Identification and Partial Purification of a Dipeptidyl Aminopeptidase from \textit{Streptococcus faecalis}

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A dipeptidyl aminopeptidase was identified in \textit{Streptococcus faecalis} JH2SS and was partially purified (approximately 245-fold) by HPLC. Gel filtration chromatography indicated an \(M_r\) of 140000. The partially purified enzyme exhibited a requirement for \(\text{Co}^{2+}\). The pH optimum for the hydrolysis of L-Val-L-Ala-p-nitroanilide was approximately 9.5. The apparent \(K_m\) for this substrate was 0.22 mM. The enzyme preferentially hydrolysed X-Ala-Y substrates, but also utilized X-Pro-Y substrates, and therefore is most closely related to the mammalian dipeptidyl aminopeptidase I (EC 3.4.14.20).

The enzyme was inhibited by \(p\)-chloromercuribenzoate, but not by iodoacetate, \(N\)-ethylmaleimide or the serine protease inhibitor phenylmethyisulphonyl fluoride.

\section*{INTRODUCTION}

Recipient strains of \textit{Streptococcus faecalis} excrete small peptides which induce mating responses in donors harbouring certain conjugative plasmids (Dunny \textit{et al.}, 1978, 1979). These sex pheromones give rise to the synthesis of a proteinaceous adhesin which uniformly coats the donor cell surface and facilitates the formation of mating aggregates (Kessler \& Yagi, 1983; Yagi \textit{et al.}, 1983). Suzuki \textit{et al.} (1984) have recently purified and characterized the pheromone cPD1 (induces donors harbouring pPD1) and found it to be a hydrophobic octapeptide (H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH). The pheromone cAD1 (induces donors harbouring pAD1) is also a hydrophobic octapeptide with a sequence different from cPD1 (Mori \textit{et al.}, 1984). Mutants defective in the production of cPD1 have been found to be similarly defective in the production of cAD1 (Ike \textit{et al.}, 1983), suggesting a possible linkage in synthesis, processing or transport.

Inhibitors of RNA or protein synthesis immediately stop any increase in pheromone activity (Clewell \textit{et al.}, 1984b). Thus, pheromone synthesis on ribosomes, rather than non-ribosomal enzymic condensation, appears likely. However, the small size of the pheromone molecule and the absence of an N-terminal methionine residue suggest that synthesis occurs as a larger precursor polypeptide, which is then processed to yield mature pheromone. Julius \textit{et al.} (1983) have demonstrated an essential role for a membrane bound dipeptidyl aminopeptidase (DPAPase) in the processing of a yeast peptide sex pheromone (alpha factor). Furthermore, physiological studies and precursor sequence analyses indicate that DPAPase processing is probably a common feature in the synthesis of many secreted peptides in various higher and lower eukaryotic systems (Elovson, 1980; Julius \textit{et al.}, 1983; Kato \textit{et al.}, 1978; Kreil \textit{et al.}, 1980).

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Abbreviation: THB, Todd-Hewitt Broth.
Steiner et al., 1980). As a first step in examining the potential for this type of processing in the synthesis of \(S\). faecalis sex pheromones, we have attempted to identify and partially characterize DPAPase activity in a plasmid-free strain. The results of this study are presented here.

**METHODS**

**Bacteria and media.** The bacterial strains JH2SS, Y11 and FA1001 used in this study have been described previously (Clewell et al., 1984a; Ike et al., 1983; Tomich et al., 1980). Unless otherwise noted the medium used was Difco Todd-Hewitt Broth (THB). Optical density was monitored using a Klett-Summerson colorimeter with a no. 54 (green) filter.

**Enzyme assay.** Enzyme activity of DPAPase involved a minor modification of the procedure of Suarez-Rendueles et al. (1981) using L-Val-L-Ala-p-nitroanilide as the substrate. The reaction mixture contained, in a total volume of 0.5 ml, 0.1 M-glycine/NaOH buffer (pH 9.5), 0.45 mM-substrate, added cofactors and an appropriate amount of enzyme (or whole cells). Samples were incubated at 37°C for 5 min before initiation of the reaction by the addition of substrate. Enzyme assays were linear with respect to time (0-60 min) and protein (0-10 mg) as long as the \(A_{405}\) did not exceed 0.7 (about 25% hydrolysis of the substrate added). One Unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1.0 pmol product min\(^{-1}\) (measured as \(A_{405}\); the molar absorption coefficient of \(p\)-nitroanilide is 9.62 \(\times\) 10\(^5\) M\(^{-1}\) cm\(^{-1}\) at 37°C. All activities reported are the mean and SD of at least six independent experiments. To prepare whole cells for assay, the strains to be tested were grown to late exponential phase (100 Klett Units) at 37°C in THB. The cells were then harvested by centrifugation, washed once with 0.9% NaCl (15 ml (g wet weight)-\(^{-1}\)) and resuspended in cold 50 mM-sodium acetate (pH 5.0). The protein concentration of whole cell preparations was determined by the Lowry method using bovine serum albumin as the standard. The cells were then assayed for DPAPase activity. The protein concentration in cell extracts and during purification was measured by the method of Kalb & Bernlohr (1977).

**Enzyme purification.** A 1% inoculum of an overnight culture of \(S\). faecalis strain JH2SS was added to 81 THB (prewarmed to 37°C) and grown with gentle agitation (100 r.p.m.) to approximately 100 Klett Units (5 \(\times\) 10\(^8\) cells ml\(^{-1}\)). Glycine (3%, w/v) was added and the culture incubated for a further 1 h. Cells were harvested by centrifugation at 16000 \(g\) for 10 min and then washed once with 0.9% NaCl (15 ml (g wet weight)-\(^{-1}\)). The cells were suspended in 40 ml of a solution containing 30 mM-Tris/HCl (pH 8.0), 5 mM-EDTA, 50 mM-NaCl and 5 mg lysozyme ml\(^{-1}\). Mutanolysin (10 ml; Sigma; 5000 U ml\(^{-1}\)) was added and the cell suspension incubated at 37°C with shaking for 75 min to effect lysis. After 45 min, DNAase and RNAase (each at a final concentration of 5 \(\mu\)g ml\(^{-1}\)) were added to degrade nucleic acids. A clear supernatant fluid (crude cell extract) was obtained by centrifugation at 104000 \(g\) for 2 h at 4°C. The crude cell extract was dialysed against 210-1 M-Tris/HCl (pH 7.5) at 4°C for 18 h with one change of buffer, and then fractionated by ammonium sulphate precipitation using cold saturated ammonium sulphate solution (pH 5.5). The ammonium sulphate fraction containing the DPAPase activity (35-70% saturation) was redissolved in 50 mM-sodium phosphate (pH 6.0) and dialysed against 4 l of the same buffer for 24 h with one buffer change. This sample (volume 11 ml) was then fractionated by HPLC using a DEAE-HPLC column (Spherogel-TSK IEX-540 DEAE, 4 mm \(\times\) 30 cm) that had been equilibrated with 50 mM-sodium phosphate (pH 6.0), at a flow rate of 0.85 ml min\(^{-1}\). The protein was eluted with a linear 0-0.5 M-NaCl gradient in 100 min. Fractions containing DPAPase activity were pooled and applied to the same DEAE-HPLC column, equilibrated with 50 mM-sodium phosphate (pH 5.0), at a flow rate of 0.85 ml min\(^{-1}\). The protein was eluted as described above. The fractions containing enzyme activity were combined and further purified by applying to a TSK 3000SW HPLC gel filtration column (7.5 mm \(\times\) 30 cm) that had been equilibrated with 50 mM-HEPES/Tris (pH 7.0) containing 0.1 M-NaCl, at a flow rate of 0.5 ml min\(^{-1}\). The pooled fractions containing DPAPase activity were used to characterize the enzyme. All HPLC was done using a Beckman model 342 HPLC apparatus, and protein elution was monitored using a Beckman model 160 Detector set at 254 nm.

**PAGE.** This was done using a modification of the disc gel electrophoresis method of Davis (1964). The 7-5% (w/v) polyacrylamide gel was cast and run as a 1.5 mm slab gel. SDS-PAGE was done using the modification of the O'Farrell method described by White et al. (1981). After electrophoresis the gels were stained (assayed) for DPAPase activity by the method of Mort & Leduc (1982) using L-Lys-L-Ala-4-methoxy-ß-naphthylamide (4.5 mM) as the substrate and 0.1 mM-glycine/NaOH (pH 9.5) containing 1 mM-Co\(^{2+}\) as the buffer. The gels were stained for protein with Coomassie brilliant blue R-250.

**Effect of divalent metal ions and inhibitors on the partially purified enzyme.** Partially purified enzyme (3-8 \(\mu\)g) was incubated in the presence or absence of metal ions as chloride salts and inhibitors (p-phenanthroline, iodoacetate,
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Reagents. Inhibitors and nitroanilide and naphthylamide peptide substrates were purchased from Sigma, except for succinyl-L-Ala-p-nitroanilide and succinyl-L-Ala-L-Ala-p-nitroanilide, which were from Peninsula Laboratories. Protein Mr standards for gel filtration chromatography were from Boehringer-Mannheim. High and low range protein standards for SDS-PAGE were from Bio-Rad. All other reagents were of the highest grade commercially available.

RESULTS

Identification of DPAPase activity in S. faecalis

*S. faecalis* strain JH2SS was tested for the presence of DPAPase activity by incubating washed whole cell preparations with various p-nitroanilide peptide substrates and determining if p-nitroanilide was released. JH2SS cells hydrolysed L-Val+ Ala-p-nitroanilide efficiently under the conditions used and were also capable of hydrolysing Gly-L-Pro-p-nitroanilide at a much reduced efficiency. The levels of DPAPase activity detected in JH2SS were not reduced when this strain was harbouring the plasmids PAD1 or pPD1. When crude cell extracts of JH2SS were prepared (see Methods), DPAPase activity was detected in the soluble fraction, but not in the membrane particulate fraction.

Partial purification of DPAPase

The enzyme was partially purified by ion exchange chromatography of the 35 to 70% ammonium sulphate fraction from *S. faecalis* at pH 6.0 (Fig. 1a), followed by separation of the pooled fractions containing DPAPase activity at pH 5.0 (Fig. 1b). Further purification by gel filtration is shown in Fig. 1 (c). The fractions eluting from 16 to 18 min were pooled; this material represented the partially purified enzyme used for further characterization. The final purification was about 245-fold, and the overall yield of activity was 27.5%. The partially purified enzyme could be stored at -20 °C for at least one month without loss of activity. A summary of the purification is presented in Table 1.

Since the ammonium sulphate fractionation resulted in a substantial increase in total activity relative to the crude cell extract (Table 1), the degree of purification and the final yield of enzyme activity relative to crude extract reported in Table 1 may be overestimated.

The *M* of the enzyme was estimated as 140000 by gel filtration chromatography using a TSK 3000SW HPLC column equilibrated with 50 mM-HEPES/Tris (pH 7.0) containing 0-1 M-NaCl. The proteins in the final preparation were examined by non-denaturing and SDS-PAGE. Non-denaturing PAGE showed three bands of protein (data not shown), of which only one had DPAPase activity. SDS-PAGE (data not shown) showed the presence of at least twelve polypeptides, all of *M* < 140000. None of these polypeptides exhibited DPAPase activity upon staining, suggesting that the DPAPase is made of subunits.

Properties of the partially purified enzyme

The effect on the DPAPase activity of various metal ions was examined by adding them to the standard assay mixture. It should be noted that the buffer in which the crude cell extract was prepared contained 5 mM-EDTA which would chelate any endogenous divalent metal ions, and then subsequently remove them during the purification. Co^{2+} (1.0 mM) stimulated the DPAPase activity approximately 7.5-fold, but none of the other metal ions tested significantly stimulated activity, although Cu^{2+} (1.0 mM) and Zn^{2+} (1.0 mM) gave rise to a slight increase (Table 2). These results suggest that the enzyme has a specific requirement for Co^{2+}.

The influence of several known inhibitors on the Co^{2+}-stimulated DPAPase activity was then tested (Table 3). *N*-Ethylmaleimide and the thiol inhibitor iodoacetate had no effect, whereas p-chloromercuribenzoate inhibited the enzyme activity by almost 50%. Phenylmethylsulphonyl fluoride, a serine protease inhibitor, had little effect on the enzyme under the conditions used. The DPAPase activity was inhibited more than 50% by *o*-phenanthroline, a result consistent with the apparent requirement of the enzyme for Co^{2+}.
Fig. 1. Chromatography of the dipeptidyl aminopeptidase on an IEX-540 DEAE-HPLC column at pH 6.0 (a), then on an IEX-540 DEAE-HPLC column at pH 5.0 (b), and finally on a TSK 3000SW gel filtration HPLC column (c). ---, Abs280 of the eluate; ----, NaCl gradient. Chromatography was done as described in Methods. The bars represent DPAPase activity with L-Val-L-Ala-p-nitroanilide as substrate. Assay conditions were as described in Methods with the addition of 1 mM-Co2+.

Table 1. Purification of dipeptidyl aminopeptidase from Streptococcus faecalis strain JH2SS

Units are defined as pmol L-Val-L-Ala-p-nitroanilide hydrolysed min⁻¹. Assays were done at 37 °C in 0.1 M-glycine/NaOH (pH 9.5) with 1 mM-Co2⁺.

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (ml)</th>
<th>Protein (mg)</th>
<th>Total Units</th>
<th>Units (mg protein)⁻¹</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>43</td>
<td>731</td>
<td>614000</td>
<td>840</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (35–70%)</td>
<td>11</td>
<td>170</td>
<td>2070000</td>
<td>12200</td>
<td>337</td>
<td>14.3</td>
</tr>
<tr>
<td>DEAE-HPLC pH 6.0</td>
<td>15</td>
<td>12.3</td>
<td>1400000</td>
<td>114000</td>
<td>228</td>
<td>136</td>
</tr>
<tr>
<td>DEAE-HPLC pH 5.0</td>
<td>6.8</td>
<td>4.62</td>
<td>693000</td>
<td>130000</td>
<td>113</td>
<td>179</td>
</tr>
<tr>
<td>TSK 3000SW HPLC</td>
<td>4.3</td>
<td>0.82</td>
<td>169000</td>
<td>206000</td>
<td>27.5</td>
<td>245</td>
</tr>
</tbody>
</table>
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Table 2. Effect of metal ions on partially purified dipeptidyl aminopeptidase

Units are defined as pmol L-Val-L-Ala-p-nitroanilide hydrolysed min⁻¹. Assays were done at 37 °C in 0·1 M-glycine/NaOH (pH 9·5) with 1 mM-Co²⁺ and 3·8 µg partially purified enzyme.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Activity (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>97·0 ± 1·5</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>86·6 ± 3·3</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>86·6 ± 3·1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>97·0 ± 5·8</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>107·0 ± 15</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>709·0 ± 42</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>114·0 ± 9·8</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>125·0 ± 4·3</td>
</tr>
</tbody>
</table>

Table 3. Effect of inhibitors on partially purified dipeptidyl aminopeptidase

Assays were done at 37 °C in 0·1 M-glycine/NaOH (pH 9·5) with 1 mM-Co²⁺ and 3·8 µg partially purified enzyme. 100% activity equals 740 ± 52 pmol L-Val-L-Ala-p-nitroanilide hydrolysed min⁻¹.

<table>
<thead>
<tr>
<th>Addition (1 mM)</th>
<th>Enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Phenylmethylsulphonyl fluoride</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>110 ± 1</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>45 ± 5</td>
</tr>
</tbody>
</table>

The pH dependence for activity of the partially purified enzyme in the presence of Co²⁺ is shown in Fig. 2. Activity toward L-Val-L-Ala-p-nitroanilide was greatest over the pH range 7·5 to 10·0 with a sharp optimum at approximately pH 9·5.

The substrate saturation kinetics for the hydrolysis of L-Val-L-Ala-p-nitroanilide by the partially purified enzyme in the presence of 1·0 mM-Co²⁺ were determined at pH 9·5. The saturation curve for this peptide substrate was hyperbolic and the double reciprocal plot (not shown) yielded an apparent $K_m$ of 0·22 mM.

The partially purified enzyme was incubated at various temperatures in 0·1 M-glycine/NaOH (pH 9·5). The enzyme was stable below 45 °C, but activity was lost after 20 min incubation at 50 °C. All detectable activity was lost in 10 min when the enzyme was incubated at 65 °C.

The partially purified enzyme was tested for the ability to hydrolyse various p-nitroanilide derivatives of amino acids and peptides in the presence of 1·0 mM-Co²⁺ in 0·1 M-glycine/NaOH (pH 9·5). The peptide that was hydrolysed most readily was L-Val-L-Ala-p-nitroanilide (Table 4). The enzyme hydrolysed Gly-L-Pro-p-nitroanilide and L-Ala-L-Ala-L-Val-L-Ala-p-nitroanilide at approximately 50% of the rate of L-Val-L-Ala-p-nitroanilide. L-Ala-p-nitroanilide, succinyl-L-Ala-L-Ala-p-nitroanilide and succinyl-L-Ala-L-Ala-p-nitroanilide were not hydrolysed under the conditions used in these experiments. From these results, the enzyme resembles a DPAPase Type II.

**DPAPase activity in pheromone mutants**

Two independently isolated mutants that excreted 30-fold reduced levels of both cPD1 and cAD1 were assayed for DPAPase activity in order to determine if this enzyme might be involved in pheromone processing. In whole-cell assays one mutant, FA1001 (Clewell et al., 1984a), had levels of DPAPase comparable to the wild-type (JH2SS). In contrast, the mutant Y11 (Ike et al., 1983) showed a 25 to 40% reduction in DPAPase specific activity. When crude cell extracts were prepared from both the mutant and the wild-type, however, no difference in DPAPase specific activity was detected. Furthermore, when cell extracts of the mutant Y11 were partially purified (ion exchange and gel filtration HPLC) in parallel with cell extracts of JH2SS, no detectable difference in the enzyme characteristics or activities could be detected. These results, however, do not rule out the possibility that the mutation in Y11 is in another cell component that effects or modulates DPAPase activity.
Fig. 2. Effect of pH on the partially purified DPAPase activity with L-Val-L-Ala-p-nitroanilide as substrate. Assays contained 3.8 μg protein, 0.1 M-buffer, 0.45 mM-substrate and 1 mM-Co²⁺, and were done as described in Methods.

Table 4. Substrate specificity of partially purified dipeptidyl aminopeptidase

<table>
<thead>
<tr>
<th>Substrate (0.45 mM)</th>
<th>Enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Val-Ala-p-nitroanilide</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Gly-L-Pro-p-nitroanilide</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>L-Ala-L-Ala-L-Val-L-Ala-p-nitroanilide</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>L-Ala-p-nitroanilide</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

DISCUSSION

Dipeptidyl aminopeptidase II (EC 3.4.14.-) was first identified by McDonald et al. (1968b) in bovine pituitary extracts that could hydrolyse L-Lys-L-Ala-2-naphthylamide, a substrate that was poorly utilized by DPAPase IV. The enzyme has been purified from bovine pituitary extracts (McDonald et al., 1968a), rat testes (Vanhala-Perttula, 1973) and bovine dental pulp (McDonald & Schwabe, 1980).

Although peptidases are widely distributed among bacteria and have been studied extensively in Gram-negative bacteria (Miller, 1975) and in the streptococci (Andersson et al., 1984; Exterkate 1975, 1977; Johnson, 1974; Law et al., 1974; Mou et al., 1975; Thomas et al., 1974), we are aware of only two reports in which bacterial DPAPases have been identified and characterized. In these cases, a DPAPase IV from Streptococcus mitis (Fukasawa & Harada, 1981), and a glycylprolyl DPAPase from Bacteroides gingivalis (Abiko et al., 1985) were found to be very similar to the enzyme from mammalian sources. In the present study, we have described the partial purification and characterization of a DPAPase II-like activity from S. faecalis.

The enzyme from S. faecalis differs from the type II enzyme of mammalian origin in several ways. First, although the mammalian and S. faecalis enzymes are of similar Mᵣ, the pH optimum for the former has been reported to be pH 5-5 (McDonald et al., 1968a, b; Vanha-Perttula, 1973), whereas the latter has a pH optimum of pH 9-5 with only minimal activity at pH 5-5. Furthermore, the enzyme from S. faecalis specifically requires Co²⁺ for maximal activity and is inhibited by p-chloromercuribenzoate, a thiol reagent. The mammalian enzyme has not been
shown to have a metal ion or thiol requirement. Finally, whereas the DPAPase II from bovine dental pulp is inhibited by phenylmethylsulphonyl fluoride (McDonald & Schwabe, 1980), the enzyme from S. faecalis is not.

The physiological role of DPAPase II in S. faecalis is, as yet, unknown. Peptidases in bacteria have been reported to be involved in the utilization of peptides as sources of amino acids (Miller, 1975; Sussman & Gilvar, 1971). DPAPase precursor processing may be widespread in the production of eukaryotic bioactive peptides. Although to our knowledge there is no report in the literature of a DPAPase II that is involved in peptide processing, it is conceivable that this enzyme could have a role in the processing of S. faecalis sex pheromones. In this regard, however, two mutants with reduced levels of cPD1 and CAD1 were shown not to have a clear deficiency in this activity. It will be of interest to screen additional pheromone deficient mutants for enzyme activity; such experiments are planned.

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