Indian Journal of Biochemistry & Biophysics
Vol. 48, December 2012, pp. 421-427

Propeptide processing and proteolytic activity of proenzymes of the Staphylococcal and Enterococcal GluV8-family protease

S M A Rouf[a], Y Ohara-Nemoto[a], Y Shimoyama[b], S Kimura[b], T Ono[a] and T K Nemoto[a*]

[a]Department of Oral Molecular Biology, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan
[b]Division of Molecular Microbiology, Iwate Medical University, Yahaba-cho 028-3694, Japan

Received 21 January 2012; revised 30 August 2012

Proenzymes with various lengths of propeptides have been observed in GluV8 from *Staphylococcus aureus* and GluSE from *S. epidermidis*. However, the production mechanism of these proenzymes and roles of truncated propeptides have yet to be elucidated. Here we demonstrate that shortening of propeptide commonly occurs in an auto-catalytic manner in GluV8-family members, including those from coagulase negative *Staphylococci* and *Enterococcus faecalis*. Accompanied with propeptide shortening, the pro-mature junction (Asn/Ser_{-1}-Val_1) becomes more susceptible towards the hetero-catalytic maturation enzymes. The auto-catalytic propeptide truncation is not observed in Ser169Ala inert molecules of GluV8-family members. A faint proteolytic activity of proenzymes from *Staphylococcus caprae* and *E. faecalis* is detected. In addition, proteolytic activity of proenzyme of GluV8 carrying $Arg_{-3}AlaAsn_{-1}$ is demonstrated with synthetic peptide substrates LLE/Q-MCA. These results suggest that GluV8-family proenzymes with shortened propeptides intrinsically possess proteolytic activity and are involved in the propeptide shortening that facilitates the final hetero-catalytic maturation.

Keywords: *Enterococcus faecalis*, GluV8, Propeptide, *Staphylococcus aureus*

GluV8 from *Staphylococcus aureus* is produced as a zymogen (pGluV8) composed of an N-terminal propeptide (Leu_{-39}-Asn_{-1}) and a mature portion (Val_1-Ala_{268}) and the maturation process is mediated by a thermolysin-family metalloprotease[1,2]. The cleavage of Asn_{-1}-Val_1 bond of GluV8 expressed in *Escherichia coli* has been successfully achieved by thermolysin *in vitro*, resulting in the acquisition of proteolytic activity[3]. Besides GluV8, several GluV8-family members from coagulase negative Staphylococci have been cloned and characterized[4-6].

The proteolytic activity of GluV8 is suppressed in a chimeric molecule, where the propeptide is interchanged to that of GluSE from *Staphylococcus epidermidis*[3]. Furthermore, it has been demonstrated that an N-terminal attachment of even one amino acid residue, i.e. Asn_{-1} or Ser_{-1} efficiently inhibits the proteolytic activity[6]. Liberation of exogenous residue Asn_{-1} or Ser_{-1} by thermolysin causes full activation of GluV8. These observations suggest that mature region in pGluV8 is properly folded, independent of the structure of propeptide.

The length of propeptide of GluV8-family proteases ranges at 25-40 residues, which appears to be substantially longer than that is required for the suppression of catalytic activity. Therefore, some additional roles of propeptide are speculated. To date, proenzymes carrying various lengths of propeptide have been detected in GluV8, as well as GluSE. Nickerson *et al.*[7] reported that sequential cleavage of propeptide of GluV8 occurs at a Gln-rich segment. Similarly, we reported that processing in propeptide of GluSE occurs at Glu_{-35}-Ser_{-34} and Asp_{-19}-Ile_{-18} bonds[8]. These findings suggest a common maturation mechanism of GluV8-family proteases that accompanies the truncation of propeptide, leading to the final hetero-catalytic maturation cleavage between the Xaa_{-1}-Val_1 bond.

In the present study, we have investigated the mechanism of processing in the GluV8-family proteases and proteolytic activity of proenzymes, which would shed light on the role of propeptide.

*Author for correspondence
E-mail: tnemoto@nagasaki-u.ac.jp
Tel: +81-95-819-7640
Fax: +81-95-819-7642

Materials and Methods

Expression and purification of GluV8 family proteases and chimeric molecules

Wild type of GluV8 (GluV8-wt) and GluV8 with five substitutions in its propeptide (D-31H, E-7Q, E-4S, A-2P and N-1S) (designated as GluV8-mut)[5] and a truncated form of GluV8-mut starting from I_{17} (GluV8-ΔN)[8] were expressed as reported previously. GluSE (AB096695), GluSW from *Staphylococcus warneri* (AJ293885), GluScpr from *S. caprae* (AB544444), GluScoh from *S. cohnii* (AB544445), GluScpr-mut and GluScoh-mut introduced appropriate substitutions in the propeptide were expressed as described previously[6,8]: The propeptide of GluSW was replaced by the corresponding sequence of GluV8-mut to achieve stable expression[5].

The gene of glutamyl endopeptidase from *E. faecalis* (Z12296)[9] (GluEF) was amplified with a primer set(5'-GATGGATCCAAAAAGTTCTCCA TACGAAAAATTAG-3'and5'-GGTGGATCCCGCT GCAGGCACAGCGGATAAACG-3', *BamH*I sites are underlined) using the genomic DNA of *E. faecalis* NCTC 775 and KOD Plus DNA polymerase (Toyobo). The amplified 0.8 kb fragment was cleaved with *BamH*I and then inserted into the *BamH*I site of pQE60 (Qiagen). Subsequently, three amino acid substitutions (E-16S, E-15K and E-9I) were introduced (GluEF-mut).

Substitution of amino acids and construction of plasmids for chimeric molecules were carried out by the PCR-based methods[3]. All mutations were confirmed by DNA sequencing. Recombinant proteins were expressed in *E. coli* XL1-blue and purified by Talon affinity resin (Clontech) as reported previously[3]. Purified proteins were stored at -80°C until used.

SDS-PAGE and zymography

Proteins were separated by SDS-PAGE with a 12.5% (w/v) polyacrylamide gel. Zymography was performed using a polyacrylamide gel containing 1% (w/v) azocasein (Sigma) as previously reported[3]. The band intensity of an active protease was quantified by a PDQuest software (Bio-Rad).

In vitro maturation and measurement of proteolytic activity

Proteins (10 µg) were incubated in 0.1 ml of 10 mM sodium borate (pH 8.0) containing 0.005% (v/v) Triton X-100 and 2 mM $CaSO_4$ with thermolysin at 37°C. After 4 h, glutamyl endopeptidases (0.25 µg as proform) treated with or without thermolysin were incubated with 20 mM Z-Leu-Leu-Glu-MCA (LLE-MCA) (Peptide Institute, Osaka, Japan) or Z-Leu-Leu-Gln-MCA (LLQ-MCA) (synthesized by Thermo Fisher Scientific, Ulm, Germany) in 0.2 ml of the reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA) at 37°C for 1 h. The fluorescence was measured with excitation at 380 nm and emission at 460 nm.

N-Terminal amino acid sequencing

N-terminal amino acid sequence was determined after separation of proteins by SDS-PAGE and transference to a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad). After having been stained, bands were excised and sequenced with a Procise 49XcLC protein sequencer (ABI).

Results

Effect of the length of propeptide on hetero-catalytic maturation of GluV8

To study maturation process achieved in hetero-catalytic manner, a series of pGluV8 possessing various lengths of propeptide were prepared. When GluV8-wt was expressed in *E. coli*, a mixture of 31-kDa species starting from Gln_{-21} (Q_{-21}pGluV8) and 29-kDa species (R_{-3}pGluV8) was obtained (Fig. 1A and see Fig. 3). GluV8-ΔN was expressed as a 30-kDa pro-enzyme starting from I_{-17} with N-terminal GGS derived from the plasmid sequence (I_{-17}pGluV8). A 32-kDa pGluV8 (L_{-39}pGluV8) possessing the entire 39 residues of pro-peptide was obtained by expression of GluV8-mut.

When these pGluV8 were incubated with thermolysin, the 31-kDa Q_{-21}pGluV8 readily converted first to the 29-kDa R_{-3}pGluV8 with a minimum dose of thermolysin, then the 29-kDa species expeditiously converted to the 28-kDa active GluV8. The 30-kDa I_{-17}pGluV8 converted to the mature enzyme in presence of higher dose of thermolysin than that for the 29-kDa R_{-3}pGluV8. While the 32-kDa L_{-39}pGluV8 first converted to a 30-kDa I_{-17}pGluV8 and then to the 28-kDa mature enzyme with the highest dose of thermolysin (Fig. 1B). Dose of thermolysin required for the 50% activation of GluV8 proenzymes was lowest for GluV8-wt (270:1 as a molar ratio), followed by GluV8-ΔN (160:1) and GluV8-mut (60:1). Thus, it was evident that the Asn/Ser_{-1}-Val_1 bond became more labile to thermolysin processing along with the propeptide shortening.

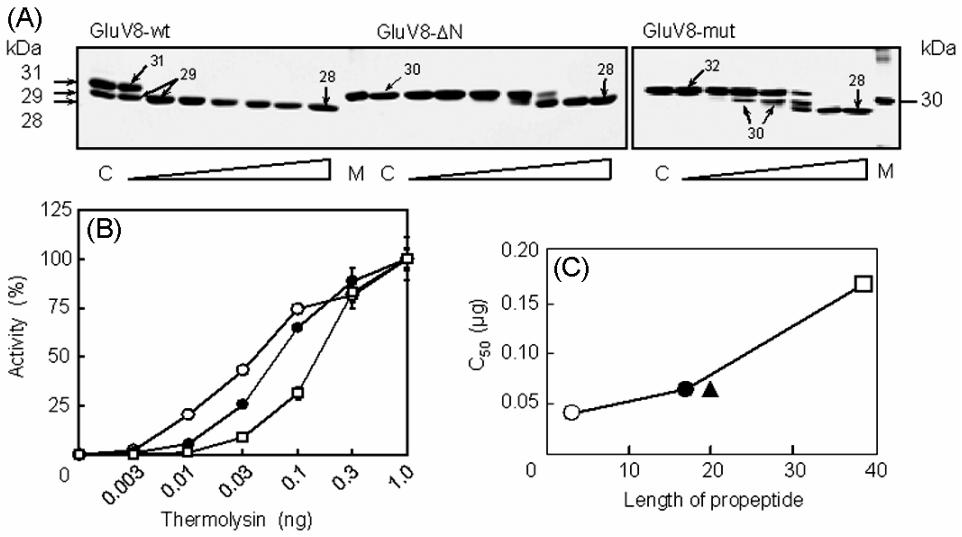

Fig. 1—Effects of the length of propeptide on the maturation processing by thermolysin [(**A**): GluV8-wt, -ΔN, and -mut (10 μg) were incubated at 0°C (lane C) or at 37°C with 0-1 μg of thermolysin. Aliquots (1 μg) were separated by SDS-PAGE. M, marker; (**B**): The proteolytic activity toward LLE-MCA was measured after thermolysin treatment (mean ± SD, n = 3). pGluV8-wt (open circle), -ΔN (filled circle) and -mut (square); and (**C**) Doses of thermolysin giving 50% of the maximal proteolytic activity (C_{50}) were plotted *versus* the length of propeptide. The length of pGluV8-wt was set to three residues, because the 31-kDa I_{-17}pGluV8 was readily converted to the 29-kDa R_{-3}pGluV8. The amino acid number 17 of the propeptide of GluV8-DN could be considered to 20, if the additional GGS derived from the vector sequence is counted (filled triangle)]

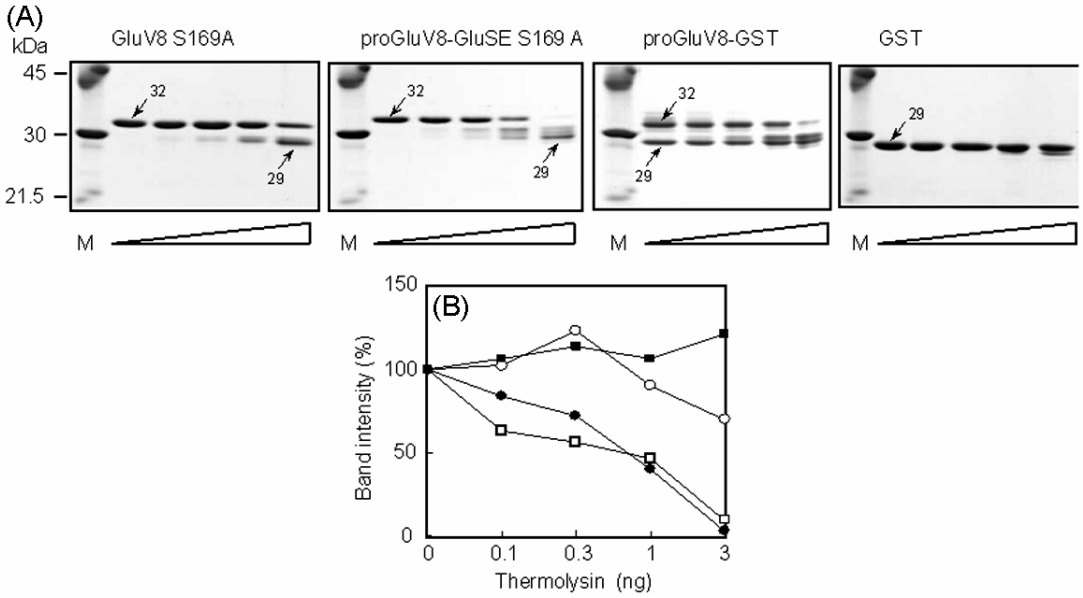

Fig. 2—Effect of exogenous propeptide on thermolysin susceptibility of the pro-mature junction [(**A**): GluV8 S169A, proGluV8-GluSE S169A, proGluV8-GST, and GST (10 μg) were incubated at 37°C for 4 h with 0-3 ng of thermolysin. Aliquots (1 μg) were separated by SDS-PAGE; and (**B**): Band intensities of 32-kDa GluV8 S169A (open circle), 32-kDa GluV8-GluSE S169A (closed circle),-32 kDa GluV8-GST (open square) and 29-kDa GST (closed square) were quantified]

Thermolysin susceptibility of the pro-mature border in chimeric molecules

The susceptibility of pro-mature junction towards thermolysin was studied with two different chimeras: proGluV8-GluSE carrying propeptide of GluV8-mut fused to mature GluSE, and proGluV8-GST consisting of GluV8-mut propeptide fused to glutathione S-transferase (the border was Ser_{-1}-Met_1). For this study, the active Ser_{169} of GluV8 and proGluV8-GluSE was substituted to Ala for eliminating the effect of massive difference in their specific activities[3]. The N-termini of both 32-kDa molecules from GluV8 S169A and proGluV8-GluSE S169A (Fig. 2) were found to be L_{-39}, indicating that

propeptide processing did not occur. Therefore, the propeptide processing could not be mediated by endogenous *E. coli* proteases, but was an autocatalytic event. On the other hand, proGluV8-GST was purified as doublets of a 32-kDa molecule starting with L_{-39} and a 29-kDa GST. The pro-mature junctions of both 32-kDa chimeric species were cleaved by thermolysin with 10-fold higher sensitivity than the 32-kDa pGluV8 S169A. GST alone was apparently resistant to thermolysin. These results suggested that the structure around the pro-mature junction in chimeric molecules was in an unfolded state and thus the Ser_{-1}-Val/Met_1 bond was more susceptible to thermolysin. Thus, there was a close relationship between the tertiary structure and thermolysin sensitivity of the pro-mature junction.

Cleavage sites in the propeptide of purified GluV8-family proenzymes

Cleavage sites on GluV8, GluSE, GluScpr and GluScoh determined in the previous studies[6] and those on GluEF, which were determined by amino acid sequencing, are summarized in Fig. 3. In GluEF, Glu_{-15} was identified as the N-terminus of 33-kDa species. When substitutions of E-16S, E-15K and E-9I were introduced (GluEF-mut), the 32-kDa species with the Gln_{-4} as N-termini was purified, indicating that the protection from the cleavages at the Glu_{-16}-Glu_{-15}, Glu_{-15}-Tyr_{-14} and Glu_{-9}-Ser_{-8} bonds seemed to trigger the Arg_{-5}-Gln_{-4} cleavage. Hydrolysis at Ala-Xaa bond should be mediated by signal peptidase[10]. From these observations, cleavage sites on the propeptides are summarized as follows: (i) the cleavages at the Glu-Arg/Ser/Glu and Asp-Ile would be mediated in an auto-proteolytic manner; (ii) those at Gln-Gln/Ala/Asn might also be explained through auto-proteolytic cleavages, because GluV8 can cleave the Gln-X bond; and (iii) those at His-Ser of GluSE, Lys-Lys of GluScpr-mut, and Arg-Gln of GluEF-mut seemed to be catalyzed by a protease preferential to basic amino acids at the P1 position.

Proteolytic activity of the GluV8-family proenzymes

Since the cleavage at Glu/Asp-Xaa and Gln-Xaa bonds in propeptides seemed to be carried out auto-catalytically, we investigated whether the proenzymes truly possessed the activity (Fig. 4). Zymography revealed the caseinolytic activity of 32- to 29-kDa pGluScpr-mut, pGluEF and pGluEF-mut. The proteolytic activity was not found in the migrating zone of respective mature enzymes, indicating the absence of mature proteases. Furthermore, LLE-MCA-hydrolyzing activity was detected with pGluScpr-mut, pGluEF and pGluEF-mut, suggesting that propeptide trimming could be mediated by the intrinsic proteolytic activity of proenzymes (Fig. 4). We also tested the activity with another partially unfolded protein substrate and found that carboxymethyl α-lactalbumin was degraded at the Glu_{68}-Tyr_{69} bond by pGluScpr-mut and pGluEF-mut (data not shown).

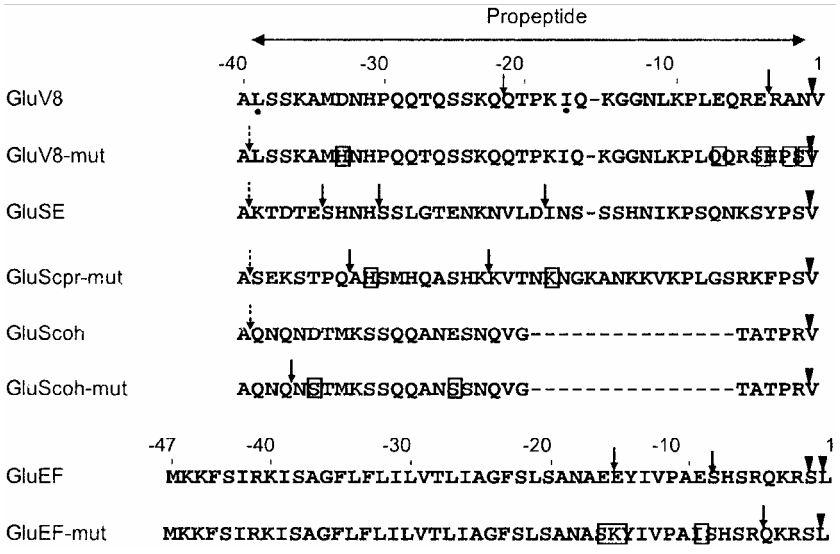

Fig. 3—Cleavage sites in propeptide of GluV8-family proteases [Arrows with dotted line represent the pre-pro borders cleaved by signal peptidase. Cleavage sites in propeptides are shown by arrows. Dots indicate Leu_{-39} and Ile_{-17} of GluV8. Arrowheads represent the pro-mature borders. In GluEF, two potential maturation sites of R_{-2}-S_{-1} and S_{-1}-L_1 have been reported[9]. Substituted amino acids are boxed. The position -3 of GluV8 was His in V8 strain[14] or Arg in ATCC25923 used for the plasmid construction (GluV8-wt)[6]]

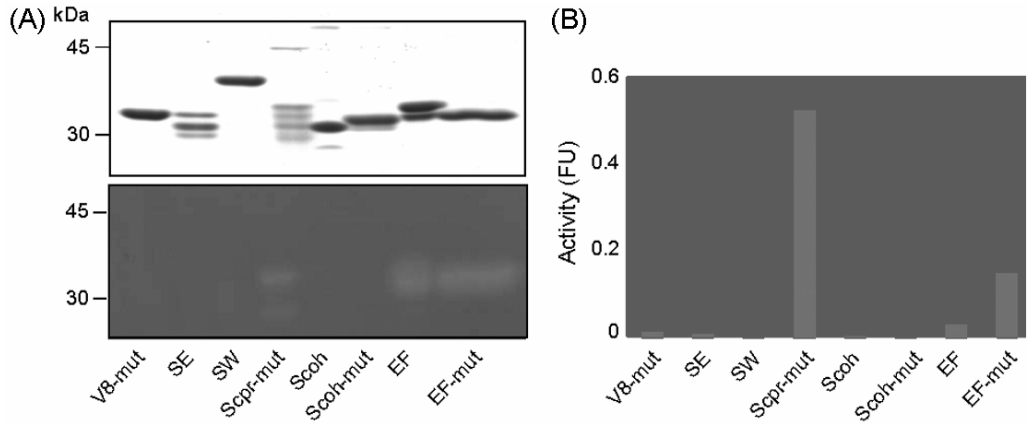

Fig. 4—Caseinolytic activity of GluV8-family proenzymes [(A): Proenzymes (1 µg) of GluV8-mut, GluSE, GluSW, GluScpr-mut, GluScoh, GluScoh-mut, GluEF and GluEF-mut were separated by SDS-PAGE (upper panel), or analyzed by casein zymography (lower panel); and (B): The proteolytic activity of proenzymes in panel A was measured with LLE-MCA (mean ± SD, n = 3)]

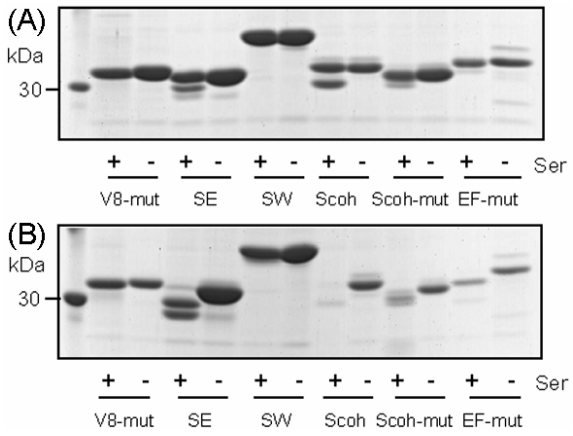

Fig. 5—Auto-degradation of GluV8-family proenzymes [(A): Proenzymes (1 µg) of GluV8-mut, GluSE, GluSW, GluScoh, GluScoh-mut and GluEF with the active Ser (wild type) (+) or S169A substitution (-) were separated by SDS-PAGE; and (B): The proenzymes were kept at 25°C for 72 h in the presence of 0.05% NaN_3 and then subjected to SDS-PAGE]

Auto-catalytic processing of proenzymes was further ascertained by studies with wild type and S169A inert mutants of six members of the family proteases (Fig. 5). When they were allowed to keep at 25ºC for 72 h, degradation was observed for wild type of GluSE, GluScoh and GluScoh-mut. On the other hand, no degradation was found for their S169A equivalents. The auto-degradation of wild type proenzymes was reinforced upon the prolonged incubation. Therefore, we concluded that cleavages at Glu/Asp-Xaa and Gln-Xaa bonds in propeptides were mediated by the intrinsic proteolytic activity of the GluV8-family proenzymes.

Proteolytic activity of pGluV8 toward peptides carrying Glu and Gln

Since pGluV8-mut starting from Leu_{-39} did not show proteolytic activity towards casein, it was

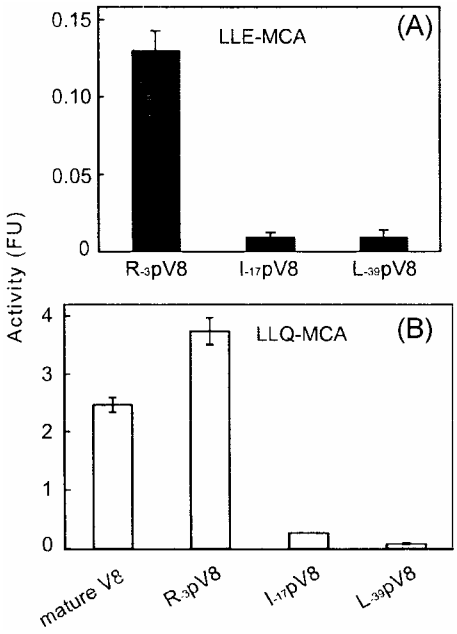

Fig. 6—Proteolytic activity of proenzyme of GluV8 with three types of propeptides. R_{-3}pGluV8, I_{-17}pGluV8 and L_{-39}pGluV8 (Fig. 1) were expressed [(A): The proteolytic activity toward LLE-MCA was measured with 0.5 µg of pGluV8 for 1 h; and (B): The proteolytic activity toward LLQ-MCA was measured with 20 µg of proteins for 6 h. (mean ± S.D., n=3). The activity of mature GluV8 (0. 25 µg) to LLE-MCA was 934.0 ± 52.7 FU]

suspected that the activity of proenzymes might exert along with shortening of propeptide. To address this possibility, the activity towards LLE/Q-MCA was carefully measured with pGluV8 possessing different lengths of propeptide. As shown in Fig. 6, a faint but convincing activity to LLE-MCA was eventually detected with a GluV8-wt fraction mainly composed of R_{-3}pGluV8. In contrast, GluV8-ΔN starting from Ile_{-17} and GluV8-mut from Leu_{-39} showed a negligible

activity. Furthermore, the hydrolysis of LLQ-MCA became detectable, when incubation was prolonged with larger amounts of proenzymes. Notably, R_{-3}pGluV8 showed a higher activity towards LLQ-MCA than mature GluV8 and the activities of proenzymes starting from Ile_{-17} and Leu_{-39} were again negligible.

Discussion

This study demonstrated that the propeptide processing commonly occurred in the GluV8-family proteases, including that from *E. faecalis*. Moreover, shortening of propeptide facilitated the hetero-catalytic maturation process of proenzymes in the family enzymes. As judged by C_{50} (Fig. 1), pro-mature border in H_{-3}pGluV8 was 5-fold more susceptible to thermolysin than L_{-39}pGluV8. The thermolysin susceptibility seemed to be closely related to the tertiary structure around the Xaa_{-1}-Val_1 bond, which probably became more exposed with a shortened propeptide. This assumption was confirmed by the finding that the border between chimeric pro-mature enzyme and propeptide-GST molecule was in unfolded structures (Fig. 2).

Hydrolysis of LLE/Q-MCA was detected on R_{-3}pGluV8, but not with pGluV8 possessing longer propeptides, which was in good agreement with the observation that Q_{-4}pGluEF had a higher proteolytic activity toward LLE-MCA than S_{-8}pGluEF. The Glu-specific activity of Q_{-4}pGluEF was also demonstrated with partially unfolded protein substrates, i.e., casein and carboxymethyl-α-lactalbumin. Therefore, cleavages of the propeptide at Glu/Asp/Gln-Xaa bond on the GluV8-family proteases could be auto-catalytically mediated by proenzymes.

Structural relationship between propeptide and mature portion has been extensively studied on subtilisin. It is reported that propeptide is folded, depending on the association with its mature portion[11]. Subbian *et al.*[12] further reported that propeptide is intrinsically unstructured and adopts an α-β structure only in the presence of mature moiety and modulates stochastic activation of subtilisin. Similarly, the present results suggested that folding state of GluV8 propeptide was rather loose, enabling the auto-catalytic propeptide processing and facilitating the final hetero- catalytic maturation cleavage.

The sequential auto-catalytic intra-molecular cleavage of pGluV8 in the Gln-rich segment (Q_{-29}QTQSSKQQTPKIQ$_{-16}$) has been proposed by Nickerson *et al.*[7]. However, the present study demonstrated that L_{-39}pGluV8 seemed to possess no proteolytic activity. Instead, we observed that R_{-3}pGluV8 carrying the shortest propeptide exhibited the activity towards LLQ-MCA, which was even higher than that of mature GluV8 (Fig. 6). Thus, it was reasonably deduced that propeptide cleavages at Gln-Xaa bonds were mediated in an auto-catalytic intermolecular manner by pGluV8 with truncated propeptide, such as R_{-3}pGluV8. This finding was consistent with the modeling, indicating that glutamine fits into the pocket of pGluV8[7].

In addition to cleavage at Glu/Gln-Xaa bonds, cleavage at Lys/Arg/His-Xaa bonds was observed as propeptide processing. These cleavages were suspected not to be catalyzed by mature enzymes, but also by proenzymes because of the following reasons: Firstly, no such cleavage was observed on the constitutive inactive forms (Figs 2 and 5); Secondly, substitution of Glu_{-9}, Glu_{-15} and Glu_{-16} to Ser, Lys and Ile, respectively, induced new cleavage at the Arg_{-5}-Gln_{-4} bond of pGluEF-mut. Since the protonated α-amino group of N-terminal Val is proposed to interact with carboxy group of Glu in a substrate[13], non-protonated imino group (Val_1) of pGluV8 may access more closely to basic amino acid residues than that of the mature form. Accordingly, Arg_{-5} could become a target and similarly, the non-acidic substrate LLQ-MCA was more efficiently hydrolyzed by R_{-3}pGluV8 than by mature GluV8.

In conclusion, the present study demonstrated that gradual cleavage in the propeptide region commonly occurred in the GluV8-family proteases and that some truncated proenzyme species possessed the convincing proteolytic activity towards Glu and Gln. These activities might mediate the propeptide shortening, facilitating the final hetero-catalytic maturation process.

Acknowledgements

This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to YO-N, TKN and SK).

References

1 Drapeau G R (1978) *J Bacteriol* 136, 607-613
2 Shaw L, Golonka E, Potempa J & Foster S J (2004) *Microbiology* 150, 217-228
3 Nemoto T K, Ohara-Nemoto Y, Ono T, Kobayakawa T, Shimoyama Y, Kimura S & Takagi T (2008) *FEBS J* 275, 573-587

4 Ohara-Nemoto Y, Ikeda Y, Kobayashi N, Sasaki M, Tajika S & Kimura S (2002) *Microb Pathog* 33, 33-41
5 Ono T, Nemoto T K, Shimoyama Y, Kimura S & Ohara-Nemoto Y (2008) *Anal Biochem* 381, 74-80
6 Ono T, Ohara-Nemoto Y, Shimoyama Y, Okawara H, Kobayakawa T, Baba T T, Kimura S & Nemoto T K (2010) *Biol Chem* 391, 1222-1232
7 Nickerson N N, Prasad L, Jacob L, Delbaere L T & McGavin M J (2007) *J Biol Chem* 282, 34129-34138
8 Ohara-Nemoto Y, Ono T, Shimoyama Y, Kimura S & Nemoto T K (2008) *Biol Chem* 389, 1217-1219
9 Kawalec M, Potempa J, Moon J L, Travis J & Murray B E (2005) *J Bacteriol* 187, 266-275
10 Chang C N, Blobel G & Model P (1978) *Proc Natl Acad Sci* (*USA*) 75, 361-365
11 Strausberg S, Alexander P, Wang L, Schwarz F & Bryan P (1993) *Biochemistry* 32, 8112-8119
12 Subbian E, Yabuta Y & Shinde U P (2005) *J Mol Biol* 347, 367-383
13 Prasad L, Leduc Y, Hayakawa K & Delbaere L T (2004) *Acta Cryst* D60, 256-259
14 Carmona C & Gray G L (1987) *Nucleic Acids Res* 15, 6757