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Original article

Purification and characterisation of an intracellular aminopeptidase from *Brevibacterium linens* ATCC 9174

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Summary — An intracellular aminopeptidase from *Brevibacterium linens* ATCC 9174 was purified 4300-fold to homogeneity using ammonium sulphate fractionation, anion exchange chromatography, hydrophobic interaction chromatography and anion exchange chromatography. The pH and temperature optima were 8.5 and 35 °C, respectively. The purified aminopeptidase was stable over the range pH 8 to 10, and was thermally stable up to 20 °C at pH 8.5. The molecular mass of the enzyme was found to be 59 kDa by SDS–PAGE and 69 kDa by gel filtration, indicating that the native enzyme exists as a monomer. The aminopeptidase was strongly inhibited by the thiol blocking agent, *p*-hydroxymercuribenzoate, and by Co^{2+} and Zn^{2+}; activity was unaffected by metal chelators, reducing agents or phenylmethylsulphonyl fluoride. K_m and k_{cat} values for L-Ala-*p*-NA were 3.3 mmol/L and 4.3 s^{-1}, respectively, while the corresponding values for L-Gly-*p*-NA were 0.2 mmol/L and 7.6 s^{-1}, respectively. The aminopeptidase hydrolysed dipeptides with an alanine residue at the N-terminal but tripeptides were not hydrolysed. The sequence of the first 19 N-terminal amino acids was NH_2-Pro-Phe-Asp-Gly-Pro-Asp-Thr-Ala-Ala-Ile-Ile-Asp-Arg-Leu-?-Asn-Ala-?-Thr.

aminopeptidase / *Brevibacterium linens* / cheese / purification

Résumé — Purification et caractérisation d'une aminopeptidase intracellulaire de *Brevibacterium linens* ATCC 9174. Une aminopeptidase intracellulaire de *Brevibacterium linens* ATCC 9174 a été purifiée 4300 fois par homogénéisation et fractionnement au sulphate d'ammonium, chromatographie d'échange d'anions, chromatographie par interactions hydrophobes et chromatographie d'échange d'anions. Le pH et la température optimale étaient respectivement de 8,5 et 35 °C. L'aminopeptidase était stable, de pH 8 à 10. De plus, elle était stable thermiquement à 20 °C à pH 8,5. La masse moléculaire de l'enzyme, determinée par SDS–PAGE et filtration sur gel était respectivement de 59 kDa et 69 kDa, ce qui indique que l'enzyme native existe sous forme de

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monomère. L'activité enzymatique est fortement inhibée par l'agent bloquant les groupements thiols, le *p*-hydroxymercuribenzoate, et par les ions Co^{2+} et Zn^{2+}; l'activité enzymatique n'est pas inhibée par les chélateurs de métal, les agents réducteurs ou le fluorure de phenylmethylsulphonyle. Les valeurs K_m et k_{cat} pour le L-Ala-*p*-NA étaient respectivement de 3,3 mmol/L et 4,3 s^{-1}, tandis que les valeurs correspondantes pour L-Gly-*p*-NA étaient respectivement 0,2 mmol/L et 7,6 s^{-1}. L'aminopeptidase hydrolysait les dipeptides possédant un résidu alanine en N-terminal mais les tripeptides n'étaient pas hydrolysés. L'ordre des 19 premiers acides aminés N-terminaux était NH_2-Pro-Phe-Asp-Gly-Pro-Asp-Thr-Ala-Ala-Ile-Ile-Asp-Arg-Leu-?-Asn-Ala-?-Thr.

aminopeptidase / *Brevibacterium linens* / fromage / purification

INTRODUCTION

The surface microflora of various smear surface-ripened cheeses, such as Limburger, Gruyère, Münster, Brick, Appenzeller and Tilsiter, includes yeasts, moulds and bacteria (Kelly, 1937; Langhus et al, 1945; El-Erian, 1969). During ripening, yeasts grow initially on the surface of the cheese and raise the curd pH by a combination of lactate utilisation and ammonia production. This increase in pH enables the growth of *Brevibacterium linens*, which eventually dominates the surface microflora of these cheeses. The growth of *B linens* on the surface is an essential prerequisite for the development of the characteristic flavour, colour, aroma and texture of smear surface-ripened cheeses (Ades and Cone, 1969; El-Erian, 1969; Sharpe et al, 1977; Hemme et al, 1982).

The purification and characterisation of extracellular proteinases and aminopeptidases from *B linens* has been described (Foissy, 1974, 1978a–c; Hayashi and Law, 1989; Hayashi et al, 1990; Juhász and Skárka, 1990; Rattray et al, 1995, 1996). However, the level of information on the intracellular activities of this microorganism is quite limited. At least six different peptide hydrolases were identified using a zymogram technique in the intracellular fraction of *B linens* ATCC 9174 (Torgersen and Sørhaug, 1978). Using the same technique (Sørhaug, 1981), a total of 18 peptide hydrolases with different substrate specificities were tentatively identified in six strains of *B linens*.

A partially purified aminopeptidase from an intracellular fraction of *B linens* HS was found to have pH and temperature optima at 7.5 and 30 °C, respectively; the aminopeptidase was inhibited by metal chelators and *p*-hydroxymercuribenzoate (PHMB) (El-Soda et al, 1991). Intracellular peptidase activity (unspecified) from a cell-free extract of *B linens* was reported (Wong and Cone, 1964) to be optimally active at pH 7.9 and 45 °C, and was strongly inhibited by Hg^{2+} and PHMB.

This paper reports the purification and characterisation of an intracellular aminopeptidase from *B linens* ATCC 9174.

MATERIALS AND METHODS

Microorganism and growth conditions

Brevibacterium linens ATCC 9174 was obtained from the culture collection of the Department of Microbiology, University College, Cork, Ireland. The organism was precultured in 250 mL of medium [1.0% trypticase peptone (w/v), 0.3% Bacto peptone (w/v), 0.25% yeast extract (w/v), 0.5% glucose (w/v), 0.25% K_2HPO_4 (w/v) and 0.02% $MgSO_4.7H_2O$ (w/v)] in a 500 mL flask on an orbital shaker at 200 cycles per min at 23 °C. After 48 h, 100 mL of preculture were used to inoculate 10 L of the same medium in a 10 L fermenter (B Braun Diessel Biotech GmbH, Melsungen, Germany). The temperature was

maintained at 23 °C, with 10% O_2 saturation; the pH was not regulated.

Preparation of cell lysate

After cultivation for 65 h, ie, late log phase, cells were harvested by centrifugation at 14 500 *g* for 25 min at 4 °C. The cells were then washed with 20 mmol/L Tris–HCl, pH 8.0, and re-centrifuged. The cells (80 g) were suspended in 500 mL of 20 mmol/L Tris–HCl, pH 8.0, containing 10 mmol/L $MgCl_2$ and 240 mg lysozyme (Sigma; from chicken egg white, 3 × crystallized), then incubated with gentle shaking for 2.5 h at 37 °C. To the resulting cell lysate, 10 mg of DNase 1 (Boehringer-Mannheim; grade II, from bovine pancreas) and 10 mg of RNase A (Sigma; type I-AS, from bovine pancreas) were added and incubated for 30 min at 37 °C. The lysate was then centrifuged at 14 500 *g* for 25 min at 4 °C, and the resulting supernatant used for aminopeptidase purification.

Measurement of aminopeptidase activity

Aminopeptidase activity was measured spectrophotometrically using L-alanine-*p*-nitroanalide (L-Ala-*p*-NA) as substrate; 50 µL of enzyme solution were added to 400 µL of 50 mmol/L Tris–HCl buffer, pH 8.5, and the reaction initiated by adding 50 µL substrate (10 mmol/L in water). The reaction mixture was incubated at 35 °C for 30 min, after which 1 mL of 30% (v/v) acetic acid was added to terminate the reaction. The absorbance of the mixture was then measured at 410 nm using a Cary 1E UV-Visible Spectrophotometer (Varian Australia Pty Ltd, Victoria, Australia). A standard curve was generated using *p*-nitroaniline. One unit of enzymatic activity was defined as the amount of enzyme that liberates 1 nmol of *p*-NA per min at 35 °C, pH 8.5. Specific activity was defined as units per mg protein. Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard.

Enzyme purification

Prior to chromatographic separation, the lysate was fractionated using $(NH_4)_2SO_4$. The lysate was adjusted to 45% saturation with $(NH_4)_2SO_4$, then centrifuged at 14 500 *g* for 25 min at 4 °C. After centrifugation, the 45% soluble fraction was adjusted to 80% saturation, and re-centrifuged. The 80% insoluble pellet was dissolved in 80 mL of 20 mmol/L Na phosphate buffer, pH 7.0, and dialysed overnight against 5 L of the same buffer.

The dialysed $(NH_4)_2SO_4$ fraction was applied to a DEAE–Sepharose Fast Flow anion exchange column (5 × 20 cm gel bed); (Pharmacia LKB Biotechnology, Uppsala, Sweden) connected to a Bio-Rad Econo System (Bio-Rad Laboratories Ltd, Hertfordshire, United Kingdom). The column was washed initially with 600 mL of 20 mmol/L Na phosphate buffer, pH 7.0, followed by a linear NaCl gradient (0 to 0.4 mol/L) in the same buffer (1400 mL); the NaCl concentration was then maintained at 0.4 mol/L for a further 500 mL, then followed by a linear gradient (0.4 to 0.5 mol/L) for 360 mL, and maintained at 0.5 mol/L for 420 mL. The flow rate was 12 mL per min and 15 mL fractions were collected. Active fractions were pooled and dialysed against 20 mmol/L Na phosphate buffer, pH 7.0. The dialysed solution was then adjusted to 2 mol/L $(NH_4)_2SO_4$ and applied to a Phenyl–Sepharose High Performance hydrophobic interaction column (1.6 × 14 cm gel bed); (Pharmacia LKB Biotechnology) connected to a Bio-Rad Econo System (Bio-Rad Laboratories Ltd). The column was washed initially with 150 mL of 20 mmol/L Na phosphate buffer, pH 7.0, containing 2.0 mol/L $(NH_4)_2SO_4$, followed by a linear $(NH_4)_2SO_4$ gradient from 2.0 to 0 mol/L for 300 mL and then maintained at 0 mol/L $(NH_4)_2SO_4$ for a further 100 mL. The flow rate was 1 mL per min and the fraction size was 5 mL. Pooled active fractions were desalted and concentrated using a Centricon-3 concentrator (Amicon Ltd, Gloucestershire, UK), and then diluted using 20 mmol/L Na phosphate buffer, pH 7.0, and injected onto a Mono-Q

HR5/5 column connected to FPLC System (Pharmacia LKB Biotechnology). The column was washed with 4 mL of 20 mmol/L Na phosphate buffer, pH 7.0, followed by a linear NaCl gradient, 0 to 0.37 mol/L, in the same buffer (41 mL), then maintained at 0.37 mol/L for 2 mL, followed by a linear NaCl gradient, 0.37 to 0.4 mol/L, over 8 mL and finally by a gradient of 0.4 to 0.5 mol/L over 10 mL. The flow rate was 1 mL per min, and 1 mL fractions were collected.

Polyacrylamide gel electrophoresis

Purity and molecular weight were determined by SDS–PAGE (Laemmli, 1970) in a gel containing 10% (w/v) polyacrylamide. The gel was stained for protein with 0.125% (w/v) Coomassie brilliant blue R-250 in methanol–acetic acid–water (40:7:53, v/v) and destained in methanol–acetic acid–water (25:10:65). Molecular mass markers were: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

Estimation of molecular mass by gel filtration

The molecular mass of the purified enzyme was also estimated by gel filtration on a TSK-Gel G3000SW column (TosoHaas GmbH, Stuttgart, Germany), using a 0.1 mol/L Na phosphate buffer, pH 7.0, containing 0.1 M Na sulphate. Bovine thyroglobulin (666 kDa), bovine γ-globulin (156 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), β-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa) were used as reference proteins for standard curve calibration.

Temperature and pH optima

Optimum temperature was determined by assaying activity over the range 4 to 60 °C in Tris–HCl, pH 8.5. The optimum pH was determined at 35 °C using Na acetate (pH 5–5.5), Na phosphate (pH 6–7), Tris–HCl (pH 7.5–8.5), and Na carbonate (pH 9–11). Buffer concentration in all assays was 50 mmol/L.

Temperature and pH stability

Temperature stability was determined by incubating 50 μL enzyme solution in 50 mmol/L Tris–HCl, pH 8.5, at 4, 20, 30, 40 or 50 °C for up to 24 h. Residual activity was then measured at the optimum temperature (35 °C). pH stability was determined by incubating 30 μL of enzyme solution with 70 μL of buffer (Na acetate, pH 5, Na phosphate, pH 6–7, Tris–HCl, pH 8–9, or Na carbonate, pH 10–11) for 24 h at 20 °C. The pH was then re-adjusted to 8.5 by adding 350 μL of 50 mmol/L Tris–HCl, and the residual aminopeptidase activity was measured as described.

Effect of activators and inhibitors

To 25 μL of dialysed purified enzyme solution, 400 μL of 50 mmol/L Tris–HCl, pH 8.5 were added, followed by 25 μL of activator or inhibitor (10 mmol/L in water), and pre-incubated for 30 min at 25 °C. Residual activity was then assayed by the addition of 50 μL substrate (10 mmol/L L-Ala-*p*-NA in water) and incubation for 30 min at 35 °C. The final concentration of activator or inhibitor was either 0.1 or 1.0 mmol/L. For reactivation of *p*-hydroxymercuribenzoate-treated enzyme (1.0 mmol/L), 1.0 mmol/L dithiothreitol was added, and the activity then measured as described.

Kinetic studies

The K_m and k_{cat} of the purified aminopeptidase were determined for L-Ala-*p*-NA or L-Gly-*p*-NA as substrate. The aminopeptidase was incubated in 50 mmol/L Tris–HCl, pH 8.5, with substrate at final concentrations ranging from 0.2 to

5.0 mmol/L for L-Ala-*p*-NA and 0.05 to 1.6 mmol/L for L-Gly-*p*-NA. After incubation for 15 min, the reaction was terminated and the activity measured as described previously. K_m values were calculated from Lineweaver–Burk plots using an Enzfitter computer programme (BIOSOFT, Cambridge, UK).

Substrate specificity studies

Substrate specificity on a range of peptides was determined by the addition of 50 µL of peptide substrate (10 mmol/L in water) to 400 µL of 50 mmol/L Tris–HCl, pH 8.5, followed by 50 µL of enzyme solution. The reaction mixture was incubated for 30 min at 35 °C. Liberated free amino groups were reacted with 1 mL of cadmium–ninhydrin reagent (0.8 g ninhydrin in 80 mL ethanol and 10 mL acetic acid, to which was added 1 g $CdCl_2$ in 1 mL of water) and heating at 84 °C for 5 min (Doi et al, 1981). After cooling, the developed colour was measured at 507 nm. Activity on various nitroanilide substrates was also assayed, as described previously.

N-terminal amino acid sequence

The purified aminopeptidase was centrifuged through a ProSpin Sample Preparation Cartridge (Applied Biosystems Inc, Foster City, CA, USA) onto a ProBlott polyvinylidene difluoride (PVDF) membrane. The N-terminal amino acid sequence was determined at the National Food Biotechnology Centre, University College, Cork, Ireland, by Edman degradation on an automated pulsed liquid-phase protein-peptide sequencer (Applied Biosystems Inc, model 477A). Liberated amino acids were detected as their phenylthiohydantoin derivatives using a 120A analyser (Applied Biosystems Inc).

RESULTS

Purification of aminopeptidase

Purification of the aminopeptidase was achieved using four purification steps. Ammonium sulphate precipitation, in addition to concentration, removed a considerable amount of orange pigmentation from the lysate. In the first chromatographic step (DEAE–Sepharose Fast Flow), the aminopeptidase activity was eluted at approximately 0.4 mol/L NaCl with a 25-fold increase in specific activity (fig 1a). The enzyme was eluted from the hydrophobic interaction column at 0.0 mol/L $(NH_4)_2SO_4$ (fig 1b) and specific activity was increased 713-fold. In the final chromatographic step (Mono-Q), activity was eluted at 0.37 mol/L NaCl (fig 1c), with a 4307-fold increase in specific activity. The results of the purification protocol are summarised in table I.

SDS–PAGE and molecular mass

Purity of the isolated enzyme was confirmed by SDS–PAGE; a single band with a molecular mass of 59 kDa was observed (fig 2, lane 6). HPLC gel filtration indicated a single peak corresponding to a molecular mass of 69 kDa, suggesting that the enzyme exists as a monomer in its native state.

Temperature and pH optima and stability

The optimum temperature of the aminopeptidase was determined to be 35 °C (fig 3a), while the optimum pH was 8.5 under the assay conditions used (fig 3b). The aminopeptidase was stable for 24 h at temperatures up to 20 °C (fig 4a), but at 30 °C, 25% activity was lost after 24 h. The aminopeptidase was unstable at 40 and 50 °C; no activity remained after 8 h and 30 min, respectively. The enzyme was relatively unstable at both acidic and alkaline pH; it was totally inactivated after 24 h at pH 5–6 and pH 11 (fig 4b); maximum stability was observed at pH 9.

Effect of activators and inhibitors

The effects of chemical reagents and divalent cations on aminopeptidase activity are sum-

marised in table II. The sulphydryl reducing agents, dithiothreitol (DTT), β-mercaptoethanol and cysteine, and the metal chelators, EDTA, ethylene glycol-bis(β-amino-ethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) and 1,10-phenanthroline, had no effect on activity at 0.1 or 1.0 mmol/L (table II). The serine proteinase inhibitor, phenylmethylsulfonyl fluoride (PMSF), and the alkylating agent, *N*-ethylmaleimide, also had no effect on activity. Iodoacetate caused a slight reduction in activity at 1.0 mmol/L, but the thiol-blocking agent, *p*-hydroxymercuribenzoate (PHMB), caused strong inhibition at 0.1 and 1.0 mmol/L. The PHMB-treated enzyme was almost fully reactivated (88%) by 1.0 mmol/L DTT.

None of the divalent cations activated the aminopeptidase but reduced activity in the order Zn^{2+} >, Co^{2+} >, Cd^{2+} >, Ni^{2+} >, Hg^{2+} >, Cu^{2+} >, Mn^{2+} >, Ca^{2+} > and Mg^{2+}; Fe^{2+} had no effect on activity (table II).

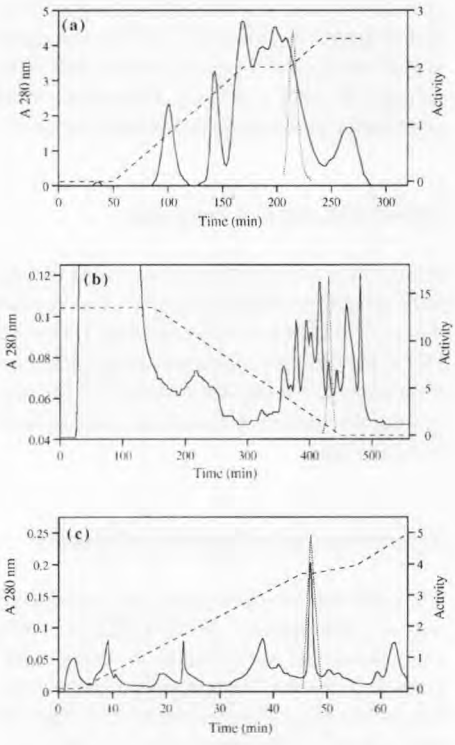

Fig 1. Purification of the intracellular aminopeptidase from *B linens* ATCC 9174. **a:** DEAE–Sepharose Fast Flow anion exchange chromatography; **b:** phenyl–Sepharose high performance hydrophobic interaction chromatography; **c:** Mono-Q anion exchange chromatography. $A_{280\ nm}$ (——); activity (•••••); NaCl or $(NH_4)_2SO_4$ (– – –).
Purification de l'aminopeptidase intracellulaire de B linens *ATCC 9174.* **a** *: chromatographie d'échange d'anions (DEAE–Sepharose Fast Flow) ;* **b** *: chromatographie d'interactions hydrophobes (Phenyl–Sepharose) ;* **c** *: chromatographie d'échange d'anions (Mono-Q).* $A_{280\ nm}$ *(——) ; activité (•••••); NaCl ou* $(NH_4)_2SO_4$ *(– – –).*

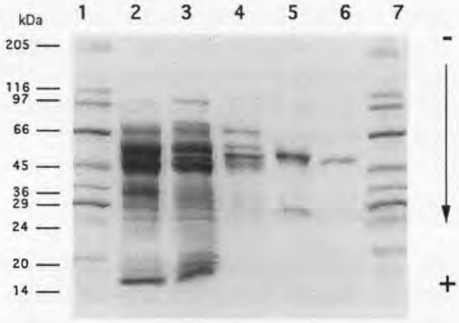

Fig 2. SDS–PAGE of the intracellular aminopeptidase from *B linens* ATCC 9174 after different purification steps. Lanes 1 and 7, molecular mass markers; lane 2, crude intracellular extract; lane 3, ammonium sulphate precipitation; lane 4, DEAE–Sepharose Fast Flow anion exchange chromatography; lane 5, Phenyl–Sepharose High Performance hydrophobic interaction chromatography; lane 6, Mono-Q anion exchange chromatography.
SDS–PAGE de l'aminopeptidase intracellulaire de B linens *ATCC 9174 après différentes étapes de purification. Voies 1 et 7, standards de masse moléculaire ; voie 2, extrait intracellulaire brut ; voie 3, précipitation sulphate d'ammonium ; voie 4, chromatographie d'échange d'anions (DEAE–Sepharose Fast Flow) ; voie 5, chromatographie d'interactions hydrophobes (Phenyl–Sepharose) ; voie 6, chromatograhie d'échange d'anions (Mono-Q).*

Substrate specificity

The relative activity of the aminopeptidase on L-Ala-*p*-NA and L-Gly-*p*-NA was 100 and 164%, respectively (table III). The aminopeptidase had no activity on any of the remaining aminopeptidase, iminopeptidase or dipeptidyl aminopeptidase substrates (table III). The relative activity of the aminopeptidase on dipeptides showed that only those with an alanine residue in the N-terminal position were hydrolysed. Maximum activity was observed on DL-Ala-DL-Ala. No activity was detected on any of the tripeptides studied.

Kinetic studies

K_m and k_{cat} values for L-Ala-*p*-NA were 3.3 mmol/L and 4.3 s^{-1}, respectively, while the corresponding values for L-Gly-*p*-NA were 0.2 mmol/L and 7.6 s^{-1}, respectively.

N-terminal amino acid sequence

The sequence of the first 19 N-terminal amino acids was NH_2-Pro-Phe-Asp-Gly-Pro-Asp-Thr-Ala-Ala-Ile-Ile-Asp-Arg-Leu-?-Asn-Ala-?-Thr. Homogeneity of the enzyme preparation was

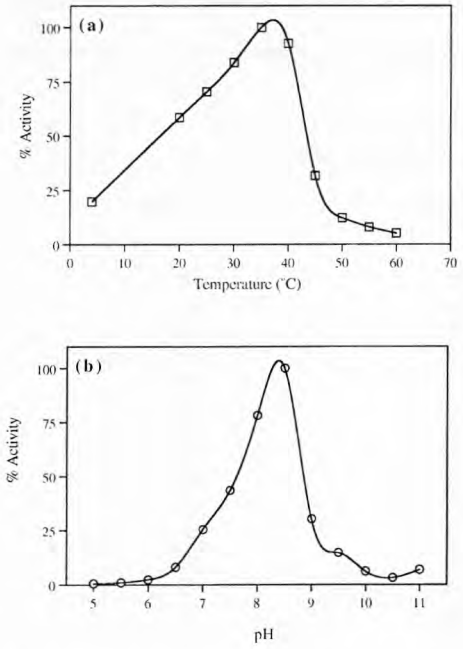

Fig 3. Effect of temperature (**a**) and pH (**b**) on the activity of the intracellular aminopeptidase from *B linens* ATCC 9174.
*Effet de la température (**a**) et du pH (**b**) sur l'activité de l'aminopeptidase intracellulaire de* B linens *ATCC 9174.*

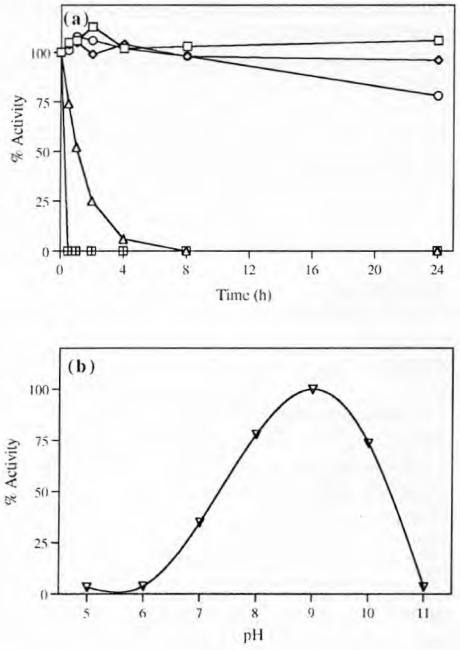

Fig 4. Effect of (**a**) temperature [4 (□), 20 (◇), 30 (○), 40 (Δ), or 50°C (⊞)] and (**b**) pH on the stability of the intracellular aminopeptidase from *B linens* ATCC 9174.
*Effet (**a**) de la température [4 (□), 20 (◇), 30 (○), 40 (Δ), ou 50 °C (⊞)] et (**b**) du pH sur la stabilité de l'aminopeptidase intracellulaire de* B linens *ATCC 9174.*

Table I. Purification of an intracellular aminopeptidase from *B linens* ATCC 9174.
Purification d'une aminopeptidase intracellulaire de B linens *ATCC 9174.*

Purification step	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
Cell-free extract	3332	57418	17	100	1.0
Ammonium sulphate fractionation	743	30896	42	54	2.5
DEAE–Sepharose	27.6	11727	425	20	25
Phenyl–Sepharose	0.55	6662	12113	12	712
Mono-Q	0.05	3661	73220	6	4307

Table II. Effect of chemical reagents and divalent cations on the activity of an intracellular aminopeptidase from *B linens* ATCC 9174.
Effet des réactifs chimiques et des cations bivalents sur l'activité d'une aminopeptidase intracellulaire de B linens *ATCC 9174.*

Reagent or cation [a]	*Aminopeptidase activity (%)*	
	0.1 mmol/L	*1.0 mmol/L*
Dithiothreitol	105	105
β-Mercaptoethanol	101	101
Cysteine	105	106
EDTA	104	101
EGTA	104	101
1,10-Phenanthroline	103	98
PMSF	100	99
N-Ethylmaleimide	103	99
Iodoacetic acid	98	96
PHMB	67	25
Fe^{2+}	104	96
Mg^{2+}	100	90
Ca^{2+}	96	85
Mn^{2+}	98	78
Cu^{2+}	97	48
Hg^{2+}	95	41
Ni^{2+}	75	41
Cd^{2+}	73	37
Co^{2+}	37	23
Zn^{2+}	23	15

[a] EDTA: ethylenediaminetetraacetic acid; EGTA: ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF: phenylmethylsulfonyl fluoride; PHMB: *p*-hydroxymercuribenzoate.

Table III. Relative activities[a] of intracellular aminopeptidase from *B linens* ATCC 9174 on various substrates. *Activités relatives d'une aminopeptidase intracellulaire de* B linens *ATCC 9174 sur différents substrats.*

Substrate	*Relative activity (%)*	*Substrate*	*Relative activity (%)*
Ala-*p*-NA	100	DL-Ala-DL-Ala	100
Gly-*p*-NA	164	D-Ala-D-Ala	22
Val-*p*-NA	0	Ala-Ala	10
Phe-*p*-NA	0	DL-Ala-DL-Phe	8
Pro-*p*-NA	0	Ala-Leu	4
Glu-*p*-NA	0	Ala-Phe	1
Leu-*p*-NA	0	Tyr-Leu	0
Lys-*p*-NA	0	Tyr-Phe	0
Arg-*p*-NA	0	Leu-Gly	0
Gly-Phe-*p*-NA	0	Leu-Leu	0
Ala-Pro-*p*-NA	0	Leu-Pro	0
		Leu-Ala	0
		Phe-Ala	0
		Phe-Gly	0
		Glu-Val	0
		His-Leu	0
		Pro-Gly	0
		Ala-Ala-Ala	0
		Leu-Gly-Gly	0
		Leu-Gly-Pro	0
		Phe-Gly-Gly	0

[a] Rates of L-Ala-*p*-NA or DL-Ala-DL-Ala hydrolysis were taken as 100%.

[a] *Les taux d'hydrolyse de* L-*Ala-p-NA ou* DL-*Ala-*DL-*Ala ont été fixés à 100 %.*

confirmed by no significant background interference during sequencing. The N-terminal amino acid sequence was compared against Swiss-prot Release 31, PIR Release 43 and GenBank Release 87 data banks (DNASTAR Inc, Madison, Wisconsin, USA) using the Lipman–Pearson protein alignment technique, but no significant homology was found.

DISCUSSION

An intracellular aminopeptidase from *B linens* ATCC 9174 was purified to homogeneity using four purification steps, ie, ammonium sulphate fractionation, anion exchange, hydrophobic interaction and anion exchange chromatography. The purified aminopeptidase had pH and temperature optima of 8.5 and 35 °C, respectively, which are comparable to the values pH 7.5 and 30 °C found for a partially purified aminopeptidase from *B linens* HS (El-Soda et al, 1991). The molecular mass of the aminopeptidase determined by SDS–PAGE and HPLC gel filtration was 59 and 69 kDa, respectively, indicating that the enzyme exists as a monomer in its native state.

The aminopeptidase was strongly inhibited by the thiol blocking agent, PHMB, with reactivation by the reducing agent DTT, indicating that a thiol group is present at the active site of the enzyme. The partially purified aminopeptidase from *B linens,* HS, was likewise inhibited by PHMB, but also by chelating agents and by the

serine proteinase inhibitor, PMSF (El-Soda et al, 1991). In contrast, the vast majority of aminopeptidases from lactic acid bacteria are metalloenzymes (Visser, 1993), with the notable exception of an aminopeptidase from *Lactobacillus delbrueckii* ssp *bulgaricus* B14 (Wohlrab and Bockelmann, 1993), which was inhibited by thiol blocking agents. Of the metals assessed, Zn^{2+} was the most inhibitory; aminopeptidases isolated from various lactic acid bacteria have also been shown to be extremely sensitive to Zn^{2+} (Bockelmann et al, 1992; Midwinter and Pritchard, 1994; Rul et al, 1994).

The aminopeptidase from *B linens* ATCC 9174 had a narrow substrate specificity; it hydrolysed both aminopeptidase and dipeptidase substrates, but not dipeptidylaminopeptidase or tripeptidase substrates. Activity on aminopeptidase substrates was confined to L-Ala- and L-Gly-*p*-NA, with highest activity on the latter; no activity was recorded on L-Val-, L-Phe-, L-Glu-, L-Leu-, L-Lys- or L-Arg-*p*-NA. Evidently, the aminopeptidase is specific for small, non-polar amino acids. The partially purified aminopeptidase from *B linens* HS (El-Soda et al, 1991) was also active on L-Ala- and L-Gly-, with the highest activity on L-Gly-*p*-NA, and no activity on L-Leu-, L-Lys-, L-Pro- or L-Arg-*p*-NA. The aminopeptidase hydrolysed peptides containing alanine at the N-terminal position. It was twice as active on D-Ala-D-Ala as on L-Ala-L-Ala, with the highest activity on DL-Ala-DL-Ala. It was not possible to assay the aminopeptidase on glycyl-peptides such as Gly-Gly and Gly-Gly-Gly, due to high substrate blanks; this is an acknowledged limitation with the method of Doi et al (1981).

This substrate specificity may indicate a potential role for the aminopeptidase in the formation of the peptidoglycan in the cell wall, which in the case of *B linens* is type A1γ (Schleifer and Kandler, 1972). During the formation of peptidoglycan, chains of the acylated amino sugars containing the peptide subunit are initially produced (in the case of type A1γ, the peptide subunit is L-Ala-D-Glu-diaminopimelic acid-D-Ala-D-Ala). The next step in peptidoglycan formation is the chemical cross-linking of these chains, which involves the amino group of the D-asymmetric carbon of the diaminopimelic acid, forming a peptide bond with the carboxyl group of the penultimate D-Ala of an adjacent peptide subunit. The energy for the formation of this peptide bond is derived from the hydrolysis of the D-Ala-D-Ala peptide bond in the peptide subunit. It may be that the aminopeptidase has a role in this hydrolysis reaction, which may indicate that it is more associated with the cell envelope rather than with the cytoplasm of the bacterium.

Another possible in vivo significance of this intracellular aminopeptidase may be in the utilisation of oligopeptides that may be transported across the cell envelope of the bacterium; such oligopeptides can be utilised for energy and growth requirements. However, due to the limited specificity of this aminopeptidase, it is unlikely that the microorganism depends entirely on it for intracellular oligopeptide hydrolysis. It is highly probable that other intracellular aminopeptidases and dipeptidases are present in this microorganism.

The influence of this aminopeptidase on the ripening of smear surface-ripened cheese is probably quite limited due to its intracellular location; however, upon autolysis of the cells, the released aminopeptidase may act on casein-derived peptides and therefore contribute to the ripening process. *B linens* does lyse upon nutrient limitations (Boyaval et al, 1985); however, whether such conditions exist on the cheese surface is not known. Clearly, the extracellular proteinases and aminopeptidases produced by *B linens* and other microorganisms in the surface smear will have a much larger affect on the ripening process than intracellular peptidases.

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