Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 139–150
DOI: 10.31883/pjfns/113532
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Hybrid Approach in the Analysis of Bovine Milk Protein Hydrolysates as a Source of Peptides Containing Di- and Tripeptide Bitterness Indicators

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Key words: bitter peptides, BIOPEP-UWM database, protein hydrolysates, hybrid approach

The aim of this study was to employ a hybrid approach combined with a fragmentomic idea of research used to analyze bovine milk protein hydrolysates as a source of peptides with a potential bitter taste. Firstly, selected sequences of bovine milk proteins were *in silico* hydrolyzed using bromelain, ficin, papain, and proteinase K. Hydrolysis was simulated using the BIOPEP-UWM "Enzyme(s) action" tool. Potentially released peptides (called parent peptides) were analyzed for the presence of shorter peptide regions with bitter taste. Some of them were defined as peptide bitterness indicators. Then, *in silico* results were verified in the *in vitro* experiments with the use of a bovine milk protein concentrate (MPC) as a substrate. The verification included the MPC hydrolysis and identification of peptides in MPC hydrolysates using RP-HPLC and RP-HPLC-MS/MS, respectively.

The hybrid analysis of bovine milk protein hydrolysates showed that all released peptides contained fragments with bitter taste and some of them were bitterness indicators, which could potentially determine the taste of a whole sequence. However, the results of *in silico* and *in vitro* hydrolysis were divergent. It was also reflected by the ranking of enzymes acting *in silico* and *in vitro*. Despite above discrepancies, our predictions concerning the release of peptides that may affect the bitter taste of a hydrolysate, contribute to bringing more insights into the taste of foods, especially if unwanted. However, before introducing a food product to the market, sensory studies are required to confirm (or not) its taste.

LIST OF ABBREVIATIONS

BSA, bovine serum albumin; B, bromelain; B-MPC, bromelain hydrolysate of milk protein concentrate; F, ficin; F-MPC, ficin hydrolysate of milk protein concentrate; MLR, multivariate linear regression; MPC, milk protein concentrate; O-MPC, non-hydrolyzed milk protein concentrate; P, papain; P-MPC, papain hydrolysate of milk protein concentrate; PK, proteinase K; PK-MPC, proteinase K hydrolysate of milk protein concentrate; RP-HPLC, reversed-phase high performance liquid chromatography; RP-HPLC-MS/MS, reversed--phase high performance liquid chromatography and mass spectrometry; Rcaf., the ratio of caffeine (the threshold concentration for 1 mM caffeine solution as a standard (Rcaf. = 1.0); $t_{R\ predicted}$, theoretical retention time; $t_{R\ experimental}$, experimental retention time; α_{s1}, casein; α_{s2}-CN, α_{s2}- casein; α-La, α–-lactalbumin; β-Lg, β– lactoglobulin; β-CN, β– casein; κ-CN, κ-casein; and TFA, trifluoracetic acid.

INTRODUCTION

Peptides derived from food proteins exhibit a variety of biological functions, *e.g.*, they contribute to the reduction of blood pressure, glucose and cholesterol levels as well as act as antioxidative, antibacterial, antithrombotic, immunomodulating *etc.* agents [Li *et al.*, 2019]. There are five taste sensations, *i.e.*, bitter, salty, sour, sweet, and umami, and some of them are more attributable to peptides, which is due to their specific amino acids [Ding *et al.*, 2017]. It especially concerns sweet, bitter, and umami peptides [Temussi, 2012].

Milk and dairy products represent sources of valuable nutrients like proteins, sugar (lactose), fat, micro- and macroelements [Guetouache *et al.*, 2014]. It is also well-known that bovine milk proteins are precursors of biopeptides and some of them are components of functional foods aiming to, *e.g.*, reduce blood pressure [Sánchez & Vázquez, 2017]. Despite this fact, hydrolysis of milk proteins leads to the release of bitter-tasting peptides [Kilara & Panyam, 2003]. Bitterness can be then regarded as a problematic property of peptides representing an additional, *e.g.*, health-beneficial function [Iwaniak *et al.*, 2016a]. Thus, the choice of an appropriate debittering method that would not compromise the particular bioactivity of a peptide is a challenge for food scientists and technologists producing protein hydrolysates [Lafarga & Hayes, 2017].

Loads of information on the physiological functions of compounds, including peptides, can be found in biological and chemical databases [Minkiewicz *et al.*, 2013; Bucholska *et al.*, 2018]. Sequences found in databases as well as the computer software dedicated to peptide analyses are helpful in predicting, *e.g.*, the possible mechanisms of peptide

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action, the structure-bioactivity relationship [Iwaniak *et al.*, 2015], and bioactivity [Mooney *et al.*, 2012]. The analysis of bioactive peptides based on computer predictions is called an *in silico* approach and, according to Udenigwe [2014], it is one of the preferred methodological approaches when studying peptides derived from food proteins. Another approach that combines *in silico* studies with classical ones (*i.e*., experimental) is called an integrated or hybrid approach [Udenigwe, 2014; Iwaniak *et al.*, 2019a]. This term was firstly introduced by Udenigwe [2014] who described the limitations of classical and bioinformatic approaches used "separately" to analyze bioactive peptides. The integrated approach may offer an efficient solution to problems encountered during, *e.g*., detection of bioactive peptides using sets of data provided in databases in terms of identification of some structural motifs associated with already "known" bioactivities [Udenigwe, 2014]. It can also be referred to the presence of specific amino acids in a peptide sequence which may determine its bitter taste. For example, peptides composed of phenylalanine (F) and tyrosine (Y) were found as bitter-tasting [Kim & Li-Chan, 2006]. Pripp & Ardö [2007] reported that the bitter taste of peptides depends on the presence of N-terminal basic and bulky residue as well as C-terminal amino acid with the hydrophobic side chain. To conclude, the motif(s) assigned to the particular (*i.e*., known) peptide bioactivity found in a fragment with the "unknown" function may define its biological activity. This way of establishing the function of an unknown fragment is consistent with the fragmentomic idea introduced by Zamyatnin [2009]. Taking into account the fact that such a rule can also be applicable to peptides, we have advanced a novel idea of introducing the bitter-tasting indicators defined as shorter motifs with known bitterness, which when found in the sequences of peptides may potentially determine their taste. Thus, the aim of this study was to employ the hybrid approach to identify peptides likely to be bitter due to the presence of bitter peptidic fragments, especially those called bitter-tasting indicators, in bovine milk protein hydrolysates.

MATERIALS AND METHODS

In silico analysis

Sequences of proteins and bitter-tasting peptides including peptide indicators

The following sequences of bovine (*Bos taurus*) milk proteins were derived from the UniProt database (http://www.uniprot.org/uniprot) [The UniProt Consortium, 2019] (accessed July 2018): αs_1-casein (P02662; 199), αs_2-casein (P02663; 207), β-casein (P02666; 209), κ-casein (P02668; 169), β-lactoglobulin (P02754; 162), α-lactalbumin (B6V3I5; 123), and serum albumin (P02769; 583). Their UniProt accession numbers and number of amino acid residues in the chain (excluding signal peptide), respectively, are provided in brackets. The sequences of bitter-tasting di- and tripeptides were found in the BIOPEP-UWM database (formerly BIOPEP) of sensory peptides and amino acids (51 dipeptides and 51 tripeptides; accessed in July 2018) [Minkiewicz *et al.*, 2008]. They were subjected to analysis according to the multivariate linear regression (MLR) protocol described by Iwaniak *et al.* [2019b]. Based on MLR results [Iwaniak *et al.*, 2019b], di- and tripeptides whose predicted measure of bitterness approximated that of the experimental ones achieved the status of bitter-tasting indicators. The measure of bitterness was bitterness intensity (the ratio of caffeine – Rcaf) value defined as follows [Otagiri *et al.*, 1983]:

$$Rcaf = 1mM/TV$$

TV is defined as detection threshold value of a substance, (the lowest concentration causing detectable bitterness), expressed in mM. The threshold value of caffeine is 1 mM.

Finally, the following twenty dipeptides: LG(0.05/0.1), VD(0.08/0.12), AD(0.17/0.12), IG(0.22/0.14), VI(0,17/0.19), VE(0.17/0.19), RG(0.13/0.21), VL(0.17/0.21), FG(0.17/0.23), GV(0.22/0.23), EY(0.25/0.21), YG(0.33/0.27), VY(0.33/0.29), LE(0.33/0.35), GY(0.33/0.37), LL(0.40/0.37), FP(0.67/0.58), IF(0.67/0.62), FL(0.67/0.67), and FF(0.83/0.70); and nineteen tripeptides: GLG(0.10/0.16), PGR(0.04/0.05), PGP(0.11/0.09), GGL(0.1/0.13), VVV(0.22/0.08), LGL(0.2/0.16), PPG (0.11/0.26), FGG(0.22/0.24), GVV(0.22/0.23), FPK(0.33/0.22), KPK(0.33/0.43), PPP(0.50/0.49), YGG(0.43/0.53), GGF (0.67/0.57), PIP(0.70/0.79), GLL(0.67/0.75), LLL(0.83/0.79), GRP(1.25/1.25), and GYY(2.50/2.41), were the bitterness indicators. Their experimental/predicted Rcafs. are given in brackets.

Theoretical hydrolysis and identification of bitter-tasting indicators

Milk proteins were theoretically hydrolyzed using: bromelain (EC 3.4.22.33), ficin (EC 3.4.22.3), papain (EC 3.4.22.2), and proteinase K (EC 3.4.21.64). Hydrolysis was performed with the BIOPEP-UWM tool called "Enzyme(s) action" [Minkiewicz *et al.*, 2008]. This option is provided when opening the "Analysis" tab of the BIOPEP-UWM engine. The hydrolysis was carried out by selecting the option "one substrate *(i.e.* milk protein sequence): one enzyme". Each product of potential proteolysis (*i.e.* peptide fragment; single amino acids were excluded) was then copied and pasted to the window called "For your sequence" found in the "Profiles of potential biological activity" tab (see "Analysis" panel of BIOPEP-UWM tool). This way, all potentially released fragments were searched for the presence of: bitter-tasting indicators, bitter peptides with no such status as well as their additional bioactivity (if any).

Theoretical retention times

Theoretical (*i.e.* predicted) retention times ($t_{R\ predicted}$) of peptides to be then identified using LC-MS/MS were calculated using the Sequence Specific Retention Calculator (SSRCalc). Correction of retention time predictions was firstly introduced by Dziuba *et al.* [2011] and included corrections made taking into account the type of column, apparatus, and mobile phase composition used for the experimental part of analyses being different from these used to construct reference dataset in SSRCalc software [Spicer *et al.*, 2007]. The following equation [Darewicz *et al.*, 2014] was used for peptide retention time prediction:

$$t_{Rpredicted} = 0.0002 \times t_{R\ SSRCalc}{}^{3} - 0.0085 \times t_{R\ SSRCalc}{}^{2} + 1.0415 \times t_{R\ SSRCalc} + 8.6434$$

where: $t_{RSSRCalc}$ – retention time (min) calculated with the Sequence Specific Retention Calculator (SSRCalc, available at: http://hs2.proteome.ca/SSRCalc/SSRCalc.html, accessed: December 2018) [Spicer *et al.*, 2007].

To calculate the retention times with SSRCalc, peptides were provided in a one-letter code and implemented to the software. The following parameters were set up: a = 2.02, *i.e.* the retention time of the substance not adsorbed on the column; and b = 0.94, *i.e.* the parameter dependent on the acetonitrile gradient (0.66% per min) [Dziuba *et al.*, 2011]. The mathematical algorithm provided by the SSRCalc software was adjusted to the column, the same was done with separation parameters like pore diameter: 100 Å, column: C18, and TFA concentration: 0.1% [Krokhin *et al.*, 2004; Krokhin, 2006].

In vitro analyses

Materials and reagents

A commercial milk protein concentrate (MPC) called TMP80 (containing 80% protein in a proportion of casein to whey proteins at 8:2 (w/w)) was produced by Milei GmbH (Leutkirch, Germany) and was donated by Nordmann, Rasmann, Poland Ltd. (Warsaw, Poland). Bromelain (EC 3.4.22.32, Sigma-Aldrich No. B5144; 5–15 units/mg protein), proteinase K from *Tritiarachium album* (EC 3.4.21.64, Sigma-Aldrich No. P2308; ≥30 units/mg protein), papain (EC 3.4.22.2, Sigma-Aldrich No. P4762; 10 units/mg protein), ficin (EC 3.4.22.3, Sigma-Aldrich No. F4125; ≥1 unit/mg protein), trifluoroacetic acid (TFA), acetonitrile, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (Bis-Tris), 2-mercaptoethanol, and urea were purchased from Sigma-Aldrich® (Poznań, Poland). All chemicals were of analytical grade. Water used to formulate solutions and buffers was prepared using a Milli-Q PLUS system (Millipore Corp., New York, NY, USA).

Hydrolysis of milk proteins

MPC was dissolved in distilled water to obtain 5 separate solutions containing 3% protein (w/v) each [Cheung *et al.*, 2015] and having the non-adjusted pH of 6.9±0.1. All MPC solutions were continuously and gently stirred as well as preheated for 5 min using an Heidolph Unimax Modular Incubator 1010 (Heidolph Instruments GmbH & CO. KG, Schwabach, Germany). Pre-incubation temperatures were typical of enzyme activities specified by manufacturers, *i.e.* 50°C (bromelain; B), 37°C (proteinase K; PK), 65°C (papain; P), and 50°C (ficin; F). Afterwards, 3-h hydrolysis of four samples of MPC solutions was carried out [Cheung *et al.*, 2015; Lacroix *et al.*, 2016] under continuous stirring and the enzyme-to-substrate ratio (protein) of 1:100 (w/w) [Al-Shamsi *et al.*, 2018]. The pH values of MPC solutions taken for proteolysis were typical of enzyme activity, *i.e.* 7.0 (B, P, PK) and 6.5 (F). After the hydrolysis, the mixtures were heated at 90°C for 15 min to inactivate the enzymes and then freeze-dried [Lacroix *et al.*, 2016]. Four MPC hydrolysates (bromelain-, ficin-, papain-, and proteinase-K MPC hydrolysates, named B-MPC, F-MPC, P-MPC, and PK-MPC, respectively) as well as one non-hydrolyzed MPC (O-MPC; reference sample) were prepared in duplicate.

Separation of MPC and products of its hydrolysis by reversed-phase high performance liquid chromatography (RP-HPLC)

The reversed-phase high performance liquid chromatography (RP-HPLC) was used to separate MPC and products of its hydrolysis. The Shimadzu® system (Tokyo, Japan) was used for sample separation. It comprised of: two LC-20AD pumps, an SIL-20AC HT autosampler, a CBM-20A controller, a CTO-10AS VP thermostat, an SPD-M20A photodiode detector, and a DGU-20A5 degasser. The Jupiter Proteo Phenomenex® column (Torrance, CA, USA) with the following parameters: 250×2 mm, particle diameter – 4 μm and pore diameter – 90Å, was used. Mobile phase consisted of solvent A – 0.01% (v/v) TFA solution in water and solvent B – 0.01% (v/v) TFA dissolved in acetonitrile. The gradient of solvent B increased from 0 to 40% during 60 min. Then, the column was washed with solvent B in the mobile phase as follows: 40–100%, 60–65 min; 100%, 65–70 min, 100–0%, 70–71 min; 0%, 71–80 min. The injection volume was 30 μL, flow rate was 0.2 mL/min, and column temperature was 30°C [Bucholska & Minkiewicz, 2016]. Chromatograms were acquired at the wavelength of 220 nm [Visser *et al.,* 1991]. Data was analyzed using Lab Solution (LC Solution) software provided by Shimadzu®. RP-HPLC analyses were performed in duplicate.

The samples of MPC and its enzymatic hydrolysates were prepared as follows: 2 mg of a freeze-dried sample was dissolved in 300 μL of a buffer containing 0.1 M Bis-Tris and 4 M urea. Then, 20 μL of 2-mercaptoethanol were added and the mixture was vortexed and next incubated at a room temperature for 1 h. After the incubation, 680 μL of 6 M urea solution in a mixture of acetonitrile and water (at a ratio of 1: 9 (v/v); pH 2.2 adjusted by the addition of TFA) were added to the sample and stirred. Samples were then centrifuged (10 min, 10,000×g) (Hermle Z 233, M-2, HERMLE LaborTechnik GmbH, Wehingen, Germany) [Visser *et al.,* 1991; Dziuba *et al.,* 2011].

Identification of peptides using liquid chromatography and mass spectrometry (RP-HPLC-MS/MS)

The samples of MPC hydrolysates were prepared identically like for RP-HPLC analysis. The only difference was the weight of the freeze-dried hydrolysate taken for sample preparation (10 mg instead of 2 mg).

LC-MS/MS identification analysis was carried out using the VARIAN® 500-MS (Agilent Technologies, Santa Clara, CA, USA) ion trap mass spectrometer with an electrospray ion source and an HPLC assembly comprising two 212-LC pumps, a ProStar 410 autosampler, and a Degassit degasser (MetaChem Technologies®, Torrance, CA, USA) as well as a nitrogen generator (Parker Domnick Hunter Scientific®, Gateshead, UK). Gradient of the mobile phase, column type, and column parameters were identical as those described in the subchapter above. Data was registered between 5 and 60 min. The other parameters for mass spectrometry were as follows: needle and shield voltages: 5000 and 600 V re-

spectively; spraying and drying gas (nitrogen) pressure: 55 and 30 psi, respectively; drying gas temperature 390°C; and flow rate of damping gas (helium) 0.8 mL/min. The other parameters were as follows: positive polarity with current ionization 600 V, capillary voltage 100 V, retardation factor loading 100%, isolation window 3.0, excitation storage level m/z = 100–2000 Da, flow rate 0.2 mL/min, injection volume 15 μL, frequency data recording 0.05–0.07 Hz single scan averaged from five microscans, options such as: use of air segment, headspace pressure and alarm buzzer were included [Darewicz *et al.,* 2014; Bucholska & Minkiewicz, 2016]. For peptide retention times determination, all chromatograms were smoothed using Savitzky & Golay method [1964] implemented from MS WorkStation v. 6.9 software. All identification analyses were performed in duplicate.

Mass to charge ratios of fragment ions were theoretically calculated using the Fragment Ion Calculator available at: http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html, accessed: December 2018. After loading the peptide of interest into a window "Peptide:" the following software options were ticked: "+1", "+2", and "+3" (function called "Charge state") referring to mono-, double-, and triple-ionized ions, respectively as well as "A, B, C, X, Y, Z" [Roepstorff & Fohlman, 1984; Paizs & Suhai, 2005]. Submitted results included sequences of peptides to be potentially identified, their monoisotopic masses, and m/z of fragment ions (A, B, C, X, Y, Z).

RESULTS AND DISCUSSION

There are several aspects to consider when thinking about bitter taste of food resulting from the presence of peptides. They include, *e.g*., amino acid composition of peptides, physicochemical properties of amino acids constituting the peptide, conditions of hydrolysis of food proteins, enzymes used for proteolysis, taste-taste interactions between peptides, and methodologies applied to evaluate the taste of foods [Iwaniak *et al.*, 2016a]. It is also well-known that tastant peptides exhibit a variety of biological functions. It especially concerns short sequences (di- and tripeptides). Among them, inhibition of proteolytic enzymes is the main activity correlated with food taste [Iwaniak *et al.*, 2016a]. The most extensively studied so far has been the correlation between ACE inhibition and bitterness of peptides [Daskaya-Dikmen *et al.*, 2017]. The latest studies showed also that bitter peptides derived from a pepsin hydrolysate of peeled shrimp (*Litopenaeus vannamei*) exhibited lymphocyte and lysozyme activity contributing to the improvement of fish survival in aquaculture [Deng *et al.*, 2019].

The above-mentioned aspects related to bitterness of peptides indicate them to be the best-described group of tastant peptides. It can also be reflected by the number of bitter peptides in the BIOPEP-UWM database of sensory peptides and amino acids [Iwaniak *et al.*, 2016b]. Currently, 483 sequences of sensory peptides (including some amino acids) can be found in this database. This number includes 305 bitter peptides (excluding bitter amino acids), *i.e.* ~63% of all sensory sequences (accessed: October 2019). To recapitulate, considering the methodological aspects of analysis of peptides derived from food proteins, which is costly and time consuming, bitter peptides are in the focus of interests of scientists trying to develop new methods that may contribute to the extension of knowledge about food bitterness. These new methods involve also bioinformatic analyses. According to Gallego *et al*. [2019], the bioinformatic-assisted approach affords the opportunity to predict the production of bitter peptides from food sources in a cheaper and faster way. Our idea of employing predictive (*i.e. in silico*) methods combined with experimental analysis, which is presented in this paper, inscribes into this trend.

Initially (data not shown), 15 proteases were taken for the *in silico* hydrolysis of bitter peptides. Taking into account the number of peptides predicted to be released from the individual milk protein sequence by an enzyme, four proteases were reported as the "potentially most effective" ones when producing peptidic motifs. They were as follows: bromelain, papain, ficin, and proteinase K. According to literature data, these enzymes were used for the production of protein hydrolysates as well as peptides exhibiting the biological activity. For example, Choopinham *et al.* [2015] used papain to produce hydrolysates with ACE-inhibiting and antioxidative properties derived from gelatin extracted from tilapia skin. Arihara [2006] used papain for the hydrolysis of porcine actin, which allowed identifying a DAQEKLE peptide exhibiting the antioxidative activity. In turn, bromelain was expected to release from clam proteins peptides rich in cysteine which induces the antibacterial effect [Zambrowicz *et al.*, 2013]. Antibacterial peptides were also derived from goat casein hydrolyzed by ficin [Esmaeilpour *et al.*, 2016]. In turn, proteinase K was used for the hydrolysis of beef proteins to release a GFHI sequence with the ACE-inhibiting effect [Ryan *et al.,* 2011].

Our results of *in silico* hydrolysis of milk protein sequences revealed that many peptides exhibiting various biological and physiological activities, including the bitter-tasting activity, can be released using B, F, P, and PK. However, we focused on the results of simulated proteolysis which showed such peptide products that were composed of at minimum 4 residues and were not defined by BIOPEP-UWM database search options as "bitter/bioactive". They were defined as parent (*i.e.* precursor peptides) and were planned to be identified in the experimental samples of MPC hydrolysates. Moreover, released precursor peptides were searched for the presence of bitterness indicators as well as bitter peptides without such a status (see Methods). This strategy was consistent with Zamyatnin's [2009] fragmentomic idea (see above). Our results are shown in Table 1.

The number of potentially released fragments fulfilling the above criteria ranged from 0 (β-lactoglobulin treated with PK) to 27 (serum albumin hydrolyzed using B). Bromelain was the most effective enzyme in terms of production of parent peptides, while proteinase K was the least effective one. Comparable results were obtained for papain. The effectiveness of enzymes in releasing the highest number peptides was ranked in the following order: bromelain>papain>ficin>proteinase K. The total number of potentially released peptides from all milk proteins treated with four enzymes was 226. All peptides released from the milk proteins contained shorter fragments with a documented bitter taste.

TABLE 1. Peptide fragments released *in silico* from bovine milk proteins containing bitterness indicators (**in bold**) or peptides without the status of the indicator (normal font).

Milk protein	Bromelain	Ficin	Papain	Proteinase K
κ-Casein	VESTVA/VE IPPK/**PP, PK** VLSRY/**VL** TEIPTINTIA/**EI** DERFFSDK/**FF**, RF TLEDSPEVIESPPEINTVQVTSTA/ **PP, VI**, LE, **EI** LINNQFLPY/**FL**, LI QILQWQVLSNTVPA/**VL**, IL	IPPK/**PP, PK** IESPPEIN/**PP, EI** TEIPTIN/ **EI** DERFFSDK/**FF**,RF	IPPK/**PP, PK** VLSRY/**VL** FFSDK/**FF** LINNQFLPY/**FL,** LI QILQWQVLSNTVPA/**VL**, IL	ASGEP/GE KKNQDKTEI/**EI** RCEKDERF/RF
αs_1-Casein	VNELSK/EL VNQELA/EL VPLG/**LG** EPMIG/**IG** ESISSSEEIVPNSVEQK/VE, IV**, EI** PSFSDIPNPIG/**IG** VPQLEIVPNSA/IV, LE, **EI** LEQLLRLK/LE, LL TTMPLW/PL, LW PFPEVFG/FG, FP, VF, PF, PFP PELFRQFY/LF, EL LPQEVLNENLLRFFVA/**FF**, RF, FV, **VL**, LL	EPMIG/**IG** TTMPL/L ESISSSEEIV/IV, **EI** PFPEV/FP, PF, PFP RFFV/**FF**, RF, FV	VFG/FG, **VF** VLNE/**VL** IVPNSA/IV VPQLE/LE IVPNSVE/VE, IV PFPE/**FP,** PF, PFP NLLR/LL PMIG/**IG** PSFSDIPNPIG/**IG** FFVA/**FF**, FV TTMPLW/PL, LW	TDAP/DA KHQGL/GL KEGI/**GI**, EG SSSEEI/**EI** NQEL/EL
αs_2-Casein	NEEEY/EYFP ITVDDK/**VD** FPQY/**FP** VIPY/**VI** PWIQPK/**PK** VPITPTLNREQLSTSEENSK/EEN TVDMESTEVFTK/**VF, VD** NRLNFLK/**FL** NTMEHVSSSEESIISQETY/II LNEINQFY/**EI** PIVLNPWDQVK/**VL, IV**	STSEEN/EEN EEEY/EY FPQY/**FP** PWIQPK /**PK** SSSEESIISQETY/II	ITVDDK/**VD** TVDME/**VD** VFTK/**VF** SIISQE/II FPQY/**FP** VIPY/**VI** PWIQPK/**PK** LNFLK/**FL** PIVLNPWDQVK/**VL, IV**	TEEEKNRL/EE, EE STSEENSKKTV/EEN RNANEEEY/EY
β-Casein	PVRG/RGFP PFPG/**FP**, PF, PFP VLPVPQK/**VL** QEPVLG/**VL**, LG IHPFA/PF EMPFPK/**PK, FP**, FPK, PF, PFP RELEELNVPG/LE, EL, EL EIVESLSSSEESITRINK/VE, **IV, EI** FLLY/**FL**, LL PFPIIV/PFPIIV, **FP,** II PIPNSLPQNIPPLTQTPVVVPPFLQPEVMG/ **PP, PP, FL**, PF, PPF, VVV, **PIP**, VPPFL PVEPFTESQSLTLTDVENLHLPLPLLQSWM HQPHQPLPPTVMFPPQSVLSLSQSK/ **PP, PP, FP**, FPP, PF, **VL**, VE, VE, LL	PPTV/**PP** PIPN/**PIP** PFPG/**FP**, PF, PFP IPPL/**PP** EMPFPK/**PK, FP**, FPK, PF, PFP EPFTESQSL/PF MFPPQSV/**PP, FP**, FPP PPFL/**PP, FL**, PF, PPF PFPIIV/PFPIIV, **FP,** II	PFTE/PF PVLG/**VL, LG** PFPG/**FP**, PF VLPVPQK/**VL** QTQSLVY/VY, LV MPFPK/**PK, FP**, FPK, PF, PFP SQSLTLTDVE/VE FLLY/**FL**, LL PFPIIV/PFPIIV, **FP,** II QPLPPTVMFPPQS VLSLSQSK/**PP, PP, FP**, FPP, **VL** PIPNSLPQNIPPLTQTPVVVPPFLQPE/ **PP, PP**, FL, PF, PPF, VVV, **PIP**, VPPFL LPLPLLQSWMH/LL	–

TABLE 1. Continued...

Milk protein	Bromelain	Ficin	Papain	Proteinase K
β-Lactoglobulin	PTPEG/EGFP VEELK/VE, EL LDIQK/LD EPEQSLA/SLA PLRVY/VY CQCLVRTPEVDDEA/**VD**, LV LIVTQTMK/LI, **IV** SDISLLDA/LL, LD, DA VLVLDTDY/**VL, VL**, LD, DY LLFCMENSA/LL, LF DLEILLQK/LL, LE, IL, DL, **EI** LPMHIRLSFNPTQLEEQCHI/LE	PTPEG/EG	LDIQK/LD ILLQK/IL, LL LIVTQTMK/LI, **IV** LSFNPTQLE/LE SDISLLDA/LL, LD, DA VLVLDTDY/**VL, VL**, LD, LV LLFCME/LL, LF	DAQSAP/DA
α-Lactalbumin	ILDK/LD, ILFP IVQNNDSTEY/**IV, EY** CEVFRELK/**VF**, EL ILFHA/LF, IL LDQWLCEK/LD FLDDDLTDDIMCVK/**FL**, LD, DL MMSFVSLLLVG/FV, LL, LL, LLL, LV	MMSFV/FV DSTEY/**EY**	IVQNNDSTE/**IV** ILDK/LD, IL LFQINNK/LF ILFH/LF, IL LDQWLCE/LD FLDDDLTDDIMCVK/**FL**, LD, DL MMSFVSLLLVG/FV, LL, LL, LLL, LV	HTSGY/GY QNNDSTEY/**EY** SCDKF/KF
Serum albumin	HRFK/RF FP SRRHPEY/RR, **EY** RRHPY/RR DELCK/EL DDSPDLPK/**PK**, DL VFQECCQA/**VF** STVFDK/**VF** QEPERNECFLSHK/**FL** CLLPK/**PK**, LL LVTDLTK/LV, DL EFVEVTK/FV, VE, EF LQQCPFDEHVK/PF DLLECA/**DLL**, LE, LL, DL CCTESLVNRRPCFSA/RR, RP, LV LIVRY/IV, LI HLVDEPQNLIK/**VD**, LV, LI PDPNTLCDEFK/EF SLHTLFG/FG, LF LVLIA/**VL**, LV, LI TVMENFVA/FV PELLY/**ELL,** LL, EL LFTFHA/LF LVELLK/VE, **ELL**, LL, LV, EL IPENLPPLTA/**PP** VPQVSTPTLVEVSRSLG/VE, **LG**, LV VSVLLRLA/**VL**, LL LVNELTEFA/LV, EL, EF	SRRHPEY/RR, **EY** RRHPY/RR CDEFK/EF DDSPDL/DL RRPCFSA/RR, RP QQCPFDEHV/PF ECFL/**FL**	FVDK/FV, VD SLVNR/LV DDSPDLPK/**PK**, DL PQNLIK/LI STVFDK/VF LVVSTQTA/LV LIVR/**IV**, LI NFVA/FV CLLPK/**PK**, LL LVTDLTK/LV, DL CFLSH/**FL** DLLE/**DLL**, LE, LL, DL LCVLH/**VL** PLLE/LL, LE LQQCPFDE/PF NLPPLTA/**PP** VSVLLR/**VL**, LL VPQVSTPTLVE/VE, LV LSLILNR/LI, IL LVLIA/**VL**, LV, LI SFLY/**FL** LFTFH/LF	GTRCCTKP/KP ARRHP/RR KECCDKP/KP KHKP/KP NRRP/RR, RP AKEY/**EY** ADCCEKQEP/AD DTHKSEI/**EI** KADEKKF/KF, AD, ADE HKECCHGDL/DL ADESHAGCEKSL/AD, ADE GERAL/GE GEEHF/GE QEAKDAF/AF, DA ECADDRADL/AD, AD, DL CDEF/EF

Some of them, mainly dipeptides, had the status of bitterness indicators. Tripeptide bitterness indicators were present occasionally in the parent peptides. They were typical of precursor peptides composed of at minimum 5 amino acids (*e.g.* DLLECA containing **DLL** indicator and PELLY with **ELL**; sources: B hydrolysate of serum albumin). One of the exceptions was precursor PIPN with **PIP** indicator in its structure (F hydrolysate of β-casein). According to Iwaniak & Dziuba [2011], the shorter the peptide chain, the higher the probability of finding it in their protein precursor. This rule can also be referred to our study concerning parent peptides with the potential to be bitter. Another observation made by Iwaniak & Dziuba [2009] regarding the impact of the length of a parent sequence on the higher probability of finding the functional fragment in it (briefly, the longer the precursor, the higher the probability of detecting the shorter motif in it) was not applicable to our study. Although some theoretically released peptides were composed of over a dozen of amino acids, the bitterness indicators detected in them were not as numerous as expected. Thus, the potential of parent peptide to be the source of bitterness indicators was evaluated considering the frequency of occurrence of a bitterness indicator in a precursor (data not shown). It was defined as A and was used for, *e.g.*, *in silico* evaluation of animal and plant proteins as the precursors of bioactive peptides [Iwaniak & Dziuba, 2009]. The A is calculated automatically by BIOPEP-UWM using the algorithm A=a/N, where: a is the number of peptides with a given activity, and N is the number of amino acids in a parent protein (in our case – parent peptide). The higher the A is, the better source of bitterness indicators the parent peptide is. According to A, the best potential sources of peptide indicators were, *e.g.*, IPPK (source: B, F, and P hydrolysates of κ-casein containing **PP** and **PK**), PPFL with **PP**, **FL** indicators (from F hydrolysate of β-casein) or VLSLSQSK with indicators: **PP**, **PP**, **FP**, and **VL** (from P hydrolysate of β-casein). For parent peptides, *i.e.* IPPK, PPFL, and VLSLSQSK, found in the *in silico* hydrolysates of κ- and β-caseins (first and other two sequences, respectively) A was 0.5. For example, A calculated for 30-mer parent peptide PIPNSLPQNIPPLTQTPVVVPPFLQPEVMG (from B hydrolysate of β-casein) containing **PP**, **PP**, **FL**, and **PIP** indicators was 0.133. To summarize, all bovine milk proteins had the potential to release peptides containing bitter peptides, including bitterness indicators. Among them, β-casein seemed to be the richest source of parent peptides likely to be bitter due to the presence of bitter-tasting peptides with and without the status of an indicator. Bumberger & Belitz [1993] isolated and then hydrolyzed bovine β-casein using trypsin and found regions of this sequence (fragments: 49–97, 203–209) as the sources of shorter fragments with confirmed bitterness (*e.g.* segment 49–68). According to BIOPEP-UWM computations (data not shown), the majority of potentially released parent peptides were also present in the above-mentioned regions of β-casein.

Results concerning the *in silico* prediction of "bitterness potential" of parent peptides released due to the action of proteases were the premise to verify them in laboratory conditions. Then, MPC protein solutions (3%; w/v) were hydrolyzed using the above-mentioned enzymes (see Methods). Such concentrations of milk protein solutions, enzyme-to-substrate ratio, and proteolysis time were used by Cheung *et al.* [2015] for the analysis of milk whey protein hydrolysates as sources of ACE (*i.e.* angiotensin converting enzyme; EC 3.4.15.1) inhibitors as well as for the sensory evaluation of the obtained hydrolysates after additional exopeptidases treatment. Lacroix *et al.* [2016] successfully applied such parameters for the hydrolysis of commercial milk whey protein isolate to analyze the hydrolysates as sources of ACE and DPP IV (dipeptidyl peptidase IV; EC 3.4.14.5) inhibitors. Our O-MPC solution as well as respective 4 hydrolysates were analyzed using RP-HPLC to separate MPC and products of its hydrolysis. The results are shown in Figure 1 and Table 2. When looking at the chromatograms of O-MPC and its hydrolysates (Figure 1a and 1b-e, respectively), two retention time intervals referring to the process of molecules separation can be distinguished, *i.e.*: 14.00–39.99 min and 40.00–60.00 min. The first, biggest peak that was eluted at about 10 min, is the so-called "injection peak" representing

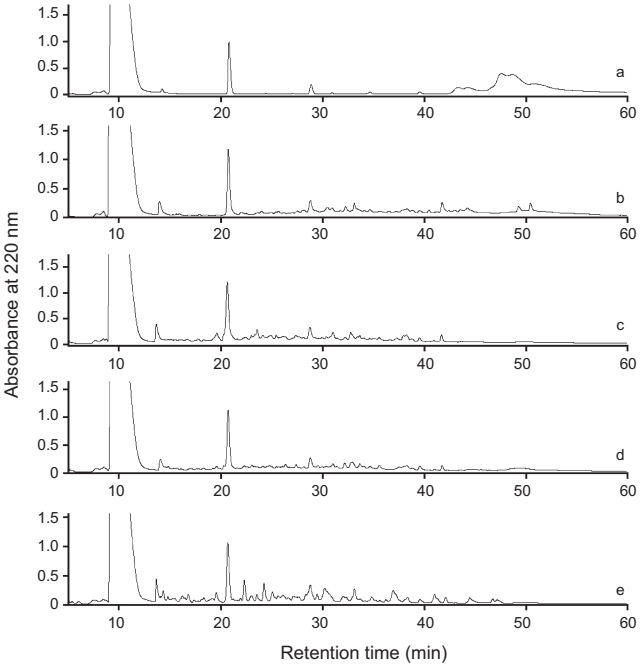

FIGURE 1. RP-HPLC chromatograms of milk protein concentrate (MPC) and its hydrolysates: a) non-hydrolyzed MPC; b) bromelain hydrolysate of MPC; c) ficin hydrolysate of MPC; d) papain hydrolysate of MPC; e) proteinase K hydrolysate of MPC.

TABLE 2. Relative peak areas (%)[1] of RP-HPLC separations of milk protein concentrate and its enzymatic hydrolysates.

Time interval (min)	O-MPC	B-MPC	F-MPC	P-MPC	PK-MPC
14.00–39.99	1.2	18.0	21.4	16.1	22.8
40.00–60.00	20.5	10.7	1.1	1.9	1.1

[1]Area of all peaks between 0 and 60 min is 100%.
O-MPC – non-hydrolyzed milk protein concentrate; B-MPC, F-MPC, P-MPC, PK-MPC – milk protein concentrate hydrolyzed by bromelain, ficin, papain and proteinase K, respectively.

non-retained substances like, *e.g.*, components of buffers used to dissolve proteins or peptides [Bucholska & Minkiewicz, 2016]. Such compounds could also be present in the peak eluted between 20.00 and 20.99 min. Thus, peaks eluted between 00.00–14.00 and 20.00–20.99 min were not subjected to further interpretation. The highest number of peaks was observed in the time interval between 14.00 and 39.99 min and they were characteristic for all hydrolysates. Some peaks eluted between 40.00 and 60.00 min appeared only in O-MPC chromatogram (Figure 1a) and can be considered as typical of the high molecular mass molecules, like proteins. They disappeared in the chromatograms of hydrolysates. Changes in peaks distribution during the RP-HPLC separation were also confirmed by the relative peak areas in the above-specified time intervals (Table 2). The changes in the distribution of peak areas confirmed that MPC was hydrolyzed by 4 enzymes. The content of compounds with long retention times (above 40.00 min) in a bromelain hydrolysate was higher than in the other hydrolysates. Long retention times may be tentatively attributed to intact proteins and/or polypeptides being products of proteolysis. If there is no significant difference in hydrophobicity, peptides with longer chains have usually longer retention times than these with shorter chains [Krokhin *et al.,* 2004; Krokhin, 2006; Spicer *et al.*, 2007; Dziuba *et al.*, 2011]. In the light of results presented in Figure 1 and Table 2, bromelain seems to be less efficient in the hydrolysis of proteins form milk powder than other enzymes used in this experiment.

Identification of parent peptides was based on the analysis of their fragment ions, according to the nomenclature introduced by Roepstorff & Fohlman [1984]. Peptides were shown as the groups of ions detected at the same retention time. Fragmentation of peptides may occur due to, *e.g.*, the non-sequential charge-directed pathway. It is related to the formation of fragment ions involving neutral loss of neutral molecules like water or ammonia [Paizs & Suhai, 2005]. The following requirements had to be fulfilled to enable identification of a specific peptide in a hydrolysate sample: the presence of fragment ions detected in a specific retention time, and the difference between predicted ($t_{R\ predicted}$) and experimental ($t_{R\ experimental}$) retention times of *ca*. ±10% [Bucholska & Minkiewicz, 2016].

An example of a chromatogram with the identified peptide is shown in Figure 2. It concerns the parent sequence KEGI identified in α_{s1}-casein. KEGI was identified in PK hydrolysate of MPC. The *m/z* of precursor ion $[M+H]^+$ was 446.26. Eight fragment ions: Y_3^+, Z_4^+, X_1^+, C_1^+, B_4^+, B_3^+, A_4^+, and A_3^+, were eluted between 13.27 and 14.00 min of the separation process. Based on the presence of the group of fragment ions eluted at the same time interval as well as their intensity expressed in thousands of counts (kCounts), KEGI was classified to the group of identified parent peptides. The differences between $t_{R\ predicted}$ (14.15 min) and $t_{R\ experimental}$ (13.64 min – average value from these presented above) was 5.6%.

Results concerning the identification of other peptides in MPC hydrolysates are summarized in Table 3. Twenty eight peptides were identified in MPC *in vitro* hydrolysates, which accounted for 12.38% of the total number of parent sequences "identified" *in silico* (226; see Table 1). Ficin and papain were

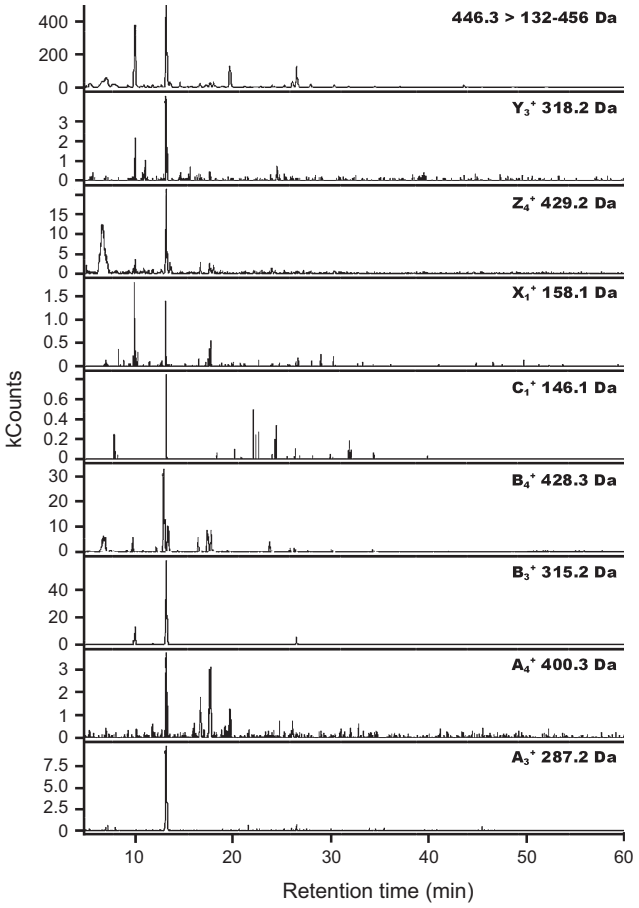

FIGURE 2. Exemplary LC-MS/MS chromatogram of peptide KEGI (from proteinase K hydrolysate of milk protein concentrate) containing: *m/z* of precursor ion $[M+H]^+$ (the top of the chromatogram), *m/z* range of investigated fragment ions (the top of the chromatogram), and types of fragment ions including their *m/z* (chromatograms 2–9).

the most effective enzymes *in vitro* – they both released 9 parent peptides. Bromelain and proteinase K released 5 such peptides both. Thus, enzyme effectiveness in releasing the highest number of peptides could be ranked as follows: ficin = papain > bromelain > proteinase K, and it differed from the ranking based on *in silico* predictions (bromelain > papain > ficin > proteinase K). The fact that hydrolysis of milk proteins by papain and ficin hydrolysis led to obtaining more identified peptides of interest than the hydrolysis by proteinase K is consistent with *in silico* predictions. The discrepancy between the predicted and the determined effectiveness of bromelain in producing oligopeptides may be explained taking into account incomplete hydrolysis of proteins by this enzyme (Figure 1 and Table 2). Although, both rankings of enzymes were different, β-casein was the protein in which the highest number of peptides was detected during *in silico* experiment. The highest number of peptides referring to those present in β-casein (8) were also identified in MPC hydrolysates. All *in vitro* identified peptides contained shorter bitter fragments, including bitterness indicators. For example, PFPIIV peptide was known as bitter itself, and contained the following fragments, including two indicators (in bold): PFP, PF, IV, **FP, II**, PFPIIV. This peptide was identified in B, F, and P hydrolysates of MPC. Two identified parent peptides, *i.e.* TTMPLW (P-hy-

TABLE 3. Peptides identified in hydrolysates of milk protein concentrate (MPC) using LC-MS/MS.

MPC hydrolysate	Identified peptide	$[M+H]^+$ (*m/z*)	$t_{R\ predicted}$ (min)	$t_{R\ experimental}$ (min)	Bitter peptide	Protein source
B-MPC	VLPVPQK (antioxidative)	780.50	24.6	26.80 -27.81	**VL**	β-casein
	EIVESLSSSEESITRINK	2021.04	35.43	38.99–41.00	VE, **IV, EI**	
	FLLY	555.32	39.05	34.80 – 35.70	**FL**, LL	
	PFPIIV	685.43	40.60	40.28 – 40.90	PFP,PF, IV, **FP, II**, PFPIIV	
	SDISLLDA	833.43	34.71	38.10 -39.88	LL, LD, DA	β-lactoglobulin
F-MPC	TTMPL	562.29	27.11	28.67 -28.10	**FP,** PF, PFP	α_{s1}-casein
	ESISSSEEIV	1079.51	28.15	30.20 – 31.46	PL, LW	
	FPQY	554.26	25.62	24.98 – 25.20	FP	α_{s2}- casein
	PPFL	473.28	32.99	34.57 – 35.20	**PP, FL**, PF, PPF	β-casein
	PFPIIV	685.43	40.60	40.28 – 40.90	PFP,PF, IV, **FP, II**, PFPIIV	
	IESPPEIN	898.45	26.45	26.04 – 26.10	**PP, EI**	κ-casein
	DERFFSDK	1043.48	27.77	23.53 – 25.30	**FF**, RF	
	MMSFV	614.27	34.00	33.83 -34.10	FV	α-lactalbumin
	PTPEG	500.24	11.70	12.87 -13.01	EG	β-lactoglobulin
P-MPC	TTMPLW (opioid, immunomodulating, ACE inhibitor)	748.37	38.39	39.83 -40.10	PL, LW	α_{s1}-casein
	MPFPK	619.33	27.49	30.23 -33.11	**PK, FP**, FPK, PF, PFP	β-casein
	PFPIIV	685.43	40.60	40.28 – 40.70	PFP,PF, IV, **FP, II,** PFPIIV	
	VLSR	474.30	17.00	15.50–16.10	**VL**	κ-casein
	SDISLLDA	833.43	34.71	38.10 -39.88	LL, LD, DA	β-lactoglobulin
	LLFCME	755.35	39.05	40.01 – 40.60	LL, LF	
	NLPPLTA	725.42	29.09	32.28- 32.66	**PP**	serum albumin
	SFLY	529.27	34.00	34.62–34.80	**FL**	
	LCVLH	584.32	27.39	24.69 -25.20	**VL**	
PK-MPC	KHQGL	582.34	11.80	9.62 – 10.80	GL	α_{s1}-casein
	KEGI	446.26	14.15	13.27 – 14.00	**GI**, EG	
	KKNQDKTEI	1103.61	14.34	15.77 -16.24	**EI**	κ-casein
	DAQSAP	588.26	9.78	9.96 – 10.20	DA	β-lactoglobulin

B-MPC – milk protein concentrate hydrolyzed by bromelain, F-MPC – milk protein concentrate hydrolyzed by ficin, P-MPC – milk protein concentrate hydrolyzed by papain, PK-MPC – milk protein concentrate hydrolyzed by proteinase K; additional biological activity of peptide (if any) was given in brackets; peptide bitterness indicators (**bold**) and bitter peptides without the status of the indicator (normal font).

drolysate of MPC) and VLPVPQK (B-hydrolysate of MPC), served additional biofunctions. According to data found on these sequences in the BIOPEP-UWM database, the first acted as an opioid (ID 3127) as well as an immunomodulating (ID 8172) and ACE-inhibiting (ID 3530) agent, while the second one exhibited antioxidative activity (ID 7877). More detailed information about additional bioactivities of these peptides can be found in the BIOPEP-UWM database under the accession numbers provided in the brackets. The fact that peptides with health-beneficial effects may have an undesired taste may be considered important by food manufacturers and scientists when producing foods attractive for the consumers [Iwaniak *et al.*, 2018]. Additionally, the process of food production like, *e.g.*, production of milk protein hydrolysates, may require additional technological procedures aiming to reduce/debitter the unwanted taste [Lafarga & Hayes, 2017]. Our idea of research based on the fragmentomic approach enables predicting whether, due to the presence of specific bitter-tasting fragments, the released biopeptides may have the potential to taste bitter.

The number of parent peptides successfully identified in protein sequences during *in silico* and in *vitro* experiment varied. The discrepancies between predictions and actual results are the common fact in the literature [Mallick *et al*., 2007]. Firstly, the experiment carried out in laboratory conditions has its own specificity. It takes *e.g.* time for reagent and sample preparation, time involved in experiment, and time for results' analysis. *In silico* hydrolysis is relatively easy and cost-effective to perform but peptides produced with this method match those already present in the database. Moreover, the *in silico* prediction may differ when made during different time intervals [Udenigwe, 2014]. The BIOPEP-UWM is a curated database, *i.e.* systematically updated by the experienced staff in the field of bioactive peptides. No new bitter peptide sequences have been found in the literature and uploaded to the BIOPEP-UWM database between running the experiment and submission of this article. Thus, "not-updating" the database, could have not been the reason of *in silico* and *in vitro* discrepancies in peptides identification. Nevertheless, the necessity of the regular update of databases is a very important aspect that should be considered when comparing the results of *in silico* and *in vitro* analyses [Udenigwe, 2014].

According to Bucholska & Minkiewicz [2016], the following factors may be found responsible for the unsuccessful identification of peptides: the absence of a detectable amount of peptide in the hydrolysate (*e.g.* if some peptide bonds predicted to be cleaved are actually resistant to proteolysis), and the absence of a detectable fragmentation in an ion trap mass spectrometer. Moreover, possible peptides to be identified are defined as proteotypic peptides and may vary depending on the mass spectrometer used (*e.g.* matrix-assisted laser desorption ionization – MALDI or electrospray) [Bucholska & Minkiewicz, 2016]. To recapitulate, there is no method that would enable identifying all products of protein hydrolysis [Bucholska & Minkiewicz, 2016].

The differences between *in silico* and *in vitro* results may also stem from the issue referring to the hydrolysis of protein. According to Panjaitan *et al.* [2018], results of theoretical and practical analyses of proteases may not always be comparable. Programs for *in silico* hydrolysis are based on the specificity of enzymes. Moreover, theoretical hydrolysis assumes that peptide bonds of a substrate are 100% cleavable by the enzyme [Panjaitan *et al.*, 2018]. Complete enzyme characteristics is more vast than the information about the peptide bonds cleaved by an appropriate protease [Vermeirssen *et al.*, 2004]. Moreover, during the *in silico* hydrolysis, the peptide bond is cleaved by the enzyme relatively easily, especially when some amino acids in a substrate (protein) are not modified, *e.g.* by glycosylation. Under experimental conditions, glycosylated residues may impede the cleavage of the peptide bond [Khaldi, 2012]. Additionally, the complexity of the protein structure might hinder protease–protein interactions, which may also affect the divergence in the predicted and experimentally obtained results of proteolysis aimed at producing peptides [Panjaitan *et al.*, 2018]. Discrepancies concerning the release of peptides from proteins were also observed in experiments carried out *in vitro* and *in vivo/ex vivo*. For example, Darewicz *et al.* [2014] analyzed ACE-inhibiting peptides from salmon (*Salmo salar*) proteins hydrolyzed *in vitro* (with commercial enzymes) and *ex vivo* (using digestive juices from volunteers) and compared the results obtained using *in silico* analyses. They found that some *in silico* identified ACE inhibitors were common for both hydrolysates, however there were peptides identified either in the *ex vivo* or in the *in vitro* hydrolysate. According to Rawlings [2009], the likely reasons behind the differences between the results obtained in different experimental conditions include: involvement of inhibitors, and susceptibility of substrate peptide bonds to enzyme resulting from the protein-protein interactions.

To summarize, it is well-known that taste evaluation is more comprehensive than bioinformatic-assisted studies, several aspects need to be considered to get the reliable results when evaluating taste. The reliability of taste evaluation requires, *e.g.* panelist fatigue [Li-Chan, 2015], usage of an appropriate scale for sample evaluation (*e.g.* point or hedonic) [Lim, 2011], and/or limitations resulting from the usage of "machines" (*e.g.* e-tongue) [Ciosek & Wróblewski, 2011]. Thus, *in silico* studies combined with empirical methodologies enable the complete search for tasting-peptides derived from foods. However, all theoretical predictions of food taste must be confirmed [Gallego *et al.,* 2019].

CONCLUSIONS

The hybrid analysis of bovine milk protein hydrolysates confirmed that all released parent peptides contained shorter fragments with bitter taste and that some of them had the status of bitterness indicators. The presence of bitter regions in a parent peptide may determine the taste of the whole sequence. β-Casein turned out to be the richest source of peptides with potential to be bitter, as confirmed both *in silico* and *in vitro*. However, the results of *in silico* and *in vitro* hydrolysis concerning the number of released peptides as well as the effectiveness of enzyme applied differed. Twenty eight peptides with potential bitterness were identified in MPC *in vitro* hydrolysates while *in silico* hydrolysis enabled releasing 226 such sequences. The most effective enzymes in releasing peptides *in silico* were: bromelain > papain > ficin > proteinase K, whereas in the experimental conditions these were: ficin = papain > bromelain > proteinase K.

Summing up, the fragmentomic idea of research has so far been successfully employed in other scientific disciplines. We found it useful in food science when coupled with the hybrid approach. Despite the limitations of *in silico* analyses that do not fully reflect the *in vitro* results, the knowledge about the bitterness of peptide indicators, selection of enzymes for protein hydrolysis, and predictions of possible peptide products that may affect the bitter taste of a hydrolysate, contribute to providing more insights on the taste of foods, especially if unwanted. However, before introducing a food product to the market, sensory analyses are required to confirm (or not) its bitter taste.

RESEARCH FUNDING

Project financially supported by Minister of Science and Higher Education in the range of the program entitled "Regional Initiative of Excellence" for the years 2019–2022, Proj-

ect No. 010/RID/2018/19, amount of funding 12.000.000 PLN as well as the funds of the University of Warmia and Mazury in Olsztyn (Project No. 17.610.014–300).

CONFLICT OF INTEREST

The authors do not declare the conflict of interest.

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Submitted: 6 September 2019. Revised: 24 October 2019. Accepted: 28 October 2019. Published on-line: 2 January 2020.