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Purification and characterization of a dipeptidase from *Lactobacillus curvatus* DPC2024

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Abstract

A dipeptidase was purified to homogeneity from a cell-free extract of *Lactobacillus curvatus* DPC2024 by chromatography on diethylaminoethyl-sephacel, phenyl sepharose, chelating sepharose fast flow and MonoQ. The purified dipeptidase was a monomer of ~52 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis and gel filtration chromatography. The enzyme was optimally active at pH 8 and 50°C and retained ~10% of its maximum activity after pre-heating for 10 min at 70°C. The enzyme was a metallopeptidase, strongly inhibited by 0.1 mM ethylenediaminetetraacetic acid and *o*-phenanthroline and reactivated by a number of divalent metal ions. The enzyme was also inhibited by *p*-chloromercuribenzoate and β-mercaptoethanol. The enzyme was a strict dipeptidase, capable of hydrolysing a range of dipeptides but not tri-, tetra- or pentapeptides, *p*-nitroanilide derivatives of amino acids nor N- and C-terminal-blocked dipeptides. The N-terminal amino acid sequence of the first 20 residues showed significant homology with dipeptidases from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290 and *Lactococcus lactis* subsp. *cremoris* MG1363.

1. Introduction

Lactic acid bacteria (LAB) are a very important group of microorganisms for the production of fermented dairy products. They are extremely fastidious for a number of nutrients and require, in addition to a carbohydrate source, exogenous sources of nucleotides, vitamins and many amino acids (Pritchard & Coobear, 1993; Thomas & Pritchard, 1987). However, milk contains only low concentrations of free amino acids and small peptides which are not sufficient to support growth of LAB to high cell densities (Pritchard & Coobear, 1993; Thomas & Pritchard, 1987). Only by means of their proteolytic system, which consists of proteinases and peptidases, are lactic acid bacteria able to release the amino acids and small peptides necessary to grow to high populations in milk. Additionally, proteolysis of the caseins is also important to the development of flavour and texture in cheese during ripening (Fox, 1989; Visser, 1993). The components of the proteolytic system of lactococci and some lactobacilli have been investigated and a large number of peptidases have been purified and characterized from LAB, and several peptidase genes have been cloned and sequenced (see Kunji, Mierau, Hagting, Poolman & Konings, 1996; Law & Haandrikman, 1997). Dipeptidases of LAB are metallopeptidases with a broad specificity towards dipeptides. A number of dipeptidases with similar properties have been purified and characterized from *Lactococcus lactis* subsp. *cremoris* H61 (Howang, Kaminogawa & Yamauchi, 1981), *Lactococcus lactis* subsp. *cremoris* Wg2 (van Boven, Tan & Konings, 1988), *Lactobacillus delbrueckii* subsp. *bulgaricus* B14 (Wohlrab & Bockelmann, 1992), *Lactobacillus sake* L110 (Montel, Seronie, Talon & Hebraud, 1995), *Lactobacillus helveticus* SBT 2171 (Tan, Sasaki, Bosman & Iwasaki, 1995), *Lactobacillus sanfrancisco* CB1 (Gobbetti, Smacchi & Corsetti, 1996), *Lactobacillus helveticus* 53/7 (Vesanto, Peltoniemi, Purtsi, Steele & Palva, 1996) and *Lactobacillus casei* subsp. *casei* IFPL 731 (Fernandez-Espla & Martin-Hernandez, 1997).

Although the proteolytic system of lactobacilli has been studied less extensively than that of lactococci, interest in the proteolytic system of mesophilic lactobacilli has increased over the last few years. The facultatively heterofermentative lactobacilli are the predominant components of the adventitious non-starter lactic acid bacteria

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(NSLAB) which grow during ripening of Cheddar and Dutch-type cheeses (Jordan & Cogan, 1993; Williams & Banks, 1997). The role of NSLAB in the development of cheese flavour still remains unclear although addition of viable or attenuated strains of *Lactobacillus* spp. has a positive influence on cheese quality and accelerates cheese ripening (Williams & Banks, 1997). *Lactobacillus curvatus* is a component of the NSLAB flora of both raw and commercial pasteurized milk Cheddar cheese (Jordan & Cogan, 1993; McSweeney et al., 1993).

Metal-independent, PepN-like and X-prolyldipeptidyl aminopeptidases have been isolated from *Lb. curvatus* DPC2024 in order to elucidate the role that this microorganism might play in the development of cheese flavour during ripening or its suitability as an adjunct to accelerate cheese ripening (Magboul & McSweeney, in press-a, b, c). This study describes the purification and characterization of a dipeptidase from *Lb. curvatus* DPC2024.

2. Materials and methods

2.1. Reagents

Diethylaminoethyl (DEAE)-sephacel, phenyl sephrose and chelating sepharose fast flow were purchased from Pharmacia LKB Biotechnology Inc., Uppsala, Sweden. Ninhydrin was obtained from BDH Biochemical, England. 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*-carbobenzyloxy (CBZ)-blocked dipeptides were obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland or from the Sigma Chemical Co., St Louis, MO. 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 also of analytical grade obtained from different companies.

2.2. Microgranism, growth conditions and preparation of a cell-free extract

Lb. curvatus DPC2024, which was originally isolated from a commercial pasteurized milk Irish Cheddar cheese (Jordan & Cogan, 1993), was obtained from the Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. The strain was propagated twice in de Man, Rogosa and Sharpe (MRS) broth (Unipath Ltd., Basingstoke, Hampshire, UK) and then cultured in 20 l of the same medium (1% inoculum) at 30°C for ~15 h. The cells were harvested by centrifugation (5000 *g*, 15 min, 4°C) and washed with 50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl buffer, pH 7.

The cell-free extract was prepared from the washed cells by sonication as described by Magboul and McSweeney (1999d).

2.3. Measurement of dipeptidase activity

Dipeptidase activity was determined using the Cd-ninhydrin assay to follow the hydrolysis of Leu-Leu. The reaction mixture consisted of 50 μl enzyme preparation, 50 μl substrate (10 mM in methanol or deionized water) and 400 μl 50 mM Tris-HCl buffer, pH 8.0. The mixture was incubated for 30 min at 40°C and the reaction terminated by the addition of 1 ml Cd-ninhydrin reagent (Folkerstma & Fox, 1992). The contents were thoroughly mixed and heated at 84°C for 5 min, cooled immediately on ice and the absorbance at 507 nm was measured. One unit (U) of enzyme activity was defined as the amount of enzyme required to give an increase of one absorbance unit at 507 nm per min under the assay conditions. Enzyme activity on *p*-nitroanilide derivatives of amino acids was determined as described by Magboul and McSweeney (1999a).

2.4. Determination of protein

The protein content of column effluents was determined by measuring the absorbance at 280 nm and the protein content of the cell-free extract and pooled active fractions was determined by the BioRad Protein Assay (BioRad Laboratories GmbH, Müchen, Germany) using bovine serum albumin (Sigma Chemical Co.) as standard.

2.5. Enzyme purification

The cell-free extract was concentrated by ultrafiltration (UF) using a Minitan UF unit fitted with polysulfone membranes with 10 kDa nominal molecular weight cutoff (Millipore Corp., Bedford, MA). The concentrated cell-free extract was dialysed for 6 h at 4°C against 50 mM Tris–HCl buffer, pH 7.

The concentrated cell-free extract was first applied to a DEAE-sephacel column (70×1.6 cm) connected to a Gradifrac system (Pharmacia). The column was previously equilibrated with 50 mM Tris–HCl buffer, pH 7 and elution was performed using a linear NaCl gradient (0–0.35 M) in the same buffer. Fractions with Leu-Leu hydrolase activity were pooled and concentrated using a Centriprep-3 concentrator (Amicon, Beverly, MA).

A phenyl sepharose column (20×1 cm) was equilibrated with 20 mM Tris–HCl, pH 7 containing 1 M $(NH_4)_2SO_4$. The concentrated active fraction from chromatography on DEAE-sephacel was brought to

1 M $(NH_4)_2SO_4$ and then applied to the column, which was eluted with a gradient from 1 to 0 M $(NH_4)_2SO_4$. Fractions with Leu-Leu hydrolase activity were pooled and concentrated as described above.

Pooled active fractions from chromatography on phenyl sepharose were dialysed for ~4 h against 20 mM Tris–HCl buffer, pH 7, containing 0.5 M NaCl. A chelating sepharose column (20×1.6 cm) loaded with Cu^{2+} (15 ml of 0.4 M $CuCl_2$), connected to an FPLC® system (Pharmacia), was equilibrated with 20 mM Tris–HCl buffer, pH 7.0, containing 0.5 M NaCl. The dialysed sample was loaded onto the column at a flow rate of 2 ml per min. The column was eluted with a linear gradient from 0.0 to 0.12 M glycine in the same buffer. Fractions with Leu-Leu hydrolase activity were pooled and concentrated as described above.

Active fractions from metal affinity chromatography were dialysed against 20 mM Tris–HCl buffer, pH 7.0 for ~3 h and then applied to a MonoQ HR5/5 column (Pharmacia) connected to an FPLC® system (Pharmacia). The enzyme was eluted with a linear gradient from 0.0 to 0.3 M NaCl in the same buffer. Fractions with the highest dipeptidase activity were collected and stored for enzyme characterization.

2.6. Determination of molecular mass

The molecular mass of the purified dipeptidase was estimated by gel filtration chromatography and by SDS–PAGE. Concentrated preparations of the purified dipeptidase (20 μL) were applied to a TSK G2000 SW column (TosoHAAS, Cambridge, UK). The column was equilibrated with 50 mM Tris–HCl buffer, pH 7 containing 100 mM NaCl and for molecular weight estimation the column was calibrated using MW-GF-200 molecular mass standard kit (Sigma). SDS–PAGE was performed according to the procedure of Laemmli (1970) with gels containing 12% acrylamide. The apparent molecular mass was estimated by reference to molecular weight markers (SDS-VII-L; Sigma Chemical Co.).

2.7. Effect of pH and temperature on dipeptidase activity

The effect of pH on the enzyme activity in the range of pH 5–11 was examined in universal buffer composed of boric acid (57 mM), citric acid (33 mM), NaH_2PO_4 (33 mM), NaOH (1 M) and adjusted to different pHs with various amounts of 100 mM HCl (Gobbetti et al., 1996). The temperature optimum for dipeptidase activity was determined in the temperature range 20–70°C in 50 mM Tris–HCl buffer, pH 8. To determine the thermal stability of the dipeptidase, purified enzyme was pre-incubated in 50 mM Tris–HCl buffer, pH 8 at temperatures from 50 to 70°C for 0, 10, 20, 30, 60 and 100 min and then cooled immediately in ice. Residual dipeptidase activity was then determined using Leu-Leu as substrate.

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

Purified enzyme was pre-incubated in 50 mM Tris–HCl buffer, pH 8 at 50°C for 30 min, with each reagent at 0.1, 1 and 10 mM. The reaction was initiated by addition of the susbstrate (Leu-Leu) and the extent of hydrolysis was determined as described above. For reactivation of the dipeptidase inhibited by *o*-phenanthroline, ethylenediaminetetraacetic acid (EDTA) or *p*-chloromercuribenzoate (pCMB), divalent metal ions or thiol reducing agents (0.1, 1 and 10 mM final concentration) were added and the mixture was incubated at 50°C for a further 30 min and the recoverable dipeptidase activity was determined as described above.

2.9. Substrate specificity

Substrate specificity of the purified dipeptidase was determined as described previously on a number of peptides and *p*-nitroanilide derivatives of amino acids and peptides.

2.10. N-Terminal amino acid sequencing

The purified aminopeptidase was electroblotted from an SDS–PAGE gel onto polyvinylidene difluoride membranes (Applied Biosystems Inc., Foster City, CA) according to a procedure described by Singh, Fox, and Healy (1995). The protein band was excised from the blot and the N-terminal 20 amino acid residues were sequenced using a pulsed liquid phase Procise 494 Protein Sequencer with a 785A programmable absorbance detector (Applied Biosystems Inc.) interfaced with a Macintosh Quadra 650 computer.

3. Results

Chromatography on DEAE-sephacel, phenyl sepharose, chelating sepharose fast flow and MonoQ was used to purify the dipeptidase to homogeneity from a concentrated cell-free extract of *Lb. curvatus* DPC2024 (Fig. 1). Two peaks with Leu-Leu hydrolase activity (Peaks I and II, Fig. 1A) were eluted at ~0.23 and 0.28 M NaCl from DEAE-sephacel. Peak I contained two aminopeptidases the purification of which are described in Magboul and McSweeney (in press-a, b). Peak II was concentrated and applied to phenyl sepharose. The dipeptidase activity was eluted at ~0.88 M $(NH_4)_2SO_4$ (Fig. 1B). Pooled active fractions from this step were concentrated and applied to a chelating sepharose column

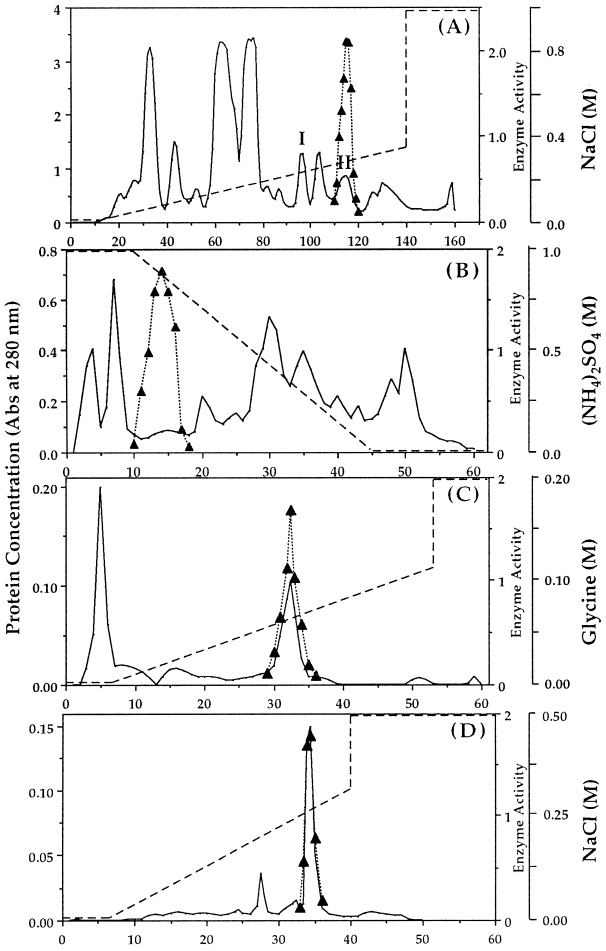

Fig. 1. Purification of a dipeptidase from *Lactobacillus curvatus* DPC2024; protein concentration (———, A280); salt gradient (- - - - - -) dipeptidase activity on Leu–Leu (·····▲·····, A_{507}). Elution profiles from (A) anion-exchange chromatography (DEAE-sephacel); (B) hydrophobic interaction chromatography (Phenyl sepharose); (C) metal chelating affinity chromatography loaded with Cu^{2+} (chelating sepharose fast flow); (D) anion-exchange chromatography (MonoQ HR 5/5).

immobilized with Cu^{2+} and the dipeptidase activity was eluted at ~0.07 M glycine (Fig. 1C). Active fractions were then concentrated and applied to a MonoQ column. The enzyme activity was eluted as a sharp peak at ~0.25 M NaCl. The cell-free extract and the pooled active fractions after each purification step were analysed by SDS-PAGE to determine enzyme purity (Fig. 2). After the final chromatographic step on MonoQ, the enzyme was purified to homogeneity (Lane 6, Fig. 2) with approximately 106-fold concentration over the concentrated cell-free extract with an activity yield of 10.5% (Table 1). The purified enzyme was used for biochemical characterization.

3.1. Determination of molecular mass

The molecular weight of the dipeptidase was estimated to be ~52 kDa by SDS–PAGE (Fig. 2) and gel filtration chromatography on a TSK SW 3000G column (not shown).

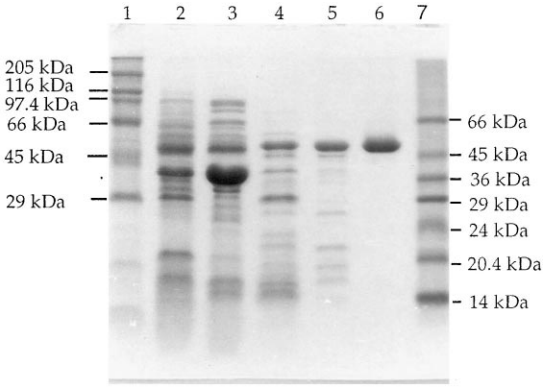

Fig. 2. SDS polyacrylamide gel electrophotograms of fractions obtained during the purification of a dipeptidase from *Lactobacillus curvatus* DPC2024. Lane 2: concentrated cell-free extract; lanes 3–6 are pooled active fractions after chromatography on DEAE-sephacel, phenyl sepharose, chelating sepharose fast flow and MonoQ, respectively; lanes 1 and 7 are high and low molecular weight standards, respectively.

Table 1
Purification of a dipeptidase from *Lactobacillus curvatus* DPC2024

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification factor	Activity yield (%)
CCFE[a]	3138	42 366	13.5	1	100
DEAE-sephacel[b]	392	16 099	41	3	38
HIC[c]	28	10 422	372.2	27.6	24.1
IMAC[d]	8	7838	979.8	72.6	18.5
MonoQ[e]	3.1	4448	1434.8	106	10.5

[a] Concentrated cell-free extract.
[b] Anion-exchange chromotography on DEAE-sephacel.
[c] Hydrophobic interaction chromotography on phenyl sepharose.
[d] Chelating sepharose fast flow loaded with Cu^{2+}.
[e] High performance anion-exchange chromotography on monoQ HR5/5.

3.2. Effect of pH and temperature on dipeptidase activity

The enzyme showed high activity in the pH range of 7.5–9.5 with an optimum activity at pH 8 (Fig. 3A). Very little activity was observed at pH 5.5 but ~20% of the maximum activity was found at pH 11. At pH 8, the enzyme showed high activity at temperatures from 35 to 65°C with maximum activity at 50°C (Fig. 3B). More than 30% of the maximum activity was observed at 20 and 70°C. The enzyme retained ~61, 34 and 1% of its activity when it was pre-incubated for 100 min at 55, 60 and 65°C, respectively (Fig. 4). The enzyme also retained 10% of its activity after pre-heating for 10 min at 70°C indicating that the enzyme was more stable when it was heated with the substrate (Fig. 3B).

3.3. Effect of inhibitors, reducing agents and metal ions

The effect of various compounds on dipeptidase activity is shown in Table 2. The enzyme was almost completely inhibited by EDTA, *o*-phenanthroline and pCMB at 0.1 mM concentration while phenylmethylsulphonyl fluoride, *N*-ethylmaleimide and iodoacetic acid were strongly inhibitory only at 10 mM concentration. The dipeptidase was strongly inhibited by dithiothreitol (1 mM) and β-mercaptoethanol (10 mM). The enzyme activity was strongly inhibited by Fe^{3+} and Hg^{2+} at 0.1 mM, Cd^{2+}, Cu^{2+} and Ni^{2+} at 1.0 mM and Zn^{2+} at 10 mM concentration and slightly by Cd^{2+} and Cu^{2+} at 0.1 mM and Zn^{2+} at 1 mM concentration. Slight activation was observed by treatment with 0.1 mM of Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} and strong activation was caused only by 1 and 10 mM Co^{2+} while Mg^{2+} and Ca^{2+} had no effect at all concentrations tested. Dipeptidase activity, lost due to the treatment with EDTA and *o*-phenanthroline (0.1 mM), was completely restored by 0.1 mM Mn^{2+} and Zn^{2+} and 1 mM Co^{2+} and partially by 0.1 mM Ni^{2+} while no reactivation was observed by the other divalent metal ions tested (e.g., Ca^{2+}, Cu^{2+}, Hg^{2+}). On the other hand, inhibition by pCMB was partially restored (50%) by 1 mM *β*-mercaptoethanol but not by dithiothreitol.

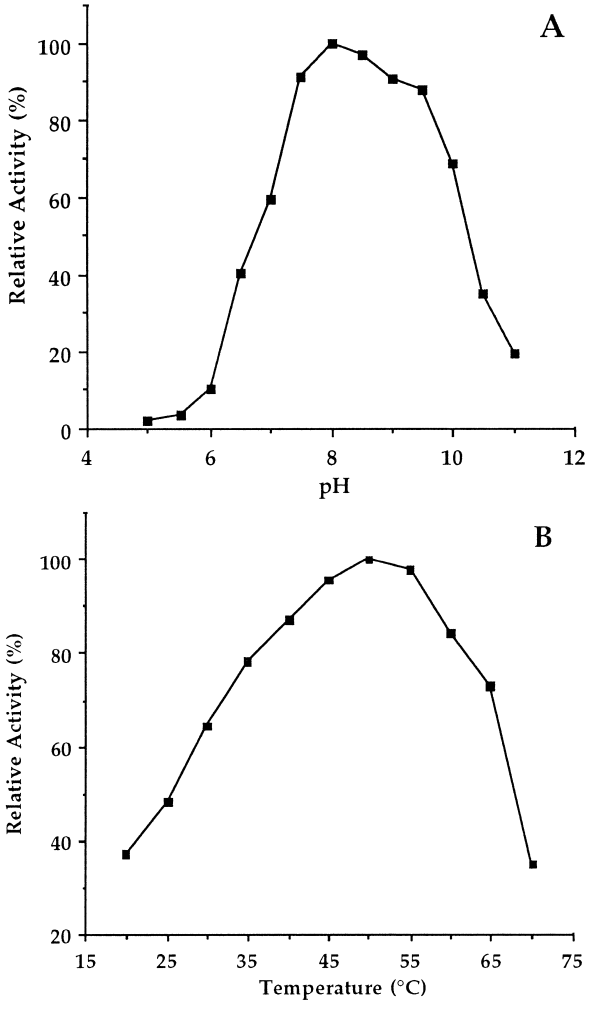

Fig. 3. Effect of environmental conditions on the activity of the purified dipeptidase from *Lactobacillus curvatus* DPC2024 determined on Leu–Leu. Effect of (A) pH in the pH range 5–11 was determined at 50°C and (B) temperature was determined between 20 and 70°C at pH 8.

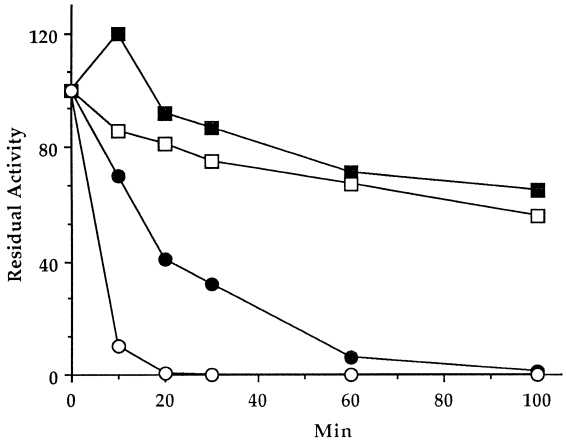

Fig. 4. Thermal stability of the dipeptidase purified from *Lactobacillus curvatus* DPC2024. After pre-heating for 0–100 min at 55 (■), 60 (□), 65 (●) and 70°C (○), residual activity of the dipeptidase was determined at pH 8 and 50°C on Leu–Leu.

Table 2
Effect if different compounds on the activity of the dipeptidase purified from *Lactobacillus curvatus* DPC2024

	Residual activity (%) Concentration (mM l^{-1})		
Compounds	0.1	1	10
None	100	100	100
Ethylenediminetetraacetic acid	0	0	0
o-Phenanthroline	4	0	0
p-Chloromercuribenzoate	2	0	0
N-Ethylmaleimide	93	78	43
Phenylmethylsulphonyl fluoride	100	101	14
Iodoacetic acid	102	98	2
β-Mercaptoethanol	100	66	7
Dithiothreitol	92	12	6
$CaCl_2$	102	101	101
$CoCl_2$	112	231	294
$CdCl_{12}$	73	20	4
$CuCl_2$	79	26	0
$FeCl_3$	5	0	0
$HgCl_2$	0	0	0
$MgCl_2$	101	100	102
$MnCl_2$	120	99	99
$NiCl_2$	112	28	0
$ZnCl_2$	142	71	1

3.4. Substrate specificity

The ability of the dipeptidase to hydrolyse different substrates was determined and results are shown in Table 3. The enzyme was active towards a number of dipeptides with relatively hydrophobic amino acids at their N-termini but did not hydrolyse dipeptides containing proline or dipeptides with Glu and Gly at their N-termini nor N- and C-terminal-blocked dipeptides (CBZ-Phe-Gly, CBZ-Ala-Leu or Leu-Leu-amide). Also, the enzyme did not hydrolyse tri-, tetra-, pentapeptides nor *p*-nitroanilide derivatives of amino acids and peptides.

3.5. N-terminal amino acid sequence

The sequence of the first 20 amino acids was determined for the purified enzyme and is compared with sequences for dipeptidases from *Lb. delbrueckii* subsp. *lactis* DSM 7290, *Lb. helveticus* CNRZ32, *Lb. helveticus* 53/7 and *Lc. lactis* subsp. *cremoris* MG1363 (Dudley, Husgen, He & Steele, 1996; Hellendoorn, Franke-Fayard, Mierau, Venema & Kok, 1997; Vesanto et al., 1996; Vongerichten, Klein, Matern & Plapp, 1994), in Table 4.

4. Discussion

A metal-independent aminopeptidase, a PepN-like aminopeptidase and an X-prolyl dipeptidyl aminopeptidase have been purified and characterized from the cell-free extract of *Lb. curvatus* DPC2024 (Magboul & McSweeney, in press-a, b, c). In this study, a dipeptidase from the cell-free extract of this strain was purified to homogeneity about 106-fold over the concentrated cell-free extract with an activity yield of 10.5%.

The dipeptidase was found to be a monomer with a molecular mass of ~52 kDa. Similar molecular masses have been reported for monomeric dipeptidases from *Lc. lactis* subsp. *cremoris* Wg2 (van Boven et al., 1988), *Lb. delbrueckii* subsp. *bulgaricus* B14 (Wohlrab & Bockelmann, 1992), *Lb. sake* L110 (Montel et al., 1995), *Lb. helveticus* SBT2171 (Tan et al., 1995) and *Lb. casei* subsp. *casei* IFPL 731 (Fernandez-Espla & Martin-Hernandez, 1997). However, dipeptidases from *Lc. lactis* subsp. *cremoris* H61 (Howang et al., 1981), *Lb. sanfrancisco* CB1 (Gobbetti et al., 1996) and *Lb. helveticus* 53/7 (Vesanto et al., 1996) had native molecular masses of 100, 65 and 420 kDa, respectively. While there were

Table 3
Substrate specificity of the dipeptidase purified from *Lactobacillus curvatus* DPC2024

Substrate	Relative activity (%)	Substrate	Relative activity (%)	Substrate	Relative activity (%)
Ala-Ala	29	Phe-Gly	32	Val-Pro-Leu	0
Ala-Leu	99	Pro-Ala	0	Pro-Phe-Gly-Lys	0
Ala-Lys	55	Pro-Gly	0	Ala-Ala-Ala-Ala	0
Ala-Met	98	Pro-Leu	0	$(Ala)_5$	0
Ala-Phe	98	Pro-Phe	0		
Ala-Pro	0	Pro-Pro	0		
Asp-Leu	18	Tyr-Phe	81	Leu-pNA	0
Glu-Val	0	N-CBZ-Phe-Gly	0	Ala-pNA	0
Gly-Leu	0	N-CBZ-Ala-Leu	0	Gly-pNA	0
His-Leu	6	Leu-Leu-amide	0	Glu-pNA	0
His-Val	8	Ala-Ala-Ala	0	Lys-pNA	0
Leu-Ala	91	Ala-Leu-Gly	0	Pro-pNA	0
Leu-Arg	98	Arg-Pro-Pro	0	Val-pNA	0
Leu-Leu[a]	100	Gly-Pro-Arg	0	Phe-pNA	0
Leu-Pro	0	Leu-Ala-Pro	0	Met-pNA	0
Leu-Trp	26	Leu-Gly-Gly	0	His-pNA	0
Leu-Val	31	Leu-Leu-Leu	0	Gly-Phe-pNA	0
Lys-Leu	4	Met-Leu-Gly	0	Arg-Pro-pNA	0
Met-Ala	94	Phe-Gly-Pro	0	Ala-Ala-pNA	0
Phe-Ala	82	Pro-Gly-Gly	0	Ala-Ala-Pro-pNA	0

[a] Leu-Leu hydrolysis was considered as 100%.

Table 4
Sequence alignment of the first 20 amino acid residues determined for the dipeptidase purified from *Lactobacillus curvatus* DPC2024 (A) and deduced N-terminal amino acid sequences for dipeptidases from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290 (B), *Lactococus lactis* subsp. *cremoris* MG1363 (C), *Lactobacillus helveticus* CNRZ32 (D), *Lactobacillus helveticus* 53/7 (E)

(A)			**T_1**	**L**	**N**	**W**	**Q**	**Q**	**E**	**A**	**A**	**K**	**Y**	**K**	**D**	**Q**	**M**	**L**	**A**	**D**	**L**	**T_{20}**
(B)		M_1	D	**L**	**N**	F	K	E	L	**A**	E	A	K	**K**	**D**	A	I	**L**	K	**D**	**L**	E_{21}
(C)	M_1	T	**T**	I	D	F	K	A	**E**	V	E	**K**	R	**K**	**D**	A	L	M	E	**D**	**L**	F_{22}
(D)		M_1	K	Q	T	E	C	T	T	I	L	V	G	**K**	K	A	S	I	D	G	S	**T_{21}**
(E)		M_1	K	Q	T	E	C	T	T	I	L	V	G	**K**	K	A	S	I	D	G	S	**T_{21}**

no data on the quaternary structure for the dipeptidase from *Lc. lactis* subsp. *cremoris* H61, the dipeptidase from *Lb. sanfrancisco* CB1 was a monomer and that from *Lb. helveticus* 53/7 had eight subunits of ~53.5 kDa.

The dipeptidase from *Lb. curvatus* DPC2024 had an optimum activity at pH 8 and 50°C. Optima between pH 7 and 8 have been reported for other dipeptidases purified from LAB (Fernandez-Espla & Martin-Hernandez, 1997; Gobbetti et al., 1996; Montel et al., 1995; Tan et al., 1995; van Boven et al., 1988; Wohlrab & Bockelmann, 1992), with the exception of a dipeptidase from *Lb. helveticus* 53/7 (Vesanto et al., 1996) which had optimum pH of 6. While most of the dipeptidases purified from LAB had optimum temperatures between 40 and 55°C, dipeptidases from *Lb. sanfrancisco* CB1 (Gobbetti et al., 1996) and *Lb. casei* subsp. *casei* IFPL 731 (Fernandez-Espla & Martin-Hernandez) showed maximum activity at 30 and 60–75°C, respectively. The dipeptidase from *Lb. curvatus* DPC2024 was relatively stable to pre-heating for 100 min at 55 and 60°C and retained ~10% of its activity after 10 min pre-incubation at 70°C. A dipeptidase from *Lb. helveticus* SBT 2171 retained 20% of its initial activity when pre-heated for 30 min at 75°C (Tan et al., 1995) while a dipeptidase from *Lb. casei* subsp. *casei* IFPL 731 retained 100% of its activity after pre-heating for 30 min at 60°C and lost 90% activity following preheating for 15 min at 70°C (Fernandez-Espla & Martin-Hernandez).

The effect of inhibitors showed that the dipeptidase characterized in this study was a metalloenzyme, as were all the other dipeptidases reported so far from LAB with the exception of the dipeptidase from *Lb. helveticus* 53/7 which was unaffected by metal chelators (Vesanto et al., 1996). The dipeptidase from *Lb. curvatus* DPC2024 was stimulated by Co^{2+}, Zn^{2+} and Mn^{2+} and the activity lost by treatment with EDTA and *o*-phenanthroline was fully restored by Co^{2+}, Mn^{2+} and Zn^{2+} and partially by Ni^{2+}. The divalent metal ions, Co^{2+} and Mn^{2+}, restored the activity of the apoenzymes from other metallo-dipeptidases from LAB formed as a result of treatment with metal chelators (Fernandez-Espla & Martin-Hernandez, 1997; Montel et al., 1995; Tan et al., 1995; van Boven et al., 1988; Wohlrab & Bockelmann, 1992). However, Co^{2+} was found to inhibit dipeptidases from *Lc. lactis* subsp. *cremoris* Wg2 and *Lb. delbrueckii* subsp. *bulgaricus* B14 at concentrations greater than 0.1 mM. Dithiothreitol and β-mercaptoethanol increased the activity of a dipeptidase from *Lb. delbrueckii* subsp. *bulgaricus* B14 at concentrations of 0.1–1 mM. These reagents, however, were inhibitory to other dipeptidases purified from LAB including the enzyme characterized in this study. Also, the enzyme characterized in this study was strongly inhibited by pCMB as were other dipeptidases from LAB (Fernandez-Espla & Martin-Hernandez; Gobbetti et al., 1996; Montel et al., 1995; Tan et al., 1995; van Boven et al., 1988; Vesanto et al., 1996).

The dipeptidase from *Lb. curvatus* DPC2024 hydrolysed a number of dipeptides with relatively hydrophobic amino acids but not tri-, tetra-, pentapeptides, *p*-nitroanilide derivatives of amino acids and peptides, dipeptides containing proline or dipeptides with glutamine and glycine as N-terminal amino acid. Similar specificities were observed for most of the dipeptidases isolated so far from LAB. Some of the dipeptides hydrolysed by the dipeptidase purified from *Lb. curvatus* DPC2024 were also hydrolysed by two aminopeptidases purified from the same strain (Magboul & McSweeney, in press-a, b).

Sequence alignment of the first 20 amino acid residues determined for the dipeptidase purified from *Lb. curvatus* DPC2024 revealed 40 and 35% homology with dipeptidases from *Lb. delbrueckii* subsp. *lactis* DSM 7290 (Vongerichten et al., 1994) and *Lc. lactis* subsp. *cremoris* MG1363 (Hellendoorn et al., 1997) while only 10% homology was observed with dipeptidases from *Lb. helveticus* CNRZ32 and *Lb. helveticus* 53/7 (Dudley et al., 1996; Vesanto et al., 1996).

The contribution of mesophilic lactobacilli to cheese flavour is equivocal but there has been increased interest in recent years in their proteolytic system. Therefore, the biochemical and genetic characterization of the peptidiolytic system of NSLAB in combination with the recent developments in chromosomal integration systems for LAB to construct (multiple) peptidase negative mutants, would be one way to determine the role these microorganims might play in cheese flavour development. The enzyme purified from the cell-free extract of *Lb. curvatus* DPC2024 might contribute to cheese during ripening if the cells of this microorganism lyse during the ripening process since most of the peptidases of LAB have an intracellular location (Kunji et al., 1996).

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