Cloning and Expression of an Oligopeptidase, PepO, with Novel Specificity from *Lactobacillus rhamnosus* HN001 (DR20)

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Received 8 May 2001/Accepted 12 October 2001

Oligopeptidases of starter and nonstarter lactic acid bacteria contribute to the proteolytic events important in maturation and flavor development processes in cheese. This paper describes the molecular cloning, expression, and specificity of the oligopeptidase PepO from the probiotic nonstarter strain *Lactobacillus rhamnosus* HN001 (DR20). The *pepO* gene encodes a protein of 70.9 kDa, whose primary sequence includes the HEXXH motif present in certain classes of metallo-oligopeptidases. The *pepO* gene was cloned in *L. rhamnosus* HN001 and overexpressed in pTRKH2 from its own promoter, which was mapped by primer extension. It was further cloned in both pNZ8020 and pNZ8037 and overexpressed in *Lactococcus lactis* subsp. *cremoris* NZ9000 from the *nisA* promoter. The purified PepO enzyme demonstrated unique cleavage specificity for α_{s1} -casein fragment 1–23, hydrolyzing the bonds Pro-5–Ile-6, Lys-7–His-8, His-8–Gln-9, and Gln-9–Gly-10. The impact of this enzyme in cheese can now be assessed.

Lactic acid bacteria (LAB) produce a wide repertoire of proteolytic enzymes that are involved in metabolizing casein for cell growth in milk, and these proteolytic systems have been extensively reviewed (7, 17, 33). Essentially, the cell surface proteinase releases oligopeptides from the milk caseins (as well as a limited amount of small peptides) that are transported into the cell. Intracellular peptidase activities combine to supply the cell with critical amino acids. The activities of the proteolytic enzymes are therefore essential for the growth of LAB in milk, which itself contains only low levels of peptides and free amino acids.

In cheese, the growth of nonstarter LAB is perhaps partially facilitated by the residual proteolytic activity of the starter LAB (21), but it is still dependent on the nonstarter proteolytic capability. The activities of the various proteolytic enzymes originating from the cheese flora determine the rate of ripening of cheeses such as Cheddar, and the balance of the various enzymes elaborated also affects the flavor of the final product. The enzymes of LAB that hydrolyze the oligopeptide products of proteinase activity have been investigated both biochemically and genetically (27–30). The name PepO was originally used for a specific oligopeptidase from lactococci (38). This designation is now used for metallo-oligopeptidases from LAB with significant amino acid sequence similarity to the lactococcal enzyme. However, the name oligopeptidase O (derived from oligoendopeptidase) was recently suggested as a suitable name for general use (26). PepO has not yet received an enzyme classification number (26).

PepO from *Lactococcus lactis* subsp. *cremoris* Wg2 (39) and

other oligopeptidases identified in LAB are metalloenzymes, including the oligopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* B14 (3), and PepF from *L. lactis* subsp. *cremoris* NCDO763 (29). This has been determined by activity studies on the purified enzymes with metal ions and chelators such as EDTA, 1,10-phenanthroline, and phosphoramidon and the identification of the Zn²⁺-binding motifs HEISH (28) and HETGH (29). Sequence data from oligopeptidase genes have identified a number of characteristic motifs present in zinc-dependent metallo-oligopeptidases (2).

Although several oligopeptidases have been purified, only a few oligopeptidase-encoding genes have been cloned and sequenced from lactococci (28, 29, 42) and lactobacilli (6). However, the manipulation of LAB peptidases is a potentially useful approach to changing cheese flavor. In a preliminary investigation, Cheddar cheese was made with a lactococcal starter overproducing different peptidases (E. Johansen, L. U. Guldeldt, H. Behrndt, D. Williams, and P. Strøman, 6th Symposium on Lactic Acid Bacteria, Genetics, Metabolism and Applications, poster presentation, 1999). Overproduction of PepO did not affect the organoleptic properties of the cheese, but the significance of this result is unclear, as neither the expression level of PepO in the mutant strain nor the susceptibility of the strain to autolysis was reported. Autolysis is a critical factor governing both the balance of proteolytic enzymes in cheese ripening and the timing of their introduction into the cheese matrix (8).

There are in fact a number of reports that indicate an important role for oligopeptidase activity in cheese ripening. Exterkate and Alting (13) showed that PepO is active in Gouda cheese and indeed affects the peptide composition. Baankreis et al. (1) demonstrated that a neutral oligopeptidase from *L. lactis* is active under cheese conditions and concluded that it probably plays a crucial role in the degradation of the very bitter cheese peptide β -casein fragment from position 193 to 209 [β -casein f(193–209)]. Recent results show that in model

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics or further modifications	Reference
Strains		
<i>L. rhamnosus</i> HN001 (DR20)	Wild-type industrial nonstarter <i>L. rhamnosus</i> HN001 with pTRKH2 <i>L. rhamnosus</i> HN001 with pLB001	32 This study This study
<i>L. lactis</i> subsp. <i>lactis</i> NZ9000	Strain MG1363 with <i>pepN::nisR nisK</i> <i>L. lactis</i> subsp. <i>lactis</i> NZ9000 with pNZ8020 <i>L. lactis</i> subsp. <i>lactis</i> NZ9000 with pLB002 <i>L. lactis</i> subsp. <i>lactis</i> NZ9000 with pLB003 <i>L. lactis</i> subsp. <i>lactis</i> NZ9000 with pNZ8037 <i>L. lactis</i> subsp. <i>lactis</i> NZ9000 with pLB004	19 This study This study This study This study This study
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 Δ lacZ Δ M15 Δ (<i>lacZYA-argF'</i>) U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ , r _K ⁺) <i>supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	
Plasmids		
pTRKH2	High-copy-number shuttle vector between <i>E. coli</i> , <i>Lactococcus</i> , <i>Lactobacillus</i> , <i>Streptococcus</i> , and <i>Enterococcus</i> , with erythromycin resistance marker	31
pLB001	<i>pepO</i> from <i>L. rhamnosus</i> HN001 with its own promoter and terminator cloned in pTRKH2	This study
pNZ8020	Transcriptional fusion vector with <i>nisA</i> promoter and chloramphenicol resistance marker	11
pLB002	<i>pepO</i> from <i>L. rhamnosus</i> HN001 with its own terminator and fused to <i>nisA</i> in pNZ8020	This study
pLB003	<i>pepO</i> from <i>L. rhamnosus</i> HN001 with its own terminator cloned in pNZ8020	This study
pNZ8037	Translational fusion vector with <i>nisA</i> promoter and chloramphenicol resistance marker	11
pLB004	<i>pepO</i> from <i>L. rhamnosus</i> HN001 with its own terminator cloned in pNZ8037	This study

systems with water activities, pHs, and NaCl concentrations similar to those in Cheddar cheese, PepO from *L. lactis* remains active and hydrolyzes several peptides known to contribute to bitterness (J. R. Reid, S. A. Harvey, and T. Coolbear, unpublished results). Together, these findings strongly suggest that the release of sufficient levels of PepO by autolysis early in the ripening process would modify the peptide composition, and therefore the organoleptic properties, of the cheese. This means that differences in the specificity of action of oligopeptidases from different sources could be important in cheese flavor development.

In this study, we focused on the PepO enzyme of *Lactoba-*

cillus rhamnosus HN001 (DR20). This is a probiotic strain that was first isolated from a dairy environment (32), and its genome has been sequenced (unpublished data). The *pepO* gene was cloned and overexpressed in both *L. rhamnosus* HN001 and *L. lactis* subsp. *cremoris* NZ9000. PepO was purified to homogeneity, and the N-terminal sequence was determined. The specificity for α_{s1} -casein f(1–23), α_{s1} -casein f(1–17), and bradykinin was analyzed by reverse-phase high-pressure liquid chromatography (RP-HPLC) and mass spectroscopy and compared with previously published data for oligopeptidases in other strains. The possible role of this PepO in reducing cheese bitterness during ripening is discussed.

TABLE 2. Primers

Primer	Sequence (5'-3') ^a	Feature(s)
PepO2	TTTTTT CTCGAGT GGCTACATTATTACCACGACCTG	Reverse primer downstream of the two inverted repeats downstream of <i>pepO</i> , with recognition site for <i>XhoI</i> for cloning in pTRKH2, pNZ8020, and pNZ8037
PepO3	TTTTTT GTCGACC CTTGATGTTATGATCAATGTCTGC	Forward primer upstream of <i>pepO</i> promoter, with recognition site for <i>SalI</i> for cloning in pTRKH2
PepO4	GTTGATGCTGATATGCACGATG	Sequencing primer, forward
PepO5	GCTTTTGGGATTGCCAACGAG	Sequencing primer, forward
PepO6	AGCAGGCATTTGATCAGCTGG	Sequencing primer, forward
PepO7	CGAGATTTTCATGACCGATCGTTG	Sequencing primer, reverse
PepO8	CATCCATGCGTGCCATTCTG	Sequencing primer, reverse
PepO9	GCTTATCCGATCACGAACCTGG	Sequencing primer, reverse
PepO10	TTTTTT AGATC TTGAGATTACGACATGAAAACGGAG	Forward primer including ribosome binding site and recognition site for <i>BglII</i> for cloning in pNZ8020
PepO11	TTTTTT AGATC TTGAGATAACGACATGAAAACGGAG	Forward primer including ribosome binding site, recognition site for <i>BglII</i> , and site-directed mutagenesis to create a stop codon in <i>nisA</i> upstream of <i>pepO</i> for cloning in pNZ8020
PepO12	AAAAAGAGGCAATT ACATG TCATTACCAAG	Forward primer with the translational start of <i>pepO</i> and site-directed mutagenesis to create a recognition site for <i>Bsp</i> LU11I for cloning in pNZ8037

^a Recognition sites for restriction endonucleases are underlined; nucleotides different from those in the *pepO* sequence are in bold.

A. pLB002

nisa -35 *nisa* -10

TAATAATATTATTGTCGATAACGCGAGCATAATAAACGGCTCTGATTAAATTTCTGAAGTTTGTAGATACAATGATTTCGTTCTGAAGGAACACAAAATA

nisa RBS

AATTATAAGGAGGCACTCAAAATGAGTACAAAAGATTTTAACTTGGATTGGTATCTGTTTCTGAAGAAAGATTTCAGGTGCATCACCACGCATTACAAGTA

M S T K D F N L D L V S V S K K D S G A S P R I T S I

NisA

Bam HI | Bgl II *pepO* RBS

TTTCGCTATGTACACCCGGTTGTAAAACAGGAGACTCTGCATGGATCTTGAGATTACGACATGAAACGGAGGCAATTGCATGACATTACCAAGAATTCA

S L C T P G C K T G D S A W I L R L R H E N G G N C M T L P R I Q

PepO

B. pLB003

nisa -35 *nisa* -10

TAATAATATTATTGTCGATAACGCGAGCATAATAAACGGCTCTGATTAAATTTCTGAAGTTTGTAGATACAATGATTTCGTTCTGAAGGAACACAAAATA

nisa RBS

AATTATAAGGAGGCACTCAAAATGAGTACAAAAGATTTTAACTTGGATTGGTATCTGTTTCTGAAGAAAGATTTCAGGTGCATCACCACGCATTACAAGTA

M S T K D F N L D L V S V S K K D S G A S P R I T S I

NisA

Bam HI | Bgl II *pepO* RBS

TTTCGCTATGTACACCCGGTTGTAAAACAGGAGACTCTGCATGGATCTTGAGATAACGACATGAAACGGAGGCAATTGCATGACATTACCAAGAATTCA

S L C T P G C K T G D S A W I L R * M T L P R I Q

PepO

C. pLB004

nisa -35 *nisa* -10

TAATAATATTATTGTCGATAACGCGAGCATAATAAACGGCTCTGATTAAATTTCTGAAGTTTGTAGATACAATGATTTCGTTCTGAAGGAACACAAAATA

nisa RBS Nco I | Bsp LU11I

AATTATAAGGAGGCACTCACCATGTTCATTACCAAGAATTCAAGATGATTGTACCTAGCCGTCATGGCGAATGGCAAGCGAAGACGCCGATTCCACCTG

M S L P R I Q D D L Y L A V N G E W Q A K T P I P P D

PepO

FIG. 1. Cloning strategies in pNZ8020 and pNZ8037. The *nisa* promoter and ribosome binding sites for *nisa* and *pepO* are in bold, primers used to amplify *pepO* are double underlined, and nucleotides changed by site-directed mutagenesis are in bold italics. (A) pNZ8020 was cut with *Bam*HI and ligated to the PCR product of *pepO* with forward primer PepO10 cut with *Bgl*II. This generated a NisA-PepO fusion protein. (B) pNZ8020 was cut with *Bam*HI and ligated to the PCR product of *pepO* with forward primer PepO11 cut with *Bgl*II. Primer PepO11 introduced a stop codon for *nisa* by site-directed mutagenesis. (C) pNZ8037 was cut with *Nco*I and ligated to the PCR product of *pepO* with forward primer PepO12 cut with *Bsp*LU11I. An A instead of a T was introduced by primer PepO12 by site-directed mutagenesis, resulting in a codon for serine rather than threonine as the second amino acid.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. Strains and plasmids used in this study are listed in Table 1. Freeze-dried cultures of *L. rhamnosus* HN001 were inoculated into 100 ml of MRS broth (10) in 100-ml bottles and incubated at 37°C for 18 to 24 h. For selection of transformants of *L. rhamnosus*, 5 µg of erythromycin was added per ml of medium. *L. lactis* was grown in M17 with 10 g of glucose per liter (40) at 30°C, and transformants were selected by addition of 5 µg of erythromycin or 5 µg of chloramphenicol per ml of medium. *Escherichia coli* was grown at 37°C in LB medium (35). Transformants were plated on medium containing 200 µg of erythromycin, 40 µg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.5 mM IPTG (isopropyl-β-D-galactopyranoside) per ml of medium. For solid media, agar was added to 15 g/liter.

Cloning of *pepO*. Genomic sequence data for *pepO* were previously obtained (unpublished data). Forward primers PepO10, PepO11, and PepO12 and reverse primer PepO2 (Table 2) were constructed to amplify fragments including the ribosome binding site, the coding sequence, and two inverted repeats downstream of the coding sequence. The *pepO* reading frame continued 129 bp upstream of the likely translational start (inferred from sequence alignment), and forward primer PepO3 was constructed to anneal upstream of this region (Table 2). All custom primers were produced by Life Technologies (Auckland, New Zealand).

Total genomic DNA of *L. rhamnosus* HN001 was extracted and purified as described elsewhere (32). The sequence containing *pepO* was amplified by PCR with *Pwo* DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) and a Perkin Elmer GenAmp 9600 thermocycler (Applied Biosystems,

Foster City, Calif.). PCR products and vectors were cut with *Sal*I, *Bgl*II, *Bsp*LU11I, *Xho*I, and *Nco*I (Table 2) according to the manufacturer's recommendations (Boehringer Mannheim). The PepO3-PepO2 product was ligated to pTRKH2, the PepO10-PepO2 and PepO11-PepO2 products were ligated to pNZ8020, and the PepO12-PepO2 product was ligated to pNZ8037.

E. coli DH5α was transformed (35) with the pTRKH2 ligations. The recombinant plasmids were prepared with a plasmid kit (Qiagen GmbH, Hilden, Germany). *L. rhamnosus* HN001 was made competent by growth in the presence of glycine (43) and then transformed with pLB001 by electroporation. To verify the transformants of *L. rhamnosus*, plasmids were prepared essentially as described previously (25) but on a smaller scale and with a commercial kit (Concert; Life Technologies). *L. lactis* subsp. *cremoris* NZ9000 was made competent by growth in the presence of glycine (15) and then transformed with the pNZ8020 and pNZ8037 ligations by electroporation.

Sequencing of *pepO* clones. Both strands of four different *pepO* clones were sequenced by using a BigDye terminator cycle sequencing ready-reaction kit (Applied Biosystems, Melbourne, Australia) and an MJ Research (Boston, Mass.) PTC-100TM thermal cycler and analyzed with an ABI Prism 377 automated sequencer. To assemble the data, the sequencing project in the software GeneWorks 2.5 (Oxford Molecular Group Inc., Campbell, Calif.) was used.

Northern hybridization and primer extension. *L. rhamnosus* HN001 was grown in 50 ml of MRS medium in 50-ml tubes at 37°C. Total RNA was prepared as described previously (24). For primer extension, the avian myeloblastosis virus reverse transcriptase kit from Promega (Madison, Wis.) was used to end-label 10 pmol of the oligonucleotide 5'TTACTATCCGCACTCACAAACAC with 30 µCi of [γ-³²P]ATP (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, United

FIG. 2. Nucleotide and protein sequences of *pepO* in *L. rhamnosus* HN001. The promoter, ribosome binding site (RBS), and zinc motif are shown in bold. The translated protein is shown, and the full open reading frame is indicated by italics. Arrows show the inverted repeats.

Kingdom) and to perform primer extension reactions on 20 µg of total RNA. Sequencing reactions were generated by using the same ³²P-end-labeled primer with pLB001 as the template and the fmol cycle sequencing kit (Promega). The products were separated on a 7% acrylamide–7 M urea sequencing gel and visualized by autoradiography. Northern hybridization using pLB001 labeled with ³²P as the probe was done as described previously (24).

Production of PepO. Overnight cultures of *L. rhamnosus* HN001 (wild type) and strains harboring the vector only or vector with the cloned *pepO* gene were inoculated (1% vol/vol) into fresh MRS and grown to late log phase. Overnight cultures of *L. lactis* subsp. *cremoris* NZ9000 (host strain) and strains harboring the vectors only or vectors with the cloned *pepO* gene were inoculated (1% vol/vol) into fresh M17 and grown to an optical density at 600 nm of 0.5. For induction of the nisin promoter, nisin was added to a final concentration of 0.15 µg/ml, and the cells were further cultured until the optical density at 600 nm was 1.20. As a background level control, all lactococcal cultures were also grown without addition of nisin. The bacteria were harvested by centrifugation at 4°C and washed in 50 mM bis-Tris propane, pH 6.4.

Purification of PepO. The washed pellets were resuspended in the bis-Tris propane washing buffer, and the cells were disrupted by two passes through a French press. The cell debris was removed by centrifugation at 27,500 × g for 30 min at 4°C, and the supernatant was stored at –80°C until needed. Cell lysis was

checked by measuring the release of an intracellular marker enzyme, tagatose 1,6-bisphosphate aldolase (EC 4.1.2.40), using the assay procedure described previously (9) and by determining the soluble protein content with the bicinchoninic assay kit (36) (Sigma Chemical Co., St. Louis, Mo.), with bovine serum albumin as the standard.

The supernatant was loaded onto a 5- by 100-mm Protein-Pak Q column (Waters New Zealand Ltd, Auckland, New Zealand) equilibrated with 20 mM bis-Tris propane buffer, pH 8.0 (buffer A), and linked to a PC-controlled fast protein liquid chromatography system (Amersham Pharmacia Biotech Europe GmbH, Uppsala, Sweden). The column was washed with 15 ml of buffer A to remove unbound material. Buffer B was 20 mM bis-Tris propane (pH 8.0) containing 1 M NaCl. Bound protein was eluted at room temperature with a linear gradient of 0 to 30% buffer B over 30 min at a flow rate of 0.7 ml per min. Fractions containing high oligopeptidase activity [i.e., hydrolyzed α₁-casein f(1–23)] eluted at approximately 22% buffer B and were pooled. NaCl was slowly added to the pooled fraction to a final NaCl concentration of 3 M. The sample was loaded onto an HR 5/5 phenyl-Superose fast protein liquid chromatography column (Amersham Pharmacia Biotech) equilibrated with 20 mM bis-Tris propane, pH 6.8, containing 3 M NaCl (buffer C). The column was washed with 10 ml of buffer C to elute unbound material. Buffer D was 20 mM bis-Tris propane, pH 6.8. Bound protein was eluted with a linear gradient of 0 to 100% buffer D

FIG. 3. *L. rhamnosus* HN001 *pepO* transcript analysis. (A) Northern hybridization of total RNA with a *pepO* probe. (B) Primer extension analysis of *pepO*. Sequence reactions (A, G, C, and T) and primer extension (PE) were done with the same primer. The arrows indicate the primer extension products. (C) Interpretation of the primer extension results. The putative *pepO* -35 and -10 promoter hexamers (bold), transcription start site (underlined), and putative ribosome binding site and ATG start codon (italics) are shown.

in 40 min at a flow rate of 0.5 ml per min. Fractions containing high oligopeptidase activity (eluting at approximately 2.7 M NaCl) were concentrated 10-fold with Centricon 10 concentrators (Amicon Division, WR Grace, Beverly, Mass.). Fresh 20 mM bis-Tris propane buffer, pH 6.8, was added to reduce the concentration of NaCl to 150 mM. The purified enzyme was stored at -80°C until required.

PAGE. Samples for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by heating (100°C) for 2 min with an equal volume of 0.125 M Tris-HCl (pH 6.8) containing 4% (wt/vol) SDS, 20% (wt/vol) glycerol, and 10% (vol/vol) β -mercaptoethanol. SDS-PAGE was carried out according to the procedure of Laemmli (20) with 12% (wt/vol) acrylamide Ready Gel precast gels (Bio-Rad New Zealand, Auckland, New Zealand).

Determination of molecular mass. The molecular mass of the denatured monomer was determined by SDS-PAGE with the following protein standards (Amersham Pharmacia Biotech): myosin (220 kDa), phosphorylase *b* (97.3 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (30 kDa), and lysozyme (14.3 kDa).

N-terminal sequence analysis. The N-terminal amino acid sequence of the purified oligopeptidase was determined by the automated Edman method using a 476A protein sequencer (Applied Biosystems).

Enzyme assays, peptide hydrolysis, and identification of peptide products. The chymosin-generated peptides α_{s1} -casein *f*(1–17) and α_{s1} -casein *f*(1–23), comprising the first 17 and 23 residues of α_{s1} -casein, respectively, and bradykinin (Sigma Chemical Co.) were used as oligopeptidase substrates for specificity studies. α_{s1} -Casein *f*(1–23) was generated and purified by the method of Exterkate and Alting (12) and used as the substrate to detect oligopeptidase activity during the enzyme purification procedure. The peptide α_{s1} -casein *f*(1–17) was extracted from an 8-week-old Cheddar cheese by the hot water extraction method of Kuchroo and Fox (18) and purified by the RP-HPLC method of Exterkate and Alting (12). The method used for the hydrolysis of all substrates was essentially identical to that described by Pritchard et al. (34). Enzyme activity was deduced from the decreasing concentrations of substrate as detected by peptide absorbance at 214 nm after separation of substrate and products by RP-HPLC. After separation, the peptide products were collected, dried under vacuum, and identified by mass spectrometry with a triple-quadrupole model API 300 mass spectrometer (Applied Biosystems).

RESULTS

Cloning of *pepO*. All *E. coli* colonies that appeared after transformation with pTRKH2/*pepO* ligations were blue. Sequence analysis showed that the cloned fragment contained a

promoter and translational start in the opposite orientation upstream of *pepO*, thus promoting read through in-frame to *lacZ*. We originally planned to clone *pepO* into pNZ8020 by using *SalI* or *BglII*, which have been reported to have unique sites in this vector (11). However, we found that both of these endonucleases cut the plasmid twice (data not shown), so the vector was restricted with *BamHI* instead. Attempts were made to transform *E. coli* with pNZ8020/*pepO* ligations, but all transformants screened either contained the pNZ8020 alone or contained the pNZ8020 with the *pepO* insert in the opposite orientation with respect to the *nisA* promoter. To avoid apparent instability in *E. coli*, the ligations were introduced directly into *L. lactis* subsp. *cremoris* NZ9000. All cloned fragments were sequenced and found to be identical to the HN001 genomic sequence of *pepO*. By using the forward primer PepO10, the cloned fragment in pNZ8020 was found to encode a fusion protein between *nisA* and *pepO* (Fig. 1). Therefore, alternative primers PepO11 and PepO12 were constructed (Table 2), where primer PepO11 introduced a stop codon in *nisA* upstream of *pepO*.

The *pepO* gene and gene product. The *pepO* gene of *L. rhamnosus* HN001 was 1,896 bp, encoding a putative protein of 632 amino acids (Fig. 2). The sequence had a GC content of 49.5%, which is close to the average overall GC content of 46 to 48% observed for several other *L. rhamnosus* HN001 genes (unpublished data). The molecular mass and pI were calculated from the sequence to be 70.9 kDa and 4.86, respectively. The fusion protein NisA-PepO encoded by pLB002 had a calculated mass of 76.6 kDa. The amino acid sequence of PepO was 37% identical to PepO in *Lactobacillus helveticus* (accession number AF019410) and 36% identical to PepO in *L. lactis* (accession number L18760). The *pepO* gene product contained the sequence HEISH, which is a characteristic HEXXH-motif present in zinc metalloproteases (2). North-

A **B**

FIG. 4. SDS-PAGE showing the molecular mass and purity of PepO. (A) Cell extract of *L. lactis* subsp. *cremoris* NZ9000 with pLB003 induced with nisaplin (lane 1) and the final purified PepO (lane 2). (B) Cell extract of *L. lactis* subsp. *cremoris* NZ9000 with pLB003 obtained without induction (lane 1) and induced with nisaplin (lane 2). Lane 3, standard proteins.

ern hybridization showed that the *pepO* transcript was about 1.9 kilonucleotides, indicating that it is monocistronic (Fig. 3A). Primer extension identified the start of the transcript (Fig. 3B), and a promoter with the configuration TTGACG-N₁₇-TATGCT was inferred (Fig. 3C).

Purification of oligopeptidase PepO. RP-HPLC was used to quantify the oligopeptidase activity obtained from cell extracts of the various cultures standardized to a known cell dry weight by culture density at 600 nm. PepO activity was twofold higher in the *L. rhamnosus* HN001 transformant harboring pLB001 than in wild-type HN001. However, in *L. lactis* subsp. *cremoris* NZ9000, it was estimated that PepO activity was 10- to 15-fold higher in the induced transformant harboring pLB003 and 20- to 25-fold higher in the induced transformant harboring pLB004 than in the host strain (data not shown). The increased expression level of PepO upon induction of *L. lactis* subsp. *cremoris* NZ9000 harboring pLB003 was clearly observable following SDS-PAGE analysis of the cell extract obtained before and after induction (Fig. 4B). The plasmid encoding NisA-PepO (pLB002) also overproduced PepO, but to a lesser extent. It was estimated that the uninduced lactococcal transformants harboring either pLB003 or pLB004 expressed about twofold more PepO activity than either the uninduced host strain or the uninduced strain harboring the vectors only. This showed that the *nisA* promoter is turned on at a reasonable level even without nisin. Since overproduction of *L. rhamnosus* PepO was much higher in *L. lactis* subsp. *cremoris* NZ9000 than in *L. rhamnosus* HN001, the enzyme was purified from the lactococcal transformant.

Determination of the size, N-terminal amino acid sequence, and substrate specificity of PepO. The molecular mass of the denatured monomer of the PepO from the lactococcal transformant was estimated to be 70 kDa by SDS-PAGE (Fig. 4A), which was in agreement with the mass calculated from the nucleotide sequence. The chemically determined sequence of amino acid residues 1 to 20 of PepO purified from the clone harboring pLB003 was Thr-Leu-Pro-Arg-Ile-Gln-Asp-Asp-Leu-Tyr-Leu-Ala-Val-Asn-Gly-Glu-Trp-Gln-Ala-Lys. This amino acid sequence was in agreement with that predicted from the nucleotide sequence following the removal of the N-terminal methionine (Fig. 2). The sequence of amino acid residues 1 to 20 of the purified PepO from the clone harboring pLB004 was identical except for the first amino acid residue, which was Ser, and confirmed the single-residue substitution necessitated by the translational fusion design (Fig. 1). The purified PepO from both clones hydrolyzed α_{s1} -casein f(1-23) and bradykinin in an identical manner. The specific bonds hydrolyzed are shown in Fig. 5 and are compared with those hydrolyzed by other oligopeptidases from LAB. The primary sites of α_{s1} -casein f(1-23) hydrolysis by the cloned *L. rhamnosus* PepO were in the N-terminal half of the peptide at Pro-5-Ile-6, Lys-7-His-8, His-8-Gln-9, and Gln-9-Gly-10. This is in direct contrast with the specificities of all other oligopeptidases of LAB shown in Fig. 5A. The bonds Pro-5-Ile-6 and Gln-9-Gly-10 were hydrolyzed more rapidly by the *L. rhamnosus* PepO than were the bonds Lys-7-His-8 and His-8-Gln-9. After extended incubation times (4 h or more), all of the peptides initially produced by hydrolysis of the primary sites were themselves hydrolyzed, except for the peptide α_{s1} -casein f(1-5). This peptide was resistant to further hydrolysis by PepO and there-

The purified PepO cleaved the Pro-7-Phe-8 bond in bradykinin, a specificity similar to that of a neutral oligopeptidase from *L. lactis* (23). In addition, PepO slowly hydrolyzed the Gly-4-Phe-5 bond of bradykinin. This has also been shown for lactic acid bacterium endopeptidase II (LEP II) from *L. lactis* subsp. *cremoris* H61 (44) and for the oligopeptidases from *L. lactis* subsp. *cremoris* SK11 (34) and *L. delbrueckii* subsp. *bulgaricus* B14 (3).

However, the specificity for α_{s1} -casein *f*(1–23) was dramatically different from that of previously described oligopeptidases from lactococci and lactobacilli. The bonds in α_{s1} -casein *f*(1–23) that were hydrolyzed by the *L. rhamnosus* HN001 PepO, i.e., Pro-5-Ile-6, Lys-7-His-8, His-8-Gln-9, and Gln-9-Gly-10, were all located in the N-terminal part of the substrate. In contrast, all of the bonds hydrolyzed by the other oligopeptidases studied to date (shown for comparison with *L. rhamnosus* HN001 PepO in Fig. 5) are located in the C-terminal part of the substrate, with the exception of one bond (Gln-9-Gly-10) that is hydrolyzed by LEP II (44).

The differences in the specificity of the *L. rhamnosus* HN001 PepO have ramifications for the flavor of the peptides produced. The peptide α_{s1} -casein *f*(1–13) has been shown to be bitter and to contribute to the bitterness of Cheddar cheese (22). α_{s1} -Casein *f*(1–14) is also likely to be bitter, since it has a very similar structure and hydrophobicity, and work by our research group has shown that α_{s1} -casein *f*(1–17) is also very bitter (unpublished data). Each of these peptides is known to accumulate during cheese ripening (13, 14).

The specificities reported for oligopeptidases from other LAB and detailed in Fig. 5 indicate that if these enzymes are released into the cheese matrix during the early stages of ripening, they will hydrolyze α_{s1} -casein *f*(1–23) to produce the bitter cheese peptides discussed above and other closely related bitter peptides. Indeed, Exterkate and Altling (13) detected α_{s1} -casein *f*(1–14) in Gouda cheese and concluded that it was produced from hydrolysis of α_{s1} -casein *f*(1–23) by an intracellular endopeptidase released by early starter lysis. However, the *L. rhamnosus* HN001 PepO described in the present study is very active against the N-terminal region of α_{s1} -casein *f*(1–23), and it is highly likely, therefore, that this enzyme will efficiently degrade the bitter breakdown products of this casein fragment that may be produced by the action of either lactocepin or PepO from other LAB. This suggestion is strongly supported by the rapid hydrolysis of α_{s1} -casein *f*(1–17) by the *L. rhamnosus* HN001 PepO reported in the present study. Therefore, if a sufficient quantity of active PepO is released from *L. rhamnosus* HN001 by autolysis during ripening, it could play a role in controlling cheese bitterness.

ACKNOWLEDGMENTS

We thank NIZO Food Research, The Netherlands, for supplying *L. lactis* subsp. *cremoris* NZ9000 and plasmids pNZ8020 and pNZ8037 and Todd Klaenhammer, North Carolina State University, for the kind gift of pTRKH2.

C. Christensson was partly financed by scholarships from the Swedish Institute and Bengt Lundqvists minne, SE Banken, Sweden. This study was supported by the New Zealand Foundation for Research, Science and Technology.

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