St-PepA, a *Streptococcus thermophillus* aminopeptidase with high specificity for acidic residues

Francoise Rul, Jean-Claude Gripon and Véronique Monnet

The proteolytic system of lactic acid bacteria has been extensively studied over the past 10 years and peptidases from lactococci are now well known. The situation is, however, different for *Streptococcus thermophilus* from which only a few peptidases have been purified and characterized. The present work was conducted to characterize an aminopeptidase of *S. thermophilus* CNRZ 302, called St-PepA, with high specificity for acidic amino acids. St-PepA was purified by a three-step procedure from a spheroblast extract of *S. thermophilus* CNRZ 302. Its molecular mass was estimated to be 360 kDa by gel filtration and 45 kDa by SDS-PAGE, indicating that it had an octameric structure. Its activity against aspartate-p-nitroanilide was maximal at pH 8.5 and 62 °C and highly enhanced by Zn²⁺, Co²⁺ and Mg²⁺. St-PepA was inhibited by metal-chelating reagents and, to a lesser extent, by agents modifying sulfhydryl groups. It showed an activity towards p-nitroanilide derivatives, di- and tripeptides, and larger peptides such as fragment 43-58 of αs1-casein. It had a high substrate specificity towards N-terminal acidic amino acid residues but it could also release serine and malic acid, the α-hydroxy acid homologue of aspartic acid. Kinetic studies revealed that the affinity of St-PepA was more than 18-fold higher for aspartic acid-p-nitroanilide (Kₘ = 0.42 mM) than for glutamic acid-p-nitroanilide (Kₘ = 765 mM) with a similar Vₘₐₓ for both substrates [about 40 μmol min⁻¹ (mg enzyme)⁻¹]. St-PepA is heat stable, with a maximal loss of activity of 15% after incubation for 120 min at 50 °C and 60 °C. It is likely to be involved in the nitrogen metabolism of *S. thermophilus* and in the development of the organoleptic characteristics of cheese and yoghurt.

**Keywords**: *Streptococcus thermophilus*, aminopeptidase A, glutamic and aspartic acids, metallopeptidase

**INTRODUCTION**

Lactic acid bacteria are widely used as starter cultures in industrial milk fermentations. They require an exogenous nitrogen source for optimal growth (Law & Kolstad, 1983; Thomas & Pritchard, 1987). Because milk is relatively poor in free amino acids and small peptides, nitrogen mostly comes from caseins. Thus, the optimal growth of lactic acid bacteria depends on their proteolytic system composed of proteinases and peptidases which can produce these amino acids and small peptides from milk caseins (Kok, 1990; Laan et al., 1989). This proteolytic system is also important because it contributes to the development of texture and flavour during cheese ripening.

The cell-envelope-associated proteinase and a variety of peptidases with different specificities have been described for the genus *Lactococcus* (for reviews see Monnet et al., 1993; Tan et al., 1993). Less information is available about the proteolytic enzymes of *Streptococcus thermophilus*, although it is widely used in the dairy industry. *S. thermophilus* exhibits low levels of proteinase activity and it develops in milk in association with lactobacilli species, the latter producing small peptides which are substrates for peptidases. Several of these peptidases have been described in *S. thermophilus*; a neutral intracellular endopeptidase was purified and characterized from *S. thermophilus* CNRZ 160 (Desmazeaud, 1974). A dipeptidase

**Abbreviations**: pNa, p-nitroanilide; β-Na, β-naphthylamide.
(Rabier & Desmazeaud, 1973), specific for dipeptides with a large amino acid residue at the N terminus, and a X-prolyl-dipeptidyl-aminopeptidase (Meyer & Jordi, 1987) were also described. In addition, two general aminopeptidases similar to those isolated from lactococci were purified from *S. thermophilus*: PepN (Tsakalidou & Kalantzopoulos, 1992; Midwinter & Pritchard, 1994; Rul et al., 1994) and PepC, whose gene has recently been cloned (Chapot-Chartier et al., 1994).

As *S. thermophilus* is widely used in the dairy industry in cheese making and yoghurt manufacturing, it is of interest to know more about its peptidasic system. After a screening of aminopeptidase activities, we detected four aminopeptidases in extract of *S. thermophilus* CNRZ 302 spheroplasts. Three are general aminopeptidases (Rul, 1994); the fourth, St-PepA, is an aminopeptidase with high specificity for acidic amino acids. To investigate further the potential role of St-PepA in nitrogen metabolism and organoleptic development of yoghurt and cheese, we purified it and determined its biochemical properties.

**METHODS**

**Bacterial strain and culture conditions.** *Streptococcus thermophilus* CNRZ 302 was obtained from the collection of the Station de Recherches Laitières (INRA, Jouy-en-Josas, France). It was grown at 37 °C in 5 l of M17 broth (Difco) containing 5 g lactose l−1. Growth was assessed by measurement of OD₅₅₀.

**Intracellular extract preparation.** Cells were collected at the end of the exponential growth phase by centrifugation (6000 g, 15 min, 4 °C). The pellet was washed twice with 50 mM β-glycerophosphate and then incubated for 2 h at 30 °C in 50 mM triethanolamine (TEA) buffer (pH 7) containing 30% (w/v) sucrose, 0·1 mg lysozyme ml⁻¹ (Sigma) and 42 μM mutanolysin ml⁻¹ (Sigma). The spheroplasts obtained were collected by centrifugation (12000 g, 30 min, 4 °C) and resuspended in the same buffer without sucrose. After the spheroplasts had burst, the supernatant was recovered by centrifugation (20000 g, 30 min, 4 °C).

**Protein assay.** Proteins were determined in the extracts and purified fractions by the method of Bradford (1976) with bovine serum albumin (Pierce) as standard.

**Aminopeptidase activity assay.** St-PepA activity was assayed against three types of substrates.

Firstly, against amino acid-p-nitroanilides (p-Na) and β-naphthylamides (β-Na). The purification of St-PepA was followed by monitoring the release of p-nitroanilide from Asp-pNa (Bachem) at 410 nm as described by Zevaco et al. (1990). In the case of β-Na derivatives, the reaction mixture contained 0·3 mM substrate in 50 mM Tris/HCl (pH 8) in a final volume of 1 ml and incubation was performed at 37 °C. The reaction was stopped by adding 0·5 ml of 1 mg Fast Garnet ml⁻¹. After 25 min incubation at 37 °C, the release of β-naphthylamine was monitored by measuring A₅₅₀. The unit of activity used was the katal (the quantity of enzyme releasing 1 mol p-nitroanilide or β-naphthylamine s⁻¹).

Secondly, with the purified aminopeptidase, against several di-, tri- and tetrapeptides (Sigma, or Bachem for Asp-Tyr, Asp-Tyr-Met and Asp-Tyr-Met-Gly). Asp-Phe-methyl ester (Asp-Phe-OME; Aspartame), Asp-Phe-ethyl ester (Asp-Tyr-OME) and Mal-Tyr-ethyl ester (Mal-Tyr-OEt; Mal, maleyl) were provided by BioEurope. The enzyme fraction was incubated at 37 °C in the presence of 82 μM substrate in 50 mM TEA (pH 8) and 1 mM CoCl₂. After 1 h incubation, the reaction was stopped by addition of 10% 1 M HCl. The free amino acids present in the hydrolysates of the different peptides were directly identified with a LC3000 amino acid analyser (Biotronik).

Thirdly, against oligopeptides. Angiotensin I and leucokinin were from Sigma. Fragment 43–58 of bovine α₁-casein and fragment 115–124 of caprine α₁-casein (with free or phosphorylated serine at the N terminus) were gifts from G. Brignon (INRA, Station de Recherches Laitières, Jouy-en-Josas, France). The enzymatic reaction was achieved as described above with 1 h incubation (or 24 h for the α₁-casein fragments). The samples were analysed by RP-HPLC using a C18 column in a trifluoroacetic acid (TFA)/acetonitrile solvent system (solvent A, 0·115% TFA; solvent B, 0·1% TFA, 60%, v/v, acetonitrile). A linear gradient of 20–80% solvent B was used over 15 min for leucokinin and angiotensin and over 20 min for fragment 43–58 of bovine α₁-casein. A linear gradient of 10–50% solvent B was used over 30 min for fragment 115–124 of caprine α₁-casein. The peptides collected were then identified with a sequencer (model 477A; Applied Biosystems).

**Aminopeptidase purification.** Nucleic acids in the cell extract were hydrolysed by adding RNase (2·75 mg per 100 ml extract; Sigma) and DNase (0·05 mg per 100 ml extract; Sigma) in the presence of 1 mM MgCl₂. Nucleic acids were then precipitated in the presence of 60 mM MnSO₄ and the solution was centrifuged at 10000 g for 30 min. The extract was then dialysed overnight against 10 mM TEA (pH 7) and filtered through a 0·45 μm filter (type HVLP, Millipore).

The first ion exchange chromatography was done on a Q-Sepharose Fast Flow XK 26/40 column (26 × 15 cm; Pharmacia) with a FPLC system. The column was equilibrated with 50 mM TEA (pH 7). The extract (317 mg protein) was applied to the column and eluted with a linear NaCl gradient of 0–0·5 M over 5 h at a flow rate of 5 ml min⁻¹ and 10 ml fractions were collected. Fractions active against Asp-pNa were recovered and dialysed against 10 mM sodium phosphate buffer.

The second ion-exchange chromatography was performed by FPLC on a MonoQ HR 10/10 column (1 × 10 cm; Pharmacia) equilibrated with 50 mM TEA (pH 7·5). The proteins were eluted with a linear NaCl gradient of 0·0–0·5 M over 4 h at a flow rate of 5 ml min⁻¹. Three millilitre fractions were collected and the fractions active against Asp-pNa were pooled.

The following hydrophobic interaction chromatography was done with an Alkyl Superose HR5/5 column (0·5 × 5 cm; Pharmacia). The column was equilibrated with 50 mM sodium phosphate buffer (pH 7·5) containing 1·7 M (NH₄)₂SO₄. This was added to the sample which was applied to the column and eluted with a linear NaCl gradient of 1·7–0 M (NH₄)₂SO₄ in 50 mM sodium phosphate (pH 7·5) over 3·5 h at a flow rate of 0·5 ml min⁻¹. 0·5 ml fractions were collected. Fractions active against Asp-pNa were recovered and dialysed against 10 mM sodium phosphate buffer.

**Aminopeptidase characterization**

**Electrophoresis.** The purified fraction was electrophoresed under denaturing and reducing conditions (SDS-PAGE) using 10% acrylamide gels (acylamide/bisacrylamide, 29:1, v/v) according to Laemmli (1970). The proteins were stained with Coomassie Brilliant Blue R-250. The following markers were...
used to determine the molecular mass of St-PepA: myosin (205 kDa), \( \beta \)-galactosidase (116:25 kDa), phosphorylase b (97:4 kDa), bovine serum albumin (66:2 kDa) and ovalbumin (45 kDa). Native electrophoresis (in the absence of SDS and \( \beta \)-mercaptoethanol) allowed the detection of the aminopeptidase activity under the following incubation conditions: 30 ml 0·2 M Tris buffer (pH 7·5) containing 10 mg Asp-pNa at room temperature. Hydrolysis of the substrate was reflected by the appearance of an unstable yellow colour at the level of the enzyme band.

**Effect of pH on aminopeptidase activity.** The effect of pH on the aminopeptidase activity was determined on Asp-pNa at 37 °C over a pH range of 4–10 using the following 0·1 M buffers: sodium acetate pH 4–5·5; Bis-Tris, pH 6–7; TEA, pH 7·5–8; Tris, pH 8–9·5 and CAPS, pH 10.

**Effect of temperature on aminopeptidase activity.** The effect of temperature on the aminopeptidase activity was determined at temperatures ranging from 20 to 75 °C with Asp-pNa as a substrate. After incubation of the enzyme at the desired temperature in 50 mM TEA buffer (pH 8) for 5 min, substrate was added and the reaction mixtures were incubated for 0–20 min at 20–75 °C. The enzymic activity was then measured as described above.

To determine the thermostability of St-PepA activity, a solution of the purified aminopeptidase in 50 mM TEA buffer (pH 8) containing 1 mM MgCl\(_2\) was incubated at 30, 40, 50 or 60 °C. After 20, 30, 60, 120 and 480 min, samples were taken from each incubation mixture to determine the activity as described above.

**Effect of bivalent cations on the aminopeptidase activity.** Bivalent cations were assayed firstly for their effect on aminopeptidase activity and secondly for their ability to restore aminopeptidase activity after inactivation by EDTA. The aminopeptidase was preincubated with various bivalent cations: CaCl\(_2\), CuCl\(_2\), CoCl\(_2\), MgCl\(_2\), MnCl\(_2\) and ZnCl\(_2\) for 20 min at 37 °C at final concentrations of 1 mM in 50 mM TEA buffer (pH 8). Its activity was then assayed at 37 °C for 20 min using Asp-pNa as a substrate. The rate of hydrolysis of Asp-pNa in the absence of metal ion was taken as 1. After inactivation with 1 mM EDTA, the aminopeptidase was incubated with each cation preparation for 20 min at 37 °C before the restored activity was measured as described above.

**Effect of inhibitors on aminopeptidase activity.** The aminopeptidase was preincubated at various concentrations of 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefablock-SC), iodoacetic acid, \( p \)-hydroxymercuribenzoate, EDTA, \( \alpha \)-phenanthroline or DTT to determine the activity of the enzyme after 20 min at 37 °C in 50 mM TEA buffer (pH 7·5). Its activity was measured following the standard procedure and the rate of hydrolysis of Asp-pNa in the absence of any inhibitory agent was taken as 100%.

**Determination of kinetic parameters.** \( K_m \) and \( V_{max} \) values for Asp-pNa and Glu-pNa were determined from a Lineweaver–Burk plot generated from the initial reaction velocity obtained with substrate concentrations ranging from 0·5 to 15 mM and 95 ng purified enzyme ml\(^{-1}\). Each assay was carried out at 37 °C in 50 mM TEA buffer (pH 8), 1 mM MgCl\(_2\) and the variation in \( A_{110} \) was monitored continuously for 10 min using a 931 Uvikon spectrophotometer (Kontron Instruments).

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**RESULTS**

**Aminopeptidase purification**

St-PepA was purified about 130-fold from the cell-free extract in three chromatography steps, with an activity recovery of 27% as shown in Table 1. The last purification step (Alkyl Superose) resulted in a peak which showed a single protein band upon examination by SDS-PAGE (Fig. 1) and by PAGE; after non-denaturing PAGE, the enzyme activity was detected at the same position as the protein band.

**Aminopeptidase properties**

The molecular mass of St-PepA was estimated to be 360 kDa by gel filtration and 45 kDa by SDS-PAGE. These data suggest that St-PepA is an octamer, with subunits of 45 kDa. It was active over a pH range of 6·5–10 with maximal hydrolysis of Asp-pNa at pH 8·5; 75% of its activity remained at pH 10. At pH 8, with Asp-pNa as a substrate, the optimum temperature was 62 °C (Fig. 2). In fact St-PepA showed a rather high degree of thermal stability since more than 82% of the initial activity was retained after incubation at 30 or 40 °C for 8 h, or at 50 or 60 °C for 2 h (Fig. 3).

Activity was measured in the presence of different classes of inhibitors (Table 2) and different levels of inhibition were observed. Strong inhibition (62–90%) was observed with DTT and metal-chelating reagents such as \( \alpha \)-phenanthroline or EDTA which suggests St-PepA is a metallopeptidase. Moderate inhibition (7–34%) was observed with inhibitors of aminopeptidases (amastatin and bestatin) and inhibitors of cysteine proteases (iodoacetic acid and \( p \)-hydroxymercuribenzoate). There was no inhibition at all with Pefablock, a serine protease inhibitor.

St-PepA was also inhibited by 1 mM CuCl\(_2\), while incubation with 1 mM CaCl\(_2\), ZnCl\(_2\), CoCl\(_2\) and MgCl\(_2\) resulted in an increase of activity (> twofold with ZnCl\(_2\), CoCl\(_2\) and MgCl\(_2\); Table 3). After treatment with 1 mM EDTA, activity was only recovered with the addition of Zn\(^{2+}\).

**Substrate specificity**

St-PepA’s activity was tested with several substrates (Table 4). The enzyme had three properties as follows.

Firstly, it was observed to release only acidic amino acids (Glu and Asp), Asp being preferentially released, among all the amino acid derivatives (pNA or -\( \beta \)-NA) tested (Arg, Phe, Lys, Pro, Asp, Glu).

Secondly, it hydrolysed di-, tri- and tetrapeptides with a N-terminal acidic amino acid but no preference for Asp or Glu was observed. The best Asp-X dipeptide substrates were those containing an aromatic residue (Tyr or Phe) at position \( P_1 \) (according to the nomenclature of Schechter & Berger, 1967). St-PepA could also hydrolyse dipeptides with N-terminal malic acid, the \( \alpha \)-hydroxy acid homologue of Asp; the highest rate of hydrolysis was
Table 1. Purification of aminopeptidase St-PepA from \textit{S. thermophilus} CNRZ 302

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (nkat)</th>
<th>Specific activity (nkat mg$^{-1}$)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>317</td>
<td>72</td>
<td>0.23</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>10.5</td>
<td>50</td>
<td>4.8</td>
<td>69</td>
<td>21</td>
</tr>
<tr>
<td>MonoQ</td>
<td>1.2</td>
<td>21</td>
<td>17.5</td>
<td>29</td>
<td>77</td>
</tr>
<tr>
<td>Alkyl Superose</td>
<td>0.068</td>
<td>2</td>
<td>29.4</td>
<td>2.7</td>
<td>130</td>
</tr>
</tbody>
</table>

Fig. 1. SDS-PAGE of different fractions obtained during the purification of aminopeptidase St-PepA from \textit{S. thermophilus} CNRZ 302. Lanes: 1, molecular mass standards (kDa); 2, cell-free extract; 3 and 4, fractions from first and second anion-exchange chromatography, respectively; 5, fraction from hydrophobic interaction chromatography.

Fig. 2. Effect of temperature on the activity of St-PepA of \textit{S. thermophilus} CNRZ 302. Temperature (°C).

Fig. 3. Effect of temperature on the stability of St-PepA of \textit{S. thermophilus} CNRZ 302. Samples containing 95 ng of purified enzyme in 50 mM TEA buffer (pH 8), were incubated at 30 (△), 40 (●), 50 (■) and 60 °C (○) prior to assaying at 37 °C using Asp-pNa as substrate. Initial activity was taken as 100%.

Table 2. Effect of inhibitors on St-PepA activity of \textit{S. thermophilus} CNRZ 302

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amastatin</td>
<td>0.01</td>
<td>10</td>
</tr>
<tr>
<td>Bestatin</td>
<td>0.01</td>
<td>7</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>0.1</td>
<td>12</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>Pefablock</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.1</td>
<td>62</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>DTT</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>70</td>
</tr>
</tbody>
</table>

measured on Mal-Tyr-OEt. Serine was released from fragment 1–4 of adrenocorticotropic hormone (SYSM) but both fragments 115–124 of caprine \(\alpha_{s1}\)-casein with free or phosphorylated N-terminal serine were not hydrolysed. Dipeptides with an additional group at the C-terminus were better substrates than those without that additional group, as demonstrated with Asp-Phe, Asp-Phe-NH\(_2\) and Asp-Phe-OMe (aspartame).

Finally, it was observed to hydrolyse larger peptides. N-Terminal Asp was slowly released from fragment 43–58 of bovine \(\alpha_{s1}\)-casein (15 residues). Peptides with N-
terminal Asp-Pro or Asp-Arg (leucokinin, angiotensin) were not hydrolysed. Liberation of serine was observed from SYSM but not from larger peptides like fragment 115–124 (10 residues) of caprine αs1-casein. These results show that St-PepA preferentially degrades small (<5 residues) rather than large peptides.

Neither carboxypeptidase activity on carbobenzoxy-Glu-Tyr nor endopeptidase activity on leucokinin, angiotensin I, fragment 43–58 of bovine αs1-casein or fragment 115–124 of caprine αs1-casein were detected.

**Kinetic parameters**

Kinetic parameters were determined for the interaction of St-PepA with Asp-pNa and Glu-pNa as substrates. Lineweaver–Burk plots showed that $K_m$ for Asp-pNa and Glu-pNa were of 0.42 mM and 7.65 mM, respectively, thus indicating a better affinity of St-PepA for Asp-pNa. Maximal rates of hydrolysis were similar for both substrates ($V_{max}$ of 41.7 and 38.5 μmol min$^{-1}$ mg$^{-1}$ for Asp-pNa and Glu-pNa, respectively).

**DISCUSSION**

Our work demonstrates the existence in *S. thermophilus* CNRZ 302 of an aminopeptidase, St-PepA, specific to peptide bonds in which a N-terminal acidic amino acid is involved.

All the aminopeptidases A described up to now are metalloenzymes and specifically liberate Glu and Asp residues. Only a few of them have been described in bacteria, the others being from human or animal origin. St-PepA differs from aminopeptidases A of human origin (Lalu *et al.*, 1984; Mizutani *et al.*, 1981; Nagatsu *et al.*, 1970; Yamada *et al.*, 1988) or of animal origin (Cheung & Cushman, 1971; Danielson *et al.*, 1980; Feracci *et al.*, 1981; Petrovic & Vitale, 1990; Tobe *et al.*, 1980) in many ways, including biochemical properties such as molecular mass, multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to the bacterial aminopeptidases A which have been purified from *Lactococcus lactis* (Bacon *et al.*, 1994; Niven, 1991; Exterkate & De Veer, 1987) and *Staphylococcus chromogenes* (Yoshpe-Besanson *et al.*, 1993). All of these have a multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to the bacterial aminopeptidases A which have been purified from *Lactococcus lactis* (Bacon *et al.*, 1994; Niven, 1991; Exterkate & De Veer, 1987) and *Staphylococcus chromogenes* (Yoshpe-Besanson *et al.*, 1993). All of these have a multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to the bacterial aminopeptidases A which have been purified from *Lactococcus lactis* (Bacon *et al.*, 1994; Niven, 1991; Exterkate & De Veer, 1987) and *Staphylococcus chromogenes* (Yoshpe-Besanson *et al.*, 1993). All of these have a multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to the bacterial aminopeptidases A which have been purified from *Lactococcus lactis* (Bacon *et al.*, 1994; Niven, 1991; Exterkate & De Veer, 1987) and *Staphylococcus chromogenes* (Yoshpe-Besanson *et al.*, 1993). All of these have a multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to the bacterial aminopeptidases A which have been purified from *Lactococcus lactis* (Bacon *et al.*, 1994; Niven, 1991; Exterkate & De Veer, 1987) and *Staphylococcus chromogenes* (Yoshpe-Besanson *et al.*, 1993). All of these have a multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to the bacterial aminopeptidases A which have been purified from *Lactococcus lactis* (Bacon *et al.*, 1994; Niven, 1991; Exterkate & De Veer, 1987) and *Staphylococcus chromogenes* (Yoshpe-Besanson *et al.*, 1993). All of these have a multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to the bacterial aminopeptidases A which have been purified from *Lactococcus lactis* (Bacon *et al.*, 1994; Niven, 1991; Exterkate & De Veer, 1987) and *Staphylococcus chromogenes* (Yoshpe-Besanson *et al.*, 1993). All of these have a multimeric structure, optimal conditions of activity and the nature of the metal ion it requires. It is closer to

### Table 3: Effect of 1 mM bivalent cations on the aminopeptidase St-PepA activity of *S. thermophilus* CNRZ 302

<table>
<thead>
<tr>
<th>Ion</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>3.7</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>3.0</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>2.2</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1.4</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.8</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Table 4: Substrate specificity of St-PepA from *S. thermophilus* CNRZ 302**

The rate of hydrolysis of Asp-Tyr was taken as 100%. OMe and -OEt, methyl ester and ethyl ester, respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp-pNa</td>
<td>15</td>
<td>Glu-Val-Phe</td>
<td>139</td>
</tr>
<tr>
<td>Glu-pNa</td>
<td>5</td>
<td>Ser-Tyr-Ser-Met</td>
<td>70</td>
</tr>
<tr>
<td>Asp-β-Na</td>
<td>6</td>
<td>Bovine αs1-casein</td>
<td>4</td>
</tr>
<tr>
<td>Glu-β-Na</td>
<td>0 (fragment 43–58)</td>
<td>(DIGSEŠTEDQAMEDIK)</td>
<td></td>
</tr>
<tr>
<td>Asp-Gln</td>
<td>37</td>
<td>Asp-Lys</td>
<td>13</td>
</tr>
<tr>
<td>Glu-Lys</td>
<td>34</td>
<td>Angiotensin I</td>
<td>0</td>
</tr>
<tr>
<td>Glu-Ser</td>
<td>100</td>
<td>(DRVYHPFHL)</td>
<td></td>
</tr>
<tr>
<td>Asp-Phe</td>
<td>52</td>
<td>Leucokinin</td>
<td>0</td>
</tr>
<tr>
<td>Asp-Phe-OMe</td>
<td>120</td>
<td>(DPAFNWSG)</td>
<td>0</td>
</tr>
<tr>
<td>Asp-Phe-NH$_2$</td>
<td>100</td>
<td>Caprine αs1-casein</td>
<td>0</td>
</tr>
<tr>
<td>Asp-Tyr</td>
<td>100</td>
<td>(fragment 115–124)</td>
<td>0</td>
</tr>
<tr>
<td>Mal-Tyr-OEt</td>
<td>266</td>
<td>(SAEQLHSMK)</td>
<td></td>
</tr>
<tr>
<td>Asp-Tyr-Met</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp-Tyr-Met-Gly</td>
<td>94</td>
<td></td>
<td></td>
</tr>
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</table>
Veer, 1987) after treatment with EDTA and, secondly, St-PepA and PepA from L. lactis HP (Exterkate & De Veer, 1987) and AM2 (Bacon et al., 1994) exhibit especially good temperature stability.

St-PepA has a high substrate specificity towards acidic amino acid residues at the N terminus of peptides but it can also release serine. St-PepA shares with the aminopeptidase A from S. chromogenes (Yoshpe-Besanson et al., 1993) the ability to liberate N-terminal malic acid, the α-hydroxy acid homologue of Asp. Thus, streptococcal and staphylococcal aminopeptidases A show no strict requirement for a free α-amino group, contrary to the amino-1987) peptidase A from S. thermophilus. St-PepA shares with the amino-1993) requirement for a free α-amino group, contrary to the amino-

The specificity of St-PepA and especially its capacity to liberate Glu is interesting for two reasons.

Firstly, because St-PepA is most probably important in the nitrogen metabolism of S. thermodus: S. thermophilus generally does not possess a substantial extracellular proteolytic activity (Shahal et al., 1991) and peptides from caseins must be released by the cell-wall-associated proteinase of lactobacilli and by the rennet, in the case of cheese manufacturing. These peptides are rich in Asp and Glu as caseins are and could be transported into the cytoplasm of lactobacilli. However, St-PepA (Rul, 1994; Rul, 1994; Tsakalidou & Kalantopoulou, 1992).

Secondly, because St-PepA could contribute to flavour development during cheese ripening by liberating free amino acids. Glutamic acid is the major free amino acid in Swiss-type cheese and Biede & Hammond (1979) suggested that it can play a role in cheese flavour because of its broth taste and its flavour-enhancing properties. Furthermore, it has been shown recently that Glu can contribute to lowering the bitterness of a trypsic hydrolysate of casein (Warmke & Belitz, 1993). St-PepA could also participate to the decrease of bitterness by hydrolysing, with other peptidases we have described (Rul, 1994), hydrophobic peptides responsible for bitterness.

Three aminopeptidases have now been purified from S. thermodus: S. thermodus: St-PepA and two general aminopeptidases, St-PepC and St-PepN. Biochemical studies and sequence data supported the evidence of a striking resemblance between the streptococcal aminopeptidases and PepC, PepN and PepA from lactococci. The aminopeptidases isolated from the two species indeed have similar biochemical properties and substrate specificities. Moreover, the N terminus sequences we obtained from the purified proteins St-PepN (Rul et al., 1994) and St-PepA (data not shown) are 67% (19 residues) and 77% (13 residues) homologous, respectively, to the sequences of the corresponding lactococcal aminopeptidases PepN (Tan & Konings, 1990) and PepA (F. Müllerholland, personal communication). Finally, the gene encoding St-PepC was sequenced by our group (Chapot-Chartier et al., 1994) and was found to be 82% similar to the gene pepC encoding PepC from L. lactis.

ACKNOWLEDGEMENTS
This work was supported by the French Ministry of Research and Technology (programme Aliment 2002, contract no. 90T0927). We thank P. Anglade and G. Brignon for the N-terminal sequencing of peptides.

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Received 16 March 1995; revised 5 May 1995; accepted 15 May 1995.