Interaction of Enzymes and Hide/Leather Based on Microwave Use

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Abstract

With the characteristics of high efficiency, specificity and mild reaction conditions, enzymes are able to catalyse many biochemical reactions. Hence, they are widely used in the soaking, unhairing, degreasing and bating processes in the leather industry. At present, there are a lot of studies on the interaction laws between protease and collagen under conventional water bath heating; however, under microwave irradiation with both thermal and non-thermal effects, corresponding research has not yet been reported. In this work, enzymatic unhairing (EU), bating (EB) and wet-blue leather treating (ET) processes were investigated under microwave irradiation heating compared with water bath heating. Hydroxyproline concentration and chrome content were analysed by ultraviolet-visible absorption spectroscopy. The grain of the sample was observed with a microscope. Thermal performance and physical and mechanical properties of the collagen composites was characterised by the shrinkage temperature, softness, tensile and tear strength, respectively. The results showed that microwave could increase EU and EB rates, EU was completed 2 hours earlier and the end of EB was 1 hour in advance. After microwave irradiation, the grain of the skin or leather had better flatness and softness. It also promoted enzymatic proteolysis, in which the hydroxyproline concentration in EB and ET was decreased by 0.26 mg/L and 0.12mg/L, respectively. In addition, microwave use lowered the removal of chromium in ET, and the chrome content was reduced by 11.96mg/L. Quite a long exposure to microwave irradiation would inactivate enzymes, slightly weakening the tensile and tear strength of the leather. The results indicated that microwave treatment could promote the leather manufacturing process and improve the performance of collagen composites, which may lay a foundation for the application of microwaves in the leather industry.

摘要：由于具有高效、专一、反应条件温和等特点，酶能催化许多生物化学反应。因此，酶被广泛用在制革的浸水、脱毛、脱脂、软化等过程。在常规水浴加热条件下，对制革用蛋白酶与胶原蛋白的作用规律已经研究得很多，但是目前在微波这种同时具有热效应和非热效应的热源条件下，对制革用蛋白酶与胶原蛋白作用的研究还未见报道。本文通过与水浴加热对比，研究了微波辐照下的酶脱毛、酶软化以及蓝湿革酶处理过程中酶与胶原蛋白的相互作用规律。通过紫外-可见吸收光谱分析了废液的羟脯氨酸浓度以及铬含量。同时分别采用收缩温度，柔软度，抗张强度与撕裂强度表征了微波辐照皮胶原材料的热稳定性以及物理机械性能。结果表明：微波辐照能提高酶脱毛和酶软化的速率，酶脱毛过程提前2h完成，酶软化终点提前1h；微波辐照后的皮或革，粒面的平细性和皮的柔软度更好；微波辐照能促进蛋白的酶解，表现为酶软化和蓝湿革酶处理废液中羟脯氨酸浓度分别降低了0.26 mg/L和0.12mg/L；蓝湿革酶处理时，微波辐照能减少脱铬现象的发生,废液中铬含量降低了11.96mg/L。较长时间的微波辐照酶会失活，使得蓝湿革的抗张撕裂强度有少许降低。这些结果证明了微波辐照可以促进制革过程并改善皮胶原复合材料的物理机械性能，将为微波在制革中的应用提供参考。

1 INTRODUCTION

Development of microwave use stems from the radar technology in the 1930s. Following on from that, it is now widely used in food, communications, medical, remote sensing, non-destructive testing, energy and in the biochemistry field because of its mild, environmentally friendly and efficient features.[1] In the leather industry, microwaves have been used in sludge treatment,[2] digestion,[3] drying,[4] tanning[5] and dyeing.[6] In these processes, scientists found that microwave use, not only shows thermal effects, but also presents some non-thermal effects. The results have fully demonstrated that the use of microwaves to accelerate or reform a manufacturing process has become a potential innovation of leather technology.

Enzymes play an important role in the leather industry, dating back to the 1910 unhairing method using trypsin.[7] Uses of enzymes in the processes of soaking, unhairing, degreasing, bating and so on is becoming increasingly widespread, mainly because it helps the depilation and fleshing of raw hide and removal of excess intradermal components so that, the cortical fibres can be properly loosened, providing the basic conditions for the combination of hides with chemicals. Furthermore, enzymes will not undermine the organisational structure of the hide surface, which is the most satisfactory for leather chemists. Generally, the enzymes commonly used for leather-making include alkaline, neutral and acid proteases. Owing to the effectiveness of depilation with minimal collagen damage, an alkaline protease is usually served for

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unhairing, and there are even a few products used for soaking and liming.[8,9,10] Neutral proteases are also applied in the soaking, unhairing and liming. Liu *et al.*[11] found that a neutral protease produced by *Bacillus subtilis* could be used in unhairing which was as effective as alkali depilation and did not affect leather quality. Acid proteases in the tanning industry, due to their good acid resistance, are used more in the bating of pickled skin and wet-blue leather. Some chemists[12,13] have done a comparatively systematic study about the impact of acid protease on wet-blue leather bating, and the results indicated that an acid protease could effectively disperse collagen fibre and reduce skin location differences. Therefore, acid protease bating has become an important process for wet-blue leather processing at present. In addition, Kanth *et al.*[14] applied an acid protease to the pretreatment of dyeing, and the results showed that the enzyme-treated leather had an increased dye-uptake and absorption.

It seems that these studies were all carried out with conventional water bath heating and corresponding research on the condition of microwave irradiation has not yet been reported. Due to the polarity of both enzymes and collagen, their interactions may change to some extent with the influence of a microwave field. These changes are perhaps able to help to improve the macroscopic mechanical properties of leather and further promote the development of the leather industry. In this study, microwave power was applied to enzymatic unhairing, bating and wet-blue leather treating processes in order to investigate the interaction of collagen and enzymes, which may lay a foundation for the development of microwave technology in leather processing.

2. EXPERIMENTAL PROCEDURES

2.1 Materials

537 acid protease (ACP) was obtained from Shanghai New Enzyme Preparation Plant, China. 115 neutral protease (NEP) was produced by Novozyme, China. 2709 alkaline protease (ALP) was made by Jiangyin Aidun Dayton Biological Engineering Co. Ltd., China. Non-pickle chrome tanning agent (NPC) was obtained from Guangdong Shengfang Chemical Co. Ltd., China. All the other compounds used were analytical reagents, purchased from Chengdu Kelon Chemical Reagent Factory, China. Soaked and delimed goatskins and wet-blue goat leather were made in our laboratory according to a traditional process.

2.2 Enzymatic unhairing (EU) process

Two soaked goatskin pieces were sampled (13 x 7cm) along the backbone. One was used for the experiment with microwave irradiation heating (MIH, produced by a Xian Yuhui MCR-3 microwave chemistry reactor), the other acted as a control for water bath heating (WBH). The piece was depilated with 250 U/mL of ALP in a 400% float at 35°C for 10 minutes. Then ammonium sulfate (0.3%) was used to adjust the pH to 9-9.5. After 3 hours, hair removal condition was checked every 30 minutes. 3mL of the reaction solution was sampled per 30 minutes during the process, centrifuged for 10 minutes at 4000rpm, and 2mL of the supernatant was used to test the hydroxyproline concentration (HC) according to the literature.[15] The final depilated skin was tanned with NPC, dried and have observed the grain using a SZX12 Stereo Microscope (Olympus, Japan).

2.3 Enzymatic bating (EB) process

2.3.1 Preparation

Two delimed goatskin pieces were sampled (13 x 10cm) along the backbone. One test was performed using MIH for the experiment, and the other was used as a control when WBH was used. The piece was bated with 100U/mL of NEP in a 200% float at 37°C. And the sample was neutralised to pH7-8 with ammonium sulfate (0.5%). After 30 min, the bating condition was examined every 10 min. The final reaction solution (3mL) was sampled, centrifuged for 10 minutes at 4000rpm, and 2mL of the supernatant was used to test the HC (hydroxyproline content).

2.3.2 Microscopy of bated skin

Tissue staining microscopy is one of the more effective techniques for investigating the removal of elastic fibres. 2 x 2cm pieces of the delimed and bated skin were first fixed in 10% neutral formaldehyde solution (1000mL, v/v) containing sodium dihydrogen phosphate (4g) and disodium hydrogen phosphate (6.5g) for 36 hours. Next, the fixed piece was washed completely and sectioned (thickness, 15μm) using a CM-1950 freezing microtome (Leica, Germany); after this step the section was attached to the slide with protein glycerin, and dried for 72 hours. In the staining step, the section was placed in a solution containing basic fuchsin (2g), resorcinol (4g), ferric chloride solution (29%, 25mL), ethanol (95%) and distilled water (200mL) for 40 minutes, followed by differentiation with 1% acid alcohol containing ethanol (70%, 100mL) and concentrated hydrochloric acid (1mL), and immersion in 95% alcohol, pure alcohol and xylene, respectively (2 times each of the alcohol liquids and once for xylene, 2 minutes each time). The final stained piece was observed under the microscope.

2.3.3 Grain and softness of bated skin tanned with NPC

Bated samples were tanned with NPC, and dried in the air. The morphology of the leather grain was observed using the stereo microscope. The softness determination temperature was 20°C (65% relative humidity, RH), and the sample was measured with a GT-303 softness tester (GOTECH Testing Machines Inc., China). When the sample reached the weight of 500g using a standard ring of 20mm, the top height of the leather sample was recorded.

2.4. Enzymatic treatment (ET) of wet-blue goat leather

2.4.1 Preparation

Two wet-blue goat leather pieces were sampled (15 x 10cm) along the back line. One test was carried out under MIH for the experiment, and the other served as a control being treated by WBH. The piece was treated with 200U/mL of ACP in a 600% float at 40°C for 5 hours. Before this, the sample was neutralised to pH3.5 with formic acid (2M).

2.4.2 Analysis of enzymatic treatment effluent

HC of the effluent was tested as mentioned above. Spectrophotometry is a more accurate method to determine the chrome content. Because the reaction of Cr^{6+} with diphenyl carbamide produces a magenta complex, a strong absorbance appears at 540nm. In the digestion process, 2mL of concentrated nitric acid was added to the effluent (10mL), heated on a hotplate and cooled after the solution was concentrated to below 10mL. After this step, 2mL of concentrated sulfuric acid was added, heated until white smoke was produced and the solution became clear and colourless, it was then cooled to room temperature. The final digested solution was diluted to 100mL. In the oxidation process, 25mL of the digested effluent was treated with sulfuric acid (1:1, 0.2mL), phosphoric acid (1:1, 0.2mL) as well as potassium permanganate (0.5%, 1mL), boiled until the purple solution did not fade, and cooled. Next, urea solution (20%, 1mL) was added to the cooled solution, and again sodium nitrite (0.5%) was added dropwise until the red just faded away. Finally, to the solution was added, diphenylcarbazide acetone solution (2mL), made up to 50mL with distilled water and mixed evenly. The mixture was determined by a UV1900 spectrophotometer (Shanghai Jinghua Technology Instrument Co. Ltd., China) at the wavelength of 540 nm. The quantitative standard curve for chrome content was prepared according to the operation mentioned above. The content was calculated using Formula 1:

$$\text{Cr } (mg/L) = \frac{V_3 \text{ x } 0.01 \text{ x } 1000}{V_1 \text{ x } \frac{V_2}{100}} \qquad (1)$$

where V_1 is volume of the effluent (10mL), V_2 is volume of the digested effluent (25mL) and V_3 is volume of chrome in the standard curve (mL).

2.4.3 Analysis of physical and mechanical properties

The enzyme-treated leather grain was observed using the stereo microscope and differences of the grain and pores were compared between the experiment and control samples. To determine the shrinkage temperature (Ts), a 50 x 3mm leather piece was heated using a MSW-YD4 digital shrinkage temperature meter (Institute of Sunshine Electronics, Shaanxi University, China) in glycerin solution at 2°C/min rate. The measurement of leather softness was the same as in the operation mentioned above. The tensile and tear strength samples were conditioned at 20 ± 2°C (RH, 65 ± 2%). The 45 x 10mm leather piece was measured with a thickness gauge and an AI-7000S automatic tensile machine (GOTECH testing Machines Inc.), China was used for the tests. The tear strength was calculated using Formula 2:

$$T = \frac{F}{t} \qquad (2)$$

where T is tear strength (N/mm), F is tear force (N) and t is average thickness of sample (mm).

3 RESULTS AND DISCUSSION

3.1 Microwave effect on EU

It was found that the hair roots began to loosen after 3.5 hours unhairing under MIH, which was 1.5 hours earlier than for the control. After reacting for 6 hours, the hair removal in microwave sample was completely clean; however, a little hair remained on the skin surface and could not be taken off even after 8 hours of the reaction under WBH. It inferred that microwave-assisted enzymatic unhairing could promote enzyme hydrolysis of hair root membrane and surrounding tissue protein owing to the uniformity of microwave heating. Microwave treatment might have an influence on protein structure such as a sticky protein, so the enzyme can combine with it more easily.

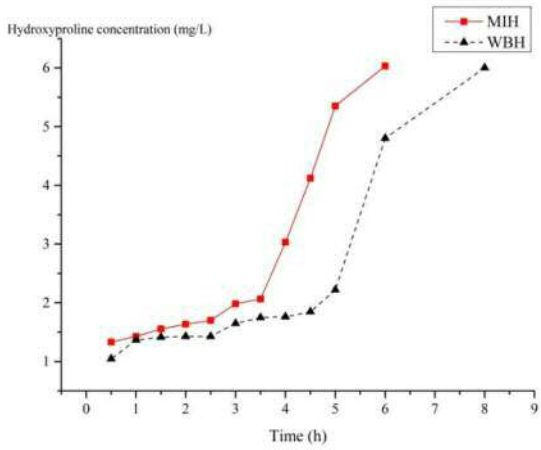

Figure 1. HC in the enzymatic unhairing process.

Figure 1 shows the HC in the enzymatic unhairing process. Apparently, all the HC levels of the samples are very low before the hair roots began to loosen (MIH, 3.5 hours; WBH, 5 hours), and exhibit a tendency to rise slowly. Once unhairing ocurred, the HC in the solution rapidly increased. With gradual penetration into the skin, enzymatic hydrolysis initially occurs between the epidermis and papillary layer and only a small amount of enzymes interact with collagen in the beginning of the process. When a large amount of enzyme is present in the skin, it can quickly react with collagen to produce hydroxyproline. However, the HC level is slightly higher than that of control, and enzymatic hydrolysis under microwave action is 1.5 hours ahead of time, indicating that microwave may

display a facilitating role in the penetration of enzymes into the skins.

Enzymatic unhaired skin was tanned with NPC, and observation of the leather grain was carried out. The results of the grain feature are shown in Figure 2. Grain and pores of the leather depilated under microwave is similar to the matching leather and both of them are quite clear, reflecting that microwave would not damage the grain and pores of the skin during the enzymatic unhairing.

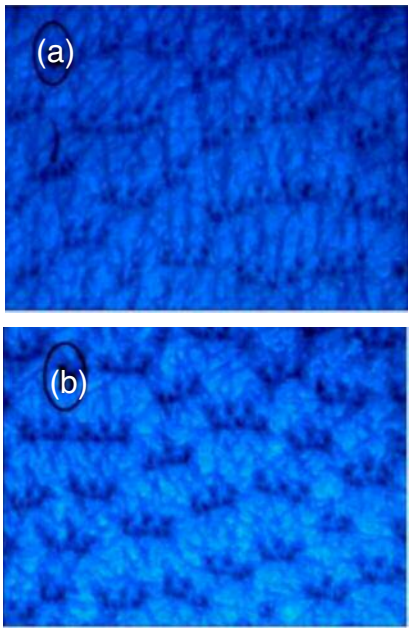

Figure 2. Grain of enzymatic depilated leather; (**a**, EU under WBH; **b**, EU under MIH).

3.2 Microwave effect on EB

When a silky grain and clear fingerprint of the skin were reached and remained unchanged for a long time this was taken as the endpoint for the enzymatic bating. As found from the experiment, the enzymatic bating time was 1 hour earlier than that of the control, indicating that microwave could improve the bating rate. HC in the solution was determined after the process was completed. Results showed that 3.83mg/L of HC was in the MIH group and 3.57mg/L in the WBH group. Combined with the difference of HC and bating time, microwave could not only shorten the enzymatic bating time, but also hydrolyse the substrate to a greater extent.

Tissue staining samples were observed under microscope with 50x magnification. As seen in Figure 3, before and after enzymatic bating, the structure and amount of elastic fibres varies greatly. The elastic fibres show a dendritic distribution, forming a dense web around the hair follicles before bating. However, after bating, the distribution of elastic fibres is obviously loosened and the number has been greatly reduced, which proved that enzymatic bating weakened the function of elastic fibres in the dermal tissue structure. Comparing b and c, there is a difference in the number of fibres. The skin bated under MIH has a lower value, pointing out that microwave was helpful for the enzyme to destroy the elastic fibres. Both results for the HC amount in the effluent and elastic fibres inferred that microwave has the ability to promote the hydrolysis of the protein.

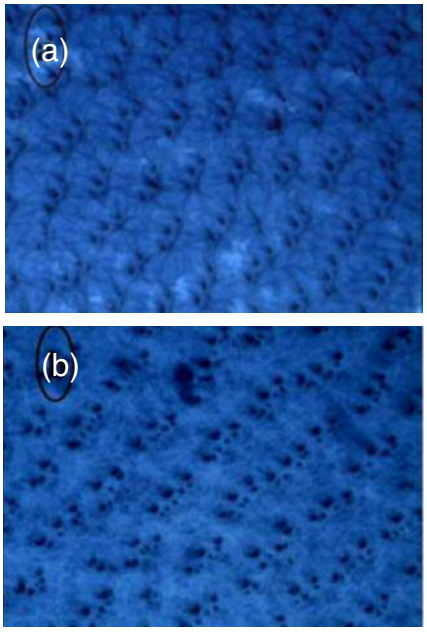

Figure 4. Grain of enzymatic bated leather (**a**, EB under WBH; **b**, EB under MIH).

After the bated skin was tanned with NPC, the grain and pores were relatively clear as exhibited in Figure 4. The purpose of EB is to improve the softness of the leather and the grain flatness. As microwave could promote the hydrolysis of protein, the flatness of the skin bated under MIH was obviously better than for the control. After the softness determination, the results showed that the top height of the leather bated under MIH was 3.62mm which was 0.4mm higher than the matched samples, due to the catalytic role of microwave on the hydrolysis of collagen.

Comprehensive comparison of EB under MIH and WBH shows that the bating rate increases and the bating effect becomes better under MIH due to microwave's homogeneous heating and proteolysis property. At the same time, it also reflects that microwave is more inclined to promote enzymatic hydrolysis of collagen rather than the inhibition of NEP activity.

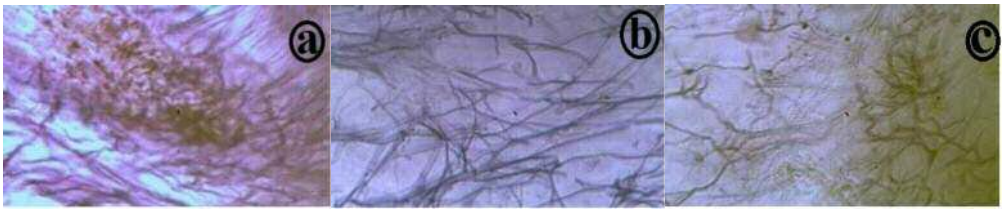

Figure 3. Tissue staining micrograph of elastic fibres (**a**, before EB; **b**, EB under WBH; **c**, EB under MIH)

3.3 Microwave effect on ET

As seen in Figure 5, both of the treated leather grains are clear and the pores have not been damaged, showing that ET is not likely to affect the leather grain. Compared with the untreated leather, the enzymatic one shows a flatter grain. However, the experimental sample has the best grain fineness among the samples. Thus, microwave might give an improvement to leather grain fineness.

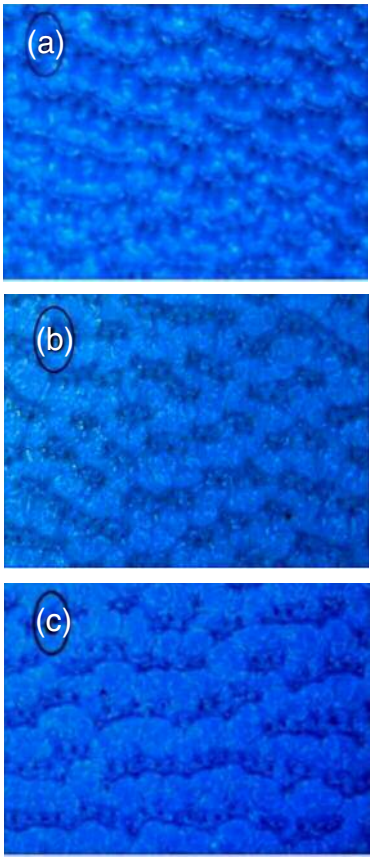

Figure 5. Grain of enzymatic treated wet-blue goat leather (**a**, before ET; **b**, ET under WBH; **c**, ET under MIH.

Chromium concentrations in the effluent were measured using ultraviolet-visible spectrophotometry, HC and Results showed that 0.89mg/L of HC and 10.00mg/L of chrome content were present in the MIH group and 1.01mg/L and 21.96mg/L in the WBH group. Therefore, in ET, microwave promoted the hydrolysis of collagen and reduced the occurrence of chromium removal. These may be because of the non-thermal effect of microwave, which causes part of the free and single-point combined chromium to be converted into multi-point fixed chrome and to form chrome complexes. Thus, the chromium content in the effluent was slightly lower in acidic condition under MIH.

After ET, Ts shows a reduction to varying degrees (Table I). This is due to the occurrence of chromium removal during the treatment, this removes some of the free chromium and the poorly bound chromium from the fibre. Ts is related to the chrome content in the leather. The higher the chrome content is, the higher the Ts will be. Compared with that of control, the Ts of the leather treated under MIH increased by about 2.5°C. The results are consistent with the previous study on the chrome content in the effluent and also demonstrate that microwave irradiation could effectively reduce chromium removal during ET in an acidic condition.

TABLE I
Physical and mechanical properties of the enzymatic treated wet-blue leather

	Before ET	ET under WBH	ET under MIH
Ts (°C)	112.95	107.90	110.45
Softness (mm)	3.22	3.30	3.40
Tensile process			
Thickness (mm)	1.16	1.05	0.99
Tensile strength (MPa)	19.30	24.65	21.30
Elongation at break (%)	48.60	44.20	44.10
Maximum strength (N)	223.70	264.00	211.25
Tear process			
Thickness (mm)	1.08	0.87	0.96
Maximum strength (N)	34.65	41.35	39.80
Tear strength (MPa)	32.00	47.25	41.35

Wet-blue leather, after treating it with ACP, shows that the softness has been slightly increased; however, the MIH group shows a higher value. Further, tear and tensile strength are likewise a little higher after ET as exhibited in Table I. The results show that the degree of interlacing of collagen fibre bundles increased after ET. Because it had a loosening effect on collagen fibres, the homogeneity of the leather was improved, which avoided the phenomenon of stress concentration in a certain cross section and enhanced the mechanical properties. It is found that the increase of the leather's mechanical strength in the MIH group is less than that of the control, indicating that ACP still has the loosening effect on the fibres when the microwave is applied to the heterogeneous system of ACP and collagen, but the loss of enzyme activity becomes even more pronounced due to the lengthy microwave irradiation.

CONCLUSIONS

In this work, the microwave effect on the heterogeneous system of enzymes and collagen was evaluated by using microwave in enzymatic unhairing, bating and wet-blue leather processing in comparison with water bath heating. The results presented here showed that microwave use could improve enzymatic unhairing and the bating rate and have good unhairing and bating effects. Moreover, it could increase the grain fineness and softness as well as the physical and mechanical properties of enzymatic treated leather. In addition, the occurrence of chromium removal was reduced effectively during the enzymatic treatment of the wet-blue leather in acidic conditions under microwave use. Therefore, microwave has the potential to be used in leather manufacturing process in the future.

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