Purification and characterization of aminopeptidase A from *Lactococcus lactis* subsp. *lactis* NCDO 712

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An aminopeptidase A (EC 3.4.11.7) was purified from *Lactococcus lactis* subsp. *lactis* NCDO 712. Of the 18 aminoacyl-alanine dipeptides tested, the enzyme hydrolysed Asp-Ala, Glu-Ala and Ser-Ala. It was also active against tripeptide substrates but did not hydrolyse Ala-Asp or Ala-Glu. The kinetics of dipeptide hydrolysis were allosteric with positive cooperativity of substrate binding. The Hill constants were 1.52 for Asp-Ala, 1.51 for Glu-Ala and 1.61 for Ser-Ala. The M_r was found to be 245 000 by gel-filtration chromatography. A single band corresponding to an M_r of 41 000 was detected by SDS-PAGE of the purified enzyme which indicated that the enzyme is hexameric. Activity against glutamate *p*-nitroanilide was optimum at 65 °C and pH 8. Activity was inhibited by 1 mM-EDTA, implying that the enzyme is metal-containing. Cu^{2+} , Mn^{2+} and Zn^{2+} (all at 1 mM) inhibited activity and Co^{2+} was stimulatory.

Introduction

Lactic acid bacteria are widely used as starter cultures in the manufacture of cheese, where they are involved not only in the initial fermentation of lactose but also in subsequent flavour development. Amino nitrogen in milk is available mainly in the form of proteins and lactic acid bacteria possess a wide array of proteolytic and peptidolytic enzymes in order to utilize this source of nitrogen (Thomas & Mills, 1981; Law & Kolstad, 1983). The activity of these enzymes over several months, even after cessation of active cell growth, contributes to the development of the flavour of mature cheese (Grappin *et al.*, 1984; Rank *et al.*, 1984; Kamaly & Marth, 1989), and the addition of extracts of starter cultures during cheese manufacture accelerates cheese ripening (Law & Wigmore, 1983).

The purification and characterization of peptidolytic enzymes is fundamental to the understanding of starter culture nutrition and the improvement of starter culture strains. Several such enzymes have been purified from strains of *Lactococcus lactis* subsp. *cremoris* (formerly *Streptococcus cremoris*) including an aminopeptidase A (Exterkate & De Veer, 1987), a dipeptidase (Van Boven *et al.*, 1988), a tripeptidase (Bosman *et al.*, 1990), a

X-prolyl dipeptidyl aminopeptidase (Kiefer-Partsch *et al.*, 1989; Booth *et al.*, 1990) and aminopeptidases with activity against a wide range of dipeptides and tripeptides (Neviani *et al.*, 1989; Tan & Konings, 1990). In the case of *Lactococcus lactis* subsp. *lactis* (formerly *Streptococcus lactis*), only a X-prolyl dipeptidyl aminopeptidase has been purified and characterized (Zevaco *et al.*, 1990; Lloyd & Pritchard, 1991), although this subspecies is also widely used in cheese manufacture.

Using cluster analysis, Kaminogawa *et al.* (1983) demonstrated that *cremoris* and *lactis* subspecies of *L. lactis* formed distinct groups on the basis of peptidase activities against 54 substrates, including 10 different N-terminal amino acid residues. The elution profiles of peptidase activities on ion-exchange chromatography were different between these groups. It is therefore possible that there are significant differences in the characters and types of these enzymes between subspecies which contribute to their different cheese making properties. Further studies of the enzymes of *L. lactis* subsp. *lactis* are required if an adequate comparison is to be made.

In this paper the purification and characterization of a peptidase from *L. lactis* subsp. *lactis* is described; this enzyme shows specific activity against acidic N-terminal amino acid residues (aminopeptidase A, EC 3.4.11.7).

Abbreviation: GNA, L-glutamate *p*-nitroanilide.

Table 1. Purification of aminopeptidase A from *L. lactis* subsp. *lactis* NCDO 712

The data shown are the results of a single purification but are typical of several similar purifications.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification (-fold)	Yield (%)
Cell-free extract	1076	10.45	0.0097	1.0	100
Heat treatment	264	9.20	0.0349	3.6	88
Ammonium sulphate precipitation	50.4	6.29	0.125	12.8	60
Phenyl-Sepharose	3.25	5.64	1.73	178	54
Mono Q	0.595	4.20	7.06	727	40

Methods

Organism and preparation of cell extracts. *Lactococcus lactis* subsp. *lactis* NCDO 712 [obtained from the National Collection of Dairy Organisms (now the National Collection of Food Bacteria, NCFB), Shinfield, Reading, UK] was grown in 10 l batch cultures of M17 broth (Difco) containing 5 g lactose l⁻¹. These were inoculated to 15% (v/v) and incubated for 4 h at 30 °C prior to harvesting.

Cultures were washed in 50 mM-Tris/HCl, pH 7.5, and concentrated to approximately 150 ml by cross-flow filtration using a Millipore Pellicon system fitted with a 0.45 µm membrane. Cells were disrupted by two passes through a French pressure cell at 100 MPa and debris was removed by centrifugation at 20000 g for 30 min.

Assay of aminopeptidase A activity. Activity was determined by measuring the formation of *p*-nitroanilide at 50 °C in a 1 ml reaction mixture containing 1 mM-L-glutamate *p*-nitroanilide (GNA) (Bachem Feinchemikalien), 50 mM-Tris/HCl, pH 8.0, and 50 µl of enzyme solution. Samples were incubated for 10–20 min, over which time the reaction rate was linear. The reaction was stopped by the addition of 0.5 ml 30% (v/v) acetic acid and samples were centrifuged for 15 min at 10000 g. Absorbance at 410 nm was measured and *p*-nitroanilide concentration was determined using a molar absorption coefficient of 8800 M⁻¹ cm⁻¹. All assays were done in duplicate and the mean variation between samples was approximately 4%. One unit of activity was defined as the amount of enzyme required to produce 1 µmol *p*-nitroanilide in 1 min under the conditions described above.

Purification of aminopeptidase A. Approximately 150 ml of cell-free extract was incubated in a waterbath at 60 °C for 30 min with constant stirring. It was then cooled to 4 °C and centrifuged at 20000 g for 30 min.

The supernatant was subject to precipitation by the addition of solid (NH₄)₂SO₄ at 4 °C. Proteins precipitating between 20% and 40% saturation were collected by centrifugation at 10000 g for 30 min and the pellet was resuspended in 0.5 M-(NH₄)₂SO₄ in 50 mM-Tris/HCl, pH 7.5.

Hydrophobic interaction chromatography of the resuspended precipitate was carried out using a 22 × 1.5 cm phenyl-Sepharose column (Pharmacia). A gradient of 0.5 M to 0 M-(NH₄)₂SO₄ in 50 mM-Tris/HCl, pH 7.5, was run over 200 ml at 1 ml min⁻¹. Eluent fractions containing aminopeptidase A activity were pooled and dialysed against 50 mM-Tris/HCl, pH 7.5.

The dialysate was applied to a Pharmacia HR 5/5 Mono Q anion-exchange column. A gradient of 0 M to 0.5 M-NaCl in 50 mM-Tris/HCl, pH 7.5, was run over 25 ml at 0.5 ml min⁻¹. Collected fractions which contained aminopeptidase A activity were pooled and dialysed against 50 mM-Tris/HCl, pH 7.5.

M_r determination. The M_r of purified native enzyme was determined by gel filtration using a Pharmacia HR 10/30 Superose 12 column.

Samples (100 µl) containing 10–20 µg protein were loaded and eluted at 0.4 ml min⁻¹ of 50 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl. The column was calibrated using the following Sigma M_r standards: lysozyme, myoglobin, β-lactoglobulin, bovine serum albumin, alcohol dehydrogenase and β-amylase.

Subunit M_r was determined by SDS-PAGE using a Pharmacia Phast System. Samples (3 µl) containing 0.3 µg protein were run on 20% homogeneous gels for 100 V h at 15 °C. Gels were stained using the Pharmacia silver staining kit by the manufacturer's recommended procedure.

Hydrolysis of dipeptides. The hydrolysis of alanine-containing dipeptides was determined at 25 °C in a coupled assay with alanine dehydrogenase (EC 1.4.1.1). The formation of NADH was constantly monitored at 340 nm in a reaction mixture containing 1 U alanine dehydrogenase ml⁻¹, 2 mM-NAD⁺, 1 mM-dipeptide and 2 mU purified aminopeptidase A ml⁻¹ in 50 mM-Tris/HCl, pH 8.0. The rate of NADH formation was calculated using a molar absorption coefficient of 6220 M⁻¹ cm⁻¹. Control assays containing no enzyme were also carried out and the rate of NADH formation for each substrate was subtracted from that in the presence of enzyme. The mean variation between duplicate samples was 1.5%.

Hydrolysis of tripeptides. Samples (1 ml) containing 0.4 mM-tripeptide and 2 mU purified aminopeptidase ml⁻¹ in 50 mM-Tris/HCl, pH 8, were incubated for 60 min at 50 °C. After this time the reaction was terminated by the addition of 0.5 ml 30% (v/v) acetic acid and samples were centrifuged for 15 min at 10000 g. Control samples which contained no enzyme were incubated in the same way. Tripeptide hydrolysis was determined by capillary electrophoresis using an Applied Biosystems 270A system and a Drew Scientific Roseate data collection and integration package. Samples were loaded for 3 s under vacuum and run for 10 min in 20 mM-sodium citrate buffer, pH 2.5, at 45 °C with a positive potential of 30 kV. Eluent absorbance was constantly monitored at 200 nm.

Protein determination. Protein concentrations were determined by the method of Bradford (1976).

Results

An enzyme which was capable of hydrolysing GNA was detected in cell-free extracts of *L. lactis* subsp. *lactis* and was purified to homogeneity by the method described above. The results of each step of a typical purification are shown in Table 1. The enzyme eluted from the phenyl-Sepharose column at approximately 0.18 M-(NH₄)₂SO₄ which implies a high degree of hydrophobi-

Table 2. Hydrolysis of dipeptides by aminopeptidase A

Liberation of alanine was detected via the synthesis of NADH by alanine dehydrogenase. Each assay contained 1 mM-dipeptide and 2 mU purified aminopeptidase A ml⁻¹.

Substrate	NADH produced (nmol min ⁻¹ ml ⁻¹)	Substrate	NADH produced (nmol min ⁻¹ ml ⁻¹)
Ala-Ala	0.346*	Met-Ala	0.068
Arg-Ala	0.116	Phe-Ala	0.077
Asp-Ala	6.482	Pro-Ala	0.000
Cys-Ala	0.154	Ser-Ala	2.691
Glu-Ala	8.740	Thr-Ala	0.048
Gly-Ala	0.125	Trp-Ala	0.135
His-Ala	0.087	Tyr-Ala	0.174
Ile-Ala	0.164	Val-Ala	0.125
Leu-Ala	0.145	Ala-Asp	0.010
Lys-Ala	0.125	Ala-Glu	0.277

* The actual rate of Ala-Ala hydrolysis was half that of NADH formation due to the formation of 2 mol alanine per mol of dipeptide hydrolysed.

city. A NaCl concentration of 0.3 M was required to elute the enzyme from the Mono Q ion-exchange column. The resulting eluent gave a single band on SDS-PAGE corresponding to an M_r of 41 000. The M_r of the native enzyme as determined by gel-filtration chromatography on Superose 12 was 245 000. These data are consistent with the enzyme being a hexamer of identical subunits.

Substrate specificity of the purified enzyme was investigated by determining the rate of hydrolysis of a series of alanine-containing dipeptides in a coupled assay with alanine dehydrogenase (Table 2). Of the 18 aminoacyl-alanine dipeptides tested, only Asp-Ala, Glu-Ala and Ser-Ala were hydrolysed to a significant extent. The rates of hydrolysis of all other aminoacyl-alanine dipeptides were less than 2% of the rate of hydrolysis of Glu-Ala. No significant hydrolysis of Ala-Glu or Ala-Asp was observed which indicates that this enzyme is not capable of hydrolysing acidic C-terminal amino acid residues. The purified enzyme was also active against tripeptide substrates. Samples of Glu-Glu-Glu, Glu-Glu-Asp and Glu-Val-Phe were analysed by capillary electrophoresis after incubation for 60 min in the presence and in the absence of aminopeptidase (Table 3). In this time Glu-Glu-Glu was completely hydrolysed and no dipeptide peak was detected. Glu-Glu-Asp was partially hydrolysed and 20% of the substrate remained after incubation in the presence of the enzyme. A single product peak was also detected which possibly corresponded to Glu-Asp although this peak was small and it is likely that most of the dimer product was further hydrolysed to single amino acids. Glu-Val-Phe was also completely hydrolysed and a single product peak which accounted for approximately all of the substrate was detected. It is therefore likely that the enzyme cleaved the N-terminal glutamate residue, leaving Val-Phe

Fig. 1. The thermal stability of aminopeptidase A. Samples containing 4 mU purified enzyme ml⁻¹ in 50 mM-Tris/HCl buffer, pH 8, were preincubated at 70 °C (●) and 80 °C (○) prior to assaying at 50 °C using GNA as substrate.

which was not hydrolysed further. This spectrum of activity is consistent with the designation of the enzyme an aminopeptidase A (EC 3.4.11.7).

The effects of pH and temperature on the activity of the purified enzyme were investigated using GNA as substrate. Activity optima were at approximately pH 8 and 65 °C. The enzyme also showed a high degree of thermal stability. Samples were preincubated for varying times in waterbaths at 70 °C and 80 °C. Activity was assayed after rapid cooling of samples in iced water followed by equilibration at 50 °C: 85% of activity remained after preincubation for 60 min at 70 °C and 24% remained after preincubation for the same time at 80 °C (Fig. 1). The thermal stability of the enzyme was made use of as part of the purification procedure described previously. The purified enzyme was stored at 4 °C in 50 mM-Tris/HCl, pH 7.5, and showed no appreciable loss of activity after 3 months under these conditions.

The activity of aminopeptidase A was determined in the presence of various compounds which are known to

Table 3. Hydrolysis of tripeptides by aminopeptidase A

Samples containing 0.4 mM-tripeptides were incubated for 60 min at 50 °C in the presence of 2 mU purified aminopeptidase A ml⁻¹ and in the absence of enzyme. Samples were analysed by capillary electrophoresis. These data are the result of a single experiment but are representative of several replicate analyses.

Substrate	Peaks detected			
	- Enzyme		+ Enzyme	
	Retention time (min)	Area (units)	Retention time (min)	Area (units)
Glu-Glu-Asp	7.52	58.8	7.50 6.85	13.16 8.70
Glu-Glu-Glu	7.35	55.2	—	—
Glu-Val-Phe	6.84	202	6.08	189

Table 4. Effects of enzyme inhibitors on aminopeptidase A activity

Activities were determined using GNA as substrate with 2 mU purified enzyme ml⁻¹ and 1 mM inhibitor.

Inhibitor	Activity (% control)
None	100
EDTA	14
Iodoacetamide	98
Iodoacetic acid	98
Phenylmethylsulphonyl fluoride	111
Leucine chloromethylketone	104

inhibit certain types of enzymes (Table 4). The inclusion of 1 mM-EDTA in the assay resulted in a substantial inhibition of activity which implied that the enzyme may be metal-containing. None of the other compounds which were tested, including the sulphhydryl reagents iodoacetamide and iodoacetic acid, the serine protease inhibitor phenylmethylsulphonyl fluoride and the leucine aminopeptidase inhibitor leucine chloromethylketone had any inhibitory effect.

The effects of metal salts on enzyme activity are shown in Table 5. Aminopeptidase A was strongly inhibited by 1 mM-Cu²⁺ and, to a lesser extent by 1 mM-Mn²⁺ and Zn²⁺. Co²⁺ had a stimulatory effect on enzyme activity.

A study of the effect of substrate concentration on enzyme activity using Glu-Ala, Asp-Ala and Ser-Ala substrates revealed allosteric kinetics. Fig. 2 shows Hill plots for each substrate and the kinetic parameters, which were determined using the Enzfitter data analyses computer program (Elsevier-Biosoft), are listed in Table 6. The gradients of each line are similar and are greater than 1 which indicates positive cooperativity of substrate binding. The cooperativity indices (Taketa & Pogel, 1965) of this enzyme were calculated to be 18 for Glu-Ala and Asp-Ala and 15 for Ser-Ala, compared with a theoretical value of 81 for Michaelis-Menten kinetics.

Table 5. Effect of metal salts on the activity of aminopeptidase A

Activities were determined using GNA as substrate with 2 mU purified enzyme ml⁻¹ and 1 mM salts.

Addition	Activity (% control)	Addition	Activity (% control)
None	100	MnCl ₂	46
CaCl ₂	106	NaCl	126
CoCl ₂	143	Na ₂ SO ₄	121
CuSO ₄	7	ZnSO ₄	39
MgCl ₂	112

Fig. 2. Hill plots of aminopeptidase A activity against substrate concentration. The units of substrate concentration ($[S]$) are μM and the units of enzyme activity (V) are $\text{mU min}^{-1} (\text{mg protein})^{-1}$. The substrates used were Asp-Ala ($\bullet$), Glu-Ala ($\blacksquare$) and Ser-Ala ($\blacktriangle$). Activities were determined by monitoring the formation of NADH in a coupled assay with alanine dehydrogenase.

Table 6. Kinetic parameters of aminopeptidase A

These data are derived from the plots shown in Fig. 2. Activities were determined by measuring the rate of NADH formation in a coupled assay with alanine dehydrogenase.

Substrate	V_{max} [$\mu\text{mol min}^{-1}$ ($\text{mg protein})^{-1}$]	K_m (mM)	Hill constant
Asp-Ala	36.3	0.212	1.52
Glu-Ala	42.6	0.283	1.51
Ser-Ala	11.9	27.3	1.61

The values of V_{max} and K_m for Glu-Ala and Asp-Ala were similar but the V_{max} of Ser-Ala was lower and the value of K_m was higher. The affinity of the enzyme for Ser-Ala and its ability to hydrolyse this substrate were significantly lower than for Glu-Ala or Asp-Ala.

Discussion

The enzymes forming the peptidolytic system of *L. lactis* have been most thoroughly studied in strains of *L. lactis* subsp. *cremoris*. The only aminopeptidase previously purified from *L. lactis* subsp. *lactis* was a X-prolyl dipeptidyl aminopeptidase (Zevaco *et al.*, 1990; Lloyd & Pritchard, 1991). The purification and characterization of an acidic aminopeptidase from that subspecies is described in this report. The purification procedure described made use of the high thermal stability and hydrophobicity of the enzyme and resulted in a 700-fold purification and a recovery of 40%.

The substrate specificity of this enzyme was tested against a substantial range of aminoacyl-alanine dipeptides, and the enzyme was shown to be active against N-terminal aspartyl and glutamyl residues. It was also

active against tripeptide substrates but did not cleave acidic C-terminal residues. It was therefore designated as an aminopeptidase A (EC 3.4.11.7). In addition, this enzyme also hydrolysed seryl-alanine. An aminopeptidase A has previously been purified from *L. lactis* subsp. *cremoris* (Exterkate & De Veer, 1987) but activity against seryl residues was not reported; nor has this activity been observed in aminopeptidase A purified from mammalian sources (Nagatsu *et al.*, 1970; Danielsen *et al.*, 1980; Benajiba & Maroux, 1980; Tobe *et al.*, 1980). Many of the serine residues in caseins are phosphorylated (Eigel *et al.*, 1984) but it is possible that this enzyme may have activity against phosphorylated serine residues if the acidic side chain causes them to act as aspartate analogues. This activity may therefore be of importance in the utilization of casein-derived peptides by lactic acid bacteria. Further work is in progress to investigate this hypothesis.

The aminopeptidase A purified from *L. lactis* subsp. *cremoris* by Exterkate & De Veer (1987) was reported to be a trimer of M_r 130 000 compared to the M_r 245 000 hexamer described here. However, both enzymes showed a high degree of thermal stability, were metallo-proteins and had similar subunit M_r values. Kaminogawa *et al.* (1983) studied the spectrum of peptidase activities in extracts of 11 strains of *S. lactis* and *S. cremoris* after ion-exchange chromatography using 54 different substrates. They showed that the strains investigated could be divided into three groups on this basis which differentiated between *cremoris* and *lactis* strains. Kolstad & Law (1985) demonstrated differing spectra of peptidase activities between *S. lactis* and *S. cremoris* using electrophoretic separation. *S. lactis* and *S. cremoris* have been shown to have a high degree of DNA homology (Jarvis & Jarvis, 1981) and have since been removed from the genus *Streptococcus* and reclassified as subspecies of *Lactococcus lactis* (Schleifer *et al.*, 1985). Despite the broad similarities between these strains, there are therefore specific differences in their peptidolytic enzymes which may have some significance for their use in the dairy industry. Further studies, particularly of the enzymes from *L. lactis* subsp. *lactis*, are required to determine the influence of such differences on the use of these strains in cheese starter cultures.

In this report, allosteric kinetics for the hydrolysis of dipeptides by aminopeptidase A were observed which indicated a degree of positive cooperativity of substrate binding. Studies have been made of the kinetics of action of peptidases from *L. lactis* subsp. *cremoris* against aminoacyl *p*-nitroanilide substrates (Neviani *et al.*, 1989; Tan & Konings, 1990) and peptide substrates (Van Boven *et al.*, 1988; Bosman *et al.*, 1990) and in each case Michaelis-Menten saturation kinetics were described.

There is a great deal of interest in the peptidolytic

systems of lactic acid bacteria because of their suspected role in the development of cheese flavour (Law & Wigmore, 1983). Aminopeptidase A may be significant in this respect as glutamate salts have flavour-enhancing properties (Kirimura *et al.*, 1969) and this enzyme has high stability and substrate affinity. It may be possible to improve strains of starter bacteria by cloning and controlling the expression of such enzymes.

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