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Purification, Characterization, Gene Cloning, Sequencing, and Overexpression of Aminopeptidase N from *Streptococcus thermophilus* A

FREDERIC CHAVAGNAT,* MICHAEL G. CASEY, AND JACQUES MEYER

Department of Biochemistry, Swiss Dairy Research Institute, 3097 Liebefeld-Bern, Switzerland

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The general aminopeptidase PepN from *Streptococcus thermophilus* A was purified to protein homogeneity by hydroxyapatite, anion-exchange, and gel filtration chromatographies. The PepN enzyme was estimated to be a monomer of 95 kDa, with maximal activity on *N*-Lys–7-amino-4-methylcoumarin at pH 7 and 37°C. It was strongly inhibited by metal chelating agents, suggesting that it is a metallopeptidase. The activity was greatly restored by the bivalent cations Co^{2+}, Zn^{2+}, and Mn^{2+}. Except for proline, glycine, and acidic amino acid residues, PepN has a broad specificity on the *N*-terminal amino acid of small peptides, but no significant endopeptidase activity has been detected. The *N*-terminal and short internal amino acid sequences of purified PepN were determined. By using synthetic primers and a battery of PCR techniques, the *pepN* gene was amplified, subcloned, and further sequenced, revealing an open reading frame of 2,541 nucleotides encoding a protein of 847 amino acids with a molecular weight of 96,252. Amino acid sequence analysis of the *pepN* gene translation product shows high homology with other PepN enzymes from lactic acid bacteria and exhibits the signature sequence of the zinc metallopeptidase family. The *pepN* gene was cloned in a T7 promoter-based expression plasmid and the 452-fold overproduced PepN enzyme was purified to homogeneity from the periplasmic extract of the host *Escherichia coli* strain. The overproduced enzyme showed the same catalytic characteristics as the wild-type enzyme.

Lactic acid bacteria (LAB) are widely used as starter cultures in industrial milk fermentation. Their proteolytic system is of fundamental importance for their growth in milk: indeed, milk is poor in small peptides and free amino acids (55) and so the proteolytic system supplies LAB with the nitrogen necessary for their proper growth by degrading the casein of milk. Proteolysis is also involved in the development of texture and flavor in dairy products. The proteolytic system of LAB and especially *Lactococcus lactis* has been broadly investigated (20, 39, 51, 54). Proteolysis is initiated by hydrolysis of casein by cell wall proteinases; the resulting oligopeptides are transported into the bacterial cell by an oligopeptide transport system (9, 15, 19, 21, 37, 59) and then broken down into amino acids by intracellular oligopeptidases, tripeptidases, dipeptidases, and aminopeptidases (34, 36). The enzymatic properties of the general aminopeptidase PepN and other peptidases from various *Streptococcus thermophilus* strains have been determined (8, 30, 33, 40, 42, 43, 56, 58), but only the cysteine aminopeptidase-encoding *pepC* gene has been cloned and sequenced (6). The large specificity of PepN from LAB (1, 3, 16, 17, 35, 52, 57), including *Streptococcus thermophilus* strains (33, 43, 56), has been demonstrated. Thus, PepN certainly plays an important role for bacterial growth in cheese by supplying the necessary free amino acids and probably later contributes to the development of texture and taste by producing precursors of flavor compounds. However, since the strains used in starter cultures in Switzerland have been shown to be different from those used elsewhere for the manufacture of dairy products (14), the identification of the PepN-encoding gene of *S. thermophilus* and the characterization of the corresponding enzyme are of interest for the Swiss dairy industry. In this study, we describe the characterization of the purified enzyme as well as the cloning, nucleotide sequence, and overexpression of the *pepN* gene encoding the general aminopeptidase N from *S. thermophilus* A, a strain which is widely used in the Swiss hard- and semihard-cheese industry.

* Corresponding author. Mailing address: Department of Biochemistry, Swiss Dairy Research Institute, Schwarzenburgstrasse 161, 3097 Liebefeld-Bern, Switzerland. Phone: 41-31-323-81-52. Fax: 41-31-323-82-27. E-mail: frederic.chavagnat@mbox.fam.admin.ch.

MATERIALS AND METHODS

Bacterial strains, media, plasmids, and oligonucleotides. *S. thermophilus* A was isolated from a mixed starter culture used in the Swiss dairy industry and supplied by the Federal Dairy Research Institute. It was grown at 38°C in LS5 medium (5) in which lactose was replaced by glucose. *Escherichia coli* Max Efficiency DH5α (Gibco BRL/Life Technologies, Inc.) and *E. coli* BL21 (Novagen) were used as hosts for recombinant plasmids. The *E. coli* transformants were grown at temperatures ranging from 28 to 42°C in Luria-Bertani (LB) broth containing, when required, 70 μg of ampicillin, 50 μg of kanamycin, and 50 μg of carbenicillin per ml. pUC18 (Gibco BRL) was used to subclone DNA fragments and to prepare double-stranded DNA for sequencing. pET12a is a vector for the T7 RNA polymerase-induced expression of recombinant proteins in *E. coli*; the *omp*T leader allows the expression product of a target gene cloned between *Sal*I and *Bam*HI sites to be exported into the periplasmic compartment. pET12a confers ampicillin resistance and was purchased from Novagen. pT7POL23, which confers kanamycin resistance, was used for the controlled expression of the target gene and was from Mertens et al. (29). Oligonucleotides were synthesized by Microsynth, Balgach, Switzerland.

Crude extract and enzyme assay. Bacterial cell disruption was performed as described elsewhere (5) but with a different buffer (30 mM sodium phosphate, pH 7.0; 50 mM NaCl; 5 mM $MgCl_2$) for cell suspension. After incubation with 15 U of RNase A (EC 3.1.27.5; Sigma) per ml for 30 min at 37°C and 1.7 U of DNase I (EC 3.1.21.1; Sigma) per ml for 30 min at 20°C, the crude extract was collected by centrifugation at 20,000 × *g* for 20 min. Protein concentration was estimated according to the method of Bradford (4) with bovine serum albumin as a standard. The standard enzyme assay for the determination of PepN activity was performed in 0.1 M sodium phosphate (pH 7.0) at 37°C on 118 μM Lys–7-amino-4-methylcoumarin (AMC) (Bachem) by a fluorimetric method (5). The sample volume was 2 to 100 μl, and the activity was expressed in micromoles of substrate hydrolyzed per minute per milligram of protein.

Purification of *S. thermophilus* A aminopeptidase PepN. The three chromatography purification steps were performed with a fast-protein liquid chromatography system from Pharmacia, Uppsala, Sweden.

(i) Chromatography on hydroxyapatite column. A Bio-Rad Bio-Gel HTP column (1.6 by 9.2 cm) was equilibrated with 30 mM sodium phosphate (pH

7.0)–50 mM NaCl–5 mM $MgCl_2$ and, after application of the crude extract (54 mg of protein from 2 liters of culture), the column was washed with the same buffer. The adsorbed aminopeptidase was eluted at 74 mM sodium phosphate–50 mM NaCl with a linear gradient (10 bed volumes) of sodium phosphate (pH 7.0), from 50 to 100 mM (flow rate, 60 ml · h^{-1}). The active fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 7.3)–300 mM NaCl.

(ii) Ion-exchange chromatography. Active fractions were applied to a MonoQ HR 10/10 column (Pharmacia), equilibrated, and washed with the same buffer. The adsorbed aminopeptidase was eluted at 398 mM NaCl by using a step-by-step NaCl gradient in 20 mM Tris-HCl (pH 7.3) (flow rate, 180 ml · h^{-1}). Active fractions were pooled and dialyzed against 50 mM sodium phosphate (pH 7.0)–140 mM NaCl.

(iii) Gel filtration chromatography. Active fractions were applied (flow rate, 30 ml · h^{-1}) to a Superdex 75 column (Pharmacia) equilibrated with the same buffer. The aminopeptidase was eluted in a sharp and symmetrical active peak (elution volume, 52 ml). After each step of purification, active fractions were analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE), and protein was estimated according to the Bradford method.

Assay for enzyme characterization. The effect of temperature on enzyme activity was determined in the same buffer as described above by using 118 μM Lys-AMC as a substrate. The effect of pH was determined in 0.1 M buffers, sodium acetate from pH 4 to 5.5, Tris-maleate from pH 5.5 to 8.0, and Tris-HCl from pH 7.5 to 8.8. For inhibition studies, the enzyme was incubated in the presence of inhibitors at 37°C for 20 min and, when necessary, for a second incubation (also at 37°C for 20 min) in the presence of a metallic ion to study a putative restoration of activity. The relative activity was expressed as a percentage of the noninhibited PepN activity. Aminopeptidase specificity was studied with several amino acid-AMC substrates, and the activity was expressed as a percentage of the PepN activity with Lys-AMC. Activity of purified PepN was also tested with several peptides (2.5 mM) at 37°C in the same buffer. Samples (10 μl) were taken at various intervals, and the enzymatic reaction was stopped by cooling to 4°C and by the addition of 20 μl of 0.2 M Tris-HCl (pH 9.0). The mixture and control samples were spotted onto a precoated 25-cm-thick silica gel 60 plate (20 by 20 cm), and thin-layer chromatography was performed in *n*-butanol–acetic acid–water (40:20:20), in *n*-propanol–25% amonium hydroxide–water (60:30:10), or in *n*-butanol–water–pyridine–acetic acid (15:12:10:3). The chromatogram was visualized under UV light after being stained with 0.04% fluorescamine in 99% acetone.

SDS-PAGE. SDS-PAGE (10%) was carried out according to the method of Laemmli and Favre (22). Gels were stained with PAGE blue 83.

Amino acid sequencing. For amino acid sequence determination, purified PepN (8 μg) was electroblotted onto an Immobilon polyvinylidene difluoride (PVDF) Millipore membrane by using an LKB Multiblot apparatus as described by Matsudaira (26). From membrane strips, the N-terminal amino acid sequence of the enzyme was determined by amino acid microsequencing on an Applied Biosystems 473A Sequencer. Two internal peptides, P1 and P2, were obtained by proteolysis at 35°C of the enzyme with 0.4 μg of endoprotease-Lys-C for 18 h and separated by DEAE-C_{18} high-performance liquid chromatography with an acetonitrile–0.1% trifluoroacetic acid gradient. The N-terminal amino acid sequences of P1 and P2 were determined by amino acid microsequencing on an Applied Biosystems Procise Sequencer. The N-terminal sequences of PepN and P1 and P2 peptides were used to synthesize nucleotides O, O1, and O2, respectively (see Results).

***S. thermophilus* A genomic DNA preparation.** Bacterial cells pelleted from 10 ml of culture were suspended in 10 ml of 100 mM sodium borate buffer (pH 8.0)–10 mM EDTA–25% sucrose and incubated at 37°C for 1 h in the presence of 2 mg of lysosyme per ml. Spheroplasts were collected at 4°C by centrifugation at 4,430 × *g* for 10 min, suspended in 20 mM EDTA (pH 7.0), and incubated at room temperature in the presence of 1% SDS for 15 min (final volume, 5 ml). Then, 1.25 ml of 5 M sodium perchlorate and 3 ml of choroform (4% isoamyl alcohol) were added and mixed by inverting the mixture several times. After a 15-min incubation at room temperature, the upper phase was collected by centrifugation at 10,000 × *g* for 10 min, and the DNA was precipitated with 3 ml of 2-propanol, incubated at room temperature for 15 min, collected by centrifugation at 10,000 × *g* for 10 min, and redissolved in 200 μl of TE buffer. After treatment with 50 μg of RNase A DNase-free (Serva 34390) at 37°C for 30 min and subsequent incubation with 0.6 mAnson units of proteinase K (Merck 1.07393) at 37°C for 30 min, DNA was precipitated with ice-cold alcohol, pelleted at 4°C by centrifugation at 10,000 × *g* for 10 min, and resuspended in 200 μl of TE buffer.

PCR amplification. Mixtures (100 μl) containing DNA template (1 to 2 μg), deoxynucleotide triphosphates (20 nmol of each), and oligonucleotide primers (0.5 to 1 μg of each) were treated with Vent DNA polymerase (2 U) in Vent buffer (New England Biolabs) or with Expand High Fidelity PCR system (2 U) in Expand HF buffer (Boehringer), both in the presence of 1.5 mM Mg^{2+}. The reactions were carried out in a GeneAmp PCR system 9600 (Perkin-Elmer) for 30 cycles, each with a 1-min denaturation at 94°C, a 1-min annealing at a $T_m - T_m - 5$°C, and a 180-s extension at 72°C. The final elongation step at 72°C was for 10 min. The T_m values of the primers were calculated by using Primer Analysis Software OLIGO version 4.1 (National Biosciences, Hamel, Minn.). The PCR products were purified by using the EasyPrep PCR Product Prep Kit (Pharmacia Biotech) or extracted from an agarose gel with the Sephaglas BandPrep Kit (Pharmacia Biotech).

TABLE 1. Amino acid sequencing data of PepN from *S. thermophilus* A

Peptide	N-terminal amino acid sequence	Amino acid positions
PepN	TASVARFIES	2–11
Peptide P1	TFTGNVAITG	29–38
Peptide P2	ALERNILMGI	814–823

Plasmid preparation and nucleotide sequencing. Plasmids were extracted from *E. coli* cells by the alkaline lysis method (2) and purified by using the FlexiPrep Kit (Pharmacia Biotech). Recombinant plasmids were constructed and agarose gel electrophoresis was performed according to the method of Sambrook et al. (44). Restriction enzymes (Boehringer; NEB), T4 DNA ligase (NEB), and other nucleic acid-modifying enzymes were used with the buffers provided and under the conditions recommended by the suppliers. DNA segments cloned into pUC18 were sequenced by the dideoxynucleotide chain termination method (45) with the M13 universal and reverse oligonucleotides or synthetic oligonucleotides as primers. Denaturation of double-stranded DNA was performed as described by Zhang et al. (64). Sequencing reactions were carried out by using the T7 sequencing kit (Pharmacia LKB Biotechnology) with [^{35}S]dATP labelling (Amersham). For computer-assisted sequence analysis, the PC-Gene (Intelli Genetics) and OMIGA 1.0 (Oxford Molecular Group) softwares were used.

Inverse PCR. *S. thermophilus* A genomic DNA was digested by *Ava*I under the conditions recommended by the supplier and used as a template (500 ng) in an inverse PCR (47).

Overexpression of *pepN* gene. *Hpa*II and *Bam*HI sites were created by PCR at the 5′ and 3′ ends, respectively, of *pepN*. The DNA Polymerase I Klenow fragment was used to fill-in the 3′ recessed end of *Sal*I-digested pET12a and the 5′ recessed end of *Hpa*II-digested *pepN*. After digestion with *Bam*HI, *pepN* was cloned into pET12a by ligation of the blunt ends on the one hand and the *Bam*HI cohesive ends on the other hand. In the resulting pET-*pepN* recombinant vector, the *pepN* sequence was in the open reading frame of the *ompT* leader. The pair supercoiled pET-*pepN*/pT7POL23 was used to transform *E. coli* BL21 as described by Sambrook et al. (44). *E. coli* BL21 pET-*pepN*/pT7POL23 cells were grown with vigorous aeration at 28°C in LB broth containing 50 μg of carbenicillin and 50 μg of kanamycin per ml until an optical density at 600 nm of 0.6 was obtained. Target gene expression was induced by a rapid increase of culture temperature to 42°C, and growth was continued for 20 h at this temperature.

Purification of the overproduced PepN aminopeptidase. Bacterial cells were pelleted at 4°C by centrifugation at 5,000 × *g* for 10 min, and the periplasmic extract was prepared as described by Linquist et al. (24). The purification of the overproduced PepN enzyme involved the two first chromatography steps used for the PepN purification, and elution of the overproduced PepN occurred exactly under the same conditions as for PepN.

Sequence accession number. The nucleotide sequence of the *pepN* gene from *S. thermophilus* A and the encoded amino acid sequence have been deposited in the EMBL nucleotide database under accession number AJ007700.

TABLE 2. Effect of inhibitors on the activity of the purified PepN enzyme of *S. thermophilus* A

Reagent	Final concn (mM)	Relative activity (%)
Control		100
Iodoacetic acid	1	96
Phenylmethylsulfonyl fluoride	1	98
EDTA	0.1	14
EDTA	1	10
EDTA	10	0
o-Phenanthroline	0.1	2
o-Phenanthroline	1	0
Ca^{2+}	1	100
Co^{2+}	1	100
Cu^{2+}	1	38
Mg^{2+}	1	97
Mn^{2+}	1	98
Zn^{2+}	1	95

TABLE 3. Effect of bivalent ions on the activity of the purified PepN enzyme of *S. thermophilus* A inactivated with 0.1 mM EDTA

PepN	Added ion (1 mM)	Relative activity (%)
Control		100
Inactivated		14
Inactivated	Ca^{2+}	45
Inactivated	Co^{2+}	80
Inactivated	Cu^{2+}	15
Inactivated	Mg^{2+}	12
Inactivated	Mn^{2+}	98
Inactivated	Zn^{2+}	87

RESULTS

Purification of PepN aminopeptidase. PepN aminopeptidase produced by *S. thermophilus* A was purified to protein homogeneity from the crude extract by a three-step chromatography procedure. The yield was 54 μg of purified enzyme, the activity yield was 25.2%, and the purification factor was 243-fold. Peptides P1 and P2, produced by endoprotease-Lys-C, were isolated. Table 1 shows the N-terminal amino acid sequences of PepN and of peptides P1 and P2. The N-terminal methionine was absent from the mature form of PepN.

Properties of PepN. The molecular mass of PepN was estimated by SDS-PAGE to be approximately 95 kDa, both under reductive and nonreductive conditions, suggesting that PepN had a monomeric structure. The optimal temperature of PepN was 37°C; the relative activity was >95% between 34 and 40°C and ca. 50% at 17 or 53°C. The optimal pH was 7.0, and PepN is active at between pH 4.5 and 9. Several inhibitors were tested (Table 2); neither 1 mM iodoacetic acid nor 1 mM phenylmethylsulfonyl fluoride had any effect on PepN activity. However, EDTA and *o*-phenanthroline strongly or completely inhibited PepN activity. Among the bivalent cations directly tested, such as Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, only Cu^{2+} exhibited partial inhibition (relative activity, 38%) of PepN activity, and none induced an increase of PepN activity. After strong inhibition of PepN (relative activity, 14%) by 0.1 mM EDTA (Table 3), PepN activity was partially restored by Ca^{2+} and largely restored by either Co^{2+}, Zn^{2+}, and Mn^{2+} (relative activity, 80, 87, and 98%, respectively). Both the crude extract and purified PepN were incubated for 30 days at pH 7.0 or 5.5 at both 20 and 14°C, and the residual activity was determined at the pH and temperature of incubation. The residual activities of purified PepN incubated at pH 7.0 at 20 and 14°C were 85 and 89%, respectively. The residual activities of crude extracts incubated at pH 7.0 at 20 and 14°C were 97 and 98%, respectively, whereas the residual activities of crude extracts incubated at pH 5.5 at 20 and 14°C were both 91%. In 20 mM Tris-HCl (pH 7.3) buffer, the activity of purified PepN depends on the NaCl concentration. The activity is 2.3 times higher in the presence of between 100 mM and 280 mM NaCl than without NaCl. At 500 mM NaCl the activity is still twice as high as that in the absence of the salt.

PepN activity and nitrogen source for *S. thermophilus* A growth. *S. thermophilus* A was grown in LS5 or in LS5 where Bacto-Tryptone was replaced by the equivalent of free amino acids, and the PepN activity of crude extracts was measured. In the presence of free amino acids the PepN activity was only 49% of that in the presence of peptides.

Specificity of PepN. Several amino acid-AMCs were tested as substrates. Lys-AMC appeared to be the best substrate for PepN, whereas Pro-AMC, Gln-AMC, Glu-AMC, and Gly-AMC were not degraded (Table 4). With the other peptides tested, di-, tri-, tetrapeptides and Met-enkephalin (Tyr-Gly-Gly-Phe-Met), the aminopeptidase specificity of PepN was confirmed. However, X-Pro bonds were not cut by PepN, and the PepN activity on X-Gly dipeptides was lower than when glycine was replaced by another amino acid (Table 4). PepN exhibited no degrading activity on bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg).

Gene cloning and sequencing: amino acid sequence of *S. thermophilus* A PepN. The resulting nucleotide sequence of the *pepN* gene and its flanking regions, as well as the translated amino acid sequence of PepN from *S. thermophilus* A, are shown in Fig. 1. The strategy of gene cloning involved the generation of three PCR products. The degenerate primers O (coding for the N-terminal amino acid sequence of PepN [Table 1]) and O2 (complementary to the encoding sequence of the internal peptide P2 [Table 1]) were used for a first amplification with *S. thermophilus* A genomic DNA as a template. The nucleotide sequence of both strands was determined. This resulted in the sequence from nucleotide 194 to nucleotide 2643. The second product was amplified by inverse PCR with the *Ava*I-digested genomic DNA as a template and the oligonucleotides iO3 (nucleotides [nt] 2554 to 2573) and iO4 (complementary to nt 231 to 250) as primers. From this second product, the nucleotide sequences 1 to 250 and 2554 to 2910

TABLE 4. Substrate specificity of the purified PepN enzyme from *S. thermophilus* A

Substrates	Relative activity (%)	Substrate	Relative activity (%)	Hydrolyzed substrates[a]	Nonhydrolyzed substrates
Lys-AMC	100	Gly-Arg-AMC	<1	Lys-▲Tyr	Glu-Lys
Leu-AMC	93				
Arg-AMC	80	Pro-Lys-AMC	<1	Leu-▲Leu	Lys-Pro
Met-AMC	28	Pro-Phe-Arg-AMC	0	Leu-▲Arg	Leu-Pro
Ala-AMC	20			Leu-▲Tyr	Arg-Pro
Phe-AMC	12	bz-Phe-Val-Arg-AMC	0	Leu-△Gly	
Tyr-AMC	7	suc-Ala-Phe-Lys-AMC	0		Arg-Pro-Pro
Ser-AMC	3			Met-▲Leu	
Val-AMC	1			Tyr-▲Leu	Tyr-Pro-Leu-Gly
Pro-AMC	0				
Gln-AMC	0			Leu-▲Leu-▲Leu	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Glu-AMC	0				
Gly-AMC	0			Ala-▲Leu-▲Ala-△Gly	
				Tyr-▲Gly-Gly-Phe-Met	

[a] Solid black arrows indicate bonds cut by PepN. White arrows indicate slower hydrolysis.

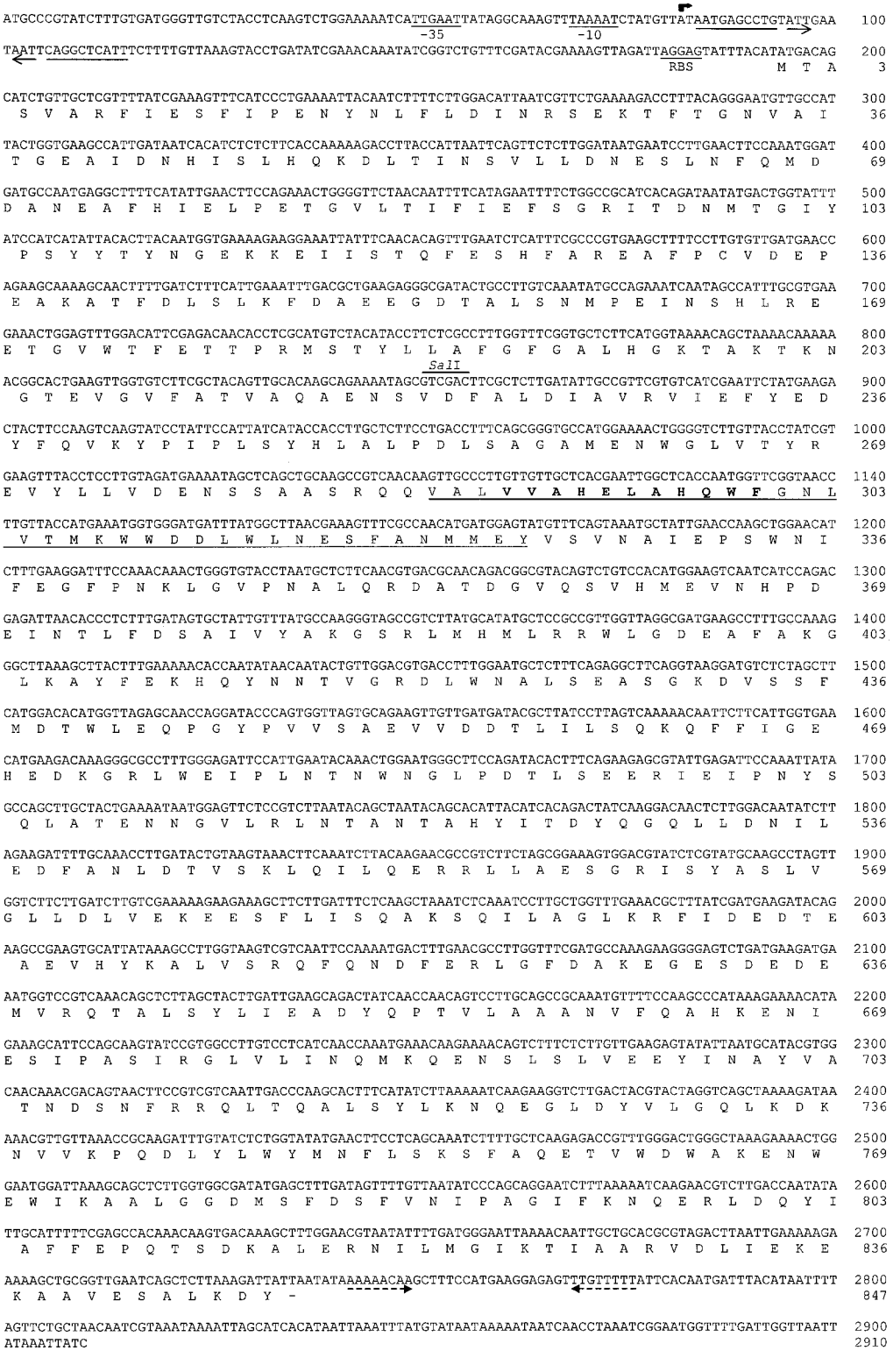

FIG. 1. Nucleotide sequence and deduced amino acid sequence of *pepN* from *S. thermophilus* A. In the nucleotide sequence, a putative RBS is indicated. The −10 and −35 regions are also indicated. The two horizontal arrows show an inverted repeat sequence. The bent arrow indicates the putative transcriptional start site. The two horizontal dotted arrows indicate the putative transcriptional terminator. In the amino acid sequence, the zinc-metallopeptidase signature is in boldface type. The underlined sequence indicates the highly conserved region, including the catalytic site, as defined by computer alignment of the amino acid sequence of PepN from *S. thermophilus* A with those of other PepN enzymes from *L. lactis* subsp. *lactis* (52) (S39955), *L. lactis* subsp. *cremoris* Wg2 (49) (X61230), *L. delbrueckii* subsp. *lactis* DSM7290 (17) (Z21701), *L. helveticus* 53/7 (60) (Z30323), and *L. helveticus* CNRZ32 (7) (U08224).

were determined on both strands. The full sequence was confirmed on the third product amplified by PCR by using genomic DNA as a template and oligonucleotides O19 (nt 1 to 20) and O20 (complementary to nt 2882 to 2910) as the primers.

The 2,541-nt open reading frame started with an ATG codon at position 194 and terminated with a TAA stop codon at position 2735. A putative ribosome-binding site (RBS) occurred 9 bp upstream from the start ATG codon with a pentanucleotide sequence AGGAG complementary to the 3′ end of *S. thermophilus* 16S rRNA (GenBank sequence accession number X68418). About 120 bp upstream from the RBS, the TTGAat and TAaAAT sequences located at positions 50 and 69, respectively, probably correspond to the −35 and −10 regions of a promoter. The putative +1 transcriptional start site was located 8 bp downstream from the −10 region and was immediately followed by a 32-bp region with an almost perfect palindromic structure. The stem-loop area located at position 2742 might be a putative transcription terminator.

The 2,541-nt sequence of *pepN* gene encoded an 847-amino-acid protein (Fig. 1) with a theoretical molecular mass of 96,252 Da and a pI of 4.34. Sequence alignment analysis by the Myer and Miller method of PepN from *S. thermophilus* A showed 55.5 and 55.3% identities with the corresponding sequences from *L. lactis* strains (49, 52), 48.3 and 48% identities with those from *Lactobacillus helveticus* strains (7, 60), and 47.9% identity with that from a *Lactobacillus delbrueckii* strain (17). Multialignment analysis performed with the CLUSTAL program showed a 34.1% identity and a 37% of similarity between the amino acid sequences of the six PepN enzymes. The signature sequence of neutral zinc metallopeptidases was recovered in each of them (Fig. 1).

Overproduction of PepN, purification, and properties of the overproduced enzyme. The production of overproduced PepN by the (carbenicillin- and kanamycin-resistant) transformants was performed. The water-soluble and active overproduced PepN was purified to protein homogeneity from the *E. coli* periplasmic extract by a two-step chromatography procedure. The specific activity (11.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was similar to that of the purified enzyme from *S. thermophilus* A (11.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$). From 1 liter of culture, the activity yield was 87%, and the yield was 12.3 mg of purified overproduced PepN. Compared to 27 μg of PepN production by 1 liter of *S. thermophilus* A culture, the overproduction factor was 452-fold. Overproduced PepN activity was studied by using the same substrates as for PepN and showed that both enzymes exhibited the same properties, specificity, and susceptibility to inhibitors (data not shown).

DISCUSSION

The aminopeptidase PepN from the *S. thermophilus* A strain used in the Swiss dairy industry was purified and characterized, and the encoding gene *pepN* was cloned, sequenced, and overexpressed in *E. coli*. PepN from *S. thermophilus* A has characteristics in common with those from other LAB, such as *L. lactis* (10, 49, 50, 52), *L. helveticus* (7, 16, 35, 60), *L. delbrueckii* (1, 3, 17, 57), and *S. thermophilus* (33, 43, 56, 57). It is a metallopeptidase strongly inhibited by metal-chelating agents such as EDTA and *o*-phenanthroline, and it is insensitive to serine-protease inhibitors but is inhibited by Cu^{2+}. It has a monomeric structure and a molecular mass of 95 kDa, a theoretical pI value of 4.34, and an optimal activity at pH 7.0. The optimal temperature at 37°C is similar to that of PepN from other *S. thermophilus* (33, 43, 56, 57) and *L. lactis* (10, 50) strains. The optimal temperature of PepN from lactobacilli is usually between 40 and 55°C.

PepN from *S. thermophilus* A, like PepN enzymes from *L. delbruecki* or other *S. thermophilus* strains, except NCDO573 (33), is not inhibited by sulfydryl group inhibitors and thus no thiol radical seems to be involved at its catalytic site. Except for Cu^{2+}, which also inhibits PepN from other LAB (3, 16, 33, 35, 43, 50, 56, 57), other bivalent cations have no effect on PepN from *S. thermophilus*, whereas they have various effects, from strong inhibition to strong activation, on PepN enzymes from other LAB. After inhibition by metal-chelating agents, only PepN enzymes from *S. thermophilus* A and *L. helveticus* (17) are largely restored by either Co^{2+}, Mn^{2+}, or Zn^{2+}. The activity is also partially restored by Ca^{2+}.

All PepN enzymes from LAB are intracellular. The cytoplasmic localization of PepN from *S. thermophilus* has been described previously (31), and the sequence analysis of the PepN enzyme from *S. thermophilus* A revealed neither a signal peptide nor a transmembrane domain. In the 5′ flanking region of the *pepN* gene, the putative transcription initiation site is closely followed downstream by a quasi-perfect 32-bp inverted repeat sequence, suggesting an operator-type structure. This suggests a repressor binding structure for the control at the transcription initiation level of *pepN* gene expression of *S. thermophilus* A. When *S. thermophilus* A was grown in a medium containing amino acids instead of peptides as a nitrogen source, lower PepN activity was detected in the crude extracts. This poorer PepN production might be explained by lower *pepN* gene expression due to increased transcription control. This should be investigated further since the existence of such a regulation mechanism has been reported for *prtP* and *prtM* genes from *L. lactis* SK11 (25) but has not been observed for other LAB *pepN* genes whose structure has been determined.

By multialignment analysis, the amino acid sequence of PepN from *S. thermophilus* A was compared with those of PepN enzymes from *L. lactis* (49, 52), *L. helveticus* (7, 60), and *L. delbrueckii* (17). The signature sequence of neutral zinc metallopeptidases, with the zinc-binding domains at the histidine positions and the putative catalytic site at the glutamic acid position, was found in one of the most conserved regions of the six PepN enzymes. The most conserved region of 38 amino acids of PepN from *S. thermophilus* A (V_{287} to Y_{324}) shows 87% identity with the corresponding regions of other LAB, 76% identity with those of the mouse (63) and rabbit (38), 74% identity, with that of *Saccharomyces cerevisiae* APE2 (12), 63% identity with that of insects (18), and 34% identity with that of *E. coli* (27). It would seem that the PepN enzyme from *E. coli* and LAB PepN enzymes have diverged during evolution, whereas mammalian and insect PepN enzymes are more closely related to LAB PepN enzymes, even though they are larger and possess a membrane-associated domain.

The high homogeneity of the LAB PepN family is indicated by sequence homology scores; indeed, there is 87% sequence identity in the most conserved region containing the catalytic site and up to 72% identity of the full sequence. However, it should also be pointed out that, first of all, PepN enzymes from *S. thermophilus* A and *L. lactis* exhibit 55% sequence identity and second, PepN enzymes from *L. helveticus* and *L. delbrueckii* exhibit 72% sequence identity, but enzymes from the first two only exhibit 48% sequence identity with those of the latter. This distinction between lactobacilli and *S. thermophilus* together with *L. lactis* was also observed when the optimal temperature of PepN enzymes was used and has already been suggested in a study of 16S rRNA (48) and the 72% sequence homology observed between the PepC enzymes from *S. thermophilus* and *L. lactis* (6).

With regards to the putative thiol radical involved in the catalytic mechanism of PepN from *L. lactis* and *L. helveticus*, no immediate explanation is provided with multialignment sequence analysis. Indeed, none of the three cysteine residues of PepN from *L. lactis* shares a common position with either of the two cysteines of PepN from *L. helveticus*.

The *pepN* gene was cloned in pET12a vector so as to permit the product of expression to be exported into the periplasm. Consequently, the N-terminal end of the overproduced PepN is STASVAR- instead of TASVAR- for PepN. In spite of this, the activity characteristics and specificities of the overproduced PepN enzyme were found to be similar to those of PepN. The N-terminal amino acid seems to have no effect on PepN activity.

The activity of PepN from *S. thermophilus* A was studied on both amino acid-AMC derivatives and small peptides. PepN from *S. thermophilus* A, like those from most of LAB, does not exhibit significant endopeptidase activity but has a broad specificity for the N-terminal amino acid of small peptides, except for proline, glycine, and acidic amino acids or when a proline residue is present at the penultimate position.

Peptidases in LAB, used as starter cultures in the dairy industry, are important for breaking down casein-derived peptides (21, 34) to supply free amino acids to bacterial cells for their growth in milk. During the manufacture of Swiss cheeses, *S. thermophilus* in starter cultures is the first bacterium to grow and is responsible for the early acidification of milk. Therefore, because of its broad amino acid specificity, PepN activity is of foremost importance because it supplies most of the free amino acids for *S. thermophilus* growth, whereas the acidic residues are most probably provided by the aminopeptidase PepA (42), and proline may be provided by the combined action of several enzymes, the X-prolyl-dipeptidyl-aminopeptidase (30, 58), and several proline-specific peptidases (41).

Later on during cheese ripening, after bacterial cell lysis, intracellular peptidases are released into the cheese medium, where peptide degradation to produce free amino acids contributes to cheese ripening. Indeed, it has been demonstrated elsewhere (23, 28, 53) that hydrolysis of casein-originating peptides by peptidases, and particularly PepN, is involved in a decrease of bitterness. Moreover, the addition of appropriate levels of amino acids and particularly of methionine may improve flavor development in cheeses (61). PepN from *S. thermophilus* certainly plays an important role in Swiss cheese manufacture: first, because of its wide amino acid specificity for debittering, and second, for its release of free amino acids such as phenylalanine, methionine, leucine, and tyrosine, which are precursors of components involved in flavor and taste.

The contribution of peptidases and particularly PepN from LAB to flavor development during cheese ripening is supported by the stability of PepN under cheese-ripening conditions. Indeed, it was demonstrated in the presence of NaCl and reduced pH in cheddar cheese (62) that the main residual aminopeptidase activity is due to PepN and PepC. In Gruyère cheese (32), aminopeptidase activities from *S. thermophilus* were detected throughout the cheese-ripening period. The aminopeptidase was not identified, but it is certainly due to PepN since there were leucine-aminopeptidase, arginine-aminopeptidase, and phenylalanine-aminopeptidase activities. In another study on Emmental cheese (11), it was suggested that aminopeptidases from thermophilic starters degraded peptides during ripening to produce free amino acids and that PepN may be involved in the production of Lys, Leu, Val, and Ala.

In our study, the activity and stability of the PepN enzyme from *S. thermophilus* A were tested in vitro under conditions of temperature (20 and 14°C) and pH (5.5) similar to those encountered during Swiss-type cheese ripening. PepN was still active at low pH (4.5), and the relative activity at 15°C was >50% of the optimal activity. The stability of purified PepN at cheese-ripening temperatures for 30 days was demonstrated, as well as its stability in crude extracts of *S. thermophilus* A at cheese-ripening pH and temperatures. These in vitro data concerning PepN from *S. thermophilus* A substantiate the above-mentioned studies made in cheese, suggesting that PepN activity from LAB participates in peptide degradation to free amino acids throughout the cheese-ripening process and thus is involved in flavor and taste development. Furthermore, the increased activity of purified PepN from *S. thermophilus* A in the presence of NaCl makes this enzyme very interesting for cheese ripening.

Because of its significant peptidase activity, *S. thermophilus* is certainly predominant in proteolysis during cheese ripening, and PepN plays a major role in this process. Thus, this species is of great importance to the dairy industry, and particularly for the manufacture of hard cheeses since it is common to starter cultures. Its potential use for debittering of cheese and the development of flavor and taste should be considered in the manufacture of other cheeses, as illustrated elsewhere (13).

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