κ -Casein as a source of short-chain bioactive peptides generated by *Lactobacillus helveticus*

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Abstract This paper explores the ability of *Lactobacillus helveticus* strains to release sequences of short biologically active peptides (containing 2–10 amino acid residues) from casein. The proteolytic enzymes of the tested strains exhibit different patterns of cleavage of CN fractions. The modification of κ -casein (κ -CN) with pyrrolidone carboxylic acid inhibits the proteolytic activity of strains *L. helveticus* 141 and the reference strain (DSMZ 20075), while the modification with phosphothreonine inhibits enzymes of all the tested bacteria. The peptide sequencing analysis indicated that the examined strains produced functional peptides very efficiently. κ -CN proved to be the main source of short peptides released by bacterial enzymes, and the hydrolysis of κ -CN yielded eighty-two bioactive peptides. The hydrolysis of α_{S2} -casein, α_{S1} -casein, and β -casein yielded six, two, and one short-chain bioactive peptides, respectively. The isolated bioactive peptides exhibited antioxidative, opioid, stimulating, hypotensive, immunomodulating, antibacterial, and antithrombotic activities. A vast majority of the isolated bioactive peptides caused inhibition of the angiotensin-converting enzyme and dipeptidyl peptidase IV. The role of

hydrolysis products as neuropeptides is also pointed out. The highest number of cleavage sites in κ -casein and functional activities of short-chain peptides were obtained in hydrolyzates produced by *L. helveticus* strain T105.

Keywords *Lactobacillus helveticus* · Biologically active peptides · κ -Casein (κ -CN)

Introduction

The development of functional foods and nutraceuticals is connected with the increasing consumers' awareness of the impact of the diet on health. Considerable attention is being paid to biologically active food ingredients that contribute to beneficial health effects.

Therefore, a great effort has been made to study milk and whey proteins as valuable precursors of bioactive components. However, the active functional properties can be released from casein only by proteolytic degradation. This can be obtained through enzymatic hydrolysis during milk fermentation involving a complex proteolytic system of bacterial starter cultures (Michaelidou 2008). Casein proteolysis is initiated by bacterial extracellular proteases, while the transporting system enables intracellular peptidases inside the cell to conduct further hydrolysis (Savijoki et al. 2006).

The proteolysis process in food industry has particular importance during cheese ripening, where the texture and the sensory and organoleptic characteristics are developed.

Bioactive peptides are defined as fragments of proteins that exert a positive influence on body systems, improving their functioning and leading to health-promoting effects (Kitts and Weiler 2003). The size of the peptides is very diverse and ranges from 2 to 20 amino acid residues.

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However, their functional properties are associated with the composition and amino acid sequences; furthermore, many of these peptides exhibit simultaneously a wide range of very different bioactivities (Korhonen and Pihlanto 2006; Dziuba and Dziuba 2014; Bhat et al. 2015). Moreover, peptides released during the fermentation process and their functional properties are affected by the types of bacterial cultures used (Szwajkowska et al. 2011).

Due to their high proteolytic activity, lactic acid production, rate of milk acidification, and ability to form the flavor and texture, *Lactobacillus helveticus* are often used for the production of fermented milk beverages and hard cheeses. Furthermore, the great diversity of LAB proteolytic enzymes and the specificity of enzyme activity are still a wide field of study focusing on their biotechnological applications (Budiarto et al. 2016).

Moreover, lactic acid fermentation is a promising method for acquisition of bioactive peptides; therefore, analysis of the proteolytic profile exhibited by new strains sets a wide path to obtaining functional food (Dziuba and Dziuba 2014; Bhat et al. 2015; Pessione and Cirrincione 2016). Strains of *L. helveticus* exhibit strong extracellular proteinase activity and capacity to liberate a significant number of various peptides during milk fermentation (Pihlanto 2013).

The number of cell envelope proteinases (CEPs) varies among *L. helveticus* strains and ranges from 1 to 4 enzymes (Sadat-Mekmene et al. 2013). CEPs are very specific and differ among *L. helveticus* strains; moreover, casein cleavage sites depend on the primary structure of the protein fraction under hydrolysis (Jensen et al. 2009; Sadat-Mekmene et al. 2011a). So far, three different types of cell envelope proteinases have been identified: P_I-type that exhibit preference to β -CN; P_{III}-type, which preferentially hydrolyze all casein fractions (but different patterns of β -CN hydrolysis have been noticed), and P_{I/III} type, in which enzymes exhibiting intermediate properties of the P_{III}-type and P_I-type have been classified (Visser et al. 1986; Exterkate et al. 1993; Juillard et al. 1995).

Identification of peptide sequences generated by *L. helveticus* as a result of casein hydrolysis may lead to selection of a particular strain able to produce peptides with defined bioactivity. This might yield new biologically active substances or contribute to a more efficient use of *L. helveticus* strains in the production of functional foods, nutraceuticals, or even pharmaceuticals. Therefore, the aim of this study was to identify the sequence of peptides released from casein by selected *L. helveticus* strains. It should be mentioned that these strains have not been previously used for this purpose on an industrial scale. Another aim of the research was to analyze the potential biological activities of the short amino acid sequences

obtained. Moreover, an attempt was made to analyze the ability of the bacterial strains to hydrolyze CN and to compare the patterns of κ -CN cleavages sites caused by the enzymes produced by the tested bacterial strains.

Materials and methods

Bacterial strains and culture conditions

Two strains of *L. helveticus* 141 and T105 were kindly provided by Prof. Łucja Łaniewska-Trokenheim from the University of Warmia and Mazury in Olsztyn, Poland. The strains were deposited in the Polish Microorganism Collection (Wrocław, Poland). Both strains and *L. helveticus* DSMZ 20075 (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) used as a reference strain were cultured in de Man, Rogosa and Sharpe broth (BTL, Poland) according to the method proposed by Waśko et al. (2014).

Casein hydrolysis

After 12 h of culture incubation, bacterial cells were harvested by centrifugation (10.000×g, 10 min, 4 °C) and washed twice with distilled sterile water. A volume of 200 μ l of whole washed cell suspensions (OD₆₀₀ = 1) were mixed with 5 ml of 1% (vol/vol) solution of casein from bovine milk (Sigma-Aldrich). The hydrolysis of proteins was carried out at 37 °C for 12 h. The hydrolyzates obtained were centrifuged (13.000×g, 8 min, 5 °C). Supernatants were immediately collected, filtered through 0.45 μ m syringe filters, and gel filtration chromatography was directly performed as described below.

Gel filtration chromatography

The BioLogic DuoFlow system (Bio-Rad, USA) consisting of two BioLogic pumps, a QuadTec UV–Vis detector (280 nm), and a BioFrac fraction collector was used in the preparative mode. For separations, Sephacryl S-100 HR gel (Sigma-Aldrich, USA) was packed in a Bio-Rad Econo glass column (2.5 × 50 cm); this was followed by equilibration with deionized water containing 0.01% sodium azide (0.85 cm³/min, 24 h). For separations, a volume of 4 cm³ of the sample (casein hydrolyzate) was loaded onto the column and this was followed by separation using deionized water with 0.01% NaN₃ (0.7 cm³/min). Collected fractions (2 mL) were dialyzed against deionized water, frozen at −80 °C for 2 h, and lyophilized (Labconco, Kansas City, USA).

Liquid chromatography–high-resolution mass spectrometry (LC–HRMS) and peptide sequencing

Twenty-five fractions from gel filtration were loaded onto the column and sequencing. The analyzes were performed using an Agilent nano-HPLC chromatograph series 1200 coupled to Agilent LC/MS QTOF 6538 equipped with a chip-cube ion source. Agilent HPLC-Chip G4240-62001 was composed of a 40 nl enrichment column and a 75 $\mu\text{m} \times 43$ mm separation column packed with Zorbax 300 SB-C18 5 μm material, which was used for the separation of peptides. A linear gradient from 3 to 95% B over 9 min was applied with the mobile phase composed of solvent A: aqueous 0.1% formic acid and B: 0.1% formic acid in acetonitrile (0.5 $\mu\text{l min}^{-1}$). The ion source fragmentor voltage was set at 200 V. Data were acquired in a full scan MS mode, and positive ions were generated and registered at the m/z range of 100–1700. Agilent Mass Hunter acquisition B.05.01 software was used for

data acquisition, and Agilent Mass Hunter qualitative analysis B.07 with integrated Bioconfirm add-on software was used for data analysis and peptide mapping.

Assay of the biological activities of peptide sequences

The profiles of potential biological activities of the peptide sequences obtained were determined according the procedure included in the BIOPEP database (Perkins et al. 1999; Minkiewicz et al. 2008; Dziuba et al. 2009).

Results and discussion

Casein hydrolysis

The significance of casein in food industry is associated with its technological properties and presence of a wide

Fig. 1 Peptide bonds of bovine κ -CN hydrolyzed after 12 h of incubation with the proteolytic system of *Lactobacillus helveticus*. **a** Strain T105, **b** strain 141, **c** strain DSMZ 20075

range of bioactive sequence precursors in the native milk protein structure (Wang et al. 2013).

This study demonstrated that κ -CN was susceptible to the action of proteases produced by all examined strains. *L. helveticus* T105 was the most effective strain that hydrolyzed κ -CN at 73 cleavage sites (Fig. 1). 55 and 35 cleavage sites were identified strains *L. helveticus* 141 and DSMZ 20075, respectively. Furthermore, 18 different residue sites in position P1 (i.e. the residue on the primary sequence of the protein preceding the amino-terminal residue of the peptide) underwent cleavage, including hydrophobic residues (Ala, Leu, Phe, and Val), non-charged polar residues (Ser, Thr, and Gln), and negatively charged hydrophilic residues (Glu and Asp). Peptide bonds located after the residues of Cys, Trp, Asx, Glx, Gly, and His underwent few or no cut. In addition, in the case of T105 and DSMZ 20075 strains, most of the cleavage sites of κ -CN were located in the hydrophobic C-terminal fragment. Our results also revealed that the signal peptide of κ -CN was hydrolyzed by two strains: *L. helveticus* T105 and 141.

It has been suggested that the occurrence of various CEPs in *L. helveticus* (PrTH, PrTH2) is a strain-dependent property (Broadbent et al. 2011; Sadat-Mekmene et al. 2011a, b). It has been revealed that α_{s1} -casein was hydrolyzed much more slowly by strains exhibiting the presence of only one CEP (Sadat-Mekmene et al. 2011a). In turn, *L. helveticus* BGRA43 possessing only PrTH was able to hydrolyze completely all casein fractions, but α_{s1} -CN and β -CN were hydrolyzed faster than κ -CN (Strahinic et al. 2013). However, cell envelope proteinase may exhibit

some differences in terms of affinity and specificity to particular casein fractions (Kunji et al. 1996). It was also concluded that CEPs involved in the formation of ACE inhibitory peptides are strain-specific (Xing et al. 2012). Therefore, it can be supposed that the affinity and specificity to different casein fractions exhibited by CEPs of *L. helveticus* strains might be related to the observed differences in the amounts of products obtained after hydrolysis as well as the variety of amino acid sequences. Our results confirm the high variability of the strains in terms of their ability to hydrolyze casein fractions.

The results also indicate that the glycosylated C-terminal fragment (called macropeptide or glycomacropeptide) is hydrolyzed by *L. helveticus* strains (Fig. 2). Moreover, the presence of modified phosphoserine as well as glycosylation of the protein did not affect the hydrolysis.

Our study confirmed that the regions containing phosphoserine and phosphothreonine residues were always very resistant to hydrolysis by CEP produced by the tested *L. helveticus* strains (Fig. 2).

Studies on the biological activities of obtained peptide sequences

Milk is a widespread food product and a valuable source of bioactive components that can be released by bacterial proteolysis (FitzGerald and Murray 2006; Jäkälä and Vapaatalo 2010).

Over the last two decades, increasing importance of protein in food science has been observed, which is connected with research on the properties of bioactive peptides

10	20	Di	30	40	50
MMKSFFLVVT	ILALTLPFLG	A ^{Di} EQNQEQPI	RCEKDERFFS	DKIAKYIPIQ	
60	70	80	90	100	
YVLSRYPSTYG	LNYYQQKPVA	LINNQFLPYP	YYAKPAAVRS	PAQILQWQVL	
110	120	130	140	150	
SNTVPAKSCQ	AQPTTMRHP	HPHLSFMAIP	PKKNQDKTEI	PTINTIASGE	
160	170	180	190		
PTSTPTTEAV	ESTVATLED ^S	PEVIESPPEI	NTVQVTS ^S AV		

The explanations notes:

Signal peptide

Glycosylation

■ modified residue phosphothreonine

■ modified residue pyrrolidone carboxylic acid

■ modified residue phosphoserine

■ disulphide

Fig. 2 Kappa casein sequence (Based on: <http://www.uniprot.org/uniprot/P02668>) with marked different types of modifications

Table 1 Peptides identified in the casein hydrolysates released by the CEP activity of *L. helveticus* strains

Casein precursor	Peptide identity ^a	Mass (Da)	Sequence	Score ^b	Activity ^c	Strain
α_{S1} -casein	f(209–214)	747.3	TTMPLW	29.2	ACE inhibitor, immunomodulating, opioid, antioxidative, dipeptidyl peptidase IV inhibitor	T105, DSM ^d
	f(106–115)	1266.6	YLGYLEQLLR	29.0	ACE inhibitor, opioid	141
α_{S2} -casein	f(130–140)	1194.6	NAVPTPTLNR	24.7	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(168–173)	743.3	LTEEEK	29.4	Dipeptidyl peptidase IV inhibitor	141
	f(204–212)	1199.6	AMKPWQPK	23.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(204–214)	1326.7	AMKPWQPKTK	28.3	ACE inhibitor, dipeptidyl peptidase IV inhibitor	DSM
	f(213–222)	1250.7	TKVIPYVRYL	29.0	ACE inhibitor, antibacterial, neuropeptide, antioxidative, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(215–222)	1021.5	VIPYVRYL	29.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM, 141
β -casein	f(218–224)	741.4	GPFPPIIV	29.1	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
κ -Casein	f(4–11)	924.5	SFFLVVTI	20.0	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM, 141
	f(5–6)	312.1	FF	29.9	–	141
	f(6–7)	278.1	FL	29.9	Dipeptidyl peptidase IV inhibitor	141
	f(7–10)	430.2	LVVT	26.7	–	141
	f(17–25)	1002.4	PFLGAQEQN	21.7	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(19–26)	886.4	LGAQEQNQ	21.5	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(22–23)	275.1	QE	29.9	Dipeptidyl peptidase IV inhibitor	141
	f(25–32)	986.4	NQEPIRC	25.1	ACE inhibitor, hypotensive	141
	f(26–32)	872.4	QEPIRC	25.2	ACE inhibitor, hypotensive	DSM
	f(26–34)	1129.5	QEPIRCEK	19.5	ACE inhibitor, hypotensive, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(35–40)	799.3	DERFFS	29.9	ACE inhibitor	T105
	f(35–41)	914.3	DERFFSD	29.5	ACE inhibitor	T105
	f(35–44)	1226.5	DERFFSDKIA	29.8	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(37–38)	321.1	RF	29.6	ACE inhibitor	141
	f(37–39)	468.2	RFF	29.9	ACE inhibitor	T105
	f(40–47)	936.5	SDKIAKYI	27.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(41–48)	946.5	DKIAKYIP	27.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(43–51)	1107.6	IAKYIPIQY	29.5	ACE inhibitor, dipeptidyl peptidase IV inhibitor	DSM
	f(44–49)	703.4	AKYIPI	29.8	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(46–53)	1007.5	YIPIQYVL	21.2	ACE inhibitor, antibacterial, stimulating, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(56–58)	365.3	YPS	27.2	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(57–58)	202.0	PS	29.1	Dipeptidyl peptidase IV inhibitor	T105
	f(59–62)	465.2	YGLN	27.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(59–69)	1371.6	YGLNYYQQKPV	28.0	ACE inhibitor, antibacterial, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(66–72)	767.4	QKPVALI	23.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(67–73)	753.4	KPVALIN	27.1	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(74–80)	887.4	NQFLPYP	26.3	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(74–83)	1274.5	NQFLPYPYYA	30.0	ACE inhibitor, antioxidative, opioid, dipeptidyl peptidase IV inhibitor	T105
	f(76–86)	1328.0	FLPYPYYAKPA	27.1	ACE inhibitor, antioxidative, opioid, dipeptidyl peptidase IV inhibitor	T105
	f(77–82)	814.3	LPYPYY	28.8	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, 141

Table 1 continued

Casein precursor	Peptide identity ^a	Mass (Da)	Sequence	Score ^b	Activity ^c	Strain
	f(76–81)	798.3	FLPYPY	29.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(80–84)	640.3	PYYAK	30	ACE inhibitor	141
	f(80–85)	737.3	PYYAKP	29.2	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(81–89)	1037.5	YYAKPAAVR	24.4	ACE inhibitor, antibacterial, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(82–85)	477.2	YAKP	29.0	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(82–88)	718.3	YAKPAAV	24.1	ACE inhibitor, antioxidative, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(89–92)	429.2	RSPA	25.6	Dipeptidyl peptidase IV inhibitor	141
	f(100–106)	700.3	LSNTVPA	29.8	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(102–110)	946.4	NTVPAKSCQ	29.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(102–112)	1143.5	NTVPAKSCQAQ	28.4	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(103–111)	903.4	TVPAKSCQA	24.3	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(105–113)	928.4	PAKSCQAQP	27.9	Dipeptidyl peptidase IV inhibitor	T105, DSM
	f(110–119)	1139.5	QAQPTTMARH	26.8	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(114–118)	678.2	TTMAR	28.5	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM, 141
	f(115–122)	945.4	TMARHPHP	19.9	ACE inhibitor, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(116–121)	747.3	MARHPH	29.3	ACE inhibitor, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(118–127)	1257.6	RHPHPLSFM	28.1	ACE inhibitor, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(119–127)	1101.5	HPHPLSFM	20.1	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(121–132)	1373.7	HPHLSFMAIPPK	29.0	ACE inhibitor, antitrombotic, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(124–126)	365.1	LSF	29.6	ACE inhibitor	141
	f(124–127)	496.2	LSFM	28.9	ACE inhibitor	141
	f(130–135)	710.4	PPKKNQ	24.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(136–142)	802.4	DKTEIPT	29.8	ACE inhibitor, antibacterial, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(136–147)	1315.7	DKTEIPTINTIA	29.4	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(137–147)	1199.6	KTEIPTINTIA	29.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(138–144)	786.4	TEIPTIN	29.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141
	f(139–147)	970.5	EIPTINTIA	29.0	ACE inhibitor, antibacterial, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(139–148)	1057.5	EIPTINTIAS	29.0	ACE inhibitor, antibacterial, dipeptidyl peptidase IV inhibitor	T105
	f(141–144)	443.2	PTIN	29.8	ACE inhibitor	DSM
	f(141–151)	1098.5	PTINTIASGEP	20.6	ACE inhibitor, antioxidative, dipeptidyl peptidase IV inhibitor	T105
	f(146–149)	346.6	IASG	29.3	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(154–164)	1133.5	TPTEAVESTV	22.4	ACE inhibitor, dipeptidyl peptidase IV inhibitor	DSM
	f(155–161)	745.3	PTTEAVE	30.0	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(155–162)	832.3	PTTEAVES	29.7	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(155–164)	1032.4	PTTEAVESTV	29.4	ACE inhibitor	DSM
	f(157–167)	1119.5	TEAVESTVATL	21.9	ACE inhibitor, antibacterial, dipeptidyl peptidase IV inhibitor	T105
	f(158–167)	1018.5	EAVESTVATL	29.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	141

Table 1 continued

Casein precursor	Peptide identity ^a	Mass (Da)	Sequence	Score ^b	Activity ^c	Strain
	f(159–167)	889.9	AVESTVATL	29.3	ACE inhibitor, antibacterial, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(164–169)	646.3	VATLED	26.8	Dipeptidyl peptidase IV inhibitor	T105, DSM
	f(168–178)	1197.5	EDSPEVIESPP	24.9	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(171–173)	343.1	PEV	29.4	–	141
	f(171–180)	1108.5	PEVIESPPEI	24.4	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(173–181)	996.5	VIESPPEIN	29.4	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(173–182)	1097.5	VIESPPEINT	30.0	Dipeptidyl peptidase IV inhibitor	T105, 141
	f(174–185)	1324.6	IESPPEINTVQV	28.4	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105
	f(176–181)	655.3	SPPEIN	29.8	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(176–184)	983.4	SPPEINTVQ	29.0	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(176–186)	1183.6	SPPEINTVQVT	28.4	ACE inhibitor, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(182–190)	904.4	TVQVTSTAV	29.8	ACE inhibitor, antibacterial, antioxidative, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(183–189)	704.3	VQVTSTA	29.6	Antioxidative, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(183–190)	803.4	VQVTSTAV	29.8	ACE inhibitor	DSM, 141
	f(185–190)	576.3	VTSTAV	29.9	ACE inhibitor, antioxidative, dipeptidyl peptidase IV inhibitor	T105, DSM
	f(187–190)	376.1	STAV	29.1	ACE inhibitor	DSM

^a Numbers refer to amino acid positions in the casein protein sequence

^b Based on Perkins et al. (1999)

^c Based on Minkiewicz et al. (2008)

^d Referring to strain *L. helveticus* DSMZ 20075

and their functionalities (Chakrabarti et al. 2014; Dziuba and Dziuba 2014; Bhat et al. 2015).

The investigated *L. helveticus* strains exhibited a varied ability to hydrolyze casein, leading to the production of bioactive peptides. Out of the 1002 short peptides derived from casein enzymatic hydrolysis performed by the tested strains, 91 peptides were clearly identified as bioactive according to the BIOPEP database (Table 1). Short sequences (containing 2–10 amino acids) formed during 12 h of casein hydrolysis are summarized in Table 1. Two of the bioactive sequences derived from α_{S1} -casein hydrolysis, 6 from α_{S2} -casein, 1 from β -casein, and 82 from κ -casein.

The analysis indicates that β -CN and α_{S1} -CN were more resistant to hydrolysis than the other fractions, which can be related to the presence of phosphoserine in their structures (Chang et al. 2014; Ha et al. 2015).

The results indicate that κ -CN is a dominant precursor for short-chain peptides, which may be related to the casein

structure. The κ -fraction is responsible for stabilization of the molecular structure of casein micelles (Horne 2006). The localization on the exterior surface of micelles might affect the susceptibility of κ -CN to proteolytic enzymes, leading to the release of a large number of short-chain peptides. Interestingly, only one bioactive sequence was generated from the β -CN region by *L. helveticus* 141, which suggests the lowest ability of the tested strains to generate short-chain peptides.

Several different functional activities have been identified in the products obtained (Table 1). A vast majority of the peptides exerted inhibitory activity towards the angiotensin-converting enzyme (ACE) and dipeptidyl peptidase IV.

High ACE inhibitory activities have been confirmed in peptides containing Phe, Pro, Tyr, or Trp at C-terminal amino acid sequences and Ser, Val, Ile, and Ala at the N-terminus (Jao et al. 2012). The analysis of the sequence indicates that the tested *L. helveticus* strains are able to

Fig. 3 LC/MS-extracted compound chromatogram (a–d) of peptide TVQVTSTAV

release short fragments produced from α_{S1} - and α_{S2} -CN with preferences to the C-terminal regions (Table 1).

The identified sequences TTMLPW produced by strain T105 from α_{S1} -CN and VIESPPEIN produced by strains DSMZ 20075 and T105 from κ -CN were also found in hydrolyzates obtained with the use of *L. helveticus* NCC 619 and were described as peptides inhibiting the

angiotensin-converting enzyme (ACE) (Robert et al. 2001). A strong antihypertensive effect of TTMLPW was observed in spontaneously hypertensive rats orally fed with a dose of 100 mg/kg ($IC_{50} = 16 \mu M$) of this peptide (Wakai and Yamamoto 2012). The analysis of the sequence of the peptide produced by strains T105 and DSMZ 20075 (Table 1) indicates a wider range of potential activities

(inhibitory activity towards ACE and dipeptidyl peptidase IV as well as immunomodulating, opioid, and antioxidative activity).

The κ -CN-derived sequence VIESPPEIN was produced by all the analyzed *L. helveticus* strains. This peptide was previously detected in milk fermented by a *Lactococcus lactis* wild-type strain (Algaron et al. 2004). Similarly, Robert et al. (2001) obtained the VIESPPEIN peptide using *L. helveticus* NCC 619 and confirmed the ACE inhibitory activity of this sequence.

Among all the studied strains, *L. helveticus* T105 was able to produce the highest number of short peptides possessing the most diverse functional activities (Table 1).

Figure 3 presents an example of an LC/MS-extracted compound chromatogram of the TVQVTSTAV peptide. This peptide derived from κ -CN f(182–190) and was identified in samples containing casein and inoculated with strains T105 or DSMZ 20075 (Table 1). Interestingly, the same sequence was identified in whey protein concentrate hydrolyzed by the extract of *Cynara cardunculus* (Tavares et al. 2011) or in milk fermented by specific *Lactococcus lactis* wild type strains (Rodríguez-Figueroa et al. 2012).

Conclusion

L. helveticus strains exhibit various caseinolytic activities as well as various patterns of casein degradation. The present investigation has demonstrated that κ -CN glycosylation or the presence of modified phosphoserine did not affect the hydrolysis.

The variety of cleavage patterns of the casein fractions resulted in a considerable number of peptide sequences generated during the study. After 12 h of casein incubation with the proteolytic system of *L. helveticus* strains, many different bioactive peptides were obtained. The potential biological properties include the inhibitory activity towards ACE and dipeptidyl peptidase IV as well as antibacterial, antioxidant, opioid, stimulating, hypotensive, immunomodulating, and antithrombotic activity. The role of the hydrolysis products as neuropeptides is also pointed out.

Among all the tested strains, *L. helveticus* T105 most efficiently hydrolyzed κ -casein most efficiently. This result suggests that strain T105 has great potential to be used in fermented dairy products with functional properties or for production of new pharmaceutical formulations. The presented results of the research have practical relevance. *L. helveticus* strain T105 can be used as an effective tool for production of bioactive peptides obtained from casein hydrolysis. Our results prompt the use of a new method for production of new health-oriented food products. Based on the promising findings presented in this paper, further

investigations verifying the biological activity of the peptides obtained in vivo should be conducted.

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