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Purification and characterization of an X-prolyl-dipeptidyl aminopeptidase from *Lactobacillus curvatus* DPC2024

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Abstract — An X-prolyl-dipeptidyl aminopeptidase (PepX) was purified ~176-fold from the cell-free extract of *Lactobacillus curvatus* DPC2024. The native enzyme appeared to be a dimer with a subunit molecular mass of ~97.4 kDa as determined by sodium dodecyl sulphate gel electrophoresis. Optimal activity of the purified enzyme on Ala-Pro-*p*-nitroanilide (pNA) was at pH 7.5 and 45 °C. The enzyme retained more than 60% of its activity after pre-incubation for 30 min at 45 °C but the activity decreased sharply following pre-incubation at temperatures above 45 °C. The enzyme was activated by Co^{2+}, Mn^{2+}, Ni^{2+} at 0.1 and 1.0 mmol·L^{-1} and by Cu^{2+}, Cd^{2+} and Zn^{2+} at 0.1 mmol·L^{-1} but it was strongly inhibited by 1.0 mmol·L^{-1} phenylmethylsulphonyl fluoride, Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} and partially by ethylenediaminetetraacetic acid, *o*-phenanthroline and *p*-chloromercuribenzoate. The enzyme hydrolysed Ala-Pro-pNA, Arg-Pro-pNA, Gly-Pro-Arg, Val-Pro-Leu at a faster rate and slowly hydrolysed Ala-Ala-pNA but it was not active on Ala-Ala-Ala, Ala-Leu-Ala, Pro-Pro-Pro, Arg-Pro-Pro, dipeptides and N-terminally blocked *p*-nitroanilide derivatives and other peptides. The sequence of the first 20 amino acid residues was determined and showed 40% homology to X-prolyl-dipeptidyl aminopeptidases from *Lactobacillus helveticus* CNRZ 32, *Lactobacillus helveticus* 53/7, *Lactococcus lactis* ssp. *cremoris* P8-2-47 and *Lactococcus lactis* ssp. *lactis* NCDO 763 and 35% homology to a PepX from *Lactobacillus delbrueckii* ssp. *lactis* DSM 7290.

***Lactobacillus curvatus* / X-prolyl-dipeptidyl aminopeptidase / purification**

Résumé — **Purification et caractérisation d'une X-prolyl-dipeptidyl aminopeptidase isolée à partir de *Lactobacillus curvatus* DPC2024.** Une X-prolyl-dipeptidyl aminopeptidase (PepX) a été purifiée environ 176 fois à partir d'un extrait intracellulaire de *Lactobacillus curvatus* DPC2024. L'enzyme native est apparue être de la forme dimère avec des sous-unités de masse moléculaire ~97,4 kDa déterminée par électrophorèse SDS-PAGE. L'activité optimale de l'enzyme purifiée, déterminée sur le fragment Ala-Pro-*p*-nitroanilide (pNA), a été obtenue à pH 7,5 et à 45 °C. L'enzyme

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a conservé plus de 60 % de son activité après une pré-incubation de 30 min à 45 °C mais cette activité a diminué brutalement après pré-incubation à des températures supérieures à 45 °C. L'enzyme a eu son activité augmentée par une concentration de 0,1 et 1,0 mmol·L^{-1} en Co^{2+}, Mn^{2+}, Ni^{2+} mais a été inhibée fortement par une concentration de 1,0 mmol·L^{-1} en phénylméthylsulfonyl fluoride et en Hg^{2+}, Cu^{2+}, Cd^{2+} et Zn^{2+} et partiellement inhibée par l'acide éthylènediaminetétraacétique, la *o*-phénanthroline et le *p*-chloromercuribenzoate. L'enzyme a hydrolysé les fragments Ala-Pro-pNA, Arg-Pro-pNA, Gly-Pro-Arg, Val-Pro-Leu à une vitesse plus élevée et le fragment Ala-Ala-pNA à vitesse lente mais n'a pas été active sur les fragments Ala-Ala-Ala, Pro-Pro-Pro, Arg-Pro-Pro, les dipeptides, les peptides et les dérivés de *p*-nitroanilide bloqués du côté N-terminal. La séquence des 20 premiers acides aminés a été déterminée et a révélé 40 % d'homologie avec les X-prolyl-dipeptidyl aminopeptidases de *Lactobacillus helveticus* CNRZ32, *Lactobacillus helveticus* 53/7 et *Lactobacillus lactis* ssp. *lactis* NCDO 763, 35 % d'homologie avec la PepX de *Lactobacillus delbrueckii* ssp. *lactis* DSM 7290.

***Lactobacillus curvatus* / X-prolyl-dipeptidyl aminopeptidase / purification**

1. INTRODUCTION

Lactic acid bacteria (LAB) are fastidious microorganisms which depend on a complex proteolytic system comprised of proteinases, peptidases and protein transport systems to supply essential amino acids from caseins during growth in milk [30, 31]. This system is also believed to play an important role in the development of cheese flavour [6, 29].

X-Prolyl-dipeptidyl aminopeptidases (PepX) are peptide hydrolases capable of releasing X-Pro and sometimes X-Ala dipeptides from the N-terminus of oligopeptides. In addition to peptidase activity, PepX has amidase and esterase activities [37]. X-Prolyl-dipeptidyl aminopeptidases have been demonstrated in several genera of lactic acid bacteria [4] and recently a number of them have been purified from strains of *Lactococcus* and *Lactobacillus* [see 12, 14]. Also, PepX genes have been sequenced from *Lc. lactis* ssp. *cremoris* P8-2-47 [19], *Lc. lactis* ssp. *lactis* NCDO 763 [28], *Lb. delbrueckii* ssp. *lactis* DSM7290 [24], *Lb. helveticus* 53/7 [33] and *Lb. helveticus* CNRZ 32 [38].

Caseins, the major proteins in bovine milk, are rich in the imino acid proline [6]. To hydrolyse peptide bonds involving proline, specialised peptidases are required because of its unique structure [34]. Therefore, proline-specific peptidases, including PepX, are important components of the proteolytic systems of the dairy LAB enabling them to degrade caseins to free amino acids.

Mesophilic lactobacilli are adventitious bacteria in cheese and dominate the non-starter lactic acid bacterial (NSLAB) flora of many cheese varieties during ripening [9, 35]; they gain access to the cheesemilk through pre- or post-pasteurization contamination and grow to high cell densities (>10^7 cfu/g cheese) during ripening [7]. *Lactobacillus curvatus* is a component of the facultatively heterofermentative NSLAB which contributes to the ripening of Cheddar cheese made from raw or pasteurized milk [9, 21, 35]. An understanding of the proteolytic system of mesophilic lactobacilli will provide valuable information on their contribution to flavour development and their potential as adjuncts to accelerate cheese ripening. A metal-independent aminopeptidase, a PepN-like aminopeptidase and a dipeptidase have isolated from *Lb. curvatus* DPC2024 [16–18]. This study describes the purification and characterization of an X-prolyl-dipeptidyl aminopeptidase from the cell free extract of *Lb. curvatus* DPC2024

which was originally isolated from a commercial pasteurized milk Cheddar cheese [9].

2. MATERIALS AND METHODS

2.1. Reagents

Diethylaminoethyl (DEAE)-Sephacel, Phenyl Sepharose and Chelating Sepharose Fast Flow were purchased from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Ninhydrin was obtained from BDH Biochemical (Poole, UK). Deoxyribonuclease (DNase) I and ribonuclease (RNase) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Di-, tri- and oligopeptides, *p*-nitroanilide derivatives of amino acids and peptides and N-terminal-blocked substrates were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland) or from the Sigma Chemical Co. (St-Louis, MO, USA). Molecular weight standards for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography were purchased from the Sigma Chemical Co. Other chemicals and reagents used were of analytical grade.

2.2. Culture, growth conditions and preparation of cell extracts

Lb. curvatus DPC2024 was obtained from the Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. The organism was precultured twice before growing in 20 L of MRS broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK) at 30 °C for ~15 h. Cells were harvested by centrifugation at 5 000 *g* for 15 min at 4 °C and washed with 50 mmol·L^{-1} tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.0. The cell-free extract was prepared by sonication as described by Magboul and McSweeney [18].

2.3. Measurement of enzyme activity

PepX activity was assayed on Ala-Pro-*p*-nitroanilide (pNA) at pH 7.0 and 40 °C as described by Magboul and McSweeney [18]. The reaction mixture consisted of 50 μL enzyme preparation, 400 μL buffer (50 mmol·L^{-1} Tris-HCl, pH 7.0) and 50 μL substrate (10 mmol·L^{-1} solution in deionized water). One unit (U) of PepX activity was defined as the amount of enzyme which produced 1 μmol *p*-nitroaniline per min at 40 °C and pH 7.0.

2.4. Protein determination

Protein contents of the cell extract and pooled fractions were determined by the Bio-Rad protein assay (BioRad) with bovine serum albumin as standard. Protein concentration in chromatographic elution profiles was monitored by measuring absorbance at 280 nm.

2.5. Purification of PepX

Cell free extract was concentrated by ultrafiltration as described by Magboul and McSweeney [18]. The concentrated cell-free extract was first fractionated by salting out with ammonium sulphate (80%). The precipitate was collected by centrifugation at 10 000 *g* for 20 min at 4 °C, dissolved in 50 mmol·L^{-1} Tris-HCl, pH 7.0 and then dialysed for 24 h against the same buffer.

DEAE-Sephacel anion-exchange chromatography column (70 × 2.6 cm) was equilibrated with 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0. The dialysed fraction from the ammonium sulphate precipitation step was applied to the column at a flow rate of 60 mL·h^{-1}. The column was washed with equilibration buffer and the proteins were eluted with a linear gradient from 0.0 to 0.35 mol·L^{-1} NaCl and then strongly-bound protein was removed from the column by maintaining the salt gradient at 1.0 mol·L^{-1} NaCl. Fractions with PepX activity were

pooled, concentrated by Centriprep [18] and dialysed against 20 mmol·L^{-1} Tris-HCl buffer, pH 7.0 containing 0.5 mol·L^{-1} NaCl.

The dialysed active fraction from an ion-exchange chromatography was applied to a Chelating Sepharose column (1.0 × 20 cm) immobilized with Cu^{2+} (15 mL of 4 mg·mL^{-1} $CuCl_2$) prior its equilibration with 20 mmol·L^{-1} Tris-HCl buffer, pH 7.0 containing 0.5 mol·L^{-1} NaCl. The column was first washed with the equilibration buffer and then the proteins were eluted competitively with a linear gradient from 0.0 to 0.2 mol·L^{-1} glycine. The active fractions were pooled, concentrated as above and dialysed against 10 mmol·L^{-1} sodium phosphate buffer, pH 6.8.

The concentrated enzyme fraction from the previous step was loaded onto a hydroxyapatite column (1.0 × 10 cm) which was equilibrated with 10 mmol·L^{-1} sodium phosphate buffer, pH 6.8. The column was washed with the equilibration buffer and proteins were eluted with a linear sodium phosphate gradient from 10 to 100 mmol·L^{-1}. Fractions with PepX activity were pooled, concentrated and dialysed against 20 mmol·L^{-1} Tris-HCl, pH 7.0.

The concentrated active fraction from chromatography on hydroxyapatite was applied to a MonoQ HR 5/5 column (Pharmacia) using an FPLC system (Pharmacia). The column was washed with 20 mmol·L^{-1} Tris-HCl buffer, pH 7.0 for 7 min after which the NaCl concentration was raised from 0.0 mol·L^{-1} to 0.25 mol·L^{-1} in the same buffer and the proteins eluted from the column with a linear gradient of 0.25 to 0.36 mol·L^{-1} NaCl over 43 min. The fractions with PepX activity were pooled and stored at ~0 °C.

2.6. Determination of molecular mass

The molecular mass of the native enzyme was determined by gel filtration chromatography using a TSKG2000 SW column (TosoHAAS, Cambridge, UK). The column was equilibrated with 50 mmol·L^{-1} Tris-HCl buffer, pH 7.0 containing 0.15 mol·L^{-1} NaCl. The column was calibrated with MW-GF-200 molecular weight standard kit (Sigma Chemical Co.). The molecular mass of the enzyme under denaturing conditions was estimated by SDS-PAGE according to a procedure of Laemmli [13] with a 4% acrylamide stacking gel and 12% acrylamide separating gel using SDS-VII-L as molecular weight standards (Sigma Chemical Co.). Protein samples were prepared and stained by Commasie Brilliant Blue R-250 as described by Magboul and McSweeney [18].

2.7. Effect of pH and temperature

The effect of pH in the range 4.0 to 9.0 was determined at 45 °C, using 100 mmol·L^{-1} sodium acetate buffer, pH 4.0 to 6.0 and 100 mmol·L^{-1} 1,3-bis [tris(hydroxymethyl)-methylamino]propane (bis-Tris) buffer, pH 6.5 to 9.0. The effect of temperature was determined in the range 20 to 60 °C in 50 mmol·L^{-1} Tris-HCl buffer, pH 7.5. For determining the thermal stability, aliquots of enzyme were pre-incubated at 45, 50, 55, 60 and 65 °C for 0 to 100 min intervals and the residual activity determined at pH 7.5 and 45 °C. In all cases Ala-Pro-pNA was used as substrate.

2.8. Effect of metal ions and inhibitors

The purified enzyme was dialysed for 24 h against 20 mmol·L^{-1} Tris-HCl buffer pH 7.5 at 4 °C before pre-incubation with various metal ions and inhibitors at 45 °C for 30 min at a final concentration of 0.1, 1 or 10 mmol·L^{-1}. The enzyme activity was determined using Ala-Pro-pNA as a substrate.

2.9. Substrate specificity

The ability of the purified PepX to hydrolyse *p*-nitroanilide derivatives of amino

acids and peptides was determined by the enzyme assay procedure described above. Enzyme activities on dipeptides, tripeptides and N-CBZ-blocked peptides were assayed using Cd-ninhydrin method as described by Magboul and McSweeney [18].

2.10. N-Terminal amino acid sequencing

The first 20 amino acid residues of the purified enzyme were determined as described by Magboul and McSweeney [18].

3. RESULTS

3.1. Purification of PepX

Purification of PepX from *Lb. curvatus* DPC2024 is summarized in Table I. Elution profiles of the enzyme from chromatographies on DEAE-Sephacel, metal Chelating Sepharose Fast Flow, hydroxyapatite and MonoQ are shown in Figure 1. In the final purification step on MonoQ the enzyme was purified about 176-fold over the concentrated cell-free extract with an activity yield of 15%.

3.2. Enzyme purity and molecular mass

SDS-PAGE electrophoretograms of active fractions obtained at different purification steps are shown in Figure 2. Only a single band was detected after the final chromatographic step on MonoQ (Fig. 2, lane 6). The molecular mass of PepX was estimated to be 97.4 kDa by SDS-PAGE (Fig. 2) and ~200 kDa by gel filtration (data not shown).

3.3. Effect of pH and temperature

The enzyme showed high activity over a wide pH range, from 6.0 to 9.0 with

Table I. Purification of an X-prolyl-dipeptidyl aminopeptidase from *Lb. curvatus* DPC2024.

Tableau I. Purification de la X-prolyl-dipeptidyl aminopeptidase purifiée à partir de *Lb. curvatus* DPC2024.

Purification step	Total protein (mg)	Total activity (U)	Specific activity ($U \cdot mg^{-1}$)	Purification-fold	Yield (% activity)
CCFS[1]	3 488	31 649	9.1	1	100
$(NH_4)_2SO_4$[2]	2 095	21 047	10	1.1	66.5
DEAE-Sephacel[3]	171	14 456	109	12.1	59.3
IMAC[4]	95	9 028	155	17	45
CHT[5]	11.5	6 559	824	90.5	29
MonoQ[6]	3	4 811	1 603	176	15

[1] Concentrated cell-free supernatant.
[2] Ammonium sulphate precipitation.
[3] Anion-exchange chromatography on DEAE-Sephacel.
[4] Immobilized metal affinity chromatography on Chelating Sepharose Fast Flow.
[5] Chromatography on ceramic hydroxyapatite.
[6] High performance anion-exchange chromatography on MonoQ HR5/5.

[1] Surnageant intracellulaire concentré.
[2] Précipitation au sulfate d'ammonium.
[3] Chromatographie d'échange d'anions DEAE-Sephacel.
[4] Chromatographie d'affinité d'ions métalliques sur Chelating Sepharose Fast Flow.
[5] Chromatographie sur colonne d'hydroxyapatite.
[6] Chromatographie d'échange d'anions haute performance sur MonoQ HR5/5.

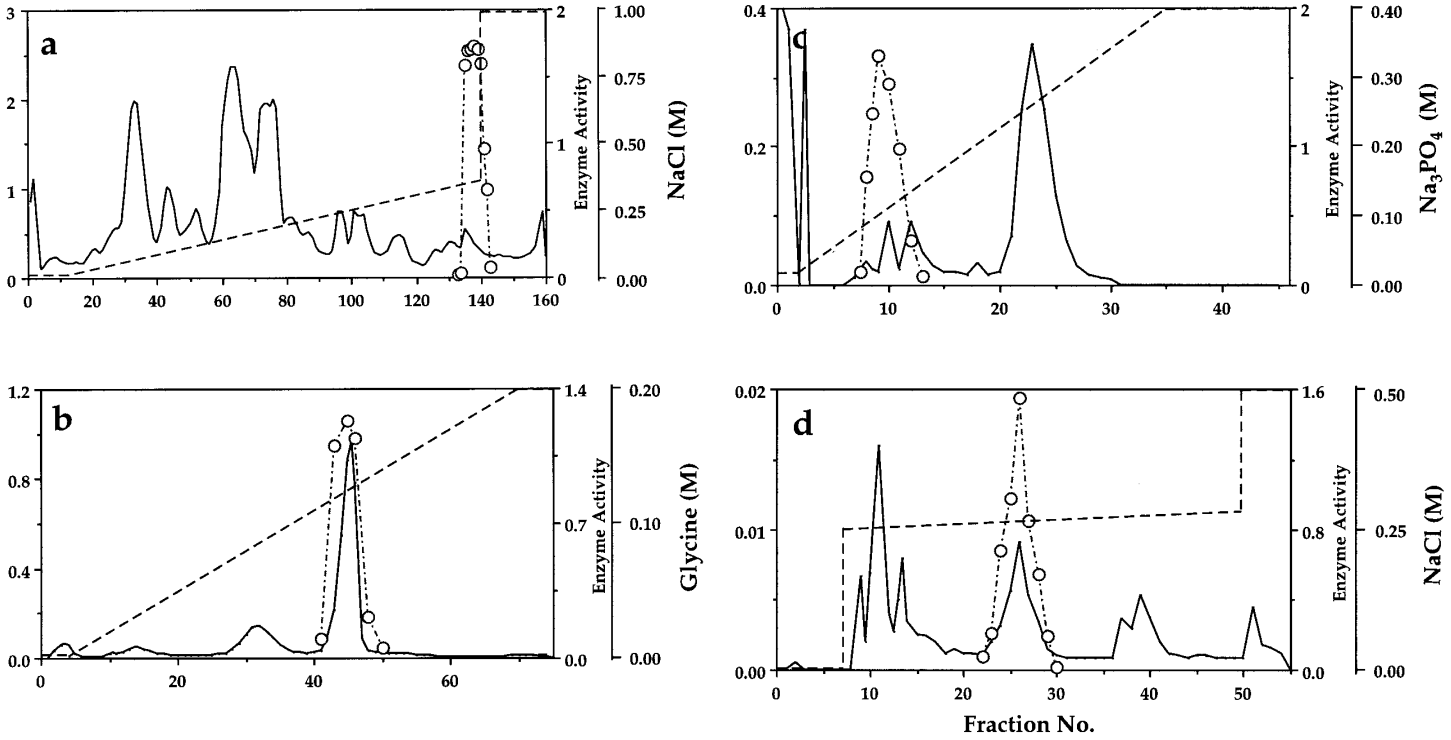

Figure 1. Chromatographic elution profiles of an X-prolyl-dipeptidyl aminopeptidase from *Lb. curvatus* DPC2024 using (**a**) DEAE-Sephacel (anion-exchange chromatography); (**b**) Chelating Sepharose (immobilized with $CuC1_2$); (**c**) Hydroxyapatite and (**d**) MonoQ HR5/5 (high-performance anion-exchange chromatography). Protein concentration (——A_{280}), PepX activity (.....○..... A_{410}) and salt gradient (– – – –).

Figure 1. Profils chromatographiques de la X-prolyl-dipeptidyl aminopeptidase issue de *Lb. curvatus* DPC2024 avec (**a**) DEAE-Sephacel (chromatographie d'échange d'anions), (**b**) Chelating Sepharose (immobilisée $CuCl_2$), (**c**) Hydroxyapatite et (**d**) MonoQ HR5/5 (chromatographie d'échange d'anions haute performance). Concentration en protéine (——A_{280}), activité de la PepX (.....○..... A_{410}) et gradient de sel (– – – –).

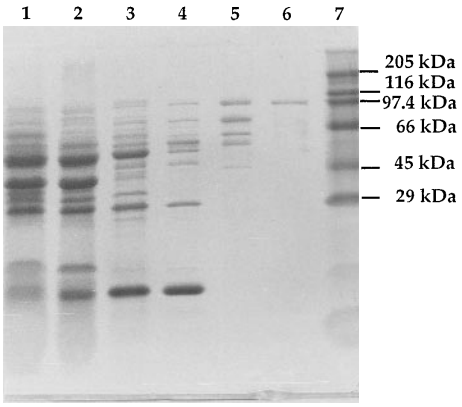

Figure 2. SDS-PAGE electrophoretograms of cell free-extract from *Lb. curvatus* DPC2024 (1) and fractions containing X-prolyl-dipeptidyl aminopeptidase activity obtained after ammonium sulphate precipitation (2) and chromatography on DEAE-Sephacel (3), Chelating Sepharose (4), hydroxyapatite (5) and MonoQ HR 5/5 (6). Lane (7): molecular weight markers.

Figure 2. Gels d'électrophorèse SDS-PAGE de l'extrait intracellulaire de *Lb. curvatus* DPC2024 (1) et des fractions possédant une activité X-prolyl-dipeptidyl aminopeptidasique obtenues : après précipitation au sulfate d'ammonium (2), après chromatographie DEAE-Sephacel (3), après Chelating Sepharose (4), après Hydroxyapatite (5) et après MonoQ HR5/5 (6). Puits (7) : marqueurs de poids moléculaires.

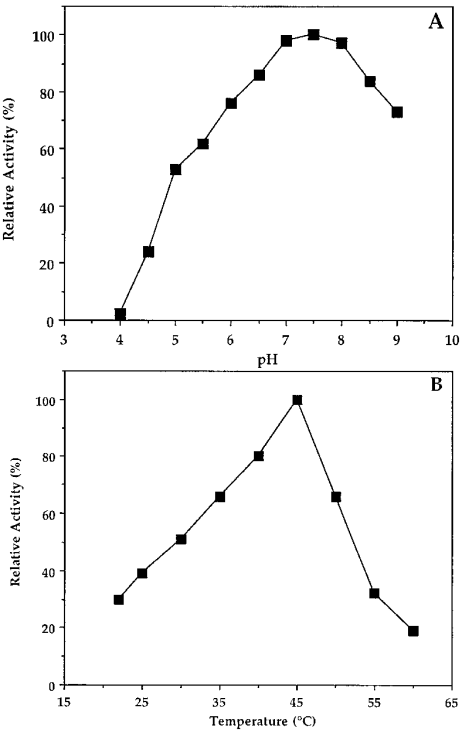

Figure 3. Effect of pH (**A**) and temperature (**B**) on the activity of the X-prolyl-dipeptidyl aminopeptidase purified from *Lb. curvatus* DPC2024.

Figure 3. Effet du pH (**A**) et de la température (**B**) sur l'activité de la X-prolyl-dipeptidyl aminopeptidase purifiée à partir de *Lb. curvatus* DPC2024.

optimum activity at pH 7.5 (Fig. 3A). While very little activity was observed at pH 4.0, more than 50% of optimum activity was observed at pH 5.0. Optimum PepX activity was at 45 °C with more than 20% at 20 °C and less than 20% at 60 °C (Fig. 3B). Preheating the enzyme in the absence of substrate at 45 and 50 °C for 30 min reduced its activity by 40 and 80%, respectively, indicating that the enzyme was more stable in the presence of substrate.

3.4. Effect of metal ions and inhibitors

The effect of different compounds on PepX activity is summarized in Table II. Phenylmethylsulphonyl fluoride at 1.0 and 10 mmol·L^{-1} and *p*-chloromercuribenzoate at 10 mmol·L^{-1}, strongly reduced the enzyme activity. Metal chelators, ethylenediminetetraacetic acid and *o*-phenanthroline slightly decreased enzyme activity at 1.0 and caused considerable reduction at 10 mmol·L^{-1} concentration while very little effect on the enzyme activity was observed at all concentrations of N-ethylmaleimide, dithiothreitol and β-mercaptoethanol. The divalent metal ions, Ba^{2+}, Ca^{2+} and Mg^{2+} showed no significant effect on the enzyme activity while a pronounced inhibitory effect on PepX activity was observed with 0.1,

Table II. Effect of inhibitors and metal ions on the activity of X-prolyl-dipeptidyl aminopeptidase purified from *Lb. curvatus* DPC2024.

Tableau II. Effet des inhibiteurs et des ions métalliques sur l'activité de la X-prolyl-dipeptidyl aminopeptidase purifiée à partir de *Lb. curvatus* DPC2024.

Compounds	Residual activity (%) concentration ($mmol \cdot L^{-1}$)		
	0.1	1.0	10
Ethylenediaminetetraacetic acid	112	73	49
o-Phenanthroline	86	76	54
N-Ethylmaleimide	95	110	114
p-Chloromercuribenzoate	86	66	56
Phenylmethylsulphonyl fluoride	54	2	0
Dithiothreitol	104	105	84
β-Mercaptoethanol	94	89	90
$BaCl_2$	100	101	100
$CaCl_2$	102	120	111
$CdCl_2$	165	18	2
$CoCl_2$	198	181	90
$CuCl_2$	163	16	2
$HgCl_2$	2	0	0
$MgCl_2$	105	96	102
$MnCl_2$	159	126	74
$NiCl_2$	216	119	38
$ZnCl_2$	122	8	0

1.0 and 10 $mmol \cdot L^{-1}$ Hg^{2+} and with 1.0 and 10 $mmol \cdot L^{-1}$ Cu^{2+}, Cd^{2+} and Zn^{2+} and with 10 $mmol \cdot L^{-1}$ Ni^{2+}. Some activation effect of PepX activity was caused by 0.1 $mmol \cdot L^{-1}$ Cd^{2+}, Cu^{2+} and Zn^{2+} and by 0.1 and 1.0 $mmol \cdot L^{-1}$ Co^{2+}, Mn^{2+} and Ni^{2+}.

3.5. Substrate specificity

The purified enzyme was incubated with several substrates (Tab. III). Among *p*-nitroanilides, the enzyme was most active on Ala-Pro-pNA followed by Arg-Pro-pNA and Gly-Pro-pNA. Low activity (18%) was observed on Ala-Ala-pNA as substrate. The enzyme also hydrolysed Gly-Pro-Arg and Val-Pro-Leu but it was unable to hydrolyse Ala-Ala-Ala, Ala-Pro-Gly, Ala-Leu-Ala, Arg-Pro-Pro, Pro-Pro-Pro or dipeptides, *p*-nitroanilide derivatives of amino acids and peptides or N-terminally blocked substrates.

3.6. Amino acid sequencing

The first 20 amino acid residues sequence of the purified PepX showed 40% identity with PepX from *Lc. lactis* ssp. *cremoris* P8-2-47, *Lc. lactis* ssp. *lactis* NCDO 763, *Lb. helveticus* CNRZ 32 and *Lb. helveticus* 53/7 and 35% identity with PepX from *Lb. delbrueckii* ssp. *lactis* DSM 7290 (Fig. 4).

4. DISCUSSION

In this study, an X-prolyl-dipeptidyl aminopeptidase (PepX) was purified to

Table III. The relative activity on different substrates of the X-prolyl-dipeptidyl aminopeptidase purified from *Lb. curvatus* DPC2024.

Tableau III. Activité relative déterminée sur différents substrats de la X-prolyl-dipeptidyl aminopeptidase purifiée à partir de *Lb. curvatus* DPC2024.

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Ala-Pro-pNA*	100	Ala-Ala-Ala	0
Arg-Pro-pNA	90	Ala-Pro-Gly	0
Gly-Pro-pNA	79	Ala-Leu-Ala	0
Ala-Ala-pNA	18	Leu-Leu-Leu	0
Gly-Phe-pNA	0	Arg-Pro-Pro	0
NCBZ-Gly-Pro-Arg-pNA	0	Gly-Pro-Arg*	100
Ala-Ala-Pro-pNA	0	Pro-Pro-Pro	0
N-Succ-Phe-pNA	0	Pro-Gly-Gly	0
Ala-pNA	0	Leu-Gly-Pro	0
Lys-pNA	0	Leu-Gly-Gly	0
Leu-pNA	0	Val-Pro-Leu	53
Pro-pNA	0	*NCBZ*-Ala-Pro-Leu	0
Glu-pNA	0	Leu-Leu	0
Phe-pNA	0	Ala-Pro	0
His-pNA	0	Pro-Leu	0
Val-pNA	0	Ala-Ala	0

* Hydrolysis of Ala-Pro-pNA and Gly-Pro-Arg was considered 100%.

* L'hydrolyse de Ala-Pro-pNA et de Gly-Pro-Arg a été considérée comme équivalente à 100 % d'activité.

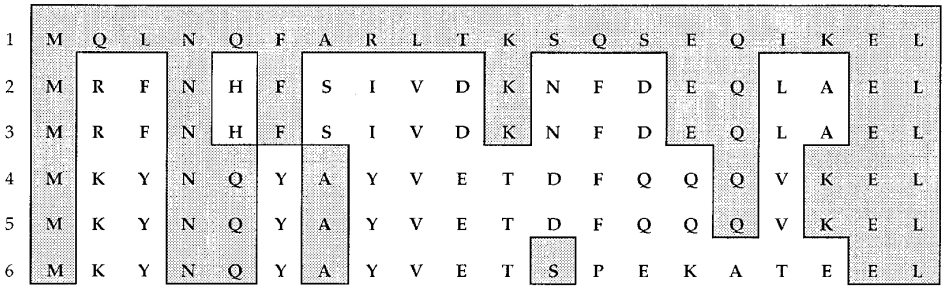

Figure 4. Sequence alignments of the first 20 amino acid residues of the X-prolyl-dipeptidyl aminopeptidase (PepX) purified from *Lb. curvatus* DPC2024 (1) and PepXs from *Lc. lactis* ssp. *cremoris* P8-2-47 (2), *Lc. lactis* ssp. *lactis* NCDO 763 (3), *Lb. helveticus* CNRZ 32 (4), *Lb. helveticus* 53/7 (5) and *Lb. delbrueckii* ssp. *lactis* DSM7290 (6).

Figure 4. Alignement de la séquence primaire des 20 premiers acides aminés de la X-prolyl-dipeptidyl aminopeptidase (PepX) purifiée à partir de *Lb. curvatus* DPC2024 (1) et des PepXs issues de *Lc. lactis* ssp. *cremoris* P8-2-47 (2), *Lc. lactis* ssp. *lactis* NCDO 763 (3), *Lb. helveticus* CNRZ 32 (4), *Lb. helveticus* 53/7 (5) et *Lb. delbrueckii* ssp. *lactis* DSM 7290 (6).

homogeneity from the cell-free extract of *Lb. curvatus* DPC2024 by five chromatographic steps. A metal independent aminopeptidase, PepN-like aminopeptidase and a dipeptidase have also been purified from this strain [16–18].

The PepX purified from *Lb. curvatus* DPC2024 had a sharp temperature optimum at 45 °C and a broad pH optimum between 6.0 and 9.0. At temperatures higher than 45 °C, the enzyme activity decreased sharply and the enzyme was more stable in the presence of substrate. Similar pH optima to this enzyme were reported for PepXs from *Lc. lactis* ssp. *cremoris* AM2 and *Lc. lactis* ssp. *lactis* H1 [3, 15] while similar temperature optima were reported by Zevaco et al. [39], Bockelmann et al. [2] and Vesanto et al. [33] for similar enzymes from *Lc. lactis* ssp. *lactis* NCDO 763, *Lb. delbrueckii* ssp. *bulgaricus* B14 and *Lb. helveticus* 53/7, respectively. PepXs from *Lc. lactis* ssp. *lactis* P8-2-47 [11], *Lb. delbrueckii* ssp. *bulgaricus* CNRZ397 [1], *Lb. delbrueckii* ssp. *bulgaricus* LBU-147 [26], *Lb. casei* ssp. *casei* LLG [8], *Lb. helveticus* LHE-511 [27] and *Streptococcus thermophilus* ACA-DC4 [32] had optimum temperatures around 50 °C.

The structure of the native PepX purified from *Lb. curvatus* DPC2024 was found to be dimeric with subunits having an identical molecular mass in the vicinity of 100 kDa as estimated by gel filtration chromatography and SDS-PAGE. These results are in agreement with molecular masses reported for PepXs from *Lc. lactis* ssp. *cremoris* P8-2-47 [11], *Lc. lactis* ssp. *lactis* NCDO 763 [39], *Lb. delbrueckii* ssp. *bulgaricus* B14 and *Lb. acidophilus* 357 [2]. Dimeric PepXs with molecular masses between 117-175 kDa were also reported for some strains of *Lactococcus* and *Lactobacillus* [1, 3, 5, 15, 22, 36]. In addition to dimeric PepXs, there are also reports of monomeric PepXs [8, 10, 24, 27] and a trimeric PepX isolated from *Lb. delbrueckii* ssp. *bulgaricus* LBU-147 [26].

The purified enzyme was able to release dipeptides with the sequence X-Pro and hydrolysed Ala-Pro-pNA, Arg-Pro-pNA, Gly-Pro-pNA, Gly-Pro-Arg and Val-Pro-Leu. This is in agreement with the substrate specificity of all PepXs isolated so far from both mammalian and bacterial sources [12]. However, the enzyme was unable to release a dipeptide from Arg-Pro-Pro or Pro-Pro-Pro suggesting that proline residues at the third position prevent the removal of the N-terminal X-Pro dipeptides. Similar results were observed for PepXs from *Lc. lactis* ssp. *cremoris* AM2 [3] and *Lc. lactis* ssp. *lactis* H1 [15]. Also, this enzyme was able to hydrolyse Ala-Ala-pNA at a slower rate but not Ala-Ala-Ala. Although there are reports that PepX hydrolyses oligopeptides [11, 15, 39], the specificity of the PepX isolated in this study was tested only on *p*-nitroanilide derivatives, di- and tripeptides.

Inhibition of the purified PepX from *Lb. curvatus* DPC2024 by phenylmethylsulphonyl fluoride indicated that the enzyme was a serine peptidase as are the other microbial PepXs studied to date [12]. However, some PepXs from LAB were strongly inhibited by *p*-chloromercuribenzoate suggesting that they have a sulphydryl group near their active sites [10, 26], this enzyme was slightly inhibited by this reagent.

The divalent metal ions, Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} and Mn^{2+}, activated the PepX purified from *Lb. curvatus* DPC2024 at 0.1 mmol·L^{-1} concentration, but Hg^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} had strong inhibitory effect on this enzyme at 1.0 mmol·L^{-1}. The inhibitory effect of Hg^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} on PepXs characterized from other LAB has been reported [1, 8, 10, 22, 33].

The N-terminal amino acid sequence of the purified peptidase showed 40% homology with PepXs from *Lc. lactis* ssp. *cremoris* P8-2-47 [19], *Lc. lactis* ssp. *lactis* NCDO 763 [28], *Lb. helveticus* CNRZ 32 [38] and *Lb. helveticus* 53/7 [33] and 35% homology with PepX from *Lb. delbrueckii* ssp. *lactis* DSM 7290 [24].

The role played by PepX in the growth of LAB remains unclear, but the concerted action of all peptidases in releasing free amino acids, appears to be necessary for their growth [23, 25]. However, in recent study, PepX from *Lb. delbrueckii* ssp. *lactis* influenced proteolysis and the sensorial characteristics of Gruyère cheese but it was not important for the growth of the microorganism [20].

In conclusion, the results obtained indicate that the X-prolyl-dipeptidyl aminopeptidase purified from *Lb. curvatus* DPC2024 had close resemblance to PepXs characterized from other LAB strains. The purification of this enzyme from a NSLAB strain might be of significance in elucidating the role played by these bacteria during ripening.

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