BACTERIAL PATHOGENICITY

Formation of a dipeptidyl arylamidase by Bacteroides splanchnicus NCTC 10825 with specificities towards glycylprolyl-x and valylalanine-x substrates

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Bacteroides splanchnicus in common with several members of the B. fragilis group constitutively produced a number of protein and peptide hydrolysing enzymes. Amongst the most active was an arylamidase, which specifically hydrolysed the dipeptidyl chromogenic substrates glycylprolyl p-nitroanilide (GPRPNA), glycylprolyl β-naphthylamide (GPβNA) and valylalanine p-nitroanilide (VAPNA), and had some proteolytic activity towards azocasein. No activity was detected against proline β-naphthylamide, glycine, valanline or alanine p-nitroanilides. Physiological studies showed that the enzyme was largely cell-associated during exponential growth in batch culture, but was progressively released by the bacteria before the cells entered stationary phase. Glycylprolyl arylamidase (GPA) was completely cell-bound during growth in continuous culture, where synthesis increased concomitantly with dilution rate (specific growth rate) in both carbon- and nitrogen-limited chemostats. Gel-filtration chromatography of B. splanchnicus cell extracts yielded a single peak of GPA activity, with an apparent molecular mass of c. 160 kDa, while one peak of enzyme activity was eluted by 0.3 M NaCl during cation-exchange chromatography. Activity staining of SDS polyacrylamide gels showed a single GPA band at 80 kDa, suggesting that the enzyme was a dimer. Two fractions of GPA activity were recorded during preparative isoelectric focusing with apparent isoelectric points of pH 3.51 (fraction 3) and 3.95 (fraction 6), indicating the possible existence of GPA isoenzymes. GPRPNA, VAPNA and azocasein were hydrolysed by the major fraction (fraction 3), while only the p-nitroanilide substrates were hydrolysed by fraction 6. Studies with the partially purified enzyme obtained from gel filtration columns showed a relatively broad pH optimum at 7.5–8.2. Inhibition experiments demonstrated that while aspartic (pepstatin A), thiol (iodoacetate) and metalloprotease (EDTA, cysteine) inhibitors had little effect on hydrolysis of glycylproline p-nitroanilide, GPA was strongly inhibited (c. 80%) by 5 mM phenylmethylsulphonyl fluoride (PMSF), indicating it to be a serine enzyme.

Introduction

Bacteroides splanchnicus is an anaerobic gram-negative rod and is a common inhabitant of the human large intestine, occurring in numbers in excess of 10^{10}/g dry weight of gut contents, in some individuals [1, 2]. The bacterium is not a member of the B. fragilis group (genus Bacteroides sensu stricto), which it resembles metabolically, and is differentiated from these organisms on the basis of its inability to grow on sucrose and certain other disaccharides [3]. Previous studies have shown that B. splanchnicus, together with some members of the B. fragilis group, is strongly proteolytic [4, 5], and is nutritionally versatile with respect to its nitrogen requirements, being able to utilise a variety of inorganic and organic N-containing compounds for growth, including pancreatic endopeptidases such as trypsin and chymotrypsin [6].

At present, little is known of the factors that control proteolysis in the large intestine, or the ecological significance of proteolysis. The ability of various bacteroides to scavenge pancreatic and other host secretions may confer a competitive advantage on these organisms during growth in the large bowel [6]. The synthesis and activities of peptidolytic enzymes by human colonic bacteroides is of interest both from

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the viewpoint of the turnover of organic N-containing compounds in the large bowel [7–9] and also because of their potential role in pathogenicity [10, 11]. For example, in bacterial overgrowth syndromes, peptidolytic enzymes produced by colonic bacteroides have been reported to destroy brush border glycosidases [12].

Previous work has shown that B. splanchnicus NCTC 10825 forms several peptide hydrolases of the thiol, serine, and possibly, the metalloprotease type, and that these enzymes exhibited arylamidase activity against chromogenic substrates such as valylalanine p-nitroanilide (VAPNA) and glycylprolyl p-nitroanilide (GPRPNA) [6]. The objectives of this study were to investigate the physiological factors affecting formation of dipeptidyl arylamidases in this organism, with a view to understanding how their synthesis might be regulated in the large intestine, and to study certain of the biochemical attributes of the enzymes.

Materials and methods

Organisms

B. splanchnicus NCTC 10825, B. vulgatus NCTC 11154, B. fragilis NCTC 10584, B. thetaiotaomicron NCTC 10582 and B. ovatus NCTC 11153 were obtained from the National Collection of Type Cultures, Public Health Laboratory Service, 61 Collindale Avenue, London. B. fragilis NCFB 2217 was purchased from the National Collection of Food Bacteria, Reading.

Arylamidase and β-naphthylamidase activities in Bacteroides spp.

Bacteria were grown anaerobically in Wilkins-Chalgren broth. Mid-exponential phase cells were pelleted by centrifugation (20,000 g, 30 min, 4°C) and were washed and resuspended in anaerobic sodium phosphate buffer (0.1 M, pH 7.4). The abilities of washed bacteria to hydrolyse a range of p-nitroanilide and β-naphthylamide substrates (see Table 1) was investigated by methods described by Gibson and Macfarlane [13].

Growth and arylamidase formation by B. splanchnicus in batch culture

B. splanchnicus was grown in 280-ml (working volume) glass fermentation vessels at 37°C. Cultures were stirred magnetically and pH was automatically controlled at 6.5 by procedures described previously [14]. Anaerobic conditions were maintained by sparging the cultures with oxygen-free CO2 (2.4 L/h). The culture medium consisted of (g/L): glucose, 10.0; peptone water, 0.25; NH4Cl, 0.5; NaCl, 4.5; KH2PO4, 4.0; MgCl2.6H2O, 0.10; CaCl2.2H2O, 0.10; CoCl2.6H2O, 0.10; MnCl2.4H2O, 0.10; cysteine, 0.80; NaHCO3, 1.5; haemin, 0.01; vitamin B12, 0.005; FeSO4.7H2O, 0.001; resazurin, 0.001. The culture medium was autoclaved (121°C, 15 min) and cooled under a stream of oxygen-free CO2 before use. Bacterial growth was monitored by following culture absorbance changes at 650 nm.

Samples were taken periodically from the fermenters for measurements of glycylprolyl arylamidase (GPA) activities during the growth cycle. Bacteria were harvested as above. Cell-free culture supernates were also retained. Crude cell extracts were prepared by disrupting the bacteria during two passages through a French pressure cell (1.1 × 106 kPa). The efficiency of cell destruction was confirmed by microscopy.

GPA activities associated with whole cells, in culture supernates and bacterial cell extracts were determined as before. All activities were corrected for different sample dilutions to allow quantitative comparisons to be made between cell-associated and extracellular enzyme activities.

Growth in continuous culture

B. splanchnicus was grown in 500-ml (working volume) chemostats. Temperature, pH and anaerobic conditions were regulated in the same way as in the batch culture experiments described earlier. The culture medium was that used in the batch culture studies; however, glucose and NH4Cl concentrations in nitrogen- and carbon-limited growth media were 10.0 and 0.5 g/L, and 5.0 and 1.0 g/L, respectively. Dilution rates ranged between 0.025 and 0.29/h. Cultures were allowed to equilibrate for at least eight complete volume changes in the chemostats, at each dilution rate, before sampling to ensure that steady state conditions had been achieved. Different dilution rates were applied to the chemostats at random. Samples were taken at different specific growth rates for measurements of cell-associated and extracellular arylamidase activities (GPRPNA substrate), as outlined above.

Inhibitor experiments

The effects of various natural and synthetic protease inhibitors (see Table 2) on arylamidase (GPRPNA and VAPNA substrates) and general protease (azocasein substrate) activities were investigated with crude cell extracts from exponential phase B. splanchnicus, employing procedures described in earlier studies [15].

Isoelectric focusing studies

Preparative isoelectric focusing of B. splanchnicus GPA was performed with the Rotofor system (BioRad). Bacterial cell extracts (20 ml) were dialysed under anaerobic conditions for 12 h at 4°C against distilled water (5 L) that had been boiled and cooled under oxygen-free nitrogen. They were subsequently diluted
to 60 ml with distilled water and ampholytes (BioRad), pH 2.0–6.5, were added to a concentration of 0.5% v/v. Samples were run on the Rotofor cell at 2°C for between 3 and 4 h at a constant power setting of 12 W. Initial and final currents ranged between 24–26 and 13–15 mA. Initial and final voltages were 420–460 and 870–940 V, respectively. The electrolytes were 0.075 M H3PO4 (anode) and 0.075 M NaOH (cathode). After harvesting, the pH in each sample tube was recorded immediately, before arylamidase (GPRPNA and VAPNA substrates) and protease (azocasein substrate) measurements were made.

Gel-filtration chromatography

GPA in cell extracts of B. splanchnicus was separated by HPLC, with an 0.8 × 30 cm TSK 3000 SW Ultrapac column (LKB). The samples were isocratically eluted with 0.1 M sodium phosphate buffer containing 0.1 M NaCl and 1 mM mercaptoethanol, at a flow rate of 0.2 ml/min. Column effluent was monitored for protein at 208 nm. Fractions (0.2 ml) were collected and assayed for activity against GPRPNA. The system was calibrated for estimations of mol. wt with a range of protein standards obtained from BioRad: amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and lysozyme (14.5 kDa).

Cation-exchange chromatography

Samples from the gel-filtration column were loaded on to a 20-cm glass column containing CM 52 (Whatman). Samples were eluted with 0.05 M sodium acetate buffer (pH 4.5), with a linearly increasing concentration of NaCl (0.1–0.4 M) at a flow rate of 80 ml/h. Protein was detected in the eluant with an UV monitor at 208 nm. Arylamidase activity against GPRPNA and VAPNA was detected as before.

SDS-PAGE

This was carried out in the presence of SDS 0.1% w/v, by procedures described by Laemmli [16]. A polyacrylamide 4% stacking gel and separating 7.5% gel were used. After running, the gels were reanimated by incubation in 25 mM Tris buffer (pH 8.0) containing Lubrol 0.5% w/v and Triton X100 0.5% v/v for 2 h at room temperature. The buffer was discarded and replaced, and the gels left for a further 14 h at 4°C. The gels were then incubated in the Tris buffer with Lubrol 0.05% w/v for 1 h. This was followed by another incubation (1 h) with 0.1 M Tris buffer (pH 6.5). The gels were subsequently cut into two pieces: one half was conventionally stained for protein with Coomassie Brilliant Blue R 0.2%, the other was stained for arylamidase activity by flooding with a solution of GPRPNA (5 mM) in the Tris buffer. The gels were wrapped in clear plastic film and incubated for 4–6 h at 37°C. Enzyme activity was visualised by the appearance of faint yellow bands, resulting from release of p-nitroaniline. The following mol. wt markers (Sigma) were used: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (445 kDa) and carbonic anhydrase (29 kDa).

Effects of pH and PMSF on GPA

These studies were done with partially purified enzyme preparations obtained from gel-filtration chromatography samples. In the pH experiments, samples were pre-incubated at 37°C for 15 min in 0.1 M Tris buffer at a range of pH values from 6.0 to 10.0, before addition of GPRPNA substrate. Measurements of enzyme activity were made after incubation for 2 h at 37°C as outlined previously.

To confirm that GPA was sensitive to PMSF, samples from the gel-filtration column were pre-incubated with various concentrations of the inhibitor (1–5 mM) for 15 min at 37°C before adding GPRPNA substrate. Arylamidase assays were then done as before.

Protein measurements

Protein concentrations in bacterial cell extracts were estimated by the Lowry method, with bovine serum albumin as standard.

Determinations of culture dry weights

These were made as described by Degnan and Macfarlane [17].

Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma. Bacteriological culture media were purchased from Oxoid.

Results

Arylamidase activities in human colonic bacteroides

Data in Table 1 show arylamidase and β-naphthylamidase activities of proteolytic enzymes formed by several human intestinal Bacteroides isolates. Notably, all species examined, with the exception of B. thetaotaomicron, exhibited high levels of hydrolytic activity against the dipetidyl substrates GPRPNA and VAPNA, while B. splanchnicus also hydrolysed glycylprolyl β-naphthylamide (GPβNA). No significant activity was detected when the dipetidyl moieties were substituted with individual amino acids, or when glycine p-nitroanilide, alanine p-nitroanilide, valine p-nitroanilide and prolyl β-naphthylamide were used as substrates, although leucine p-nitroanilide was hydrolysed to some extent by all species. No activity

B. SPLANCHNICUS GLYCYPROLYL ARYLAMIDASE 549
Table 1. Arylamidase activities of proteolytic enzymes formed by human intestinal bacteroides

<table>
<thead>
<tr>
<th>Strain</th>
<th>LPNA</th>
<th>VAPNA</th>
<th>GPRPNA</th>
<th>BAPNA</th>
<th>GPPNA</th>
<th>SA3PNA</th>
<th>GPNA</th>
<th>APNA</th>
<th>VPNA</th>
<th>GPmA</th>
<th>PpNA</th>
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<tr>
<td>B. fragilis NCFB 2217</td>
<td>22</td>
<td>368</td>
<td>291</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>B. fragilis NCTC 10584</td>
<td>10</td>
<td>140</td>
<td>125</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>B. vulgatus NCTC 11154</td>
<td>2</td>
<td>142</td>
<td>131</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>B. ovatus NCTC 11153</td>
<td>1</td>
<td>141</td>
<td>146</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>B. thetaiotaomicron NCTC 10582</td>
<td>1</td>
<td>48</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>B. splanchnicus NCTC 10825</td>
<td>17</td>
<td>296</td>
<td>281</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>276</td>
<td>-</td>
</tr>
</tbody>
</table>

LPNA, leucine p-nitroanilide; VAPNA, valylalanine p-nitroanilide; GPRPNA, glycylprolyl p-nitroanilide; BAPNA, benzoylarginine p-nitroanilide; GPPNA, glutarylphenylalanine p-nitroanilide; SA3PNA, succinylalanylalanylalanine p-nitroanilide; GPNA, glycyl p-nitroanilide; APNA, alanine p-nitroanilide; VPNA, valine p-nitroanilide; GPmA, glycylprolyl β-naphthylamide; PpNA, prolyl β-naphthylamide; -, no activity detected; NT, not tested; results are means of two determinations.

was observed against benzoylarginine p-nitroanilide, glutarylphenylalanine p-nitroanilide or succinylalanyllalanyllalanine p-nitroanilide.

**Batch culture studies on production of glycylprolyl arylamidase in B. splanchnicus**

Studies on glycylprolyl arylamidase (GPA) formation in batch cultures of *B. splanchnicus* demonstrated that high levels of the enzyme accumulated intracellularly during exponential growth (Fig. 1), while lower activities were detected with whole cells. However, towards the end of exponential growth, considerable amounts of GPA were released into the culture medium. Enzyme activities associated with whole bacteria changed little as intracellular enzymes were released.

**Expression of B. splanchnicus GPA in continuous culture**

In chemostat culture, higher levels of GPA synthesis were observed in nitrogen-limited vessels at low specific growth rates (μ), but enzyme formation was growth rate-associated in both carbon- and nitrogen-limited bacteria, and increased concomitantly with dilution rate (Fig. 2). Extracellular GPA activity was not detected in the chemostats, except at very low dilution rates (D = < 0.03 h) where trace levels were found.

**Inhibitor studies**

Studies with a variety of protease inhibitors (Table 2) showed that hydrolysis of GPRPNA and VAPNA was strongly inhibited by PMSF and to a lesser degree by thimerosal. No effects were observed with aspartic or metalloprotease inhibitors. General protease activity, as indicated by azocasein hydrolysis, was also inhibited to a notable degree by PMSF; however, thimerosal was the most effective protease inhibitor, whilst significant reductions in proteolysis were also seen with iodoacetate, EDTA and cysteine.

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**Fig. 1.** Glycylprolyl arylamidase activities in batch cultures of *B. splanchnicus*; A_{650} (■), enzyme activity associated with whole cells (■), enzyme activity in bacterial cell extracts (○), extracellular enzyme activity (●). Results are mean values obtained from two separate experiments.

**Fig. 2.** Glycylprolyl arylamidase production in carbon- (●) and nitrogen-(○) limited *B. splanchnicus* at different specific growth rates (μ) in continuous culture. Results are means of between two-to-four measurements and SD.
Table 2. Effect of inhibitors on proteolytic and arylamidase activities of *B. splanchnicus*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor concentration</th>
<th>GPRPNA</th>
<th>VAPNA</th>
<th>Azocasein</th>
<th>Protease inhibited</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>100 µg/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Trpysin-like Hazlewood and Edwards [18]</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>250 µg/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Chymotrypsin-like Umezawa and Aoyagi [19]</td>
</tr>
<tr>
<td>PMSF</td>
<td>5 mM</td>
<td>76.0 ± 5.8</td>
<td>78.1 ± 4.3</td>
<td>49.3 ± 4.0</td>
<td>Serine proteases</td>
<td>Barrett [20]</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>50 µg/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Aspartic proteases Umezawa and Aoyagi [19]</td>
</tr>
<tr>
<td>Elastin</td>
<td>8 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Elastase Umezawa and Aoyagi [19]</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
<td>-</td>
<td>-</td>
<td>18.3</td>
<td>Metalloproteases Matsubara and Feder [21]</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>2 mM</td>
<td>-</td>
<td>-</td>
<td>14.7</td>
<td>Metalloproteases Sieffer and Harper [22]</td>
<td></td>
</tr>
<tr>
<td>Thimerosal</td>
<td>5 mM</td>
<td>52.1 ± 5.1</td>
<td>63.7 ± 2.9</td>
<td>70.7 ± 6.2</td>
<td>Thiol proteases</td>
<td>Webb [23]</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>5 mM</td>
<td>7.4 ± 1.8</td>
<td>-</td>
<td>26.3 ± 3.5</td>
<td>Thiol proteases</td>
<td>Barrett [20]</td>
</tr>
</tbody>
</table>

Organisms were harvested from batch cultures during the exponential phase of growth. Measurements of protease and arylamidase activities were made with cell extracts, as described in Materials and methods. Results are means of three determinations ± SD. GPRPNA, glycylproline p-nitroanilide; VAPNA, valylalanine p-nitroanilide; PMSF, phenylmethylsulphonyl fluoride; −, no inhibition.

Protein purification experiments

In experiments aimed at further characterising *B. splanchnicus* GPA, preparative isoelectric focusing of cell extracts showed a major peak of enzyme activity in tube 3, corresponding to a pH of 3.51, for both GPRPNA and VAPNA hydrolysis (Fig. 3). A minor peak of GPRPNA, and VAPNA activity also occurred in tube 6 (pH 3.95). Azocasein was hydrolysed by tube 3 only.

Gel filtration of *B. splanchnicus* cell extracts by HPLC gave only one peak of activity against both GPRPNA and VAPNA, with an approximate mol. wt of 160 kDa (Fig. 4), while cation-exchange chromatography yielded one peak of activity against GPRPNA, which was eluted from the column by 0.3 M NaCl (Fig. 5). This was also the only peak of VAPNA hydrolysis (data not shown). SDS-PAGE (Fig. 6) resulted in one band of GPA activity on the gels, with an estimated molecular mass of c. 80 kDa.

Effects of PMSF and pH on partially purified GPA

Inhibitor studies with partially purified enzyme preparations obtained from gel filtration chromatography columns demonstrated that PMSF was inhibitory to GPA in a concentration-dependent way, with 3–5 mM inhibitor reducing enzyme activity by c. 80% (Fig. 7). Measurements to determine the pH optimum of GPRPNA hydrolysis with these enzyme preparations (Fig. 8) showed a broad band of GPA activity in the neutral to slightly alkaline pH range (7.5–8.2).

Discussion

Bacterial arylamidases catalyse hydrolysis of N-terminal amino acid residues of p-nitroanilide and p-naphthylamide substrates. A number of arylamidase activities were found to be associated with *B. splanchnicus*, which hydrolysed four of 11 p-nitroanilide and p-naphthylamide test substrates (Table 1). A similar pattern of hydrolysis was also observed in several members of the *B. fragilis* group. The inability of these organisms to hydrolyse BAPNA, GPPNA and SA3PNA demonstrated that peptidolytic enzymes formed by human colonic bacteroides do not manifest trypsin-, chymotrypsin- or elastase-like properties.

Various factors are known to be involved in regulating peptidolytic activities in micro-organisms, including end-product inhibition of enzyme activities, induction of enzyme synthesis and catabolite repression of enzyme synthesis [24]. Although GPA formation was constitutive in *B. splanchnicus*, the process was markedly influenced by nutrient availability and cultural conditions. Batch culture experiments showed...
Fig. 4. Gel-filtration chromatography of cell extracts; GPRPNA hydrolysis (●), VAPNA hydrolysis (○), protein absorbance at 208 nm shown by the solid line. Mol. wt standards (kDa): A, amylase (200); B, alcohol dehydrogenase (150); C, bovine serum albumin (66); D, ovalbumin (45). Lysozyme (14.5), which is not shown, was also used to construct the standard curve.

Fig. 5. Cation-exchange chromatography of glycylylprolyl arylamidase; enzyme activity (●), NaCl gradient (○), the solid line shows protein absorbance at 208 nm. Data shown are representative results from two experiments.
that large amounts of GPA accumulated within the bacteria during exponential growth, and that approximately 40% of intracellular arylamidase activity was released from the bacteria before cells entered stationary phase (Fig. 1).

Studies with B. fragilis have shown that protease secretion at the end of active growth may be a response to N-limitation, and that release of these enzymes is a discrete process that is not associated with cell lysis [25]. These observations are supported to some extent by continuous culture data obtained in this study, where GPA synthesis by B. splanchnicus was shown to occur optimally during N-limited growth (Fig. 2). The chemostats were used to control bacterial growth rates and regulate nutrient (glucose, ammonia) availability. Extracellular GPA activity was not detected in continuous cultures of B. splanchnicus, except for trace levels at the lowest dilution rates (D = < 0.03/h). This was probably a result of cell lysis or leakage, as the bacteria were growing under extremely nutrient-limiting culture conditions. The observation that GPA activity was completely cell-bound in the chemostats indicates that release of the enzyme may have required a specific signal that was not present in very rapidly growing bacteria. As would be expected with a constitutive enzyme, GPA synthesis was growth rate-associated over the range of dilution rates examined (D = 0.025–0.29/h), which correspond to cell doubling times of 27.7 and 2.4/h, respectively.

Inhibition experiments indicated that B. splanchnicus NCTC 10825 constitutively produced a number of peptidolytic enzymes of the thiol, serine, and possibly, the metalloprotease type (Table 2). These enzymes exhibited arylamidase activity against a restricted number of chromogenic substrates, particularly VAPA and GPRPNA, together with β-naphthylamidase activity towards glycylprolyl β-naphthylamide, demonstrating the existence of a glycylprolyl arylamidase. Thus, B. splanchnicus GPA manifests similar substrate specificities to dipeptidyl peptidase IV described in Streptococcus mitis [26] and glycylprolyl dipeptidylaminopeptidase in Porphyromonas gingivalis [27]. No significant activity was detected with glycine p-nitroanilide, proline naphthylamide, alanine p-nitro-
Fig. 8. Effect of pH on hydrolysis of GPRPNA by partially purified (gel filtration) B. splanchnicus glycylprolyl arylamidase. Data are mean values from three experiments and SD.

anilide or valine p-nitroanilide, demonstrating a high degree of specificity towards C-terminus substituted dipeptidyl substrates. The absence of inhibitory effects by elastinial, soybean trypsin inhibitor and chymostatin confirmed that peptidolytic enzymes produced by B. splanchnicus, including GPA, did not manifest elastase, trypsin or chymotrypsin-like characteristics.

Preparative isoelectric focusing of cell extracts gave a major peak (fraction 3, pH 3.51) and a minor peak (fraction 6, pH 3.95) of activity for both GPRPNA and VAPNA hydrolysis. Azocasein was also hydrolysed by fraction 3, which together with the sensitivity of azocasein hydrolysis to PMSF (Table 2), indicated a degree of general protease activity by GPA.

Gel filtration of cell extracts by HPLC gave only one peak of activity against both GPRPNA and VAPNA, with an approximate molecular mass of 160 kDa. Activity staining of SDS-PAGE gels resulted in one band of activity with an estimated molecular mass of c. 80 kDa, indicating GPA to be a dimer. Activity against VAPNA was also found here. In cation-exchange chromatography to further investigate the enzyme, one peak of activity against GPRPNA was eluted from the column by 0.3 M NaCl. This sample also contained the major peak of VAPNA hydrolysis which, with the isoelectric focusing and HPLC data, provided good evidence for a single enzyme that was active against both substrates.

Inhibitor studies with partially purified enzyme preparations from the gel-filtration column demonstrated strong inhibition by low concentrations of PMSF (Fig. 7). Measurements to determine the pH optimum of GPRPNA hydrolysis (Fig. 8) showed a broad band of activity in the slightly alkaline pH range (7.5–8.2) which, in conjunction with the inhibitor data, is consistent with B. splanchnicus GPA being a serine enzyme. Although GPRPNA hydrolysis was also inhibited by thimerosal (Table 2), the absence of inhibitory effects by iodoacetate would preclude it being a thiol protease. These results show that B. splanchnicus GPA shares a number of other characteristics with the glycylprolyl dipeptidylaminopeptidase produced by P. gingivalis, which is an extracellular serine enzyme that is sensitive to low concentrations of PMSF, and has a molecular mass of c. 160 kDa [27].

A large but variable proportion of proteolytic and peptidolytic activity in the human large intestine is of bacterial origin [15], and these cell-associated and extracellular enzymes, particularly those produced by Bacteroides spp., play an important role in the microecology of the bowel [6, 8]. The broad pH optimum
of *B. splanchnicus* GPA, 7.5–8.2 (Fig. 8), is similar to the pH optima for general proteolytic activities in the large gut [28]. However, as intestinal contents are usually acid to neutral in pH [29], these enzymes must function sub-maximally in the colon. Therefore, factors that control intestinal pH, such as short chain fatty acid production during carbohydrate fermentation, may be important in regulating proteolysis in the large intestine.

In conclusion, results presented here provide evidence for constitutive production of a glycylprolyl arylamidase by *B. splanchnicus*. In many respects, *B. splanchnicus* proteases appear to be very similar to those produced by members of the *B. fragilis* group [10, 13, 25]. The mechanism of release of intracellular GPA by this organism at the end of active growth is of particular interest, as the bacterium is a gram-negative species. Further work at the molecular and genetic level is needed to elucidate the mechanisms involved in GPA synthesis and secretion and, in particular, to identify the factors that signal arylamidase release.

References
