An aminopeptidase nutritionally important to
Fusobacterium nucleatum

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The properties of an aminopeptidase (AP) from Fusobacterium nucleatum were
studied in view of the fact that this organism, along with other Gram-negative
anaerobes involved in periodontal diseases, survives in the subgingival
environment by obtaining energy via the fermentation of a small number of
peptide-derived amino acids. The AP was found to be cell-associated and was
isolated from disrupted chemostat-grown cells. It was purified by (NH₄)₂SO₄
fractionation, two column chromatographic steps and IEF. The enzyme was
found to have a molecular mass of 54 kDa, a pl of 5.1, a pH optimum between
7.5 and 8.0 and, using Leu-Ala as substrate, it gave $K_{m}$ and $V_{max}$ values of
0.66 mM and 0.12 pmol min⁻¹ mg⁻¹, respectively. No complete homology was
found between the N-terminal sequence of the first 20 amino acids
(MDXKXYVDLKERFLRYVKFN ...) and any other published sequence, but residues
8-20 gave a 62% match with residues 9-21 of an AP from Haemophilus
influenzae. The enzyme was inactivated by chelating agents, bestatin,
$p$-hydroxymercuribenzoate and some heavy metals. Cobalt ions restored EDTA-
inactivated activity but did not reverse inhibition by 1,10-phenanthroline. In
addition, bestatin and 1,10-phenanthroline had an inhibitory effect on the
batch growth of F. nucleatum in a complex medium in which peptidase
activities would be nutritionally essential. No such inhibition was observed in
a chemically defined medium in which growth was not dependent upon
peptidase activities. The peptidase described in this paper therefore appears to
be a cobalt-activated metallo-AP which, together with other peptidases, is
considered to be important in the survival of F. nucleatum in the subgingival
environment of the mouth.

Keywords: Fusobacterium nucleatum, oral cavity, aminopeptidase, nutritional role

INTRODUCTION

Fusobacterium nucleatum has been isolated from both
active and inactive periodontitis sites in the human
mouth (e.g. Dzink et al., 1988) and is one of the most
frequently detected cultivable bacteria in subgingival
dental plaque (Moore et al., 1991; Moore & Moore,
1994). Its taxonomy, biology and possible role in
periodontal diseases have recently been reviewed by
Bolstad et al. (1996).

In a previous study, we showed that F. nucleatum can
obtain energy via the fermentation of simple sugars such
as glucose, or of amino acids such as glutamate,
histidine, serine and lysine (Rogers et al., 1991). Such
metabolic versatility may well explain its ubiquity in the
mouth.

Contrary to earlier studies (Gharbia & Shah, 1988;
Gharbia et al., 1989) we also showed that peptides were
not essential for the growth of F. nucleatum strains since
they grew in a peptide-free chemically defined medium
(Rogers et al., 1992). However, they were unable to
grow on casein or albumin as a sole source of nitrogen
and energy, primarily because they lack endopeptidase
activities (Rogers et al., 1992). On the other hand, an
organism such as Porphyromonas gingivalis, often
found together with F. nucleatum in diseased sites
A. glutamate, histidine, serine and lysine and that resulting peptides would be potential carbon and energy growth and peptides of molecular mass <1 kDa were hydrophilic fraction of a commercial peptone promoted peptides containing up to six residues and that the four key energy-yielding amino acids were rapidly taken up in the subgingival environment.

In the present study, we report on the isolation, purification and properties of an aminopeptidase (AP) from F. nucleatum.

METHODS

Micro-organism. Fusobacterium nucleatum ATCC 10953 was maintained in a locally prepared cooked meat medium containing 0.3% glucose, and in glycerol broth stored at -80 °C.

Growth conditions. The organism was grown under continuous culture conditions as described previously (Rogers et al., 1991). Briefly, it was grown in BM medium (Shah et al., 1976) at 37 °C, pH 7.4 and a dilution rate of D = 0.1 h⁻¹ under an atmosphere of N₂/CO₂ (95:5).

Preparation of cell-free extracts. The culture overflow was collected at 4 °C and the cells were harvested by centrifugation at 6000 g for 15 min at 4 °C. Deposited cells were resuspended in saline to 1/10th of their original volume and disrupted using a French pressure cell. After centrifugation at 10000 g to remove unbroken cells, the supernatant was lyophilized and the powder stored at 4 °C in a desiccator. To determine the cellular location of the peptidase, a small aliquot of the 10000 g supernatant was centrifuged at 100000 g sediment cell envelope material. These various fractions were then tested for (amino) peptidase activity (see below).

AP assay. During purification, enzyme activity was monitored by a modification of the L-amino-acid oxidase method of Lewis & Harris (1967), using the tripeptide Leu-Gly-Gly as the substrate. Briefly, about 1 μl test sample was loaded onto a cellulose acetate strip and laid in a staining solution (containing 5 mg substrate ml⁻¹, 4 mg o-dianisidine dihydrochloride ml⁻¹, 1 mg L-amino-acid oxidase ml⁻¹, 1 mg peroxidase ml⁻¹ and 100 μM MgCl₂ in 100 mM Tris/HCl buffer, pH 8/0) for 2-3 min. The strip was then removed, lightly blotted, placed between two pieces of plastic film and incubated at 37 °C. Peptidase activity was shown by the appearance of a brown stain after about 30 min incubation. The method was made semi-quantitative by testing serial double dilutions of a sample and visually estimating the end point. For example, if a sample gave a visible reaction when diluted 1/8, but not 1/16, then it was deemed to contain 8 units (U) of activity; i.e. the reciprocal of the end point.

Protein assay. Protein was determined spectrophotometrically by the BCA reagent (Pierce Chemical) with BSA as standard.

Enzyme purification

(NH₄)₂SO₄ fractionation. To approximately 4 g freeze-dried cell extract (see above) was added 340 ml 40 mM Tris/HCl buffer, pH 7.4, and the mixture was stirred at 4 °C for 30 min. Protamine sulphate to 0.4% (w/v) was then added and stirring was continued for a further 60 min. The (inactive) proteins precipitating at 60% and 70% saturation with (NH₄)₂SO₄ at 4 °C were sequentially removed by centrifugation (5000 g, 30 min, 4 °C) and the peptidase was recovered by precipitation at 80% saturation. The precipitate was dissolved in 3 ml distilled water.

Column chromatography. The chromatographic purification of this crude enzyme preparation was accomplished using Bio-Rad Econo-Pac columns. In the first step, the aequous preparation was applied to an Econo-Pac HIC (hydrophobic interaction chromatography) column equilibrated with 100 mM sodium phosphate buffer, pH 6.8, containing 1.5 M (NH₄)₂SO₄. The enzyme was eluted with a reverse linear gradient of (NH₄)₂SO₄ (1.5-0 M) at a flow rate of 1 ml min⁻¹. The active fractions were pooled, concentrated [using a Centriprep filtration unit (exclusion limit 30 kDa; Amicon)] and equilibrated with 1.5 M (NH₄)₂SO₄ in 100 mM sodium phosphate buffer, pH 6.8.

In the second step, the concentrated fraction was applied to an Econo-Pac Q, IEC (ion exchange chromatography) column equilibrated with 50 mM Tris/HCl buffer, pH 8.0. The enzyme was eluted with a linear gradient of NaCl (0-1 M) in 50 mM Tris/HCl, pH 8.0, at a flow rate of 1 ml min⁻¹. Active fractions were pooled, concentrated using the Centriprep unit as described above and desalted against MilliQ water prior to freeze-drying; they were stored desiccated at -80 °C. It was subsequently found that this material gradually lost activity after storage at -80 °C but that this could be avoided by addition of 1 mM EDTA to the pooled active fractions obtained following ion exchange chromatography. Prior to freeze-drying and storage, these fractions were desalted against MilliQ water.

IEF. Pre-cast gels, pH range 3-10, were obtained from Novex; they were 1.0 mm thick and contained 5% acrylamide with 2% ampholytes. Gels were run on a 'Mighty Small II' dual-cycled vertical slab gel electrophoresis unit (Hoeffer). The anode buffer was 10 mM phosphoric acid and the cathode buffer consisted of 20 mM each of the free bases of lysine and arginine. Lyophilized preparation (see above) was dissolved in double-strength sample buffer (Novex) at a concentration of 100 μg protein ml⁻¹. Samples (25 μl) were loaded into two lanes of the gel, which was then electrophoresed at 100 V for the first hour, 200 V for the second hour and finally at 500 V for 30 min. After electrophoresis, the gel was blotted onto Problott (Applied Biosystems), using a Hoeffer TE42 Transfer Electrophoresis Unit, over 2 h using 250 mA constant current. The membrane was then washed and cut to separate the two lanes. One lane was stained for AP activity as described above; the other lane was stained with Coomassie blue (0.025% Coomassie blue R-250, 40% methanol, 10% acetic acid) for 1 min before destaining with 10% acetic acid in 50% methanol. The band having AP activity was identified by matching the two halves. It was excised from the Coomassie-blue-stained half of the membrane, air-dried and the N-
An aminopeptidase from *Fusobacterium nucleatum*

**RESULTS**

Peptidase activity could not be detected in either culture supernatants or cytoplasmic contents and was associated with the cell envelope fraction.
A. H. ROGERS and OTHERS

Table 1. Purification of F. nucleatum AP

Data are means of three separate experiments; differences did not exceed about 10% between experiments.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity (U)*</th>
<th>Total protein (mg)</th>
<th>Specific activity [U (mg protein)⁻¹]</th>
<th>Purification factor (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract (powder)</td>
<td>340</td>
<td>5400</td>
<td>2250.0</td>
<td>2.4</td>
<td>1.0</td>
<td>100.00</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>44</td>
<td>1840</td>
<td>88.6</td>
<td>20.8</td>
<td>8.7</td>
<td>3.90</td>
</tr>
<tr>
<td>Hydrophobic interaction chromatography</td>
<td>26</td>
<td>1656</td>
<td>12.0</td>
<td>138.0</td>
<td>58.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>2</td>
<td>512</td>
<td>24</td>
<td>256.0</td>
<td>107.0</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* One unit of AP activity is defined as the reciprocal of the double dilution end point giving a positive L-amino-acid oxidase reaction when incubated in the presence of Leu-Gly-Gly (see text).

Table 2. Relative rates of breakdown of various peptides by an F. nucleatum peptidase

Data represent the means of three separate experiments in which differences did not exceed 10%. Rate of breakdown using Leu-Gly-Gly as substrate was taken as 1. This was equivalent to a rate of 0.09 μmol min⁻¹ mg⁻¹.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rate</th>
<th>Peptide</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Ala</td>
<td>244</td>
<td>Val-Leu-Ser</td>
<td>0.8</td>
</tr>
<tr>
<td>Arg-Gly</td>
<td>157</td>
<td>His-Gly-Gly</td>
<td>0.3</td>
</tr>
<tr>
<td>Phe-Ala</td>
<td>14.5</td>
<td>Ser-Gly-Gly</td>
<td>0.3</td>
</tr>
<tr>
<td>Ser-Ala</td>
<td>8.3</td>
<td>Pro-Gly-Gly</td>
<td>0.2</td>
</tr>
<tr>
<td>Glu-Ala</td>
<td>8.2</td>
<td>Glu-Gly-Phe</td>
<td>0.1</td>
</tr>
<tr>
<td>Lys-Ala</td>
<td>6.9</td>
<td>Bz-Gly-Arg</td>
<td>0.0</td>
</tr>
<tr>
<td>Arg-Ala</td>
<td>5.8</td>
<td>Met-Arg-Phe-Ala</td>
<td>0.0</td>
</tr>
<tr>
<td>Pro-Ala</td>
<td>4.3</td>
<td>Leu-Trp-Met-Arg</td>
<td>0.0</td>
</tr>
<tr>
<td>Gly-Ala</td>
<td>2.4</td>
<td>Gly-Lys-Arg-Trp</td>
<td>0.0</td>
</tr>
<tr>
<td>His-Ala</td>
<td>0.7</td>
<td>Tyr-Glu-Glu-Trp</td>
<td>0.0</td>
</tr>
<tr>
<td>Gly-Gly-Gly</td>
<td>1.5</td>
<td>Gly-Lys-Ala-Ala</td>
<td>0.1</td>
</tr>
<tr>
<td>Arg-Gly-Gly</td>
<td>1.4</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
<td>0.0</td>
</tr>
<tr>
<td>Gly-Ser-Phe</td>
<td>1.3</td>
<td>Leu-Trp-Met-Arg-Phe</td>
<td>0.0</td>
</tr>
<tr>
<td>Ala-His-Lys</td>
<td>1.2</td>
<td>Gly-Lys-Trp-Gly</td>
<td>0.0</td>
</tr>
<tr>
<td>Lys-Gly-Gly</td>
<td>1.1</td>
<td>Leu-Trp-Met-Arg-Phe-Ala</td>
<td>0.0</td>
</tr>
<tr>
<td>Leu-Gly-Gly</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enzyme purification

An AP was partially purified by the methods described above and the results of each step of a typical purification protocol are summarized in Table 1. In the (NH₄)₂SO₄ fractionation steps, two-thirds of the activity was lost, mostly at the 70% saturation level. However, it resulted in a nine-fold purification factor. The two column chromatography steps ultimately resulted in a purification factor of 107. When run on a 12% acrylamide SDS-PAGE gel (4% stacking gel) the material obtained after ion exchange chromatography showed two well-separated bands of 64.9 and 54 kDa with a minor band of 21.2 kDa. This partially purified enzyme preparation was used in the subsequent characterization tests.

Properties of the peptidase

pI and molecular mass. IEF showed that the enzyme had a pI of pH 5.1. Elution of the active band from the Western blot and subsequent SDS-PAGE revealed that it had a molecular mass of 54 kDa.

Effect of pH on enzyme activity. The optimum pH for peptidase activity was shown to be between 7.5 and 8.0 in the presence of 30 mM Tris/HCl.

Substrate specificity. The partially purified enzyme hydrolysed a variety of unsubstituted di- and tripeptides but did not attack larger peptides or the N-terminally blocked dipeptide Bz-Gly-Arg (Table 2). The dipeptides were broken down more rapidly than the tripeptides, which were attacked N-terminally. In addition, the enzyme failed to hydrolyse the β-naphthylamide or p-nitroanilide derivatives of a variety of amino acids.

Effects of inhibitors and metal ions. The chelating agents EDTA and 1,10-phenanthroline totally inhibited enzyme activity, the latter being effective at 0.1 mM. As noted above, EDTA-inactivated activity could be restored most effectively by 0.1 mM Co²⁺, less so by Mn²⁺ and not at all by Ca²⁺ and Mg²⁺. However, as also noted above, inhibition by 1,10-phenanthroline was irreversible. Amastatin and bestatin, each at a final concentration of 0.1 mM, also totally inhibited enzyme activity. Iodoacetate and p-hydroxymercuribenzoate showed at least 90% inhibition while leupeptin, PMSF and 2-mercaptoethanol showed only 35, 26 and 13% inhibition, respectively. Of the divalent cations, Zn²⁺, Hg²⁺ and Ni²⁺ reduced activity by 93, 82 and 43%, respectively, Mg²⁺ and Mn²⁺ were slightly stimulatory.

Kinetic properties. Using the substrates Leu-Ala and Leu-Gly-Gly, the respective Kₘ values were 0.66 and 4.0 mM; the respective Vₘₐₓ values were 0.12 and 0.09 μmol min⁻¹ mg⁻¹.
**Table 3. Effect of various protease inhibitors on the batch culture growth of F. nucleatum ATCC 10953**

Experiments were performed on three separate occasions; differences did not exceed 10% between experiments. Broth cultures were incubated anaerobically at 37 °C in either a chemically defined medium (CDM) or BHI, both containing the inhibitor as indicated. Optical density readings were taken at about 3 h intervals up to 21 h. Figures in parentheses represent the change in OD560 expressed as a percentage of the change in OD560 in the control.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Change in OD560</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDM</td>
</tr>
<tr>
<td></td>
<td>0–9 h</td>
</tr>
<tr>
<td>Bestatin, 0-4 mM</td>
<td>0.25 (90)</td>
</tr>
<tr>
<td>Iodoacetate, 0.2 mM</td>
<td>0.05 (14)</td>
</tr>
<tr>
<td>1,10-Phenanthroline, 0-1 mM</td>
<td>0.27 (97)</td>
</tr>
<tr>
<td>None (control)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

N-terminal sequence of the enzyme. The first 20 amino acid residues were determined to be MDKXXYVDL-KERFLRYVKFN. As can be seen, residues 3 and 5 could not be identified. Complete N-terminal sequence homology could not be identified through the MPsrch protein–protein database search but a 62% match was found between residues 8–20 of the enzyme and residues 9–21 of *Haemophilus influenzae* strain Rd peptidase T (pep T) (EC 3.4.11.12).

**Effect of various protease inhibitors on the batch culture growth of F. nucleatum.** Most evident was the fact that iodoacetate caused significant depression of CDM-based growth while the remaining inhibitors had little or no effect (Table 3). However, BHI-based growth was markedly reduced in the presence of bestatin (an AP inhibitor) and 1,10-phenanthroline (a metalloprotease inhibitor), as well as by iodoacetate, which probably has wider inhibitory properties against other metabolic (enzyme) functions.

**DISCUSSION**

The finding that the peptidase activity appeared to be associated with the cell envelope fraction is in agreement with our earlier studies on the processing of small peptides by resting cells of *F. nucleatum* (Rogers et al., 1992) and the findings of Bakken et al. (1989), who suggested that small peptides may be cleaved external to the inner membrane of this organism. The APs, particularly those of *Escherichia coli* and the lactic acid bacteria, are usually cytoplasmic but this does not exclude the possibility that some may be associated with the cell envelope (Gonzales & Robert-Baudouy, 1996). Indeed, for those peptidases involved in bacterial nutrition, location close to membrane systems involved in the transport of exogenous peptides would facilitate reaction with relevant substrates as soon as they enter the cell (Gonzales & Robert-Baudouy, 1996). It is also worth noting that the apparent location of enzyme activities can be affected by growth rate (Minhas & Greenman, 1989; Spratt et al., 1995).

Since the optimal pH for activity of the peptidase and the pH of periodontal pockets and subgingival plaque are within the range 7.5–8.0 (Bickel & Cimasoni, 1986; Eggert et al., 1991), the peptidase, if produced *in vivo*, would be active against natural substrates, particularly those in gingival crevicular fluid.

The finding that the peptidase N-terminally attacked a variety of small peptides and lacked both endo- and carboxypeptidase activities indicates that it is an AP of broad substrate specificity. Based on their catalytic mechanisms (sensitivity to various inhibitors), bacterial APs can be divided into three main groups, the largest of which is the metallo-APs. The activity of these enzymes is regulated by the presence of divalent metal cations and they are inhibited by chelating agents and by bestatin and amastatin. While Zn\(^{2+}\) is the most frequently associated cation, there is a group of Co\(^{2+}\)-requiring metallo-APs (Gonzales & Robert-Baudouy, 1996) which includes a methionine AP from *E. coli* (Roderick & Matthews, 1993), a dipeptidase from *Lactobacillus sake* (Montel et al., 1995) and an AP from *Streptococcus mitis* (Andersson et al., 1992). Based upon its substrate specificity and sensitivity to various inhibitors, especially metal ion chelators, the *F. nucleatum* AP could therefore be categorized as a Co\(^{2+}\)-activated metallo-AP. Like most enzymes of this type (e.g. Montel et al., 1995), the sensitivity of the AP to *p*-hydroxymercuribenzoate and iodoacetate indicated that thiol groups are involved in catalysis. Also, the ability of Mn\(^{2+}\), albeit less than that of Co\(^{2+}\), to restore the activities of EDTA-inactivated enzyme is consistent with the behaviour of other metallo-APs (e.g. Montel et al., 1995).

In terms of its enzymic properties, the AP displayed Michaelian saturation kinetics with regard to the substrates tested. The values obtained indicate a mod-
erate or weak substrate affinity, common for bacterial APs. The low pH value and alkaline pH optimum are also typical (Gonzales & Robert-Baudouy, 1996).

The use of a wide variety of peptides as nutrients is among the important physiological roles of bacterial APs (Lazdunski, 1989). For example, the proteolytic system of organisms such as Lactococcus lactis is of importance to the dairy industry and has been well characterized. Peptide breakdown by these organisms is accomplished by the combined action of a number of peptidases displaying broad specificities and overlapping activities. With the exception of a methionine-specific AP that removes N-terminal Met residues from proteins (Miller et al., 1989), none of them is essential for the cells' physiology and survival (Lazdunski, 1989). Allowing for the fact that different strains were used, it appears that F. nucleatum may contain more than one peptidase, since our previous studies (Rogers et al., 1992) showed that resting cells could attack peptides containing up to five residues while the AP described here could not. The co-operative effects of such peptidases would be vital to the growth and survival, in the subgingival environment, of potential pathogens such as F. nucleatum. The notion that peptidase activities are of nutritional importance is supported by the present findings that both bestatin and L-phenylalanine inactivated the isolated metallo-AP and that these inhibitors also markedly affected the organism's growth in BHI but not CDM. The ability of these two compounds to inhibit the peptidase activities and growth of Gram-negative anaerobes has previously been reported (Grenier, 1992; Grenier & Michaud, 1994; Wallace & McKain, 1996). Growth in BHI but not CDM would be reliant on peptides to provide the necessary energy-yielding amino acids; this, in turn, would be dependent upon functional (amino) peptidase activities. Metabolizable peptides could be provided from the degradation of proteins and oligopeptides via the trypsin-like protease (endopeptidase) activities of organisms such as Capnocytophaga gingivalis (Spratt et al., 1995) and P. gingivalis (Marsh et al., 1994; Pike et al., 1994). Among the key determinants of the microbial ecology, and thus pathogenic potential, of organisms in periodontal pockets may be the efficiency of peptide utilization among the various species (Tang-Larsen et al., 1995).

Finally, we have also isolated, purified and characterized a Co²⁺-activated metallo-AP from F. nucleatum ATCC 25386. Its characteristics are almost identical to those described here for strain ATCC 10953.

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REFERENCES


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