

Removal of Proteoglycans in Animal Hides by Glycosidase to Promote Opening-Up of Collagen Fiber Bundles: Optimization of Glycosidase and its Application in the Soaking Process

by

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Abstract

The degree of proteoglycan removal from animal hides is closely related to the opening-up of collagen fibers and the properties of leather, but conventional leather-making processes are not very effective at removing proteoglycans. By rationally selecting and utilizing glycosidases during the leather-making process, proteoglycans can be efficiently removed without causing collagen damage due to glycosidases specifically hydrolyzing glycosidic bonds. In spite of this, glycosidases are highly specific and come in a wide variety of types. To solve the blindness of optimizing glycosidases and its application in the soaking process, in this study, seven typical commercial glycosidases capable of hydrolyzing β -glycosidic bonds were selected according to the types of major glycosidic bonds in animal hide proteoglycans. The basic properties of the selected glycosidases and its effectiveness in removing proteoglycans from the hide during the soaking process were investigated. Then, the effects of the dosage of the two optimal glycosidases on the dissolution of polysaccharide, total soluble protein and hydroxyproline, as well as the properties of leather during the soaking process were further studied. The results showed that although the optimal pH of the selected glycosidases was between pH 5 - 7, they maintained more than 50% of their activity under weak alkaline conditions and could be applied to the soaking process. The removal of proteoglycan by different glycosidases is closely related to their specificity for glycosidic bonds, and the penetrability of the enzyme proteins, namely the molecular weight and isoelectric point of the enzymes. Among them, β -Mannase-AKT and Xylanase-AKT are more effective in removing proteoglycans. When the dosage of β -Mannase-AKT and Xylanase-AKT reached 47 U/mL, almost all the glycosaminoglycan was removed from the hide, the opening-up of collagen fibers and the softness and physical properties of the crust leather were significantly improved without damage to the structural proteins.

Introduction

Proteoglycans (PG) are primarily found between the epidermis, hair follicles and collagen fibers. Although its content is not high,

they play a significant role in the growth of animals. Once the raw hides lose moisture, glycoconjugates will adhere the collagen fibers together, negatively affecting the penetration of water and other chemical materials into the hides, as well as the opening-up of collagen fibers and the softness of the leather.¹⁻³ Therefore, proteoglycans should be removed as early as possible in the leather-making process. Since proteoglycans are chemically stable, only half of them can be removed in strongly liming processes.⁴ Although the application of proteases during the soaking process can effectively remove inter-fibrillary substances, it is important to realize that the use of proteases may lead to the destruction of structural proteins in the leather, resulting in loose and damaged grain.⁵

Glycosidase can specifically hydrolyze glycosidic bonds and the sugar chain, thus removing the proteoglycan from the hides. Studies showed that the use of glycosidases, such as galactosidase, amylase, glucosidase and cellulase, in the soaking, enzymatic dehairing and bating processes is beneficial for the removal of proteoglycan and the improvement of leather properties.^{6,7} In addition, glycosidases do not hydrolyze collagen and can be properly selected and used in the leather-making process to achieve efficient removal of proteoglycans without causing damage to collagen.^{8,9} Therefore, glycosidases have great application potential in the leather making process.

There are a wide variety of glycosidase enzymes, each of which has a specific substrate specificity. The cleavage sites and action mechanisms to proteoglycan chains of different glycosidases differ considerably, and their optimal conditions of use also vary considerably. Additionally, animal hides are primarily composed of collagen fibers, which are formed in a three-dimensional network. Consequently, there is some mass transfer resistance to the penetration of enzyme molecules into the hide, which is influenced by the molecular size and charge state of the enzyme molecules. Therefore, the removal effect of glycosidase on proteoglycan depends on the specificity of the glycosidic bond, the molecular weight and the isoelectric point of the enzyme, as well as the conditions of application. Although there are some reports regarding the application of glycosidases in the leather-making process, however, there has been a lack of in-depth research and blindness in the

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optimization of glycosidases and the development of application technologies.

In this paper, seven typical commercial glycosidases were selected to hydrolyze animal hides β -glycosidic bonds according to the glycosidic bond specificity and the types of main PG glycosidic bonds in animal hide. The hydrolytic properties of the selected glycosidases were investigated by using proteoglycan-containing hide powder as substrate, and the molecular weight and isoelectric point of the enzyme molecule were also determined. Based on this, proteoglycan removal effects of different glycosidases in the soaking process were compared, the optimal enzymes were screened out and the relationship between the dosage of enzyme and the degree of proteoglycan removal and leather properties were investigated. This study attempts to provide methods and a basis for the development of glycosidase application technologies in the leather making processes.

Materials and Methods

Materials

Wet-salted cattle hides were purchased from the local market in Chengdu, China. Alison Blue, 1,6-dimethyl methylene blue and bovine serum protein was purchased from Sigma, USA. Proteoglycan-containing hide powder was made by our laboratory.¹⁰ Glycosidases were all commercial grade and supplied by Qactive Bio-products Co. Ltd. and Longda Bio-products Co. Ltd, China. Leather chemicals were all industrial grade and supplied by Sichuan Dowell Science and Technology Co., Ltd, China. Other analytical grade reagents were purchased from local suppliers in Chengdu, China. The weight of leather making chemicals used for soaking was based on the weight of the wet-salted hide.

Assay of glycosidase activity

The activity of glycosidases was determined through our laboratory-established polysaccharide-containing hide powder substrate method.¹⁰ In detail, 2.000 g \pm 0.001 g of hide powder was accurately weighed and added to a 150 mL conical flask. 40 mL of Britton-Robinson buffer (0.1 mol/L, pH 7.5) was added and stirred at 30°C for 2 h. Then, 2 mL of enzyme solution was added to the experimental group and stirred at 30°C for 2 h, relatively, 2 mL of hyperthermia (100°C) inactivated enzyme solution was added to the control group under the same conditions. The reaction was terminated by adding 2 mL of sulfuric acid (6 mol/L) and the mixture was filtered with a qualitative filter paper. The concentration of total sugar in the filtrate was determined by the phenol sulfate method¹¹ and the total sugar content in the reaction liquor was calculated.

One unit of glycosidase activity is defined as the amount of enzyme required to produce 1 μ g of glucose per hour for the hydrolysis of polysaccharides in hide powder at specific conditions.

Application of glycosidase in the soaking process

After pre-soaking and fleshing according to the conventional method, wet-salted cattle hides were symmetrically sampled and weighed. The soaking process was carried out in 100 % (w/w) of water at 25°C, a certain amount of glycosidase was added to the experimental groups and 0.2% of soaking auxiliary SWA was added to the control group. The drum was rotated continuously for 1 h at the beginning of the soaking; then, rotated for 30 min and stopped for 30 min 2 times; then, rotated for 10 min and stopped for 50 min 2 times; finally, soaked for 13 h overnight at room temperature. After 30 min of rotation, the produced amount of soluble protein, total sugar, hydroxyproline and glycosaminoglycan at the end of the soaking process were measured, respectively. The residual sugar in the hide was observed after being adjacently and symmetrically sampled from the hides and stained by the periodate-Schiff-Alixin blue staining method.

Soaked samples were treated according to the conventional leather making procedures. The softness, physical properties and organoleptic properties of the crust leather were evaluated as per the standard methods.

Analysis of soluble protein content in the soaking liquors

The soaking liquors were centrifuged at 3500 r/min for 10 min and the content of soluble protein in the supernatant of the soaking liquor was measured by Lowry's method.¹² The absorbance of the colored mixture was determined at 680 nm to measure the concentration of soluble protein using bovine serum protein as the standard substrate.

Analysis of total sugar content in the soaking liquors

The content of total sugar in the supernatant of the soaking liquor was determined by the sulfuric acid-phenol method.¹¹ In detail, 1 mL of the supernatant and 0.5 mL of phenol solution (6 %) were mixed in a test tube; then, 2.5 mL of concentrated sulfuric acid was added and reacted at 90°C for 30 min; finally, the mixture was cooled in cold water for 3 min and the absorbance of the mixture was measured at 490 nm. The content of total sugar was calculated using glucose as the standard substrate.

Analysis of glycosaminoglycan content in the soaking liquors

The content of glycosaminoglycan (GAG) in the supernatant of the soaking liquor was determined by the DMB method.¹³ In detail, 100 μ L of the supernatant and 2.5 mL of DMB reagent were mixed in a colorimetric tube and the absorbance was measured at 525 nm, immediately. The content of GAG was calculated using chondroitin sulfate as the standard substrate.

Analysis of hydroxyproline content in the soaking liquors

The content of hydroxyproline (Hypro) in the supernatant was determined by the Chloramine-T oxidation method.^{14,15} The

absorbance of the colored mixture was determined at 560 nm and the content of Hypro was calculated using analytical grade Hypro as the standard substrate.

Histological staining of polysaccharides

Samples were fixed in a neutral formaldehyde solution (10%, v/v) for 24 h. Sections of 12 μm thickness were obtained using a CM1950 freezing microtome (Leica, Germany) and stained according to the Periodic acid-Schiff-Alcine blue method.¹⁶ In detail, sections were stained in Alcine Blue staining solution for 40 min, followed by thorough washing with deionized water. Then, samples were oxidized in a periodic acid solution for 8 min and washed with deionized water and stained with Schiff's staining solution for 8 minutes. Next, the sections were dehydrated by 70% alcohol, 95% alcohol, 100% alcohol, xylene-ethanol solution (1:1), and pure xylene for 3 min each, respectively, and sealed with a neutral resin. The residue of polysaccharides was observed with a Nexcope NE900 optical microscope (Ningbo Yongxin Optics Co. Ltd., China) and the stained polysaccharide was in red.

Molecular weight and isoelectric point of the glycosidases

The molecular weight (Mr) and isoelectric point (pI) of the selected glycosidases were determined through SDS-PAGE and IEF-PAGE methods, respectively, and stained by the Coomassie Brilliant Blue method.^{17,18}

Stereoscopic microscope and scanning electron microscope analysis of the crust leather

The grain surface and reticular layer fibers of the crust leather were observed using a stereoscopic microscope and a scanning electron microscope (SEM), respectively.

Pore diameter of the crust leather

The pore diameter of the crust leather was determined using a Gemini VII 2390 Surface Area Analyzer (Micrometrics Instrument Corp., USA) after cutting the samples into approximately 0.125 cm³ pieces.

Physical properties of the crust leather

Crust leather was sampled in the adjacent and symmetrical parts and conditioned as per the IUP method (IUP 2, 2000). The physical properties such as tear strength, tensile strength and bursting strength were tested as per the standard procedures (IUP 8, 2000; IUP 6, 2000; IUP 9, 2000). The softness of the crust leather was tested using a GT-303 Leather Softness Tester (Gotech Testing Machines Inc., China).

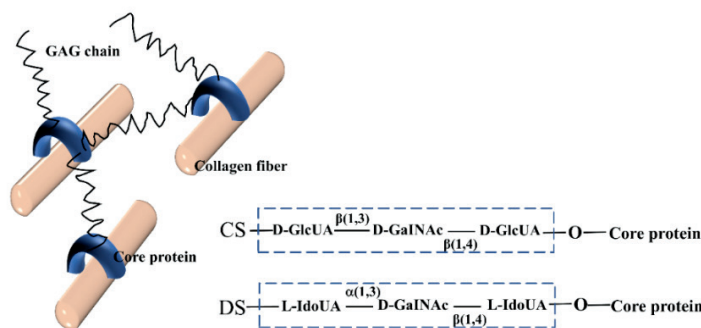


Figure 1. Schematic diagram of proteoglycan structure in cattle hide³⁰

Results and Discussion

Performance of glycosidase on proteoglycan

There are various types of glycosidases and the substrate specificity is very strict. Therefore, the substrates for different types of glycosidase activity assessment are different. For example, starch, xylan, and barley dextran are usually used as the substrates for amylase, xylanase, and dextranase activity assessment, respectively.¹⁹⁻²¹ However, the glycosidic bonds in these substrates are different than that of the proteoglycans in cattle hide. Enzyme activities measured by these methods can not reflect their actual effects on the glycosidic bond in animal hide proteoglycan (PG). The proteoglycan in cattle hide is diverse and structurally complex. PG consists of a core protein chain and one or more glycosaminoglycan (GAG) chains. The GAG chain of cattle hides PG is mainly composed of disaccharide structural units of chondroitin sulfate (CS) and dermatan sulfate (DS), namely the glycosyl groups are mainly connected by β -glycosidic bonds, such as β -1,3 glycosidic bonds and β -1,4 glycosidic bonds (as is shown in Figure 1).²²

Based on the above analysis, several commercial glycosidases (including β -Mannase-AKT, Cellulase-AKT, Hyaluronate lyase-AKT, β -Glucosidase-AKT, Xylanase-AKT, Xylanase-LKT and β -Glucanase-AKT) capable of hydrolyzing β -glycosidic bonds were selected based on the types of major glycosidic bonds in cattle hide PG (Table I). The hydrolysis effect on the PG glycosidic chain was investigated by using a glycosidase activity assessment method, which was established by our laboratory using PG-containing hide powder as the substrate.¹⁰

Basic properties of typical glycosidases

Effect of pH on the activity of glycosidases

Most of the optimum pH of glycosidases is acidic, and the pH of the main treatment in the beamhouse of leather making is in an alkaline environment, except for the pickling process. Therefore, the variation of several glycosidases' activity with pH was investigated in the pH range of 4 - 9 using the hide powder substrate method, and the results are shown in Figure 2.

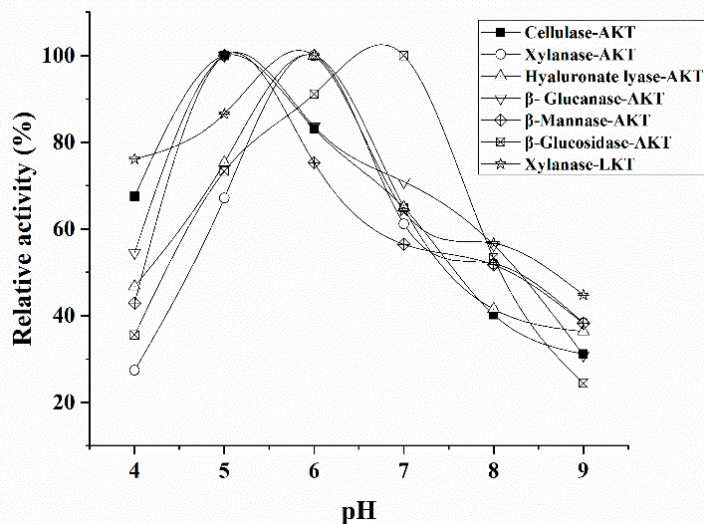


Figure 2. Effect of pH on the glycosidase activity (30°C)

The result in Figure 2 shows that, for hydrolyzing proteoglycans in the hide powder substrate, the optimum pH of β -Mannase-AKT, β -Glucanase and Cellulase-AKT is around 5.0, the optimum pH of Xylanase-LKT, Hyaluronate lyase-AKT, and Xylanase-AKT is around 6.0, β -Glucosidase has the highest activity at pH 7.0. Although the optimum pH of the selected glycosidases is acidic (pH 5 - 7), most of the enzymes still maintain more than 50 % of activity in the pH range of 7 - 8. Therefore, they have the potential to be applied in weakly alkaline processes, such as soaking and bating.

Effect of temperature on the activity of glycosidases

Collagen in rawhide is sensitive to heat, and high temperature can easily cause collagen denaturation. Therefore, the treatment temperature of the beamhouse generally does not exceed 40°C. The variation of the selected glycosidase activity with temperature was

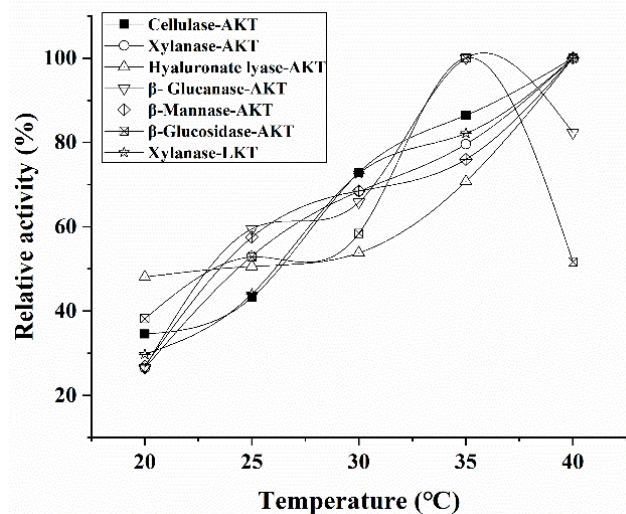


Figure 3. Effect of temperature on the glycosidase activity (pH 7.5)

investigated in the range of 20°C - 40°C, and the results are shown in Figure 3.

As is shown in Figure 3, the thermal resistance of β -Glucanase-AKT and β -Glucosidase-AKT was poor, and their optimum temperature was 35°C, which decreased sharply when the temperature was increased further. The activity of other glycosidases was increased with the increase of temperature in 25°C - 40°C. Remarkably, Hyaluronate lyase-AKT shows little activity change from 20°C to 30°C. The activity of glycosidases changes greatly with temperature, so its dosage should be adjusted according to the temperature of different leather making processes. For example, the dosage of enzyme used in the soaking process at a lower temperature (25°C) should be higher than that of bating process at a higher temperature (35°C).

Table I
Molecular weight, isoelectric point and activity of glycosidases

Glycosidase	Mr (kDa)			pI		Activity* (U/g)
	M ₁	M ₂	M ₃	P ₁	P ₂	
β -Mannase-AKT	40.9	-	-	4.40	-	5546 \pm 57
Cellulase-AKT	64.3	56.1	45.2	4.85	7.50	2888 \pm 30
Hyaluronate lyase-AKT	80.4	-	-	4.60	-	2788 \pm 35
β -Glucosidase-AKT	123.3	-	-	4.85	-	2599 \pm 28
Xylanase-AKT	11.3	-	-	6.72	-	2580 \pm 33
Xylanase-LKT	70	60.5	17.7	4.80	8.30	2484 \pm 16
β -Glucanase-AKT	25.7	-	-	4.55	-	2253 \pm 23

*30°C, pH 7.5

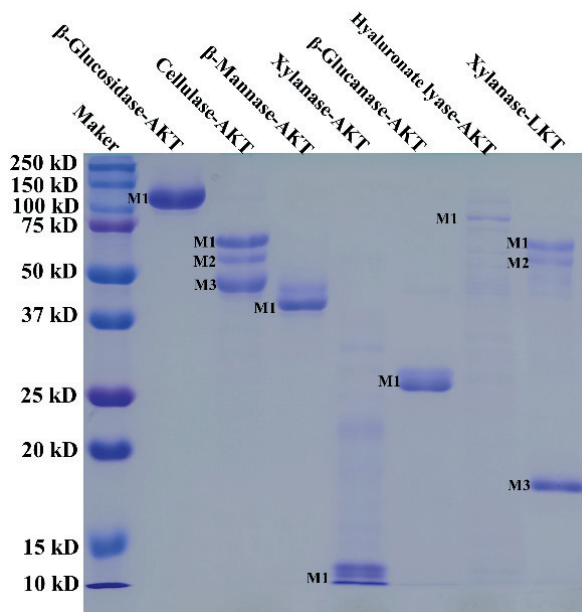


Figure 4. SDS-PAGE electropherogram of glycosidases

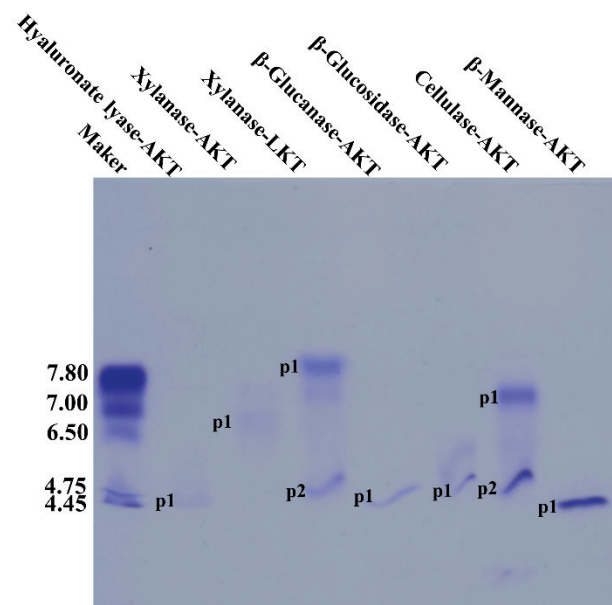


Figure 5. IEF-PAGE electropherogram of glycosidases

Usually, the soaking temperature is below 30°C and the pH is in the range of 8 - 10, however, the optimum pH of the selected glycosidases is close to acidic. Considering the effective utilization of enzyme activity, the glycosidase-based soaking stage should be controlled at pH 7 - 8 and 30°C. Table I shows that β -Mannase-AKT possesses of highest enzyme activity to proteoglycan, and other glycosidases show similar activity.

Molecular weight, isoelectric point and activity properties of the selected glycosidases

The penetration of glycosidase protein molecules into the hides is very important during the treatment process. The penetration property of enzymes mainly depends on their molecular weight and charge state. Therefore, the molecular weight (Mr) and isoelectric point (pI) of the selected glycosidases were analyzed using SDS-PAGE and IEF-PAGE methods, and the results are shown in Figure 4, Figure 5 and Table I, respectively.

As is shown in Figure 4 and Table I, except for Cellulase-AKT and Xylanase-LKT, most of the glycosidases contain only one enzyme protein component. The Mr of these glycosidases is different. The Mr of β -Glucosidase (123.3 kDa) is the largest, and Xylanase-AKT has the smallest Mr (11.3 kDa). The Mr of β -Mannase-AKT, β -Glucanase-

AKT and Hyaluronate lyase-AKT are 40.9 kDa, 25.7 kDa and 80.4 kDa, respectively. Cellulase-AKT contains three different protein components in the range of 45.2 - 64.3 kDa. Xylanase-LKT contains three different protein components with the Mr of 70.0 kDa, 60.5 kDa and 17.7 kDa, it's worth noting that the smallest component (17.7 kDa) doesn't have activity.

As is shown in Figure 5 and Table I, Xylanase-LKT and Cellulase-AKT contain two different pI components. The pI of Xylanase-LKT is 8.3 and 4.8, and the pI of Cellulase-AKT is 7.5 and 4.85. The other five glycosidases only have one protein component with a pI in the range of 4 - 5, except Xylanase-AKT has a relatively higher pI of 6.72.

The penetration of enzyme molecules into cattle hide is closely related to its molecular size. Among the selected glycosidases, β -Glucosidase-AKT has a larger molecule weight and may have an obvious penetration barrier. Furthermore, the penetration of enzyme molecules is also influenced by the charge state of hide collagen and enzyme proteins. When it takes unlike charge at a certain pH value, a strong bond on the surface of hides between collagen fiber and enzyme may affect the penetration of enzyme proteins.

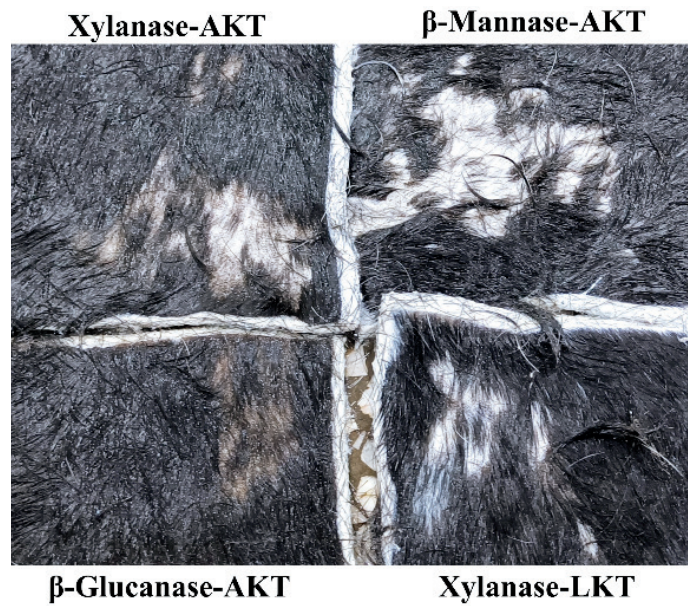


Figure 6. Residue of the epidermis after the soaking process

Comparison of the proteoglycan removal effect by different glycosidases

Effect of glycosidase on the epidermis

Seven kinds of glycosidases were applied in the soaking process with the same dosage of enzymes (0.6%, w/w), and the removal effect of the epidermis at the end of soaking is shown in Figure 6.

The results showed that the hair root of β -Mannase-AKT, Xylanase-AKT and Xylanase-LKT treated hide were loosed and easily pulled out by hand. Most of the epidermis was removed, especially for the β -Mannase-AKT treated sample. However, the hair and epidermis were still tightly bound after being treated with β -Glucanase-AKT, Cellulase-AKT, Hyaluronate lyase-AKT and β -Glucosidase-AKT.

The inner layer of the hair follicle is the inner root sheath, which is formed by the depression of keratinized epidermis cells; Its outer layer is the outer root sheath, which consisted of the basal layer

of the epidermis; The outermost layer is connective tissue sheath; Hair bulbs and hair papilla are bonded mainly by proteoglycan.³¹ The epidermis, hair papillae, hair root sheath and connective tissue sheath contain a large number of proteoglycans, and the action of glycosidase on proteoglycans leads to the destruction of the epidermis and the loosening of the hair root. Due to the high substrate specificity of glycosidase, different glycosidases have different hydrolysis properties to the glycosidic bond of the specific proteoglycan, GAG. The above experimental results show that β -Mannase-AKT, Xylanases-AKT and Xylanases-LKT have stronger proteoglycans removal effects on the epidermis and hair follicle.

Produced amount of total sugars and glycosaminoglycans in soaking liquors

At the end of the soaking, the produced amount of total sugar in the soaking liquor was used to characterize the removal effect of polysaccharides by glycosidase. The content of total sugar and GAG

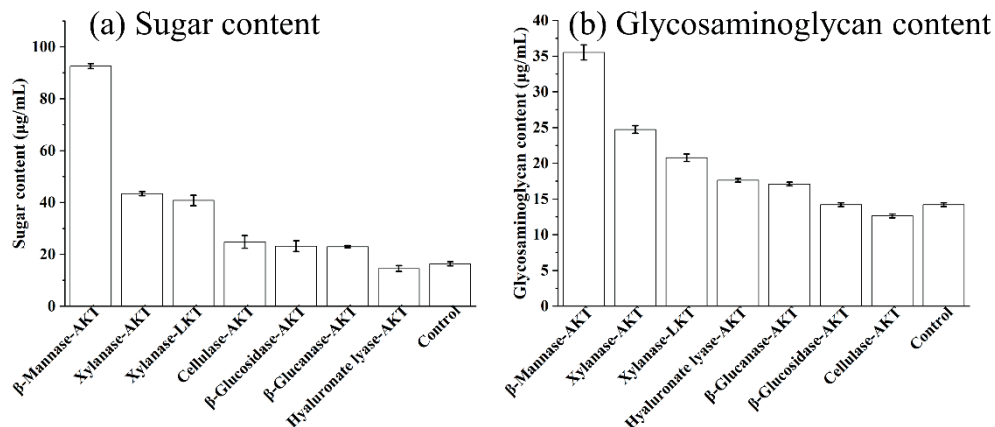


Figure 7. Total sugar and glycosaminoglycan content in the soaking liquors

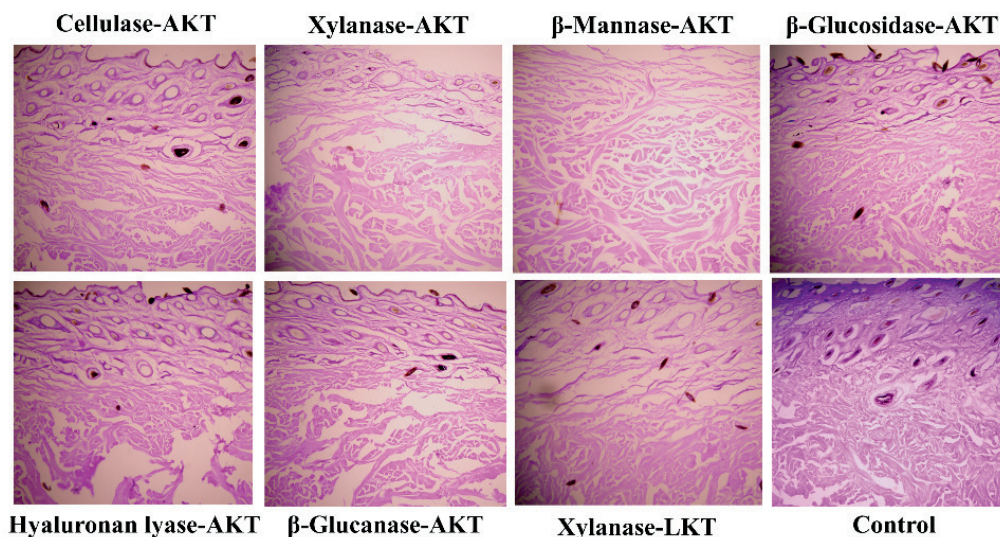


Figure 8. Residues of polysaccharides in the soaked hide (40 X)

treated with the same dosage (0.6 %, w/w) of enzymes were shown in Figure 7.

In the soaking process, glycosidases specificity hydrolyzes the glycosidic bond of the polysaccharide chain and destroys the polysaccharide structure, then, small sugar chains are dissolved from the hide. As is shown in Figure 7, β -Mannase-AKT has the highest activity and produced the highest content of total sugar and GAG. Although there is a little difference in the activity of other glycosidases to the proteoglycan-containing hide powder substrate, the produced amount of total sugar shows a significant difference. Xylanase-AKT and Xylanase-LKT produced a higher amount of total sugar and GAG. β -Glucosidase-AKT and Hyaluronate lyase-AKT have poorly effective in the removal of proteoglycan.

The types and structures of proteoglycan in the cattle hide are complicated, and the hydrolysis specific to glycosidic bonds of glycosidases is very high. Therefore, different glycosidases have significantly different performances on the proteoglycan. In addition, the effect of glycosidases on hides proteoglycan is also influenced by its penetration ability in the hides. Figure 4 and Table I showed that β -Glucosidase-AKT, Hyaluronate lyase-AKT and Cellulase-AKT have relatively larger molecular weights, which may have penetration barriers, exhibiting poor polysaccharide removal effect. Furthermore, the solubility, agglomeration and charge state of enzyme molecules also affect their penetrability. Figure 5 and Table I showed that the pI value of the major component of Cellulase-AKT was 7.5, its solubility is poor under pH 7 - 8, and enzyme proteins may be agglomerated together. The molecular weight of Xylanase-LKT is significantly higher

than that of Xylanase-AKT. Moreover, Xylanase-LKT (pI 8.3) and collagen fiber (pI 7) take an unlike charge at pH 7 - 8, causing a strong affinity on the surface of the hide. All of these make the penetration of Xylanase-LKT not easy. All of these indicated that, although Xylanase-AKT and Xylanase-LKT have almost a similar activity on the hide powder substrate, the proteoglycan removal effect of Xylanase-LKT is not good as Xylanase-AKT. Furthermore, β -Mannase-AKT has the highest activity, small molecular weight and acidic isoelectric point, which make it easy to penetrate the cattle hide and exhibit a higher proteoglycan removal effect.

Staining results of polysaccharides

To further observe the effect of glycosidase on the removal of proteoglycan, the residue of polysaccharides in the hides was stained using the Periodic acid-Schiff and Alcine blue method (PAS-AB), and the results are shown in Figure 8.

Acidic glycosaminoglycan can be colored blue using the PAS-AB staining method, other sugars with red color, and purple color represent the presence of both types of polysaccharides. As is shown in Figure 8, the epidermis, basement membrane and hair follicle of the control group showed a deep purple color, indicating that it contains a large amount of GAG. The hides treated with different glycosidases showed lighter color than the control group, indicating that parts of the acidic GAG were removed. β -Mannase-AKT and Xylanase-AKT treated hides showed the lightest color, and the color is uniform throughout the vertical section and without any purple color, which means that all of the GAG has been removed. The above staining results are consistent with the quantitative results shown in Figure 7.

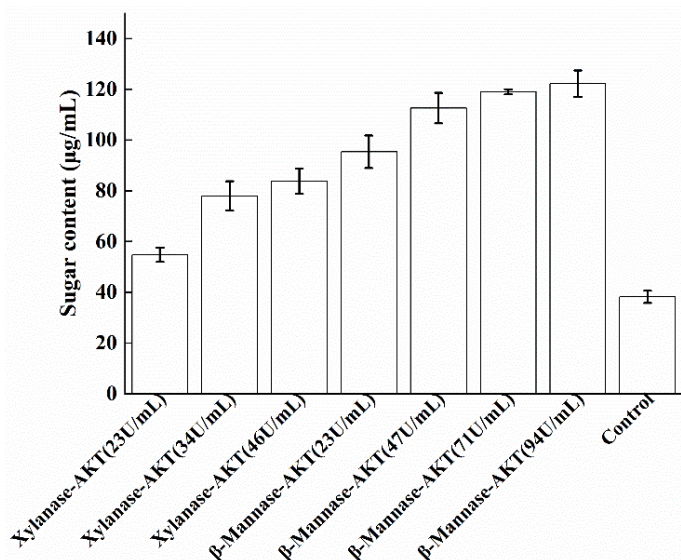


Figure 9. Content of total sugar in the soaking liquors

GAG was almost completely removed from the epidermis, basement membrane and hair follicle after being treated with β -Mannase-AKT and Xylanase-AKT. In particular, β -Mannase-AKT treated hides almost have no epidermis, basement membrane, hair follicle and hair root (Figure 8). These results are consistent with the results shown in Figure 6. It proved that the presence of proteoglycans in the hair follicle has a binding effect on the hair root, and the hair root could be loose after proteoglycans are destroyed. Although the GAG in other glycosidases treated hides showed a different extent of degradation, the hair follicle is still clearly visible, and an obvious proteoglycan circle around the hair root is observed around the hair follicles.

Meanwhile, β -Mannase-AKT and Xylanase-AKT treated hides showed the lightest color on the dermis and the reticular layer, indicating that it contains less GAG. The removal of proteoglycan can eliminate the adhesion of collagen fibers, making hides more open and uniform.

Effect of glycosidase dosage on the soaking

The above result shows that β -Mannase-AKT and Xylanase-AKT have better proteoglycan removal effects in the soaking process. Therefore, the effect of the dosage of these two glycosidases on the soaking and the properties of crust leather was further studied.

Produced amount of total sugar in the soaking liquors

Glycosidases hydrolyze insoluble large sugar chains into soluble small sugar, therefore, the content of total sugar in the soaking liquor can be used to characterize the effect of glycosidases on the removal of proteoglycan from the hide. After being treated with different dosages of glycosidase, the produced amount of total sugar at the end of the soaking is shown in Figure 9.

The result in Figure 9 shows that the concentration of total sugar in the soaking liquors increased with the increase of enzyme dosage. However, the concentration of total sugar was close to the maximum value when the dosage of β -Mannase-AKT and Xylanase-AKT reached 47 U/mL and 34 U/mL, respectively. Staining results of polysaccharides also showed that almost all of the GAG has been removed when the concentration of β -Mannase-AKT reached up to 47 U/mL. Furthermore, at the same concentration of glycosidase activity, β -Mannase-AKT is more effective than Xylanase-AKT in removing polysaccharides from cattle hide. The reason for this is that the enzyme molecule of β -Mannase-AKT has a low isoelectric point, which makes it dissolve well at pH 7 - 8 and it possesses a weak affinity for collagen fibers, making it easier to penetrate the cattle hide.

Produced amount of soluble protein and hydroxyproline in the soaking liquors

The produced amount of soluble protein and Hydro in the soaking liquors after being treated with different amounts of glycosidases are shown in Figure 10.

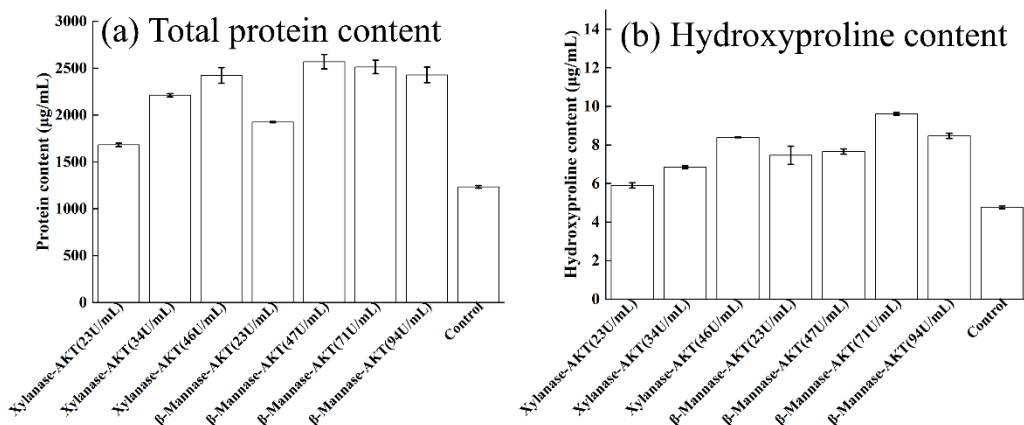


Figure 10. Content of soluble protein and hydroxyproline in the soaking liquors

The result in Figure 10 (a) shows that the content of soluble protein in the glycosidases-soaked liquors was significantly higher than that of the control. The hydrolysis of sugar chains in the proteoglycans by glycosidase can break the proteoglycan structure, which is a benefit for the solubilization of core proteins and increases the soluble protein content in the soaking liquor.²³ The content of soluble protein in Xylanase-AKT treated liquor increased with the increase of enzyme dosage. However, when the concentration of β -Mannase-AKT reached up to 47 U/mL, the content of soluble protein in the soaking liquor was close to maximum value because the GAG in the cattle hide was almost all removed by β -Mannase-AKT. The result in Figure 10 (b) shows that the content of Hypro in both glycosidases treated liquors was slightly higher than that of the control and has not increased with the increase of glycosidase dosage. Overall, the concentration of Hypro in the soaking liquor was low (less than 10 μ g/mL), indicating that glycosidases just have a weak effect on collagen.

The grain surface of the wet blue was observed after being soaked with different dosages of glycosidases and conducted the conventional leather making procedures, and the results are shown in Figure 11.

The result in Figure 11 shows that there was no obvious difference in the grain surface of the wet blue after being soaked by glycosidase when compared to the control. Even with a higher glycosidase dosage and longer duration time (94 U/mL of β -Mannase-AKT for 18 h), the grain of the crust leather was still full, intact and undamaged. These results proved once again that the application of glycosidases in the soaking process is a safe and efficient method for raw hides to rehydrate and open the fibers because it has nearly no effect on collagen fibers compared to proteases.

Opening up of collagen fibers

SEM result of the reticular layer fibers in Figure 12 shows that, after being treated with glycosidase, the opening-up of collagen fibers significantly improved, especially, when the dosage of glycosidases reached 47 U/mL, the opening-up of collagen fibers was more uniformly. Oppositely, collagen fibers of the crust leather treated without glycosidase (Figure 12, Control) showed obvious adhesion and poor opening up effect.

Generally, the opening up of collagen fibers is closely related to the pore diameter of the leather. The result in Table II shows that the average pore diameter of the crust leather treated by glycosidases was larger than that of the control. The average pore diameter of 23 U/mL β -Mannase-AKT (52.8 nm) treated sample was significantly larger than that of 46 U/mL higher dosage of Xylanase-AKT (43.5 nm). When the dosage of β -Mannase-AKT reached 47 U/mL, the average pore diameter of the crust leather reached maximum value and did not increase with further increase of the glycosidase dosage.

The opening up degree of the collagen fibers and the average pore diameter of the crust leather is consistent with the results of the amount of sugar dissolved in the soaking liquor, namely the higher the amount of sugar dissolved from the cattle hide, the higher the degree of collagen fiber opening up and average pore diameter. All of these results further demonstrated that glycosidases can break the proteoglycans and eliminate the bonding between collagen fibers, which could promote the opening up of collagen fibers and improve the softness of the crust leather. Furthermore, at the same concentration of glycosidase activity, β -Mannase-AKT can remove more proteoglycan and preferably opening up collagen fibers.

Physical and organoleptic properties of the crust leather

The physical and organoleptic properties of the crust leather are shown in Table III. The result shows that, compared with the

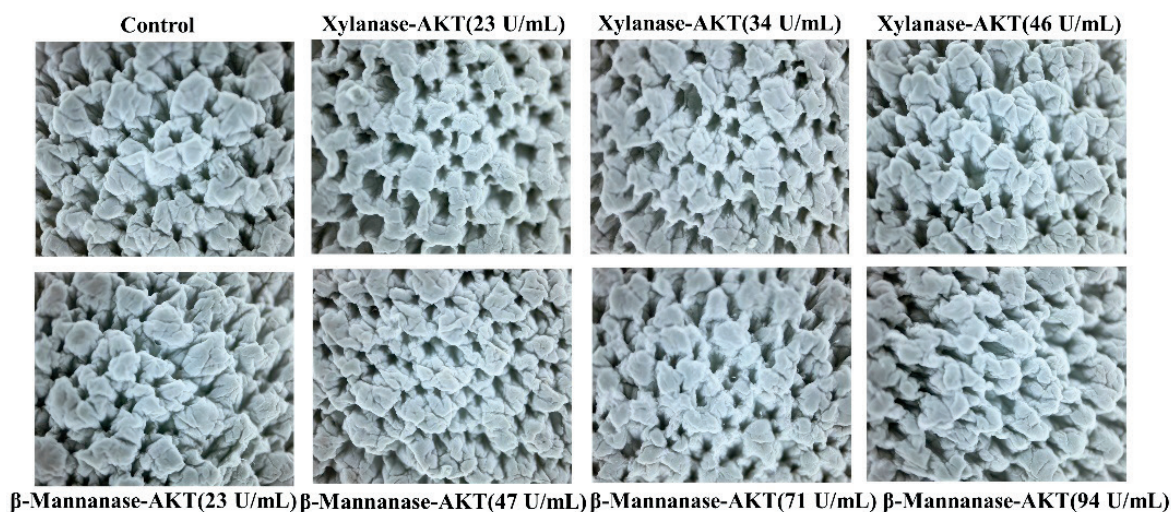


Figure 11. Grain surface of wet blue leather (100 X)

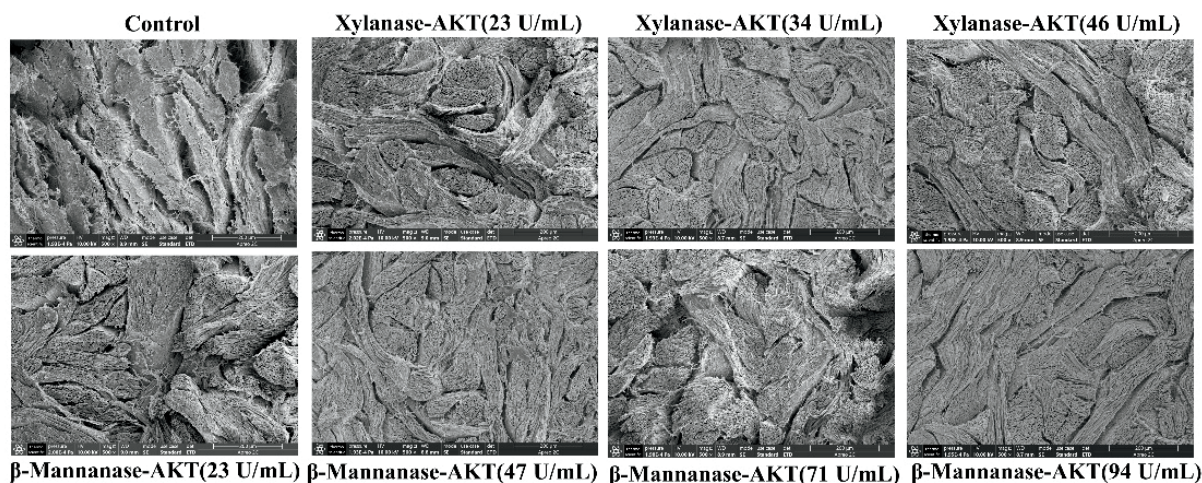


Figure 12. SEM images of the reticular layer of crust leather (500 X)

Table II
Average pore diameter of the chrome tanned crust leather

Glycosidase	Average pore diameter (nm)
Xylanase-AKT (23 U/mL)	38.3 ± 0.24
Xylanase-AKT (34 U/mL)	42.2 ± 0.41
Xylanase-AKT (46 U/mL)	43.5 ± 0.18
β -Mannase-AKT (23 U/mL)	52.8 ± 0.27
β -Mannase-AKT (47 U/mL)	67.9 ± 0.30
β -Mannase-AKT (71 U/mL)	66.2 ± 0.48
β -Mannase-AKT (94 U/mL)	68.1 ± 0.25
Control	34.3 ± 0.39

control, the softness of the crust leather was significantly improved after being treated with glycosidase. Moreover, the main physical properties of the crust leather were also improved. The improvement of softness and physical properties of the crust leather was caused by the removal of proteoglycan and better opening up of the collagen fibers after being treated with glycosidases in the soaking process. The properties of the crust leather were obviously improved when the concentration of β -Mannase-AKT reached up to 47 U/mL; however, the physical properties of the crust leather decreased when the enzyme activity reached 94 U/mL, which is probably caused by the excessive opening up of the collagen fibers.

Conclusion

Based on the types of major PG glycosidic bonds in animal hide, seven typical commercial glycosidases capable of hydrolyzing β -glycosidic bonds were selected and their basic properties

and the effect on the removal of proteoglycans in soaking were investigated. Glycosidase β -Mannase-AKT and Xylanase-AKT with good proteoglycan removal effect were selected to investigate the relationship between the dosage of enzyme and the dissolution value of polysaccharide, total soluble protein and hydroxyproline, as well as the overall performance of the leather. The results showed that the seven glycosidases were able to maintain more than 50% of their activity under weak alkaline conditions and have the potential for application in the soaking process. The molecular weight, isoelectric point and specificity to the glycosidic bonds of glycosidases affected the penetration of the enzymes into the hides and the removal of proteoglycans. Among them, β -Mannase-AKT and Xylanase-AKT were more effective in removing proteoglycans, and almost all glycosaminoglycan was removed from the hides when the enzyme dosage reached 47 U/mL. The removal of proteoglycans by glycosidase can significantly improve the opening-up of collagen fibers and the pore diameter of the crust leather, the softness and physical properties of the crust leather were also improved. The

Table III
Physical and organoleptic properties of the crust leather

Enzyme	Tensile strength (N/mm ²)	Breaking strength (N/mm)	Breaking height (mm)	Tear strength (N/mm)	Softness (mm)
Xylanase-AKT (23 U/mL)	9.64 ± 0.21	246.07 ± 0.41	15.79 ± 0.38	46.73 ± 0.71	7.51 ± 0.38
Xylanase-AKT (34 U/mL)	10.02 ± 0.23	267.49 ± 0.77	20.10 ± 0.45	52.36 ± 0.83	7.57 ± 0.32
Xylanase-AKT (46 U/mL)	9.42 ± 0.30	257.97 ± 0.25	15.48 ± 0.26	51.17 ± 0.71	7.40 ± 0.24
β-Mannase-AKT (23 U/mL)	9.52 ± 0.31	281.10 ± 0.46	18.64 ± 0.32	51.05 ± 0.43	7.27 ± 0.14
β-Mannase-AKT (47 U/mL)	10.36 ± 0.24	329.60 ± 0.62	17.15 ± 0.51	63.20 ± 0.60	7.72 ± 0.33
β-Mannase-AKT (71 U/mL)	10.72 ± 0.17	338.67 ± 0.17	19.26 ± 0.52	61.38 ± 0.46	7.84 ± 0.23
β-Mannase-AKT (94 U/mL)	9.56 ± 0.23	254.84 ± 0.34	16.91 ± 0.38	54.06 ± 0.25	7.54 ± 0.31
Control	9.26 ± 0.18	238.36 ± 0.89	17.94 ± 0.42	46.61 ± 0.71	6.93 ± 0.13

use of glycosidase in the soaking process does not cause excessive damage to the structural proteins due to the weak effect on the structural proteins. Therefore, through the rational selection and application of glycosidases in leather making process, it is possible to safely and effectively remove proteoglycans from the hides, promote the opening of collagen fibers and improve the softness and overall properties of the leather.

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