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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^{1} through cleavage between Arg^{-1} and Ser^{1} bond, which was subsequently auto-degraded into inactive species through the cleavage at the Glu^{6}-Asp^{7} and Glu^{11}-Val^{12} bonds. Although thermolysin could produce SprE with the N-terminus Leu^{2}, but possessed no proteolytic activity. In contrast to the absolute requirement of the N-terminal Val^{1} in *staphylococcal* glutamyl endopeptidases, the N-terminal Ser^{1} of mature SprE could be substituted by other amino acids despite that Ser showed the maximal activity. Substitution of penultimate Leu^{2} of SprE to Val^{2} 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^{1} 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 (aggregation 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 $Ser^{1}$and Leu^{2} from wild-type *E. faecalis* OG1RF (TX4002) and a gelatinase-null mutant TX5264, among which SrpE starting with $Ser^{1}$was super active. Moreover, they suspected the instability of active SprE (Ser^{1}-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 *Staphylococcal* GluV8-family protease was reported to sequentially remove to shorter proenzymes in an autocatalytic manner by cleaving at Glu-X and Gln-X in the prosegment and finally matured by aureolysin at N-terminal Asn^{-1}-Val^{1} [23, 24]. Consistently recombinant GluV8 expressed in *E. coli* was efficiently processed to mature GluV8 by thermolysin in vitro [25]. Val^{1} is conserved at the N-termini of mature glutamyl endopeptidases from *Staphylococcus epidermidis* [26, 27], *Staphylococcal warneri* [28], *Staphylococcal cohnii* and *Staphylococcal caprae* [29].

The N-terminus amino acid residue of mature GluV8-family endopeptidases was found to be critical for maturation by aureolysin and for the stabilization of mature structure resistant to further processing at the Val^{1}-Ile^{2} and Ile^{2}-Leu^{3} bonds [25]. Furthermore, Val^{1} is directly involved in the protease activity itself, because Val^{1} cannot be substituted by other amino acids, even if the processing was correctly mediated with Arg^{-1}-X^{1}-substituted forms by trypsin (X=Ala, Phe, Gly and Ser) [25]. Exceptionally, the Val^{1} substitution to Leu^{1} could partially (30%) retained proteolytic activity of the wild type, further indicating the significance of Val^{1} for the protease activity [29]. Sequence alignment indicates that Val^{1} of GluV8 corresponds to $Leu1^{2}$ of SprE, whereas it has been reported that SprE with N-terminal Leu^{2} (Leu^{2}-SprE) had a significantly negligible activity compared to that with N-terminal Ser^{1} [18]. 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. Materials and Methods

2.1. Materials

The materials used and their sources were as follow: expression vector pQE60, from Qiagen Inc. (Chatsworth, CA, USA); low-molecular-weight markers, from GE Healthcare (Buchinghamshire, 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 thermoproteolyticusrokko*, 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'-GTGGATCCCGCTGCAGGCACAGCGGATAAACG-3') containing *Bam*HI 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 *Bam*HI, and then inserted into the *Bam*HI 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 preprosequence of SprE, the expression vector pQE60 that encoded an affinity tag, [Gly-Ser-Arg-Ser-$(His)_6$] at the C-terminus was used (Fig. 1A). In addition Gly-Gly-Ser derived from the vector was present between 1st-Met and 2^{nd}-Lys of the N-terminal prepropeptide. *Escherichia coli* XL1-blue was transformed with the pQE60-SprE expression plasmids. C-terminal His_6-tagged recombinant proteins were expressed and purified as described previously [25].Protein concentrations were determined by the bicinchoninic acid method using BSA as the standard (Pierce, Rockford, IL, USA). Purified proteins were stored at -80 ˚C until used.

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 (Glu^{-15}Ser, Glu^{-14}Lys, Glu^{-8}Ile, designated as SprE-mut), 4 amino acids in the mature region (Glu11Gln, Glu6Gln, Ser1Thr/Ala/Val and Leu2Val), and an essential Ser^{180} 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

(CBB). Band intensities wer quantified with *Image J* software [31]. Zymography was performed by using a polyacrylamide gel containing 1mg/ml of gelatin (instead of azocasein) according to the method previously described [32]. For zymography, thermolysin- or trypsin-treated samples (0.25µg) were separated on a 12.5% polyacrylamide gel containing 1% (w/v) of gelatin by SDS-PAGE, the gel was incubated twice with 100 ml of 2.5% (v/v) of Triton X100 at 25 ˚C for 20 min, twice for 10 min each time with 100 ml of 50 mMTris-HCl (pH 7.8) containing 30 mMNaCl, and then incubated overnight at 37 ˚C in 100 ml of the new batch of the same buffer containing 0.001% NaN_3. Finally, non-hydrolyzed gelatin in the gel was visualized by CBB staining.

2.6. In Vitro Processing of Recombinant Proteases and the Measurement of the Protease Activity

Recombinant proteins (10 µg) were incubated for 2 h in 0.1 ml of buffer A [10 mM sodium borate (pH 8.0) containing 0.005% (v/v) Triton X100, and 2 mM $CaSO_4$] without or with 0.3 µg of thermolysin (molar ratio of 33:1) 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 mMTris-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 Membrane, 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 Glu^{-14} and 34-kDa one at Ser^{-7}, indicating the autocatalytic prosequence cleavage between the Glu^{-15}-Glu^{-14} and Glu^{-8}-Ser^{-7} 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 Asn^{-1}-Val^{1} 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 Arg^{-1}-Ser^{1} and at Ser^{1}-Leu^{2} 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).

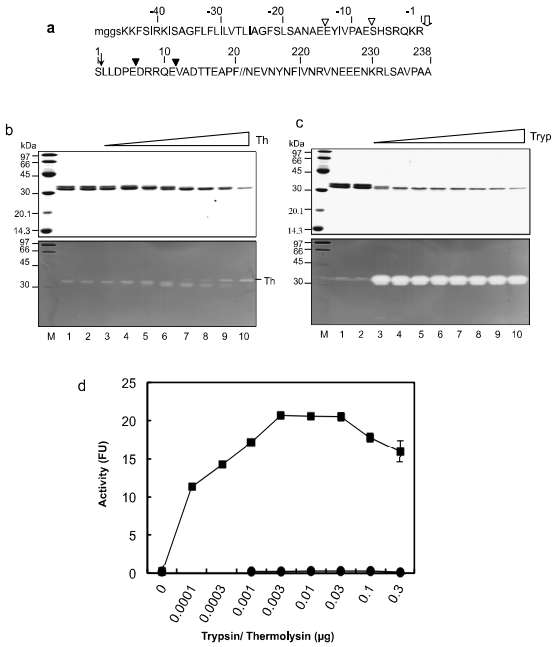

Fig 1. *A schematic presentation of pro-SprE expressed in E. coli. (a) Amino acid sequences of Pre-pro and N-terminal regions of mature SprE. The open (▽) and closed (▼) arrow-head showing the trypsin (Tryp) and thermolysin (Th) processing site respectively, arrow(↓) indicates the site of autodegradation 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 0ºC (Lane1) or at 37ºC (lane2) without Thermolysin (Th)/Trypsin (Tryp) and with Th- 0.001µg/Tryp- 0.0001µg (lane3), Th- 0.003/ Tryp- 0.0003µg (lane4), Th- 0.01µg/Tryp- 0.001 (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.*

N-terminal sequencing of thermolysin-processed 31-kDa species revealed a cleavage at the Ser^{1}-Leu^{2} bond, whereas the N-terminus of trypsin-processed species was a mixture of SprE with N-terminus Val^{12} (major) and Ser^{1} (minor) (Table 1). Since the Glu^{11}-Val^{12} bond should not be processed by trypsin, but by glutamyl endopeptidase itself, it was reasonably suggested that mature SprE with N-terminal

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}-SprEquantitatively 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 proseuqence region was efficiently suppressed by the substitution of Glu and Asp in the proseqquences, 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-Glu11Gln), 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).

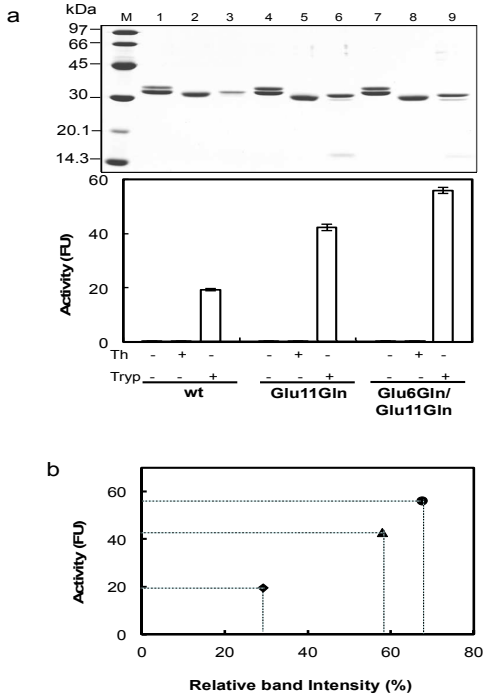

Fig 2. *Prevention of auto-degradation and recovery enhancement of mature SprE. Proenzymes(10µg) of SprE wild-type (wt), single and double amino acid substitution mutants Glu6Gln and Glu6Gln/Glu11Gln respectively were incubated for 2 h at 37ºC without thermolysin (Th)/ trypsin (Tryp) or with Th-0.3µg (Th)/ Tryp- 0.003µg. (Tryp) as described in the materials and methods section. (a) Proteins (1µg) were separated by SDS-PAGE, stained with CBB and proteolytic activities (mean ± SD; n=3) of the samples were determined with LLE-MCA as per materials and methods section. (b) The band intensities of the trypsin treated mature SprE wild-type (wt) ( ), Glu6Gln (▼) and Glu6Gln/Glu11Gln (●) (1µg) were determined by using image J software [31]. Band intensities of pro-SprE set as 100%.*

Because there was Glu^{6} at position nearer to the N-terminus, this residue was also substituted by Gln (SprE-Glu6Gln/Glu11Gln). 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-Glu11Gln was a mixture of Ser^{1} and Asp^{7} and that of SprE-Glu6Gln/Glu11Gln 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).

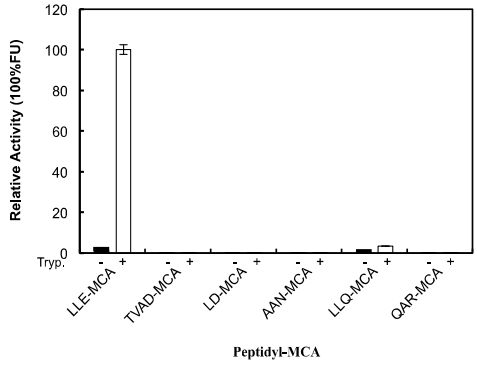

Fig 3. *Substrate specificity of SprE. Substrate specificity of pro- and mature SprE was determined with different MCA-peptides as shown in the figure bellow. Proteolytic activity towards Z-LLE-MCA of trypsin processed mature SprEwt set as positive control and considered 100%. Pro-SprE (10µg) was incubated for 2h at 37ºC without or with 0.003µg trypsin (molar ratio 3300:1), there after 0.25µg of each sample was used for different MCA-peptides proteolytic assay as discussed in the materials and methods.*

Table 1. *N-terminal amino acid sequences of recombinant SprE and its derivatives*

SprE derivatives	Thermolysin (Th)/Trypsin (Tryp)	Cleavage site (X-/-X)
pro-SprE	-[a]	E^{-15}/$\underline{E}^{-14}$ $\underline{YIVPAE}^{-8}$/$\underline{S}^{-7}\underline{RQKRSLLDPED}$
pro-SprEmut	-	$SHSQ^{-3}$/$\underline{K}^{-2}\underline{RSLLDPEDRRQ}$
	Th	$SHSQKRS^{1}$/$\underline{L}^{2}\underline{LDPED}RRQ$
SprEwt	Tryp[b]	$SHSQKR^{-1}$/$\underline{S}^{1}\underline{LLDPE}DRRQ$
		$RRQE^{11}$/$\underline{V}^{12}\underline{ADTTE}A$
SprE-Glu11Gln	Th	KRS^{1}/$\underline{L}^{2}\underline{LDPED}RRQ$
	Tryp[c]	$SHSQKR^{-1}$/$\underline{S}^{1}\underline{LLDPE}DRRQ$
		$KRSLLDPE^{6}$/$\underline{D}^{7}\underline{RRQQV}ADT$
SprE-Glu6Gln/Glu11Gln	Tryp	$SHSQKR^{-1}$/$\underline{S}^{1}\underline{LLDPQD}RRQQ$
SprE-G6Gln/Glu11Gln/Ser1Thr	Tryp	$SHSQKR^{-1}$/$\underline{T}^{1}\underline{LLDPQD}RRQQ$
SprE-G6Gln/Glu11Gln/Ser1Ala	Tryp	$SHSQKR^{-1}$/$\underline{A}^{1}\underline{LLDPQD}RRQQ$
SprE-G6Gln/Glu11Gln/Ser1Val	Tryp	$SHSQKR^{-1}$/$\underline{V}^{1}\underline{LLDPQD}RRQQ$
SprE-Leu2Val	Th	$SHSQKRS^{1}$/$\underline{V}^{2}\underline{LDPED}RRQ$
	Tryp[b]	$SHSQKR^{-1}$/$\underline{S}^{1}\underline{VLDPE}DRRQ$
		$RRQE^{11}$/$\underline{V}^{12}\underline{ADTTE}A$

[abc] 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^{-15}-Glu^{-14} or Glu^{-8}-Ser^{-7} 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^{-1}-Ser^{1} in a hetero catalytic manner.

3.4. Significance of Ser^1 and Leu^2 in the Catalytic Processes

As Val^1 is highly conserved in the mature form of all *Staphylococcal* glutamyl endopeptidases and is indispensible for enzyme catalysis [26, 29], we examined the requirement of the N-terminal Ser^1 on the activity of SprE. Because the wild-type SprE subsequently underwent auto-degradation after trypsin treatment, we used SpreE-Glu6Gln/Glu11Gln instead of wild-type SprE as control and for mutagenesis. When Ser^1 of SpreE-Glu6Gln/Glu11Gln 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^1 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^1 requirements of GluV8 [26,29].The substitution of Leu^2 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.

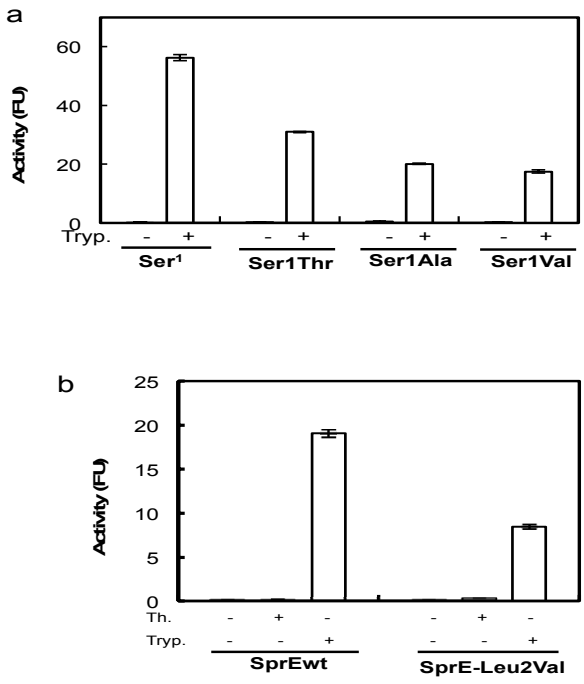

Fig 4. *Comparison of proteolytic activities of SprE substitution mutants. (a)Proenzymes of SprE-Glu6Gln/Glu11Gln and it's Ser^1substitution derivatives (Ser1Thr, Ser1Ala and Ser1Val) (10μg) were incubated at 37℃ without or with 0.003μg trypsin (Tryp), as described in the materials and methods section. The proteolytic activities (mean±SD; n=3) of the samples were determined with LLE-MCA. (b)Proenzymes of SprEwt and SprE-Leu2Val (10μg) were incubated at 37℃ 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^{-14} and $Ser^{-7}$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^{-15}, Glu^{-14} and Glu^{-8}] to stop auto degradation in the prosequence, then only one proenzymes starting with Lys^{-2} (Lys^{-2} pro-SprE) was purified (data not shown). However, no such cleavage was found when catalytically inert SprE (active site Ser^{180} 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 shorter zymogens (Glu^{-14} pro-SprE, Ser^{-7} pro-SprE or Lys^{-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^{1}-Leu^{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^{-1}-Ser^{1}(Table 1) leaving fully active SprE, however the wild-type SprE subsequently underwent auto degradation preferentially between Glu^{11}-Val^{12} and Glu^{6}-Asp^{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-SprE zymogens 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^{1} truncated GluV8 (GluV8 starting with Ile^{2}) loose enzyme activity [29]. Moreover, it is known that docking of an N-terminal residue of a chymotrypsin family serine protease affected substrate-binding sites of the enzyme [36]. Thus the length of N-terminal region was indispensible for the glutamyl endopeptidase activity and in absence of trypsin like protease; other thermolysin like protease(s) might processes SprE at Ser^{1}-Leu^{2} leaving Leu^{2} as N-terminal amino-acids that still retained very faint residual geltinase activity.

As the wild-type active SprE rapidly underwent autodegradation at Glu^{11}-Val^{12} and Glu^{6}-Asp^{7} (Table-1), we mutated the susceptible Glu- residues ($Glu^{11}$and Glu^{6}) to Gln- and found that the recovery of mature SprE following trypsin treatment was greatly enhanced (approximately two-fold increase when Glu^{11} was mutated and about 2.4-fold when both Glu^{11} and Glu^{6} were mutated) (Fig-2). However we did not find any auto-degradation between Glu^{17}-Ala^{18} that was reported to be the primary auto-degradation site [18]. We supposed that auto degradation first attacked at Glu^{11}-Val^{12} and then the enzymetically inactive degraded products underwent further sequential degradation at many points including Glu^{17}-Ala^{18}.

Staphylococcal GluV8 family proteases were reported to conserve Val^{1} as N-terminal amino acid and indispensable in enzyme catalysis [14,25,29].To investigate such importance of Ser^{1} of mature SprE, we mutated Ser^{1} to Thr, Ala and Val. In our surprise we found such substitutions were not significantly affect the enzyme activity as in the case of Val^{1} of GluV8. More over Leu^{2} substitution to Val also reduced Glu-specific activity (SprE-Leu2Val showed 44% activity of that of the SprEwt) after maturation by trypsin. However, thermolysin again could not raise protease activity even after processing at Val^{2} position. The possible effects of substitution of N-terminal Ser^{1}or Leu^{2} on SprE activity might be due to the difference in structure as well as in hydrophobicity with respect to Ser^{1} and Leu^{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 endoppetidase 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

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