Enzymatic Bating Technology for Wet Blue: II. The Basic Properties and Application Effectiveness of Typical Acidic Proteases

by

Xu Zhang,[1,2] Xiang Zhong,[2] Mengchu Gao,[1,2] Biyu Peng[1,2]* and Chunxiao Zhang[1,2]**

[1]*National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University,* Chengdu, Sichuan 610065, P.R. China

[2]*Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University,* Chengdu, Sichuan 610065, P.R. China

Abstract

Most of the reported bating technologies for wet blue are based on the usage of acidic protease, which takes a long time and needs large enzyme dosage. A thorough understanding of the basic characteristics of typical acidic proteases and the interaction between enzyme proteins and wet blue fibers will help to improve bating technology for wet blue by selecting the suitable proteases. In this paper, the enzymatic characteristics, molecular weight (M_r) and isoelectric point (pI) of several proteases and their bating effectiveness were investigated. The results indicated that there are two main factors which may affect the wet blue bating effectiveness of acidic proteases. First, the common acidic proteases exhibited low activity towards chrome-tanned collagen fiber which lead to inefficient bating effect through normal dosage. Nonetheless, when the dosages of chrome-tanned collagen fiber activity reached up to 50 U/mL, these acidic proteases also can achieve a good bating effect, the caseinolytic activity has been reached up to 1000 U/mL-4000 U/mL. Second, because of the large molecular weight and the charge repulsion between enzyme proteins and wet blue fibers, the enzymatic hydrolysis process, the penetration and distribution of acidic protease proteins, into wet blue is very difficult. Additionally, neutral proteases have more prospects in wet blue bating process due to the higher chrome-tanned collagen fiber activity and less charge repulsion than acidic proteases.

Introduction

Currently, to reduce pollution and control tannery beamhouse waste disposal costs, more and more tanneries are tending to purchase wet blue as raw materials. However, purchased wet blue generally has some obvious defects, such as insufficient opening up of fiber structure, vein marks, scars, wrinkles, hardened fibers from long-term transportation and storage, especially, evident quality difference from different tanneries, and so on, which seriously affects the unification of the subsequent processing technology and the performance and use value of the leather.[1-4] Hence, enzymatic bating operation is usually the first stage of the post-tanning process to resolve the problems and improve the quality of purchased wet blue in most tanneries. In conventional enzymatic wet blue bating process, protein fiber structure is further opened, grease and inter-fibrillary substances are removed, defects are alleviated, complimentary, by using enzyme preparations, especially proteases.[5-9] Consequently, wet blue quality difference from different tanneries is reduced and the subsequent processing can be standardized. Hence, the evenness, softness, physical and mechanical properties and organoleptic performances of finished leather are improved.

The main components of most of the commercial enzyme preparations for wet blue bating are acidic proteases. This is restricted by the traditional thinking pattern that post-tanning processing is carried on acidic conditions. It is well accepted that through properly selecting bating enzymes and bating conditions according to wet blue situations and the property requirement to finished leather, the use of acidic protease preparations in the post-tanning process is useful in improving the quality of finished leather.[10-12] However, it takes a long time and needs a large dosage of enzyme to hit the spot of wet blue bating in the traditional acidic enzymatic bating technology, which does not only defect the production efficiency but increases the cost of production. Further, most of the enzymatic bating technologies only involved the bating effectiveness of some specific enzyme preparations to wet blue and the optimization of the using conditions, but rarely mentioned the mechanism of enzymes in wet blue bating process, especially the interaction between enzyme proteins and wet blue fiber substrates. This makes it difficult to select suitable and efficient protease for wet blue bating process.

Proteolytic activity is the main parameter during the application of proteases. Many methods were used to assay the activity of protease in tanneries, and the commonest one is the Folin method by using casein as substrate,[13,14] which is distinctively different from the chrome-tanned protein fibers in wet blue. Therefore, the protease selected based on the proteolytic activity by these traditional methods cannot reflect the actual proteolytic ability to chrome-

*Corresponding author email: pengbiyu@scu.edu.cn
**Corresponding author email: chunxiaozhang@scu.edu.cn
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tanned protein fibers. Previously, we have established a quantitative method for characterizing the proteolytic activities towards chrome-tanned elastin and collagen fibers through measuring the produced amount of the unique amino acid Desmosine (DES) and Hydroxyproline (Hypro) in the reaction liquor, respectively.[4] This method can be used as an available tool to correctly select proteases and optimize process parameters for wet blue bating.

Furthermore, wet blue is a porous material woven from chrome-tanned collagen fibers, the enzymatic bating of wet blue is a solid-liquid heterogeneous reaction. The collagen fibers would not be opened up evenly if the proteases cannot penetrate into the inner layers of the wet blue. The mass transfer characters of enzyme proteins would be related to the molecular weight of enzyme protein molecules and charge interactions between the enzyme proteins and hide/leather protein fibers, for both collagen fibers and proteases are amphiprotic substances. Many studies such as fluorescent tracer technology,[15-18] protease protein purification technology[19] and enzymatic hydrolysis properties regulation technology[20] have been conducted to investigate the mass transfer and reaction character of proteases in the leather manufacturing. But the influence role of enzyme molecular weight (M_r) and isoelectric point (pI) on bating effectiveness is still indistinct and should be further investigated.

In this paper, the basic properties of several typical proteases, including molecular weight (M_r), isoelectric point (pI) and hydrolyzing ability to chrome-tanned collagen fiber, were analyzed. The bating effectiveness of these proteases on wet blue was studied comparatively. Based on this we can provide some useful scientific methods and enzyme information to guide the choosing of highly efficient proteases and optimizing process parameters for wet blue bating process.

Materials and Methods

Materials

All protease preparations (Table I) were purchased from the market. Hydroxyproline (Hypro) standard was obtained from MembraPure GmbH (Germany). Shaved cowhide wet blue (1.2 mm) was supplied by Tongtianxing Group Co.Ltd., China. Chrome-tanned collagen fiber powder was prepared by our laboratory from the reticular layer of bovine hide.[4] All the other chemicals used for the analysis were of analytical grade and other chemicals used for leather processing were of commercial grade.

Thermal stability of proteases

Protease preparations were diluted into a certain concentration by 0.1 mol/L of Britton-Robinson buffer (B-R buffer, pH 3.5 for acidic proteases and pH 6.5 for neutral protease) and incubated at 40° for 12 h. The caseinolytic activity was determined according to the modified Folin method[13,14] at intervals of 2 h at pH 3.5 or pH 6.5 and 40°.

Assay of proteolytic activity on casein substrate

The caseinolytic activity was determined by the Folin method under certain conditions. First, the proteolysis was performed by incubating 1mL of diluted enzyme solution with 1 mL of 1 % (m/v) casein in 0.1 mol/L B-R buffer (pH 3.5 for acidic proteases and pH 6.5 for neutral proteases) at 40° for 10 min. Then, the reaction was quenched by adding 2 mL of trichloroacetic acid (0.4 mol/L) and allowed to centrifuged at 3500 r/min for 10 min. Finally, 1 mL of the supernatant was transferred into a 15 mL test tube and reacted with 5 mL of Na_2CO_3 solution (0.4 mol/L) and 1 mL of Folin-Phenol reagent at 40° for 20 min. After the reaction, the absorbance of the reaction mixture was measured at 680 nm to determine the amount of tyrosine released during the proteolysis. One unit of caseinolytic activity is defined as the amount of enzyme capable of digesting the casein substrate to produce 1 μg of tyrosine in 1 min under certain conditions.

Assay of proteolytic activity on chrome-tanned collagen fiber substrate

Chrome-tanned collagen fiber activity was determined according to the established method by our laboratory, the chrome-tanned collagen fiber with 2.11% of chrome content was chosen to be the substrate.[4] The amount of Hypro in the digested reaction liquor was tested to represent the performance of proteases hydrolyzing chrome-tanned collagen fiber. In detail, 100 mg ± 1 mg of chrome-tanned collagen fiber was accurately weighed in a test tube, followed by adding 5 mL of B-R buffer (0.1 mol/L) and stirred in an incubator for 10 min. Then, 1 mL of enzyme solution was added and stirred for another 4 h at 150 r/min, then the concentration of Hypro in the digested reaction liquor was tested. One unit of chrome-tanned collagen fiber activity is defined as the amount of enzyme capable of

Table I

Selected wet blue bating protease preparations

Proteases	Characterization	Company
YNU-A	Acidic bacteria protease	Qactive Bio-technology Co. Ltd.
LKT-A	Acidic bacteria protease	Longda Bio-products Co. Ltd.
ABG	Acidic bacteria protease	Novozymes Investment Co. Ltd.
TP	Acidic bacteria protease	Denykem Co. Ltd.
EW01	Complex neutral bacteria protease	Longda Bio-products Co. Ltd.

digesting the chrome-tanned collagen fiber substrate to produce 1 μg of Hypro in 1 hour under certain conditions.

Determination the molecular weight and isoelectric point of acidic proteases

The molecular weight (M_r) and isoelectric point (pI) of these proteases were tested through SDS-PAGE and IEF-PAGE method, respectively, and stained by Coomassie Brilliant Blue R-250.[21]

Bating wet blue with typical protease preparations

Shaved cowhide wet blue (1.2 mm) from a supplier in China was chosen as the raw materials for this study. Pieces of wet blue (50 cm ×50 cm) were symmetrically taken along the backbone in a piece of shaved cowhide wet blue. Samples were wetted, bleached and adjusted the pH to 3.5 (for acidic proteases) or 6.5 (for neutral protease) by using sodium bicarbonate solution (1:10, w/v). Samples were treated by different protease preparations with a certain dosage of proteolytic activity at 40°, pH 3.5 or 6.5, run for 4 h then left overnight. After bating, the concentration of soluble protein and Hypro in the reaction liquors were tested according to the steps described in the following measuring method section. The opening-up of collagen fiber was observed with an optical microscope after staining per the Weigert-Van Gieson method. Then, samples were neutralized, retanned, fatliquored, squeeze-spread, toggle-dried and milled as per the same standard post-tanning procedures. The softness and main physical properties of the crust leathers from the adjacent and symmetrical parts of the same wet blue were evaluated. The organoleptic properties of these crust leathers were evaluated by 10 professional skilled tanners.

Determination of the concentration of soluble protein in the reaction liquor

After the end of the reaction, the concentration of soluble protein was determined according to the modified Lowry method.[22] First, the reaction liquor was filtered with a qualitative filter paper and diluted into a certain concentration by ultrapure water. 1 mL of filtrate was mixed with 5 mL of Folin-phenol reagent-A at room temperature for 10 min, then 0.5 mL of Folin-phenol reagent-B was added and incubated at 30° for 30 min. After the reaction, the absorbance of the mixture was measured at 660 nm to determine the amount of soluble protein.

Determination of the concentration of Hypro in the reaction liquor

After the end of the reaction, the reaction liquor was filtered with a qualitative filter paper and 2 mL of filtrate was mixed with 2 mL of concentrated hydrochloric acid (12 mol/L) in a 10 mL COD digestion tube (HACH, America). The mixture was hydrolyzed at 120° for 12 h and 2 mL of EDTA-Na_2 (20 g/L) solution was added to avoid the interference of chromium.[23] Then the acid hydrolysate was evaporated to dryness with a Vacuum Concentrator (TC-8F, TAITEC, Japan). A certain amount of amino acid analysis sample dilution buffer was added to dissolve the dry sample. The dissolved liquor was filtered with an aqueous filter head (D=0.22 um). The concentration of Hypro was tested with an Amino Acid Analyzer (A300, MembraPure GmbH, Germany).

Histological analysis of collagen fiber

Samples of 1 cm^2 were cut from identical official sampling portions of the corresponding bated wet blue. Sections of 12 μm thicknesses were obtained using CM1950 freezing microtome (Leica, Germany) and stained with the Weigert-Van Gieson staining method. The opening-up of collagen fiber was observed with an optical microscope (CX41, Olympus, Japan).

Test of softness and physical properties of crust leather

Dried crust leather samples of each enzyme bating group were taken out in the adjacent and symmetrical parts of the same leather for testing softness and physical properties. The softness of the crust leather was determined with a Leather Softness Tester (GT-303, Gotech Testing Machines Inc., China). The physical properties such as tensile strength, elongation at break, tear strength and bursting strength were examined as per the standard procedures.[24]

Results

Characteristics of typical proteases

Caseinolytic and chrome-tanned collagen fiber activities of proteases

Wet blue bating is usually conducted on the pH is around 3.5 to improve the opening up of chrome-tanned collagen fibers by using acidic proteases. To evaluate the viabilities of the typical acidic proteases, the caseinolytic and chrome-tanned collagen fiber activities of several protease preparations were assayed at pH 3.5 (for acidic proteases) or pH 6.5 (for neutral proteases) and 40°. The results in Table II shows that the proteolytic activities determined by different methods were found to be significantly different. All of these acidic proteases exhibit much higher activity to casein substrate than that of chrome-tanned collagen fiber substrate. The relative activity of the acidic protease defined as the chrome-tanned collagen fiber activity to 1 unit of casein hydrolysis activity (H_{Cr}/F)[4] is

Table II

Enzymatic characteristics of proteases

Protease (40°)	Caseinolytic activity (F, U/g)	Chrome-tanned collagen fiber activity (H_{Cr}, U/g)	H_{Cr}/F
ABG (pH 3.5)	4426	18	0.004
LKT-A (pH 3.5)	2091506	25758	0.012
TP (pH 3.5)	694716	10745	0.015
YNU-A (pH 3.5)	49605	2064	0.042
EW01 (pH 6.5)	6768	3749	0.554

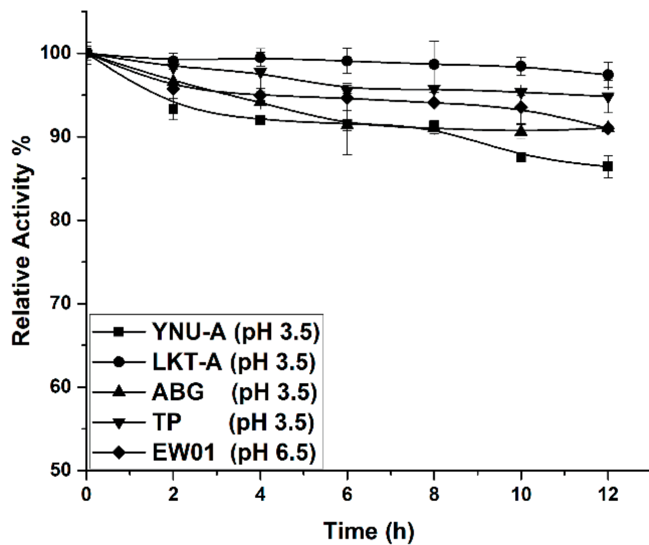

Figure 1. Thermal stability of proteases

at a very low level. As a comparison, the activity of a complex neutral protease EW01 was also evaluated. The results indicate that EW01 exhibit significantly higher H_{Cr}/F than all of the acidic proteases.

The thermal stability of proteases

As mentioned above, it usually takes a long time to bate the wet blue with proteases. Therefore, the thermal stability of the proteases was measured at the conditions of pH 3.5 for acidic protease (pH 6.5 for neutral protease) and 40°. The results in Fig. 1 illustrated that protease LKT-A has the most stable activity for there was almost no activity lost after incubation at 40° for 12 hours. The thermal stability of protease YNU-A is relatively weaker than the other proteases, its activity kept dropping for 12 hours. The activities of proteases ABG, TP and EW01 tended to be stable after incubation for 12 hours.

Analyzation of the M_r and pI of acidic proteases

As mentioned above, wet blue is a porous material woven from chrome-tanned collagen fibers and the collagen fibers would not be opened up evenly if the proteases cannot penetrate into the inner layers of the wet blue. The mass transfer characteristics of enzyme proteins would be related to the molecular weight of the enzyme molecules and charge interactions between the enzyme proteins and leather protein fibers. To further analyze the characters of acidic proteases in wet blue bating process, the molecular information, such as molecular weight (M_r) and isoelectric point (pI), of these acidic proteases were investigated through SDS-PAGE and IEF-PAGE methods, respectively. Fig. 2A shows that all of these acidic proteases contain a variety of protein molecules, the M_r of the major components is approximately 45 kDa. Fig. 2B shows that all of these acidic proteases contain at least two different electrophoretic bands, and all of the pI values are higher than 4.0, even higher than 4.5, which is approximately 4.45-5.10. Hence, at acidic wet blue bating conditions (the pH is approximately 3.5), acidic proteases and wet blue protein fibers (pI is approximately 6.5-7.5[25-28]) are all carrying in large amounts of positive charge. Additionally, the M_r and pI values of commercial complex neutral protease EW01 is different than all of the acidic proteases, which is approximately 25 kDa-35 kDa and 7.5-8.5, respectively. It is supposed that the charge interaction between enzymes and collagen fibers may have some effect on the penetration, distribution and reaction of proteases and thus influence the bating process, that like charges repel each other but opposite charges attract.

Effects of activities of acidic proteases on wet blue bating

Bating effectiveness of wet blue with same caseinolytic activity concentration

Typical acidic protease preparations YNU-A, LKT-A, ABG and TP were chosen for bating wet blue as the following conditions: 40°, pH 3.5, run for 4 h then left overnight. The dosages of the acidic proteases were the same based on the caseinolytic activity (cF) was 90 U/mL reaction liquor, which is equal to the normal dosage of commercial acidic protease preparations. Table III shows that the concentration of chrome-tanned collagen fiber activity (cH_{Cr}) in the bating liquor for each protease was significantly different. After bating, the concentrations of soluble protein (SP) and hydroxyproline (Hyp)

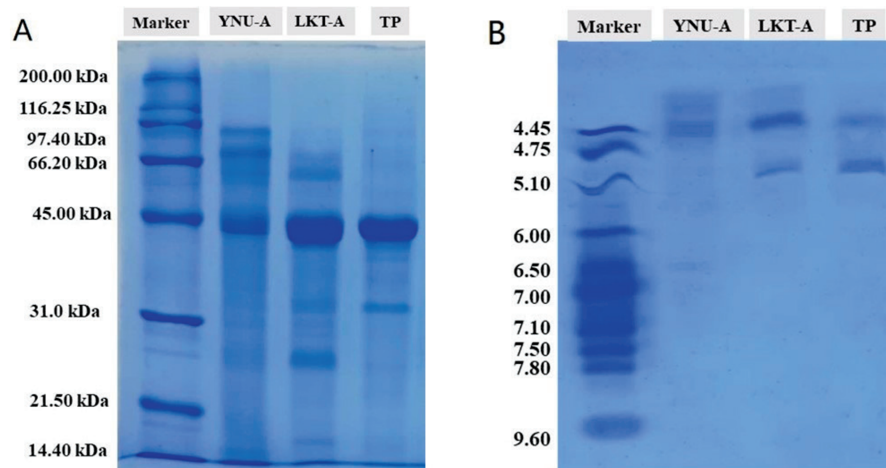

Figure 2. SDS-PAGE **(A)** and IEF-PAGE **(B)** electrophoresis diagrams of acidic proteases

Table III

Amounts of proteases, soluble protein and hydroxyproline in wet blue bating liquor treated by same dosage of caseinolytic activity

Proteases	cF*1 (U/mL)	cH_{Cr}*2 (U/mL)	SP*3 (μg/mL)	Hyp*4 (μg/mL)	SP/cF* (μg/U)	Hyp/cH_{Cr}*6 (μg/U)
YNU-A	90.0	3.75	454	3.72	5.03	0.99
LKT-A	90.0	1.11	356	1.86	3.94	1.67
ABG	90.0	0.36	463	1.21	5.13	3.37
TP	90.0	1.40	363	2.17	4.02	1.56

*1: cF represents the concentration of caseinolytic activity in the bating liquor.

*2: cH_{Cr} represents the concentration of chrome-tanned collagen fiber activity in the bating liquor.

*3: SP represents the concentration of produced soluble protein in the bating liquor.

*4: Hyp: represents the concentration of produced hydroxyproline in the bating liquor.

*5: SP/cF represents the amount of soluble protein hydrolyzed by 1 unit of caseinolytic activity.

*6: Hyp/cH_{Cr} presents the amount of hydroxyproline hydrolyzed by 1 unit of chrome-tanned collagen fiber activity.

in the bating liquors were measured. Although some differences of the produced amounts of SP and Hyp have existed between different enzymes, the total amount of SP and Hyp is small. With the same caseinolytic activity concentration, the content of soluble protein hydrolyzed by 1 unit of caseinolytic activity (SP/cF) is approximately 4.5 μg; the content of hydroxyproline hydrolyzed by 1 unit of chrome-tanned collagen fiber activity (Hyp/cH_{Cr}) is approximately 1.7 μg.

The opening-up of the collagen fiber in the vertical-section of bated wet blue was observed through Weigert-Van Gieson staining method. Fig. 3 shows that after treatment with these acidic protease preparations, the collagen fibers have a higher degree of opening-up than the control. Compared with the analysis data in Table III, it is obvious that the higher the chrome-tanned collagen fiber activity, the larger the concentration of hydroxyproline produced in the reaction liquor and, further, the higher the degree of collagen fibers opening-up.

The softness and main physical properties of the crust leathers from the adjacent and symmetrical parts of a same wet blue were evaluated. Table IV shows that acidic proteases YNU-A and LKT-A gives the crust leather the highest softness, but ABG and TP enzyme scarcely improving the softness of the crust leathers. Besides, the tear strength, tensile strength, bursting strength and elongation at break of these protease preparations treated wet blue are higher or comparable to the control.

Further, the shaved wet blue bated with different protease preparations were neutralized, retanned, fatliquored, squeeze-spread, toggle-dried and milled as per the same standard procedures. The organoleptic properties of these crust leathers were evaluated by 10 professional skilled tanners. They thought that the crust leather treated by YUN-A and LKT-A enzyme is a little better than ABG and TP enzyme treated in the respect of softness, hand feeling, and so on. However, the whole quality of crust leathers treated by these acidic proteases has no obvious improvement over the control.

Table IV

Softness and physical properties of crust leather treated by same dosage of caseinolytic activity

Proteases	YNU-A	LKT-A	ABG	TP	Control
Softness (mm)	8.57	8.65	7.43	7.70	7.43
Tear strength (N/mm)	143	139	149	143	145
Tensile strength (N/mm^2)	75	85	81	84	86
Elongation at break (%)	122	121	105	114	129
Bursting strength (N/mm)	443	479	496	486	458
Bursting height (mm)	11.8	12.3	11.2	11.7	12.3

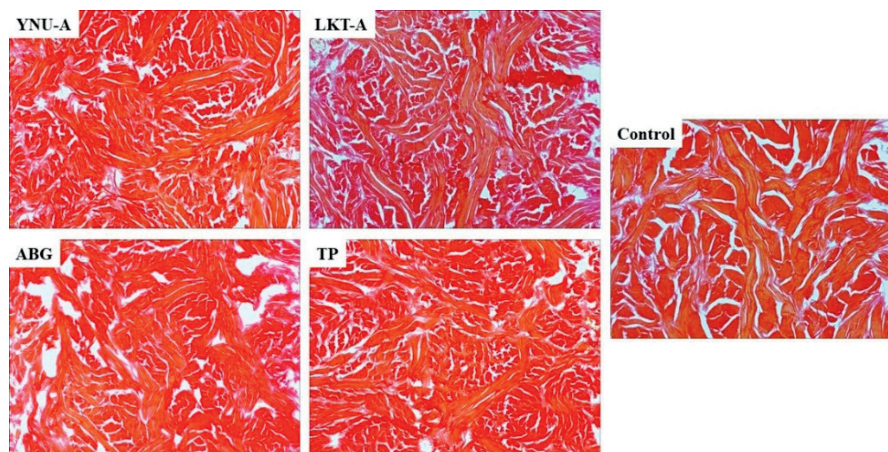

Figure 3. Staining results of collagen fiber in wet blue treated by same dosage of caseinolytic activity (100× whole ver. sec.)

Bating effectiveness of wet blue with same chrome-tanned collagen fiber activity concentration

For improving the bating effect of acidic proteases, a larger dosage of acidic proteases is needed, in other words, the concentration of chrome-tanned collagen fiber activity in the bating liquor should be increased. YNU-A, LKT-A and TP enzyme were chosen for bating wet blue as the following conditions with larger enzyme dosages: 40°, pH 3.5, run for 4 h then left overnight. The dosages of the acidic proteases were the same based on the chrome-tanned collagen fiber activity (cH_{Cr}) was 50 U/mL reaction liquor, which is based on the experiences of wet blue bating technology in our laboratory. Also, the produced amounts of soluble protein and Hyp in the bating liquors were measured.

Table V shows that with same chrome-tanned collagen fiber activity concentration, the content of soluble protein hydrolyzed by 1 unit of caseinolytic activity is approximately 3.1 µg; the content of hydroxyproline hydrolyzed by 1 unit of chrome-tanned collagen fiber activity is approximately 0.58 µg. Compared with Table III, the dosages of the acidic protease preparations in the bating liquors are approximately 13-45 times higher, the produced amount of soluble protein is approximately 13-26 times higher and the produced amount of hydroxyproline is approximately 6-19 times higher than above bating operation.

Table V

Amounts of proteases, soluble protein and hydroxyproline in wet blue bating liquor treated by same dosage of chrome-tanned collagen fiber activity

Proteases	cF (U/mL)	cH_{Cr} (U/mL)	SP (µg/mL)	Hyp (µg/mL)	SP/cF (µg/U)	Hyp/cH_{Cr} (µg/U)
YNU-A	1171	50	5690	23.33	4.86	0.47
LKT-A	4044	50	9307	34.56	2.30	0.69
TP	3242	50	6861	29.63	2.12	0.59

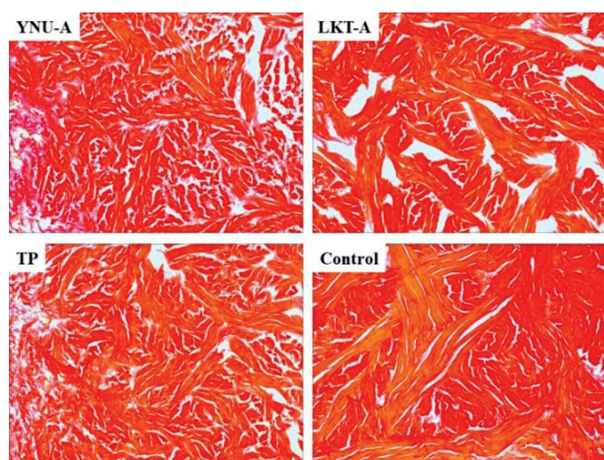

Figure 4. Staining results of collagen fiber in wet blue treated by same dosage of chrome-tanned collagen fiber activity (100× whole ver. sec.)

Table VI

Softness and physical properties of crust leather treated by same dosages of chrome-tanned collagen fiber activity

Proteases	YNU-A	LKT-A	TP	Control
Softness (mm)	8.38	8.40	8.37	7.74
Tear strength (N/mm)	145	128	167	162
Tensile strength (N/mm^2)	56	52	60	49
Elongation at break (%)	133	124	103	117
Bursting strength (N/mm)	421	457	462	454
Bursting height (mm)	12.5	13.4	12.1	12.2

Fig. 4 shows that all of these protease preparations opening-up most of the collagen fibers in the wet blue. Meanwhile, the opening-up degree of collagen fiber is higher than the results shown in Fig. 3.

Table VI shows that all of these protease preparations give the crust leathers better softness than control. Besides, the tear strength, tensile strength, bursting strength and elongation at break of these proteases treated wet blue are higher or comparable than control. Also, all of these wet blues were carried out the same standard post-tanning procedures, and the organoleptic properties of these crust leathers were evaluated. The result shows that the crust leather treated by these acidic proteases is better than control. Besides, the organoleptic properties and the whole quality of the finished leathers are significantly better than above-bated crust leathers.

In summary, with larger acidic proteases dosage in wet blue bating liquors can significantly improve the softness, organoleptic properties and the whole quality of the finished leather without affecting the physical properties. However, the dosage of acidic protease preparations is 13-45 times higher than normal dosage, as a result, the costs of the post-tanning process increased a lot, which is unacceptable by the tanneries.

Comparison of the bating effectiveness of acidic and neutral proteases

As mentioned above, caused by low chrome-tanned collagen fiber activity and large charge repulsion between enzyme proteins and wet blue fiber substrates at acidic bating conditions, the enzymatic hydrolysis process and the penetration of acidic protease proteins in wet blue are difficult. All of this makes an inefficient bating effect of acidic proteases even under a large dosage. Whereas, neutral protease EW01 have higher chrome-tanned collagen fiber activity than all of the acidic proteases. In addition, wet blue protein fibers are close to electric neutrality at neutral conditions, which means that less charge repulsion exists between neutral proteases and wet blue fiber substrates. For comparing the wet blue bating effectiveness of neutral and acidic proteases, neutral protease EW01 and acidic protease YNU-A were chosen for bating wet blue as the following

Table VII
Amounts of proteases, soluble protein and hydroxyproline in wet blue bating liquor

Proteases	cF (U/mL)	cH_{Cr} (U/mL)	SP (μg/mL)	Hyp (μg/mL)	SP/cF (μg/U)	Hyp/cH_{Cr} (μg/U)
YNU-A	1171	50	5533	23.08	4.73	0.46
EW01	90	50	6117	66.60	67.78	1.33

conditions: 40° and pH 6.5 or pH 3.5, run for 4 h then left overnight. The dosages were the same based on the chrome-tanned collagen fiber activity was 50 U/mL reaction liquor. The produced amount of soluble protein and Hyp in the bating liquors were measured after bated, the softness and main physical properties of the crust leathers from the adjacent and symmetrical parts were also evaluated.

Table VII shows that with same concentration of chrome-tanned collagen fiber activity, the caseinolytic activity of acidic protease YNU-A is 13 times higher than neutral protease EW01. However, the produced amount of soluble protein and Hyp by EW01 enzyme was 1.1 and 2.9 times higher than YNU-A, respectively. Besides, the content of soluble protein hydrolyzed by 1 unit of caseinolytic activity of YNU-A and EW01 enzyme is 4.7 μg and 67.8 μg, respectively; the content of hydroxyproline hydrolyzed by 1 unit of chrome-tanned collagen fiber activity of YNU-A and EW01 enzyme is 0.46 μg and 1.33 μg, respectively.

Table VIII shows that the softness of EW01 enzyme treated crust leather is better than that of the YNU-A enzyme. The tear strength, tensile strength, bursting strength and elongation at break is comparable than that of the YNU-A enzyme treated crust leather. Furthermore, Fig. 5 shows that EW01 enzyme opening-up more collagen fibers than YNU-A enzyme, which makes the softness of the finished leather better. Additionally, all of the professional skilled tanners also thought that the whole quality of the finished leather treated by neutral protease EW01 is better than that of acidic protease YNU-A.

Discussion

Generally, the use of acidic proteases in wet blue bating processing required a long time and large enzyme dosage. To find the causes and solve this problem, studies have been conducted by us for a long time. Firstly, an accurate method for the quantitative characterization of proteases activity towards chrome-tanned elastin and collagen fibers by determining the unique amino acid, namely desmosine and hydroxyproline, in the reaction liquors was successfully established.[4] Our research found that acidic proteases almost have no effect on chrome-tanned elastin fiber, therefore, the proteolysis abilities of acidic proteases against chrome-tanned collagen fiber may have large influences on the bating effectiveness of wet blue. Hence, a thorough understanding of the basic characteristics of acidic proteases in wet blue bating process is necessary, especially the interaction mechanism between protease proteins and chrome-tanned collagen fiber substrates.

Previously, we proved that well-tanned protein fibers exhibit highly protease-resistance ability,[4] therefore, the enzymatic wet blue bating process is usually sustained over long periods. Fig. 1 shows that all of the selected typical proteases have excellent thermal stability under 40° and pH 3.5 or pH 6.5, which is preliminarily adapted to the wet blue bating conditions. Then, the enzymatic characteristics of these proteases towards casein and chrome-tanned collagen fiber

Table VIII
Softness and physical properties of crust leather

Proteases	Softness (mm)	Tear strength (N/mm)	Tensile strength (N/mm²)	Elongation at break (%)	Bursting strength (N/mm)	Bursting height (mm)
YNU-A	8.20	154	56	143	424	12.5
EW01	8.56	151	50	117	483	12.8

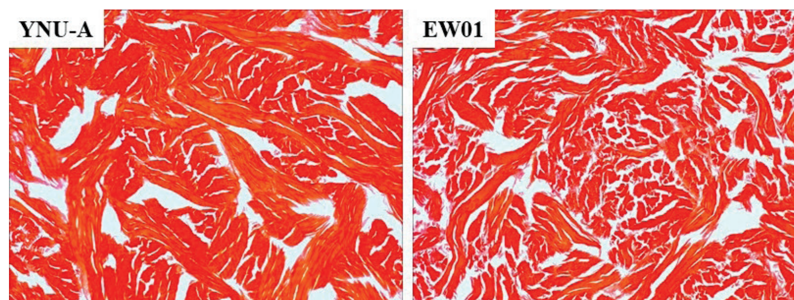

Figure 5. Staining results of collagen fiber in wet blue treated by acidic (YNU-A) or neutral (EW01) protease (100× whole ver. sec.)

substrates were investigated. The results in Table II shows that acidic proteases exhibit rather higher activity to casein substrate than chrome-tanned collagen fiber substrate. The chrome-tanned collagen fiber activity per 1 unit of caseinolytic activity is approximately 0.018 U, however, it is extremely lower than neutral protease EW01, which is 0.554 U.

One of the main factors affecting the acceptability of a wet blue bating enzyme preparation by tannery is whether it can improve the softness, the whole quality and keep the original physical properties of the finished leather. The highly cross-linked collagen fiber structure can be further opened up by using proteases, thus the inter-fibrillary substances hidden in collagen fiber bundles can be removed effectively. The bating effectiveness of proteases on wet blue was positively related to their chrome-tanned collagen fiber activity, proteases with highly chrome-tanned collagen fiber activity could significantly improve the softness of crust leather. However, excessive proteolysis of collagen structure is harmful to the leather matrix and significantly affects the properties of the final leather product. Nevertheless, the proteolysis degree of collagen fiber at mentioned enzyme dosage and wet blue bating conditions have a negligible effect on the mechanical properties but a positive effect on the softness of the crust leather, as shown in Table IV, Table VI and Table VIII. Therefore, the deficiency of acidic proteases in the wet blue bating process is most probably related to its insufficient to chrome-tanned collagen fiber. Table III, Table IV and Fig. 3 shows that the bating effectiveness and fiber opening-up degree of the crust leathers treated by acidic proteases are not obvious under the dosage of caseinolytic activity is 90 U/mL. Although, acidic proteases can obviously improve the softness, fiber opening-up degree and hand feelings of the crust leather under the caseinolytic activity is 1000 U/mL-4000 U/mL, the chrome-tanned collagen fiber activity is 50 U/mL, (Table V, Table VI and Fig. 4), but the cost of the post-tanning process increased a lot as well due to the large dosage of enzymes, which is unacceptable by the tannery.

Apart from the enzymatic characteristics of acidic proteases, Fig. 2(A) shows that the M_r of acidic proteases is approximately 45 kDa, which is much larger than the reported commercial leather making neutral and alkaline proteases.[19,29] The molecular weight of the main component of EW01 enzyme is approximately 25 kDa-35 kDa. Therefore, it can be speculated that the penetration and distribution of acidic protease molecules into wet blue are harder than neutral and alkaline proteases. Furthermore, as mentioned above, at normal acidic wet blue bating conditions (the pH is approximately 3.5), acidic protease molecules (pI is approximately 4.45-5.10) and wet blue protein fibers (pI is approximately 6.5-7.0) are all carrying a large amount of positive charge. Caused by large charge repulsion between protease proteins and wet blue fibers, the penetration and distribution of acidic protease molecules in wet blue are difficult. Hence, the bating effect of acidic proteases is insufficient even at large enzyme dosage. Additionally, although acidic proteases may have less charge repulsions at pH 4.5-5.0, the caseinolytic and chrome-tanned collagenolytic activity of acidic proteases is extremely low, which is also unacceptable.

As mentioned above, the isoelectric point of chrome-tanned collagen fibers in wet blue is approximately 6.5-7.5,[25-28] which is close to electric neutrality at neutral wet blue bating conditions. The isoelectric point of the main component of EW01 enzyme is approximately 7.5-8.5, which is carrying a trace amount of positive charge at neutral condition. Considering less charge repulsion exists between enzyme proteins and wet blue fiber substrates, it can be speculated that the penetration and distribution of neutral proteases may be easier than acidic proteases at neutral wet blue bating conditions.

Finally, the bating effectiveness of neutral protease EW01 was investigated and compared with acidic protease YNU-A. Table VII shows that the chrome-tanned collagen fiber activity per 1 unit of caseinolytic activity of the EW01 enzyme (0.556 U) is 13 times higher than the YNU-A enzyme (0.042 U). The produced amount of hydroxyproline per 1 unit of chrome-tanned collagen fiber activity by EW01 (1.33 μg) is 2.9 times higher than YNU-A (0.46 μg). Moreover, the results in Table VIII and Fig. 5 shows that the wet blue bating effectiveness of neutral protease EW01 is better than that of acidic protease YNU-A. These results suggest that neutral proteases may have more prospects in wet blue bating process.

Conclusions

Based on the investigation of the thermal stability, enzymatic characteristics and molecular information of several proteases, the bating effectiveness of acidic and neutral proteases on wet blue was studied comparatively. The mechanism of enzyme penetration and distribution in wet blue was also preliminarily investigated. The results showed that there are two main factors may affect the wet blue bating effectiveness of acidic proteases. First, the common acidic proteases exhibited low activity towards chrome-tanned collagen fiber leads to inefficient bating effect through normal dosage. Second, caused by large molecular weight and charge repulsion between enzyme proteins and wet blue fibers, the enzymatic hydrolysis process, the penetration and distribution of acidic protease proteins into wet blue are very difficult. Additionally, the results suggested that neutral proteases have more prospects in wet blue bating process due to the higher chrome-tanned collagen fiber activity and less charge repulsion than acidic proteases. The results can provide some useful scientific methods and enzyme information to guide the choosing of high-efficient proteases and optimizing process parameters for wet blue bating process.

Of course, the mechanism of enzyme in acidic and neutral wet blue bating conditions should be further investigated. As mentioned above, the enzymatic hydrolysis process, the penetration and distribution of acidic protease molecules into wet blue are harder

than neutral protease molecules. Therefore, correctly application of neutral proteases, even alkaline proteases, should be detailly studied. We speculated that these problems can be solved by investigating the mass transfer and reaction mechanism of enzyme proteins in wet blue bating process, and this work is undertaken.

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