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Original article

Purification and characterization of the X-prolyl-dipeptidyl aminopeptidase (PepX) from *Streptococcus macedonicus* and cloning of the *pepX* gene

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Abstract – The X-prolyl-dipeptidyl aminopeptidase from *Streptococcus macedonicus* ACA-DC 191 was purified by anion exchange and hydrophobic interaction chromatography. A single band of a molecular mass of about 84 000 g·mol^{-1} appeared in SDS-PAGE; by gel filtration it was shown that the native enzyme was dimeric. The enzyme showed optimum activity on glycyl-prolyl-4-nitroanilide at pH 7.0, with a K_M = 0.42 mmol·L^{-1} and a V_{max} = 12.8 µmol·mg^{-1}·min^{-1}. It was active over a temperature range of 10–60 °C. Over 60 °C, the enzyme activity declined rapidly. The peptidase was completely inactivated by PMSF, DTNB and Cu^{2+}, while metal chelators had no effect on enzyme activity. By using the PCR technique with synthetic primers, the *pepX* gene was amplified, cloned and sequenced. This 2 289 nucleotide gene encodes a protein of 763 amino acids with a molecular mass of 86 866 g·mol^{-1}. The deduced amino acid sequence analysis of the *pepX* gene shows a high identity with PepX enzymes from other lactic acid bacteria and contains a motif around the active site serine (G-K-S-Y-L-G) that is well conserved among the PepX enzymes.

***Streptococcus macedonicus* / X-prolyl-dipeptidyl aminopeptidase / PepX purification / enzyme characterization / cloning**

Résumé – **Purification et caractérisation de la X-prolyl-dipeptidyl aminopeptidase (PepX) de *Streptococcus macedonicus* et clonage du gène *pepX*.** Une X-prolyl-dipeptidyl aminopeptidase extraite de *Streptococcus macedonicus* ACA-DC 191 a été purifiée par chromatographie échangeuse d'anions et chromatographie d'intéractions hydrophobes. Une seule bande de masse moléculaire de 84 000 g·mol^{-1} a été detectée sur SDS-PAGE ; par chromatographie d'exclusion de taille il a été démontré que l'enzyme native était dimérique. L'enzyme a une activité maximale sur le substrat glycyl-prolyl-4-nitroanilide à pH 7,0, avec K_M = 0,42 mmol·L^{-1} et V_{max} = 12,8 µmol·mg^{-1}·min^{-1}. Elle est active dans une gamme de températures de 10–60 °C. Au-dessus de 60 °C, l'activité enzymatique

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diminue rapidement. La peptidase est complètement inactivée par le PMSF, le DTNB et les ions Cu^{2+}, mais des chélateurs de métaux n'ont aucun effet sur l'activité enzymatique. En utilisant la technique de PCR avec des primers synthétiques, le gène *pepX* a été amplifié, cloné et séquencé. Ce gène, de 2 289 nucléotides code pour une protéine de 86 866 $g \cdot mol^{-1}$. La séquence en acides aminés déduite du gène *pepX* présente une forte identité avec les enzymes PepX des autres bactéries lactiques. La séquence entourant la sérine du site actif (G-K-S-Y-L-G) est assez bien conservée chez les enzymes PepX.

***Streptococcus macedonicus* / X-prolyl-dipeptidyl aminopeptidase / purification de PepX / caractérisation / clonage**

1. INTRODUCTION

Lactic acid bacteria possess a large number of metabolic properties, which are responsible for their successful use as starter cultures in the food and feed industry, and as probiotics and dietary additives for nutritional and health purposes [11, 13, 18, 30, 48, 49]. They have limited abilities to synthesize amino acids, which are essential for their growth, and milk contains insufficient amounts of free amino acids and low molecular mass peptides to sustain growth [1, 25, 50]. Although they are considered as weak proteolytic bacteria compared with other groups of microorganisms, it has been shown that lactic acid bacteria possess a complex proteolytic system capable of hydrolyzing milk proteins to peptides and amino acids [23, 37, 41, 43, 45]. Furthermore, it is generally accepted that their proteolytic system contributes to the degradation of milk proteins and hence to the texture, taste and aroma of dairy fermented products [2, 15, 33, 47].

During a survey of the lactic acid bacterial flora of naturally fermented Greek Kasseri cheese, a group of 26 strains phenotypically assigned to *Streptococcus thermophilus* was isolated. SDS-PAGE analysis of whole-cell proteins revealed that the group was quite different from *S. thermophilus*. Comparative 16S and 23S rRNA sequence analyses showed that the isolates represented a new species within the genus *Streptococcus*, which was named *S. macedonicus* [52]. Strains of *S. macedonicus* were screened for technologically important metabolic properties, among them their ability to degrade milk casein [17]. One of the most interesting findings was that, compared with all the other peptidases examined, the X-prolyl-dipeptidyl aminopeptidase was the dominant peptidase activity determined for the majority of the strains. *S. macedonicus* does not seem to possess the rich pool of peptidases of *S. thermophilus*, the peptidase system of which appears to be as complex as that of lactococci or even more [45]. However, the prevailing X-prolyl-dipeptidyl aminopeptidase in the *S. macedonicus* strains is of great technological interest, since milk casein is rich in proline and this activity could be important in view of using these strains in milk fermentations.

There are several reports in the literature on the biochemical characterization of the X-prolyl-dipeptidyl aminopeptidase activity from various lactic acid bacteria [4, 6–8, 12, 16, 20, 21, 28, 34, 39, 51, 53, 55, 57]. Additionally, there are reports concerning the characterization of PepX-deficient mutants and the sequence analysis of the *pepX* gene from lactic acid bacteria. The nucleotide sequences of the *pepX* genes from *Lactococcus lactis* [32, 42], *Lactobacillus delbrueckii* ssp. *lactis* [35], *Lb. helveticus* [54, 56], *Lb. rhamnosus* [53], *S. pyogenes* SF370 [14] and *S. gordonii* (Goldstein J.M., Banbula A., Kordula T., Mayo J.A., Travis J., unpublished sequence in GenBank. Accession number: AY032733, 2001) have been determined.

The present work describes the purification and biochemical characterization of the X-prolyl-dipeptidyl aminopeptidase from *S. macedonicus* ACA-DC 191, as well as the cloning and sequence analysis of the corresponding gene, designated *pepX*.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

S. macedonicus ACA-DC 191 was obtained from the ACA-DC Collection of the Laboratory of Dairy Research at the Agricultural University of Athens, Greece. It was isolated from Greek Kasseri cheese and stored at –30 °C in sterile skim milk (10% w/v). Before experimental use, the strain was subcultured twice in milk and finally in MRS broth (Biokar Diagnostics, Beauvais, France) at 37 °C. Growth was assessed by measurement of pH and the optical density at 600 nm. *L. lactis* ssp. *lactis* MG1363 carrying the plasmid pBM329 [32] was kindly provided by Prof. J. Kok, University of Groningen, The Netherlands. It was grown at 30 °C in M17 broth supplemented with 0.5% (w/v) glucose and 5 μg·mL^{-1} erythromycin. The 9.7 kb pBM329 plasmid contains the *pepX* gene from *L. lactis* ssp. *cremoris* P8-2-47. The *Escherichia coli* TOP10F' competent cells supported with the AdvanTAge™ PCR Cloning Kit (Clontech, Palo Alto, CA, USA) were used for transformation according to the manufacturer's instructions.

2.2. Purification of the enzyme

Late logarithmic phase cells (9 h) of *S. macedonicus* ACA-DC 191 were collected from the growth medium (1 L) by centrifugation (12 000 *g*, 10 min, 4 °C). The pellet obtained was washed twice with 0.9% NaCl. Subsequently, it was resuspended in 50 mmol·L^{-1} Tris-HCl buffer, pH 8.5, containing 2 mg·mL^{-1} lysozyme, and incubated for 2 h at 37 °C. The supernatant obtained after centrifugation (12 000 *g*, 10 min, 4 °C) was designated the crude cell-free extract. The crude cell-free extract was firstly applied on a DEAE-cellulose column (2 × 8 cm), equilibrated with 50 mmol·L^{-1} Tris-HCl buffer, pH 8.5. Elution was performed at a flow rate of 2 mL·min^{-1} with a linear gradient of 0–0.5 mol·L^{-1} NaCl in the same buffer. The active fractions were pooled, properly diluted and applied on a Resource Q column (16 × 30 mm), equilibrated with 50 mmol·L^{-1}. Tris-HCl buffer, pH 8.5, and subsequently on the same column, this time equilibrated with 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0. Elution was performed under the same conditions as before. The last purification step was performed on a Phenyl Superose HR 5/5, equilibrated with 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0, containing 1.5 mol·L^{-1} $(NH_4)_2SO_4$, at a flow rate of 0.5 mL·min^{-1} with a linear gradient of 1.5–0 mol·L^{-1} $(NH_4)_2SO_4$ in the same buffer. The active fractions were pooled, dialyzed against 50 mmol·L^{-1} phosphate buffer, pH 7.0, and used for the enzyme characterization.

2.3. Enzyme assay

Enzyme solution (50 μL) and glycyl-prolyl-4-nitroanilide (50 μL; 20 mmol·L^{-1} in methanol) were incubated in 400 μL 50 mmol·L^{-1} phosphate buffer, pH 7.0, at 50 °C. In all cases, activity was assayed at 410 nm by measuring in a continuous assay the degree of substrate hydrolysis. A unit of enzyme activity was defined as the amount of the enzyme producing 1 μmol 4-nitroaniline per min (A_{410} = 8 800 L·mmol^{-1}·cm^{-1}). Specific activity was defined as the number of units per mg protein.

Recombinant *E. coli* colonies were screened for PepX activity by an enzymatic plate assay, using glycyl-prolyl-4-methoxy-β-naphthylamide as substrate [42].

2.4. Protein determination

The method of Lowry et al. [29] was used with bovine serum albumin as a standard.

2.5. Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (10% acrylamide gel; [24]) was used to control the purification steps and to determine the molecular mass of the purified enzyme. Myosin (205 000 g·mol^{-1}), α-galactosidase (116 000 g·mol^{-1}), phosphorylase b (97 400 g·mol^{-1}), bovine serum albumin (66 000 g·mol^{-1}), egg albumin (45 000 g·mol^{-1}) and carbonic anhydrase (29 000 g·mol^{-1}) were used as marker proteins. The same electrophoretic system, but in the absence of SDS and β-mercaptoethanol, was used for the post-electrophoretic detection of the enzyme, using glycyl-prolyl-4-methoxy-β-naphthylamide as substrate [38].

2.6. Molecular mass determination

The purified enzyme was filtered on a Sephacryl S-300 16/60 column equilibrated with 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0, containing 0.1 mol·L^{-1} KCl, at a flow rate of 0.5 mL·min^{-1}. The column was standardized with the following proteins of known molecular mass: α-amylase (200 000 g·mol^{-1}), bovine serum albumin (66 000 g·mol^{-1}), carbonic anhydrase (29 000 g·mol^{-1}) and cytochrome c (12 400 g·mol^{-1}). Blue dextran (2 000 000 g·mol^{-1}) was used for the determination of the void volume of the column.

2.7. Effect of pH

Enzyme activity measurements were carried out in 50 mmol·L^{-1} acetate buffer (pH 4.0–5.0), 50 mmol·L^{-1} phosphate buffer (pH 6.0–7.0) and 50 mmol·L^{-1} Tris-HCl buffer (pH 8.0–9.0), at 30 °C, using glycyl-prolyl-4-nitroanilide as substrate.

2.8. Effect of temperature

Enzyme activity measurements were carried out in 50 mmol·L^{-1} phosphate buffer, pH 7.0 at various temperatures from 10 to 60 °C, using glycyl-prolyl-4-nitroanilide as substrate.

Additionally, enzyme solution was preincubated in the absence of substrate for 2 min at various temperatures from 50 to 80 °C. The measurement of the remaining enzyme activity was carried out in 50 mmol·L^{-1} phosphate buffer, pH 7.0 at 50 °C, using glycyl-prolyl-4-nitroanilide as substrate.

2.9. Michaelis constant

Enzyme activity measurements were carried out in 50 mmol·L^{-1} phosphate buffer, pH 7.0 at 50 °C, using various concentrations of glycyl-prolyl- and arginyl-prolyl-4-nitroanilide from 0.125 to 3 mmol·L^{-1}.

2.10. Substrate specificity

Enzyme activity measurements were carried out in 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0 at 50 °C, with various substrates, each against the respective blank. For the derivatives of 4-nitroanilide, such as alanyl-, arginyl-, glycyl-, leucyl-, lysyl-, methionyl-, prolyl-, phenylalanyl-, N-acetyl-alanyl-, arginyl-prolyl-, glycyl-phenylalanyl- and glycyl-prolyl-4-nitroanilide, the method described under measurement of enzyme activity was used; the substrate concentration in the assay mixture was 2 mmol·L^{-1}. When peptides were used as substrates, substrate solution (50 μL; 4 mmol·L^{-1} in 50 mmol·L^{-1} phosphate buffer, pH 7.0) was incubated with 50 μL of enzyme solution at pH 7.0 and at 50 °C. The reaction was stopped with 100 μL of 12% (w/v) TCA. The release of amino groups was estimated by the o-phthaldialdehyde method [10]. The following peptides were tested: glycyl-phenylalanine, methionyl-glycine, tyrosyl-alanine, glycyl-tyrosine, alanyl-prolyl-

glycine, isoleucyl-prolyl-isoleucine, glycyl-prolyl-arginyl-proline and alanyl-leucyl-alanyl-leucine.

2.11. Effect of inhibitors

Diisopropylfluorophosphate (DFP), N-ethylmaleimide, iodoacetic acid, iodoacetamide and EDTA (5 mmol·L^{-1} in 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0) were incubated with an equal volume of enzyme solution at 30 °C for 60 min. Then, 50 µL of the solution were added to the assay mixture and the remaining activity was measured. Phenylmethylsulfonylfluoride (PMSF), 5,5-dithio-bis-(2-nitrobenzoate) (DTNB) and 1,10-phenanthroline (50 mmol·L^{-1} in isopropanol) were diluted to 5 mmol·L^{-1} in 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0 and then incubated with the enzyme as before. The effect of isopropanol on the enzyme was also considered.

2.12. Effect of metals

Metal ion solutions (5.8 µL) of $MnSO_4 \times H_2O$; $MgSO_4 \times 7H_2O$; $CuSO_4 \times 5H_2O$; $BaCl_2 \times 2H_2O$; $CaCl_2 \times 2H_2O$; $ZnSO_4 \times 7H_2O$; $MgCl_2 \times 4H_2O$; LiCl, $HgCl_2$ and $FeSO_4 \times 7H_2O$ (100 mmol·L^{-1} in 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0), and $AgNO_3$ and $(CH_3COO)_2Pb \times 3H_2O$ (100 mmol·L^{-1} in distilled H_2O) were incubated with 110 µL of enzyme solution at 30 °C for 60 min. Then, 50 µL of the solution was added to the assay mixture and the remaining activity was measured.

2.13. Extraction of DNA

Chromosomal DNA of *S. macedonicus* ACA-DC 191 was isolated from a late log-phase culture (10 mL) according to Leenhouts et al. [27]. Plasmid DNA from *L. lactis* subsp. *lactis* MG1363 was isolated according to Leenhouts et al. [26]. Plasmid DNA from *E. coli* TOP10F' and its clones was isolated by using the Quantum Prep®, Plasmid Miniprep Kit (Bio-Rad, Hercules, CA, USA). The general procedures for DNA manipulation were essentially those described by Sambrook et al. [46].

2.14. Amplification of DNA by PCR

Chromosomal DNA from *S. macedonicus* ACA-DC 191 was amplified by PCR in reaction conditions recommended by the manufacturer of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland). Two DNA primers (supplied by MWG Biotech AG, Ebersberg, Germany) were used for PCR amplification. Primer FOR (5'-TTT CAC AGT CCA CGT AAC ATC C-3') has been designed according to the 215–236 region of the *pepX* gene from *S. mutans* GS-5 Kuramitsu (Peruzzi F., Piggot P.J., Daneo-Moore L. unpublished sequence in GenBank. Accession number: U78605, 1996). Primer REV (5'-GAG TTT AGA TTG AGA TAA ATC AAT CTC GTA-3') has been designed according to the 3 445–3 474 region of the *pepX* gene from *L. lactis* ssp. *cremoris* P8-2-47 (GenBank Accession Number: M58315, [32]). The PCR conditions included a hot start at 94 °C (4 min), an annealing temperature of 52 °C (1 min) and polymerization at 72 °C (4 min); the reaction was repeated for 32 cycles.

2.15. Southern hybridization

The primer pair used gave rise to a 4.8 kb DNA fragment, which was checked by Southern hybridization for carrying the *pepX* gene. DNA was transferred to a Nylon Membrane Positively Charged (Roche, Mannheim, Germany) as described by Sambrook et al. [46]. Hybridization was performed at 42 °C in the presence of 10% (v/v) formamide, and of a *pepX*-specific probe labeled with digoxigenin-dUTP by using a random primed DIG DNA labeling and detection kit (Roche, Mannheim, Germany); hybridization and

immunological detection were performed as recommended by the supplier. The 1.8 kb fragment obtained after *Hind*III digestion of the pBM329 plasmid, which corresponds to the *pepX* gene from *L. lactis* ssp. *cremoris* P8-2-47, was used as a *pepX*-specific probe.

2.16. DNA cloning and sequencing

The PCR product was cloned in the plasmid vector pT-Adv supported with the AdvanTAge™ PCR Cloning Kit (Clontech, Palo Alto, CA, USA), using the manufacturer's ligation instructions. Sequencing of the cloned gene was performed by the MWG Biotech AG (Ebersberg, Germany), using the ABI3700 system (Perkin Elmer Biosystems, Tucson, AZ, USA) with the standard sequencing protocols suggested by PE Biosystems, and pT-Adv-specific primers and sequence-specific oligonucleotides for primer walking.

2.17. Computer analysis

The MBS-translator software was used for the translation of the nucleotide sequence. Protein homology searches were carried out with the NCBI and Swiss-Prot databases, using the BLAST network service [3].

2.18. Nucleotide sequence accession number

The GenBank nucleotide sequence accession number for the *pepX* gene from *S. macedonicus* ACA-DC 191 is AF373012.

3. RESULTS AND DISCUSSION

3.1. Purification of the enzyme

Three steps of anion exchange and one step of hydrophobic interaction chromatography were applied for the separation of the X-prolyl-dipeptidyl aminopeptidase. On the anion exchange chromatography columns, the enzyme was eluted at a concentration of 0.28 mol·L^{-1} (DEAE-cellulose column at pH 8.5), 0.38 mol·L^{-1} (Resource Q column at pH 8.5) and 0.31 mol·L^{-1} (Resource Q column at pH 7.0) in the NaCl gradient. On the hydrophobic interaction chromatography (Phenyl Superose column at pH 7.0) the enzyme was eluted as a single peak at a concentration of 0.4 mol·L^{-1} in the $(NH_4)_2SO_4$ gradient. These active fractions were pooled, dialyzed against 50 mmol·L^{-1} phosphate buffer, pH 7.0, and used for the enzyme characterization. The recovery and degree of purification are summarized in Table I.

Table I. Purification and recovery of the X-prolyl-dipeptidyl aminopeptidase activity from *Streptococcus macedonicus* ACA-DC 191.

Purification step	Volume (mL)	Protein (mg)	Specific activity (U·mg^{-1})	Total activity (U)	Yield (%)	Purification fold
Crude extract	100	151	0.05	7.6	100	1
DEAE-cellulose	44	46.6	0.2	9.3	100	4
Resource Q pH 8.5	12	6.0	1.1	6.6	87	22
Resource Q pH 7.0	8	2.5	2.1	5.3	70	42
Phenyl Superose	4	0.3	15	4.5	59	300

One unit (U) is defined as the amount of enzyme producing 1 μmol 4-nitroaniline per min.

The post-electrophoretic detection of the purified enzyme, under native conditions and using glycyl-prolyl-4-methoxy-β-naphthylamide as substrate, revealed a single colored band (data not shown). This fact, combined with the detection of a single protein band after the SDS-PAGE, and a single symmetrical peak after the gel filtration chromatography (see below) of the purified enzyme, confirmed the presence of a single protein.

3.2. Molecular mass determination

A molecular mass of 167 000 g·mol^{-1} was estimated by gel filtration on Sephacryl S-300, where the purified enzyme was eluted as a symmetrical peak. By SDS-PAGE in the presence of β-mercaptoethanol, the molecular mass of the purified enzyme was estimated to be 84 000 g·mol^{-1} (Fig. 1). These results indicate that the X-prolyl-dipeptidyl aminopeptidase activity consists of two identical subunits. This molecular mass correlates well with the molecular mass of the *S. thermophilus* enzyme described by Meyer and Jordi [34] and Tsakalidou et al. [51]. Dimeric configuration has also been reported for the X-prolyl-dipeptidyl aminopeptidase of *L. lactis* ssp. *cremoris* [21], *L. lactis* ssp. *lactis* [28, 57], and *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. acidophilus* [6]. However, there are reports on monomer enzyme configuration for *Lb. helveticus* [20, 40] and *Lb. casei* [19], while Miyakawa et al. [39] described a trimer enzyme from *L. delbrueckii* ssp. *bulgaricus*.

3.3. Effect of pH

The *S. macedonicus* X-prolyl-dipeptidyl aminopeptidase was active over a pH range of 5.0–9.0. The optimum pH for the hydrolysis of glycyl-prolyl-4-nitroanilide was 7.0, when phosphate buffer was used. The enzyme showed 75% of its maximum activity at pH 9.0, only 9% at pH 5.0 and no activity at pH 4.0. In this respect, it was similar to the *S. thermophilus* and the *L. lactis* ssp. *cremoris* enzymes previously described [7, 21, 34, 51].

3.4. Effect of temperature

The *S. macedonicus* X-prolyl-dipeptidyl aminopeptidase was active on glycyl-

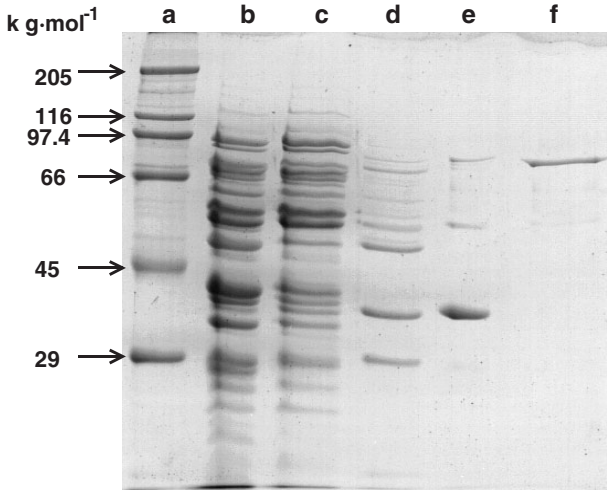

Figure 1. SDS-PAGE (10% acrylamide) analysis of the fractions obtained in the course of the enzyme purification. Lanes: a, marker proteins; b, crude cell-free extract; c, DEAE-cellulose column at pH 8.5; d, Resource Q column at pH 8.5; e, Resource Q column at pH 7.0; f, Phenyl Superose column at pH 7.0.

prolyl-4-nitroanilide over a temperature range of 10–60 °C. Even at 50 °C, the reaction rate was constant for 60 min. At 60 °C, hydrolysis of the substrate proceeded in a linear way for the first 5 min; afterwards the reaction stopped due to the denaturation of the enzyme. The initial rate at 60 °C corresponded to the maximum reaction rate, and it was used to calculate the remaining activity at 10, 20, 30, 40 and 50 °C as follows: 13.4, 28.5, 47.3, 75.8 and 96.8%, respectively.

When the enzyme was pre-incubated in the absence of substrate for 2 min at 50 °C, it preserved 100% of its activity, while at 60 °C 87% of the activity was retained. Over 60 °C, the enzyme activity declined rapidly. At 70 °C in the absence of substrate, the enzyme retained only 5% of its original activity, while after 2 min at 80 °C it was completely inactivated. All X-prolyl-dipeptidyl aminopeptidases from lactic acid bacteria reported up to now showed an optimum activity around 50 °C. However, a rapid enzyme inactivation above this temperature has been described in most cases [4, 6, 19, 21, 34, 39, 51]. Only Miyakawa et al. [40] described a thermostable enzyme isolated from *Lb. helveticus*.

3.5. Michaelis constant

From the linear Lineweaver-Burk plot, similar K_M values, and thus similar affinity, were determined for glycyl-prolyl- and arginyl-prolyl-4-nitroanilide, namely, 0.42 and 0.44 mmol·L^{-1}, respectively. However, the V_{max} for arginyl-prolyl-4-nitroanilide, 243.6 μmol·mg^{-1}·min^{-1}, was 19 times higher than that determined for glycyl-prolyl-4-nitroanilide, 12.8 μmol·mg^{-1}·min^{-1}. Similar K_M values have already been reported in the literature for X-prolyl-dipeptidyl aminopeptidases from other lactic acid bacteria [4, 6, 19, 28, 34, 39, 55]. Only Tsakalidou et al. [51] reported higher K_M values for the *S. thermophilus* enzyme.

3.6. Substrate specificity

As shown in Table II, the enzyme hydrolyzed substrates with an X-Pro N-terminal sequence almost specifically, but the activities depended on the type of the X amino acid. Hydrolysis of glycyl-prolyl-4-nitroanilide proceeded with 77% of the rate determined for arginyl-prolyl-4-nitroanilide, which was not the case for the *S. thermophilus* enzyme [34, 51], or the *L. lactis* ssp. *cremoris* enzyme [55]. However, higher hydrolysis rates for arginyl-prolyl- or alanyl-prolyl- than for glycyl-prolyl- have

Table II. Substrate specificity of the X-prolyl-dipeptidyl aminopeptidase activity from *Streptococcus macedonicus ACA-DC 191.*

Substrate	Relative activity (%)
4-nitroaniline derivatives	
alanyl-4-nitroanilide	5
arginyl-4-nitroanilide	0
glycyl-4-nitroanilide	0
leucyl-4-nitroanilide	4
lysyl-4-nitroanilide	0
methionyl-4-nitroanilide	0
prolyl-4-nitroanilide	0
phenylalanyl-4-nitroanilide	8
N-acetyl-alanyl-4-nitroanilide	0
arginyl-prolyl-4-nitroanilide	100
glycyl-phenylalanyl-4-nitroanilide	0
glycyl-prolyl-4-nitroanilide	77
Peptides	
glycyl-phenylalanine	0
methionyl-glycine	20
tyrosyl-alanine	13
glycyl-tyrosine	42
alanyl-prolyl-glycine	100
isoleucyl-prolyl-isoleucine	49
glycyl-prolyl-arginyl-proline	93
alanyl-leucyl-alanyl-leucine	11

often been reported in the past [6, 20, 39, 40, 57]. Among the peptides tested, alanyl-prolyl-glycine was hydrolyzed with the highest rate, followed by glycyl-prolyl-arginyl-proline, while hydrolysis of isoleucyl-prolyl-isoleucine and alanyl-leucyl-alanyl-leucine proceeded much more slowly. Very low or no aminopeptidase, dipeptidase or endopeptidase activity was observed, as has also been reported for other X-prolyl-dipeptidyl aminopeptidase activities [20, 40, 51, 57].

3.7. Effect of inhibitors and metal ions

As shown in Table III, metal chelators such as 1,10-phenanthroline and EDTA did not affect enzyme activity. PMSF inhibited the X-prolyl-dipeptidyl aminopeptidase activity completely, thus suggesting the presence of essential OH groups in the active site of the enzyme. Nevertheless, DFP, which has the same specificity with PMSF, showed no inactivation effect. Sulfhydryl group reagents such as N-ethylmaleimide and iodoacetamide had no inhibitory effect, although iodoacetic acid and DNTB caused 31% and complete inactivation, respectively. This could also be an indication of the involvement of functional sulfhydryl group(s) at or near the active site of the enzyme. Similar behavior has been reported for the *S. thermophilus* enzyme described by Meyer and Jordi [34] and Tsakalidou et al. [51]. Although all X-prolyl-dipeptidyl aminopeptidase activities from lactic acid bacteria reported in the literature are considered as serine enzymes, many authors claim that sulfhydryl groups may be important for the activity of the enzyme [20, 39, 40, 54]. Metal ions such as Mn^{2+}, Mg^{2+}, Ba^{2+}, Ca^{2+} and Li^{+} had no inhibitory action on the X-prolyl-dipeptidyl aminopeptidase activity (Tab. III). Under the same conditions Ag^{+}, Zn^{2+}, Pb^{2+} and Fe^{2+} caused a loss of 28 up to 64% of the activity. Higher inhibition was observed with Hg^{2+}, while Cu^{2+} inhibited the enzyme almost completely.

Table III. Effect of inhibitors and metal ions on the X-prolyl-dipeptidyl aminopeptidase activity from *Streptococcus macedonicus* ACA-DC 191.

Reagent	Remaining activity (%)
EDTA	109
1,10-phenanthroline	96
DFP	111
PMSF	0
N-ethylmaleimide	113
Iodoacetic acid	69
Iodoacetamide	108
DTNB	0
$MnSO_4 \times H_2O$	112
$MgSO_4 \times 7H_2O$	104
$MgCl_2 \times 4H_2O$	99
$CuSO_4 \times 5H_2O$	2
$BaCl_2 \times 2H_2O$	102
$CaCl_2 \times 2H_2O$	104
$Zn\ SO_4 \times 7H_2O$	70
LiCl	114
$HgCl_2$	22
$Fe\ SO_4 \times 7H_2O$	36
$AgNO_3$	72
$(CH_3COO)_2Pb \times 3H_2O$	42

Although metal ions are not considered as specific inhibitors, similar results concerning the inactivation of X-prolyl-dipeptidyl aminopeptidase by heavy metal ions such as Cu^{2+} and Hg^{2+} or Zn^{2+} has often been reported in the past [19, 20, 54, 55].

3.8. Cloning and sequence analysis of *pepX* gene

The size of the PCR product amplified from the chromosomal DNA of *S. macedonicus* ACA-DC 191, using the FOR and REV primers, was approximately 4.8 kb. This size was higher than the expected one (2.4 kb). As was shown from the

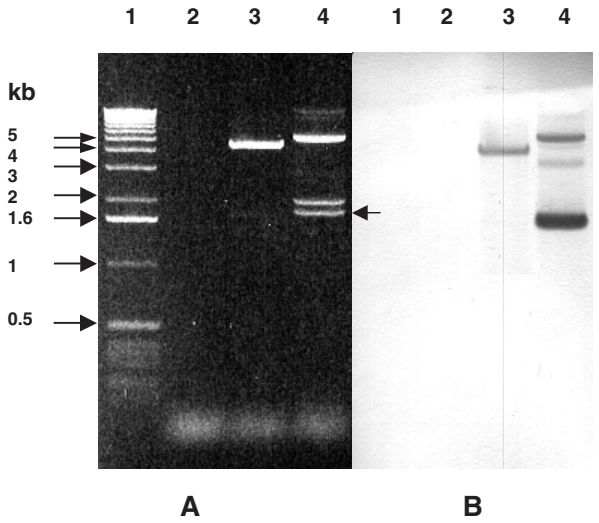

Figure 2. (A) Agarose gel electrophoresis (1% agarose). Lanes: 1, 1 kb DNA Ladder (Gibco BRL); 2, negative control (PCR without target DNA); 3, PCR amplification product of the *Streptococcus macedonicus* ACA-DC 191 chromosomal DNA; 4, plasmid pBM329 digested with *Hind*III. (B) Southern hybridization of agarose gel with the *pepX*-specific probe. Arrow indicates the 1.8 kb *Hind*III fragment of the pBM329 plasmid used as probe.

subsequent nucleotide sequence analysis, this was due to the mismatch of the REV primer. More specifically, the REV primer annealed 2 400 bp downstream of the expected annealing region, because of its low homology to the target sequence.

Indeed, hybridization analysis of the 4.8 kb PCR product, using the 1.8 kb fragment containing the *pepX* gene from *L. lactis* ssp. *cremoris* P8-2-47 as a specific probe, strongly indicated that the *pepX* gene was located in this PCR product (Fig. 2). This product was cloned in the pT-Adv plasmid and the recombinant plasmid was designated pMac. The *E. coli* clones carrying pMac were confirmed to synthesize PepX after detection of the enzyme activity by the in situ enzymatic plate assay. Two positive clones were selected for nucleotide sequence analysis.

The nucleotide sequence for a 2 725 bp region of pMac plasmid, downstream of the FOR primer, was determined and an open reading frame (ORF) was found, which starts at position 121 (ATG) and stops at the termination codon TGA at position 2 410 (Fig. 3). A putative ribosome binding site (GAAGGG, nucleotides 107–112) is located upstream of the start codon. Around position 50 a potential promoter region is present. The –10 sequence (TATAAT, nucleotides 56–61) is identical to the –10 box of the consensus *E.coli* and vegetative *B. subtilis* promoters [44]. The corresponding putative –35 box (TAGACA) is located 17 nucleotides upstream of the –10 box, while a stretch of A and T residues is present a few nucleotides upstream of the –35 promoter hexanucleotide. Whether this A+T-rich region is important for promoter activity in vivo remains to be established, especially since the overall A+T content in the lactic acid streptococci is already relatively high (the mean value of A+T for four different lactic streptococcal strains is 63%; [22]). The exact initiation of transcription has to be determined by primer extension analysis. Downstream of the stop codon a region of dyad symmetry is present (ACAAAAACCTCCTCT nucleotides 2 531–2 545, AGAGGAGGTTTTTGT nucleotides 2 560–2 574) that could act as a rho-independent transcriptional terminator.

The 2 289 bp ORF encodes a protein of 763 amino acids with a calculated molecular mass of 86 866 g·mol^{-1} and a theoretical

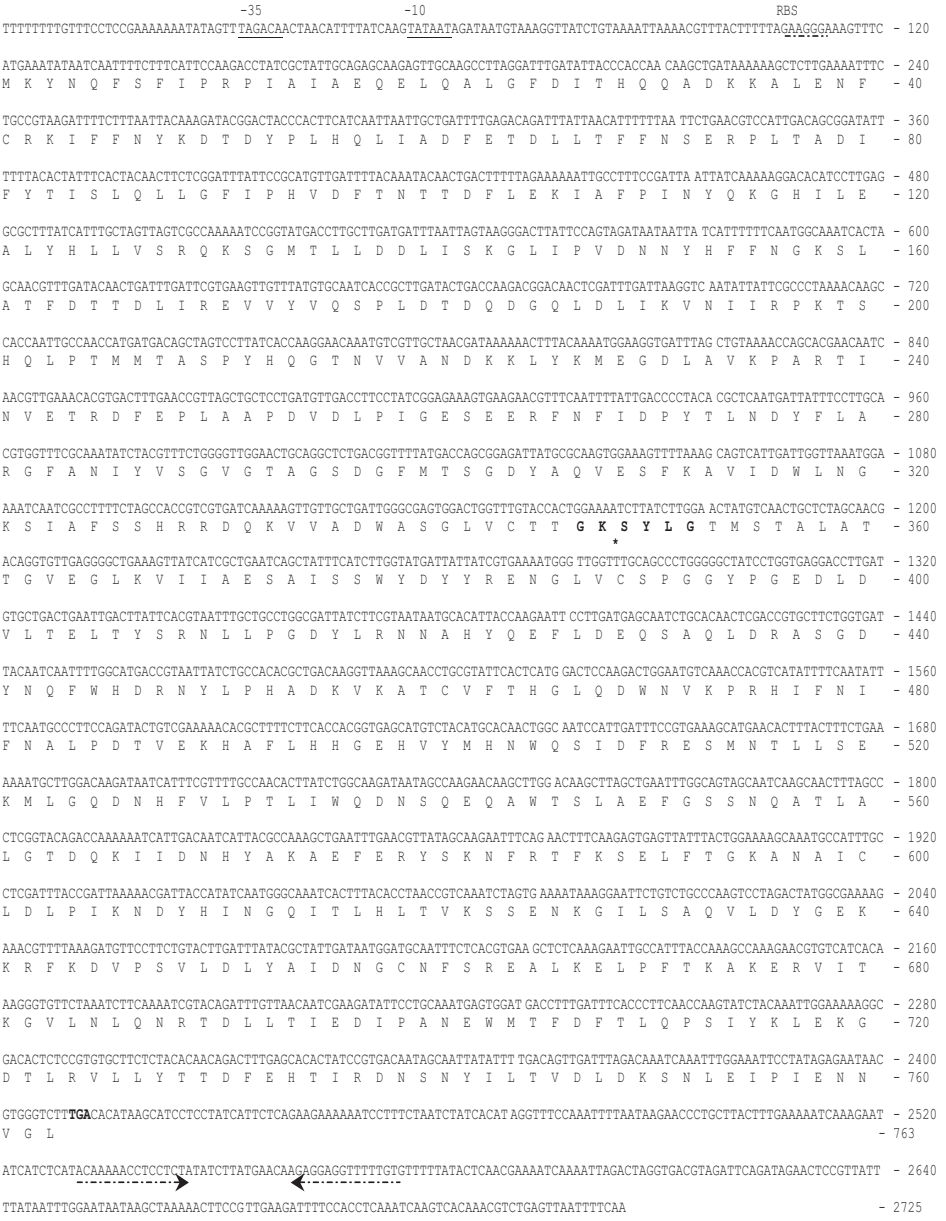

Figure 3. Nucleotide and deduced amino acid sequences of the *Streptococcus macedonicus* ACA-DC 191 *pepX* gene. The predicted –35 and –10 regions of the putative promoter are underlined. RBS refers to the putative ribosome binding site, which is indicated by a dashed line. Conserved amino acid residues involved in the active site are indicated by bold type and the active site serine with an asterisk. The stop codon is written in bold type. The putative transcription terminator is shown with dashed arrows.

isoelectric point of 5.2. This calculated molecular mass agrees well with that of the purified enzyme estimated from SDS-PAGE for the monomeric form of the enzyme (84 000 g·mol^{-1}). The protein databases of NCBI and Swiss-Prot were searched (BLAST search) for proteins homologous to the deduced one from the ORF, and it was found to be 65%, 56%, 49%, 48%, 34% and 33% identical to the PepX proteins of *S. pyogenes* SF370 [14], *S. gordonii* (Goldstein J.M., Banbula A., Kordula T., Mayo J.A., Travis J., unpublished sequence in GenBank. Accession number: AY032733, 2001), *L. lactis* ssp. *cremoris* P8-2-47 [32], *L. lactis* ssp. *lactis* NCDO763 [42], *Lb. helveticus* CNRZ32 [56] and *Lb. delbrueckii* ssp. *lactis* DSM7290 [35], respectively. Additionally, there is a 36% identity over a stretch of 676 amino acids (region 81–756) with the PepX protein of *Lb. rhamnosus* 1/6 [53], and a 51% identity over a stretch of 187 amino acids (region 1–187) with the PepX protein of *S. mutans* GS-5 Kuramitsu (Peruzzi F., Piggot P.J., Daneo-Moore L., unpublished sequence in GenBank. Accession number: U78605, 1996).

The fact that *E. coli* clones used for sequence analysis exhibited PepX activity in combination with the results obtained by the BLAST search allowed the characterization of the ORF determined as the *pepX* gene of *S. macedonicus* ACA-DC 191. Preliminary sequencing and BLAST analysis of the rest pMac plasmid revealed the presence of two complete and one partial ORF in opposite orientation to the *pepX* gene. These three ORFs encode proteins with high similarity to the two-component regulatory system consisting of a histidine protein kinase and a response regulator (data not shown).

Most of the PepXs isolated from lactic acid bacteria as well as those isolated from other bacteria, yeasts and eucaryotic sources have been classified as serine proteinases on the basis of the effect of several substances known to inhibit this type of enzyme [4, 6, 21, 34, 51]. The fact that the sequence of the PepX protein from *S. macedonicus* ACA-DC 191 did not show significant similarity to any serine proteinase suggests that this protein has a different evolutionary origin. The active site serine of PepX from *L. lactis* has been identified [9]. Sequence comparison of active sites of different PepXs with other serine proteases shows that the consensus motif: G-x-S-x-G-G is not exactly conserved. PepXs are characterized by the motif G-x-S-x-x-G. PepXs from streptococci and lactococci (formerly named N streptococci) display the motif G-K-S-Y-L-G whereas the enzymes from some lactobacilli strains (*Lb. helveticus*, *Lb. delbrueckii* ssp. *lactis*) exhibit R instead of K (Tab. IV).

Table IV. Sequence comparison of the region surrounding the active site serine of the X-prolyl-dipeptidyl aminopeptidases of different lactic acid bacteria.

Species	Motif of the serine active site
S. macedonicus ACA-DC 191 (present work)	G-K-S-Y-L-G
S. pyogenes SF370 [14]	G-K-S-Y-L-G
L. lactis [9]	G-K-S-Y-L-G
Lb. helveticus CNRZ32 [56]	G-*R*-S-Y-L-G
Lb. delbrueckii ssp. *lactis* DSM7290 [35]	G-*R*-S-Y-L-G
Lb. rhamnosus 1/6 [53]	G-K-S-Y-L-*A*
S. gordonii (Accession number: AY032733)	G-*I*-S-Y-L-G

Further work will be the determination of the role of the streptococcal PepX during casein degradation. Cloning of *pepX* into *Streptococcus* vectors and subsequent growth experiments will be instructive, if overproduction of the enzyme could improve cheese ripening. Studies on PepX-negative mutants of *L. lactis* [31, 36] have shown that in milk the growth of the PepX-negative mutant was clearly affected. However, wider investigation on *L. lactis* showed that bacterial growth might depend on the activity of not only one peptidase but a combination of several important peptidases [36]. Inactivation of the *pepX* gene in *S. macedonicus* using genetic tools [5] should allow the evaluation of the role of this peptidase in bacterial growth as well as during cheese ripening.

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