EFFECTIVE COMPONENT IN α-AMYLASE PREPARATION FOR UNHAIRING

by

YUNHANG ZENG,[1,2] XIAN KONG,[1] YA-NAN WANG,[1,2] XUEPIN LIAO,[1] WENHUA ZHANG[1] AND BI SHI[1,2]*
[1]*Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University,*
CHENGDU, 610065, P. R. CHINA

[2]*National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University,*
CHENGDU 610065, P. R. CHINA

ABSTRACT

The use of α-amylase preparations (AP) in sulfide and lime free unhairing systems has recently received renewed interest. However, the mechanism of AP action on hides or skins remains unclear. AP is generally a mixture of α-amylase and concomitant protease(s), and it is not yet known which components of AP are effective in unhairing. To investigate whether "pure" α-amylase or concomitant protease(s) are mainly responsible for unhairing, a protease-free α-amylase preparation (PFAP) and an amylase-free α-amylase preparation (AFAP) were prepared by selective inactivation of protease(s) and α-amylase in AP respectively. AP, PFAP and AFAP were employed for unhairing of cattle hide for 4 h at 25°C, and their effectiveness of unhairing was evaluated by analyzing the extent of removal of hair, epidermis and protein from hide. The results indicated that rather than "pure" α-amylase, the concomitant protease(s) in AP were the effective components for unhairing. This suggests that the mechanism of AP action on hides should be consistent with the mechanism of enzymatic unhairing with proteases.

RESUMEN

El uso de preparados α-amilasa (AP) en sistemas de pelambre libres de sulfuro y cal ha recibido recientemente un renovado interés. Sin embargo, el mecanismo de acción de AP en las pieles sigue siendo poco clara. AP es generalmente una mezcla de α-amilasa y proteasa(s) concomitantes, y todavía no se conoce cuál de los componentes de AP son eficaces en el depilado. Para investigar si la α-amilasa "pura" o proteasa(s) concomitantes son responsables principalmente del depilado, una preparación de α-amilasa libre de proteasa (PFAP) y una preparación libre de α-amilasa (AFAP) fueron preparados mediante la inactivación selectiva de las proteasa(s) y α-amilasa en AP, respectivamente. AP, PFAP y AFAP fueron empleados en el depilado de cuero vacuno durante 4 horas a 25°C, y su eficacia en el depilado se evaluó analizando el grado de eliminación de pelo, epidermis y proteínas de la piel. Los resultados indicaron que en lugar de la α-amilasa "pura", la proteasa(s) concomitantes en AP fueron los componentes eficaces en el depilado. Esto sugiere que el mecanismo de acción de AP en pieles debe ser consistente con el mecanismo de depilado enzimático con proteasas.

---

*Corresponding author e-mail address: sibitannin@vip.163.com

Manuscript received October 8, 2012, accepted for publication December 4, 2012.

INTRODUCTION

Conventional sulfide-lime unhairing system, which results in high sulfide and COD content in tannery wastewater and a large quantity of lime sludge, has received considerable attention due to increasingly stringent environmental regulations. Therefore, much research recently has focused on the development of environmentally friendly unhairing systems, such as unhairing with protease or peroxide, and opening-up of collagen fibers with sodium hydroxide or sodium metasilicate.[1-6] In the last decade, some researchers have shown renewed interest in the use of α-amylase for non-sulfide or non-lime unhairing systems. They found that unhairing with the composite of α-amylase and protease resulted in a higher amount of hair removal in comparison with unhairing using protease alone.[7, 8] Furthermore, α-amylase has fiber opening-up action and is considered to be a potential substitute for lime.[9, 10]

However, the mechanism of action of α-amylase on hides/skins remains unclear. Burton, Reed and Flint observed that unhairing was completed by using amylase within approximately three days at room temperature, and they suggested that this phenomenon may be due to the removal of mucoid material.[11] Bose, Madhava Krishna and Das supported the suggestion of Burton *et al.* and reported that unhairing using amylase depended essentially on the hydrolysis and removal of mucoid.[12] But Gillespie argued that amylase could neither unhair nor act on mucoid material, and that bacteria may have been primarily responsible for the unhairing observed by Burton *et al.*.[13] On the basis of Burton's and Gillespie's discussions, Cordon stated that commercial amylases could effectively remove hair from pretreated hides in a sterile unhairing process. However, Cordon also pointed out that commercial amylase preparations are not single enzymes and that the effective components of the amylase preparations for unhairing is not yet known.[14] In fact, α-amylase preparations are generally mixtures of amylases and concomitant proteases, and the concomitant proteases are difficult and expensive to remove.[15-17] In addition, it has been shown that many proteases are useful for unhairing,[18] and Yates even reported that the only enzyme activity identified with unhairing is a proteolytic activity involving proteases of the endopeptidase type.[19] Therefore, for understanding the action mechanism of α-amylase preparation on hides or skins, it is necessary to first determine whether "pure" α-amylase or concomitant protease(s) are mainly responsible for unhairing.

In this study, a protease-free α-amylase preparation (PFAP) and an amylase-free α-amylase preparation (AFAP) were prepared by selectively inactivating protease(s) and α-amylase in α-amylase preparation (AP) respectively. The effectiveness of unhairing using AP, PFAP and AFAP were investigated by analyzing the extent of hair, epidermis and protein removal from hide.

EXPERIMENTAL

Materials

Conventional soaked cattle hide was used for unhairing trials. The commercial bacterial α-amylase preparation (AP) supplied by Youtell Biochemical Co. Ltd. (Shanghai, China) was employed for the preparation of PFAP and AFAP and the unhairing trials. All the chemicals used for processes in leather manufacture were of commercial grade, and the chemicals used for the analyses were of analytical grade.

Preparation of PFAP and AFAP

α-Amylase Activity Assay

α-Amylase activity was assayed by the method described in literature with some modifications.[20] The reaction mixture containing 1 mL of 1.0 mg/mL enzyme solution, 1 mL of Briton-Robinson buffer (pH 7.0) and 2 mL of 1% (w/v) starch solution was incubated at 25°C for 5 min. The reaction was stopped by adding 4 mL of 0.4 mol/L NaOH solution. Then 2 mL of properly diluted mixture was reacted with 2mL of dinitrosalicylic acid reagent (100 mL of solution contained 1 g of 3,5-dinitrosalicylic acid, 30 g of potassium sodium tartrate, and 20 mL of 1 mol/L NaOH) in boiling water for 5 min. After reaction, the mixture was cooled to room temperature and diluted to 25 mL. The absorbance of the diluted solution was then measured at 520 nm using an Ultraviolet Visible Spectrophotometer (UV-Vis, Lambda 25, PerkinElmer, USA) to calculate the amount of maltose released during enzymolysis. One unit of α-amylase activity was defined as the amount of enzyme, which releases 1 mg maltose per minute under the assay conditions.

Protease Activity Assay

Protease activity was assayed by the method described in standard.[21] The enzymatic hydrolysis reaction was performed by incubating 1 mL of 1.0 mg/mL enzyme solution with 1 mL of 2% (w/v) casein in Briton-Robinson buffer (pH 7.0) at 25°C for 10 min. The reaction was then stopped by adding 2 mL of 0.4 mol/L trichloroacetic acid. The mixture was settled at 25°C for 20 min and successively filtered. Then, 1mL of filtrate was reacted with 5 mL of 0.4 mol/L Na_2CO_3 solution and 1 mL of Folin-Ciocalteu reagent at 40°C for 20 min. After reaction, the absorbance of the mixture was measured at 660 nm to obtain the amount of tyrosine released during enzymolysis. One unit of protease activity was defined as the amount of enzyme that releases 1 μg tyrosine per minute under the assay conditions.

Effect of pH on Enzyme Activity of AP

The effect of pH on α-amylase activity and protease activity of AP was investigated. A series of Briton-Robinson buffers were prepared, where the pH values of buffers were 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, respectively. The α-amylase activity and the protease activity of AP were determined by the same procedures described above except that the Briton-Robinson buffer of different pH was used for each assay.

Preparation of PFAP

In order to obtain PFAP (viz. "pure" α-amylase), the protease(s) present in AP were inactivated by heating AP solution according to the method described by De Stefanis and Turner with minor modification.[22] The operating temperature for protease inactivation was optimized by heating 10 g/L AP solutions at different temperatures for 15 min and then immediately cooling the solutions to room temperature, where the temperatures were 25 °C (control), 40°C, 50°C, 60°C and 70°C. The residual relative α-amylase activity and protease activity were calculated as:

$$\text{residual relative enzyme activity} = \frac{\text{enzyme activity after inactivation}}{\text{enzyme activity before inactivation}} \times 100\%$$

According to said experiments, PFAP was obtained after heating 10 g/L AP solution at the optimum temperature (70°C) for 15 min and then cooling to room temperature.

Preparation of AFAP

For achieving AFAP (viz. concomitant proteases), the α-amylase in AP was inactivated using sodium hypochlorite according to the method established by Hoerle.[23] The amount of sodium hypochlorite was primarily optimized. The inactivation of α-amylase was conducted by adding different amounts of sodium hypochlorite solution (4% weight of available chlorine) into 10% (w/v) aqueous solution of AP, followed by constant shaking in 130 rpm at 25°C for 2 h, where the amounts of sodium hypochlorite solution were 0 (control), 2.5%, 5.0%, 10.0% and 20.0% of AP weight respectively. After inactivation, the residual relative α-amylase activity and protease activity were analyzed. According to said experiments, AFAP was obtained by adding the optimum amount (20% of AP weight) of sodium hypochlorite solution into 10% (w/v) AP solution, followed by shaking at 25°C for 2 h.

Effectiveness of Unhairing by Enzymes

Unhairing Processes

Six pieces of soaked cattle hide without hair slip were prepared for the following unhairing trials. Each of them was approximately 500 g. Enzymatic unhairing was performed in the solution containing X% enzyme (X represents amount of enzyme, as listed in Table I) and 50% water at 25°C for 4 h. After enzymatic unhairing, the pelts were treated using the procedures given in Table II.

Analyses of Proteins and Hyp Concentrations in Enzymatic Unhairing Liquors

After enzymatic unhairing, the unhairing liquors were sampled and centrifuged at 8000 rpm for 10 min. The supernatant liquors were taken for measurement of proteins and Hyp concentrations as reported in documents.[24, 25]

TABLE I
Enzymes used for unhairing trials[a].

Number of group	Offer of enzyme
1 (control)	no enzyme
2	0.25% PFAP
3	0.50% PFAP
4	0.25% AP
5	0.25% AFAP
6	0.50% AFAP

a - Percentage is based on weight of soaked hide.

TABLE II
Chemical unhairing and deliming processes[a].

Process	Offer of agent	Remarks
Washing	200% water	Run 5 min
Chemical Unhairing	100% water, 1% lime	Run 60 min, drain 50% water
	1% Na_2S, 1% NaCl	Run 60min
Washing	200% water	Run 5 min
Deliming	100% water, 4% $(NH_4)_2SO_4$	Run 50 min

a - Percentage is based on weight of soaked hide.

Observation of Unhaired Pelts

In order to analyze the extent of hair, epidermis and pigment removal, the grain of enzymatic unhaired pelts and delimed pelts was captured using a digital camera, respectively. Furthermore, after enzymatic unhairing, samples (dimensions 1 cm × 1 cm) were cut from pelts and fixed in 10% neutral buffered formalin for 48 h. Then, the samples were cut into sections of 15μm thickness using a freezing microtome (CM1950, Leica, Germany). The sections were stained with Weigert's iron hematoxylin and then counterstained with Van Gieson's stain to differentiate collagen fibers, muscle fibers, hair roots and epidermis. After staining, the histological sections were observed using a biologic microscope (CX41, Olympus, Japan).

RESULTS AND DISCUSSION

PFAP and AFAP

Since α-amylases are mostly derived from microorganisms, especially bacteria, due to their stable performances and low-cost production,[18, 26] a commercial bacterial α-amylase preparation (AP) was employed in this study. It is evident that AP is a mixture of α-amylase and protease(s) as shown in Figure 1, which is consistent with previous studies.[15, 16] The increase of pH exhibits negative effect on the α-amylase activity of AP, and the effect of pH on the protease activity is very similar with that on the α-amylase activity. This should be due to the fact that amylases and proteases are concomitant enzymes in AP.

As mentioned previously, the purpose of this study was to investigate which enzymes in AP are mainly responsible for unhairing. For this purpose, we first need to obtain "pure" α-amylase and "pure" protease(s) from AP. Compared with some purification methods, such as ion exchange chromatography, separation on columns of CMC or DEAE cellulose, and Sephadex gel filtration, selective inactivation of protease(s) or α-amylase in AP is more economical and simpler to achieve "pure" α-amylase or "pure" protease(s). Although undesirable enzymes are still present in AP after selective inactivation, they are effectively neutralized as if they were physically removed. [23]

In our study, a thermal treatment of AP solution was used for selectively inactivating protease(s) in AP. The effect of temperature on the extent of protease inactivation is given in Figure 2(a). The residual relative protease activity of AP decreased with the rise of temperature. After heating at 70°C for 15 min, the AP solution was almost free of protease activity while simultaneously full of α-amylase activity. These results suggest that PFAP can be prepared by heating AP solution at 70°C.

In addition, AP solution was incubated with sodium hypochlorite for an irreversible inactivation of α-amylase in AP as reported by Hoerle.[23] As can be seen in Figure 2(b), the increasing amount of sodium hypochlorite is accompanied by a remarkable decrease in residual relative α-amylase activity and a slight decrease in residual relative protease activity. When the amount of sodium hypochlorite solution increased to 20% of AP weight, the relative α-amylase activity remained about 5% and the residual relative protease activity was nearly 60%. In spite of this, the residual α-amylase activity was negligible and the residual protease activity was acceptable. Therefore, 20% sodium hypochlorite solution can be used to obtain AFAP.

As a result of using the optimum conditions described above, we obtained PFAP and AFAP by inactivating protease(s) and α-amylase in AP respectively for the following unhairing trials.

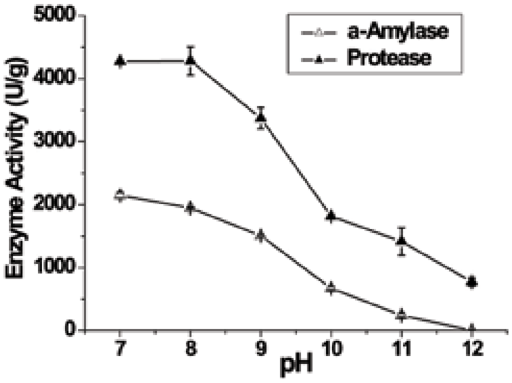

Figure 1. Effect of pH on enzyme activity of AP.

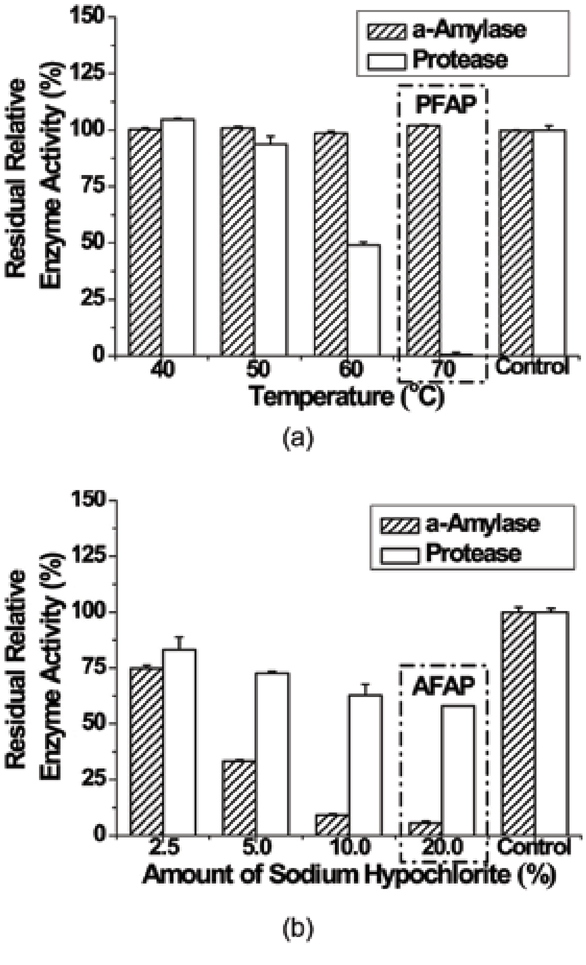

Figure 2. Effects of temperature (a) and amount of sodium hypochlorite solution (b) on residual relative enzyme activity of AP.

Unhairing Effectiveness of AP, PFAP and AFAP

In this section, the unhairing effectiveness of PFAP, AFAP and AP was investigated to determine whether "pure" α-amylase or concomitant protease(s) are the effective components of AP for unhairing. Because the main purpose of an unhairing system is to remove hair, epidermis and noncollagen proteins from hides,[27] the effectiveness of enzymatic unhairing was evaluated by analyzing the extent of hair, epidermis and protein removal.

Extent of Hair Removal

The digital photos of grain of enzymatic unhaired pelts and the photomicrographs of Van Gieson stained horizontal sections from enzymatic unhaired pelts are shown in Figure 3. It can be observed that no hair was removed from the control hide (Figure 3(1)), which indicates that microorganisms on hides cannot cause loosening of hair after unhairing for 4 h at 25°C. The extent of hair removal from the pelts unhaired by using 0.25% or 0.50% PFAP for 4 h is negligible and extremely similar with that of the control (Figure 3(2) and 3(3)). These results suggest that "pure" α-amylase cannot remove hair. According to Figure 3(4), it is obvious that AP removes a majority of hair from soaked hides, which was consistent with the results obtained in previous studies.[11, 14] Unhairing with 0.25% or 0.50% AFAP achieves a satisfactory extent of hair removal (see Figure 3(5) and 3(6)), suggesting that concomitant

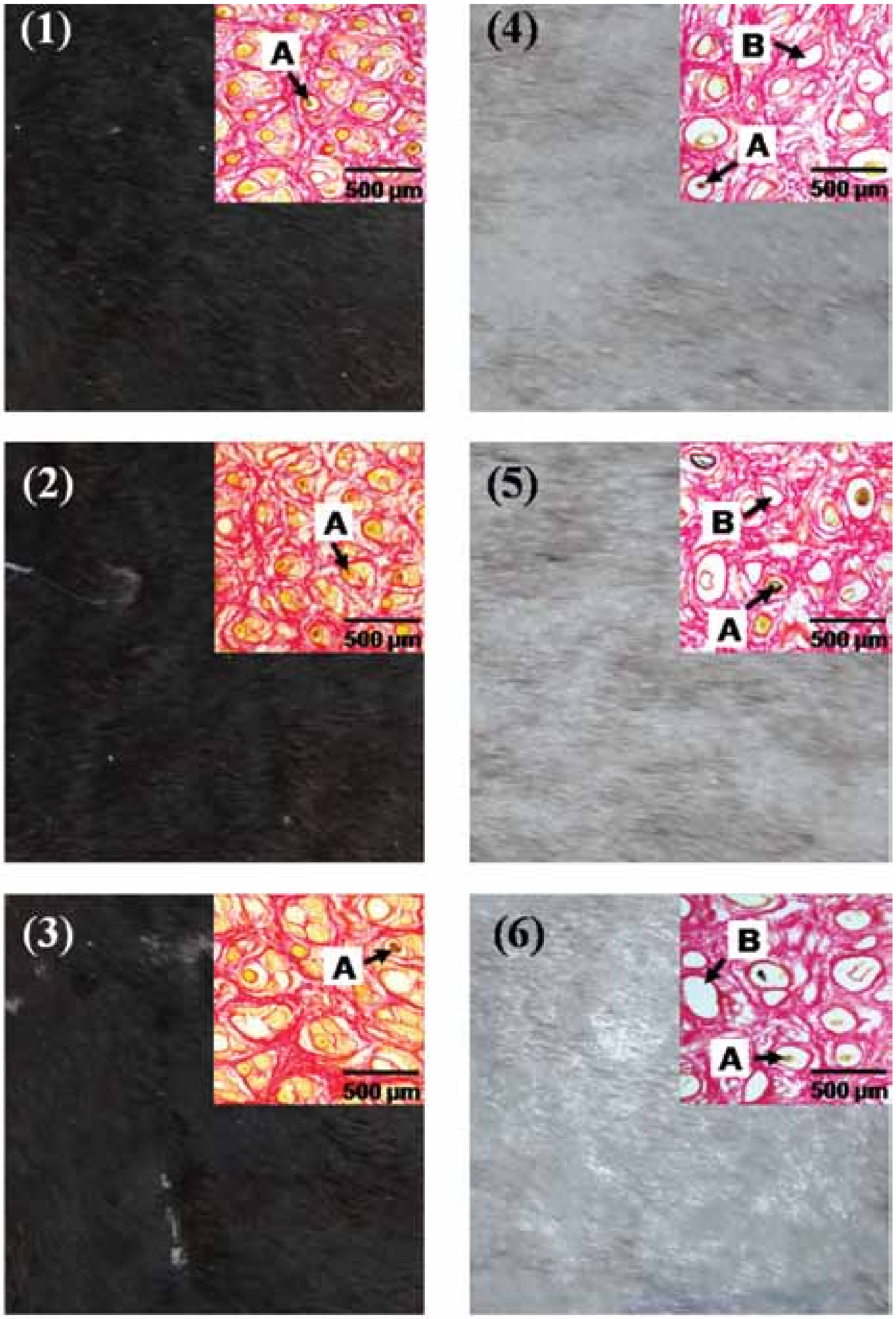

Figure 3. Grain of enzymatic unhaired pelts captured by digital camera and horizontal sections (Van Gieson stain) from enzymatic unhaired pelts using (1) control, no enzymes; (2) 0.25% PFAP; (3) 0.50% PFAP; (4) 0.25% AP; (5) 0.25% AFAP; (6) 0.50% AFAP. A – hair root; B – no hair.

protease in AP, like some other proteases used in leather manufacture,[18] has an ability to remove hair. Comparing the extent of hair removal from pelts unhaired with PFAP and AFAP, it can be inferred that rather than "pure" α-amylase, concomitant protease(s) are the effective components of AP for removing hair.

Extent of Epidermis Removal

The histological photomicrographs of vertical sections from enzymatic unhaired pelts are illustrated in Figure 4, where the sections were stained with Weigert's iron hematoxylin and counterstained with Van Gieson's stain to differentiate collagen fibers (red) and epidermis (black or brown). The control pelt had an intact epidermis. After unhairing using 0.25% or 0.50% PFAP, almost all epidermis remained on the surface of pelt. In contrast, the epidermis of pelts unhaired using AP or AFAP was removed completely. These facts imply that rather than "pure" amylase, protease(s) present in AP resulted in the removal of epidermis.

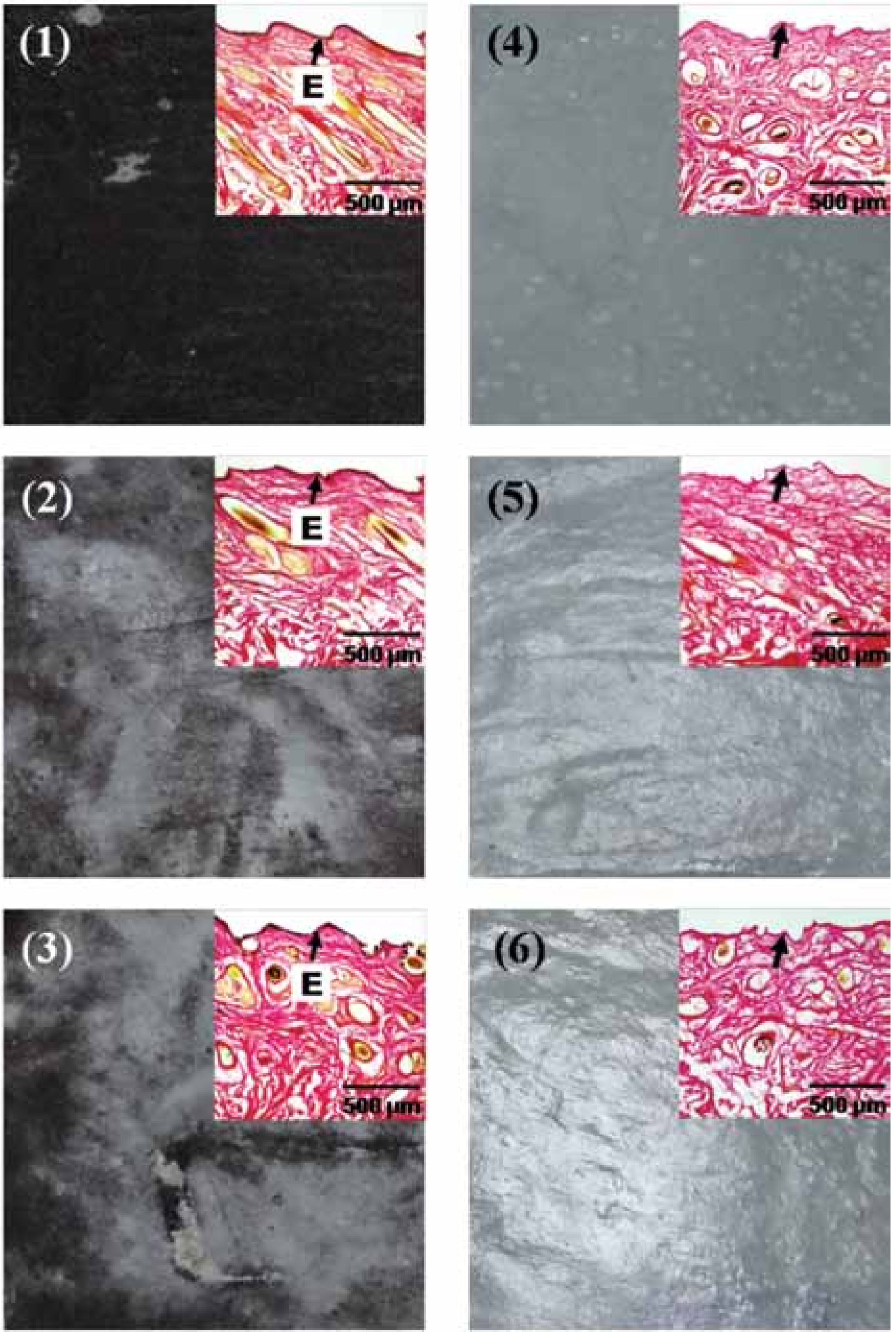

Figure 4. Grain of delimed pelts captured by digital camera and vertical sections (Van Gieson stain) from enzymatic unhaired pelts using (1) control, no enzymes; (2) 0.25% PFAP; (3) 0.50% PFAP; (4) 0.25% AP; (5) 0.25% AFAP; (6) 0.50% AFAP. E – epidermis.

Furthermore, the cleanliness of grain of pelts was observed. Before observation, the residual hair on pelts was removed by using a low sulfide unhairing system as described in Table II. Then the pelts were immediately delimed using ammonium sulfate to eliminate the interference of color caused by commercial sodium sulfide. As a result, a black surface of the control pelt is observed in Figure 4(1), suggesting that the cattle hide employed for unhairing was rich in pigment such as melanin and that a short-time treatment with low sulfide cannot remove the pigment. A part of pigment is removed by using 0.25% or 0.50% PFAP, while all pigment is removed by using AP or AFAP as shown in Figure 4. These results indicate that protease is more effective in removing melanin than "pure" amylase, which might be due to the fact that melanins are derived from dopaquinone formed following the oxidation of tyrosine by tyrosinase.[28]

Extent of Protein Removal

The concentrations of protein and Hyp in enzymatic unhairing liquors are shown in Figure 5. It was found that the concentrations of protein in PFAP unhairing liquors are higher than the control (see Figure 5(a)). This could be attributed to the addition of enzymes which themselves are proteins. The concentrations of protein in AP and AFAP unhairing liquors were much higher than those in PFAP unhairing liquors, which indicated that protease(s) in AP are more beneficial to the removal of protein than "pure" amylase. There is only a very small increase in the concentrations of Hyp in PFAP unhairing liquors compared with the control (Figure 5(b)), which may be caused by the little residual protease in PFAP. In contrast, the concentrations of Hyp in AP and AFAP unhairing liquors were significantly higher than that in the control unhairing liquor, suggesting that the protease(s) could lead to inevitable damage to collagen.

According to the results and discussions above, it can be found that the removal of hair and epidermis is related to the removal of protein. This is in agreement with the conclusion of Bose *et al*. that enzymatic unhairing depended on the removal of mucoid materials.[12] However, it is affirmative that rather than "pure" α-amylase, protease(s) in AP are the effective components for the removal of proteins including mucoid as well as the removal of hair. These results support the theory of Yates that the unique type of enzyme activity related to the unhairing effectiveness is a proteolytic activity.[19] The mechanism of action of α-amylase preparation on hides should be consistent with that of unhairing using a protease.

It is known from the literature that α-amylase preparations obtained from different suppliers usually have various ratios of α-amylase activity to protease activity.[21] Our research confirms that the protease activity of α-amylase preparation is the most important factor that influences the unhairing effectiveness. Therefore, it is reasonable to speculate that the differences in the unhairing effectiveness of amylase preparations observed by Burton *et al*.,[11] Gillespie[13] and Cordon[14] should be due to the varying protease activities of amylase preparations used by them.

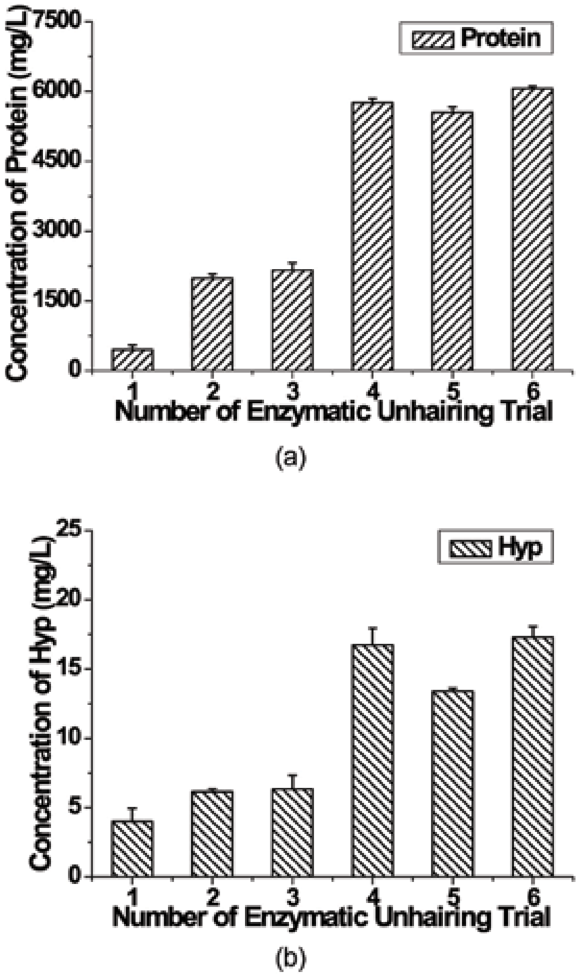

Figure 5. Concentrations of protein (a) and Hyp (b) in enzymatic unhairing liquors. 1 - control, no enzyme; 2 - 0.25% PFAP; 3 - 0.50% PFAP; 4 - 0.25% AP; 5 - 0.25% AFAP; 6 - 0.50% AFAP.

CONCLUSIONS

In consideration of the effectiveness of removing hair, epidermis and protein from hides using different enzymatic components of α-amylase preparation, it is clear that the concomitant protease(s) in α-amylase preparation are mainly responsible for unhairing rather than "pure" α-amylase. The action mechanism of α-amylase preparation on hides/skins should be consistent with that of proteases. To make better use of α-amylase preparation in non-sulfide or non-lime unhairing systems, a possible direction would be to produce cost-effective preparations with high activity of efficient proteases by culturing the genetically modified α-amylase producing microorganisms.

ACKNOWLEDGEMENT

This work was supported by the National Key Technologies R&D Program (2011BAC06B11) and the National Natural Science Foundation of China (21076130).

REFERENCES

1. Paul, R. G., Mohamed, I., Davighi, D., Covington, A. D., and Addy, V. L.; The use of neutral protease in enzymatic unhairing. *JALCA* **96**, 180-185, 2001.
2. Thangam, E. B., Nagarajan, T., Rajkumar, G. S., and Chandrababu, N. K.; Application of alkaline protease isolated from *Alcaligenes faecalis* for enzymatic unhairing in tanneries. *JALCA* **96**, 127-132, 2001.
3. Shi, B., Lu, X. F., and Sun, D. H.; Further investigations of oxidative unhairing using hydrogen peroxide. *JALCA* **98**, 185-192, 2003.
4. Marmer, W. N., and Dudley, R. L.; Oxidative dehairing by sodium percarbonate. *JALCA* **100,** 427-431, 2005.
5. Thanikaivelan, P., Rao, J. R., Ramasami, T., and Ramasami, T.; Approach towards zero discharge tanning: exploration of NaOH based opening up method. *JALCA* **96,** 222-233, 2001.
6. Saravanabhavan, S., Thanikaivelan, P., Rao, J. R., Nair, B. U., and Ramasami, T.; Sodium metasilicate based fiber opening for greener leather processing. *Environ. Sci. Technol.* **42**, 1731-1739, 2008.
7. Song, J., Tao, W. Y., and Chen W. Y.; Mathematical analysis of depilation using combinations of amylase and protease. *JSLTC* **91**, 212-215, 2007.
8. Zeng, Y. H., Liao, X. P., Lu, J. H., He, Q., and Shi, B.; Enzymatic unhairing of cattle hide by protease and α-amylase. XXXI IULTCS Congress, Valencia, 2011.
9. Aravindhan, R., Saravanabhavan, S., Rao, J. R., and Nair, B. U.; A bio-driven lime and pickle free tanning paves way for greener garment leather production. *JALCA* **99**, 53-66, 2004.
10. Thanikaivelan, P., Rao, J. R., Nair, B. U.,and Ramasami, T.; Zero discharge tanning: A shift from chemical to biocatalytic leather processing. *Environ. Sci. Technol.* **36**, 4187-4195, 2002.
11. Burton, D., Reed, R., and Flint, F. O.; The unhairing of hides and skins without lime and sulphide. – The use of mucolytic enzymes. *JSLTC* **37**, 82-87, 1953.
12. Bose, S. M., Madhava Krishna, W., and Das, B. M.; Mechanism of unhairing skins and hides by means of certain proteolytic or amylolytic enzymes. *JALCA* **50**, 192-199, 1955.
13. Gillespie, J. M.; The depilation of sheepskins with enzymes. *JSLTC* **37**, 344-353, 1953.
14. Cordon, T. C; Unhairing of hides and skins by amylase preparations. *JALCA* **50**, 270-274, 1955.
15. Moseley, M. H., and Keay, L.; Purification and characterization of the amylase of *B. subtilis* NRRL B3411. *Biotechnol. Bioeng.* **12**, 251-271, 1970.
16. Leach, H. W., and Hebeda, R.; Protease inactivated α-amylase preparations. US patent 4,235,970, 1980.
17. Hmidet, N., Ali, N. E. H., Haddar, A., Kanoun, S., Alya, S. K., and Nasri, M.; Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive. *Biochem. Eng. J.* **47**, 71-79, 2009.
18. Choudhary, R. B., Jana, A. K., and Jha, M. K.; Enzyme technology applications in leather processing. *Indian J. Chem. Technol.* **11**, 659-671, 2004.
19. Yates, J. R.; Studies in depilation. Part X. The mechanism of the enzyme depilation process. *JSLTC* **56**, 158-177, 1972.
20. Bernfeld, P.; Amylase α and β. In: Colowick, S. P., and Kaplan, N. O., eds; Methods in enzymology. New York: Academic Press Inc., pp. 149-158, 1955.
21. SB/T 10317-1999 (Chinese). Measurement of proteinase activity.
22. De Stefanis, V. A., and Turner, E. W.; Modified enzyme system to inhibit bread firming method for preparing same and use of same in bread and other bakery products. US patent 4,299,848, 1981.
23. Hoerle, R. D.; Differential inactivation of amylase in amylase-protease mixtures. US patent 4,086,139, 1978.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.; Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275, 1951.
25. Reddy, G. K., and Enwemeka, C. S.; A simplified method for the analysis of hydroxyproline in biological tissues. *Clin. Biochem.* **29**, 225-229, 1996.
26. de Souza, P. M., and Magalhaes, P. D. E.; Application of microbial α-amylase in industry - a review. *Braz. J. Microbiol.* **41**, 850-861, 2010.
27. Cantera, C. S., Goya, L., Galarza, B., Garro, M. L., and Lopez, L. M. I.; Hair saving unhairing process. Part 5 Characterisation of enzymatic preparations applied in soaking and unhairing processes. *JSLTC* **87**, 69-77, 2003.
28. Wakamatsu, K., and Ito, S.; Advanced chemical methods in melanin determination. *Pigment Cell Res.* **15**, 174-183, 2002.