



FRAGMENTOMIC ANALYSIS OF BIOPEPTIDES IN SILICO RELEASED FROM MILK PROTEINS*

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Abstract

The fragmentomic-assisted method was employed to predict the biological potential of peptides derived from milk proteins hydrolyzed by papain and bromelain. Firstly, protein sequences were acquired from the BIOPEP-UWM database and then hydrolyzed by the above enzymes using a BIOPEP-UWM tool called “Enzyme(s) action”. The released peptides were defined as parent peptides and further analyzed for the presence of shorter peptidic regions with documented bioactivity as well as their likelihood to be bioactive.

The results revealed the bioactive potential of the released parent peptides. β -Casein was found as the best source of biopeptides. Although this finding is consistent with literature data, the new parent peptide i.e., PVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK, produced by the action of bromelain might be considered as a new strategic zone due to the presence of multi-active regions. Some parent peptides theoretically produced from milk proteins turned out to be fully bioactive. Despite the usefulness of the tools for peptide bioactivity prediction, the critical thinking while planning the application of such data in future experiments would thus appear to be a worthwhile line of inquiry.

Introduction

It is well known that food affects our well-being (ROZIN et al. 1999) due to the presence of, i.a., proteins, sugars, fats, and vitamins that are respon-

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sible for the regulation of body functions (CARREIRO et al. 2016). The first serve several crucial functions in all biological processes as, e.g., catalysts, transporters, immune protectors, growth and differentiation controllers (BERG et al. 2002). Proteins are additionally sources of biologically active (bioactive) peptides (BHANDARI et al. 2020), i.e., non-active fragments encrypted in protein sequences, which serve various biological functions after enzymatic release and interactions with appropriate body receptors, including e.g., reduction of blood pressure, reduction of glucose and cholesterol levels as well as immunomodulating, antioxidative, antibacterial, antithrombotic etc. effects (MOHANTY et al. 2016). According to the scientific reports, milk proteins derived from different animal species represent the richest sources of biopeptides (IWANIAK et al. 2020a).

Once the computers had been invented, they were used for data storage. The popularity of Internet facilitated the remote access to scientific data (DATE 2005), including databases (CHANPUT et al. 2010). Databases useful in food science contain information about nutritional characteristics of individual foods, meals, diets, dishes and food-derived compounds (MARCONI et al. 2018). They are repositories of the molecules, like e.g., peptides, carbohydrates, enzymes etc. The first include the databases of biologically active peptides, like BIOPEP-UWM, EROP-Moscow or AHTPDB. They provide the sequences of biopeptides with various bioactivities (first two) and peptides exhibiting the antihypertensive effect (the latter one) (MINKIEWICZ et al. 2019).

Depending on the nature of a molecule, databases are the core of bio- and cheminformatics. These both disciplines deal with the elaboration of databases for categorization and data storage as well as employment of computer technologies for data analysis (IWANIAK et al. 2019). The coupled use of computer programs that are suitable to study biomolecules from foods and databases enables the bioinformatic-assisted approach. It is the one of the three approaches employed in food science to analyze biopeptides derived from food proteins. The second one is called a classical approach and relies on biological material (protein or food protein source) selection, its hydrolysis, identification of peptides in hydrolysates, and assessment of biological activity of both hydrolysate and peptides. Finally, the combination of the above methodologies is defined as hybrid/integrated approach, which helps getting more insights on the nature of biopeptides. Regardless the approach applied, each of them has its own pros and cons (IWANIAK et al. 2019).

According to RIEDER et al. (2010), potency is a key quality attribute of a biological material. The potential of a molecule can be determined using different bioassays (RIEDER et al. 2010). One of the attractive approaches

to determine the biological potency of a compound is by computing its similarity (both chemical and structural) to the molecule with a known bioactivity (PERIWAL et al. 2020). This similarity-assisted concept is consistent with the fragmentomic idea of research introduced by ZAMYATNIN et al. (2009). Briefly, it relies on the rule according to which the presence of shorter motifs with a known biological function in the fragment with an unknown bioactivity may decide about the potency of the whole molecule (ZAMYATNIN et al. 2009).

Many peptides derived from food proteins were studied using classical or hybrid approach (IWANIAK et al. 2019). The latter one enabled identifying a biopeptide sequence in the hydrolysate and then predicting its bioactivity based on the peptide search in specific peptide databases. If there were no such sequences in the specific database, it was presumed that peptide might not be bioactive (IWANIAK et al. 2020a). Thus, the aim of this study was to apply a fragmentomic-assisted approach for the prediction of the biological potency of bioactive peptides *in silico* released from bovine milk proteins using papain and bromelain.

Materials and Methods

The following protein sequences derived from bovine milk (*Bos taurus*) were acquired from the BIOPEP-UWM database (<https://biochemia.uwm.edu.pl/biopep-uwm/>; MINKIEWICZ et al. 2019) and then analyzed: α_{s1} -casein, genetic variant B (ID 1087); α_{s2} -casein, genetic variant A (ID 1090); β -casein, genetic variant A1 (ID 1097); κ -casein, genetic variant A (ID 1117); α -lactalbumin, genetic variant B (ID 1115); β -lactoglobulin, genetic variant A (ID 1116); and serum albumin (ID 1729). The numbers in brackets denote the accession numbers of these sequences in the BIOPEP-UWM database providing the following data: the full sequence of the protein, the number of amino acid residues, references, cross-references to other databases, and additional information (if possible).

The analyses of the above-mentioned proteins involved: their hydrolysis simulation using bromelain (EC 3.4.22.32) and papain (EC 3.4.22.2), followed by the fragmentomic analysis of potentially released peptides (defined as parent peptides). Theoretical hydrolysis of milk proteins was computed using the BIOPEP-UWM option called “Enzyme(s) action” available in the tab entitled “Analysis”. The predicted proteolysis was performed by selecting one out of the three possible options, i.e., one protein sequence: one enzyme at one computation. Finally, the fragmentomic analysis involved the prediction of potential bioactivity of the theoretically

released peptides. It was achieved by calculating the Score parameter for each parent peptide predicting the presence of shorter motifs with known bioactivities in the released peptides. The Score was computed using PeptideRanker program (<http://distilldeep.ucd.ie/PeptideRanker/>; MOONEY et al. 2012). All computations were carried out in April-June 2021.

Results and Discussion

Loads of articles concerning the discovery of new biologically active peptides are published every year. The Web of Science database (https://apps.webofknowledge.com/WOS_GeneralSearch; accession date: 22 April 2021] showed 2,384 records when typing the following words: “bioactive peptides”, “foods”, and “proteins” in search via “topic”. It concerned the period of 1991–2021. More detailed statistics showed that the number of the released articles in 1991–1999 varied between 1 (1991) and 6 (1999). Years 2000 (12) – 2009 (56) provided more papers on this issue (see in brackets). In the next decade, there were 72 (2010) and 320 (2019) articles, and finally 390 publications were released in 2020. In turn, 62 papers were published between January and April 2021. Such a dynamics in the research concerning the bioactive peptides shows that, despite the new information appearing on a daily basis, it is rather impossible to predict all possible sequences in the protein of interest. Such an amount of data also requires the regular update of databases (UDENIGWE 2014) used for the so-called *in silico* analyses. Thus, although bovine milk proteins have been known as the source of biopeptides for decades (CAPRIOTTI et al. 2016), their continued analyses are found reasonable.

Peptides theoretically released from bovine milk proteins using papain and bromelain are shown in Table 1 and Table 2, respectively. When analyzing the results, the main attention was paid to the released sequences that were composed of at least 4 amino acids. They were called parent peptides. A similar terminology was applied by IWANIAK et al. (2020a) who harnessed the fragmentomic approach to study the presence of bitter-tasting motifs in peptides (i.e., parent peptides) released from a milk protein concentrate hydrolyzed by different proteolytic enzymes, including the two above. As potential products of milk proteins' hydrolysis by papain and bromelain, di- and tripeptides were excluded from our studies. The fragmentomic analysis of milk protein sequences was carried out using a BIOPEP-UWM tool called the profile of potential biological activity of protein. It is defined as the type (understood as bioactivity) and location of a peptide in a protein chain (MINKIEWICZ et al. 2019). This function is

available in the BIOPEP-UWM database and enables inserting shorter fragments instead of the protein. “Technically”, this analysis shows the exact matches of some sequential motifs in a sample (i.e., parent peptide in this case). In the case of potentially released dipeptides, the exact match would show the same sequence, whereas in the case of tripeptides, it would be the same sequence and/or dipeptide match. This means that the shorter the parent sequence is, the smaller number of bioactive motifs can be found in it. This regularity is also applicable to the substrate (protein and/or parent peptide sequence) as the relatively short substrate sequences produce a smaller number of peptides than the longer ones (IWANIAK et al. 2020a). It needs to be noted that we did not include in our analysis the repetitions of bioactive motif in the parent sequence because, regardless of the number of motif repetitions, it would show the same bioactivity and the value of PeptideRanker Score.

Table 1
Peptides theoretically released from bovine milk proteins due to the action of papain

<i>Substrate for hydrolysis: α-lactalbumin</i>	
Parent peptide	Encrypted bioactive motif
MMSFVSLLLVG	MM ^{ACEi, DPP4i, AOX} ; SF ^{ACEi, DPP4i} ; LV/LL ^{DPP4i, sti-glu} ; LLL ^{sti-sub} ; SL ^{DPP4i} ; VG ^{ACEi, DPP4i}
ILFH	LF ^{ACEi} ; IL ^{ACEi, sti-glu}
QLTK	QL/LT/TK ^{DPP4i}
VSLPE	LP/SL/VS ^{DPP4i}
WVCTTFH	TF ^{ACEi, DPP4i} ; WV/VT ^{DPP4i}
DTQA	QA ^{DPP4i} ; TQ ^{ACEi, DPP4i}
IVQNNDSTE	NN/QN/ND/VQ ^{DPP4i} ; IVQ/ST ^{ACEi} ; TE ^{ACEi, DPP4i} ; IV ^{sti-glu}
LFQINNK	LF/NK ^{ACEi} ; FQ ^{ACEi, DPP4i} ; IN/QI/NN ^{DPP4i}
IWCK	WC ^{DPP4i} ; IW ^{ACEi, DPP4i}
DDQNPH	NP/QN/DQ ^{DPP4i} ; PH ^{ACEi, DPP4i}
FLDDDLTDDIMCVK	FL/IM ^{DPP4i} ; LT/TD/VK ^{DPP4i}
ILDK	IL ^{ACEi, DPP4i, sti-glu}
LCSE	SE ^{sti-sub}
LDQWLCE	WL ^{ACEi, DPP4i} ; QW/DQ ^{DPP4i}
<i>Substrate for hydrolysis: β-lactoglobulin</i>	
CLLLA	LL ^{DPP4i, sti-glu} ; LLL ^{sti-sub} ; LA ^{ACEi, DPP4i, ubi}
LTCG	LT ^{DPP4i}
LIVTQTMK	TW/LIVTQ ^{ACEi} ; LI ^{DPP4i, sti-glu} ; IV ^{sti-glu} ; MK/TM/QT/TQ/VT ^{DPP4i}
LDIQ ^{K(score = 0.11)}	IQ ^{DPP4i} ; LDIQ/QK ^{ACEi}
SDISLLDA	LL ^{DPP4i, sti-glu} ; SL ^{DPP4i} ; DA ^{ACEi}

Cont. Table 1

PTPE	TP/PT ^{ACEi} , DPP ⁴ⁱ
ILLQK	LL DPP ⁴ⁱ ,sti-glu, IL ^{ACEi} , DPP ⁴ⁱ , sti-glu, LQ/QK ^{ACEi}
VLVLDTDY	DY ^{ACEi} , reg-ion; LVL ^{ACEi} ; VL/LV DPP ⁴ⁱ , sti-glu, TDY ^{AOX} ; TD DPP ⁴ⁱ
LLFCME	LF/LLF ^{ACEi} ; LL DPP ⁴ⁱ ,sti-glu, ME ^{ACEi} , DPP ⁴ⁱ
QSLA	SL/QS DPP ⁴ⁱ ; LA ^{ACEi} , DPP ⁴ⁱ ,ubi
CQCLVR	LVR ^{ACEi} ; VR ^{ACEi} , DPP ⁴ⁱ ; LV DPP ⁴ⁱ , sti-glu
VDDE	VD DPP ⁴ⁱ
LPMH	PM/LP/MH DPP ⁴ⁱ
LSFNPTQLE	FN/NP/QL DPP ⁴ⁱ i; SF/PT/TQ ^{ACEi} , DPP ⁴ⁱ
<i>Substrate for hydrolysis: κ-casein</i>	
SFFLVVTTILA	FFL ^{ACEi} ; FL DPP ⁴ⁱ ; SF ^{ACEi} , DPP ⁴ⁱ ; IL ^{ACEi} , DPP ⁴ⁱ ,sti-glu; LA ^{ACEi} , DPP ⁴ⁱ ,ubi; LV DPP ⁴ⁱ ,sti-glu; TI/VV/VT DPP ⁴ⁱ
LTLPFLG	PF/FL/LP/TL/LT DPP ⁴ⁱ ; LPF/LG ^{ACEi}
QNQE	QN/NQ/QE DPP ⁴ⁱ
QPIR	PI/QP DPP ⁴ⁱ ; IR ^{ACEi} , DPP ⁴ⁱ ,AOX,i-ren, CaMPDEi
IPIQ ^Y (score = 0.36)	PI/PI/IPIQY/QY/IQ DPP ⁴ⁱ ; IP ^{ACEi} , DPP ⁴ⁱ ; IQY ^{ACEi} , AOX, ab
VLSR	VL DPP ⁴ⁱ ,sti-glu
LINNQFLPY	FL/QF/LP/PY/IN/NN/NQ DPP ⁴ⁱ ; LI DPP ⁴ⁱ ,sti-glu
QILQWQVLSNTVPA	QW/WQ/PA/QI/NT/QV/TV DPP ⁴ⁱ ; IL ^{ACEi} , DPP ⁴ⁱ , sti-glu;VP ^{ACEi} , DPP ⁴ⁱ ; LQ ^{ACEi} ; VL DPP ⁴ⁱ , sti-glu
SCQA	QA DPP ⁴ⁱ
QPTTMA	MA/QP/TM/TT DPP ⁴ⁱ ; PT ^{ACEi} , DPP ⁴ⁱ
LSFMA	SF ^{ACEi} , DPP ⁴ⁱ ; MA DPP ⁴ⁱ
IPPK	PP/IP ^{ACEi} , DPP ⁴ⁱ ; IPP ^{ACEi} ; PK DPP ⁴ⁱ ; PPK ^{ACEi} , at
NQDK ^(score = 0.07)	NQDK ^{at} ; NQ/QD DPP ⁴ⁱ
IPTINTIA	IP/PT/IA ^{ACEi} , DPP ⁴ⁱ ;IN/TI/NT DPP ⁴ⁱ
PTSTPTTE	TP/PT/TE ^{ACEi} , DPP ⁴ⁱ ; TS/TT DPP ⁴ⁱ ; ST ^{ACEi}
STVA	ST ^{ACEi} ; VA/TV DPP ⁴ⁱ
DSPE	SP DPP ⁴ⁱ
SPPE	PP ^{ACEi} , DPP ⁴ⁱ / SP DPP ⁴ⁱ
INTVQVTSTA	TSTA ^{AOX} ; ST/VQV ^{ACEi} ; IN/TA/NT/TS/QV/VQ/TV/VT DPP ⁴ⁱ
<i>Substrate for hydrolysis: β-casein</i>	
LNVPG	PG ^{ACEi} , DPP ⁴ⁱ , anm, at, reg-sto; LN/VP ^{ACEi} , DPP ⁴ⁱ ; NV DPP ⁴ⁱ
SLSSSE	SL DPP ⁴ⁱ ;SSS ^{sti-sub} ; SE ^{reg-ion}
SITR	SI/TR DPP ⁴ⁱ
FQSE	FQ ^{ACEi} , DPP ⁴ⁱ ; QS DPP ⁴ⁱ ; SE ^{sti-glu}
QQQTE	QQ/OT DPP ⁴ⁱ ; TE ^{ACEi} , DPP ⁴ⁱ

Cont. Table 1

LQDK	LQ ^{ACEi} ; QD ^{DPP4i}
QTQSLVY	VY/TQ ^{ACEi, DPP4i} ; LVY ^{ACEi} ; LV ^{DPP4i, sti-glu} ; VY ^{AOX} ; SL/QS/QT ^{DPP4i}
PFFPG	FP ^{ACEi, DPP4i} ; PF ^{DPP4i} ; PG ^{ACEi, DPP4i, anm, at, reg-sto}
NSLPQNIPPLTQT PVVVPPFLQPE	PF/FL/PPL/FLQP/LP/LPQNIPPL/QP/SL/IPPLTQTPV/ VV/IPPLTQT- PV/PV/LQ/LT/QN/QT^{DPP4i}; PP/PL/IP/LQP/PQ/NP/TQ^{ACEi, DPP4i}; IPP/VVPPF/VPP/TPVVPPFLQP/SLPQN/TP/ NIPPLTQTPV/VVPP/ LTQTPVVPPF^{ACEi, VV^{vacan}}
MPFPK	FP ^{ACEi, DPP4i} ; PF/MP/PK ^{DPP4i}
PVQPFTE	TE ^{ACEi, DPP4i} ; PF/QP/PV/VQ ^{DPP4i}
SQSLTLTDVE	VE ^{ACEi, DPP4i} ; LTLTDVE ^{ACEi} ; SL/TL/LT/QS/TD ^{DPP4i}
LPPLLLQSWMH	WM/PL/PP ^{ACEi, DPP4i} ; SW/PPL/LP/MH/QS ^{DPP4i} ; LPP/LQSW/LQ ^{ACEi} ; LLL ^{sti-sub} ; LL ^{DPP4i, sti-glu}
QLPPTVMFPPQSVL- SLSQSK	MF/FP/PP/PL/PQ/PT ^{ACEi, DPP4i} ; VL ^{DPP4i, sti-glu} ; PLP/LPP ^{ACEi} ; LP/QP/SL/VM/QS/SK/SV/TV ^{DPP4i}
VLPVPE	VL ^{DPP4i, sti-glu} ; LP/PV ^{DPP4i} ; VP ^{ACEi, DPP4i} ; VL ^{PACEi}
DMPIQA	MP/PI/QA/IQ ^{DPP4i} ; DM ^{ACEi}
FLLY	LL ^{DPP4i, sti-glu} ; FL ^{ACEi, DPP4i} ; LY ^{ACEi, AOX} ; LLY ^{ist}
QQPVLG	VL ^{DPP4i, sti-glu} ; QQ/QP/PV ^{DPP4i} ; LG ^{ACEi}
PPPIIV	FP ^{ACEi, DPP4i} ; PI ^{DPP4i, sti-glu} ; IV ^{sti-glu} ; PI/PF ^{DPP4i} ; FPIIV ^{ACEi}
<i>Substrate for hydrolysis: α_{S2}-casein</i>	
FFIFTCLLA	LL ^{DPP4i, sti-glu} ; LA ^{ACEi, DPP4i, ubi} ; IF ^{ACEi}
NTME	ME ^{ACEi, DPP4i} ; TM/NT ^{DPP4i}
VSSSE	SSS ^{sti-sub} ; SE ^{reg-ion} ; VS ^{DPP4i}
SIISQE	II ^{DPP4i, sti-glu} ; SI/QE ^{DPP4i}
INPSK	NP/PS/IN/SK ^{DPP4i}
NLCSTFCK	TF ^{ACEi, DPP4i} ; NL ^{DPP4i} ; ST ^{ACEi}
SSSE	SSS ^{sti-sub} ; SE ^{reg-ion}
ITVDDK	VD/TV ^{DPP4i}
INQFY	QF/IN/NQ ^{DPP4i} ; FY ^{ACEi}
FPQY	FP ^{ACEi, DPP4i} ; PQ/QY ^{DPP4i}
PIVLNPWDQVK	PW ^{AOX, DPP4i} ; PWD ^{AOX} ; IV ^{sti-glu} ; VL ^{DPP4i, sti-glu} ; LN/VK ^{ACEi, DPP4i} ; WD/PL/NP/DQ/QY ^{DPP4i} ; LNP ^{ACEi}
VPITPTLNR	LN/TP/PT/VP ^{ACEi, DPP4i} ; PI/NR/TL/VPITPT ^{DPP4i}
QLSTSE	SE ^{reg-ion} ; QL/TS ^{DPP4i} ; ST ^{ACEi}
TVDME	DM ^{ACEi} ; ME ^{ACEi, DPP4i} ; TV/VD ^{DPP4i}
VFTK	VF ^{ACEi, DPP4i} ; TK ^{DPP4i}
LNFLK	NF/FL/LN ^{ACEi, DPP4i} ; LNF ^{ACEi} ; LK ^{AOX}
LPQY	PQ ^{ACEi, DPP4i} ; LP/QY ^{DPP4i}
PWIQPK	PW ^{AOX, DPP4i} ; PWI ^{AOX} ; IQP ^{ACEi, DPP4i} ; WI/WIQP/QP/IQ/PQ ^{DPP4i}



Cont. Table 1

VIPY	IP ^{ACEi,DPP4i} ; IPY ^{ACEi} ; PY ^{VI} ^{DPP4i}
<i>Substrate for hydrolysis: α_{S1}-casein</i>	
LPQE	LP/PQ/QE ^{DPP4i}
VLNE	VL ^{DPP4i, sti-glu} ; LN ^{ACEi,DPP4i} ; NE ^{DPP4i}
NLLR	LL ^{DPP4i, sti-glu} ; LR ^{ACEi} ; LLR ^{AOX} ; NL ^{DPP4i}
FFVA	VA ^{DPP4i}
PPFQVFG	FP/VF/PQ ^{ACEi,DPP4i} ; FG ^{ACEi} ; PF/QV ^{DPP4i}
SISSSE	SSS ^{sti-sub} ; SE ^{reg-ion} ; SI ^{DPP4i}
IVPNSVE	VP/VE ^{ACEi,DPP4i} ; IV ^{sti-glu} ; PN/SV ^{DPP4i}
DVPSE	SE ^{reg-ion} ; VP ^{ACEi,DPP4i} ; PS ^{DPP4i}
QLLR	LL ^{DPP4i, sti-glu} ; LR ^{ACEi} ; LLR ^{AOX} ; QL ^{DPP4i}
VPQLE	PQ/VP ^{ACEi,DPP4i} ; QL ^{DPP4i}
IVPNSA	VP ^{ACEi,DPP4i} ; IV ^{sti-glu} ; PN ^{DPP4i}
PMIG	PM/MI ^{DPP4i} ; IG ^{ACEi}
VNQE	NQ/QE/VN ^{DPP4i}
QLDA	DA ^{ACEi} ; QL ^{DPP4i}
VPLG	VPL ^{DPP4i, anm, sti-sub} ; PL/VP ^{ACEi,DPP4i} ; LG ^{ACEi} ; PLG ^{ACEi, op}
PSFSDIPNPIG	SF/IP ^{ACEi,DPP4i} ; IG ^{ACEi} ; PI/NP/PN/PS ^{DPP4i}
TTMPL ^{W(score = 0.74)}	TTMPLW ^{ACEi, mod, op} ; LW ^{ACEi,DPP4i, AOX} ; PLW ^{ACEi} ; MP/TM/TT ^{DPP4i}
<i>Substrate for hydrolysis: serum albumin</i>	
WVTFISLLLLFSSA	LL ^{DPP4i, sti-glu} ; LLL ^{sti-sub} ; TF ^{ACEi,DPP4i} ; LF/LLF ^{ACEi} ; WV/SL/V ^T ^{DPP4i}
LVLIA	LI/VL/LV ^{DPP4i, sti-glu} ; IA ^{ACEi,DPP4i} ; LVL ^{ACEi}
FSQY	QY ^{DPP4i}
LQQCPFDE	LQ/LQQ ^{ACEi} ; PF/QQ ^{DPP4i}
LVNE	NE/VN ^{DPP4i} ; LV ^{DPP4i, sti-glu}
TCVA	VA ^{DPP4i}
TLFG	LF/FG ^{ACEi} ; TL ^{DPP4i}
CFLSH	CF/FL ^{ACEi} ; SH ^{DPP4i}
DDSPDLPK	DLP ^{ACEi} ; LP/SP/PK ^{DPP4i}
PDPNTLCDE	DP/PN/TL/NT ^{DPP4i}
VFQE	FQ/VF ^{ACEi,DPP4i} ; QE ^{DPP4i}
CCQA	QA ^{DPP4i}
CLLPK	LL ^{DPP4i, sti-glu} ; LLP ^{ACEi} ; LP/PK ^{DPP4i}
SIQK	QK ^{ACEi} ; SI/IQ ^{DPP4i}
WSVA	WS/VA/SV ^{DPP4i}
LSQK	QK ^{ACEi}
LVTDLTK	LV ^{DPP4i, sti-glu} ; LT/TD/TK/VT ^{DPP4i}

Cont. Table 1

DLLE	LL DPP4i,sti-glu
ICDNQDTISSK	DN/NQ/QD/SK/TI DPP4i
PLLE	LL DPP4i,sti-glu, PL ACEi,DPP4i
NLPPLTA	PP/PL ACEi,DPP4i, LLP ACEi, PPI/LP/NL/LT/TA DPP4i
SFLY	LY ACEi,AOX
VSVLLR	LL/VL DPP4i,sti-glu, LR ACEi, LLR AOX, SV/VS DPP4i
DDPH	PH ACEi,DPP4i, DP DPP4i
STVFDK	FDK/ST ACEi, VF ACEi,DPP4i, TV DPP4i
LVDE	LVDP4i,sti-glu, VD DPP4i
PQNLIK	LI ^{DPP4i,sti-glu} , PQ ^{ACEi,DPP4i} , NL/QN ^{DPP4i}
QNCDQFE	QF/QN/DQ DPP4i
FQNA	FQ/NA/QN DPP4i
LIVR	LI ^{DPP4i,sti-glu} , IV ^{sti-glu} , VR ^{ACEi,DPP4i} , IV ^{RACEi}
VPQVSTPTLVE	PQ/TP/PT/VP/VE ^{ACEi,DPP4i} , TL/QV/VS/ ^{DPP4i} , LV ^{DPP4i,sti-glu} , LVE/ ^{STACEi}
CCTK	TK DPP4i
MPCTE	TE ^{ACEi,DPP4i} , MP DPP4i
LSLILNR	IL DPP4i,sti-glu, ACEi, LI ^{DPP4i,sti-glu} , LN ^{ACEi,DPP4i} , SL/NR ^{DPP4i}
LCVLH	LH ^{AOX,DPP4i} , VL ^{DPP4i,sti-glu}
TPVSE	SE ^{reg-ion} , TP ^{ACEi,DPP4i} , PV/VS DPP4i
CCTE	TE ^{ACEi,DPP4i}
SLVNR	LV ^{DPP4i,sti-glu} , SL/NR/ ^{VN} DPP4i
PCFSA	CF ACEi
LTPDE	TP ^{ACEi,DPP4i} , LT ^{DPP4i}
LFTFH	TF ACEi,DPP4i, LF ^{ACEi}
DICTLPDTE	TE ^{ACEi,DPP4i} , LP / ^{TL} DPP4i
TVME	ME ^{ACEi,DPP4i} , TV/ ^{VM} DPP4i
NFVA	NF ACEi,DPP4i, VA ^{DPP4i}
FVDK	VD ^{DPP4i}
LVVSTQTA	LV ^{DPP4i,sti-glu} , TQ ^{ACEi,DPP4i} , ST ^{ACEi} , TA/ ^{QT/VS/VV} DPP4i

ACEi – angiotensin converting enzyme inhibitor; DPP4i – dipeptidyl peptidase IV inhibitor; AOX – antioxidative; sti-glu – stimulating absorption of glucose; sti-sub – stimulating the release of vasoactive substances; ubi – activator of ubiquitin-mediated proteolysis; reg-ion – regulator of ion flow; i-ren – renin inhibitor, acan-anticancer; CaMPDEi – inhibitor CaMPDE (calmodulin-dependent cyclic nucleotide phosphodiesterase); ist – immunostimulating; mod – immunomodulating; ab – antibacterial; at – antithrombotic; anm – anti-amnestic; op – opioid; reg-sto – regulator of stomach mucosal membrane action; **bold** – peptide with Score > 0.5; grey – peptide with confirmed bioactivity

Table 2

Peptides theoretically released from bovine milk proteins due to the action of bromelain

Substrate for hydrolysis: α -lactalbumin	
Parent peptide	Encrypted bioactive motif
MMSFVSLLLVG	MM ^{ACEi,DPP4i,AOX} ; LL/LV ^{DPP4i,sti-glu} ; LLL ^{sti-sub} ; SF/VG ^{ACEi,DPP4i} ; SL/V ^S ^{DPP4i}
ILFHA	IL ^{ACEi,DPP4i,sti-glu} ; LF ^{ACEi} ; HA ^{DPP4i}
EQLT ^{K(score = 0.05)}	EQLTK ^{ab}
CEVFRELK	FR/VF/EV ^{ACEi,DPP4i} ; LK/EL ^{AOX}
VSLPEWVCTTFHTSG	TF/EW/EV ^{ACEi,DPP4i} ; LP/WV/LPEWVCTTFH/SL/HT/T ^S /VS/T ^T ^{DPP4i} ; SG ^{ACEi}
DTQA	TQ ^{ACEi,DPP4i} ; QA ^{DPP4i}
IVQNNSTHEY	EY/TE ^{ACEi,DPP4i} ; IV ^{sti-glu} ; ST/IVQ ^{ACEi} ; NN/QN/ND/VQ ^{DPP4i}
LFQINNK	FQ ^{ACEi,DPP4i} ; LF/NK ^{ACEi} ; NN/IN/QI ^{DPP4i}
IWCK	IW ^{ACEi,DPP4i} ; WC ^{DPP4i}
DDQNP ^{HSSNICNI-SCDK}	PH ^{ACEi,DPP4i} ; PHS ^{AOX} ; NP/HS/QN/DQ/QNP ^{HSSNICNI} ^{DPP4i}
FLDDDLTDDIMCVK	VK ^{ACEi,DPP4i} ; FL/IM/LT/TD ^{DPP4i}
ILDK	IL ^{ACEi,DPP4i,sti-glu}
LCSEK	SE ^{reg-ion} ; EK ^{ACEi,DPP4i}
LDQWLCEK	VL/EK ^{ACEi,DPP4i} ; QV/DQ ^{DPP4i}
Substrate for hydrolysis: β -lactoglobulin	
CLLLA	LL ^{DPP4i,sti-glu} ; LLL ^{sti-sub} ; LA ^{ACEi,DPP4i,ubi}
LTCG	LT ^{DPP4i}
LIVTQTMK	LI ^{DPP4i,sti-glu} ; IV ^{sti-glu} ; MK/TM/QT/TQ/V ^T ^{DPP4i} ; TW/LIVTQ ^{ACEi}
LDIQ ^{K(score = 0.11)}	LDIQK/QK ^{ACEi} ; IQ ^{DPP4i}
SDISLLDA	LL ^{DPP4i,sti-glu} ; SL ^{DPP4i} ; DA ^{ACEi}
PLRVY	VY ^{ACEi,DPP4i,AOX} ; PL ^{ACEi,DPP4i} ; LR/RVY ^{ACEi}
VEELK	EE ^{sti-sub} ; LK/EL ^{AOX} ; VE ^{ACEi}
PTPEG	TP/PT/EG ^{ACEi,DPP4i}
DLEILLQK	LL ^{DPP4i,sti-glu} ; EI ^{ACEi,DPP4i} ; IL ^{ACEi,DPP4i,AOX} ; LQ/OK ^{ACEi}
WENDECA	WE/ND ^{ACEi,DPP4i}
LNENK	LN ^{ACEi,DPP4i} ; NE ^{DPP4i} ; NK ^{ACEi}
VLVLDTDY	VL/LV ^{DPP4i,sti-glu} ; DY ^{ACEi,sti-ion} ; TDY ^{AOX} ; TD ^{DPP4i} ; LVL ^{ACEi}
LLFCM ^{ENSA}	LL ^{DPP4i,sti-glu} ; ME ^{ACEi,DPP4i} ; LLL/LL/CM ^{ENSA} ^{ACEi}
EPEQSLA	LA ^{ACEi,DPP4i,ubi} ; EP/QS/SL ^{DPP4i}
CQCLVRTPEVDDEA	LV ^{DPP4i,sti-glu} ; LVR/LVRT/EA ^{ACEi} ; TP/VR/EV ^{ACEi,DPP4i} ; VD ^{DPP4i}

Cont. Table 2

LPMHIRLSF-NPTQLEEQCHI	IR ^{ACEi} , DPP ⁴ⁱ , AOX, i-ren, CaMPDE ⁱ ; SF/PT/TQ ^{ACEi} , DPP ⁴ⁱ ; PM/FN/LP/MH/NP/QL/HI ^{DPP4i} ; MHIRL ^{AOX} ; HIRL/HIR/LEE ^{ACEi} ; EE ^{sti-sub}
<i>Substrate for hydrolysis: κ-casein</i>	
SFFLVVTLA	LA ^{ACEi} , DPP ⁴ⁱ , ubi; IL ^{ACEi} , DPP ⁴ⁱ , sti-glu; LV ^{DPP4i} , sti-glu; SF ^{ACEi} , DPP ⁴ⁱ ; TI/VT/VV ^{DPP4i} ; FFL ^{ACEi}
LTLPFLG	LPF/LG ^{ACEi} ; PF/FL/LP/TL/LT ^{DPP4i}
QEQNQEPIRCEK	IR ^{ACEi} , DPP ⁴ⁱ , AOX, i-ren, CaMPDE ⁱ ; EK ^{ACEi} , DPP ⁴ⁱ ; PI/QP/QN/NQ/QE ^{DPP4i}
DERFFSDK	RF ^{ACEi}
IPIQY	IP ^{ACEi} , DPP ⁴ⁱ ; IQY ^{AOX, ACEi, ab} ; PI/PI/PIQY/QY/IQ ^{DPP4i}
VLSRY	VL ^{DPP4i} , sti-glu; RY ^{ACEi}
LINNQFLPY	LI ^{DPP4i} , sti-glu; FL/QF/LP/PY/IN/NN/NQ ^{DPP4i}
VRSPA	VR ^{ACEi} , DPP ⁴ⁱ ; PA/SP ^{DPP4i} ; VRSP ^{ACEi}
QILQWQVLSNTVPA	VL ^{DPP4i} , sti-glu; IL ^{ACEi} , DPP ⁴ⁱ , sti-glu; QW/WQ/PA/QI/NT/QV/TV ^{DPP4i} ; VP ^{ACEi} , DPP ⁴ⁱ ; LQ ^{ACEi}
SCQA	QA ^{DPP4i}
QPTTMA	PT ^{ACEi} , DPP ⁴ⁱ ; MA/QP/TM/TT/PT ^{DPP4i}
RHPHPHLSFMA	SF/HP/PH ^{ACEi} , DPP ⁴ⁱ ; RHPHP ^{AOX, ACEi} ; PHL/HPHL/HPH ^{AOX} ; HL ^{ACEi} , DPP ⁴ⁱ , AOX; MA/RH ^{DPP4i} ; PHPHLSF ^{chymi}
IPPK	PP/IP ^{ACEi} , DPP ⁴ⁱ ; IPP ^{ACEi} , PK ^{DPP4i} ; PPK ^{ACEi} , at
NQDK ^(score = 0.07)	NQDK ^{at} ; NQ/QD ^{DPP4i}
TEIPTINTIA	IP/PT/LA/EI/TE ^{ACEi} , DPP ⁴ⁱ ; IN/TI/NT ^{DPP4i} ; EIPT ^{ab}
EPTSTPTTEA	TP/PT/TE ^{ACEi} , DPP ⁴ⁱ ; EP/TS/TT ^{DPP4i} ; ST/EA ^{ACEi}
VESTVA	VE ^{ACEi} , DPP ⁴ⁱ ; VA/ES/TV ^{DPP4i} ; ST ^{ACEi}
TLEDSPEVIESPPEIN-TVQVTSTA	PP/EI/EV ^{ACEi} , DPP ⁴ⁱ ; TSTA ^{AOX} ; VQV/IE ^{ACEi} ; SP/IN/TL/ST/TA/VI/TS/NT/QV/ES/VQ/VT ^{DPP4i}
<i>Substrate for hydrolysis: β-casein</i>	
RELEELNVPG	PG ^{ACEi} , DPP ⁴ⁱ , anm, at, reg-sto; LN/VP ^{ACEi} , DPP ⁴ⁱ ; VE ^{ACEi} , DPP ⁴ⁱ ; EL ^{AOX} ; NV ^{DPP4i} ; LEE ^{ACEi} ; EE ^{sti-sub}
EIVESLSSEESI-TRINK	EI/VE ^{ACEi} , DPP ⁴ⁱ ; RI/SL/SI/IN/TR/ES ^{DPP4i} ; SSS/SE/EE ^{sti-sub} ; NK ^{ACEi} ; IV ^{sti-glu}
FQSEEQQTE-DELQDK	FQ/TE ^{ACEi} , DPP ⁴ⁱ ; SE/EE ^{sti-sub} ; LQ ^{ACEi} ; EL ^{AOX} ; QS/QD/QQ/QT ^{DPP4i}
IHPFA	HP ^{ACEi} , DPP ⁴ⁱ ; PF/FA/IH ^{DPP4i}
QTQSLVY	VY/TQ ^{ACEi} , DPP ⁴ⁱ ; LVY ^{ACEi} ; LV ^{DPP4i} , sti-glu; VY ^{AOX} ; SL/QS/QT ^{DPP4i}
PFPG	FP ^{ACEi} , DPP ⁴ⁱ ; PF ^{DPP4i} ; PG ^{ACEi} , DPP ⁴ⁱ , anm, at, reg-sto
PIHNSLPQNIPPLTQT-PVVVPPFLQPEVMG	MG/PP/PL/IP/LQP/LQ/PQ/TP/VP/TQ/EV ^{ACEi, DPP4i} ; IPP/VVVPPF/VPP/TPVVVPPFLQP/SLPQN/NIPPLTQTPV/VVPP/LTQTPVVVPPF ^{ACEi} ; PF/FL/PPL/FLQP/LP/LPQNIPPL/LPQNIPP/PI/QP/VM/SL/IPPLTQTPV/PV/IH/LT/QN/QT/VV ^{DPP4i} ; VVV ^{vacan}

Cont. Table 2

EMPFPK(score = 0.77)	FP ^{ACEi, DPP4i} ; EMPFK ^{ACEi} ; PF/MP/PK ^{DPP4i}
FLLY	LL ^{DPP4i, sti-glu} ; FL ^{ACEi, DPP4i} ; LY ^{ACEi, AOX} ; LLY ^{ist}
PFPIIV	FP ^{ACEi, DPP4i} ; PI ^{DPP4i, sti-glu} ; IV ^{sti-glu} ; PI/PI ^{DPP4i} ; FPIIV ^{ACEi}
QQPVLG	VL ^{DPP4i, sti-glu} ; QQ/QP/PV ^{DPP4i} ; LG ^{ACEi}
PVQPFTEQSLSLTDVENLHLP PLLLQSWMHQ PHQPLPPTVMFP PQSVLSLSQSK	WM/MF/FP/PP/PL/PH/PQ/PT/VE/TE ^{ACEi, DPP4i} ; PF/SW/PPL/LP/MH//VM/SL/NL/PV/TL/LT/QS/SK/SV/VQ/TD/ES/TV ^{DPP4i} ; PLP/LPP/QSWM-HQPHQ/LQSW/LHLP/NLHLP/LQ/TEQSLSL/LTLTDVE ^{ACEi} ; LLL ^{sti-sub} ; LL ^{DPP4i, sti-glu} ; HL ^{ACEi, DPP4i, AOX} ; LH ^{AOX, DPP4i} ; LHL/PHQ ^{AOX} ; VL ^{DPP4i, sti-glu}
VLPVPEK	VP/EK ^{ACEi, DPP4i} ; VL ^{ACEi} ; LP/QT ^{DPP4i}
PQRDMPIQA	PQ ^{ACEi, DPP4i} ; DM/PQR ^{ACEi} ; RDMPIQ ^{AOX} ; MP/PI/IQ/QA ^{DPP4i}
PVRG	VR ^{ACEi, DPP4i} ; RG/PV ^{DPP4i}
<i>Substrate for hydrolysis: a₅₅-casein</i>	
FFIFTCLLA	LL ^{DPP4i, sti-glu} ; LA ^{ACEi, DPP4i, ubi} ; IF ^{ACEi}
NTMEHVSSSE-ESIISQETY	ME ^{ACEi, DPP4i} ; II ^{DPP4i, sti-glu} ; TY ^{DPP4i, AOX} ; SSS ^{sti-sub} ; SE ^{reg-ion} ; EE ^{sti-sub} ; TM/SI/HV/NT/EH/VS/QE/ES/ET ^{DPP4i}
INPSK	NP/PS/IN/SK ^{DPP4i}
ENLCSTFCK	ST ^{ACEi} ; TF ^{ACEi, DPP4i} ; NL ^{DPP4i}
EVVRNA	VR/EV ^{ACEi, DPP4i} ; RN/LA/VV ^{DPP4i}
NEEEY	EY ^{ACEi, DPP4i} ; NE ^{DPP4i} ; EE/EEE ^{sti-sub}
SSSEESA	SSS ^{sti-sub} ; SE ^{reg-ion} ; EE ^{sti-sub} ; ES ^{DPP4i}
TEEVK	VK/EV/TE ^{ACEi, DPP4i} ; EE ^{sti-sub}
ITVDDK	VD/TV ^{DPP4i}
FPQY	FP ^{ACEi, DPP4i} ; PQ/QY ^{DPP4i}
LNEINQFY	LN/EI ^{ACEi, DPP4i} ; FY ^{ACEi} ; QF/IN/NQ/NE ^{DPP4i}
PIVLNPWDQVK	PW ^{AOX, DPP4i} ; PWD ^{AOX} ; IV ^{sti-glu} ; VL ^{DPP4i, sti-glu} ; LN/VK ^{ACEi, DPP4i} ; WD/PL/NP/DQ/QV ^{DPP4i} ; LNP ^{ACEi}
VPITPTLNREQLST-SEENSK	TP/LN/PT/VP/ACEi, DPP4i; SE^{reg-ion}; EE^{sti-sub}; PI/QL/NR/VPITPT/TL/ST/TS^{DPP4i}
TVDMESTEVEFTK	VF/ME/EV/TE ^{ACEi, DPP4i} ; DM/ST ^{ACEi} ; ES/VD/TK/TV ^{DPP4i}
LTEEEK	EE/EEE ^{sti-sub} ; TE/EK ^{ACEi, DPP4i} ; LT ^{DPP4i}
LPQY	PQ ^{ACEi, DPP4i} ; LP/QY ^{DPP4i}
PWIQPK	PW ^{AOX, DPP4i} ; PWI ^{AOX} ; IQP ^{ACEi, DPP4i} ; WI/WIQP/QP/IQ/PQ ^{DPP4i}
VIPY	IP ^{ACEi, DPP4i} ; IPY ^{ACEi} ; PY/VI ^{DPP4i}
NRLNFLK	NF/RL/LN ^{ACEi, DPP4i} ; LNF ^{ACEi} ; FL/NR ^{DPP4i} ; LK ^{AOX}
ISQRY	RY ^{ACEi}
QHQB	QH ^{ACEi} ; QB ^{DPP4i}

Cont. Table 2

<i>Substrate for hydrolysis: a₁-casein</i>	
HPIK	HP ^{ACEi} ; PI ^{DPP4i}
LPQEVLNENLLRFFVA	FP / VF / PQ ^{ACEi,DPP4i} ; LL ^{DPP4i, sti-glu} ; LR ^{ACEi} ; LLR ^{AOX} ; VL ^{DPP4i, sti-glu} ; VLNENLLR ^{ab} ; RF ^{ACEi} ; LP/NL/VA/QE/NE ^{DPP4i}
PPFQVFG	PQ/LN/EV ^{ACEi,DPP4i} ; FG ^{ACEi} ; PF/QV ^{DPP4i}
VNELSK	EL ^{AOX} ; SK/VN/NE ^{DPP4i}
SESTEDQA	TE ^{ACEi,DPP4i} ; SE ^{reg-ion} ; ST ^{ACEi} ; QA/DQ/ES ^{DPP4i}
MEDIK	ME ^{ACEi,DPP4i}
EMEA	ME ^{ACEi,DPP4i} ; EA ^{ACEi}
ESISSEEIVPNSVEQK	VP/EI/VE ^{ACEi,DPP4i} ; SE ^{reg-ion} ; EE ^{sti-sub} ; IV ^{sti-glu} ; SSS ^{sti-sub} ; QK ^{ACEi} ; PN/SI/SV/ES ^{DPP4i}
HIQK	HI/IQ/QK ^{DPP4i}
EDVPSERY	VP ^{ACEi,DPP4i} ; SE ^{reg-ion} ; RY ^{ACEi} ; PS ^{DPP4i}
LEQLLRK	RL ^{ACEi,DPP4i} ; LL ^{DPP4i, sti-glu} ; LR ^{ACEi} ; LLR/LK ^{AOX} ; QL ^{DPP4i}
VPQLEIVPNSA	PQ/VP/EI ^{ACEi,DPP4i} ; IV ^{sti-glu} ; PN/QL ^{DPP4i}
TTMPLW ^(score = 0.74)	TTMPLW ^{ACEi, mod, op} ; LW ^{ACEi,DPP4i,AOX} ; PLW ^{ACEi} ; MP/TM/TT ^{DPP4i}
VPLG	VPL ^{DPP4i, ann, sti-sub} ; PL/VP ^{ACEi,DPP4i} ; LG ^{ACEi} ; PLG ^{ACEi, op}
QLDA	DA ^{ACEi} ; QL ^{DPP4i}
PSFSDIPNPIG	SF/IP ^{ACEi,DPP4i} ; IG ^{ACEi} ; PI/NP/PN/PS ^{DPP4i}
EERLHSMK	RL ^{ACEi,DPP4i} ; EE ^{sti-sub} ; LHS ^{AOX} ; LH ^{AOX,DPP4i} ; MK/HS ^{DPP4i}
EPMIG	IG ^{ACEi} ; PM/MI/EP ^{DPP4i}
VNQELA	LA ^{ACEi, DPP4i, ubi} ; EL ^{AOX} ; NQ/VN/QE ^{DPP4i}
PELFRQFY	PEL/EL ^{AOX} ; FR ^{ACEi,DPP4i} ; LF/FY/LFR/LFRQ ^{ACEi} ; QF ^{DPP4i}
SENSEK	SE ^{reg-ion} ; EK ^{ACEi, DPP4i}
<i>Substrate for hydrolysis: serum albumin</i>	
WVTFISLLLLFSSA	LL ^{DPP4i, sti-glu} ; LLL ^{sti-sub} ; TF ^{ACEi,DPP4i} ; LF/LLF ^{ACEi} ; WV/SL/VT ^{DPP4i}
LVLIA	LI/VL/LV ^{DPP4i, sti-glu} ; IA ^{ACEi,DPP4i} ; LVL ^{ACEi}
VFRRDTHK	FR / VF / RR ^{ACEi,DPP4i} ; HK ^{ACEi} ; TH ^{DPP4i}
TCVA	VA ^{DPP4i}
SEIA	IA/EI ^{ACEi,DPP4i} ; SE ^{reg-ion}
HRFK	RF ^{ACEi} ; HR ^{DPP4i}
EEHFK	HF/EH ^{DPP4i} ; EE ^{sti-sub}
LVLIA	LI/VL/LV ^{DPP4i, sti-glu} ; IA ^{ACEi,DPP4i} ; LVL ^{ACEi}
FSQY	QY ^{DPP4i}
LQQCPFDEHVK	VK ^{ACEi,DPP4i} ; HK ^{ACEi} ; PF/LQQ/QQ/HV/EH ^{DPP4i}

Cont. Table 2

LVNELTEFA	TE ^{ACEi,DPP4i} ; LV ^{DPP4i,sti-glu} ; EF ^{i-ren, CaMPDEi} ; EL ^{AOX} ; FA /LT/VN/NE ^{DPP4i}
DESHA	HA/SH/ES ^{DPP4i}
SLHTLFG	LH ^{AOX,DPP4i} ; LHT ^{AOX} ; LF/FG ^{ACEi} ; TL/FL/HT ^{DPP4i}
DELCK	EL ^{AOX}
SLRETY	TY ^{AOX,DPP4i} ; LR ^{ACEi} ; SL/ET ^{DPP4i}
DCCEK	EK ^{ACEi,DPP4i}
QEPERNECFLSHK	CF /HK ^{ACEi} ; FL /RN/EP/SH/QE/NE ^{DPP4i}
DDSPDLPK	DLP ^{ACEi} ; LP /SP/PK ^{DPP4i}
PDPNTLCDEFK	EF ^{i-ren, CaMPDEi} ; DP/PN/TL/NT ^{DPP4i}
RRHPY	RR /HP ^{ACEi,DPP4i} ; PY /RH ^{DPP4i}
PELLY	PEL/EL ^{AOX} ; LL ^{DPP4i,sti-glu} ; LLY ^{ist} ; LY ^{ACEi,AOX}
VFQECCQA	FQ / VF ^{ACEi,DPP4i} ; QA/QE ^{DPP4i}
CLLPK	LL ^{DPP4i,sti-glu} ; LLP ^{ACEi} ; LP /PK ^{DPP4i}
IETMREK	EK ^{ACEi,DPP4i} ; IE ^{ACEi} ; MR /TM/ET ^{DPP4i}
RQRLRCA	RL ^{ACEi,DPP4i} ; LR ^{ACEi}
SIQK	QK ^{ACEi} ; SI/IQ ^{DPP4i}
WSVA	WS /VA/SV ^{DPP4i}
RLSQK	RL ^{ACEi,DPP4i} ; QK ^{ACEi}
EFVEVTK	EF ^{i-ren, CaMPDEi} ; EV/VE ^{ACEi,DPP4i} ; TK/VT ^{DPP4i}
LVTDLTK	LV ^{DPP4i,sti-glu} ; LT/TD/TK/VT ^{DPP4i}
ECCHG	HG ^{ACEi}
DLLECA	LL ^{DPP4i,sti-glu}
DDRA	RA ^{ACEi, DPP4i, ubi} ; DR ^{DPP4i}
ICDNQDTISSK	DN/NQ/QD/SK/TI ^{DPP4i}
PLLEK	LL ^{DPP4i,sti-glu} ; PL /EK ^{ACEi,DPP4i} ; LEK ^{ACEi}
SHCIA	IA ^{ACEi,DPP4i} ; SH ^{DPP4i}
EVEK	EV/VE/EK ^{ACEi,DPP4i}
IPENLPPLTA	PL / PP / IP ^{ACEi,DPP4i} ; LPP ^{ACEi} ; PPL / LP /NL/LT/TA ^{DPP4i}
SFLY	LY ^{ACEi,AOX} ; SF ^{ACEi} ; FL ^{DPP4i}
SRRHPEY	RR /HP/EY ^{ACEi,DPP4i} ; RH ^{DPP4i}
VSVLLRLA	RL ^{ACEi,DPP4i} ; LL /VL ^{DPP4i,sti-glu} ; LLR ^{AOX} ; LA ^{ACEi, DPP4i, ubi} ; LR ^{ACEi} ; SV/VS ^{DPP4i}
TLEECCA	EE ^{sti-sub} ; LEE ^{ACEi} ; TL ^{DPP4i}
DDPHA	PH ^{ACEi,DPP4i} ; PHA ^{AOX} ; DP/HA ^{DPP4i}

Cont. Table 2

STVFDK	FDK /ST ^{ACEi} ; VF ^{ACEi,DPP4i} ; TV ^{DPP4i}
HLVDEPQNLIK	HL ^{ACEi} , DPP4i, AOX; LV ^{DPP4i,sti-glu} ; LI ^{DPP4i,sti-glu} ; PQ ^{ACEi,DPP4i} ; NL/EP/QN/VD ^{DPP4i}
QNCDQFEK	EK ^{ACEi,DPP4i} ; QF /QN/DQ ^{DPP4i}
FQNA	FQ /NA/QN ^{DPP4i}
LIVRY	RY ^{ACEi} ; VR ^{ACEi,DPP4i} ; LI ^{DPP4i,sti-glu} ; IV ^{sti-glu} ; VRY/IVR ^{ACEi}
VPQVSTPTLVEVSR-SLG	PQ/TP/PT/VP/EV/VE ^{ACEi,DPP4i} ; LV ^{DPP4i,sti-glu} ; LG /STE/LVE ^{ACEi} ; SL/TL/VS/QV ^{DPP4i}
TRCCTK	TR/TK ^{DPP4i}
PESERMPCTEDY	TE ^{ACEi,DPP4i} ; SE ^{reg-ion} ; DY ^{ACEi, sti-ion} ; MP/RM/ES ^{DPP4i}
LSLILNRLCVLHEK	RL /LN/EK ^{ACEi,DPP4i} ; LHE ^{AOX} ; IL ^{ACEi, sti-glu} ; LI/VL ^{DPP4i,sti-glu} ; LH ^{AOX,DPP4i} ; SL/NR/HE ^{DPP4i}
TPVSEK	TP/EK ^{ACEi,DPP4i} ; SE ^{reg-ion} ; PV/VS ^{DPP4i}
CCTESLVNRRPCFSA	RPRR /TE ^{ACEi,DPP4i} ; LV ^{DPP4i,sti-glu} ; CF ^{ACEi} ; SL/NR/VN/ES ^{DPP4i} ;
LTPDETY	TP ^{ACEi,DPP4i} ; TY ^{AOX,DPP4i} ; LT/ET ^{DPP4i}
FDEK	EK ^{ACEi,DPP4i}
LFTFHA	TF ^{ACEi,DPP4i} ; LF ^{ACEi} ; HA ^{DPP4i}
DICTLPDTEK	TE/EK ^{ACEi,DPP4i} ; LP /TL ^{DPP4i}
LVELLK	LL /LV ^{DPP4i,sti-glu} ; LK/EL ^{AOX} ; VE ^{ACEi,DPP4i} ; LVE ^{ACEi}
TEEQLK	TE ^{ACEi,DPP4i} ; LK ^{AOX} ; EE ^{sti-sub} ; QL ^{DPP4i}
TVMENFVA	NF /ME ^{ACEi,DPP4i} ; VM/VA/TV ^{DPP4i}
FVDK	VD ^{DPP4i}
LVVSTQTA	LV ^{DPP4i,sti-glu} ; TQ ^{ACEi,DPP4i} ; ST ^{ACEi} ; TA/QT/VS/VV ^{DPP4i}

ACEi – angiotensin converting enzyme inhibitor; DPP4i – dipeptidyl peptidase IV inhibitor; AOX – antioxidative; sti-glu – stimulating absorption of glucose; sti-sub – stimulating the release of vasoactive substances; ubi – activator of ubiquitin-mediated proteolysis; reg-ion – regulator of ion flow; i-ren – renin inhibitor; chymi – chymosin inhibitor; acan-anticancer; CaMPDEi – inhibitor CaMPDE (calmodulin-dependent cyclic nucleotide phosphodiesterase); ist – immunostimulating; mod – immunomodulating; ab – antibacterial; at – antithrombotic; anm – anti-amnesic; op – opioid; reg-sto – regulator of stomach mucosal membrane action; **bold** – peptide with Score > 0.5; grey – peptide with confirmed bioactivity

All milk proteins were theoretically hydrolyzed using papain and bromelain. These enzymes were proven potent to produce bioactive peptides from foods in vitro (IWANIAK et al. 2020a). For example, papain was effective in producing ACE inhibitors and antioxidative peptides from gelatin-derived tilapia skin (CHOOPINHAM et al. 2015), whereas bromelain was applied to hydrolyze clam proteins to generate antibacterial peptides (ZAMBROWICZ et al. 2013). Moreover, papain and bromelain can be used as substitutes for rennet in the cheesemaking (ARLENE et al. 2015). They also

offer an alternative to animal-derived coagulants when taking into account ethic, religious, and economic concerns (AKTAYEVA et al. 2018). Additionally, papain and bromelain were tested during the hydrolysis of goat and bovine milks to produce peptidic ACE inhibitors (SHU et al. 2018). Thus, our predictions involving these enzymes might be suitable in the design of food rich in bioactive peptides. However, the scientists highlight that successful prediction of peptides' release from proteins depends on the regular update of the database with the new sequences and/or completing the information about the bioactivities of peptides and specificity of enzymes (UDENIGWE 2014).

SOŁOWIEJ et al. (2016) highlighted the role of casein as a valuable ingredient that may be incorporated to foods due to its functional properties, like e.g., consistency and fat emulsification ability. *In silico* hydrolysis of milk proteins with two enzymes also revealed that caseins were good sources of biopeptides. It especially concerned β -casein, being the richest source of parent peptides and, thus, abundant in bioactive motifs. These proteins produced the longest parent sequences (see above rules). Among them, the longest fragment (i.e. PVQPFTEESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK) was produced during the hydrolysis of β -casein with bromelain (Table 2). It consisted of 55 residues and contained 10 peptides with a dual bioactivity (ACE/DPP-IV inhibitors), 9 ACE inhibitors, 18 DPP-IV inhibitors, and 1 peptide with anticancer function. Multiple bioactive motifs found in the PVQPFTEESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK parent peptide suggest its potential to be another strategic zone of β -casein. So far, the term "strategic zone" concerned the region between the 60th and 70th residue (PFAQTQSLVYPPFGPIHNSL) of β -casein. According to the scientific reports, β -casein is the richest source of biopeptides, including its strategic zone, due to the presence of the motifs eliciting ACE-inhibiting, immunostimulating, and opioid effects (HAQUE and CHAND 2008). When looking at our results, the part of this strategic zone (PIHNSL-motif) was encrypted in another parent peptide i.e., PIHNSLPQNIPPLTQTPVVVP-PFLQPEVMG, being a product of β -casein hydrolysis with bromelain (Table 2). The PIHNSL-motif contained two sequences, namely IH and PI, acting as DPP-IV inhibitors.

Regardless of the enzyme applied, the function of the great majority of parent peptides that were released from all milk proteins remained unknown (Table 1 and Table 2). This dearth of data encouraged us to further employ the fragmentomic analysis of milk proteins hydrolyzed (*in silico*) by the two tested enzymes. It showed that the released parent peptides were abundant in the motifs with ACE and DPP-IV inhibiting activ-

ities. Briefly, these peptides are involved in the regulation of blood pressure and glucose level, respectively, thereby eliciting antihypertensive and antidiabetic effects (RIVERO-PINO et al. 2020). These motifs could be detected in the parent sequences due to their abundance in the BIO-PEP-UWM database, which increases the probability of finding them encrypted in a parent peptide, especially when they are di- or tripeptides.

When looking at the structure (sequence) of parent peptides being the sources of ACE-inhibiting motifs, they were rich in amino acids with non-polar side chains, like glycine, valine, leucine, isoleucine or those possessing a ring, like proline, phenylalanine and/or tryptophan. The studies on the structure-activity analysis of peptides demonstrated the impact of N-terminal glycine, isoleucine, leucine, or valine on the ACE inhibitory activity. In turn, C-terminus of these peptides was composed of “ring containing amino acids” (ABACHI et al. 2019). Thus, the abundance of parent peptides in such residues affected the presence of ACE inhibitors. Moreover, loads of ACE inhibitors composed of the above-mentioned residues had a PeptideRanker Score > 0.5 (bold sequences). The PeptideRanker program estimates the likelihood of a peptide to be bioactive (with no indication of particular bioactivity). Its value ranges from 0.00 to 1.00, and the Score of a potentially active peptide should exceed 0.5 (MOONEY et al. 2012). According to the scientific reports, amino acids, like tryptophan or proline, were typical of peptides with DPP-IV inhibitory activity (RIVERO-PINO et al. 2020). It was also confirmed in the present study, where the majority of DPP-IV-inhibiting motifs having a PeptideRanker Score > 0.50 were abundant in such residues.

The fragmentomic analysis of the products of milk protein hydrolysis by bromelain and papain revealed that the antioxidant bioactivity was the third dominant bioactivity of the parent peptides. Antioxidant peptidic motifs were mostly rich in the amino acids possessing a ring or an apolar side chain (N-end) and proline or histidine, leucine, and valine (C-end). These observations were consistent with findings obtained by other authors who employed the chemometric analysis (i.e. multivariate regression) to find the “structure-activity” relationships of antioxidant peptides identified in the food-derived protein hydrolysates (UDENIGWE and ALUKO 2011).

The other motifs present in parent peptides acted mostly as antiemetic, glucose absorption-simulating, antibacterial, antithrombotic, enzyme inhibiting agents etc. Their full list is found in Table 1 and Table 2. To the best of our knowledge, there are no literature works providing information on the structural nature of such peptides, which is probably due to several factors that impede the structure-function analysis of molecules.

These include, e.g., difficulties with the collection of an appropriate number of samples (understood as peptide sequences), variables to form a data matrix as well as problems with applying the appropriate measure of activity to run QSAR (i.e., quantitative structure-activity relationship) analysis. More details related to QSAR studies of peptides, including the pros and cons of methods used, were described by IWANIAK et al. (2015). Thus, the fragmentomic analysis of parent peptides may be useful for the brief finding of some regularities in motifs with specific activities assuming there is a plenty of peptides representing the specific activity. Moreover, calculation of PeptideRanker Scores might be one of the steps of the hybrid approach (see above), like the initial selection of relatively strong peptides (*in silico* part of the study) followed by their identification in the hydrolysate and determination of their bioactivity *in vitro*.

According to DALIRI et al. (2007), many scientific reports provide the data about peptides showing one biological effect, whereas relatively small amount of data refer to the multifunctional sequences. Thus, due to the multiple health benefits, peptides exhibiting more than one biological activity are in the focus of the scientific interests. It especially concerns the identification of such sequences in hydrolysates (DALIRI et al. 2007). Therefore, databases can offer a supportive tool to acquire the knowledge on multi-active peptides. Several functions were ascribed to the motifs encrypted in parent peptides theoretically produced from milk proteins hydrolyzed with papain and bromelain (see Table 1 and Table 2). A great majority of them had dual functions, like e.g. IW acting as ACE/DPP-IV inhibitor (parent source: IWCK released from α -lactalbumin, enzyme used: papain/bromelain). One peptide, PG (see below, bold font), showing 5 bioactivities, was encrypted in the following parent peptides: LNV**PG** and PF**PG** (source: β -casein hydrolyzed with papain, see Table 1) and RELEEL-NV**PG** (source: β -casein hydrolyzed with bromelain, see Table 2). PG was confirmed as ACE/DPP-IV inhibitor, stimulator of the action of stomach mucosa membrane as well as antiemetic and antithrombotic peptide.

It needs to be highlighted that, although the bioinformatic analysis of multifunctional peptides is useful and easy, it has some limitations. They were discussed by IWANIAK and MOGUT (2020). Briefly, such an analysis is based on the so-called positive selection assuming that a peptide of interest matches the sequences present in the database used (IWANIAK and MOGUT 2020). At the time of data analysis, the BIOPEP-UWM database contained the information about 3,200 bioactive peptide sequences. Currently, it contains over 4,000 sequences. Thus, some authors postulate the regular update of the database (UDENIGWE 2014) to get more knowledge on the additional functions of peptides before running the *in vitro* part of

the experiment. Regular update of databases should be a golden standard to ensure the high quality of data when performing any type of computations, including those applied in our protocol.

Several parent peptides that were *in silico* released from milk proteins were known as bioactive themselves (Table 1 and Table 2), i.e.: LDIQK^{0.11} (ACE inhibitor, source: β -lactoglobulin, enzyme applied: papain and bromelain), IPIQY^{0.36} (DPP-IV inhibitor, source: κ -casein, enzyme applied: papain and bromelain), NQDK^{0.07} (antithrombotic peptide, source: κ -casein, enzyme applied: papain and bromelain), TTMPLW^{0.74} (ACE inhibitor/immunomodulator/opioid, source: α _{s1}-casein, enzyme applied: papain and bromelain), EQLTK^{0.05} (antibacterial peptide, source: α -lactalbumin, enzyme applied: bromelain), and EMPFPK^{0.77} (ACE inhibitor, source: β -casein, enzyme applied: bromelain). The superscripts mean the PeptideRanker Score of each peptide. All the above-mentioned peptides were identified experimentally in food sources. In the case of peptides acting as enzyme inhibitors, their bioactivity measured in experimental conditions was expressed as IC₅₀ understood as the concentration of a molecule (i.e., peptide) corresponding to its half-maximal inhibition (PRIPP and ARDÖ 2007). Comparison of theoretical vs. experimental bioactivity of these peptides (i.e., PeptideRanker Scores vs. IC₅₀) enabled various options of data interpretation: *a*) peptide with theoretically strong but experimentally weak activity; *b*) peptide with theoretically weak but experimentally strong activity; *c*) peptide with theoretically and experimentally strong activity, and *d*) peptide with theoretically and experimentally weak activity.

The first option was exemplified by the EMPFPK parent peptide (see Table 2). Its weak bioactivity was reported when studying the ACE inhibitory potential of the EMPFPK peptide (IC₅₀ = 432.0 μ g/mL) (HAYES et al. 2007), whereas its high potency was indicated by the PeptideRanker Score (0.77).

The LDIQK peptide (Table 1 and Table 2) is an example of a sequence with weak theoretical bioactivity but a strong experimental potency. According to the literature, LDIQK was an ACE inhibitor with IC₅₀ = 27.6 μ M (relatively potent) (HERNÁNDEZ-LEDESMA et al. 2006), whereas its PeptideRanker Score (0.11) suggested its weak potential. Similar regularity was reported for the IPIQY sequence (DPP-IV inhibitor) with IC₅₀ = 35.2 μ M (NONGONIERMA et al. 2014) and 0.36 (PeptideRanker Score).

A strong theoretical and experimental potential was ascribed to the TTMPLW (Table 1 and Table 2) parent peptide acting as an ACE inhibitor. Its IC₅₀ value was 16.0 μ M (strong potential) (FUGLSANG et al. 2003), which was also confirmed by a high PeptideRanker Score (0.74). This peptide was identified by IWANIAK et al. (2020a) in bovine milk protein concentrate hydrolyzed both *in silico* and *in vitro* by papain.

Finally, the parent peptide with a sequence NQDK exhibited weak potency according to the PeptideRanker Score (0.07) and literature data. According to literature findings, it inhibited the ADP-induced human platelet aggregation ($IC_{50} = 400 \mu M$) (FIAT et al. 1993). Weak theoretical and experimental bioactivity was also ascribed to the EQLTK sequence. Its PeptideRanker Score was 0.05 and, according to the literature, this peptide exhibited an antibacterial function. Its antibacterial potential was expressed as $\log N_0/N_1$ (N_0 – control number of colonies without antibacterial material; N_1 – the number of colonies containing antibacterial agent after an incubation period of 2 h), and the values calculated against different microbial strains showed its weak activity against Gram-negative bacteria compared to the other peptides (PELLEGRINI et al. 1999).

To recapitulate, according to FU et al. (2016), the PeptideRanker Score might be useful for the structure-activity analysis of peptides, but the “exact” prediction of bioactivity is rather impossible. The discrepancies between the theoretical and experimental bioactivities of peptides were also observed by FU et al. (2016) who assessed the potency of peptides derived from patatin (potato). Based on the literature search, it was found that FP had a weak ACE inhibitory potency ($IC_{50} = 1215.7 \mu M$), which was in opposition to the PeptideRanker Score (0.99) suggesting strong bioactivity. Another peptide (WG) had the same PeptideRanker Score as FG peptide, but there was no literature data about its potential measured *in vitro* (FU et al. 2016). It needs to be noted that the measure of the bioactivity of peptides might also be an important feature when comparing their predicted and experimental activities. No units are provided by PeptideRanker Scores, whereas experimental bioactivity is expressed in different units, which might affect the interpretation of results. Nevertheless, biological activity prediction may prove useful while selecting peptides for their synthesis in order to determine their effect *in vitro* (FU et al. 2016). Finally, our approach shows how to determine the potential of proteins as the sources of biopeptides. However, some discrepancies may appear when comparing the results of *in silico* and *in vitro* hydrolysis of proteins. Such phenomenon is quite common and possible factors affecting such discrepancies were discussed by IWANIAK et al. (2020b). However, the analyses of large datasets involving bioinformatic-assisted methods enable to preselect the protein and protease candidates to produce peptides before their identification in the laboratory conditions.

Final remarks

The fragmentomic approach applied in this study showed the potential of milk protein-derived parent peptides to be bioactive. β -Casein was considered as the best source of biopeptides, which is the common fact. Despite this, the new parent peptide, i.e., PVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK produced by the action of bromelain, showed the likelihood to act as a new strategic zone of β -casein due to the presence of plenty of motifs with various activities. Several parent peptides theoretically released from milk proteins possessed experimentally confirmed bioactivity. However, the analysis of their predicted and experimental potency showed some discrepancies. Despite the usefulness of the tools for peptide bioactivity prediction, critical thinking while planning the application of such data in future experiments would thus appear to be a worthwhile line of inquiry. It results from immense structural diversity of natural compounds and the complexity of structure-activity relationships. Nevertheless, the scientists highlight the suitability of bioinformatic-assisted analyses of large datasets to preselect the protein and protease candidates to produce peptide before discovering bioactive peptides in the laboratory conditions.

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