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Investigation of the Impact of Proteases for leather manufacture on Elastin

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Abstract

In recent years, tanners pay more attention to biotechnology of leather-making, so proteases are more generally used in various process stages, such as soaking, unhairing and bating, instead of traditional chemicals to reduce pollution. But excessive enzyme treatment will cause loose grain and even damaged grain, especially in enzyme-unhairing process. One of the keys to successfully carry out bioprocesses of leather-making is how to control the balance between the desirable effect and the proteolytic degree of proteases to structure proteins in skins and hides. Elastin is one of the important fibrous proteins, especially for grain layer, and the excessive degrading of elastin is an important factor causing grain damaged. Elastin is more sensitive to proteases, so our serials of investigations are focusing on evaluating the impacts of commercial proteases for leather manufacture on Elastin and leather quality.

This paper mainly involved in evaluating the specificity of typical and widely used proteases in leather-making processed to elastin. Firstly, a suitable and sensitive method for determining the elastase activity at the broad spectrum of pH, based on Remazol Brilliant blue Elastin powder (Elastin-RBB) substrate, was established. The main commercial proteases were selected, including microorganism and pancreas, alkaline, acid and neutral proteases widely used in the processes of soaking, unhairing, liming and bating, for assaying elastolytic activity. The specificities of these proteases to collagen were also evaluated, based on hide powder-RBB substrate. The results show that most commercial alkaline and neutral proteases exhibit elastolytic ability, while acid proteases almost show very low activity against elastin. The optimum pH of elastase activity of these proteases is about 9.0. The optimum temperature is about 65°C, and elastinase activity linearly increases with temperature rise from 30-65°C. Comparing to bacterial enzymes, pancreatic proteases show lower activity against elastin, especially, a purified typsinase. Most of bacterial proteases show higher activity against elastin, and several alkaline proteases used in soaking and liming processes exhibit much higher ratio of activity to elastin against collagen. So it should be very careful to select proteases in leather-making processes to avoid to grain loosing and damage from the excessive removal of elastin.

Keywords: protease; elastolytic activity; leather manufacture

1. Introduction

Elastin is a kind of insoluble protein that always appears in conjunction with collagen. It is one of the major non-collagen fibrous proteins mostly present within grain layer and surrounding blood vessels in hides and skins. Elastic fibers have important influence on the leather quality. Although present at low concentration in the skin, it is known that it can contribute to the elastic properties of the skin [1], enhance the strength of leather, especially grain layer, and impart leather good grain tightness. But in the meantime, the existence of elastic fibers can also limit the softness, extensibility and area of leather[2].

Elastin is rather stable, and it is difficult to degrade elastin with chemicals, heat and extreme pH, but it is sensitive to some proteases with elastolytic activity[3]. The findings of some early researches indicated that conventional leather-making beamhouse operations canot significantly damage elastic fibers, such as liming and bating. The removal of elastin from hides and skins during processing by protease has a variety of effects on the properties of leather. Appropriately damaging elastic fiber by elastase can improve the softness, extensibility and area of leather and it is necessary in soft leather processing, such as sofa leather and garment leather[4]. Excessive breakdown of elastic fiber by elastase will result in the looseness of leather and even grain damage[5]; hence, the digestion of elastin should be strictly controlled during shoe-upper leather making. In recent years, proteases as a cleaning reagent have been widely applied at various process stages of leather-making, such as soaking, liming, unhairing and bating of hides and skins[6]. So it should be careful to use proteases with elastase activity to avoid to the excessive loss of elastin in grain layer in the bioprocesses of leather-making, especially enzyme-unhairing. The main objective of this paper is to establish a new sensitive method for determining the elastase activity at the broad spectrum of pH, and investigate the elastolytic ability of the main commercial proteases for leather manufacture.

2. Materials and methods

2.1 Materials

Remazol Brilliant Blue R (RBB) was purchased from sigma. Proteases were chosen from commercial leather enzymes, and supplied by Novozymes and Dowell Technology Co., Ltd., etc.

2.2 Methods

2.2.1 Preparation of Remazol Brilliant Blue-elastin powder (Elastin-RBB) [7]:

Elastin powder was extracted by hot alkaline process from bovine ligamentum nuchae or aorta [8]. 5g elastin powder was soaked in 50 ml of distilled water at 60°C in a Erlenmeyer flask and stirred for 30min. Then 0.5% (w/v) RBB solution was added to the elastin powder suspension. After stirred for another 10min, 10g of sodium sulfate was added in four sequential additions over a 20min interval, in 2.5g increments, and then alkaline solution was used to adjust the suspension pH to 11. The mixture was stirred for another 30min at 60°C. The product was neutralized to pH 7 and then centrifuged to

separate the supernatant solution. The stained elastin powder was thoroughly washed with distilled water until the supernatant was quite colorless, dehydrated with acetone and then air dried at room temperature. The dried powder was milled and the Elastin-RBB powder was obtained.

2.2.2 Dye-eluting characteristics of Elastin -RBB and Elastin-Congo red

Test tubes (1.5 ml) were respectively charged with 5mg of Elastin-RBB or Elastin-Congo red. 1ml of 0.05M Britton-Robinson buffers with pH range from 3.0 to 11.0 were added, individually. The tubes were stirred in an incubator at 40°C for 30min, and then centrifuged to separate the supernatant solution. The optical density of each elution was determined immediately at 596 nm or 495nm.

2.2.3 Optical absorption characteristics of the elastin-RBB hydrolysate

A protease with high elastolytic activity was chosen to hydrolyze the stained elastin. 0.5g of RBB-elastin powder was put into a beaker and soaked in 10ml of 0.05 M Britton-Robinson buffer of pH 9.0 at 40°C for 20min, and then the protease solution with sufficient amount was added. The mixture was incubated for 60min, and the stained elastin was completely degraded to a clear solution. Part of the hydrolysate solution was taken and diluted with buffers of different pH, respectively, and then the absorbance of these solutions was detected in wave-length range from 200nm to 800nm.

2.2.4 Determination of Elastin-RBB calibration curve

Increasing amounts (2mg to 12mg) of elastin-RBB were incubated in 1ml of 0.05 M Britton–Robinson buffer of pH 9.0 with excess amount elastase solutions until completely degraded to clear solutions. Then part of the hydrolysate solution was taken and diluted with buffers of different pHs, respectively. The optical density of each sample was plotted against the respective amount of substrate dissolved. Then the real amount of hydrolyzed elastin during the elastase activity analyzing was obtained according to the calibration curve.

2.2.5 Elastase activity assaying

Test tubes (1.5 ml) were charged with 5±0.1 mg of stained substrate and 1ml of buffer with differing pH. The tubes were placed in an incubator at 40°C for preheating 5 minutes, and then 0.2 ml of each enzyme solution was added. The tubes were stirred for accurate 30 minutes at the rate of 800 rpm. The tubes were then centrifuged immediately for 5 minutes at the rate of 1200 rpm. The optical density of the supernatant was determined immediately at 596 nm in a spectrophotometer. Blanks were obtained similarly by adding enzyme solutions at the end of the incubation period, immediately before centrifugation. The amount of enzyme digested elastin was calculated by comparing the OD value with the calibration curve.

One unit of elastase activity was defined as the amount of elastase capable of digesting 1μg of substrate by incubation at 40°C for 1 min under standard conditions.

2.2.6 Collagenase activity determination

The collagenase activities of the above chosen proteases were also detected, and the testing procedure is similar to that of elastase activity determination, just using hide powder-RBB as the substrate. One unit of collagenase activity was defined as the amount of collagenase capable of digesting 1μg of

substrate by incubation at 40°C for 1 min under standard conditions.

3. Results and discussion

3.1 Optical absorption characteristics of Elastin-RBB enzymatic hydrolysate

The Elastin-RBB can be completely digested by an elastinase. Fig.1 shows that the maximum absorption peak of prepared Elastin-RBB enzymatic hydrolysate is located at around 596 nm, and the OD curves of the hydrolysate at differing pH from 3 to 11 almost have no move.

Proteases are used in different pH processes of leather-making, so the evaluating the impact of proteases on elastin should be undertaken at a broad spectrum of pH, and it needs the dyestuff do not release from the substrate; otherwise, it will affect the accuracy of the determining results. The comparison of color-elution characteristics of Elastin-RBB and Elastin-Congo red is illustrated in Fig. 2. It can be seen that Congo red dyestuff is easily eluted from the substrate at alkaline pH, and be fixed well at low pH, especially lower than 3. And the color of Elastin-Congo red will change at different pH, because of Congo red dyestuff with bright-red colour in alkali state and with blue-purple color in acid state. So it is not an appropriate substrate to assay elastase activity at a broad spectrum of pH condition.

Remazol Brilliant Blue R is a kind of active dyestuff with vinyl sulfone group, which can bind with substrate through covalent bond, so the stained substrate has excellent colorfastness, and the dyestuff cannot be easily released from the substrate even if at extreme pH. Fig 1 and fig 2 show that RBB can not be eluted and its colour is stable at the broad spectrum of pH, so Elastin-RBB is a suitable substrate for assaying elastolytic activity of proteases used in different processes of leather-making.

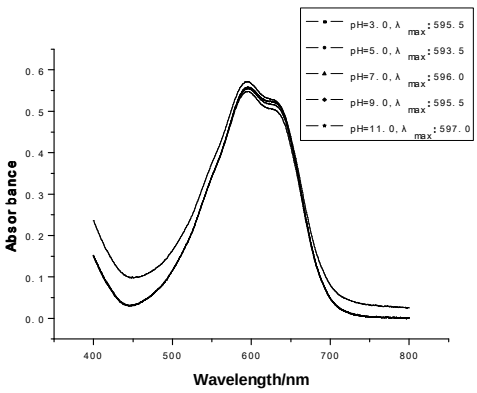

Fig.1 The wavelength scaning of Elastin-RBB hydrolysate

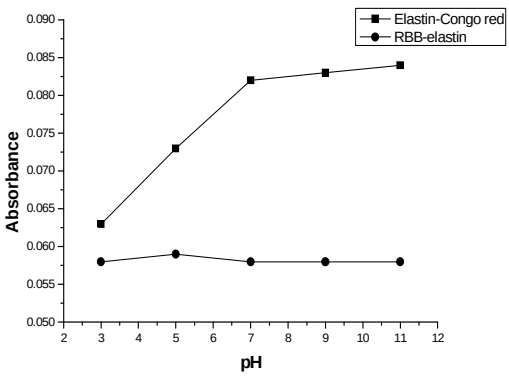

Fig.2 The elution characteristics of RBB-elastin and Elastin-Congo red

3.2 Determination of Elastin-RBB calibration curve

As mentioned before, one unit of elastase activity was defined as the amount of elastase capable of digesting 1μg of substrate by incubation at 40°C for 1 min under standard conditions. The relationship between the amount of Elastin-RBB and the OD value of its digestion solution should be established. Graded amounts of elastin-RBB were digested to clear solutions with adequate elastase. The relationship of optical density values of the digested solutions at different pH with the amount of substrate are illustrated in Fig. 3. It shows the calibration curves under different pH are coincident and indicates that pH has no influence on the standard curve. The linear regression equation is obtained as y=0.38764*x (R=0.99989), which is very close to the documentary report [7].

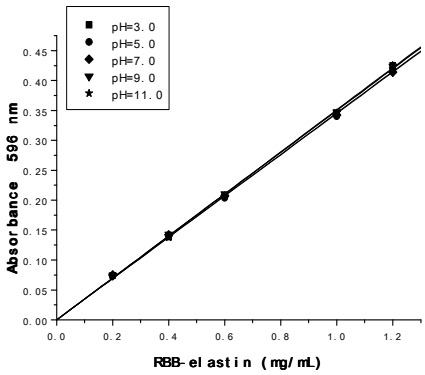

Fig.3 RBB-elastin calibration under different pH

3.3 Reacting characteristics of elastase with Elastin-RBB

A kind of leather protease from Novozymes Company, named AX and declaimed behaving relatively high elastolytic activity, was chosen to study the reacting properties as the representative of leather elastases.

3.3.1 Effect of reaction time on elastolytic reaction

The effect of reaction time on elastase activity of AX enzyme was studied over a range of reaction time (10-40min) respectively under the condition of 40°C and pH 9.0. The experimental results, presented in Fig.4, demonstrates that under the determination conditions, the relationship between reaction time and the absorbance is linear (R=0.99946) within 30min. The enzyme activity is measured by determining the initial velocity of enzymatic reaction, consequently, 30 minutes is reasonably chosen as reaction time for assaying elastase activity in consideration of the shorter reaction time, the larger the experimental error.

3.3.2 Effect of elastase concentration on elastolytic reaction

The effect of enzyme concentration on elastase activity was studied over a range of enzyme concentration (1.0-4.0mg/mL of AX enzyme) respectively under the determination condition of 40°C and pH 9.0. Fig. 5 shows that when the reaction system was charged with a fixed amount of

Elastin-RBB substrate (5mg) and the enzyme concentration is between 1.5 and 3.0 mg/mL, the relationship between the enzyme concentration and the absorbance is linear (R=0.99975). Consequently, in order to obtain correct value of elastase activity in this reaction system, the absorbance values are more reliable in the range of 0.18-0.32 and the elastolytic activity is in the range of 18-36 U/mL.

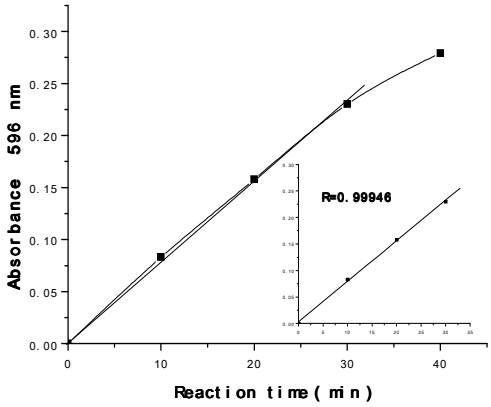

Fig. 4 Effect of time on elastolytic reaction

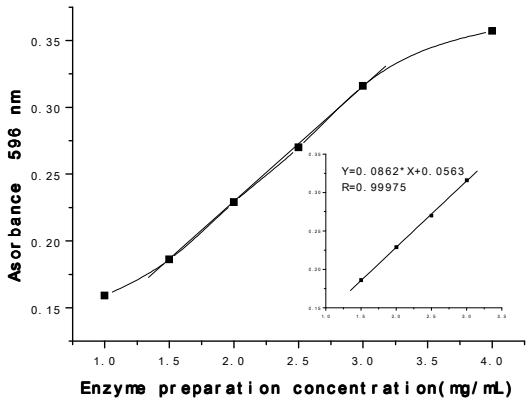

Fig.5 Effect of elastase concentration on elastolytic

3.3.3 Effect of pH on elastolytic reaction of several leather proteases

Several leather proteases, including neutral and alkaline enzyme, were chosen to investigate their impact on elastin under different pH. AX is a neutral protease used in bating, PY is an alkaline protease used in soaking, MP is an alkaline protease used in liming and PTN is a partially purified pancreatic protease. The optimum pHs of these proteases were studied over a broad spectrum of pH (3.0-11.0) with Elastin-RBB respectively at the determination conditions of 40°C and 30 min. Fig. 6 shows that the selected three bactrial proteases behave the largest elastolytic activity at around pH9.0, but the partially typsin's optimum pH is about 8.5, which indicate that elastinase is a kind of alkaline proteases. Elastase activity significantly decreases as pH value below 7.0 or above 10.0. This result is consistent with the result reported in several literatures [9-11]. The adsorption of elastase to elastic fibres directly impacts on elastase activity through the side-chain carboxyl groups [12].

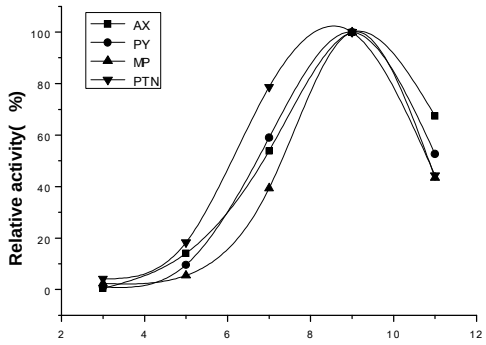

Fig.6 Effect of pH on elastolytic activity

3.3.4 Effect of temperature on elastolytic activity

The effect of temperature on elastase activity of AX enzyme was examined at various temperatures (40-70°C) for 30min at pH 9.0. The enzyme is active at a broad range of temperatures with an optimum temperature around 65°C, and this is very similar to the other research results [9]. Elastase activity linearly increases with temperature raised from 40-65°C. The experimental result was illustrated in Fig.7.

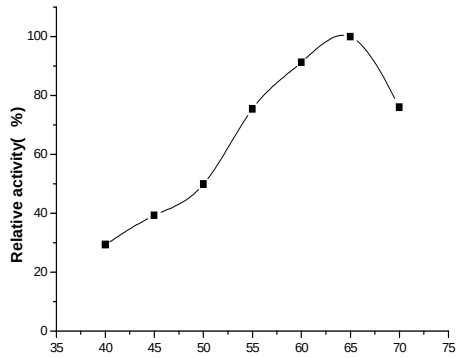

Fig.7 Effect of temperature on elastolytic

3.4 Reproducibility of elastase activity assay method

For verifying the reproducibility of the proposed method, it was designed to determine the elastase activity at least six times using two batches of Elastin-RBB prepared at different time respectively as the substrates. The results are shown in Table 1. It is found that the each result of elastase activity measured is relatively close and the error is within the limit of experiment. The reproducibility of the proposed method for determining the elastase activity based on RBB-elastin substrate was satisfactory.

3.6 Comparison of elastase activity measured with three different substrates

Further, three kinds of stained elastin substrates, bovine ligamentum nuchae elastin-RBB (substrate1), bovine aorta elastin-RBB (substrate2) and elastin-Congo Red (substrate3), were used to evaluate the impact of typical leather proteases on elastin in order to compare the effect of the selected proteases on elastins with different origins. PY, SG and AX, three proteases usually used in soaking, unhairing and bating process, were chosen as the evaluated objects.

Tab.1 The reproducibility of the proposed assay method (AX enzyme)

Different batches of RBB-elastin	Elastase activity (U/g)						average value	RSD (%)
RBB-elastin(0322)	59363	60912	59429	58847	59621	60266	59740 ±735	1.2
RBB-elastin(0510)	58613	58365	60064	59220	61031	58733	59338 ±1024	1.7

The results (Table 2) show that all of the three selected leather proteases behave considerable elastolytic activity, especially PY. They also show the different hydrolyzing ability to different original elasin, and the elastin of bovine aorta is more sensitive to elastinase than that of ligamentum nuchae. Although the absolute values of elastase activity of these three enzymes to different substrates are quite different, the activity ratios of each enzyme between two of the three substrates among these three proteases are substantially similar. Consequently, when comparing the relative ability of different proteases to digest elastin at pH9.0, any of these three substrates should be feasible.

Tab.2 Results of elastase activity measured with three different substrates*

	PY	SG	AX	Ratio(PY/AX)	Ratio(SG/AX)
substrate1	114684	39728	60064	1.910	0.661
substrate 2	557651	195085	265691	2.100	0.734
substrate 3	355056	114132	175911	2.020	0.649
Ratio(1/2)	0.206	0.204	0.226		
Ratio(1/3)	0.323	0.348	0.341		

*: Elastase activity was determined at 40 ^{o}C , pH9.0.

3.7 Investigation of elastase activity of commercial proteases

The main proteases used in leather processes were chosen for evaluating their impact on elastin, including microorganism and pancreas, alkaline, acid and neutral proteases widely used in the processes of soaking, unhairing, liming and bating. In order to ensure the accuracy of the testing results, all of the selected proteases are in the concentrated form, which are the diluted leather enzyme products' raw materials. The activities of these proteases to collagen were also evaluated, based on hide powder-RBB substrate. The results are show in Table 3.

It can be seen that most of commercial alkaline and neutral proteases from bacteria exhibit rather high activity to elastin, pancreatic proteases showed lower activity against elastin, especially, a purified typsin (PTN-P), and acid proteases from fungi have very low activity against elastin. It is well accepted by tanners that bating effect of pancreatic enzyme is mild and safety, relatively. Maybe the main reason is from its low activity against the elastin in skin or hide's grain layer. Table 3 also shows that the ratio of collagenase activity to elastinase activity. Most of selected proteases exhibit both two kinds of activity and several alkaline proteases used in soaking and liming processes exhibited much

higher activity to elastin than to collagen. So it should be very careful to select proteases in leather-making processes for different aims to avoid to grain loosing and damage from the excessive removal of elastin.

Tab.3 The investigation results of commercial proteases

Commercial proteases		The different substrates for assaying enzyme activity		Ratio(2/1)
		1（hide powder-RBB）	2（Elastin-RBB）	
Alkaline proteases	SG	87896	40830	0.46
	PY	50966	114539	2.25
	TAN G	44259	58838	1.33
	MP	71608	496496	6.93
	LIME GS	84766	131565	1.55
	LIME G	43004	27305	0.63
neutral proteases and Trpsin	BATE GS-G	31031	90935	2.93
	1398	24119	9493	0.39
	AX	33706	60064	1.78
	PTN	47813	17597	0.37
	PTN-P	43696	4538	0.10
	BATE 100	18520	14759	0.80
	BATE 115	8876	4024	0.45
Acid proteases	WB	/	3147	/
	ABG	/	929	/
	537	/	1135	/

4. Conclusions

A sensitive method for determining elastase activity at the broad spectrum of pH, based on elastin-RBB substrate, was established. The stained elastin (Elastin-RBB) was derived from elastin powder labeled covalently with Remazol brilliant Blue R, which is highly stable and is a suitable substrate for assaying elastolytic activity of leather-using enzymes at the broad spectrum of pH. The reproducibility of the proposed method was satisfactory.

The impact of proteases on elastin applied in leather-making processes was also evaluated. The results show that most commercial alkaline and neutral proteases exhibited specificity to elastin, and acid proteases almost have no activity against elastin. Most of bacterial proteases show higher activity against elastin than pancreatic proteases. Several alkaline proteases used in soaking and liming processes exhibite much higher activity to elastin against collagen. So it should be very careful to select proteases in leather-making processes to avoid to grain loosing and damage from the excessive removal of elastin.

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Acknowledgments

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