Detergent-independent in vitro activity of a truncated Bacillus signal peptidase

Maarten L. van Roosmalen, Jan D. H. Jongbloed, Anne de Jong, Jaap van Eerden, Gerard Venema, Sierd Bron and Jan Maarten van Dijl†

INTRODUCTION

Secretory proteins are synthesized as pre-proteins with an N-terminal signal peptide. Type I signal peptidases (SPases) remove these signal peptides during, or shortly after, pre-protein translocation across the cytoplasmic membrane, thereby releasing the mature proteins from the trans side of the membrane (for reviews, see Pugsley, 1993; Dalbey et al., 1997). The largest number of type I SPases has, thus far, been found in the Gram-positive eubacterium Bacillus subtilis, which contains five chromosomally encoded type I signal peptidases (SPases) for the processing of secretory pre-proteins. Even though four of these SPases, denoted SipS, SipT, SipU and SipV, are homologous to the unique SPase I of Escherichia coli, they are structurally different from that enzyme, being almost half the size and containing one membrane anchor instead of two. To investigate whether the unique membrane anchor of Bacillus SPases is required for in vitro activity, soluble forms of SipS of Bacillus subtilis, SipS of Bacillus amyloliquefaciens and SipC of the thermophile Bacillus caldolyticus were constructed. Of these three proteins, only a hexa-histidine-tagged soluble form of SipS of Bacillus amyloliquefaciens could be isolated in significant quantities. This protein displayed optimal activity at pH 10, which is remarkable considering the fact that the catalytic domain of SPases is located in an acidic environment at the outer surface of the membrane of living cells. Strikingly, in contrast to what has been previously reported for the soluble form of the E. coli SPase, soluble SipS was active in the absence of added detergents. This observation can be explained by the fact that a highly hydrophobic surface domain of the E. coli SPase, implicated in detergent-binding, is absent from SipS.

Keywords: Bacillus subtilis, protein secretion, SipS, SipC

The Gram-positive eubacterium Bacillus subtilis contains five chromosomally encoded type I signal peptidases (SPases) for the processing of secretory pre-proteins. Even though four of these SPases, denoted SipS, SipT, SipU and SipV, are homologous to the unique SPase I of Escherichia coli, they are structurally different from that enzyme, being almost half the size and containing one membrane anchor instead of two. To investigate whether the unique membrane anchor of Bacillus SPases is required for in vitro activity, soluble forms of SipS of Bacillus subtilis, SipS of Bacillus amyloliquefaciens and SipC of the thermophile Bacillus caldolyticus were constructed. Of these three proteins, only a hexa-histidine-tagged soluble form of SipS of Bacillus amyloliquefaciens could be isolated in significant quantities. This protein displayed optimal activity at pH 10, which is remarkable considering the fact that the catalytic domain of SPases is located in an acidic environment at the outer surface of the membrane of living cells. Strikingly, in contrast to what has been previously reported for the soluble form of the E. coli SPase, soluble SipS was active in the absence of added detergents. This observation can be explained by the fact that a highly hydrophobic surface domain of the E. coli SPase, implicated in detergent-binding, is absent from SipS.

INTRODUCTION

Secretory proteins are synthesized as pre-proteins with an N-terminal signal peptide. Type I signal peptidases (SPases) remove these signal peptides during, or shortly after, pre-protein translocation across the cytoplasmic membrane, thereby releasing the mature proteins from the trans side of the membrane (for reviews, see Pugsley, 1993; Dalbey et al., 1997). The largest number of type I SPases has, thus far, been found in the Gram-positive eubacterium Bacillus subtilis, which contains five chromosomally encoded enzymes of this type (SipS, SipT, SipU, SipV and SipW) (van Dijl et al., 1992; Bolhuis et al., 1996; Tjalsma et al., 1997, 1998). In addition, certain strains of Bacillus subtilis (natto) contain plasmid-encoded type I SPases, denoted SipP (Meijer et al., 1995; Tjalsma et al., 1999a). Even though the presence of paralogous SPases is not unusual in eubacteria, archaea and eukaryotes, the number of paralogous SPases in Bacillus subtilis is unusually high, suggesting that some of these SPases may have specialized functions. Indeed, it was recently shown that SipS, SipT and SipP are of major importance for the secretion of degradative enzymes and cell viability, unlike SipU, SipV and SipW (Tjalsma et al., 1998, 1999a). Furthermore, SipW is of major importance for the processing of precursors of certain proteins, such as TasA (Stöver & Driks, 1999a) and YqxM (Stöver & Driks, 1999b). Taken together, these observations indicate that there are significant differences in the substrate specificities of the various SPases of Bacillus subtilis. Nevertheless, SipS, SipT, SipU and SipV were shown to process the hybrid precursor pre-(A13i)-β-lactamase (pre-A13i-Bla; Tjalsma et al., 1997), and all five chromosomally encoded SPases were involved in the processing of the Bacillus amyloliquefaciens α-amylase AmyQ (Tjalsma et al., 1998). The latter observations showed...
Fig. 1. Alignment of deduced amino acid sequences of signal peptidases of Gram-positive eubacteria and E. coli. The alignment includes the following type I SPases: SipC (Bca) from B. caldolyticus, SipV (Bsu) from B. subtilis (Tjalsma et al., 1997), SpsB (Sau) from Staphylococcus aureus (Cregg et al., 1996), SipT (Bsu) from B. subtilis (Tjalsma et al., 1997), SipP (pTA1015) from B. subtilis (natto) (Meijer et al., 1995), SipS (Bsu) from B. subtilis (van Dijl et al., 1992), SipS (Bam) from B. amyloliquefaciens (Meijer et al., 1995), SipU (Bsu) from B. subtilis (Tjalsma et al., 1997) and the E. coli SPase I [Lep (Eco)] (Wolfe et al., 1983). Residues that are critical for activity are labelled with a γ. The conserved arginine residue, which is the second residue in the soluble forms (sf) of SipC, SipS (Bsu) and SipS-His (Bam), is indicated by an arrow. Regions of Lep (Eco) that are absent from the aligned SPases are not shown, but their relative positions and the corresponding residue numbers in the Lep (Eco) sequence are indicated below the alignment. Note that residues 106–115 of Lep (Eco), which represent a very hydrophobic β-hairpin, are not conserved in SipS (Bam) or other Bacillus SPases. Identical amino acids (*) or conservative replacements (.) are marked. Residues printed in bold are predicted to be part of the membrane anchors of the SPases.

that the B. subtilis SPases have at least partly overlapping substrate specificities (Tjalsma et al., 1997).

Recent studies have provided compelling evidence that the type I SPase of Escherichia coli, also known as leader peptidase, makes use of a serine–lysine catalytic dyad (Tschantz et al., 1993; Paetzel et al., 1998). This is also probably true for SipS, SipT, SipU and SipV of B. subtilis and for the SPases from other eubacteria and organelles that are related to the SPase I of E. coli (van Dijl et al., 1995; Dalbey et al., 1997). Nevertheless, apart from the residues involved in catalysis (Paetzel et al., 1998; Bolhuis et al., 1999c), very little is known of those factors that determine the activity of SPases from eubacteria other than E. coli. Considering the fact that the type I SPases of B. subtilis display different substrate specificities in vivo, these enzymes appeared to be attractive models for the identification of important determinants of SPase activity. Further study of these enzymes is necessary to understand their function in vivo.
to be solubilized with detergents. In the present studies, we constructed soluble forms of SipS of B. subtilis, SipS of B. amyloliquefaciens and, with the aim of obtaining a presumably more stable enzyme, SipC of the thermophile Bacillus caldolyticus. This last organism can grow and secrete proteins at a temperature of 70 °C. The results showed that only a truncated, hexa-histidine-tagged soluble form of SipS of B. amyloliquefaciens could be isolated in significant quantities for in vitro characterization. Strikingly, as demonstrated with pre-(A2)-α-amyrase (pre-A2-Amyl), a precursor that is efficiently processed in vivo and synthesized in vitro (Smith et al., 1987, 1988; Bolhuis et al., 1999b), this soluble form of SipS was active in the absence of added detergents or phospholipids, unlike the soluble form of the E. coli Spase I (Tschantz et al., 1995). This observation is of general importance, because the highly hydrophobic surface domain of the E. coli Spase that was recently implicated in detergent-binding (Paetzol et al., 1998) is not conserved either in SipS of B. amyloliquefaciens, or in the large majority of known eubacterial SPases. It thus seems that, at least with respect to its in vitro requirement for added detergents or phospholipids, the E. coli enzyme could represent a relatively small subgroup of the known SPases.

**METHODS**

**Plasmids, bacterial strains and media.** Table 1 lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto-tryptone (1%, w/v), Bacto yeast extract (0.5%, w/v) and NaCl (1%, w/v). If required, the medium for E. coli was supplemented with ampicillin (50 μg ml⁻¹) and kanamycin (40 μg ml⁻¹).

**DNA techniques.** Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and transformation of E. coli were carried out as described by Sambrook et al. (1989). Enzymes were from Roche Molecular Biochemicals. PCR was carried out with Vent DNA polymerase (New England Biolabs) as described by van Dijl et al. (1995). DNA and protein sequences were analysed with the PCGene Analysis Program (version 6.7; IntelliGenetic) and CLUSTAL W version 1.74 (Thompson et al., 1994).

The sipC gene of B. caldolyticus was cloned as previously described for the cloning of the sipS gene of B. subtilis (van Dijl et al., 1992). First, chromosomal DNA of B. caldolyticus was cleaved with SphI and ligated into the unique BclI site of plasmid pGDL46. Subsequently, E. coli MC1061 was transformed with the ligation mixture and kanamycin- and ampicillin-resistant transformants were selected. Finally, transformants that were able to process the hybrid precursor pre-A13i-Bla, and release mature A13i-Bla into the surrounding growth medium, were selected using a plate (halo) assay for β-lactamase activity (van Dijl et al., 1992). Notably, the E. coli Spase I is unable to process pre-A13i-Bla in vivo. Consequently, this hybrid precursor remains attached to the membrane and no mature A13i-Bla is released into the surrounding medium, unless a heterologous SPase is expressed in the surrounding medium, unless a heterologous SPase is expressed. As shown by DNA sequencing, the plasmid pGDL46.36 thus selected contained the sipC gene of B. caldolyticus (SWISS-PROT # P41027).

Plasmid pT7dC, specifying a soluble form of SipC, was constructed by ligating an EcoRI- and BamHI-cleaved PCR-amplified fragment of sipC into the corresponding sites of pT1713. The sipC-specific fragment was amplified with the primers SipCO02 (5'-GGATTTCTTGCGAGGAACAAAATGCTTGTTTTCGACAAATTAGC-3') and SipCO04 (5'-GGATTTCTTGCTACAGAAGCGGAAGAACGTTGTGATAAACGGC-3'), using pGDL46.36 DNA as a template. Plasmid pT7dCH, specifying a hexa-histidine-tagged soluble form of SipC, was constructed by ligating an EcoRI- and SalI-cleaved, PCR-amplified fragment of sipC into the corresponding sites of pT1712. The sipC-specific fragment was amplified with the primers SipCO02 and SipCHis004 (5'-GTCTTAGATTTCT- TAGTATGTGTGTGATGATAAAGCAGCCAACCCTAGTTTTAACGGC-3'), using pGDL46.36 DNA as a template. Plasmid pT7dS, specifying a soluble form of SipS from B. subtilis (Bsu), was constructed by ligating a SalI- and BamHI-cleaved, PCR-amplified fragment of sipS (Bsu) into the corresponding sites of pT1712. The sipS (Bsu)-specific fragment was amplified by PCR with the primers SipSO01 (5'-GACTAIGTCAGCCAGGACCAATAATGCTTACATTGCGAAATTACATTGCGACTTTTGGCC-3') and Lbs91 (5'-CGGGATCCCGGGACTAATTTGTGTTTGGCG-3') using B. subtilis 168 chromosomal DNA as a template. Plasmid pT7dAH, specifying a hexa-histidine-tagged soluble form of SipS from B. amyloliquefaciens (Bam), was constructed by ligating an EcoRI- and SalI-cleaved PCR-amplified fragment of sipS (Bam) into the corresponding sites of plasmid pT1712. The sipS (Bam)-specific fragment was amplified by PCR with the primers SipA001 (5'-GGAATTCCGCGAGGACACATTAGCGCAACTTATTGCTCC-3') and SipAHS02 (5'-GGGATTCCTAGTATGTGTGTGATGATAAAGCAGCCAACCCTAGTTTTAACGGC-3') using pGDL46.21 as a template. To prevent the selection of non-overexpressing variants of pT7dC, pT7dCH, pT7dS and pT7dAH, these plasmids were first constructed using E. coli MC1061, which does not contain the gene for the T7 RNA polymerase. Subsequently, E. coli BL21 (de3) was used for the production of soluble SPases specified by pT7dC, pT7dCH, pT7dS and pT7dAH.

**Protein overproduction and purification.** For overproduction and subsequent purification of the hexa-histidine-tagged soluble form of SipS (Bam), denoted sf-SipS-His (Bam), E. coli BL21 (de3) was transformed with plasmid pT7dAH. Transformants were grown overnight in TY medium at 37 °C. Next, 1 litre of fresh TY medium was inoculated with 10 ml of this overnight culture and incubated at 37 °C. When the culture reached an OD₆₀₀ of 0.6–0.9, the production of sf-SipS-His (Bam) was induced by adding IPTG to a final concentration of 0.5 mM. Cells were collected by centrifugation about 3 h after induction. The cell pellet was resuspended in 10 ml lysis buffer (50 mM Tris/HCl, 300 mM NaCl, 1 mM phosphoramidon, 1 mM PMSF, pH 8.0) and disrupted by three passages through a chilled French pressure cell at 10000 p.s.i. (69 MPa). Cells and debris were removed from the extract by centrifugation at 5000 g (15 min, 4 °C). To separate the soluble sf-SipS-His (Bam) from the membrane-bound E. coli Spase I, membranes were removed from the supernatant by two subsequent ultracentrifugation steps (100000 g, 30 min, 4 °C). Finally, sf-SipS-His (Bam) was isolated by metal-affinity chromatography, using a column containing 5 ml TALON resin (CLONTECH Laboratories) that was pre-equilibrated with lysis buffer. The column was washed with 25 ml lysis buffer and sf-SipS-His (Bam) was eluted with elution buffer (i.e. lysis buffer with 300 mM imidazole). To verify the level of purification, samples from 0.5 ml fractions were analysed by SDS-PAGE and subsequent staining with Coomassie brilliant blue R (CBR). Fractions containing the purified sf-SipS-His (Bam) were pooled and transferred to a buffer containing 50 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT.
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Genotype/relevant properties</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
</tr>
<tr>
<td>MC1061</td>
<td>F' araD139 Δ[ara-leu]7696 galE15 galK16 Δ[lac]X783 rpsL. hsdR2 mcrA mcrB1</td>
</tr>
<tr>
<td>BL21(d3)</td>
<td>F' ompT r(c) m(m) DE3</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>tepC2</td>
</tr>
<tr>
<td>WB600</td>
<td>tepC2 nprE nprB apr epr mpr bpr Em(R)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pGDL42</td>
<td>pWVO1-derived plasmid; encodes pre-A13t-Bla; 7.5 kb; Ap(R) Em(R) Km(R)</td>
</tr>
<tr>
<td>pGDL46</td>
<td>pGDL42 derivative; contains no EcoRI and SalI sites; 7.5 kb; Ap(R) Em(R) Km(R)</td>
</tr>
<tr>
<td>pGDL46.21</td>
<td>pGDL42 derivative; encodes SipS of <em>B. amyloliquefaciens</em>; 8/8 kb; Ap(R) Em(R) Km(R)</td>
</tr>
<tr>
<td>pGDL46.36</td>
<td>pGDL42 derivative; encodes SipC of <em>B. caldolyticus</em>; 87 kb; Ap(R) Em(R) Km(R)</td>
</tr>
<tr>
<td>pT712</td>
<td>pUC12c derivative; contains the M13mp10/pUC12 polynucleotide downstream of the T7 promoter; 28 kb; Ap(R)</td>
</tr>
<tr>
<td>pT713</td>
<td>As pT712; but with the M13mp10/pUC12 polynucleotide reversed</td>
</tr>
<tr>
<td>pT7dS</td>
<td>pT712 derivative; encodes sf-SipS (Bsu) from <em>B. subtilis</em>; 3.3 kb</td>
</tr>
<tr>
<td>pT7dAH</td>
<td>pT712 derivative; encodes sf-SipS-His (Bam) from <em>B. amyloliquefaciens</em>; 3.3 kb</td>
</tr>
<tr>
<td>pT7dC</td>
<td>pT713 derivative; encodes sf-SipC from <em>B. caldolyticus</em>; 3.3 kb</td>
</tr>
<tr>
<td>pT7dCH</td>
<td>pT712 derivative; encodes sf-SipC-His from <em>B. caldolyticus</em>; 3.3 kb</td>
</tr>
</tbody>
</table>

(PH 8.0) by gel filtration with a PD-10 Sephadex G-25 M column (Amersham Pharmacia Biotech). Pure sf-SipS-His (Bam) was stored either at 4 °C or at -80 °C in the presence of 20% (v/v) glycerol.

**Signal peptidase activity.** The *in vitro* assay for *Bacillus* SPase activity was performed essentially as described previously (Vehmaanperä et al., 1993). An S-135 extract for the *in vitro* synthesis of [35S]methionine-labelled pre-A2-AmyL (Smith et al., 1987, 1988) was prepared from *B. subtilis* WB600, a sixfold protease-negative strain (Wu et al., 1991). The energy-regenerating system for *in vitro* transcription–translation was from the Promega *E. coli* S-30 Extract System for Linear Templates. To assay SPase activity, 2 µl of the *in vitro* transcription–translation mix, with labelled pre-A2-AmyL, was diluted in 16 µl reaction buffer (50 mM HEPES, 5 mM MgSO4, pH 7.5, 37 °C). Next, 2 µl purified sf-SipS-His (Bam) (7.5 µM) or reaction buffer (negative control) was added. If required, 0.5% (v/v) Triton X-100 was added to the reaction buffer. Reactions were terminated by the addition of sample buffer for SDS-PAGE (20 µl) and subsequent boiling for 5 min. Finally, the samples were analysed by SDS-PAGE, fluorography and scanning of films as described by Vehmaanperä et al. (1993).

Extracts of *E. coli* that were used to verify the absence of SPase-like activities in the cytoplasm were prepared as described by van Dijl et al. (1991a). The *in vitro* assay for SPase activity, based on the pre-A2-AmyL synthesized in extracts of *E. coli*, was performed as described by van Dijl et al. (1991b).

**RESULTS**

Construction and overexpression of soluble SPases

The biochemically well-characterized soluble form of *E. coli* SPase I lacks residues 2–75 (Kuo et al., 1993). The second residue of this mutant enzyme is a conserved arginine residue that is absent from only a few SPases, such as SipV from *B. subtilis* and SpsB from *Staphylococcus aureus* (Fig. 1). Notably, this conserved arginine residue is critical for the activity of the intact *E. coli* SPase I (Bilgin et al., 1990). Therefore, mutant soluble forms of SipC of *B. caldolyticus* (sf-SipC and sf-SipC-His), SipS of *B. subtilis* (sf-SipS [Bsu]) and SipS of *B. amyloliquefaciens* (sf-SipS-His [Bam]) were constructed in such a way that the conserved arginine residue was located at the +2 position relative to the methionine residue specified by the new start codon (Fig. 2). Thus, sf-SipC (Bca) and sf-SipC-His (Bca) lack residues 2–24, sf-SipS (Bsu) lacks residues 2–29 and sf-SipS-His (Bam) lacks residues 2–31. Two of these enzymes, sf-SipC-His (Bca) and sf-SipS-His (Bam), were provided with a C-terminal hexa-histidine tag to facilitate their purification.
Soluble form of a *Bacillus* signal peptidase

**Fig. 2.** Schematic presentation of constructs for overproduction of truncated soluble signal peptidases. All constructs used for the overproduction of sf-SipC, sf-SipC-His, sf-SipS (Bsu) and sf-SipS-His (Bam) are based on the plasmids pT712 or pT713 (filled parts of the bar). PCR-amplified sequences specifying the sf-Sip proteins were cloned downstream of the T7 promoter of pT712/pT713, using restriction sites (indicated by MCS) in the multiple cloning sites of these plasmids. All truncated genes contained the ribosome-binding site and start codon of the *B. subtilis* *obg* gene as indicated. Residues printed in bold are remnants of the membrane anchors of the corresponding wild-type SPases. The catalytic serine residue of these enzymes is underlined. The sf-SipC-His and sf-SipS-His (Bam) contained a C-terminal hexa-histidine tag.

**Fig. 3.** Overproduction and purification of a truncated soluble *Bacillus* signal peptidase. (a) Cells of *E. coli* BL21(de3), transformed with plasmid pT7dAH, were grown in TY medium and the overproduction of sf-SipS-His (Bam) was induced with IPTG as described in Methods. Cells containing the ‘empty vector’ (pT712) were used as a negative control. Samples, collected 3 h after induction, were separated by SDS-PAGE and, subsequently, the gel was stained with CBB. The positions of sf-SipS-His (Bam) (arrow) and molecular mass reference markers are indicated. (b) The sf-SipS-His (Bam) was purified by metal-affinity chromatography from a cytosolic fraction of IPTG-induced cells of *E. coli* BL21(de3) containing plasmid pT7dAH as described in Methods. Samples obtained from the different fractions, before and after metal-affinity chromatography, were analysed by SDS-PAGE and subsequent CBB staining. Cytosol, the cytosolic fraction of IPTG-induced cells; wash, the wash fraction; elution, the fraction obtained by elution with buffer containing imidazole. The positions of sf-SipS-His (Bam), the specific degradation products d1 and d2 of sf-SipS-His (Bam) and molecular mass reference markers are indicated.

by metal-affinity chromatography. To overproduce the soluble *Bacillus* SPases, the corresponding 5′-truncated *sip* genes were provided with the efficient ribosome-binding site and start codon of the *obg* gene of *B. subtilis* (Welsh et al., 1994), cloned downstream of the T7 promoter on plasmids pT712 or pT713 (Studier et al., 1990) and introduced into an *E. coli* strain that produces the T7 RNA polymerase upon induction with IPTG. The IPTG-induced expression of these truncated genes resulted in the production of sf-SipC, sf-SipS (Bsu) and sf-SipS-His (Bam) to levels allowing their detection in CBB-stained SDS-PAGE gels. In contrast, sf-SipC-His was barely visible in CBB-stained SDS-PAGE gels (Fig. 3a; only the results for sf-SipS-His [Bam] are shown). Notably, compared to the production of intact SipS (Bsu) or SipS (Bam) with the same T7 expression system, the soluble forms of these enzymes were produced at significantly lower levels (our unpublished observations).

**Characterization of sf-SipS**

As our attempts to purify sf-SipC or sf-SipS (Bsu) to homogeneity by conventional chromatography (e.g. ion exchange, gel filtration) met with little success, and sf-SipC-His production levels were too low (data not shown), our efforts to purify a soluble form of a *Bacillus* SPase were focused on sf-SipS-His (Bam). Due to its C-terminal hexa-histidine tag, sf-SipS-His (Bam) could be isolated in one step by metal-affinity chromatography.
cytoplasm of AmyL. (b) To verify the absence of SPase-like activities in the SDS-PAGE and fluorography. p, pre-A2-AmyL; m, mature A2-AmyL. Samples were analysed by Triton X-100 (TX-100). In parallel, labelled pre-A2-AmyL was unprocessed in extracts of cursor pre-A2-Bla (Smith et al. 1987, 1988) remaining, showing that SPase-like activities are absent from the cytoplasm of E. coli (data not shown), showing that SPase-like activities are present in the cytoplasm of E. coli. Similarly, the wild-type β-lactamase (Bla) precursor and the hybrid precursor pre-A2-Bla (Smith et al., 1987, 1988) remained unprocessed in extracts of E. coli (van Dijl et al., 1991b), confirming the absence of cytoplasmic SPase-like activities in E. coli. Taken together, these observations demonstrate that the purified sf-SipS-His (Bam) was active, even in the absence of added phospholipids or detergents.

To characterize the enzymic activity of sf-SipS-His (Bam), a hybrid precursor, denoted pre-A2-AmyL (Smith et al., 1987, 1988) was used. This precursor contains the first 60 residues of the YvcE protein of B. subtilis, including a typical signal peptide (Bolhuis et al., 1999b). The 35S-labelled pre-A2-AmyL was generated by in vitro transcription–translation in a B. subtilis S-135 extract and incubated for 16 h with purified sf-SipS-His (Bam), in the absence of added phospholipids and in the absence or presence of 0.5% Triton X-100. As shown in Fig. 4(a), a significant fraction of the labelled pre-A2-AmyL was processed to the mature form, irrespective of the presence of Triton X-100. Notably, no mature A2-AmyL was produced upon synthesis and incubation of the corresponding precursor (which was competent for processing by the SPase I of E. coli) in extracts of E. coli (Fig. 4b). This was true even when pre-A2-AmyL was incubated for 16 h in an E. coli extract (data not shown), showing that SPase-like activities are absent from the cytoplasm of E. coli. Similarly, the wild-type β-lactamase (Bla) precursor and the hybrid precursor pre-A2-Bla (Smith et al., 1987, 1988) remained unprocessed in extracts of E. coli (van Dijl et al., 1991b), confirming the absence of cytoplasmic SPase-like activities in E. coli. Taken together, these observations

![Detergent-independent precursor processing by purified sf-SipS-His (Bam).](image)

**Fig. 4.** Detergent-independent precursor processing by purified sf-SipS-His (Bam). (a) 35S-labelled pre-A2-AmyL, synthesized in vitro with a B. subtilis S-135 extract, was incubated (16 h at 37 °C) with 7.5 µM purified sf-SipS-His (Bam) in 50 mM HEPES (pH 7.5), 5 mM MgSO4 in the presence or absence of 0.5% Triton X-100 (TX-100). In parallel, labelled pre-A2-AmyL was incubated without sf-SipS-His (Bam). Samples were analysed by SDS-PAGE and fluorography. p, pre-A2-AmyL; m, mature A2-AmyL. (b) To verify the absence of SPase-like activities in the cytoplasm of E. coli, 35S-labelled pre-A2-AmyL was synthesized in vitro, using an E. coli S-30 extract. Next, this precursor was incubated (60 min at 37 °C) in a lysate of E. coli as previously described (van Dijl et al., 1991a). As a control for the processing competence of pre-A2-AmyL under these conditions, this precursor was also incubated in the presence of purified SPase I of E. coli (Lep).

![In vitro processing of pre-A2-AmyL by sf-SipS-His (Bam).](image)

**Fig. 5.** In vitro processing of pre-A2-AmyL by sf-SipS-His (Bam). (a) The pH-dependent activity of sf-SipS-His (Bam). In vitro-synthesized, 35S-labelled pre-A2-AmyL was incubated (30 min at 37 °C) with 7.5 µM purified sf-SipS-His (Bam) in 50 mM acetic acid/NaOH (pH 4.0 and pH 5.0), 50 mM Tris/Cl (pH 6.0, pH 7.0 and pH 8.0), or 50 mM glycine/NaOH (pH 9.0, pH 9.5 and pH 10.0). Samples were analysed by SDS-PAGE, fluorography and scanning of films. The percentage of pre-A2-AmyL processing was calculated as the amount of labelled mature AmyL divided by the total amount of labelled A2-AmyL (precursor + mature A2-AmyL). The results of two independent experiments are indicated with filled or open bullets. About 10% of the labelled A2-AmyL was mature after in vitro synthesis and no additional processing was observed in mock-treated samples. (b) Temperature dependence of the activity of sf-SipS-His (Bam). In vitro-synthesized, 35S-labelled pre-A2-AmyL was incubated for 30 min with 7.5 µM purified sf-SipS-His (Bam) in 50 mM glycine/NaOH buffer (pH 10.0) at the temperatures indicated. Processing of pre-A2-AmyL was analysed as in (a). (c) Processing of pre-A2-AmyL as a function of time. In vitro synthesized 35S-labelled pre-A2-amyL was incubated at 30 °C with 7.5 µM purified sf-SipS-His (Bam) in 50 mM glycine/NaOH buffer (pH 10.0) for different periods of time. Processing of pre-A2-AmyL was analysed as in (a).
In vitro processing of pre-A2-AmyL was used to determine the pH and temperature optima of sf-SipS-His (Bam). As shown in Fig. 5(a, b), optimal activity of sf-SipS-His (Bam) was observed at pH 10 and at incubation temperatures between 30 °C and 37 °C. At pH values above 10, the activity of sf-SipS-His (Bam) was significantly reduced (data not shown). No SPase activity was detected at pH values lower than 5 or temperatures below 4 °C (data not shown) or above 50 °C. Finally, as pre-A2-AmyL processing by sf-SipS-His (Bam) was frequently incomplete (irrespective of the presence or absence of Triton X-100), varying between about 40 and 80% of the labelled AmyL, the processing of pre-A2-AmyL was determined as a function of the time of incubation at optimal pH and temperature. As shown in Fig. 5(c), maximal levels of pre-A2-AmyL processing were observed after approximately 20 min of incubation. As can be inferred from the comparison of Figs 4(a) and 5(a), the presence of MgSO₄ did not affect the level of pre-A2-AmyL processing. Notably, the remaining pre-A2-AmyL was not even processed upon the addition of fresh sf-SipS-His (Bam) or the intact SPase I of E. coli (data not shown), suggesting that this fraction of the labelled pre-protein was incompetent for processing.

**DISCUSSION**

In the present studies, we have shown that a soluble variant of the B. amyloliquefaciens SPase, SipS, is active even in the absence of added detergents or phospholipids. In fact, the activity of this so-called sf-SipS-His (Bam) protein was not stimulated by the addition of Triton X-100. The latter observation is remarkable, in view of the previously documented observation that the activity of a soluble derivative of the E. coli SPase strongly depends on the addition of Triton X-100 (Tschantz et al., 1995; van Klompenburg et al., 1998). On the basis of the crystal structure of the soluble derivative of the E. coli SPase, Paetzel et al. (1998) postulated that the stimulation of SPase activity by Triton X-100 is due to the presence of a large, highly hydrophobic surface domain of this enzyme. A significant part of this exposed hydrophobic surface is provided by a β-hairpin between residues 106 and 124 of the E. coli SPase (Paetzel et al., 1998). Strikingly, the most hydrophobic part of this β-hairpin (residues 106–115) is not conserved in SipS (Bam) or other Bacillus SPases (Fig. 1). Thus, the lack of a stimulating effect of Triton X-100 on sf-SipS-His (Bam) activity supports the view (Paetzel et al., 1998) that this hydrophobic β-hairpin is responsible for the detergent requirement of the E. coli SPase. Interestingly, the region between residues 106 and 124 of the E. coli SPase is only conserved in nine known type I SPases from Bordetella pertussis, Bradyrhizobium japonicum, Haemophilus influenzae, Helicobacter pylori, Pseudomonas fluorescens, Rhodobacter capsulatus, Salmonella typhimurium and Rickettsia prowazekii. The fact that this region is absent from the remaining 53 known SPases of this type (our unpublished observation) suggests that the E. coli SPase I could be atypical with respect to its detergent requirement in vitro. We consider it unlikely that the observed detergent-independent in vitro activity of sf-SipS-His (Bam) relates to the particular pre-protein that was used in our studies (i.e. pre-A2-AmyL), rather than to the structural features of sf-SipS-His (Bam). Dalbey and co-workers were recently able to show that even the intact SipS (Bsu), which was produced from a fusion with the maltose-binding protein (Carlos et al., 2000), was capable of efficient processing of a pro-OmpA nuclease A hybrid precursor in the absence of detergent (R. E. Dalbey, personal communication). This is the same precursor that was used to demonstrate the detergent-dependent activity of the soluble derivative of the E. coli SPase I (Tschantz et al., 1995). Moreover, SipS (Bsu) and SipS (Bam) are highly similar proteins (91% identical residues and conservative replacements; Meijer et al., 1995). Finally, pre-A2-AmyL is a typical pre-protein that is efficiently processed, both in B. subtilis and in E. coli (Smith et al., 1987, 1988; van Dijl et al., 1991b; Bolhuis et al., 1999b).

The soluble derivative of SipS (Bam) was shown to have optimal activity at alkaline pH and a similar result was obtained with the wild-type form of this enzyme (our unpublished observations). These observations are consistent with the proposed catalytic mechanism of type I SPases, involving a serine–lysin catalytic dyad (Dalbey et al., 1997; Paetzel & Dalbey, 1997; Paetzel et al., 1998), as an alkaline pH would allow the active-site lysine residue to act as a general base in catalysis. Compared to the E. coli SPase, which has optimal activity between pH 8.5 and 9 (Zwizinski et al., 1981), sf-SipS-His (Bam) displayed a slightly higher pH optimum (pH 10). Similarly, the lipoprotein-specific (type II) SPase of E. coli, which appears to belong to a novel family of aspartic proteases (Tjalsma et al., 1999b), was shown to have optimal activity at alkaline pH (Tokunaga et al., 1985). Even though the high pH optima of these SPases are consistent with their proposed catalytic mechanisms, they are in apparent conflict with the fact that the catalytic sites of type I and type II SPases are probably located at the outer surface of the cytoplasmic membrane (Dalbey et al., 1997; Tjalsma et al., 1999b), which has an acidic pH. In fact, the pH at the outer surface of the membrane is likely to be lower than 6, due to the transmembrane H⁺ gradient that is present in living cells. At such low pH values, the type I SPases at least are barely active, suggesting that their activity could be regulated by the pH. Thus, the potentially deleterious proteolysis of membrane proteins by SPases would be minimized in the acidic environment at the outer membrane surface, until such enzymes are activated to perform their specific task by interactions with pre-proteins or other, as yet unidentified, components of the protein export machinery. Alternatively, the pKa of the active-site lysine residue of type I SPases could be lowered by the hydrophobic interactions with membrane components, such as phospholipids. Consistent
with the latter hypothesis, the crystal structure of the E. coli SPase indicates that hydrophobic interactions between the side-chain of the active-site lysine residue and various other residues, such as phenylalanine 133 and tyrosine 143, are important for catalysis (Paetzel et al., 1998). This view is supported by the recent observation that the equivalents of the latter two residues in the B. subtilis SPase SipS (i.e. leucine 74 and tyrosine 81) are required for the catalytic activity of SipS (Bolhuis et al., 1999a). Whether the hydrophobic environment of the membrane contributes to the lowering of the $K_a$ of the active-site lysine residue of type I SPases is presently unknown.

Zwizinski et al. (1981) reported that Mg$^{2+}$ can have an inhibitory effect on the activity of the E. coli SPase I. Interestingly, 5 mM Mg$^{2+}$ does not seem to inhibit sf-SipS (Bam). Thus, the presence of MgSO$_4$ cannot be the reason for the observed incomplete processing of pre-A2-AmyL shown in Fig. 4(a). Similar levels of processing were detected in the same buffer (HEPES, pH 7.0 or pH 8.0) without MgSO$_4$, as shown in Fig. 5(a). The most likely explanation of why pre-A2-AmyL preparations are not completely processed is that some molecules of this precursor become incompetent for processing. In vitro this can be due to (micro)aggregation of the precursor, which leads to translocation and processing incompetence (van Wely et al., 1998). In fact, this would explain why the use of different precursor preparations resulted in different maximum levels of processing. Moreover, even in the living cell, pre-proteins can become incompetent for processing due to folding of the mature protein before processing (van Dijl et al., 1991b; Bolhuis et al., 1999c). Notably, the pH-, temperature- and time-dependence of processing was not influenced when different pre-protein preparations were used (data not shown), indicating that the incomplete processing of pre-A2-AmyL, as observed in the present studies, has no bearing on our conclusions.

Finally, our present results show that sf-SipS-His (Bam) is prone to degradation and that this seems to be even worse for sf-SipC, sf-SipC-His and sf-SipS (Bsu). This suggests that the latter three proteins are more sensitive to degradation than sf-SipS-His (Bam). However, we cannot exclude the possibility that the different production levels reflect different expression levels. Most likely, the degradation of the soluble SPase mutant proteins used in these studies is due both to self-cleavage and to cleavage by cytosolic proteases of E. coli (van Roosmalen et al., 2000). In fact, as exemplified by sf-SipS-His (Bam), proteolysis is a major bottleneck for the overproduction, purification and structural analysis of soluble derivatives of Bacillus SPases. At present, we are trying to develop novel strategies to overcome this problem.

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