***Capnocytophaga gingivalis* aminopeptidase: a potential virulence factor**

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The production and properties of an aminopeptidase from *Capnocytophaga gingivalis* were studied. *C. gingivalis* was grown in continuous culture over a range of dilution rates and the cell-bound and extracellular levels of aminopeptidase and trypsin-like protease (TLPase) measured. At high growth rates (0.6 μ_{rel}) TLPase specific activity was low and found exclusively as cell-bound activity; at low growth rates (0.0375 μ_{rel}), specific activity was high and 26% was found as extracellular activity. In contrast, aminopeptidase specific activity was highest at 0.3 μ_{rel} and the ratio of cell-bound to extracellular activity was relatively constant at all growth rates. Only about 5% of the total activity was extracellular. The aminopeptidase, which has a wide specificity towards artificial substrates, was purified to homogeneity, as judged by SDS-PAGE, from the supernatant fluid of cells grown in continuous culture in a tryptone/glucose/thiamine medium. The enzyme has a molecular mass of 61 kDa, a pI of 6.3, a pH optimum close to 7.5 and showed a requirement for magnesium or calcium ions. The N-terminal sequence of the first 10 amino acids (Asp-Val-Asn-Met-Leu-Trp-Tyr-Val-x-Arg...) showed no similarity to any published sequence. This enzyme in its cell-bound or extracellular form may be important in the nutrition and pathogenesis of *C. gingivalis* in the human oral cavity.

Keywords: *Capnocytophaga gingivalis*, oral cavity, aminopeptidase, trypsin-like protease, extracellular and cell-bound enzymes

INTRODUCTION

The genus designation of *Capnocytophaga* was conferred by Leadbetter *et al.* (1979) who characterized some non-flagellated, gliding, Gram-negative fusiform bacteria isolated from the human oral cavity. *C. gingivalis* is thought to form part of the normal oral flora (Holdeman *et al.*, 1985), and is present in both supragingival and subgingival plaque. Members of the genus have been implicated in juvenile periodontal disease (Newman *et al.*, 1976; Slots, 1976) and periodontal disease associated with insulin-dependent diabetes mellitus (Mashimo *et al.*, 1983). In mono-infected rats, *Capnocytophaga* spp. cause periodontal disease between the first and second maxillary molars, with accompanying migration of the gingival margin and destruction of alveolar bone (Irving *et al.*, 1978). Their

pathogenic potential has been attributed in part to the production of tissue-destroying hydrolytic enzymes (Nakamura & Slots, 1982; Shibata *et al.*, 1992). In addition to aminopeptidase activity, a trypsin-like protease has been identified, purified and characterized (Shibata *et al.*, 1992). However the biochemical basis of the pathogenicity of these organisms has yet to be determined.

The aminopeptidase of *Capnocytophaga* may be an important virulence factor in associated pathological conditions owing to its postulated involvement in bradykinin formation (Hopsu-Havu *et al.*, 1966) and degradation of collagen fragments (Ando, 1980; Nakamura & Slots, 1982). Aminopeptidases of both bacterial and fungal origin have been implicated in the mechanism of pathogenesis by reason of association (Green *et al.*, 1967; D'Amato *et al.*, 1978), but there is little information on aminopeptidase production by *Capnocytophaga gingivalis*.

As part of our studies on the physiology and biochemistry of the oral flora, we decided to define optimum conditions for production and activity of the aminopeptidase of *C.*

Abbreviations: BAPNA, *N*- α -benzoyl-L-arginine-*p*-nitroanilide; TLPase, trypsin-like protease.

The EMBL accession number for the N-terminal amino acid sequence data reported in this paper is P80474.

gingivalis. Continuous culture using chemostats has been used to study the physiology of periodontal micro-organisms by various workers (Greenman *et al.*, 1981; Marsh *et al.*, 1983). Here we also describe the properties of the purified enzyme, including the molecular mass, specificity towards artificial chromogenic substrates, cation requirement and N-terminal amino acid sequence analysis. This work may provide a basis for further investigations of the pathogenicity of *C. gingivalis*, since purified enzyme and N-terminal sequence data can be used for the production of specific antibodies and oligonucleotide probes, respectively.

METHODS

Organisms and culture maintenance. *Capnocytophaga gingivalis* strain ATCC 33624 was obtained from the American Type Culture Collection Rockville, MD, USA. This Gram-negative fusiform bacterium requires a CO₂-enhanced (> 5%) atmosphere for growth. Cultures were maintained on fastidious anaerobe agar (FAA) supplemented with horse blood (5% v/v). Plates were incubated in an anaerobic cabinet at 37 °C, in an atmosphere of N₂/H₂/CO₂ (80:10:10 by vol.). Cultures were stored at -70 °C on porous beads (Microbank).

Continuous culture. *C. gingivalis* was grown in a chemostat vessel (1 l) with control modules for temperature, pH and stirrer rate (LH Fermentation). The culture volume (750 ml) was maintained at 37 °C (±0.1 °C), and the pH at 7.0 (±0.05 units) by automatic addition of 2 M NaOH or 1 M H₂SO₄. The impeller speed was 500 r.p.m. (±10 r.p.m.). The vessel was sparged with N₂/CO₂ and air to give an N₂/O₂/CO₂ ratio of 85:10:5 (by vol.). The growth media consisted of brain heart infusion (18.5 g l⁻¹) and yeast extract (5 g l⁻¹) for growth rate studies, and tryptone (18.5 g l⁻¹), glucose (2 g l⁻¹) and thiamine (0.5 mg l⁻¹) for the production of aminopeptidase for purification. The maximum specific growth rate ($\mu_{\max}$) was determined by the washout method (Tempest, 1970) and a dilution rate was set to give the required growth rate. To study the effect of dilution rate on aminopeptidase and trypsin-like protease (TLPase) production, the medium addition pump was adjusted to give a range of growth rates between 0.0375 and 0.6 μ_{rel} . At each dilution rate, once steady state was achieved, standard assays were carried out for aminopeptidase and TLPase. Assays were carried out, in quadruplicate, on three consecutive samples taken from steady-state culture over a 3 d period and means of the 12 determinations calculated.

Enzyme assays. Assays for aminopeptidase and TLPase were measured using the chromogenic substrates, L-leucine-*p*-nitroanilide and *N*- α -benzoyl-L-arginine-*p*-nitroanilide (BAPNA), respectively, which upon hydrolysis produce the coloured end-product, *p*-nitroaniline ($\lambda_{\max}$ = 410 nm). Both assays were carried out in a total volume of 300 μ l in the wells of a microtitre plate. The aminopeptidase assay mixture consisted of 230 μ l MOPS buffer (0.1 M, pH 7.5) and 50 μ l leucine-*p*-nitroanilide substrate (8 mM). A 20 μ l aliquot of enzyme preparation or cell suspension was added to the well and the contents mixed to start the reaction. The TLPase assay consisted of 180 μ l MOPS buffer (0.1 M, pH 7.5), 100 μ l BAPNA (0.2 mM) and 20 μ l enzyme. The absorbance change was followed using a microtitre plate reader (Anthos ht II) at a wavelength of 410 nm, taking absorbance readings at 1 min intervals for 10 min and calculating the rate of reaction. All assays were conducted at room temperature (19 ± 2 °C).

Assays were carried out using culture supernatant with a range

of 0.1 M zwitterionic buffers to determine the pH optimum for catalytic activity for both enzymes. The buffers used were: MES (pH 5.5–6.7), MOPS (pH 6.5–7.9), HEPES (pH 6.8–8.2), Bicine (pH 7.6–9.0), CHES (pH 8.9–10.0) and CAPS (pH 9.7–11.1). The pH of each assay mixture was checked before and after incubation to ensure that no pH change had occurred (19 ± 2 °C). The optimum assay pH for both enzymes was used for all subsequent assays.

Units of enzyme activity. A unit of activity is defined as μ mol product formed (under the conditions of the assay) min⁻¹. Specific activity is defined as enzyme units (mg dry wt of bacterial cells)⁻¹. Specific activity in the supernatant fraction was related to the dry weight of cells in the culture sample before centrifugation. During purification procedures, specific activity is defined as enzyme units (mg protein)⁻¹. The quantity of product formed was calculated using the molar extinction coefficient (ϵ) of *p*-nitroaniline at 410 nm (10000 M⁻¹ cm⁻¹). Dry weights were determined by the method of Holland *et al.* (1979) and culture absorbances were related to dry weight with reference to a calibration curve.

Purification of the aminopeptidase. Large samples (500 ml) of the culture grown in tryptone/glucose/thiamine medium, from the chemostat (grown at 0.3 μ_{rel}), were centrifuged for 20 min at 5000 *g* (Beckman J2-21) at 4 °C. The supernatant was concentrated by ultrafiltration at 4 °C using a 10 kDa filter (PM 10, 76 mm diameter) with a pressure of 2.4 × 10⁵ Pa provided by N₂ gas. The concentrate was termed crude enzyme preparation. Protein content was estimated by the Coomassie blue method for all samples (Bradford, 1976).

The crude enzyme preparation was dialysed against 20 mM Tris/HCl buffer (pH 8.0) overnight at 4 °C, filtered through a 0.2 μ m filter (Acrodisk, Gelman Sciences) and applied to an ion exchange column (Mono Q HR 5/5) equilibrated with 20 mM Tris/HCl (pH 8.0). The proteins were eluted from the column with a linear gradient of 0.4–0.6 M NaCl in equilibration buffer. Fractions (0.5 ml) were collected and assayed for aminopeptidase activity. Fractions showing significant activity were pooled and concentrated by filtration (Cetricon-10 concentrators, Amicon) at 5000 *g*. The concentrated fractions from the ion exchange step were applied to a gel filtration column (Superose 12 10/30) equilibrated with buffer (50 mM Tris, 100 mM NaCl, pH 7.4) or buffer and Triton X-100 (0.5% v/v). Fractions (0.5 ml) were collected and assayed for aminopeptidase activity. The fractions showing significant aminopeptidase activity were pooled and concentrated (Microcon-10 microconcentrator, Amicon) in a microcentrifuge at 15000 *g*. All chromatography was performed using FPLC equipment (Pharmacia).

Electroelution was carried out on native PAGE gels to recover the aminopeptidase. Identical samples were loaded in all of the wells of a non-denaturing (native) PAGE gel. The aminopeptidase was visualized by activity staining the gel and a strip corresponding to the activity was excised from the gel. The protein within this gel matrix was electroeluted from the gel using Miniprotein II electroelution apparatus. A 10 kDa molecular mass cut-off membrane was used to capture the protein during the electroelution step. A concentration step was carried out to reduce the volume of the sample to approximately 100 μ l (Microcon-10).

Native PAGE gels were subjected to electroblotting (Milliblot) to enable the aminopeptidase (in an active form) to be immobilized on an inert membrane (Immobilon-P). The electroblotting was carried out at 2 mA cm² gel area for 30 min. Following the blotting step, the Immobilon was stained with a

Coomassie stain consisting of Coomassie Brilliant blue R-250 (10 mg), methanol (5 ml) and water (5 ml).

Characterization of the aminopeptidase. Preparative isoelectric focusing (LKB 8101 Ampholine Electrofocusing Equipment) was carried out on samples of crude enzyme. A solution of ampholytes (Pharmacia) was used to produce a pH range of pH 3–10. The electrofocusing was performed at 450 V for 30 h whilst being cooled by means of a water jacket. Fractions (1 ml) of the electrofocused contents of the column were collected, each fraction was assayed for aminopeptidase activity and the pH measured.

Denaturing and non-denaturing (native) PAGE techniques (Laemmli, 1970) were utilized in characterizing and determining the purity of the aminopeptidase enzyme. Resolving gels of 7.5% acrylamide with stacking gels of 4% acrylamide were cast. Myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20.1 kDa) were used as molecular mass markers.

Gels were fixed and stained by one of three techniques; (i) Coomassie Brilliant blue R-250 staining, (ii) silver staining (Bio-Rad) according to manufacturer's instructions or (iii) activity staining. Activity staining for aminopeptidase was carried out as follows (Dr Anthony Rogers, University of Adelaide, Australia, unpublished). N-terminal leucine liberated from the tripeptide substrate Leu-Gly-Gly (5 mg ml^{-1}) was oxidized by L-amino acid oxidase (1 mg ml^{-1}) to release hydrogen peroxide. This was reacted with *o*-dianisidine dihydrochloride (4 mg ml^{-1}) in the presence of horseradish peroxidase (1 mg ml^{-1}) to produce a colour change. The reagents were made up in 2 ml Tris/HCl (0.1 M , pH 8.0). The gel was wrapped in plastic film and incubated at 37°C . Activity was shown by a brown colouration after incubation for 4–8 h. All reagents used in the staining techniques were prepared with purified water from a system consisting of a Reverse Osmosis De-ioniser and a Still Plus 'Water Polisher' (Purite).

The substrate specificity of the aminopeptidase enzyme was investigated using LRA ZYM AP strips (bioMérieux) which allowed the simultaneous study of the 20 substrates contained in the AP-I and AP-III strips. Aliquots of aminopeptidase which had been purified by both ion exchange and gel filtration, and crude enzyme were added to the cupules on the strips. The samples were standardized by diluting to give the equivalent activity with respect to leucine *p*-nitroanilide.

Experiments were performed on the aminopeptidase using a variety of enzyme inhibitors (see Table 4) and the metal ions Ca^{2+} , Mg^{2+} , Mn^{2+} , Ba^{2+} , Co^{2+} , Cd^{2+} , Ni^{2+} , V^{3+} and Zn^{2+} . The standard aminopeptidase assay was modified and consisted of 700 μl buffer, 170 μl leucine *p*-nitroanilide substrate (8 mM), 30 μl enzyme and 100 μl inhibitor in a 1 ml cuvette. Two types of experiments were carried out: (i) the reaction mixture (without substrate) was incubated (room temperature $19 \pm 2^\circ\text{C}$) with inhibitor or metal ion salt for 30 min, substrate was then added and the rate of reaction measured as above; (ii) the enzyme was incubated with EDTA (1 mM) for 20 min, a metal ion solution (5 mM final concentration) was then added and the reaction mixture was incubated for a further 20 min before finally adding substrate and following the reaction rate as previously stated.

A sample of the aminopeptidase enzyme, eluted from a native gel, was used for the determination of the N-terminal amino acid sequence (Immune Systems Ltd). The N-terminal sequence

was compared to other protein sequences using the following databases: Brookhaven Protein Data Bank, Swiss-Prot 26.0, PIR 38.0 (complete), CDS translations from GenBank (R) release 79.0, TDF transcription factor (protein) database release 7.0, six-frame translations of representative human Alu repeats and Kabat sequence of proteins of immunological interest. The sequence comparisons were performed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service.

RESULTS

Effect of dilution rate on aminopeptidase and TLPase production

The effect of dilution rate on the specific activity of aminopeptidase and TLPase is shown in Table 1. A steady increase occurred in aminopeptidase activity with increasing growth rates until $0.3 \mu_{\text{rel}}$ was reached, whereafter it was severely reduced. The total TLPase activity was characterized by a general decrease in activity as dilution rate increased. The relative proportion of cell-bound to extracellular aminopeptidase activity was constant regardless of dilution rate, whilst for TLPase a higher proportion of extracellular activity occurred at lower dilution rates.

Purification of aminopeptidase

The aminopeptidase was purified from the culture supernatant. Although cell-bound aminopeptidase activity was higher than that of culture supernatant, the activity in the latter contained less extraneous protein than the cell lysate. The supernatant fluid contained about $0.1 \text{ mg protein ml}^{-1}$. Aliquots of the concentrated crude enzyme were applied to an ion exchange column. Fractions showing significant activity (Fig. 1) were pooled, concentrated and applied to a gel filtration column. Fractions collected were assayed for aminopeptidase activity (Fig. 2). Two major peaks of aminopeptidase activity were eluted, one between fractions 16 and 18, and the other between fractions 24 and 28. The first peak was in the void volume of the column and therefore represents molecules with a molecular mass above 300 000 Da. The second peak represents aminopeptidase activity from molecules with lower molecular masses. There was no TLPase activity associated with the peaks.

A non-ionic detergent (Triton X-100) was used to investigate the possibility that the high-molecular-mass-associated activity was due to aggregated enzyme molecules. If the high-molecular-mass activity was due to aggregation of enzyme, the Triton would serve to disaggregate it (Ingham *et al.*, 1983). However, if the activity was due to a true high molecular mass form of the aminopeptidase, then it would be unaffected. The experiment was repeated equilibrating both buffer and sample with Triton X-100. The peak associated with the void volume disappeared (Fig. 2) leaving only one peak between fractions 24 and 28. From these results, the approximate molecular mass of the aminopeptidase was shown to be 64 kDa. Subsequently, fractions showing

Table 1. The effect of specific growth rate on biomass, total enzyme production and relative proportion of cell-bound and extracellular enzyme activity for the aminopeptidase and TLPase of *C. gingivalis*

Assays were carried out, in quadruplicate, on three consecutive samples taken from steady-state culture over a 3 d period. Data are the means of 12 determinations.

μ_{rel}	Dilution rate (h ⁻¹)	Biomass (mg dry wt ml ⁻¹)	Specific activity [μ mol product formed min ⁻¹ (mg dry wt cells ⁻¹)]		Relative proportions of cell-bound/extracellular activity	
			Apase	TLPase	Apase	TLPase
0.0375	0.0105	0.3	104	1.6	94/6	74/26
0.075	0.021	0.295	109	0.4	96/4	99/1
0.15	0.042	0.29	144	0.5	95/5	100/0
0.3	0.084	0.285	359	0.7	95/5	100/0
0.6	0.168	0.24	20	0.5	95/5	100/0

Fig. 1. Anion exchange chromatography of concentrated crude enzyme showing aminopeptidase activity in the fractions collected. $\circ$, A_{280} ; ----, NaCl concentration; ▨, aminopeptidase activity.

Fig. 2. Aminopeptidase activity in fractions from a gel filtration column when equilibrated with or without Triton X-100 (0.5% v/v). $\circ$, equilibrated with Triton X-100; $\bullet$, equilibrated without Triton X-100.

activity from the ion exchange chromatography step were pooled, concentrated by ultrafiltration and dialysed against a buffer containing Triton X-100 prior to gel filtration chromatography. The purification of the aminopeptidase is summarized in Table 2. A purification factor of 113 was achieved for the enzyme derived from the culture supernatant.

Properties of the aminopeptidase

pH optimum for catalytic activity. The pH optimum for catalytic activity for both the aminopeptidase and the TLPase was shown to be pH 7.5.

pI and molecular mass. The pI of the aminopeptidase was shown to be pH 6.3. Samples of enzyme which had undergone ion exchange chromatography and gel filtration chromatography were concentrated by ultrafiltration and subjected to native PAGE and subsequently stained for protein, using Coomassie blue, or for aminopeptidase activity. The activity-stained band corresponded to the major Coomassie-blue-stained band. The band containing the enzyme was excised and subjected to SDS-PAGE. One band was detected by silver staining, thus confirming the purity of this preparation. Using molecular mass markers, the mass of the aminopeptidase was calculated to be 61 kDa.

Substrate specificity. The specificity of the aminopeptidase towards β -naphthylamide derivatives of 20 amino acids was investigated (Table 3). The partially purified aminopeptidase (after ion exchange and gel filtration) hydrolysed all of the derivatives with a free N-terminal amino acid, but not the two benzyloxycarbonyl-blocked derivatives.

Effects of inhibitors and metal ions on aminopeptidase activity. The enzyme activity was inhibited by chelating

Table 2. Purification of *C. gingivalis* aminopeptidase

Results are the means of three experiments.

Fraction	Volume (ml)	Total activity (μmol product formed min^{-1})	Total protein (mg)	Specific activity [μmol product formed min^{-1} (mg protein) $^{-1}$]	Purification factor	Yield (%)
Crude culture supernatant	5000	458	450	60	1	100
Ultrafiltration	50	356	142.5	150	2.5	78
Ion exchange*	2	90	10.8	500	8.2	19
Gel filtration*	0.5	30	0.43	4200	69	7
Electroelution†	0.4	16	0.14	6800	113	3.4

* Relevant fractions were concentrated by ultrafiltration.

† Sample concentrated by ultrafiltration.

Table 3. Hydrolysis of β -naphthylamide derivatives of 20 amino acids (AP-I and AP-III strips) by crude enzyme concentrate and purified aminopeptidase

Data represent the API score after a 3 h incubation period, and are approximate being equivalent to nmol β -naphthylamine liberated. The experiment was repeated twice and the results were confirmed. N-CB2, benzyloxycarbonyl group.

β -Naphthylamide substrate	Crude enzyme	Purified aminopeptidase
(a) AP-I		
L-Tyrosyl-	1	1
L-Pyrrolidonyl-	1	1
L-Phenylalanyl-	2	3
L-Lysyl-	2	2
L-Hydroxypropyl-	0.5	4
L-Histidyl-	2	2
Glycyl-	2	4
L-Aspartyl-	2.5	3
L-Arginyl-	2.5	2
L-Alanyl-	2	2
(b) AP-III		
N-CBZ-L-argininyl-4-methoxy-	1	0
L-Glutaminyl-	2.5	2.5
α -L-Glutamyl-	2.5	5
L-Isoleucyl-	1	1
L-Ornithyl-	5	2.5
L-Prolyl-	3	2
L-Seryl-	5	2
L-Threonyl-	2	2
L-Tryptophyl-	2.5	3.5
N-CBZ-glycyl-glycyl-L-arginyl	1	0

Table 4. Effect of pre-incubation with various enzyme inhibitors on the activity of aminopeptidase

The residual activity shown is the amount of activity retained after a 30 min incubation at room temperature with the inhibitor. All assays were carried out in triplicate and the mean values are shown.

Inhibitor	Final concentration (mM)	Residual activity (%)	Percentage inhibition
Control	—	100	0
EDTA	10	21	79
1,10-Phenanthroline	1	14	86
PHMB	10	34	66
Iodoacetamide (IAA)	10	92	8
AEBSF	1	100	0
Bestatin	10	100	0

activity. EDTA-inactivated enzyme activity could be reactivated 38 and 22 %, respectively, by Mg^{2+} or Ca^{2+} ions.

N-terminal sequence of the aminopeptidase

The sequence data were limited to the first 10 amino acids which were determined to be Asp-Val-Asn-Met-Leu-Trp-Tyr-Val-x-Arg.... The sequence of 10 amino acids has an unidentified residue (x in the above sequence), possibly due to masking by high background levels of other residues. No N-terminal sequence homology could be identified by reference to databases (see Methods) and consequently no match with any other aminopeptidases was found.

agents (Table 4) with 100 mM EDTA giving a 50 % reduction in activity. Heavy metal ions (Co^{2+} , Cd^{2+} , Ni^{2+} and Zn^{2+}) showed a greater than 90 % inhibition of

DISCUSSION

A continuous culture system was used to study the production of aminopeptidase and TLPase enzymes. It has been reported that *C. gingivalis* cells produce various enzymes when grown in batch culture (Nakamura & Slots, 1982; Slots, 1982) but the extent to which the activities were cell-bound or extracellular remained unstudied. Since the chemostat is the only way of controlling growth rate, it was used in an early experiment to study the effects of growth rate on the production and export of hydrolytic enzymes. The results show two different trends between growth rate and hydrolytic enzyme production. The aminopeptidase specific activity peaked at $0.3 \mu_{rel}$ and showed a constant ratio of cell-bound to extracellular activity. However, the TLPase specific activity increased with decreasing growth rate and clearly showed that the ratio of cell-bound enzyme to extracellular enzyme decreases with decreasing relative growth rate. This finding is consistent with results for TLPase of *Porphyromonas gingivalis* where the ratio of cell-bound to extracellular activity decreases with decreasing growth rate (Minhas & Greenman, 1989).

The pH optimum for catalytic activity of both the aminopeptidase and TLPase was pH 7.5. This suggests that, if the enzymes are produced *in vivo*, they would be active against their natural substrates in the periodontal tissues since the pH of subgingival plaque and the periodontal pocket is in the range pH 7.0–8.0 (Bickel & Cimasoni, 1986).

The molecular masses of aminopeptidases characterized from various sources in nature extend over a wide range, from 390 kDa (Cueva *et al.*, 1989) to 29 kDa (Ben-Basset *et al.*, 1987). The aminopeptidase of *C. gingivalis* had an estimated molecular mass of 61 kDa (SDS-PAGE) or 64 kDa (gel filtration) which corresponds closely with other Gram-negative aminopeptidases such as the periplasmic prolyl aminopeptidase (ca. 65 kDa) isolated from *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* (Fricke & Aurich, 1993).

The aminopeptidases produced by various strains of *Capnocytophaga* show wide substrate specificities (Nakamura & Slots, 1982). In this study, comparison of the substrate specificities of the crude and purified aminopeptidase showed that the purified enzyme was capable of hydrolysing all of the substrates with free N-terminal amino acid residues, but not the N-terminal blocked ones. In contrast the crude enzyme preparation hydrolysed all the substrates, including the N-terminal blocked ones, implying that other enzymes were present. It is possible that TLPase, which can hydrolyse N-terminal blocked amino acid substrates (Shibata *et al.*, 1992), was present in small amounts in the crude enzyme preparation. The partially purified aminopeptidase was purified by both cation exchange and gel filtration chromatography. A silver-stained native PAGE gel of purified aminopeptidase showed one main band, which was attributed to the aminopeptidase (by activity staining) and three other faint unidentified bands. The main band was aminopeptidase and had been selected for throughout

the purification procedure by assays using L-leucine *p*-nitroanilide and the tripeptide Leu-Gly-Gly (activity stain) as substrates.

Circumstantial evidence suggests that *C. gingivalis* produces only a single aminopeptidase since: (i) the activity profile with four substrates on all fractions from gel filtration chromatography were similar (Spratt, 1994); and (ii) the substrate specificity of the purified aminopeptidase, as tested against 20 substrates, was shown to be wide and similar to that of the crude enzyme (Table 3).

The action of various inhibitors on the aminopeptidase activity indicated that it should be classed as a metallo-protease or a metal-activated protease. It can be deduced from the results that the aminopeptidase was not a cysteine or a serine protease since the action of IAA, a cysteine protease inhibitor, and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor, was negligible. The effect of chelating agents (EDTA and 1,10-phenanthroline) and metal ions identified the enzyme as a metallo-protease or a metal-activated protease with a requirement for Mg^{2+} or Ca^{2+} . The effect of *p*-hydroxymercuribenzoate (PHMB) indicated a requirement for free sulphhydryl groups for normal catalytic function of the aminopeptidase. Bestatin insensitivity indicates that the aminopeptidase of *C. gingivalis* is structurally different to the mammalian-derived aminopeptidase M (Umezawa, 1982) and aminopeptidase N (Kumano & Sugawara, 1992), both of which are inhibited by bestatin.

The aminopeptidase is produced in substantial amounts by *C. gingivalis* suggesting an important role in the cells natural habitat. Other oral bacteria, for example *Streptococcus mitis* (Andersson *et al.*, 1992) and *Treponema vincentii* (Mäkinen *et al.*, 1988) also produce aminopeptidases and, together or separately, these enzymes could provide a steady supply of amino acids to the consortium of organisms in the mouth by the stepwise hydrolysis of proteins or peptides. The TLPases of *C. gingivalis* and *Porphyromonas gingivalis*, which are endopeptidases, may ensure that proteins or large peptides are degraded to give an increase in the oligopeptides available for aminopeptidase action. Assuming that the enzymes can work in concert, we can envisage co-operation and competition between members of the oral consortium for the production and use of free amino acids.

REFERENCES

- Andersson, C., Linder, L. E., Sund, M. & Lönnies, H. (1992). Purification and characterization of an aminopeptidase from *Streptococcus mitis* ATCC 903. *Curr Microbiol* **25**, 261–267.
- Ando, K. (1980). Collagenase, dipeptidylpeptidase IV and cathepsin D activities in gingival fluid and whole saliva from patients with periodontal disease. *Jap J Periodont* **22**, 387–402.
- Ben-Basset, A., Bauer, K., Sheng-Yung, C., Myambo, C., Boosman, A. & Chang, S. (1987). Processing of the initiation methionine from proteins: properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. *J Bacteriol* **169**, 751–757.
- Bickel, M. & Cimasoni, G. (1986). Recent advances in gingival crevicular fluid research. In *The Borderland Between Caries and*

Periodontal Disease III. Edited by T. Lehner & G. Cimisoni. Geneva: Médecine et Hygiène.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Cueva, R., García-Alvarez, N. & Suárez-Rendueles, P. (1989). Yeast vacuolar aminopeptidase yscI. Isolation and regulation of the *APE1* (*LAP4*) structural gene. *FEBS Lett* **259**, 125–129.

D'Amato, R. F., Eriquez, L. A., Tomfohrde, K. M. & Singerman, E. (1978). Rapid identification of *Neisseria gonorrhoeae* and *Neisseria meningitidis* by using enzymatic profiles. *J Clin Microbiol* **7**, 77–81.

Fricke, B. & Aurich, H. (1993). Periplasmic aminopeptidases in *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa*. *J Basic Microbiol* **33**, 291–299.

Green, S. S., Goldberg, H. S. & Blenden, D. C. (1967). Enzyme patterns in the study of *Leptospira*. *Appl Microbiol* **15**, 1104–1113.

Greenman, J., Holland, K. T. & Cunliffe, W. J. (1981). Effects of glucose concentration on biomass, maximum specific growth rate and extracellular enzyme production by three species of cutaneous *Propionibacteria* grown in continuous culture. *J Gen Microbiol* **127**, 371–376.

Holdeman, L. V., Moore, W. E., Cato, E. P., Burmeister, J. A., Palcanis, K. G. & Ranney, R. R. (1985). Distribution of *Capnocytophaga* in periodontal microfloras. *J Periodont Res* **20**, 475–483.

Holland, K. T., Greenman, J. & Cunliffe, W. J. (1979). Growth of *Propionibacteria* on synthetic medium: growth yields and exoenzyme production. *J Appl Bact* **47**, 383–394.

Hopsu-Havu, V. K., Mäkinen, K. K. & Glenner, G. G. (1966). Purification of a mammalian peptidase selective for N-terminal arginine and lysine residues: aminopeptidase B. *Arch Biochem Biophys* **114**, 557–566.

Ingham, E., Holland, K. T., Gowland, G. & Cunliffe, W. J. (1983). Studies of the extracellular proteolytic activity produced by *Propionibacterium acnes*. *J Appl Bact* **54**, 263–271.

Irving, J. T., Socransky, S. S. & Tanner, A. C. R. (1978). Histological changes in experimental periodontal disease in rats mono-infected with Gram-negative organisms. *J Periodont Res* **13**, 326–332.

Kumano, N. & Sugawara, S. (1992). Ubenimex (Bestatin), an aminopeptidase inhibitor, modulates protein kinase C in K562 cells. *J Biol Regulat Homeostat Agents* **6**, 116–120.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Leadbetter, E. R., Holt, S. C. & Socransky, S. S. (1979). *Capnocytophaga*: new genus of Gram-negative gliding bacteria. I. General characteristics, taxonomic considerations and significance. *Arch Microbiol* **122**, 9–16.

Mäkinen, K. K., Syed, S. A., Loesche, W. J. & Mäkinen, P.-L. (1988). Proteolytic profile of *Treponema vincentii* ATCC 35580 with special reference to collagenolytic and arginine aminopeptidase activity. *Oral Microbiol Immunol* **3**, 121–128.

Marsh, P. D., Hunter, J. R., Bowden, G. H., Hamilton, I. R., McKee, A. S., Hardie, J. M. & Ellwood, D. C. (1983). The influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. *J Gen Microbiol* **129**, 755–770.

Mashimo, P. A., Yamamoto, Y., Slots, J., Park, B. H. & Genco, R. J. (1983). The periodontal microflora of juvenile diabetics: culture, immunofluorescence and serum antibody studies. *J Periodontol* **54**, 420–430.

Minhas, T. & Greenman, J. (1989). Production of cell-bound and vesicle-associated trypsin-like protease, alkaline phosphatase and N-acetyl- β -glucosaminidase by *Bacteroides gingivalis* strain W50. *J Gen Microbiol* **135**, 557–564.

Nakamura, M. & Slots, J. (1982). Aminopeptidase activity of *Capnocytophaga*. *J Periodont Res* **17**, 597–603.

Newman, M. G., Socransky, S. S., Savitt, E. D. & Crawford, A. (1976). Studies in the microbiology of periodontitis. *J Periodontol* **47**, 373–379.

Shibata, Y., Fujimura, S. & Nakamura, T. (1992). Isolation and characterization of enzymes hydrolyzing chymotrypsin synthetic substrate (enzyme 1) and trypsin synthetic substrate (enzyme 2) from the envelope fraction of *Capnocytophaga gingivalis*. *Med Microbiol Immunol* **181**, 107–115.

Slots, J. (1976). The predominant cultivable organisms in juvenile periodontitis. *Scand J Dent Res* **84**, 1–10.

Slots, J. (1982). Importance of black-pigmented *Bacteroides* in human periodontal disease. *Etiol Microb Spec* **2**, 27–45.

Spratt, D. A. (1994). *A study of aminopeptidase and other hydrolytic enzymes produced by Capnocytophaga gingivalis*. PhD Thesis, University of the West of England, Bristol, UK.

Tempest, D. W. (1970). The continuous cultivation of micro-organisms. I. Theory of the chemostat. *Methods Microbiol* **2**, 259–276.

Umezawa, H. (1982). Low molecular weight enzyme inhibitors of microbial origin. *Annu Rev Microbiol* **36**, 75–99.

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