN | 59–60 | bitter |
| | | β-LG | 41–42 | bitter |
| | | αS2-CN | 189–190 | bitter |

Peptide mapping of the products of hydrolysis of the milk whey protein matrix identified 57 peptides with ACE-inhibitory function and 15 peptides with antioxidant function. In addition, 68 peptides with DPP-IV-inhibitory function were found in the bioinformatically simulated hydrolysate. Furthermore, 42 peptides (out of 95 identified) had at least dual-functionality, and 17 bioactive peptides were characterized by a bitter taste. The safety assessment of the hydrolysis products revealed no allergenic sites capable of triggering an immune response mechanism in the organism, but 7 potentially toxic peptides (ADCCE, CCAKDDP, CCDKP, CCHGDL, VDKCCAADDKE, VGTRCCTKP, VTKCCTE) were identified. Toxic peptides have molecular weights from 539 to 1196 Da and localize in the BSA precursor protein.

Based on the protein profile of whey (Table 1) and data from Table 5, we calculated the concentrations of potentially released bioactive peptides from the bioconversion of various types of whey Table 6.

**Table 6.** Potential concentration of bioactive peptides in hydrolysates of different types of whey.

| Whey | Content of Bioactive Peptides in Hydrolysates of Different Types of Whey (in Terms of Standardized Whey with a Mass Fraction of Protein 1%), mg/100 g | | | | | |
|---|---|---|---|---|---|---|
| | Antioxidative | ACE-Inhibitory | DPP-IV-Inhibitory | Bitter | Toxin | Allergen Epitopes |
| CTW (AC) | 28.29 (50.08) | 76.54 (136.38) | 86.68 (154.04) | 47.20 (84.32) | 1.26 (1.89) | 0.00 (0.00) |
| CTW (A/RC) | 23.42 (53.75) | 61.54 (141.53) | 71.40 (164.29) | 38.62 (88.80) | 1.89 (4.41) | 0.00 (0.00) |
| CHW (RC) | 41.29 (45.97) | 115.24 (127.42) | 130.90 (145.10) | 71.94 (79.25) | 1.26 (1.89) | 0.00 (0.00) |
| CHW (A/RC) | 43.23 (51.95) | 108.26 (130.05) | 124.14 (149.25) | 68.91 (82.75) | 1.89 (2.52) | 0.00 (0.00) |
| CHW (TAC) | 7.43 (25.24) | 50.26 (171.30) | 55.05 (188.46) | 24.00 (100.52) | 0.34 (1.26) | 0.00 (0.00) |
| CPW (TCC) | 6.79 (29.02) | 41.10 (177.04) | 45.53 (197.07) | 29.72 (103.09) | 0.35 (1.26) | 0.00 (0.00) |

The highest concentration of antioxidant, ACE-, and DPP-IV-inhibitory peptides was observed in CHW (RC) and CHW (A/RC) types of whey. This fact correlated with their higher protein content. The potentials of CHW (TAC) and CPW (TCC) types of whey were validated by proportional recalculation of the protein profile to standardize protein values (1 g/100 g). However, the concentration of antioxidative peptides was 1.5–2 times lower compared to the other types of whey. At the same time, the whey obtained from the production of cottage cheese had higher concentrations of ACE- and DPP-IV-inhibitory peptides; the content of antioxidant peptides was approximately similar. It was also noted that as the concentration of bioactive peptides increased, the content of peptides possessing bitter flavor also increased.

### *3.5. Modeling (In Silico) of the Digestion of Enzymatic Hydrolysis Products from Milk Whey Proteins*

Stability to GI enzymes action of annotated functional properties is one of the key points of the targeted modeling of the hydrolysis. Another important one is the ability of released peptides to pass the bloodstream unimpeded through the intestinal epithelial barrier and reach the target areas of the body unchanged. At the same time, as a result of peptide digestion in the gastrointestinal tract, no allergenic and toxin-like fragments should be formed. Thus, at the next stage, bioinformatic modeling of the digestion of bioactive peptides by GI enzymes (pepsin, chymotrypsin A, and pancreatic elastase) was performed in silico. The results of modeling the process of digestion in the gastrointestinal tract are presented in Table 7.

A total of 33 peptides were identified guaranteed not to undergo enzymatic degradation by pepsin, chymotrypsin A, and pancreatic elastase (Table 7). There were also identified 20 peptides with ACE- and 19 peptides with DPP-IV-inhibitory activity. Furthermore, 6 peptides (out of 20 peptides) had dual functionality. At the same time, the effective concentration of the half-maximal inhibition of the biological process (IC50) for most peptides has not been established to date. Based on the available data, the lowest IC50 for ACE inhibition was observed for peptides IW (0.7 μM), IY (2.1 μM), and VY (7.1 μM) and for DPP-IV for peptides VL (74.0 μM), IP (149.6 μM), and VA (168.2 μM). Peptides with antioxidant activity underwent enzymatic degradation during in silico digestion modeling. This fact does not exclude their technological use before in vivo consumption. Additionally, 61% of identified bioactive peptides referred to sensory peptides, and 90% of them were bitter-tasting peptides. The identified bioactive peptides were ranked by molecular weight ranging from 174 to 303 Da. It suggests passing into the bloodstream through the intestinal epithelial barrier. The isoelectric point of all the identified bioactive peptides except IH (pI 7.10) was in the acidic (pI $\leq$ 5.88) and alkaline (pI $\geq$ 9.11) ranges. Analysis of the hydropathicity index showed that the bulk of the non-degraded peptides had medium to high levels of hydrophobicity except GR and VR, which were characterized by hydrophilic properties. The absence of precursor protein sites with allergenic and toxic properties among both bioactive peptides and other hydrolysis products was also shown.

**Table 7.** Peptide mapping of the results of bioinformatic modeling of digestion in the gastrointestinal tract.

| Peptide | Protein Precursor (Location) | Bioactivity | IC50, μM | Sensory Characteristics | Allergenicity | Toxicity | Hydropathicity | PI | Molecular Mass, Da |
|---|---|---|---|---|---|---|---|---|---|
| AF | BSA (516–517) | ACE inhibitor | NA | bitter | - | - | 2.30 | 5.88 | 236.26 |
| CF | BSA (384–385) | ACE inhibitor | NA | - | - | - | 2.65 | 5.85 | 285.18 |
| GL | BSA (21–22) | ACE inhibitor<br>DPP-IV inhibitor | NA<br>NA | bitter | -<br>- | -<br>- | 1.70 | 5.80 | 188.21 |
| GR | LF (113–114) | ACE inhibitor | NA | bitter | - | - | −2.45 | 10.11 | 231.24 |
| IA | β-LG (74–75)<br>BSA (25–26; 297–298; 7–8;145–146)<br>LF (49–50)<br>κ-CN (22–23; 129–130) | ACE inhibitor | NA | bitter | - | - | 3.15 | 5.88 | 202.24 |
| IE | BSA (187–188)<br>κ-CN (157–158)<br>β-CN (30–31) | ACE inhibitor | NA | bitter | - | - | 0.50 | 4.00 | 260.31 |
| IG | $\alpha S_1$-CN (44–45; 140–141; 192–193)<br>$\alpha S_2$-CN (54–55) | ACE inhibitor | NA | bitter | - | - | 2.05 | 5.88 | 188.21 |
| IH | β-CN (49–50)<br>$\alpha S_1$-CN (131–132) | DPP-IV inhibitor<br>DPP-III inhibitor | NA<br>NA | - | -<br>- | -<br>- | 0.65 | 7.10 | 268.30 |
| IL | β-LG (56–57)<br>α-LA (97–98)<br>BSA (469–470)<br>LF (135–136) | ACE inhibitor | NA | bitter | - | - | 4.15 | 5.88 | 244.32 |
| IM | κ-CN (75–76)<br>α-LA (91–92) | ACE inhibitor | NA | - | - | - | 3.20 | 5.88 | 262.39 |
| IN | α-LA (55–56; 103–104)<br>κ-CN (51–52; 163–164; 126–127)<br>β-CN (26–27)<br>$\alpha S_2$-CN (87–88) | DPP-IV inhibitor | NA | bitter | - | - | 0.50 | 5.88 | 245.26 |
| IP | β-LG (80–81)<br>BSA (305–306)<br>LF (131–132; 320–321;483–484)<br>κ-CN (110–111; 26–27; 121–122)<br>β-CN (68–69; 76–77)<br>$\alpha S_1$-CN (188–189)<br>$\alpha S_2$-CN (207–208) | ACE inhibitor<br>DPP-IV inhibitor | 130.0<br>149.6 | bitter | - | - | 1.45 | 5.88 | 228.28 |

**Table 7.** *Cont.*

| Peptide | Protein Precursor (Location) | Bioactivity | IC50, μM | Sensory Characteristics | Allergenicity | Toxicity | Hydropathicity | PI | Molecular Mass, Da |
|---|---|---|---|---|---|---|---|---|---|
| IQ | β-LG (12–13)<br>BSA (208–209)<br>κ-CN (28–29)<br>β-CN (193–194)<br>$\alpha S_1$-CN (83–84)<br>$\alpha S_2$-CN (200–201) | DPP-IV inhibitor | NA | bitter | - | - | 0.50 | 5.88 | 259.29 |
| IR | β-LG (151–152)<br>LF (46–47)<br>κ-CN (9–10) | ACE inhibitor | 695.0 | - | - | - | 0.00 | 10.11 | 287.35 |
| IW | α-LA (59–60)<br>LF (275–276) | ACE inhibitor | 0.7 | bitter | - | - | 1.80 | 5.88 | 317.37 |
| IY | LF (83–84; 411–412) | ACE inhibitor | 2.1 | - | - | - | 1.60 | 5.88 | 294.33 |
| VA | β-LG (15–16)<br>BSA (221–222; 54–55; 79–80; 569–570)<br>LF (79–80; 97–98; 153–154; 212–213; 264–265; 450–451)<br>κ-CN (48–49; 147–148)<br>$\alpha S_2$-CN (66–67) | DPP-IV inhibitor | 168.2 | bitter | - | - | 3.00 | 5.88 | 188.21 |
| VD | β-LG (132–133)<br>BSA (392–392; 572–573)<br>LF (245–246; 268–269; 324–325; 475–476)<br>$\alpha S_2$-CN (75–76; 143–144) | DPP-IV inhibitor | NA | umami, bitter, salty | - | - | 0.35 | 3.80 | 232.22 |
| VE | $\alpha S_1$-CN (78–79)<br>β-LG (83–84)<br>α-LA (8–9) | DPP-IV inhibitor | NA | bitter | - | - | 0.35 | 4.00 | 246.25 |
| VF | BSA (167–168; 384–385)<br>LF (66–67; 220–221)<br>$\alpha S_1$-CN (31–32)<br>$\alpha S_2$-CN (150–151)<br>α-LA(101–102) | ACE inhibitor | NA | bitter | - | - | 3.50 | 5.88 | 264.31 |
| VG | BSA (446–447)<br>LF (360–361) | ACE inhibitor | NA | umami, bitter | - | - | 1.90 | 5.88 | 174.19 |
| VH | BSA (246–247)<br>α-LA (94–95) | DPP-IV inhibitor | NA | - | - | - | 0.50 | 7.10 | 254.28 |
| VK | BSA (40–41)<br>LF (215–216; 453–454;100–101)<br>β-CN (100–101)<br>$\alpha S_2$-CN (114–115) | ACE inhibitor | NA | - | - | - | 0.15 | 9.11 | 245.31 |

**Table 7.** *Cont.*

| Peptide | Protein Precursor (Location) | Bioactivity | IC50, μM | Sensory Characteristics | Allergenicity | Toxicity | Hydropathicity | PI | Molecular Mass, Da |
|---|---|---|---|---|---|---|---|---|---|
| VL | β-LG (94–95; 96–97)<br>BSA (23–24; 194–195; 354–355; 475–476)<br>LF (394–395; 396–397; 422–423; 440–441)<br>κ-CN (31–32; 80–81)<br>β-CN (166–167; 174–175; 203–204)<br>$\alpha S_1$-CN (15–16)<br>$\alpha S_2$-CN (107–108) | DPP-IV inhibitor | 74.0 | bitter | - | - | 4.00 | 5.88 | 230.29 |
| VM | BSA (564–565)<br>β-CN (94–95; 159–160) | ACE inhibitor<br>DPP-IV inhibitor | NA<br>NA | - | - | - | 3.05 | 5.88 | 248.33 |
| VN | BSA (43–44; 497–498]<br>LF (489–490)<br>$\alpha S_1$-CN (37–38; 142–143) | DPP-IV inhibitor | NA | - | - | - | 0.35 | 5.88 | 231.24 |
| VP | BSA (426–427; 513–514)<br>LF (162–163; 258–259; 420–421)<br>κ-CN (85–86) | ACE inhibitor<br>DPP-IV inhibitor | 420.0<br>NA | sour | - | - | 1.30 | 5.88 | 214.25 |
| VQ | β-CN (8–9; 86–87; 177–178; 182–183)<br>$\alpha S_1$-CN (74–75; 88–89; 108–109; 114–115; 171–172)<br>$\alpha S_2$-CN (119–120)<br>α-LA (42–43)<br>κ-CN (85–86) | DPP-IV inhibitor | NA | - | - | - | 0.35 | 5.88 | 245.26 |
| VR | β-LG (127–128)<br>BSA (420–421)<br>LF (6–7; 37–38)<br>κ-CN (69–70) | ACE inhibitor<br>DPP-IV inhibitor | 52.8<br>826.1 | salty | - | - | −0.15 | 10.11 | 273.32 |
| VS | β-CN (207–208)<br>$\alpha S_2$-CN (44–45; 210–211)<br>α-LA (21–22)<br>BSA (352–353; 429–430; 439–440; 82–483; 594–595) | DPP-IV inhibitor | NA | - | - | - | 1.70 | 5.88 | 204.21 |
| VT | β-CN (97–98)<br>$\alpha S_2$-CN (7–8)<br>β-LG (3–4)<br>BSA (240–241; 486–487; 236–237)<br>LF (57–58; 381–382)<br>κ-CN (168–169) | DPP-IV inhibitor | NA | - | - | - | 1.75 | 5.88 | 218.24 |
| VW | LF (356–357) | ACE inhibitor<br>DPP-IV inhibitor | NA<br>NA | - | - | - | 1.65 | 5.88 | 303.34 |
| VY | β-LG (41–42)<br>β-CN (59–60)<br>$\alpha S_2$-CN (189–190) | ACE inhibitor | 7.1 | bitter | - | - | 1.45 | 5.88 | 280.13 |

Comparative analysis of the data in Tables 6 and 8 showed that for protein-standardized whey (1 g/100 g), 100% of peptides with antioxidant functions and 50–56% of peptides with ACE- and DPP-IV-inhibitory activity were degraded under the influence of digestive enzymes. In addition, complete degradation of toxin-like peptides was observed as a result of in silico digestion modeling. It is also shown that in one case, degradation of the peptides under the action of digestive enzymes resulted in loss of their activity, whereas in the other case, hydrolysis resulted in the formation of new bioactive peptides from inactive protein residues.

**Table 8.** Potential concentration of bioactive peptides in hydrolysates of different whey types after modeling of digestion in the gastrointestinal tract.

| Whey | Content of Bioactive Peptides in Hydrolysates of Different Types of Whey (in Terms of Standardized Whey with a Mass Fraction of Protein 1%), mg/100 g | | | | | |
|---|---|---|---|---|---|---|
| | Antioxidative | ACE-Inhibitory | DPP-IV-Inhibitory | Bitter | Toxin | Allergen Epitopes |
| CTW (AC) | 0.00 (0.00) | 42.34 (75.40) | 44.92 (79.95) | 49.47 (88.16) | 0.00 (0.00) | 0.00 (0.00) |
| CTW (A/RC) | 0.00 (0.00) | 34.82 (80.07) | 38.29 (88.14) | 41.99 (96.53) | 0.00 (0.00) | 0.00 (0.00) |
| CHW (RC) | 0.00 (0.00) | 64.52 (71.18) | 68.60 (75.80) | 74.10 (81.98) | 0.00 (0.00) | 0.00 (0.00) |
| CHW (A/RC) | 0.00 (0.00) | 61.80 (74.04) | 66.99 (80.44) | 75.95 (91.40) | 0.00 (0.00) | 0.00 (0.00) |
| CHW (TAC) | 0.00 (0.00) | 25.46 (86.08) | 29.22 (99.35) | 24.64 (83.54) | 0.00 (0.00) | 0.00 (0.00) |
| CPW (TCC) | 0.00 (0.00) | 21.80 (93.15) | 25.11 (108.04) | 20.55 (88.52) | 0.00 (0.00) | 0.00 (0.00) |

## 4. Discussion

The type of milk coagulation in the production of a wide range of dairy products has a significant influence on the formation of the whey protein profile. The protein composition of whey plays a determining role in the bioactive potential of the substrate. A review [12] previously showed a difference in the distribution of bioactive functions for peptides in the structure of whey proteins and casein fractions. This difference is caused by their individual amino acid sequences. It is also confirmed by the present study. We have determined the optimal directions for processing each type of milk whey by evaluation of the distribution and yield of protein fractions in the different whey types and the results of in silico analysis. Previously, in silico studies of milk proteins have not observed a combined assessment of prognostic and production data of milk whey. For example, work [24] described the use of in silico approaches (QSAR, molecular docking) in the production of BAP from milk proteins without assessing the technological factors influencing the protein profile of milk raw materials. We combined in silico analysis and assessment of the influence of technological factors on the formation of protein fractions distribution in milk whey. Thus, we determined that the whey obtained by rennet and acid-rennet coagulation was better for producing hydrolysates with high antioxidant and antihypertensive potential. This fact was based on a higher protein content in these milk whey types. Differences in the protein composition of sweet and acid whey types have also been confirmed in [25,26]. The authors reported that sweet whey obtained in cheese production by the rennet method of coagulation was characterized by significantly higher protein content than acid whey obtained under isoelectric coagulation. Heat in the presence of $Ca^{2+}$ coagulation and heat-acid coagulation produced whey with reduced BSA content. BSA has the highest number of toxin-like peptides (ADCCE, CCAKDDP, CCDKP, CCHGDL, VDKCCAADDKE, VGTRCCTKP, VTKCCTE), according to in silico results. Toxic properties of BSA were previously mentioned in the literature in the context of the ABBOS protein segment [27]. This segment was not determined by us. Heat in the presence of $Ca^{2+}$ coagulation and heat-acid coagulation whey were characterized by a small yield of whey proteins, but the protein profile of these whey showed a high antihypertensive and antidiabetic potential relative to other types of whey (when standardized to the same protein content). This fact was proven in our study and is consistent with earlier studies [13,28]. In the process of screening the frequency of BAP in silico, we found that milk whey proteins were characterized by

three main bioactive functions: antidiabetic, antihypertensive, and antioxidant. This fact correlates with the results obtained in [29,30]. At the same time, the authors did not estimate the potential biological activity of these peptides. Biological activity has an important role in determining the optimal processing direction of whey proteins. The number of antidiabetic peptides prevails, but their predicted bioactivity is low. The number of antihypertensive peptides is lower but with greater bioactivity. Thus, the cumulative release efficiency of antihypertensive BAPs appeared to be the most perspective.

Producing a hydrolysate with targeted functional action and optimal safety and sensory characteristics depends on the potential of peptides in the protein structure and on the substrate specificity of enzymes. Enzyme specificity and combining enzymes in compositions can provide hydrolysates with higher degree of hydrolysis and different functional properties. In our study, the enzyme composition consisting of chymotrypsin C and subtilisin proved to be the most effective in releasing the optimal set of peptides. The released sites correlated with the distribution of functional properties with the overall potential of the whey proteins and provided a lower yield of free amino acids compared to other enzyme compositions. Free amino acids can impart bitterness to the hydrolysates. This is consistent with the results of a study [31], which found that when whey was hydrolyzed with subtilisin in vitro, the substrate concentration (15%) and ES (1:100) produced an optimal peptide profile characterized by high oligopeptide content and low free amino acids. The researchers studied six different variations of substrate concentration and ES for subtilisin hydrolysis, which resulted in products with different peptide compositions. This indicates that in addition to substrate characteristics and enzyme specificity, which can be studied in silico, the final peptide profile is also strongly influenced by technological parameters. In earlier studies using only casein fractions as a substrate, Carreira R.L. et al. found that using several enzymes (pepsin and trypsin) mixed with subtilisin increased the nutritional value of hydrolysates. Additionally, the researchers emphasized that the sequence in which the enzymes are added to the compositions and the time significantly affect the peptide profile of the hydrolysates. This confirms the fact that in silico analysis is a significant practical tool in understanding the potential of both substrate and enzyme compositions and provides for further in vitro studies. The other enzyme compositions, trypsin and pepsin, according to our study in the context of all milk whey proteins, are not optimal for protein substrate conversion. Pepsin was found to have the highest relative ACE-inhibitory activity with an absence of specificity for the release of antioxidant peptides and a tendency to release large amounts of free amino acids for the prevailing protein in whey, β-LG. Trypsin was also unacceptable in the aspect of the release of antioxidant peptides from β-LG. The occurrence of ACE-inhibitory β-LG peptides released under its action in the model was 37% lower compared with chymotrypsin C.

It has previously been proven that the protein substrate is subject to digestion by gastrointestinal enzymes in the human body. For this reason, Ref. [32] notes the importance of assessing the stability of BAP structures because part of them may be subject to digestion, and the bioactive effect is reduced. This is confirmed by the data obtained after modeling digestion. Thus, out of 95 peptide types obtained in the model of hydrolysis, only 33 types remained after digestion (almost 3-time reduction). At the same time, it should be noted that the reduction in the concentration of DPP-IV-inhibitory and ACE-inhibitory peptides was only two times. Antioxidant peptides were completely degraded after modeling digestion in the gastrointestinal tract. This suggests the need to use encapsulation technologies to protect antioxidant bioactive structures. Considering this fact, Ref. [33] encapsulated antioxidant and antihypertensive sheep whey peptides in phosphatidylcholine liposomes. It allowed the protection of antioxidant activity at a certain level without its complete loss. Thus, the design of the hydrolysis of milk whey proteins should include the analysis of the entire protein profile based on safety requirements and reduction of potential risks of toxin-like and immunoreactive peptides. In some cases, preselective filtration using membranes with a 50 kDa delay level should be used to isolate BSA in order to eliminate the formation of toxin-like peptides. Additionally, it is important to improve approaches in

the preparation and purification of bioactive peptides in order to reduce bitterness in the obtained hydrolysates and to intensify the development of technologies of their delivery to target organs.

## 5. Conclusions

The implementation of a bioinformatic approach in targeted bioconversion of complex protein matrices of secondary raw materials allowed to substantiate the choice of functions (ACE-inhibitory and antioxidant) based on the frequency of bioactive fragments in the substrate and their potential biological activity.

Summarizing the results of computer modeling in silico with mono- and bi-enzyme compositions, the maximum efficiency of the combined release of peptides with antioxidant and ACE-inhibitory activity by the mixture chymotrypsin C-subtilisin was established. At the same time, the potential activity of hydrolysates varies with the type of whey and its protein profile. On the basis of this model, program software was developed for quantitative calculation of predictably released biopeptides from non-standardized secondary raw materials. It was noted that bioactive peptides obtained as a result of in silico hydrolysis can be considered safe in terms of allergic reactions and toxicological manifestations. It was shown that a large group of bioactive peptides (ACE- and DPP-IV-inhibitory) were resistant to degradation under the action of digestive enzymes. Destruction of peptides with antioxidant function by digestive enzymes was noted. This fact does not reduce their prospective use as natural antioxidants in the technological aspect.

It should be noted that in silico is only a preliminary stage of the wide research for studying biologically active milk protein peptides. It is caused by the impossibility of prediction of directed theoretical enzymatic digestion considering optimal technological modes (temperature, duration, system acidity, substrate-enzyme ratio, conformation state of protein, and activators and inhibitors action). This fact is the reason for research in optimizing the conditions of enzymatic hydrolysis, taking into account technological factors in vitro. However, as our study has shown, the proficient incorporation of in silico analysis into the experimental procedure helps to dramatically reduce the number of in vitro assays required. Our study examined the effect of 16 enzymes on the potential efficiency of whey protein hydrolysis on the models of "digital twins". This eliminated assays to determine the degree of hydrolysis, amount of free amino acids, and ACE-inhibitory and antioxidant activity for hydrolysates produced by each individual enzyme preparation. Additionally, obtained data on the synergistic effects of enzymes resulting from in silico analysis made it possible to avoid carrying out a separate block of in vitro experiments on paired enzyme compositions. The reduction of in vitro assays required to perform reduced the financial and labor costs for further experimentation. In this regard, other researchers can use the algorithm of the combined use of physicochemical analysis of raw materials and bioinformatic tools presented in this paper to optimize their studies and increase their efficiency. Data on the bioactive potential of each whey type can also be used to determine the necessary technological operations for efficient and directed processing of raw materials in order to obtain safe, sensorially acceptable, and bioactive products.

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