Investigation of Mass Transfer and Action Mechanism of Enzymes in Unhairing and Bating Processes using Fluorescence Tracing

1. Introduction

Application of enzymes for reduction in pollutants and for improvement of production efficiency has attracted widespread interest in leather industry.[1] For example, much research recently has focused on the development of enzymatic unhairing systems to reduce sulfide and lime pollutions.[2,3] As we know, the effectiveness of enzymatic treatment is closely related to the mass transfer of enzymes in skin/hide, since skins/hides have certain thicknesses with complex weaves of collagen fiber bundles. However, the mass transfer and reaction mechanism of enzymes in leather processing remains unclear due to lack of an appropriate method that is able to accurately visualize and quantify enzymes in skin/hide, which makes the application of enzymes mainly depend on the experience of tanners as well as restricts a wide use of enzymes in commercial scale.

Fluorescent tracer technique makes it possible to accurately locate proteins labeled with fluorescein in cell or tissue.[4-7] So it is reasonable to speculate that the fluorescent tracer technique can be used to detect enzymes in skin/hide as long as the enzymes are endowed with fluorescent properties. In this project, we chose trypsin (biological reagent), a kind of protease with satisfactory unhairing[8] and bating effects[9], as a research model of commercial unhairing and bating enzymes, since it can be easily fluorescein-labeled and purified. First, a fluorescent trypsin (FITC-trypsin) with both high fluorescence intensity and high biological activity was prepared. Then, to investigate the mass transfer characteristics of enzymes in unhairing and bating processes, soaked cattle hides and delimed pelts were treated with FITC-trypsin, respectively, and the distribution of FITC-trypsin in the hides and the pelts was visualized with fluorescence microscope and quantified with Image J software.

2. Materials and Methods

2.1 Materials

Trypsin from bovine pancreas purchased from Shanghai Aladdin Biochemical Technology Co., Ltd., fluorescein isothiocyanate isomer I (FITC, ≥90% (HPLC)) and Sephadex G-25 (fine)

purchased from Sigma-Aldrich Co. LLC. were used for preparation of FITC-trypsin. Bovine serum albumin (BSA, molecular biology grade) purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. and Rhodamine B isothiocyanate (RBITC) purchased from Sigma-Aldrich Co. LLC. were used for preparation of RBITC-BSA. Conventional soaked cattle hides (pH 8.0) that had tight hair were used for unhairing trials. Delimed cattle pelts prepared by treating conventional limed pelts (3 mm in thickness) with 3% ammonium sulfate for 2 hours were used for bating trials. Collagen fiber (hide powder) was prepared according to the method described in the literature.[10] Commercial neutral protease produced by culturing *Bacillussubtilis* was purchased from Nanning Pangbo Enzyme Co., Ltd. (China). Commercial sodium sulfide ($Na_2S \geq 60\%$) was purchased from Kangxin Sichuan Chem Co., Ltd. (China). A commercial fatty alcohol polyoxyethylene ether (JFC) was purchased from Jiangsu Haian Petrochemical Plant (China). All the other chemicals used for leather processing were of commercial grade, and the chemicals used for the analyses were of analytical grade.

2.2 Preparation of FITC-labeled trypsin (FITC-trypsin)

2.2.1 Preparation of FITC-trypsin

Trypsin solution (20.0 mg/mL) and FITC solution (10.0 mg/mL) were prepared by dissolving trypsin and FITC into carbonate-bicarbonate buffer (0.1 mol/L, pH 9.16), respectively. Then, 9 mL of the trypsin solution was mixed with 3 mL of the FITC solution, and the labeling reaction was performed in the dark at $4^{o}C$ for 10 h. After labeling, the mixture was concentrated to about 3 mL using Amicon Ultra-15 centrifugal filter devices (10 kDa MWCO, Millipore). Subsequently, the concentrated solution was loaded onto a Sephadex G-25 gel-filtration column (3.5 x 85 cm) for removal of unreacted FITC. The column was equilibrated and eluted with ultrapure water at a flow rate of 1.0 mL/min. The eluate was collected with an automatic fraction collector (5.0 mL per fraction), and the absorbance of each fraction was measured at 495 nm (the absorbance maximum of FITC) using an ultraviolet-visible spectrophotometer (UV-Vis, UV-1800PC, Mapada, China). Then, the fractions containing FITC-trypsin without FITC were collected and freeze-dried. As a result, the purified FITC-trypsin that retained 72% of its original proteolytic activity was obtained, whose molecular ratio of fluorescein to protein (F/P) was 2.81.

Additionally, RBITC-BSA was prepared by the same procedures described above except that trypsin and FITC were replaced with BSA and RBITC, respectively.

2.2.2 Analysis of fluorescence emission spectra

The fluorescence emission spectra of trypsin (2.0 mg/L), FITC-trypsin (2.0 mg/L) and FITC (2.0 μg/L) solutions were measured with an excitation wavelength of 495 nm (the excitation maximum of FITC) using a fluorescence spectrophotometer (Cary Eclipse, Agilent, USA).

2.2.3 Analysis of molecular weight

The molecular weight of trypsin and FITC-trypsin was determined by SDS-PAGE as described by Laemmlli[11] using a Mini-PROTEAN Tetra Cell system (Bio-Rad, USA). Protein bands were observed after staining with a solution of Coomassie Brilliant Blue R-250.

2.3 Visualization and quantification of FITC-trypsin in cattle hide during enzymatic unhairing

Four pieces of soaked cattle hides (5 mm in thickness) were treated by 2% enzyme containing 0.15% FITC-trypsin and 1.85% trypsin (800 units/g hide) and 100% water (based on weight of soaked cattle hide) at 25°C for 60, 150 and 180 min, respectively. After enzymatic treatment, the hides were cut into vertical sections of 20 μm thickness using a freezing microtome (CM1950, Leica, Germany). The sections were observed using a fluorescence microscope (Ti-U, Nikon, Japan) to locate FITC-trypsin in the hides, and then the fluorescence micrographs were processed with Image J software to semi-quantify the relative content of FITC-trypsin in the hides. Additionally, the sections were stained with Weigert's iron hematoxylin and counterstained with Van Gieson's stain to distinguish epidermis, hair roots and collagen fibers. After staining, the sections were observed using an optical microscope (CX41, Olympus, Japan). Moreover, the surfaces of treated cattle hides were captured using a digital camera.

2.4 "pH-controlled" enzyme assisted low-sulfide hair-save unhairing (EALS unhairing)

2.4.1 Effect of pH on proteolytic activity of neutral protease

The effect of pH on proteolytic activity of the neutral protease was investigated by the method described in the literature[12]. A series of 1.0 mg/mL neutral protease solutions and 2% (w/v) casein solutions were prepared by using Briton-Robinson buffers at pH 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, respectively. One mL of neutral protease solution was mixed with 1 mL of casein solution (both at the same pH) and then incubated at 25°C for 10 min. Subsequently, the enzymatic hydrolysis reaction was stopped with trichloroacetic acid, and the amount of tyrosine released during enzymolysis was measured with Folin-Ciocalteu reagent. One unit of

proteolytic activity was defined as the amount of protease that releases 1 μg tyrosine per minute under the assay conditions. The relative proteolytic activities of the neutral protease at different pH values were calculated as:

$$\text{relative proteolytic activity} = \frac{\text{proteolytic activity at a certain pH}}{\text{proteolytic activity at pH 7.0}} \times 100\%$$

2.4.2 Effect of pH on hydrolysable action of neutral protease on collagen fiber

Fifty mg of collagen fiber was suspended in 25 mL of 0.15 mg/mL neutral protease solution, where the pH values of the protease solutions were 7.0 and 12.0, respectively. The mixtures were constantly shaken in 130 rpm at 25°C for 1 h. Then, to evaluate the damage to collagen caused by protease, the mixtures were filtered and the filtrates were taken for measurement of Hyp concentration as reported in the document[13]. Moreover, the control experiments were conducted simultaneously by the same procedures except addition of neutral protease.

2.4.3 Comparison between low-sulfide hair-save unhairing (LS unhairing) and EALS unhairing

Two pieces of soaked cattle hide, approximately 1 kg for each, were treated by using LS unhairing and EALS unhairing, respectively, and hide/pelt samples (No. 1-6) were collected as shown in Table I. The samples from the hides (No. 1 and 2) and the pelts (No. 5 and 6) were cut into sections of 15 μm thickness using a freezing microtome. The sections were observed using an optical microscope after staining with Weigert's iron hematoxylin and Van Gieson's stain. Moreover, the surfaces of pelts (No. 3, 4, 5 and 6) were captured using a digital camera.

After unhairing, the pelts were both processed by using 2% lime, 2% swelling agent (containing sodium silicate (30 wt.%), magnesium chloride (5 wt.%), sodium hydroxide (5 wt.%) and water (60 wt.%)) and 250% water for 18 h. Subsequently, the limed pelts were delimed, bated, pickled and chrome tanned by using common procedures. Then, the chrome tanned leathers were piled for 24 h and their grain was observed with stereomicroscope (SZX12, Olympus, Japan).

Table I Procedures of LS unhairing and EALS unhairing

Process	Offer of agent[a] and remarks[b]	
	LS unhairing	EALS unhairing
Enzymatic treatment	–	+100% water, neutral protease (20 units/g hide) Run 40 min, pH 8
Hair immunization /protease inactivation	+100% water, 1% lime Run 90 min, pH>12 Hide sampling (No. 1)	+1% lime Run 90 min, pH>12 Hide sampling (No. 2)
Hair removal	Drain 50% water +0.8% Na_2S Run 10min +0.8%NaCl Run 15 min, pelt sampling (No. 3) Run 45 min, pelt sampling (No. 5)	Drain 50% water +0.8% Na_2S Run 10min +0.8%NaCl Run 15 min, pelt sampling (No. 4) Run 45 min, pelt sampling (No. 6)

a - Percentage of chemicals was based on weight of soaked hide.

b - All the processes were performed at 22°C.

2.4.4 Comparison between conventional sulfide-lime unhairing and EALS unhairing

Four soaked cattle hides (approximately 100 kg) were cut along the backbone in halves. The left sides were treated with the conventional sulfide-lime unhairing and liming procedures (control process) listed in Table II, and the right sides were treated by using the EALS unhairing and low-lime liming procedures (experimental process) given in Table III. After liming, the limed pelts were captured using a digital camera. As shown in Table II and Table III, effluent samples (No. 1-3) were collected for analyses of concentrations of S^{2-}, total solids (TS), suspended solids (SS) and chemical oxygen demand (COD) according to the methods reported in documents[14]. Loads of these pollutants were calculated by multiplying their concentrations by volumes of the effluents per ton of raw hides and expressed as kg/t of raw hides.The control and experimental limed pelts were then further processed to obtain crust leathers by using the common techniques of leather processing. The crust leathers were sampled for analyses of physical properties such as tensile strength[15], percentage elongation at break[15], tear load[16] and softness[17]. Moreover, their grain was observed with stereomicroscope.

Table II Procedures of conventional sulfide-lime unhairing and liming (control process)[a,b]

Process	Chemical	Remark
Unhairing	100% Water, 0.8% Sodium sulphide, 0.3% Degreasing agent	Run 30 min
	1.0% Sodium sulfide	Run 30 min
	1.0% Sodium sulphide, 0.5% Lime	Run 20 min, stop 40 min
Liming	7.0% Lime	Run 90 min
	50% Water	Run 15 min
	50% Water	Run 30 min
	50% Water	Run 60 min, pH 13.3; Run 5 min per hour for another 12 hours; Overnight.
Next day, run 30 min. Effluent sampling (No. 1).		

a - Percentage of chemicals was based on weight of soaked hide.

b - All the processes were performed at 22°C.

Table III Procedures of EALS unhairing and low-lime liming (experimental process)[a,b]

Process	Chemical	Remark
Enzymatic treatment	100% Water, Neutral protease (15 units/g hide)	Run 40 min, pH 8.5
Hair immunization /Protease inactivation	1.0% Lime	Run 20 min, stop 20 min, run 20 min; pH 12.9
Drain 50% water. Effluent sampling (No. 2).		
Hair removal	0.8% Sodium sulphide, 0.3% Degreasing agent	Run 10 min
	0.8% Sodium chloride	Run 50 min. Hair was completely removed.
Hair was filtered out, dried and weighed. Run 30 min, stop 30 min.		
Liming	2.0% Lime	Run 60 min
	1.0% Swelling agent	Run 15 min, stop 30 min
	1.0% Swelling agent	Run 15 min, stop 30 min
	50% Water	Run 30 min
	50% Water	Run 30 min
	50% Water	Run 60 min, pH 13.3; Run 5 min per hour for another 12 hours; Overnight.
Next day, run 30 min. Effluent sampling (No. 3).		

a - Percentage of chemicals was based on weight of soaked hide.

b - All the processes were performed at 22°C.

2.5 Visualization and quantification of FITC-trypsin in pelt during bating

2.5.1 Effect of bating time on distribution of enzyme in pelt

Three pieces of delimed pelts were bated by 0.50% enzymes containing 0.25% FITC-trypsin and 0.25% trypsin and 100% water (based on weight of delimed pelt) at 30°C for 5, 30 and 90 min, respectively. After bating, the pelts were cut into vertical sections of 20 μm thickness. The FITC-trypsin in the sections were visualized and quantified by the same procedures described in Section 2.3. Additionally, the sections were stained with hematoxylin and eosin (HE) stain to identify collagen fibers[18] and then observed using a biologic microscope.

2.5.2 Effect of amount of enzyme on mass transfer of enzyme in pelt

Five pieces of delimed pelts were bated with different amount of enzymes and 100% water (based on weight of delimed pelt) at 30°C for 30 min, respectively. The enzymes used herein were 0.10% FITC-trypsin, 0.25% FITC-trypsin, 0.50% enzyme mixture (0.25% FITC-trypsin + 0.25% trypsin), 1.0% enzyme mixture (0.25% FITC-trypsin + 0.75% trypsin) and 2.0% enzyme mixture (0.25% FITC-trypsin + 1.75% trypsin), respectively. After bating, the FITC-trypsin in the pelts was observed using a fluorescence microscope. Moreover, the grain surfaces of the bated pelts were observed using a scanning electron microscope (SEM, Phenom Pro, Phenom, China), and the concentration of Hyp in bating liquors was determined as reported in the document[13].

2.5.3 Effect of molecular weight of enzyme on mass transfer of enzyme in pelt

One piece of delimed pelt was treated with 0.25% FITC-trypsin, 0.25% trypsin, 0.25% RBITC-BSA, 0.25% BSA and 100% water (based on weight of delimed pelt) at 30°C for 90 min. After treatment, the FITC-trypsin and the RBITC-BSA in the pelt were observed using a fluorescence microscope.

2.5.4 Effect of surfactant on mass transfer of enzyme in pelt

Four pieces of delimed pelt were bated with 0.10% FITC-trypsin, 0.50% JFC and 100% water (based on weight of delimed pelt) at 30°C for 5, 15, 30 and 60 min, respectively. After bating, the FITC-trypsin in the pelts was observed using a fluorescence microscope, and the concentration of Hyp in bating liquors was determined as reported in the document[13]. Moreover, the control experiments were conducted simultaneously by the same procedures except addition of JFC.

3. Results and Discussion

3.1 Characteristics of FITC-trypsin

As mentioned previously, the purpose of this project was to investigate the mass transfer characteristics of enzymes in unhairing and bating processes. For this purpose, we first need to fluorescently label enzymes. Hence, the trypsin (biological reagent), as a model of commercial unhairing and bating enzymes, was fluorescently labeled using FITC as a fluorophore. Here it is worth noting that after the labeling reaction, the unreacted FITC that remains in the product of FITC-trypsin will interfere with accurate fluorescence detection of FITC-trypsin in hide, since it can easily adhere to hides in unhairing/bating process. Therefore, after labeling, the mixture containing FITC-trypsin and the unreacted FITC was subjected to a Sephadex G-25 gel-filtration column for removal of FITC. As shown in Figure 1, the FITC was well separated from FITC-trypsin using gel filtration chromatography, and the FITC-trypsin with high purity was obtained. Moreover, the purified FITC-trypsin was analyzed by fluorescence spectroscopy. As shown in Figure 2, while trypsin had no emission following excitation at 495 nm, FITC-trypsin had an emission maximum at 519 nm, which was almost the same as that of FITC. The emission spectra of trypsin, FITC and FITC-trypsin demonstrated that FITC was successfully incorporated into trypsin, providing it with detectable fluorescence.

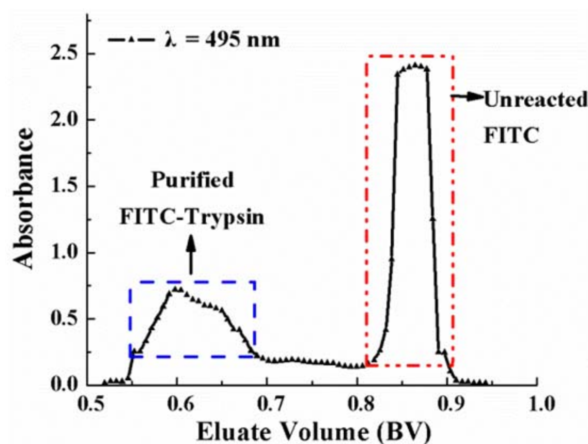

Figure 1. Chromatogram of FITC-trypsin on a Sephadex G-25 gel-filtration column (3.5 x 80 cm). The column was eluted with ultrapure water at a flow rate of 1.0 mL/min.

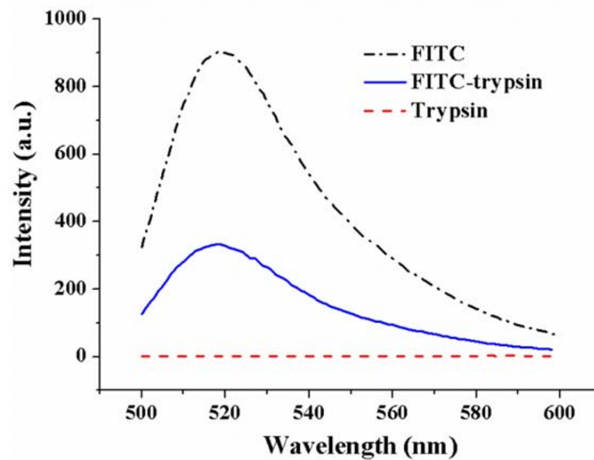

Figure 2. Fluorescence emission spectra of FITC, FITC-trypsin and trypsin obtained using an excitation wavelength of 495 nm (the excitation maximum of FITC).

As we know, the molecular size is an important factor influencing the mass transfer of chemicals in skin/hide. Therefore, the effect of labeling with FITC on the molecular weight of trypsin was investigated. As shown in Figure 3, the molecular weight of FITC-trypsin was slightly larger than that of trypsin, which was due to the incorporation of FITC into trypsin (F/P = 2.81). Despite this, the change in the molecular weight of trypsin was negligible.

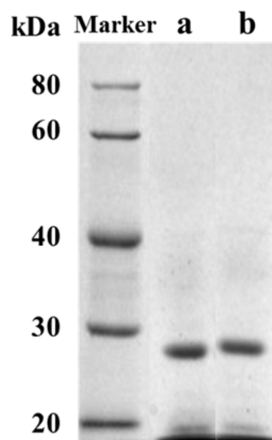

Figure 3. SDS-PAGE electrophoretogram of trypsin (a) and FITC-trypsin (b).

3.2 Mass transfer of enzyme in hide during enzymatic unhairing process

In this section, the mass transfer of enzyme in enzymatic unhairing was investigated by fluorescent tracer technique. Soaked cattle hide without hair slip was treated by using 0.15% FITC-trypsin and 1.85% trypsin at 25°C. The digital photos of enzymatic treated hide surfaces, the photomicrographs of Van Gieson stained vertical sections cut from enzymatic treated hides and the fluorescence micrographs of FITC-trypsin (green) in the vertical sections are

shown in Figures 4(a), 4(b) and 4(c), respectively. Furthermore, after processing Figures 4(c-1), 4(c-2) and 4(c-3) with Image J software, the quantitative distribution of FITC-trypsin in the enzymatic treated hides was obtained as shown in Figure 4(d). It was obvious that enzyme penetrated very slowly in hide and could not penetrate through the whole cattle hide even after most of the hair had been removed. The fact that protease remains on the grain/papillary layer and the lower reticular layer for a long time in unhairing process, should be the main reason why enzymatic unhairing may cause grain damage or loose grain. From these phenomena, we infer that shortening enzymatic treating time is necessary to avoid risk of grain damage or loose grain. Besides, as shown in Figures 4(a) and 4(b), epidermis was effectively removed from hide in a short time prior to removal of hair. Additionally, little protease penetrated into grain when hide still had tight hair (see Figures 4(a-1), 4(c-1) and 4(d)). These results suggest that inactivation of protease, after cleavage of epidermis but before removal of hair, might be crucial for guaranteeing quality of resutant leather.

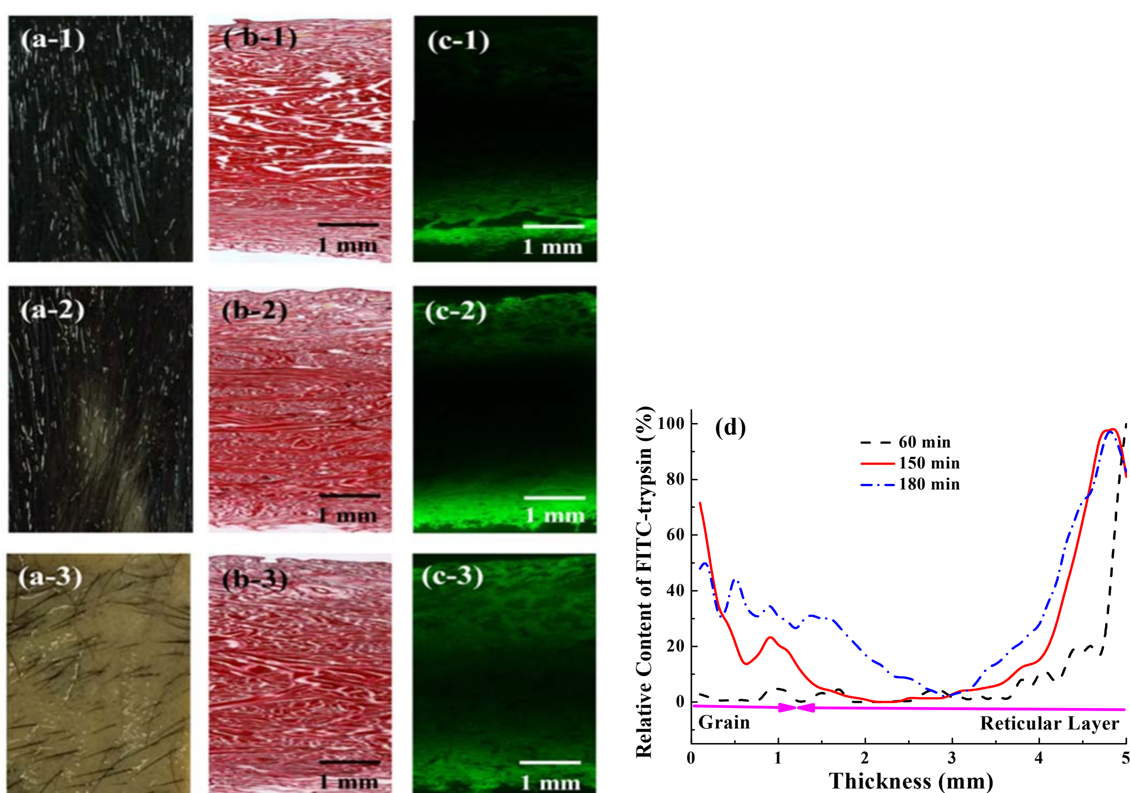

Figure 4. (a) Surfaces of hides captured by digital camera after enzymatic treating for 60 min(a-1), 150 min (a-2) and 180 min (a-3); (b) Photomicrographs of vertical sections (Van Gieson stain) from hides that were treated by enzyme for 60 min (b-1), 150 min (b-2) and 180 min (b-3); (c) Fluorescence micrographs of FITC-trypsin (green) in the vertical sections of cattle hides that were treated by enzyme for 60 min (c-1), 150 min (c-2) and 180 min (c-3); (d) Distribution of FITC-trypsin in the enzymatic treated cattle hides (the relative content of FITC-trypsin in hide was quantified by analysis of Figures (c-1), (c-2) and (c-3) using Image J software).

3.3 Enzyme assisted low-sulfide hair-save unhairing (EALS unhairing)

Based on the mass transfer characteristics of protease in hide during enzymatic unhairing, we developed a "pH-controlled" enzyme assisted low-sulfide hair-save unhairing (EALS unhairing). To avoid risk of hide damage, soaked cattle hide (pH 8) was first treated with neutral protease (20 units/g hide) for 40 min at 22°C to cleave epidermis but not to unhair, and then 1% lime was immediately added both for inactivation of the neutral protease (pH>12) and for hair immunization. (When pH was above 12, the neutral protease was nearly free of proteolytic activity (Figure 5(a)) and almost lost ability to damage collagen (Figure 5(b)).) Finally, the hide was completely unhaired by using 0.8% sodium sulfide with intact hair shaft.

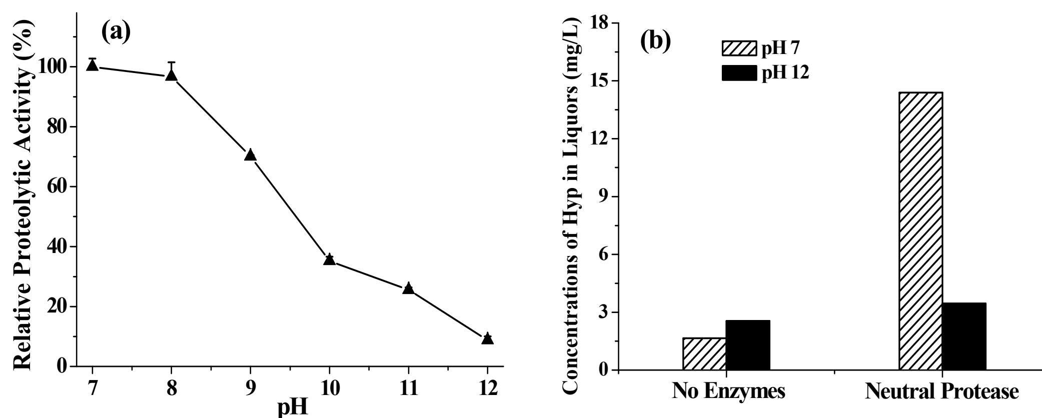

Figure 5. Effect of pH on the proteolytic activity of neutral protease (a) and the damage to collagen fiber caused by neutral protease (b)

Comparing Figures 6(a-LS) and 6(a-E) shows that, after hair immunization by lime, epidermis (black or brown) was nearly entirely remained in hide in LS unhairing system, while a majority of epidermis was removed from hides in EALS unhairing system. These results demonstrated that a mild treatment of neutral protease (20 units/g hide, 40 min, 22°C) was quite effective in hydrolyzing and removing epidermis from soaked hides (pH 8). For both of the systems, hair (yellow) was well remained in hides after hair immunization. According to Figures 6(b-LS), 6(b-E), 6(c-LS) and 6(c-E), it is obvious that the cleavage of epidermis by using neutral protease was very beneficial to a rapid and complete removal of hair and epidermis by using a small amount (0.8%) of sodium sulfide. This proves that the cleavage of epidermis in hide by neutral protease can largely improve the unhairing function of sodium sulfide. From Figures 6(d-LS) and 6(d-E) it can be seen that the grain surface of chrome tanned leather processed by EALS unhairing was considerably cleaner compared with that processed by LS unhairing. This suggests that the EALS unhairing can guarantee good quality

of resultant leather based on complete removal of hair and epidermis from hide and timely inactivation of protease.

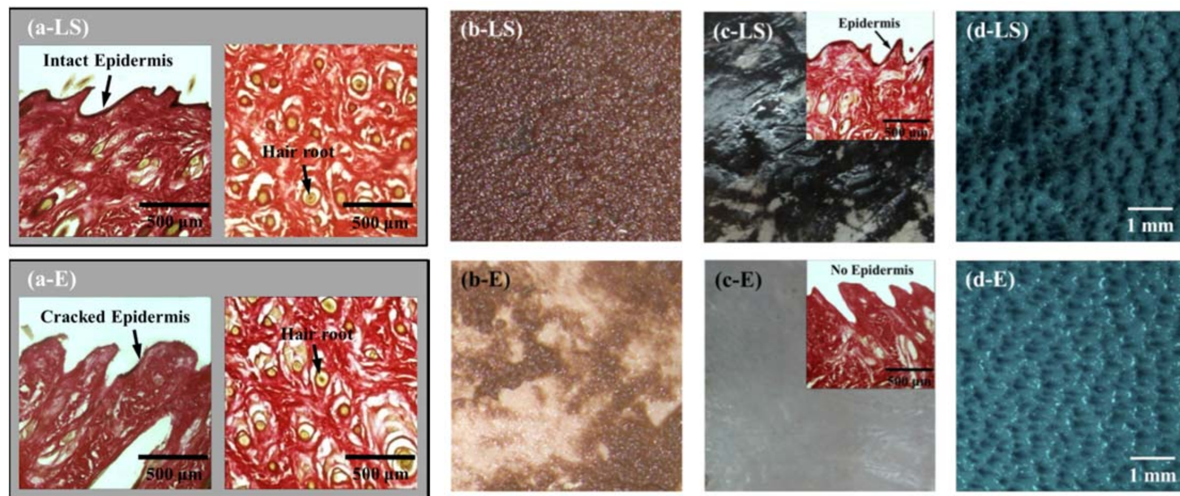

Figure 6. (a) Photomicrographs of vertical sections and horizontal sections (Van Gieson stain) from cattle hides after hair immunization with 1% lime for 90 min: (a-LS) LS technique; (a-E) EALS technique. (b) Digital photos of surfaces of the hides after treating by 0.8% NaCl for 15 min: (b-LS) LS technique; (b-E) EALS technique. (c) Digital photos of surfaces and photomicrographs of vertical sections (Van Gieson stain) from unhaired pelts after treating by 0.8% NaCl for 50 min: (c-LS) LS unhaired pelt; (c-E) EALS unhaired pelt. (d) Grain of chrome tanned leathers observed by stereomicroscope: (d-LS) LS technique; (d-E) EALS technique.

Moreover, the limed pelt as well as the crust leather processed by using EALS unhairing had a cleaner grain surface compared with that using conventional sulfide-lime unhairing (see Figures 7 and 8). Additionally, the physical properties of the leather processed with EALS unhairing, such as tensile strength, percentage elongation at break, tear load and softness, were comparable to those of conventional leather (Table IV). These results demonstrated that an appropriate replacement for sulfide soudium and lime by neutral protease and swelling agent, along with a smart process control in the experimental process, could guarantee a better quality of resultant leather. S^{2-}, TS, SS and COD in the EALS unhairing effluent were markedly reduced (Table V) due to a dramatic decrease in the input of sodium sulfide and lime as well as the recovery of hair.

Figure 7. Limed pelts captured by digital camera: (a) control; (b) experiment.

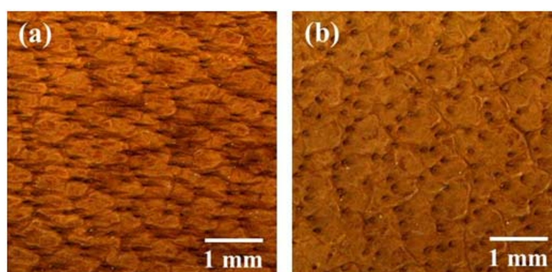

Figure 8. Grain of curst leathers observed by stereomicroscope: (a) control; (b) experiment.

Table IV Physical properties of control (C) and experimental (E) crust leathers

Sample	Tensile strength (N/mm^2)	% Elongation at break	Tear load (N)	Softness (mm)
C	12.7	44.5	81.0	6.3
E	13.6	40.8	73.7	6.2

Table V Pollution loads in control (C) and experimental (E) unhairing and liming effluents (Unit: kg/t of raw hide)

Sample	S^{2-}	TS	SS	COD
C (No. 1)	3.64	96.05	46.75	70.00
E (No. 2+No. 3)	0.66	54.31	3.06	33.14

In summary, based on the visual findings of the mass transfer and action characteristics of protease in enzymatic unhairing, the EALS unhairing has been developed, which favors production of high quality leather as well as a remarkable decrease in S^{2-}, TS, SS and COD in effluent. This suggests that a thorough investigation of the mass transfer and reaction mechanism of enzymes in unhairing by using the fluorescent tracer technique is beneficial to a rational control of the action of enzymes on hide, which can prevent leather from defects and improve unhairing effectiveness.

3.4 Mass transfer of enzyme in pelt during bating process

The vertical section of bated pelt (HE stain) is shown in Figure 9(a), where collagen fibers are red. The fluorescence micrographs of FITC-trypsin (green) in the bated pelts are presented in Figures 9(b)-9(d). It can be observed that the fluorescence intensity of the middle layer improved with an increase in bating time, suggesting that FITC-trypsin gradually penetrated into the pelt. After processing Figures 9(b)-9(d) with Image J software, the quantitative distribution of FITC-trypsin in the pelt was obtained as shown in Figure 9(e). The relative content of FITC-trypsin in the grain was higher than that in the lower reticular layer, indicating that compared with the reticular layer, FITC-trypsin penetrated faster into the grain. This is because many pores exist in the grain, which is beneficial to the mass transfer of enzyme.

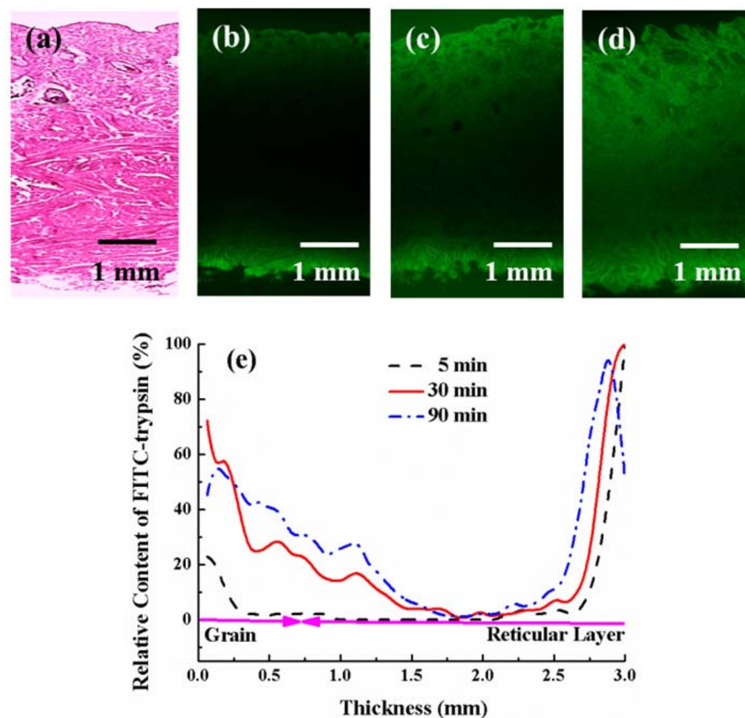

Figure 9. (a) Photomicrograph of vertical section (HE stain) cut from bated pelt; (b)-(d) Fluorescence micrographs of FITC-trypsin (green) in the vertical sections from pelts that were bated for 5 min (b), 30 min (c) and 90 min (d); (e) Relative content of FITC-trypsin in pelts quantified by analysis of Figures 9(b), 9(c) and 9(d) using Image J software. (The delimed pelts were bated with 0.5% enzyme.)

It should be noted that FITC-trypsin could not penetrate through the whole pelt in a short time, even when using 0.5% enzyme in bating (Figure 9). Therefore, the enhancement of mass transfer of enzyme in bating is vital for a uniform treatment of pelt and a high quality of resultant leather. As seen in Figure 10(a), an increase in the amount of trypsin can enhance its

mass transfer in pelt. This is consistent with the typical effect of concentration on mass transfer. But the increase in the amount of trypsin led to a negative effect on the grain surface of pelt (Figure 10(b)) and a more considerable damage to hide collagen (Figure 11). So the amount of trypsin should be limited, and a true understanding of how to enhance mass transfer of enzyme in pelt when using a desired small amount is of great theoretical and practical importance.

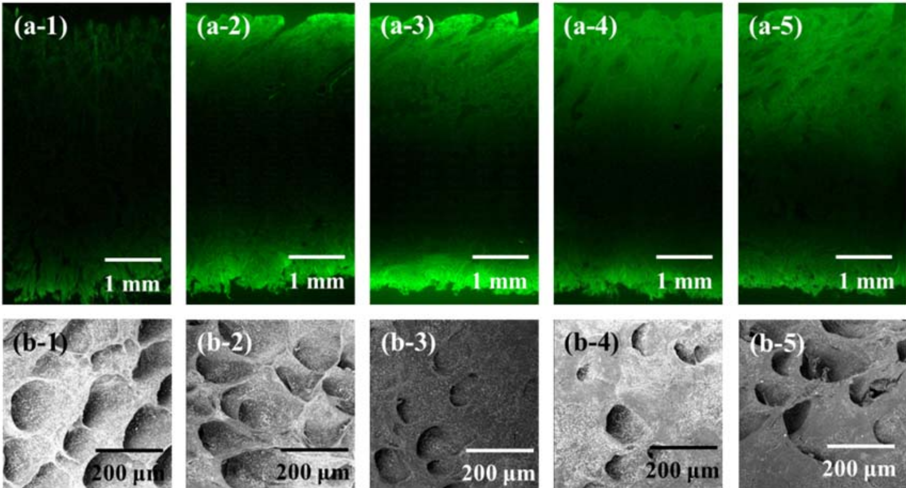

Figure 10. (a) Fluorescence micrographs of FITC-trypsin (green) in the vertical sections of pelts that were bated with 0.10% enzyme (a-1), 0.25% enzyme (a-2), 0.50% enzyme (a-3), 1.0% enzyme (a-4) and 2.0% enzyme (a-5); (b) SEM images of grain surfaces of pelts that were bated with 0.10% enzyme (b-1), 0.25% enzyme (b-2), 0.50% enzyme (b-3), 1.0% enzyme (b-4) and 2.0% enzyme (b-5). (The delimed pelts were bated for 30 min.)

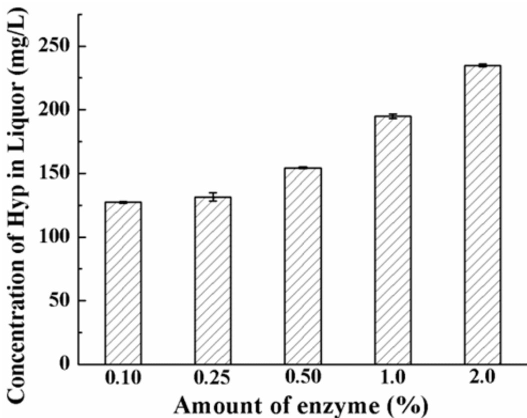

Figure 11. Effect of amount of enzyme on damage to hide collagen

As can be seen in Figure 12, the penetration depth of trypsin (24 kDa) in pelt was much greater than that of BSA (66 kDa), meaning that a relatively lower molecular weight is

beneficial to the mass transfer of enzyme in bating. Moreover, it is exciting to observe that when using trypsin together with surfactant such as JFC, the penetration rate of FITC-trypsin in pelt was markedly increased (Figure 13), and the damage to hide collagen caused by trypsin was reduced (Figure 14). These results imply that addition of proper surfactants in bating process can enhance mass transfer of enzymes, which favors a uniform bating performance and production of high quality leather.

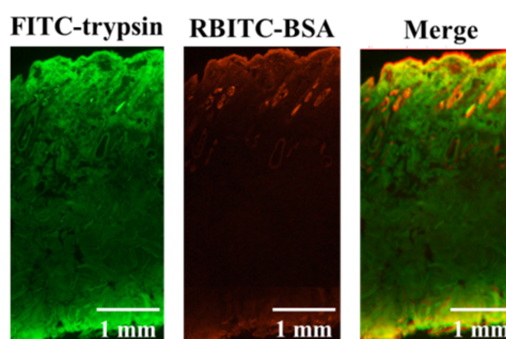

Figure 12. Vertical section from the pelt treated with FITC-Trypsin (green) and RBITC-BSA (red) for 90 min.

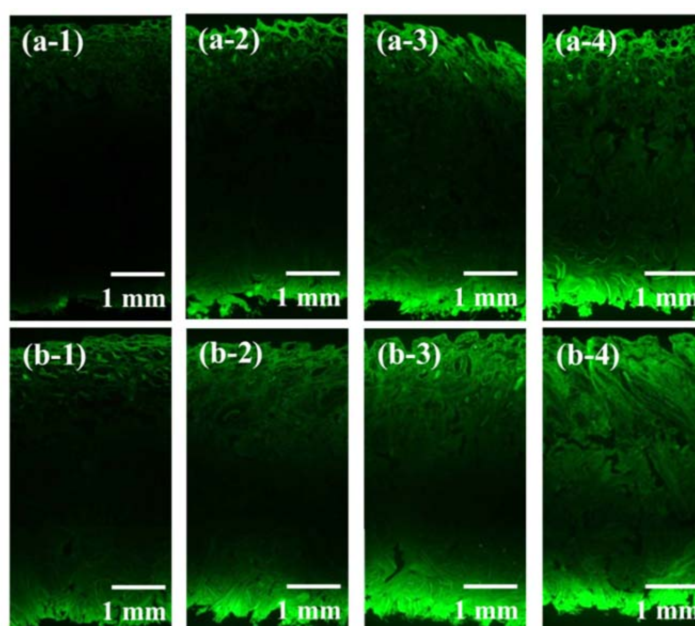

Figure 13. Fluorescence micrographs of FITC-trypsin (green) in the vertical sections of pelts bated with 0.10% enzyme (a) and bated with 0.10% enzyme and 0.50% JFC (b); (a-1), (b-1) bating for 5 min; (a-2), (b-2) bating for 15 min; (a-3), (b-3) bating for 30 min; (a-4), (b-4) bating for 60 min.

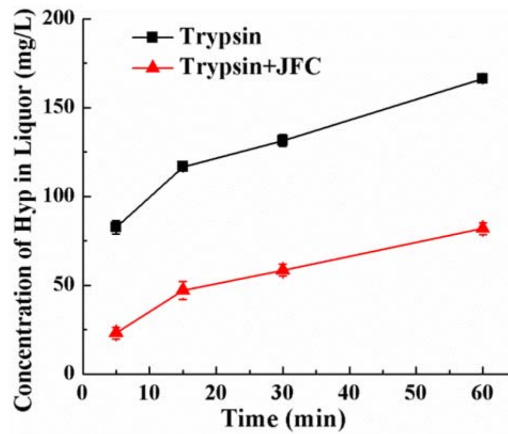

Figure 14. Effect of addition of surfactant on damage to hide collagen

Above all, enzymes in pelt can be well visualized and quantified by using fluorescent tracer technique together with image processing technique, which is useful for us to investigate the mass transfer characteristics of enzyme in bating and to enhance mass transfer of enzyme for a uniform bating performance.

4. Conclusions

The fluorescent tracer technique is effective in real-time monitoring the distribution of enzymes in hide, by which we can systematicly investigate the mass transfer and reaction mechanism of enzymes in leather processing. Furthermore, the information provided by this technique is helpful to optimize conditions of enzymatic treatment and to choose optimal enzymes for leather processing.

5. Suggestion for Future Work

(1) This project provides visual evidence that protease penetrates very slowly into hide and mainly remains on the grain/papillary layer and the lower reticular layer even after removal of hair. This is the main reason why enzymatic unhairing may cause grain damage or loose grain. Therefore, when developing an eco-friendly unhairing based on protease in future, care should be taken to rationally control protease activity for preventing leather from defects.

(2) For the sake of uniform bating performance, future work will focus on the effects of auxiliaries, temperature and mechanical action on the mass transfer of enzymes in bating.

(3) The fluorescent tracer technique is effective in investigating the mass transfer of protease in unhairing and bating. In fact, amino, carboxyl and hydroxyl groups of materials can form

covalent bond with fluorescence, endowing the materials with fluorescent properties. Therefore, future work will investigate the mass transfer and reaction mechanism of many other leather chemicals in leather processing by fluorescent tracer technique, such as lipase, amino resins, acrylic resins and syntans.

6. References

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