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 Asn_{43}, Val_{46}, and Ile_{56}; and that at a large dose (lane 6) consisted of a single species with the N-terminus of Ile_{56} (Table 1). N-termini of 38- and 40-kDa forms were Val_{69}, 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 Glu_{279}-Asp_{280} 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 Leu_{30} (Table 1), which was equivalent to the N-terminus (Lys_{28}) of the 44-kDa proGluSE-matGluV8. The Ala_{27}-Lys_{28} bond of proGluSE-matGluV8 and the Ala_{29}-Leu_{30} 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 Ile_{49}, Ile_{56}, Asn_{61}, Ser_{63}, Pro_{65} or Ser_{66} (Fig. 5a). The minimal one possessed the last amino acid (Ser_{66}) of the GluSE propeptide. The expression levels were varied among constructions, of which the forms starting from Pro_{65} and Ser_{66} 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 Pro_{65}-matGluV8 as well as GluSE Ser_{66}-matGluV8 was thoroughly degraded by thermolysin treatment (lanes 6 and 7 Th+). This finding may cause the low expression of GluSE Pro_{65}-matGluV8 and GluSE Ser_{66}-matGluV8. After the thermolysin processing, the proteolytic activities of truncated molecules containing the sequence from Ile_{49}, Ile_{56}, Asn_{61} or Ser_{63} to the last amino acid residue Ser_{66} of the GluSE prosegment acquired the protease activities comparable to that of proGluSE-matGluV8. To the contrary, GluSE Pro_{65}-matGluV8 showed significantly lower activity, and GluSE Ser_{66}-matGluV8-C scarcely possessed the activity (Fig. 6c). Therefore, the C-terminal tetra-peptide of the propeptide (Ser_{63}-Tyr-Pro-Ser_{66}), which was almost sufficient for the suppression of the protease activity, was also adequate for the intra-molecular chaperone function. We also expressed GluSE-Ser_{66}-matGluV8 with the long N-terminal tag (Met-Arg-Gly-Ser-His_6-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.*, Asn_{61}–Ser_{66} and Ser_{63}–Ser_{66}, could not be ignored (Fig. 6c, columns 4 and 5). Concerning this result, we should note that the recombinant GluSE Asn_{61}-matGluV8 and GluSE Ser_{63}-matGluV8 were expressed in consideration of the auto-proteolytic sites of GluV8 propeptide, *i.e.,* Glu_{62}-Gln_{63} and Glu_{65}-His_{66} 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 Ser_{237}

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, Ser_{237} and Val_{69}. GluV8 is a serine protease, the active site of which consists of the His_{119}, Asp_{161}, and Ser_{237} triad [19]. To confirm the role of Ser_{237}, 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 Val_{69} in processing of the GluV8 proform

We finally investigated the role of the N-terminal Val_{69} of mature GluV8. It has been proposed that

α-amino group of the N-terminal Val_{69} 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 Val_{69} is simply required for processing with thermolysin, which hydrolyzes the amino-side peptide bond of hydrophobic amino acids. To test this idea, we substituted Val_{69} of proGluSE-matGluV8 to Phe. Additionally, Val_{69} 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 Ile_{70}. These results show that thermolysin attacked the Xaa_{69}-Ile_{70} bond of the mutant, instead of Ser-Xaa_{69} (Xaa=Phe, Gly, or Ala) bonds. In consequence, we unexpectedly found that Val_{69} was indispensable for correct processing at the Ser-Val_{69} bond by thermolysin and that GluV8 with N-terminal Ile_{70} had essentially no proteolytic activity.

Role of the N-terminal Val_{69} in the proteolytic activity

Since Val_{69} was indispensable for the precise processing at the Ser_{66}-Val_{69} bond, it was impossible to investigate the role of Val_{69} in the enzymatic reaction. To overcome this difficulty, we prepared mutant proGluSE-matGluV8, the Ser_{66} of which was replaced by Arg (designated proGluSE Arg_{66}-matGluV8), because the peptide bond between Arg_{66} and Val_{69} can be degraded by trypsin. Indeed, trypsin processing of proGluSE Arg_{66}-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 Arg_{66}-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 Ser_{66} by non-preferable Arg. Hence, it became possible to utilize trypsin as the processing enzyme.

Trypsin cleavage of proGluSE Arg_{66}-matGluV8 with Val_{69} 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 Val_{69} (Fig. 9b). Therefore, we conclude that Val_{69} plays an important role in the enzyme reaction itself, although other amino acids, such as Ala, could partially substitute for Val_{69}.

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 (Gln_{63}-Asn_{68} and His_{66}-Asn_{68}) 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 Ala_{67}-Asn_{68} to Pro-Ser further suppressed the degradation. We currently speculate that an endogenous protease in *E. coli* cleaves the Ala_{67}-Asn_{68} or Asn_{68}-Val_{69} bond of GluV8. The substitution of Asn_{67} 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 *Aeromoas 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 Ser_{66} of proGluSE-matGluV8 to Arg_{66} (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 His_{119}, Asp_{161} and Ser_{237} residues of which form an active triad. In fact, Ser_{237} 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 (Ser_{63}-Tyr-Pro-Ser_{66}) 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 Val_{69}, 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 (Ser_{63}-Tyr-Pro-Ser_{66}) 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.*, Ser_{63}-Ser_{66} and Asn_{61}-Ser_{66} were not completely zero. In contrast, the proteolytic activities of GluV8 with longer GluSE propeptides (Ile_{56}-Ser_{66} and Ile_{49}-Ser_{66}, and Ser_{33}-Ser_{66}) 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 Val_{69}. Val_{69} was essential for the precise processing at the peptide bond between Ser_{66} of GluSE propeptide and Val_{69} of GluV8 mature sequence for protease maturation. When the N-terminal Val_{69} was substituted to Ala, Phe, Gly or Ser on GluV8 with Arg_{66} substituted by Ser_{66}, 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 Val_{69}. Furthermore, we demonstrated that GluV8 starting from Ile_{70} was inactive. These findings indicated that Val_{69} 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 Lys_{94}-Ser-Val-Ile-Gly_{98} around the processing site, the sequence similar to that of GluSE (Pro_{65}-Ser-Val-Ile-Leu_{71}), the N-terminus of the mature form is reported not to be Val_{96}, but Ser_{95}, 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 Ser_{-1} possesses the proteolytic activity much higher than that starting from Leu_1. 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 (Buchinghamshire, 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 (Met_1-Gln_{282}) was amplified with a pair of primers, 5'-TATGGATCCAAAAAGAGATTTTTATCTATATGTAC-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 (Met_1-Ala_{336}) was amplified with a pair of primers, 5'-ATGGGATCCAAAGGTAAATTTTTAAAAGTTAGTTCT-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 (Met_1-Ser_{66}) and

the mature sequence of GluV8 (Val_{69}-Ala_{336}) was amplified with a pair of primers, 5'-GTTATATTACCAAATAACGATCGTCACC-3' and 5'-ACTTGGGTAACTTTTATTTTGACTTGGT-3'. The former targeted the mature sequence of GluV8 (Val_{69}-Ala_{336}); and the latter, the prepropeptide of GluSE (Met_1-Ser_{66}). 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 Met_1-Ser_{66}/GluV8 Val_{69}-Ala_{336} chimeric protein became predominant. After *Dpn*I 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 (Val_{69}-Ala_{336}) fused to truncated propeptides of GluSE at the N-terminus, *i.e.*, Ile_{49}-Ser_{66}, Ile_{56}-Ser_{66}, Asn_{61}-Ser_{66}, Ser_{63}-Ser_{66}, Pro_{65}-Ser_{66} and Ser_{66} 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'-CGTAGTCACGCAAATGTTATATTCCCAAATAACG-3') and an antisense primer (5'-TTGTTGTAATGGTTTGTTACCGCCTTTTT-3') were used as PCR primers. The resulting plasmid was designated pQE60-GluV8 2mut. (ii) Nucleotides (GCAAAT) encoding Ala_{67}-Asn_{68} of GluV8 were further substituted to those (CCAAGT) encoding Pro_{65}-Ser_{66} of GluSE at equivalent positions. The plasmid pQE60-GluV8 2mut was used as a template. A sense primer (5'-CGTAGTCACGCAAATGTTATATTCCCAAATAACG-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 Ser_{237} of pQE60-GluV8 4 mut were substituted to those encoding Ala with a sense primer (5'-GGTTCACCTGTATTTAATGAAAAAA-3') and an antisense primer (5'-TGCATTACCACCAGTTGTACTTAAATC-3'). (iv) Nucleotides (AGT) encoding Ser_{66} of pQE60-proGluSE-matGluV8 were substituted to CGT encoding Arg. A sense primer (5'-GTTATATTACCAAATAACGATCGTCACC) and an antisense primer (5'-ACGTGGGTAACTTTTATTTTGACTTGGTTTG-3') were used as PCR primers. The resulting plasmid was designated pQE60-proGluSE Arg_{66}-matGluV8. (vi) Nucleotides (GTT) encoding Val_{69} of pQE60-proGluSE-matGluV8 and pQE60-GluSE Arg_{66}-matGluV8 were substituted to those encoding Phe (TTT), Ala (GCG), Gly (GGT) or Ser (AGC) with appropriate primers.

Expression and purification of recombinant proteins

His_6-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 $CaCl_2$ 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 μg/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 μg/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 μl) 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^{o}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 μg (lane 7), 0.3 μg (lane 8) or 1 μg (lane 9) of thermolysin. As a control, thermolysin (1 μg) was incubated in the absence of GluV8 (lane Th/35 kDa). Aliquots (0.5 μg) 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 μg) 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 μM Z-Leu-Leu-Glu-MCA. Values are means ± 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 K_{28}. (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 ± S.D. (n=4). (d) The glutamic acid-specific protease activity of aliquots (1 mg) incubated without thermolysin was measured. Values are means ± S.D. (n=4). Nos. 1-7 are identical in panels a-d.

Fig. **7.** Effect of the amino acid substitution at Ser_{237} 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 μg (+) 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 ± S.D. (n=3).

Fig. 8. Effect of amino acid substitutions at Val_{69} on thermolysin processing. ProGluSE-matGluV8 or its mutants at Val_{69} 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": Val_{69} (open circle), Val69Phe (closed circle), Val69Ala (open square), and Val69Gly (open triangle; identical to Val69Phe).

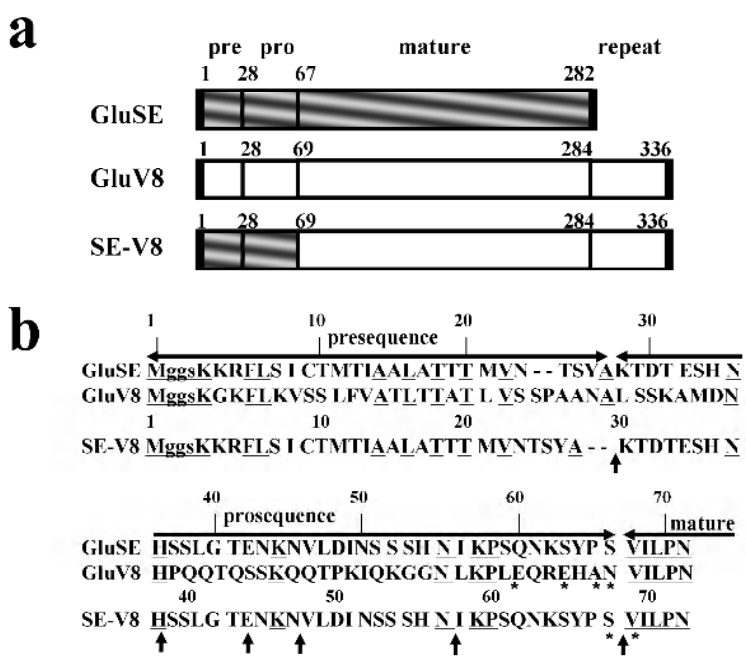
Fig. 9. Involvement of Val_{69} in the protease activity. (a) Ser_{66} of proGluSE-matGluV8 was substituted to Arg (GluSE Arg66-GluV8). ProGluSE-matGluV8 (wt) and proGluSE Arg_{66}-matGluV8 (Ser66Arg) were incubated at 0˚C without protease (lanes 1 and 4), at 37˚C with 0.3 mg of thermolysin (lanes 2 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) Val_{69} of proGluSE Arg_{66}-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 Arg_{66}-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 preproseuence of GluSE. [a]A mixture of three fragments, and their amounts were a > b >>c. [b]Ser_{68} was the amino acid of GluV8 4mut substituted from Asn_{68}.

Species	Detected amino acids	Determined sequence
proGluSE-matGluV8		
44 kDa (Fig. 3, lane 1)[a]		
a	KTDTESHNHS	*A_{27}/K_{28}TDTESHNHS*
b	NKNVLDINSS	*E_{42}/ N_{43}KNVLDINSS*
c	SSLGTENKNV	*H_{36}/S_{37}SLGTENKNV*
42 kDa (lane 3)[a]		
a	VLDINSSSHN	*N_{45}/V_{46} LDINSSSHN*
b	IKPSQNKSYP	*N_{55}/I_{56} KPSQNKSYP*
c	NKNVLDINSS	*E_{42}/N_{43} KNVLDINSS*
42 kDa (lane 6)	IKPSQNKSYP	*N_{55}/I_{56} KPSQNKSYP*
40 kDa	VILPNNDRHQ	*S_{66}*/V_{69}ILPNNDRHQ
38 kDa	VILPNNDRHQ	*S_{66}*/V_{69}ILPNNDRHQ
GluV8 4mut		
44 kDa	LSSKAMDNHP	A_{29}/L_{30}SSKAMDNHP
40 kDa	VILPPNN	S_{68}/V_{69}ILPNN[b]

Fig. 1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pre pro mature repeat
1 28 67 282
GluSE
1 28 69 284 336
GluV8
1 28 69 284 336
SE-V8
b
presequence
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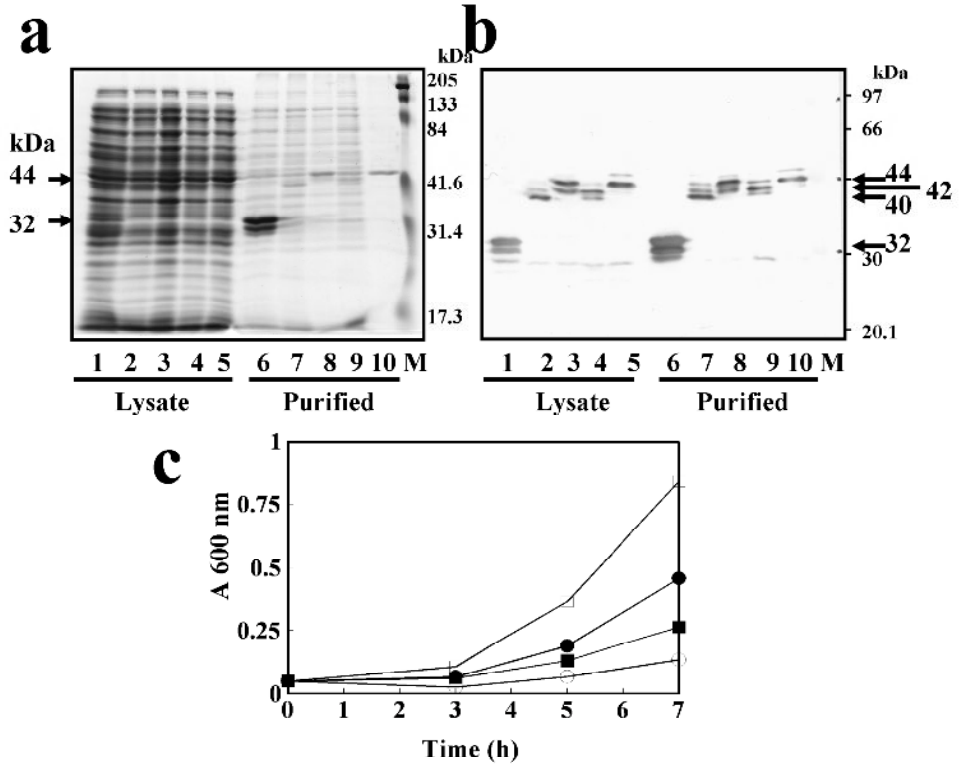
GluV8 MggsKGKFLKVSS LFVATLTTAT L VS SPAANAL SSKAMDN
SE-V8 MggsKKRFLS I CTMTIAALATTT MVNTSYA - - KTDTESH N
prosequence
mature
GluSE HSSLG TENKNVLDINS S SH N I KPSQNKSYP S VILPN
GluV8 HPQQTQSSKQQTPKIQKGGN LKPLEQREHAN VILPN
SE-V8 HSSLG TENKNVLDI NSS SH N I KPSQNKSYP S VILPN

Fig. 2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kDa
44
32
205
133
84
41.6
31.4
17.3
1 2 3 4 5 6 7 8 9 10 M
Lysate
Purified
b
kDa
97
66
44
42
40
32
30
20.1
1 2 3 4 5 6 7 8 9 10 M
Lysate
Purified
c
A 600 nm
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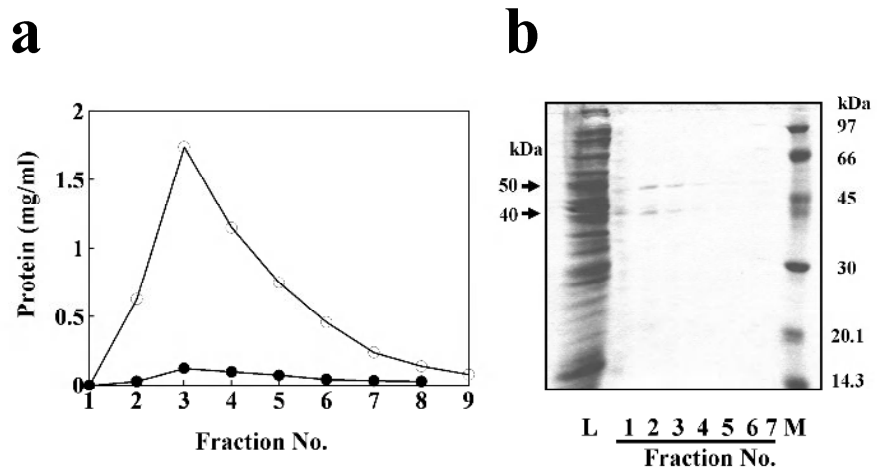
0.75
0.5
0.25
0
0 1 2 3 4 5 6 7
Time (h)

Fig. 3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b
Protein (mg/ml)
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66
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30
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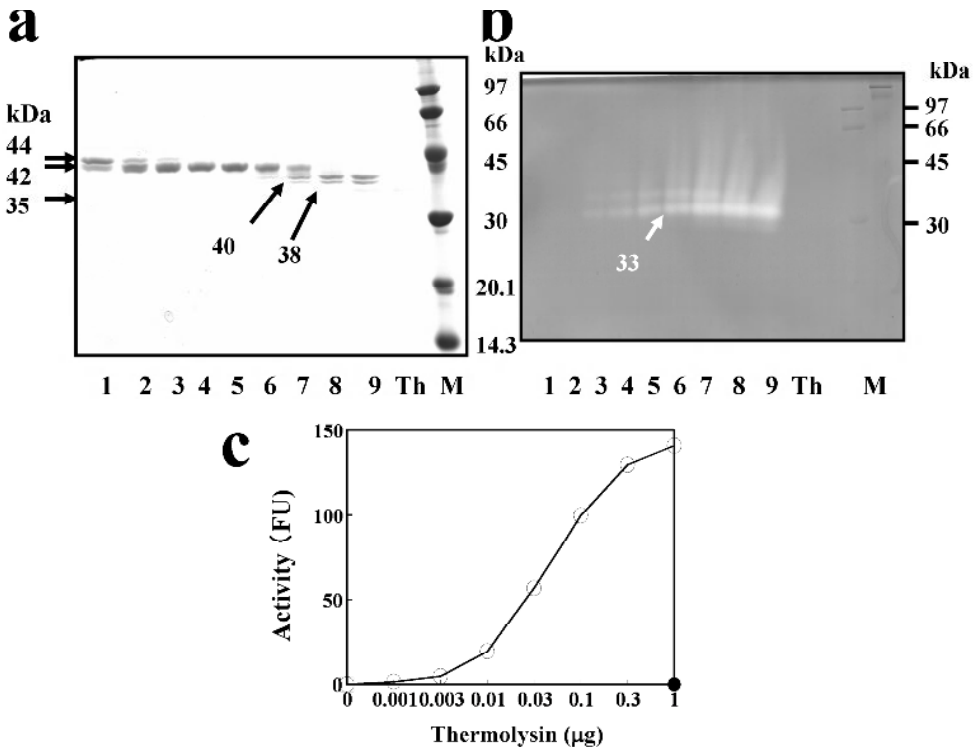
14.3
L 1 2 3 4 5 6 7 M
Fraction No.

Fig. 4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42
35
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kDa
97
66
45
30
20.1
14.3
33
1 2 3 4 5 6 7 8 9 Th M
c
Activity (FU)
Thermolysin (μg)
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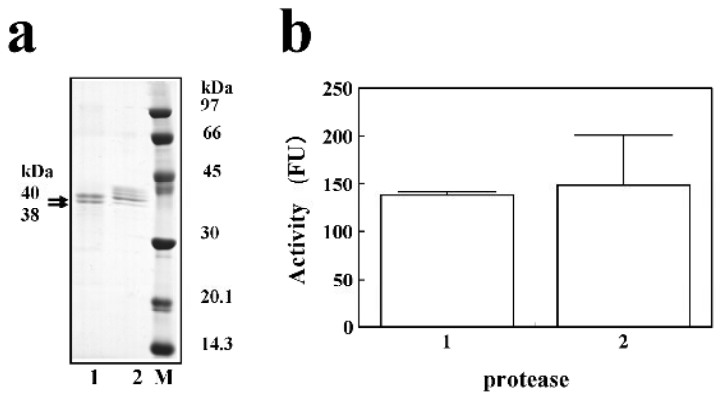
Fig. 5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kDa
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66
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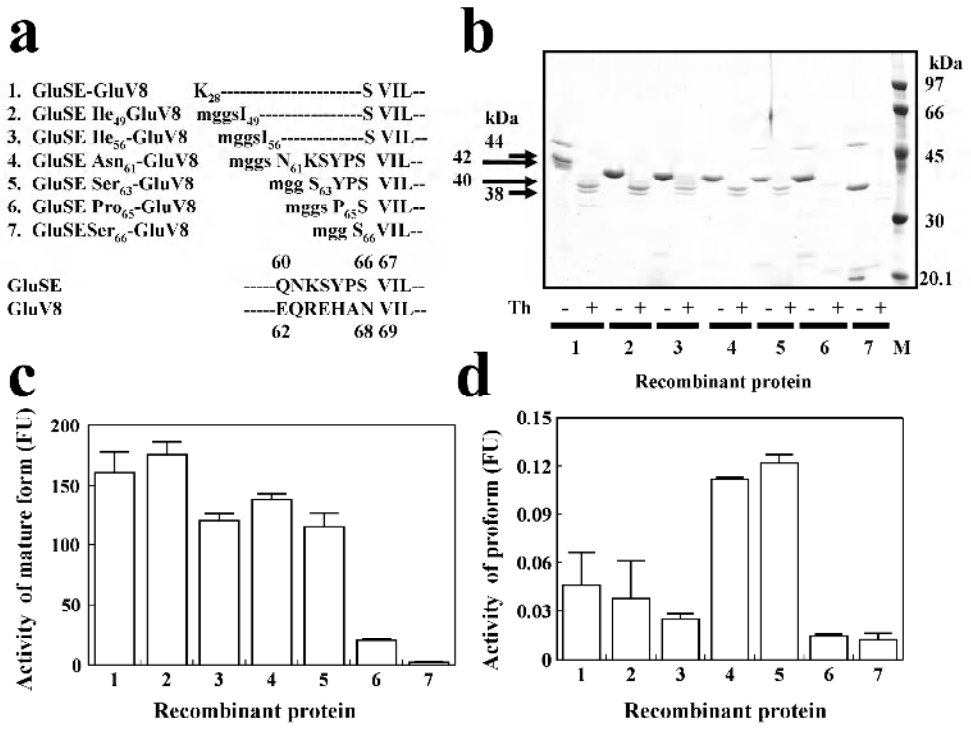
100
50
0
1
2
protease

Fig. 6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3. GluSE Ile56-GluV8 mggsI56----------S VIL--
4. GluSE Asn61-GluV8 mggs N61KSYPS VIL--
5. GluSE Ser63-GluV8 mgg S63YPS VIL--
6. GluSE Pro65-GluV8 mggs P65S VIL--
7. GluSESer66-GluV8 mgg S66VIL--
60 66 67
GluSE -----QNKSYPS VIL--
GluV8 -----EQREHAN VIL--
62 68 69
b
kDa
44
42
40
38
kDa
97
66
45
30
20.1
Th - + - + - + - + - + - + - + - +
1 2 3 4 5 6 7 M
Recombinant protein
c
Activity of mature form (FU)
200
150
100
50
0
1 2 3 4 5 6 7
Recombinant protein
d
Activity of proform (FU)
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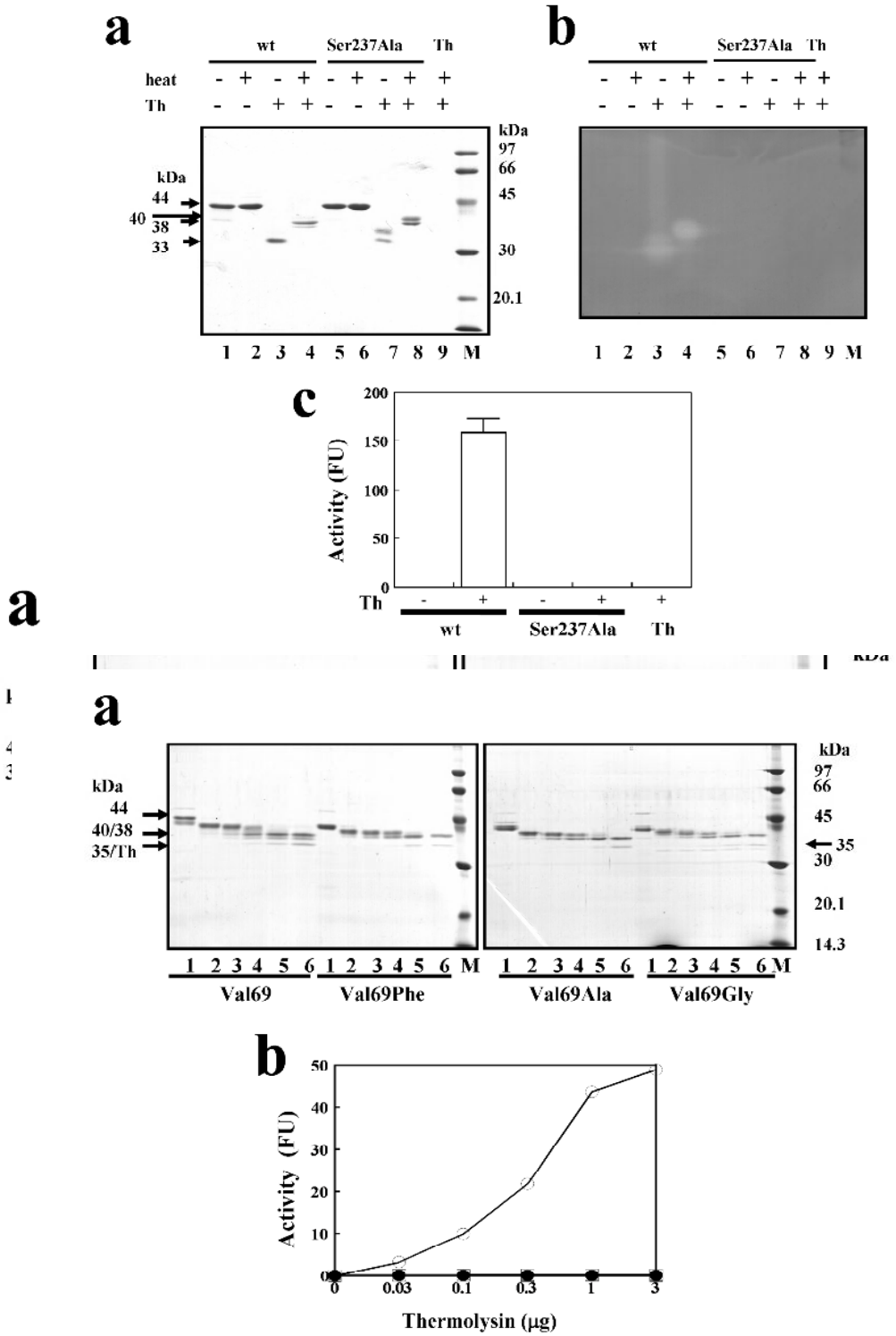
0.09
0.06
0.03
0
1 2 3 4 5 6 7
Recombinant protein

Fig. 7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Th
heat
Th
kDa
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40
38
33
97
66
45
30
20.1
1 2 3 4 5 6 7 8 9 M
b
wt
Ser237Ala
Th
1 2 3 4 5 6 7 8 9 M
c
Activity (FU)
200
150
100
50
0
Th
wt
Ser237Ala
Th
a
kDa
44
40/38
35/Th
97
66
45
35
30
20.1
14.3
1 2 3 4 5 6 1 2 3 4 5 6 M 1 2 3 4 5 6 1 2 3 4 5 6 M
Val69
Val69Phe
Val69Ala
Val69Gly
b
Activity (FU)
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20
10
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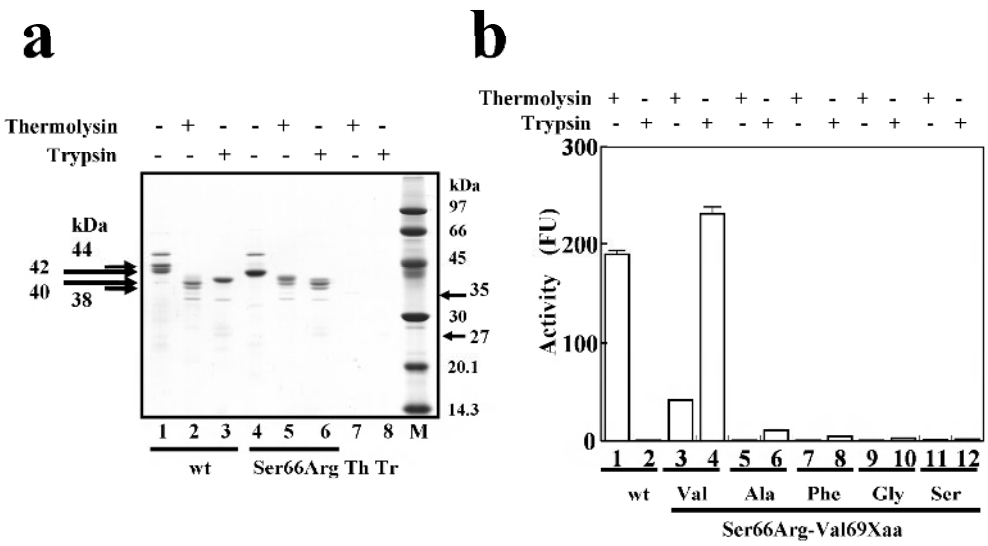
0
0.03
0.1
0.3
1
3
Thermolysin (μg)

Fig. 9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Thermolysin - + - - + - + -
Trypsin - - + - - + - +
kDa
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42
40
38
kDa
97
66
45
35
30
27
20.1
14.3
1 2 3 4 5 6 7 8 M
wt
Ser66Arg Th Tr
b
Thermolysin + - + - + - + - + - + -
Trypsin - + - + - + - + - + - +
300
200
100
0
Activity (FU)
1 2 3 4 5 6 7 8 9 10 11 12
wt
Val
Ala
Phe
Gly
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Ser66Arg-Val69Xaa