

Characterization of the Glutamyl Endopeptidase from *Staphylococcus aureus* Expressed in *Escherichia coli*

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Running title

V8 protease prosegment in chaperoning and enzyme suppression

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Abstract V8 protease, a member of glutamyl endopeptidase I family, of *Staphylococcus aureus* V8 strain (GluV8) is widely used for proteome analysis because of its unique substrate specificity and resistance to detergents. We here developed an *Escherichia coli* expression system for GluV8 as well as its homolog from *Staphylococcus epidermidis* (GluSE), and investigated the roles of the prosegments and two specific amino acid residues, Val₆₉ and Ser₂₃₇. C-terminal His₆-tagged pro-GluSE was successfully expressed from the full-length sequence as a soluble form. In contrast, GluV8 was poorly expressed by the system due to auto-degradation; however, it was efficiently obtained by swapping its prosegment with that of GluSE or by the substitution of four residues in the GluV8 prosequence to those of GluSE. The purified proGluV8 was converted to the mature one *in vitro* by thermolysin treatment. The prosegment was essential not only for the suppression of the proteolytic activity, but also for proper folding of GluV8, indicating its role of intra-molecular chaperoning. Furthermore, the four amino acid residues at the C-terminus of the prosegment were sufficient for both of these roles. *In vitro* mutagenesis revealed that Ser₂₃₇ was essential for the proteolytic activity and that Val₆₉ was indispensable for the precise cleavage by thermolysin and was involved in the proteolytic reaction itself. This is the first study that quantitatively expressed GluV8 in *E. coli* and explicitly demonstrated the intra-molecular chaperone activity of the prosegment of glutamyl endopeptidase I.

Keywords

V8 protease; glutamyl endopeptidase; chaperone; *Staphylococcus aureus*; *Staphylococcus epidermidis*

Abbreviations

GluV8, glutamyl endopeptidase I of *Staphylococcus aureus*; GluSE, GluV8 homologue of *Staphylococcus epidermidis*; CBB, Coomassie brilliant blue; PAGE, polyacrylamide gel electrophoresis; and SDS, sodium dodecyl sulfate.

Introduction

Staphylococcus aureus produces extracellular proteases, which are regarded as important virulence factors. One of the classically defined exoproteases is a serine protease, GluV8, known also as V8 protease/SspA [1]. GluV8 contributes to the growth and survival of this microorganism in animal models [2], and plays a key role in degrading the cell-bound *Staphylococcus* surface adhesion molecules of fibronectin-binding proteins and protein A [3]. This protease specifically cleaves the peptide bond after negatively charged residues, glutamic acid and less potently aspartic acid, and belongs to the glutamyl endopeptidase I (EC 3.4.21.19) family [4]. The nucleotide sequence encodes a protein of 336 amino acids that includes a prepropeptide consisting of 68 residues (Met₁-Asn₆₈) and a C-terminal tail of 52 residues consisting of a 12-fold repeat of the tripeptide Pro-Asp/Asn-Asn [5]. Drapeau [6] first reported that activation of GluV8 precursor is achieved by a neutral metalloprotease. Shaw *et al.* [7] have recently demonstrated that the GluV8 activation process involves the proteolytic cascade of major extracellular pathogenic proteases of *S. aureus*, including metalloprotease/aureolysin, GluV8/SspA, and cysteine protease, SspB.

Expression of recombinant GluV8 in *Escherichia coli* would be useful to elucidate in detail the roles of the prepro- and C-terminal repeated segments as well as specific amino acid residues involved in the processing and enzymatic activity. One expression study has been reported to date [8], in which mature GluV8 was expressed as a sandwiched fusion protein and recovered from inclusion bodies. The mature protein was obtained by cleavage of the exogenous peptides, denaturation-renaturation, and then ion-chromatography for purification. By using this expression system, they showed that GluV8 with its prepro- and C-terminal repeated sequences deleted was able to fold by itself, although the yield at the denaturation-renaturation step was limited to 20%. Besides the *E. coli* expression, the

expression of a GluV8-family protease from *Bacillus licheniformis* was achieved by use of *Bacillus subtilis* as a host [9]; and that of *Streptomyces griseus*, by *Streptomyces lividans* expression system [10].

A prosegment of proteases is known to function as an intra-molecular chaperone as well as its inhibitory function of protease activity. Winther *et al.* [11] reported that the prosequence of carboxypeptidase Y functions as a chaperone and reduces the rate of nonproductive folding or aggregation. O'Donohue and Baumont [12] demonstrated dual roles of the prosequence of thermolysin in enzyme inhibition and folding *in vitro*. The group further demonstrated that the prosequence of thermolysin acts as an intramolecular chaperone even when expressed in *trans* with the mature sequence in *E. coli* [13]. For GluV8, Drapeau [6] demonstrated that accumulation of proteolytically inactive GluV8 precursor occurred in mutants of an *S. aureus* strain V8 lacking the metalloprotease. That study strongly suggested an inhibitory function of the GluV8 prosequence. However, there is no direct evidence demonstrating the role of the GluV8 prosequence in enzyme inhibition. The intra-molecular chaperone activity of the GluV8 propeptide has been even less characterized. The study by Yabuta *et al.* [8] demonstrated the renaturation of GluV8 without the propeptide, which could be interpreted to mean that the preprosequence is not required for folding of GluV8 [4]. These puzzles will be settled after the establishment of an appropriate expression and activation of a latent form of GluV8 *in vitro*.

We previously characterized a major extracellular protease of *Staphylococcus epidermidis*, designated GluSE [14]. Subsequently, Ohara-Nemoto *et al.* [15] and Dubin *et al.* [16] cloned its structural gene, *gseA*. GluSE consists of 282 amino acids composed of preprosequence (Met₁-Ser₆₆) and mature portion (Val₆₇-Gln₂₈₂). The amino acid sequence of mature GluSE is most similar to that of GluV8 (59.1%) among the glutamyl endopeptidase-family members, whereas the prepropeptide has

only limited similarity, *i.e.*, 23.5% [15]. In the present study, we show that it is possible to express the C-terminal His₆-tagged GluV8 in *E. coli*, the prosegment of which is swapped for that of GluSE. Furthermore, by use of this expression system, we investigated the roles of the propeptide and specific amino acid residues of GluV8. The method described herein should be valuable for studying the properties of glutamyl endopeptidase I in detail.

Results

Expression of the full-length forms of GluSE and GluV8 in *E. coli*

In order to minimize the modification of the N-terminal preprosequence, we used the expression vector pQE60, which encodes an affinity tag [Gly-Ser-Arg-Ser-(His)₆] at the C-terminus of the expressed protein. Also, Gly-Gly-Ser derived from the vector was present between Met₁ and Lys₂ of the N-terminal prepropeptide (Fig. 1). When the full-length of GluSE was expressed in *E. coli*, 29-32-kDa bands were abundant in the purified fraction upon protein staining on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2a, lane 6). For large-scale preparation, it was purified by one-step Talon affinity chromatography; and approximately 18 mg of the recombinant protein was obtained from a 1-liter culture (Fig. 3a).

When the full-length of GluV8 was expressed on a small scale (10 ml) and batch purified by affinity chromatography, a 40-kDa band was found on the immunoblot (Fig. 2b, lane 2). This 40-kDa species was discernable as one of the bands from the purified fraction (Fig. 2a, lane 7). However, our trial of large-scale purification resulted in poor recovery of the GluV8 recombinant protein, i.e., \approx 0.1 mg/l culture (Fig. 3a); and the purity was only approximately 50% (Fig. 3b). Therefore, there was a crucial difference in the recovery between recombinant GluSE and GluV8.

Expression of the preproGluSE-mature GluV8 chimeric protein (proGluSE-matGluV8) in *E. coli*

In contrast to the kinship of the mature portion between GluV8 and GluSE, the identity of their preprosequences is restricted (23.5%), as shown in Fig. 1 [15]. Thus, we suspected that some alteration within the prepro-region was relevant to the poor expression of GluV8. If so, we reasoned

that swapping of the preprosequence of GluV8 with that of GluSE might overcome this difficulty. To test this supposition, the chimeric protein proGluSE-matGluV8 was expressed (Fig. 1). On SDS-PAGE, it migrated to the 44-kDa position, indicating an apparent molecular weight larger than the 40 kDa of the wild-type GluV8 (Fig. 2b, lane 8). Moreover, the Coomassie Brilliant Blue (CBB)-stained band intensity was apparently increased (Fig. 2a, compare lanes 7 and 8). Indeed, in large-scale preparation, it was purified by the one-step Talon affinity chromatography; and 3-6 mg of the recombinant protein were obtained from a 1-liter culture. The purified fraction contained 44-kDa major and 42-kDa minor species (Fig. 4a, lane 1).

Expression of the full-length form of GluV8 with amino acid substitutions

Why was proGluSE-matGluV8 more stably expressed in *E. coli* than was genuine GluV8 full-length form? Noticeably, Glu₆₂ and Glu₆₅ are localized near the processing site Asn₆₈-Val₆₉ of GluV8 and are converted to Gln₆₀ and Ser₆₃, respectively, in GluSE. Hence, if a small amount of active GluV8 happened to be produced during the expression, the Glu₆₂-Gln₆₃ and Glu₆₅-His₆₆ bonds could have been auto-proteolysed. The resulting products, which carried a few residues of the propeptide, potentially could have acquired some proteolytic activity and the cascade activation of the protease could be toxic to host cells.

To test this hypothesis, we expressed the full-length form of GluV8 with substitutions from Glu₆₂ and Glu₆₅ to amino acids of GluSE at equivalent positions, *i.e.*, Gln and Ser, respectively (designated GluV8 2mut). As a result, the appearance of 40-, 42-, and 44-kDa forms in GluV8 2mut did not vary from that of intact GluV8 qualitatively (Fig. 2b, compare lanes 7 and 9), whereas the 42-kDa form became predominant in contrast to the predominance of the 40-kDa form of the wild-type GluV8 (lane

9). Thus, these mutations certainly prevented GluV8 from degradation.

Referring to the prosequence of GluSE, we further introduced 2 additional substitutions, from Ala₆₇ to Pro and from Asn₆₈ to Ser, into GluV8 2mut. The resulting form possessed 4 substitutions, from Glu₆₂, Glu₆₅, Ala₆₇, and Asn₆₈ of GluV8 to Gln, Ser, Pro, and Ser, respectively, of GluSE (Fig. 1b, asterisks, designated GluV8 4mut). Consequently, a 44-kDa species, the value identical to that of proGluSE-matGluV8, was detected on the immunoblot and was even obvious on CBB staining (Fig. 2a and b, lane 10). Taken the electrophoretic profiles, we concluded that the proteolysis of GluV8 was most efficiently suppressed in GluV8 4mut, followed by proGluSE-matGluV8 and then GluSE 2mut.

We assumed that the proteolytic degradation of GluV8 caused the activation of GluV8 and thus was toxic for host cells. To confirm this assumption, we compared the growth rates of the *E. coli* expressing the full-length form of GluV8 and its three derivatives (Fig. 2c). The cells expressing the wild type GluV8 proliferated most slowly at 30°C. The growth was partially accelerated by two amino acid substitutions in the GluV8 propeptide (GluV8 2mut), and further by four substitutions (GluV8 4mut). The cells with the proGluSE-GluV8 chimeric form showed the moderate growth rate between GluV8 2mut and 4mut. This result of the bacterial growth was in accord with the degree of suppression on the auto-proteolytic degradation, indicating the toxicity of the activated proteases for *E. coli* cells.

Indeed, GluV8 4mut as well as proGluSE-matGluV8 could be purified by large-scale preparation, yielding approximately 3-6 mg/l culture. From these data taken together, we concluded that the full-length form of GluV8 could be quantitatively recovered by suppression of the self-degradation either by use of the GluSE prepropeptide or the GluV8 prepropeptide with 4 amino acid substitutions. In the subsequent experiments, we used proGluSE-matGluV8 and GluV8 4mut as the source of

recombinant GluV8. Essentially identical results were obtained on the enzyme activity with either of these recombinant GluV8 species. However, most data presented herein were obtained from proGluSE-matGluV8, because the protein became available at the early stage of our study.

Maturation processing of proGluSE-matGluV8 and GluV8 4mut

It has been reported that native GluV8 is processed to its mature form through cleavage by a thermolysin-family metalloprotease, aureolysin [6, 17]. Hence, proGluSE-matGluV8 was incubated with serial doses of thermolysin. As a result, the 44-kDa protein was converted to a 42-kDa species, and finally 38- and 40-kDa species accumulated (Fig. 4a). The 42-kDa band appearing at a small dose of thermolysin (lane 3) was composed of multiple species with the N-termini of Asn₄₃, Val₄₆, and Ile₅₆; and that at a large dose (lane 6) consisted of a single species with the N-terminus of Ile₅₆ (Table 1). N-termini of 38- and 40-kDa forms were Val₆₉, which coincided with the N-terminus of native GluV8 [5].

Thermolysin-processed recombinant proteins were then subjected to zymography. The caseinolytic activity emerged in a thermolysin dose-dependent manner (Fig. 4b). The major band with the caseinolytic activity was 33 kDa (Fig. 4b), indicating that the non-heated sample of mature GluV8 migrated faster than heated one on SDS-PAGE. We further examined this phenomenon below (see Fig. 7). The proteolytic activity toward the peptide substrate also emerged by thermolysin treatment (Fig. 4c). Thermolysin itself did not possess these activities even at the maximal dose used (Fig. 4b and c). Therefore, we concluded that the 40-kDa form represented the mature form. The 38-kDa form that possessed the identical N-terminus seemed to be further processed at the C-terminal side. We suspected that the Glu₂₇₉-Asp₂₈₀ bond of GluV8 was degraded by auto-proteolytic process. Taken

together with these findings, we concluded that GluV8 mature peptide fused to the GluSE prosegment properly folded, and thus was correctly processed to the mature form by thermolysin *in vitro*.

Next we compared biochemical properties and proteolytic activities between native and recombinant mature forms of GluV8. Native GluV8 was present as two forms of 38- and 40-kDa forms (Fig. 5a). The profile of recombinant GluV8 was essentially identical to that of native GluV8, except for the presence of non-degraded 41-44-kDa bands of the recombinant form, presumably due to insufficient cleavage with thermolysin.

We also determined the N-terminal sequence of the 44-kDa GluV8 4mut. Its N-terminus was Leu₃₀ (Table 1), which was equivalent to the N-terminus (Lys₂₈) of the 44-kDa proGluSE-matGluV8. The Ala₂₇-Lys₂₈ bond of proGluSE-matGluV8 and the Ala₂₉-Leu₃₀ bond of GluV8 4mut appeared to match with the recognition site of signal peptidase I [18]. However, because the borders between the pre- and pro-sequences of GluSE and GluV8 remain to be established, it should be determined that these sites are actually processed in GluSE and GluV8 expressed in *S. epidermidis* and *S. aureus*, respectively.

Role of the prosequence

In order to investigate the role of the propeptide, we expressed GluV8 with a series of truncated propeptides of GluSE. Their N-terminus started from Ile₄₉, Ile₅₆, Asn₆₁, Ser₆₃, Pro₆₅ or Ser₆₆ (Fig. 5a). The minimal one possessed the last amino acid (Ser₆₆) of the GluSE propeptide. The expression levels were varied among constructions, of which the forms starting from Pro₆₅ and Ser₆₆ were poorly recovered. However, all of them could be purified to near homogeneity as 40- to 44-kDa bands. The proteolytic activities of the non-processed molecules were trivial in all cases (Fig. 6d).

When recombinant proteins were processed with thermolysin, the 38- and 40-kDa mature forms were produced in most cases (Fig. 6b, lanes 1-5, Th+). Exceptionally, GluSE Pro₆₅-matGluV8 as well as GluSE Ser₆₆-matGluV8 was thoroughly degraded by thermolysin treatment (lanes 6 and 7 Th+). This finding may cause the low expression of GluSE Pro₆₅-matGluV8 and GluSE Ser₆₆-matGluV8. After the thermolysin processing, the proteolytic activities of truncated molecules containing the sequence from Ile₄₉, Ile₅₆, Asn₆₁ or Ser₆₃ to the last amino acid residue Ser₆₆ of the GluSE prosegment acquired the protease activities comparable to that of proGluSE-matGluV8. To the contrary, GluSE Pro₆₅-matGluV8 showed significantly lower activity, and GluSE Ser₆₆-matGluV8-C scarcely possessed the activity (Fig. 6c). Therefore, the C-terminal tetra-peptide of the propeptide (Ser₆₃-Tyr-Pro-Ser₆₆), which was almost sufficient for the suppression of the protease activity, was also adequate for the intra-molecular chaperone function. We also expressed GluSE-Ser₆₆-matGluV8 with the long N-terminal tag (Met-Arg-Gly-Ser-His₆-Gly) encoded by pQE9 expression vector. The recombinant protein possessed trace proteolytic activity both before and after thermolysin treatment (data not shown). Thus, the length of the propeptide was not critical, but the sequence itself was important for folding and activity suppression of the mature portion.

When analyzed carefully, the proteolytic activities of the non-processed forms were not entirely zero. In particular, the activities of GluV8 with shorter propeptides, *i.e.*, Asn₆₁-Ser₆₆ and Ser₆₃-Ser₆₆, could not be ignored (Fig. 6c, columns 4 and 5). Concerning this result, we should note that the recombinant GluSE Asn₆₁-matGluV8 and GluSE Ser₆₃-matGluV8 were expressed in consideration of the auto-proteolytic sites of GluV8 propeptide, *i.e.*, Glu₆₂-Gln₆₃ and Glu₆₅-His₆₆ bonds, respectively (Fig. 1b). Accordingly, GluV8 auto-processed at these sites may possess weak proteolytic activity as postulated in the experiment of Fig. 2.

Mutation of the essential amino acid Ser₂₃₇

Establishment of the *E. coli* expression system of GluV8 enabled us to investigate by *in vitro* mutagenesis the roles of certain amino acids comprising the protease. As an initial approach, we chose two key amino acids, Ser₂₃₇ and Val₆₉. GluV8 is a serine protease, the active site of which consists of the His₁₁₉, Asp₁₆₁, and Ser₂₃₇ triad [19]. To confirm the role of Ser₂₃₇, its substitution to Ala was introduced to proGluSE-matGluV8 (designated GluV8 Ser237Ala). As a result, GluV8 Ser237Ala showed no caseinolytic or glutamic acid-specific activities (Fig. 7b and c).

As described in Fig. 4, we noticed that the mobility of mature GluV8 on SDS-PAGE was altered by heating of samples in the SDS-sample buffer. Unprocessed GluV8 Ser237Ala as well as the wild type migrated to the 44-kDa position (Fig. 7a). After thermolysin treatment, the mobility of the wild type was shifted to 33 and 38/40 kDa under non-heated and heated conditions in the presence of SDS, respectively (Fig. 7a). The profile of GluV8 Ser237Ala was similar to that of the wild type, although 35- (lane 7) and 41-kDa (lane 8) intermediate forms were additionally observed. The faster migration of processed and non-heated GluV8 strongly suggested its more compact conformation. However, this conformation was not prerequisite for renaturation of the protein, because GluV8 exposed to heat could renature under the conditions of zymography (Fig. 7b, lane 4). This finding indicated that, although the zymography experiment used samples not treated with heating, mature form of GluV8 could be renatured even after the exposure to heat in the presence of SDS.

Role of the N-terminal Val₆₉ in processing of the GluV8 proform

We finally investigated the role of the N-terminal Val₆₉ of mature GluV8. It has been proposed that

α -amino group of the N-terminal Val₆₉ of mature GluV8 interacts with the γ -carboxyl group of Glu of a substrate peptide [19]. If so, since any N-terminal residue except the imino acid Pro possesses an α -amino group, we speculated that Val₆₉ is simply required for processing with thermolysin, which hydrolyzes the amino-side peptide bond of hydrophobic amino acids. To test this idea, we substituted Val₆₉ of proGluSE-matGluV8 to Phe. Additionally, Val₆₉ was replaced by Ala and Gly, as thermolysin cleavage of peptide bonds with these amino acid residues was reported [20]. The 44-kDa mutant forms as well as the wild form were processed to 42-kDa intermediate forms, and further to 40-kDa ones, which indicated that the mutation did not modify the steric structure of GluV8. However, these molecules showed no proteolytic activities (Fig. 8). Strikingly, we found that the N-termini of the processed forms were not the 69th substituted amino acids, but entirely Ile₇₀. These results show that thermolysin attacked the Xaa₆₉-Ile₇₀ bond of the mutant, instead of Ser-Xaa₆₉ (Xaa=Phe, Gly, or Ala) bonds. In consequence, we unexpectedly found that Val₆₉ was indispensable for correct processing at the Ser-Val₆₉ bond by thermolysin and that GluV8 with N-terminal Ile₇₀ had essentially no proteolytic activity.

Role of the N-terminal Val₆₉ in the proteolytic activity

Since Val₆₉ was indispensable for the precise processing at the Ser₆₆-Val₆₉ bond, it was impossible to investigate the role of Val₆₉ in the enzymatic reaction. To overcome this difficulty, we prepared mutant proGluSE-matGluV8, the Ser₆₆ of which was replaced by Arg (designated proGluSE Arg₆₆-matGluV8), because the peptide bond between Arg₆₆ and Val₆₉ can be degraded by trypsin. Indeed, trypsin processing of proGluSE Arg₆₆-matGluV8 faithfully mimicked the thermolysin processing of proGluSE-matGluV8 (Fig. 9a, compare lanes 2 and 6). Concomitantly, its glutamic acid-specific

proteolytic activity was enhanced (Fig. 9b). Although thermolysin treatment of proGluSE Arg₆₆-matGluV8 also increased the activity (Fig. 8b, column 3), the efficiency was less than that of the trypsin treatment (column 4), reflecting the predominance of the non-degraded 42-kDa intermediate (Fig. 9a, lane 5). This should be due to substitution of the P1' site Ser₆₆ by non-preferable Arg. Hence, it became possible to utilize trypsin as the processing enzyme.

Trypsin cleavage of proGluSE Arg₆₆-matGluV8 with Val₆₉ substituted to Ala, Phe, Gly or Ser generated the 40-kDa form with designed N-termini (data not shown). Their glutamic acid-specific proteolytic activities were 4.5% (Ala), 1.4% (Phe), 1.1% (Gly), and 0.6% (Ser) of that of Val₆₉ (Fig. 9b). Therefore, we conclude that Val₆₉ plays an important role in the enzyme reaction itself, although other amino acids, such as Ala, could partially substitute for Val₆₉.

Discussion

The present study succeeded in the expression of GluV8 in *E. coli* as a soluble proform for the first time. This study shed light on the reason why GluV8 has hardly been expressed in *E. coli* to date. The propeptide of GluV8 possesses Glu at positions 62 and 65, and their C-terminal sides undergo auto-proteolysis and the resultant GluV8 with truncated propeptides (Gln₆₃-Asn₆₈ and His₆₆-Asn₆₈) were partially active, though the least so. This may have induced the cascade reaction of GluV8 activation, because recombinant proteins remain inside of *E. coli* cells, in contrast to being secreted from *S. aureus*. The conversion of amino acids adjacent to the processing site from Ala₆₇-Asn₆₈ to Pro-Ser further suppressed the degradation. We currently speculate that an endogenous protease in *E. coli* cleaves the Ala₆₇-Asn₆₈ or Asn₆₈-Val₆₉ bond of GluV8. The substitution of Asn₆₇ to Pro could prevent this

proteolysis, because Pro-Xaa and Xaa-Pro bonds (Xaa=any amino acid) are highly resistant to most proteases.

A chimeric protease has been previously expressed on pro-aminopeptidase-processing protease, *i.e.*, a thermolysin-like metalloprotease produced by *Aeromonas caviae* T64 [21]. The propeptide of the protease could be replaced by that of vibriomysin, a homologue of the protease, which shared 36% amino acid identity. We here demonstrated that the propeptide of GluV8 could be replaced by that of GluSE, although the identity (15.4%) of their prosequences was much lower than the case of the thermolysin-like protease. Taken together, it may be proposed that the amino acid requirement of prosequences for assisting protein folding and inhibiting catalytic activity is lower than requirement for the proteolytic entity. This is further indicated by the finding that the last four residues of the propeptide of GluSE, which are completely varied from those of GluV8, were sufficient for the role of the propeptide of GluV8 (Fig. 1b).

Among glutamyl endopeptidase family members, GluV8 and GluSE are processed by thermolysin-family metalloprotease, aureolysin [6, 17, 22, and this study]. On the other hand, the N-terminus of glutamic acid-specific endopeptidase from *Bacillus licheniformis* was Ser, indicating the processing of the Lys-Ser bond by a protease with trypsin-like specificity [9]. This may be not surprising, because the processing enzyme could be replaced from thermolysin to trypsin by substitution of Ser₆₆ of proGluSE-matGluV8 to Arg₆₆ (Fig. 9). This result indicated that any proteolytic enzyme can activate the glutamyl endopeptidase, if it can properly cleave the processing site.

GluV8 is a serine protease, the His₁₁₉, Asp₁₆₁ and Ser₂₃₇ residues of which form an active triad. In fact, Ser₂₃₇ was essential for the protease reaction. Because GluV8 Ser237Ala was normally processed by thermolysin, its overall structure appeared not to be altered from the active form. Therefore, to

elucidate the mechanism of the suppression of the protease activity and the alteration in the proteolytic activities between the two proteases, crystallographic analyses are now under way in our laboratory by use of GluV8 Ser237Ala and GluSE Ser235Ala.

The prosegment of bacterial proteases, such as thermolysin [12, 13] and subtilisin [23], is indispensable not only for the suppression of the protease activity but also for the proper folding of the protease. An inhibitory role of the propeptide has been also postulated for GluV8, because the GluV8 precursor was specifically activated by metalloprotease, aureolysin [6]. However, its direct evidence has not been presented to date. The present study confirmed this role. To the contrary, the intra-molecular chaperone activity of the GluV8 propeptide has been scarcely investigated to date, primarily because an appropriate expression system of GluV8 had not been available. A previous study indicated that the prosequence of GluV8 is dispensable for the folding, as the active enzyme was recovered after denaturation-renaturation of a mature polypeptide [8]. However, the present study clearly demonstrated the intra-molecular chaperone activity of the GluSE propeptide toward the mature portion of GluV8. Moreover, we demonstrated that only four residues of the propeptide (Ser₆₃-Tyr-Pro-Ser₆₆) were almost sufficient for the chaperone function. It was impossible to completely segregate the regions responsible for the dual roles, indicating that the two functions may be tightly connected to each other. On the two roles of the propeptide, the inhibitory effect on the protease activity may be explained by the propeptide amino acids attached to N-terminal Val₆₉, because of the essential role of the α -amino group of the N-terminal amino acid [19]. However, it remains unknown how the prosequence, especially the tetrapeptide (Ser₆₃-Tyr-Pro-Ser₆₆) of the GluSE propeptide, supports the folding of mature portion of GluV8. We suppose that the tetrapeptide may form a scaffold for the folding of the mature sequence. For instance, it has been reported that the

intrinsically unstructured propeptide of subtilisin adopts an arranged structure only in the presence of the mature form of the protease [23]. It should be investigated whether or not a similar mechanism works on folding of the glutamyl endopeptidase family.

Our result on zymography reproduced the renaturation of the mature polypeptide reported by Yabuta *et al.* [8]. However, this finding did not exclude the need for the intra-molecular chaperone activity of the propeptide. Similar results were observed on proteins that are folded by general molecular chaperones. That is, even if a protein can fold spontaneously under *in vitro* conditions, it may be unable to fold under *in vivo* conditions without molecular chaperones. In particular, the folding of nascent polypeptides is substantially distinct from the renaturation process of a polypeptide *in vitro*. Like general molecular chaperone Hsp70, which immediately binds to nascent polypeptides [24], the GluV8 propeptide may associate with subsequently-synthesized nascent polypeptide, and suppress misfolding of the mature portion. In contrast, the entire mature portion of GluV8 may be ready to fold under *in vitro* denaturation and renaturation conditions spontaneously.

We found that mature GluV8 polypeptide was more resistant to denaturation in the presence of SDS than was the non-processed form. The faster electrophoretic mobility of mature GluV8 indicated a more compact structure. This strongly suggested that the conformation of the non-processed GluV8 is distinct from the simple summation of the pro- and mature polypeptides. Hence, the propeptide seems to prevent the mature polypeptide from conversion to a more compact structure. Non-covalent association of an intra-molecular chaperone propeptide with the mature portion has been reported for subtilisin [23] and furin [25].

Prasad *et al.* [19] proposed that the positively-charged α -amino group of the N-terminus is involved in the substrate recognition of GluV8. In this same line, Popowicz *et al.* [26] reported that a

recombinant form of SplB, a GluV8-family member, possessed proteolytic activity, while that carrying an additional Gly-Ser dipeptide left was devoid of activity; although they did not present any data for this conclusion. The present study clearly demonstrated the inhibitory effect of the prosegment on the proteolytic activity. The proteolytic activities of GluV8 with truncated GluSE propeptides, *i.e.*, Ser₆₃-Ser₆₆ and Asn₆₁-Ser₆₆ were not completely zero. In contrast, the proteolytic activities of GluV8 with longer GluSE propeptides (Ile₅₆-Ser₆₆ and Ile₄₉-Ser₆₆, and Ser₃₃-Ser₆₆) were more rigorously inhibited. The α -amino group of the N-terminus of shorter propeptides might function as a weak acceptor of the negative charge of a substrate peptide.

In the present study, we investigated the role of Val₆₉. Val₆₉ was essential for the precise processing at the peptide bond between Ser₆₆ of GluSE propeptide and Val₆₉ of GluV8 mature sequence for protease maturation. When the N-terminal Val₆₉ was substituted to Ala, Phe, Gly or Ser on GluV8 with Arg₆₆ substituted by Ser₆₆, they showed low but substantial protease activities, *i.e.*, 0.6-4.5% of the wild type. Therefore, the enzyme activity was varied according to the N-terminal amino acids and was much lower than that with Val₆₉. Furthermore, we demonstrated that GluV8 starting from Ile₇₀ was inactive. These findings indicated that Val₆₉ was more than a supplier of an α -amino group for the substrate recognition, but was important, if not essential, for the proteolytic reaction.

The N-terminal Val is conserved among GluV8, GluSE, and glutamic acid-specific proteases from *Streptomyces griseus* [10] and *Streptomyces fradiae* [27]. In contrast, the N-termini of six serine proteases Spl from *S. aureus* are Glu [28]. Although a glutamyl endopeptidase from *Bacillus licheniformis* possesses the sequence Lys₉₄-Ser-Val-Ile-Gly₉₈ around the processing site, the sequence similar to that of GluSE (Pro₆₅-Ser-Val-Ile-Leu₇₁), the N-terminus of the mature form is reported not to be Val₉₆, but Ser₉₅, presumably being dependent upon their processing enzymes [9]. Moreover,

Kawalec *et al.* [29] reported that the processed glutamyl endopeptidase of *Enterococcus faecalis* with an additional Ser₁ possesses the proteolytic activity much higher than that starting from Leu₁. Therefore, the requirement of Val at the N-terminus might be dependent on the conformation of each protease. It is interesting to test whether the substitution of the N-terminal amino acids of non-Val type glutamic acid-specific proteases to Val enhances the proteolytic activity or not.

Glutamyl endopeptidases from *S. fradiae* [27] and *S. griseus* [10] are supposed to be activated through auto-proteolysis at Glu-Xaa bonds. Similarly, bacterial proteases, *e.g.*, Arg- and Lys-specific proteases, from *P. gingivalis* [29-32] appear to be auto-processed by the cleavage at Arg-Xaa or Lys-Xaa bonds. Therefore, as shown in the present study, the modification of the processing sites by *in vitro* mutagenesis may be useful for suppression of the auto-proteolytic cascade of these proteases for their expression in *E. coli*.

Experimental procedures

Materials- The materials used and their sources were the following: Expression vector pQE60 and plasmid pREP4, from Qiagen Inc. (Chatsworth, CA, USA.); low-molecular-weight markers, from GE Healthcare (Buckinghamshire, England); kaleidoscope prestained molecular standard, from Bio-Rad (Richmond, CA, USA); restriction enzymes and DNA-modifying enzymes, from Nippon Gene (Tokyo, Japan); KOD plus DNA polymerase, from Toyobo (Tokyo, Japan); fluorescent peptide, Z-Leu-Leu-Glu-MCA, from Peptide Institute (Osaka, Japan); trypsin and azocasein, from Sigma; protease V8/GluV8 from *S. aureus* V8 strain, from Roche Diagnostics (Mannheim, Germany); Talon metal affinity resin, from Clontech Laboratories Inc. (Palo Alto, CA, USA); anti-penta-His monoclonal antibody, from Qiagen Inc.; and alkaline-phosphatase-conjugated rabbit anti-mouse Ig(G+A+M), from

Zymed Laboratories Inc. (San Francisco, CA, USA). Oligonucleotide primers were purchased from Genenet (Fukuoka, Japan).

Bacterial expression vector for GluSE- GluSE was expressed in *E. coli* with a histidine hexamer tag at the C-terminus by use of the pQE60 expression vector (Qiagen). The DNA fragment carrying the full-length GluSE (Met₁-Gln₂₈₂) was amplified with a pair of primers, 5'-TATGGATCCAAAAGAGATTTTTATCTATATGTAC-3' and 5'-ATTGGATCCCTGAATATTTATATCAGGTATATTG-3'. *Bam*HI sites introduced in the primers are indicated by underlines. Genomic DNA of *S. epidermidis* (ATCC 14990) was used as a template. PCR was performed for 30 cycles by use of the KOD plus system, which did not tag any nucleotide at the 3'-OH end of PCR fragments. A PCR product was then cut with *Bam*HI and then inserted into a *Bam*HI site of pQE60. Y1090[pREP4] cells were transformed with the plasmid (designated as pQE60-GluSE), and the transformants were selected on LB broth agar plates containing 50 µg/ml of ampicillin and 25 µg/ml of kanamycin.

Expression vectors for the full-length form and chimeric form of GluV8

The DNA fragment encoding the full-length form of GluV8 (Met₁-Ala₃₃₆) was amplified with a pair of primers, 5'-ATGGGATCCAAAGGTAATTTTTAAAAGTTAGTTCT-3' and 5'-ATTGGATCCCTGAATATTTATATCAGGTATATTG-3', and then processed as described above. *Bam*HI sites introduced in the primers are indicated by underlines. Genomic DNA of *S. aureus* V8 strain was used as a template. The resulting plasmid was designated as pQE60-GluV8.

A DNA fragment encoding a chimeric protein, *i.e.*, the prepropeptide of GluSE (Met₁-Ser₆₆) and

the mature sequence of GluV8 (Val₆₉-Ala₃₃₆) was amplified with a pair of primers, 5'-GTTATATTACCAAATAACGATCGTCACC-3' and 5'-ACTTGGGTAAC TTTTATTTGACTTGGT-3'. The former targeted the mature sequence of GluV8 (Val₆₉-Ala₃₃₆); and the latter, the prepropeptide of GluSE (Met₁-Ser₆₆). A mixture of pQE60-GluSE and pQE60-GluV8 (45 ng each) was used as templates. During the PCR cycles, a 5-kb PCR fragment encoding the vector and the GluSE Met₁-Ser₆₆/GluV8 Val₆₉-Ala₃₃₆ chimeric protein became predominant. After *DpnI* digestion of the templates, the 5'-end of the fragment was phosphorylated by T4 polynucleotide kinase and self-ligated by T4 DNA ligase simultaneously. Y1090[pREP4] cells were transformed with the resulting plasmid (designated pQE60-proGluSE-matGluV8). Production of the chimeric plasmid was confirmed by DNA sequencing.

Expression vectors for truncated forms of GluV8

Expression plasmids encoding the mature protein of GluV8 (Val₆₉-Ala₃₃₆) fused to truncated propeptides of GluSE at the N-terminus, *i.e.*, Ile₄₉-Ser₆₆, Ile₅₆-Ser₆₆, Asn₆₁-Ser₆₆, Ser₆₃-Ser₆₆, Pro₆₅-Ser₆₆ and Ser₆₆ were amplified by PCR with appropriate primers carrying *Bam*HI sites by use of pQE60-proGluSE-matGluV8 as a template (Fig. 6). The amplified fragments were inserted into a *Bam*HI site of pQE60 as described above.

***In vitro* mutagenesis by PCR**

In vitro mutagenesis was performed by the PCR technique as described above with mutated primers with the altered nucleotides indicated below as underlines. (i) Nucleotides (GAA) encoding Glu at

positions 62 and 65 of pQE60-GluV8 were substituted to nucleotides encoding Gln and Ser, respectively. The plasmid pQE60-GluV8 was used as a template. A sense primer (5'-CGTAGTCACGCAAATGTTATATCCCAAATAACG-3') and an antisense primer (5'-TTGTTGTAATGGTTTGTACCGCCTTTTT-3') were used as PCR primers. The resulting plasmid was designated pQE60-GluV8 2mut. (ii) Nucleotides (GCAAAT) encoding Ala₆₇-Asn₆₈ of GluV8 were further substituted to those (CCAAGT) encoding Pro₆₅-Ser₆₆ of GluSE at equivalent positions. The plasmid pQE60-GluV8 2mut was used as a template. A sense primer (5'-CGTAGTCACGCAAATGTTATATCCCAAATAACG-3') and an antisense primer (5'-ACTTGGGTGACTACGTTGTTGTAATGGTTT-3') were used as PCR primers. The resulting plasmid was designated pQE60-GluV8 4mut. (iii) Nucleotides (TCA) encoding Ser₂₃₇ of pQE60-GluV8 4 mut were substituted to those encoding Ala with a sense primer (5'-GGTTCACCTGTATTTAATGAAAAAA-3') and an antisense primer (5'-TGCATTACCACCAGTTGTAAGTTAAATC-3'). (iv) Nucleotides (AGT) encoding Ser₆₆ of pQE60-proGluSE-matGluV8 were substituted to CGT encoding Arg. A sense primer (5'-GTTATATTACCAAATAACGATCGTCACC) and an antisense primer (5'-ACGTGGGTAACCTTTTATTTTACTTGGTTTG-3') were used as PCR primers. The resulting plasmid was designated pQE60-proGluSE Arg₆₆-matGluV8. (vi) Nucleotides (GTT) encoding Val₆₉ of pQE60-proGluSE-matGluV8 and pQE60-GluSE Arg₆₆-matGluV8 were substituted to those encoding Phe (TTT), Ala (GCG), Gly (GGT) or Ser (AGC) with appropriate primers.

Expression and purification of recombinant proteins

His₆-tagged recombinant proteins were expressed and purified as described previously [33]. Briefly,

Y1090[pREP4] carrying pQE9- or pQE60-derived expression plasmids was cultured in LB broth containing 50 µg/ml of ampicillin and 25 µg/ml of kanamycin at 37°C overnight. Protein expression was induced by dilution of the culture with 2 volumes of LB broth containing 0.2 mM isopropyl β-D-thiogalactopyranoside and incubation at 30°C for 3 h. Bacterial cells were harvested by centrifugation and lysed with lysis/washing buffer (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl containing 10 mM imidazole) to which 0.5 mg/ml of lysozyme and 10 µg/ml of leupeptin had been added. Recombinant proteins were recovered in the cell lysate fraction and then purified by an affinity chromatography with Talon metal affinity resin (Clontech Lab. Inc., Palo Alto, CA) according to the manufacturer's protocol, except that 10 mM imidazole was included in the lysis/washing buffer. After extensive washing, bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol. Purified proteins were stored at -80°C until used.

***In vitro* processing and measurement of the protease activity**

Unless otherwise stated, the *in vitro* processing of recombinant proteins and subsequent protease assay were performed as follows: Recombinant proteins (10 µg/0.1 ml) were incubated in 10 mM sodium borate, pH 8.0, 0.005% (v/v) Triton X100 containing 2 mM CaCl₂ with thermolysin (0.3 or 1 µg) at 37°C for 4 h. Thereafter, aliquots were incubated with 10 mM Z-Leu-Leu-Glu-MCA in 0.2 ml of 50 mM Tris-HCl (pH 8.0) and 5 mM EDTA at 25°C for 2 h. Fifty-seven pmol of proteins (0.18 µg for 32-kDa GluSE proform and 0.25 µg for 44-kDa GluV8 proform) were used for each protease assay unless otherwise stated. EDTA was added to the reaction mixture to inactivate thermolysin [34]. The fluorescence was measured with an excitation at 380 nm and emission at 460 nm with a Fluorescence Photometer F-4000 (Hitachi, Tokyo, Japan). The activity was presented as fluorescent units (FU).

SDS-PAGE and zymography

Samples (0.5 or 1 μg) were separated by electrophoresis in the presence of 0.1% SDS at a polyacrylamide concentration of 12.5% (w/v), and then stained with CBB. For zymography, SDS-PAGE was performed by using 12% polyacrylamide gels containing 1 mg/ml of azocasein [35]. Samples (1 μg) were loaded onto the gel without heat treatment unless otherwise stated. After SDS-PAGE, the gel was incubated twice at 25°C with 100 ml of 2.5% (w/v) Triton X100 for 20 min each time, and then twice at the same temperature with 100 ml of 50 mM Tris-HCl, pH 7.8, containing 30 mM NaCl for 10 min each time. Thereafter, the gel was incubated in 100 ml of the latter buffer at 37°C overnight. Finally, non-hydrolyzed azocasein in the polyacrylamide gel was stained with CBB.

Immunoblotting

Bacterial lysates containing recombinant proteins were prepared as reported previously [36]. The purified fraction used for the immunoblotting was obtained by batch purification of 1 ml of bacterial lysate with 30 ml of suspension (resin/buffer=1/1) of Talon affinity resin pre-equilibrated with lysis buffer, followed by 5 washings with 1 ml of washing buffer. Bound proteins were then extracted and denatured with 30 ml of 3x SDS-sample buffer. The bacterial lysate or an affinity purified fraction (5 ml) was loaded onto a polyacrylamide gel. Following electrophoresis and transference of proteins to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA), the membrane was incubated with 0.2 $\mu\text{g}/\text{ml}$ of anti-penta-His monoclonal antibody (Qiagen Inc., Chatworth, CA, USA), and then with rabbit-anti-mouse Ig(G+A+M) antibody-alkaline phosphatase conjugate at 0.1 $\mu\text{g}/\text{ml}$ (Zymed Laboratories Inc., San Francisco, CA, USA). Blots on the membrane were visualized by

immersion in a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Nakarai, Kyoto, Japan).

N-terminal amino acid sequencing

N-terminal amino acid sequences were determined after separation of recombinant proteins on SDS-PAGE and transference to a polyvinylidene difluoride membrane (Sequi-Blot PVDF Membrane, Bio-Rad). After having been stained with CBB, the bands were excised and directly sequenced with a model Precise 49XcLC protein sequencer (ABI).

Protein Concentration

Protein concentrations were determined by the CBB dye method (Bio-Rad).

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FIGURE LEGNEDS

Fig. 1. Comparison of the amino acid sequences between GluSE and GluV8. (a) The sequences of GluSE, GluV8 and proGluSE-matGluV8 (SE-V8) are schematically illustrated. Open and shadow boxes represent amino acid sequences derived from GluV8 and GluSE, respectively. Closed areas at the N- and C-termini represent 3 and 10 amino acids, respectively, derived from the vector pQE60. pre, presequence; pro, prosequence; mature, mature sequence; and repeat, the C-terminal 12-fold repeat of a tripeptide (Pro-Asp/Asn-Ala). (b) Amino acids of GluSE and GluV8 preprosequences are aligned. Small letters (ggs) represent amino acids derived from the vector; hyphens represent deletions introduced for maximal matching. Identical amino acids between GluSE and GluV8 are underlined. Amino acid numbers on the top are for GluSE, and ones at the middle are for GluV8. Proteolytic sites observed in the purified preparation and thermolysin-treated sample of proGluSE-matGluV8 (SE-V8) are indicated by arrowheads (see Table 1). Asterisks indicate amino acids substituted in this study.

Fig. 2. SDS-PAGE of GluSE, GluV8, and their derivatives. The lysates (lanes 1-5) and batch-purified fractions (lanes 6-10) of recombinant GluSE (lanes 1 and 6), GluV8 (lanes 2 and 7), proGluSE-matGluV8 (lanes 3 and 8), GluV8 2mut (lanes 4 and 9), and GluV8 4mut (lanes 5 and 10) were prepared. Aliquots (10 μ l) of them were separated by PAGE and stained with CBB (a) or immunoblotted with anti-penta-His monoclonal antibody (b). M, molecular weight markers. The apparent molecular masses of major products are shown on the left (a) and right (b). (c) Growth curves of GluV8 (open circle), proGluSE-matGluV8 (closed circle), GluV8 2mut (closed square), and GluV8 4mut (open square) cultured at 30°C in the presence of 0.2 mM

isopropyl- β -thiogalactopyranoside.

Fig. 3. Talon affinity chromatography of recombinant proteins. (a) The bacterial lysate (50 ml) of a 500-ml culture expressing the full-length form of GluSE (open circle) or GluV8 (closed circle) was separated on a Talon affinity resin (1x5cm) as described in “Experimental procedures.” One-ml fractions were collected. (b) Aliquots (10 ml) of the eluates of GluV8 were separated on SDS-PAGE and then stained with CBB. L, bacterial lysate expressing GluV8. M, low-molecular-weight markers.

Fig. 4. *In vitro* processing of proGluSE-matGluV8 by thermolysin. proGluSE-matGluV8 was incubated at 0°C (lane 1) or 37°C (lane 2) without protease or at 37°C with 1 ng (lane 3), 3 ng (lane 4), 10 ng (lane 5), 30 ng (lane 6), 0.1 μ g (lane 7), 0.3 μ g (lane 8) or 1 μ g (lane 9) of thermolysin. As a control, thermolysin (1 μ g) was incubated in the absence of GluV8 (lane Th/35 kDa). Aliquots (0.5 μ g) of thermolysin-treated samples were separated by SDS-PAGE and stained with CBB (a) or visualized by zymography (b). M, molecular weight markers. Apparent molecular masses of major bands are indicated. (c) After incubation with thermolysin as described in “Experimental procedures,” the proteolytic activity toward Z-Leu-Leu-Glu-MCA was measured (open circle). Fluorescence units (FU) of the sample incubated at 0°C (open square) and thermolysin without GluV8 at 37°C (closed circle) were measured.

Fig. 5. Comparison of the active forms between native and recombinant GluV8. (a) Aliquots (0.5 μ g) of native GluV8 (lane 1) and recombinant GluV8 treated with thermolysin (lane 2), were separated

by SDS-PAGE. M, low-molecular weight markers. (b) The proteolytic activities of native GluV8 (column 1) and recombinant GluV8 (2) were measured with 10 μ M Z-Leu-Leu-Glu-MCA. Values are means \pm S.D. (n=3). Samples for columns 1 and 2 are identical to those for lanes 1 and 2, respectively, of panel a.

Fig. 6. Minimal region of the prosequence responsible for chaperoning and enzyme inhibition. (a) N-terminal sequences of proGluSE-matGluV8 and its N-terminally truncated forms are shown. ProGluSE-matGluV8 was expressed as the full-length form, but its N-terminus was processed up to K₂₈. (b) Recombinant proteins shown in panel a were incubated without protease at 0°C (-) or with thermolysin (1 mg) at 37°C (+) as described in “Experimental procedures.” Thereafter, aliquots (0.5 mg) were separated by SDS-PAGE. (c) The glutamic acid-specific protease activity of aliquots (0.25 mg) pretreated with thermolysin was measured. Values are means \pm S.D. (n=4). (d) The glutamic acid-specific protease activity of aliquots (1 mg) incubated without thermolysin was measured. Values are means \pm S.D. (n=4). Nos. 1-7 are identical in panels a-d.

Fig. 7. Effect of the amino acid substitution at Ser₂₃₇ on the proteolytic activity. ProGluSE-matGluV8 (wt), or its mutant (Ser₂₃₇Ala) was incubated at 0°C without protease (-) or at 37°C with 0.3 μ g (+) of thermolysin. (a) Thereafter, aliquots (1 mg) were separated on SDS-PAGE and stained with CBB (left) or subjected to zymography (right). Samples were mixed with a half volume of SDS-sample buffer and subjected to SDS-PAGE without (heat -) or after heat denaturation (heat +). M, low-molecular weight markers. Apparent molecular masses of major bands are indicated on the left. (b) Aliquots of the thermolysin-treated samples were subjected to the protease assay using Z-Leu-Leu-Glu-MCA. Values are means \pm S.D. (n=3).

Fig. 8. Effect of amino acid substitutions at Val₆₉ on thermolysin processing. ProGluSE-matGluV8 or its mutants at Val₆₉ were incubated at 0°C without protease (lane 1) or at 37°C with 0.03 mg (lane 2), 0.1 mg (lane 3), 0.3 mg (lane 4), 1 mg (lane 5) or 3 mg (lane 6) of thermolysin. Thereafter, aliquots (0.5 mg) were separated on SDS-PAGE (a) or subjected to the protease assay with Z-Leu-Leu-Glu-MCA (b). M, low-molecular weight markers. Apparent molecular masses of major bands and 35-kDa thermolysin are indicated. Symbol designations in “b”: Val₆₉ (open circle), Val69Phe (closed circle), Val69Ala (open square), and Val69Gly (open triangle; identical to Val69Phe).

Fig. 9. Involvement of Val₆₉ in the protease activity. (a) Ser₆₆ of proGluSE-matGluV8 was substituted to Arg (GluSE Arg₆₆-GluV8). ProGluSE-matGluV8 (wt) and proGluSE Arg₆₆-matGluV8 (Ser₆₆Arg) were incubated at 0°C without protease (lanes 1 and 4), at 37°C with 0.3 mg of thermolysin (lanes 2 and 5) or at 37°C with 0.3 mg of trypsin (lanes 3 and 6) as described in “Experimental procedures.” As controls, 0.3 mg of thermolysin (lane 7/Th) and trypsin (lane 8/Tr) were incubated without recombinant protein. Thereafter, aliquots (0.75 mg) were separated by SDS-PAGE. M, low-molecular weight markers. Apparent molecular masses of major bands are indicated on the left. (b) Val₆₉ of proGluSE Arg₆₆-matGluV8 was mutated, and the glutamic acid-specific protease activity of the mutated forms was measured with aliquots of the samples after the incubation with thermolysin or trypsin. wt, proGluSE-matGluV8 (columns 1 and 2). Val69Xaa: Amino acid at position 69 of GluSE Arg₆₆-GluV8 was substituted to Val (columns 3 and 4), Ala (columns 5 and 6), Phe (columns 7 and 8), Gly (columns 9 and 10) or Ser (columns 11 and 12). Values are means ± S.D. (n=3).

Table 1. N-terminal sequences of GluV8 derivatives

N-terminal sequences of the bands of proGluSE-matGluV8 obtained by SDS-PAGE (Fig. 4a), those of GluV8 4mut were determined. *Italic letters represent amino acids derived from the preprosequence of GluSE.* ^aA mixture of three fragments, and their amounts were a > b >>c. ^bSer₆₈ was the amino acid of GluV8 4mut substituted from Asn₆₈.

Species	Detected amino acids	Determined sequence
proGluSE-matGluV8		
44 kDa (Fig. 3, lane 1) ^a		
a	KTDTESHNHS	<i>A₂₇/K₂₈TDTESHNHS</i>
b	NKNVLDINSS	<i>E₄₂/N₄₃KNVLDINSS</i>
c	SSLGTENKNV	<i>H₃₆/S₃₇SLGTENKNV</i>
42 kDa (lane 3) ^a		
a	VLDINSSSHN	<i>N₄₅/V₄₆LDINSSSHN</i>
b	IKPSQNKSY	<i>N₅₅/I₅₆KPSQNKSY</i>
c	NKNVLDINSS	<i>E₄₂/N₄₃KNVLDINSS</i>
42 kDa (lane 6)	IKPSQNKSY	<i>N₅₅/I₅₆KPSQNKSY</i>
40 kDa	VILPNDRHQ	<i>S₆₆/V₆₉ILPNDRHQ</i>
38 kDa	VILPNDRHQ	<i>S₆₆/V₆₉ILPNDRHQ</i>
GluV8 4mut		
44 kDa	LSSKAMDHP	<i>A₂₉/L₃₀SSKAMDHP</i>
40 kDa	VILPPNN	<i>S₆₈/V₆₉ILPPNN^b</i>

Fig. 1

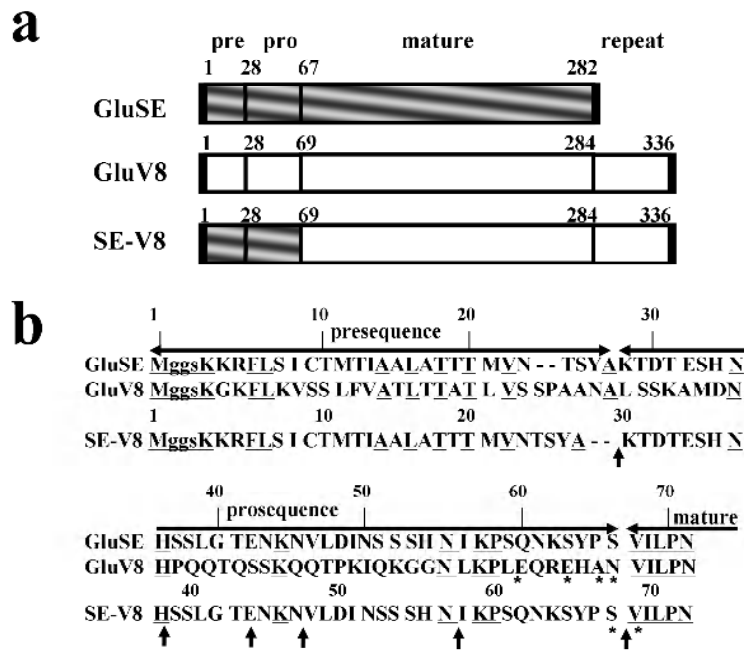


Fig. 2

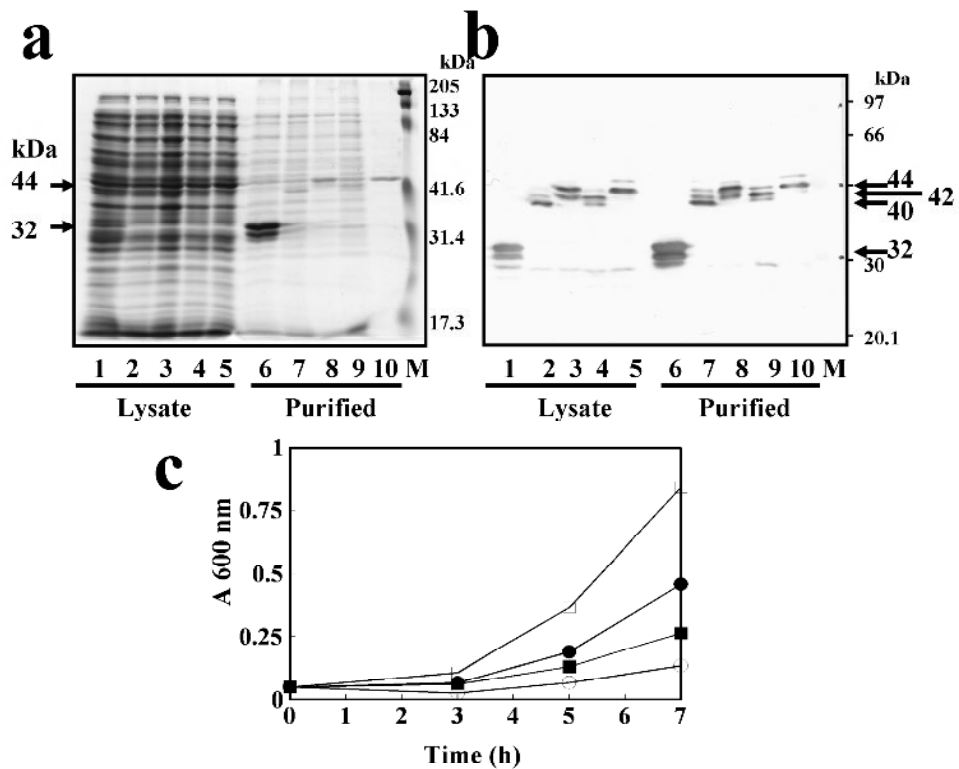


Fig. 3

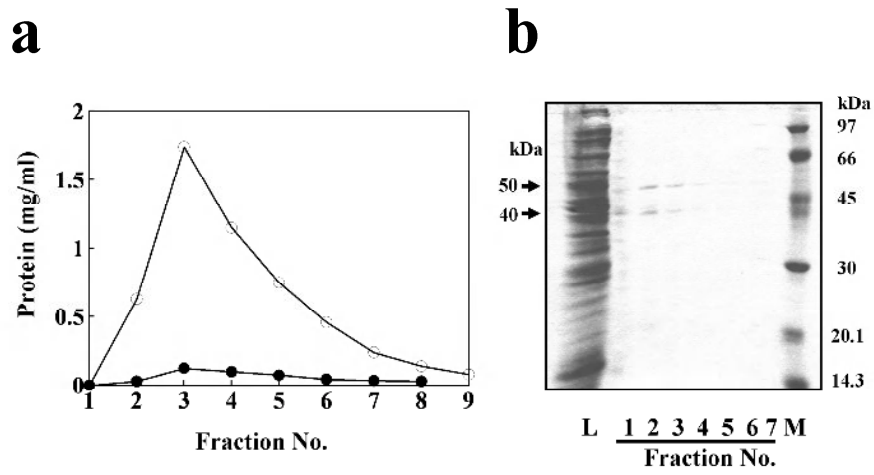


Fig. 4

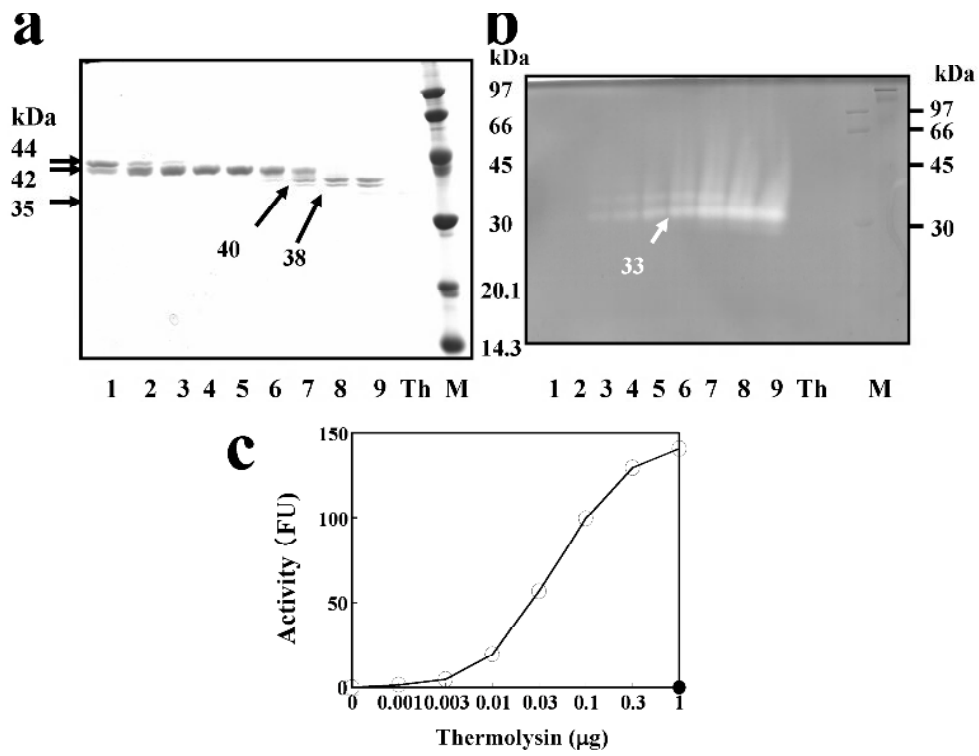


Fig. 5

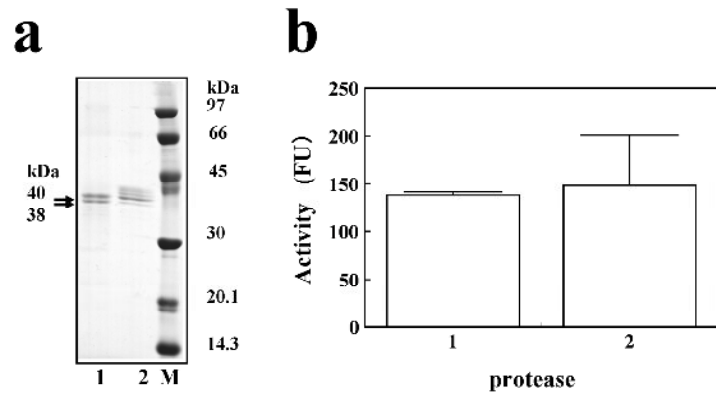


Fig. 6

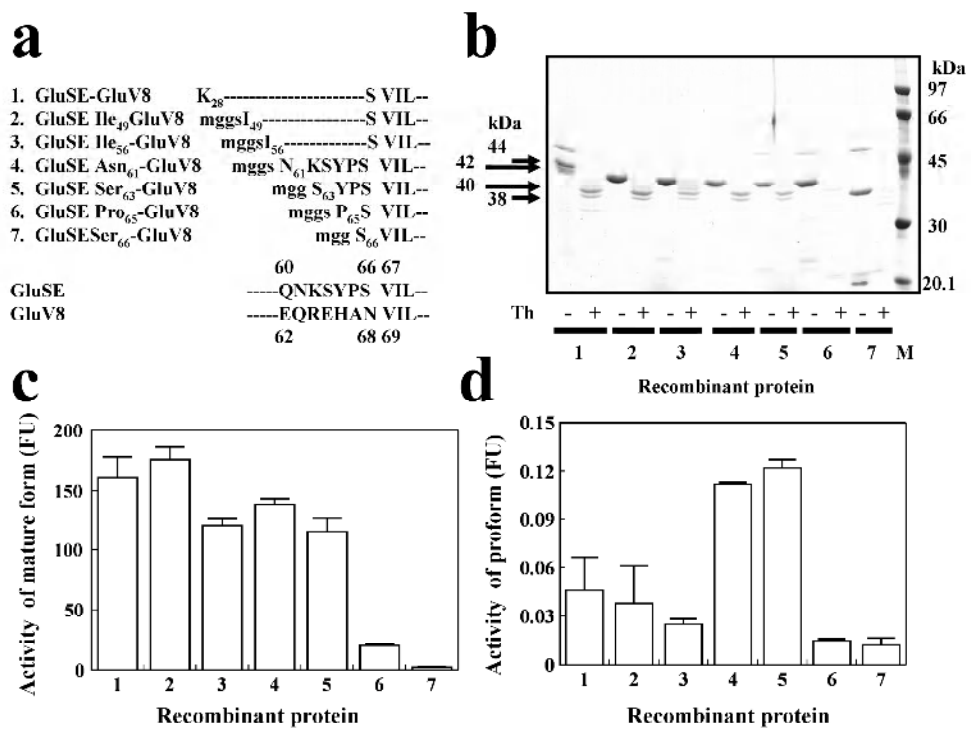


Fig. 7

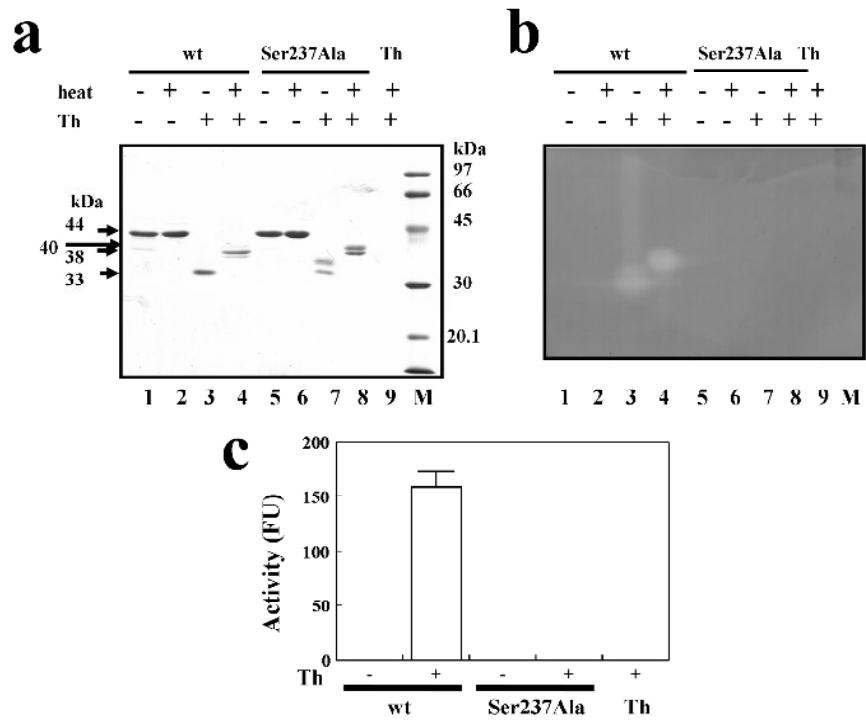


Fig. 8

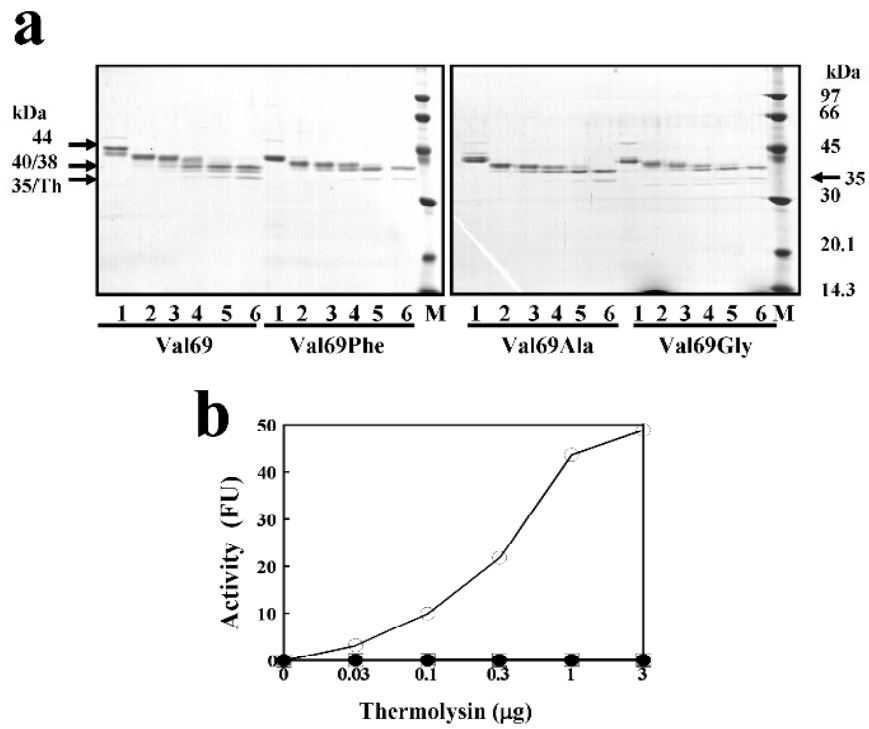


Fig. 9

