# Quantification of Human Tissue Kallikreins in the Stratum Corneum: Dependence on Age and Gender

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***Human tissue kallikreins*** **are a family of 15 trypsin or chymotrypsin-like secreted serine proteases (hK1–hK15). hK5, hK6, hK7, hK8, and hK13 have been identified in the stratum corneum (SC), stratum granulosum, and skin appendages. It has been reported that hK5 and hK7 degrade desmosomes/corneodesmosomes, suggesting that kallikreins are responsible for desquamation. We report the quantification of hK5, hK6, hK7, hK8, hK10, hK11, hK13, and hK14 in the SC by ELISA and their variation among age groups. The total SC trypsin and chymotrypsin-like activities were also measured. The amount of hK7, hK8, and hK11 (ng per mg dry weight) were high, and varied from 6 to 14, hK5 (2.0–4.0) was present at intermediate levels, and hK10 (0.65–1.0), hK14 (0.1–0.3), hK6 (0.1–0.3), and hK13 (0.02–0.1) were present at lower levels. hK6 and hK14 were significantly lower in females between 20 and 59 y. hK5, hK7, hK10, hK11, and hK14 were not significantly different across the age groups. hK8 was lowest at extremes of age (highest at 30–39 y), hK6 was lower at $>30$ y, and hK13 was lower at $>20$ y. Overall trypsin-like activity did not differ across age groups but was higher in subjects $<11$ y. Overall chymotrypsin-like activity was not related to age. In conclusion, we found multiple kallikreins in the SC and suggest that these enzymes may be responsible for desquamation through an enzymatic cascade pathway.**

Key words: aging/desquamation/human kallikreins/serine proteases/stratum corneum
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Numerous studies during the last 30 y have demonstrated that the stratum corneum (SC) is structurally and biochemically diverse and plays key roles in skin barrier function and aging. The SC structure, the SC lipids, the number of SC layers, and the transepidermal water loss have been shown to be important in these processes (Harding, 2004; Marks, 2004). In addition, the desquamation process has also been recognized to be important for the maintenance of skin barrier function. The proliferation rate of keratinocytes and their transformation into corneocytes is matched by the shedding of old corneocytes at the SC regardless of age (Ya-Xian *et al*, 1999; Harding *et al*, 2000), with a turnover time through the SC estimated to be 14 d in normal skin (Halprin, 1972; Pierard *et al*, 2000). The SC appears to desquamate at a fixed rate that is uninfluenced by external forces (Kligman, 1964). The desquamation of corneocytes requires both "trypsin-like" and "chymotrypsin-like" serine protease activities (Suzuki *et al*, 1996) for desmosome and/or corneodesmosome degradation (Chapman and Walsh, 1990). It is likely that there is a regulatory system maintaining serine protease activity in order to sustain a steady skin barrier function.

The *human tissue kallikrein* (*KLK*) gene family localizes as a cluster to chromosome 19q13.4 and encodes 15 secretory serine proteases (hK1–hK15) (Yousef and Diamandis, 2001) that have been suggested to function as an enzymatic cascade pathway in many tissues (Yousef and Diamandis, 2002). In the case of skin tissue, several kallikreins, including hK5 (previously known as SC trypsin-like enzyme, SCTE; Brattsand and Egelrud, 1999; Yousef and Diamandis, 1999), hK7 (previously known as SC chymotrypsin-like enzyme, SCCE; Hansson *et al*, 1994), as well as hK6, hK8, and hK13 (Komatsu *et al*, 2005), have been identified immunohistochemically in the SC, the stratum granulosum, and the skin appendages, and are predicted to be secretory serine proteases involved in skin desquamation. Functional studies have shown that hK7 cleaves corneodesmosin and desmocollin 1 (Caubet *et al*, 2004), and that the pro-forms of hK7 and hK14 are activated by hK5. Activation of pro-hK5 is either autocatalytic or is mediated by hK14 (Brattsand *et al*, 2005). hK5 also degrades corneodesmosin (Simon *et al*, 2001).

hK5, hK7, and hK8 have been found to be transported from the trans-Golgi network within lamellar granules, and the granules are released from the apical surface of the

Abbreviations: Ab, antibody; ALP, alkaline phosphatase; AMC, 7-amino-4-methyl-commarin; BSA, bovine serum albumin; hK, kallikrein protein; KLK, kallikrein gene; pNA, para-nitroanilide; SA-ALP, streptavidin-alkaline phosphatase; SC, stratum corneum; SCCE, stratum corneum chymotrypsin-like enzyme; SCTE, stratum corneum trypsin-like enzyme; SD, standard deviation

most-superficial granular cells (Ishida-Yamamoto *et al*, 2004, 2005). In addition, previous *in situ* hybridization studies have identified *KLK1*, *KLK4*, *KLK9*, *KLK10*, *KLK11*, and *KLK14* as additional serine protease genes that are candidate players in desquamation (Komatsu *et al*, 2003).

But it has not as yet been determined if these kallikreins are detectable at the protein level in the SC, if their concentration changes with aging, and to what extent each kallikrein contributes to the total SC serine protease activity. Here, we report quantification of hK5, hK6, hK7, hK8, hK10, hK13, and hK14 in the SC of individuals of various ages by ELISA assays and correlate these amounts with total SC trypsin- and chymotrypsin-like enzymatic activities.

## Results

**Kallikrein levels in the SC among age groups** We used in-house developed immunofluorometric assays (ELISA) to quantitatively determine the amounts of hK5, hK6, hK7, hK8, hK10, hK11, hK13, and hK14 in normal SC extracts. In Table I, kallikrein concentrations are reported in groups stratified by age (per decade from age 20 to 59 y) and further subdivided by gender. Figure 1 displays the kallikrein concentrations among age groups, in which male and female values are combined, and includes subjects less than 11 y and older than 70 y. The same specimens were also analyzed for hK4 using a method described in Table II, but as 60% of the samples had undetectable hK4 concentration, this kallikrein was excluded from further analysis.

**Table I. Kallikrein quantification in the stratum corneum among age groups[a]**

| hK | Sex | 20s: mean ± SD | M (n = 15) F (n = 15) (%) | 30s: mean ± SD | M (n = 15) F (n = 15) (%) | 40s: mean ± SD | M (n = 15) F (n = 15) (%) | 50s: mean ± SD | M (n = 15) F (n = 15) (%) |
|---|---|---|---|---|---|---|---|---|---|
| Chymotrypsin-like hK | | | | | | | | | |
| hK7 | M | 13.7 ± 8.0 | | 11.1 ± 6.4 | | 12.9 ± 11.7 | | 7.4 ± 7.8 | |
| | F | 11.3 ± 12.3 | | 13.8 ± 12.6 | | 11.7 ± 6.6 | | 10.5 ± 7.2 | |
| Average | | 12.5 | | 12.4 | | 12.3 | | 8.9 | |
| Trypsin-like hK | | | | | | | | | |
| hK8 | M | 8.1 ± 2.9 | 42.8 | 9.0 ± 3.9 | 41.0 | 8.4 ± 4.4 | 37.4 | 7.6 ± 4.6 | 39.0 * |
| | F | 9.8 ± 3.3 | 42.8 | 14.4 ± 4.6 | 53.7 | 11.6 ± 3.5 | 45.8 | 6.1 ± 1.6 | 32.9 * |
| hK11 | M | 7.5 ± 3.1 | 39.8 | 9.0 ± 4.5 | 41.1 | 8.8 ± 3.5 | 39.4 | 8.5 ± 6.4 | 44.0 |
| | F | 9.2 ± 6.7 | 40.2 | 8.5 ± 4.1 | 31.6 | 10.1 ± 3.8 | 39.9 | 8.3 ± 4.4 | 44.9 |
| hK5 | M | 1.9 ± 0.85 | 10.1 | 2.8 ± 1.0 | 12.8 | 3.8 ± 3.5 | 16.8 | 2.0 ± 1.8 | 10.3 |
| | F | 2.4 ± 0.98 | 10.5 | 2.9 ± 1.5 | 10.7 | 2.6 ± 1.2 | 10.1 | 3.2 ± 1.7 | 17.5 |
| hK10 | M | 0.70 ± 0.47 | 3.7 | 0.69 ± 0.39 | 3.2 | 0.98 ± 0.58 | 4.4 | 0.93 ± 0.80 | 4.8 |
| | F | 0.94 ± 0.34 | 4.1 | 0.68 ± 0.35 | 2.5 | 0.70 ± 0.42 | 2.7 | 0.66 ± 0.32 | 3.6 |
| hK14 | M | 0.31 ± 0.20 | 1.6 | 0.23 ± 0.21 | 1.0 | 0.24 ± 0.15 | 1.1 | 0.18 ± 0.16 | 0.9 ** |
| | F | 0.22 ± 0.14 | 1.0 | 0.18 ± 0.15 | 0.7 | 0.22 ± 0.086 | 0.8 | 0.12 ± 0.083 | 0.6 ** |
| hK6 | M | 0.31 ± 0.054 | 1.6 | 0.14 ± 0.075 | 0.6 | 0.16 ± 0.096 | 0.7 | 0.13 ± 0.088 | 0.7 ** |
| | F | 0.26 ± 0.048 | 1.1 | 0.10 ± 0.032 | 0.4 | 0.11 ± 0.058 | 0.4 | 0.087 ± 0.048 | 0.5 ** |
| hK13 | M | 0.033 ± 0.021 | 0.2 | 0.054 ± 0.044 | 0.2 | 0.033 ± 0.046 | 0.1 | 0.045 ± 0.038 | 0.2 |
| | F | 0.063 ± 0.040 | 0.3 | 0.12 ± 0.072 | 0.4 | 0.046 ± 0.054 | 0.2 | 0.018 ± 0.013 | 0.1 |
| Total of trypsin-like hK | | | | | | | | | |
| | M | 18.9 ± 5.4 | | 21.9 ± 8.0 | | 22.4 ± 9.9 | | 19.4 ± 11.2 | |
| | F | 22.8 ± 11.4 | | 26.8 ± 7.2 | | 25.4 ± 6.8 | | 18.5 ± 5.8 | |
| Average | | 20.9 | | 24.3 | | 23.9 | | 18.9 | |

[a]The values indicate the mean ± SD (ng per mg dry weight). The percentage represents the concentration of each trypsin-like kallikrein compared with the total concentration of all trypsin-like kallikreins.

Two-way ANOVA showed significant differences among age groups for hK8 (*$p < 0.05$) and between genders for hK6 and hK14 (**$p < 0.05$). More details are described in "Results."

M, male; F, female; n, number of samples.

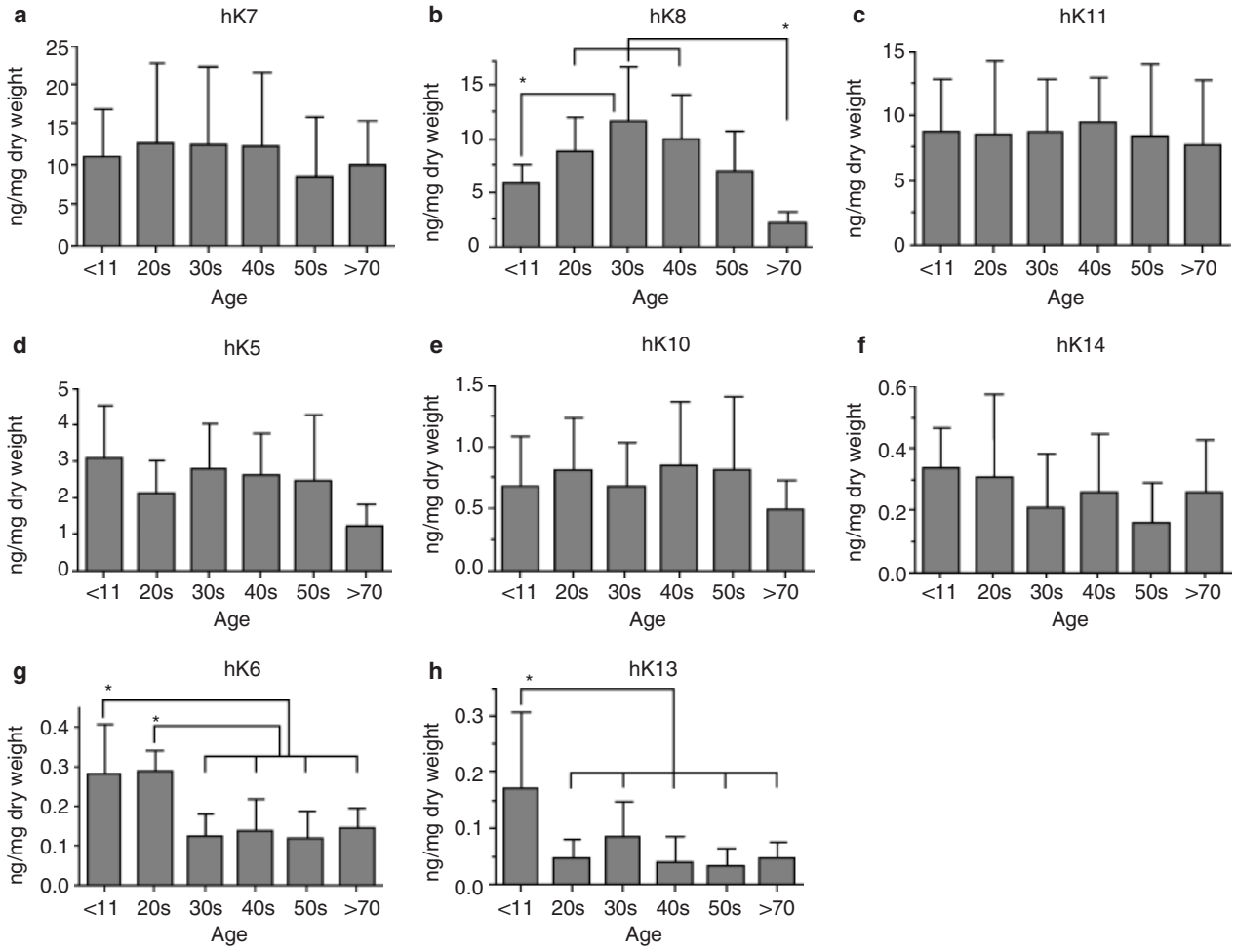


**Figure 1**
**Kallikrein quantification in the stratum corneum among age groups.** The Y-axes represent mean concentrations of each kallikrein, and the error bars indicate ± SD, for each age group, combining males and females. Lines and (*) indicate significant differences ($p < 0.05$) between the specified age groups. For discussion, see text.

**Table II. Experimental conditions for the immunofluorometric assays[a]**

| | hK4 | hK5 | hK6 | hK7 | hK8 | hK10 | hK11 | hK13 | hK14 |
|---|---|---|---|---|---|---|---|---|---|
| Sample dilution (fold) | None | 5 | None | 5 | 5 | None | 5 | None | None |
| Capture Ab | Mono | Mono | Mono | Mono | Mono | Mono | Mono | Mono | Mono |
| anti-hK detection Ab | Poly | Biot mono | Biot mono | Biot mono | Biot mono | Biot mono | Poly | Biot mono | Poly |
| Dilution (fold)[b] | 2,000 | 2,000 | 500 | 1,000 | 1,000 | 2,000 | 2,000 | 2,000 | 2,000 |
| Secondary detection | GARIg-ALP | SA-ALP | SA-ALP | SA-ALP | SA-ALP | SA-ALP | GARIg-ALP | SA-ALP | GARIg-ALP |
| Dilution (fold)[b] | 3,000 | 20,000 | 20,000 | 20,000 | 20,000 | 20,000 | 3,000 | 20,000 | 3,000 |
| Diluted in | Assay buffer | BSA buffer | BSA buffer | BSA buffer | BSA buffer | BSA buffer | Assay buffer | BSA buffer | Assay buffer |
| Incubation time (min) | 45 | 15 | 15 | 15 | 15 | 15 | 45 | 15 | 45 |

[a]Ab, antibody; SA, streptavidin; ALP, alkaline phosphatase; BSA, bovine serum albumin; GARIg, goat anti-rabbit Ab; mono, monoclonal; poly, polyclonal; biot, biotinylated. All monoclonal antibodies were developed in mice and all polyclonal antibodies in rabbits. All capture antibodies were used at amounts of 500 ng per 100 μL per well except for the hK11 assay (250 ng per well). See also cited literature for more details on these ELISA assays.
[b]All stock antibodies or streptavidin solutions were 1 mg per mL.

As the hKs have been predicted to have either chymotrypsin-like (hK7) or trypsin-like activity (hK5, hK6, hK8, hK10, hK11, hK13, and hK14) (Yousef and Diamandis, 2001), they have also been subdivided according to this classification. In this study, "trypsin-like activity" refers to the activity of an enzyme towards a Boc-Phe-Ser-Arg-AMC (FSR-) substrate. "Chymotrypsin-like activity" refers to the activity of an enzyme towards a MeO-Suc-Arg-Pro-Tyr-pNA-HCl (RPY-) substrate.

The mean concentration of the chymotrypsin-like kallikrein hK7 ranged from 7.5 to 14 ng per mg dry weight, but no significant differences were observed among any of the age groups (Table I and Fig 1*a*). Among trypsin-like kallikreins, hK8 and hK11 were abundant (mean values of 6–14 ng per mg dry weight for each) in all age and gender groups, representing 80%–85% of the total amounts of trypsin-like kallikreins detected (Table I). hK8 was present at lower levels at the extremes of age (highest in the 30s), and the amount of hK8 was significantly different between groups $<11$ y and 30s ($p<0.01$), and between $>70$ y and 20s, 30s, and 40s ($p<0.01$ for each comparison) (Table I and Fig 1*b*). In contrast, hK11 did not differ between subjects stratified by age or gender (Table I and Fig 1*c*). The mean concentrations of hK5 (2.0–4.0 ng per mg), representing 10%–17% of total trypsin-like kallikreins, and hK10 (0.65–1.0 ng per mg), representing 2.5%–5.0% of total trypsin-like kallikreins, did not differ significantly among age and gender groups (Table I and Fig 1*d* and *e*). hK14 and hK6 levels in females were significantly lower than in males in all age groups ($p<0.01$) (Table I), and their quantity was 0.1–0.3 ng per mg (0.4%–1.6% of total trypsin-like kallikreins) for each. In addition, the level of hK6 was lower at ages over 30 y ($p<0.01$ for each comparison) (Fig 1*g*). Although hK13 was detectable in all cases, its levels were consistently very low in all samples, and represented only 0.02–0.2 ng per mg, corresponding to 0.1%–0.3% of total trypsin-like kallikreins (Table I and Fig 1*h*). hK13 levels in subjects $<11$ y were significantly higher than in other age groups ($p<0.01$ for each comparison) (Fig 1*h*).

**Total SC trypsin- and chymotrypsin-like kallikrein concentration among age groups** The overall concentrations of chymotrypsin-like kallikreins (i.e., hK7) and trypsin-like kallikreins (i.e., the sum of the concentrations of all other kallikreins) are shown in Fig 2. The amount of the chymotrypsin-like kallikrein (hK7) did not differ significantly across age groups (as also shown in Fig 1*a*). However, the total concentration of trypsin-like kallikreins was higher in the 30s and 40s age groups compared with subjects $>70$ y ($p<0.05$). On the other hand, the total concentration of chymotrypsin-like plus trypsin-like hK did not differ among groups. The total concentration of trypsin-like kallikreins was approximately 2-fold higher than the chymotrypsin-like kallikrein.

**SC trypsin-like enzymatic activity among age groups** The SC trypsin-like enzymatic activity was measured in normal skin samples from each individual (Fig 3). When the SC samples were boiled for 5 min, the activity was completely lost (data not shown). The activities displayed no significant differences among the 20s to 50s age and gender groups (data not shown). Figure 3*a* displays the activity among age groups in which male and female values are combined and includes subjects $<11$ and $>70$ y. Subjects $<11$ y display the highest trypsin-like activity at any time point. According to a trypsin calibration curve, the trypsin-like activity per milligram of SC was comparable with $\sim 1$ ng of trypsin (data not shown). Although the trypsin-like activity among subjects $>70$ y of age was slightly lower than any other age group, statistically significant differences were not observed, except for subjects $<11$ y.

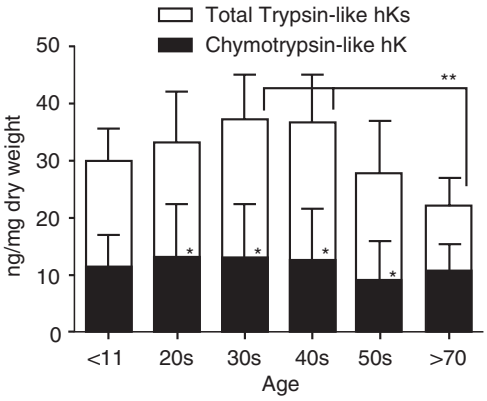


**Figure 2**
**Differences in the total concentrations of trypsin-like and chymotrypsin-like kallikreins in the stratum corneum among age groups.** The solid bars and open bars indicate the concentrations of chymotrypsin-like kallikrein (hK7) and trypsin-like kallikreins (hK5, hK6, hK8, hK10, hK11, hK13, and hK14) (mean $\pm$ SD), respectively. The error bars indicate the standard deviation of trypsin- or chymotrypsin-like kallikrein in each age group, females and males combined. *Significant differences ($p<0.05$) between the total concentration of trypsin-like kallikreins and of chymotrypsin-like kallikrein in the specified age groups. Lines and **significant differences ($p<0.05$) in the total concentration of trypsin-like kallikreins between the specified age groups.

In Fig 3*b*, the correlation between the total concentrations of trypsin-like kallikreins and the trypsin-like activities in the samples is shown. The total concentrations of trypsin-like kallikreins and trypsin-like activity were weakly correlated ($r=0.40$, $p<0.01$). As the number of samples in the $<11$ and $>70$ y age groups were small, and subjects $<11$ y had significantly elevated trypsin-like activity (Fig 3*a*), these points were excluded from the regression analysis.

**SC chymotrypsin-like enzymatic activity among age groups** As shown in Fig 4, the SC chymotrypsin-like enzymatic activity in normal skin samples was also measured in a subset of subjects ($n=6$ for each age group). In this assay, measurements were taken at 2 and 4 h. When the SC samples were boiled for 5 min, the activity was completely lost (data not shown). The pNA release was insufficient to generate standard chymotrypsin curves. Nonetheless, when the samples were categorized by age groups, the chymotrypsin-like activity for all groups increased in a time-dependent manner with no significant differences at any time point (Fig 4*a*).

The correlation between the concentration of the chymotrypsin-like kallikrein (i.e., hK7) and the chymotrypsin-like activity is shown in Fig 4*b*. The concentration and activity were moderately correlated ($r=0.62$, $p<0.01$). Another chymotrypsin substrate with phenylalanine in the P1 position was inactive against the SC samples (data not shown).

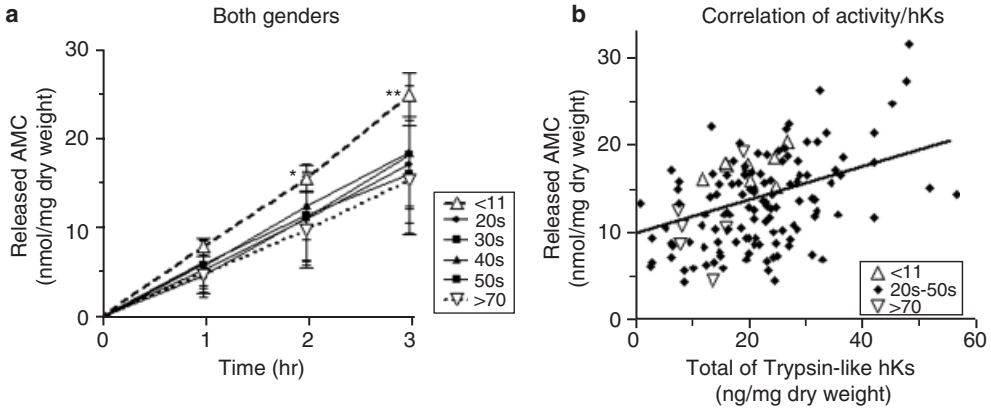


**Figure 3**
**The stratum corneum (SC) trypsin-like enzymatic activity among age groups.** The SC trypsin-like activities (mean ± SD) represent released AMC from the synthetic substrate. (*a*) Activity among age groups when male and female subjects are combined, plus subjects <11 and >70 y. *Significant difference ($p < 0.05$) between <11 and >70 y age groups at 2 h and **significant differences ($p < 0.05$) between <11 y and all the other age groups at 3 h; (*b*) correlation between the total concentrations of trypsin-like kallikreins measured by ELISA and trypsin-like enzymatic activities. Data from 20s to 50s age groups inclusive were used for the regression analysis. Although the data points are shown (*triangles*), the <11 and >70 y age groups were small and subjects <11 y had significantly elevated trypsin-like activity; for these reasons, these points were excluded from the regression analysis. The equation of the regression line is $y = 0.20x + 9.0$.

## Discussion

This study aimed to quantitatively measure human tissue kallikreins, hK5, hK6, hK7, hK8, hK10, hK11, hK13, and hK14, as well as the total trypsin-like and chymotrypsin-like activities in the normal SC. The data were further compared between genders and various age groups.

Despite their similarities, kallikreins were detected at varying amounts in different organs and displayed different types of enzymatic activity, e.g. hK3, hK7, and hK9 have chymotrypsin-like activity, whereas the rest possess trypsin-like activity (Yousef and Diamandis, 2001). KLK3 and KLK9 mRNA have not been conclusively shown to be expressed in the skin (Gan *et al*, 2000; Harvey *et al*, 2000; Komatsu *et al*, 2003). hK7 is likely the major chymotrypsin-like hK in the SC; this is in accord with our data showing consistently high hK7 levels in the SC across all age groups and a significant correlation between hK7 immunoreactivity by ELISA and chymotrypsin-like activity in the SC (Fig 4*b*). In addition, we observed strong similarities between hK7's activity toward the RPY substrate (Franzke *et al*, 1996) and the overall SC chymotrypsin-like activity profile, which further suggests that hK7 is largely responsible for this activity.

Our immunofluorometric ELISA assays confirmed that at least seven trypsin-like hK are detectable in the normal SC: hK5, hK6, hK8, hK10, hK11, hK13, and hK14. The concentrations of trypsin-like kallikreins range widely; the concentration difference between the least (hK13) and the most abundant (hK8 or hK11) kallikreins is approximately 200-fold. The total concentration of trypsin-like hK is about 2-fold greater than that of chymotrypsin-like hK. The total concentration of trypsin-like kallikreins showed significant differences across age groups (decreasing with age), whereas the chymotrypsin-like kallikrein (i.e., hK7) was present at more uniform levels (Fig 2). Therefore, the SC

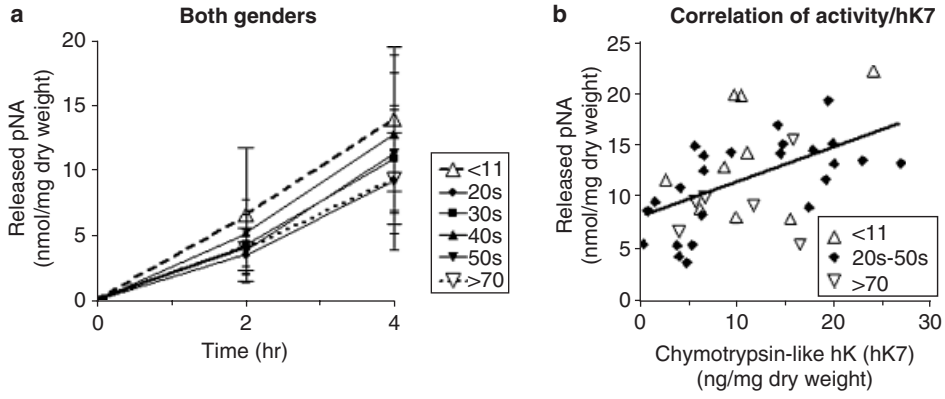


**Figure 4**
**The stratum corneum (SC) chymotrypsin-like enzymatic activity among age groups.** The SC chymotrypsin-like enzymatic activities (mean ± SD) represent released pNA from the synthetic substrate. (*a*) Subjects are divided into groups by age. The activity among age groups in which male and female values are combined and include subjects <11 and >70 y; (*b*) correlation between the total concentration of chymotrypsin-like kallikreins measured by ELISA and the chymotrypsin-like enzymatic activities. Data from the 20s to 50s age groups inclusive were used for the regression analysis. Although the data points are shown (*triangles*), the <11 and >70 y age groups were excluded from the regression analysis (see also legend of Fig 3). The equation of the regression line is $y = 0.34x + 7.6$.

trypsin-like activity may play a more significant role with regard to skin aging than the SC chymotrypsin-like activity.

We report for the first time that hK8 and hK11 are the most abundant trypsin-like skin kallikreins; consistent with the high expression of KLK11 mRNA previously detected in skin (Komatsu *et al*, 2003), hK11 protein levels were relatively high, regardless of age or gender. hK11 could therefore be an important trypsin-like kallikrein in the SC. hK8 levels were significantly different among age groups (Fig 1*b*), and this variation contributes to the changes seen with age with respect to total trypsin-like kallikrein levels. Our results and those from another report describing that *KLK8*-deficient mice showed a reduced rate of recovery of the epidermis and corneocytes after ultraviolet B irradiation suggest that hK8 might be involved in skin differentiation (Kirihara *et al*, 2003). However, a previous RT-PCR study amplified only KLK8 mRNA splice variants that are predicted to code for non-functional hK8 (Komatsu *et al*, 2003). Further work is necessary to elucidate whether the hK8 detected in this study is, in fact, a functional enzyme.

hK5, another trypsin-like kallikrein, was originally identified in skin (Brattsand and Egelrud, 1999). We show here that hK5 is the third most abundant hK in the SC, after hK8 and hK11. hK5 accounts for 10%–17% of total trypsin-like kallikreins and is one of the major trypsin-like kallikreins in the SC.

hK10, hK14, hK6, and hK13 were found at relatively low levels in the SC, together totalling less than 10% of the SC trypsin-like kallikreins. hK14 and hK6 levels were significantly lower in females of the 20s to 50s age groups, inclusive *KLK14* (Yousef *et al*, 2003a), *KLK6* (Yousef *et al*, 1999), and other kallikreins are upregulated by steroid hormones (Yousef and Diamandis, 2001). Therefore, expression of kallikreins could be steroid hormone dependent in the SC. The amount of hK6 in subjects $<11$ y and in their 20s, and hK13 in subjects $<11$ y suggests that these kallikreins might be associated with SC differentiation at early ages. Although KLK4 mRNA was detected in skin tissue (Komatsu *et al*, 2003), our immunofluorometric assay did not detect hK4 in a large proportion of specimens. Further studies are necessary to examine hK4 presence in skin. Even though levels of hK6, hK10, hK13, and hK14 were low in the normal SC, these kallikreins could provide diversity to the SC serine protease activity and could target as yet unidentified specific substrates.

The number of SC layers is known to be unaffected by aging (Ya-Xian *et al*, 1999), and this study demonstrated that the overall SC trypsin- and chymotrypsin-like activities were largely similar in subjects in their 20s to $>70$ y of age. As increases and decreases in the SC protease activity seen in Netherton syndrome (Komatsu *et al*, 2002) and ichthyosis vulgaris (Suzuki *et al*, 1996), respectively, may cause aberrant desquamation, maintenance of a stable SC serine protease activity may be essential for retaining a stable number of SC layers and proper skin barrier function.

As hK7 exists in the SC as a mixture of the pro-form and the active form (Ekholm and Egelrud, 1999), trypsin-like kallikreins may also be present in both pro- and active forms.

It is likely, as previously suggested (Caubet *et al*, 2004; Yousef and Diamandis, 2002), that pro-forms of some kallikreins are activated by other kallikreins, thus creating a cascade reaction. This possibility merits further investigation.

Subjects under 11 y old always had the highest trypsin- and chymotrypsin-like activities, whereas the total concentration of trypsin- and chymotrypsin-like kallikreins was lower than those of the 20s to 50s age groups. This implies that younger subjects might possess a higher proportion of hK in the active form, or their SC contains as yet unidentified proteases.

Recent studies demonstrated the lamellar granular localization of hK5, hK7, and hK8, and the secretion of granules into intercellular spaces (Ishida-Yamamoto *et al*, 2004, 2005) and the degradation of desmosomes and/or corneodesmosomes by hK5 and hK7 (Simon *et al*, 2001; Caubet *et al*, 2004). These data strongly implicate these two kallikreins in desquamation. In addition, kallikreins tend to be detected in more highly differentiated cells, i.e., the stratum granurosum, SC, and appendages, which implies that kallikreins might also be related to skin maturation and differentiation (Ekholm *et al*, 2000, Komatsu *et al*, 2005). Our study detected the kallikreins in the superficial SC. Kallikreins are likely present at higher concentrations in the deeper SC, and could be present in all SC layers as reactive proteases against external stimuli. Following our original suggestion for kallikrein involvement in a proteolytic cascade pathway (Yousef and Diamandis, 2002), others proposed similar pathways in the skin for kallikreins hK5, hK7, and hK14 (Brattsand *et al*, 2005). Our data suggest that this pathway is likely more complex and involves at least eight kallikreins (hK5, hK6, hK7, hK8, hK10, hK11, hK13, and hK14).

In summary, we report for the first time the quantification of numerous kallikreins in normal SC. The hK family is an important protease group within SC, with a large diversity in both quantity and activity. The data presented here may aid in the better understanding of skin barrier function and aging, especially in conjunction with desquamation. It will be of interest to quantitatively compare kallikrein levels and activity of normal and diseased skin.

## Materials and Methods

**Skin samples and preparation of extracts** The SC samples were obtained from the forearm of 135 normal volunteers (15 females and 15 males) in each of four groups with ages 20–29, 30–39, 40–49, and 50–59 y, respectively, nine (four females and five males) with ages less than 11 y, and six (five females and one male) with ages over 70 y). Informed consent was obtained from all participants and our study was conducted according to the Declaration of Helsinki. The medical ethical committee of the Graduate School of Medical Science, School of Medicine, Kanazawa University approved all described studies. SC was obtained by stripping using Nichiban tape (organic solvent-stable tape with organic solvent-soluble adhesive, Nichiban, Tokyo, Japan). For optimization purposes, samples collected from several subjects using various tape brands were weighed and tested with an hK6 ELISA. Nichiban tape yielded the highest levels of hK6 among all samples tested, which indicated the lowest contamination of adhesive material after purification. The tape stripping was performed as follows: subjects took a shower the night before and used no topical agents

afterwards. The following afternoon, the tape was applied to the skin surface of the subjects' forearm and upper arm. As the number of SC layers in the forearm is known to be consistent regardless of age (Ya-Xian *et al*, 1999), we performed the stripping at different places until around 2 mg of SC had been visibly obtained. With this procedure, we assumed that a similar number of SC layers had been removed in each case. The SC samples on the tape were immediately stored at $-20^{\circ}$C until toluene treatment was performed. When the tape was dipped in 10 mL of toluene, all adhesive was dissolved and any attached SC was suspended. After the insoluble tape backing was removed, the sample was centrifuged at $1700 \times g$ for 15 min. The precipitate was washed with 5 mL of toluene six times in order to remove any residual adhesive. The amount of SC obtained was typically around 2 mg regardless of subjects' age and gender. After toluene treatment, the purified samples were air dried, weighed, and kept at $-20^{\circ}$C until immunofluorometric assays and enzymatic activity measurements could be performed. As the stripping was less well tolerated by children and seniors, the sample numbers for these subjects were limited and data were not classified by gender.

For the immunofluorometric assays, 0.5 mg dry weight of the SC samples were mixed with 20 μL of *N,N*-dimethylformamide, 480 μL of 0.1% Triton X-100, 350 μL of 0.2 M Tris-HCl buffer (pH 8.0), and 100 μL of $H_2O$. The mixtures were incubated at 37°C for 1 h on a shaker. Shorter or longer incubation times can cause lower extraction efficiencies or degradation of hK, respectively. After incubation, samples were then centrifuged at $1700 \times g$ for 10 min and the supernatants retrieved. Fifty microliter aliquots were used in each immunofluorometric assay for hK4, 6, 10, 13, and 14. For hK5, 7, 8, and 11 assays, 10 μL aliquots were diluted with 40 μL of the same mix solution, as described above. To test our extraction efficiency, the SC samples were incubated in the extraction solution for 1 h and then centrifuged. The supernatants were collected as the "first extraction." Next, the insoluble SC material was re-extracted as above. The supernatant from the second extraction was then collected after centrifugation. The first and second extracts were assayed for kallikreins by ELISA and the results compared. The ratio of major hK in the first *versus* second extract was approximately 40:1 (97.5%). In the case of the minor hK, the concentration became undetectable in the second extract.

**Immunofluorometric assays for human tissue kallikreins** With the exception of mouse monoclonal FB6MA53 anti-hK11 antibody (Ab), which was purchased (Fuso, Osaka, Japan); all other monoclonal and polyclonal anti-kallikrein Ab described in Table II were developed in our laboratory. All ELISA assays displayed negligible cross-reactivity with other kallikreins (data not shown).

Sandwich-type, non-competitive immunoassays (Dickson *et al*, 1995; Ferguson *et al*, 1996) were generally performed as follows (more details are shown in Table II). White polystyrene microtiter plates were coated with 100 μL per well (250 or 500 ng) of coating Ab solution (50 mM Tris, pH 7.8) and incubated overnight at room temperature. On the following day, the plates were washed three times with the washing buffer (9 g per liter NaCl and 0.5 g per liter Tween 20 in 10 mM Tris buffer, pH 7.4). Fifty microliters of assay calibrators and undiluted or diluted samples were then pipetted into each well, in duplicate, along with 50 μL of assay buffer (60 g per liter bovine serum albumin (BSA), 50 mM Tris (pH 7.8), 0.5 g per liter $NaN_3$, 0.5 M KCl, 100 mg per liter goat IgG (Sigma, St Louis, Missouri, USA), 20 mg per liter mouse IgG (Fortron, Morrisville, North Carolina, USA), 1.0 g per liter bovine IgG (Sigma), and 0.5% Tween 20, and incubated for 2 h with shaking at room temperature. The plates were then washed with washing buffer six times. Subsequently, 100 μL of the diluted anti-hK detection Ab (monoclonals were biotinylated) in assay buffer were applied to each well and incubated for 1 h with shaking at room temperature. The plates were then washed six times. Finally, 100 μL of alkaline phosphatase (ALP)-conjugated goat anti-rabbit (H + L fragment-specific) IgG antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) or streptavidin (SA)–ALP conjugate (Jackson ImmunoResearch), diluted in assay buffer or BSA (60 g per liter BSA, 50 mM Tris, pH 7.8, 0.5 g per liter $NaN_3$) were added to each well and incubated for 45 or 15 min, respectively. The plates were then washed three times as described above. One hundred microliters of 1 mM diflunisal phosphate in substrate buffer (0.1 M Tris (pH 9.1), 0.1 M NaCl, and 1 mM $MgCl_2$) were added to each well and incubated for 10 min. Finally, 100 μL of a developing solution containing 1 M Tris base, 0.4 M NaOH, 2 mM Terbium (III) chloride ($TbCl_3$), and 3 mM EDTA was pipetted into each well and mixed for 1 min. The fluorescence was then measured with the Cyberfluor 615 time-resolved fluorometer (MDS Nordion, Kanata, ON, Canada). The calibration and data reduction were performed automatically as described elsewhere (Dickson *et al*, 1995; Ferguson *et al*, 1996).

Further details for each kallikrein ELISA immunoassay have been published for hK4 (Obiezu *et al*, 2002), hK5 (Yousef *et al*, 2003b), hK6 (Diamandis *et al*, 2000), hK7 (Kishi *et al*, 2004), hK8 (Kishi *et al*, 2003), hK10 (Luo *et al*, 2001), hK11 (Diamandis *et al*, 2002), hK13 (Kapadia *et al*, 2003), and hK14 (Borgono *et al*, 2003).

**Assay of trypsin- and chymotrypsin-like enzymatic activities** The synthetic peptide substrate Boc-Phe-Ser-Arg-AMC (7-amino-4-methyl-coumarin) (Peptide Institute, Osaka, Japan) was used for the assay of trypsin-like activity, and MeO-Suc-Arg-Pro-Tyr-pNA-HCl (3-carbomethoxypropionyl-L-arginyl-L-prolyl-L-Tyrosine-*p*-nitroaniline hydrochloride) (Chromogenix, Milano, Italy) was used for the assay of chymotrypsin-like activity. The reaction mixtures consisted of 0.5 mg dry weight of SC, 10 μL of *N,N*-dimethylformamide, 240 μL of 0.1% Triton X-100, 175 μL of 0.2 M Tris-HCl buffer (pH 8.0), and 50 μL of either 1 mM trypsin-like activity substrate or 4 mM of chymotrypsin-like activity substrate (Komatsu *et al*, 2002). The mixtures were incubated at 37°C for 1–4 h with shaking. Released AMC was measured using a fluorescence spectrophotometer (FluoroScan Ascent FL, Labsystems, Helsinki, Finland; excitation/emission = 355/460 nm), calibrated using reference standard AMC (Molecular Probes, Eugene, Oregon). Porcine trypsin type II (Trypsin tablets, Sigma; molecular weight 23.8 kDa) was used as a positive control. Released pNA by chymotrypsin-like activity was measured spectrophotometrically at 405 nm (Wallac Victor$^2$ 1420 Multilabel counter, Perkin Elmer, Boston, Massachusetts). All measurements were performed in triplicate.

**Statistical analysis** Differences in either the concentrations of each kallikrein or enzyme activities between groups of subjects were determined by either two-way or one-way ANOVA, respectively. The reported p-values were adjusted by the Bonferroni method to reflect multiple comparisons. These tests were performed using GraphPad Prism 4, version 4.02 software (GraphPad Software Inc.).

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