A cleaning and efficient approach to improve wet-blue sheepleather quality by enzymatic degreasing

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Abstract: Enzyme is a green, efficient and easily degradable substance. In the present investigation, the papain and 100-c enzyme instead of part of surfactants were used in degreasing process. The results indicated that when wet-blue sheep leather was treated by the enzymatic compound, protein content in the effluent was about 9 times more than that of the non-ionic degreasing agent with less damage to collagen. Moreover, the degreasing rate for enzyme degreasing was 40.1% which was much higher than that of non-ionic degreasing agent. Mechanical properties of the crust leather treated by enzymatic compound were matchable to those treated by non-ionic degreasing agent. Color difference values of the leather indicated that the wet-blue sheep leather was easy to be dyed after being treated by enzymatic compound. The K/S value of the leather treated by enzymatic compound was much higher than that treated by non-ionic degreasing agent. Histological analysis of wet-blues visually showed efficient fat removal by enzymatic compound. Enzymatic compound used in degreasing of greasy wet-blues could improve the leather' quality.

Key words: enzyme, wet-blue, degreasing, bating, leather

1 Introduction

Leather-making processing is one of the earliest industrial activities taken up by mankind. Leather industry is making significant contributions to economic development, however it is globally facing challenges owing to pollution it causes to the environment (George N. et al., 2014; Saran S. et al., 2013; Jia L. et al., 2016). However, qualities of wet-blues from different batches or different manufacturers are not consistent with each other. In order to improve the quality of finished leather, wet-blue sheepskin need further centralized processing before retanning, especially for some wet-blue leathers which degreased inadequately. For example, fat or grease remaining in the wet-blue sheepskin will influence the binding of the subsequent chemicals (Sivakumar, V. et al., 2009). The residual fat always results in high fatty acid content. In chrome tanning process, the unsaturated fatty acid and chromate salt are easy to be combined, thus chrome soap can be formed, which is poorly soluble in water. Fatty acids consume chromium salt, and can be integrated with other lipids, thus leading to unevenly dyed leather. On the other hand, fat can gradually migrate to the leather surface at room temperature, which will reduce the quality of leather.

Surfactant used in degreasing process has a good effect. However, the traditional surfactant has pollution problem due to difficult degradation. Cleaner-preservation techniques using biological agents have been developed to reduce pollution in leather processing operations (Kanagaraj J. et al., 2014). Enzyme is a green, efficient and easy degradable substance. The use of enzyme-based products has been explored in many areas. For example, in leather manufacture enzymes are used during soaking,

unhairing and liming, bating, degreasing, and area expansion (Saravanan, P. et al., 2014; Song, J. et al., 2011). The use of enzymes is considered as one of the most promising methods for improving environmental conditions related to leather processing (Saran S. et al., 2013; Kanth S V. et al., 2009). Moreover, enzymes have been used in the treatment of wet-blues to improve the quality of leather. Although numerous studies have been done to study enzyme treatment of wet-blue sheep leather (Pfleiderer E., 1974; Wei S. L. et al., 1991), most of them focused on the effect of proteases on the collagen fibres and the softness of leather. In the late 1960's, Pfeleiderer studied the softness of wet-blue sheep leather treated by enzyme. Chen Haiming (Chen H.M. and Liao L.L., 2000) used acid lipase, acid protease to treat chrome tanned leather. It was found that grease on the leather surface and other pollution on the neck wrinkles were reduced and uniformly dyed leather was produced. However, few researchers have studied the degreasing performance of enzyme for greasy wet-blue sheep leather.

Our preliminary experiments indicated that the combination of two or more enzymes could efficiently degrade the interfibrillar component in the soaking process (Ma J.Z. et al., 2014). In the present study, Papain, 100-C enzyme and OTAC were selected to prepare enzymatic compound via blending method. 100-C enzyme, as a class of glycerol ester hydrolysis enzyme, has good hydrolysis effect to the triglycerides ester formed by advanced fatty acid and glycerol. Papain is a kind of proteolytic enzyme, which has better hydrolysis effect to the fat cell membrane, thus facilitate 100-C enzyme to hydrolyze more fat. OTAC is a type of cationic surfactant,

which can promote chemicals used in leather process permeability. OTAC was mixed with 100-C and papain to improve permeation effect of the two enzymes and the cooperative degreasing action. This degreasing process was compared with the conventional surfactant degreasing process. A comprehensive degreasing effect of enzyme was investigated by detecting hydroxyproline content in the wastewater, collagen content, degreasing rate and mechanical properties of leather samples (tensile strength and elongation) as the indexes .

2 Material and method

2.1 Materials

Foline-Phenol (Sigma F-9252) and papain were purchased from Hefei Bomei Biotechnology Co., Ltd, China. Octadecyl dimethyl ammonium chloride (OTAC) is purchased from Henan Provincial Dao Chun Chemical Technology Co., Ltd. Enzyme 100-C was provided by Beijing Fanbo Chemicals Co., Ltd. Bovine serum albumin (BSA) and hydroxyproline were biological reagents used for the production of protein and hydroxyproline standard curve, respectively. Greasy sheep wet-blues were provided by a tannery. Other materials used for the analysis were of analytical grade. Other retanning materials were of industrial grade.

TU-1900 Double Beam UV-vis Spectrophotometer (Beijing Persee General Instrument Co., Ltd.) and thermostatic water bath were used to determine the activity of enzymes, protein and hydroxyproline. AI-3000 Universal Testing Machine (Gaotie Detection Instruments Co., Ltd.) and GT-303 Softness Tester were used to test the mechanical properties and softness of the crust leather. SZF-06C Fat Tester (Zhejiang

Top Instrument Co., Ltd) was used to determine the degreasing rate.

2.2 Preparation of enzymatic compound

100-C and papain enzymes in different mass ratio was weighed and dissolved in 100mL water via stirring to mix evenly. Then 0.2% octadecyl trimethyl ammonium chloride (OTAC) was introduced into the mixture system to prepare the compound enzyme which was applied in wet-blues' degreasing process .

2.3 Degreasing effect of enzymatic compound for greasy wet-blue sheepleather

The greasy wet-blue sheepleather was cut into two halves along the backbone. The left half was used for control and the right half was used for enzymatic compound degreasing. The degreasing process was conducted as the following. The shaved wet-blue sheepleathers with 0.6-0.7 mm thickness were weighed and used for degreasing process. Chemical dosages were calculated on the basis of the weight of shaved wet-blue sheepleathers. The degreasing temperature was 40 °C. Weighed wet-blue sheepleathers, 200% water and 0.2% formic acid were added into the drum. And then 0.5% non-ionic degreasing agent was added as the control degreasing process for 40min. As for the enzyme degreasing process, enzyme or the compound were fed into the drum allowing for 40min. After degreasing, the effluen was drawn and filtered using filter paper to remove the gross solids.

In order to evaluate the effect of enzyme on the wet-blue sheepleather properties, the degreased wet-blues were processed with conventional retanning, dyeing and fatliquoring methods.

2.4 Determination

2.4.1 Protein and hydroxyproline in the effluent

Hydroxyproline is one of main amino acids of collagen, so testing hydroxyproline concentration in the effluent is an effective method for measuring hydrolysis degree of collagen. The higher hydroxyproline concentration in the effluent, the greater extent collagen hydrolysis, thus indicating the more serious damage degree to the collagen, which is not beneficial to leather. The hydrolysis degree to collagen directly and greatly affects the physical and mechanical properties of wet-blue sheepskin, so we must consider it in every process.

The degreasing liquor was collected to determine the total protein and hydroxyproline content. The total protein was determined by Lowry's method (Lowry O.H. et al., 1951) using bovine serum albumin as the standard.

The determination of hydroxyproline was carried out according to the methods given by Neuman and Logan (Neuman R.E. and Logan M.A., 1950) using UV-Vis spectrophotometry. In order to avoid the influence caused by enzyme as a kind of protein in UV testing process, the UV absorption curves of experimental enzyme and hydroxyproline were determined separately. Their maximum UV absorption wavelengths were 194 nm and 558 nm respectively. The two UV absorption curves were not overlapping. In other words, the UV absorption of hydroxyproline could not be affected by the UV absorption of enzyme.

Collagen concentration and collagen proportion accounting for total protein can be calculated according to the formula below.

Collagen concentration ($\mu\text{g/mL}$) = Hydroxyproline $\times 7.4$ (Krishnamoorthy G. et al.,

2013)

Collagen proportion accounting for total protein (%)=(Collagen/Total protein)×100%

2.4.2 Degreasing rate

Around 10±0.1g of samples were extracted for 4h with methylene chloride using a SZF-06C Fat Tester. Then the extract was dried at 102±2°C to constant weight. Degreasing rate can be calculated according to the formula below.

Degreasing rate (%)=(extract weight/sample weight)×100%**Error! Reference source not found.**

2.4.3 Mechanical properties and softness of the crust wet-blue sheepleather

The crust wet-blue sheepleather samples were conditioned under standard atmospheric conditions (65±5% relative humidity and 20±2°C) for 48 h prior to analysis of the mechanical properties. The tensile strength, tear strength and elongation at break of the leather samples were tested by a Universal Testing Machine (AI-3000) following the standard methods (IUP 6., 2000). Softness was tested on a GT-303 Softness Tester, which was the average of six testing points.

2.4.4 Color difference

The crust wet-blue sheepleather treated by the non-ionic degreasing agent and enzymatic compound were dyed in black and the dyed wet-blue sheepleathers were subjected to a reflectance measurements called Data Color SF-600 Plus CT.

2.5 Characterization

2.5.1 Histological analysis of wet-blue sheepleathers

Samples of 1 cm² were cut from identical locations on treated wet-blue sheepleathers, washed and fixed with 10% formaldehyde solution. The samples were then embedded in gelatin to cut sections of 10 µm using microtome. Sections were then stained using Trichrome and Sudan IV before analysing the histological features on a multimedia microscope. In this method, fat is stained in red and collagen fibres were stained in blue.

2.5.2 Scanning electron microscopy

The chrome tanned wet-blue sheepleather was air-dried and then cut into thin slices and sprayed with platinum. Hitachi's S-4800 scanning electron microscope was used to observe the fibre dispersion of wet-blue sheepleather.

3 Results and discussion

3.1 Effect of enzyme ratios on degreasing of wet-blue

The 100-c enzyme and papain were selected in our experiment. 100-c enzyme, a pale yellow powder, is a kind of glycerol ester hydrolase. It can not only be decomposed or synthesized advanced fat section and glycerol, which can form triglyceride ester. It has a highly efficient hydrolysis of oils and fats and can remove the natural oils. Papain, a proteolytic enzyme, can hydrolyze adipose cell membrane to release fat from adipose cell, which was beneficial for 100-c enzyme to hydrolyze fats.

The degradation of protein and collagen can be indicated by the total protein and hydroxyproline concentration in the degreasing effluent. The total protein and hydroxyproline can explain the action of the enzyme to the interfibrillar protein

indirectly. Figure 1 shows the total protein concentration in the effluent from the control and different ratios of 100-C to papain degreasing. It can be seen that the trend of protein concentration decreased with the increase of papain proportion. The total protein in the effluent in any ratios of 100-C to papain was higher than that of control because that enzyme can not only promote the solubility of water-soluble protein but also degrade some insoluble proteins. While the control degreasing process using non-ionic degreasing agent alone just acted on some water-soluble proteins, which resulted in lower total protein concentration. Correspondingly, figure 2 provides that the trend of hydroxyproline concentration increased and then decreased with the increase of papain proportion, but the hydroxyproline concentration in the effluent from the degreasing by 7:3 of 100-C to papain was comparatively lower.

However, it was not enough to describe the effect of enzyme degreasing only via the total protein and hydroxyproline concentration. In fact, the collagen content can be calculated by hydroxyproline (Krishnamoorthy G. et al., 2013). Collagen was included in the total protein. The total protein contains collagen and other interfibrillar proteins. So, concentration of total protein and the ratio of collagen to total protein can better illustrate the degradation of protein and the damage to collagen. Table 1 shows collagen and collagen proportion accounting for total protein degreasing by the control and compound in different ratios of 100-C to papain. It was observed that collagen proportion of leather degreased by the compound was much lower than that of control. When the ratio of 100-C to papain was 7.0: 3.0, the collagen proportion accounting for total protein was the lowest and the total protein was the highest

(figure 1). In all, the strong hydrolysis of enzymatic compound leads to high total protein. However, there was no significant difference in hydroxyproline between enzymatic compound and control process. Therefore, the collagen proportion accounting for total protein of enzymatic compound was much lower than that of control.

Another purpose of enzyme degreasing for greasy wet-blue sheepleather was to remove the residual fat in wet-blue sheepleathers. Removal of fat can promote the penetration and combination of other chemicals. Therefore, degreasing rate is an important indicator to estimate the enzyme degreasing performance. Figure 3 exhibits the degreasing rates of wet-blue sheepleather degreased by the control and enzymatic compound. It can be found that the degreasing rate decreased with the increase of papain proportion. The degreasing rate of wet-blue sheepleather treated by enzymatic compound with 7:3 ratio of 100-C to papain was higher than that of control.

Based on the above results, the enzymatic compound with 7:3 ratio of 100-C to papain was selected as the combination enzyme. And the following experiments were carried out according to this combination.

3.2 Effect of OTAC on the enzyme degreasing

Surfactant OTAC was introduced in the enzyme (100-C: Papain=7:3) to further improve the degreasing performance. In order to study the effect of OTAC, composition of the effluent obtained from wet-blue sheepleathers treated by control, enzyme, OTAC and the combination of enzyme and OTAC is shown in table 2, separately. The total protein in the effluent treated by the combination of enzyme and

OTAC was the highest and even higher than that of the sum of enzyme and OTAC. The trend of collagen content was in agreement with the total protein content. Nevertheless, collagen proportion accounting for total protein was very different. The comprehensive results of total protein and collagen proportion accounting for total protein can thus illustrate the degradation of wet-blue sheepleathers. Higher total protein and lower collagen proportion were expected. The effluent after degreasing by the combination of enzyme and OTAC resulted in the highest total protein and comparatively lower collagen proportion accounting for total protein, which indicated that there was less damage to collagen. This may be attributed to cooperative action of the enzyme and OTAC.

The degreasing rates of wet-blue sheepleather treated by control, enzyme, OTAC and the combination of enzyme and OTAC are shown in figure 4. It was evident that the degreasing rate was much higher for wet-blue sheepleather treated by the combination of enzyme and OTAC than that of the wet-blue sheepleather treated by enzyme or OTAC individually, and even higher than that of the control. This indicated that the combination of enzyme and OTAC had outstanding degreasing rate because of the highly efficient degradation of fat. Moreover, combination of enzyme and OTAC made full use of the synergy effect. The surfactant OTAC had excellent emulsifying performance. On the one hand, OTAC itself can emulsify fat in the wet-blue sheepleather and this can open channels for the permeation of enzyme (Eriksson T. et al., 2002). On the other hand, the fatty acid and fat fragments degraded by enzyme could be emulsified by OTAC. Synergistic effect of the enzyme and

OTAC on the fat resulted in higher degreasing rate.

3.3 Mechanical properties of the crust leather

Mechanical properties are very important for wet-blue sheep leather. In order to investigate the effect of enzyme degreasing on the properties of crust leather, mechanical properties and softness were characterized (Parka M. et al., 2014). Table 3 shows the data obtained from the control and enzymatic compound degreasing. It can be seen that the enzyme degreasing did not affect the strength properties of the wet-blue sheep leather. Mechanical characteristics of the crust leathers treated by enzymatic compound *viz.* tensile strength, elongation at break and softness were in good agreement with that of the control. And an enhancement of tear strength of the crust leather treated by enzymatic compound was observed compared with that of the control. This may be explained by the less damage to collagen of enzymatic compound since collagen is the major component of wet-blue sheep leather. In the degreasing process, the damage to collagen may lower the mechanical properties of the crust leather. In this research, it indicated that the enzymatic compound used for degreasing greasy wet-blue sheep leather brought less damage to collagen. Moreover, degreasing process under the action of enzymatic compound removed the interfibrillar proteins and some residual fat, which could make the collagen fibres open and disperse well. The mechanical and bulk properties of crust leather were not reduced.

3.4 Color difference analysis of the dyed wet-blue sheep leather

Variation in color of the dyed wet-blue sheep leather was obtained. The color

difference values between the wet-blue sheepleather treated by enzymatic compound and the control are presented in table 4. In the color difference test, the control wet-blue sheepleather was tested as a standard sample, so the color difference values were zero. The total color difference ΔE was higher for the wet-blue sheepleather treated by enzymatic compound. For the dyed wet-blue sheepleather treated by enzyme, $\Delta L < 0$ indicated that the color of wet-blue sheepleather was darker, and $\Delta C < 0$ indicated that the color of wet-blue sheepleather was grayer. ΔH of dyed wet-blue sheepleather treated by enzyme was comparable to that of the control. This indicated that the color of wet-blue sheepleather has not deviated from the control.

Figure 5 and figure 6 present K/S value and reflectivity of dyed wet-blue sheepleather treated by the control and enzymatic compound, respectively. Higher K/S value and lower reflectivity mean that the color was darker. It can be seen that K/S value from 400nm to 700nm was significantly higher for the dyed wet-blue sheepleather treated by enzymatic compound than that for the control (figure 5). Accordingly, the reflectivity was comparatively lower (figure 6). That is, after the wet-blue sheepleather was treated by enzymatic compound, the crust wet-blue sheepleather was easier to be dyed and the color was more uniform due to the removal of fat. The fact is that removal of fat and interfibrillar proteins gave more active groups in collagen fibres, thus react with dyes to improve the dyes adsorption (Kanth S V. et al., 2009). Meanwhile, collagen fibers with less fat had good affinity with dyes and resulted in strong dyes fixation on the fibres.

3.5 Histological analysis

To further visually understand the removal of fat, Trichrome and Sudan IV stained sections of the wet-blue sheepleather were analysed for the histological features. The histological micrographs of wet bluea are shown in figure 7. The dark red parts in the micrographs indicated the presence of fat and the blue parts indicated collagen fibres. As figure 7(a) is shown, untreated wet-blue sheepleather was dyed by trichrome and Sudan IV staining. The fat cells and the free fat between fibers were red. There are much red zone in figure 7(a), which indicated that fat content was very high in the wet-blue, and the fibers closed to each other were slight purple . However, in figure 7(b), the wet-blue sheepleather treated with DN was stained by trichrome and Sudan IV. The red area of leather treated with DN was significantly reduced compared with that of the wet-blue, which indicated that fat content of the wet-blue sheepleather decreased greatly. Fibers in blue are shallow and discontinuous, which indicate that fiber dispersed better compared with 7(a). In order to achieve better degreasing effect, wet-blue sheepleather treated with enzymatic compound was dyed as figure 7(c) shown.. Compared with figure 7(b), after wet-blue sheepleather was treated by composite enzyme, the majority zone was blue, and the fiber loose lines are clearer. This results suggested that enzymatic compound has good greasing effect and beneficial to collagen dispersing. And there was less fat in the wet-blue sheepleather treated by enzymatic compound than that of the wet-blue sheepleather treated by the control. The collagen fibres splitting was more uniform than that of the control. These results were all in agreement with the data of total protein (table 2) and degreasing rate (figure 4).

3.6 Scanning electron microscopy

The crust leather samples were also viewed under SEM to understand the collagen fibres' structure, and the micrographs of crust leather are provided in figure 8. Figure 8b shows better separated and opened-up fibres in the case of the crust leather treated by enzymatic compound. Hence, the crust leather obtained from enzymatic compound degreasing are favored for increasing the contact surface areas in the collagen fibre network and thus more reaction sites could be exposed to interact with dyes. The increased interaction between collagen fibres and dyes resulted in the increase of dyes exhaustion. This may be contributed to the composition of the enzymatic compound. Commonly, a multi-enzyme system may be more suitable for wet-blue sheepskin processing (Jayakumar G. C. et al., 2014). In this study, the enzymatic compound was a composite component comprised of protease, lipase and surfactant. The synergy between the enzymes and surfactant could remove the residual protein and fat on the collagen fibres efficiently and brought better and finer collagen fibres splitting. The superior fibres splitting can facilitate the mechanical properties of crust leather.

4 Conclusions

The enzymatic compound in combination of 100-C, papain and octadecyl dimethyl ammonium chloride had stronger action on protein and obvious application performance when applied in greasy wet-blues' degreasing process. This as-prepared enzyme could give higher degreasing rate compared with that of the control degreasing agent, and the collagen damage of the wet-blue treated by enzymatic

compound and OTAC was significantly reduced. Moreover, the wet-blue sheepleather treated with enzymatic compound was easy to be dyed since it gave a uniform color to leather. The wet-bule sheepleather treated by enzymatic compound degreasing also had impressive mechanical properties. So this approach is a clean and efficient method for degreasing greasy wet-blues and it could improve wet-blue sheepleathers' quality and dye absorption, thus reduce surfactant in the conventional degreasing process.

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Abbreviations

OTAC	Octadecyl dimethyl ammonium chloride
BSA	Bovine serum albumin

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Table captions

Table 1 Collagen and the ratio of collagen to total protein degreasing by control and different ratios of 100-C to papain

Table 2 Composition analysis of the degreasing effluent

Table 3 Mechanical properties and softness of the crust leather obtained by control and enzymatic compound degreasing

Table 4 Color difference values of dyed leather obtained by control and enzymatic compound degreasing

Figure captions

Figure 1 Total protein concentration in the effluent degreasing by different ratios of 100-C to papain

Figure 2 Hydroxyproline concentration in the effluent degreasing by different ratios of 100-C to papain

Figure 3 Degreasing rate of the wet-blue sheepleather by control and different ratios of 100-C to papain

Figure 4 Degreasing rates of wet-blue sheepleather by different exp.

Figure 5 K/S curve of dyed leathers degreasing by control and enzymatic compound

Figure 6 Reflectivity of dyed leathers degreasing by control and enzymatic compound

Figure 7 Trichrome and Sudan IV stained sections of wet-blue, (a) wet-blue sheepleather before degreasing; (b) wet-blue sheepleather degreasing by control; (c) wet-blue sheepleather degreasing by enzymatic compound.

Figure 8 Scanning electron microscopy of crust leather, (a) crust leather obtained by control degreasing (b) crust leather obtained by enzymatic compound degreasing.

Table 1

Exp.	Collagen ($\mu\text{g/mL}$)	The ratio of collagen to total protein (%)
control	0.93	1.29
7.0: 3.0	0.62	0.12
6.0: 4.0	2.11	0.49
5.0: 5.0	2.15	0.53
4.0: 6.0	0.48	0.13
3.0: 7.0	0.78	0.18

Table 2

Total protein	Hydroxyproline	Collagen	The ratio of collagen to
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	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	total protein (%)
Control	111.31	0.13	1.30	1.16
Enzyme	495.30	0.18	0.88	0.18
OTAC	217.44	0.25	1.84	0.84
Enzyme+OTAC	975.72	0.37	2.77	0.28

Note: Enzyme-7:3 ratio of 100-C to papain; OTAC-0.5% OTAC; Enzyme+OTAC-7:3 ratio of 100-C to papain and 0.2% OTAC.

Table 3

Tensile strength	Tear strength	Elongation at break	Softness
(N / mm^2)	(N/mm)	(%)	(mm)

Control	9.73	31.27	56.41	6.92
Enzymatic compound	8.14	45.32	56.02	6.97

Table 4

	ΔE	ΔL	ΔC	Δa	Δb	ΔH
Control	0	0	0	0	0	0
Enzymatic compound	6.421	-5.948	-2.349	0.654	2.326	0.568

Note: The leather degreasing by control was tested as standard sample, so the color difference values were zero.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Efficient degreasing by enzyme

Wet-blue before treating

Wet-blue treated by enzyme