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PepN-like aminopeptidase from *Lactobacillus curvatus* DPC2024: purification and characterization

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Abstract — An enzyme with a PepN-like aminopeptidase activity was purified 127-fold with 4.3 % recovery from a cell-free extract of *Lactobacillus curvatus* DPC2024, a component of the non-starter lactic acid bacterial flora of Cheddar cheese. The purified enzyme exhibited maximum activity on leucyl-*p*-nitroanilide (Leu-pNA) at pH 7.0 and 40 °C. The enzyme had a molecular mass of ~98 kDa as estimated by gel permeation chromatography, and showed one band corresponding to a molecular mass of ~95 kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), indicating that the native enzyme existed as a monomer. The aminopeptidase appeared to be a metalloenzyme since it was strongly inhibited by ethylenediaminetetraacetic acid (EDTA) and *o*-phenanthroline at 0.1 mmol·L^{-1} concentration and was partially reactivated by Zn^{2+} and Co^{2+}. The enzyme was also partially inhibited by *p*-chloromercuribenzoate and iodoacetic acid, suggesting the involvement of sulphydryl groups in the reaction mechanism. The enzyme had a broad substrate specificity, hydrolysing a number of *p*-nitroanilide derivatives of amino acids and peptides and di-, tri-, tetra- and pentapeptides. The N-terminal amino acid sequence of the first 20 amino acid residues was determined (AELMRFYQSFQPEHYQVFLD), and showed 40–55 % homology with Zn^{2+}-dependent aminopeptidases from *Streptococcus thermophilus* NCDO53, *Lactobacillus delbrueckii* subsp. *lactis* DSM7290, *Lactococcus lactis* subsp. *lactis* MG1363 and *Lactococcus lactis* subsp. *cremoris* Wg2.

aminopeptidase / PepN / *Lactobacillus curvatus* / purification

Résumé — Aminopeptidase de type PepN de *Lactobacillus curvatus* DPC2024 : purification et caractérisation. Une enzyme avec une activité aminopeptidasique de type PepN a été purifiée 127 fois avec un taux de récupération de 4,3 % à partir d'un extrait sans cellule de *Lactobacillus curvatus* DPC 2024, un composant de la flore lactique non-levain du cheddar. L'enzyme purifiée possède une activité maximale sur le substrat leucyl-*p*-nitroanilide (Leu-pNA) à pH 7.0 et à 40 °C. La masse moléculaire de l'enzyme, déterminée par chromatographie d'exclusion par la taille, est de ~98 kDa. Par SDS–PAGE, une bande correspondant à une masse moléculaire de ~95 kDa a été mise en évidence, indiquant que l'enzyme native existe sous forme de monomère. Cette aminopeptidase

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semble être une métalloenzyme car elle est fortement inhibée par l'EDTA et l'*o*-phenanthroline à la concentration de 0,1 mmol·L^{-1} et est partiellement réactivée par Zn^{2+} et Co^{2+}. L'enzyme est aussi partiellement inhibée par le *p*-chloromercuribenzoate et l'acide iodoacétique, suggérant l'implication de groupements sulfydryle dans le mécanisme de la réaction. L'enzyme a une large spécificité de substrat, hydrolysant un certain nombre d'acides aminés, de peptides, di-, tri-, tetra- et pentapeptides dérivés du *p*-nitroanilide. Les 20 premiers acides aminés de la séquence N terminale ont été identifiés (AELMRFYQSFQPEHYQVFLD). Une homologie de 40–55 % avec l'aminopeptidase Zn^{2+} dépendante des *Streptococcus thermophilus* NCDO53, *Lactobacillus delbrueckii* subsp. *lactis* DSM7290, *Lactococcus lactis* subsp. *lactis* MG1363 et *Lactococcus lactis* subsp. *cremoris* Wg2, a été observée.

aminopeptidase / PepN / *Lactobacillus curvatus* / purification

1. INTRODUCTION

Lactobacilli, like most lactic acid bacteria (LAB) used in dairy fermentations, are characterized by their multiple requirements for essential growth factors, including vitamins and amino acids [21]. As milk does not contain sufficient free amino acids and peptides to allow growth to high populations, lactobacilli possess a complex proteolytic system consisting of proteinases and peptidases, which enable these microorganisms to use the caseins in milk as an additional source of nitrogen [24, 31].

Several endo- and exopeptidases from lactobacilli have been isolated and studied biochemically and genetically [15, 17]. Peptidases of lactobacilli are similar, but not identical, to corresponding enzymes from *Lactococcus lactis* which have been studied in more detail [15]. Amongst the most thoroughly studied of the exopeptidases from lactococci is a general aminopeptidase, PepN [12, 30, 35]. Aminopeptidases from other lactic acid bacteria with similar properties have also been characterized [1, 4–6, 10, 13, 14, 19, 20, 25, 26, 32, 36]. In most strains studied, the enzyme is a monomeric metallopeptidase of 87–97 kDa. Sequence alignments have shown that the gene encoding this enzyme (*pepN*) is conserved amongst the dairy lactic acid bacteria [6, 14, 30]. PepN is a broad specificity aminopeptidase capable of hydrolyzing a wide range of peptide substrates differing in both size and amino acid composition [1, 20, 23, 26, 29, 30].

Mesophilic lactobacilli constitute most of the non-starter lactic acid bacteria (NSLAB) in Cheddar and Dutch-type cheeses [11, 37]. These microorganisms typically gain entrance to the milk through post-pasteurization contamination, but they also constitute part of the raw milk microflora and may survive pasteurization [34]. The typical strains of NSLAB found in Irish Cheddar cheese are facultatively heterofermentative lactobacilli and include *Lb. casei*, *Lb. plantarum*, *Lb. paracasei* subsp. *paracasei*, *Lb. curvatus* and *Lb. brevis* [11]. The contribution of NSLAB to cheese flavour and flavour development is equivocal, but acceleration of cheese ripening occurs when certain strains are used as adjuncts [9].

A dimeric metal-independent aminopeptidase with high affinity for dipeptides has been purified and characterized from *Lb. curvatus* DPC2024 [18]. In this study, purification and characterization of a second aminopeptidase (PepN-like) from *Lb. curvatus* DPC2024 is described.

2. MATERIALS AND METHODS

2.1. Chemicals

Diethylaminoethyl (DEAE)–Sephacel, Phenyl Sepharose and Chelating Sepharose Fast Flow

were purchased from Pharmacia, LKB Biotechnology Inc., Uppsala, Sweden. *p*-Nitroanilide derivatives of amino acids and peptides, di- tri-, oligopeptides and N-carbobenzyloxy (N-CBZ)-blocked peptides were obtained from Bachem, Feinchemikalien, Bubendorf, Switzerland or Sigma Chemical Co., Saint-Louis, MO, USA. Deoxyribonuclease (DNase) I and ribonuclease (RNase) were obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Molecular weight standards for gel permeation chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from the Sigma Chemical Co. All other chemicals and reagents used were of analytical grade.

2.2. Microorganism and growth conditions

Lb. curvatus DPC2024, which was originally isolated from a commercial pasteurized-milk Cheddar cheese [11], was obtained from the Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. The organism was pre-cultured twice in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK) at 30 °C. After ~15 h, pre-culture (1 % v/v) was used to inoculate 20 L of the same medium. After incubation for ~15 h, cells were harvested by centrifugation (5 000 *g*, 15 min, 4 °C) washed with 50 mmol·L^{-1} tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (pH 7.0), and recentrifuged.

2.3. Preparation of cell-free extract

The cell-free extract was prepared from the washed cells by sonication as described by Magboul and McSweeney [18].

2.4. Measurement of aminopeptidase activity

Aminopeptidase activity was measured spectrophotometrically using leucyl-*p*-nitroanilide (Leu-pNA) as substrate as described by Magboul and McSweeney [18]. One unit (U) of aminopeptidase activity was defined as the amount of enzyme required to liberate 1 µmol·L^{-1} *p*-nitroaniline per min at 40 °C and pH 7.0.

2.5. Enzyme purification

Protein contents of cell-free extract and pooled fractions were determined by the BioRad protein assay (BioRad Laboratories GmbH, Munich, Germany) with bovine serum albumin as standard. The protein concentration in chromatographic effluents was measured by determining absorbance at 280 nm.

Prior to chromatographic separation, the cell-free extract was concentrated by ultrafiltration (UF) using a Minitan UF unit fitted with polysulfone membranes with a 10 kDa nominal molecular mass cutoff (Millipore Corp., Bedford, MA, USA). The concentrated cell-free extract was then dialyzed for 6 h at 4 °C against 50 mmol·L^{-1} Tris–HCl buffer (pH 7.0).

The concentrated, dialyzed, cell-free extract was applied to a column (70 × 1.6 cm) of DEAE–Sephacel, connected to a Gradifrac system (Pharmacia). The column was washed initially with 180 mL of 50 mmol·L^{-1} Tris–HCl buffer (pH 7.0), followed by a linear NaCl gradient (0–0.35 mol·L^{-1}) in the same buffer. The flow rate was 1.0 mL·min^{-1} and 15-mL fractions were collected.

Active fractions from ion-exchange chromatography on DEAE–Sephacel were pooled and concentrated using a Centriprep-3 concentrator (Amicon, Beverly, MA, USA). Ammonium sulphate was added to the concentrated sample to a final concentration of 1 mol·L^{-1} and then the sample was applied to a Phenyl Sepharose column (20 × 1.0 cm) connected to a Gradifrac System (Pharmacia), which had previously been equilibrated with 20 mmol·L^{-1} Tris–HCl (pH 7.0) containing 1 mol·L^{-1} $(NH_4)_2SO_4$. The column was washed with the equilibration buffer (50 mL) and the proteins were eluted using a linear gradient from 1.0 to 0.0 mol·L^{-1} $(NH_4)_2SO_4$ for 150 mL, and then maintained at 0 mol·L^{-1} $(NH_4)_2SO_4$ for 100 mL. The flow rate was 0.5 mL·min^{-1} and 5-mL fractions were collected.

Pooled active fractions from hydrophobic interaction chromatography were concentrated as described above and dialyzed for 4–6 h against 20 mmol·L^{-1} Tris–HCl buffer (pH 7.0), containing 0.5 mol·L^{-1} NaCl. A chelating Sepharose column (20 × 1.6 cm) immobilized with Cu^{2+} (15 mL of 4 mg·mL^{-1} $CuCl_2$), connected to an FPLC system (Pharmacia), was equilibrated with 20 mmol·L^{-1} Tris–HCl buffer (pH 7.0), containing 0.5 mol·L^{-1} NaCl. The dialyzed sample was applied to the column at a flow rate of 2 mL

per min. The column was washed with twice its volume of equilibration buffer, and then proteins were eluted with a linear gradient from 0.0 to 0.12 mol·L^{-1} glycine in the same buffer. Fractions (6 mL) were then collected.

Active fractions from metal affinity chromatography were pooled and concentrated as described above. The concentrated sample was dialyzed against 20 mmol·L^{-1} Tris–HCl buffer, pH 7.0 for ~3 h and then applied to a MonoQ HR5/5 column (Pharmacia) connected to an FPLC system. The column was washed with the starting buffer (20 mmol·L^{-1} Tris–HCl, pH 7.0) for 7 min, and then proteins were eluted from the column for 40 min by a linear gradient from 0.0 to 0.4 mol·L^{-1} NaCl in the same buffer.

2.6. Effect of pH and temperature on enzyme activity

The effect of pH on the activity of the purified enzyme was determined in the range 4.0–9.5 for 30 min at 40 °C, using 100 mmol·L^{-1} Na-acetate, (pH 4.0–6.0) or 100 mmol·L^{-1} 1,3-bis[tris-(hydroxymethyl)-methylamino]-propane (bis-Tris), (pH 6.5–9.5) buffers using Leu-pNA as substrate. The effect of temperature on Leu-pNA hydrolase activity was determined in the range 20–55 °C in 50 mmol·L^{-1} Tris–HCl buffer (pH 7.0). For both pH and temperature optima, the activity was expressed as a percentage of the maximum activity.

2.7. Heat stability

The purified enzyme was incubated in 50 mmol·L^{-1} Tris–HCl buffer (pH 7.0) at 40, 45 or 50 °C; aliquots (50 mL) of the enzyme solution at each temperature were withdrawn after 0, 10, 20, 30, 60 and 100 min. The residual activity was then determined on Leu-pNA at pH 7.0 and 40 °C.

2.8. Determination of purity and molecular mass

The homogeneity of the active fractions after each purification step was examined by SDS–PAGE according to the method of Laemmli [16] with a 4 % (w/v) acrylamide stacking and 12 % (w/v) acrylamide separating gels. Proteins were stained using Coomassie brilliant blue R-250 (0.1 % w/v). The molecular mass of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and by gel filtration chromatography on a TSK G2000 SW as described by Magboul and McSweeney [18].

2.9. Effect of inhibitors, reducing agents and metal ions on enzyme activity

The effect of metal ions, inhibitors and reducing agents was determined on the purified enzyme. The enzyme was dialyzed against water (6 h, 4 °C) and pre-incubated in the presence of various compounds for 30 min at 40 °C to a final concentration of 0.1, 1.0 or 10.0 mmol·L^{-1} in 50 mmol·L^{-1} Tris–HCl buffer, pH 7.0. The enzyme activity was then determined on Leu-pNA (40 °C, 30 min).

2.10. Substrate specificity

The relative activity of the purified aminopeptidase against several pNA derivatives was determined as described above. Hydrolysis of peptide substrates was determined using a Cd–ninhydrin assay as described by Magboul and McSweeney [18].

2.11. N-terminal amino acid sequencing

The first 20 amino acid residues of the purified aminopeptidase were determined as described by Magboul and McSweeney [18]. The EMBL database was searched for amino acid sequence alignments and homology using a PlastP search tool [40].

3. RESULTS

3.1. Enzyme purification

The purification of an aminopeptidase from the cell-free extract of *Lb. curvatus* DPC2024 is summarized in *table I* and chromatographic separations are shown in *figure 1*. Following anion-exchange chromatography on DEAE–Sephacel (*figure 1A*),

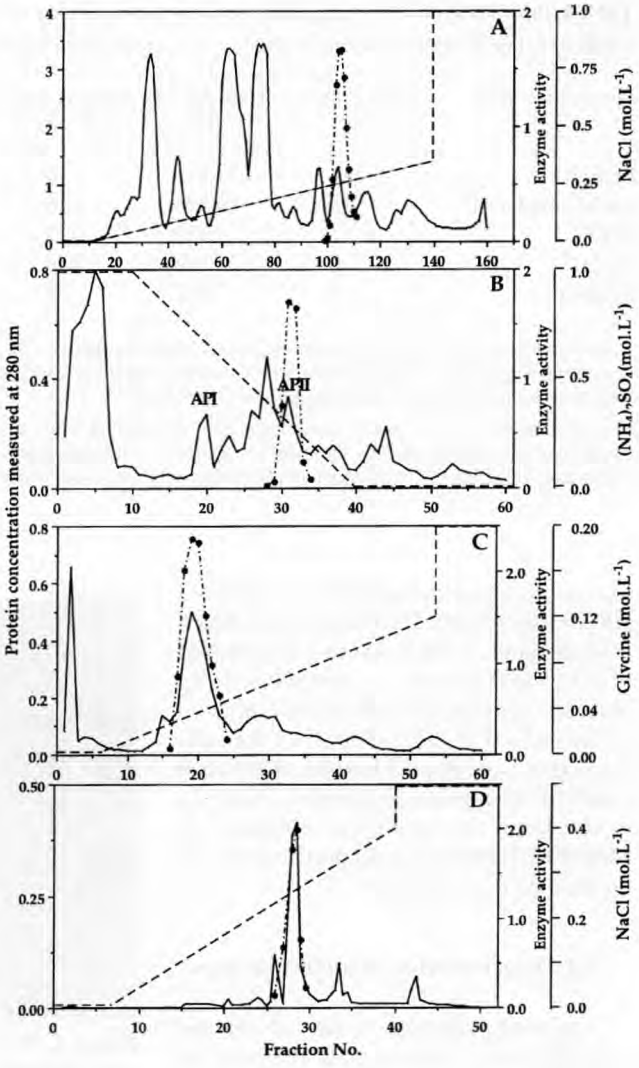

Figure 1. Purification of an aminopeptidase from *Lb. curvatus* DPC2024; protein concentration (—, A_{280}); salt gradient (— —); aminopeptidase activity on Leu-pNA (– – –•– – –, A_{410}). Elution profiles from (**A**) anion-exchange chromatography (DEAE–Sephacel); (**B**) hydrophobic interaction chromatography (Phenyl Sepharose); (**C**) metal-chelating affinity chromatography with immobilized Cu^{2+} (Chelating Sepharose Fast Flow); (**D**) FPLC anion-exchange chromatography (MonoQ HR 5/5).

Figure 1. Purification d'une aminopeptidase à partir de *Lb. curvatus* DPC2024 ; concentration en protéine (—, A_{280}) ; gradient de sel (— —) ; activité aminopeptidasique sur Leu-pNA (– – –•– – –, A_{410}). Profils d'élution (**A**) d'une chromatographie échangeuse d'ions ; (**B**) d'une chromatographie à interactions hydrophobes ; (**C**) d'une chromatographie d'affinité avec du Cu^{2+} immobilisé (Sepharose Fast Flow) ; (**D**) d'une chromatographie échangeuse d'anions FPLC (MonoQ HR 5/5).

Leu-pNA hydrolase activity was eluted at ~0.23 mol·L^{-1} NaCl with a yield of ~64 % activity and 3-fold increase in specific activity. Chromatography on Phenyl Sepharose gave two Leu-pNA aminopeptidase activities (designated API and APII) eluted at ~0.64 and 0.3 mol·L^{-1} $(NH_4)_2SO_4$, respectively (*figure 1B*). The purification and characterization of API is described elsewhere [18] and that of APII is described in this study. Hydrophobic interaction chromatography on Phenyl Sepharose increased APII activity 22-fold over the concentrated cell-free extract with a yield of 19 % (*table I*). Further purification of APII on chelating Sepharose resulted in a single peak with Leu-pNA activity which eluted at ~0.04 mol·L^{-1} glycine (*figure. 1C*) with an activity yield of 17 % and 107-fold increase in specific activity over the concentrated cell-free extract (*table I*). Finally, the enzyme was purified by high-performance anion-

Table I. Purification of an aminopeptidase from *Lactobacillus curvatus* DPC2024.
Tableau I. Purification d'une aminopeptidase à partir de *Lactobacillus curvatus* DPC2024.

Purification step	Total protein (mg)	Total activity (U)	Specific activity ($U \cdot mg^{-1}$)	Purification factor	Activity yield (%)
CCFE[a]	3 744	22 734	6	1	100
DEAE–Sephacel[b]	877	14 456	16.5	3	63.6
HIC[c]	32	4 329	135.2	22	19
IMAC[d]	6	3 869	644.8	107	17
MonoQ[e]	1.3	987	759	126.5	4.3

[a] Concentrated cell-free extract; [b] anion-exchange chromatography on DEAE–Sephacel; [c] hydrophobic interaction chromatography on Phenyl Sepharose; [d] Chelating Sepharose Fast Flow immobilized with Cu^{2+}; [e] high performance anion-exchange chromatography on MonoQ HR5/5.

[a] Extrait concentré sans cellules ; [b] chromatographie échangeuse d'anions sur DEAE–Sephacel ; [c] chromatographie d'interactions hydrophobes sur phényl Sepharose ; [d] chromatographie d'affinité avec du Cu^{2+} immobilisé (Sepharose Fast Flow) ; [e] chromatographie échangeuse d'ions haute performance sur MonoQ HR 5/5.

exchange chromatography on MonoQ HR5/5 (*figure 1D*). The enzyme was eluted at ~0.26 $mol \cdot L^{-1}$ NaCl, and this step resulted in a 127-fold increase in specific activity over the concentrated cell-free extract with a yield of 4.3 %. SDS–PAGE of the cell-free extract and pooled fractions after each purification step was performed (*figure 2*). A single protein band was obtained on SDS–PAGE after the final purification step on MonoQ (lane 5, *figure 2*).

3.2. Determination of molecular mass

Apparent molecular masses of ~95 and ~98 kDa were estimated for the purified aminopeptidase by SDS–PAGE (*figure 2*) and high performance gel permeation chromatography (data not shown) respectively.

3.3. pH, temperature optima and heat stability

The optimum pH for aminopeptidase activity was at pH 7.0 and it retained less than 40 % of its maximum activity at pH 5.5 and 8.0 (not shown). The optimum temperature for aminopeptidase activity was

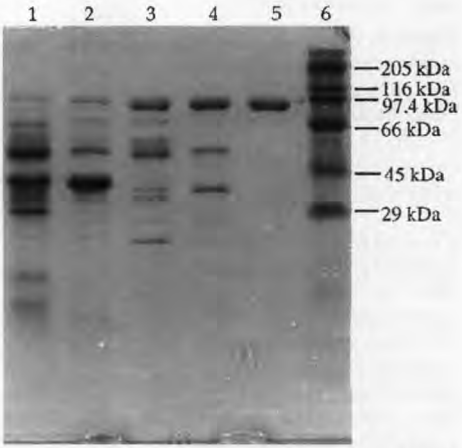

Figure 2. SDS–PAGE electrophotograms of fractions obtained during the purification of an aminopeptidase from *Lb. curvatus* DPC2024. Lane 1: cell-free extract; lanes 2–5 are pooled active fractions after chromatography on DEAE–Sephacel, Phenyl Sepharose, Chelating Sepharose Fast Flow and MonoQ, respectively; lane 6: molecular weight standards.

Figure 2. Électrophorégrammes SDS–PAGE de fractions obtenues pendant la purification de l'aminopeptidase de *Lb. curvatus* DPC2024. Bande 1 : extrait sans cellules ; bandes 2–5 : fractions actives regroupées après chromatographie sur DEAE–Sephacel, phényl Sepharose, Sepharose Fast Flow et MonoQ, respectivement ; bande 6 : standards de poids moléculaire.

40 °C at pH 7.0 (not shown). The enzyme had more than 30 % of its maximum activity at 20 °C and less than 10 % at 55 °C. The purified enzyme retained ~78 % of its maximum activity after heating at 45 °C for 100 min, but lost more than 95 % of its activity after heating at 50 °C for 10 min.

3.4. Effect of inhibitors, reducing agents and metal ions

The effects of inhibitors, reducing agents and metal ions are shown (*table II*). Metal chelating agents (ethylenediaminetetraacetic [EDTA] acid and *o*-phenanthroline) strongly inhibited enzyme activity. Complete inhibition and 70 % reduction in enzyme activity were observed after pre-incubation of enzyme solutions in 0.1 mmol·L^{-1} EDTA and *o*-phenanthroline, respectively. Aminopeptidase activity, inhibited by EDTA and *o*-phenanthroline, was partially restored by 0.05 and 0.1 mmol·L^{-1} Co^{2+} and Zn^{2+} (data not shown). Thiol blocking agents (e.g., *p*-chloromercuribenzoate and iodoacetic acid) at a concentration of 1 and 10 mmol·L^{-1} caused pronounced inhibition of aminopeptidase activity. Phenylmethylsulfonyl fluoride (PMSF) and N-ethylmaleimide had no effect on the enzyme activity at concentrations of 0.1 or 1 mmol·L^{-1}, although the serine protease inhibitor, PMSF, caused 93 % inhibition of the aminopeptidase activity at 10 mmol·L^{-1}. The reducing agents dithiothreitol and β-mercaptoethanol caused sig-

Table II. Effect of different compounds on the activity of an aminopeptidase purified from *Lactobacillus curvatus* DPC2024.

Tableau II. Effet de différents composés sur l'activité de l'aminopeptidase purifiée à partir de *Lactobacillus curvatus* DPC2024.

Compounds	Residual activity (%) Concentration (mmol·L^{-1})		
	0.1	1	10
None	100	100	100
Ethylenediaminetetraacetic acid	0	0	0
o-Phenanthroline	32	0	0
p-Chloromercuribenzoate	82	39	2
N-Ethylmaleimide	95	79	78
Phenylmethylsulfonyl fluoride	110	104	7
Iodoacetic acid	74	58	0
β-Mercaptoethanol	98	60	38
Dithiothreitol	69	55	26
$CaCl_2$	92	83	80
$CoCl_2$	338	410	557
$CdCl_2$	40	9	0
$CuCl_2$	39	6	0
$FeCl_3$	29	0	0
$HgCl_2$	0	0	0
$MgCl_2$	126	150	186
$MnCl_2$	62	31	22
$NiCl_2$	98	54	0
$ZnCl_2$	139	94	19

Table III. Substrate specificity of the aminopeptidase purified from *Lb. curvatus* DPC2024.
Tableau III. Spécificité de substrat de l'aminopeptidase purifiée à partir de *Lb. curvatus* DPC2024.

Substrate	Relative activity (%)[a]	Substrate	Relative activity (%)[b]	Substrate	Relative activity (%)[b]	Substrate	Relative activity (%)[b]
Leu-pNA*	100	Ala-Pro	0	Ala-Ala	10	Val-Pro-Leu	0
Ala-pNA	6	Asp-Leu	0	Ala-Leu	14	Pro-Phe-Gly-Lys	0
Gly-pNA	0	Glu-Val	0	Ala-Lys	20	Ala-Ala-Ala-Ala	24
Glu-pNA	0	His-Leu	27	Ala-Met	17	$(Ala)_5$	79
Lys-pNA	63	His-Val	0	Ala-Phe	10		
Pro-pNA	0	Leu-Ala	17	Pro-Pro	0		
Val-pNA	0	Leu-Arg	52	Tyr-Phe	48		
Phe-pNA	58	Leu-Gly	17	N-CBZ-Phe-Gly	0		
Met-pNA	26	Leu-Leu*	100	N-CBZ-Ala-Phe	0		
His-pNA	0	Leu-Pro	0	Ala-Ala-Ala	265		
Arg-Pro-pNA	0	Leu-Trp	55	Ala-Leu-Gly	62		
Ala-Ala-Pro-pNA	0	Leu-Val	31	Arg-Pro-Pro	0		
		Lys-Leu	24	Gly-Pro-Arg	0		
		Met-Ala	17	Leu-Ala-Pro	345		
		Phe-Ala	65	Leu-Gly-Gly	145		
		Phe-Gly	20	Leu-Leu-Leu	76		
		Pro-Ala	0	Met-Leu-Gly	231		
		Pro-Gly	0	Phe-Gly-Pro	262		
		Pro-Leu	0	Met-Leu-Pro	345		
		Pro-Phe	0	Pro-Gly-Gly	0		

* Activity was expressed relative to the rates of hydrolysis of Leu-pNA ([a]) or Leu-Leu ([b]) which were taken as 100 %.
* L'activité est exprimée en fonction des taux d'hydrolyse du Leu-pNA ([a]) ou Leu-Leu ([b]) qui peuvent atteindre 100 %.

nificant inhibition of the enzyme activity at 1 and 10 mmol·L^{-1}.

The aminopeptidase purified from *Lb. curvatus* DPC2024 was slightly activated by 0.1 mmol·L^{-1} Zn^{2+} and by 0.1, 1 and 10 mmol·L^{-1} Mg^{2+}. Very strong activation was observed following treatment with 0.1, 1 and 10 mmol·L^{-1} Co^{2+}. Complete inhibition of enzyme activity was caused by Hg^{2+} at 0.1 mmol·L^{-1}, Fe^{3+} at 1 mmol·L^{-1} and by Cu^{2+}, Cd^{2+} and Ni^{2+} at 10 mmol·L^{-1}. Strong inhibition of aminopeptidase activity was observed for Fe^{3+}, Cu^{2+}, Cd^{2+} and Ni^{2+} at 0.1 or 1 mmol·L^{-1} and by Zn^{2+} at 10 mmol·L^{-1}.

3.5. Substrate specificity

The relative rates of hydrolysis of several substrates by the aminopeptidase purified from *Lb. curvatus* DPC2024 were determined (*table III*). The highest rates of hydrolysis for *p*-nitroanilide derivatives were observed with Leu-pNA, followed by Lys-pNA, Phe-pNA and Met-pNA. A lower hydrolysis rate was observed for Ala-pNA, while no hydrolysis was observed using Gly-pNA, Glu-pNA, Asp-pNA, Pro-pNA, Val-pNA, His-pNA, Arg-Pro-pNA or Ala-Ala-Pro-pNA as substrate.

The enzyme showed broad specificity and hydrolyzed a range of di-, tri-, tetra- and pentapeptides. The enzyme hydrolyzed some of the tripeptides (e.g., Leu-Ala-Pro, Met-Leu-Pro, Ala-Ala-Ala, Phe-Gly-Pro, Met-Leu-Gly and Leu-Gly-Gly) at a faster rate than Leu-Leu (*table III*). N-Terminal-blocked dipeptides and dipeptides with Asp and Glu at the N-terminus or di- and tripeptides with Pro at the first or second position were not hydrolyzed by this enzyme.

3.6. N-Terminal amino acid sequencing

The amino acid sequence of the first 20 residues of aminopeptidase purified from *Lb. curvatus* DPC2024 and the corresponding amino acid sequence of aminopeptidases from *Streptococcus thermophilus* NCDO53 [19], *Lb. delbrueckii* subsp. *lactis* DSM7290 [14], *Lactococcus lactis* subsp. *lactis* [33], *Lc. lactis* subsp. *lactis* MG1363 [30, 35] and *Lc. lactis* subsp. *cremoris* Wg2 [27] are shown in *table IV*.

4. DISCUSSION AND CONCLUSION

Lactobacillus curvatus DPC2024 is one of the mesophilic *Lactobacillus* strains which were originally isolated from a commercial pasteurized-milk Irish Cheddar cheese [11]. Facultatively heterofermentative

Table IV. Amino acid sequence alignments of the N-terminal residues of the aminopeptidase purified from *Lb. curvatus* DPC2024 (A) and PepN-type aminopeptidases from *Sc. thermophilus* NCDO53 (B), *Lb. delbrueckii* subsp. *lactis* DSM 7290 (C), *Lc. lactis* subsp. *lactis* (D) and *Lc. lactis* subsp. *lactis* MG1363 and *Lc. lactis* subsp. *cremoris* Wg2 (E).

Tableau IV. Séquence d'acides aminés des résidus N-terminaux de l'aminopeptidase purifiée à partir de *Lb. curvatus* DPC2024 (A) et de l'aminopeptidase de type PepN à partir de *Sc. thermophilus* NCD053 (B), *Lb. delbrueckii* subsp. *lactis* DSM7290 (C), *Lc. lactis* subsp. *lactis* (D) et *Lc. lactis* subsp. *lactis* MG1363 et *Lc. lactis* subsp. *cremoris* Wg2 (E).

(A)			A_1	E	L	M	R	F	Y	Q	S	F	Q	P	E	H	Y	Q	V	F	L	D_{20}
(B)		T_1	A_2	S	V	A	R	F	I	E	S	F	I	P	E	N	Y	X	L	F	L_{20}	
(C)			M_1	A	V	K	R	F	Y	E	T	F	H	P	D	H	Y	D	L	Y	I	D_{20}
(D)	M_1	T_2	A_3	S	V	A	R	F	I	E	S	F	I	P	E	N	Y	N	L	F	L	D_{22}
(E)			M_1	A_2	V	K	R	L	I	E	T	F	V	P	E	N	Y	K	I	F	L	D_{20}

lactobacilli dominate the NSLAB flora of Cheddar and Dutch-type cheeses [11, 37] and it is believed that they make a positive contribution to cheese flavour when used as adjuncts [9]. Two aminopeptidases active on Leu-pNA (API and APII) have been purified and characterized from the cell-free extract of *Lb. curvatus* DPC2024. Purification and characterization of API has been described by Magboul and McSweeney [18], and APII is described in this study.

APII was purified to homogeneity in four chromatographic steps with ~127-fold increase in specific activity over the concentrated cell-free extract. The native enzyme existed as a monomer of ~95 kDa, as suggested by SDS–PAGE and gel permeation chromatography. This compares well with the molecular masses of PepN-type aminopeptidases characterized from *L. lactis* subsp. *cremoris* (strains Wg2, MG1363 and HP), *Lb. delbrueckii* subsp. *lactis* DSM7290, *Lb. delbrueckii* subsp. *bulgaricus* B14 and *Lb. helveticus* (strains LHE511, CNRZ32, ITGL1 and SBT2171) [8, 15].

The aminopeptidase described in this study had maximum activity on Leu-pNA at pH 7.0 and 40 °C. Heating at pH 7.0 and 45 °C for up to 100 min caused ~23 % decrease in enzyme activity, but the enzyme lost more than 80 % of its activity after preincubation at 50 °C for 10 min. Most of the PepN and PepN-like aminopeptidases characterized from *Lactococcus*, *Streptococcus* and *Lactobacillus* have maximum activity at pH values around neutrality and at temperatures between 35–50 °C [8, 15]. The aminopeptidase from *Lb. delbrueckii* subsp. *bulgaricus* B14 showed maximum activity at pH 7.0 and 50 °C, and retained 90 % of its activity after heating at 48 °C for 5.5 h.

The aminopeptidase from *Lb. curvatus* DPC2024 characterized in this study appeared to be a metallopeptidase, since it was strongly inhibited by 0.1 mmol·L^{-1} EDTA and *o*-phenanthroline and was activated by Co^{2+}, Mg^{2+} and Zn^{2+}. The apoenzyme formed by the treatment with 0.1 mmol·L^{-1} EDTA and *o*-phenanthroline was partially reactivated by 0.05 and 0.1 mmol·L^{-1} Co^{2+} and Zn^{2+}. All of the PepN-type aminopeptidases characterized to date from LAB are metalloenzymes, strongly inhibited by EDTA and *o*-phenanthroline and, in some cases, this inhibition may be fully or partially restored by different divalent metal ions [5, 38]. The enzyme was also partially inhibited by thiol reducing or blocking agents, indicating that thiol groups are important for aminopeptidase activity. Inhibition by thiol reagents was observed for a number of PepN and PepN-like aminopeptidases [1, 2, 5, 7, 13, 20, 26, 28].

Like most general aminopeptidases, the enzyme purified in this study showed broad specificity and hydrolyzed *p*-nitroanilide derivatives of amino acids (e.g. Leu-pNA, Lys-pNA), di-, tri-, tetra- and pentapeptides. The enzyme did not hydrolyze di- and tripeptides with proline residues at the first or second position, in agreement with the substrate specificity of many general aminopeptidases [1, 5, 19, 22, 25, 28] with the exceptions of metallopeptidases from *Lb. delbrueckii* subsp. *bulgaricus* B14 [39] and *Lb. helveticus* SBT 2171 [26].

The sequence of the first 20 amino acid residues of the purified aminopeptidase showed 40–55 % homology with N-terminal amino sequences of PepN-type aminopeptidases from *S. thermophilus* NCDO53 [19], *Lb. delbrueckii* subsp. *lactis* DSM7290 [14], *Lc. lactis* [33], *Lc. lactis* subsp. *lactis* MG1363 [30, 35] and *Lc. lactis* subsp. *cremoris* Wg2 [27].

These results indicated that the aminopeptidase characterized in this study is closely related to PepN-type aminopeptidases isolated from strains of *Lactobacillus*, *Streptococcus* and *Lactococcus*.

The aminopeptidase characterized in this study was dissimilar to the other aminopeptidase [API; 18] characterized from the same strain with respect to hydrophobicity, metal dependency, molecular mass, substrate

specificity and N-terminal amino acid sequence. The presence of more than one leucyl-aminopeptidase activity was suggested for *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 397 and *Lb. helveticus* ITGL2/3 by Atlan et al. [3] and Blanc et al. [4], respectively.

The function of peptidases in LAB is partly to provide the organism with a suitable source of amino acids, but the action of peptidases during cheese ripening is also important in contributing directly to cheese flavour by releasing single amino acid residues or indirectly when these free amino acids act as flavour precursors or when peptidases hydrolyze bitter peptides to shorter non-bitter peptides. Due to the presumed involvement of mesophilic lactobacilli in the development of cheese flavour during ripening, and since the NSLAB grow to high cell densities ($>10^7$ cfu·g^{-1} cheese [8]) during ripening, purification and characterization of individual peptidases from these strains may help to elucidate the role of their peptidolytic system in the development of cheese flavour.

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