Preventing Enzymatic Damage to Hides by Timely Inhibition of Trypsin Activity with Soybean Flour during Bating Process

by

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Abstract

Enzymatic bating is important for producing clean, soft, and high-yield leather. However, it is prone to cause damage to hide collagen fibers and result in loose grain or damaged grain surface due to the longer enzymatic hydrolysis time of the grain layer than the middle layer caused by the slow mass transfer and the rapid hydrolysis reaction of the bating agent in hide. Considering trypsin is the most common bating agent, soybean flour (SF) that contains environmental friendly Kunitz trypsin inhibitor (KTI) was used after delimed hides were treated with trypsin for a certain time to opportunely inhibit its activity in the grain and avoid bating defects. The fluorescence micrographs of hides bated with fluorescently labelled trypsin and KTI showed that KTI could cover only the surface layers when it was added after bating for a certain time, whereas trypsin could penetrate the hide completely. When sufficient SF was added to the bating float at 2 h, the damage to hide collagen caused by trypsin was effectively reduced. In particular, the grain surface remained intact after bating for 8 h. The decrease in the strength of leather caused by bating was also reduced. In addition, the usage of SF scarcely affected the fiber dispersion of the middle layer, thereby ensuring the softness of leather. In summary, opportunely inhibiting the activity of trypsin in the grain could prevent hide damage and improve the quality of leather.

Introduction

Enzymatic bating is an indispensable process for leather making because it can remove non-collagenous proteins from hide and disperse hide collagen fibers, which is important for producing clean, soft, and high-yield leather.[1-4] As known, if the bating time is too short, hide collagen fibers cannot be well dispersed (viz. inadequate bating). However, if the bating time is too long, the hide grain may be damaged, thereby resulting in loose grain and even damaged surface (viz. overbating or bate runs). Extreme caution is required for tanners throughout the bating process to prevent enzymatic damage to hides, such as loose grain and damaged surface.[5,6] Unfortunately, the bating endpoint is difficult to be assessed because it unavoidably depends on subjective judgment. Therefore, an efficient method that can make the bating easy to operate and especially avoid enzymatic damage to hides is crucial for high-quality leathers and the economic benefit of tannery.

Identifying why enzymatic bating is prone to damage hide is vital for developing an easy bating method to prevent damage. The slow mass transfer and the rapid hydrolysis reaction of bating agent in the bating process are known to be the main reasons.[5] Trypsin is the most common bating agent.[2] It has high catalytic activity under a weak alkaline condition (e.g., bating pH 8–9);[7] a molecular weight of 23.3 kDa, which is much higher than those of other leather chemicals; and an isoelectric point of approximately 10.5.[8,9] The high molecular weight and the alkaline isoelectric point make trypsin penetrate the hide slowly and prone to hydrolyze the grain layer stronger than the middle layer of hide during the bating process (Figure 1).

Properly controlling the catalytic activity of trypsin and accelerating the penetration of trypsin into the hide have been proven to be effective in alleviating the problem caused by the stronger enzymatic hydrolysis of the grain.[5,10,11] For example, Wang et al. proposed an interesting method by using Kunitz trypsin inhibitor (KTI, isoelectric point of 4.5[12]) at the beginning of the bating process to reduce the hydrolysis time of the hide surface by trypsin and accelerate the penetration of trypsin into the hide.[11] KTI, which was first crystallized from soybean by Kunitz in 1945,[13] is a common and environmental friendly competitive inhibitor for trypsin.[14] KTI can prevent protein substrates from combining with trypsin through binding itself to the active site of trypsin.[15] Wang et al. found that when the mass ratio of trypsin to KTI was 5:2 (1:0.4), the trypsin activity was less than 37% within 5 min but it returned to more than 88% after 100 min. Inspired by this phenomenon, they used a trypsin–KTI complex to bate hide, prevented trypsin from rapidly hydrolyzing the hide surface, and reduced the grain damage.[11] However, inhibiting the trypsin activity with KTI at the beginning of bating results in a weak bating performance, because although the trypsin activity could be mostly restored after a certain bating time, some trypsin molecules already lost their activities in the bating float over time. Therefore, continuing to develop preventive methods to avoid bating defects is necessary.

As known, bating defects are mainly caused by the longer enzymatic hydrolysis time of the grain layer than the middle layer. Hence, decreasing the hydrolysis time of the grain is undoubtedly able to prevent bating defects. If a sufficient inhibitor (e.g., KTI) is added

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after trypsin penetrates the hides completely, the activity of trypsin on the surface could be adequately inhibited. As a result, the excessive hydrolysis of the hide surface could be avoided, and the bating performance, especially the dispersion of collagen fibers in the middle layer, could not be affected (Figure 2).

Crops like soybeans that have a high yield and low cost contain the environmental friendly trypsin inhibitor KTI.[16,17] Therefore, in this study, soybean flour (SF) was used to opportunely inhibit the activity of trypsin in the grain and avoid bating defects. The SF amount for bating was first optimized by analyzing the inhibitory effects of KTI and SF on the trypsin activity. Subsequently, the mass transfer behaviors of trypsin and KTI in hides were observed to determine the adding time of SF. Finally, SF was used after bating with trypsin for 2 h, and the performance of bating system was analyzed according to the hide damage, the dispersion of hide collagen fibers, and the leather properties.

Experimental

Materials

Trypsin (the proteolytic activity at pH 8.5 and 32°C was 270,000 U/g; trypsin activity is defined as how much tyrosine is released per minute by trypsin under given conditions)[11] and KTI were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Soybean was ground into SF by using a blender (7011HS, Waring Commercial, USA). Fluorescein isothiocyanate-labeled trypsin (FITC-trypsin) was prepared as described in the authors' previous study.[19] Rhodamine B isothiocyanate-labeled KTI (RBITC-KTI) was prepared using the same method for preparing FITC-trypsin, except that FITC and trypsin were replaced with RBITC and KTI, respectively (Figure 3). Delimed cattle hides (pH 8–9) were prepared by deliming conventional limed hides (2.2 mm in thickness) with 3.0% ammonium sulfate and 100% water at 32°C for 90 min and used for bating trials. Analytical-grade chemicals

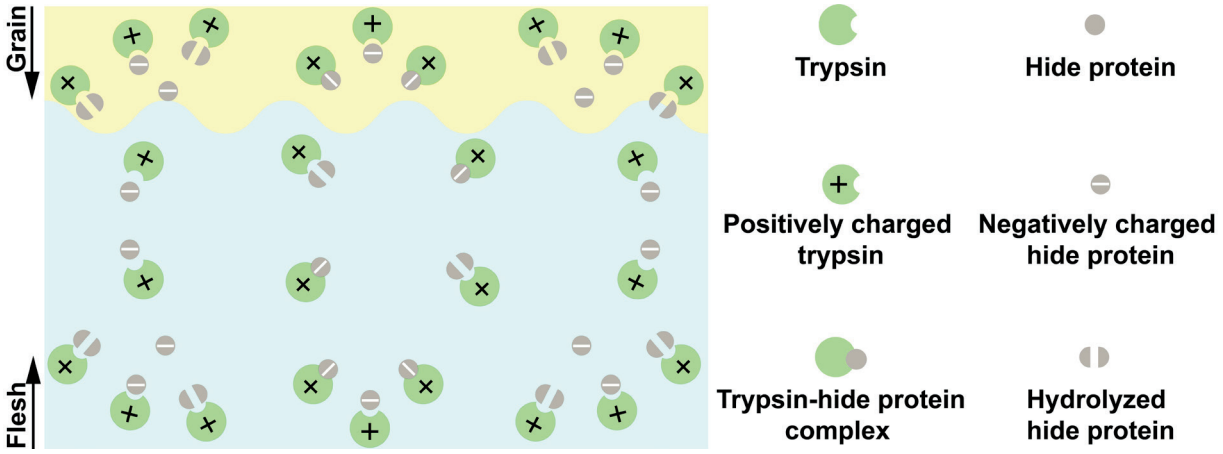

Figure 1. Schematic of traditional bating with trypsin. Hide protein (isoelectric point of 6–7[18]) is negatively charged, and trypsin (isoelectric point of 10.5[8,9]) is positively charged at the bating pH of 8–9.

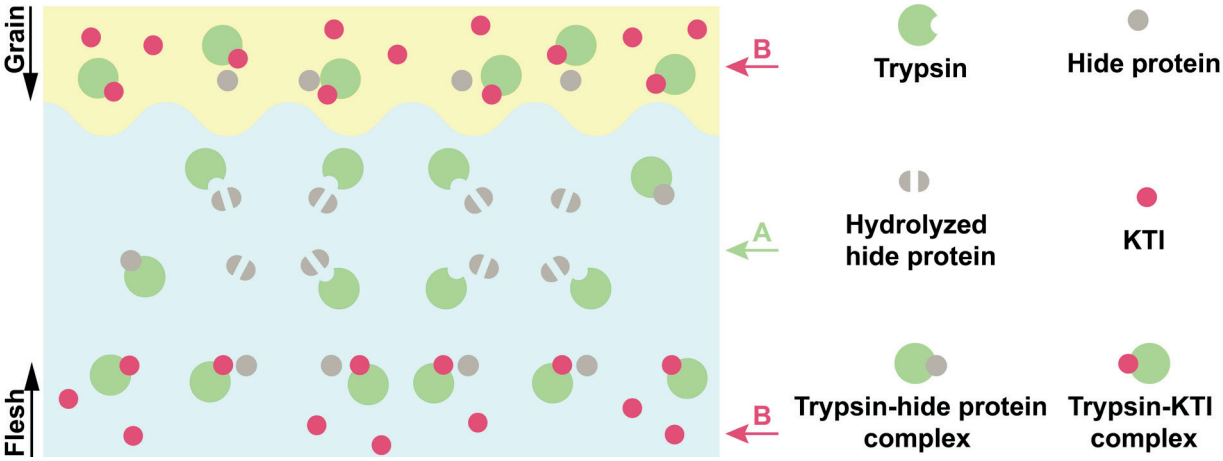

Figure 2. Schematic of trypsin activity inhibition on hide surface with KTI after trypsin penetrates hide in bating

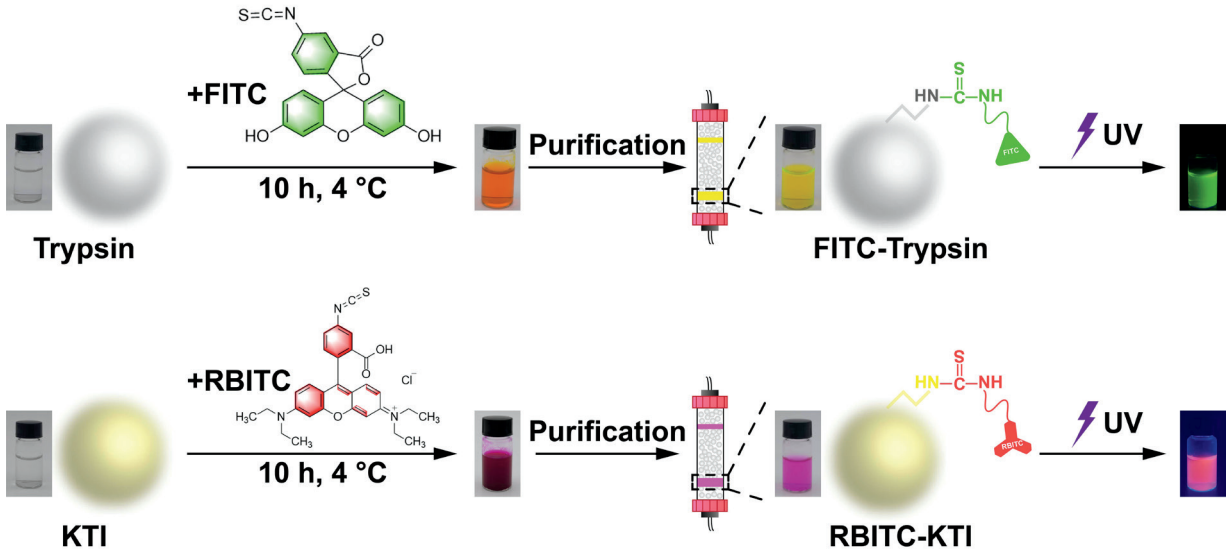

Figure 3. Schematic of the preparation of FITC-trypsin and RBITC-KTI.

were used for analyses, and commercial-grade chemicals were used for making leather.

Assay of trypsin activity in the presence of KTI and SF

The effects of KTI and SF on the trypsin activity were investigated by the method reported in our previous study with some modifications.[11] A trypsin, three trypsin–KTI complex (trypsin was incubated with KTI for 30 min), seven trypsin–SF mixture (trypsin was incubated with SF for 30 min) solutions (pH 8.5) were prepared. The concentration of trypsin in all the solutions was 0.05 g/L. The concentrations of KTI in the three trypsin–KTI complex solutions were 0.022, 0.043, and 0.086 g/L separately. The concentrations of SF in the seven trypsin–SF mixture solutions were 0.43, 0.86, 1.72, 2.58, 3.44, 5.16, and 6.88 g/L separately. Trypsin, trypsin–KTI complex, and trypsin–SF mixture solutions (1 mL each) were separately incubated with casein solution (1 mL, 20 g/L, pH 8.5) at 32°C for 10 and 120 min. After the incubation was performed, trichloroacetic acid (2 mL, 0.4 mol/L) was used to terminate the enzymatic reaction. The mixture was settled for 10 min and filtered. The filtrate (1 mL) was reacted with sodium carbonate (5 mL, 0.4 mol/L) and Folin-Ciocalteu's phenol reagent (1 mL). The color reaction was performed at 40°C for 20 min. The absorbance of the filtrate was measured at 680 nm with a microplate reader (Multiskan Go, Thermo Fisher Scientific, USA) after cooling to room temperature. A blank test was conducted by adding trichloroacetic acid before the casein solution was added. Equation (1) was used to calculate the relative proteolytic activities of the trypsin–KTI complex and trypsin–SF mixture.

$$\text{relative proteolytic activity} = \frac{A_1}{A_2} \times 100\% \quad (1)$$

where A_1 is the increment in the absorbance of the trypsin–KTI complex or trypsin–SF mixture solution compared with the blank, and A_2 is the increment in the absorbance of trypsin solution compared with the blank.

Analyses of trypsin and KTI penetrations during bating

The delimed hides were bated with 0.1% FITC-trypsin and 100% water at 32°C. After bating was performed for 2.0 h, 0.17% RBITC-KTI was added into the float. The above percentages were based on the weight of delimed hide. The hides were sampled after bating for 0.5, 1.0, 2.0, 3.0, 4.0, and 8.0 h, and sample sections were prepared on a freezing microtome (CM1950, Leica, Germany) at a thickness of 20 μm. The sections were observed using a fluorescence microscope (DMi8, Leica, Germany) with a FITC/RHOD filter cube for FITC-trypsin /RBITC-KTI localization. The fluorescence micrographs of the hides were analyzed by LAS X software to obtain brightness induced by FITC-trypsin/RBITC-KTI. The penetration rate of FITC-trypsin/RBITC-KTI in hide was calculated using Equation (2) as follows:

$$\text{penetration rate} = \frac{T_1}{T_2} \times 100\% \quad (2)$$

where T_1 is the thickness of hide with brightness (mm), and T_2 is the total thickness of hide (mm).

Bating trials by using trypsin and KTI/SF

Three groups of delimed hides (Nos.1–3) were bated with 0.1% trypsin and 100% water at 32°C for 2.0 h. KTI (0.17%) and SF (5.16%) were then added to the Nos.2 and 3 groups. The above percentages were based on the weight of limed hide. The three groups of hides were subsequently bated for another 6.0 h at 32°C. The bated hides (Nos.1 and 3) were then pickled, chrome tanned and post-tanned to obtain crust leathers (Figure 4).

After bating for 2.0, 3.0, 4.0, and 8.0 h, the bate-liquors and the hides were sampled. The hydroxyproline (Hyp) concentration of bate-liquor was measured to evaluate the damage to hide collagen. The grain surface of hide was observed using field-emission scanning electron microscopy (FESEM, Nova NanoSEM 450, FEI, USA). The

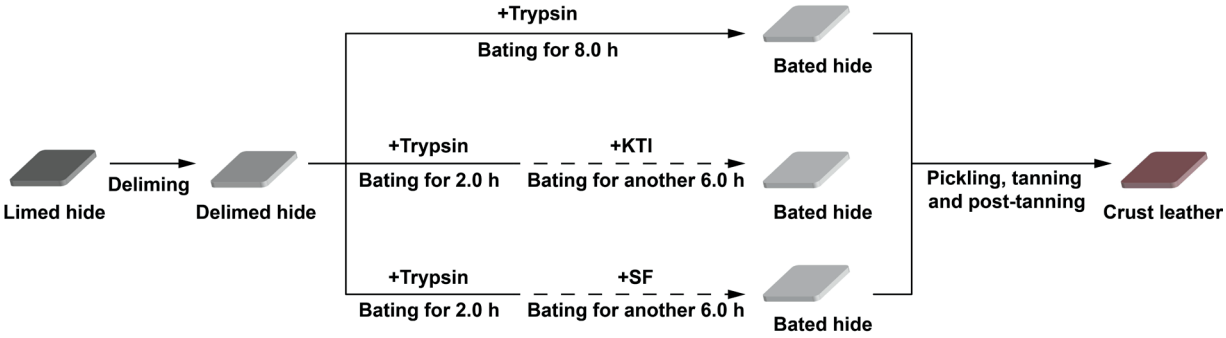

Figure 4. Schematic of bating trials.

hide sample was fixed in 10% neutrally buffered formalin for 48 h, and then the fixed sample was dehydrated, infiltrated, embedded in paraffin, and cut into 4 μm-thick sections by using a pathology slicer (RM2016, Leica, Germany). The sections were stained with hematoxylin and eosin (HE) to observe the dispersion of collagen fibers by using a brightfield microscope (DMi8, Leica, Germany).

The crust leathers were sampled, and their grain surfaces and middle layers were observed using a stereo microscope (M205C, Leica, Germany) and FESEM, respectively. Moreover, the physical properties, such as softness,[20] tensile strength,[21] tear strength[22] of the crust leathers were measured after conditioning at 20°C and 65% relative humidity for 24 h.[23]

Results and discussion

Effects of KTI and SF on trypsin activity

In this work, we aimed to achieve an opportune inhibition of trypsin on the hide surface to prevent excessive grain hydrolysis without weakening the bating performance. For this purpose, the inhibitory effects of KTI and SF on trypsin activity were first investigated. When the molar ratio of trypsin:KTI was 1:0.5, the relative trypsin activity was approximately 41% over 10 min and restored to more than 87% in 120 min; when the trypsin:KTI ratio was 1:1, the relative activity remained lower than 3% after reacting for 10 min and lower than 20% at 120 min; when the trypsin:KTI ratio increased to 1:2, the activity was still lower than 3% after reacting for 10 min and remained lower than 12% at 120 min [Figure 5(a)]. The results indicated that the trypsin:KTI molar ratio of 1:2 was sufficient to inhibit the trypsin activity.

In accordance with the content of KTI in SF, the effect of SF concentration from 0.43 g/L to 6.88 g/L on trypsin activity was studied to obtain the optimal SF amount for subsequent experiments. Figure 5(b) shows that the inhibitory effect of SF on trypsin activity increased with increasing SF concentration. When the concentration of SF was 2.58 g/L, the relative trypsin activity was lower than 5% after reacting for 10 min and returned to lower than 11% in 120 min. When the concentration of SF was 6.88 g/L, the relative activity was nearly 0% after reacting for 10 min and remained lower than 4% at 120 min. Considering the inhibitory effect and economic benefits, the SF concentration of 2.58 g/L was found to be more suitable. Therefore, the mass ratio of trypsin to SF of 1:51.6 was selected for the following bating trials.

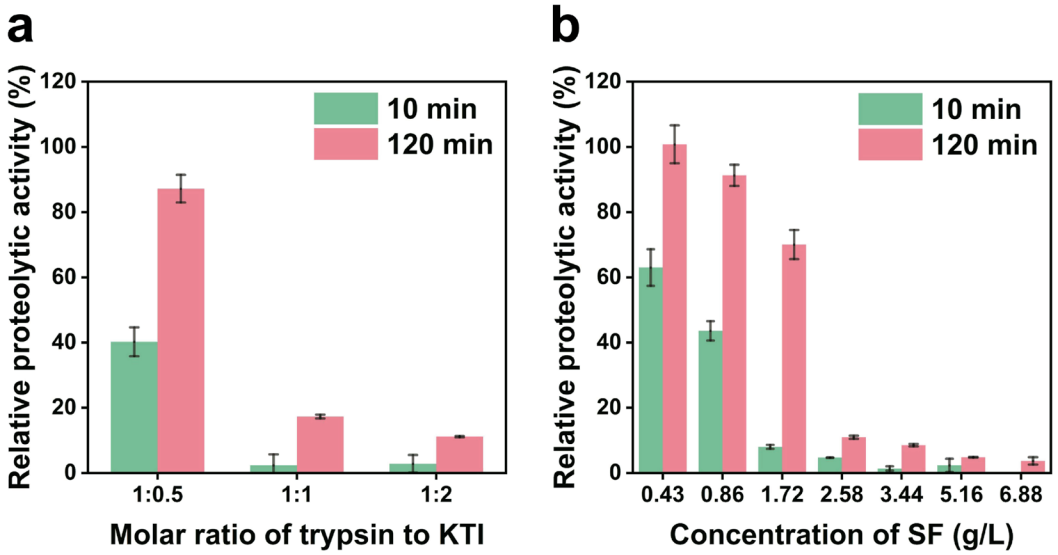

Figure 5. Effects of KTI (a) and SF (b) on trypsin activity.

Penetrations of trypsin and KTI in hide

The penetration behaviors of trypsin and KTI in hides are shown in Figure 6. The fluorescence micrographs in Figure 6(a) show that FITC-trypsin (green) gradually penetrated the hide over time. The penetration rate of FITC-trypsin was 54.88% after bating for 2.0 h [Figure 6(b)], indicating that considerable trypsin existed in the grain within 2.0 h. Hence, RBITC-KTI was added to the bating float at 2.0 h. The penetration rate of FITC-trypsin only increased to 59.86% after bating for 3.0 h, indicating that trypsin penetrated the hide slowly and hydrolyzed the grain for a longer time than the hide's middle layer during the bating process. RBITC-KTI (red) also gradually penetrated the hide over time. Interestingly, after bating for 4.0 h (viz. after adding KTI for 2.0 h), the penetration rates of FITC-trypsin and RBITC-KTI were 100% and 60.16%, respectively. The green and red fluorescence micrographs were merged. The merged image of hide bated for 4.0 h shows that the upper and lower surface layers are yellow due to green and red overlay, while the middle layer is green. The phenomenon indicated that RBITC-KTI was in the grain, whereas FITC-trypsin was in the whole hide. These results illustrated that adding KTI after bating for a certain time could make the inhibitor cover only the surface layers, thereby preventing grain damage without affecting the enzymatic hydrolysis of the hide's middle layer. After bating for 8.0 h, FITC-trypsin and RBITC-KTI completely penetrated the hide, implying that KTI may even inhibit the trypsin activity in the whole hide at the end of bating.

Effects of KTI and SF on hide damage

This research aimed to avoid enzymatic damage to hide by opportunely adding SF containing KTI to inhibit the trypsin activity. Therefore, the Hyp concentrations of bate-liquors and the morphology of bated grain surfaces were analyzed to evaluate the SF prevention effect. As shown in Figure 7(a), the Hyp concentration of bate-liquor increased over time, and the increase followed the order: control (trypsin) > KTI (trypsin + KTI) > SF (trypsin + SF). The results revealed that KTI and SF effectively reduced the damage to hide collagen caused by trypsin. In addition, the SF group had less collagen damage than the KTI group, which may be attributed to the fact that SF has other proteins besides KTI.[16] The proteins in SF could also be hydrolyzed by trypsin, thereby reducing the hydrolysis of hide protein and the damage to hide collagen. The SEM images of bated grain surfaces are shown in Figure 7(b). It is obvious that the hair pores were gradually damaged over time when only using trypsin for bating. Here, it should be mentioned that the trypsin activity used in this study was much higher than that usually used in tannery, so that the hide damage could inevitably occur in a short time. When the bated hides were treated with KTI and SF after bating for 2.0 h, the hair pores became more intact than those bated with only trypsin. In particular, the grain surfaces remained intact after bating for 8.0 h. These phenomena proved that SF greatly reduced the enzymatic damage to the grain because SF containing sufficient KTI effectively inhibited the activity of trypsin in the grain.

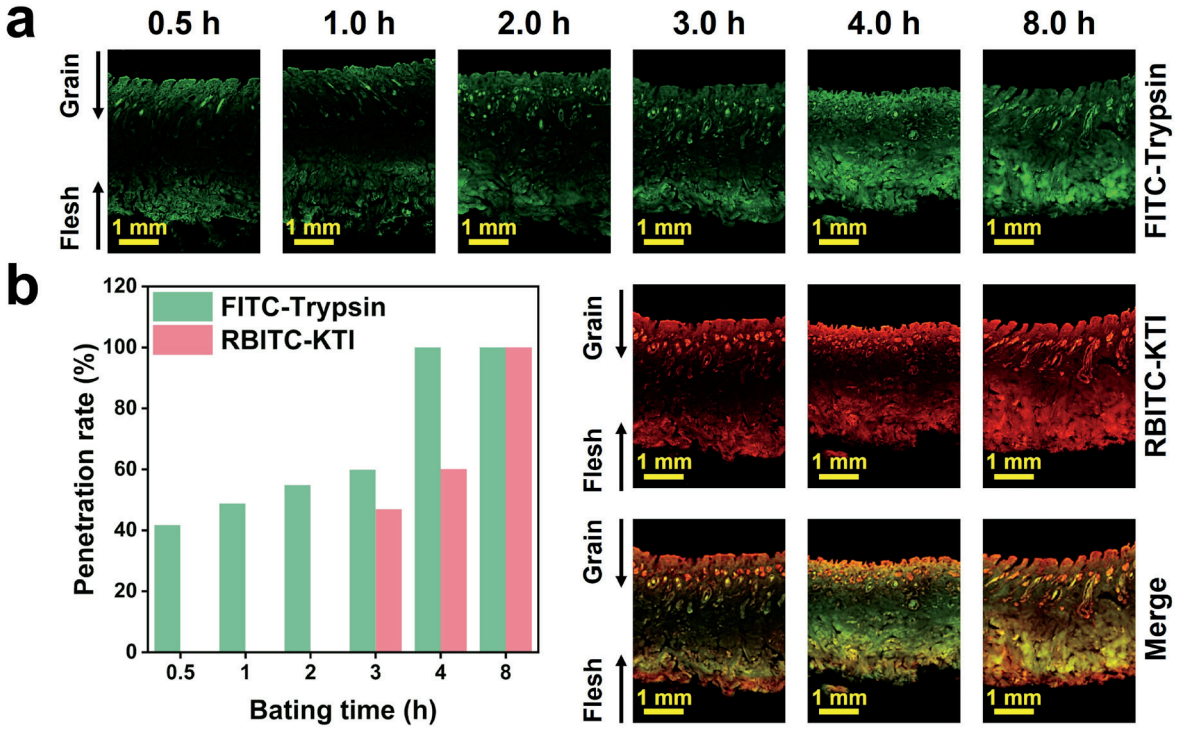

Figure 6. (a) Fluorescence micrographs of longitudinal sections from hides bated with FITC-trypsin and RBITC-KTI (scale bars, 1 mm) and (b) penetration rates of FITC-trypsin and RBITC-KTI in bated hides.

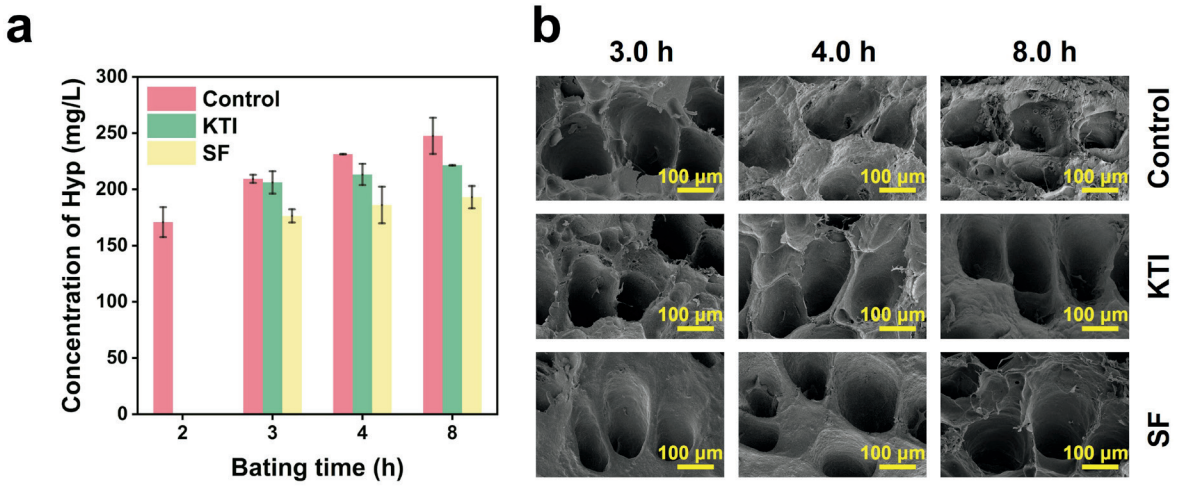

Figure 7. (a) Concentration of Hyp in bate-liquor and (b) SEM images of grain surfaces of bated hides.

Effects of KTI and SF on fiber dispersion

As mentioned earlier, an important purpose of adding SF after bating with trypsin for a certain time was to inhibit the activity of trypsin in the grain without weakening the bating performance. Therefore, in this section, the dispersion of collagen fibers in hide, especially in the middle layer, was analyzed to determine whether SF affected the bating performance. The collagen fibers were stained with HE and observed using a microscope. It was found that the collagen fibers in the middle layer were similar in the control, KTI, and SF bating groups (Figure 8). The results of fiber dispersion indicated that the method proposed in this study could ensure no attenuation of the bating performance.

Effect of SF on leather properties

The effect of SF on leather quality was further evaluated by comparing the surface morphology, the fiber dispersion, and the physical properties of the SF- and control crust leathers. The stereomicroscopic images of grain surfaces in Figure 9(a) showed that the hair pores were more intact and clearer in the SF group than in the control group (bating with only trypsin). The middle layers of the two leathers presented similar fiber dispersion degree, as shown in Figure 9(b). These results indicated that opportunely inhibiting the activity of trypsin in the grain protected the hide surface without weakening the bating performance.

The data in Table I showed that the softness of the two crust leathers increased as the bating time prolonged, and the increments in the softness of the control and SF groups were similar, which proved that opportunely inhibiting the activity of trypsin in the grain did not weaken the bating performance, including the fiber dispersion of the middle layer. In addition, the tensile and tear strengths of

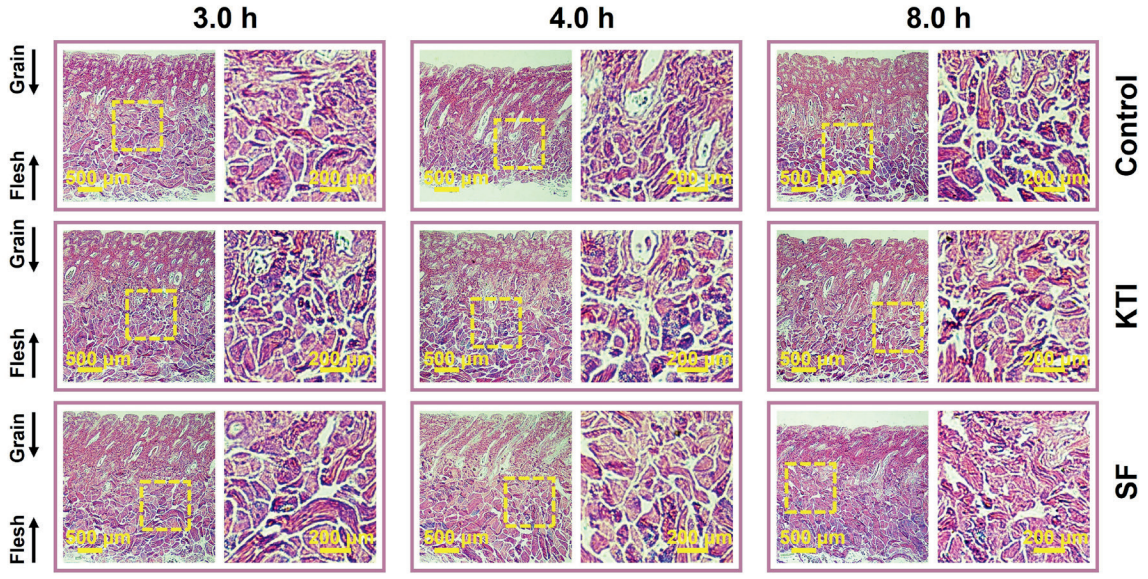

Figure 8. Microscopic images of HE-stained longitudinal sections of bated hides.

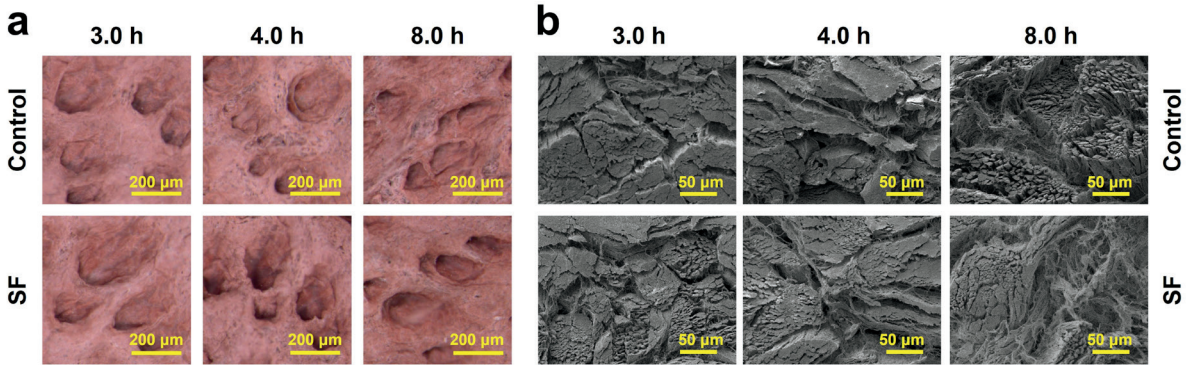

Figure 9. (a) Stereo microscopic images of grain surfaces of crust leathers and (b) SEM images of middle layers of crust leathers.

Table I

Physical properties of crust leathers

Sample	Bating time (h)	Softness (mm)	Tensile strength (N/mm^2)	Tear Strength (N/mm)
Trypsin	2.0	7.73 ± 0.43	11.40 ± 0.61	54.62 ± 1.10
Control (Trypsin)	3.0	8.52 ± 0.11	9.06 ± 0.10	39.49 ± 1.07
	4.0	8.55 ± 0.15	6.66 ± 1.42	32.11 ± 4.95
	8.0	8.76 ± 0.18	5.14 ± 0.09	28.52 ± 3.04
SF (Trypsin+SF)	3.0	8.80 ± 0.08	8.10 ± 0.82	42.07 ± 2.68
	4.0	8.73 ± 0.25	8.01 ± 0.12	32.96 ± 0.06
	8.0	8.86 ± 0.16	7.57 ± 0.43	39.54 ± 4.33

Values are means ± standard deviations of six determinations.

the crust leathers decreased with increasing bating time. However, it was interesting that the decrement in the strengths of the SF-crust leather was less than that of the control leather. These results also proved that adding SF during the bating process reduced the damage to hide collagen, especially the damage to the grain, which is important to the leather's strengths.

Conclusion

The addition of sufficient inhibitor after bating for a certain time could effectively inhibit the activity of trypsin in the grain layer and not affect the enzymatic hydrolysis of the middle layer. As a result, the surface morphology (clearness and intactness) and the grain strength of the bated hide could be protected. The proposed bating method using SF to opportunely inhibit the trypsin activity exhibited simple, environmental friendly, and low-cost advantages and huge potential for producing high-quality leathers. New insights are provided into the development of bating methods that can avoid the enzymatic damage to hide and not affect the performance of bating system.

Acknowledgement

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