EFFECTS OF DIFFERENT SALT-ENZYMES ON OPENING UP OF COLLAGEN FIBER BUNDLES FOR LEATHER MAKING

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Abstract. Traditional leather industry involves mechanical, chemical and biological processes, and a lot of leather chemicals are widely used annually. In the beamhouse, especially in liming and re-liming, enormous pollution is usually released because of the traditional use of Na_2S and lime. Many researchers have devoted to clean production for leather making. In this study, salt-enzyme liming process was studied in modern leather process to remove the inter-fibrillary matter. Three such salts as Na_2SO_4, NaCl, and $MgCl_2$ were used with such enzymes as neutral protease and cellulase. The enzyme activity was evaluated by Folin Method. The opening up degree of collagen fiber bundles was observed by SEM and microscopic image of histological staining. The waste water was analyzed. The tannin absorptivity of the samples was evaluated by colorimetry. It was demonstrated that enzyme activity is not affected by salt, but it helps the action of enzymes on hides. Salt might accelerate the penetration of enzymes into the hide to promote the removal of inter-fibrillary and the opening up of collagen fiber bundles. The best fiber opening result was found by SEM at the $MgCl_2$ content of more than 0.4 wt.% in liming. Microscopic observation by histological staining as well as waste water analysis indicated a good removing effect for the inter-fibrillary. This work may provide a cleaner leather making technology.

1 Introduction

Traditional processing industries directly cause adverse changes for environment and are, therefore, being challenged severely by mankind[1]. Among them that leather industry as a traditional industry in the world[2], many leather products are produced each year. However, the environment has been seriously polluted and people's health also threatened due to the use of many chemicals and lime during leather making processes.

The important stages in the leather-making process are beam-house operation, tanning operation and finishing operation[3], and the beam-house operation is the most critical stage among them. Traditional beam-house operation involves lots of water, lime, sulfide and other chemicals, which produced waste water, silt, solid waste and so on. About 60%-70% of total pollution such as biological oxygen demand (BOD), chemical oxygen demand (COD) and total dissolved solids (TDS) are released from beam-house operation[4; 5]. The preparation sections for unhairing and removal of interfibrillar substance cause nearly 40% of biochemical oxygen demand (BOD) and 50% of chemical oxygen demand (COD)[6]. In past few decades' years, many scientific workers have been committed to the cleaner production of leather the sake of protecting the environment from harm and study the leather making method sustainability. With the growth of biotechnology has achieved remarkable results in the production and application such as biological products in leather-making processing such as unhairing, liming and re-liming[4; 7]. Several bio-products have been developed for leather making, in which such as neutral protease, keratinase, amylase and lipase been used for unhairing, fiber opening and fat removal from hide and the leather produced by these methods have achieved the similar properties to those traditional leather properties, which can absolutely replace lime and sodium sulfide[8]. In leather making, more and more enzyme-based processes have been reported recent years[9].

Enzyme have been widely used in many industries especially for leather process due to its characteristics of high efficiency and specificity. Usually, enzyme-assisted beam-house process is being used in many leather factories to replace lime, sulfide and calcium hydroxide [10]. Over the past decade, enzyme-assisted materials such as ionic liquids[11; 12], hydrogen peroxide[13] and organic solvents[14] have been developed for leather making and achieved the similar result to traditional methods. In present study, an attempt that salt-assisted enzymatic for fiber opening has been studied to as far as possible dispersed the collagen fiber, and then tanning experiments been carried out to explore its absorption in tannic acid by leather. The results proved that salt could promote fiber dispersion without destroying enzyme's activity. This method of beam-house operation make it possible to achieve the clean production.

2 Materials and methods

2.1 Materials

Dry salted cow skin chosen from Henan Prosper Skin & Leather Enterprise Co., Ltd., Jiaozuo 454791, P. R. China was used for leather process. Neutral protease with its CAS of 9068-59-1 (Commercial grade, from Nanning Donghenghuadao Biotechnology Co., Ltd., activity 100000U/g), cellulase (BR, from Macklin, activity 10000U/g), sodium chloride (NaCl, AR, Aladdin), sodium sulphate (Na_2SO_4, AR, Macklin), magnesium chloride ($MgCl_2$, AR, Macklin). All the other chemicals in leather process were analytical purity bought by Macklin. Tannic acid bought from Macklin was used as tanning operation.

2.2 Activity of neutral protease

The activity of neutral protease with different sodium sulphate concentrations and different times were assessed by the Folin-reagent method in this study[13]. 2.0 wt.% enzyme solution was mixed with different concentration of sodium sulfate solutions. 1 mL samples were collected from the enzyme mixed solution, added 1 mL of casein and 2 mL of trichloroacetic acid in sequence. After standing for 15 minutes, the supernatant was filtered and 1 mL was collected to add 5 mL sodium carbonate solution and 1 mL Folin reagent to incubate for 20 minutes, and then the absorbance at 660 nm was measured compared to the blank group. The concentration of tyrosine was further calculated by standard curve. 1 U of protease activity is defined as the amount of enzyme required to produce 1 µmol of tyrosine/min[7].

2.3 Fiber opening

The soaked cow pelt was taken for unhairing and fiber opening. After unhairing and fat removal from hide operation the hide was divided into several uniform pieces. 2.0 wt.% of protease and 0.1 wt.% of cellulase of mixed solution were prepared, and then six different concentrations of sodium sulphate solutions (which 0, 0.2 wt.%, 0.4 wt.%, 0.6 wt.%, 0.8 wt.% and 1.0 wt.%) were added to the mixed solutions. The other mixed solutions of magnesium chloride and sodium chloride with enzyme were prepared the same as the method of mixed solution of sodium sulfate with enzyme. The hide pieces were treated by above solutions for removal of interfibrillar substance and fiber opening for 24 h. These samples treated by different salt-enzyme solutions were thoroughly washed and subjected for tanning operation using tannic acid.

2.4 Tanning

The salt-enzyme treated samples were washed thoroughly and taken for tanning process. Tannin tanning was done to turn the pelt to leather in this study. All the experimental samples were tanned using tannic acid of 45 wt.% of the sample mass, which the tannic acid was added in three portions (10 wt.%, 15 wt.% and 20 wt.%). The time interval for each addition was 2 hours, and the tanning continued for 48 hours after the last addition.

2.5 Proteins dissolution analysis

The proteins dissolution from hide samples treated with salt-enzyme at different time intervals (which 4, 8, 12, 20 and 24 h) were measured during the fiber opening process. All the liquors were collected the filtrate and tested. The quantification of the proteins was done by matching the absorbance values at 562 nm with the standard curve derived by the mucin standards.

2.6 Scanning electron microscope (SEM)

For scanning electron microscope analysis, the samples were prepared after treatment with above salt-enzyme solutions and stored in a deep freezer (-50℃) for 24 hours, and thereafter lyophilized. The freeze dried samples were cut into 2×2 mm^2 sections and placed on the conductive adhesive of sample stand and then gold coated. The surface morphology of fiber-opened samples was observed using FEI's field scanning electron microscopy, and SEM images were obtained in the working voltage of 10 kV, at a room temperature 20℃ and 50% relative humidity.

2.7 Histological staining evaluation

In order to evaluate the effect of different salt-enzyme in fiber opening of the skin, the samples of 1 cm^2 were cut from pelt treated with different methods at similar positions and fixed in 4% formaldehyde solution for more than 24 hours. Samples sections of 6 µm were obtained using microtome. The tissue specimens obtained were dehydrated with ethanol from low to high (up to 100%) and stained with hematoxylin and eosin. The images were observed and collected under a bright field microscopy.

2.8 Estimation of Absorption of Tannic acid

Subsequent to tanning, Ultraviolet-visible spectrophotometry was carried out to estimate the absorbance of tannic acid at 275 nm. Tannic acid solutions were diluted to 10000 times and the absorbance was measured by standard curve, and the absorption amount of leather to tannin tanning reagent was calculated.

3 Results and discussion

3.1 Enzyme activity

The activity of neutral protease in different sodium sulfate solutions were evaluated firstly in this study in order to assess the effect of different salt concentrations on enzyme-based fiber opening. Hence, to study the influence of sodium sulfate on enzyme activity, the changes of activity of the mixed salt-enzyme solutions with the increased of time were tested and combined the results in

Figure 1. It is very interesting to conclude that the enzyme activity would not obviously affected when different concentrations of sodium sulfate were mixed with protease. As time goes on, the activity of the mixed solution decreased and 70% of enzyme activity was preserved after 10 days. Nevertheless, there was somewhat different from previous reported[15] that the activity of enzymes decreases considerably in the presence of salt. This situation might be due to the lower concentration of salt used in this study so that there was not evidently reduction at protease activity. Protease essentially is protein, the prolongation of the retention time of the mixed solution will cause partial denaturation of the protein, resulting in partial loss of the enzyme activity, which is consistent with common sense. This activity study proved that the protease activity would not affected greatly when sodium sulfate was mixed with the neutral protease, so the efficiency of the enzyme in the fiber opening process will not be affected.

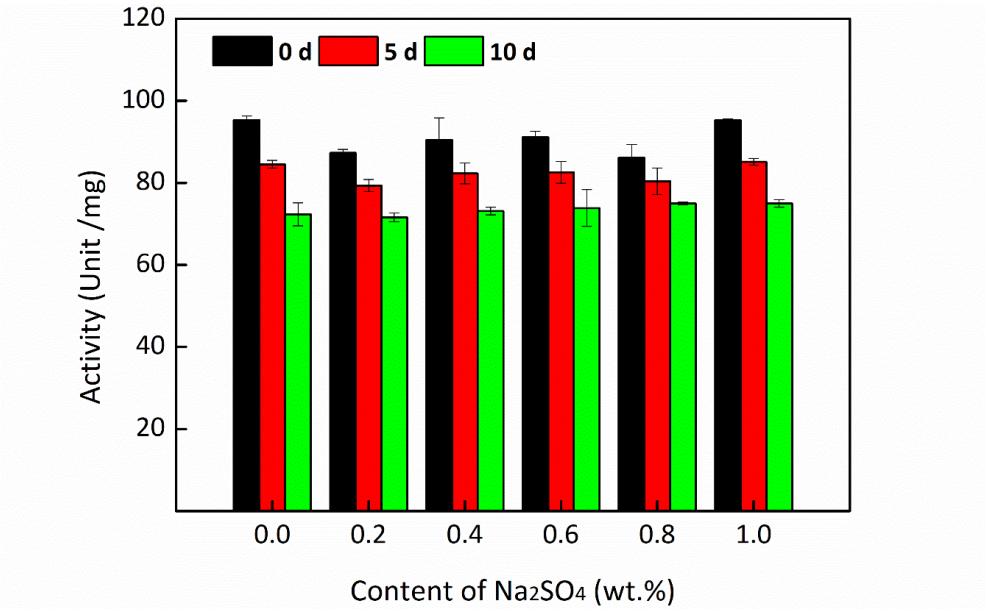

Fig. 1. Neutral protease activities at different sodium sulphate concentrations and different times.

3.2 Scanning electron microscope (SEM)

In general, SEM is a very important method to characterize the dispersion of collagen fibers in leather study. Great majority of the reported about leather industry have used this method. The dispersion of collagen fiber could be observed by SEM. In present study, three different salt-enzyme (which sodium sulfate-enzyme, magnesium chloride-enzyme and sodium chloride-enzyme) systems had been used for removal of interfibrillar substance and fiber opening. The effects of different kinds of salt-enzyme and different salt concentrations on fiber opening were discussed.

The results of SEM images of fiber opened by different concentrations of sodium sulfate (which 0, 0.2 wt.%, 0.4 wt.%, 0.6 wt.%, 0.8 wt.% and 1.0 wt.%) with 2.0 wt.% protease and 0.1 wt.% cellulase were showed in **figure 2**. It can be obviously inferred from the **figure 2** that the enzyme-based fiber opening would be improved distinctly at the presence of sodium sulfate compared with the method of without sodium sulfate, and the maximum dispersion of collagen fiber was observed when the concentration of sodium sulfate was 0.4 wt.%. Nevertheless, an appearance of inhibiting fiber opening was appeared when the sodium sulfate arrived above 0.6 wt.%.

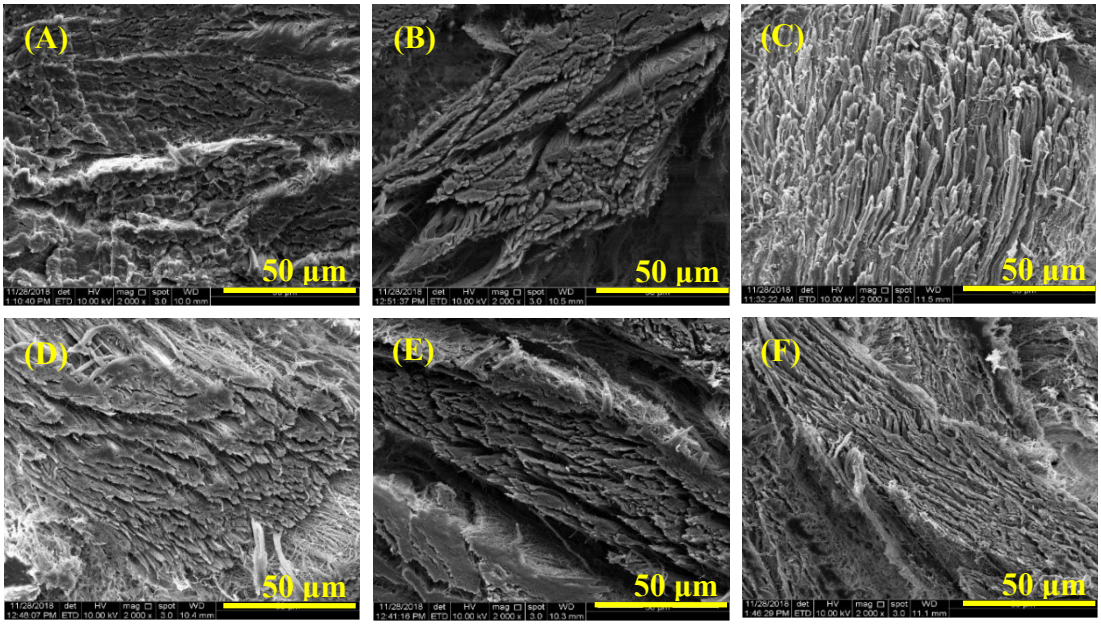

Fig. 2. Scanning electron microscopy images after fiber opening. (Content of Na2SO4: (A) 0 (B) 0.2 wt.% (C) 0.4 wt.% (D) 0.6 wt.% (E) 0.8 wt.% (F) 1.0 wt.%. Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

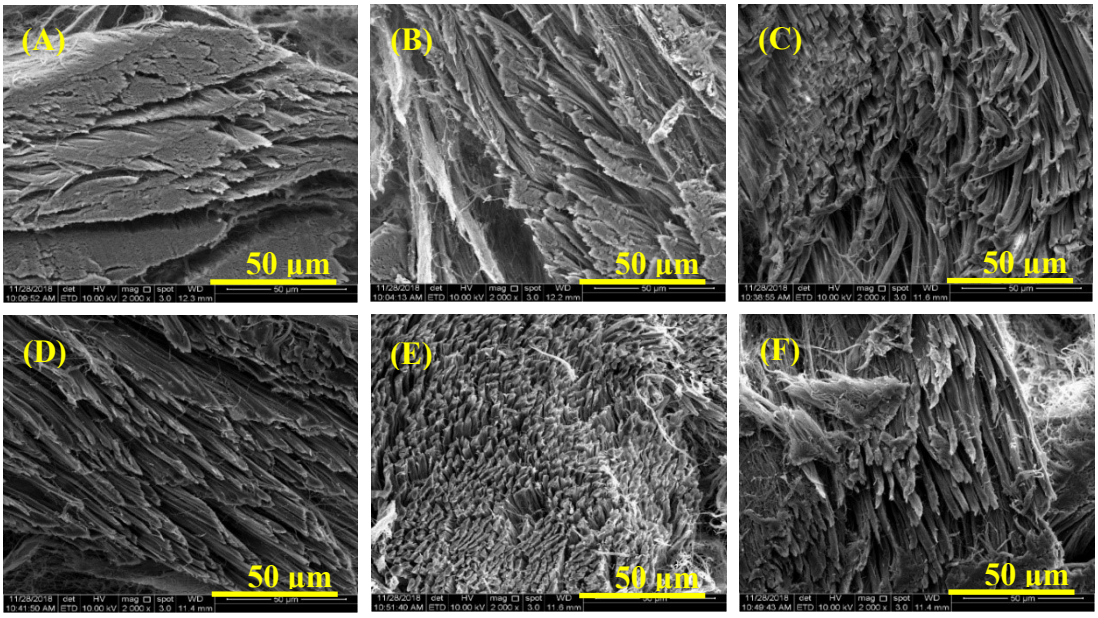

Fig. 3. Scanning electron microscopy images after fiber opening. (Content of $MgCl_2$: (A) 0 (B) 0.2 wt.% (C) 0.4 wt.% (D) 0.6 wt.% (E) 0.8 wt.% (F) 1.0 wt.%. Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

The SEM images showing the different concentrations of magnesium chloride (which 0, 0.2 wt.%, 0.4 wt.%, 0.6 wt.%, 0.8 wt.% and 1.0 wt.%) mixed with 2.0 wt.% protease and 0.1 wt.% cellulase for fiber opening were showed in **figure 3**. The magnesium chloride assisted enzyme-based treated skin showed better opened up structure of collagen fiber and formed the single bundle of fibers with the increase of salt concentration. Hence, the maximum dispersion of collagen fiber would be observed when the concentration of magnesium chloride was 0.4 wt.%. On the contrary, with the concentration of salt was further increased, the degree of opened collagen fiber has been not increased significantly. This results may be due to the fact that magnesium chloride could promote

the protease into the skin to open the collagen fiber more thoroughly, and the most effect of the enzyme-based opened up structure was exhibited in the SEM when the concentration of salt was 0.4 wt.%. Therefore, the dispersion of collagen fiber opened by enzyme would not be further improved through the concentration of salt was increased.

The cross section of leather produced by experiments with different concentrations of sodium chloride (which 0, 0.2 wt.%, 0.4 wt.%, 0.6 wt.%, 0.8 wt.% and 1.0 wt.%) mixed with 2.0 wt.% protease and 0.1 wt.% cellulase for fiber opening were still observed by a SEM, and the results were showed in **figure 4**. As can be seen from **figure 4** that sodium chloride promoted the protease on the opening of collagen fiber less than sodium sulfate and magnesium chloride. However, it is worthwhile mentioning that the obviously opened up fiber structure has been observed by SEM when the concentration was 0.8 wt.% and with the concentration of sodium chloride further increased until to 1.0 wt.%, the opened up structure of fiber could not be observed clearly.

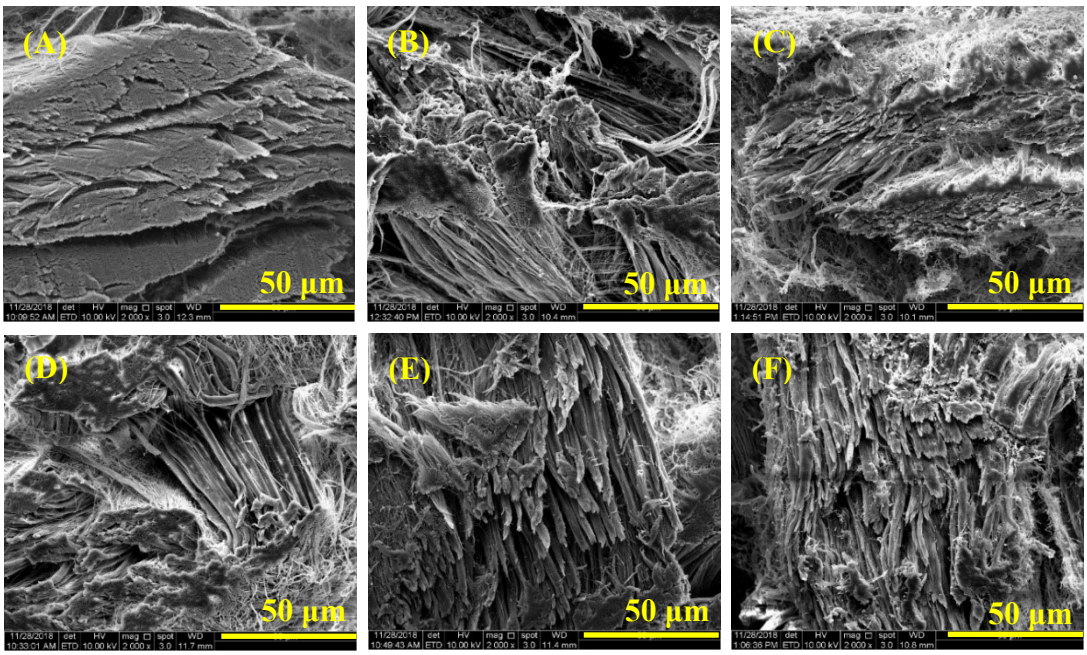

Fig. 4. Scanning electron microscopy images after fiber opening. (Content of NaCl: (A) 0 (B) 0.2 wt.% (C) 0.4 wt.% (D) 0.6 wt.% (E) 0.8 wt.% (F) 1.0 wt.%. Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

In general, the effect of different salt-assisted enzyme on fiber opening were different. It can be clearly inferred from SEM images above that magnesium chloride had a better effect compared with other two salts. And sodium sulfate also could promote fiber opening as we could observed in **figure 2**. On the contrary, the effect of sodium chloride-assisted enzyme was not as well as that of the other two salts. It can be concluded that different salt-assisted enzymes had a same rule on fiber opening, which was that when the salt concentration reached a certain value, there was a little difficult to increase the salt concentration on promote the enzyme-based fiber opening, somewhat even inhibit the dispersion of fiber. The reasons are related to the ion radius of salt and the effect of different kinds of salt on the skin.

3.3 Total proteins dissolution analysis

The interfibrillar substance is mainly composed by interstitial-protein and proteoglycan, which is not conducive to the later tanning operation, so it is necessary to remove it. In beamhouse, the dissolution of interstitial-protein indirectly reflects the effect of salt-enzyme system on hide. Hence, it is need to determine the amount of protein released from hide. By measuring the protein content in solution before and after the experiment, which could indirectly determine the situation of

protein dissolution from hide and the effect of enzymes on removal of interfibrillar substance. In present study, the enhanced BCA Protein Assay Kit[16] was used to determine the content of protein.

Figure 5 (A) shows the mass of protein dissolution per gram of skin after fiber opening using different concentrations of sodium sulfate mixed with 2.0 wt.% protease and 0.1 wt.% cellulase and **Figure 5** (B) shows the mass of protein dissolution in different treatment periods. It could be clearly concluded from results that different concentrations of salt have the different effects on protein dissolution. It could be observed the maximum dissolution of proteins when the concentration of sodium sulfate was 0.4 wt.%, however, with further increased the concentration of sodium sulfate, the dissolution of protein was inhibited. From which can be indicated that a low concentration of sodium sulfate could promote the enzyme on dissolution of proteins, while if the concentration was more than 0.4 wt.%, it will inhibit them. It could be extracted that low concentration of sodium sulfate accelerates the enzymatic penetration into the skin and accelerate the enzymatic action on removal of interfibrillar substance, which dynamic process can proved from **Figure 5** (B).

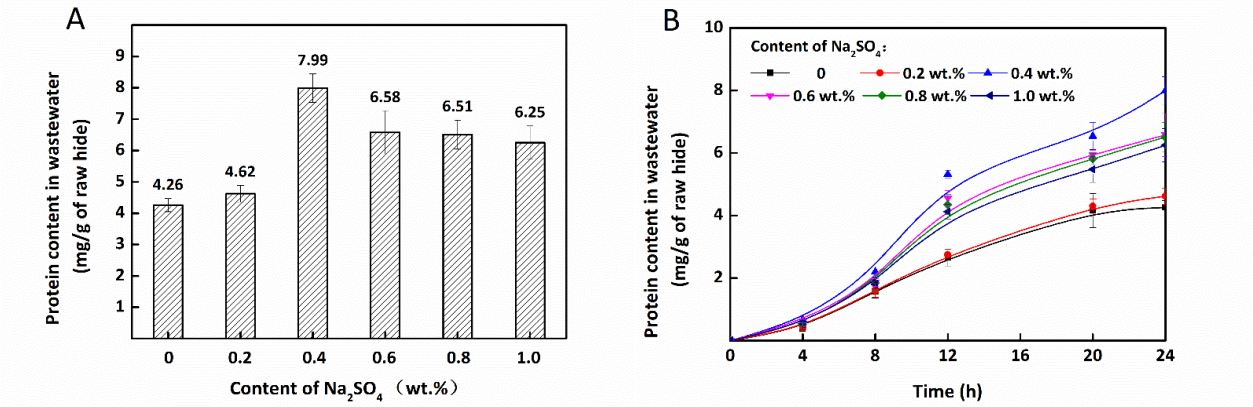

Fig. 5. Values of proteins of the liming process (A) and dynamic process (B) at different sodium sulphate concentrations. (Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

Figure 6 (A) shows the mass of protein dissolution per gram of skin after fiber opening using different concentrations of magnesium chloride solution mixed with 2.0 wt.% enzymes and 0.1 wt.% cellulase, and **Figure 6** (B) shows the dissolution of protein at different periods. It can be seen from **Figure 5** and **Figure 6** that the effect of protein dissolution as similar as sodium sulfate that different concentrations of magnesium chloride also had the different effect on protein dissolution. Nevertheless, there were also some difference that when the concentration of magnesium chloride was 0.4 wt.%, the protein dissolution was 10.73 mg/g and when the concentration of magnesium chloride was further increased, the phenomenon of neither accelerating protein dissolution nor inhibiting protein dissolution occurred, reaching a relatively balanced amount. Enzymatic fiber opening played a full role when the concentration of magnesium chloride was 0.4 wt.%, hence, the enzymatic action could not be improved further more by increasing the concentration of salt. Compared with sodium sulfate, magnesium chloride mixed with enzyme had a better removal of interfibrillar substance, which was indicated that magnesium chloride had better effect on promoting enzyme into the skin. From **Figure 6** (B), it can be seen that the dissolution ratio of proteins in the first 12 hours was improved evidently when using magnesium chloride assisted enzyme for fiber opening faster than that with using sodium sulfate, which indicated that the mixed action of magnesium chloride with enzyme can not only promoted the increase of the amount of protein dissolution, but also improved the dissolution rate. When this mixture was mixed for fiber opening, magnesium chloride could quickly promote the enzyme into the skin. The slope of the protein dissolution curve for the first eight hours shown in **Figure 6** (B) also proved this. With time goes on, the dissolution of protein gradually reaches a saturation value.

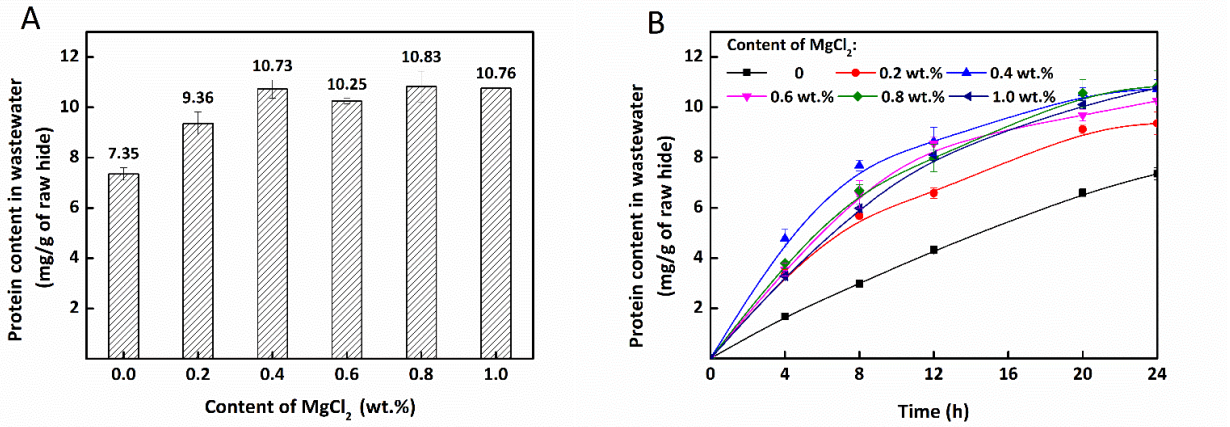

Fig. 6. Values of proteins of the liming process (A) and dynamic process (B) at different magnesium chloride concentrations. (Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

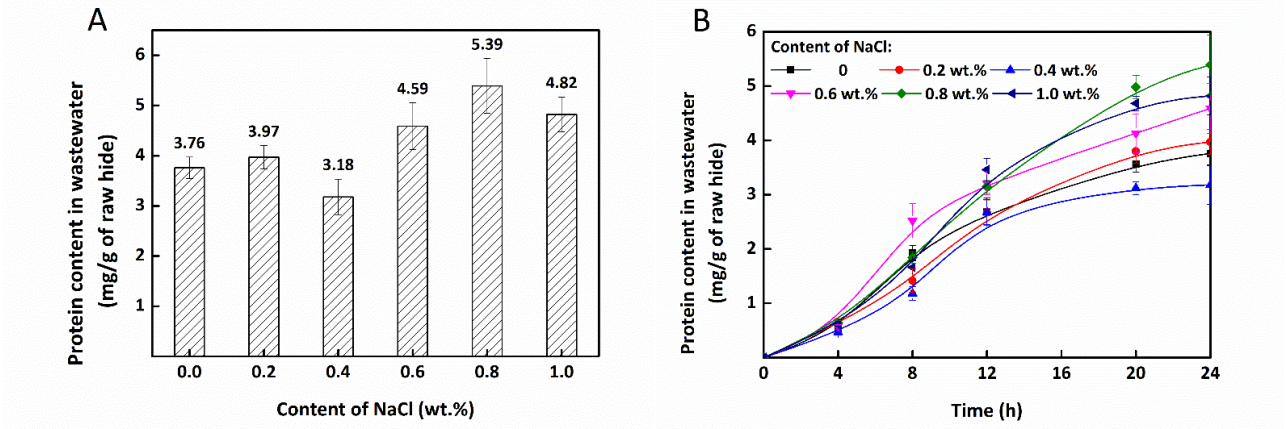

Fig. 7. Values of proteins of the liming process (A) and dynamic process (B) at different sodium chloride concentrations. (Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

Different concentrations of sodium chloride mixed with enzyme were used to removal of interfibrillar substance and the situation of the protein dissolution were shown in **Figure 7**. By comparing **Figure 5**, **Figure 6** and **Figure 7**, it can be easily concluded that the effect of sodium chloride with enzyme on removing interfibrillar substance was obviously weaker than that of sodium sulfate and magnesium chloride mixed with enzyme. The maximum dissolution of protein was only 5.39 mg/g as the concentration of sodium chloride was 0.8 wt.%. Besides, with the further increased of NaCl concentration, protein dissolution was inhibited.

3.4 Histological staining

The histological staining analysis method is a very practical method for characterizing the dispersion of collagen fiber. After fiber opening, the degree of removal of interfibrillar substance could be judged by histological staining. In present study, histological stained cross section of skins after fiber opening were performed and observed using EVG staining. This stained method can provide the information about collagen and non-collagen materials present in the skin. **Figure 8** (A) shows the tissue staining image of the raw skin without fiber opening, while **Figure 8** (B), (C) and (D) show the histological stained cross section of skin after fiber opening using three different salts mixed with enzyme.

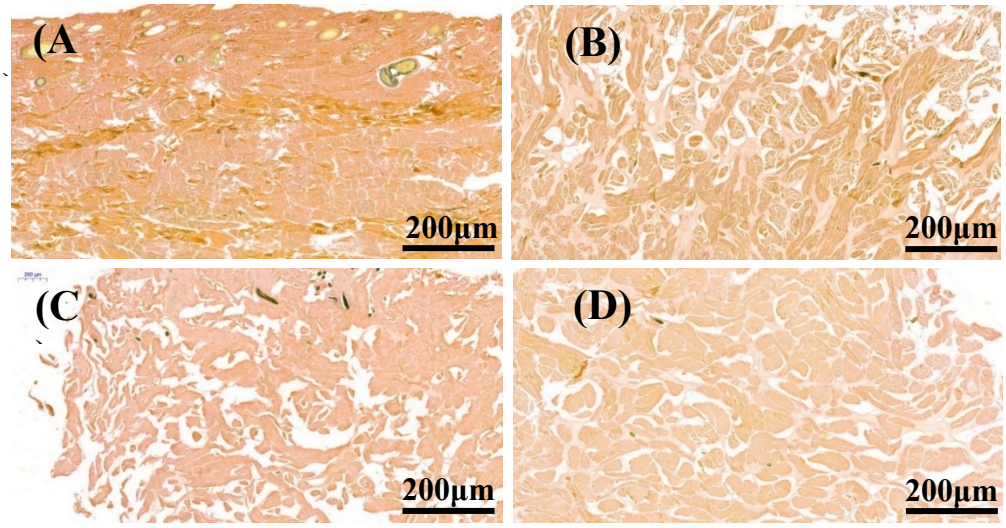

Fig. 8. EVG staining images of hides. ((A) Untreated (B) Na2SO4 (C) MgCl2 (D) NaCl Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

The non-collagen materials present in the skin could be clearly seen from **Figure 8** (A). Compared with the raw skin without fiber opening, **Figure 8** (B), (C) and (D) clearly demonstrate the effectiveness of enzyme-driven fiber opening process and different salt-assisted enzyme have different effects on fiber opening. The dispersion of collagen fiber could be assessed in terms of the interfibrillar spacing. It can be obviously inferred from **Figure 8** (C) that, after fiber opening using magnesium chloride mixed enzyme, the collagen fiber was split apart, which could be observed in the opened up structure. And this is consistent with the observation from SEM (**Figure 3**). On a comparative note, the fiber opening of samples using sodium sulfate assisted enzymes and sodium chloride assisted enzymes for fiber opening were slightly worse than those with magnesium chloride-assisted enzymes for fiber opening, as it could be seen from **Figure 8** (C) and (D).

3.5 Tannin Absorption

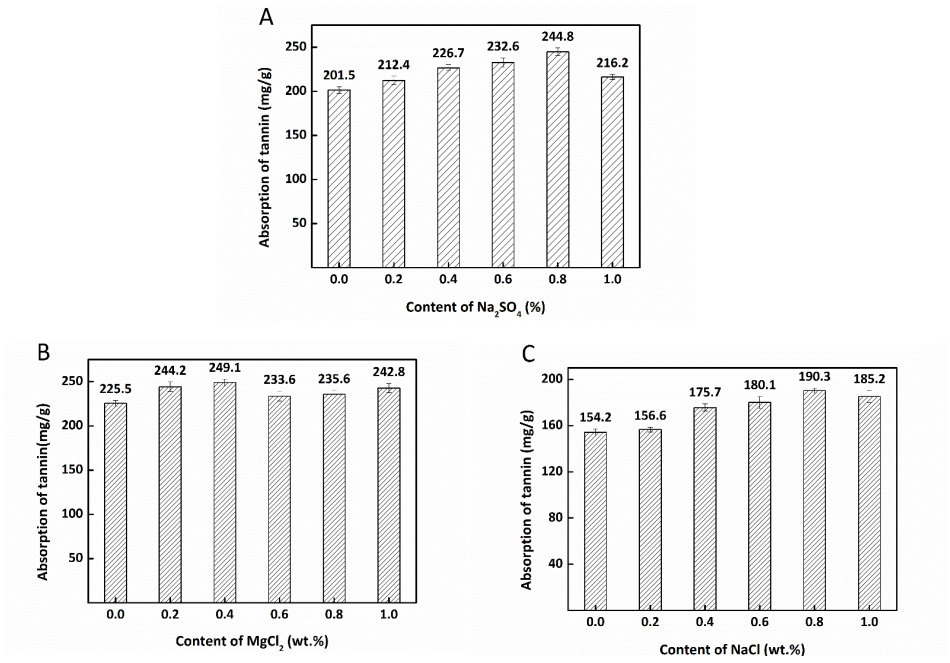

Fig. 9. Absorption of tannin after fiber tanning using different salt-enzyme for fiber opening. (Neutral protease: 2.0 wt.%. Cellulase: 0.1 wt.%)

Tanning process is a very important operation in which animal skin turn into leather[17]. After fiber opening operation, many active groups would be exposed on the collagen molecule. Tanning process usually is mainly through the penetration of tanning agent molecules into the skin and combine with the active group of collagen molecule in which would happen the qualitative change. In present study, the tannic acid was used to tanning operation. In the tanning process, the tannin molecule and the collagen molecule were linked by hydrogen bonds and formed a stable large network structure[18]. To some extent, the better the dispersion of collagen fiber, the more active groups exposed on the collagen molecule, and the more tannic acid molecules which could bind to collagen molecules. Therefore, the dispersion of collagen fiber can be indirectly judged by evaluating the amount of tannic acid binding to collagen molecules. **Figure 9** shows the amount of tannic acid absorbed by skin after tanning of the experiment samples. The more tannic acid was absorbed by per unit mass of skin, the better opened up structure of collagen fiber. It can be observably concluded from **Figure 9** that the absorption of tannic acid by skin was exactly related to the degree of fiber opening. The experiment samples which after fiber opening treated with sodium sulfate assisted enzyme and magnesium chloride assisted enzyme, the absorption of tannic acid absorbed by skin reached more than 200 mg/g, which indicated that the collagen fiber had a preferable opened structure and the evidence also could be obtained from the SEM (**Figure 2** and **Figure 3**). When the concentration of magnesium chloride was 0.4 wt.%, the content of tannic acid absorbed by the skin reached to 249.1 mg/g, indicating the best opened up structure, which was also verified by SEM (**Figure 3** (C)). On the contrary, the tannic acid absorbed by the sample treated by sodium chloride mixed enzyme was less than above two methods, and the poor opened up fiber structure was also observed.

4 Conclusion

In this study, three kinds of salts (sodium sulfate, magnesium chloride and sodium chloride) were selected to assist enzyme for fiber opening operation in the leather making. The results have shown that salt can be used to promote enzyme into skin to remove the interfibrillar substance and open the collagen fiber, furthermore the degree of fiber opening was related to the kind and concentration of salt assisting. Magnesium chloride had the best effect of promoting fiber opening, and the enzyme could play its full role in the process when the concentration of salt was 0.4 wt.% and at the same time the maximum protein dissolution and tannic acid could be measured. Compared with sodium sulfate and magnesium chloride, sodium chloride had a slightly poor effect on promoting fiber opening, nevertheless the dispersed fiber structure could be observed at 0.8 wt.% concentration. The degree of dispersion of fiber was related to the amount of tannin tanning agent absorbed by skin. The better dispersed structure could absorb more tannic acid. This beamhouse method provides a more efficient reference for the removal of interfibrillar substance and fiber opening by use salt-enzyme mixture.

Acknowledgement

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References

1. *Thanikaivelan P, Rao J R, Nair B U, et al. Progress and recent trends in biotechnological methods for leather processing. TRENDS BIOTECHNOL, 22(4), 181-188, 2004*
2. *Durga J, Ranjithkumar A, Ramesh R, et al. Replacement of lime with carbohydrases – a successful cleaner process for leather making. J CLEAN PROD, 112, 1122-1127, 2016*
3. *Saravanabhavan S, Aravindhan R, Thanikaivelan P, et al. Green solution for tannery pollution: effect of enzyme based lime-free unhairing and fibre opening in combination with pickle-free chrome tanning. GREEN CHEM, 5(6), 707-714, 2003*
4. *Pandi A, Ramalingam S, Rao J R, et al. Inexpensive α-amylase production and application for fiber splitting in leather processing. RSC ADV, 6(39), 33170-33176, 2016*
5. *Nazer D W, Al-Sa'ed R M, Siebel M A. Reducing the environmental impact of the unhairing–liming process in the leather tanning industry. J CLEAN PROD, 14(1), 65-74, 2006*
6. *Rao R J, Thanikaivelan P, Nair B. An eco-friendly option for less-chrome and dye-free leather processing: in situ generation of natural colours in leathers tanned with Cr–Fe complex. CLEAN TECHNOL ENVIR, 14(2), 115-12, 2002*
7. *Saran S, Mahajan R V, Kaushik R, et al. Enzyme mediated beam house operations of leather industry: a needed step towards greener technology. J CLEAN PROD, 54, 315-322, 2013*
8. *Murugappan G, Zakir M J A, Jayakumar G C, et al. A Novel Approach to Enzymatic Unhairing and Fiber Opening of Skin Using Enzymes Immobilized on Magnetite Nanoparticles. ACS SUSTAIN CHEM ENG, 4(3), 828-834, 2016*
9. *Durga J, Ramesh R, Rose C, et al. Role of carbohydrases in minimizing use of harmful substances: leather as a case study. CLEAN TECHNOL ENVIR, 19(5), 1567-1575, 2017*
10. *Ranjithkumar A, Durga J, Ramesh R, et al. Cleaner processing: a sulphide-free approach for depilation of skins. ENVIRON SCI POLLUT R, 24(1), 180-188, 2017*
11. *Vijayaraghavan R, Vedaraman N, Muralidharan C, et al. Aqueous ionic liquid solutions as alternatives for sulphide-free leather processing. GREEN CHEM, 17(2), 1001-1007, 2015*
12. *Alla J P, Rao J R, Fathima N N. Integrated Depilation and Fiber Opening Using Aqueous Solution of Ionic Liquid for Leather Processing. ACS SUSTAIN CHEM ENG, 5(10), 8610-8618, 2017*
13. *Andrioli E, Petry L, Gutterres M. Environmentally friendly hide unhairing: Enzymatic-oxidative unhairing as an alternative to use of lime and sodium sulfide. PROCESS SAF ENVIRON, 93, 9-17, 2015*
14. *Aravindhan R, Monika V, Balamurugan K, et al. Highly clean and efficient enzymatic dehairing in green solvents. J CLEAN PROD, 140, 1578-1586, 2017*
15. *Yongchao Liang. Effects of silicon on enzyme activity and sodium, potassium and calcium concentration in barley under salt stress.pdf. PLANT SOIL, 209, 217–224, 1999*
16. *Piercer. BCA Protein Assay Kit product information (Fisher Scientific GmbH, 2010.*
17. *Zhang H, Chen X, Wang X, et al. A salt-free pickling chrome tanning approach using a novel sulphonic aromatic acid structure. J CLEAN PROD, 142, 1741-1748, 2017*
18. *Tambi L, Frediani P, Frediani M, et al. Hide tanning with modified natural tannins. J APPL POLYM SCI, 108(3), 1797-1809, 2008*