UNHAIRING AND FIBER BUNDLE-OPENING OF COWHIDES USING KCL AND LIBR/[AMIM]CL ASSISTED NEUTRAL PROTEASE FOR LEATHER MAKING

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Abstract. Nowadays, tannery pollution is of great concern worldwide. The unhairing and fiber bundle-opening processes produce the majority of the pollution by the use of sodium sulfide and calcium hydroxide, which were proposed to be replaced by neutral protease combined with KCl, LiBr/[AMIm]Cl in the present work. Proper amount of KCl can speed up the unhairing with the grain not destroyed by the neutral protease. Four methods for unhairing and fiber bundle-opening were used as follows: 1#. Two steps in different float as KCl/neutral protease unhairing, followed by LiBr/[AMIm]Cl for fiber bundle-opening; 2#. Two steps in different float as neutral protease unhairing, followed by LiBr/[AMIm]Cl for fiber bundle-opening; 3#. One step in the same float as neutral protease for unhairing firstly and then LiBr/[AMIm]Cl for fiber bundle-opening. 4#. One step in the same float as neutral protease/KCl for unhairing firstly and then LiBr/[AMIm]Cl for fiber bundle-opening. It was found that using neutral protease/KCl solution for unhairing and LiBr/[AMIm]Cl solution for fiber bundle-opening is the best in fiber bundle-opening at the liming process. Besides, all the methods used here are better than the conventional liming processes (C) from the viewpoints of unhairing and fiber bundle-opening.

Keyword: Ionic liquid/LiBr, KCl, unhairing, fiber bundle opening, leather, neutral protease

1 Introduction

The beamhouse processing of leather production involves soaking, unhairing, liming, reliming, deliming, bating, picking and more water solution unit operations[1], involving many biochemical reagents. Its objective is to remove dirt, hairs, epidermis layer, non-collagenous proteins (proteoglycan) and grease from rawhide, and open up collagen fiber bundles so as to favor the subsequent tanning process. During this processing, the conventional unhairing and fiber-opening processes involve the use of calcium hydroxide and sodium sulfide. And the low solubility of calcium hydroxide leads to the formation of lime sludge and the liberation of toxic hydrogen sulfide through the use of sodium sulfide[2]. What's more, the mechanism of unhairing using calcium hydroxide and sodium sulfide is the way of damaging hair, which brings suspended solids, carbon and nitrogen pollution. To address these problems, various approaches have been tried to reduce or avoid the use of calcium hydroxide and sodium sulfide in leather processing[3].

Nowadays, enzyme and enzyme technology are widely applied to unhairing and liming of leather manufacturing, which have specificity, efficiency, selectivity and environmental friendly such as alkaline protease, neutral protease, cellulase, α-amylase[4], β-glucanase, α-glactosidase, etc.. Many researcher and leather industries have obtained the leather manufacturing technology with enzymes and made great progress, but many problems were found with a lot of reports read. For instance, while proteolytic enzymes attack the proteoglycan of the hair root, the collagen of the grain layer may be partly destroyed, resulting in poor quality and inferior appearance of final leather. Besides, although many studies were focused on the development of enzymatic unhairing technology, very few techniques have been applied at industrial level, due to various obstacles such as damage of collagen[5] and grain surface, incomplete removal of fine hair[6] and epidermis[7], and inefficient manual operation[8], which will lead to low yield of leather. Hence, how to unhairing quickly, reduces the destruction of the pelt grain layer and accelerate the hydrolysis and dissolution

of glycosaminoglycan (proteoglycan and mucopolysaccharide) for collagen fiber bundle-opening are the key points to leather researcher. Similarly, neutral protease is widely employed in unhairing and fiber bundle-opening with above same problems. Therefore, it is necessary to improve the efficiency of the neutral protease for unhairing and liming.

The enzyme is a macromolecule protein with highly specific and catalytic, which the molecular weight is more than 10 kDa. And, in unhairing/liming processing, the cowhide is a huge blocky tissue with dense orientation structure, which increase the difficulty of the active center of enzyme to contact and catalyze the proteoglycan of the hair root. However, the catalytic rate of enzyme is depended on the type of binding such as hydrogen bond and metal ionic bond. And the binding ability of metal ionic bond with enzyme/ proteoglycan is stronger than the hydrogen bond in the water solution. Hence, adding metal ions can improve the binding ability of the enzyme with proteoglycan, which named salt bridge. Neutral protease usually is used for unhairing, which is very easy coagulation in water solution lead to a decline in catalytic rate. Therefore, adding metal ions can increase the stability of neutral protease solution and the binding ability of the enzyme and zymolyte, which improving the catalytic rate of neutral protease.

Up to now, most of researcher has reported about using salt to improve the catalytic activity[9] and the stability of enzyme solution[10-13]. For instance, the correlation of the effect of ions on the stability of protein/enzyme conforms to the typical ordering of the anion/cation series (Hofmeister series). The metal cation usually as an activator and adjuvant to build a salt bridge between the enzyme and zymolyte for improving the catalytic efficiency of the enzyme. In this study, potassium chloride was chosen as a salt bridge to improve the catalytic efficiency of neutral protease owing to the same function with sodium chloride for leather manufacture. Besides, the wastewater containing potassium chloride can be discharged into the soil to provide potassium ions for plant growth, and will directly absorbed by crops. In liming processing, LiBr and [AMIm]Cl have good function of opening up hydrogen bond owing to strong ionic bonds, especially [AMIm]Cl. [AMIm]Cl is a low temperature molten salt and can easily bond with the hydrogen bonds. In this work, the effect of potassium chloride on neutral protease activity and unhairing rate, the stability and permeation rate of the neutral protease solution was studied. The results of fiber bundle-opening through one step, two steps and traditional methods were analyzed by Verhoeff's Van Gieson (EVG) staining technique. The contents of protein, carbohydrate, hydroxyproline and COD_{Cr} in wastewater were also investigated. Potassium chloride as a salt bridge to improve unhairing rate and further reduce enzyme hydrolysis of collagen is expected to improve the yield of leather.

2. Materials and methods

2.1 Materials

Salt cowhides were kindly provided by Xinxiang Huixian Leather co., LTD. (Henan, China). Neutral protease (Dispase) (BR, 50u/mg), sodium sulfide nonahydrate (ACS), calcium hydroxide (ACS, ≥ 95.0%) , lithium bromide solution(LiBr, 99%) and silver nitrate (AR, 99.8%) were purchased from Aladdin reagent co., LTD. Potassium chloride(KCl, AR) was purchased from Tianjin hengxing chemical reagent manufacturing co., LTD. 1-allyl-3-methylimidazolium chloride([AMIM]Cl, ≥99%) was supplied by Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences. An enhanced BCA protein assay reagent kit was purchased from the Beyotime Institute of Biotechnology, China. Distilled water.

2.2 The treatment of cow hides

Dried salt cowhide with a uniform thickness from the same body part were immersed in quintuple distilled water in volume, and then transited and rolled in the rollers of leather tanning machine (DJDØ350, Xishan, Beitang mine Leather Machinery Factory, Jiangsu, China). The soaking liquid was substituted every hour until no white precipitation when the liquid was instilled silver nitrate solution. Thereafter, the hairs and cuticle of obtaining cow hides were scraped by scraper, while the subcutaneous tissue of cow hides was excised by fillet knife. The hides were washed with distilled water. Next, the bovine hides were cut into square pieces with 2 g and stored in 4 ℃.

Table 1. Unhairing and Fiber-Opening Processes.

Process	Materials	Percentages (%)					Temperature (℃)	Time (h)	Remarks
		Two steps		One step					
		1#	2#	3#	4#	C			
Unhairing	Cowhides [a]	100	100	100	100	100	30	13	Magnetic stirring (about 200r/min)
	Distilled water [b]	1000	1000	1000	1000	1000			
	Neutral protease	8	8	8	8	-			
	Potassium chloride	8	-	-	8	-			
	Sodium sulfide nonahydrate	-	-	-	-	8			
Fiber opening	Distilled water	1000	1000	-	-	-	30	19	Magnetic stirring (about 200r/min)
	Lithium bromide	8	8	8	8	-			
	[AMIm]Cl	8	8	8	8	-			
	Calcium hydroxide	-	-	-	-	50			

[a] The weight of hides is 2 g in each method.

Unhairing and fiber opening of cowhides using KCl and LiBr/[AMIM]Cl assisted neutral protease for leather making are denoted as 1#, 2#, 3#, 4#, and C. Unhairing of me thod 1# was carried out using the solution of KCl/neutral protease and was followed by the solution of LiBr/[AMIM]Cl for the fiber-opening process. For 2#, unhairing was accomplished using the solution of the neutral protease without KCl and was followed by fiber-opening using the solution of LiBr/[AMIM]Cl. Unhairing of method 3# was carried out using the solution of the neutral protease without KCl, then adding LiBr/[AMIM]Cl for the fiber-opening process into the same solution. For 4#, unhairing was accomplished using the solution of KCl/neutral protease, and adding LiBr/[AMIM]Cl for the fiber-opening process directly into the same solution. The conventional unhairing and fiber-opening method was C. The sodium sulfide solution was used to unhairing for 13h and then calcium hydroxide was added into the same solution for 19h. As detailed a description of the unhairing and fiber-opening processes is provided in **Table 1**. The percentages reported in the table are based on cowhide weight.

2.3 Neutral protease activity

For the measurement of the activity of the neutral protease, the universal protease activity assay with casein as the substrate and tyrosine was used as the standard. 0.8 g of the neutral protease was dissolved in 100 mL of phosphate buffer solution (pH 7.2), and this enzyme solution 1 mL was diluted 10 times to be measured. Next, measured 1mL and added casein solution 1 mL, and kept 10 min in the 40 ℃. After that, the reaction was inhibited using trichloroacetic acid, the solution was then filtered and used for colorimetric analysis. The optical density was measured at 680 nm after the addition of sodium carbonate and Folin's phenol reagent. Detailed experimental steps referred to the national standard of China SB/T10317-1999.

In addition, the activity of the neutral protease was analyzed in the presence of the KCl at different concentrations and temperature was the central composite design (CCD) of response surface methodology (RSM). The selection of CCD was made based on preliminary experiments in order to identify the minimum number of experimental runs and fitting surface model based on the second order polynomial equation[14, 15]. At last, achieved the regularization of the neutral protease activity at different KCl concentration and temperature. The independent variables of KCl concentration (x_1) and temperature (x_2), which were varied at the real levels and coded levels presented in **Table 2**. The activity of neutral protease (y) was selected as dependent variables. And the data was analyzed with RSM based on the CCD of experiments via Design Expert Software 8.0.6. Experiments were conducted up to 13 trial runs involving the analysis of variance (ANOVA) applied to analyze the results and the following second order polynomial model was used for the observations:

$$y = \alpha_0 + \sum_{i=1}^{2} \alpha_i x_i + \sum_{i=1}^{2} \alpha_{ii} x_i^2 + \sum_{i=0}^{1} \sum_{j=i+1}^{2} \alpha_{ij} x_i x_j \qquad (1)$$

Where y is the predicted response i.e. the activity of the neutral protease, α_0 is the constant coefficient, α_i is the ith linear coefficient of the input factor x_i, α_{ii} is the ith quadratic coefficient of the input factor of x_{ii}, and α_{ij} is the different interaction coefficient between the input factors x_i and x_j.

The interactions between the process variables and response were obtained from ANOVA. The correlation coefficient (R^2) demonstrated the goodness-of-fit of the second-order polynomial model and an F-test was performed to determine the statistical significance of the model. The ANOVA of the model was analyzed using the 95% confidence level ($P < 0.05$).

Table 2. Real and coded levels of variables (α=1.5).

Variable	Symbol	Coded levels				
		-α	-1	0	+1	+α
KCl content (%)	x_1	0.00	0.20	0.60	1.00	1.20
Temperature (℃)	x_2	20.00	26.00	38.00	50.00	56.00

2.4 Turbidity, transmittance, zeta potential and the contact angle of neutral protease solution

The turbidity, transmittance and zeta potential of the neutral protease solution were determined through turbidity meter (HACH, 2100Q, USA), ultraviolet spectrograph (UVS) (PERSEE, TU-1950, China) and zeta potentiometer (Malcern Panalytical, UK) respectively. The contact angle of neutral protease solution on the cowhide surface via the contact angle and interfacial tension tester (KINO, C60, USA)

2.5 Protein and carbohydrate analysis

Bicinchoninic acid (BCA) was used to determine the protein content of the solution after unhairing and fiber-opening processes. The concentration of carbohydrate was measured by anthrone colorimetry and glucose was used as the standard. 2mL of solution after unhairing and fiber-opening processes and added to the solution of anthrone (2g/L), concentrated sulfuric acid as solvent) 4 mL. Cool to room temperature after boiling water for 10 minutes and measured at 652 nm.

2.6 Verhoeff's Van Gieson (EVG) staining techniques

Samples were embedded using paraffin and then cut into thin slices (about 4μm). Slices were dewaxed using dimethylbenzene and ethanol absolute, and then cleaned up with distilled water. Next, slices were stained using Verhoeff's solution for 15-30min until they became dark black. These slices were differentiated using ferric trichloride solution for 10-20s until elastic fibers were

becoming black and background was becoming gray using upright optical microscope (NIKON ECLIPSE CI-L, Japan). Excess iodine of slices was removed by sodium thiosulfate, then counterstained by Van Gieson's for 3-5min and finally cleaned by ethanol absolute. Then it was dehydrated by ethanol absolute and dimethylbenzene, and sealed with neutral balsam. Finally, the surface morphologies were observed by digital slice scanning system (Pannoramic 250/MIDI, Hungary). Elastic fibers are black blue, collagen fibers are red, and other tissues are yellow.

2.7 Wastewater characteristics

To understand the environmental implications of neutral protease-based leather processing, wastewaters from the unhairing and fiber bundle-opening processes (1#, 2#, 3#, 4#, and C) were collected and analyzed for chemical oxygen demand (COD).

3. Results and discussion

3.1 KCl and LiBr/[AMIM]Cl assisted neutral protease for unhairing and fiber-opening processes

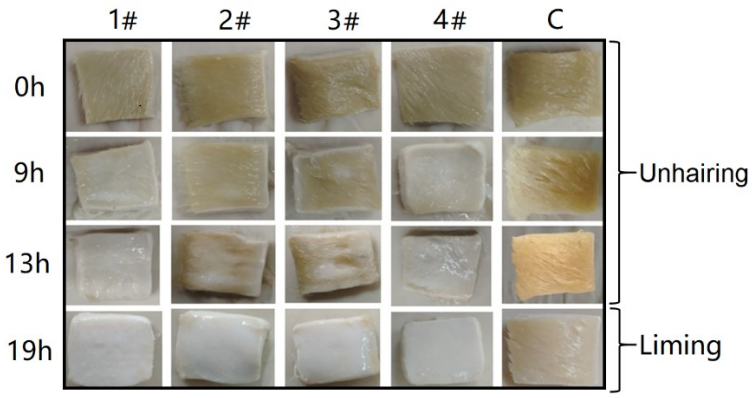


Fig. 1. The digital images of samples of cowhides during unhairing and fiber-opening processes.

In unhairing processing, the method 1# and 4# used potassium chloride to assist neutral protease for unhairing. The method 2# and 4# used neutral protease for unhairing. The method of C used conventional calcium hydroxide/sulfide for unhairing. And the results are shown in **Fig. 1.** The samples were not processed by mechanical or manual unhairing in order to maintain the appearance of cowhides surface at different time in whole unhairing process. It is found that the method 1# and 4# unfair completely after 13h, the method 2# and 3# were not. And the method C still has many hairs. Therefore, it is suggested that potassium chloride as salt bridge can successfully realize the goals of accelerating unhairing and loosing hair root. In liming processing, the method 1# and 2# used LiBr/[AMIm]Cl for liming, the methods 3# and 4# used LiBr/[AMIm]Cl together with unhairing solution for liming. In method C, calcium hydroxide was added in the solution of unhairing for liming processing. The method C still has many hairs, but the method 1#, 2#, 3# and 4# are not, which are white color on their surface.

3.2 Effect of potassium chloride concentration and temperature on neutral protease activity

Suitable potassium chloride can speed up the unhairing rate. Therefore, the effect of potassium chloride and temperature on neutral protease activity using CCD was studied. The results of the CCD experiments to investigate the effects of the two independent variables together with the

predicted and actual responses are shown in **Table 3**. In this study, the experimental data fit well with the empirical second-order polynomial models. Final equation in terms of actual factors:

$$y = -17.72701 - 25.61260x_1 + 2.16475x_2 + 0.14709x_1x_2 + 16.13318x_1^2 + (2.3239E-003)x_2^2 \quad (2)$$

Analysis of variance (ANOVA) was conducted to test the significance of fit of the second order polynomial equation for the experimental data as shown in **Table 4**. ANOVA for neutral protease activity indicated that model terms were significant because of the values of 'Prob>F' less than 0.05. Therefore, the variables x_2 and $x_1^2$are significant in neutral protease activity, but x_1, x_1x_2, x_1x_2 and x_2^2 are not significant.

Furthermore, the correlation coefficient R^2 (**Table 5**) of 0.9962 indicated that only 0.38% of the total variation could not be explained by the empirical model. The value of Adeq. Precision higher than 4 was desirable[16]. Adeq. Precision measures the signal to noise ratio, which in this case the value of 64.980 was obtained, indicating an adequate ratio. Besides, low coefficient of variation (C.V. % is 3.12 less than 10%) and the standard deviation (Std. Dev. is 1.98) values proved that this model is efficient for navigating the design space[17], implying that this model is good.

Table 3. Experimental results of CCD central composite design and predicted responses.

Run	Variable				Actual value(U/mg)	Predicted value(U/mg)
	x_1	*Coded*	x_2	*Coded*		
1	0.60	0	56.00	1.5	103.88	102.24
2	1.20	1.5	38.00	0	66.66	65.31
3	1.00	1	26.00	-1	32.89	34.47
4	0.20	-1	26.00	-1	33.93	40.65
5	0.20	-1	50.00	1	94.52	90.86
6	0.60	0	38.00	0	61.70	61.68
7	0.60	0	38.00	0	61.70	64.14
8	1.00	1	50.00	1	96.30	91.74
9	0.60	0	38.00	0	61.70	59.26
10	0.60	0	20.00	-1.5	21.33	21.13
11	0.60	0	38.00	0	61.70	61.68
12	0.60	0	38.00	0	61.70	61.68
13	0.00	-1.5	38.00	0	68.66	64.53

Table 4. ANOVA results for response surface quadratic model for neutral protease activities.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Status
Model	7283.27	5	1456.65	371.06	< 0.0001	significant
x_1	0.60	1	0.60	0.15	0.7078	Not significant
x_2	7225.32	1	7225.32	1840.54	< 0.0001	significant
x_1x_2	1.99	1	1.99	0.51	0.4991	Not significant
x_1^2	55.20	1	55.20	14.06	0.0072	significant
x_2^2	0.93	1	0.93	0.24	0.6417	Not significant
Residual	27.48	7	3.93			
Lack of Fit	27.48	3	9.16			
Pure Error	0.000	4	0.000			
Cor Total	7310.75	12				

Table 5. Statistical parameters of ANOVA of the neutral protease activities predicted model

Statistics	Value	Statistics	Value
Std. Dev.	1.98	R-Squared	0.9962
Mean	63.59	Adj R-Squared	0.9936
C.V. %	3.12	Pred R-Squared	0.9729
PRESS	197.91	Adeq Precision	64.980

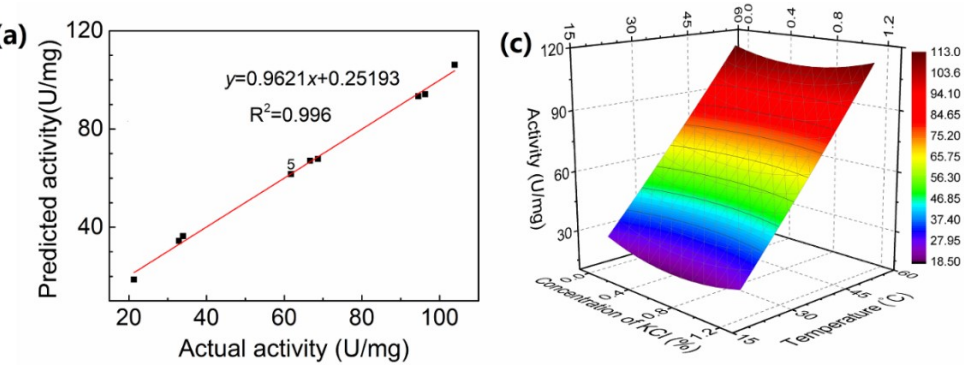


Fig 2. (a) Comparison of predicted and experimental responses; (b)three-dimensional response plots of neutral protease activities at different potassium chloride concentrations and temperature.

Fig. 2 shows the relationship between potassium chloride content (insignificant, independent variables) and temperature (significant, independent variables) and the activity of neutral protease (dependent variable). The activity of neutral protease shows a slightly concave trend with the potassium chloride concentration (0-1.2%) raise. But potassium chloride concentration was not significant on the activity of neutral protease, which has been demonstrated in (**Table 4**). At a suitable temperature, it does not affect the depilatory activity of the neutral protease. In contrast, the variable of temperature is a very significant factor influencing neutral protease activity. As is shown in the **Fig. 2**, the activity of neutral protease raised with the increasing temperature (20-56°C) and has linear relationship between the activity of neutral and temperature in same content of potassium chloride.

3.3 Turbidity, transmittance, zeta potentials and the contact angle of neutral protease solution

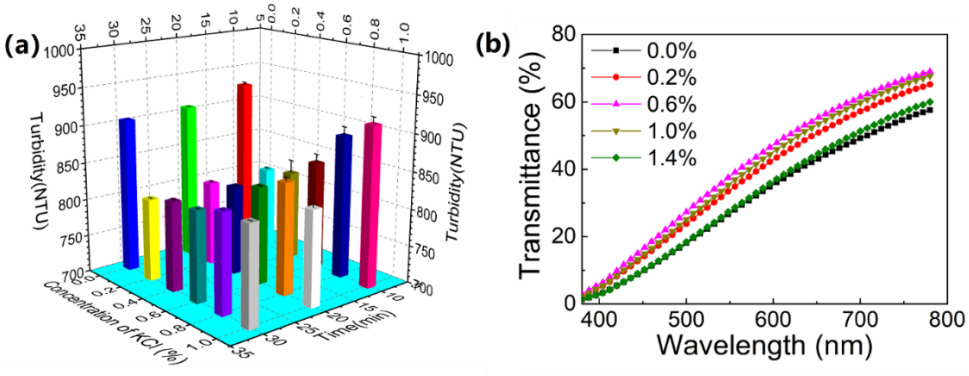


Fig. 3. (a) Effect of KCl concentration on turbidity of neutral protease solution; (b) Effect of KCl content on visible light transmittance of neutral protease solution.

The turbidity of neutral protease decreases after adding suitable KCl, and it can be seen in **Fig. 3**(a). In the beginning, the turbidity increases with the increasing concentration of KCl (0-1%), but it is lower

compared with the solution of the neutral protease without KCl. The turbidity of the neutral protease solution tends to be stable after 30min. However, the turbidity is still very high. Therefore, it is beneficial to improve the solubility and dispersibility of water solution after adding suitable KCl for neutral protease.

It was investigated that the transmittance of neutral protease solution when adding different concentration of KCl by ultraviolet spectrograph (UVS). The transmittance of the neutral protease solution increased with the KCl concentration raised and then decreased over 1% as **Fig. 3**(b) showed. This also indicated that KCl can adjust the solubility and dispersibility of water solution.

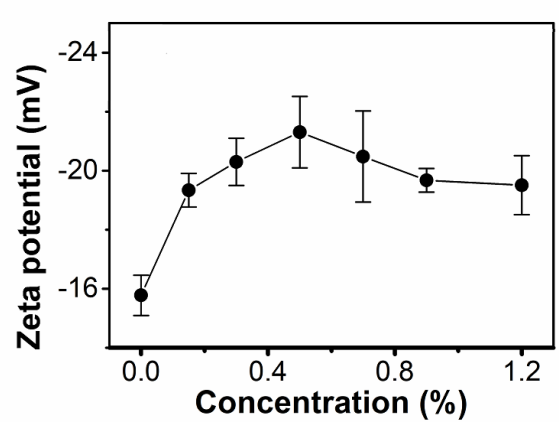


Fig. 4. Effect of KCl on zeta potential of the neutral protease solution.

The zeta potential of KCl/neutral protease solution was investigated, which is shown in **Fig. 4**. Increasing the concentration of KCl is beneficial to improve the stability of neutral protease solution owing to the increased zeta potential of the neutral protease solution (**Fig. 4**). However, the stability of neutral protease solution decreased when the concentration of KCl is greater than 0.5%.

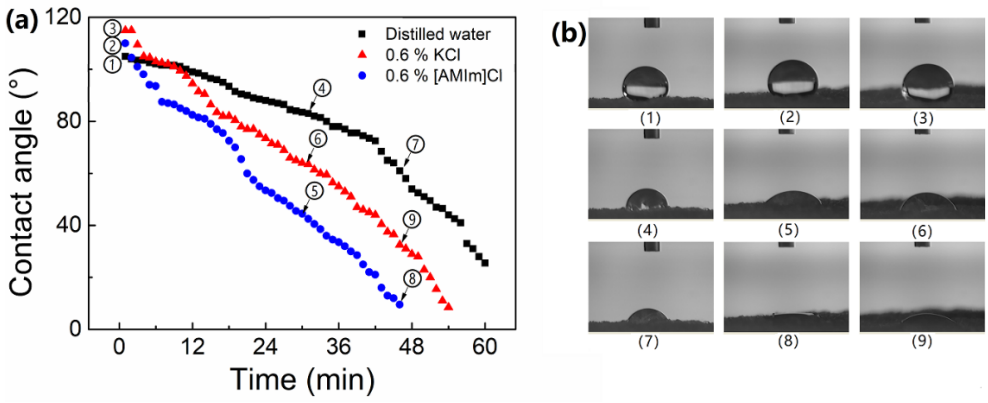
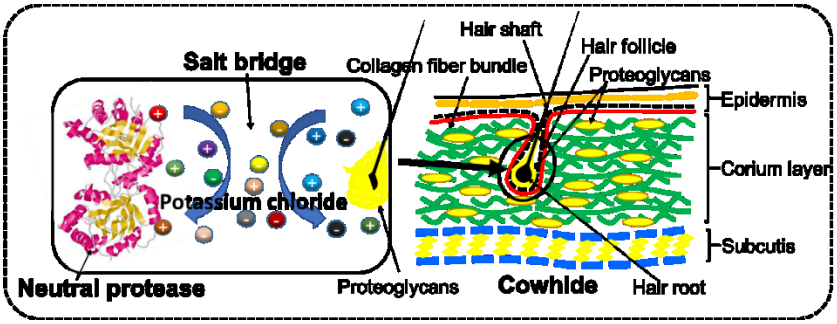


Fig. 5. (a) Effect of potassium chloride and [AMIm]Cl on the contact angle of water on the surface of cowhide; (b) the images of contact angle corresponding to the number in the left figure.

The penetration of water solution was improved when adding KCl and [AMIm]Cl, and the results are shown in **Fig. 5**(b). Both of them can accelerate the penetration rate of the neutral protease solution and shorten the time of binding between neutral protease and zymolyte (proteoglycan/ mucopolysaccharide), improving the catalytic efficiency of neutral protease.

Therefore, KCl was introduced into the neutral protease solution. On the one hand, it can improve the dispersibility of the water solution and the stability of the neutral protease solution. On the other hand, the penetration of the neutral protease solution is improved, and further increasing the catalytic efficiency of neutral protease.



Scheme 1. Potassium chloride as a salt bridge between neutral protease and proteoglycans in cowhide for unhairing.

The catalytic mechanism diagram of the neutral protease on polysaccharide was preliminarily obtained, according to the above results analysis of improving the stability and subcutaneous penetrating quality of neutral protease solution and the dispersibility of the water solution after adding KCl, as well as result of improving the unhairing rate of the neutral protease (**Scheme 1**). However, the catalytic mechanism of protease activity center on polysaccharides needs further study.

3.4 The protein, carbohydrate, hydroxyproline and COD_{Cr} of wastewater analysis

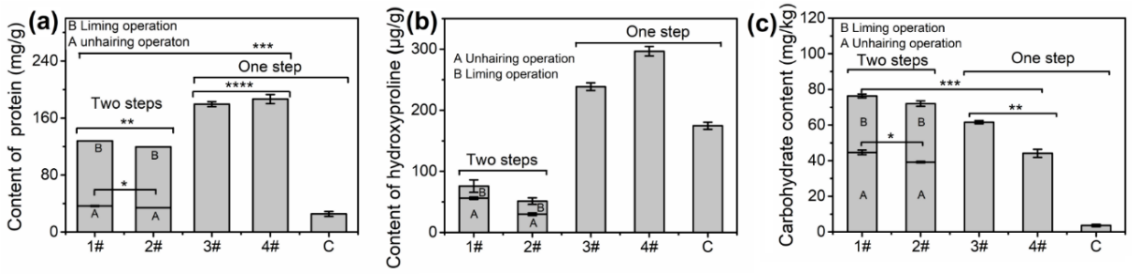


Fig. 6. Comparison of protein (a), hydroxyproline content (b) and carbohydrate content (c) in wastewater of method 1#, 2#, 3#, 4# and C. (*, ** and *** significance (P) less than 0.05; ****P > 0.05)

The contents of protein, carbohydrate and hydroxyproline of wastewater after unhairing and liming processing can be used to evaluate the degree of collagen and neutral protease to polysaccharides/ mucopolysaccharide hydrolysis, which the results are shown in **Fig. 6**. The content of protein in wastewater of method 1# is higher than method 2# when adding KCl in method 1#. The addition of KCl increases the catalytic efficiency of the neutral protease to polysaccharides (**Fig. 6**(b)) and collagen (**Fig. 6**(c)) especially polysaccharides. The protein of method 3# and 4# is higher than method 1# and 2# owing to neutral protease in whole operation of unhairing and liming. Compared with method 2# and 3#, the contents of hydroxyproline in the wastewater of method 1# and 4# are higher. The results indicated that KCl can expedite hydrolysis of cowhide collagen (**Fig. 6**(b)). The contents of hydroxyproline in wastewater of method 3# and method 4# are much higher than method 1# and 2# because neutral protease always exists in the unhairing and the liming solution of method 3# and 4#. The content of carbohydrate in wastewater of method C is lower than others (**Fig. 6**(c)). It is suggested that calcium hydroxide/sodium sulfide system is bad for the dissolution of polysaccharides/mucopolysaccharide. The content of carbohydrate in wastewater of method 1# is higher than method 2# and the method 3# is higher than method 4#, indicating that adding KCl is good for the dissolution of polysaccharides/mucopolysaccharide.

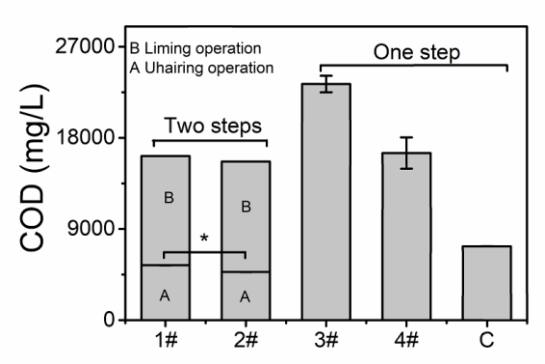


Fig. 7. Comparison of CODCr content in wastewater of method 1#, method 2#, method 3#, method 4# and method C. (* significance (P) less than 0.05)

COD_{Cr} is an indicator to measure the amount of reducing substances in wastewater. The reducing substances mainly are organic matter (protein and polysaccharides/mucopolysaccharide) in wastewater of the unhairing and liming solution. The COD_{Cr} content in wastewater of method 1#, method 2#, method 3#, method 4# and method C are shown in **Fig. 7**. It is found that COD_{Cr} content in wastewater of method 3# is higher than other methods science the summation contents of protein and carbohydrate is the highest (**Fig. 6** (a) and (c)).

3.5 Verhoeff's Van Gieson (EVG) staining analyses

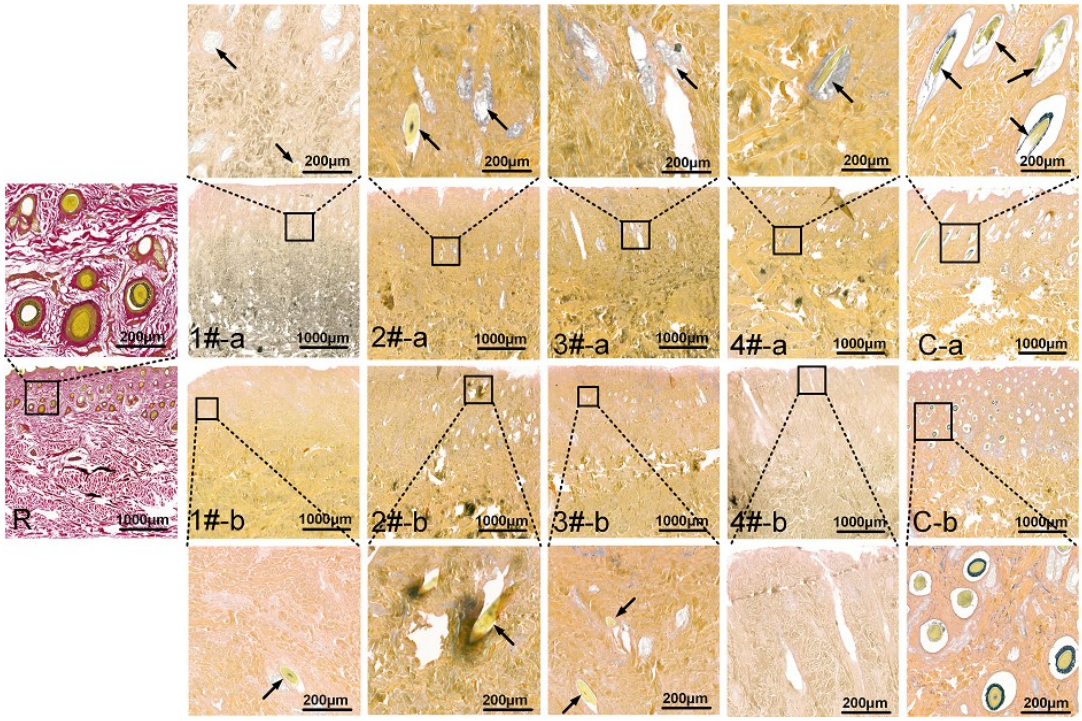


Fig. 8. The cross-section images of cowhides by the EVG staining slice after (a) unhairing and (b) liming through method 1#, 2#, 3#, 4# and C; (R) Raw cowhide.

The cross-section images of cowhides by the EVG staining slice after unhairing and liming processing are shown in **Fig. 8.** Red is cowhide collagen fiber bundles. Brownish yellow is polysaccharides/mucopolysaccharide. The content of polysaccharides/mucopolysaccharide in hair follicle using method C is higher than others, the fiber bundles swelling and their gaps increase after

unhairing and liming (**Fig. 8**(C)). It is concluded that the dissolved ability of calcium hydroxide/sodium sulfide solution system to polysaccharides/mucopolysaccharide was not excellent for unhairing and liming operation. Adding KCl (**Fig. 8**(2# and 3#)) is good for dissolution of polysaccharides/mucopolysaccharide owing to the effect of salt bridge. Strong ability of the ions will open hydrogen bonds to promote the hydrolysis of polysaccharides/mucopolysaccharide.

The open degree, mechanical and thermal properties of collagen fibers will be further analyzed by SEM, texture analyzer (TA) and thermogravimetry (TG) after tanning operation in our future work, as well as recycling technology of wastewater after unhairing and liming.

4 Conclusions

In this study, KCl as a salt bridge assist neutral protease to accelerate unhairing rate and reduce the hydrolysis of cowhides collagen. Neutral protease can increase the hydrolysis of collagen and decrease leather yield, if it is continue to be used in liming processing. Choosing KCl, neutral protease and LiBr/[AMIm]Cl to open collagen fiber bundles for leather making can reduce the pollution of the environment. This novel technology exhibits great potential in commercial exploitation of cleaner unhairing/liming process in leather industry for eco-friendly production of leather.

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