Effects of Calcium Content on the Enzymatic Bating of Delimed Hides

by
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Abstract

The effects of calcium content on the enzymatic bating of delimed hide were analyzed by amino acid analysis, tissue staining, and scanning electron microscopy–elemental energy spectrometry. Results show that the content and form of calcium in pelt can affect enzyme activity. The content of calcium in pelt can be adjusted by phosphoric-phosphate deliming to reduce the degree of damage caused by enzymes to elastin and collagen. The calcium bound in pelt can be further removed by deliming using a calcium chelator, thus effectively improving the efficiency of enzyme action. When the calcium content is lower than 0.6%, the removal degree of elastin by enzymes and the loosening effect to collagen fiber can be significantly improved. This research offers important guidance to improve the leather bating process.

Introduction

Tanning is the process of processing the collagen matrix of animal skin into leather products of practical value. To obtain high-quality leather products, the hair, epidermis, fibrous stroma, grease, and other non-collagen components in the skins have to be removed through processes such as soaking, degreasing, unhairing and liming, deliming, bating, and pickling, while collagen fiber could be opened up moderately according to the requirements for finished leather.[1] Then, the pelts are prepared for tanning and promoting the infiltration and bonding of leather chemicals in collagen matrix to improve the properties of crust leather.

Bating is the process of deliming pelts through enzyme preparations (mainly trypsin), and it is the only biotechnology that, as of now, cannot be replaced by chemical treatment.[2] The essence of its biochemical reaction is that, with the use of proteases, non-collagen components such as elastin, keratin, glycoprotein, albumin, and globulin in skins and hides are further destroyed or removed, the collagen is hydrolyzed and the collagen fibers are further opened up to the desired degree,[3,4] determining the overall style and basic sensory properties of the finished leather. Therefore, bating is one of the essential operations in the conventional leather manufacturing process and one of the most difficult processes to control in leathermaking.

In general, the bating operation is conducted under pH conditions of 7.0–9.0 at 30°–40°C.[5] However, the properties of leathers, including softness, fullness, and tightness, always vary with raw hides and the varieties and styles of finished leather. The enzyme dosage, bating temperature, pH, and time are usually adjusted to fulfill the requirements of different leather properties according to the tanners' experience. For example, upper leathers and other leathers with a high degree of tightness can easily soften, while leathers with higher softness requirements, such as sofas and clothing, needs a longer time to satisfy basic requirements, easily causing damage to the grain.[6,7]

At present, trypsin with high specificity to peptide bonds is mainly selected for use in the bating process of leather.[8] For a long time, the main goal has been to find a new protease to replace trypsin. Therefore, the research emphasis of bating technology is the screening of biological enzymes and the exploration of the optimal operating conditions in the bating process.[9-10] However, the influence of the substrate characteristics (the state of delimed pelt) on the enzymatic bating has not received sufficient attention. Studies have shown that the calcium ion (Ca^{2+}) can combine with the carboxyl group of collagen side chain under alkaline conditions, which changes the surface charge of collagen fiber. It can improve the resistance to enzymatic hydrolysis of collagen fiber and effectively protect the grain surface during enzymatic unhairing.[11] Through a simulation of the biomineralization mechanism, a porous and insoluble calcium phosphate protective layer can form on the surface of collagen fiber, which can also improve the enzyme resistance of collagen fiber.[12] In the unhairing-liming process, to loosen collagen fibers effectively, adding a large amount of lime is often necessary for the treatment of alkali expansion, resulting in a large calcium content of limed pelt.[13] In the deliming process, the calcium in the limed skin will be partially dissolved and removed under the action of acid-base neutralization, dehydration deswelling, and so on. However, its removal degree will be greatly different because of the type of delimed agent and degree of deliming, thus affecting the effect of enzymatic bating.

Therefore, in this research, the degree of calcium removal within the delimed pelt was purposefully controlled, and the effect of calcium removal degree during deliming on the effect of enzymatic bating

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was investigated by various means, such as protein (desmosine and hydroxyproline) analysis, histological staining, and scanning electron microscopy–energy dispersive X-ray spectroscopy (SEM-EDS) analysis. This research provides a scientific basis for improving the controllability of the bating process for different styles of leather.

Materials and Methods

Materials

Salted-wet cattle hides from Sichuan, China, were purchased from a local tannery (Chengdu Xinshi Leather Industry CO., Ltd.), and were carefully chosen from reliming stage and the rest of the beamhouse operations were carried as per commercial leather making procedure reported previously.[15] Ethylenediaminetetraacetic acid disodium solution (EDTA-Na, 10 g/L, pH=8.0-8.5), Elastase (210,000 U/g, pH8.0, 40°C, Novozymes Co.), Trypsin (360,000 U/g, pH8.0, 40°C, Novozymes Co.), the chrome tannage, syntans, fatliquors, polymers and filling agents used in this experiment were industrial grade while the rest of the chemicals were analytical grade.

Methods

Preparation of delimed pelt with different calcium contents

Four pieces of relimed cow hides were taken from symmetrically adjacent parts along the back line, marked and weighed, and washed twice with 200% (the weight of limed hide) water at 30°C for 30 min each time. Then, the water dosage was controlled to 50% of the mass of limed hides, and the deliming process was performed at 33°C in four thermal cycle stainless steel quadrupole drums (GSD, Wuxi Xinda Light Industry Machinery Co., LTD., China). The methods are as follows:

(1) The appropriate amounts of sodium dihydrogen phosphate and 10% phosphoric acid solution were added in the drum and rotated for 240 min to infiltrate evenly. The pH was controlled to about 8.0–8.5 (the same below), and then the samples were washed with 200% water 3 times, 30 min each time.

(2) The 2.5% ammonium sulfate was added in the drum, which was rotated for 40 min, and then drained. The samples were washed with 200% water for 30 min, redelimed with 1.5% ammonium sulfate under the same conditions, and washed with water 3 times. Other operating conditions are the same as (1).

(3) The 2.5% ammonium sulfate was added in the drum, which was rotated for 40 min, and then drained. The samples were washed with 200% water for 30 min. The redeliming was performed combined with 1.0% ammonium sulfate and 1.0% EDTA-Na under the same conditions and then washed 3 times.

(4) The samples were pre-delimed with 2.5% ammonium sulfate and 1.0% EDTA-Na to be rotated for 40 min and drained. The samples were washed with 200% water for 30 min. The redeliming was performed combined with 0.5% ammonium sulfate and 2.0% EDTA-Na under the same conditions and then washed 3 times.

Effect of calcium content of pelt on bating

The delimed pelts were dropped into four drums. The liquid ratio was 50%, and the temperature was 35°C. The activity concentration of trypsin and elastase was controlled to 20 U/mL, and the samples were rotated for 240 min. The pH of the bath solution was measured every 60 min, and 5.0 mL bath solution was extracted as reserve. Then, the samples were drained and washed with 200% water twice, 30 min each time. The hides were extracted as reserve. Subsequent processes, such as acid dipping, chrome tanning, retanning, fatliquoring, and drying, were performed according to the process of creating bovine sofa leather.[14]

Evaluation of deliming effect

(1) Evaluation of deliming degree of different methods

Ten points were marked on the pelt, and the thickness of each point was measured before and after deliming. Then, the degree of deliming was characterized by the average of thickness change rate of the 10 points.

$$\text{Change rate of thickness (\%)} = \frac{\text{the thickness of washed redelimed pelt} - \text{the thickness of washed delimed pelt}}{\text{the thickness of washed redelimed pelt}} \times 100$$

(2) Quantitative analysis of calcium content and distribution in delimed pelt

After being freeze-dried at −55°C and 20 Pa, the skin was evenly divided into three layers by a precision splitting machine (C520L, Camog (a) Inc., Italy). After cutting and ensuring a constant weight of each layer, the sample was accurately weighed and fully digested with sufficient nitric acid and hydrochloric acid at 120°C to form a clear solution. Then, the volume was fixed to 100 mL after cooling. The calcium concentration of the digested solution was measured by the AES-ICP method, and the calcium content of each layer in pelt was further calculated.[15,16]

(3) EDS analysis of calcium in delimed pelt[17]

After being freeze-dried, the delimed pelt was cut into samples of appropriate size. After being sprayed with copper, plane scanning of calcium element was performed by using an SEM-EDS energy spectrometer (Scanning Electron Microscope and X-ray Energy Dispersive Spectroscopy, JSM-7500F/X-MAX50, JEOL, Japan) with a control time of 5 min.

Evaluation of bating process

(1) Determination of soluble protein content in bating bath

The bating bath was centrifuged at 3500 rpm for 5 min every 60 min, and the supernatant was diluted with an appropriate amount. The soluble protein content of the bath was determined by Folin-phenol assay.[18]

(2) Determination of hydroxyproline and desmosine in bating bath

A total of 1.50 mL of bating bath was centrifuged at 8000 rpm for 6 min, and 1.0 mL of concentrated hydrochloric acid (12 mol/L) was added to 1.00 mL supernatant. The bath was fully dissolved at 120°C for 12 h. After deacidification, concentration, and dilution, the concentrations of hydroxyproline and desmosine in the bath were measured by using an A3000 amino acid analyzer (MembraPure, Germany).[19]

(3) Staining analysis of bated pelt tissue sections

The bated pelt was fixed with 10% neutral formaldehyde at room temperature for 24 hours, and the sections were cut into approximately 12 μm thick pieces in the longitudinal direction by using freezing microtome (CM1950, Leica, Germany). After drying, the skin was dyed by using the hematoxylin-eosin method and alkaline fuchsin method. Then, the distribution of elastic fibers and the dispersion degree of collagen fibers were observed by using an optical microscope.

(4) EDS analysis of carbon elements in bated pelt

The bated pelt was freeze-dried and cut into samples with an appropriate size. After being sprayed with copper, surface scanning of carbon elements on the cut surface was performed by using an SEM-EDS energy spectrometer with a control time of 5 min.

(5) Evaluation of grain pattern of the crust leather

The samples of the dried crust leathers of each tanning group were viewed microscopically using Desktop Phenom Pro Desktop Scanning Electron Microscope (Phenom Pro, Phenom World Inc., Netherland) to evaluate the grain pattern on the surface of leather samples.

Results

Property analysis of the delimed pelt

Bating is a heterogeneous catalytic reaction process in which the enzyme penetrates into the delimed pelt and acts on the corresponding substrate. The thickness, pH, and temperature of the delimed pelt have a great influence on the enzymatic activity. As can be seen from Table I, the four deliming methods, which use phosphoric acid-sodium dihydrogen phosphate, ammonium sulfate, and different amounts of EDTA-Na, can effectively neutralize the alkali in the limed hides. The pH of pelt can be controlled at 8.2–8.4, and the phenolphthalein was colorless in the vertical incision of pelt. After washing, the average thickness of the delimed hide was between 1.2 mm to 1.3 mm, and the thickness change rate of each delimed hide was between 31% to 37%. The four deliming methods achieved comparable deliming degrees on the limed hides, and the overall state of each delimed pelt is relatively similar, thus laying the foundation for further study on the influence of calcium content of pelt on enzyme action.

After being freeze-dried, the delimed pelt was evenly divided into three layers by using a precision layer splitting machine, and the calcium content of each layer was measured. The results shown in Figure 1 indicate that the calcium content of pelt is about 1.1% after deliming with sodium dihydrogen phosphate (method I). After two times of deliming with ammonium sulfate (method II), the calcium content of pelt was reduced to about 0.6%. When 1.0%

Table I
Deliming effectiveness of the series of methods

Deliming method*	I	II	III	IV
Deliming pH	8.2	8.3	8.4	8.4
Thickness of delimed hide (mm)	1.3	1.2	1.2	1.3
Thickness change rate of pelt (%)	33	35	37	31

*Deliming method: I - phosphate - sodium dihydrogen phosphate deliming, II - 2.5% ammonium sulfate pre-deliming, 1.5% sulfuric acid compound deliming, III - 2.5% ammonium sulfate deliming, 1.0% ammonium sulfate combined with 1.0% neutral EDTA-Na complex deliming, IV – 2.5% ammonium sulfate combined with 1.0% neutral EDTA-Na pre-deliming, 0.5% ammonium sulfate combined with 2.0% neutral EDTA-Na complex deliming,

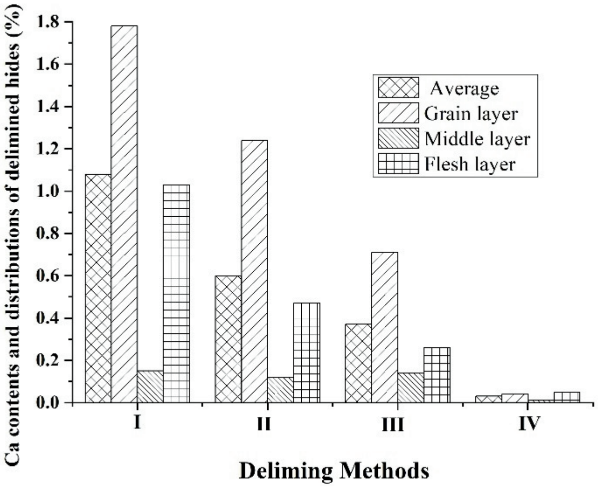

Figure 1. Ca content and distribution of delimed hide

EDTA-Na (method III) was used in redeliming with ammonium sulfate, the calcium content of pelt was further reduced to about 0.4%. When the neutral EDTA was sufficiently used in both pre-deliming and redeliming, which used ammonium sulfate (method IV), the calcium in pelt is almost completely removed, and the residual calcium content is about 0.03%. However, the calcium content in the middle layer of each delimed pelt was similar (except for method IV), and the difference of calcium content was mainly reflected in the difference of calcium content on the surface of the pelt.

The calcium ions were analyzed by using X-ray EDS (Figure 2). As the degree of calcium removal intensified, the calcium signal density at the incision was significantly reduced. When sodium dihydrogen phosphate was used in the deliming process, the distribution signal density of calcium in the papillary layer was significantly stronger than that in the middle and flesh sides, which shows that, deliming with sodium dihydrogen phosphate leads to calcium precipitation in the papillary layer. When ammonium sulfate was used alone for deliming (II), the signal density of calcium was stronger, and the signal density of calcium on both sides was relatively larger. This result occurred mainly because the calcium content on both sides of limed skin is higher than that of the middle layer, which was not effectively removed by deliming with ammonium sulfate alone. When combined with neutral EDTA-Na treatment of pelt (III and IV), the signal density of calcium in pelt was significantly reduced, and the uniformity was significantly increased, thereby again proving that EDTA could effectively remove calcium from the inside and the outside of pelt, while sodium dihydrogen phosphate mainly caused calcium precipitation on the surface of pelt. Therefore, the calcium content of pelt can be linearly changed by different deliming methods, and the bating effect of protease on delimed skin with different calcium content can be further investigated.

Effect of calcium content of pelt on enzymatic bating

After deliming, the main component of pelt is fibrous collagen, followed by elastin, which is mainly distributed in the papillary layer, and the interstitial proteins that have not been sufficiently removed. During the bating process, under the action of enzymes, non-structural proteins such as glycoprotein, albumin, and globulin within the pelt will be destroyed, and the structural proteins such as elastin and collagen will be partially dissolved, resulting in increased concentration of soluble proteins or peptides in the bath. Therefore, the combination of high-purity trypsin and microbial protease with high elastin activity was selected to bate the delimed pelt with different calcium contents. The overall effect of the enzymatic bating on the pelt could be analyzed by measuring the soluble protein content in the bath. As can be seen from Figure 3, with the decrease in the calcium content of pelt, the concentration of soluble protein in the bath increases sequentially, showing that the high calcium

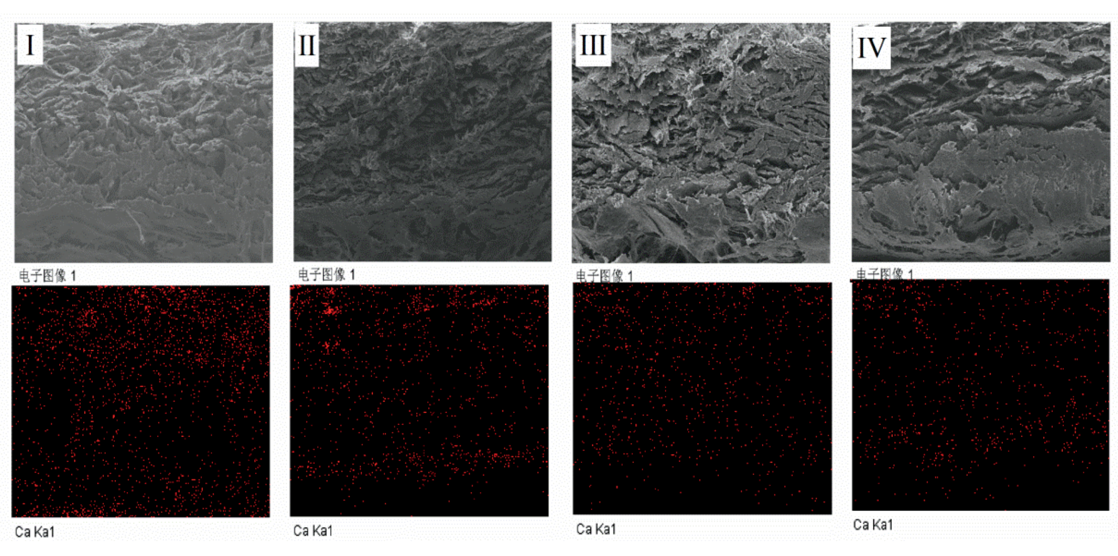

Figure 2. SEM-EDS analysis of the calcium on the bated pelts' longitudinal section

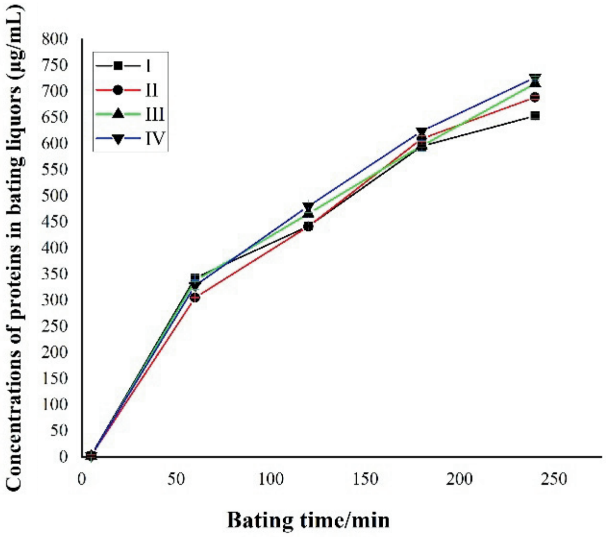

Figure 3. Concentrations of soluble protein in bating baths

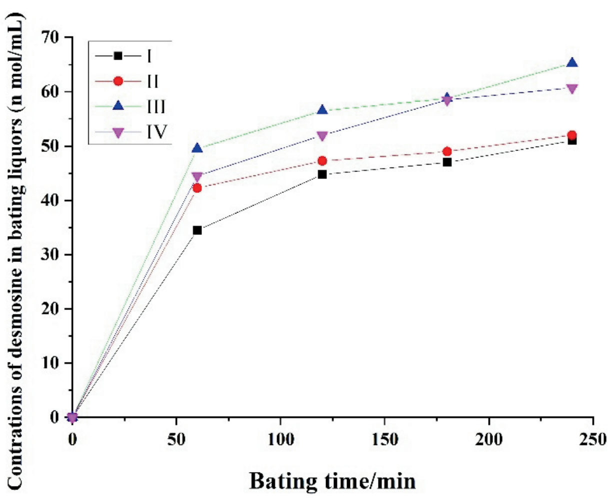

Figure 4. Effects of Ca content in grain layer on concentrations of desmosine in bating baths

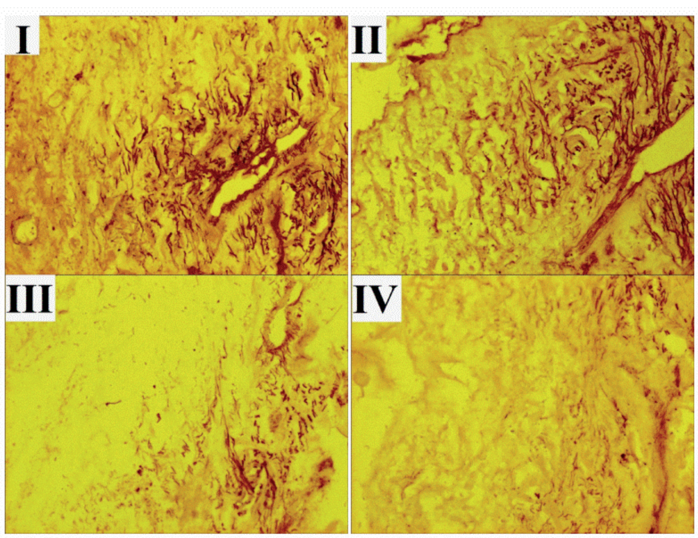

Figure 5. Elastin analyze of the bated pelt section (20 × 10)

content of delimed pelt will affect the enzymatic effect on the protein components of skin. However, when the average calcium content within delimed pelt was above 0.6%, the concentration of soluble protein in bath was relatively slow. When the calcium content of pelt was below 0.6%, the concentration of soluble protein increased rapidly.

Influence of calcium content of pelt on enzymatic bating with elastase

Elastin is a structural protein component in delimed pelt, second in abundance after collagen and is mainly distributed in the papillary layer. Its content has a great impact on the elasticity, compactness, and shaping of leather. Elastin has good acid and alkali resistance and can generally only be hydrolyzed by enzymes during the leathermaking process. Elastin contains desmosine. Thus, the hydrolysis rate of elastin by enzymes can be determined by analyzing the desmosine content in the bating bath. As can be seen from Figure 4, the concentration of desmosine in hydrolysate increased with the enhancement of the calcium removal from the grain layer. However, when the calcium content of the grain layer was above 1.2%, the calcium content of the pelt on the grain layer had no obvious influence on the action of enzyme on elastin. When the calcium content in the grain layer (Figure 1) decreased from 1.8% to 1.2%, the content of desmosine increased from 51 n mol/mL to 52 μg/mL only. When the calcium content decreased to 0.7%, the concentration of desmosine increased sharply to about 65 n mol/mL, indicating that strengthening the removal of calcium in pelt during deliming can significantly enhance the effect of protease on elastin.

The changes in the elastin fibers, which were derived by the bating of pelt with different calcium contents, were further reflected by histological staining results (Figure 5). As the degree of deliming increased, the elastic fibers of the bated pelt were destroyed to a greater extent. After deliming with sodium dihydrogen phosphate (I) and ammonium sulfate (II), the structure of elastic fibers of bated pelt remained relatively intact. The elastic fibers of bated pelt were almost completely destroyed when the calcium content within pelt was low after the deliming process combined with EDTA. This result was consistent with the trend in the concentration of hydrolysate (desmosine) in the bating bath.

Effect of calcium content of pelt on the collagenase effect

The main component of delimed pelt is collagen, which is also the main object of processing and utilization in leather production. The degree of hydrolysis directly affects the overall quality and style of leather, and is the main control point in the bating process, which is adjusted according to the performance requirements of different leather species. Therefore, the hydrolyzation of collagen were evaluated by hydroxyproline concentrations in the liquors and Histological staining, the results are shown in Figure 6 and Figure 7.

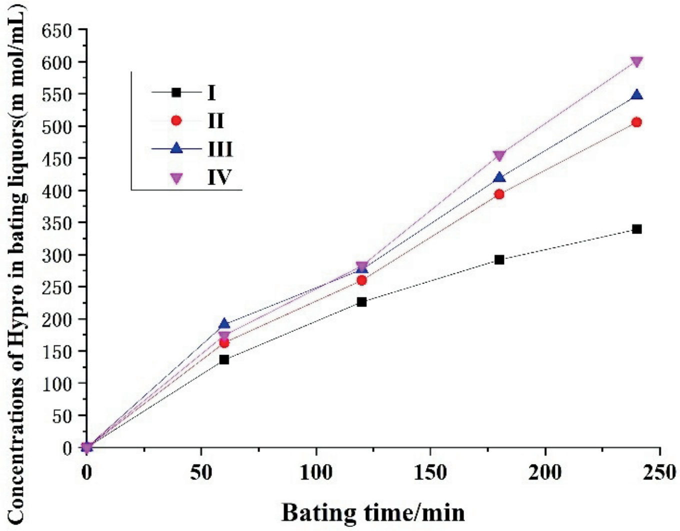

Figure 6. Effects of Ca content in delimed pelts on concentrations of hydroxyproline in bating baths

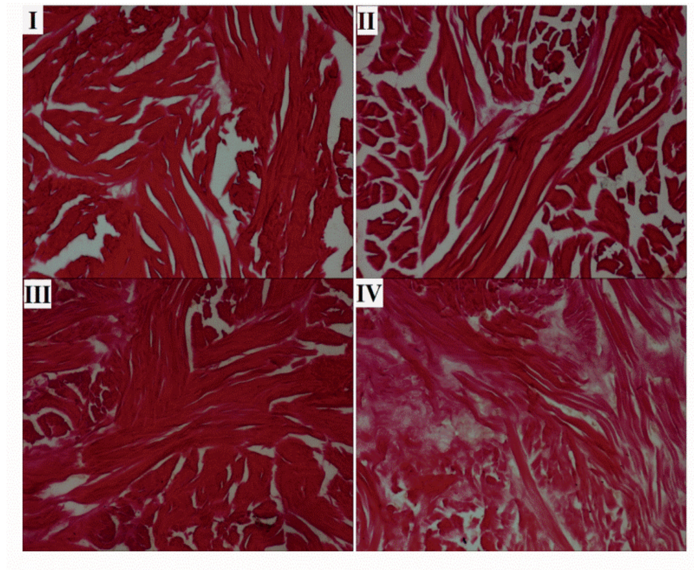

Figure 7. Collagen analysis of the bated pelts section (20 × 10)

As can be seen from Figure 6, the hydroxyproline in the bating bath increased gradually with the decrease in the calcium content of delimed pelt. Histological staining (Figure 7) showed that after enzymatic treatment, the fibrous bundles of bated pelt were thicker and showed a complete "bundle bar" when the calcium content of pelt was greater (I and II). However, with the decrease in the calcium content, the loosening of the collagen fibrous bundles gradually increased. When the calcium content was reduced to about 0.4% (III), the bunch-bar shape of the fibers started to be destroyed, and the fiber bundle showed a better loosening effect. When the calcium was completely removed from the delimed pelt (IV), the collagen fiber bundles dispersed into loose filaments. The carbon (C) elements in bated pelt were further analyzed by SEM-EDS, and the results (Figure 8) showed that when the calcium content was above 0.6% (I and II), the carbon signal density of the incision after the bating process was greater, and the difference was not obvious. This finding indicates that the dispersion degree of the fibers in the pelt had minimal difference. The carbon signal density on the incision of bated pelt decreased significantly with the further decrease in the calcium content, indicating that the fiber dispersion was enhanced.

As mentioned before, excessive hydrolysis on collagen by enzymes result in damaging grain is one of the potential risks of bating. The electron micrographs (Figure 9) illustrate that with the decrease of calcium content in the delimed pelts, the leather grains are damaged, the patterns are not smooth and clearly visible. Therefore, the grain patterns would be protected from

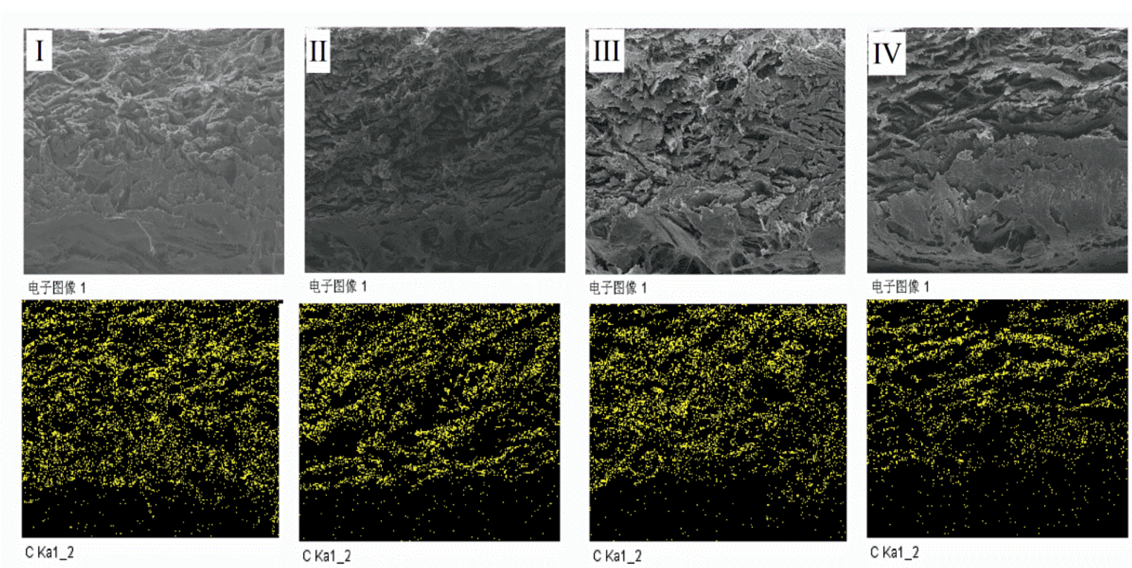

Figure 8. SEM-EDS analysis of carbon on the bated pelts' longitudinal section

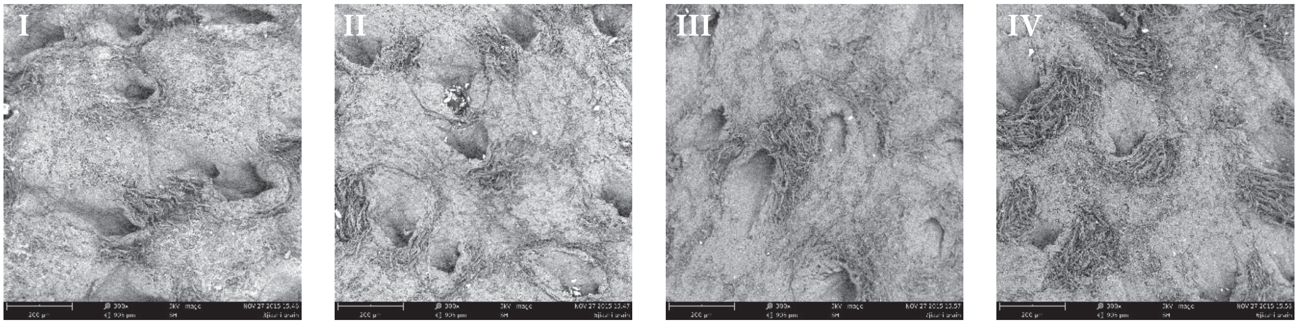

Figure 9. SEM images of grain pattern of crust leather (×300, 5KV)

being damaged by the deposited Ca with phosphate, which would be removed during pickling.[11]

Analysis of enzymatic mechanism of delimed pelt by calcium content and its practical application

Bating is one of the most difficult processes to control in the leather preparation process. The enzymatic degree on collagen and elastin needs to be adjusted in time according to the performance requirements of different leather species, and improving the controllability and safety of the bating process are always the main concern in leather production.

In the limed pelts, calcium exists in three forms, including free calcium ions, dissolved/adsorbed calcium hydroxide, and calcium bound to collagen. The calcium bound to collagen changes the surface charge of collagen fibers, and weak cross-linkages formed between collagen fibers. Thus, the enzyme resistance of collagen fibrils improved.[21] In the conventional deliming process using ammonium sulfate, the free calcium ions and calcium hydroxide in the limed skin can be effectively removed by neutralizing and dehydrating, but the calcium ions bound to collagen cannot be effectively removed. In the deliming process with phosphoric acid and phosphate, calcium phosphate precipitates are formed in the pelt (especially on the surface of the pelt) to coat the protein surface, which hinders the enzymatic effect to a certain extent (Figure 3). However, this method does not make the calcium bound to structural proteins, such as collagen and elastin within the pelt, rather it makes the enzymatic effect non-significant. In the deliming combined with ammonium sulfate and EDTA-Na (methods III and IV), the calcium bound to proteins within the pelt could be removed more effectively through the chelation of calcium by EDTA-Na.[22] This approach reduces the enzyme resistance of collagen and elastin, and significantly enhances the enzymatic effect.

For the production of leather items with high grain tightness requirements, such as shoe upper leather, it is necessary to improve the binding efficiency of calcium ion with the pelt, enhancing the enzyme resistance of collagen and elastin, which prevents the excessive enzymatic effect on elastin and collagen during the bating process. In the preparation of leather products such as garment leather, sofa leather, and leather where grain spreading and leather softness are required, a calcium chelating agent should be used to enhance the degree of calcium removal and improve the enzymatic efficiency.

Controlling the calcium content of delimed pelt can effectively regulate the effect of enzymes on elastic fibers and collagen fibers. This study is of great significance to ensure uniform enzymatic action during the bating process.

Conclusion

The calcium content of delimed pelt can be controlled effectively by using phosphoric-phosphate, ammonium sulfate, and EDTA-Na during the deliming process. The calcium content of delimed pelt can affect the performance of enzymes during the bating process. Adjusting the content and form of calcium in delimed pelt enables the regulation of the degree of enzymes' action on collagen and elastin during the bating process, thus improving the controllability of the bating process of different leather species.

Acknowledgements

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