In vitro processing of glutamyl endopeptidase proenzymes from Enterococcus faecalis and importance of N-terminal residue in enzyme catalysis

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Abstract: Glutamyl endopeptidase from Enterococcus faecalis, designated SprE, is one of the important virulence factors secreted as zymogen. In the present study we expressed recombinant SprE proenzyme (pro-SprE) in Escherichia coli and investigated the in vitro processing to mature SprE. It was found that trypsin could efficiently produce the active form of SprE with the N-terminus Ser through cleavage between Arg and Ser bond, which was subsequently auto-degraded into inactive species through the cleavage at the Glu-Asp and Glu-Val bonds. Although thermolysin could produce SprE with the N-terminus Leu, but possessed no proteolytic activity. In contrast to the absolute requirement of the N-terminal Val in staphylococcal glutamyl endopeptidases, the N-terminal Ser of mature SprE could be substituted by other amino acids despite that Ser showed the maximal activity. Substitution of penultimate Leu to Val also reduced the activity to 40% of the wild type. Taken together, we conclude that pro-SprE was converted to mature form with the N-terminus Ser by a protease with specificity of trypsin and the length of the N-terminal region rather than specific residue is absolutely required for enzyme activity.

Keywords: Glutamyl Endopeptidase, Proenzyme Processing, Spre, Enterococcus faecalis, Gluv8

1. Introduction

Enterococcus faecalis is a gram-positive cocci and common inhabitant of human gastrointestinal and genitourinary tract [1]. It causes a wide variety of diseases in human, infecting the urinary tract, endocardium, abdomen, biliary tract, burn wounds and indwelling foreign devices [2]. In dentistry, E. faecalis has been associated with caries lesions, chronic periodontitis, recurrent root canal infection and persistent apical periodontitis [3, 4]. Candidate virulence factors of E. faecalis include: cytolysin and proteolytic enzyme (gelatinase and serine protease), adhesions (agglutination substance, enterococci surface protein or Esp, collagen adhesion protein or Ace, antigen A or EfaA) and capsular and cellular wall polysaccharides [2, 5]. Serine protease, gelatinase and collagen-binding protein (Ace) facilitate E. faecalis bind to dentin [6]. Several studies have been undertaken to investigate the possible role of gelatinase and serine proteases in the disease processes caused by E. faecalis and they were found to contribute to the pathogenesis through biofilm formation, facilitating bacterial invasions, degradation of immune peptides required for host response [7, 8, 9,10]. Gelatinase (GelE) and serine protease (SprE) expressed in E. faecalis were reported to be important for enterococcal virulence in mouse peritonitis model [11]. Previous studies also indicated that the presence of extracellular proteases, GelE and SprE, in the E. faecalis raised mortality in animal models [12, 13].

Glutamyl endopeptidase (EC 3.4.21.19) from Staphylococcus aureus V8 strain (GluV8) is a serine protease with unique substrate specificity to Glu-X and Asp-X with the higher preference to the former [14,15]. This family protease from E. faecalis SprE, which has been shown to contribute to pathogenesis in animal models [16, 11, 17]. Kawalec et al. [18] purified several isoforms of
mature SprE, starting with N-terminal Ser1 and Leu2 from wild-type *E. faecalis* OG1RF (TX4002) and a gelatinase-null mutant TX5264, among which SprE starting with Ser1 was super active. Moreover, they suspected the instability of active SprE (Ser1-SprE form) apparently due to auto-degradation.

SprE shows sequence homology with GluV8 (27% identity, 49% similarity) [19] and that from *S. epidermidis* (GluSE) (26% identity, 49% similarity) [20]. It has been reported that the proenzyme of GluV8 is processed by a thermolysin-family metalloprotease, aureolysin in vivo [21,22]. The prosequence of *Staphylococcus* GluV8-family protease was reported to sequentially remove to shorter prosequence of *Staphylococcus* aureolysin at N-terminal Asn-X (X=Ala, Phe, Gly and Ser) [25]. Exceptionally, the N-terminal Ser of the active SprE was numbered as 1 and Leu2 tagged recombinant proteins were expressed and purified as described previously [25]. Protein concentrations were determined by the absorbance at 280 nm.

2. Materials and Methods

2.1. Materials

The materials used and their sources were as follows: expression vector pQE60, from Qiagen Inc. (Chatsworth, CA, USA); low-molecular-weight markers, from GE Healthcare (Buckinghamshire, England); restriction enzymes and DNA-modifying enzymes, from Nippon Gene (Tokyo, Japan); KOD Plus DNA polymerase, from Toyobo (Tokyo, Japan); Talon metal-affinity resin, from Clontech Laboratories Inc. (Palo Alto, CA, USA); Z-Leu-Leu-Glu-MCA (LLE-MCA), Ac-Thr-Val-Ala-Asp-MCA (TVAD-MCA), Z-Ala-Ala-Asn-MCA (AAN-MCA) and Boc-Gln-Ala-Arg-MCA (QAR-MCA) from the Peptide Institute Inc. (Osaka, Japan); Leu-Asp-MCA (LD-MCA) and Z-Leu-Leu-Gln-MCA (LLQ-MCA), synthesized by Thermo Fisher Scientific (Ulm, Germany); thermolysin from *Bacillus thermoproteolyticus* rokko, trypsin procine pancreas, bovine serum albumin (BSA), were from Sigma-Aldrich (St. Louis, MO, USA); and gelatin from the NacalaiTesque. Inc. (Osaka, Japan).

2.2. Expression Vector for the Expression of SprE

The SprE gene was amplified by PCR using KOD Plus DNA polymerase and chromosomal DNA (0.1 µg) of *E. faecalis* NCTC 775 as template. The synthetic oligonucleotides primers (5'-ATGGATCCAAAAAGTTCTCCATACGAAAAATTA G-3') and (5'-GTGGATCCGCTGCAGGCGAGCGGATAACG-3') containing BamHI sites (underlined) were designed on the basis of the DNA sequence of SprE (GeneBank accession no. Z12296), used for SprE gene amplification. PCR-amplified 0.8-kb fragment (without stop codon) was cleaved with BamHI, and then inserted into the BamHI site of pQE60 to yield pQE60-SprE expression vector.

2.3. Expression and Purification of Recombinant Proteases

In order to minimize the modification in the N-terminal presequence of SprE, the expression vector pQE60 that encoded an affinity tag, [Gly-Ser-Arg-Ser-(His)6]-Z-Leu2 (X=Ala, Phe, Gly and Ser) [21,22]. Exceptionally, the Val1 substitution to Leu1 could partially (30%) retained proteolytic activity of the wild type, further indicating the significance of Val1 for the protease activity [25]. Sequence alignment indicates that Val1 of GluV8 corresponds to Leu1 of SprE, whereas it has been reported that SprE has N-terminal Leu2 (Leu2-SprE) had a significantly negligible activity compared to that with N-terminal Ser1 [21,22]. Moreover, the processing mechanism of pro-SprE still remains unknown. Therefore, we here investigated the in vitro processing of pro-SprE expressed in *E. coli* and addressed the roles of N-terminal amino acids in the enzyme catalysis.

2.4. Amino Acid Numbering and In Vitro Mutagenesis

The N-terminal Ser of the active SprE was numbered as the first amino acid residue (Ser1). *In vitro* mutagenesis was performed as reported previously [30] by PCR with mutated primer(s) to substitute 3 amino acids in the prosequence (Glu15Ser, Glu14Lys, Glu8Ile, designated as SprE-mut), 4 amino acids in the mature region (Glu11Gln, Glu6Gln, Ser1Thr/Ala/Val and Leu2Val), and an essential Ser180 to Ala. All mutations were confirmed by DNA sequencing.

2.5. SDS-PAGE and Zymography

Recombinant proteins (1 µg) were separated by SDS-PAGE at a polyacrylamide concentration of 12.5% (w/v), and then stained with Coomassie Brilliant Blue
polyacrylamide gel containing 1mg/ml of gelatin (instead of proform) were incubated at 37 °C for 1 h with 20µM MCA peptides in 0.2 ml of 50 mM Tris-HCl (pH 8.0) containing 0.001% NaN₃ and 0.003µg of trypsin (molar ratio 3300:1) at 37 °C, unless otherwise stated. Thereafter, proteins (0.25 µg as proform) were incubated at 37 °C for 1 h with 20µM MCA peptides in 0.2 ml of 50 mM Tris-HCl (pH 8.0) containing 5 mM EDTA. The fluorescence was measured with excitation at 380 nm and emission at 460 nm with a Fluorescence Photometer F-4000 (Hitachi, Tokyo, Japan).

2.7. N-terminal Amino Acid Sequencing

N-terminal amino acid sequences of recombinant SprE and its derivatives were determined after separation by SDS-PAGE (2µg) and transference to a polyvinylidenedifluoride membrane (Sequi-Blot PVDF, Bio-Rad, Hercules, CA, USA). After having been stained with CBB, the bands were excised; and directly sequenced with a Procise 49XcLC protein sequencer (ABI, Foster City, CA, USA).

3. Results

3.1. Expression and In Vitro Processing of SprE

When the full-length pro-SprE was expressed in E. coli, a mixture of 34- and 35-kDa proteins was recovered by affinity purification (Fig. 1a, upper panel, lane 1). Amino acid sequencing revealed that the 35-kDa band started at Glu¹¹ and 34-kDa one at Ser⁷, indicating the autocatalytic prosequence cleavage between the Glu¹¹-Glu¹⁴ and Glu⁸-Ser⁷ bonds, respectively (Table 1). The 34-35-kDa SprE showed negligible glutamic acid-specific peptidase activity, as recombinant SprE still carried part of the propeptide (Fig.1b,c upper; d). Staphylococcal GluV8-family proteases were reported to be processed into mature form by thermolysin cleaving at Asn¹¹-Val¹² in vitro [29]. To investigate heterocatalytic maturation of pro-SprE, we incubated 34-35-kDa recombinant pro-SprE with trypsin or thermolysin, expected that may induce processing at Arg¹¹-Ser¹ and at Ser¹-Leu² sites respectively. Consequently, trypsin treatment accompanying a slight decrease in the apparent molecular mass induced an acquisition of the gelatinase activity (Fig. 1c, lanes 3-10). In contrast, thermolysin treatment did not accompany an apparent increased gelatinase activity, although the apparent molecular mass was decreased. Very faint gelatinase activities were found for thermolysin treated or untreated samples (Fig 1b lower). In consistent to these findings, the Glu-specific peptidase activity was measured for the trypsin treated SprE in a dose-dependent manner (Fig 1d).

(Fig 1. A schematic presentation of pro-SprE expressed in E. coli. (a) Amino acid sequences of pro-pro and N-terminal regions of mature SprE. The open (V) and closed (X) arrow-head showing the trypsin (Tryp) and thermolysin (Th) processing site respectively, arrow(↓) indicates the site of autodegradation in the pro- and mature region. (b,c) In vitro processing of the SprE proenzymes and Z-LLE-MCA cleavage activity of the mature SprE. Pro-SprE (10µg) were incubated for 2 h at 0°C (lane1) or at 37°C (lane2) without Thermolysin (Th)/Trypsin (Tryp) and with Thermolysin (Th)/Trypsin (Tryp) and with Th-0.001µg/Tryp-0.0001µg(lane3), Th-0.003/Tryp-0.0003µg (lane4), Th-0.01µg/Tryp-0.001µg (lane5), Th-0.03µg/Tryp-0.003µg (lane6), Th-0.1µg/Tryp-0.01µg (lane7), Th-0.3µg/Tryp-0.03µg (lane8), Th-1.0µg/Tryp-0.1µg (lane9), and Th-3.0µg/Tryp-0.3µg (lane10). Aliquots (1µg or 0.25µg) of each Thermolysin/Trypsin treated samples were subjected to SDS-PAGE and then stained with CBB (b,c upper) or subjected to collagen-Zymography (b,c lower) respectively. (d) The proteolytic activities (means ± SD; n=3) towards Z-LLE-MCA of the thermolysin (Th) and trypsin (Tryp) treated samples were determined as described in the materials and methods.)
Ser\(^1\) (Ser\(^1\)-SprE) produced by trypsin readily auto-degraded to the Val\(^{12}\)-SprE. However, the possibility that Val\(^{12}\)-SprE represents a genuine mature species with the peptidase activity was not completely eliminated, if considered that N-terminal Val\(^1\) was commonly observed in Staphylococcal mature glutamyl endopeptidases [29]. To address these possibilities, we tried to increase recovery of Ser\(^1\)-SprE quantitatively after trypsin processing by introducing amino acid substitutions in the N-terminal region of the mature SprE as in the following section.

3.2. Suppression of Auto-Degradation of Mature SprE

The autodegradation of Staphylococcal glutamyl endopeptidases occurring within the prosequence region was efficiently suppressed by the substitution of Glu and Asp in the prosequences, to Gln, Asn or other amino acids [25, 30]. Here, this strategy was introduced at the N-terminal region of mature SprE. When Glu\(^{11}\) of SprE was substituted to Gln (SprE-Glu\(^{11}\)Gln), the 31-kDa mature SprE band was more intensively recovered after trypsin treatment of the purified proenzyme (Fig-2a upper lane6), which accompanied by an increased peptidase activity in the fraction (Fig-2a lower).

Because there was Glu\(^6\) at position nearer to the N-terminus, this residue was also substituted by Gln (SprE-Glu\(^{6}\)Gln/Glu\(^{11}\)Gln). As a result, the recovery was further increased after trypsin treatment (Fig-2a upper lane9). It is noticeable that the specific activity of the mutant form was not varied (Fig-2b), whereas the recovery after the processing was increased by suppression of the auto-degradation. Even when thermolysin treatment was conducted on these mutants, no increase in recovery (Fig-2a upper lane 5 &8) as well as in the activity (Fig-2a lower panel) was observed again. The N-terminus of trypsin-processed SprE-Glu\(^{11}\)Gln was a mixture of Ser\(^1\) and Asp\(^7\) and that of SprE-Glu\(^{6}\)Gln/Glu\(^{11}\)Gln was Ser\(^1\). Taken together with the activity measurement, it was concluded that Ser\(^1\)-SprE was the active and mature form and that Val\(^{12}\)-SprE as well as Asp\(^7\)-SprE was an inactive auto-degraded product (Table 1).

3.3. Substrate Specificity of Pro- and Mature SprE

Glutamyl endopeptidase cleaved peptide bond between Glu-X with highest efficiency and much less efficiently between Asp-X [15]. We investigated the substrate specificity of pro- and mature SprE with several peptidyl-MCA substrates to know whether any possibility of propeptide processing in an autocatalytic manner. Mature Ser\(^1\)-SprE specifically hydrolyzed LLE-MCA, and did not hydrolyze TVAD-, LD-, AAN-MCA as well as GAR-MCA, whereas it possessed very faint activity for LLQ-MCA after 1h incubation (Fig-3).

![Fig 3. Substrate specificity of SprE. Substrate specificity of pro- and mature SprE was determined with different MCA-peptides as shown in the figure below. Proteolytic activity towards Z-LLE-MCA of trypsin processed mature SprEwt set as positive control and considered 100%. Pro-SprE (10µg) was incubated for 2h at 37°C without or with 0.003µg trypsin (molar ratio 3300:1), thereafter 0.25µg of each sample was used for different MCA-peptides proteolytic assay as discussed in the materials and methods.](image)
Table 1. N-terminal amino acid sequences of recombinant SprE and its derivatives

<table>
<thead>
<tr>
<th>SprE derivatives</th>
<th>Thermolysin (Th)/Trypsin (Tryp)</th>
<th>Cleavage site (X/-X)</th>
</tr>
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<tbody>
<tr>
<td>pro-SprE</td>
<td>-</td>
<td>E&lt;sup&gt;10&lt;/sup&gt;/E&lt;sup&gt;14&lt;/sup&gt;YIVPAE&lt;sup&gt;8&lt;/sup&gt;S'ROKRSLDPED</td>
</tr>
<tr>
<td>pro-SprEmut</td>
<td>-</td>
<td>SHSQ&lt;sup&gt;2&lt;/sup&gt;K&lt;sup&gt;3&lt;/sup&gt;RSLLDPEDRRQ</td>
</tr>
<tr>
<td>SprEwt</td>
<td>Tryp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SHSQKR&lt;sup&gt;1&lt;/sup&gt;S'/LLDPEDRRQ</td>
</tr>
<tr>
<td>SprE-Glu&lt;sub&gt;11&lt;/sub&gt;Gln</td>
<td>Th</td>
<td>RRQ&lt;sup&gt;1&lt;/sup&gt;/V&lt;sup&gt;12&lt;/sup&gt;ADTTEA</td>
</tr>
<tr>
<td>SprE-Glu&lt;sub&gt;6&lt;/sub&gt;Gln/Glu&lt;sub&gt;11&lt;/sub&gt;Gln</td>
<td>Tryp</td>
<td>SHSQKR&lt;sup&gt;1&lt;/sup&gt;/S&lt;sup&gt;3&lt;/sup&gt;/LLDPQDRRQ</td>
</tr>
<tr>
<td>SprE-Glu&lt;sub&gt;6&lt;/sub&gt;Gln/Glu&lt;sub&gt;11&lt;/sub&gt;Gln/Ser1Thr</td>
<td>Tryp</td>
<td>SHSQKR&lt;sup&gt;2&lt;/sup&gt;A'/LLDPQDRRQ</td>
</tr>
<tr>
<td>SprE-Glu&lt;sub&gt;6&lt;/sub&gt;Gln/Glu&lt;sub&gt;11&lt;/sub&gt;Gln/Ser1Ala</td>
<td>Tryp</td>
<td>SHSQKR&lt;sup&gt;2&lt;/sup&gt;/V'LLDPQDRRQ</td>
</tr>
<tr>
<td>SprE-Leu&lt;sub&gt;2&lt;/sub&gt;Val</td>
<td>Th</td>
<td>SHSQKR&lt;sup&gt;1&lt;/sup&gt;/V'&lt;sub&gt;12&lt;/sub&gt;LLDPQDRRQ</td>
</tr>
</tbody>
</table>

<sup>a</sup> A mixture of two polypeptides; Detected N-terminal amino acids of the SprE species were underlined.

The substrate specificity of SprE strongly suggested that pro-SprE could not be processed to active SprE as either pro- or mature SprE unable to cleave GAR-MCA. More interestingly, pro-SprE possessed a faint activity for LLE- and LLQ-MCA. These results strongly suggested that the propeptide shortening via cleavages at the Glu<sup>-15</sup>-Glu<sup>-14</sup> or Glu<sup>-8</sup>-Ser<sup>-7</sup> bond observed in the purified pro-SprE (Fig-1 a,b lane 1,2) was mediated by pro-SprE, but not by mature SprE and finally maturation achieved by cleaving between Arg<sup>-1</sup>-Ser<sup>1</sup> in a hetero catalytic manner.

3.4. Significance of Ser<sup>1</sup> and Leu<sup>2</sup> in the Catalytic Processes

As Val<sup>1</sup> is highly conserved in the mature form of all Staphylococcal glutamyl endopeptidases and is indispensable for enzyme catalysis [26, 29], we examined the requirement of the N-terminal Ser<sup>1</sup> on the activity of SprE. Because the wild-type SprE subsequently underwent auto-degradation after trypsin treatment, we used SprE-Glu<sub>6</sub>Gln/Glu<sub>11</sub>Gln instead of wild-type SprE as control and for mutagenesis. When Ser<sup>1</sup> of SprE-Glu<sub>6</sub>Gln/Glu<sub>11</sub>Gln was mutated to Thr, Ala and Val, the activity of SprE after trypsin treatment was decreased to 55%, 36%, and 31% respectively (Fig. 4a). Thus, the amino acid Ser<sup>1</sup> was required at N-terminal position for the maximal activity and amino acids similar to Ser are more preferable, whereas the extent is not so absolute compared to the Val<sup>1</sup> requirements of GluV8 [26,29]. The substitution of Leu<sup>2</sup> to Val also reduced the activity to 44% of that of wild-type (Fig.4b).

Altogether, these results indicated the N-terminal amino acids involved with the substrate interaction in the enzyme catalysis.

Fig 4. Comparison of proteolytic activities of SprE substitution mutants. (a)Proenzymes of SprE-Glu<sub>6</sub>Gln/Glu<sub>11</sub>Gln and it’s Ser<sup>1</sup>substitution derivatives (Ser1Thr, Ser1Ala and Ser1Val) (10µg) were incubated at 37°C without or with 0.003µg trypsin (Tryp), as described in the materials and methods section. The proteolytic activities (mean±SD; n=3) of the samples were determined with LLE-MCA. (b)Proenzymes of SprEwt and SprE-Leu<sub>2</sub>Val (10µg) were incubated at 37°C without or with 0.3µg thermolysin (Th) and 0.003µg trypsin (Tryp) for 2h and their proteolytic activities (mean±SD; n=3) were measured as discussed in the materials and methods.
4. Discussion

We purified mixture of two SprE proenzymes starting Glu\textsuperscript{14} and Ser\textsuperscript{6} when expressed in E. coli and these proenzymes showed trace Glu-specific activity towards LLE- and LLQ-MCA. When all of the three susceptible Glu in the prosequence [Glu\textsuperscript{15}, Glu\textsuperscript{14} and Glu\textsuperscript{6}] to stop auto degradation in the prosequence, then only one proenzymes starting with Lys\textsuperscript{2} (Lys\textsuperscript{2} pro-SprE) was purified (data not shown). However, no such cleavage was found when catalytically inert SprE (active site Ser\textsuperscript{68} was mutated to Ala) was purified from E. coli lysate (unpublished data). Taken together, we proposed that full-length pro-SprE truncated it’s prosequence to shorterzymogens (Glu\textsuperscript{14} pro-SprE, Ser\textsuperscript{6} pro-SprE or Lys\textsuperscript{2} pro-SprE) by autocatalysis to facilitate final heterocatalytic maturation. Furthermore, absences of any R-X or S-X cleavage activity by the SprE (both pro-and mature) reiterated the inability of final maturation by autocatalysis.

We have been reported for the first time the in vitro processing of glutamyl endopeptidase proenzymes from Enterococcus faecalis, (pro-SprE) expressed in E.coli. In this study we showed that trypsin (molar ratio 3300:1) could efficiently processed pro-SprE to mature SprE in dose-dependent manner, whereas thermolysin (molar ratio 33:1) could also processed leaving nearly inactive SprE (Fig-1d). Sequencing data confirmed that thermolysin processed between Ser\textsuperscript{1}-Leu\textsuperscript{2}. Whereas taken together the sequencing data of trypsin processed SprEwt, SprE-Glu11Gln and SprE-Glu6Gln/Glu11Gln, it was confirmed that trypsin processed between Arg\textsuperscript{1}-Ser\textsuperscript{4} (Table 1) leaving fully active SprE, however the wild-type SprE subsequently underwent auto degradation preferentially between Glu\textsuperscript{11}-Val\textsuperscript{12} and Glu\textsuperscript{6}-Asp\textsuperscript{7} after maturation. E. faecalis strain V583 and TX0411 were reported to carries the htrA gene that produce the trypsin-like serine protease [34,35]. Thus we hypothesize, E. faecalis strains expressing trypsin might efficiently processes secreted pro-SprEzymogens to fully active mature SprE in vivo and might be more pathogenic than strains which do not express trypsin.

It was reported that N-terminal Val\textsuperscript{1} truncated GluV8 (GluV8 starting with Ile\textsuperscript{2}) loose enzyme activity [29]. Moreover, it is known that docking of an N-terminal residue with respect to Ser\textsuperscript{1} and Leu\textsuperscript{2}. Increase hydrophobicity might bury the N-terminal residue more compactly making less available for substrate interaction to S1 site. Thus we hypothesized the N-terminal amino acid residue of the glutamyl endopeptidase is evolutionally conserved for facilitating heterocatalytic maturation and to favour substrate interaction to the active site.

In conclusion, we reported in vitro processing of SprE by trypsin, and investigated the significance of N-terminal residue that may enlighten further study on the in vivo processing and pathogenic importance of SprE.

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Abbreviations

GluV8 & SprE, glutamyl endopeptidase from Staphylococcus aureus and Enterococcus faecalis respectively; ac-, acetyl; boc, t-butyloxycarbonyl; [(2S)-2-amino-3-(benzyloxycarbonyl)propionyl]; Z-, benzyloxycarbonyl; MCA, 4-methylcoumaryl-7-amide, Th, thermolysin; Tryp, trypsin

References


