Determination of three amino acids that caused the alteration of proteolytic activities of staphylococcal glutamyl endopeptidases

Takayuki K. Nemoto[1,*], Toshio Ono[1], Yu Shimoyama[2], Shigenobu Kimura[2] and Yuko Ohara-Nemoto[1]

[1] Department of Oral Molecular Biology, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588 Japan

[2] Department of Oral Microbiology, Iwate Medical University School of Dentistry, Morioka, 020-8505 Japan

*Corresponding author

T. K. Nemoto, Department of Oral Molecular Biology, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan

Fax: +81 95 819 7640, Tel: +81 95 819 7642, E-mail: tnemoto@nagasaki-u.ac.jp

Running title: Amino acid substitutions of V8 protease

Abstract

Staphylococcus aureus, Staphylococcus epidermidis and *Staphylococcus warneri* secrete glutamyl endopeptidases, designated GluV8, GluSE and GluSW, respectively. The order of their protease activities was GluSE<GluSW<<GluV8. The present study investigated the mechanism that causes these differences. Expression of chimeric proteins between GluV8 and GluSE revealed that the difference was primarily attributed to amino acids at residues 170-195, which defined the intrinsic protease activity, and additionally to residues 119-169, which affected the proteolysis sensitivity. Among nine substitutions present in residues 170-195 of the three proteases, the substitutions at positions 185, 188 and 189 were responsible for the changes in their activities; and the combination of W185, V188 and P189, which naturally occurred on GluV8, exerted the highest protease activity. Among them, W185 and P189 were indispensable for the full activity; but V188 could be replaced by hydrophobic amino acids. These three amino acid residues appeared to create a substrate-binding pocket together with the catalytic triad and the N-terminal V1, and therefore, defined the K_m values of the proteases. This study also describes the way to produce a chimeric form of GluSE and GluV8 that was resistant to proteolysis, and therefore, possessed activity 4-fold higher than that of the wild-type recombinant GluV8.

Key words: glutamyl endopeptidase; *Staphylococcus aureus*; *Staphylococcus epidermidis*; substrate-binding pocket; V8 protease.

Introduction

Staphylococcus aureus produces extracellular proteases, which are regarded as important virulence factors. One of the classically defined extracellular proteases is a serine protease, GluV8, known also as V8 protease/Glu-C/SspA (Drapeau et al., 1972; Rice et al., 2001). GluV8 contributes to the growth and survival of this microorganism in animal models (Coulter et al., 1998) and plays a key role in degrading fibronectin-binding proteins and protein A, which are adhesion molecules on the staphylococcal surface (Karlsson et al., 2001).

GluV8 belongs to the glutamyl endopeptidase I (EC 3.4.21.19) family. It specifically cleaves the peptide bond after negatively charged residues, i.e., glutamic acid, and much less potently, aspartic acid (Stennicke and Breddam, 1998). Because of its strict and unique substrate specificity and resistance to detergents, this enzyme has been frequently used for proteome analysis in combination with N-terminal sequencing and TOF-MS analyses. The nucleotide sequence of GluV8 encodes a protein of 336 amino acids that includes a prepropeptide consisting of 68 residues (M-68 - N-1) and a mature portion (V1 - A268). The mature sequence carries a C-terminal tail of 52 residues containing a 12-fold repeat of the tripeptide P-D/N-N, the role of which remains unknown (Carmona and Gray, 1987). Drapeau (1978) reported that activation of the GluV8 precursor is mediated by a neutral metalloprotease/aureolysin that belongs to the thermolysin family. In fact, the proform of recombinant GluV8 was processed to an active form *in vitro* by thermolysin treatment (Nemoto et al., 2008).

Two other staphylococcal glutamyl endopeptidases of V8 homologues, GluSE and GluSW, have been cloned and characterized. GluSE from *Staphylococcus epidermidis* consists of 282 amino acids composed of a prepro-sequence (M-66 - S-1) and mature portion (V1 - Q216), and does not have the C-terminal repeat sequence present in GluV8 (Ohara-Nemoto et al., 2002). Deduced amino acid sequence of GluSW from *Staphylococcus warneri* was composed of 316 amino acids and to possess a 34-amino acid D/N-rich region at its C-terminus (Yokoi et al., 2001).

We recently reported the expression of GluSE in *Escherichia coli* as a full-length form (Ohara-Nemoto et al., 2008). Recombinant GluSE was recovered as a soluble and inactive proform, which maintained the entire or parts of its prosegment (L-39 - S-1). In contrast, recombinant GluV8 was poorly obtained by the identical system due to auto-degradation, but was efficiently expressed and recovered by swapping its preprosegment with that of GluSE or by the introduction of amino acid substitutions into the prosequence of GluV8 (Nemoto et al., 2008; Ono et al., 2008). This modification suppressed the degradation of GluV8 in host cells. By using the modified prosequence of GluV8 as a fusion, GluSW was also over-expressed. They were purified as inactive proforms, and thereafter converted to the active forms by thermolysin processing *in vitro*. Through the expression and characterization of these recombinant proteases, we noticed that the proteolytic activities measured with a peptide substrate were significantly different among them: the activity of GluV8 was 40-fold higher than that of GluSE, and that of GluSW was 1.5-fold higher than that of GluSE (Ono et al., 2008).

To address the mechanism of alteration of the proteolytic activities, we explored in detail the substitutions of amino acids and demonstrate that their differences are attributable to a few amino acid substitutions within the β-strand segment forming the substrate-binding pocket.

Results

Proteolytic activities of GluSE/GluV8 chimeric proteases

Comparison of the amino acid sequences at the mature portions of GluV8, GluSW and GluSE except for C-terminal extensions characteristic of GluV8 and GluSW revealed 58.8-80.1% homology (Figure 1). We recently demonstrated that the proteolytic activities of the three staphylococcal glutamyl proteases were significantly different from each other (Ono et al., 2008). For instance, GluV8 and GluV8ΔC, in which the C-terminal 52 residues was deleted, exhibited activities against a peptide substrate (156.20 ± 3.20 and 205.13 ± 4.57 FU, respectively) 40-50 times higher than that of GluSE (4.31 ± 0.08 FU, Figure 1). Their amino acid conversion should cause the alteration of the protease activities, although it is unknown (i) whether a limited number of amino acid substitutions specifically modified the active site or (ii) whether substitutions nonspecifically modified the 3D structure and the activity.

To address this issue, we first investigated whether a specific region was responsible for the difference in the proteolytic activity by expressing chimeric proteases. Because the C-terminal 52-amino acids of GluV8 appeared not to significantly affect the activity (Figure 2) (Ono et al., 2008; Yabuta et al., 1995), the remaining region of the mature sequence was dissected (Figures 1 and 2). Namely, concerning the mature sequence, Chimera A carried the N-terminal half of GluV8 (positions 1-118) and C-terminal half of GluSE (positions 119-216); Chimera B carried GluV8, in which the third quarter from the N-terminus (positions 119-169) was replaced by the sequence of GluSE; Chimera C carried GluV8, in which the C-terminal quarter (positions 170-216) was replaced by the sequence of GluSE; and Chimera D carried GluV8, in which the seventh part of 8 portions (positions 170-195) was replaced by the sequence of GluSE. The chimeric proteases as well as GluSE, GluV8 and GluV8ΔC were converted to their mature forms by thermolysin treatment (Figure 1).

The activity of Chimera A (5.88 ± 0.36 FU) was comparable to that of GluSE (4.31 ± 0.08 FU), whereas Chimera B revealed super-activity (460.43 ± 9.06 FU)

that was even higher than that of GluV8 or GluV8ΔC. This finding indicated that the C-terminal quarter (positions 170-216) of GluV8 was responsible for the greater proteolytic activity of GluV8. In contrast, the proteolytic activities of Chimera C (1.46 ± 0.02 FU) and Chimera D (0.07 ± 0.01 FU) were even lower than that of GluSE. This result further narrowed the region required for the higher activity of GluV8 into amino acids 170-195. The recovery of mature forms, such as GluV8 and Chimeras C and D, was apparently lower than that of the others; and the difference in recovery could partly account for the lower activities of some of the mature enzymes. We will further investigate the protease sensitivity of the glutamyl endopeptidases that caused the low recovery later.

W185 and P189 were responsible for the higher proteolytic activity of GluV8

Figure 2 demonstrated that the amino acid substitutions at a limited region (positions 170-195) should be responsible for the alteration of the protease activity between GluSE and GluV8. In that region, there are 8 amino acid differences, at positions 176, 179, 185, 189, 191, 192, 194 and 195, between GluSE and GluV8 (Figure 3A). On the 3D structure of GluV8, Glu176-L177 forms a protruding loop connecting two β-strands composed of G170-S-P-V-F-N175 and N178-E179-V-I-G182; and E176 and E179 are located at the surface of the molecule, being distant from the H51, D93 and S169 catalytic triad (Prasad et al., 2004). Hence, the substitutions from G176 and Q179 of GluSE to two E residues of GluV8 seemed not to significantly affect the activity. In contrast, H184-W-G-G-V-P189, which contained two substitutions (underlined), and E191-F-N-G-A195, which contained four substitutions (underlined), form an anti-parallel β-sheet structure associated with each other (Figure 4). Interestingly, the former β-strand faces the catalytic triad and V1, an important amino acid for substrate recognition (Prasad et al., 2004); and, therefore, we assumed that this region possibly takes part in a substrate-binding pocket together with the four amino acids. The latter β-strand is largely buried inside of the molecule behind the former β-strand (Figure 4).

Hence, we suspected that the 6 substitutions, especially, the two substitutions at the N-terminal side of Y185W and D189P may affect the protease activity.

When the 6 amino acids of GluSE were simultaneously replaced by those of GluV8, i.e., Y185W, D189P, L191E, Y192F, S194G and S195A (designated GluSE-WPEFGA), the proteolytic activity of GluSE-WPEFGA (820.77 ± 27.69 FU) became 3.8-fold higher than that of GuV8ΔC (215.70 ± 6.05 FU, Figure 3C), in accord with the super-activity of Chimera B. Moreover, it is of great interest that the activity of GluSE with the two substitutions, i.e., Y185W and D189P (designated GluSE-WP), was equivalent to that of GluSE-WPEFGA. In contrast, the substitution of residual four residues (designated GluSE-EFGA, 13.81 ± 0.21 FU) induced little increase (2-fold) over the activity of the wild-type GluSE (6.72 ± 1.25 FU). A single substitution of either Y185W or D189P from GluSE, producing GluSE-W (173.07 ± 2.40 FU) and GluSE-P (143.67 ± 18.30 FU), respectively, induced moderate enhancements. These results demonstrated that the two substitutions of Y185W and D189P were critical for the proteolytic activity and even sufficient for the achievement of the significantly high activity of GluV8. Therefore, we concluded that W185 and P189 were keenly involved in the proteolytic activity of GluV8, in addition to the catalytic triad and the N-terminal V1.

A proteolytically susceptible region of GluV8 and GluSW that affected the protease activity

The recovery of mature forms of Chimeras C and D after thermolysin treatment was apparently lower than that of Chimeras A and B (Figure 2). We previously demonstrated that mal-expression of wild-type GluV8 and GluSW in *E. coli* was triggered by the auto-proteolysis of the prosequence (Nemoto et al., 2008; Ono et al., 2008). In view of those studies, the low recovery of Chimeras C and D as well as GluV8 strongly suggested that region 119-169 of GluV8 was more susceptible to proteolysis than the corresponding sequence of GluSE. To confirm this assumption, we compared the thermolysin sensitivity of GluSE and GluSW, because mature GluSW was

the most susceptible to thermolysin among the three endopeptidases (Ono et al., 2008).

Firstly, when the proforms of GluSW and GluSE were incubated with thermolysin, the 32-kDa mature GluSW was produced at higher doses at 4 h, but most of it further degraded by 20 h of incubation, whereas most of the 28-kDa mature GluSE remained intact at 4 and 20 h (Figure 5). These findings clearly demonstrated higher protease sensitivity of GluSW compared with that of GluSE. After a 4-h incubation, the proteolytic activities of both GluSW and GluSE were increased concomitantly with the appearance of the mature forms; and the activity of GluSW was 2-fold higher than that of GluSE (Figure 5). After 20 h, the activity of GluSW became comparable to that of GluSE at lower doses, and even lower than that of GluSE at higher doses of thermolysin.

We then expressed a chimeric form between GluSE and GluSW (designated GluSE/SW) that mimicked the constitution of Chimera B. GluSE/SW was composed of amino acids -66 - 169 of GluSE, which carried a putative proteolytic resistant region (residues 119-169) and amino acids 170-250 of GluSW. As a result, the 32-kDa mature GluSE/SW remained un-degraded even at the highest dose of thermolysin after the 20-h incubation. Accompanied with these changes, the proteolytic activity of GluSE/SW became 4- and 2-fold higher than that of GluSE and GluSW, respectively, at 4 h, and approximately 3-fold higher than those activities of GluSE and GluSW at 20 h (Figure 5). These observations indicated that the proteolytic activity of a recombinant protease measured was the sum of the intrinsic proteolytic activity and the degree of degradation of the mature enzyme. Therefore, the super-activities observed on Chimera B and GluSE-WP (Figures 2 and 3) could be accomplished by the protease resistance owing to the sequence of 119-169 of GluSE. These findings also indicated that Chimera B and GluSE/SW represented the intrinsic activities of GluV8 and GluSW, respectively.

W185, A188 and A189 were responsible for the activity of GluSW

We here defined the amino acid substitutions that characterized the protease activity of GluSW. From the data in Figure 5, it is evident that the protease activity of GluSW was

characterized by amino acids 170-250. This region could be narrowed to amino acids 170-195, in consideration of the results on GluV8. At positions 170-195, there are nine amino acid substitutions between GluSW and either GluSE or GluV8. Among them, the importance of W185 and A189 of GluSW appeared to be clear as discussed for GluV8 (Figures 3 and 4). Furthermore, the involvement of S176, R177 and E179 could be eliminated by the reason discussed above for E176 and E179 of GluV8; and that of S191, Y192, G194 and A195 was also unlikely, since GluSE-EFGA showed little change in the activity (Figure 3). Thereby, we focused on the rest of the substitutions, of A188 in addition to W185 and A189.

Because the activity of GluSE-W, i.e., GluSE with the Y185W mutation, was 29-fold higher than that of the wild-type GluSE, it was reasonable to assume that the activity of GluSW possessing W185 was higher than that of GluSE (Figure 3). However, this magnitude was substantially higher than the difference between GluSE/SW and GluSE, suggesting that the remaining two substitutions were also involved in the modulation of the activity. Accordingly, we first substituted D189 of GluSE-W to A (designated GluSE-WA), and found that its protease activity was reduced by 55% from that of GluSE-WP (Figure 6C, columns 5 and 7). However, the activity of GluSE-WA was still 11-fold higher than that of GluSE/SW (columns 3 and 5). We then introduced the V188A substitution to GluSE-WA and GluSE-WP, creating GluSE-WAA and GluSE-WAP, respectively. The activity of GluSE-WAA was reduced by 78% from that of GluSE-WA (columns 5 and 6). A similar reduction (58%) was observed upon the substitution from GluSE-WP to GluSE-WAP (columns 7 and 8). As a consequence, the activity of GluSE-WAA (49.44 ± 10.91 FU) became comparable to that of GluSE/SW (19.79 ± 1.37 FU, 2.5-time difference). Therefore, we concluded that the activity of GluSE/SW, which may elicit the intrinsic activity of GluSW, was primarily attributable to the substitutions at positions 188 and 189 of GluV8 and the substitutions at positions 185, 188 and 189 of GluSE.

Determination of kinetic parameters

This study defined the proteolytic activities of glutamyl endopeptidases by an end-point assay at a single concentration of the peptide substrate, and therefore, there was little information on the mechanism of amino acid substitutions. Then, we here determined the kinetic parameters of recombinant proteases with altered amino acids at positions 185, 188 and 189. As a result, the K_m and k_{cat} values of Chimera B and GluSE-WP were comparable to each other (Table 1). The substitution from W185 to Y185 represented by GluSE-WP to GluSE-P induced a 12-fold increase in K_m with a constant k_{cat} value. The conversion of P189 to D189 represented by GluSE-WP to GluSE-W resulted in an 8-fold increase in K_m with a similar k_{cat}. Similarly, the conversion of V188 to A188 represented by GluSE-WP to GluSE-WAP also caused a 4-fold increase in K_m, although the magnitude was smaller than the increases at positions at 185 and 189. Taken together, the conversions of the three amino acids from W185, V188 and P189 to Y185, A188 and D189 commonly induced an increase in the K_m values, but did not modify the k_{cat} values. We also attempted to define the K_m and k_{cat} of GluSE and GluSW. Their K_m values appeared to be at the mM level or higher. However, quantitative values were not determined under our experimental conditions, because the fluorescent scores were low and not saturated at the 0.05-0.5 mM substrate concentrations (data not shown).

Roles of amino acids at positions 185, 188 and 189

To further evaluate the roles of amino acids at positions 185, 188 and 189, we substituted them with various amino acids (Figure 7). Y185 of GluSE was substituted to hydrophobic amino acids of W (GluV8 and GluSW types), L, A or Q, and the proteolytic activity for LLE-MCA of these mutants was then determined (Figure 7B, left). Among them, the highest activity was achieved with W185. GluSE-Q185 showed an activity 2.7-fold higher than that of the wild-type GluSE (Y185). The activities of GluSE-L185 and A185 were even lower than the activity of GluSE. The proteolytic activity was also

determined with AE-MCA, a less potent glutamyl substrate (2% of that for LLE-MCA) (Ono et al., 2008). The activity was the highest for GluSE-W185, followed by wild-type GluSE (Y185); GluSE-Q185 showed activity lower than GluSE-Y185; and GluSE-L185 and A185 showed no activity (Figure 7B, right). These results showed that hydrophobicity at position 185 was not primarily important but that W185 should be indispensable for the maximal proteolytic activity.

Next, D189 of GluSE was replaced by P (GluV8 type), E, A (GluSW type) or T (Figure 7C). As a result, the highest activity was achieved with P189. Increases of 2.3-, 3.2- and 5.1-fold from wild-type GluSE (D189) were observed by the substitutions to E, A and T, respectively. The activity toward AE-MCA was again the highest for GluSE-P189, and showed lowest activities with D and E. Therefore, P was the most preferable, A was less preferable and the acidic amino acids were the least preferable at position 189.

At last, we examined the effect of the substitution at V188 (Figure 7D). In this case, we used GluSE-WP (GluV8 type) as a reference. When V188 of GluSE-WP was substituted to A (GluSW type), L, I or M, L188 and I188 gave activities toward LLE-MCA similar to the activity of V188. M188 showed a slightly lower activity, followed by F188; and A188 had the lowest activity. When examined with AE-MCA, the activity was the highest with L188, F188 and V188 followed by M188, I188. Again, A188 showed the lowest activity. This finding strongly suggests that hydrophobic residues could be accepted at position 188. Interestingly, the activity of F188 accounted for 67% of the activity of the reference (GluSE-WP) toward LLE-MCA, but was 103% toward AE-MCA, indicating that F188 was acceptable for AE-MCA, but not preferable for LLE-MCA.

Taken together, the combination of W185, V188 and P189, which naturally occurred on GluV8, exerted the highest protease activity. Among them, W185 and P189 could not be replaced by other amino acids for the full activity, but V188 could be replaced by hydrophobic amino acids, such as L188 for the two substrates, F188 for

AE-MCA and I188 for LLE-MCA.

Discussion

Several amino acids involved in the proteolytic activity of the glutamyl endopeptidases have been reported. The glutamyl endopeptidases belong to the serine protease superfamily, in which three amino acids, H51, D93 and S169, forming the catalytic triad, are completely conserved (Figure 1). In fact, the substitution of S169 of GluSE (Ohara-Nemoto et al., 2008), GluV8 (Nemoto et al., 2008) and GluSW (T. Ono and T.K. Nemoto, unpublished observation) to A caused complete loss of the protease activity. In addition to the catalytic triad, Prasad *et al.* (2004) proposed the importance of the N-terminal V1, whose α-amino group interacts with the γ-carboxyl group of a substrate peptide. Similarly, the 3D structure of the glutamyl endopeptidase from *Bacillus intermedius* revealed that the N-terminal end is involved in the formation of a substrate-binding pocket (Meijers et al., 2004). Truly, the cleavage at the S-1 - V1 bond of GluSE and N-1 - V1 of GluV8 and GluSW was prerequisite for the acquisition of the protease activity (Nickerson et al., 2007; Nemoto et al., 2008; Ohara-Nemoto et al., 2008). A mutagenesis study revealed that the N-terminal V1 is beyond a simple provider of an α-amino group for a substrate, as it could not be compensated by other amino acids, such as A, F, G or S (Nemoto et al., 2008). H184 of GluV8 is located close to S169 (Figure 4) and highly conserved among endopeptidases from staphylococci (Carmona and Gray, 1987; Ohara-Nemoto et al., 2002; Yokoi et al, 2001), Bacillus species (Kakudo et al., 1992; Svendsen and Breddam, 1992; Rebrikov et al., 1999) and Streptomyces species (Suzuki et al., 1994; Svendsen et al., 1991), indicating its importance. In fact, the substitution of H186T of *B. intermedius* endopeptidase (equivalent to H184 of GluV8) resulted in a 615-fold reduction in k_{cat}, but had little effect on the K_m (4.9-fold increase) (Demidyuk et al., 2004).

This study is the first to demonstrate that the difference in the protease activities

among the three staphylococcal glutamyl endopeptidases can be characterized by only three amino acids at positions 185, 188 and 189. This was surprising, because there are 89 and 84 amino acid substitutions, respectively, at the mature sequences between GluV8 and GluSE and between GluSW and GluSE except for the C-terminal extensions (Figure 1). The substitutions of these three amino acids significantly affected K_m, but had little effect on k_{cat} of the staphylococcal endopeptidases. How are these amino acids involved in the proteolytic activities? The 3D structure of GluV8 showed that the β-strand carrying the three amino acids and H184 are exposed at the surface of the molecule and form a substrate-binding pocket together with the catalytic triad and V1 (Figure 4). In particular, W185 is located adjacent to the triad, and it could not be substituted to any amino acids tested for achieving the high proteolytic activity (Figure 7B). A simulation of the 3D structure indicated that Y185 replaced by W185 overhung D93 of the triad and narrowed the substrate-binding pocket (data not shown). This may be the main reason why Y185 was not preferable.

Among the amino acids at position 189 being substituted in this study, acidic amino acids showed the lowest activity (Figure 7C). The 3D structure showed that P189 faces against the N-terminal V1 at a 4.2Å distance (Figure 4), of which α-amino acid could be associated with the γ-carboxyl group of glutamic acid of a substrate peptide. Accordingly, the negative charge of D and E at position 189 may interefere with the substrate interaction mediated by V1.

Although the V188A substitution also reduced the protease activity of GluSW, its effect was limited compared to the substitutions at positions 185 and 189 (Figure 7D). This may reflect that V188 is located relatively distant from the catalytic triad and V1 (Figure 4). Interestingly, hydrophobic amino acids partially or fully compensated the role of V188. Moreover, the degree of the compensation appeared to be altered between substrates, i.e., LLE-MCA and AE-MCA. Taken together with these results and the fact that GluV8 prefers F at P2 and V at P3 positions (Breddam and Meldal, 1992), we propose that the hydrophobic amino acid at position 188 participates in the

interaction of P2 and P3 amino acids of a substrate. The alteration of k_{cat} as reported for the H186T mutation of *B. intermedius* endopeptidase indicates the role of H184 in the step(s) following the recognition of a substrate (Demidyuk et al., 2004). In contrast, the change in K_m accompanied by the substitutions at positions 185, 188 and 189 strongly suggests their roles in the substrate recognition.

At last, it is important to emphasize the usefulness of the recombinant endopeptidases overexpressed in *E. coli*. By use of an *E. coli* expression system, we can avoid the usage of glutamyl endopeptidase purified from the extracellular fraction of *S. aureus*, which might contain virulence factors of the infectious bacteria. Furthermore, GluSE-WP and Chimera B, which possessed the super-activity higher than the parent form due to resistance to proteolysis, are most useful for proteome analysis.

Materials and methods

Materials

The materials used and their sources were the following: expression vector pQE60 and plasmid pREP4, from Qiagen Inc. (Chatsworth, CA); low-molecular-weight markers, from GE Healthcare (Buchinghamshire, England); restriction enzymes and DNA-modifying enzymes, from Nippon Gene (Tokyo, Japan); KOD plus DNA polymerase, from Toyobo (Tokyo, Japan); Z-LLE-MCA and Suc-AE-MCA, from Peptide Institute Inc. (Osaka, Japan); and Talon metal affinity resin, from Clontech Laboratories Inc. (Palo Alto, CA). Oligonucleotide primers were purchased from Genenet (Fukuoka, Japan).

Expression plasmids for the full-length forms

Recombinant proteins were expressed in *E. coli* with a histidine hexamer tag at their C-terminus by use of the pQE60 expression vector. Methods for the expression of GluSE (designated pQE60-GluSE) (Ohara-Nemoto et al., 2008), GluV8 (pQE60-GluV8) and its C-terminal-52-amino acid deletion form (designated pQE60-GluV8ΔC) (Ono et al., 2008) were described previously. GluSW was expressed as a fusion carrying the modified prepropeptide of GluV8 and the mature form of GluSW (designated pQE60-GluSW) as reported previously (Ono et al., 2008).

Expression plasmids for chimeric forms

The DNA fragments encoding chimeric forms between GluSE and GluV8 or GluSE and GluSW were amplified by a PCR with a pair of appropriate primers with KOD Plus DNA polymerase. Two of the following plasmids, pQE60-GluSE, pQE60-GluV8ΔC, or pQE60-GluSW were used as template as mixtures. Because the preprosequence of GluSE completely compensate the function of that of GluV8 (Nemoto et al., 2008), we did not care about the difference in the preprosequences among the chimeric proteins. PCR was performed according to the following conditions: An initial denaturation at

94 ˚C for 2 min, and 34 cycles of 94 ˚C for 20 sec, 57 ˚C for 30 sec and 68 ˚C for 6 min. After *Dpn*I digestion of the templates, the 5'-end of 5-kb PCR-amplified linear plasmid was phosphorylated by T4 polynucleotide kinase and self-ligated by T4 DNA ligase. Y1090[pREP4] cells were transformed with the resulting plasmids. Production of chimeric plasmids was confirmed by DNA sequencing. The structures of the chimeric proteases are illustrated in Figures 2A and 6A.

***In vitro* mutagenesis by PCR**

In vitro mutagenesis was performed to substitute the nucleotides of pQE60-GluSE encoding the amino acids at positions 185, 189, 191, 192, 194 and 195 (designated pQE60-GluSE-WPFEGA); at positions 185 and 189 (designated pQE60-GluSE-WP); and at positions 191, 192, 194 and 195 (designated pQE60-GluSE-EFGA) by PCR-based mutagenesis technique using mutated primers. Y185 in GluSE was substituted to W, L, A or Q, and D189 to P, E, A or T. V188 in GluSE-WP, which carried Y185W and D189P substitutions, was substituted to A, F, L, I or M. Every mutagenesis was confirmed by DNA sequencing.

Expression and purification of recombinant proteases

Recombinant proteins were expressed and purified as described previously (Nemoto et al., 2008). Briefly, *E. coli* Y1090[pREP4] carrying a pQE60-derived expression plasmid was cultured overnight at 37 ˚C in LB broth containing 50 μg/ml of ampicillin and 25 μg/ml of kanamycin. Protein expression was induced by dilution of the culture with 1.5 vol of LB broth containing 0.2 mM isopropyl β-D-thiogalactopyranoside and incubation at 30 ˚C for 3 h. The following process was performed at 4 ˚C: Bacterial cells were harvested by centrifugation and lysed with lysis/washing buffer (20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 10 mM imidazole), to which 0.5 mg/ml of lysozyme and 10 μg/ml of leupeptin had been added. The bacterial lysate was obtained by centrifugation at 30,000 ***g*** for 40 min, and recombinant proteins were then purified by

chromatography with Talon metal affinity resin according to the manufacturer's protocol, except that 10 mM imidazole was included in the lysis/washing buffer. After washing out the unbound materials, bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol. The purified proteins were stored at -80 ˚C until used.

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

Unless otherwise stated, the *in vitro* processing of a recombinant proenzyme and the subsequent protease assay were performed as follows: Recombinant proteins (0.3 nmol) were incubated in 0.1 ml of buffer A [10 mM sodium borate, pH 8.0, containing 0.005% (v/v) Triton X100 and 2 mM CaSO4] with 8 pmol (ratio 37.5:1) of thermolysin at 37 ˚C for 4 h. Thereafter, aliquots of glutamyl proteases (35 nM as a proform) were incubated with 20 μM LLE-MCA in 0.2 ml of 50 mM Tris-HCl (pH 8.0) and 5 mM EDTA at 25 ˚C for 2 h. EDTA was added to the reaction mixture to inactivate thermolysin (Fontana, 1988). The fluorescence was measured with an excitation at 380 nm and emission at 460 nm with a Fluorescence Photometer F-4000 (Hitachi, Tokyo, Japan).

In the experiment of Figure 5, proenzymes were treated with various doses of thermolysin (0.1-30 pmol, ratio 3,000:1 to 10:1); and incubated for 4 or 20 h. In the experiment of Figure 7, the activity was also measured with a peptide substrate, AE-MCA. For determination of kinetic parameters, the protease reaction was performed at 25 ˚C for 1 h with thermolysin-treated samples (0.285 or 0.57 pmol) in 20 μl of buffer A containing 0.05-0.5 mM LLE-MCA. K_m and V_{max} were calculated by nonlinear regression analysis for fitting the Michaelis-Menten equation by using a GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). k_{cat} was determined from V_{max} divided by total enzyme concentrations.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified proteins (0.8 μg) were separated by PAGE in the presence of 0.1% SDS at a polyacrylamide concentration of 12.5% (w/v). Separated proteins were stained with

Coomassie Brilliant Blue.

3D structure preparation

The 3D figures of GluV8 were prepared by using the program PyMol (Delano Scientific LLC: http//pymol.sourceforge.net).

N-terminal amino acid sequencing

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

Protein concentration

Protein concentrations were determined by the bicinchoninic acid method (Pierce). Bovine serum albumin was used as a reference and was dissolved in 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol to adjust buffer compositions to affinity-purified samples.

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Table 1. Kinetic parameters of recombinant endopeptidases.

	Amino acid				
Protease	185	188	189	K_m [a] (mM)	k_{cat} [a] (sec^{-1})
Chimera B	W	V	P	0.30 ± 0.08	6147 ± 1117
GluSE-WP	W	V	P	0.35 ± 0.13	7695 ± 1200
GluSE-W	W	V	D	2.84 ± 1.49	8863 ± 1765
GluSE-P	Y	V	P	4.15 ± 1.58	7681 ± 3069
GluSE-WAP	W	A	P	1.32 ± 0.10	9059 ± 4205

[a]Mean ± SD of 4 separate experiments analyzed in duplicate.

Figure legends

Figure 1 Comparison of the amino acids at the mature region of three staphylococcal glutamyl endopeptidases.

The mature sequences of GluV8, GluSW and GluSE are aligned. Hyphens are deletions introduced for maximal matching. Amino acids identical among the three proteases are shown by asterisks below the sequences, and those identical in two endopeptidases are shown by dots. The catalytic triad (H51, D93 and S169), V1 and H184 important for the activity are indicated by arrowheads. Underlines on the C-terminal sequence of GluV8 represent deleted amino acids for the expression of GluV8ΔC. Broken vertical lines indicate the borders of the chimeric forms expressed in Figures 2 and 6. Amino acids 170-195, which defined the proteolytic activity of staphylococcal endopeptidases, are boxed.

Figure 2 Expression of GluSE, GluV8 and their chimeric proteases.

(A) The structures of recombinant proteases and their chimeric proteins are schematically illustrated. Amino acid sequences of GluSE and GluV8 are indicated by gray and open boxes, respectively. Asterisks represent the 5 amino acid substitutions introduced in the prosequence (Ono et al., 2008). Presequence, prosequence and the three amino acids forming the catalytic triad are indicated on the top. (B) The purified samples (0.3 nmol) were incubated at 0 ˚C without protease (-) or at 37 ˚C with 8 pmol (+) of thermolysin. Aliquots (0.8 μg) of non-untreated (-) and thermolysin-treated (+) samples were separated by SDS-PAGE. Lane M, low-molecular-weight markers. (C) The proteolytic activity toward LLE-MCA was determined as described in "Materials and methods," and presented as fold increase (means ± SD, n=3), with the activity of GluSE (4.31 ± 0.08 FU) set to 1. Numbers 1-7 in panels "B" and "C" coincide with those of panel "A."

Figure 3 Expression of GluSE and its derivatives that mimicked the activity of GluV8.

(A) Amino acid substitutions present at 170-195 of GluSE, GluV8ΔC, GluSE derivatives and GluSW are aligned. Amino acids identical to those of GluSE are represented by hyphens. (B) The purified samples were incubated at 0 ˚C without protease (-) or at 37 ˚C with (+) thermolysin. Aliquots (0.8 μg) of thermolysin-untreated (-) and thermolysin-treated (+) samples were separated by SDS-PAGE. Lane M, low-molecular-weight markers. (C) The proteolytic activity toward LLE-MCA was determined and expressed as fold increase (means ± SD, n=3), with the activity of GluSE (5.13 ± 0.11 FU) set to 1. Numbers 1-7 in panels "B" and "C" coincide with those of panel "A."

Figure 4 3D structure of GluV8.

(A) Overall fold of the mature form of GluV8ΔC is shown based on the data of Prasad et al. (2004). H184-W-G-G-V-P189 and E191-F-N-G-A-V-F-I198, forming an anti-parallel β-sheet, are shown in red and blue, respectively. Residual groups of H184, W185 and P189 are illustrated, and those of V1, H51, D93 and S169 are shown in green. (B) Surface structure of GluV8ΔC. Color coding is as in panel "A."

Figure 5 Thermolysin sensitivity of GluSE, GluSW and their chimera.

The chimera of GluSE and GluSW that carried residues -66-169 of GluSE and 170-250 of GluSW (designated GluSE/SW) was expressed. Purified forms of GluSE, GluSW and GluSE/SW (0.3 nmol) were incubated at 0 ˚C without thermolysin (lane 1), at 37 ˚C without thermolysin (lane 2), with 0.1 (lane 3), 0.3 (lane 4), 1 (lane 5), 3 (lane 6), 10 (lane 7), or 30 pmol (lane 8) of thermolysin for (A) 4 or (B) 20 h. Aliquots (0.8 μg) of the samples were separated on SDS-PAGE. Lane M, molecular markers. The proteolytic activity of GluSE (open circles), GluSW (closed circles), and GluSE/SW (squares) toward LLE-MCA was determined at 35 nM recombinant proteins and presented as fold increases (mean ± SD, n=3), with the activity of GluSE treated with 30 pmol of thermolysin set to 1 (7.06 ± 0.66 and 5.32 ± 0.0.11 FU at the 4- and 20-h

treatments, respectively).

Figure 6 Expression of GluSE and its derivatives that mimicked the protease activity of GluSW.

(A) The structures of the expressed proteases are illustrated. Amino acid sequences of GluSE, GluV8 and GluSW are indicated by gray, open and hyphened boxes, respectively. Asterisks in GluSW indicate the 5 amino acid substitutions introduced into the GluV8 prosequence (Ono et al., 2008). Y185, V188 and D189 of GluSE were substituted as shown in Numbers 5-8. (B) Purified samples (0.3 nmol) were incubated at 0 ˚C without protease (-) or at 37 ˚C with 8 pmol (+) of thermolysin. Aliquots (0.8 μg) of thermolysin-untreated (-) and thermolysin-treated (+) samples were separated by SDS-PAGE. Lane M, molecular markers. (C) The proteolytic activities toward LLE-MCA were determined at 35 nM recombinant proteins. The proteolytic activity was presented as fold increase (mean ± SD, n=3), with the activity of GluSE (5.13 ± 0.11 FU) set to 1. Numbers 1-8 in panels “B” and “C” coincide with those of panel “A.”

Figure 7 Effects of amino acid substitutions at Y185 and D189 of GluSE and V188 of GluSE-WP on the protease activity.

(A) The structures of the three endopeptidases are illustrated. Y185 and D189 of GluSE and V188 of GluSE-WP were substituted to amino acids X. (B) Y185 of GluSE was substituted to W, L, A or Q. (C) D189 of GluSE was substituted to P, E, A or T. (D) V188 of GluSE-WP was substituted to A, F, L, I or M. Thereafter, proteolytic activities toward LLE-MCA or AE-MCA were determined after thermolysin processing. Values of GluSE with W185, GluSE with P189 and GluSE-WP were set to 100% in panels “B”, “C” and “D”, respectively. Values are means ± SD (n=3).

Figure 1

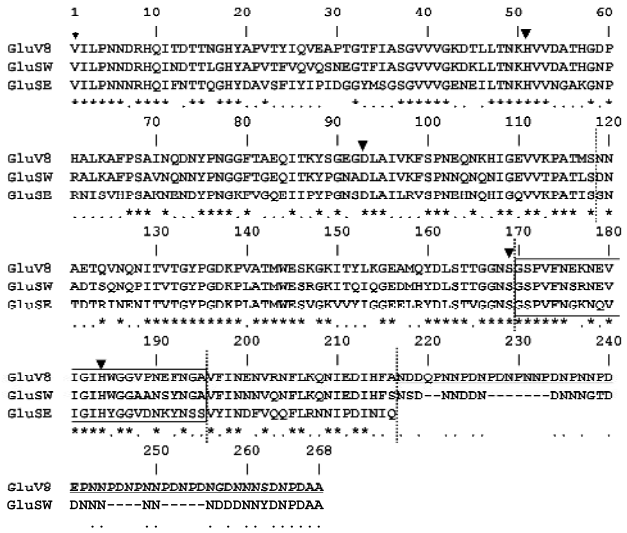


Figure 2

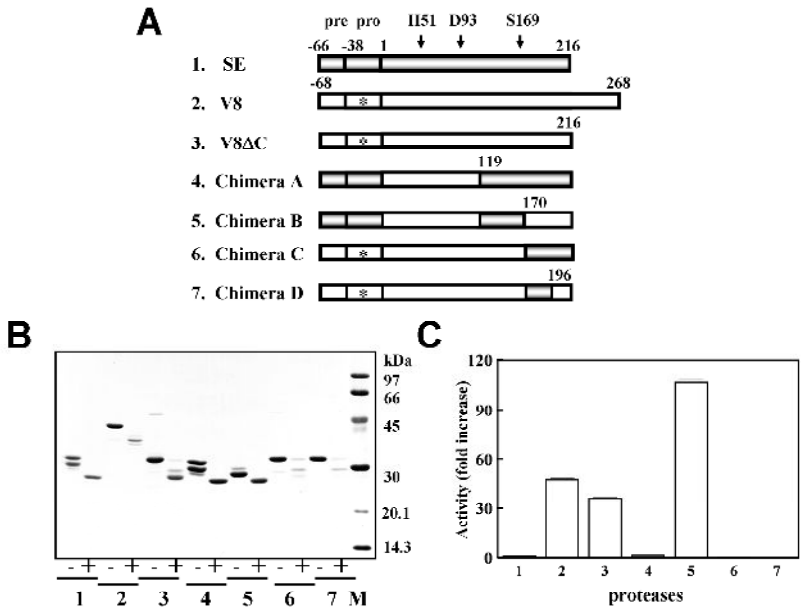


Figure 3

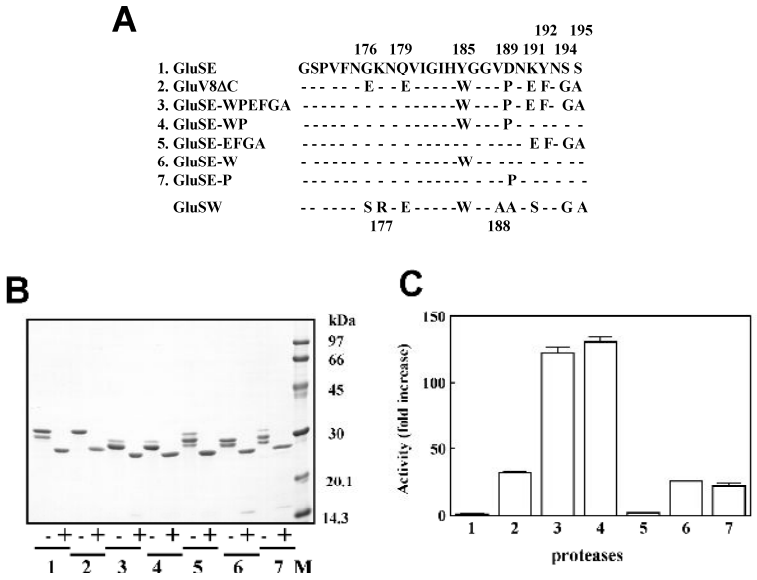


Figure 4

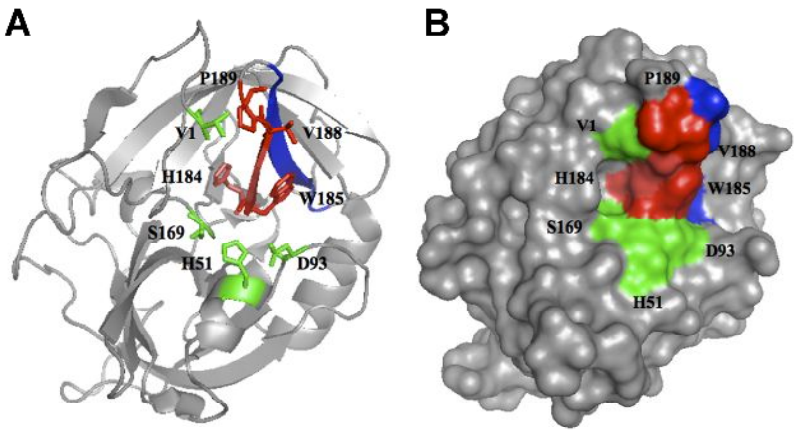


Figure 5

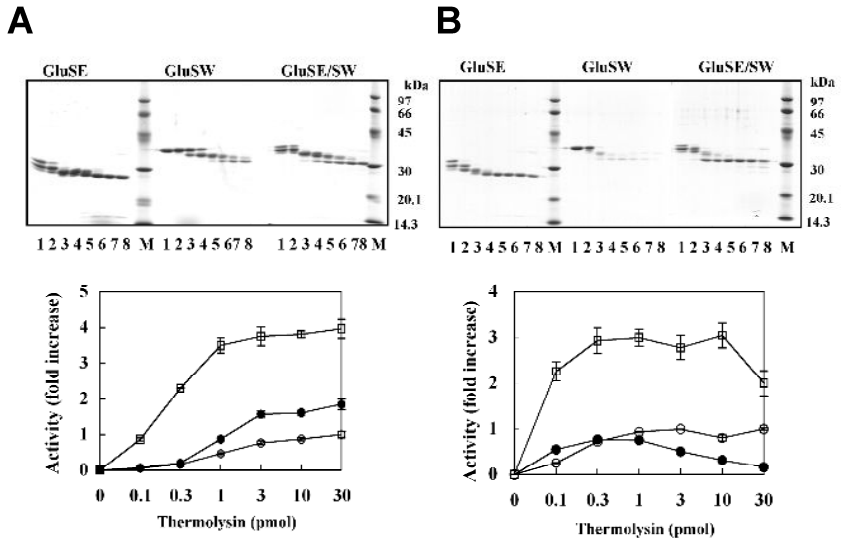


Figure 6

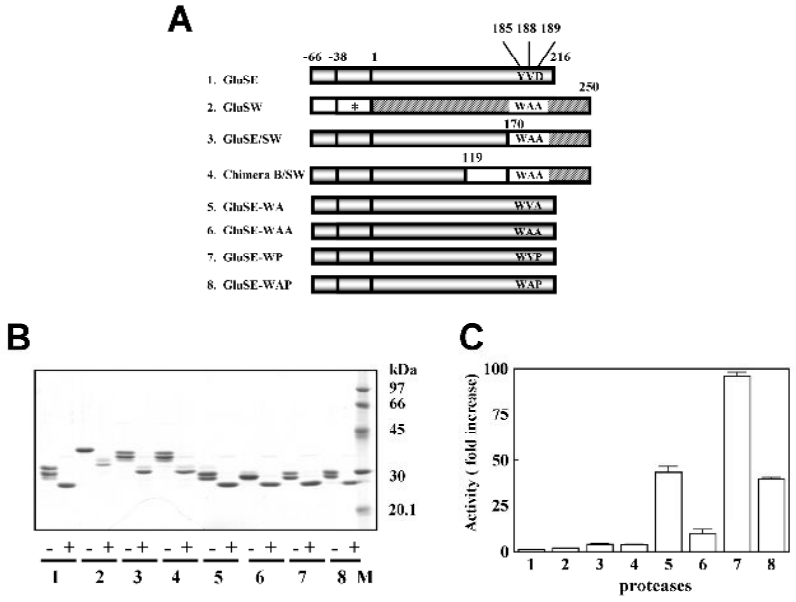
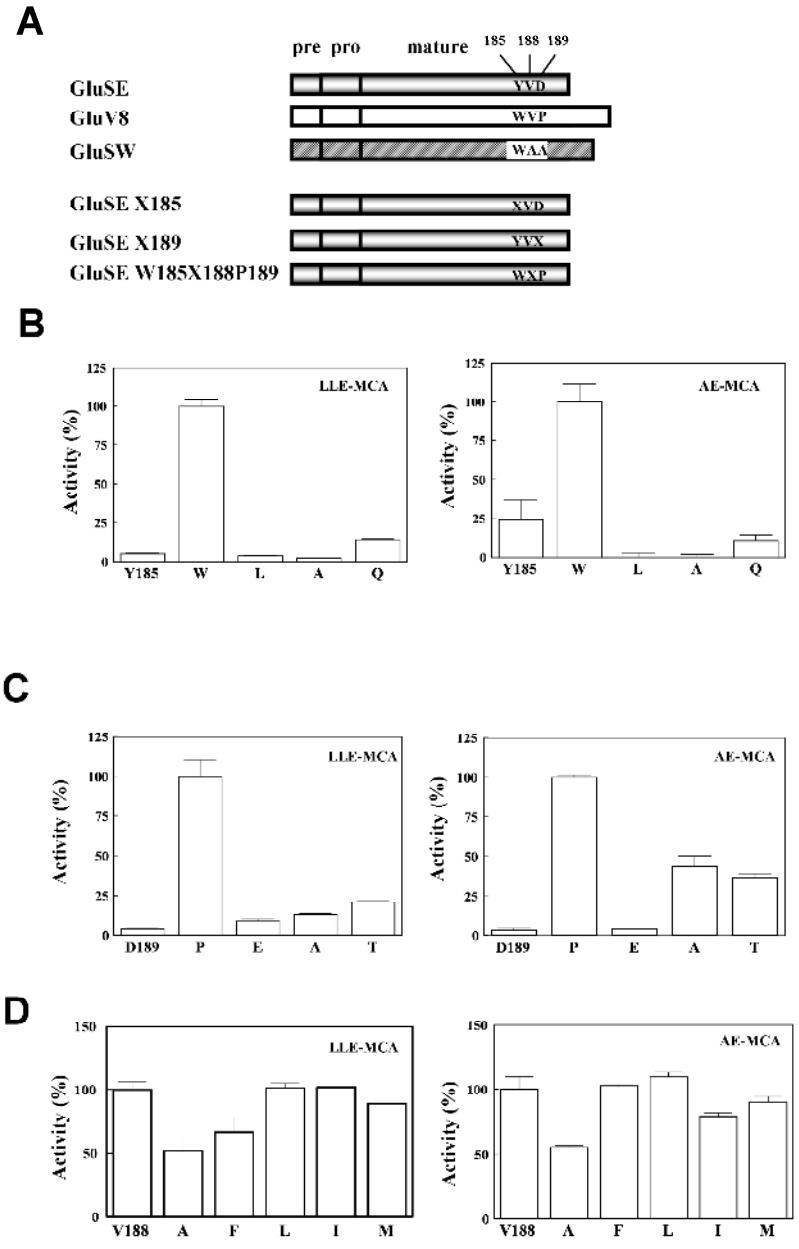


Figure 7


A
pre pro mature 185 188 189
GluSE YVD
GluV8 WVP
GluSW WAA
GluSE X185 XVD
GluSE X189 YVX
GluSE W185X188P189 WXP
B
LLE-MCA
AE-MCA
Activity (%)
Y185 W L A Q
C
LLE-MCA
AE-MCA
Activity (%)
D189 P E A T
D
LLE-MCA
AE-MCA
Activity (%)
V188 A F L I M