ANALYSIS OF THE FUNCTIONAL COMPONENTS OF ACID PROTEASE AND INVESTIGATION OF BATING MECHANISM OF WET-BLUE

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Abstract. In recent years, acid protease is widely used in bating process of wet-blue by tanneries. Therefore, it is necessary to study the mechanism between acid protease and wet-blue for better practical application in bating process. In this study, one acid protease from Aspergillus was used to study the mechanism between protease and wet-blue. Firstly, the SDS-PAGE analysis revealed that the molecular weight of it was 48KD, the Zata potential analysis showed that its pI was consistent with its optimum pH value, which was at 3.0. According to its enzymatic properties, the activated ingredients were separated from it by Tangential Flow Filtration (TFF) and used to treat wet-blue, then the enzymatic hydrolysate were obtained. On the one hand, the contents of Hydroxyproline(Hyp) and Desmosine(Des) in the hydrolysate were determined by HPLC and ELISA method, respectively, then, the biodegradation rates of collagen and elastin were calculated, the results showed collagen and elastin degraded 0.006‰ and 0.5‰ respectively. On the other hand, the changes of Elastic fibers in wet-blue before and after treated by the activated ingredients were characterized by Super Depth of Field Microscope. The results showed that the Elastic fibres are clearly dispersed by acid protease. And this paper would provide the basis and researches for the development of mechanism about acid protease in bating process.

1 Introduction

Protease is an irreplaceable biomaterial for improving leather quality and realizing cleaner production and sustainable development of leather industry, and the protease is widely used in many leather manufactures process, such as soaking[1], dehairng[2] and bating[3]. In leather industry, the processing of hides or skins to leathers involves three important stages: beam-house operations, tanning process, and finishing process.

At present, chrome-tanned leather has excellent physical and chemical properties such as high shrinkage temperature, softness, fullness and good hygienic properties, thus chrome tanning is the most popular tanning method. The beam-house operations are known to contribute more than 90% of the total pollution load from leather processing[4]. In order to reduce the cost of pollution control, most tanneries directly use wet-blue as raw material to produce finished leather with different types and styles. With a long-time transportation and preservation, the fibres of wet-blue may be further cross-linked by residual chrome. The tightly arranged fibres bundles bring a negative influence on the penetration of chemicals in subsequent process, which may result in defect of finished leather. In order to reduce grain defects and improve the quality of finished leather, a pre-treatment should be performed to homogenize wet-blue from different regions and disperse the fibres of wet-blue. Because pH of wet-blue was approximately 4.0, acid protease has potential to be applied in the bating process.

Most of currently used industrial enzymes are derived from microbial fermentation process, and the proteases remain the dominant enzyme type, so the composition of enzymes is very complex, and the enzymes were not used in any pure or well-characterized form[5]. So, in order to better explore the mechanism between acid protease and wet-blue, it is necessary to separate the active components of acid protease.

In this paper, the reaction mechanism between acid protease and wet-blue in bating process was investigated by exploring the biodegradability rate of the collagen and elastin in wet-blue. The changes of elastic fibres before and after acid protease treatment were observed. This study has a great significance t for protease application in wet-blue bating.

2 Experimental

2.1 Materials

The wet-blue were purchased from Shandong Dexin Leather Industry. Hydroxyproline (Hyp) were purchased from Shanghai yuanye Bio-Technology Co.,Ltd. Desmosine (DES) ELISA Kit were purchased from Jiangsu Kete Biological Technology Co.,Ltd. Acid protease were purchased from Longda Biotechnology (Shandong,China). Rainbow predyed wide molecular weight protein marker (10-260 KD) purchased from HeFei BoMei Biotechnology Co.Ltd. The chemicals used for analytical techniques were of analytical grade.

2.2 Determination the Enzymatic Properties of Acid protease

2.2.1 The optimum pH value and isoelectric point(pI)

The acid protease was dissolved in buffers with different pH values(pH=2-11).The activity was determined with casein solution as the substrate, from the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute[6-8]. The pI was determined by Zeta Potential and Nanoparticle Size Measuring analysers(Malvern,UK).

2.2.2 The molecular weight by SDS-PAGE method

At the same time, the molecular weight of the acid protease was analyzed by Polyacrylamide Gel Electrophoresis methods(SDS-PAGE)[9-12], the standard curve was established with relative mobility of standard protein marker and the relative mobility of acid protease were substituted for the standard curve equation to calculate its molecular mass. 12% separation gel and 5% stacking gel were separately prepared, the loading quantity of marker and acid protease are all 20 μL. The stacking gel voltage was 80 V and the separation gel voltage was 120 V and the thickness of the gel was 1mm.

2.3 Preparation of the Enzymatic Separation Hydrolysate of Acid protease

2.0 g enzyme powder was weighed accurately and dissolved in sodium lactate buffer (pH=3), filtered and fixed volume in 1 L volumetric bottle to prepare 2 g/L enzyme solution. According to the results of SDS-PAGE, the effective components of acid protease was separated by Tangential Flow Filtration (TFF) system (MinimateTM TFF Capsule with OmegaTM 50K Membrane, Pall Corporation, the USA).

A several circular samples with diameter of 5 cm were taken and divide into two groups with equal mass, A and B. Group A served as control group and the group B as experimental group. According to the conventional bating process, the group B was placed in the effective components and the group A was placed in the sodium lactate buffer(pH=3) at 40℃ treated for 4 h. The volume of the solution used in the control group and the experimental group was 100 mL. After the process, the waste liquid of the two group was taken, filtered and stored at 4 ℃ for reserve.

2.4 Analysis of Enzymatic Hydrolysate

2.4.1 Determination of Hyp by HPLC method

The Hydrolysate was mixed with 6M HCl in equal volume (v:v=1:1) and digested at 150 ℃ for 15 min by Microwave Digestion machine(Ethos. UP, Milestone, Italin). The Hyp content were determined by HPLC method[13-15] with pre-column derivation with 2,4-dinitrofluorobenzene. The mobile phase was A: ammonium acetate(30 mmol/L), B: acetonitrile. Gradient elution(0-7 min,80%A;7-10 min,60%A;10-30 min,60%A) with a flow rate of 1 mL/min. Aiglent TC-C18(5 um, 4.6×250 mm), the detection wavelength is 360 nm, loading quantity of sample was 20 uL and the temperature of chromatographic column is 27 ℃.

2.4.2 Determination of Des by ELISA method

This experiment was carried out by using the double antibody sandwich ELISA method to detect the Des content in the Hydrolysate[16-18]. A solid-phase antibody was prepared by coating the microporous plate with purified anti-desin antibody. Des was added to the micropore of the coated monoclonal antibody in turn, and then combined with HRP-labeled antides antibody to form an antibody-antigen-enzyme labeled antibody complex. After thorough washing, TMB was added to the substrate to develop the antibody. TMB is converted to blue catalyzed by HRP enzymes and finally yellow by acid. The OD value was measured at 450 nm by Enzyme Labeling Instrument (Labsystems,Multiskan,MS-352,Finland).The concentration of Des in the samples was calculated by standard curve. The specific test method is carried out according to the requirements of the instructions of the Des Element Kit.

2.5 Determination of the Biodegradation Rate of Structural Protein by Protease

Collagen accounts for three-quarters of the dry weight of skin[19],and Hyp is the unique amino acid of collagen and each 100 g of collagen contains 12.8 g of hydroxyproline[20]. Thus the constant for calculating collagen degradation rate is designed as 96. Elastin content in the skin is low, only constituted 2%-5% of the dry weight of skin[21],and the Des constant is $(2 \times 10^4)/17$ because 1 g elastin contains 17 µmol Des (M_{Des}=526.6031 g/mol) of Des[22].The wet-blue samples used for bating process were dried to constant weight in an oven at 50 ℃ and the total dry weight of the samples was determined, that is m_0. According to the concentration of Hyp and Des in enzymatic hydrolysate, that is C_{Hyp} and C_{Des}, respectively, and

according to the above relationship, then the biodegradation rates(Represent it with the letter D) of collagen and elastin during bating process were calculated according to the following formulas (1) and (2),respectively:

$$D_{collagen} = \frac{C_{Hyp} \times V}{96 m_0} \times 1000‰ \quad (1)$$

$$D_{Elastin} = \frac{20000V \times C_{Des}}{17 M_{Des} \times m_0} \times 1000‰ \quad (2)$$

2.6 Observation of the Change of Elastin Fibre by Acid Protease

The wet-blue samples were sectioned horizontal at 25 µm by the freezing microtome (CM1950 type, Leica Company, Germany). The slices were oxidized with potassium permanganate solution (5 g/L), then, the slices were bleached with oxalic acid solution (10 g/L), then, the slices were stained with aldehyde-fuchsin, and followed by separating, dehydrating, clearing, and sealing. The method of acid protease treatment for the slices is the same as the above. Then, the condition of elastic fiber treated or untreated by acid protease in wet-blue was observed by Super Depth of Field Microscope (Leica DVM6, Leica Company, Germany) with 1000 times magnification.

3 Results and Discussion

3.1 Analysing the Enzymatic Properties of Acid protease

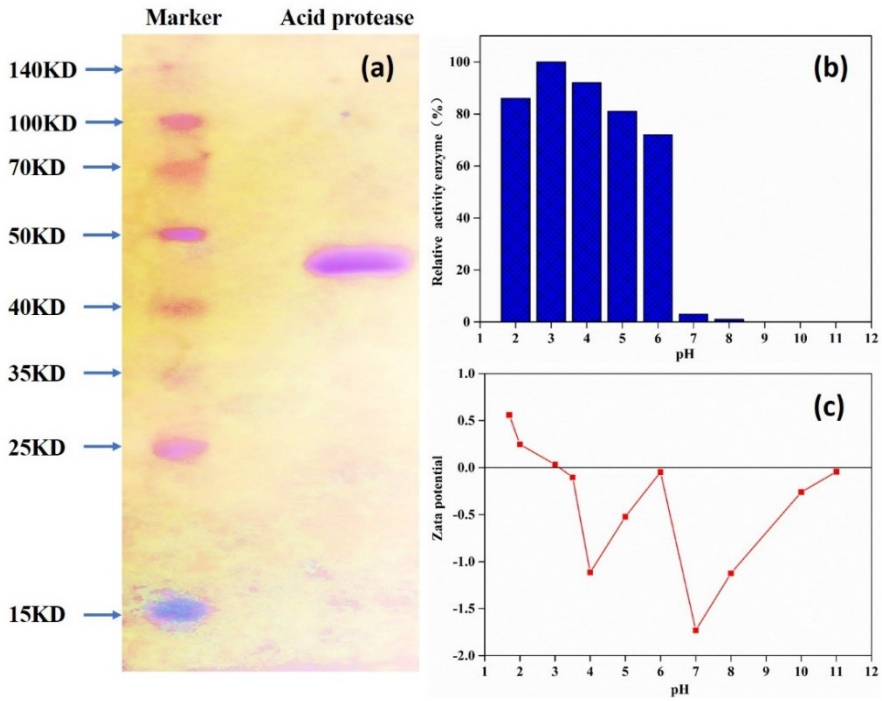

Fig. 1. The Enzymatic properties of Acid Protease. (a) the SDS-PAGE picture of acid protease;(b) the relative Enzyme activity of acid protease; (c) the isoelectric point(pI) of the acid protease.

From Fig. 1 (a), there is only one electrophoretic band, the map illustrate that the acid protease is highly expressed and the other enzymes are relatively few. According to the relative mobility standard curve equation of maker was $\lg M = -1.2316x + 2.1471$, $r = 0.9913$. and the molecular weight of acid protease was about 48 KD. Fig.1(b) and Fig.1(c) shows that the protease has stable catalytic activity under acid conditions, and the pI and the optimum pH are all around 3.0.

3.2 Analysing the Biodegradation Rate of Acid Protease

3.2.1 The content of Hyp and Des in hydrolysate

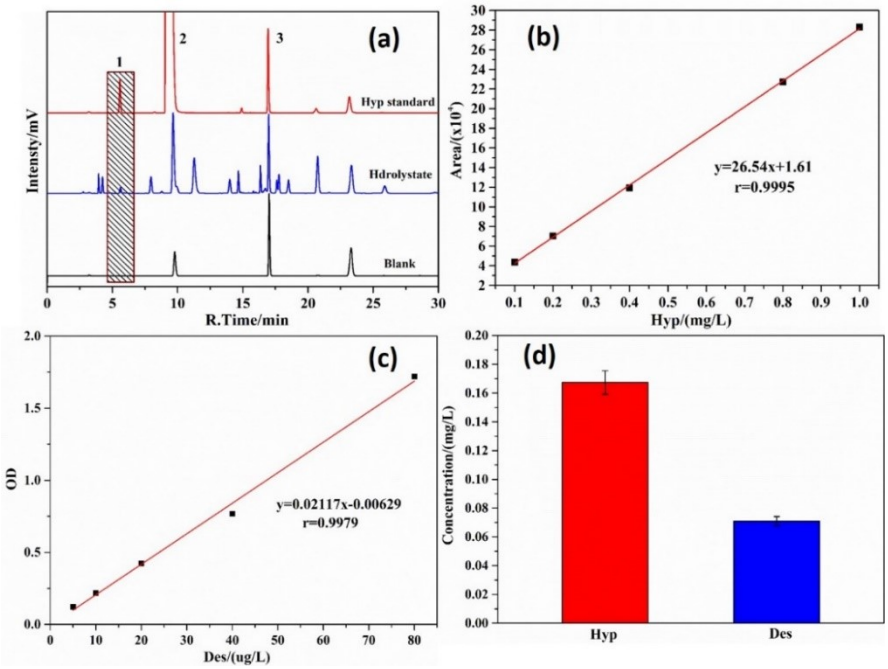

Fig 2. (a) The HPLC chromatograms. 1: Hyp; 2: 2, 4-Dinitrophenol; 3: 2, 4-Dinitrofluorobenzene (b) the standard regression curve of Hyp standard by HPLC; (c) the standard regression curve of Des by ELISA; (d) the concentration of Hyp and Des in Hydrolysate.

As shown in Fig. 2 (a), we can see that when elution time approached 6 minutes, the elution peak of Hyp appeared. Fig. 2 (b) shows that the standard curve equation of Hyp was $y = -26.54x + 1.61$, $y = A / 10000$, $r = 0.9995$. From Fig.2(d), the concentration of Hyp in enzymatic hydrolysate calculated by standard curve equation was 0.17 mg/L. In Fig. 2 (c), the standard curve equation of Des was $y = 0.02117x + 0.00629$, $r = 0.9979$. and from Fig. 2 (d), the concentration of Des in enzymatic hydrolysate calculated by standard curve equation was 0.071 mg/L.

3.2.2 The biodegradation rate of acid protease to structural protein

Table 1. The Biodegradation Rate of collagen and elastin in bating process.

Project	Structural Proteins in wet-blue	
	Collagen	Elastin
Biodegradation Rate (‰)	0.006	0.5

Table 1 shows that the biodegradation rates of collagen and elastin was 0.006‰ and 0.5‰, respectively. The result illustrated that the degradation rate of elastin is about 100 times that of collagen in bating process of wet-blue. Therefore, the degradation ratio of elastin is much larger than that of collagen, and the major reason for the results was elastin has fewer amino acids containing carboxyl group than collagen. Therefore, the cross-linking reaction of elastin with chromium is weaker than that of collagen in tanning process, so acid protease is more likely to act on elastin. Based on the above results, we can conclude that the degradation of elastin in wet-blue by acid protease is the main reason for improving the properties of crust leather.

3.3 Analysing the Change of Elastic Fibres by Acid Protease

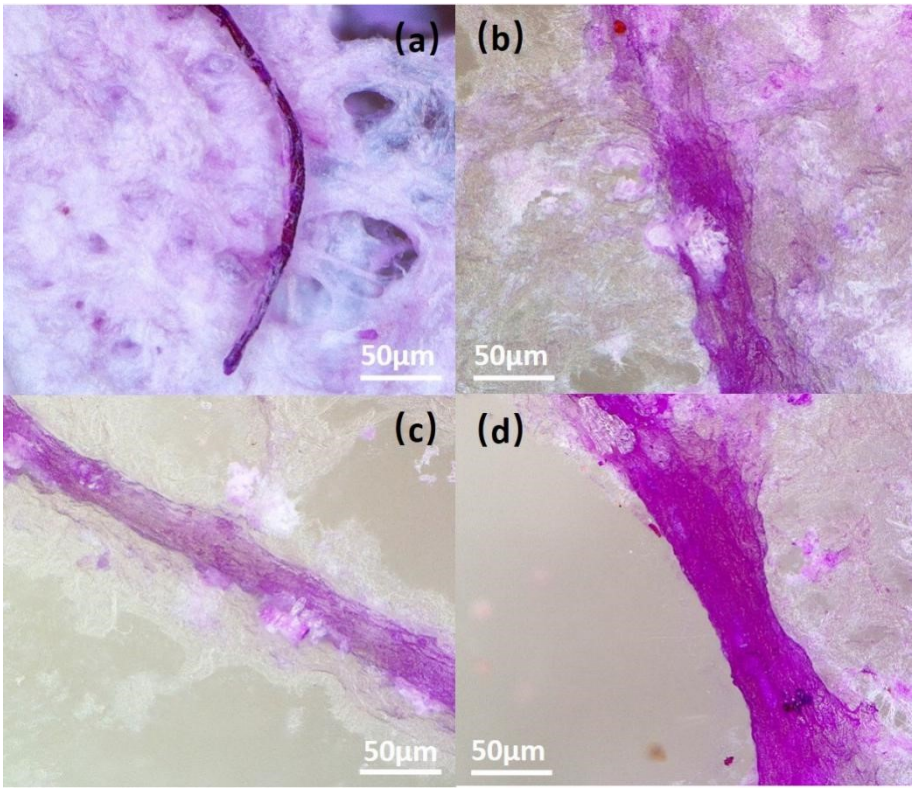

Fig. 3. The image of Elastic Fibres. (a) the Elastic Fibres untreated. (b), (c) and (d) the Elastic Fibres treated by acid protease.

Fig.3(a) shows that the elastic fibres in the wet-blue are dyed dark purple by aldehyde-fuchsin. Comparing the Fig.3(a) and (b), (c), (d), we can see the distribution range of the purple area is obviously enlarged, this indicates that elastic fibres are dispersed after treated with acid protease. Besides, the distribution of tissues (collagen, etc.) outside the elastin has also become uniform. This will make the flatness of grain surface and the softness of the crust leather be improved.

Conclusions

In the bating process, acid protease can degrade the two main structural proteins (collagen and elastin) in wet-blue and improve the performance of crust leather. However, in this process, the biodegradation rate of elastin is about 100 times that of collagen. Therefore, the conclusion that we come to is that the degradation of elastin in wet-blue by acid protease is the main factor affecting the improvement of performance of crust leather. This paper clears the degradation mechanism of wet blue to some extent which have great significance for the development of mechanism of acid protease to the bating process of wet-blue.

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