Biochemical properties of membrane-associated proteases of *Brachyspira pilosicoli* isolated from humans with intestinal disorders

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A membrane-associated, subtilisin-like, serine protease activity was found in both pathogenic and non-pathogenic strains of *Brachyspira* species in a previous study, but the biochemical properties of the enzyme were not investigated. The purpose of the present study was to characterize further the biochemical properties, including substrate specificity, of the membrane-associated protease of *Brachyspira pilosicoli* isolated from humans with intestinal disorders. Protease activity of detergent-enriched membrane protein extracts of *B. pilosicoli* was assessed using fluorescent dye-labelled synthetic peptides as substrates and determination of electrophoretic mobility of cleavage products in agarose gels. Each activity was further confirmed with class-specific protease inhibitors and thermal denaturation. The presence of a hydrophilic membrane-associated thermostable serine endopeptidase with specificity for Leu was confirmed. Two additional hydrophilic membrane-associated thermostable proteolytic activities were identified, one with a putative Ala specificity, and one a carboxypeptidase. Taken together, these data suggest that, in addition to a previously described membrane-associated subtilisin-like serine protease, the membrane of *B. pilosicoli* contains proteins with at least two other proteolytic activities.

**INTRODUCTION**

*Brachyspira pilosicoli* has been implicated as a cause of colonic spirochaetosis, an inflammatory bowel disease that affects a broad range of hosts, including humans (Duhamel, 2001; Trott et al., 1997). Infection with *B. pilosicoli* is characterized by colonization of the large intestine followed by polar attachment of the spirochaetes along the apical membrane of the colonic epithelium (Duhamel, 2001). Damage to epithelial cells and penetration of the epithelial layer have also been seen, but the mechanism(s) of invasion has not been investigated. Demonstration of a subtilisin-like serine protease activity in detergent-enriched membrane fractions of pathogenic and non-pathogenic *Brachyspira* species suggested that the enzyme might be important for survival in the intestinal environment (Muniappa & Duhamel, 1997). Alternatively, the membrane-associated serine protease might indirectly contribute to damage caused by pathogenic spirochaetes after intimate association with the colonic epithelial surface.

Proteases have been found in spirochaetes and a role in damage to host cells and the mucosal barrier has been proposed (Ellen et al., 2000; Grenier et al., 1990). Among spirochaetes, trypsin- and chymotrypsin-like proteases of *Treponema denticola*, an oral spirochaete associated with periodontitis, have been characterized (Ellen et al., 2000; Grenier et al., 1990). A role for *T. denticola* proteases in periodontal tissue invasion by degradation of host cell proteins and inactivation of bioactive peptides has been suggested (Grenier et al., 1990; Reijntjens et al., 1986). The purpose of the present study was to characterize the biochemical properties of the membrane-associated protease activity present in human strains of *B. pilosicoli*. The substrate specificities of the proteases were identified using synthetic peptides representative of all predicted target amino acid residues designed on the basis of cleavage patterns obtained in a previous study (Muniappa & Duhamel, 1997).

**METHODS**

**Bacterial strains and growth conditions.** The human *B. pilosicoli* isolates SP16 (ATCC 49776) and SP13 (Jones et al., 1986; Ramanathan et al., 1993; Lee & Hampson, 1994; Duhamel et al., 1995; Fisher et al., 1997) and HRM-5B and HRM-7 (Coene et al., 1989; Lee & Hampson, 1994; Fisher et al., 1997), respectively isolated from human beings with intestinal disorders in the United States and Italy, were investigated in...
the present study. The reference porcine B. pilosicoli strain P43/6/78\(^T\) (ATCC 51139\(^T\)) was also included in some experiments (Trott et al., 1996). Each strain was propagated in pre-reduced anaerobically sterilized Trypticase soy broth supplemented with 2 % (v/v) fetal bovine serum. Cultures were grown to late exponential phase in 5 ml Hungate tubes with constant stirring at 37 °C under an 80 % nitrogen, 10 % hydrogen and 10 % carbon dioxide atmosphere (Ramanathan et al., 1993).

Membrane protein extraction. Membrane-associated proteins of spirochaetes were extracted using the non-ionic detergents n-octyl \(\beta\)-D-glucopyranoside (ODG; Sigma), as described by Comstock et al. (1993) with modifications, and 1 % (v/v) Triton X-114 (Sigma), as described by Bordier (1981) with modifications (Brusca & Radolf, 1993) with modifications, and 1 % (v/v) Triton X-114 (Sigma), as described by Bordier (1981) with modifications (Brusca & Radolf, 1994). Spirochaete cells were harvested by centrifugation at 800 g for 10 min, washed in Tris buffer pH 7.2 (50 mM Tris-base, 27 mM KCl, 145 mM KCl, 145 mM NaCl; Sigma) and approximately 10\(^{10}\) spirochaetes in 968 μl Tris buffer were incubated with 32 μl of 25 % (w/v) ODG stock solution (8 mg final concentration) at room temperature for 15 min. The supernatant containing ODG-enriched membrane proteins was separated from protoplasmic cylinders by centrifugation at 10 000 g for 5 min at 4 °C. Both Triton X-114-aqueous and detergent phases were washed in Tris buffer and examined for the presence of protease activity.

Peptide synthesis. Synthetic peptides were assembled manually using fmoc-amino acid-pentafluorophenyl esters as described previously (Sigal & Wylie, 1996). All chemicals for peptide synthesis were from a commercial supplier (Applied Biosystems), unless indicated. Briefly, approximately 10 μmol equivalent of peptides were synthesized as peptide amides. Amino acids were added (8-fold excess) as their respective fmoc-aminoacyl-pentafluorophenyl esters dissolved in 0.1 M 1-hydroxybenzotriazole in dimethylformamide. After coupling of the final amino acid, the free peptide N terminus was coupled for 12 h to 5-(and-6)-carboxyfluorescein using 5-(and-6)-carboxyfluorescein succinimidyl ester (4-fold excess, Molecular Probes), dissolved in dimethylformamide containing 0.1 M diisopropylethylamine. The resin was washed extensively with dimethylformamide, followed by dichloromethane, dried and cleaved using trifluoroacetic acid/water/triisopropylsilane/thioanisole (94:2:2:2, by vol.). Crude peptide was precipitated into cold methyl t-butyl ether, collected by centrifugation at 500 g for 10 min, and lyophilized twice from water. Peptides were used directly or after purification by reversed phase chromatography.

Protease assay. The proteolytic activities of detergent-enriched membrane protein extracts of spirochaetes were assessed using fluorescent dye-labelled synthetic peptides as substrates and the electrophoretic mobility of cleavage products with altered size and charge in agarose gels. Assay mixtures containing 1 μl synthetic peptide (~1 mg ml\(^{-1}\)), 8 μl 0-25 M Tris buffer, pH 7.2 (Sigma) and 21 μl of either detergent-enriched membrane proteins, 0-2 μg sequencing grade trypsin (Promega) or 2 μg alkaline protease (Promega) were incubated for 3 h at 37 °C. A 10 μl aliquot of the reaction mixture was mixed with an equal volume of glycerol (Sigma) and subjected to electrophoresis in 1 % (v/v) agarose gels (FMC Bio Products) prepared with Tris-boric acid buffer, pH 8.5 at 7 V cm\(^{-1}\) for 45–60 min. Peptide cleavage products were visualized and photographed under UV illumination.

The optimal pH for protease activity was determined by incubating ODG-enriched membrane proteins of strain SP16 with peptide N (Table 1) dissolved in Tris buffer or HEPES buffer adjusted to either pH 5.0, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0 or 9.0 for 180 min at 37 °C. In subsequent experiments, the optimal time required for complete digestion of peptide N dissolved in Tris buffer, pH 7.2 at 37 °C from 15, 30, 45, 60, 90, 120, 150 and 180 min was examined. Because the maximum cleavage of peptide was observed when samples were incubated for 180 min, this incubation time was used in all subsequent experiments.

Inhibition of B. pilosicoli membrane proteases. To better define the class of protease present in detergent-enriched membrane protein extracts of strain SP16, the chelating agent EDTA (Sigma), at final concentrations of 1, 5 and 25 mM, the cysteine protease inhibitor trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (Sigma) at final concentrations of 1, 10 and 100 μM and the serine protease inhibitor 3,4-dichloroisocoumarin (Sigma) at final concentrations of 0.01, 0.1, 0.5 and 1.0 mM (Powers & Harper, 1986) were added in a volume of 3 μl to 21 μl ODG-enriched membrane proteins and 5 μl Tris buffer, pH 7.2. After incubation at 37 °C for 1 h, enzymic activities were determined by adding 1 μl peptide N followed by a further incubation for 3 h at 37 °C and gel electrophoresis.

Stability of B. pilosicoli membrane proteases. The thermal stability of ODG-enriched membrane proteases of strain SP16 was investigated by incubating extracts at either 37, 50, 75 or 95 °C for 10 min, prior to assay for protease activity. Similarly, the stability of the membrane proteases was determined at regular intervals after storage of ODG-enriched membrane proteins for a period of up to 12 days at 4 °C or 5 months at ~ 80 °C. The stability of ODG-enriched membrane proteases in 0.01, 0.1 and 1.0 % (v/v) of the non-ionic detergents Triton X-100 and Triton X-114 was also examined.

RESULTS AND DISCUSSION

Further analysis of cleavage products of two commercially available peptide substrates examined in a previous study (Muniappa & Duhamel, 1997), namely, dye-Leu-Arg-Arg-

Table 1. Amino acid sequences of synthetic peptides investigated in this study and their respective predicted sites of cleavage

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>dye-Leu-Phe-Lys-CONH(_2)</td>
<td>dye-Leu-COO(^-)</td>
</tr>
<tr>
<td>B</td>
<td>dye-Leu-Asp-Lys-CONH(_2)</td>
<td>dye-Leu-COO(^-)</td>
</tr>
<tr>
<td>C</td>
<td>dye-Leu-Ala-Lys-CONH(_2)</td>
<td>dye-Leu-COO(^-)</td>
</tr>
<tr>
<td>D</td>
<td>dye-Ala-Ala-Lys-CONH(_2)</td>
<td>dye-Ala-COO(^-)</td>
</tr>
<tr>
<td>N</td>
<td>dye-Ala(^1)-Lys(^8)-Glu(^1)-Ile(^1)-Ala(^3)-Trp(^8)-Leu(^7)-Val(^9)-Lys(^8)-Glu(^10)-Arg(^11)-CONH(_2)</td>
<td>dye-Ala-COO(^-) (N4)</td>
</tr>
<tr>
<td></td>
<td>dye-Ala-Lys-Glu-Ile-Ala-COO(^-) (N3)</td>
<td>dye-Ala-Lys-Glu-Ile-Ala-Trp-Leu-COO(^-) (N3)</td>
</tr>
</tbody>
</table>
Ala-Ser-Leu-Gly and dye-Pro-Leu-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys, suggested that the membrane of Brachyspira species contains protease activity with specificity for Leu and possibly Ala. To confirm the presence of proteolytic activity further and define the enzyme specificity, we examined the proteolytic cleavage of three newly synthesized dye-labelled peptides with the following amino acid sequences: (1) dye-Pro-Arg-Ala-Gln-Lys, (2) dye-Pro-Glu-Ala-Arg-Lys-Ala and (3) dye-Pro-Leu-Glu-Arg-Ala-Lys. Consistent with the predicted enzymic cleavage at the Leu residue, peptide 3 served as a substrate for the membrane-associated protease of human and the reference porcine B. pilosicoli strains. Peptides 1 and 2 were also cleaved, but the amounts of products were less than with peptide 3, suggesting that additional enzymic activities, perhaps at lower concentrations, were present in the membrane protein extracts.

To confirm the amino acid specificity of the membrane-associated protease further, four additional dye-labelled tripeptides were synthesized (Table 1). The presence of the non-polar aromatic amino acid Phe next to the Leu residue did not affect the enzymic cleavage of peptide A (Fig. 1). Similarly, enzymic cleavage of peptide B at the Leu residue was not affected by the presence of a negatively charged Asp residue (Fig. 1). Peptide B also confirmed the absence of a trypsin-like activity with specificity for Lys in the detergent-enriched membrane proteins of B. pilosicoli. Peptide C was designed to verify Ala specificity (Table 1). Cleavage at the Leu–Ala bond would release the dye-Leu residue, whereas cleavage at the Ala–Lys bond would release dye-Leu-Ala. Since both products have the same net charge of −2 (fluorescein dye has a charge of −1), although with slightly altered molecular masses, these potential products could not be resolved. Therefore, peptide D was designed to demonstrate the presence of a potential Ala-specific protease activity in the membrane protein extract. Cleavage at either the Ala–Ala or the Ala–Lys bond would suggest Ala-specific protease activity. Cleavage of peptide D by the membrane protein extract confirmed that Ala is a cleavage site (Fig. 1); however, the cleavage products could also have been attributable to carboxypeptidase-type activity. To differentiate between these possibilities, a larger dye-labelled peptide, designated peptide N, that contained multiple potential cleavage sites was synthesized (Table 1). On the basis of predicted cleavage sites and corresponding net charges, it was anticipated that peptide N would discriminate among the proteolytic activities when used in conjunction with class-specific protease inhibitors. To enhance electrophoretic separation of cleavage products, the locations of Leu and Ala residues were designed to produce a net charge of −2.

Incubation of peptide N with membrane protein extracts of human and the porcine reference B. pilosicoli strains revealed four cleavage products (Fig. 2a). Based on our earlier observations with the dye-labelled tripeptides A–D, it was concluded that the band exhibiting maximal mobility, designated N4, was the N-terminal dye-labelled Ala. Band N3 was consistent with peptide products of Leu cleavage at position 7 (Leu7), and possibly some peptide cleaved at Ala5, since both of these products had calculated net charges of −2 and should exhibit similar mobilities in our assay system (Table 1). Additionally, the two products at N3 were likely to serve as substrates for the enzyme that released the N-terminal dye-Ala product at N4. On the basis of gel mobility, the peptide products N1 and N2 appeared to have larger molecular masses than N3 with a more negative charge than the parent peptide N (Fig. 2a). The presence of these products

![Fig. 1. Proteolytic cleavage products of peptides A (lane 1), B (lane 3), C (lane 5) and D (lane 7) by B. pilosicoli strain SP16 membrane-associated protease separated by electrophoresis in a 1 % (w/v) agarose gel and photographed under UV illumination. Control intact peptides A, B, C and D are in lanes 2, 4, 6 and 8, respectively. In each lane, intact peptide remains closest to the loading well toward the anode (bottom), whereas cleavage products migrate toward the cathode (top).](http://jmm.sgmjournals.org)

![Fig. 2. (a) Control intact peptide N (lane 1) and cleavage pattern (lane 2) following incubation with B. pilosicoli strain SP16 membrane-associated protease and separation by agarose gel electrophoresis. (b) Control peptide N incubated with B. pilosicoli strain SP16 membrane-associated protease (lane 1), intact peptide N (lane 2) and peptide N digested with trypsin (lane 3). Intact peptide N and the resulting products N1, N2, N3 and N4 with membrane protease (a and b) and T with trypsin (b) are indicated on the right according to the order of migration from anode (bottom) to cathode (top).](http://jmm.sgmjournals.org)
may be attributable to deamidation of the C-terminal Arg and/or removal of residues close to the C terminus via the action of a carboxypeptidase-type activity.

Incubation of peptide N with the alkaline protease control generated two bands that corresponded to the four expected cleavage products, one product following cleavage at Ile\(^4\) migrating alone, and products following cleavage at Trp\(^6\), Leu\(^7\) and Val\(^8\) migrating closely together (data not shown). Furthermore, incubation of peptide N with trypsin generated the expected dye-Ala-Lys product with a net charge of -1 (Fig. 2b, lane 3), confirming that the \textit{B. pilosicoli} membrane proteins do not contain trypsin-like activity (Muniappa & Duhamel, 1997). Taken together, these observations suggested that the \textit{B. pilosicoli} membrane contains three types of proteolytic activities, a protease with specificity for Leu and possibly Ala, another with specificity for Ala, and a carboxypeptidase-type enzyme. In order to characterize each activity further, additional assays with class-specific protease inhibitors and thermal denaturation experiments were conducted.

The membrane-associated proteases of strain SP16 were not inhibited by the chelating agent EDTA. This observation is consistent with a previous report (Muniappa & Duhamel, 1997) indicating that the membrane-associated proteases of \textit{Brachyspira} species are not metalloenzymes and do not require divalent cations as co-factors for activation. Furthermore, a lack of inhibition by the cysteine-specific inhibitor, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, at any concentration, suggested the absence of cysteine proteases in the membrane of strain SP16. Absence of the N3 products after incubation of the membrane extract with increasing concentrations of the serine-specific inhibitor 3,4-dichloroisouccoumarin confirmed the presence of a serine endopeptidase activity with specificity for Leu (Table 2 and Fig. 3a). The lack of inhibition by this compound of N1, N2, and N4 cleavage product generation provided further evidence that the N3 product is predominantly the Leu\(^7\) fragment and not the Ala\(^8\), as suggested earlier. Furthermore, inhibition of the serine endopeptidase caused apparent accumulation of the other cleavage products N1, N2, and N4, suggesting that the N1 and N2 fragments are substrates for the Leu-endopeptidase, and thus are larger than the Leu\(^7\) fragment seen following cleavage at N3. The results confirmed the presence of a serine endopeptidase with Leu specificity and further suggested that additional protease activities were present in the membrane proteins of \textit{B. pilosicoli}. Because these other enzyme activities were not inhibited by the class-specific inhibitors examined in the present study, the catalytic classes of these proteases remain unknown.

In our preliminary experiments, samples incubated in HEPES buffer produced low yields of peptide N cleavage products, whereas cleavage of the same peptide was complete when detergent-enriched membrane extracts were incubated in Tris buffer at either pH 6.8 or 7.2, but not at any other pH examined. Because of the qualitative nature of band intensity estimation, it was not possible to correlate substrate preference and optimal pH; all four products N1, N2, N3 and N4 were equally intense. However, these observations further suggested that more than one protease activity, with different pH optima, were present in the membrane proteins of \textit{B. pilosicoli}.

Absence of the N3 products after incubation of peptide N with detergent-enriched membrane proteins of strain SP16 at 75 and 95 °C for 10 min (Fig. 3b) confirmed previous observations (Muniappa & Duhamel, 1997), indicating

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Concentration (mM) & Specificities/inhibition & \\
& N2 & N3 (Leu) & \\
\hline
0.01 & Complete & Partial & \\
0.1 & Partial & Complete & \\
0.5 & Partial & Complete & \\
1.0 & None & Complete & \\
\hline
\end{tabular}
\caption{Inhibition of \textit{B. pilosicoli} strain SP16 membrane-associated protease activity on synthetic peptide N by increasing concentrations of the serine-specific protease inhibitor 3,4-dichloroisouccoumarin.}
\end{table}

Note that the production of N1 and N4 ( Ala) products was not inhibited at any concentration.

\textbf{Fig. 3.} (a) Inhibition of \textit{B. pilosicoli} strain SP16 membrane-associated protease activity. Peptide N was incubated with 0.01 (lane 1), 0.1 (lane 2), 0.5 (lane 3) and 1.0 mM (lane 4) 3,4-dichloroisouccoumarin. Lane 5 shows control intact peptide N, lane 6 shows peptide N and membrane protease without inhibitor. (b) Inhibition of \textit{Brachyspira pilosicoli} strain SP16 membrane-associated protease activity by increasing temperature. Peptide N was incubated with membrane-associated protease at 37 (lane 1), 50 (lane 2), 75 (lane 3), and 95 °C (lane 4). Control intact peptide N (lane 5). Intact peptide N and the resulting products N1, N2, N3 and N4 are indicated on the right according to the order of migration from anode (bottom) to cathode (top). Note absence of N3 products with increasing concentrations of inhibitor and concurrent increase in apparent concentrations of N1, N2 and N4 products in (a).
thermal instability of the serine endopeptidase. Conversely, enhanced intensity of N1, N2 and N4 products at higher incubation temperatures suggested thermal stability of the Ala-specific (N4) protease and carboxypeptidase activities. These observations further supported the conclusion that two or more proteases are present in the membrane proteins of B. pilosicoli.

The ODG-enriched membrane proteases of strain SP16 were stable after storage for 12 days at 4°C and 5 months at −80°C. Proteolytic cleavage of peptide N by the membrane proteins of strain SP16 was also not affected by the presence of Triton X-100 or Triton X-114 at any of the concentrations examined, indicating that the proteases are stable in non-ionic detergents. Because integral membrane proteins are anchored to the hydrophobic core of the lipid bilayer by hydrophobic domains or by amphiphilic groups covalently linked to the protein, it is possible to separate them by phase partitioning in the non-ionic detergent Triton X-114 into hydrophobic and amphiphilic detergent phase and hydrophilic aqueous phase (Bordier, 1981; Brusca & Radolf, 1994). Protease activity was limited to the aqueous phase of the Triton X-114 extract, suggesting that the proteases of B. pilosicoli are hydrophilic or low-hydrophobic membrane-associated proteins rather than integral membrane proteins.

Identification of a serine endopeptidase with specificity for Leu, and at least two other protease activities in the membrane proteins of B. pilosicoli Triton X-114 extract, suggesting that the proteases of B. pilosicoli are hydrophilic or low-hydrophobic membrane-associated proteins rather than integral membrane proteins.

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