SepA, the 110 kDa protein secreted by *Shigella flexneri*: two-domain structure and proteolytic activity

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Shigellosis is characterized by a strong inflammatory response which is induced by bacteria invading the colonic mucosa. Characterization of a *sepA* mutant indicated that SepA, the major protein secreted by *Shigella flexneri* growing in laboratory media, might be involved in invasion and destruction of the host intestinal epithelium. The sequence of the first 500 residues of mature SepA (110 kDa) is homologous to that of the N-terminal region of IgA1 proteases. To investigate the potential proteolytic activity of SepA, the activity of the purified protein on a wide range of synthetic peptides was tested. SepA hydrolysed several of these substrates and the activity was inhibited by PMSF. Several peptides which were hydrolysed by SepA have been described as specific substrates for cathepsin G, a serine protease produced by polymorphonuclear leukocytes that was proposed to play a role in inflammation. However, unlike cathepsin G, SepA degraded neither fibronectin nor angiotensin I and had no effect on aggregation of human platelets. In addition, analysis of SepA hydrolysis by proteinase K suggested that the protein is composed of two domains of about 450 residues separated by a hinge region of 100 residues. The 47 kDa N-terminal domain was stable and endowed with proteolytic activity.

Keywords: *Shigella flexneri*, serine protease, cathepsin G, inflammation, protein domain

INTRODUCTION

Shigella spp. are Gram-negative bacteria responsible for shigellosis. This disease is characterized by a strong inflammatory response, which is induced by bacteria invading the colonic mucosa (LaBrec *et al.*, 1964). The acute inflammation appears to be responsible for the mucosal destruction observed upon infection of rabbit ligated ileal loops by wild-type *Shigella flexneri* (Perdomo *et al.*, 1994a). In addition, following initial uptake of *S. flexneri* by M cells (Wassef *et al.*, 1989; Sansonetti *et al.*, 1991), inflammation may also facilitate bacterial invasion of epithelial cells. Indeed, migration of polymorphonuclear neutrophils (PMN) through a cell monolayer was shown to promote entry of *S. flexneri* into epithelial cells of the monolayer (Perdomo *et al.*, 1994b) by allowing bacteria to reach their

basolateral pole where entry occurs (Mounier *et al.*, 1992).

Bacterial effectors of both entry and cell-to-cell spread are encoded by a 200 kb virulence plasmid (see Parsot & Sansonetti, 1995, for a review). Entry involves the secreted IpaA–D proteins (Ménard *et al.*, 1993, 1996) and their type III secretion apparatus, the activity of which is induced upon contact with target cells (Ménard *et al.*, 1994; Watarai *et al.*, 1995). The actin-based movement of intracellular bacteria, which is required for cell-to-cell spread, involves the outer membrane IcsA (VirG) protein (Lett *et al.*, 1989; Bernardini *et al.*, 1989). The virulence plasmid also encodes SepA, which is the major protein secreted by wild-type *S. flexneri* when it is grown in laboratory media (Benjelloun-Touimi *et al.*, 1995). SepA is synthesized as a 146 kDa precursor that is converted to a mature extracellular protein of 110 kDa by two specific cleavage events which remove the N-terminal signal peptide and a 30 kDa C-terminal segment. The secretion of SepA is achieved by a mechanism

Abbreviations: pNA, *p*-nitroaniline; PMN, polymorphonuclear neutrophils; Suc, succinyl.

similar to that first described for the *Neisseria gonorrhoeae* IgA protease (Pohlner *et al.*, 1987). Construction and phenotypic characterization of a *sepA* mutant suggested that SepA plays a role in epithelium invasion or destruction. A *sepA* mutant, which was not impaired in entry into cultured epithelial cells or in intercellular dissemination, exhibited an attenuated virulence in the rabbit ligated ileal loop model (Benjelloun-Touimi *et al.*, 1995).

SepA belongs to a family of proteins that includes IgA1 proteases from *N. gonorrhoeae* (Pohlner *et al.*, 1987) and *Haemophilus influenzae* (Poulsen *et al.*, 1989), Hap from *H. influenzae* (St Geme *et al.*, 1994), Tsh from an *Escherichia coli* strain pathogenic for avians (Provence & Curtiss, 1994), EspC from enteropathogenic *E. coli* (Stein *et al.*, 1996), EspP from enterohaemorrhagic *E. coli* (Brunner *et al.*, 1997) and PssA from Shiga-toxin-producing *E. coli* (Djafari *et al.*, 1997). IgA1 proteases cleave with a high specificity within the hinge region of human IgA1. Hap is involved in adherence of *H. influenzae* to, and internalization by, epithelial cells. Tsh can endow a non-pathogenic strain of *E. coli* K-12 with haemagglutinin activity. EspP has proteolytic activity towards pepsin and coagulation factor V and PssA is endowed with proteolytic activity towards casein and has cytotoxicity against Vero cells. These proteins are characterized by (i) their extracellular localization, (ii) their mode of secretion, which involves a C-terminal autotransporter domain that is cleaved following translocation of the precursor across the outer membrane, (iii) the size of the mature extracellular protein, about 110 kDa, (iv) the extensive similarity exhibited by the sequences of their first 500 residues, and (v) the presence, within this conserved region, of the motif GDSGSP that has been described as part of the active site of various serine proteases (Bachovchin *et al.*, 1990). These features suggest that all these proteins might be composed of two domains, each of about 500 residues, and that they should be endowed with a proteolytic activity, presumably carried by the N-terminal domain.

To investigate the potential proteolytic activity of SepA, we have tested the ability of the purified protein to cleave a wide range of synthetic peptides. SepA was able to hydrolyse several peptides, some of which have been described as specific substrates for cathepsin G, a serine protease produced by PMN. This led us to compare the specific activities of SepA and cathepsin G on the same peptides and to test whether SepA, like cathepsin G, could degrade fibronectin and induce platelet aggregation. In addition, the putative two-domain structure of SepA was investigated by subjecting it to partial proteolysis with proteinase K. This showed that the 110 kDa secreted protein appeared to be composed of two domains of about 450 residues, which are separated by a hinge region of 100 residues.

METHODS

Bacterial strains, plasmids and growth medium. Construction of pZK15 and pZK16 has been previously described (Benjelloun-Touimi *et al.*, 1995). Briefly, cloning of the entire

sepA gene into the pUC19 vector gave rise to pZK15, which encodes the 146 kDa SepA precursor and allows secretion of the 110 kDa mature protein. pZK16 was derived from pZK15 by deleting an internal fragment of *sepA* which encodes the putative serine protease active site. BS176 is a virulence plasmid-cured *S. flexneri* strain in which pZK15 and pZK16 were each introduced for production of SepA and SepAΔ1, respectively. Bacteria were grown in tryptic soy broth containing ampicillin (50 µg ml⁻¹).

Protein purification and analysis. Bacteria producing SepA or SepAΔ1 were grown at 37 °C for 16 h, harvested by centrifugation at 10000 g for 10 min and the culture supernatants filtered through 0.22 µm filters. Proteins present in the filtrates were precipitated by addition of 50% ammonium sulfate, resuspended in 0.01 vol. 50 mM Tris, 10 mM NaCl, pH 7.5, dialysed against the same buffer and applied to a DEAE-Sephadex column. Proteins were then eluted by a step gradient of NaCl at 0.05, 0.1, 0.15, 0.2 and 0.25 M. Fractions were collected and analysed by SDS-PAGE as described by Laemmli (1970). After electrophoresis, proteins were stained with Coomassie brilliant blue. Protein concentration was determined according to Bradford (1976). Cathepsin G was purified from human neutrophils as described by Baugh & Travis (1976). Purified human thrombin was purchased from Diagnostica Stago and *N. gonorrhoeae* IgA1 protease and *Tritirachium album* proteinase K were from Boehringer. Reduced-carboxymethylated egg white lysozyme and human angiotensin I were from Sigma, and human fibronectin was from Telio.

Proteolysis of SepA. Kinetics of SepA proteolysis was performed using 30 µg purified protein and 6 µg proteinase K in a 100 µl reaction mixture. After different incubation times at 37 °C, aliquots of 20 µl were withdrawn, supplemented by addition of PMSF at 2 mM and the reaction mixtures analysed by SDS-PAGE and Coomassie blue staining. The six major hydrolytic fragments were extracted from the polyacrylamide gel, transferred onto a PVDF membrane and their N-terminal sequences determined by the Edman degradation procedure. For purification of the SepA breakdown products, the proteolysis mixture resulting from incubation of 60 µg SepA with 2 µg proteinase K for 16 h at 37 °C was applied to a 500 µl DEAE-Sephadex column. The column was washed with 10 vols 50 mM Tris pH 7.5 and then eluted by a step gradient of NaCl at 0.1, 0.2, 0.3 and 1 M in the same buffer. Fractions of 500 µl were collected and tested for proteolytic activity towards each of two *p*-nitroanilide substrates. Pools of two to five fractions were then concentrated by filtration through a membrane with a cutoff of 10 kDa and samples analysed by SDS-PAGE and Coomassie blue staining. The sizes of SepA degradation products were calculated from a curvilinear line derived from the electrophoretic mobilities of protein molecular mass standards.

Enzymic assays. *p*-Nitroanilide substrates (Calbiochem-Novabiochem) were prepared as 10 mM stock solutions in 50% DMSO. Hydrolysis of these substrates was measured at 37 °C by monitoring the increase in absorption at 405 nm on a microtitre plate reader. An ϵ value of 8800 M⁻¹ cm⁻¹ was used for *p*-nitroaniline (*p*NA) (Erlanger *et al.*, 1961). Hydrolysis was assayed with 1 mM substrate in 50 mM Tris pH 7.5, unless otherwise specified. For protease inhibition assays, hydrolysis of Suc-Val-Pro-Phe-*p*NA was measured after preincubation of SepA with the inhibitor at 20 °C for 30 min. The following protease inhibitors (Sigma) were tested: PMSF (0.01–1 mM), benzamidin (400 µM), α_1 -antitrypsin (2 µM), orthophenanthroline (2 mM), phosphoramidon (1 mM), eglin C (200 µM) and α_1 -antichymotrypsin (2 µM).

For proteolysis of fibronectin, 50 ng purified human fibronectin were incubated in the presence of 50 ng SepA or 50 ng cathepsin G in a 20 µl reaction mixture containing 20 mM Tris pH 7.8, 4 mM NaCl and 10 µM SDS for 2 h at 37 °C. For proteolysis of lysozyme, 10 µg reduced-carboxymethylated egg white lysozyme were incubated with 2.5 µg SepA in a 20 µl reaction mixture containing 50 mM Tris pH 7.5 for 16 h at 37 °C. The reaction mixtures were then analysed by SDS-PAGE and Coomassie blue staining or immunoblotting using rabbit polyclonal antibodies raised against human fibronectin. Horseradish peroxidase-labelled goat anti-rabbit antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence. For proteolysis of angiotensin I, 1 mM angiotensin I was incubated with 110 nM SepA in a 50 µl reaction mixture containing 50 mM Tris pH 7.5 for 16 h at 37 °C. The reaction mixtures were then analysed by reverse phase analytical HPLC with UV detection.

Platelet activation. Blood was obtained from healthy human volunteers and collected in SAG-M solution (187.6 mM NaCl, 71 mM dextrose, 1.95 M adenine, 10.7 mM mannitol, pH 5.1). The platelet-rich plasma was isolated by centrifugation at 180 g for 20 min. Cells were resuspended in a volume of Tyrode's buffer [137 mM NaCl, 2.68 mM KCl, 11.9 mM NaHCO₃, 0.42 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose, 5 mM HEPES, 0.35% (w/v) BSA, pH 7.4] such that the final platelet concentration was 4×10^8 cells ml⁻¹. Platelet aggregation was recorded under constant stirring at 37 °C using a Dual Aggro-Meter (Chrono-Log-Corp). Activation was initiated by addition of cathepsin G or SepA and variations in light transmission were recorded continuously. For inhibition assays, platelets were preincubated for 30 s with 400 nM purified SepA prior to their exposure to 550 nM cathepsin G or to 0.5 U thrombin ml⁻¹.

RESULTS AND DISCUSSION

Proteolytic activity of SepA

The similarity between the sequences of SepA and IgA1 proteases suggested that SepA might have proteolytic activity. However, SepA failed to cleave human IgA1 and had no activity towards gelatin (Benjelloun-Touimi *et al.*, 1995). In addition, SepA had no activity towards reduced-carboxymethylated egg lysozyme (data not shown), which indicated that it had a narrow substrate specificity. Therefore, to investigate the proteolytic activity of SepA, we tested a wide range of synthetic peptides which have been described as substrates for various proteases (Table 1). These peptides contain, at their C terminus, a pNA group, the release of which can be monitored by spectrophotometry. SepA was purified by ammonium sulfate precipitation and anion-exchange chromatography from the culture medium of a derivative of *S. flexneri* strain BS176 harbouring pZK15, which carries the *sepA* gene (Fig. 1). Purified SepA was able to hydrolyse 7 substrates out of 34 tested (Table 1). Comparison of the structures of peptides which were hydrolysed and of those which were not indicated that both the length of the substrate and its P1 residue, the amino acid linked to pNA, are important for cleavage. Whereas Suc-Ala-Ala-Pro-Phe-pNA, Suc-Val-Pro-Phe-pNA and Suc-Phe-Leu-Phe-pNA were well hydrolysed, Glutaryl-Phe-pNA was poorly hydrolysed and Suc-Phe-pNA was not hydrolysed. This indicated that SepA does

Table 1. *p*-Nitroanilide substrates tested for hydrolysis by SepA

Hydrolysis was performed in a 100 µl reaction mixture containing 0.1 M MOPS pH 7.3, 10⁻⁵ M ZnSO₄, 0.2 M NaCl, 1 mM substrate and 2 µg SepA. The specific activity of SepA on Suc-Val-Pro-Phe-pNA, 6.3 pmol substrate hydrolysed min⁻¹ (µg enzyme)⁻¹ at 37 °C, was taken as the reference (100%) to express the relative activity of SepA towards other substrates. —, Non-detectable activity (i.e. relative activity < 2%); Pyr, pyroglutamic acid; Z, benzyloxycarbonyl; Boc, butoxycarbonyl; Sar, sarcosine; Suc, succinyl.

<i>p</i> -Nitroanilide substrate	Relative activity (%)
P4 P3 P2 P1	
Pyr-pNA	—
Benzoyl-Tyr-pNA	—
Z-Lys-pNA	—
Benzoyl-DL-Arg-pNA	—
Z-Arg-Arg-pNA	—
H-Gly-Arg-pNA	10
Boc-Leu-Gly-Arg-pNA	—
H-Sar-Pro-Arg-pNA	—
Z-Lys-Phe-Arg-pNA	—
H-D-Val-Ser-Arg-pNA	—
Boc-Leu-Ser-Thr-Arg-pNA	—
Benzoyl-Phe-Val-Arg-pNA	—
H-Cys-pNA	—
Ac-Phe-Gly-pNA	—
H-Glu-pNA	—
H-Leu-pNA	—
Z-Ala-Ala-Leu-pNA	—
Boc-Gly-Gly-Leu-pNA	—
Pyr-Phe-Leu-pNA	34
Suc-Phe-pNA	—
Glutaryl-Phe-pNA	14
H-Gly-Phe-pNA	—
Suc-Gly-Gly-Phe-pNA	—
Suc-Phe-Leu-Phe-pNA	80
Suc-Val-Pro-Phe-pNA	100
Suc-Ala-Ala-Pro-Phe-pNA	50
MeOSuc-Ala-Ala-Pro-Met-pNA	50
Boc-Ala-Ala-Pro-Ala-pNA	—
MeOSuc-Ala-Ala-Pro-Val-pNA	—
Pyr-Pro-Val-pNA	—
Suc-Tyr-Leu-Val-pNA	—
H-Arg-Pro-pNA	—
Z-Gly-Pro-pNA	—
H-Gly-Pro-pNA	—

not recognize only the P1 residue, Phe, in these peptides, but interacts better with a longer substrate. The nature of the P1 residue is critical, however, since the replacement of the Phe residue in the peptide N-Suc-Ala-Ala-Pro-Phe-pNA by Val or Ala residues completely prevented proteolysis by SepA (Table 1). Given the sequence similarity detected between SepA and IgA1 proteases, we also tested the hydrolytic activity of the *N. gonorrhoeae* IgA1 protease on the best synthetic sub-

Fig. 1. Purification of SepA and SepA Δ 1. Proteins present in the culture supernatant of derivatives of *S. flexneri* BS176 harbouring the pUC19 vector, pZK15 or pZK16 were concentrated 50 times and analysed by SDS-PAGE and Coomassie blue staining. The sizes (in kDa) of molecular mass markers are indicated on the left.

strate for SepA. No hydrolysis of Suc-Val-Pro-Phe-pNA was observed in the presence of purified IgA1 protease ($50 \mu\text{g ml}^{-1}$), even after 16 h incubation at 37°C (data not shown). This is consistent with the observation that sequences cleaved by IgA1 proteases are characterized by a Pro residue in P1 (Wood & Burton, 1991). In addition, among the 34 peptides which were tested as potential substrates for SepA, 3 had a Pro residue in P1 and none of these was hydrolysed by SepA. These results confirm that SepA and IgA1 protease do not have the

same substrate specificity, as was suggested by the lack of proteolytic activity of SepA on IgA1 (Benjelloun-Touimi *et al.*, 1995).

We investigated various reaction conditions on the hydrolysis rate of Suc-Val-Pro-Phe-pNA. The optimum pH for hydrolysis was found to be pH 7.5; SepA exhibited 50% activity at pH 6.0 and pH 8.4 and 10% activity at pH 5.4 and pH 10.2. The rate of hydrolysis was maximal at 37°C ; SepA exhibited 63% and 90% residual activity at 20°C and 40°C , respectively. Concentrations of cations, such as NaCl, CaCl_2 , MgCl_2 or KCl, up to 400 mM had little or no effect on the activity of SepA (data not shown). Concentrations of DMSO, the solvent in which substrates were prepared, from 1% to 25% (v/v) had no effect on the rate of substrate hydrolysis (data not shown). To characterize the activity of SepA further, hydrolysis of Suc-Val-Pro-Phe-pNA was tested in the presence of various protease inhibitors. Release of pNA was inhibited by PMSF (50% inhibition at $100 \mu\text{M}$ and 100% inhibition at $250 \mu\text{M}$), but not by other serine protease inhibitors such as benzamidine, α_1 -antitrypsin and α_1 -antichymotrypsin or by metalloprotease inhibitors such as EDTA and ortho-phenanthroline.

Inhibition of SepA activity by PMSF was consistent with the observation that the sequence of the N-terminal region of SepA contains the motif GDSGSP (from residue 266 to residue 271), which is part of the active site of a family of serine proteases (Bachovchin *et al.*, 1990). To determine whether this region of SepA was indeed involved in proteolytic activity, we tested the activity of a derivative of SepA lacking residues 203–306. This recombinant protein, SepA Δ 1, was purified from the culture medium of a derivative of BS176 harbouring pZK16 (Fig. 1). SepA Δ 1, which was eluted from the

Fig. 2. Kinetics of proteolysis of SepA by proteinase K. (a) SepA was incubated with proteinase K at 37°C for various times and digestion mixtures were analysed by SDS-PAGE and Coomassie blue staining. Numbers above the gel indicate the incubation times (in min). The molecular masses (in kDa) of molecular mass markers are indicated on the left. Proteinase K, SepA and the sizes of SepA breakdown products are indicated by arrows. (b) Schematic representation of the SepA hydrolytic fragments. Arrows indicate the positions of the four putative cleavage sites for proteinase K on the 110 kDa SepA sequence (thick line). Dashed lines represent proteolytic intermediates and narrow lines represent breakdown products which are resistant to proteinase K. Numbers indicate the molecular mass of each product as estimated by SDS-PAGE.

Fig. 3. DEAE-Sephadex chromatography and proteolytic activity of SepA degradation products. SepA was hydrolysed by proteinase K and the digestion mixture was loaded onto a DEAE-Sephadex column. The column was washed with 50 mM Tris pH 7.5 and eluted with increasing concentrations of NaCl (arrows). Fractions were collected and analysed for hydrolysis of Suc-Ala-Ala-Pro-Met-pNA (Pepl, ○) and Suc-Val-Pro-Phe-pNA (PepII, ▲). Hydrolytic activity is expressed in nmol substrate hydrolysed min⁻¹ (μl fraction)⁻¹. Fractions were pooled (A–H), concentrated and analysed by SDS-PAGE and Coomassie blue staining, together with SepA (S), proteinase K (K) and the digestion mixture before chromatography (SK). The molecular masses (in kDa) of molecular mass markers are indicated between the two panels. Proteinase K, SepA and the sizes of SepA breakdown products are indicated by arrows.

chromatography column at the same NaCl concentration as SepA, was not able to hydrolyse Suc-Val-Pro-Phe-pNA. This indicated that the N-terminal moiety of SepA was essential for proteolytic activity. Moreover, the lack of activity of SepAΔ1 confirmed that the activity detected with SepA was not due to a contaminating protease which would have been present in the culture medium of derivatives of BS176.

Two-domain structure of SepA

The N-terminal sequences of members of the IgA1 protease family are homologous whereas there is little, if any, sequence similarity between their C-terminal regions, which suggested that these proteins are com-

posed of two domains, each of about 500 amino acid residues. To investigate the putative two-domain structure of SepA, the purified protein was subjected to proteolysis by trypsin or proteinase K. No cleavage or degradation of SepA were observed when the protein was incubated in the presence of trypsin for 2 h at 37 °C, even using an enzyme:substrate ratio of 0.5 (data not shown). In contrast, incubation of SepA in the presence of proteinase K gave rise to six major fragments of 90, 85, 50, 47, 35 and 30 kDa (Fig. 2). The 90, 85 and 50 kDa fragments disappeared upon prolonged incubation with proteinase K, whereas the 47, 35 and 30 kDa fragments accumulated during proteolysis. A smaller fragment of about 15 kDa was also detected after prolonged proteolysis (Fig. 3, lane SK).

The N-terminal sequence of the 90, 85 and 47 kDa fragments was identified as ATVS, which corresponds to the N-terminal sequence of the mature protein (position 57 in the sequence of the precursor). The N-terminal sequence of the 50, 35 and 30 kDa fragments was identified as KIEA, which corresponds to positions 611–614 in the sequence of the precursor (Benjelloun-Touimi *et al.*, 1995). Accordingly, there are four potential cleavage sites for proteinase K in SepA. The 47 kDa fragment, which corresponds to the N-terminal domain, was generated by cleavage around residue 500, as calculated from the molecular mass of this fragment. The 50 kDa fragment, which corresponds to the C-terminal domain, was generated by cleavage before residue 611. The molecular mass of this fragment is consistent with our previous proposal that the SepA precursor was cleaved after residue 1089 to release the mature protein from the outer membrane. This C-terminal fragment could be further hydrolysed at two different sites to give rise to 30 and 35 kDa fragments, both of which have the same N-terminal sequence. The approximate locations of these two additional cleavage sites are around residues 880 and 930. Cleavage of the 110 kDa protein at either of these two sites generated the proteolytic intermediates of 85 and 90 kDa. The generation of a 47 kDa N-terminal and a 50 kDa C-terminal fragment strongly suggests that SepA has a structure composed of two domains separated by a hinge region of about 100 residues.

Proteolytic activity of the N-terminal domain of SepA

Prolonged treatment of SepA with proteinase K gave rise to a 47 kDa fragment that corresponds exactly to the SepA region the sequence of which is conserved with IgA1 proteases. To determine whether SepA degradation products were endowed with proteolytic activity it was necessary to separate them from proteinase K, which is also active on *p*-nitroanilide peptides that are substrates of SepA. Preliminary experiments indicated that proteinase K, the pI of which is 8.02, was not retained on a DEAE column equilibrated with 50 mM Tris pH 7.5. In contrast, SepA fragments were retained on the column and could be eluted using a buffer containing 100 mM NaCl. Furthermore, we noticed that proteinase K and SepA had different activities on two synthetic substrates. SepA had approximately the same activity on Suc-Ala-Ala-Pro-Phe-*p*NA (PepI) and Suc-Val-Pro-Phe-*p*NA (PepII) (Table 1), whereas proteinase K was found to be 2000 times more active on PepI than on PepII. These different activities could be used to discriminate between the two proteases.

After incubation of 60 µg SepA and 2 µg proteinase K for 16 h at 37 °C, the digestion mixture was loaded onto a DEAE column equilibrated with 50 mM Tris pH 7.5. Fractions eluted in the flow-through, by washes with 50 mM Tris and by increasing concentrations of NaCl, were assayed for their activity on PepI and PepII (Fig. 3). Fractions were then pooled, concentrated and analysed by SDS-PAGE and Coomassie blue staining (Fig. 3). Most of the activity (95 %) towards PepI was present in

the flow-through (fractions 2 and 3) and the remaining activity (5 %) was eluted in the first washes (fractions 4 and 5). This activity was due to proteinase K, as confirmed by SDS-PAGE analysis of the corresponding pools (pools A and B in Fig. 3). The very low activity of other fractions towards PepI indicated that they contained less than 0.1 % of contaminating proteinase K. In contrast, two peaks of activity were detected using the substrate PepII. The first peak (fractions 2 and 3) matched the peak of activity measured on PepI and was due to proteinase K. The second peak of activity on PepII was present in fractions 12–15. SDS-PAGE analysis of pools D and E corresponding to fractions 12–13 and 14–15, respectively, indicated that they contained SepA degradation products (Fig. 3). The specific activity of pool D, which contained 15 µg of proteins, towards PepII was 5.6 pmol min⁻¹ µg⁻¹, a value which is very similar to that measured with full-length SepA (6.3 pmol min⁻¹ µg⁻¹).

Although the 47 kDa fragment was not separated from the other degradation products, this result suggested that the N-terminal domain, which corresponds exactly to the SepA region that is conserved in IgA1 proteases, is sufficient for proteolytic activity. Given the similar sizes of SepA, Tsh, EspC, EspP, PssA, Hap and IgA1 proteases and the sequence conservation between their N-terminal regions, it seems likely that each of these proteins has a two-domain structure similar to that proposed here for SepA and that their N-terminal domain is endowed with proteolytic activity. The function of the C-terminal domain of these proteins remains to be elucidated. The C-terminal domain of SepA does not appear to be involved in regulation of the proteolytic activity, at least when activity is assayed on synthetic substrates.

Comparison of the activities of SepA and cathepsin G

The four most reactive substrates for SepA have been described as specific substrates for cathepsin G (Nakajima *et al.*, 1979; Tanaka *et al.*, 1985), a serine protease produced by PMN. This led us to compare the activity of SepA and cathepsin G on the same substrate, Suc-Val-Pro-Phe-*p*NA, under identical experimental conditions. Cathepsin G was purified from human PMN as described by Baugh & Travis (1976). Kinetic constants of SepA and cathepsin G were derived by using Lineweaver–Burk plots on the initial rates of hydrolysis determined at different substrate concentrations. A K_m value of 1.5 mM was obtained for both SepA and cathepsin G and k_{cat} values of 0.064 s⁻¹ and 6.9 s⁻¹ were obtained for SepA and cathepsin G, respectively. The kinetic constants determined for cathepsin G were consistent with those reported by Tanaka *et al.* (1985). Therefore, the k_{cat}/K_m value of SepA (43 M⁻¹ s⁻¹) was 100 times lower than that of cathepsin G (4600 M⁻¹ s⁻¹). The low k_{cat}/K_m value of SepA measured for hydrolysis of Suc-Val-Pro-Phe-*p*NA, which appears to be a consequence of a low turnover rate, might be due either to the nature of substrates which have been tested so far or to an intrinsic property of the purified protein. Synthetic substrates might not be

sufficient to reveal the full activity of SepA. Whereas *N. gonorrhoeae* IgA1 protease readily cleaves the hinge region of IgA1, it does not cleave synthetic peptides which are derived from the sequence of that region (Wood & Burton, 1991). Alternatively, the low activity of SepA might be due to an inadequate conformation of the protein.

We then investigated whether SepA was endowed with some biological activities similar to those described for cathepsin G. The characteristic feature of cathepsin G is its ability to induce platelet activation, which involves the proteolytic cleavage of an as yet unidentified receptor (Selak *et al.*, 1988, 1994). Purified SepA or cathepsin G were added to a suspension of human platelets and platelet activation was followed by measuring their aggregation. Whereas 80% platelet activation was obtained using 550 nM cathepsin G, no platelet activation was observed using concentrations of SepA up to 5 µM (data not shown). Thrombin is the other serine protease which can induce platelet activation by cleaving its specific receptor (Vu *et al.*, 1991). Under certain experimental conditions, cathepsin G cleaves the thrombin receptor at the surface of platelets, which inhibits their ability to be activated by thrombin (Molino *et al.*, 1995). This led us to investigate the potential ability of SepA to block the response of platelets to thrombin stimulation. Pretreatment of platelets with SepA did not inhibit their ability to be activated by thrombin. Likewise, platelets preincubated with SepA could still be activated by cathepsin G (data not shown). This suggested that SepA was not able to cleave the thrombin receptor or the cathepsin G receptor.

Cathepsin G is also able to hydrolyse components of the extracellular matrix, such as fibronectin (Vartio *et al.*, 1981) and collagen (Kielty *et al.*, 1993), and to convert angiotensin I to angiotensin II (Klickstein *et al.*, 1982). To investigate these activities, SepA and cathepsin G were each incubated in the presence of purified human fibronectin and reaction mixtures were analysed by SDS-PAGE and immunoblotting using anti-fibronectin antibodies. Whereas fibronectin was readily hydrolysed by cathepsin G, no fibronectin degradation was observed in the presence of SepA, even using equimolar amounts of enzyme and substrate (data not shown). Likewise, no angiotensin II was detected following incubation of angiotensin I in the presence of SepA (data not shown). Therefore, no cathepsin-G-like activity could be demonstrated for SepA under these assay conditions. Nevertheless, it is noteworthy that SepA and cathepsin G share the same substrate specificity on synthetic peptides, since a pro-inflammatory activity of SepA would be consistent with the phenotype exhibited by the *sepA* mutant in infected rabbit intestinal loops. In this model, the acute inflammation which is induced upon infection by wild-type *S. flexneri* appears to be responsible for both causing mucosal destruction and facilitating invasion of epithelial cells (Perdomo *et al.*, 1994a). The *sepA* mutant exhibited a reduced ability to induce mucosal atrophy and tissue inflammation as compared to the wild-type. The number of PMN infiltrating the

intestinal epithelium infected with the *sepA* mutant was also clearly reduced, suggesting a decrease in intensity of the inflammatory response (Benjelloun-Touimi *et al.*, 1995).

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