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Identification of Endopeptidase Genes from the Genomic Sequence of *Lactobacillus helveticus* CNRZ32 and the Role of These Genes in Hydrolysis of Model Bitter Peptides

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Genes encoding three putative endopeptidases were identified from a draft-quality genome sequence of *Lactobacillus helveticus* CNRZ32 and designated *pepO3*, *pepF*, and *pepE2*. The ability of cell extracts from *Escherichia coli* DH5α derivatives expressing CNRZ32 endopeptidases PepE, PepE2, PepF, PepO, PepO2, and PepO3 to hydrolyze the model bitter peptides, β-casein (β-CN) (f193-209) and α_{S1}-casein (α_{S1}-CN) (f1-9), under cheese-ripening conditions (pH 5.1, 4% NaCl, and 10°C) was examined. CNRZ32 PepO3 was determined to be a functional paralog of PepO2 and hydrolyzed both peptides, while PepE and PepF had unique specificities towards α_{S1}-CN (f1-9) and β-CN (f193-209), respectively. CNRZ32 PepE2 and PepO did not hydrolyze either peptide under these conditions. To demonstrate the utility of these peptidases in cheese, PepE, PepO2, and PepO3 were expressed in *Lactococcus lactis*, a common cheese starter, using a high-copy vector pTRKH2 and under the control of the *pepO3* promoter. Cell extracts of *L. lactis* derivatives expressing these peptidases were used to hydrolyze β-CN (f193-209) and α_{S1}-CN (f1-9) under cheese-ripening conditions in single-peptide reactions, in a defined peptide mix, and in Cheddar cheese serum. Peptides α_{S1}-CN (f1-9), α_{S1}-CN (f1-13), and α_{S1}-CN (f1-16) were identified from Cheddar cheese serum and included in the defined peptide mix. Our results demonstrate that in all systems examined, PepO2 and PepO3 had the highest activity with β-CN (f193-209) and α_{S1}-CN (f1-9). Cheese-derived peptides were observed to affect the activity of some of the enzymes examined, underscoring the importance of incorporating such peptides in model systems. These data indicate that *L. helveticus* CNRZ32 endopeptidases PepO2 and PepO3 are likely to play a key role in this strain's ability to reduce bitterness in cheese.

The proteolytic enzyme system of lactic acid bacteria (LAB) includes diverse enzymes whose primary physiological functions involve housekeeping needs and acquisition of essential amino acids to support growth (9). Intracellular peptidases of LAB consist of both endopeptidases and aminopeptidases. Endopeptidases, due to their ability to hydrolyze peptide bonds within a peptide, are of particular interest, since they promote hydrolysis of peptides that are resistant to aminopeptidase activity. The proteolytic enzymes of LAB are also of practical interest because they play a major role in cheese maturation and flavor.

Bitterness, a common flavor defect in Cheddar and Gouda cheeses, results from the accumulation of hydrophobic bitter peptides to concentrations higher than their taste threshold. Formation of these peptides during cheese ripening is directly related to the activity and specificity of the cell envelope proteinase and chymosin (2, 14, 24). Degradation of these peptides is related to the activity of peptidases derived from the starter and nonstarter bacteria present (24). Bitter peptides typically contain relatively high levels of proline (24, 36), underscoring the importance of proline-specific endopeptidases in debittering cheese.

Proteolytic systems of lactobacilli have been studied extensively due to their ability to retain activity under the low-pH and high-salt conditions found in the cheese-ripening process (8). This is particularly true for the proteolytic system of *Lactobacillus helveticus* CNRZ32, a commercially used adjunct that can reduce bitterness in Cheddar and Gouda cheeses (8, 9, 26). While numerous enzymes of the proteolytic system of *L. helveticus* CNRZ32 have been identified (8), our understanding of the specific enzymes responsible for this strain's ability to reduce bitterness in cheese is incomplete. As part of this endeavor, previously characterized *L. helveticus* CNRZ32 aminopeptidase PepN and endopeptidase PepO2 were shown to hydrolyze model bitter peptides, β-casein (β-CN) (f193-209) and α_{S1}-casein (α_{S1}-CN) (f1-9) (5, 8). However, the hydrolysis specificities of previously characterized CNRZ32 endopeptidases PepE, PepO, and PepO2 toward these peptides under cheese-ripening conditions (pH 5.1, 4% NaCl, and 10°C) and in the presence of other cheese-derived peptides is largely unknown.

Recently, a draft-quality (4×) genome sequence for *L. helveticus* CNRZ32 was assembled and screened for genes encoding additional proteolytic enzymes (3). That effort identified coding sequences for three additional endopeptidases, designated PepE2, PepF, and PepO3, that appeared to be paralogs

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TABLE 1. List of bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	Strain with high-efficiency cloning, enables α-complementation	Bethesda Research Laboratories
ABLE C	Cloning strain, reduces copy number of ColE1-based vectors by fourfold	Strategene, La Jolla, CA
L. helveticus CNRZ32	Wild type	Steele Laboratory collection
L. lactis LM0230	Plasmid-free laboratory strain	13
Plasmids		
pTRKH2	Em^r *lacZ;* 6.9 kb	30
pJDC9	Em^r *lacZ;* 6.85 kb	4
pTRKH-N	3.8-kb SmaI-SalI fragment containing *L. helveticus* CNRZ32 *pepN* ligated into pTRKH2; Em^r	J. E. Christensen (Steele Laboratory collection)
pSUWL29	3.0-kb PstI fragment containing CNRZ32 *pepO2* ligated into pJDC9	Y.-S. Chen (Steele Laboratory collection)
pSUW650	2.6-kb BamHI-KpnI fragment containing *pepO3* ligated into pJDC9	This study
pSUW651	2.5-kb BamHI-KpnI fragment containing *pepF* ligated into pJDC9	This study
pSUW652	2.1-kb BamHI-KpnI fragment containing *pepE2* ligated into pJDC9	This study
pSUW653	2.0-kb SmaI-XbaI fragment containing *pepE* ligated into pJDC9	This study
pSUW660	0.2-kb KpnI-BamHI fragment containing CNRZ32 P_{pepO3} ligated into pBlueskript II SK (+)	This study
pSUW661	2.4-kb BamHI-XbaI ORF containing CNRZ32 *pepO2* ligated into pSUW660	This study
pSUW662	1.7-kb BamHI-XbaI ORF containing CNRZ32 *pepE* ligated into pSUW660	This study
pSUW663	2.5-kb BamHI-SacI ORF containing CNRZ32 *pepF* ligated into pSUW660	This study
pSUW664	2.6-kb PCR fragment containing CNRZ32 *pepO3* from pSUW650 ligated into pTRKH2	This study
pSUW665	2.6-kb PvuII-XbaI fragment containing CNRZ32 *pepO2* from pSUW661 ligated into pTRKH2	This study
pSUW666	1.9-kb PvuII-XbaI fragment containing CNRZ32 *pepE* from pSUW662 ligated into pTRKH2	This study

or orthologs of known endopeptidases. To utilize these enzymes to reduce bitterness in cheese, it would be advantageous to overexpress them in *Lactococcus*, a common cheese starter.

In this study, we independently cloned the genes encoding these three endopeptidases into *Escherichia coli* DH5α and examined the hydrolysis specificities of these peptidases and previously characterized CNRZ32 endopeptidases PepE (16), PepO (6), and PepO2 (5) against β-CN (f193-209) and α_{S1}-CN (f1-9) under cheese-ripening conditions. Additionally, we cloned *pepO2*, *pepO3*, *pepE*, and *pepF* under the control of the *pepO3* promoter and examined the expression of these peptidases in *Lactococcus lactis* LM0230 using the high-copy vector pTRKH2. We examined the rate of hydrolysis and specific activity of the individual peptidases toward α_{S1}-CN (f1-9) and β-CN (f193-209) in single-peptide reactions, in a defined mixture of cheese-derived peptides, and in Cheddar cheese serum (CCS).

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are presented in Table 1. *E. coli* DH5α (Gibco-BRL Life Technologies, Inc., Gaithersburg, MD) and derivatives were grown in Luria-Bertani (33) medium at 37°C with aeration. *L. lactis* was grown at 30°C without aeration in M17 (Difco, Detroit, MI) supplemented with 0.5% (wt/vol) glucose (G-M17) or lactose (L-M17). *L. helveticus* CNRZ32 (23) was grown in MRS broth (Difco) (12) at 37°C without aeration. Agar plates were prepared by adding 1.5% (wt/vol) granulated agar (Difco) to liquid media. To select for *E. coli* strains carrying pBluescript II SK (+) (Stratagene, La Jolla, CA) and its derivatives, ampicillin (Sigma, St. Louis, MO) was added to media to a final concentration of 100 μg ml^{-1}. Erythromycin (Sigma) was added to liquid media or agar plates to select for pJDC9, pTRKH2, and their derivatives in *E. coli* and *L. lactis* at 500 μg ml^{-1} and 5 μg ml^{-1}, respectively. Bacteria were maintained as frozen stocks at −80°C in liquid media containing 12% glycerol.

Molecular biology techniques. DNA cloning and plasmid isolation techniques were performed according to the method of Sambrook et al. (33). Restriction and modifying enzymes were used as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Transformation of *E. coli* was performed with a Gene-Pulser following the manufacturer's recommended instructions (Bio-Rad Laboratories, Richmond, CA). For transformation of *L. lactis*, the procedure of Holo and Nes (21) was utilized. *L. helveticus* CNRZ32 chromosomal DNA was isolated as described by Marmur (27). Lactococcal plasmid DNA was isolated from a 50-ml culture using a modified alkaline lysis method (33) with an addition of lysozyme (30 mg ml^{-1}) to the resuspension buffer. DNA used for cloning was further purified by passing it through minicolumns from a Qiaquick PCR purification kit (QIAGEN, Valencia, CA) and was resuspended in a final volume of 30 to 50 μl. For isolation of DNA from gels, the Qiaquick gel extraction kit (QIAGEN) was used.

DNA amplification via PCR. The DNA primers listed in Table 2 were synthesized by Invitrogen. Amplification reactions were typically performed using *Taq* DNA polymerase; for high fidelity reactions, Platinum *Pfx* DNA polymerase (Invitrogen) was utilized. The PCR cycling conditions for amplification of DNA both from *E. coli* and *L. lactis* normally included 95°C for 5 min, followed by 25 to 30 cycles of 94°C for 30 s, 50 to 60°C for 30 s, and 72°C for 1 min per kb of the fragment amplified, followed by a single cycle of 72°C for 7 min. "Direct colony" PCR was used to screen transformants; the fragment of interest was amplified directly from the colonies without initial DNA template isolation. A sterile plastic pipette tip was used to pick colonies from plates, and cells were mixed with 20 μl of standard PCR mix containing *Taq* DNA polymerase (Invitrogen). To lyse the cells prior to standard cycling conditions, samples were heated to 98°C for 10 min.

DNA sequencing and sequence analysis. DNA sequencing was conducted with sequence-specific primers synthesized by Invitrogen (Table 2). Sequencing reactions were performed using the ABI Big Dye reaction mix (Applied Biosystems, Foster City, CA) and a Perkin-Elmer model 480 thermal cycler (Perkin-Elmer Corp., Norwalk, CT). Sequence analysis was done on an ABI 377XL DNA sequencer by the Nucleic Acid and Protein facility of the University of Wisconsin Biotechnology Center (Madison, WI). The sequences were assembled and analyzed using Lasergene (DNASTAR Inc., Madison, WI) sequence analysis software.

TABLE 2. List of sequence-specific primers

Primer name	Description	Sequence (5′–3′)[a]
PepO3-For-*Bam*HI	Forward, amplification of *pepO3*	CGGGATCCTTTTGACTTTGGGTGAAT
PepO3-Rev-*Kpn*I	Reverse, amplification of *pepO3*	GGGGTACCACGAGAAGTGGTTAGTTGA
PepF-For-*Bam*HI	Forward, amplification of *pepF*	CGGGATCCCTTAAGGGAGTTCGGAG
PepF-Rev-*Kpn*I	Reverse, amplification of *pepF*	GGGGTACCTTGGAGGAATTCATCTTTAG
PepE2-For-*Bam*HI	Forward, amplification of *pepE2*	CGGGATCCTATAACAAGAACGCTAAGAA
PepE2-Rev-*Kpn*I	Reverse, amplification of *pepE2*	GGGGTACCCAGATAATGGCAAATGATA
PepE-For-*Sma*I	Forward, amplification of *pepE*	TCCCCCGGGATTAGATTAAGCAAG
PepE-Rev-*Xba*I	Reverse, amplification of *pepE*	GCTCTAGAGAAATTCGCCCTGGTC
*Kpn*I-P_{pepO3}-For	Forward, amplification of P_{pepO3}	GGGGTACCGACTTTGGGTGAATC
*Bam*HI-P_{pepO3}-Rev	Reverse, amplification of P_{pepO3}	CGGGATCCCATTTTATTATTCAAAGAGAA
*Bam*HI-PepF-ORF-For	Forward, amplification of *pepF* ORF	CGGGATCCCCAACAAGAAGCGAAGTC
*Sac*I-PepF-ORF-Rev	Reverse, amplification of *pepF* ORF	GCTGGAGCTCGTCAGCTTTTTGTATGG
*Bam*HI-PepO2-ORF-For	Forward, amplification of *pepO2* ORF	CGCGGATCCAATTTAGCAAAAATC
*Xba*I-PepO2-ORF-Rev	Reverse, amplification of *pepO2* ORF	GCTCTAGATCAATTATATAACTGATAC
*Bam*HI-PepE-ORF-For	Forward, amplification of *pepE* ORF	CGGGATCCGAATTAACTGTGCAGG
*Xba*I-PepE-ORF-Rev	Reverse, amplification of *pepE* ORF	GCTCTAGAGAAATTCGCCCTGGTC

[a] The restriction site included in each primer is underlined.

Cloning of *L. helveticus* CNRZ32 endopeptidases. DNA and protein analyses of the draft sequence using online BLAST search engines (http://www.ncbi.nlm.nih.gov/BLAST/) detected several new putative endopeptidases (Table 3). Sequence-specific primers with added BamHI and KpnI linkers were designed for the CNRZ32 *pepE2*, *pepF*, and *pepO3* genes (Table 2) to amplify fragments including the ribosome binding site, promoter regions, coding sequence, and inverted repeats located within 300 to 400 bp downstream of the coding sequence. The respective genes were amplified from the total genomic DNA of *L. helveticus* CNRZ32 using Platinum *Pfx* DNA polymerase (Invitrogen). The resulting amplicons were purified, digested with BamHI and KpnI, ligated to similarly double-digested pJDC9 (4), and then transformed into *E. coli* DH5α. *L. helveticus pepE* (16) was amplified with its native promoter from the total DNA template of *L. helveticus* CNRZ32 using Platinum *Pfx* DNA polymerase and gene-specific primers (Table 2) with SmaI and XbaI linkers. The resulting amplicon of ~2.0 kb was digested with SmaI and XbaI, ligated to similarly digested pJDC9, and transformed into DH5α. Putative transformants for all constructs were screened using gene-specific primers by direct colony PCR. Restriction digest analysis and sequencing of the gene were performed to confirm the presence of cloned endopeptidase genes in transformants, and representative isolates containing cloned *pepO3*, *pepF*, *pepE2*, and *pepE* and were designated pSUW650, pSUW651, pSUW652, and pSUW653, respectively (Table 1). All cloned fragments were sequenced and found to be identical to the *L. helveticus* CNRZ32 genome sequence of the respective genes. The nucleotide sequences of *pepO3*, *pepF*, and *pepE2* have been deposited in GenBank (see "Nucleotide sequence accession number" below).

Construction of P_{pepO3} transcriptional fusion plasmids. The promoter region of *pepO3* (GenBank accession number AF019410) was amplified from pSUW650 using Platinum *Pfx* DNA polymerase (Invitrogen) with primers KpnI-P_{pepO3}-For and BamHI-P_{pepO3}-Rev (Table 2). The amplified *pepO3* promoter fragment was digested with BamHI and KpnI and then ligated to similarly digested pBluescript II SK(+) (Stratagene) to generate transcriptional fusion plasmid pSUW660. The ligation mixture was electroporated into *E. coli* DH5α, and blue-white screening by α-complementation using isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Invitrogen) was employed to identify putative transformants. Direct colony PCR using the above primers, restriction digest analysis, and nucleotide sequencing confirmed the presence of the promoter fragment of *pepO3* in pSUW660.

Cloning of *L. helveticus* CNRZ32 endopeptidase genes as P_{pepO3} transcriptional fusions. The open reading frame (ORF) of *L. helveticus* CNRZ32 *pepO2* starting with the second codon and with transcription terminators was amplified from pSUWL29 (Table 1) via PCR using Platinum *Pfx* polymerase and primers BamHI-PepO2-ORF-For and XbaI-PepO2-ORF-Rev (Table 2). A similar approach was used to prepare comparable fragments of the *L. helveticus* CNRZ32 *pepE* and *pepF* genes using PCR primer pairs BamHI-PepE-ORF-For–XbaI-PepE-ORF-Rev and BamHI-PepF-ORF-For–SacI-PepF-ORF-Rev, respectively (Table 2). The *pepO2* and *pepE* fragments were digested with BamHI and XbaI and ligated to similarly digested pSUW660 to generate pSUW661 and pSUW662, respectively (Table 1). The *pepF* fragment was digested with BamHI and SacI and ligated to similarly digested pSUW660 to generate pSUW663. Ligation mixtures were transformed into *E. coli* ABLE C, and then representative transformants with each plasmid were identified via direct colony PCR using the respective gene-specific primers. Endopeptidase activity was confirmed with chromogenic substrates as described by Chen et al. (5).

Cloning of *L. helveticus* CNRZ32 endopeptidases into *L. lactis* using pTRKH2. The *L. helveticus* CNRZ32 *pepO3* gene with its promoter region was cleaved from pSUW650 by digestion with BamHI and SacI and ligated to similarly digested pTRKH2 to generate pSUW664 (Table 1). *L. helveticus* CNRZ32 P_{pepO3}:*pepO2* and P_{pepO3}:*pepE* were cleaved from pSUW661 and pSUW662, respectively, using PvuII and XbaI sites and ligated to SmaI- and XbaI-digested pTRKH2 to generate pSUW665 and pSUW666, respectively. *L. helveticus* CNRZ32 P_{pepO3}:*pepF* was removed from pSUW663 via digestion with PvuII and SacI and ligated to SmaI- and SacI-digested pTRKH2 to generate pSUW667. Ligation mixtures were electroporated into *L. lactis* LM0230, and transformants were isolated from L-M17 plates containing 5 μg ml^{-1} erythromycin after 48 h of anaerobic incubation at 30°C. The presence of the constructs in the transformants was confirmed using direct colony PCR with primers specific to the promoter of *pepO3* and the pTRKH2-specific primer. However, several attempts to ligate P_{pepO3}:*pepF* to pTRKH2 followed by direct transformation into *L. lactis* LM0230 were unsuccessful. Endopeptidase activity was confirmed with chromogenic substrates as described by Chen et al. (5).

TABLE 3. *L. helveticus* CNRZ32 genes encoding known or putative endopeptidases

Gene	GenBank accession no.	Known (reference) or predicted product
pepE	AAB52540	Thiol-dependent endopeptidase (16)
pepE2	AY365130	PepE paralog, 52% identical to CNRZ32 endopeptidase PepE (16)
pepF	AY365129	PepF ortholog, 46% identical to *L. lactis* endopeptidase PepF
pepO	AF019410	Endopeptidase O ortholog (6)
pepO2	AF321529	Post-prolyl endopeptidase (5)
pepO3	AY355128	PepO/PepO2 paralog, 62% identical to CNRZ32 endopeptidases PepO and PepO2
gcp		Gcp ortholog, 63% identical to predicted O-sialoglycoprotein endopeptidase Gcp from *L. plantarum*
ydiC		Glycoprotein endopeptidase ortholog, 37% identical to predicted glycoprotein endopeptidase from *L. plantarum*

Preparation of CCS. CCS was prepared as described by Morris et al. (29) using custom-made molds designed by Hassan (20). Briefly, 850 g of a 3-week-old Cheddar cheese was grated and mixed with an equal quantity of sterile sea sand. The sea sand-cheese mixture was placed in the molds and squeezed using a Carver manual hydraulic press (model 3912; Fred S. Carver, Inc., Summit, NJ). Pressure was increased up to 10,000 lb/in^2 over 6 h and then held at that pressure for ~3 h. The CCS and cheese liquid fat were collected and kept at 4°C for 2 h. This storage temperature allowed the expressed fat to solidify as the upper layer, which was removed using a spatula. Residual fat was removed by centrifugation at 1,380 × *g* for 10 min using an induction drive centrifuge (model J2-21 M; Beckman Coulter, Fullerton, CA). Additionally, CCS was passed through a 1.6-μm glass microfiber filter (Whatman International Ltd., England), filter sterilized by sequential passages through 0.45-μm and 0.22-μm cellulose nitrate filters (Nalgene Filtration Products, Rochester, NY), and stored at −80°C until used. For peptide hydrolysis, CCS was prepared as described above, boiled for 5 min at 100°C, extracted with equal volumes of 100% ethyl ether, vacuum dried using a Savant Speed Vac (SC 210A; Global Medical Instrumentation, Inc., Albertville, MN), and then resuspended in a volume of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.0) to yield an effective 2× concentrated CCS.

Synthesis of peptide substrates. The peptides β-CN (f193-209), α_{S1}-CN (f1-6), α_{S1}-CN (f1-9), α_{S1}-CN (f1-13), and α_{S1}-CN (f1-16) were synthesized and then purified by preparatory reverse-phase high-performance liquid chromatography (RP-HPLC) by the University of Wisconsin Biotechnology Center. Peptide purity and identity was established by mass spectrometry (MS) using a Bruker Reflex II for matrix-assisted laser desorption ionization–time of flight. The peptides were lyophilized and stored at −80°C. Stock solutions were prepared in sterile, double-distilled water and stored at −80°C.

Peptide hydrolysis reactions. Cultures were grown to late log phase (A_{600}, ~2.0), and cell extracts (CFEs) were prepared in 50 mM MES buffer (pH 5.1). Cells were broken by vortexing with 300 mg of glass beads for 3 min using a Turbomix attachment to a Vortex Genie 2 (Scientific Industries, NY). CFEs from *E. coli* expressing CNRZ32 *pepE*, *pepE2*, *pepF*, and *pepO3* transcriptional fusion genes were assayed for endopeptidase activity against chromogenic substrates as described by Chen et al. (5). CFE protein concentrations were determined using the protein assay kit I from Bio-Rad with bovine serum albumin as the protein standard.

Peptide hydrolysis reactions with CFEs from *E. coli* or *L. lactis* were performed in a 50-μl total volume. Each sample contained 10 μl of CFE (2.0 to 2.5 mg of protein ml^{-1}), 10 μl of peptide substrate solution, and 30 μl of buffer. The buffer used for single-peptide reactions and defined peptide mix reactions was 120 mM MES (pH 5.1)–0.68 M NaCl (4% NaCl). CCS concentrate with a final pH of 5.2 and 4% NaCl was used for the cheese model system. The reactions were initiated by substrate addition, and then samples were incubated at 10°C for 2, 4, 6, 12, 24, and 48 h. Initial substrate concentrations in individual peptide reactions were 1 mg ml^{-1} for β-CN (f193-209) and 10 mg ml^{-1} for α_{S1}-CN (f1-9). In the defined peptide mix reactions, peptides α_{S1}-CN (f1-9), α_{S1}-CN (f1-13), α_{S1}-CN (f1-16), α_{S1}-CN (f1-6), and β-CN (f193-209) were present at 10, 5, 2.5, 1, and 1 mg ml^{-1} concentrations, respectively. α_{S1}-CN (f1-9) and β-CN (f193-209) were added to the peptide mix and CCS at levels known to accumulate in bitter cheeses (2), while the other peptides were added at levels that would mimic our spiked CCS. Reactions were stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 5%, and samples were frozen at −20°C until analyzed by HPLC.

Peptide separation and identification of hydrolysis products. Peptides were separated by RP-HPLC on an HP1100 series system (Agilent Technologies, Palo Alto, CA) using 0.1% TFA in HPLC-grade water (solvent A) and 0.085% TFA, 80% acetonitrile, 20% HPLC-grade water (solvent B). The samples were analyzed on an Ultima C_{18} column (250 by 2.1 mm, 5-μm particle size, 100-Å pore size; Alltech Associates, Inc., Deerfield, IL). The initial condition was 10% solvent B, and a linear gradient of solvent B from 10% to 60% was generated over the course of 35 min for the separation of β-CN (f193-209), the defined peptide mix, and CCS samples. A linear gradient from 10 to 20% was generated over the course of 15 min for the separation of α_{S1}-CN (f1-9) at a flow rate of 0.25 ml/min at 25°C. The eluted peaks were detected by absorbance at 214 nm using a photodiode array detector spectrometer (HP1100 series). Before injection, samples inactivated with TFA were thawed at room temperature and centrifuged (14,000 × *g* for 5 min at 25°C); 20 μl of the supernatant was injected directly into the column using an HP1100 series autosampler equipped with a dilutor module that contained a water-acetonitrile (1:1) wash solution (Agilent Technologies). A standard curve for pure peptide was generated for every run of HPLC with an R^2 of 0.99 when determining concentrations of the substrates by peak area. The substrate was hydrolyzed to ~10 to 20%, and reaction rates were verified to be in the linear range (R^2 = 0.99) when calculating specific activity. In the case of α_{S1}-CN (f1-9), the reaction rates were calculated from a nonlinear range to include at least three different time points (R^2 = 0.93 to 0.95). Average specific activities are reported for this peptide. Specific activity was calculated as nmoles of peptide hydrolyzed per h per mg of protein, and reported values were corrected by subtracting the mean values obtained in the control treatments.

Mass values for RP-HPLC-separated peptides were determined using a triple-quadrupole mass spectrometer (Quattro II; Micromass Ltd., Manchester, United Kingdom) with electrospray ionization sources at the University of Wisconsin Biotechnology Center. To identify hydrolysis products, individual mass values were compared to the calculated molecular masses of peptides and amino acids derived from β-CN (f193-209) and α_{S1}-CN (f1-9). Sample preparation and identification of CCS peptides using tandem mass spectrometry were also performed at the University of Wisconsin Biotechnology Center. Data-dependent MS/MS switching on quadrupole-time of flight was done using MassLynx software. Raw data were analyzed using MASCOT (Matrix Science Ltd., London, United Kingdom) and Spectrum Mill (Agilent Technologies) software, allowing for oxidized methionine, phosphotyrosine, and phosphoserine modifications. The identities of individual peptides were determined by comparison against the SWISS PROT database.

Statistical analysis. The rate of hydrolysis of the peptides and the specific activity were calculated in nmoles of peptide hydrolyzed per h per mg of protein. Results reported are mean values ± standard deviations of the results from three independent trials. Student's *t* test with a *P* value of ≤0.05 was used to report significant differences between values obtained from control samples and individual peptidases. A single-factor analysis of variance was performed, and the least significant differences were calculated (34) for separation of means of individual peptidases in single-peptide reactions, in the defined peptide mix, and in CCS.

Nucleotide sequence accession number. The nucleotide sequences of *pepO3*, *pepF*, and *pepE2* have been deposited in the GenBank database under accession numbers AY355128, AY365129, and AY365130, respectively.

RESULTS

Sequence analysis. The ORF of *pepO3* was 1,929 bp, encoding a polypeptide of 643 amino acid residues with a deduced mass of 71.4 kDa. PepO3 was 62% identical to PepO2 and PepO from *L. helveticus* CNRZ32 (GenBank accession number AF321529 and AF019410, respectively) and 78% identical to a predicted metalloendopeptidase from *Lactobacillus gasseri* (protein accession number ZP_00045894.1). The *pepF* ORF was 1,794 bp, encoding a polypeptide of 598 amino acid residues with a deduced mass of 66.4 kDa. PepF had 52%, and 46% identity to previously characterized PepF proteins from *Lactobacillus plantarum* (protein accession number NP_785715.1) and *L. lactis* (A55485) (28), respectively. It had 75% identity to oligopeptidase F from *L. gasseri* (protein accession number ZP_00046654.1). The *pepE2* ORF was 1,311 bp, encoding a polypeptide of 437 amino acid residues with a deduced mass of 48.5 kDa. PepE2 had 52% identity to a previously characterized PepE protein from *L. helveticus* CNRZ32 and 80% identity to aminopeptidase C from *L. gasseri* (protein accession number ZP_00047232.1). The ORFs of *pepO3* and *pepF* encode the sequences HEISH and HETGH, respectively, which are characteristic of the HEXXH motif present in zinc metallopeptidases (1). The amino acid residues (Q, H, N, and W at positions 64, 361, 382, and 384, respectively) important for substrate binding and catalysis by cysteine proteinases of prokaryotic and eukaryotic origin are conserved in PepE2 (16).

Peptide hydrolysis and specificity by *E. coli* CFE. CFE of *E. coli* derivatives expressing PepO2 or PepO3 had significantly greater activity ($P \leq 0.05$) with both β-CN (f193-209) and α_{S1}-CN (f1-9) than the control, CFE from *E. coli* DH5α (pJDC9); the highest activity was observed with PepO2 (Table 4). PepF activity was detected with β-CN (f193-209) but not

TABLE 4. Specific activities of CFEs of *E. coli* DH5α expressing *L. helveticus* CNRZ32 endopeptidases toward α_{S1}-CN (f1–9) and β-CN (f193–209)[1]

Peptidase[b]	Mean (SD) sp act toward[a]:	
	α_{S1}-CN (f1–9)	β-CN (f193–209)
Control	46 (19)‡	0.3 (0.2)§
PepE	ND	ND
PepE2	ND	ND
PepF	ND	14 (3.0)*
PepO	ND	ND
PepO2	3,700 (125)*	290 (19)†
PepO3	85 (21)†	88 (5.0)‡

[a] Specific activity is measured in nmoles of substrate hydrolyzed per h per mg of protein. Values were corrected by subtracting the mean values obtained in the control treatments from CFE of *E. coli* DH5α (pJDC9). Means with different symbols are statistically different within a column at an α value of ≤0.05. Assays were performed under cheese-ripening conditions (pH 5.0 to 5.2, 4% NaCl, 10°C). ND, not detected.

[b] CFEs were prepared from *E. coli* DH5α(pJDC9) (control), *E. coli* DH5α (pSUW653) (PepE), *E. coli* DH5α(pSUW652) (PepE2), *E. coli* DH5α (pSUW651) (PepF), *E. coli* DH5α(pSUW51) (PepO), *E. coli* DH5α(pSUW29) (PepO2), or *E. coli* DH5α(pSUW650) (PepO3).

with α_{S1}-CN (f1-9). PepE activity with α_{S1}-CN (f1-9) was not higher than the control and, therefore, is reported as not detectable (Table 4). There was no PepE activity detected with β-CN (f193-209). PepO and PepE2 activities were not detected with either peptide.

Hydrolysis specificities of PepO2, PepO3, PepE, and PepF with β-CN (f193-209) and α_{S1}-CN (f1-9) were determined. Like PepO2 (5), PepO3 was a postproline endopeptidase that hydrolyzed the Pro_{206}-Ile_{207}, Pro_{196}-Val_{197}, and Pro_{200}-Val_{201} bonds of β-CN(f193-209) and the Pro_5-Ile_6 bond of α_{S1}-CN (f1-9) (Fig. 1). PepF also demonstrated postproline specificity at the Pro_{204}-Phe_{205} and Pro_{206}-Ile_{207} bonds but additionally hydrolyzed the X-Gly Lys_{198}-Gly_{199} and Arg_{202}-Gly_{203} bonds of β-CN(f193-209). PepE hydrolyzed α_{S1}-CN (f1-9) at the Lys_3-His_4 and Lys_7-His_8 bonds.

Identification of peptides in CCS. The peptide/protein summary of CCS generated by Spectrum Mill (Agilent Technologies) and MASCOT (Matrix Science Ltd.) identified peptides and phosphopeptides from α_{S1}-CN and β-CN. The majority of the peptides identified from β-CN were from premature (uncleaved) β-CN, β-CN (f60-81) and β-CN (f107-118). β-CN (f193-209) was not identified. The peptides identified from α_{S1}-CN were α_{S1}-CN (f1-9), α_{S1}-CN (f1-13), α_{S1}-CN (f1-16), α_{S1}-CN (f1-17), α_{S1}-CN (f24-41), and α_{S1}-CN (f24-39).

Peptide hydrolysis by *L. lactis* CFE. The time course of hydrolysis by CFE of LM0230 expressing PepO2, PepO3, and PepE of α_{S1}-CN (f1-9) at a concentration of 10 mg ml^{-1} exhibited a slow initial rate (lag phase), which increased after 12 h of incubation with a single peptide and a defined peptide mix and after 24 h of incubation in CCS (Fig. 2). A lag phase (40) was not observed when lower concentrations (1 mg ml^{-1}) of α_{S1}-CN (f1-9) were used (data not shown), suggesting a possibility for substrate inhibition at higher concentrations (10 mg ml^{-1}). LM0230 derivatives expressing PepO2 and PepO3 under the control of the *pepO3* promoter hydrolyzed both β-CN (f193-209) and α_{S1}-CN (f1-9) at a significantly greater rate ($P \leq 0.05$) than the control, CFE from LM0230(pTRKH2), in all three systems (Fig. 2; Table 5). The peptide β-CN (f193-209) was almost completely hydrolyzed within 12 h when spiked CCS was incubated with CFE of strains expressing PepO2 or PepO3. The activity of PepO2 with β-CN (f193-209) was significantly higher ($\alpha \leq 0.05$) by twofold in spiked CCS than in single-peptide reactions or in the defined peptide mix (Table 5). The activity of PepO2 with α_{S1}-CN (f1-9) was lower in CCS and the defined peptide reactions compared to the activity observed on α_{S1}-CN (f1-9) in single-peptide reactions (Table 5).

The CFEs from LM0230 derivatives expressing PepE hydrolyzed α_{S1}-CN (f1-9) at a significantly greater rate ($P \leq 0.05$) than the control CFE in single-peptide and defined peptide mix reactions (Fig. 2; Table 5). In CCS, PepE activity was significantly inhibited ($\alpha \leq 0.05$) (Table 5). There was higher background activity detected in the control CFE, *L. lactis* LM0230 (pTRKH2), for peptides α_{S1}-CN (f1-9) and β-CN (f193-209) in CCS than in single-peptide reactions or in defined peptide mix reactions (data not shown); however, the peptide profiles of the control reactions were similar to those

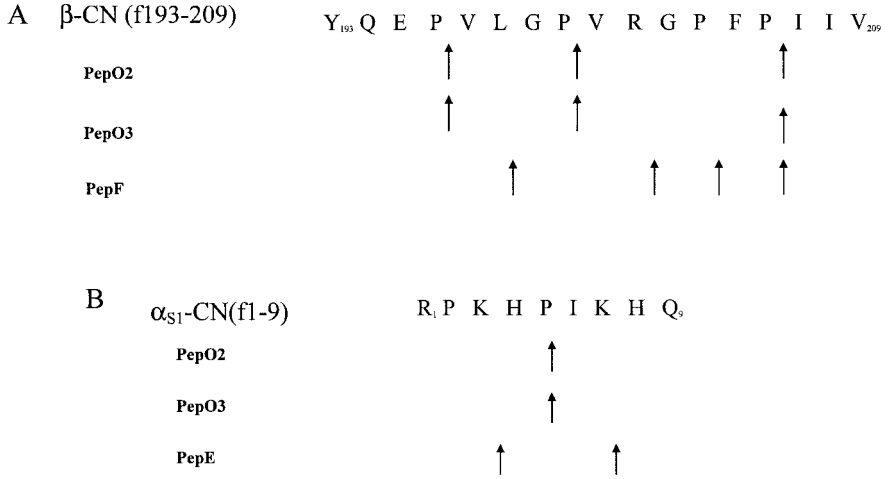

FIG. 1. Specificity of *Lactobacillus helveticus* CNRZ32 endopeptidases toward β-CN (f193-209) (A) and α_{S1}-CN (f1-9) (B) under simulated cheese-ripening conditions (pH 5.0 to 5.2, 4% NaCl, 10°C).

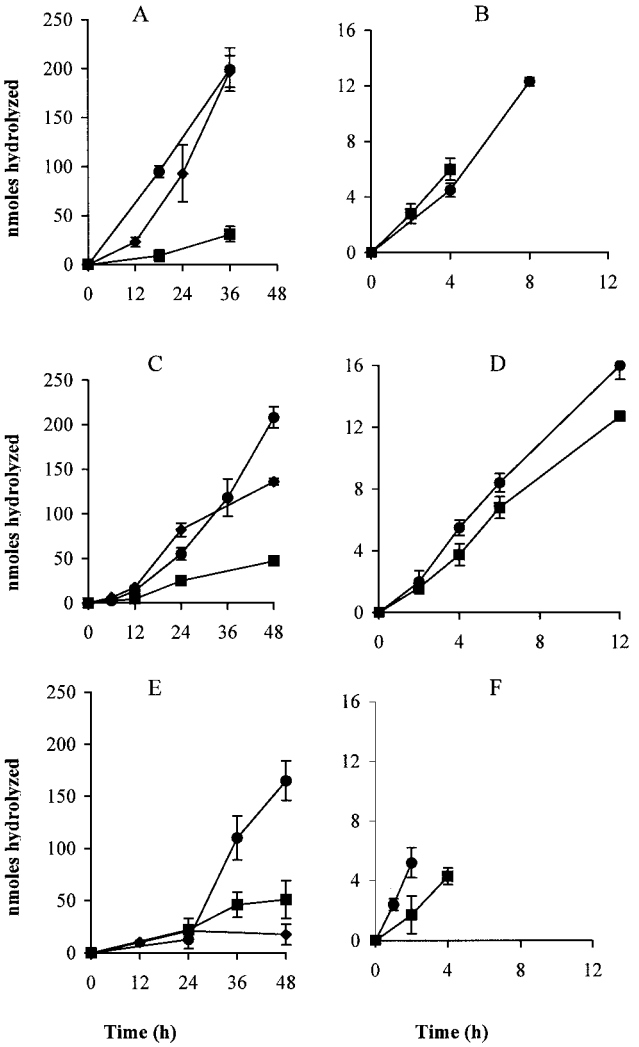

FIG. 2. Rate of β-CN (f193-209) (right panels) and α_{S1}-CN (f1-9) (left panels) hydrolysis at pH 5.2, 4% NaCl at 10°C in a single-peptide system (A, B), in a defined peptide mix system (C, D), and in Cheddar cheese serum (E, F) by cell extracts from *Lactococcus lactis* LM0230 derivatives expressing *Lactobacillus helveticus* CNRZ32 endopeptidase PepO2 (circles), PepO3 (squares), or PepE (diamonds). Values were corrected by subtracting the values obtained from the control cell extract, prepared from *L. lactis* LM0230(pTRKH2). Error bars represent the standard errors of the means ($n = 3$). Initial substrate concentrations in individual peptide reactions were 1 mg ml^{-1} for β-CN (f193-209) and 10 mg ml^{-1} for α_{S1}-CN (f1-9).

observed at time zero. The relative activity toward the other peptides in the defined peptide mix was also calculated, with activity toward α_{S1}-CN (f1-9) taken as 100% (Fig. 3). Each peptidase hydrolyzed the different peptides at different rates. Among α_{S1}-CN-derived peptides, for example, PepO2 had the highest activity toward peptides α_{S1}-CN (f1-9) and α_{S1}-CN (f1-13), PepO3 had the highest activity toward α_{S1}-CN (f1-13), and PepE had the highest activity toward α_{S1}-CN (f1-9) and α_{S1}-CN (f1-6) (Fig. 3).

DISCUSSION

Bitterness is a major flavor defect in Cheddar and Gouda cheeses (37). This defect is primarily caused by the accumulation of hydrophobic peptides, such as β-CN (f193-209) and α_{S1}-CN (f1-9), to concentrations greater than their taste thresholds (2, 14, 24). The reduction of bitterness in cheese is believed to be the result of preferential hydrolysis of bitter peptides to nonbitter hydrolysis products by specific peptidases from starter or nonstarter bacteria (2, 19, 24).

In this investigation, we conducted functional studies on three putative endopeptidase genes, *pepE2*, *pepF*, and *pepO3*, which were identified from a draft-quality genome sequence of *L. helveticus* CNRZ32. Although two additional glycoprotein endopeptidases were identified (Table 3), genes encoding the latter enzymes were not included in this study, since glycoproteins are not thought to influence bitterness. We also included three previously characterized endopeptidases, PepE, PepO, and PepO2, in this investigation, since their hydrolysis specificities had not been determined under cheese-ripening conditions (pH of 5.0 to 5.2, 4% NaCl, 10°C). Additionally, we attempted to express these CNRZ32 peptidases in *L. lactis* under the control of the *L. helveticus* CNRZ32 *pepO3* promoter on a high-copy vector. Peptide hydrolysis studies in single-peptide reactions, in a defined peptide mix, and in CCS were then conducted to evaluate the debittering potential of the genetically modified strains.

We successfully cloned and expressed the newly identified endopeptidases PepF and PepO3 from *L. helveticus* CNRZ32 in *E. coli* and determined their hydrolysis specificities toward two bitter peptides. In contrast, no measurable PepE2 activity was detected against chromogenic substrates or bitter peptides; this result may be due to either enzyme specificity or insufficient expression. *L. helveticus* CNRZ32 PepO3 appeared to be a functional paralog to the postprolyl endopeptidase PepO2 (5) and hydrolyzes β-CN (f193-209) and α_{S1}-CN (f1-9) in a manner that was indistinguishable from that observed with the

TABLE 5. Specific activities of CFEs of *L. lactis* LM0230 expressing *L. helveticus* CNRZ32 endopeptidases towards β-CN (f193–209) and α_{S1}-CN (f1–9) in a single-peptide reaction, in the defined peptide mix, and in Cheddar cheese serum

Peptide and peptidase[b]	Mean (SD) spact in[a]:		
	Single-peptide reaction	Defined peptide mix	Cheddar cheese serum
β-CN (f193–209)			
Control	16 (2.0)†,*	9.0 (2.0)†,†	27 (3.0)‡,*
PepE	ND	ND	ND
PepO2	64 (1.5)*,†	42 (10)*,*	120 (32)*,*
PepO3	81 (7.0)*,*	40 (6.0)*,*	61 (23)†,*
α_{S1}-CN (f1–9)[c]			
Control	4.0 (4.0)‡,*	4.0 (0.2)§,*	23 (3.0)‡,†
PepE	190 (14)*,*	120 (7)*,†	31 (17)‡,‡
PepO2	240 (10)*,*	84 (20)†,‡	150 (40)*,†
PepO3	41 (19)†,*	38 (9.0)‡,*	63 (25)†,*

[a] Specific activity is measured in nmoles of substrate hydrolyzed per h per mg of protein. Values were corrected by subtracting the mean values obtained in the control treatments from CFE of *L. lactis* LM0230(pTRKH2). Means with different symbols are statistically different at an α value of ≤0.05. Lightface symbols compare values within a column, and boldface symbols compare values within a row. Assays were performed under cheese-ripening conditions (pH 5.0 to 5.2, 4% NaCl, 10°C). ND, not detected.

[b] CFEs were prepared from *L. lactis* LM0230(pTRKH2) (control), *L. lactis* LM0230(pSUW666) (PepE), *L. lactis* LM0230(pSUW665) (PepO2), or *L. lactis* LM0230(pSUW664) (PepO3).

[c] In the case of α_{S1}-CN (f1–9) hydrolysis, average specific activities are reported.

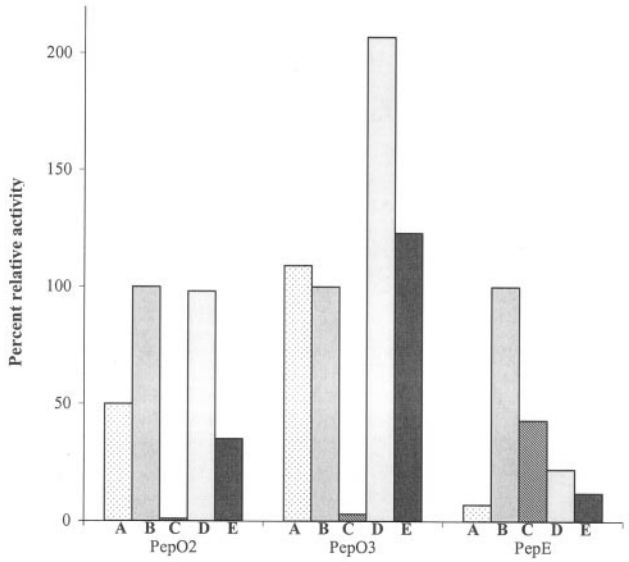

FIG. 3. Percent relative activity of *Lactococcus lactis* LM0230 derivatives expressing *Lactobacillus helveticus* CNRZ32 PepO2, PepO3, or PepE toward peptides in the defined peptide mix at pH 5.2, 4% NaCl, and 10°C. (A) β-CN (f193-209); (B) α_{S1}-CN (f1-9); (C) α_{S1}-CN (f1-6); (D) α_{S1}-CN (f1-13); (E) α_{S1}-CN (f1-16). Values were corrected by subtracting the values obtained in the control treatments. Activity with α_{S1}-CN (f1-9) was arbitrarily set to 100%.

latter enzyme. PepF was also found to be a postproline endopeptidase, but this enzyme did not hydrolyze α_{S1}-CN (f1-9). In contrast, PepE hydrolyzed α_{S1}-CN (f1-9), but not β-CN (f193-209), and does not have postproline endopeptidase activity. Proline constitutes 16.7% of β-CN and 8.7% of α_{S1}-CN and is present in the majority of the known bitter peptides (24, 38). The postproline hydrolysis of PepF, PepO2, and PepO3 toward β-CN (f193-209) and α_{S1}-CN (f1-9) under cheese-ripening conditions suggests that these enzymes may contribute to the debittering activity of *L. helveticus* CNRZ32.

Genes from lactobacilli have been previously expressed in *L. lactis* (10, 39) using the inducible *nisA* promoter, the *L. helveticus* *pepX* promoter, or their own promoters (22, 25). A previous study of *L. helveticus* CNRZ32 promoter strength, which used β-glucuronidase as the reporter gene, found that *pepO* and *pepO2* promoters were weakly expressed in *L. lactis* (7). In this study, we cloned *pepO3* on pTRKH2 and observed relatively high PepO3 activity in *L. lactis.* Therefore, the ORFs of *pepO2* and *pepE* were subsequently cloned under the control of the *pepO3* promoter, and significant PepO2 and PepE activities were observed in the respective constructs, higher than when expressed using their native promoters. These results suggest that the *pepO3* promoter may have utility for the expression of heterologous genes in lactococci.

Peptidase specificities for bitter peptides may be different in cheeses than in in vitro model systems. However, assessing the performance of the different strains expressing these peptidases during cheese ripening would be time-consuming and expensive; therefore, the development of a cheese-like system was desirable. Previously, researchers have used various types of cheese model systems that included cheese slurries, cheese pastes, and miniature cheeses (11, 15, 32, 35, 41). In this study, we used a cheese-like buffer system based on cheese serum that had been previously used for studying inorganic constituents and proteolysis in Cheddar and Emmental cheeses, respectively (18, 20, 29).

Peptides α_{S1}-CN (f1-9), (f1-13) (f1-16), and (f1-17) were identified in CCS and are known to accumulate in cheese due to the activity of the lactococcal cell envelope proteinase and a lactococcal endopeptidase on α_{S1}-CN (f1-23) (2, 17). We did not identify β-CN (f193-209) in our CCS; this was expected because accumulation of this peptide typically occurs after 1 month of ripening (2). A previous study demonstrated that peptides α_{S1}-CN (f1-6) and α_{S1}-CN (f1-16) were derived from α_{S1}-CN (f1-23) by *L. helveticus* CNRZ32 PrtH (31); therefore, we believed that CNRZ32 would likely contain peptidases capable of hydrolyzing these peptides.

Recombinant lactococcal strains expressing specific lactobacillus peptidases have been used in numerous studies to study cheese flavor development and reduce bitterness (10, 11, 22). In this study, we demonstrated that three CNRZ32 endopeptidases (PepO2, PepO3, and PepE) had relatively high specific activities toward the peptides α_{S1}-CN (f1-9) and β-CN (f193-209) when present alone, in the defined peptide mix, or in CCS under cheese-ripening conditions (pH 5.2, 4% NaCl, 10°C). Interestingly, the activities of PepO2 and PepO3 for both α_{S1}-CN (f1-9) and β-CN (f193-209) were greater in CCS than in the defined peptide mix. One reason could be less inhibition by competing peptides, α_{S1}-CN (f1-13) and α_{S1}-CN (f1-16), that were present at a higher concentration in the defined peptide mix than in CCS. Alternatively, the ions present in CCS, particularly Ca^{2+}, may activate PepO2 and PepO3, as they are metallopeptidases. In contrast, we noted significant inhibition of PepE activity in CCS. One possible explanation is competitive inhibition of PepE by peptides present in CCS.

In conclusion, we successfully expressed *L. helveticus* CNRZ32 PepO2, PepO3, and PepE under the control of P_{pepO3} in *L. lactis* LM0230 using a high-copy vector. The results indicated that PepO2 and PepO3 had equally high activities toward β-CN (f193-209), while PepO2 had greater activity for α_{S1}-CN (f1-9) and α_{S1}-CN (f1-13) in CCS at pH 5.2, 4% NaCl, and 10°C. We also determined that other cheese peptides can affect the activity of CNRZ32 endopeptidases, especially PepE, underscoring the importance of using simple but cheese-like model systems such as CCS for studying peptide hydrolysis. Moreover, we have identified two key enzymes from *L. helveticus* CNRZ32, PepO2 and PepO3, that have the potential to hydrolyze bitter peptides in cheese when expressed in a lactococcal strain. Future studies will include examining food-grade strains of lactococci that express CNRZ32 peptidases PepO2 and PepO3 in combination with aminopeptidase PepN to reduce bitterness in bacterial ripened cheeses, such as Cheddar and Gouda.

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