# A New Approach of Enzymatic Unhairing Process on Cattle Hide

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## Abstract

Unhairing, an important step for leather making process, is known as the most steps to make pollution in the beamhouse. The effluent for conventional hair-burning unhairing contains a large amount of COD, BOD, $S^{2-}$ and causes sludge. Enzymatic Unhairing method has already put forward for decades, while it was known hard to control and easily cause the leather with defects such as looseness and grain sueding. In order to solve those enzymatic unhairing problems, a new approach was build up in this paper by using the specific enzyme composites for cattle hide unhairing under lower temperatures than previous unhairing methods.

The enzymatic unhairing stage was checked by determining the amount of hair on the hide and the proteins content in the solutions. The appearance and the physical properties of the unhaired leather were determined. The samples for different unhairing process (sulfide unhairing, enzymatic unhairing under various temperatures) were investigated by histology method for deep looking into the unhairing mechanism. The epidermis, the hair follicle, the basement membrane and the corium of the unhairing samples was observed by transmission electron microscope (TEM) to investigate the changes of these structures during unhairing process.

**Keywords**: Enzymatic unhairing; Hair-saving unhairing; Cattle hide; Temperature

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## 1. Introduction

Traditional hair-burning unhairing is so far the step produced the most pollutant in beamhouse process of leather manufacturing where sodium sulfide was used to reduce the disulfide bonds of the keratin, the main component of hair and epidermis. The effluent produced in this process contains extremely high chemical oxygen demand and toxic sulfide ion (Liao 2001). To make cleaner unhairing, several technologies were put forward to displace sodium sulfide, such as oxidative unhairing by $H_2O_2$ (Shi et al. 2003; Marsal et al.1999), hair-saving unhairing by proteases (Paul et al. 2001; Song et al. 2011; Virgilijus et al. 2009),

and so on.

Enzymatic unhairing has been used as one of the alternative cleaner methods [3]. The mechanism of enzymatic process is distinct from that of the chemical process. The research shows that the non-collagenous proteins and other cementing substances were removed from the skin by enzyme, thus the hair could be easily removed by scrubbing or by machine. A number of proteases from bacterial, fungal have been studied for enzymatic unhairing (Dayanandan et al. 2003; Gehring et al. 2002; Sivasubramanian et al. 2008; Thangam et al. 2001;). The enzymatic unhairing methods reported so far were treated at 38-40℃, the optimum temperature of the enzyme to make sure that the enzyme could extremely react with its substrate. However, at this high temperature, accompany with the hair removal, the enzyme would damage the dermal collagen fiber which would cause injury to the grain. In our previously research, we found that when the enzyme unhairing at low temperature such as 25℃, very little affect to the collagen fiber was observed while its activity could be maintained for longer time. It was implied that the enzymatic unhairing processed in lower temperature may can prevent the hide from grain damage and loosen.

In this paper, the cattle hide was unhaired at 20-25℃ by a composite enzyme, DTM198. The enzymatic unhairing stage was checked by determining the amount of hair on the hide and the proteins content in the solutions. The appearance and the physical properties of the unhaired leather will be determined. The temperature and the enzyme dose will be the main investigation parameter for obtaining the ideal enzymatic unhairing process. The structure changes of the epidermis and the hair follicle during the unhairing stages were observed by TEM.

## 2 Materials and methods

### 2.1 Materials

The salt cured cattle hide was obtained from local tannery (Chengdu, China). DTM198 commercial unhairing protease (optimum pH range 7.5–9) was obtained from ShengFang Chemical Corporation (Guangdong, China). Folin phenol reagent was prepared in the lab. All the chemicals and reagents used such as casein, hydroxyproline, tyrosine, glutaraldehyde, sodium carbonate, osmium tetroxide, acetone, uranyl acetate, lead citrate were reagent grade or better.

### 2.2 Methods

***Unhairing process***

The whole process from raw hide to crust is briefly as the follows: weighting- soaking- fleshing- unhairing- alkali swelling treatment- splitting- neutralization- pickling- chrome tanning- shaving- retanning and fatliquoring-drying.

The soaked and fleshed cattle hide was enzymatic unhaired as the follows. The enzyme unhairing process was carried out under 20~25℃ with 150% float at pH 9.0, which was adjusted by sodium carbonate. DTM198 commercial unhairing enzyme was added with the dosage of 100~300 units/g hide. The drum was run for 30min with 4rpm and then it was stopped and would run 1min/h. The unhairing solution was collected for each hour and was spun down to take its supernatant for the determinations. When approximately 90% of hair was removed from the hide, the enzyme would be stopped by washing and sodium hydroxide treatment. The samples during the unhairing process were collected and fixed in 2.5% glutaraldehyde solution.

As a control, the sodium sulfide unhairing process was as the follows. It was carried out at 23-25℃ with 100% float by adding 3% sodium sulfide for 1hr continuously drumming. The samples during unhairing were collected and fixed in 2.5% glutaraldehyde solution.

As another control, the enzymatic unhairing at 38℃ was as the follows. The unhairing process was carried out under 38℃ with 150% float at pH8.5 which adjusted by sodium carbonate. DTM198 was added with the dosage of 200 units/g hide. Kept the drum running with 4rpm for 2h till the hairs were removed. The enzyme activity would be stopped by washing and sodium hydroxide treatment. The samples during unhairing were collected and fixed in 2.5% glutaraldehyde solution.

***Determination of the total protein content (TPC)***

The supernatant of the unhairing solution was diluted to 20 times, and in which total protein content was determined by the Folin phenol reagent method (Lowry et al. 1951). Tyrosine was used to make the standard curve.

***Determination of the enzyme activity***

The remaining enzyme activity (REA) of the supernatant in the unhairing solution was determined by the Folin method in which casein was used as the substrate (Pan 1988 ).

***Determination of the hydroxyproline content (HPC)***

The supernatant of the unhairing solution was diluted to 5 times, and in which hydroxyproline content was determined by the Chloramine-T method (Bergman and Loxley 1963). The collagen content in the solution was calculated according to the ratio of Hyp residue contained in a collagen molecule.

***Transmission electron microscopy (TEM) observation***

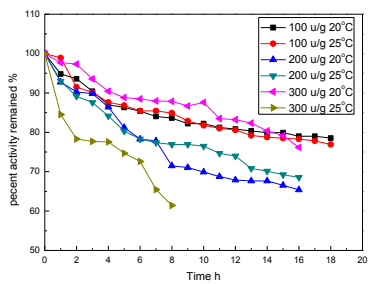
The glutaraldehyde fixed skin samples were stained with 1% osmium tetroxide. The stained samples were dehydrated gradually by acetone and then embedded in EPON812. Ultrathin sections were cut with the ultra-microtome equipped with a diamond knife, stained

with uranyl acetate and lead citrate, and examined under an H600-IV electron microscope (Zanette et al. 2011; Zhe et al. 2011).

## 3. Results and discussion

### 3.1 Investigation on the unhairing solutions

The enzymatic unhairing solutions were centrifuged and its supernatants were collected for the determinations of REA, TPC and HPC respectively. The remaining enzymatic activity (REA) in the enzymatic unhairing bath was shown in Figure 1. It was shown that the activity of the enzyme will be decreased during the unhairing processes. For the same dosage of enzyme, the higher the temperature is, the more activity will be lost during the same period. The more enzyme used, the difference will be more apparent. It indicated that the temperature plays a main role to the decrease of the activity.



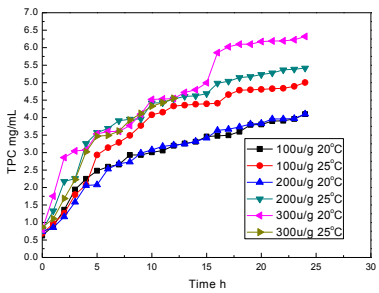




**Fig.1** the enzyme activity in unhairing bath **Fig.2** the TPC in the enzymatic unhairing bath

The amount of protein dissolved in the unhairing bath could give us the information of the trend of unhairing. According the enzyme unhairing processed, the amount of protein in the bath will be increased slowly (Fig. 2). It was shown that the higher the temperature and the more enzymes used, the more protein would be dissolved in the bath. Correspondent to this, we found when the temperature was set at 20 $^{o}$C degree, the duration for the unhairing with the dosage of enzyme at 100u/g, 200u/g and 300u/g respectively will be 19h, 16h and 16h, only 3h difference was. For 300u/g dosage of enzyme, when the temperature was set at 25 $^{o}$C degree, the unhairing duration will be 8h, which is much shorter than the time to 100u/g and 200u/g dosage of enzyme. It illustrated that unhairing at a lower temperature, increasing the dosage of enzyme could not increase the unhairing rate, while unhairing at a higher temperature, the dosage of enzyme could affect the unhairing rate remarkably.

Table 1 and Fig. 3 showed the hydrolyzed collagen content in the enzymatic unhaired bath. The results showed that the hydrolyzed collagen content in the unhairing bath at 20 $^{o}$C was much lower than at 38 $^{o}$C. When the unhairing temperature was set at 20 $^{o}$C , the collagen concentration is just about half of that at 38$^{o}$C. While the temperature was set at 25 $^{o}$C with the dosage of enzyme at 300U/g, the collagen concentration would reach 90% to that of at 38

°C, which revealed that the higher the temperature was and the higher the enzyme dosage used, the more collagen fiber would be hydrolyzed. The results clearly illustrated that enzyme unhairing at lower temperature could protect the collagen fiber from hydrolyzing by collagenous protease. It could help to reduce the risk of the finished leather from grain damage and looseness.

**Tab.1 collagen content in the enzymatic unhairing baths**

| Dosage of the enzyme（U/g） | 200 | 200 | 200 | 300 | 300 |
|---|---|---|---|---|---|
| Temperature（℃） | 38 | 20 | 25 | 20 | 25 |
| Float (%) | 150 | 150 | 150 | 150 | 150 |
| Collagen concentration（μg/mL） | 0.8983 | 0.4439 | 0.5720 | 0.4788 | 0.6257 |

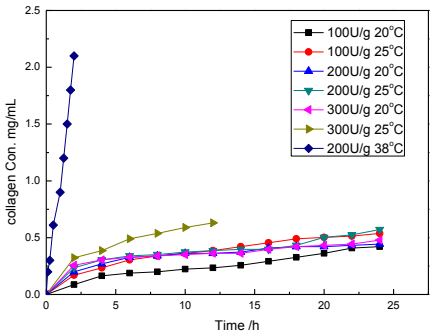



**Fig.3** hydrolyzed collagen in unhairing bath

## 3.2 physical properties of the enzymatic unhaired crust

After enzymatic unhaired, the pelt was chrome tanned and fatliquored. The physical properties of the dried crust were determined (Table 2). The results showed that the main physical properties, such as tensile strength, tear strength and elongation, of the enzymatic unhaired curst were can reach the requirement standard to the shoe upper leather.

**Table 2 Mechanical property test result**

| | Tensile strength（N/mm$^2$） | Tear strength（N/mm） | Elongation 10N（%） | Elongation at break（%） |
|---|---|---|---|---|
| A | 18.473 | 87.694 | 27.671 | 36.791 |
| B | 17.594 | 83.075 | 29.406 | 41.721 |
| C | 17.185 | 80.219 | 30.398 | 46.676 |
| D | 16.879 | 76.601 | 32.710 | 47.533 |
| E | 17.025 | 74.391 | 30.457 | 49.193 |
| F | 16.493 | 71.852 | 32.895 | 51.382 |

| | | | |
|---|---|---|---|
| **Shoe upper leather standard** | **≥15** | **≥35** | **≤55** |

### 3.3 Appearance of the grain surface by stereomicroscope

The unhaired cattle hide samples dyed to gray were investigated under stereomicroscope with 25 times amplification (Fig. 4). The samples unhaired with sodium sulfide showed very clear hair pores which could be a control to compare with the enzymatic unhaired samples. It was shown that the hair pores has been blurred and could not clearly be seen for the samples enzymatic unhaired at 38 $^{o}$C. While the enzymatic unhairing was carried out at 20 $^{o}$C with 100 to 300 U/g dosage of enzyme, the hair pores of the samples was similar to that of sodium sulfide unhaired. Unhairing at a lower temperature is benefit to prevent the grain from damage, although when the unhairing conditions was carried out at 25 $^{o}$C with 300U/g dosage enzyme has the trend to make the hair pores collapse, so that the enzymatic unhairing at a lower temperature could be thought as a much safer process.

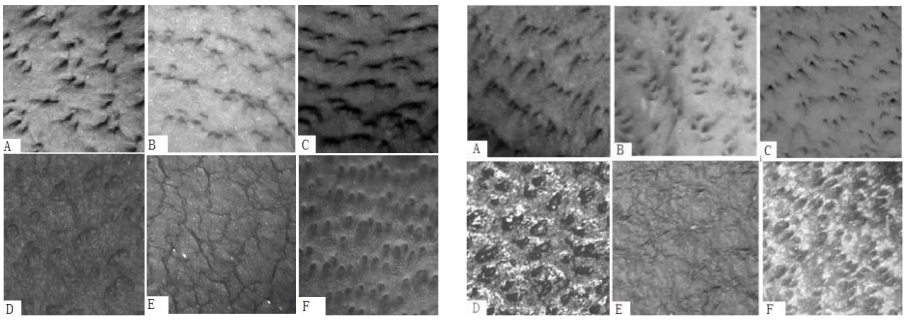



**Fig.4 Observations by stereomicroscope**

A: 200u/g, 20℃; B: 200u/g, 25℃;
C: 300u/g, 20℃; D: 300u/g, 25℃;
E: 200u/g, 38℃; F sodium sulfide unhairing

### 3.4 Histological investigation of the enzymatic unhairing

The hide or skin of an animal consists of epidermis and dermis, which is separated from each other by basement membrane (BM), which is a 50- to 100-nm layer of specialized extracellular matrix protein complex (Ito and Sato 1990). Stratum basal cells attached to BM by means of hemidesmosome. The stratum basal cells stretch out some protrusions. The other side of the BM is connected to the papillary layer, a fine and loosely collagen fiber networks. The hair was surrounded by the hair follicle which is known as a number of connected layers. Adhering to the BM are the root sheath's cells which become more keratinised and cornified as they move towards the hair shaft. Similar to the epidermal cells, these cells attached to each other by means of desmosomes.

The fine structures of cattle hide such as epidermis, hair follicle and BM were investigated during different unhairing methods (Fig.5). For sodium sulfide unhairing, the stratum basale cells of epidermis were hydrolyzed firstly, which led to a separation between the epidermis and the dermis. There were no apparent changes about the *lamina lucida* and *lamina densa* of BM during this process. For enzymatic unhairing, the hair root sheath's cells were destroyed firstly, but a few remnants of them were attached to the *lamina densa* last for certain time. This indicated that during enzymatic unhairing process，the separation between epidermis and dermis was due to the destroy of basal cells, but not the separation of stratum basale from BM.

The disruption to BM was different when the enzymatic process was carried out under different temperatures. The *lamina lucida* of BM was obseved indistinctly after 4h enzymatic treatment at 25℃, whlie the *lamina densa* was still intact after 8h. In contrast, while the hide was enzymatic unhaired at 38℃, both the *lamina lucida* and the *lamina densa* were destroyed after 2h. The disappearance of the BM may be the beginning of the grain damage.

Moreover, the fibril and microfiber morphologies were investigated during different unhairing processes (Fig. 6, Fig. 7). Fibrils in the papillary layer and the reticular layer showed little change during sodium sulfide unhairing. While fibrils in the papillary layer were split after enzymatic treatment at 25℃. The connections of fibrils in the reticular layer were weakened, which made the crystal structure of the fibril breakdown. This damage took place on the microfiber surface, and tended to run into gradually. There was no free fibril coming out from the fiber bundle during this process. After enzymatic unhairing at 38℃, fibrils in the papillary layer were split extremely, but the fiber dispersion degree in the reticular layer was less dispersed than that of the lower temperature enzymatic treatment. Several individual fibrils could be observed had been released from the microfiber.

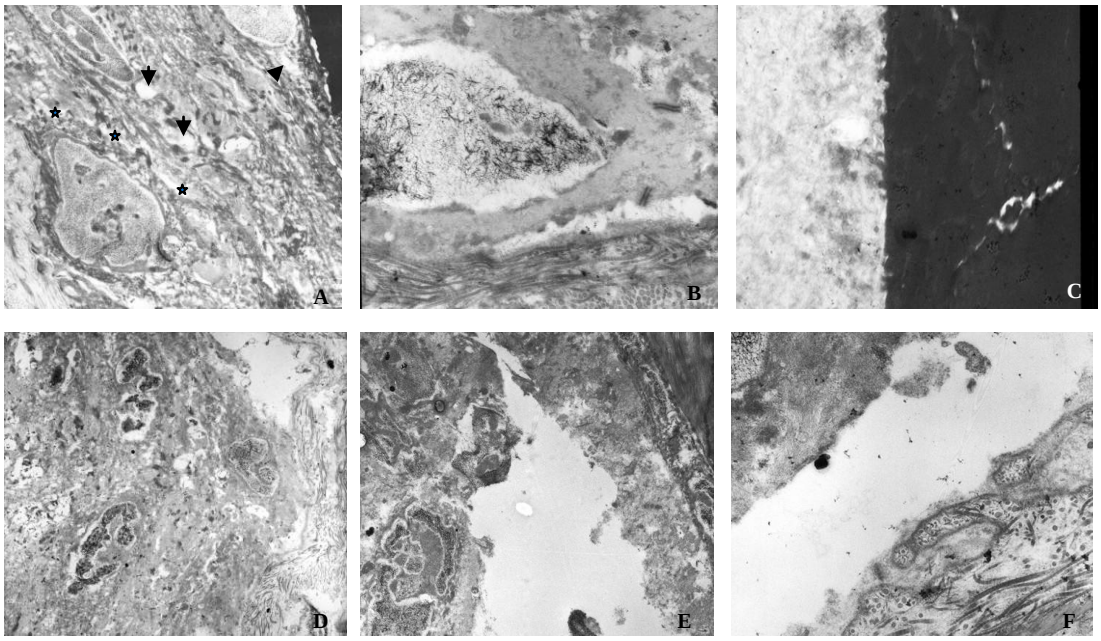



**Figure 5 the hair follicle structure of cattle hide during enzymatic unhairing at 20℃**

A: 2h×5,000；B: 4h×10,000；C: 6h×10,000; D: 6h×3,000；E: 8h×3,000；F: 8h×10,000

The histological structures of the unhaired cattle hide after liming and bating treatment were also observed. For sodium sulfide unhaired hide, the result showed that fiber dispersion degree was lowest and fibrils were not destroyed after enzymatic bating. In contrast, for enzymatic unhaired samples, the fibrils were split homogeneously and intensely after enzymatic bating. The results suggest that the enzymatic unhaired hide with enzymatic bating would lead to excessive dispersion to the fibrils, which may the cause of the grain looseness of enzymatic unhaired leather. Therefore, bating process should be cancelled for enzymatic unhaired pelt.

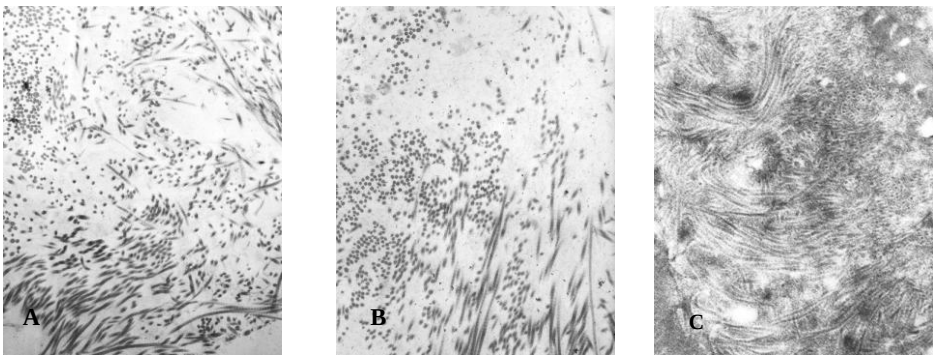



**Figure 6 the papillary layer structure of cattle hide by different unhairing**

A: enzymatic unhaired at 20 degree for 24h×8,000，

B: enzymatic unhaired at 38 dgree for 5h×10,000，

C: sodium sulfide unhaired for 1.5h×12,000

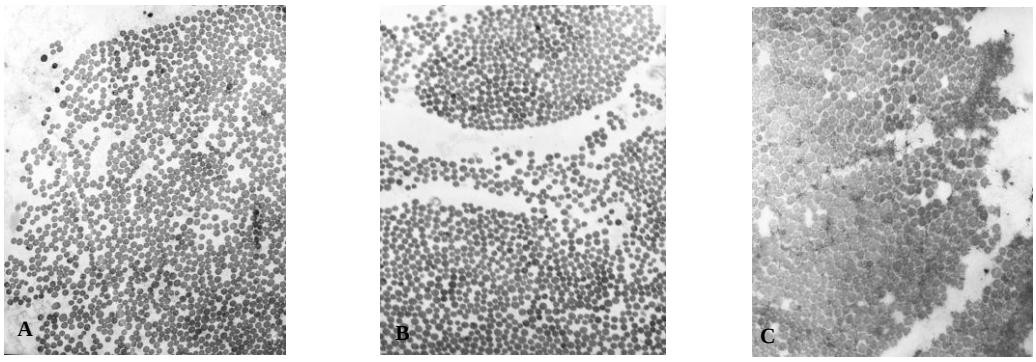



**Figure 7 the reticule structure of cattle hide by different unhairing**

A: enzymatic unhaired at 20 degree for 24h×10,000，

B: enzymatic unhaired at 38 dgree for 5h×10,000，

C: sodium sulfide unhaired for 1.5h×12,000

## 4. Conclusions

We can conclude that cattle hide unhaired by enzyme at 20℃ to 25℃ is much safer than

at higher temperatures. The unhairing process could be processed with the conditions of liquor ratio 0.5~1.5, protease 100~300 U/g of hide, pH 7.5~9.0, supplemented by intermittent rotation, the unhairing step could be finished in 8~19h. During the process the enzyme activity loss was less than 60%. The hydrolyzed collagen in unhaired bath was lower than that of enzyme unhairing at 38℃. The physical properties of curst enzymatic unhaired at lower temperature were can reach the requirement standard to the shoe upper leather.

Histology detection displayed that the unhaired grain was intact, and its pore shape was clear, with no grain looseness and grain damage. During enzymatic unhairing process，The TEM observation showed the separation between epidermis and dermis was due to the destroy of basal cells, but not the separation of stratum basale from BM. The disruption to BM was different when the enzymatic process was carried out under different temperatures. The *lamina lucida* of BM was obseved indistinctly after 4h enzymatic treatment at 25℃, whlie the *lamina densa* was still intact after 8h. while the hide was enzymatic unhaired at 38℃, both the *lamina lucida* and the *lamina densa* were destroyed after 2h. The disappearance of the BM may be the beginning of the grain damage.

## 5. Acknowledgements

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