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Felix Hoppe-Seyler (1825–1895) was a pioneer of biochemistry, remembered not only for his discovery of hemoglobin and his contributions to the chemical characterization of many other biological compounds and processes but also for having been the mentor of Friedrich Miescher and Albrecht Kossel. In his preface to the first issue of ***Zeitschrift für Physiologische Chemie,*** Felix Hoppe-Seyler coined the term ***Biochemistry*** ('Biochemie') for the then newly emerging discipline.

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Amino acid residues modulating the activities of staphylococcal glutamyl endopeptidases

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Abstract

The glutamyl endopeptidase family of enzymes from staphylococci has been shown to be important virulence determinants of pathogenic family members, such as *Staphylococcus aureus*. Previous studies have identified the N-terminus and residues from positions 185–195 as potentially important regions that determine the activity of three members of the family. Cloning and sequencing of the new family members from *Staphylococcus caprae* (GluScpr) and *Staphylococcus cohnii* (GluScoh) revealed that the N-terminal Val residue is maintained in all family members. Mutants of the GluV8 enzyme from *S. aureus* with altered N-terminal residues, including amino acids with similar properties, were inactive, indicating that the Val residue is specifically required at the N-terminus of this enzyme family in order for them to function correctly. Recombinant GluScpr was found to have peptidase activity intermediate between GluV8 and GluSE from *Staphylococcus epidermis* and to be somewhat less specific in its substrate requirements than other family members. The 185–195 region was found to contribute to the activity of GluScpr, although other regions of the enzyme must also play a role in defining the activity. Our results strongly indicate the importance of the N-terminal and the 185–195 region in the activity of the glutamyl endopeptidases of staphylococci.

Keywords: processing; *Staphylococcus aureus*; *Staphylococcus caprae*; *Staphylococcus cohnii*; *Staphylococcus epidermidis*; thermolysin.

Introduction

Staphylococcus aureus, a frequent pathogen in human disease, produces extracellular proteases, which are regarded as important virulence factors. One of these abundantly secreted extracellular proteases is a serine protease, GluV8, also known as V8 protease/SspA/Glu-C (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). *S. aureus* infection triggers the production of inhibitory antibodies against GluV8 in mice, suggesting that the immunological response to GluV8 could be an important host-defense mechanism (Calander et al., 2008).

GluV8 belongs to the glutamyl endopeptidase I family (EC 3.4.21.19), whereby members specifically cleave the carboxyl side of the peptide bond of negatively charged residues, i.e., glutamic acid and, much less potently, aspartic acid (Stennicke and Breddam, 1998). In staphylococci, GluV8 family members are expressed in *S. aureus* (Drapeau et al., 1972), *Staphylococcus epidermidis* (designated GluSE; Sasaki et al., 1998; Dubin et al., 2001; Ohara-Nemoto et al., 2002), and *Staphylococcus warneri* (GluSW; Yokoi et al., 2001). Members of the GluV8 family are also expressed in *Bacillus licheniformis* (Kakudo et al., 1992; Svendsen and Breddam, 1992), *Bacillus intermedius* (Rebrikov et al., 1999; Demidyuk et al., 2004), *Enterococcus faecalis* (Kawalec et al., 2005), *Streptomyces fradiae* (Kitadokoro et al., 1993), and *Streptomyces griseus* (Svendsen et al., 1991; Suzuki et al., 1994), suggesting important roles of these proteases in the life of these bacteria. Therefore, it is reasonable to postulate that other staphylococcal species also produce these endopeptidases. In fact, the genome project of *Staphylococcus saprophyticus* revealed the existence of the gene encoding a GluV8 family endopeptidase (Figure 1), although the protein expression of it has not been achieved yet (Kuroda et al., 2005).

Drapeau (1978) found that maturation of GluV8 zymogen is mediated by a thermolysin family enzyme, aureolysin, that exhibits a preference for hydrophobic amino acids in the P1′ position of its substrates. In accordance with this finding, recombinant GluV8 expressed in *Escherichia coli* can be efficiently processed into the mature form by thermolysin treatment *in vitro* (Nemoto et al., 2008). Because V is commonly located at the N-termini of all mature enzymes studied thus far (Ono et al., 2008; Figure 1), the thermolysin family enzymes could be responsible for processing of all staphylococcal glutamyl endopeptidases. In addition to a role of the pro-mature border, the protonated α-amino group of V1 was reported to be important for the recognition of a substrate peptide (Prasad et al., 2004). However, if these two are the entire roles of V1, any hydrophobic amino acids (L, Y, I, and F) and A can be located at the N-terminus of mature

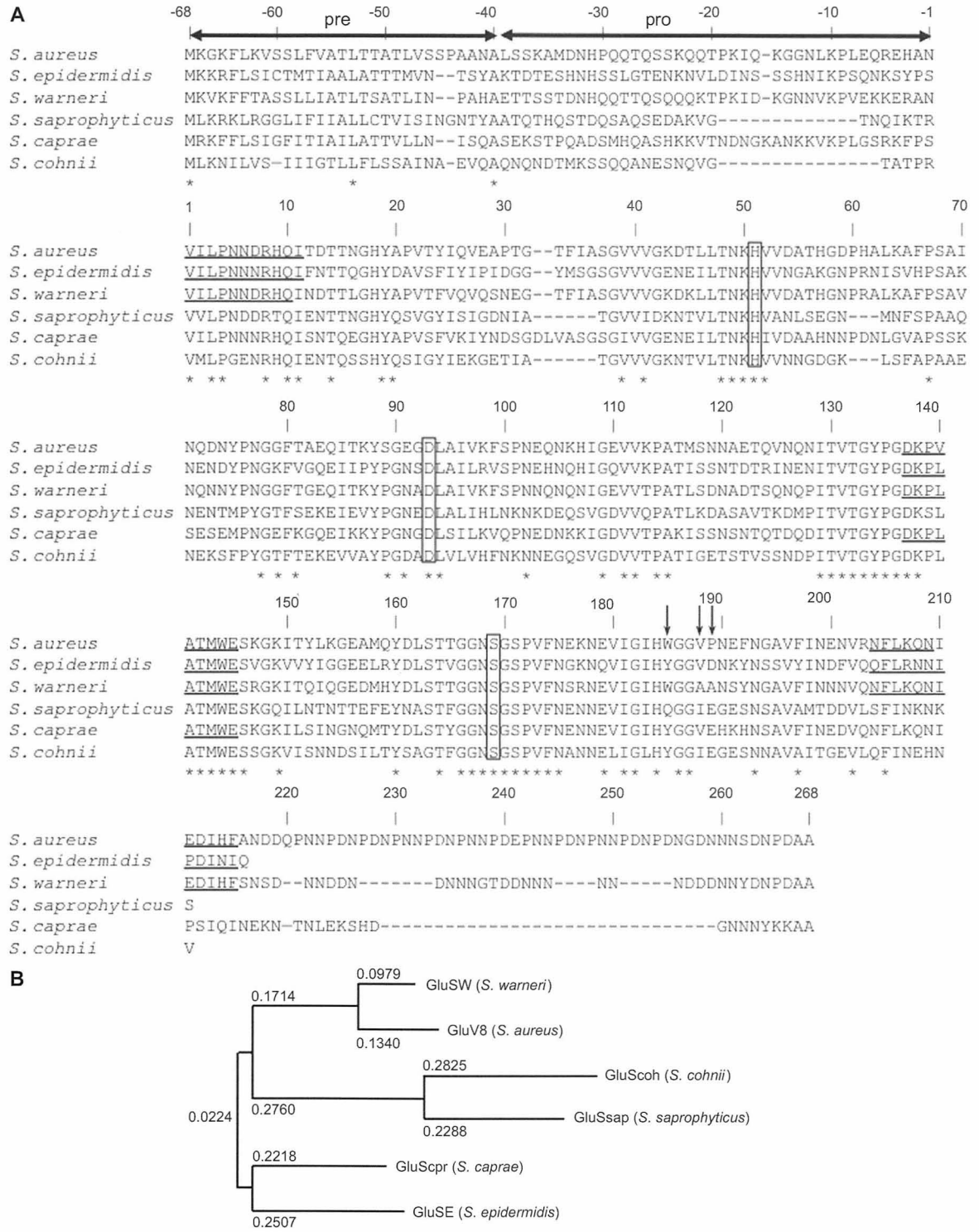

Figure 1 Alignment of the amino acid sequences of staphylococcal glutamyl endopeptidases.
(A) The deduced amino acid sequences of glutamyl endopeptidases from *S. aureus* (Carmona and Gray, 1987), *S. epidermidis* (Ohara-Nemoto et al., 2002), *S. warneri* (Yokoi et al., 2001), *S. saprophyticus* (Kuroda et al., 2005), *S. caprae* and *S. cohnii* are aligned. Hyphens are introduced for maximal matching. Identical amino acids among the six endopeptidases are indicated by asterisks. pre, presequence; pro, prosequence. Amino acids forming the catalytic triad of serine proteases are boxed. Arrows indicate the amino acids at positions 185, 188, and 189, which define the proteolytic activities of GluV8, GluSE, and GluSW (Nemoto et al., 2009). Amino acid sequences taken into consideration for the synthesis of the degenerate primers are underlined. (B) Phylogenic tree of the six staphylococcal glutamyl endopeptidases prepared by comparison with V1-A216 of GluV8 and its equivalent regions of other proteases. The tree was generated by a distant-cased method using a neighbor-joining algorithm. Numbers of the branches represent length between two taxa.

enzymes. To address this issue, we expressed GluV8 with the V1L substitution by site-directed mutagenesis and studied the processing of proGluV8 and the proteolytic activity.

In addition to V1 residue, we recently defined three amino acids at positions 185, 188, and 189 (as the numbers of mature GluV8) involved in the alteration of the proteolytic activities of GluV8, GluSE, and GluSW by modification of their K_m values (Nemoto et al., 2009). In combination of W185, V188, and P189, GluV8 exhibited the smallest K_m value. In the present study, we also aimed to elucidate whether these three amino acids are generally major determinants of the proteolytic activities of staphylococcal glutamyl endopeptidases. Toward this goal, for the first time, we cloned the homologous genes from *Staphylococcus caprae* (designated GluScpr) and *Staphylococcus cohnii* (designated GluScoh), and then compared their protease activities in detail with those of GluV8 and GluSE.

Results

Cloning of the genes encoding the glutamyl endopeptidases from *S. caprae* and *S. cohnii*

The genes encoding glutamyl endopeptidases from *S. caprae* and *S. cohnii* were cloned by performing successive PCR in combination with a pair of degenerate primers and gene-walking techniques (Table 1). The resultant 4042-bp EcoRV DNA fragment from *S. caprae* carried the open reading frame of the GluScpr gene composed of 918 bp encoding a 305-amino acid protein. A 1952-bp DNA fragment that potentially encoded a glutamyl endopeptidase GluScoh was cloned from *S. cohnii*. In the putative GluScoh gene, the ATG typical start codon did not exist at the region equivalent to that of GluV8; however, TTG instead of ATG was regarded as the start codon, because (i) TTG and GTG are able to function as the start codon in prokaryote, (ii) the Shine-Dalgarno sequence (Shine and Dalgarno, 1975) existed at the position equivalent to that of other staphylococcal glutamyl endopeptidases (data not shown), and (iii) TTG is also used as the start codon of the glutamyl endopeptidase from *B. licheniformis* (Kakudo et al., 1992). Therefore, we propose that the open reading frame of the GluScoh gene is composed of 771 bp encoding a 256-amino acid protein. This number is the smallest among the six staphylococcal glutamyl endopeptidases reported to date.

The deduced amino acid sequences of GluScpr and GluScoh were aligned together with those of the four other staphylococcal glutamyl endopeptidases (Figure 1). The pre-, pro-, and mature regions of GluScpr were predicted to possess 27, 40, and 238 amino acids, respectively, and those of GluScoh possessed 27, 25, and 204 amino acids, respectively. A 15-amino acid deletion was present in the prosequence of GluScoh starting at the position identical to the 13-amino acid deletion of glutamyl endopeptidase from *S. saprophyticus* (tentatively designated GluSsap). The amino acid identity among the proteases was maximal at the mature region; and the identity of GluScpr and GluScoh to that of GluV8 was 60.1% and 44.9%, respectively. By contrast, amino acid sequences were least conserved at the prosequence region: the identity of the prosequences of GluScpr and GluScoh to that of GluV8 was 17.5% and 18.0%, respectively, and the identity of presequences of GluScpr and GluScoh to that of GluV8 was 27.6% and 24.1%, respectively, and only three amino acid residues, M-68 (first M), L-53, and A-40, which is a putative recognition site by signal peptidase, were con-

Table 1 Primers used for degenerate PCR, cloning, and expression.

Primer	Sequence
Degenerate PCR	
5V8degV1	5′-GT(c/t)ATA(c/t)TACC(a/t)AA(c/t)(a/g)AT(a/c)G(a/t)CA(c/t)CAAAT-3′
3V8degF225	5′-A(a/t)AT(g/t)(g/a/t)ATATC(a/t)(g/t)(g/c)(a/t)ATATT(g/t)T(g/t)T(c/t)TTA(a/g)GAA-3′
3V8degS146	5′-ACTTTCCCACAT(t/a)GT(t/a/g)GC(t/a)A(c/g/a)(a/t)GGTTT(a/g)TC-3′
Cloning	
3ScprGSP1	5′-ATCTGGGTTATTATGTGCGGCATCAAC-3′
5ScprGSP2	5′-GGAGATAAACCTTTAGCAACAATGTGG-3′
5ScprEco	5′-GATATCCCCTCGACTTAACACTCACAA-3′
3ScprEco	5′-GATATCTGCCGTCGCACGTCCGTCTTG-3′
3ScohGSP1	5′-GCCGATGGATTGGTAATGACTAGATTG-3′
5ScohGSP2	5′-CCTGCCACAATTGGTGAAACTAGTACT-3′
5ScohPvu	5′-CAGCTGATCGAAAATGTTGACTTAG-3′
3ScohDra	5′-TTTAAATCAATCGCTCAATATGGTT-3′
Expression	
5GluScprM-68Bgl	5′-GGTAGATCTATGAGAAAATTTTTTTTATCTA-3′
3GluScprA237Bgl	5′-ACTAGATCTTGCTGCTTTTTTATAATTG-3′
5GluScohL-67Bam	5′-ACAGGATCCAACATTGTGTTCATTGATAAA-3′
3GluScohV211Bam	5′-ACAGGATCCAACATTGTGTTCATTGATAAA-3′

Small letters in parentheses represent mixtures of bases. Underlined sequences represent the endonuclease restriction site.

served out of 52–68 residues of the preprosequences among six endopeptidases (Figure 1).

In the mature region, 69 amino acids were conserved. These include the catalytic triad (H51, D93, and S169) of chymotrypsin family serine proteases and H184 (Figure 1). The replacement of H184 is reported to drastically reduce the k_{cat} value of the endopeptidase from *B. intermedius* (Demidyuk et al., 2004). Continuous residues at 129–148 and 166–175 are completely identical among the six endopeptidases. In the latter segment, conserved G166, G167, or G170, most likely G167, could participate in forming an oxyanion hole of serine protease family (Tamada et al., 2009).

The phylogenic analysis on the mature proteases without the C-terminal repeat demonstrated the kinship between GluV8 and GluSW, that between GluScoh and GluSsap, and that between GluScpr and GluSE (Figure 1). The kinship between GluV8 and GluSW was further ascertained by the presence of the related hydrophilic segments at the C-terminal region, although its role remains unknown. The kinship between GluScoh and GluSsap was further indicated by their 13–15-amino acid deletions in the proregion and the identical C-terminal termination. Despite the kinship between GluScpr and GluSE, GluScpr contained 20-amino acid hydrophilic tag beyond the C-terminus of GluSE. This phylogenicity of staphylococcal glutamyl endopeptidases was basically identical to that calculated by using 16S rRNA or the hsp60 gene (Ghebremedhin et al., 2008).

Role of the N-terminal amino acid for mature GluV8

Valine 1 is completely conserved among the six staphylococcal endopeptidases (Figure 1) and GluV8, GluSE, and GluSW acquire the enzymatic activity through the cleavage at the N-1/S-1-V1 bond (Ohara-Nemoto et al., 2008; Ono et al., 2008). To investigate the role of V1, K residue was introduced at -2 and -3 positions of GluV8 molecule (designated GluV8 P-2K and H-3K, respectively) (Table 2). After thermolysin treatment, 32-kDa proGluV8 converted to 28-kDa species, of which N-terminus was V1. These molecules exhibited the full activity as the case of non-mutated GluV8 entity (Figure 2A). Although trypsin treatment resulted in the conversion to 28-kDa proteases, the N-termini of these GluV8, GluV8 P-2K, and GluV8 H-3K species were S-4, S-1, and A-2, respectively, and they did not show any proteolytic activity. These results clearly demonstrated that attachment of even one residue, S-1 in this case, to V1 completely abolished the Glu-specific proteolytic activity, and thus suggested that V residue at the N-terminus of mature GluV8 was required to achieve the proteolytic activity.

We then investigated whether V1 can be compensated by other hydrophobic amino acids. Here, L was selected for substitution, because L residue is an excellent target for aureolysin as well as thermolysin (Drapeau, 1978; Stennicke and Breddam, 1998). GluV8 V1L mutants carrying a trypsin-cleavage site at positions -1, -2, and -3 (designated GluV8 S-1K/V1L, P-2K/V1L, and H-3K/V1L, respectively) were expressed. The treatment of these V1L mutants with thermolysin as well as trypsin again produced the 28-kDa forms. Unexpectedly, the 28-kDa species of V1L mutants processed by thermolysin were a mixture of molecules with the N-termini of L1 and I2 (Table 2 and Figure 2B), indicating that the L1-I2 bond became hydrolyzed. This 28-kDa species showed 5%–10% of the activity of GluV8 (Figure 2). After trypsin treatment, the peptide bonds on the C-terminal side of K or R residues were always processed. Among them, the S-1K/V1L mutant, of which N-terminus was L, exerted the

Table 2 Amino acid sequence of GluV8 derivatives and their N-terminal residues after processing.

GluV8 species	Amino acid sequence and putative cleavage state	N-terminal residue after cleavage with	
		Thermolysin	Trypsin
	-3 -1 1		
GluV8[a]	--- E Q R E H A N↓V I L P N---	-	-
mut5[b]	--- Q Q R▾ S H P S↓V I L P N---	V1	S-4
P-2K	--- Q Q R▾ S H K▾ S↓V I L P N---	V1	S-1
H-3K	--- Q Q R▾ S K▾ A S↓V I L P N---	V1	A-2
V1L	--- Q Q R▾ S H P S↓L I L P N---	L1, I2[c]	S-4
S-1K/V1L	--- Q Q R▾ S H P K▾ ↓L I L P N---	L1<<I2[c]	L1
P-2K/V1L	--- Q Q R▾ S H K▾ S↓L I L P N---	L1<I2[c]	S-1
H-3K/V1L	--- Q Q R▾ S K▾ A S↓L I L P N---	n.d.	n.d.

[a]Carmona and Gray (1987).

[b]Five substitutions (D-33H, E-7Q, E-4S, A-2P, and N-1S) were introduced into wild type GluV8 (Ono et al., 2008).

[c]A mixture of two polypeptides starting with L or I.

Amino acid substitutions introduced into prosequence of GluV8 are underlined.

Arrows and arrowheads indicate potential processing sites mediated by thermolysin and trypsin, respectively.

n.d., not determined.

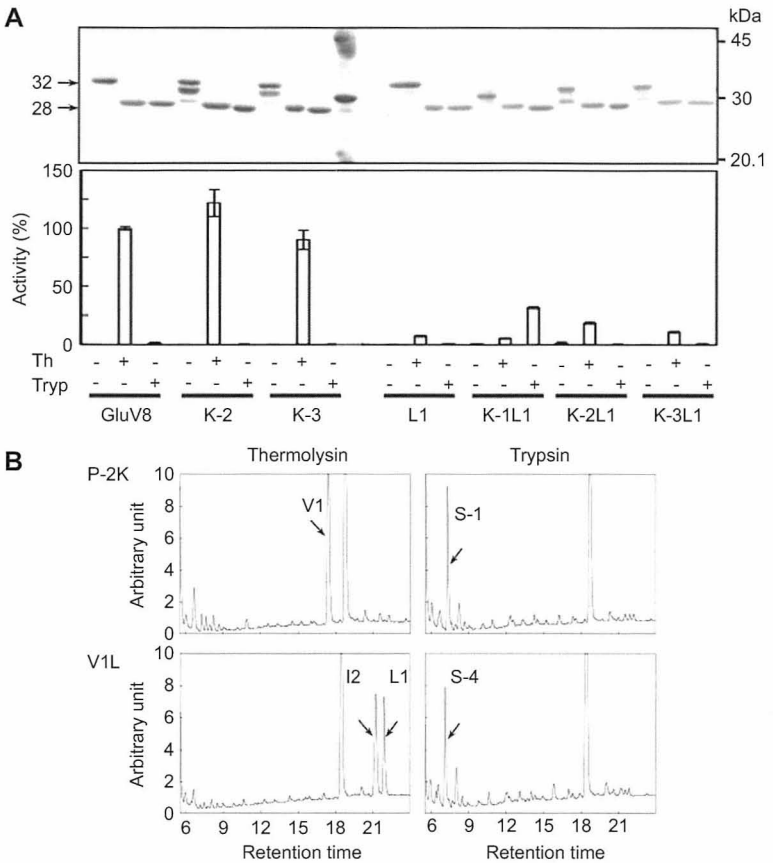

Figure 2 Processing and proteolytic activities of GluV8 and its derivatives.
GluV8 and its derivatives were incubated at 0°C without protease or at 37°C with 0.3 μg of thermolysin (Th) or trypsin (Tryp), as described in the materials and methods section. (A) Proteins (1 μg) were separated by SDS-PAGE and stained with CBB. The apparent molecular masses of major products are shown on the left. The proteolytic activities of the samples were determined with LLE-MCA and expressed as percent±SD (n=3) of the activity (4700±60 $U \cdot \mu g^{-1}$) of thermolysin-processed GluV8. (B) GluV8 P-2K and GluV8 V1L treated with either thermolysin or trypsin were subjected to the N-terminal sequencing by Edman degradation. Phenylthiohydantoin-amino acids at the first cycle were subjected to reversed phase liquid chromatography.

activity (31.8±0.9%, mean±SD, n=3). In contrast, trypsin-treated 28-kDa GluV8 P-2K/V1L and H-3K/V1L exhibited no activity due to amino acids optionally attached to L1. Therefore, these results suggested at least two roles of V1 residue in GluV8: V is more preferable to L for the precise processing event and is directly involved in the proteolytic activity.

The findings described above demonstrated that the V1-I2 bond was protected from thermolysin cleavage in GluV8. The three-dimensional structure of mature GluV8 shows that the conserved T164 and N193 are located adjacent to V1 (<2 Å) and are in contact with the α-amino group of V1 through hydrogen bonding (Prasad et al., 2004). Accordingly, we speculated that the interaction of T164 and N193 with V1 protects the V1-I2 bond from thermolysin cleavage. GluV8 mutants with either T164A or N193A and T164A/N193A substitutions were expressed to test this assumption. Although thermolysin induced the degradation from the 33-kDa form to the 28-kDa form, none of the mutants possessed any proteolytic activities. N-terminal sequencing revealed that their N-terminal amino acids were V1 in all cases (data not shown). Therefore, T164 and N193 adjacent to V1 in mature GluV8 certainly played important roles, which were distinct from the protection of V1 from the thermolysin cleavage.

Expression and processing of recombinant GluScpr and GluScoh

An attempt to express the wild type GluScpr in *E. coli* did not succeed, suggesting the toxicity of this protease due to autoproteolysis, as reported for GluV8 (Nemoto et al., 2008). To suppress the autoproteolysis, we introduced substitutions of D-18 to K (D-18K) and D-32 to H (D-32H) into the pro-sequence of GluScpr (designated GluScpr-mut). Consequently, several bands ranging at 30–38 kDa were recovered (Figure 3). Thermolysin treatment induced the conversion into two bands migrating at 29 and 30 kDa accompanied with an acquisition of the protease activity. The N-terminal amino acid of the 38-kDa form of GluScpr-mut was A-32, suggesting the cleavage at the Q-33-A-32 bond (Table 3). The 35-kDa species was a mixture of two polypeptides starting at A-32 and K-22. Valine 1 was the N-terminus of both

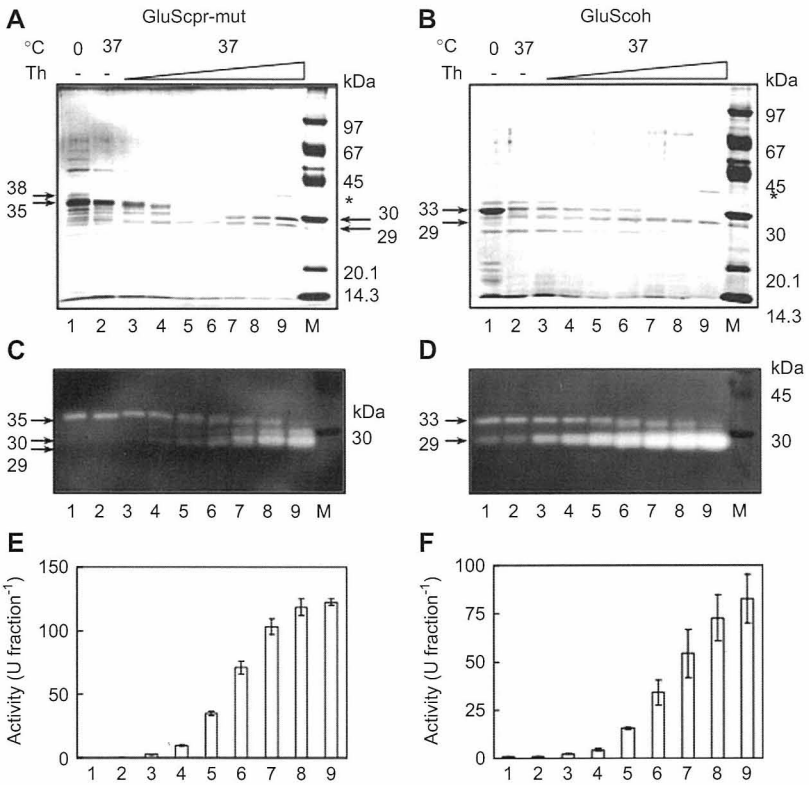

Figure 3 Thermolysin processing and protease activities of GluScpr and GluScoh.
Purified GluScpr-mut (A, C, E) and GluScoh (B, D, F) at 10 μg were incubated for 4 h without thermolysin (Th) at 0°C (lane 1), at 37°C (lane 2) or at 37°C with 0.001 (lane 3), 0.003 (lane 4), 0.01 (lane 5), 0.03 (lane 6), 0.1 (lane 7), 0.3 (lane 8) or 1 μg (lane 9) of thermolysin. Aliquots (1 μg) of the samples were separated by SDS-PAGE and stained with CBB (A and B) or subjected to zymography (C and D). Asterisks show the position of thermolysin. The apparent molecular masses of the major bands are indicated. (E and F) The proteolytic activities (means±SD, n=3) of the samples were determined as described in the materials and methods section. Samples for columns 1–9 in panels (E) and (F) are identical to those in lanes 1–9 in panels (A) and (B), respectively.

29- and 30-kDa species upon thermolysin treatment. Because GluScpr possessed two E residues at positions 217 and 224 (Figure 1), which were adjacent to the C-terminal position of GluSE (Q216), we suppose that the cleavage at either the E217–K218 or E223–K224 bond of the 30-kDa species produced the 29-kDa species.

On the expression of the full-length form of GluScoh, a 33-kDa proenzyme was obtained. When 33-kDa GluScoh was treated with thermolysin, a faint 29-kDa band showing the proteolytic activity was recovered (Figure 3). The N-terminus of the 33-kDa GluScoh was Q-39, indicating the cleavage at the A-40-Q-39 bond by signal peptidase. The N-terminus of 29-kDa GluScoh was unable to be determined because of its limited production.

Following the thermolysin treatment, the recoveries of the mature forms significantly differed among recombinant proteins, and the band intensities of mature GluScpr and GluScoh were 33.8% and 2.4% of the inputs, respectively (Figure 4). These figures were much lower than those of GluV8 (91.9%) and GluSE (82.5%). Taken this breakdown

Table 3 N-terminal sequences of recombinant GluScpr and GluScoh.

Species	kDa	Thermolysin	Detected amino acids	Determined sequence
GluScpr-mut	38	-	AHSMHQA	Q-33/A-32HSMHQA-26
	35[a]	-	AHXM	Q-33/A-32 HSM-29
			KVTN	K-23/K-22 VTN-19
	30	+	VILPNNN	S-1/V1 ILPNNN7
	29	+	VILP	S-1/V1 ILP4
GluScoh	33	-	QNQNDT	A-40/Q-39NQNDT-34

[a]A mixture of two polypeptides.
X, not identified.
The amino acid substituted in GluScpr-mut (D-31H) is underlined. Slashes represent cleavage sites.

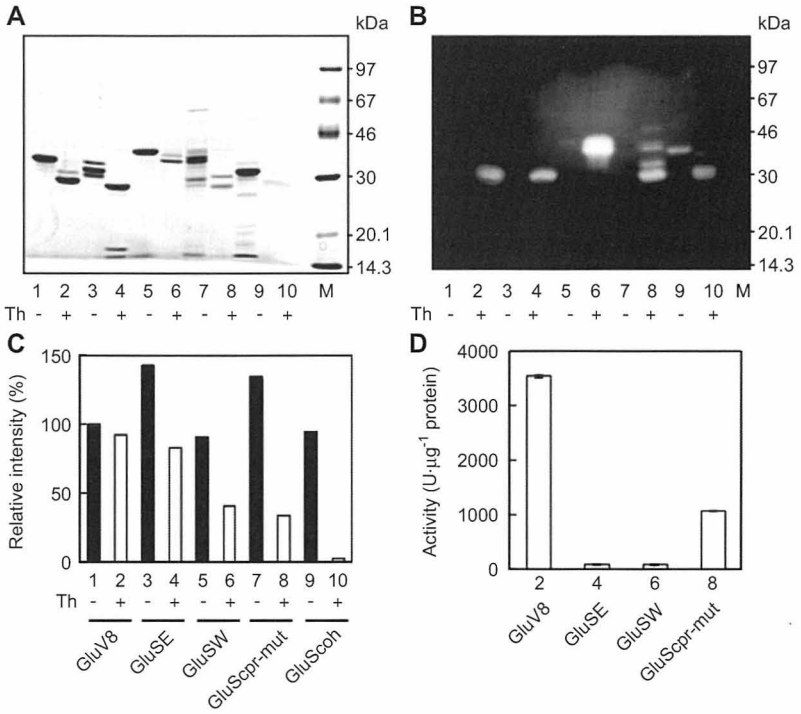

Figure 4 Comparison of the proteolytic activities of staphylococcal glutamyl endopeptidases.
GluV8 (lanes 1 and 2), GluSE (lanes 3 and 4), GluSW (lanes 5 and 6), GluScpr-mut (lanes 7 and 8), and GluScoh (lanes 9 and 10) at 10 μg were incubated at 0°C without thermolysin (Th) or at 37°C with 0.3 μg of thermolysin. Aliquots (1 μg) of the samples were separated by SDS-PAGE. Separated proteins were stained with CBB (A) or subjected to zymography (B). (C) The amounts of unprocessed and thermolysin-processed 28–29-kDa bands for GluV8, GluSE, and GluScoh, the 35-kDa band for GluSW, and the sum of 28- and 30-kDa bands for GluScpr-mut were quantified by PDQuest. (D) The proteolytic activities of thermolysin-treated enzymes toward 20 μM LLE-MCA were measured, and the specific activities (means±SD, n=3) were calculated in consideration of their recoveries determined by PDQuest. Columns 1–10 in panel (C) and 2–8 in (D) correspond to lanes 1–10 of panel (A).

in calculation, the specific activity of GluScpr-mut was defined to be 1073±2 U/μg protein, which corresponded to 30.4% of that of GluV8, but was 11-fold higher than that of GluSE. GluScpr carried Y185, V188, and E189 and GluScoh carried Y185, I188, and E189, and thus these proteases were expected to possess activities lower than GluV8 (W185, V188, and P189) and comparable to GluSE (Y185, V188, and D189). We did not calculate the specific activity of GluScoh, because reliable data could not be expected due to its intrinsic instability.

These observations indicated protease susceptibility of GluScoh. Then, this property was compared among the GluV8 family members by using constitutive-inactive S169A mutants. As a result, GluScoh S169A was completely degraded by both thermolysin and GluV8 treatments, whereas GluV8, GluSE, and GluSW mutants remained undegraded (Figure 5). Therefore, these results indicated the intrinsic structural instability of GluScoh.

Characteristics of proteolytic activities of GluScpr and GluScoh

Substrate specificity was examined in GluScpr and GluScoh. Both proteases efficiently hydrolyzed LLE-MCA, but scarcely cleaved AE-MCA (Figure 6A). Among other substrates tested, GluScpr hydrolyzed YVAD-MCA, AAN-MCA, and QAR-MCA to some extent, whereas GluScoh as well as GluV8 hardly hydrolyzed these substrates. The proteolytic activities of GluScpr and GluScoh were most effectively suppressed by diisopropyl fluorophosphate (DFP), followed by 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (Figure 5B). Tosyl-L-phenylalanine chloromethyl ketone (TPCK) (0.1 mg/ml), tosyl-L-lysine chloromethyl ketone (TLCK) (50 μg/ml), phenylmethylsulfonyl fluoride (PMSF) (1 mM), aprotinin (0.2 U/ml), leupeptin (0.1 mg/ml), pepstatin (0.01 mg/ml), and dithiothreitol (5 mM) did not inhibit the activities of GluScpr and GluScoh. Recombinant GluV8, GluScpr, and GluScoh possessed an identical pH optimum. Exceptionally, a slight shift to basic pH was observed on GluSE (Figure 6C).

Effect of amino acid substitutions at position 185 and 189 on the activity of GluScpr

We previously reported that three amino acids at positions 185, 188, and 189 are involved in the determination of the proteolytic activity of glutamyl endopeptidases (Nemoto et al., 2009). Because V188 was conserved in GluScpr, GluV8, and GluSE, here we focused on the remaining two positions at 185 and 189. The 33–35 kDa species of GluScpr-WP, which mimicked the combination of GluV8, was scarcely detected in the lysate; and the band was detected by immuno-

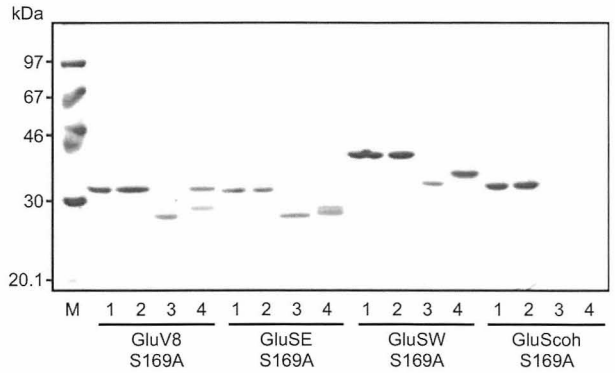

Figure 5 Protease sensitivity of GluScoh.
The S169A mutation was introduced to GluV8, GluSE, GluSW, and GluScoh. Proteases (10 μg) were incubated at 0°C (lane 1) or 37°C (lane 2) without thermolysin or at 37°C with 0.3 μg of thermolysin (lane 3) or thermolysin-activated GluV8 (lane 4). After 4 h, aliquots (1 μg) of the samples were separated by SDS-PAGE.

blotting only after Talon-affinity purification (data not shown). The growth rates of *E. coli* cells were investigated after induction of GluScpr, GluScpr-mut, and GluScpr-WP (Figure 7B). *E. coli* expressing GluScpr-mut grew fastest followed by those with GluScpr, and cells expressing GluScpr-WP did not grow under the induction conditions. These findings strongly suggested that the heavy toxicity against the host cells was caused by an enhanced protease activity of GluScpr-WP. Although the growth of GluScpr-producing cells increased after 3 h and finally overcame the growth of GluScpr-mut-producing cells, this increase did not accompany the production of GluScpr (data not shown).

Effect of amino acid substitutions at residues 190–195 of GluSE to those of GluScpr on the activity

We next investigated the reason why the specific activity of GluScpr was higher than that of GluSE. We previously reported that four amino acid substitutions of GluSE to those of GluV8 (K191E, Y192F, S194G, and S195A) induced a 2-fold increase in the proteolytic activity (Nemoto et al., 2009). By analogy, we suspected that amino acid substitutions at residues 190–195 were involved in the enhancement of the protease activity of GluScpr from that of GluSE. The substitutions of N190H, Y192H, and S195A were introduced into GluSE (designated GluSE-HHA), and the mutated protease was expressed (Figure 7A). The specific activity of GluSE-HHA was 4.5-fold increased from that of GluSE, although this activity was only 26.2% of that of GluScpr-mut. This difference was consistently observed at the substrate concentrations up to 0.2 mM (data not shown). GluSE-HHA most potently hydrolyzed LLE-MCA and scarcely cleaved the remaining MCA substrates.

Discussion

To date, four members of the glutamyl endopeptidases have been identified in staphylococci: one in *S. aureus* and three

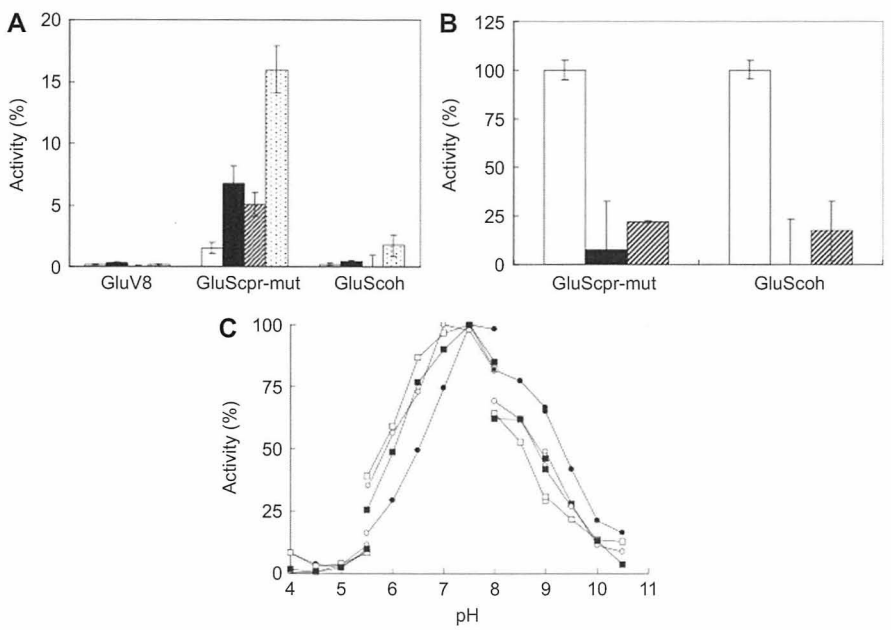

Figure 6 Proteolytic characteristics of GluScpr and GluScoh.
(A) Protease activities (mean±SD, n=3) of GluV8, GluScpr-mut, and GluScoh for AE-MCA (open), YVAD-MCA (closed), AAN-MCA (hatched), and QAR-MCA (hyphened) were compared with respective activities for LLE-MCA (100%). (B) Thermolysin-treated GluScpr-mut and GluScoh were preincubated without (open) and with 3 mM DFP (closed) or 1 mM AEBSF (hatched) at 0°C. After 30 min, the reaction was started by an addition of 20 μM LLE-MCA. (C) Proteolytic activities of GluV8 (open circle); GluSE (closed circle); GluScpr (open square); and GluScoh (closed square) at pH 4–10.5 were determined with triplicate samples in 50 mM buffers of Na-acetate (pH 4–5.5); Na-phosphate (pH 5.5–8); Tris-HCl (pH 8–9); and glycine-NaOH (pH 9–10.5).

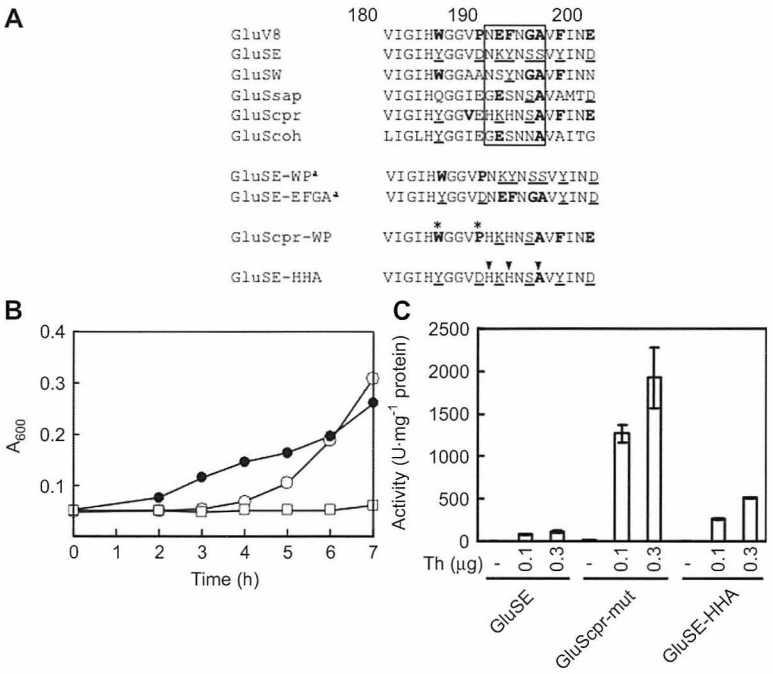

Figure 7 Effect of amino acid substitutions in staphylococcal glutamyl endopeptidases.
(A) Amino acids sequences at residues 180–200 are compared among the six staphylococcal glutamyl endopeptidases. Amino acids characteristic of GluV8 are represented by bold letters and those of GluSE are underlined. Asterisks and arrowheads show amino acids substituted in GluScpr-WP and GluSE-HHA, respectively. Residues 190–195 of GluSE, which are potentially involved in the alteration in the proteolytic activity of GluScpr, are boxed. [a]GluSE-WP and GluSE-EFGA are mutated GluSE, which mimicked residues 180–190 and 190–195 of GluV8, respectively (Nemoto et al., 2009). (B) Growth curves of *E. coli* carrying the expression plasmids for GluScpr (open circles), GluScpr-mut (filled circles), and GluScpr-WP (open squares) in Luria-Bertani broth in the presence of 0.2 mM isopropyl β-D-thiogalactopyranoside containing 50 μg/ml of ampicillin. The bacteria were cultured at 30°C with vigorous shaking. (C) Proteolytic activities (mean±SD, n=3) of aliquots (0.25 μg) of GluSE, GluScpr, and GluSE-HHA (10 μg) preincubated at 0°C without thermolysin (Th) or at 37°C with 0.1 or 0.3 μg of thermolysin for 4 h were determined.

in coagulase-negative staphylococci, i.e., *S. epidermidis*, *S. warneri*, and *S. saprophyticus*. The present study demonstrated the existence of two additional GluV8 family members in coagulase-negative staphylococci *S. caprae* and *S. cohnii*. These bacteria occasionally cause urinary tract infection and bacterial endocarditis (Kloos and Bannerman, 1994). The number of amino acids comprising GluScoh was the smallest among the six endopeptidases; 15 amino acids were deleted in the prosequence, and the typical ATG codon did not exist. However, the *E. coli* expression showed that the putative GluScoh gene was sufficient to produce a proteolytically active molecule.

Although the N-terminus of GluSW was initially reported to be R-3 (Yokoi et al., 2001), our study on recombinant GluSW demonstrated that the species starting at V1 was enzymatically active, whereas that staring at R-3 was inactive (Ono et al., 2008). Consequently, V1 was maintained in all family members. The present study demonstrated that V1 was required for limited hydrolysis between the S-1-V1 bond, because the L1-I2 bond was additionally cleaved in the V1L mutant. Moreover, mature GluV8 with the N-terminus of L exerted 32% of the activity of that harboring V1 (Figure 2). Substitutions from V to F, G, S or A completely abrogated the proteolytic activity (Nemoto et al., 2008). Therefore, taken into consideration the structural similarity between V and L, these results strongly suggested that the residual group of V1 is required for the enzyme activity itself.

The N-terminal tripeptide of staphylococcal glutamyl endopeptidases is V1-(I/V/M)2-L3 (Figure 1), implicating that the bond between V1 and (I/V/M)2, as well as (I/V/M)2 and L3 could be cleaved by thermolysin. Although this possibility had not been consciously recognized, the present study demonstrated that V1 residue prevented both cleavages. We had speculated that the T164 and N193 being located sterically adjacent to V1 ascertained the restricted processing at the N-1-V1 bond. However, GluV8 with T164A and N193A substitutions was invariably processed at the N-1-V1 bond. Thus, although the mechanism that supports the precise processing remains unknown, we currently suspect that hydrophobic interaction with the side chain of V1 could be important for the maturation processing of the GluV8 family.

GluV8 family of enzymes including GluScpr and GluScoh most potently hydrolyzed a Glu-containing peptide substrate, i.e., LLE-MCA. Noticeably, GluScpr hydrolyzed QAR-MCA as well as YVAD-MCA and AAN-MCA to some extent, indicating its broader substrate specificity. The P1 acceptance of R was also observed with GluScoh to a lesser extent (Figure 5A). In addition, it was reported that commercially sup-

plied GluV8 exhibited a subtle hydrolyzing activity toward QAR-MCA (Wildeboer et al., 2009). These findings suggest that GluV8 family members could accept positively charged amino acids at P1 position, and that this property could have some significance under certain conditions.

Among protease inhibitors tested, irreversible serine protease inhibitors, i.e., DFP and AEBSF, efficiently suppressed the protease activities of GluScpr and GluScoh. Other inhibitors that are reported to be effective on serine proteases, such as PMSF, leupeptin, aprotinin, TPLK, and TLCK did not suppress their activities. Hence, the unique inhibitory effect of DFP on GluSE (Ohara-Nemoto et al., 2002) was common to the staphylococcal endopeptidases.

In addition to V1 and the catalytic triad, the three amino acid residues at positions 185, 188, and 189 dictate the proteolytic activity of GluV8, GluSE, and GluSW (Nemoto et al., 2009). These seven amino acid residues participate in the formation of the substrate-binding pocket of GluV8. From comparison of the amino acid sequences, we speculated that the activity of GluScpr carrying Y185, V188, and E189 should be much lower than that of GluV8 and comparable to GluSE. In fact, the specific activity of GluScpr toward LLE-MCA was one-third of that of GluV8, and the mutations of Y185W and E189P mimicking the amino acids of GluV8 seemed to enhance the protease activity. This study further demonstrated that the activity of GluSE carrying triple substitutions of N190H, Y192H, and S195A (GluSE-HHA) was 4.5-fold increased from the wild type, indicating that the 190–195 region is also involved in modulation of the protease activity. However, because the activity of GluSE-HHA could reach approximately 30% of that of GluScpr, regions in addition to amino acid residues 185–195 should be certainly involved in modulation of the proteolytic activity. The three-dimensional structure of GluV8 revealed that H184WGGVP189 and E191FNGA195 form an anti-parallel β-sheet structure (Prasad et al., 2004). The former β-strand takes part in the substrate-binding pocket together with the catalytic triad and V1. In contrast, the latter β-strand is largely buried behind the former β-strand. Accordingly, the effect of the latter β-strand seems indirectly mediated possibly through the former strand.

Materials and methods

Materials

The materials used and their sources were as follows: expression vector pQE60, from Qiagen Inc. (Chatsworth, CA, USA); low-molecular-weight molecular markers, from GE Healthcare (Buchinghamshire, UK); restriction enzymes and DNA-modifying enzymes, from Nippon Gene (Tokyo, Japan); KOD Plus DNA polymerase, from Toyobo (Tokyo, Japan); MCA peptide substrates and leupeptin, from the Peptide Institute Inc. (Osaka, Japan); Genome Walker Universal kit and Talon metal affinity resin, from Clontech Laboratories Inc. (Palo Alto, CA, USA); azocasein, thermolysin from *Bacillus thermoproteolyticus rokko*, trypsin, DFP, AEBSF (Pefabloc® SC), PMSF, aprotinin, pepstatin, TPCK, and TLCK, from Sigma-Aldrich (St. Louis, MO, USA); oligonucleotide primers were purchased from Genenet (Fukuoka, Japan) and Greiner Bio-One GmbH (Frickenhausen, Germany).

Bacterial strains and culture conditions

S. caprae GTC 378, *S. cohnii* subsp. *cohnii* GTC 248, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 14990, and *S. warneri* JCM 2415 were used for this study. Staphylococci were cultured in Todd-Hewitt broth (Becton Dickinson, Sparks, MD, USA). For the expression of recombinant proteases, *E. coli* XL1Blue was used.

PCR cloning of the genes encoding glutamyl endopeptidases from *S. caprae* and *S. cohnii*

Degenerate 5′- and 3′-oligonucleotide primers (5V8degV1 and 3V8degF225) encoding V1-Ile11 and F225-A214 of GluV8, respectively, were synthesized in consideration of the nucleotide homology of GluV8, GluSE, and GluSW (Table 1). Genome DNA of *S. caprae* (100 ng) prepared as described previously (Ikeda et al., 2004) was used as a template. Following an initial denaturation at 95°C for 15 min, 50 cycles of 94°C for 20 s; 50°C for 15 s, and 72°C for 3 min were performed with a HotStar PCR system (Qiagen Inc.). As a result, a 650-bp DNA fragment was amplified. The fragment was inserted in pBluntTOPO (Invitrogen, Carlsbad, CA, USA) and sequenced. Based on the nucleotide sequence of the 650-bp fragment, two gene-specific primers (3ScprGSP1 and 5ScprGSP2; Table 1) were synthesized for amplification of the genome coupled in both directions with an adaptor primer (5′-GTA ATA CGA CTC ACT ATA GGG C-3′). Thereafter, 2-kb DNA fragments in both directions were obtained from the EcoRV-digested genome DNA by using a Genome Walker Universal kit (Clontech Laboratories Inc.). Again, the primer set (5ScprEco and 3ScprEco) was synthesized based on the sequence data from the two ends of the 2-kb DNA fragments for an overall amplification (Table 1). Finally, a 4-kb genome DNA of *S. caprae* was amplified and inserted into the SmaI site of pUC18. The nucleotide sequence of the 4042-bp insert was determined.

Degenerate primers (5V8degV1 and 3V8degS146; Table 1) corresponding to amino acids at positions V1-I11 and D137-S146 of GluV8, respectively, were used for the first PCR step with genome DNA of *S. cohnii* (100 ng), and a 450-bp DNA fragment was obtained. The fragment was inserted into pGEM-TEasy (Promega Corp., Madison, WI, USA) and sequenced. Based on the sequence of the 450-bp fragment, two gene-specific primers (3ScohGSP1 and 5ScohGSP2) were synthesized and used for gene-walking in combination with the adaptor primer (5′-GTA ATA CGA CTC ACT ATA GGG C-3′). Consequently, a 1-kb DNA fragment in the 5′-direction was obtained from the PvuII-digested genome DNA; and a 0.8-kb DNA in the 3′-direction was obtained from DraI-digested genome DNA by using the Genome Walker Universal kit (Clontech Laboratories Inc.). Again, the primer set (5ScohPvu and 3ScohDra) was synthesized for the overall amplification based on the sequence at the two ends of the DNA fragments. Finally, the 1952-bp DNA region of *S. cohnii* genome was cloned and sequenced.

Construction of the expression plasmids for glutamyl endopeptidases

Recombinant GluScpr and GluScoh were expressed in *E. coli* as the full-length form with N- and C-terminal tags (MGGS and GSRSHHHHHH, respectively) by use of the pQE60 expression vector, as described previously (Nemoto et al., 2008). Briefly, the DNA

fragment carrying the full-length form of GluScpr was amplified with 5′-primer (5GluScprM-68Bgl) and 3′-primer (3GluScprA237Bgl) (Table 1), which carried BglII sites, and then inserted into a BamHI site of pQE60 (designated pQE60-GluScpr). The DNA carrying the full-length form of GluScoh was amplified with 5′-primer (5GluScohL-67Bam) and 3′-primer (3GluScohV211Bam), which carried BamHI sites, and then inserted into a BamHI site of pQE60 (designated pQE60-GluScoh).

Full-length GluV8 with five substitutions in its prosequence (D-31H, E-7Q, E-4S, A-2P, and N-1S) and deletion of the C-terminal 52 residues was expressed in *E. coli* as described previously (Ono et al., 2008).

In vitro mutagenesis by PCR

In vitro mutagenesis was performed as described previously (Nemoto et al., 2008) by PCR with mutated primers to substitute the nucleotides of pQE60-GluScpr encoding D-31 and D-18 to H-31 and K-18 (designated pQE60-GluScpr-mut), the nucleotides of pQE60-GluScoh encoding D-35 and E-25 to S (designated pQE60-GluScoh-mut), the nucleotides of pQE60-GluScpr-mut encoding Y185 and E189 to W185 and P189 (designated pQE60-GluScpr-WP), and the nucleotides of pQE60-GluSE encoding N190, Y192, and S195 to H190, H192 and A195 (designated pQE60-GluSE-HHA), the nucleotides of pQE60-GluV8 encoding the amino acids at positions -3, -2, -1, and 1 to K at -3, -2, and -1 positions or L at position 1 (Nemoto et al., 2008). S169, T164, and N193 of GluV8 were substituted to A. Similarly, S169 of GluScoh, GluSE, and GluSW were mutated to A. All mutagenesis and truncation were confirmed by DNA sequencing.

Expression and purification of recombinant proteases

The expression and purification of recombinant proteins in *E. coli* was performed by Talon affinity chromatography as reported previously (Nemoto et al., 2008, 2009). The purified proteins were stored at -80°C until used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

Samples (1 μg) were separated by electrophoresis in the presence of 0.1% (w/v) SDS at a polyacrylamide concentration of 12.5% (w/v), and then stained with Coomassie Brilliant Blue (CBB). For zymography, SDS-PAGE was performed by using a polyacrylamide gel of 12.5% (w/v) containing 1% (w/v) of azocasein (Rice et al., 2001). After SDS-PAGE, the gel was incubated twice at 25°C with 100 ml of 2.5% (w/v) Triton X-100 for 20 min each time, twice for 10 min each time with 100 ml of 50 mM Tris-HCl (pH 7.8) containing 30 mM NaCl, and then incubated in 100 ml of the latter buffer at 37°C overnight. Finally, non-hydrolyzed azocasein in the gel was visualized with CBB.

In vitro processing and measurement of protease activity

Unless otherwise stated, *in vitro* processing of recombinant proenzymes and subsequent protease assay were performed as follows: recombinant proteins (10 μg) were incubated for 4 h in 0.1 ml of buffer A [10 mM sodium borate (pH 8.0) containing 0.005% (v/v) Triton X-100 and 2 mM $CaSO_4$] with 0.3 μg of thermolysin (weight ratio 33:1) at 37°C. Thereafter, aliquots of glutamyl endopeptidases (0.25 μg 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 37°C for 1 h. EDTA was then added to the reaction mixture to inactivate thermolysin (Fontana, 1988). The fluorescence was measured with excitation at 380 nm and emission at 460 nm with a Fluorescence Photometer F-4000 (Hitachi, Tokyo, Japan). One unit was defined as the activity that produces 1 fmol of product per min. For calculation of the specific activity, the bands visualized with CBB corresponding to mature proteases were quantified with a PDQuest software (Bio-Rad, Hercules, CA, USA) by using BSA separated on SDS-PAGE as a standard.

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 CBB, the bands were excised; and the respective materials were then directly sequenced with a model Procise 49XcLC protein sequencer (ABI, Foster City, CA, USA).

Protein concentration determination

Protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as the standard (Pierce, Rockford, IL, USA). For quantification of affinity-purified samples, bovine albumin was dissolved in either 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol to adjust buffer compositions.

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