Diffusion and Reaction Behavior of Proteases in Cattle Hide Matrix via FITC Labeled Proteases

by

Jianzhong Ma,*[1,2] Xueyan Hou,[1] Dangge Gao[1] and Jing Zhang[3]

[1]*College of Resources & Environment,*
Weiyang Campus, Shaanxi University of Science & Technology,
Xi'an 710021, Shaanxi Province, PR China

[2]*Key Laboratory of Auxiliary Chemistry and Technology for Chemical Industry,*
Ministry of Education, Shaanxi University of Science & Technology
Xi'an 710021, Shaanxi Province, PR China

[3]*College of Foreign Languages and Communications,*
Shaanxi University of Science & Technology,
Xi'an 710021, Shaanxi Province, PR China

Abstract

Enzymes play an important role in the biological treatment of animal skin collagen in the leather making process. In this work, three proteases (2709, LimeG and SoakL) with different molecular weight were used in the treatment of cattle hide. Protein and hydroxyproline absorbance was measured to evaluate the treatment effectively. Proteases labeled by Fluorescein Isothiocyanate (FITC) were used to treat the cattle hide to observe the diffusion and reaction behavior of proteases in cattle hide matrix. The results indicate that when the cattle hide was treated with smaller molecular weight protease, the degradation degree of the protein and collagen was more than that of the cattle hide treated with larger molecular weight protease. The fluorescence microscopy images demonstrate that during the early stages, proteases chiefly diffused into the cattle hide matrix through pores and hair follicles, and then diffused into the inner layer via hair follicles to hydrolyze inter-fibrillary proteins for opening up collagen fibers. In the present investigation, a visible assessment for the diffusion and reaction behavior of proteases in the enzymatic treatment of cattle hide matrix was reported.

Introduction

As a kind of biomaterials, animal skin may often have been discarded after humans ate the meat. However, animal skin collagen is the raw material for leather manufacture. Cattle hide is one of the most important leather-making raw materials. The constituents of cattle hide are very complex, including collagen fibers and many other inter-fibrillary proteins, such as albumin, globulin, mucin, mucinoid, glycosidoprotein. Among those, collagen fibers are the main constituent parts needing to be protected in the leather making process. On the contrary, other components have to be removed by chemical or physical methods. The common chemicals are surfactants, acids, alkalis and salts, but most of these chemicals lead to increased pollution of the environment.[1] As an environmentally friendly material, enzymes have been widely applied in cosmetics, medicines, food, the leather industry[2-5] and others fields[6-8] because of their excellent properties. In the leather industry, enzymes are usually used to treat animal skins to remove the non-collagen components.[9-12] Up to the present, however, essential theoretical research on enzymatic treatment of animal skins is still lacking. For example, the action mechanism of enzymes in the animal skin matrix was not clear. Cattle hide matrix, an insoluble substrate, is different from soluble substrates. For example, for some soluble substrates in enzymatic reactions, enzymes dissolve into the solution together with soluble substrates and then react regardless of the enzyme size. However it is necessary to protect cattle hide matrix in leather making process. When cattle hide is treated by enzymes to remove the non-collagen proteins, enzymes have to permeate into the cattle hide matrix and contact with the proteins inside the cattle hide matrix, and then enzymes will hydrolyze the inter-fibrillary proteins.

*Correspondence author e-mail: majz@sust.edu.cn, Tel.: +86 29 8616 8010.
Manuscript received October 3, 2013, accepted for publication February 6, 2014.

Therefore, the molecular weight of enzymes has an effect on the enzymatic treatment of cattle hide matrix. It may be difficult for larger molecular weight enzymes to enter cattle hide matrix, but the larger molecular weight enzymes hydrolyze the collagen on the cattle hide surface unduly instead. Maybe it is better to choose appropriate molecular weight enzymes in different leather making processes according to the specific collagen fiber structure. It has been reported that the lower molecular weight proteases were easier to diffuse into the wool, leading to extended fiber damage, which was the major obstacles for the use of proteases for wool finishing applications, but the larger molecular weight protease controlled enzymatic hydrolysis of wool surface.[13]

In order to determine the distribution of enzymes in animal skin matrix, researchers have already done some work. Proteases in different sections of the pelt unhaired by enzymes were transferred to casein, and the degradation reaction of enzyme and casein would produce white spots which could be used to determine the enzyme penetration rate and quantity.[14] However, the limitation was that if enzymes penetrated into the skin matrix, which had already lost activity, enzymes could not be determined. No better and easier method for detecting the presence of enzymes and their diffusion behavior in the animal skin matrix has been reported so far.

FITC is a well-known probe for labeling biologically relevant proteins.[15-16] Strong fluorescence of FITC can be observed as a yellow-green substance at excitation wavelength of 490-495nm on an epifluorescence microscope or under UV illumination.[15] FITC can react with the amino-group of proteins. Therefore, FITC labeled proteins have been used in much research to achieve visualization investigation.[13, 17-19] In this study, FITC labeled proteases were used to detect the position and penetration of proteases in the cattle hide matrix and provide a visible method to keep track of the diffusion or penetration behavior of proteases when the cattle hide matrix was treated with protease. By this means, the behaviors of several proteases with different molecular weight were compared in the diffusion and hydrolytic attack to the cattle hide. At the same time, the diffusion and reaction mechanisms of proteases in the enzymatic treatment process were proposed.

Materials and Methods

Materials

Fluorescein Isothiocyanate (Sigma) and dialysis bags were purchased from Hefei Bomei Biotechnology Co., Ltd, China. Foline-Phenol (Sigma F-9252) was purchased from Shanghai Just Scientific Co., Ltd. Proteases (Genencor LimeG and Genencor SoakL) were provided by Genencor Bio-Products Co., Ltd. Alkaline protease 2709 was purchased from Beijing Donghua Qiangsheng Biotechnology Co., Ltd. Casein, a biochemical reagent, was purchased from Beijing AOBOX Biotechnology Co., Ltd. Antiseptic KL, degreasing agent DN and auxiliaries WT-H were of commercial grade, provided by Sichuan Desaier Chemical Industry Co., Ltd. Other reagents were of analytical grade. Wet salted cattle hide was provided by a tannery.

Instrumentation

A TU-1900 Double Beam UV-vis Spectrophotometer (Beijing Persee General Instrument Co., Ltd.) was used to determine protein and hydroxyproline in the enzymatic bath. Enzymatic treatment of cattle hide matrix was performed on a SHZ-A Immersion Oscillator (Shanghai Pudong Physical Optical Instrument). A TFM-300D Trinocular Upright Fluorescence Microscope (Shanghai Tuanjie Instrument) was used to detect the FITC labeled proteases in the cattle hide matrix. The molecular weight of proteases was measured on a DIONEX ultimate 3000 Liquid Chromatograph equipped with PLaquagel-OH Mixed Column.

Determination of the Protease Activity

The activity of protease was determined by UV Spectrophotometry according to the national standard.[20] The 0.5% (w/v) protease solutions and 1% (w/v) casein solution were prepared using 0.1M Glycine-NaOH buffer (pH9.0 for SoakL and 2709, pH10.0 for LimeG). Then 1mL protease solution and 2mL casein solution were missed and incubated at 40 for 10min. The reaction was stopped by adding 4mL 0.4mol/L trichloroacetic acid (TCA). The mixture was settled at room temperature for 15min and filtered. The absorbance of the filtrate was measured at 275nm by UV Spectrophotometer. Then the concentration of tyrosine was calculated. Blanks were also prepared in the same way except that TCA was added prior to casein solution.

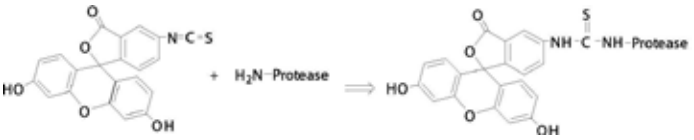
Enzymatic Treatment of Cattle Hide Matrix

Enzymatic treatment was carried out in a flask with a bath ratio of 1:200 (the mass of cattle hide to water) in an Immersion Oscillator. The dosages of chemicals were calculated on the basis of cattle hide weight. The hide in this study was fleshed pre-soaking hide. All samples were from a single hide. They were cut into 10cm×10cm pieces and soaking by using 0.1% (w/w) Antiseptic KL, 0.1% (w/w)degreasing agent DN (non-ionic surfactant), 0.3% (w/w) auxiliaries 0.4% (w/w) WT-H and Na_2CO_3 , shaken at 25°C for 1h, then 20 units of protease for per gram cattle hide were added to the bath. At intervals, the enzyme bath withdrawn was instantly kept in boiled water for 10min to inactivate proteases[21] and then the bath samples withdrawn were analyzed to determine the released protein and hydroxyproline.

FITC Labeled Protease

Proteins, including proteases, contain a large number of primary amino groups. The FITC molecule contains active isothiocyanate group (–N=C=S), which can easily react with

primary amine group of the protease molecule at alkaline pH to form thiourea bond.[22] The labeled reaction is shown as follows.



To investigate the diffusion of proteases with different molecular weights, proteases were fluorescently labeled with FITC. The cattle hide matrix was treated with FITC labeled protease solutions, and then the longitudinal sections of cattle hide were observed on a TFM-300D Trinocular Upright Fluorescence Microscope.

The FITC labeled protease reaction was carried out under alkaline and dark conditions to avoid the fluorescein loss of fluorescence. Phosphate buffer of pH 8.0 was used for the preparation of FITC solution and proteases solution. The 0.2mL 1mg/mL FITC solution was slowly drop-wise added to 10mL 2mg/mL protease solution, stirred in dark condition at 4°C for 8h. After the labeled reaction, free FITC was removed through dialyzing for 24h, and the dialysate was changed every 2h over night until the absorbance of dialysate at 495 nm was approximately zero because the maximum absorption wavelength of FITC was 495 nm. Then the FITC labeled protease solutions were diluted to 45mL and 10cm×10cm pre-soaking and fleshed cattle hide was treated in each of the FITC labeled protease solutions at 25°C, shaken for 24h. Then the treated cattle hide was washed with distilled water and then frozen and sliced. The longitudinal sections of cattle hide were analyzed on a TFM-300D Trinocular Upright Fluorescence Microscope. Figure 1 is a schematic that illustrates the idea of this research.

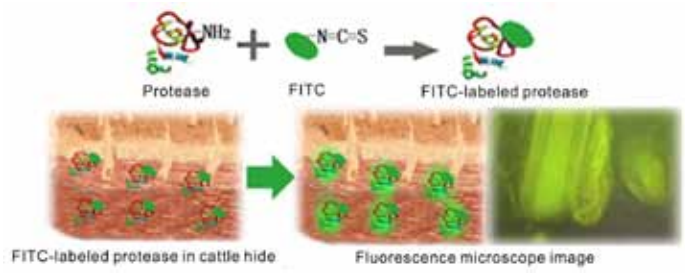


Figure 1. The schematic of FITC labeled protease to detect the behavior of protease

Analysis

Determination of protein in the enzymatic bath

The proteins hydrolyzed by proteases in the bath were determined according to Folin-Lowry's method. In this method, proteins reacted with Foline-phenol reagent to form a blue substance, which could be measured at 640 nm on a TU-1900 Double Beam UV-vis Spectrophotometer. The protein absorbance in this study was the absorbance of complexes of the protein and chemicals at 640nm. The protein absorbance was the average of two replications.

Determination of Hydroxyproline in the Enzymatic Bath

The determination of hydroxyproline was carried out according to the chloramine-T oxidation method. The sample was hydrolyzed in hydrochloric acid and release hydroxyproline, which was then oxidized by the chloramine-T to produce oxides containing pyrrole ring. Perchloric acid destroyed the excess chloramine-T. The hydroxyproline oxide reacted with dimethylaminobenzaldehyde to form red compounds, which could be determined at 558nm on a TU-1900 Double Beam UV-vis Spectrophotometer. The hydroxyproline absorbance in this study was the absorbance of complexes of the hydroxyproline and chemicals at 558nm. The hydroxyproline absorbance was the average of two replications.

Determination of Protease Molecular Weight

The molecular weight of protease was recorded on a DIONEX ultimate 3000 Liquid Chromatograph equipped with PLaquagel-OH Mixed Column, 300*7.5mm. The standard molecular weight samples were polyethylene glycol (PEG) and polyethylene oxide (PEO) in the range of 106-442800Da. The mobile phase was 0.02% $NaNO_3$ aqueous solution, and the column temperature was 40°C with flow rate of 1.0mL/min.

RESULTS AND DISCUSSION

Enzymatic Treatment of Cattle Hide Matrix

Enzymatic hydrolysis was very complicated which might be affected by many factors, including the type, specificity[11] and optimal reaction conditions of enzymes. Even the enzyme molecular weight plays an important role in the enzymatic treatment of some special insoluble substrates, such as cattle hide matrix and wool[13]. In this experiment, three proteases (LimeG, SoakL and 2709) with different molecular weights were used in the cattle hide enzymatic treatment. The molecular weight of proteases was determined by a DIONEX 3000 Liquid Chromatograph. The weight average Mws of Lime G, Soak L and 2709 are listed in Table I.

TABLE I
Molecular weights of 2709, Lime G and Soak L.

Proteases	2709	Lime G	Soak L
Weight Average Mw(kDa)	9,000	141,000	198,000

In the treatment process, proteases hydrolyzed protein in the cattle hide matrix and released protein and hydroxyproline from the hide matrix. The protein and hydroxyproline contents

in the enzymatic bath were represented as absorbance of the complexes of the protein or hydroxyproline with the chemicals at 640nm and 558nm respectively. The released protein and hydroxyproline absorbance treated with different proteases are shown in Figure 2 and Figure 3, which indicate that both protein and hydroxyproline absorbance was time-dependent whether in presence of proteases or not.

The protein absorbances in the enzymatic bath of cattle hide treated with LimeG, SoakL, 2709 and the control (without protease) were very different with the increase of treating time. For the control, the least protein was released with a slower releasing rate. The protein-releasing behavior of cattle hide treated with LimeG was very similar to that of the control. When the cattle hide was treated with 2709, more protein was released and the released rate was faster than that of the cattle hide treated with LimeG and SoakL, but the protein absorbance of cattle hide treated with SoakL was between that of the cattle hide treated with LimeG and 2709. Overall, for the four treating processes with different proteases and the control, proteins increased fast in the first 2h because most of the soluble proteins on the surface of cattle hide dissolved in water or some proteins on the surface were hydrolyzed by proteases and the proteins released in the bath rapidly. After 2h, proteases began to diffuse into the cattle hide matrix and hydrolyze the protein slowly and led to the slower increasing rate of protein. As the treatment time prolonged, the proteases gradually lost activity and had less hydrolysis action on the protein. After 15h, the released protein decreased correspondingly because there was also less soluble protein remaining after 15 hours.

As is known to all, collagen is a kind of protein and hydroxyproline is the collagen-specific amino acid. Proteases inevitably attacked collagen in the enzymatic treatment of cattle hide matrix. The higher the concentration of hydroxyproline in the enzymatic treatment bath was, the more serious the impairment in the cattle hide collagen was[21]. In this experiment, the damage to collagen was determined in terms of the concentration of hydroxyproline in the enzymatic treatment bath. Similar to the protein absorbance, the hydroxyproline absorbance in Figure 3 indicates that hydroxyproline increased rapidly in the first 4h. Subsequently, the increasing rate became slower. This may be explained by the fact that at the initial stage, the proteases had higher activity and hydrolyzed collagen. After 4h, the proteases began to lose activity and lead to a weaker hydrolysis of the collagen. The hydroxyproline concentration in the bath treating with the proteases and the control was in descending order: 2709≈LimeG>SoakL>control. The molecular weights in Table 1 indicate that the molecular weights of the three proteases were in descending order: SoakL, LimeG, and 2709. Because almost 95%-98% collagen was located at the grain layer and reticular layer of cattle hide[23] the released hydroxyproline concentration was related to the protease molecular weight to some degree. The smaller the molecular weight of protease was, the higher the concentration of hydroxyproline was. However, high molecular weight proteases could hardly penetrate into the grain layer and the reticular layer in a short time. Therefore, larger molecular weight proteases just stayed in the upper levels of the cattle hide matrix that contained less collagen, while smaller molecular weight proteases could enter the inner layer among collagen fibers to damage collagen and released hydroxyproline eventually.

The Effect of FITC on the Activity of FITC Labeled Protease

The activities of the proteases labeled by FITC and the free proteases have been determined by Folin method in order to investigate the effect of FITC on the proteases activities. Figure 4 indicates that the activities of FITC-LimeG, FITC-SoakL and 2709 were close to those of the free LimeG, SoakL and 2709. This result was consistent with the literature reported.[24] Therefore; the FITC labeled proteases would have

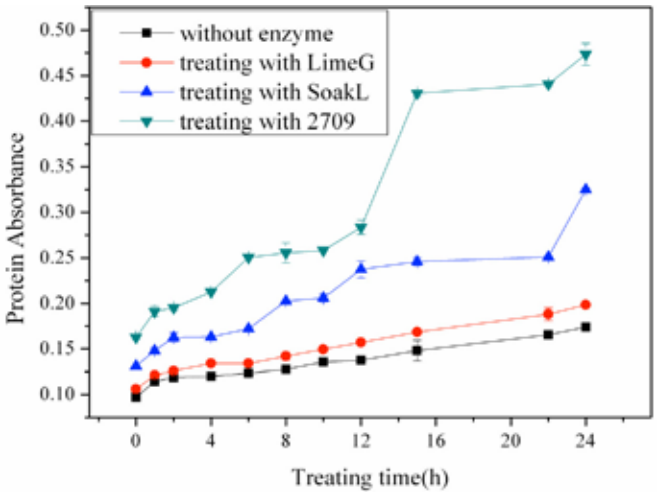


Figure 2. Protein absorbance in the bath treated with different proteases

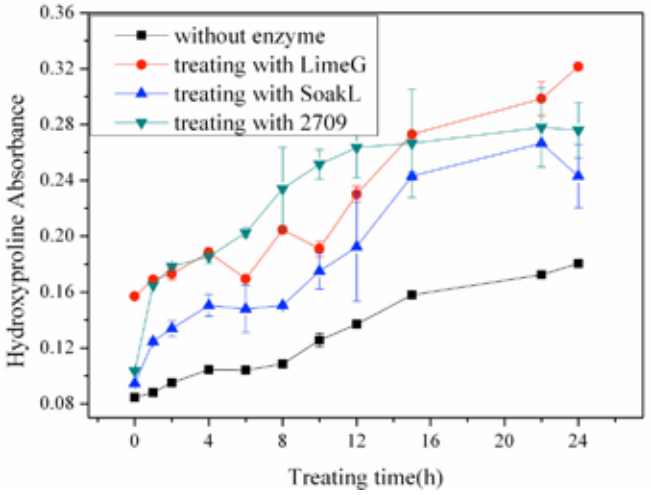


Figure 3. Hydroxyproline absorbance in the bath treated with different proteases

equivalent hydrolysis capability in the cattle hide enzymatic treatment process. On the other hand, with a low molecular weight, FITC would not affect the molecular weight of the proteases. The penetration and hydrolysis of FITC labeled proteases could reflect those of the free proteases in the cattle hide matrix equally.

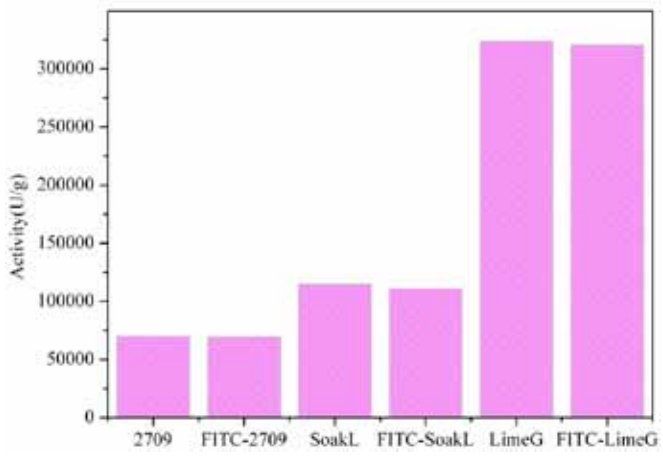


Figure 4. Activity of the free proteases and FITC labeled proteases.

Effect of Molecular Weights of Proteases on the Diffusion

In the fluorescence microscopy images (Figure 5), the green parts were FITC labeled proteases. The number A(A0,A1), B(B0,B1) and C(C0,C1) represent FITC-2709, FITC-LimeG and FITC-SoakL distributed in the different levels of the cattle hide matrix when the treating time was 12h and 24h. It is obvious that most of the proteases distributed widely in the cattle hide matrix after being treated for 24h, but the proteases only existed around the hair follicles when being treated for 12h. This indicates that during the diffusion process, most of the proteases diffused from pores into hair follicles but not through the epidermis at the initial stage because the epidermis is mainly composed of keratin, which has strong resistance to enzymes, as does collagen. General protease hardly hydrolyzes the natural keratin due to the close keratin alpha helix and a large number of peptide chains disulfide bonds on the natural keratin[23]. In the cattle hide enzymatic process, the proteases first diffused into the hair follicles might hydrolyze the protein around the hair follicles and promote the penetration of water to dissolve the soluble protein. Then the channel was opened and more proteases enter the cattle hide matrix. With the treating time prolonged, proteases diffused in the cattle hide matrix and degraded the inter-fibrillary proteins to disperse the collagen fibers.

Comparing A0, B0 and C0, we found that before the cattle hide matrix was treated with FITC-2709 for 12h, FITC-2709 had already diffused to the bottom of the hair follicles. When the cattle hide matrix was treated with FITC-LimeG for 12h, FITC-LimeG existed around the hair follicles. When the cattle hide matrix was treated with FITC-SoakL for 12h, most of

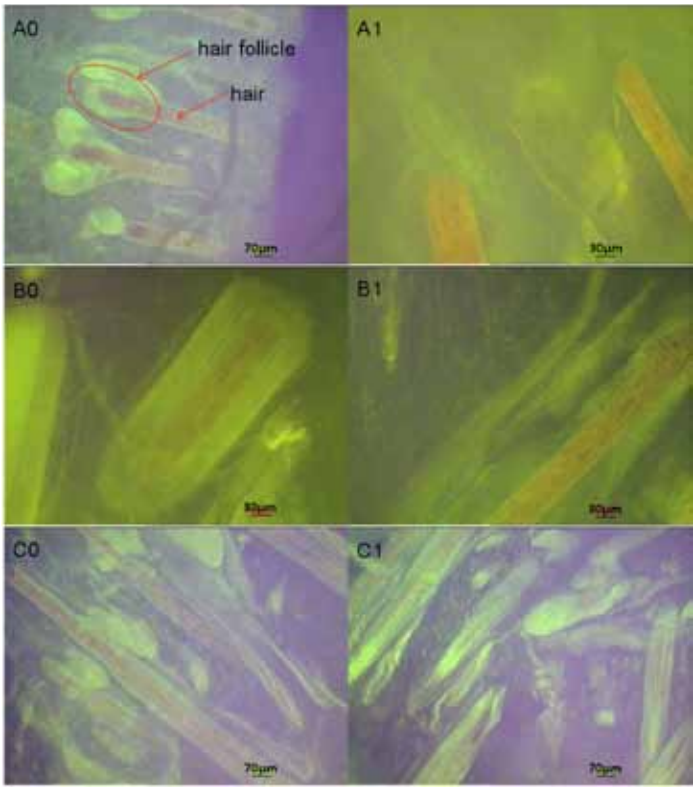


Figure 5. Fluorescence microscopy images of longitudinal sections of cattle hide treated with FITC labeled protease: (A0) FITC labeled 2709 treated cattle hide for 12h; (A1) FITC labeled 2709 treated cattle hide for 24h; (B0) FITC labeled LimeG treated cattle hide for 12h; (B1) FITC labeled LimeG treated cattle hide for 24h; (C0) FITC labeled SoakL treated cattle hide for 12h; (C1) FITC labeled SoakL treated cattle hide for 24h.

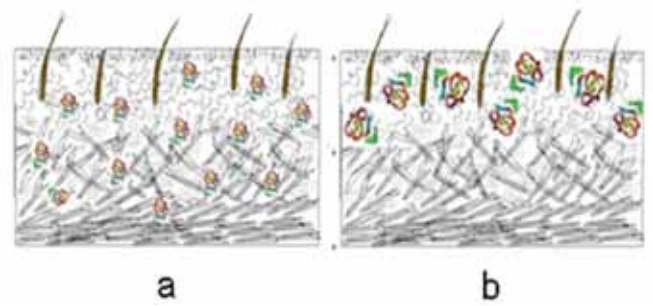


Figure 6. Schematic diagram: effect of protease molecular weight on the penetration of cattle hide matrix. a) smaller molecular weight protease diffusing into inner layer of cattle hide matrix; b) larger molecular weight protease diffusing into upper layer of cattle hide matrix.

FITC- SoakL existed around the upper half of the hair follicles and only a small part penetrated into the middle and lower parts of the hair follicles. This result indicates that proteases with smaller molecular weight were more easily to penetrate into the inner cattle hide matrix. On the contrary, larger molecular weight proteases might diffuse in the upper layer of the cattle hide matrix.[25] Figure 6 reveals the diffusion schematic diagram of larger and smaller molecular weight proteases in the cattle hide matrix. Figure 6a is the diffusion

schematic diagram of smaller molecular weight protease in the inner layer of cattle hide matrix, while figure 6b is the diffusion schematic diagram of larger molecular weight protease in the upper layer of cattle hide matrix. This may explain the preliminary result (Figure 2 and Figure 3) that the hydrolysis degree of different molecular weight proteases to the protein and collagen in the cattle hide was different.

Diffusion and Reaction Mechanism of Proteases in Cattle Hide Matrix

The cattle hide matrix is usually divided into four layers from upper epidermis to the flesh side, including epidermis, grain layer, reticular layer and subcutaneous tissues. The composition and collagen fiber weave of each layer are quite different.[26-28]Epidermis is the outmost layer of a skin. The main composition of epidermis is keratin, which has to be removed in the leather making process. Grain layer contains fibers, muscles, arteries, sweat glands, sebaceous glands, hair, et al and most of these have to be removed in different operations. In grain layer, the fibers are thin and loosely woven. Reticular layer is the most important part for leather because it contains most of collagen fibers tightly woven with the angle of weave approximately 45° that are beneficial to the strength of the leather. Subcutaneous tissues is mainly fatty adipose tissue, which must be mechanically removed at the initial stage.

According to the protein and hydroxyproline releasing behavior (Figure 2 and Digure 3) and the fluorescence microscopy images (Figure 5), the mechanism model of proteases diffusion and reaction was proposed in Figure 7. Because of the special structure of cattle hide matrix, the larger interstices between hair and hair follicles or pores made proteases first get into the matrix chiefly through hair pores or hair follicles and diffuse into the inside layer slowly. Simultaneously the penetrated proteases hydrolyzed protein around the hair follicles in stage b. As the increase of hydrolysis, some proteases entered the grain layer through hair follicles immediately but not through epidermis. In grain layer, proteases hydrolyzed more inter-fibrillary proteins, promoting the penetration of more proteases. Then from stage b to c, some soluble protein dissolved in water and the insoluble protein was hydrolyzed by the penetrated proteases. The space between the fibers became larger and larger, which was good for the later penetration of the proteases. At the same time, some proteases approached the flesh side and acted on the subcutis, but the proteases diffusion rate from flesh side was much slower than that from the grain layer because of the dense tissue structure and insoluble subcutis. From stage c to d, the fibers opened up gradually and became looser. At this time, the non-keratinised components were destroyed[29] and the epidermis was damaged to some degree because of alkaline condition for long time, and the majority of proteases dispersed in the cattle hide matrix through both epidermis and the flesh side.

Conclusion

The present study reported a visible method via FITC labeled proteases to investigate the diffusion and reaction behavior of proteases in the cattle hide matrix. The way proteases got into the cattle hide matrix was that the vast majority of proteases first entered hair follicles and then diffused through hair follicles into the grain layer and reticular layer, hydrolyzing the inter-fibrillary proteins. Moreover, the diffusion of proteases into the cattle hide matrix was dependent on molecular weight of proteases. Smaller molecular weight proteases were easier to diffuse into cattle hide matrix and degrade the inter-fibrillary proteins to disperse the collagen fibers.

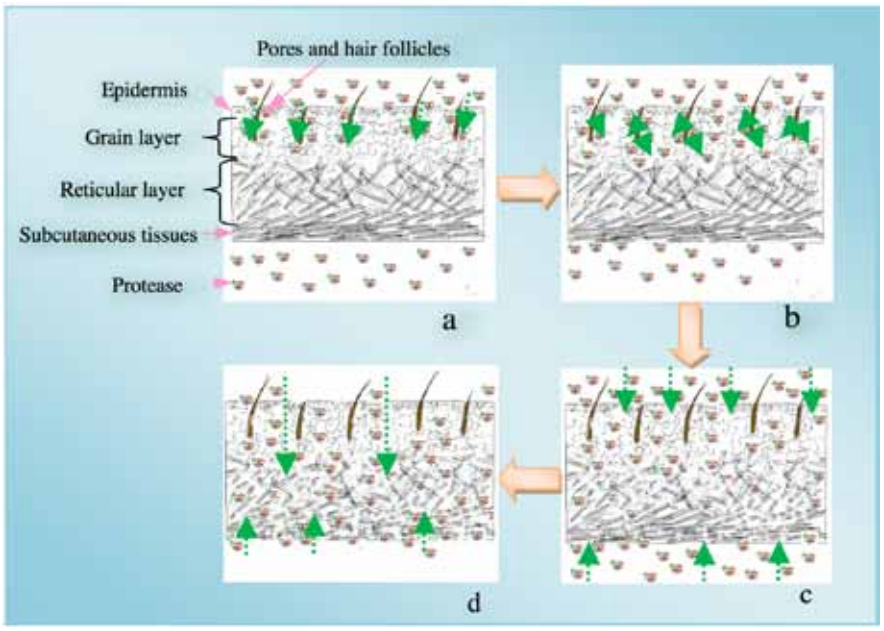


Figure 7. Mechanism model of protease penetration and reaction in cattle hide matrix. The green dashed arrows indicate the direction of protease diffusion.

ACKNOWLEDGEMENTS

This project was supported by Youth National Science Foundation of China (21104042), Doctor Science Research Foundation of the Education Ministry of China (20106125120003) and Graduate Innovation Fund of Shaanxi University of Science and Technology. We thank Shanghai Tuanjie Instruments Co., Ltd for providing TFM-300D Trinocular Upright Fluorescence Microscope, Northwest University for helping us with molecular weight measurement of proteases.

REFERENCES

1. Thanikaivelan, P., Rao, J.R., Nair U.B., Ramasami, T.; Progress and recent trends in biotechnological methods for leather processing. *Trends in Biotechnol*. **22**, 181–188, 2004.
2. Dettmer, A., Anjos, P. and Gutterres, M.; Special review paper: Enzyme in the leather industry. *JALCA* **108**, 146-158, 2013.
3. Swarnalatha, B., Sairam, G. Sekaran; Production of alkaline protease by Pseudomonas aeruginosa using proteinaceous solid waste generated from leather manufacturing industries. *Bioresour. Technol*. **99**, 1939–1944, 2008.
4. Ma, J.Z., Yang, X.Y., Gao, D.G., Lv, B., Yu, Y.; Study of JPK-1 Compound Enzyme, *Fine Chemicals* **25**, 894–899, 2008.
5. Liu, L., Liu, Q., Li, J., Du, G., Chen, l.; Characterization of Gelatin and Casein Film Modified by Microbial Transglutaminase and the Application as Coating Agents in Leather Finishing. *JALCA* **107**, 13-20, 2012.
6. Kumar, A, General, T., Bhaskar, N., Suresh,V., Sakhare, Z., Halami, M., Gowda,R., Mahendrakar, N.; Utilization of tannery fleshings: Optimization of conditions for fermenting delimed tannery fleshings using Enterococcus faecium HAB01 by response surface methodology. *Bioresour. Technol*. **101**, 1885–1891, 2010.
7. Brougher, D.S., Oleas, T.B., Kohn, R.A.; Interactions of α-Amylase and calcium chelator during neutral detergent fiber analysis. *J. Agric. Food Chem*. **53**, 5716–5718, 2005.
8. Eker, B., Zagorevski, D., Zhu, G.Y., Linhardt, R.J., Dordick, J.S.; Enzymatic polymerization of phenols in room-temperature ionic liquids. *Mol. Catal. B: Enzym*. **59**, 177–184, 2009.
9. Kanth, S.V., Venba, R., Madhan, B., Chandrababu, N.K., Sadulla, S.; Cleaner tanning practices for tannery pollution abatement: Role of enzymes in eco-friendly vegetable tanning. *J. Cleaner Prod*. **17**, 507-515, 2009.
10. Palop, R., Marsal. A., Cot, J.; Optimization of aqueous degreasing process with enzymes and its influence on reducing the contaminant load. *The Society of Leather Technol. Chem*. **84**, 170–176, 2000.
11. Puvanakrishnan, R., Manohar, B.M., Rajaram, A., Puvanakrishnan, R.; Ecofriendly lime and Sulfide free enzymatic dehairing of cattle and hides using a bacterial alkaline protease. *Chemosphere* **70,** 1015–24, 2008.
12. Foroughi, F., Keshavarz, T., Evans, C.S.; Specificities of proteases for use in leather manufacture. J. *Chem. Technol. Biot*. **81,** 257–261, 2006.
13. Araujo, R., Silva, C., Machado, R., Casal, M., Cunha, A.M., Rodriguez-Cabello, J.C., Cavaco-Paulo, A.; Proteolytic enzyme engineering: A tool for wool. *Biomacromolecules* **10**, 1655–1661, 2009.
14. Yang, Y.Z.; Test of enzyme in dehaired pelt. *China Leather* **31**, 20–22, 2002.
15. Hoffmann, C., Dudal, J.L., Patel, S., Gallet, O., Pauthe, E.; Fluorescein isothiocyanate labeled human plasma fibronectin in extracellular matrix remodeling. *Anal. Biochem*. **372**, 62–71, 2008.
16. Thiele, C, Ganzle, M.G. and Vogel, R.F.; Fluorescence labeling of wheat proteins for determination of gluten hydrolysis and depolymerization during dough processing and sourdough fermentation. *J. Agric. Food Chem*. **51**, 2745–2752, 2003.
17. Homer, K. A. and Beighton, D.; Fluorometric determination of bacterial protease activity using fluorescein isothiocyanate labeled proteins as substrates. *Anal. Biochem*. **191**, 133–137, 1990.
18. Law, B. and Tung, C.H.; Structural Modification of Protease Inducible preprogrammed nanofiber precursor. *Biomacromolecules* **9,** 421–425, 2008.
19. Chen, J., Liu, T.W., Lo, P.C., Wilson, B.C., Zheng, G.; "Zipper" molecular beacons: A generalized strategy to optimize the performance of activatable protease probes. *Bioconjugate Chem*. **20,** 1836–1842, 2009.
20. QBT 1803-1993, General methods of determination for industrial enzymes.
21. Song, J., Tao, W.Y., Chen, W.Y.; Kinetics of enzymatic unhairing by protease in leather industry. J. Cleaner Prod. 19, 325–331, 2011.
22. Miyata, S. and Morita, S.; A new method for visualization of endothelial cells and extravascular leakage in adult mouse brain using fluorescein isothiocyanate. J. Neuro. Sci. Meth. 202, 9–16, 2011.
23. Liao, L.L.; Tanning chemistry and technology. Beijing: Science Press, 2005.
24. Zhang, C., Song, X.J., Wang, J., Wen, J.; Stability and fluorescence spectra of lipase labeled with FITC, *Chinese Journal of Applied Chemis*try **25**, 1381–1384, 2008;
25. Carla J.S.M., Silvaa, M., Prabaharana, Georg Gübitzb, Artur Cavaco-Paulo; Treatment of wool fibers with subtilisin and subtilisin-PEG. *Enzyme Microbial Technol*. **36**, 917–922, 2005.

26. Basil-Jones, M.M., Edmonds, R.L., Norris, G.E. and Haverkamp, R.G.; Collagen Fibril Alignment and Deformation during Tensile Strain of Leather: A Small-Angle X-ray Scattering Study. *J. Agric. Food. Chem.* **60**, 1201–1208, 2012.
27. Basil-Jones, M.M., Edmonds, R.L., Cooper, S.M. and Haverkamp, R.G.; Collagen Fibril Orientation in Ovine and Bovine Leather Affects Strength: A Small Angle X-ray Scattering (SAXS) Study. *J. Agric. Food Chem.* **59**, 9972–9979, 2011.
28. Wang, H.R.; English for Leather Technology. Beijing: China Light Industry Press, 2005.
29. He, X.X., Cheng, H.M., Chen, M., Wu, Lian and Li, Z.Q.; Epidermis morhology: Investigation of cattle hide during unhairing by transmission electron microscope. *JSLTC* **96**, 119–122, 2012.