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Characterization of a Thiol-Dependent Endopeptidase from *Lactobacillus helveticus* CNRZ32

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An endopeptidase gene (*pepE*) was isolated from a previously constructed genomic library of *Lactobacillus helveticus* CNRZ32. The *pepE* gene consisted of a 1,314-bp open reading frame encoding a putative peptide of 52.1 kDa. Significant identity was found between the deduced amino acid sequence of *pepE* and the sequences for aminopeptidase C from *Lactobacillus delbrueckii* subsp. *lactis* DSM7290, *L. helveticus* CNRZ32, *Streptococcus thermophilus* CNRZ302, and *Lactococcus lactis* subsp. *cremoris* AM2. A recombinant PepE fusion protein containing an N-terminal six-histidine tag was constructed and purified to electrophoretic homogeneity. Characterization of PepE revealed that it was a thiol-dependent protease having a monomeric mass of 50 kDa, with optimum temperature, NaCl concentration, and pH for activity at 32 to 37°C, 0.5%, and 4.5, respectively. PepE had significant activity under conditions which simulate those of ripening cheese (10°C, 4% NaCl, pH 5.1). PepE hydrolyzed internal peptide bonds in Met-enkephalin and bradykinin; however, hydrolysis of α-, β-, and κ-caseins was not detected.

Lactic acid bacteria (LAB) are a heterogeneous family of bacteria, many of which are used as starter cultures and culture adjuncts in the manufacturer of a wide variety of fermented dairy products (26). LAB are nutritionally fastidious and cannot synthesize several essential amino acids necessary for growth; therefore, when grown in milk, LAB must obtain essential amino acids from milk (19, 21, 22, 26). However, the level of free amino acids and small peptides in milk is not sufficient for growth of LAB to high cell densities (19, 21, 22, 26). Therefore, LAB require a complex proteolytic system composed of proteinases, peptidases, and transport systems to obtain essential amino acids from the proteins present in milk, primarily caseins (19, 21, 22, 26). The casein hydrolysis products also influence cheese flavor and texture development during ripening (17, 26).

Previously, a genomic library of *Lactobacillus helveticus* CNRZ32 was constructed in *Escherichia coli* DH5α and screened for exopeptidase activities (25). This screen identified two general aminopeptidases (*pepN* and *pepC*), an X-prolyl dipeptidyl aminopeptidase (*pepX*), a general dipeptidase (*pepDA*), and a di/tripeptidase with prolinase activity (*pepPN*) (25). The genes for these five peptidases have been sequenced and further characterized (11–14, 16, 35).

Since endopeptidases of *Lactobacillus* have not been well characterized, a second screening of the genomic library was conducted to identify endopeptidases of *L. helveticus* CNRZ32. This publication describes this screening and the characterization of one of the endopeptidases identified.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and DPWC (Gold Biotechnology, Inc., St. Louis, Mo.) were grown in Luria-Bertani (LB) broth (26). *E. coli* BW26 (Gold Biotechnology) was grown in LB broth containing 50 μg of kanamycin/ml. All *E. coli* strains were grown at 37°C with aeration. *L. helveticus* strains were grown in MRS broth at 37°C without shaking. Agar plates were prepared by adding 1.5% (wt/vol) granulated agar (Difco Laboratories, Detroit, Mich.) to liquid media. The concentrations of antibiotics added to liquid media or agar plates for selection of plasmids were as follows: pJDC9 (9, 10), 1.0 mg of erythromycin/ml; pMOB (Gold Biotechnology), 100 μg of ampicillin or 100 μg of carbenicillin/ml; pQE-8 (Qiagen, Inc., Chatsworth, Calif.), 100 μg of ampicillin/ml; pREP-4 (Qiagen), 25 μg of kanamycin/ml. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). For experiments utilizing α-complementation, isopropyl-thio-β-galactoside (IPTG) (Promega Corp., Madison, Wisc.) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Gibco-BRL Life Technologies, Inc., Gaithersburg, Md.) were added to agar media at concentrations of 119 and 40 mg/liter, respectively.

Screening of *L. helveticus* CNRZ32 genomic library. Previously, a genomic library of *L. helveticus* CNRZ32 was constructed in *E. coli* DH5α by utilizing the vector pJDC9 (25). The following procedure was used to screen the library for clones encoding endopeptidase activity. Pooled cultures (10 transformants/pool) were grown overnight in LB broth containing erythromycin. Cells were pelleted at 13,000 × *g* for 5 min at 21 to 23°C, washed twice with sterile 10 mM HEPES buffer (pH 7.0) (Sigma), and suspended in 200 μl of 10 mM HEPES (pH 8.0) with 1 mg of lysozyme (Sigma)/ml. Cell suspensions were incubated at 37°C for 30 min and then subjected to three cycles of freezing in a dry ice/ethanol bath for 1 min followed by thawing at 37°C in a water bath for 5 min. The cell suspensions were vortexed for 30 s, and cell debris was removed by centrifugation for 5 min at 13,000 × *g*. Endopeptidase activity was qualitatively determined by adding 100 μl of cell extract to 2.4 ml of 10 mM HEPES (pH 7.0) containing 0.08 mM substrate and then incubating for 1.5 h at 37°C. Endopeptidase substrates (Sigma) used were *N*-benzoyl–Phe–Val–Arg–*p*-nitroanilide (pNA), *N*-benzoyl–Pro–Phe–Arg–pNA, and N-benzoyl–Val–Gly–Arg–pNA. Cell extracts obtained from mid-log and late-log cultures of *L. helveticus* CNRZ32 and *E. coli* DH5α (pJDC9) were used as positive and negative controls, respectively. The appearance of an intense yellow color (resulting from release of pNA) within 15 min was taken as a positive indication of endopeptidase activity.

Molecular cloning. Recombinant DNA and plasmid isolation techniques were performed essentially as described by Sambrook et al. (27). T4 DNA ligase, alkaline phosphatase, and restriction endonucleases were used as recommended by the manufacturer (Gibco-BRL). *E. coli* transformation was performed with a Gene Pulser following the instructions recommended by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Tn*1000* mutagenesis was performed as recommended by the manufacturer (Gold Biotechnology). Enzyme assays were conducted to determine which Tn*1000* insertions had inactivated the endopeptidase.

DNA sequencing and sequence analysis. Nested sets of Tn*1000* insertions were generated in *pepE* with the Tn*1000* Kit (Gold Biotechnology). DNA templates were isolated using the modified alkaline lysis/polyethylene glycol precipitation procedure described by Applied Biosystems, Inc. (Foster City, Calif.). Vector- and transposon-specific primers were supplied with the Tn*1000* Kit. Additional primers were designed using the Affinity program supplied by Ransom Hill Bioscience, Inc. (Ramona, Calif.), and were synthesized by Gibco-BRL Custom Primers (Grand Island, N.Y.). Cycle sequence reactions were performed in a Perkin-Elmer model 480 thermal cycler (The Perkin-Elmer Corp., Norwalk, Conn.) using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequence determination was performed by the Nucleic Acid and Protein Facility of the University of Wisconsin Biotechnology Center, using an ABI model 370/3 automated sequencer. DNA sequences

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were analyzed using the program of the Genetics Computer Group, Inc. (Madison, Wisc.). Protein homology searches were performed using the BLAST network service (1).

Purification of PepE. The *pepE* gene from pKF3 was amplified by PCR with Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.) and 5′ and 3′ *pepE* primers which contained a *Bam*HI restriction site on the 5′ end of each primer. The nucleotide sequences of the 5′ and 3′ *pepE* primers were 5′GGAT CCATGGCTCATGAATTAACTG3′ and 5′GGATCCTTAAGCAAGTGAAT CCCAT3′, respectively. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pQE-8. The ligation mixture was transformed into *E. coli* DH5α(pREP-4). Plasmids containing successful fusions between the *pepE* structural gene and the $(His)_6$ encoding region of pQE-8 were identified by restriction analysis, enzyme assays, and DNA sequencing of the 5′ and 3′ ends of *pepE*. Enzyme assays were conducted after inducing expression of the plasmid-encoded *pepE* gene by growing cells in LB broth containing 100 μg of ampicillin/ml and 2.0 mM IPTG.

Purification of PepE was performed using the QIA*expressionist* Protein Purification System (Qiagen) according to the manufacturer's instructions. Proteins from collected fractions were visualized by running vertical 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as described by Sambrook et al. (27). The most purified fractions of PepE-$(His)_6$ were pooled and dialyzed with 50 mM Na-phosphate buffer (pH 7.0) and lyophilized with a Virtis Unitrap model 10-100 vacuum/refrigeration unit, 1402 Vacuum Pump, and 10-104-LD Vacuum Controller (The Virtis Corp., Gardiner, N.Y.). Working samples of PepE were prepared by dissolving 1 mg of lyophilized PepE in 1 ml of 50 mM Na-phosphate buffer (pH 7.0).

Characterization of PepE. Purified PepE was assayed at 0.1 mg of protein/ml in a mixture of 10 mM HEPES (pH 7.0) and 0.220 mM N-benzoyl–Phe–Val–Arg–pNA that had been preequilibrated for 15 min at 35°C. Reactions were initiated by the addition of PepE and terminated after 5 min by the addition of 30% glacial acetic acid. Reaction rates were verified to be linear under these conditions and were quantified on the basis of release of pNA (extinction coefficient of 8.8 mM^{-1} cm^{-1} at 410 nm; 15). Enzyme assays were done in duplicate, and the variance was confined to ±5%. Purified PepE was also assayed as described above with 1 mM Phe-pNA, Val-pNA, and Arg-pNA, with 1 mM *N*-benzoyl–Phe–Val–Arg–pNA as a positive control. The specific activity of PepE was expressed as micromoles of pNA released per minute per milligram of protein.

For the pH study, 10 mM HEPES was replaced by a composite buffer composed of 20 mM (each) HEPES, malic acid, boric acid, and MES [2-(*N*-morpholino)ethanesulfonic acid]. This composite buffer was also used to compare PepE activity under conditions simulating those of ripening cheese (10°C, 4% NaCl, pH 5.1) to that under optimal conditions (35°C, 0.5% NaCl, pH 4.5).

Inhibitor studies employed the standard assay mixture supplemented with 1 mM inhibitor. The inhibitors (Sigma) tested were EDTA, 1,10-phenanthroline, phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, Pepstatin A, iodoacetic acid (IAA), *p*-chloromercuribenzoic acid (PCMB), dithiothreitol (DTT), and β-mercaptoethanol.

PepE specificity with Met-enkephalin, bradykinin, and β-casomorphin. Met-enkephalin (Tyr-Gly-Gly-Phe-Met), bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), and β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) samples (1 mg/ml) were incubated with purified PepE (0.1 mg protein/ml) in 50 mM Na-phosphate buffer (pH 5.0) for 10, 15, 20, and 30 min at 35°C prior to quenching reactivity by adding water/trifluoroacetic acid (1,000:1, by volume).

Hydrolytic products of Met-enkephalin, bradykinin, and β-casomorphin were analyzed by high-pressure liquid chromatography (HPLC) using a Hitachi model L-6200A pump and gradient controller, model L-4500A diode array detector, model D-6500 chromatography data system (Hitachi Ltd., Tokyo, Japan), and Gilson model 231/401 sample injector (Gilson Medical Electronics, Inc., Middleton, Wisc.) coupled with a Versapack C_{18} column (300 by 4.1 mm; Alltech Associates, Inc., Deerfield, Ill.). A 0 to 30% linear gradient of water/acetonitrile/trifluoroacetic acid (100:900:0.7, by volume) in water/trifluoroacetic acid (1,000:1, by volume) was generated over a period of 30 min with a flow rate of 1 ml/min. Eluting components were monitored from 200 to 320 nm by the DAD.

The elution times for standard fragments (Sigma) of Met-enkephalin, bradykinin, and β-casomorphin were used to assist in the identification of Met-enkephalin, bradykinin, and β-casomorphin hydrolytic products. Hydrolytic products of Met-enkephalin and bradykinin were eluted from the column and further characterized by amino acid analysis (University of Wisconsin-Madison Biotechnology Center, Madison) and mass spectroscopy (University of Wisconsin-Madison Chemistry Department, Madison).

PepE activity on α-, β-, and κ-caseins. α-, β-, and κ-caseins (Sigma) were incubated (at 0.9 mg of protein/ml) in 50 mM Na-phosphate buffer (pH 5.0) with purified PepE (0.1 mg of protein/ml) for 2 h at 35°C. Hydrolysis was qualitatively monitored by running 15% SDS-PAGE gels as described by Sambrook et al. (27). Samples of α-, β-, and κ-caseins were also incubated at 35°C in the absence of PepE for 2 h to serve as controls.

Nucleotide sequence accession number. The sequence for *pepE* has been submitted to GenBank and assigned accession number U77050.

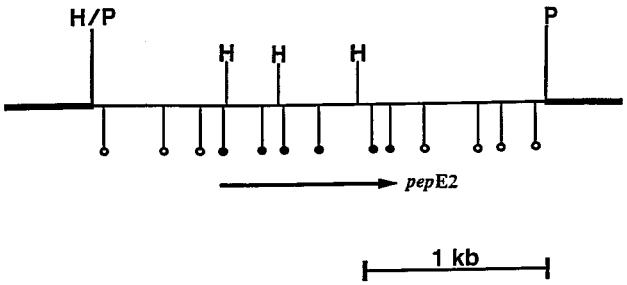

FIG. 1. Restriction endonuclease and Tn*1000* insertion map of the 2.5-kb *L. helveticus* CNRZ32 chromosomal insert of pKF3. Abbreviations for restriction enzymes used: H, *Hin*dIII; P, *Pst*I. ●, $PepE^-$ Tn*1000* insertion site; ○, $PepE^+$ Tn*1000* insertion site. The location of *pepE* on the insert is indicated by the arrow below the restriction map.

RESULTS

Screening of *L. helveticus* CNRZ32 genomic library. Two different clones from the *L. helveticus* CNRZ32 genomic library hydrolyzed *N*-benzoyl–Phe–Val–Arg–pNA, *N*-benzoyl–Pro–Phe–Arg–pNA, and *N*-benzoyl–Val–Gly–Arg–pNA. One of the clones, designated DH5α(pKF1), hydrolyzed *N*-benzoyl–Pro–Phe–Arg–pNA and *N*-benzoyl–Val–Gly–Arg–pNA but did not hydrolyze *N*-benzoyl–Phe–Val–Arg–pNA. The gene encoding this activity was later sequenced and found to have identity with PepO from *Lactococcus lactis* (10a). The other clone, designated DH5α(pKF2), hydrolyzed *N*-benzoyl–Phe–Val–Arg–pNA and *N*-benzoyl–Pro–Phe–Arg–pNA but did not hydrolyze *N*-benzoyl–Val–Gly–Arg–pNA. The endopeptidase activity encoded by pKF2 was subsequently designated PepE.

Subcloning of *pepE*. A restriction map of the 4.7-kb chromosomal insert of pKF2 was made (data not shown), and a 2.5-kb *Pst*I fragment was subcloned in pMOB in both orientations. *E. coli* DH5α containing either of these constructs, designated pKF3 and pKF4, expressed PepE activity. The plasmid pKF3 was arbitrarily chosen for further study.

Tn*1000* mutagenesis of pKF3. Inactivation of *pepE* by insertions of Tn*1000* within the 2.5-kb insert of pKF3 revealed that *pepE* was approximately 1.3 kb in length. The PepE-encoding insert, Tn*1000* insertions, and relevant restriction sites are shown in Fig. 1.

Sequence analysis. The entire 2.5-kb insert of pKF3 was sequenced, and an open reading frame (ORF) of 1,314 bp was identified and designated *pepE* (Fig. 2). The ORF could encode a polypeptide of 438 amino acid residues with a deduced mass of 52.1 kDa. The start codon of the ORF is preceded by a putative ribosome binding site (AGGAGA; nucleotides −14 to −9) and putative promoter −10 (TTAATT; nucleotides −44 to −39) and −35 (TTTATT; nucleotides −66 to −61) sequences (28). An inverted repeat (nucleotides 1331 to 1348 and 1352 to 1370) was observed in the 3′ noncoding region and may function as a *rho*-independent transcriptional terminator with a ΔG of −25.4 kcal/mol (30).

Protein sequence homology searches using BLAST revealed that PepE had a high amino acid sequence identity with thiol-dependent general aminopeptidases (PepC) from *Lactobacillus delbrueckii* subsp. *lactis* DSM7290 (20), *L. helveticus* CNRZ32 (16, 31), *Streptococcus thermophilus* CNRZ302 (8), and *Lactococcus lactis* subsp. *cremoris* AM2 (7). The amino acid sequence identities of PepE with the PepC proteins from these bacteria were 41.7, 40.8, 39.1, and 37.4%, respectively (7, 8, 16, 20, 31). A search of the PROSITE Dictionary of Protein Sites and Patterns with the deduced *pepE* amino acid sequence identified two highly conserved domains involved in substrate

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ATATTGATTAATTTATTAGATTAAGCAAGAAGTCTAATAAGTGACGGATATAATTCTCTG  -91
AAGCGAGTTCATTATTGAACCCGCTTTTATTTTGGTTTAAAATAGAATTAATTTAAGAAA  -31
AAATTAAAAATTATAAAAGGAGAATTCTTAAATGGCTCATGAATTAACTGTGCAGGAACT   29
                          M  A  H  E  L  T  V  Q  E  L
TGAAAAGTTTTCTGCTGATTTTAATAAAAATCCTAAAAATAAAGTCGTTGCTCGTGCTGC   89
 E  K  F  S  A  D  F  N  K  N  P  K  N  K  V  V  A  R  A  A
TCAACGTAGCGGTGTACTTGAAGCTTCTTATAATGACCGCGTTCAAAGCGAATTAACCCG  149
 Q  R  S  G  V  L  E  A  S  Y  N  D  R  V  Q  S  E  L  T  R
TGTCTTTTCAACTGAACTTGATACTGACAACGTTACTAACCAAAAACACTCAGGTCGTTG  209
 V  F  S  T  E  L  D  T  D  N  V  T  N  Q  K  H  S  G  R  C
CTGGTTATTTGCCACATTAAACGTTTTGCGTCATGAATTTGGCAAGAAATACAAGGCAAA  269
 W  L  F  A  T  L  N  V  L  R  H  E  F  G  K  K  Y  K  A  K
AGACTTTACTTTCTCACAAGCATACAACTTCTTCTGGGACAAGATTGAACGTGCCAACAT  329
 D  F  T  F  S  Q  A  Y  N  F  F  W  D  K  I  E  R  A  N  M
GTTCTATAACCGTATCTTAGACAGCGCTGATATGCCACTTGATTCTCGTCAAGTTAAGAC  389
 F  Y  N  R  I  L  D  S  A  D  M  P  L  D  S  R  Q  V  K  T
TGACTTAGACTTTGCAGGTACAGATGGTGGTCAATTCCAAATGGCTGCTGCCTTAGTTGA  449
 D  L  D  F  A  G  T  D  G  G  Q  F  Q  M  A  A  A  L  V  E
AAAATATGGTGTCGTACCTTCATATGCTATGCCTGAAACCTTTAACACTAACGACACTAC  509
 K  Y  G  V  V  P  S  Y  A  M  P  E  T  F  N  T  N  D  T  T
TGGTTTTGCCACTGCATTAGGCGACAAGCTTAAGAAGGATGCTTTGGTTCTTAGAAAATT  569
 G  F  A  T  A  L  G  D  K  L  K  K  D  A  L  V  L  R  K  L
AAAGCAAGAAGGCAAAGATGACGAAATCAAGAAGACTCGTGAAAAATTCTTGAGCGAAGT  629
 K  Q  E  G  K  D  D  E  I  K  K  T  R  E  K  F  L  S  E  V
TTACCAAATGACTGCTATTGCTGTTGGTGAACCACCTAAGAAGTTCGATCTTGAATACCG  689
 Y  Q  M  T  A  I  A  V  G  E  P  P  K  K  F  D  L  E  Y  R
TGATGACGATAAGAAGTACCACTTAGAAAAAGACCTTACTCCACTTGAATTCTTGCACAA  749
 D  D  D  K  K  Y  H  L  E  K  D  L  T  P  L  E  F  L  H  K
GTACTTAGGTGGCGTTGACTTTGATGACTACGTTGTTTTGACCAACGCACCAGACCACGA  809
 Y  L  G  G  V  D  F  D  D  Y  V  V  L  T  N  A  P  D  H  E
ATATGACAAGCTTTATGGTTTACCAGCAGAAGACAACGTCTCTGGTTCAATCAGAATTAA  869
 Y  D  K  L  Y  G  L  P  A  E  D  N  V  S  G  S  I  R  I  K
ACTTTTGAATGTTCCTATGGAATACTTAACCGCTGCTTCTATTGCTCAATTAAAAGACGG  929
 L  L  N  V  P  M  E  Y  L  T  A  A  S  I  A  Q  L  K  D  G
TGAAGCAGTTTGGTTCGGTAATGATGTGCTTCGTCAAATGGACCGTAAGACTGGCTACCT  989
 E  A  V  W  F  G  N  D  V  L  R  Q  M  D  R  K  T  G  Y  L
TGACACTAACCTTTACAAGTTGGATGACTTATTTGGCGTTGACCTTAAGATGTCAAAGGC 1049
 D  T  N  L  Y  K  L  D  D  L  F  G  V  D  L  K  M  S  K  A
TGACAGATTAAAGACTGGTGTCGGCGAAGTTTCTCACGCCATGACCTTAGTCGGTGTTGA 1109
 D  R  L  K  T  G  V  G  E  V  S  H  A  M  T  L  V  G  V  D
TGAAGACAACGGTGAAGTTCGTCAATGGAAAGTTGAAAACTCATGGGGCGACAAGTCCGG 1169
 E  D  N  G  E  V  R  Q  W  K  V  E  N  S  W  G  D  K  S  G
TGCAAAGGGTTACTACGTAATGAACAATGAATGGTTCAACGATTACGTTTATGAAGTTGT 1229
 A  K  G  Y  Y  V  M  N  N  E  W  F  N  D  Y  V  Y  E  V  V
CGTTCACAAGAAGTATTTGACTGATAAGCAAAAGGAACTTGCAGAAGGCCCAATTACTGA 1289
 V  H  K  K  Y  L  T  D  K  Q  K  E  L  A  E  G  P  I  T  D
TCTTCCTGCATGGGATTCACTTGCTTAATGAAAATTAAAACATAAAAATAAGCCTCAGAA 1349
 L  P  A  W  D  S  L  A  *
GTTCTGAGGCTTATTTTTTATACTTATTTAGAGAGTTTTGATTAAAGGATTAGTCTTCAT 1409
AACTAGCTGCTACTTGTTCGTAGTGCTTAACTTCATCGTCACTGCAACGAACAAAGTGAC 1469
CAGGGCGAATTTCATGCATACTGCGTTCCTTACCGTCCATTTCTTCTTTTTGAGCATCAT 1529
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FIG. 2. Nucleotide sequence of *pepE* from *L. helveticus* CNRZ32. The predicted amino acid sequence is shown in single-letter code. The putative −35 (nucleotides −66 to −61) and −10 (nucleotides −44 to −39) promoter sequences, putative ribosome binding site (nucleotides −14 to −9), and putative transcriptional termination sequence (nucleotides 1331 to 1348 and 1352 to 1370) are underlined.

binding and catalysis that are characteristic of proteinases from the cysteine proteinase family (4). The amino acid residues instrumental in substrate binding and catalysis by cysteine proteinases of prokaryotic and eukaryotic origin were found to be conserved in PepE (Gln-64, Cys-70, His-362, Asn-383, and Trp-385) (Fig. 3) (16).

Purification of PepE. Cloning of the *pepE* ORF into pQE-8 resulted in a plasmid designated pKF5. The orientation of the insert was confirmed by restriction analysis. DNA sequence analysis of the *pepE*:pQE-8 junctions confirmed that *pepE* was

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          60          74      361                          392
Lbhel     NVTNQKHSGRCWLFA.....SHAMTLVGVDE...DNGEVRQWKVENSWGDKSGAK
Lblac     SVTNQKQSGRCWMFS.....NHAMVITAVDL...VDDKPTKWKIENSWGDKSGFK
Lbhelv    KPANQKQSGRCWMFS.....DHAMVITGVDN...GDGKPTKWKIENSWGEKPGFK
Stther    EVSNQKASGRCWMFA.....THAMVLTGVDL..DADGKPIKWKIENSWGDKVGQK
Lccrem    PVTNQKQSGRCWMFA.....THAMVLAGVDL..DADGNSTKWKVENSWGKDAGQK
Yeast     PVTNQKSSGRCWLFA.....THAMLITGCHVDETSKLPLRYR.VENSWGKDSGKD
Chick     PVKDQGQCGSCWAFS.....DHGVLVVGYGFEG....GKKYWIVKNSWGEKWGDK
PAP2      PVKNQGACGSCWAFS.....DHAVTAVGYG...TS.DGKNYIIIKNSWGPNWGEK
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FIG. 3. Alignment of the amino acid sequences of the regions involved in substrate binding and catalysis of *L. helveticus* CNRZ32 PepE and several members of the cysteine proteinase family. Numbers above the sequences represent the positions of residues in the CNRZ32 PepE sequence. Conserved residues involved in the active site are indicated by boldface type. Abbreviations: Lbhel, *pepE* of *L. helveticus* CNRZ32; Lblac, *pepC* of *L. delbrueckii* subsp. *lactis* DSM7290; Lbhelv, *pepC* of *L. helveticus* CNRZ32; Stther, *pepC* of *S. thermophilus* CNRZ302; Lccrem, *pepC* of *Lactococcus lactis* subsp. *cremoris* AM2; Yeast, bleomycin hydrolase (BLH1) of *Saccharomyces cerevisiae*; Chick, cathepsin L of chicken; PAP2, proteinase II (chymopapain) of papaya.

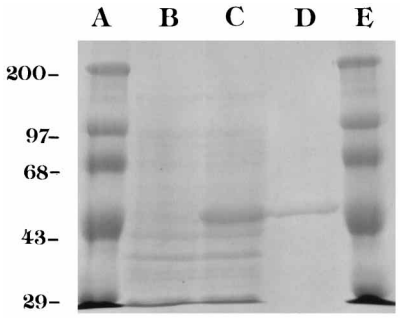

FIG. 4. SDS-PAGE gel of PepE that was purified to homogeneity. Lanes: A and E, high-molecular-mass protein standards (myosin, 200 kDa; phosphorylase B, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa); B, cell extract of DH5α(pKF5) grown in the absence of IPTG (20 μg of protein); C, cell extract of DH5α(pKF5) grown in the presence of 2.0 mM IPTG (20 μg of protein); D, purified PepE (2 μg of protein).

in frame with the upstream $(His)_6$-encoding region of pQE-8. PepE activity for *N*-benzoyl–Phe–Val–Arg–pNA was obtained after induction of DH5α(pKF5) with IPTG. PepE was purified to electrophoretic homogeneity (Fig. 4) in one step by using a Ni-nitrilotriacetic acid affinity chromatography column.

Characterization of PepE. PepE hydrolyzed the endopeptidase substrate *N*-benzoyl–Phe–Val–Arg–pNA but not the aminopeptidase substrates Phe-pNA, Val-pNA, and Arg-pNA.

The molecular mass of PepE was estimated to be 50,000 Da from an 8% SDS-PAGE gel stained with Coomassie brilliant blue (Fig. 4).

The optimum temperature for PepE was between 32 and 37°C. The specific activities of PepE at 5, 15, 25, 35, 45, and 55°C were 0.013, 0.038, 0.089, 0.140, 0.049, and 0.002, respectively. The activation energy of PepE over the range 0 to 30°C was calculated, by using an Arrhenius plot, to be 15 kcal/mol (data not shown). Similarly, the E_a for deactivation of PepE over the range 40 to 55°C was determined to be 59 kcal/mo.

The optimum NaCl condition for PepE at 35°C was 0.5%. The specific activities of PepE at NaCl concentrations of 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5% were 0.212, 0.168, 0.115, 0.080, 0.063, and 0.042, respectively.

The pH dependence of PepE, presented as a Dixon-Webb plot (Fig. 5), revealed an optimum pH of 4.5. This pattern of pH dependence could be attributed to the effect of pH on

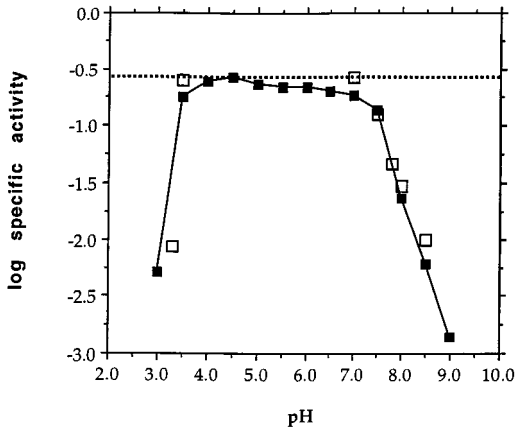

FIG. 5. Effect of pH on activity of purified PepE. Closed symbols indicate activity of PepE with respect to pH. Open symbols represent enzyme preincubated at respective pHs for 5 min and then assayed at pH 4.5.

enzyme stability. PepE instability at the pH extremes suggested that proton deactivation of PepE in the acidic range was conferred by amino acid residues with a pK_a of 3.5 to 4.0, which suggests that the ionization states of Asp/Glu residues are responsible for conferring stability in this pH range. Modulation of PepE stability in the slightly alkaline range appeared to be conferred by a prototrophic group(s) with a pK_a of 7.0 to 7.5, which suggests the importance of a Cys and/or a His residue in maintaining enzyme stability in this pH range.

Of the inhibitors analyzed, only IAA and PCMB were found to inhibit PepE activity (96.5 and 98.5% inhibition, respectively), implying that the Cys residue is essential for activity. The redox state of the residue may also be important, as the presence of DTT and β-mercaptoethanol enhanced activity by 64%. The aspartic acid, serine, and metallopeptidase inhibitors used had no effect except for EDTA, which stimulated activity by 70%.

Specificity of PepE. Met-enkephalin was determined to be hydrolyzed by PepE primarily at position Gly-3–Phe-4 and to a lesser extent at Gly-2–Gly-3 as indicated by HPLC, amino acid analysis, and mass spectroscopy. Amino acid analysis and mass spectroscopy revealed that reaction products with molecular masses of 841.1 Da and higher, consisting of Tyr and Gly residues, were produced by PepE action on Met-enkephalin. Bradykinin was hydrolyzed by PepE only at position Gly-4–Phe-5 as determined by HPLC, amino acid analysis, and mass spectroscopy. PepE did not hydrolyze β-casomorphin, and there was no evidence of digestion of intact α-, β-, and κ-caseins, as determined by SDS–15% PAGE with a gel stained with Coomassie brilliant blue (gel not shown).

DISCUSSION

The proteolytic enzyme system of *L. helveticus* CNRZ32 is of interest, because when this organism is used as a starter culture adjunct in cheese manufacture, it has been associated with reduction in bitterness, decrease in ripening time, and acceleration of flavor development (5, 6, 25). While the proteolytic activity of CNRZ32 is thought to play an important role in cheese flavor development, the role of individual proteolytic enzymes in the development of cheese flavor remains unknown (25).

This study focused on the identification and characterization of an endopeptidase identified from a genomic library of *L. helveticus* CNRZ32. Nucleotide sequencing of this endopeptidase gene, designated *pepE*, revealed a 1,314-bp ORF which could encode a protein of 52.1 kDa. Putative −10 and −35 transcriptional promoters were identified, which indicates that *pepE* may be transcribed from its own promoter (28). Also, a putative *rho*-independent transcriptional terminator (ΔG = −25.4 kcal/mol) was observed in the 3′ noncoding region (30). The presence of these putative transcriptional promoter and terminator sequences suggests that the *pepE* gene is transcribed monocistronically. The high amino acid identity of PepE with PepC from *L. delbrueckii* subsp. *lactis* DSM7290 (19), *L. helveticus* CNRZ32 (16, 31), *S. thermophilus* CNRZ302 (8), and *Lactococcus lactis* subsp. *cremoris* AM2 (7) suggests that these peptidases are evolutionally related and may have evolved from the same ancestral proteolytic enzyme. A highly conserved substrate binding and catalysis motif characteristic of cysteine proteinase family members was identified in PepE (4, 16). The presence of this motif suggests that PepE is a cysteine proteinase with a mechanism of catalytic action similar to those of other cysteine proteinases. This possibility is supported by the inhibition of the purified enzyme by IAA and PCMB, the stimulating effect of DTT and β-mercaptoethanol, and the alkaline pH (7.0 to 9.0) dependence of enzyme stability. PepE is probably located intracellularly, because no signal sequence was detected at the N terminus of the amino acid sequence deduced from *pepE* (18, 32, 33).

PepE was purified to electrophoretic homogeneity by using a one-step Ni-nitrilotriacetic acid affinity chromatography column. Sizing of PepE by SDS-PAGE revealed that PepE had a molecular mass of approximately 50 kDa, which is in agreement with the deduced molecular weight of the protein encoded by the *pepE* ORF. Optimum conditions for activity were observed to be 35°C, 0.5% NaCl, and pH 4.5. Even though PepE has high amino acid identity with general aminopeptidases (PepC) from *L. delbrueckii* subsp. *lactis* DSM7290 (19), *L. helveticus* CNRZ32 (16, 31), *S. thermophilus* CNRZ302 (8), and *Lactococcus lactis* subsp. *cremoris* AM2, the inability of PepE to hydrolyze aminopeptidase substrates such as Phe-pNA, Val-pNA, and Arg-pNA indicates that PepE is not an aminopeptidase. However, the ability of PepE to hydrolyze small peptides like *N*-benzoyl–Phe–Val–Arg–pNA, Met-enkephalin, and bradykinin but not intact α-, β-, and κ-caseins indicates that PepE is an endopeptidase with substrate size selectivity which cannot hydrolyze intact proteins.

Under conditions simulating cheese ripening (10°C, 4% NaCl, pH 5.1), PepE has 5% activity relative to that observed under optimal conditions for PepE activity (35°C, 0.5% NaCl, pH 4.5). This residual level of PepE activity suggests that PepE may play a role in peptide hydrolysis during cheese ripening. PepE is believed to be the first endopeptidase of *Lactobacillus* that has been characterized in detail. The general properties of PepE indicate that this *Lactobacillus* endopeptidase is different from the PepO, PepF, LEPI, and MEP (alkaline oligoendopeptidase) metalloendopeptidases which have been characterized in *Lactococcus* (2, 3, 23, 24, 29, 34). Further investigation is required to determine the physiological role of PepE and what, if any, role this enzyme plays in proteolysis of ripening cheese.

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