

Measurement of protease activity of exfoliative toxin A using synthetic peptidyl substrates and correlation between *in vivo* and *in vitro* activities

Mizuki T. TACHI^{1,2}, Yuko OHARA-NEMOTO¹, Tomomi T. BABA¹, Takeshi KOBAYAKAWA¹, Shuichi FUJITA³, Tohru IKEDA³, Takao AYUSE², Kumiko OI², and Takayuki K. NEMOTO¹

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 Clinical Physiology, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan

³ Department of Oral Pathology and Bone Metabolism, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan

Exfoliative toxin A (ETA) produced by *Staphylococcus aureus* causes bullous impetigo and staphylococcal scalded skin syndrome. The exfoliative activity of ETA is ascribed to its highly restricted degradation between Glu₃₈₁-Gly₃₈₂ of desmoglein 1, a component protein of desmosomes. Since the peptidase activity of ETA has been yet to be demonstrated other than desmoglein 1, the entity as a peptidase and its molecular mechanism remain to be elucidated. In the present study, we determined the peptidase activity using recombinant ETA molecules and synthetic fluorescent peptidyl substrates, while the exfoliative activity was examined by a neonatal mouse model. Although peptidase activity was trivial as compared with the *S. aureus* glutamyl endopeptidase GluV8, pro-ETA starting from Phe24 (Phe24-ETA) and the mature form from Glu39 (Glu39-ETA) exhibited the activities toward LLE-, AE-, and LE-MCA, but not toward LLQ-, LD- or AAA-MCA, indicating a Glu-specific endopeptidase activity. This activity was statistically higher in Glu39-ETA than Phe24-ETA and was inhibited by the serine protease inhibitor Pefabloc. Deletion of the $\alpha 1$ region at positions 42-56 as well as substitution of active Ser233 to Ala abrogated the peptidase activity. In accord with these results, intraepidermal blister formation and epidermolysis were induced more exclusively by Glu39-ETA than Phe24-ETA. ETAs without proteolytic activity as well as GluV8 did not cause an exfoliative reaction. These results suggest that the highly restricted Glu-specific endopeptidase activity of ETA is involved in exfoliative activity and that the $\alpha 1$ region is required for these functions.

ACTA MEDICA NAGASAKIENSIA 58: 41–48, 2013

Key words: *Staphylococcus aureus*, exfoliative toxin, desmoglein, GluV8

Introduction

Exfoliative toxins (ETs) from *Staphylococcus aureus* cause bullous impetigo and a severe blistering disease in neonates, generally called staphylococcal scalded skin syndrome (SSSS).^{1,2} A previous study using an animal model of neonatal mice demonstrated that the site of action of ETs is desmosome³, which connects adjacent granulosal cells and

becomes split on blister formation. Although ETs have long been considered as toxins and no proteolytic activity has been found, their amino acid sequence similarity^{4,5} and tertiary structure^{6,7} have strongly suggested that they belong to the chymotrypsin-like family of serine proteases and cleave peptide bonds C-terminally adjacent to acidic residues, Glu and Asp.

S. aureus produces four serologically distinct ETs, i.e.,

Address correspondence: Yuko Ohara-Nemoto, Ph.D. Department of Oral Molecular Biology, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan
Phone: +81-95-849-7643, Fax: +81-95-849-7642, E-mail: ynemoto@nagasaki-u.ac.jp

Received February 19, 2013; Accepted April 10, 2013

ETA, ETB, ETC and ETD.^{8,9} Most *et*⁺ strains isolated from human impetigo carry *eta* (68.9%) and *etb* (30.0%), while strains *etc*⁺ (1.8%) and *etd*⁺ (0%) were scarcely isolated.¹⁰ By analogy to pemphigus foliaceus, Amagai et al.^{11,12} have shown that desmoglein 1 (Dsg1), a desmosome transmembrane constituent, is a target of ETA and ETB, and cleavage of Dsg1 causes blisters. ETs cleave human and mouse Dsg1 at a single site at Glu₃₈₁-Gly₃₈₂ located in the junction region between extracellular domains 3 and 4.¹³ In addition, it has been reported that α - and β -melanocyte-stimulating hormones were cleaved by ETA and ETB.¹⁴ However, protease activity of ETs other than toward these substrates has not been shown to date, and thus the precise molecular mechanism of specific cleavage and proteolytic activity of ETs remain to be elucidated. Furthermore, it was reported that only 30-40% of *S. aureus* strains giving rise to SSSS in Europe and the United States produce ETs,^{15,16,17} and there is very little literature available on the epidemiology of ET-producing *S. aureus* strains, due to the lack of a suitable and standardized in vitro assay.¹⁸ Therefore, such assay system is keenly required to elucidate whole mechanism of *S. aureus* infectious diseases causing blister.

It has been known that *S. aureus* produces various pathogenic factors, in which Glu-specific endopeptidase, GluV8, is one of the most abundant and potent proteases mediating inflammatory reaction.¹⁹ Most human isolates possess the gene of GluV8.²⁰ GluV8 peptidase possess the Glu-specific substrate specificity and efficiently degrades Dsg1.¹³ Therefore, the reason why ETs, but not GluV8, are responsible for blister formation at diseased areas remains unknown.

In the present study, to elucidate relationship between the proteolytic and biological activities of ETA, we employed recombinant techniques for preparation of wild type ETA expressed in *Escherichia coli*, because the ambiguity of the previous data has been mainly caused by the purity of the ET preparations.¹⁴ For instance, contamination of GluV8 in either native or recombinant ETA specimen prepared from *S. aureus* may significantly influence the data due to its marked activity. Besides wild type ETA molecules possessing mature and pro-mature sequences, a single-amino acid-mutant substituted an essential Ser to Ala and an $\alpha 1$ region (Ala₄₂-Tyr₅₆)-deletion-mutant were expressed. Their proteolytic activities were measured in vitro using synthetic peptidyl substrates, and these activities were compared with their exfoliative activities examined with a mouse model.

Materials and Methods

Materials. Benzyloxycarbonyl (Z)-LLE-, succinyl (Suc)-AE-, and Suc-AAA-4-methylcoumaryl-7-amide (MCA) were obtained from the Peptide Institute (Osaka, Japan). LE-, LD-, Z-LLQ-MCA were synthesized by Thermo Fisher Scientific. Recombinant GluV8 proform (pro-GluV8) was expressed in *Escherichia coli* and converted into a mature form (mat-GluV8) by thermolysin treatment as previously reported.²¹

Construction of expression plasmids. *S. aureus* JT1 (*eta*⁺) was isolated from a region of impetigo.²² A DNA fragment encoding the full length of *eta* was amplified by PCR using *S. aureus* JT1 genomic DNA with a set of primers (5'-TGAG-GATCCATGAATAATAGTAAAATTATT-3' and 5'-TATG-GATCCCTCATTTTTCTCGTTTATAATG-3', in which the *Bam*HI sites are underlined). After *Bam*HI cleavage, a resultant fragment was inserted into the site of pQE60, producing pQE60-ETA. Expression plasmids deleting Met₁-Ala₃₈ (designated Glu39-ETA) and Met₁-Gly₅₇ (Val58-ETA) were generated by PCR from pQE60-ETA with primers (5'-GAAGTTTCAGCAGAAGAAATAAAAAAC-3' for Glu39-ETA) or (5'-GTCAATGCATTTAATTTACCAA-3' for Val58-ETA) and an antisense primer (5'-CATGGTTAATTTCTCTCTTTAATGA-3'). The Ser233Ala substitution was introduced by PCR-based mutagenesis²¹ using a set of primers (5'-GCGGGATCAGGTATATTTAATTCA-3' and 5'-ATTTCCCGGAAGTGTAAATCCATAG-3'). *E. coli* XL1-blue was transformed with the plasmids.

Induction and purification of recombinant ETAs. *E. coli* was cultured in Luria-Bertani broth in the presence of 50 μ g/ml of ampicillin. Recombinant ETAs were induced with 0.2 mM isopropyl β -D-1-thiogalactopyranoside, purified on Talon metal affinity resin (Clontech) as previously reported²¹ and then dialyzed against PBS. Recombinant proteins were sterilized by passing through a 0.45- μ m membrane filter and aliquots were stored at -80°C until being used.

Protease activity. A highly sensitive detection technique is indispensable to measure the proteolytic activity of ETA, and thus we used fluorescent substrates, i.e., commercially available and synthesized peptidyl-MCAs carrying Glu at the P1 position. Protease activity was measured according to the previous report.²¹ In brief, 0.2 ml of the reaction mixture composed of 50 mM Tris/HCl (pH 8.0) and 10 or 40 μ M MCA-peptide was preincubated at 37 °C for 5 min. The reaction was started by an addition of recombinant ETA (4 μ g) or mature GluV8 (0.25 μ g). Fluorescence intensity with

excitation at 380 nm and emission at 460 nm was measured chronologically or at 60 min after starting reaction. As negative controls, the same amounts of pure bovine serum albumin (New England Biolabs) were used instead of ETA or GluV8.

Animal model. The study protocol received approval from the Ethics Committee of Nagasaki University Graduate School of Biomedical Science (approval no. 0911170797). Neonatal ddY mice within 16 h after birth (weight 1.3-1.9 g) were subcutaneously injected in the back with either ETA or GluV8 (10 or 20 μ g) in PBS, or PBS alone. All mice were maintained at 26-28°C and covered with wet cotton to prevent dryness. They were graded on appearance and tactile examination.^{11,23} Mice were euthanized at the end of the 24-h observation period. Thereafter, tissues were carefully excised, fixed with 10% formaldehyde, and embedded in paraffin, then sections were stained with hematoxylin and eosin. To quantitate exfoliative activity of recombinant ETA and GluV8 molecules, we defined an intact index, and at least five sites of each section (933 μ m in average) were examined. Intact index was calculated as follows:

$$\text{Intact index (\%)} = \frac{\text{length of adhesion area}}{\text{length of total area appearing in a histochemical section}} \times 100$$

Statistics. Differences of proteolytic activity toward the three substrates were calculated by two-way repeated-measures ANOVA, or comparisons between two groups were assessed using Student's *t*-test.

SDS-polyacrylamide gel electrophoresis (PAGE) and N-terminal sequencing. Proteins were separated by PAGE in the presence of 0.1% (w/v) of SDS with a polyacrylamide concentration of 11% (w/v), then stained with Coomassie Brilliant Blue. For N-terminal sequencing, separated proteins were transferred to a Sequi-Blot membrane (Bio-Rad) and stained with Coomassie Brilliant Blue. The N-terminal sequences of stained bands were determined with a model Procise 49XcLC protein sequencer (Applied Biosystems) as previously described.²¹

Results

Expression, purification, and N-terminal sequences of recombinant ETAs

N-terminal 23 (Met₁-Ala₂₃) and subsequent 15 amino acid residues (Phe₂₄-Ala₃₈) of ETA were shown to correspond to prepro- and pro-sequences, respectively.⁸ When full-length ETA was expressed in *E. coli* as a C-terminal His₆-tagged protein, the N-terminal sequence was found to be Phe-Val-Ile-Asn-Asp-Glu-Leu-Met-Gln-Lys, which corresponded to Phe₂₄-Lys₃₃ of ETA (Fig. 1), hence this molecule carried pro-sequence and was designated Phe24-ETA. The cleavage at the Ala₂₃-Phe₂₄ bond could be mediated by *E. coli* signal peptidase. Glu39-ETA, a mature form, and ETA with deletion of the first α helix (α 1, Ala₄₂-Tyr₅₆) (designated Val58-ETA) were also expressed to study the inhibitory effects of the pro-sequence and α 1 region on the activity. In Val58-ETA, Gly₅₇ was additionally deleted, since the α -amino group of N-terminal Val in mature GluV8 is proposed to be

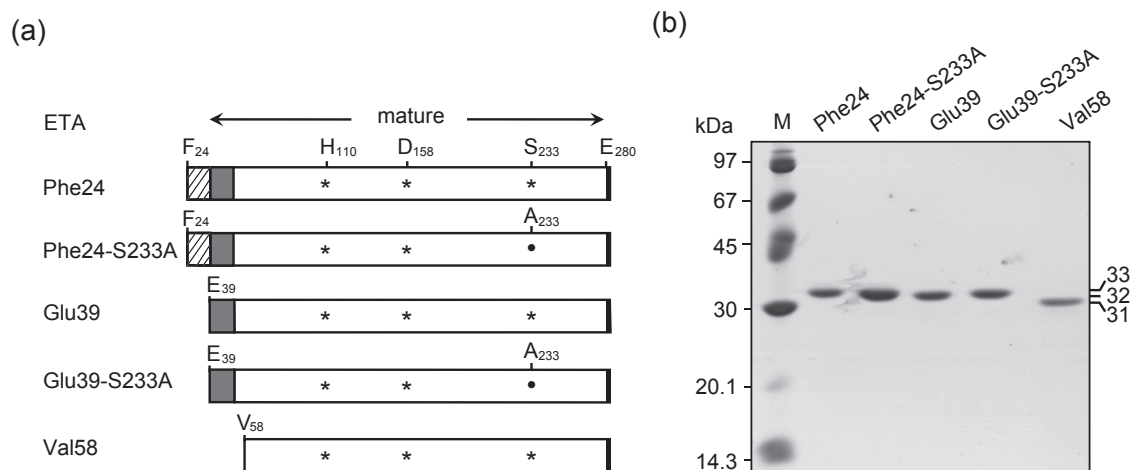


Figure 1. Schematic presentation and SDS-PAGE findings of ETA. (a) His₁₁₀, Asp₁₅₈, and Ser₂₃₃ (asterisks) are the residues to form the catalytic triad conserved in serine proteases. Dots indicate the substitution of active Ser₂₃₃ to Ala. N-terminal pro- and α 1 regions are shown by hatched and gray boxes, respectively. His₆-tag (closed boxes) is attached at the C-terminus. (b) Purified ETAs (0.3 μ g) were separated on SDS-PAGE. Lane M, low-molecular-weight marker.

associated with the γ -carboxyl group of glutamic acid of a substrate.^{21,24} The N-terminal sequences of these truncated moieties were confirmed to be Glu₃₉-Val-Ser-Ala-Glu-Glu₄₄ and Val₅₈-Asn-Ala-Phe-Asn-Leu-Pro-Lys-Glu-Leu₆₇, respectively. Additionally, Phe24- and Glu39-ETAs with a single amino acid substitution of Ser233Ala were expressed (Fig. 1). Purified Phe24-, Glu39-, and Val58-ETAs were found to be migrated as 33-, 32-, and 31-kDa bands, respectively, on SDS-PAGE, which were comparable to their calculated molecular masses (29,911, 28,157, and 25,807, respectively).

Peptidase activity of recombinant ETAs

We measured the peptidase activity using recombinant

ETAs with LLE-MCA (Fig. 2). Hydrolysis of LLE-MCA was observed in both Phe24-ETA and Glu39-ETA, and completely suppressed in the presence of 1 mM Pefabloc, a potent irreversible serine protease inhibitor. When Ser233 was substituted to Ala, these mutants of Phe24- and Glu39-ETA S233A molecules exhibited no hydrolysis. Furthermore, Val58-ETA deleting the α 1 region also lost the activity, suggesting that the region is conversely required for proteolytic activity.

In order to define the substrate specificity, the proteolytic activities of Phe24- and Glu39-ETAs were determined with five additional substrates (Fig. 3). Following LLE-MCA, ETAs hydrolyzed AE- and LE-MCA to some extents,

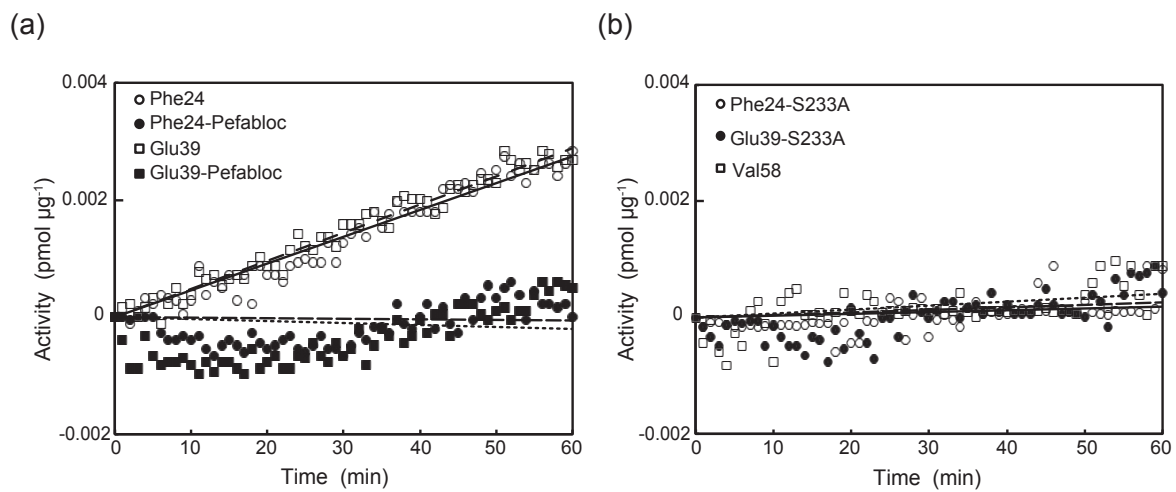


Figure 2. Proteolytic activity of ETA toward LLE-MCA. (a) Proteolytic activities of Phe24- and Glu39-ETAs in the presence and absence of 1 mM Pefabloc were measured with 10 μM LLE-MCA at 37 $^{\circ}\text{C}$ for 1 h. Lines were obtained by linear regression of the data starting from value zero at time zero. Continuous line, Phe24-ETA; dashed line, Phe24-ETA with Pefabloc; dashed spaced line, Glu39-ETA; dotted line, Glu39-ETA with Pefabloc. (b) The peptidase activities of Phe24-S233A-, Glu39-S233A- and Val58-ETAs were determined. Lines were obtained as above. Continuous line, Phe24-S233A-ETA; dashed line, Glu39-S233A-ETA; dotted line, Val58-ETA.

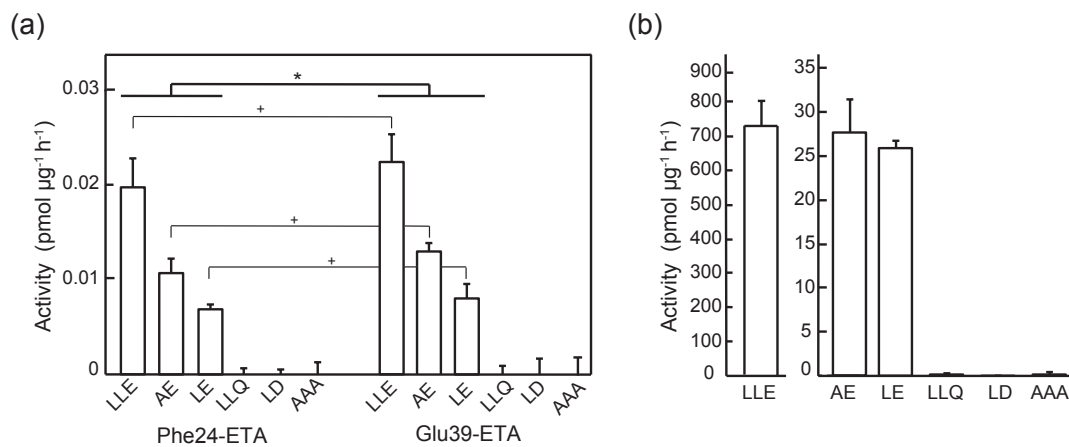


Figure 3. Substrate specificity of ETA. Proteolytic activities of (a) Phe24- and Glu39-ETAs, and (b) mature GluV8 were determined with 40 and 10 μM peptidyl MCA substrates, respectively. Values are shown as means \pm SD of triplicate samples. Representative results of three different experiments are shown. $^{\dagger}P > 0.05$, Student's *t*-test; $*P = 0.02$, two-way repeated-measures ANOVA.

whereas LLQ-, LD- and AAA-MCA were not hydrolyzed. The tendency of the substrate specificity (LLE > AE > LE) was identical to that of GluV8, though the specific activity of GluV8 was 36,000-fold higher than that of ETA. Thus, it was found that ETA is truly a Glu-specific serine endopeptidase, whose substrate specificity is basically identical to that of GluV8.

Noticeably, the hydrolyzing activities of Glu39-ETA for the three major substrates were consistently higher than those of Phe24-ETA, although the differences in the peptidase activities between two recombinant ETAs were not significant ($P=0.266$, 0.088 and 0.304 for LLE-, AE- and LE-MCA, respectively) (Fig. 3a). On the other hand, when data for the three substrates were calculated by two-way repeated-measures ANOVA, a significant difference ($p=0.024$) was noted between two ETAs.

Exfoliative activity of recombinant ETAs and GluV8

The exfoliative activities of proteolytically active and inactive ETAs and GluV8 were evaluated using a mouse model. ETAs were injected subcutaneously into the dorsal skin of neonatal mice, then exfoliation was assessed 24 h later based on gross pathology (Table 1, Fig. 4). Intraepidermal blister formation and epidermolysis were induced by

proteolytically active Phe24-ETA and Glu39-ETA, while the S233A mutants and Val58-ETA did not induce any symptoms. In histological observations of dorsal skin specimens, superficial intraepidermal blister formation without keratinocyte necrosis was also observed with Phe24-ETA and Glu39-ETA, but not with the S233A mutants or Val58-ETA.

When mice were injected with proGluV8, any changes were not detected in either gross or pathological observations of the skin, whilst mature GluV8 caused redness of the skin within 30 min after injection, and all mice (4/4) with 20 μg and two of three mice with 10 μg of GluV8 died within 24 h. Gross pathology and skin biopsy findings examined immediately after death (21 h after injection) indicated no skin separation in mature GluV8-injected mice.

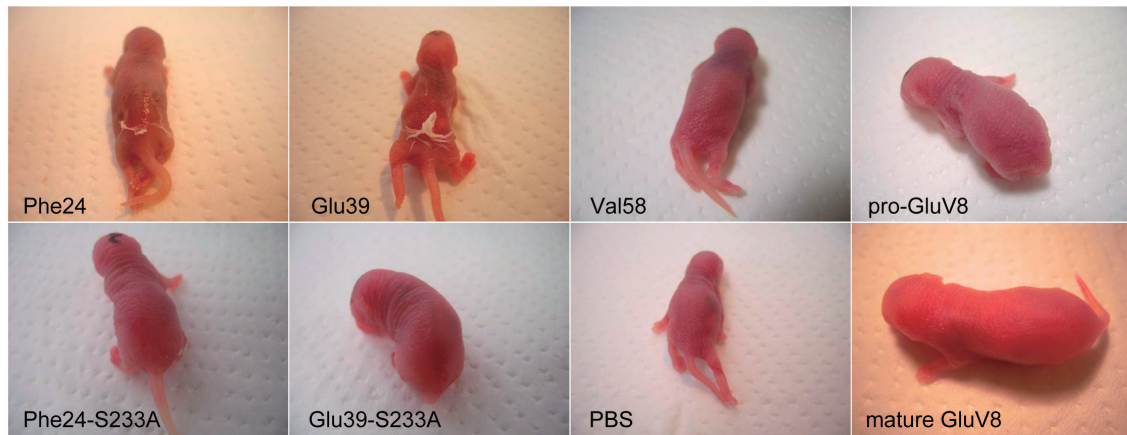
The exfoliative activity of recombinant proteins was evaluated using intact index values (Fig. 4c): The activity was more potent with Glu39-ETA ($11.6 \pm 10.2\%$, $n = 56$) followed by Phe24-ETA ($55.4 \pm 27.6\%$, $n = 46$), while no apparent exfoliation was observed with proteolytically inactive ETAs. Thus, the exfoliative activity of ETAs in the present mouse study was tightly associated with the proteolytic activity.

Table 1. Biological activities of recombinant ETAs in neonatal mice

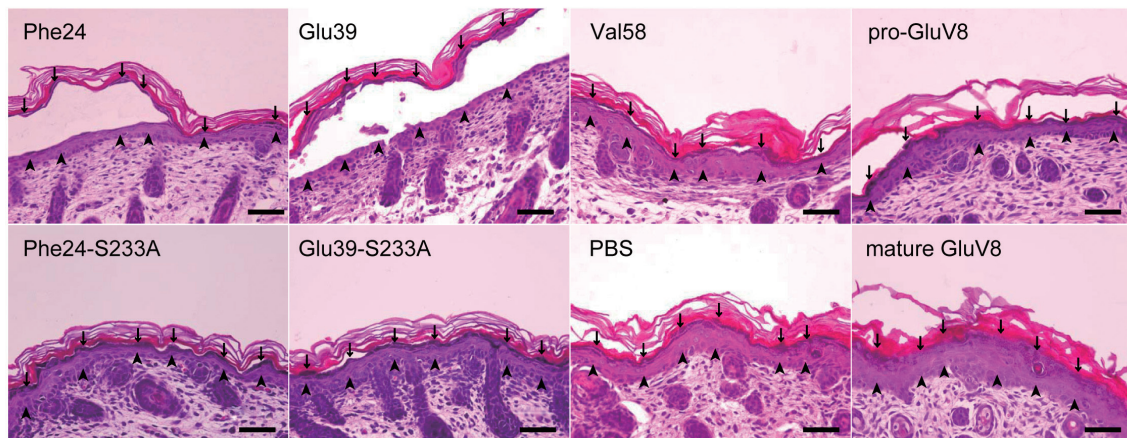
Injected agent	Dose $\mu\text{g}/\text{mouse}$	Survival/total mice	Epidermolysis*	No. of sites for histological observation [†]
Phe24-ETA	10	3/3	+	-
	20	8/8	+	46
Phe24-S233A-ETA	20	6/6	-	48
Glu39-ETA	10	3/3	+	-
	20	7/7	+	56
Glu39-S233A-ETA	20	6/6	-	48
Val58-ETA	20	4/4	-	22
pro-GluV8	10	2/2	-	-
	20	3/3	-	15
mature GluV8	10	1/3	-	-
	20	0/4	-	10
PBS	-	4/4	-	22

Neonatal ddY mice within 16 h after birth (weight 1.3-1.9 g) were subcutaneously injected in the back with either ETA or GluV8 (10 or 20 μg) in PBS, or PBS alone, and assays were performed with each group in five separate experiments. *Epidermolysis was judged by touch with a finger to determine whether epidermis peeling had occurred. [†]Randomly selected 5-8 fields of vision in one section from each mouse were examined to calculate intact index (Fig. 4c).

(a)



(b)



(c)

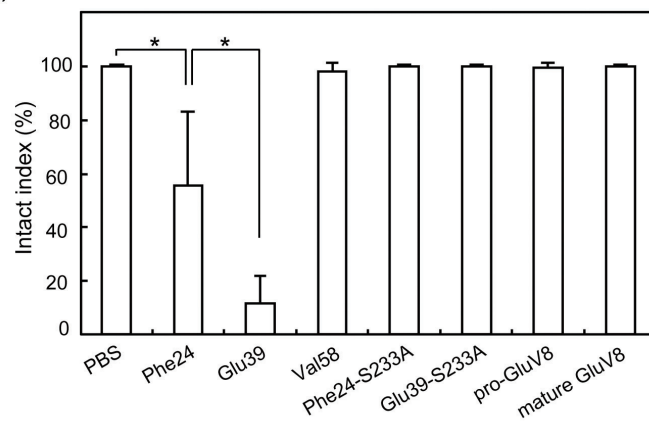


Figure 4. Exfoliative activities of ETA and GluV8. (a) ETA and GluV8 (20 μ g) were injected subcutaneously into the dorsal skin of neonatal ddY mice. After 24 (ETA) or 21 h (mature GluV8), exfoliative activity was assessed by gross pathology. (b) Blister formation was assessed based on detachment of the cornified layer mainly due to acantholysis between the granular (arrow) and prickle cell (arrowhead) layers. Sections were stained with hematoxylin and eosin. Bar = 50 μ m. (c) Exfoliative activity was quantified with intact index values (mean \pm SD) calculated using 10-56 sites in each group in five separate experiments (see Table 1). * P <0.05, Student's t -test.

Discussion

Although the specificity for Glu was suggested from the structural similarity of the active site to GluV8,^{6,7} proteolytic activity has not been measured for substrates other than Dsg1¹¹ and melanocyte-stimulating hormones.¹⁴ In the present study, Glu-specific endopeptidase activity was demonstrated with synthetic peptidyl substrates for the first time *in vitro* using *E. coli*-expressed recombinant ETAs, i.e., Phe24-ETA and Glu39-ETA. Using this *in vitro* measurement system and *E. coli*-expressed recombinant ETA mutants, we demonstrated that Ser233 is essential for the proteolytic activity in accord with previous reports showing that Ser233 is indispensable for exfoliative activity.^{25,26} Furthermore, Val58-ETA deleting the $\alpha 1$ region exhibited no activity. This result suggests that the region is not a mere "lid" of the active site²⁷ but conversely required for proteolytic activity.

In addition, it is notable that examination using recombinant *E. coli*-expressed ET molecules employed in this study is highly advantageous to assess their properties, since the possibility of contamination of GluV8 and/or other pyrogenic factors is not completely excluded in native or recombinant ET preparations obtained from *S. aureus* strains. For instance, *S. aureus* strains carrying the gene encoding GluV8 are abundantly present in clinical isolates,¹⁸ and the GluV8 activity is 36,000-fold higher than that of ETA (Fig. 3). If an ET preparation contains a trace amount of GluV8, its proteolytic activity will be considered as that of ET. Furthermore, both ETA and ETB had been reported to be bacterial superantigens exhibiting mitotic activity and stimulating proliferation of T cell^{28,29,30} possibly due to contamination by *S. aureus* real superantigens. Contamination of superantigen was also reported in a streptococcal peptidase, SpeB, fraction from *Streptococcus pyogenes*.³¹

Comparison of the hydrolyzing activities for LLE-, LE-, AE-MCA and other MCA substrates demonstrated that substrate specificity of ETA is indistinguishable from that of GluV8 (Fig. 3). The present results also indicate that the reason for apparent lack of *in vitro* protease activity is due to the extremely low peptidase activity of ETA toward substrates carrying a Glu residue at the P1 position. Indeed, the specific activity of ETA was 1/36,000 that of GluV8. Moreover, it should be notable that the more potent exfoliative activity of Glu39-ETA compared to Phe24-ETA was consistent to higher peptidase activity of this molecule.

We initially considered the possibility that pro-sequence and $\alpha 1$ might interfere with access of a substrate peptide to the active site. However, Phe24-ETA exhibited both proteolytic and exfoliative activities, though they were lower

than those of Glu39-ETA. These results suggest that the conversion of ETA from Phe24-ETA pro-form to Glu39-ETA represents the maturation step of ETA mediated by an unknown *S. aureus* peptidase. Furthermore, the present results showed that $\alpha 1$ is essential for peptidase activity. Therefore, these findings do not support an interaction between the helical $\alpha 1$ region and loop 2, which acts as a "lid" for the S1 pocket,²⁷ but suggest that $\alpha 1$ is involved in stability of formation of the enzyme pocket or in regulating the activity via interaction with a receptor molecule.^{7,27} A quantitative measurement of ETA peptidase activity *in vitro* should be critical for investigating the mechanism of impetigo or SSSS. For instance, the subtle difference in the peptidase activities between Phe24-ETA and Glu39-ETA was successfully demonstrated and was likely to be related to the significant difference in their exfoliative activities.

In conclusion, we demonstrated for the first time the proteolytic activity of ETA *in vitro* using synthetic peptidyl substrates. Our results confirm that the peptidase activity of ETA is extremely low as compared with GluV8, while the potential of ETA enzymatic activity is directly related to the development of exfoliation of skin.

Acknowledgments

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (to Y.O.-N. and T.K.N.). There are no potential conflicts of interest regarding this study.

References

- Melish ME, Glasgow LA. The staphylococcal scalded-skin syndrome. *N Engl J Med* 282: 1114-1119, 1970
- Melish ME, Glasgow LA. Staphylococcal scalded-skin syndrome: The expanded clinical syndrome. *J Pediatrics* 78: 958-967, 1971
- Melish ME, Glasgow LA, Turner MD, Lillibridge CB. The staphylococcal epidermolytic toxin: its isolation, characterization, and site of action. *Ann N Y Acad Sci* 236: 317-342, 1974
- Bailey CJ, Smith TP. The reactive serine residue of epidermolytic toxin A. *Biochem J* 269: 535-537, 1990
- Dancer SJ, Garratt R, Saldanha J, Evans R. The epidermolytic toxins are serine proteases. *FEBS Lett* 268: 129-132, 1990
- Cavarelli J, Prévost, G, Bourguet W et al. The structure of *Staphylococcus aureus* epidermolytic toxin A, an atypic serine protease, at 1.7 Å resolution. *Structure* 5: 813-824, 1997
- Vath GM, Earhart CA, Rago JV et al. The structure of the superantigen exfoliative toxin A suggests a novel regulation as a serine protease. *Biochemistry* 36: 1559-1566, 1997
- Lee CY, Schmidt JJ, Johnson-Winegar AD, Spero A, Iandolo JJ. Sequence determination and comparison of the exfoliative toxin A and toxin B genes from *Staphylococcus aureus*. *J Bacteriol* 169: 3904-3909, 1987

9. Yamaguchi T, Nishifuji K, Sasaki M et al. Identification of the *Staphylococcus aureus* *etd* pathogenicity island with encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect Immun* 70: 5853-5845, 2002
10. Nakaminami H, Noguchi N, Ikeda M et al. Molecular epidemiology and antimicrobial susceptibilities of 273 exfoliative toxin-encoding-gene-positive *Staphylococcus aureus* isolates from patients with impetigo in Japan. *J Med Microbiol* 57: 1251-1258, 2008
11. Amagai M, Matsuyoshi N, Wang ZH, Andl C, Stanley JR. Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. *Nature Med* 6: 1275-1277, 2000
12. Amagai M, Yamaguchi T, Hanakawa Y, Nishifuji K, Sugai M, Stanley JR. Staphylococcal exfoliative toxin B specifically cleaves desmoglein 1. *J Invest Derm* 118: 845-850, 2002
13. Hanakawa Y, Schechter NM, Lin C, Nishifuji K, Amagai M, Stanley JR. Enzymatic and molecular characteristics of the efficiency and specificity of exfoliative toxin cleavage of the desmoglein 1. *J Biol Chem* 279: 5268-5277, 2004
14. Rago JV, Vath GM, Tripp TJ, Bohach GA, Ohlendorf DH, Schlievert PM. Staphylococcal exfoliative toxins cleave α - and β -melanocyte-stimulating hormones. *Infect Immun* 68: 2366-2368, 2000
15. Kapral FA. Staphylococcus exfoliatin. In *Microbiology* (Schlessinger D ed; American Society for Microbiology, Washington DC) pp.263-266, 1975
16. Fleurette J, Ritter J. Prevalence des souches de *Staphylococcus aureus* productrices d'exfoliatine dans le groupe bacteriopagique II. *Ann Microbiol* 1131B: 175-183, 1980
17. Willard D, Monteil H, Piemont Y et al. L'exfoliatine dans les staphylococcies neonatales. *Nouv Presse Med* 11: 3769-3771, 1982
18. Ladhani S, Joannou CL, Lochrie DP, Evans RW, Poston SM. Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome. *Clin Microbiol Rev* 12: 224-242, 1999
19. Potempa J, Baran K, Górká M, Fedak D, Travis J, Porwit-Bóbr Z. *Staphylococcus aureus* proteases as mediators of inflammatory reaction. In *The staphylococci* (Jeljaszewicz J, Cliborowski P eds.; Gustav Fischer Verlag, Stuttgart, Germany) pp.187-188, 1991
20. Karlsson A, Arvidson S. Variation in extracellular protease production among clinical isolates of *Staphylococcus aureus* due to different levels of expression of the protease repressor *sarA*. *Infect Immun* 70: 4239-4246, 2002
21. Nemoto TK, Ohara-Nemoto Y, Ono T et al. Characterization of the glutamyl endopeptidase from *Staphylococcus aureus* expressed in *Escherichia coli*. *FEBS J* 275: 573-587, 2008
22. Nagami T, Ohara-Nemoto Y, Hirota T, Ishizaki M, Tada J. Microscopic study of experimental bullous impetigo in juvenile mice and exfoliative toxin-induced keratinocyte injury. *Antibiot Chemoth* 25: 109-116, 2009 (in Japanese)
23. Plano LRW, Gutman DM, Woischnik M, Collins CM. Recombinant *Staphylococcus aureus* exfoliative toxins are not bacterial superantigens. *Infect Immun* 68: 3048-3052, 2000
24. Ono T, Ohara-Nemoto Y, Shimoyama Y et al. Amino acid residues modulating the activities of staphylococcal glutamyl endopeptidases. *Biol Chem* 391: 1221-1232, 2010
25. Prévost G, Rifai S, Chaix ML, Piémont Y. Functional evidence that the Ser-195 residue of staphylococcal exfoliative toxin A is essential for biological activity. *Infect Immun* 59: 3337-3339, 1991
26. Redpath MB, Foster TJ, Bailey CJ. The role of the serine protease active site in the mode of action of epidermolytic toxin of *Staphylococcus aureus*. *FEMS Microbiol Lett* 81: 151-156, 1991
27. Papageorgiou AC, Plano LRW, Collins CM, Acharya KR. Structural similarities and differences in *Staphylococcus aureus* exfoliative toxins A and B as revealed by their crystal structures. *Protein Sci* 9: 610-618, 2000
28. Morlock BA, Spero L, Johnson AD. Mitogenic activity of staphylococcal exfoliative toxin. *Infect Immun* 30: 381-384, 1980
29. Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. *Science* 248: 705-711, 1990
30. Monday SR, Vath GM, Ferens WA et al. Unique superantigen activity of staphylococcal exfoliative toxins. *J Immunol* 162: 4550-4559, 1999
31. Ohara-Nemoto Y, Sasaki M, Kaneko M, Nemoto T, Ota M. Cysteine protease activity of streptococcal pyrogenic exotoxin B. *Can J Microbiol* 40: 930-936, 1994