Effect of Electrostatic Interaction between Collagen and Enzymes on Permeation of Protease into the Pelt during Leather Bating Process

by
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Abstract

The enzymatic delimed pelts bating process using proteases is critical to improving the overall performance of the leather. Bating effectiveness is determined not only by the properties but also by the permeation behavior of the proteases. Imperfect methods to control protease permeation often results in uneven distribution of enzyme proteins in the pelts, leading to excessive enzymolysis of the surface layer and inadequate opening-up of the inner layer. In this study, the relative size of proteases and delimed pelts were analyzed, the permeation behavior of fluorescein-labeled proteases in the pelt was observed using a confocal laser scanning microscope (CLSM), and the effect of electrostatic interaction between protease and collagen proteins on the permeation of protease into the pelt was investigated. The results showed that, after dehairing, liming and deliming operations, the enzyme can easily permeate into the pelts due to the formation of large cavities and interfibrillar gaps. The permeation of protease within the delimed pelt is significantly influenced by the affinity (electrostatic interactions) between the collagen and protease proteins. The isoelectric point (pI) of the protease protein, the collagen and the pH of the solution directly influence the electrostatic properties and interactions. When the enzyme and collagen are similarly charged (electrostatic repulsion), the enzyme can easily permeate into the pelts; when the enzyme and collagen are oppositely charged (electrostatic attraction), the permeation of the enzyme into the inner layer is difficult, resulting in the accumulation of protease on the grain and excessive hydrolysis of the grain layer. Therefore, the established permeation regulation mechanism of protease based on electrostatic interactions between enzyme and collagen could serve as an important basis for the selection of protease and the regulation of the enzymatic bating process.

Introduction

Enzymatic bating is an important process performed on delimed pelts to further remove scale, interfibrillar substances and opening-up collagen fibers to obtain clean, smooth, elastic and soft leather products, which cannot be replaced by any chemical and mechanical operations. As the main component of animal hides is protein, protease is the main enzyme used for pelts bating, and trypsin, an endo-protease extracted from the animal pancreas, is taken as the preferred option for pelts bating.[1] However, the disadvantages of animal trypsin limit its application, and the application of microbial proteases in the pelts bating process tends to increase.[2-4]

With the increasing demands for the softness, evenness and properties of leather products, the dosage of proteases and the duration time of the bating process, namely the intensity of the protease treatment, have been increased. The ideal bating effect is that the protease can quickly penetrate into the pelts to avoid the excessive degradation and damage of the grain surface, and to ensure the bating evenness of the inner and outer layers. However, in the practical bating process, protease tends to accumulate on the surface due to the difficulty of permeation of protease into the pelts. In addition, most of the proteases possess a stronger effect on structural proteins and wide-spread substrate specificity, resulting in damage to the grain surface at high dosages and long duration time. Therefore, the current pelts bating process usually takes a conservative method with lower protease dosage and short treatment time. However, the conservative bating method results in the insufficient opening-up of the middle layer due to the difficulty of protease permeation into the inner layer.[5] Consequently, it is crucial to improve the permeation rate and depth of proteases and avoid accumulation on the surface layers, especially on the grain layer, to achieve the desired bating effect.

Several factors may influence the permeation of proteases into the pelts,[6-8] such as the situation of the permeation channels, the relative size of the protease molecule, its aggregates and the interfibrillar space, and the affinity between the pelt fibers and the protease molecules. Our previous enzymatic dehairing research found that,[9] different proteases have significantly different isoelectric points (pI), and the surface charges of the protease molecules and collagen fibers changed with the adjustment of the pH values of the solution, resulting in the various electrostatic interactions between the proteins. The results demonstrated that it is a useful way to speed up protease permeation into the pelts by regulating the surface charges of proteins and its affinity.[9] Wang et al. [10] demonstrated that

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negatively charged materials can penetrate negatively charged skin/leather faster than positively charged materials. The above results suggest that the electrostatic interaction between the protease and delimed pelts is critical for the permeation of proteases in the pelts bating process, but the mechanism involved is not clear at present.

Fluorescence labeling and confocal laser scanning microscope (CLSM) observation is an effective method to study the permeation behavior of proteases in delimed pelt.[11] However, the purity of the selected proteases has been ignored in some of the reported studies, and the purification of the fluorescein-labeled proteases and the variations of the molecular weight and pI of the proteases have been neglected. All of these deficiencies result in the mass transfer tracking results of the labeled proteases not fully representing the permeation behavior of the unlabeled proteases.

The purpose of this study was to investigate the permeation behavior of proteases in delimed pelts during the enzymatic bating process, the effect of the relative size of proteases and pelt pores and the electrostatic interaction between protease molecules and collagen fibers on the permeation behavior of proteases. Three highly purified proteases with significantly different pI were selected and labeled with fluorescein isothiocyanate (FITC), and the effect of the degree of labeling on the properties of the proteases was evaluated. The permeation behavior of the selected proteases was investigated using fluorescein labeling and CLSM techniques, including the permeation direction, pathway and depth of the proteases in delimed pelts. Then, the effects of different electrostatic interactions on the permeation depth and rate of the proteases were analyzed by adjusting the pH of the solutions to induce the proteases and delimed pelts to carry different charges. In addition, unlabeled protease bating experiments were performed to verify the results of the fluorescein-labeled protease permeation experiments. These studies attempt to clarify the law of the permeation behavior of proteases in the pelts and provide guidance for the selection of proteases and the regulation of the pelt bating process.

Materials and Methods

Materials

Fresh wet-salted bovine hides were purchased from a tannery in Sichuan, China, and subjected to conventional soaking, dehairing, degreasing, liming and deliming processes to obtain delimed pelts. Alkaline protease P-SG-B, trypsin P-NB115-T and P-PTN110-T were purchased from Sichuan Dowell Science and Technology Co, Ltd, China. Sodium dodecyl sulfate (SDS), polyacrylamide and FITC were purchased from Shanghai Sangon Biotech, Co., Ltd, China. Chemicals used in the tanning process were of industrial grade and supplied by Sichuan Dowell Science and Technology Co., Ltd., China. All of the analytical grade reagents were purchased from local suppliers in Chengdu, China. All of the analytical experiments were repeated at least three times and the mean was reported.

Determination of protease particle size and delimed pelts pore diameter

Determination of protease particle size

The concentration of P-SG-B, P-NB115-T and P-PTN110-T was diluted to 10 mg/mL protein with deionized water. The pH of the protease solutions was then adjusted to 7.5, 8.5 and 9.5 using 0.1 mol/L hydrochloric acid and sodium hydroxide, respectively, and filtered through an aqueous filter membrane (0.1 μm). The particle size distribution of the proteases at different pH values was then determined using a particle size analyzer (Zetasizer nano zsp, Malvern, USA) under the following conditions: the dispersion medium was water, the measurement temperature was 25°C, the scattering angle was 173°, the number of measurements was automatic, the equilibration time was 120 s and the particle size calculation model was Protein Analysis.

Determination of pore diameter of delimed pelts[12]

Mercury intrusion porosimetry (MIP) was used to assess the pore diameter distribution of the delimed pelts. The delimed pelts were freeze-dried and cut into uniform pieces (approximately 1 cm^3). The pore diameter distribution of the samples was determined using a fully automated mercury porosimeter (AutoPore VI 9500, Micromeritics, USA) capable of measuring pores in the range of 5.5 nm to 120,000 nm in diameter.

Determination of zeta (ζ) potential and pI of the delimed pelts [13,14]

The delimed pelts were freeze-dried and then ground to powder and passed through a 1 mm diameter sieve. Fifteen grams of the pelt powder obtained was mixed with 500 mL of deionized water in a conical flask. The pH of the solution was adjusted to a certain value using either a 0.1 mol/L hydrochloric acid solution or a sodium hydroxide solution. The pI of the samples was determined as the pH value when the ζ potential on the curve is zero. The ζ potential of the delimed pelt powder was evaluated at different pH values using a solid zeta potential meter (SZP-10, MÜtek, Sweden). The curve of ζ potential versus pH was plotted, and the pH value at which the ζ potential on the curve was zero was determined to be the pI of the samples.

Preparation and characterization of fluorescein-labeled proteases

Preparation and purification of fluorescein-labeled protease

FITC-labeled proteases were prepared according to the reported method.[15] Specifically, P-SG-B, PTN110-T and P-NB115-T proteases were dissolved in carbonate buffer (0.5 mol/L, pH 9.0) to obtain a 5 mg/mL protein solution and mixed with a certain amount of FITC; and the mixture was gently stirred at 4°C for 12 hours under light-free conditions. Then, a certain amount of semi-saturated ammonium sulfate was added to the mixture to precipitate the labeled protease, and the precipitate was immediately separated. Next, the precipitate was dissolved in phosphate buffer solution (PBS, 0.01 mol/L, pH 7.2) and dialyzed with an 8 kDa dialysis bag at 4°C to remove the salt and uncombined fluorescein. The prepared

fluorescein-labeled proteases were designated FITC-P-SG-B, FITC-PTN110-T and FITC-P-NB115-T, respectively, and were temporarily stored at 4°C in the dark.

Determination of UV-Vis and fluorescence spectra

The unlabeled and labeled protease samples were appropriately diluted with a certain volume of PBS buffer. The PBS buffer was prepared as follows: first, 1 mol/L disodium hydrogen phosphate (liquid A) and 1 mol/L sodium dihydrogen phosphate (liquid B) were prepared. Then, 72 mL of liquid A and 28 mL of liquid B were mixed and diluted to a final volume of 1000 mL with deionized water, which was 0.01 mol/L PBS buffer (pH 7.2). The UV-Vis spectra of the unlabeled and labeled protease solutions were analyzed by using a UV-Vis spectrophotometer (V-1100D, Mapada, China) in the wavelength range of 200 nm - 800 nm at a scanning speed of 2 nm/s. The fluorescence intensity of the labeled protease solutions was analyzed by using a fluorescence spectrophotometer (F7000, Hitachi Ltd., Japan) with an excitation wavelength of 495 nm, slit width of 5 nm and integral time of 0.1 s.

Determination of the combined amount of FITC to protease protein 16

The combined rate of fluorescein was defined as the amount of combined FITC (mg) per milligram of labeled protease protein. The concentration of proteins was determined by the Lowry method [17] and the concentration of FITC was calculated according to the FITC concentration-absorbance standard curve, measured at 490 nm.

Determination of the pI and molecular weight (Mw) of the proteases

The pI of the protease was determined by isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) (Model 111 Mini IEF Cell, Bio-Rad, American). The electrophoresis steps were as follows: 100 V for 30 minutes, 200 V for 15 minutes, and 450 V for 60 minutes.

The M_w of the protease was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-PROTEAN® Tetra, Bio-rad, American) with a separating gel concentration of 12%. The electrophoresis steps were as follows: 100 V to move the samples to the boundary between the concentrate and the separating gel, then 150 V until the samples migrated to the bottom of the separating gel.

After IEF-PAGE and SDS-PAGE, the gels were fixed and stained with a Coomassie Blue solution and washed with deionized water to eliminate the background color. The pI and M_w of the proteases were calculated using Quantity One software (Bio-Rad, USA).

Permeation behavior of labeled proteases in the delimed pelts at various pH values

The pH values of the delimed pelts were adjusted to 7.5, 8.5 and 9.5 by using Britton-Robinson buffer (B-R buffer, 0.1 mol/L), respectively. Then, pieces (4 cm × 4 cm) with adjusted pH were mixed with 50% (w/w) of labeled protease solution (containing 0.5 mg/mL protein) in a 250 mL brown bottle and rotated in a drum to mimic the mechanical action of the actual pelt bating process. Samples (0.5 cm × 0.5 cm) were cut and washed at intervals of 5 min, 1 h, 3 h and 5 h, and sectioned longitudinally at 20 μm thickness using a freezing microtome (CM1950, Leica, Germany) and observed using a confocal laser scanning microscope (Stellaris, Leica, American).

The fluorescence of FITC was excited by an argon ion laser at a wavelength of 488 nm, resulting in green fluorescence. The distribution of FITC-labeled proteins on the longitudinal section of the pelts was visualized in the *xy* plane, and the confocal images were captured and analyzed; the fluorescence intensity was also quantitatively analyzed.

Delimed pelts bating effectiveness of proteases at various pH values

Six pieces of delimed pelts were sampled adjacent and symmetrically and weighed. One piece of the pelt was used as a control without subsequent bating, with only 50% (w/w) deionized water and simultaneously rotated. The rest (five pieces of pelts) were mixed with 50% (w/w) of B-R buffer solution (0.1 mol/L, containing 50% of P-NB115-T) at pH 7.5, 8.1, 8.8, 9.6 and 10.5, and rotated at 35°C for 6 h, respectively. The bated samples were then subjected to standard pickling, Cr-tanning and post-tanning operations, and the grain surface of the crust leather was observed using a stereoscopic microscope (TIPSCOPE, China).

Results and Discussion

The particle size of protease in solution and pore diameter of delimed pelts

Animal hides are primarily composed of collagen fibers. After the removal of hair and glands in the dehairing and liming processes, the pores and interfibrillar spaces provided channels for chemical permeation in the subsequent processes. While the permeation of protease molecules into the hides is very important in the enzymatic leather making process, it is still a challenge for protease proteins to permeate into the inner layer of the hides due to their relatively large molecular size. It can be speculated that the relative size of the protease molecules, their aggregations and the pore diameter of the pelts are the main factors influencing the permeation of proteases.

The particle size of protein molecules is influenced by their structure and molecular weight, temperature, pH and ionic strength of the solution, and the protein molecules may also aggregate into masses of different sizes. Protein bating is performed under weak alkaline conditions, mainly using trypsin and alkaline proteases. In this study, three widely used highly-pure proteases (animal trypsin P-PTNP110-T, microbial trypsin P-NB115-T and microbial alkaline protease P-SG-B) were selected, and the particle size of these proteases was investigated at the same pH and ionic strength of the bating conditions. The results are shown in Figure 1.

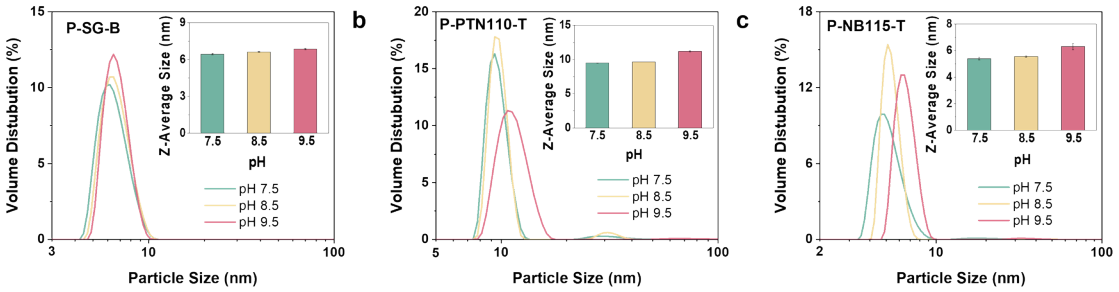


Figure 1. Particle size and distribution of proteases at various pH values (10 mg/mL of protein)

As shown in Figure 1, all of the selected proteases had similar particle sizes and distributions in an aqueous solution (pH 7.0), except for protease P-PTNP110-T, which was slightly larger but also less than 13 nm. The average size of the protease particles increased slightly with increasing pH values, with a size difference of less than 2 nm.

The relative molecular weight of the selected proteases was approximately 25 kDa (see Figure 6b), and it has been reported in the literature that the particle size of protein molecules with a relative molecular weight of around 25 kDa is approximately 5 nm.[18] Therefore, the degree of aggregation of the selected protease proteins is expected to be low at a protein concentration of 10 mg/mL (pH 7.5 - 9.5). In the practical bating process, the dosage of proteases is very low and the concentration of proteins is usually less than 0.5 mg/mL; therefore, it can be speculated that the selected proteases will not aggregate into large masses during the actual bating process.

As the enzymatic bating process uses enzymes to dispose of the delimed pelts, it was necessary to further analyze the pore diameter and distribution of the delimed pelts produced by the conventional beamhouse process. The results are shown in Figure 2.

As shown in Figure 2, the pore diameter-log differential intrusion curve shows that the most prevalent pore diameter in the delimed pelts was 12500 nm; the pore diameter cumulative curve shows that the total pore volume increased significantly between 4000 and 24000 nm, indicating that the majority of the pores in the delimed pelts are in this range. The proportion of macropores with pore diameters larger than 4000 nm was over 74% of the total pore volume, which means that the majority of the pores in the delimed pelts are microporous. In the literature,[19,20] it is reported that the pore diameter of the soaked hides is mainly in the range of 5 - 55 nm, which is much smaller than the pore diameter of the delimed pelts measured in this experiment. Despite the difference in results obtained from different methods and hides,[21] it is evident that the porosity and pore diameter of the delimed pelts are significantly higher than those of the soaked hides. This is because the removal of unwanted tissues, such as hair and glands, during dehairing, liming and deliming creates a large cavity in the grain surface layer and increases the interfibrillar gap by removing interfibrillar substances. The increased porosity and pore diameter of delimed pelts is conducive to protease permeation. During the enzymatic bating process, the small particle size of the protease (less than 13 nm) allows it to easily permeate the pelts through the large channels. However, the inner granular and reticular layers are woven by finer collagen fibers with smaller fiber gaps. When proteases pass through the small channels, the affinity interaction between the collagen fibers and the protease proteins may have a more pronounced effect on protease permeation.

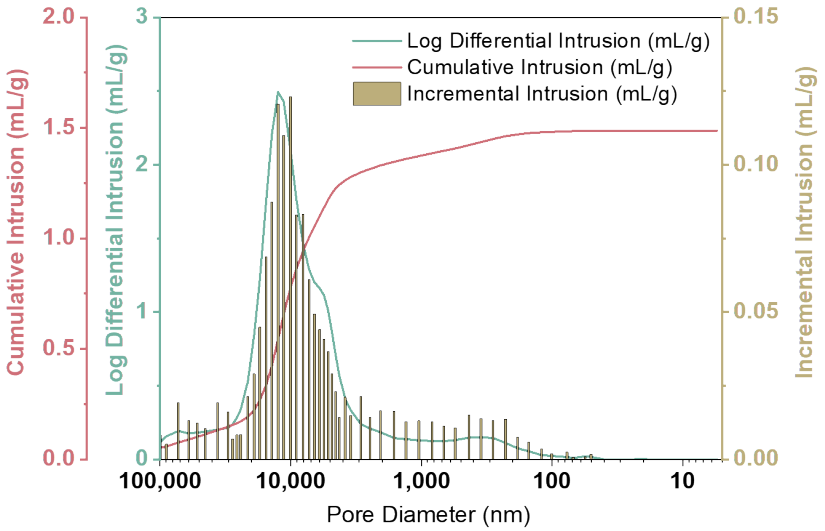


Figure 2. Pore diameter distribution of delimed pelt

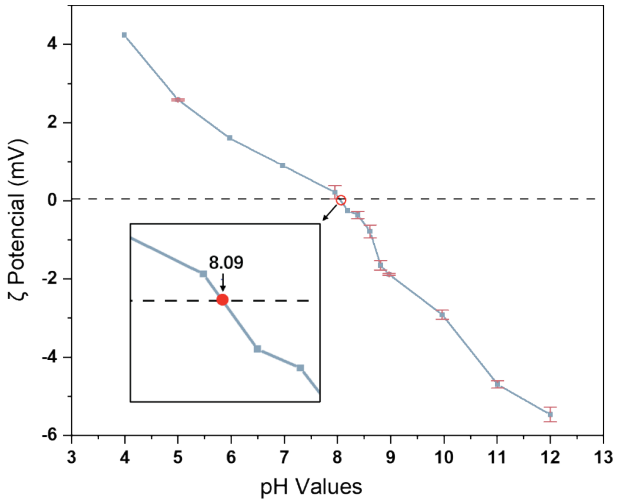


Figure 3. ζ potential of the delimed pelts at various pH values

Determination of the pI of the delimed pelts

In addition to the particle size of the protease molecules and the diameter of the permeation channels, the permeation of proteases into the pelts may also be influenced by the affinity between the protease molecules and the collagen fibers of the pelts. The charge state of the protease proteins and collagen fibers has a major influence on the affinity between them. The charge state of proteins mainly depends on their pI and the pH value of the solutions. Herein, the ζ potential of the delimed pelts was determined at various pH values and the results are shown in Figure 3.

The results in Figure 3 show that the ζ potential of the delimed pelts gradually decreased with the increasing of pH values, and the ζ potential is 0 mV when the pH of the solution is 8.09, indicating that the pI of the delimed pelts is 8.09.

Characterization of the FITC-labeled proteases

The permeation behavior of proteases in the pelts is difficult to observe directly. Fluorescein labeling is a widely used visualization technique for proteins,[22] the covalent combination of fluorescein to protease proteins imparts fluorescence properties to the proteases, allowing them to be localized and tracked. In this study, the labeling conditions and the basic properties of the labeled proteases were investigated.

The amount of fluorescein combined with a protease molecule has a large effect on the fluorescence intensity and properties of the labeled protease. Typically, the more fluorescein that binds to a protease molecule, the greater the variation in the M_w, pI and enzyme activity properties. Conversely, a low binding rate may result in a weak fluorescence signal, making it difficult to observe in subsequent permeation experiments. Therefore, as a substitute for unlabeled proteases, fluorescein-labeled proteases should have an appropriate fluorescein binding rate to allow their detection in permeation tracing experiments without dramatically affecting the properties of the protease molecules.

Labeled proteases with varying amounts of fluorescein were prepared by adjusting the amounts of both fluorescein and protease. The binding rate of fluorescein to protease is defined as the amount (mg) of combined FITC per milligram of protease protein. Based on the results of the permeation pre-experiment, when the fluorescein binding rates of FITC-P-SG-B, FITC-P-PTN110-T and FITC-P-NB115-T reached 0.009, 0.023 and 0.006, respectively, fluorescence signals were significantly observed in the labeled protease-treated pelts sections. However, if the binding rate was lower than the above values, the fluorescence signal has to be amplified by software, which might lead to the detection of autofluorescence of the collagen and affect the accuracy of the experimental results. Therefore, the fluorescein binding rates of the above-labeled proteases were used in the subsequent experiments.

UV-Vis spectroscopy characterizations of FITC-labeled proteases

The prepared fluorescein-labeled protease solutions were likely to contain uncombined fluorescein monomers and fluorescein-labeled small-molecule non-enzyme proteins, which could rapidly enter the pelts and interfere with the observation of the permeation of the actual protease molecules into the pelts, leading to erroneous conclusions. Therefore, the crude FITC-labeled protease solutions were dialyzed to remove the impurities. The proteases were examined before and after labeling using a UV-Vis spectrophotometer in the wavelength range of 200 - 800 nm.

As shown in Figure 4, The absorption peaks of the three labeled proteases are comparable. The characteristic absorption peak of FITC is known to be at 490 nm, whereas the peaks of the three labeled proteases migrate to 495 nm, indicating that FITC is combined with the proteases, which is consistent with the reported results.[23]

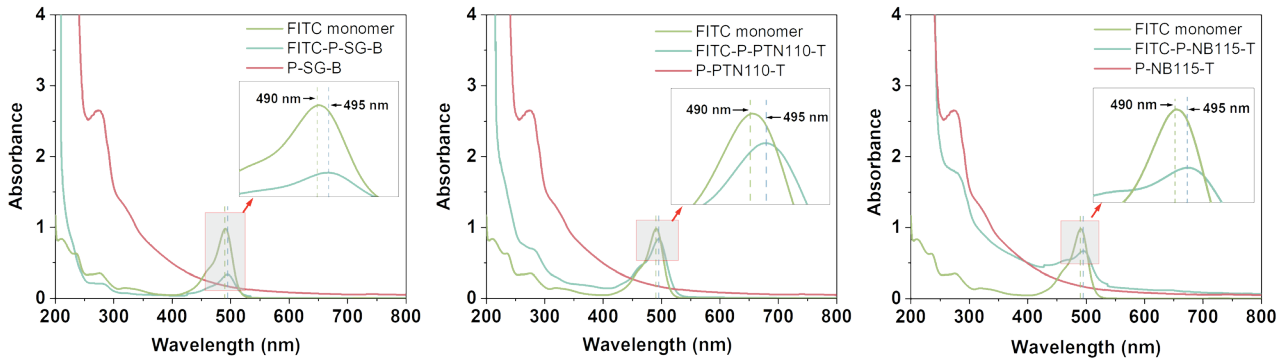


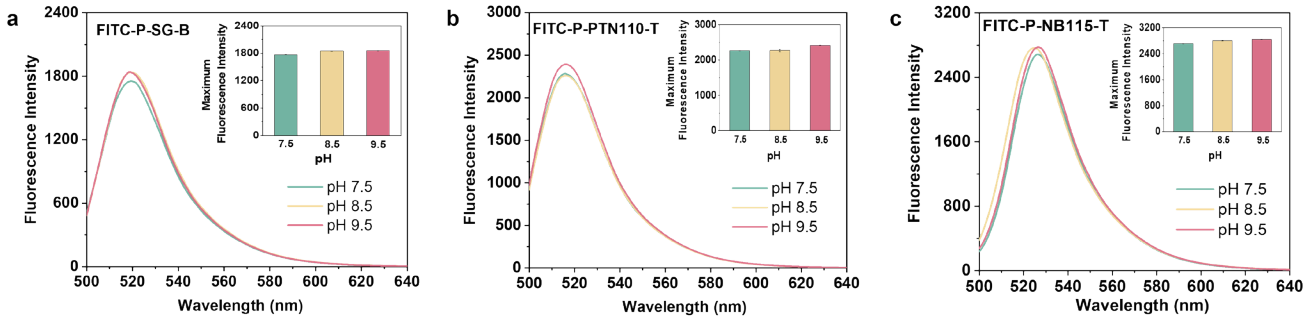
Figure 4. UV-Vis spectra of FITC monomer, proteases and FITC labeled proteases

Fluorescence spectroscopy characterizations of FITC labeled proteases

Since FITC can generate intense fluorescence under alkaline conditions and bating is typically performed in the pH range of 7.5 - 9.5, it is necessary to investigate the effect of pH on the fluorescence intensity of the labeled proteases. Figure 5 shows that the fluorescence spectra of the three labeled proteases are similar at different pH values, obtaining maximum fluorescence intensity at a wavelength of 520 nm. Although the maximum fluorescence intensity of the labeled proteases increased with the increasing of pH value, the difference is not significant.

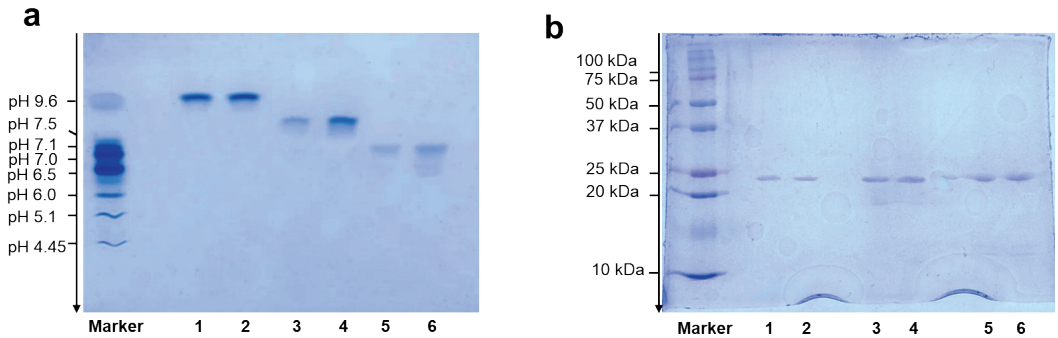
pI and relative molecular weight of labeled proteases

The isoelectric point and molecular weight of the protease proteins are critical factors that influence their mass transfer in pelts.[24,25] The covalent cross-linking of fluorescein to proteases may alter their molecular properties, therefore, it is important to compare the pI and molecular weight values of the proteases before and after fluorescein labeling. The IEF-PAGE and SDS-PAGE results in Figure 6 show that the pI and molecular weight of the labeled and unlabeled proteases are similar, and all of the selected proteases showed high purity with only a single electrophoretic band. IEF-PAGE (Figure 6a) showed that the pI of FITC-P-SG-B, FITC-P-PTNP110-T and FITC-P-NB115-T were 7.1, 8.2 and 9.6, respectively. SDS-PAGE (Figure 6b) showed that the molecular weight of the selected proteases is all close to 25 kDa. Therefore, these labeled proteases can be used to substitute the original protease to investigate the permeation behavior of the protease in the pelts through a visualization way.



(a) FITC-P-SG-B; **(b)** FITC-P-PTN110-T; **(c)** FITC-P-NB115-T

Figure 5. Fluorescence spectra and maximum fluorescence intensity of fluorescein-labeled proteases



(**1:** P-NB115-T; **2:** FITC-P-NB115-T; **3:** P-PTN110-T; **4:** FITC-P-PTN110-T; **5:** P-SG-B; **6:** FITC-P-SG-B)

Figure 6. IEF-PAGE **(a)** and SDS-PAGE **(b)** electrophoretogram of the proteases before and after fluorescein labeling

Effect of proteins affinity on proteases permeation behavior in delimed pelts

The permeation behavior of FITC-labeled proteases at various pH values in the delimed pelts was observed using a confocal laser scanning microscope (CLSM), and the results are shown in Figures 7 - 9. The results show that proteases permeated into the pelts from both the grain and flesh surfaces, and the flesh side absorbed more proteases and exhibited higher fluorescence intensity than the grain side due to its greater specific surface area than the tightly woven grain side. As the channels for protease permeation are not continuous, the proteases are mainly distributed around the channel walls at the beginning of the bating process, resulting in a discontinuous fluorescence signal in the longitudinal section. The fluorescence intensity distribution in the longitudinal section (1h) shows an obvious "fluorescent circle" around the hair follicles, and strong fluorescence signals can be detected at a distance of about 0.5 mm from the top and bottom surfaces of the pelt, which are the hair follicles and the villi of the flesh, respectively, indicating that during the early stages of bating, the permeation of proteases into the pelts through large cavities is relatively easy, such as the pores.

As the permeation time increased, the proteases transferred from both the grain and flesh sides to the inner layer of the pelts. However, pH had a significant effect on the permeation rate and depth of the proteases. Figure 7 shows that FITC-P-SG-B (pI 7.1) permeated into the pelt at pH 9.5 after 5 hours of treatment, strong fluorescein signals were detected at 1.2 mm and 2.0 mm from the grain and flesh surfaces respectively, showing a significantly better protease permeation rate and depth than those at pH 7.5 and 8.5. Figure 8 shows that the permeation rate and depth of FITC-P-PTN110-T (pI 8.2) at pH 7.5 and 9.5 were superior to those at pH 8.5. Figure 9 shows that the permeation rate and depth of FITC-P-PTN115-T (pI 9.6) at pH 7.5 were better than those at pH 8.5 and 9.5.

The pI values of the delimed pelts and FITC-P-SG-B were 8.09 (Figure 3) and 7.1 (Figure 6a), respectively. At pH 7.5, the protease molecules are negatively charged while the collagen is positively charged. As a result, the strong electrostatic attraction between the protease and the pelt surface and channel walls inhibits protease permeation. When the pH is higher than the pI of the protease and collagen, both are negatively charged and the electrostatic attraction is weakened. Therefore, protease permeation is enhanced at pH 8.5 and 9.5, especially at pH 9.5, where both molecules are more negatively charged, resulting in electrostatic repulsion that prevents protease adsorption to collagen and promotes its permeation rate and depth.

The pI of FITC-P-PTN110-T was found to be 8.2. At pH 7.5, which is significantly lower than the pI of both protease and collagen, the positively charged protease and collagen repel each other. Correspondingly, at pH 9.5, which is significantly higher than the pI of both protease and collagen, the negatively charged protease and collagen also repel each other. Therefore, at pH 7.5 or 9.5, where there is strong electrostatic repulsion between the protease and collagen, the proteases could permeate more effectively than that at pH 8.5, which is slightly higher than the pI of the protease and collagen and results in fewer negative charges, leading to weaker electrostatic repulsion.

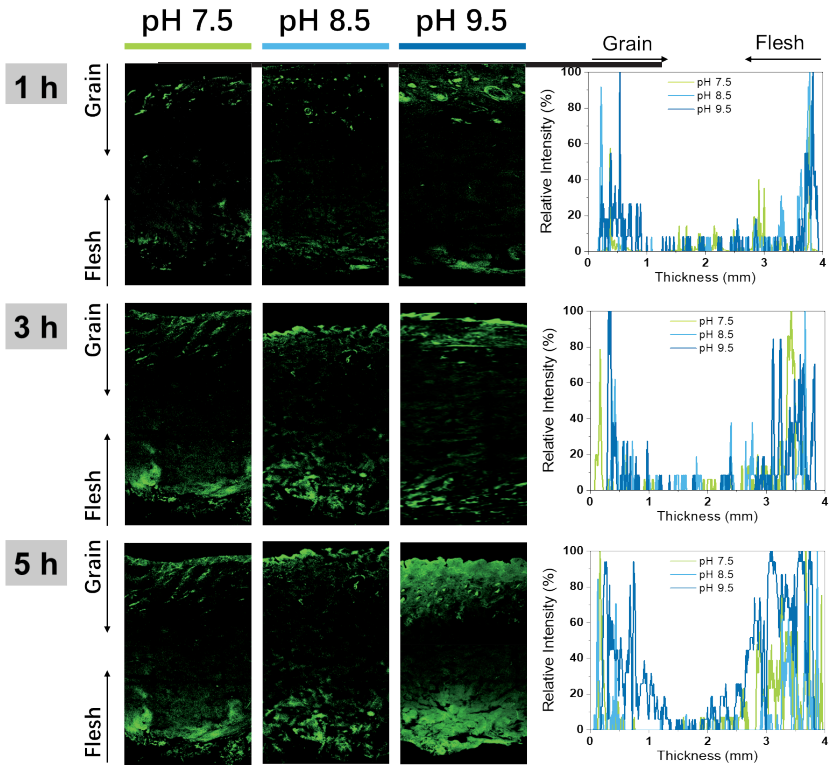


Figure 7. Permeation behavior of FITC-P-SG-B at various pH values

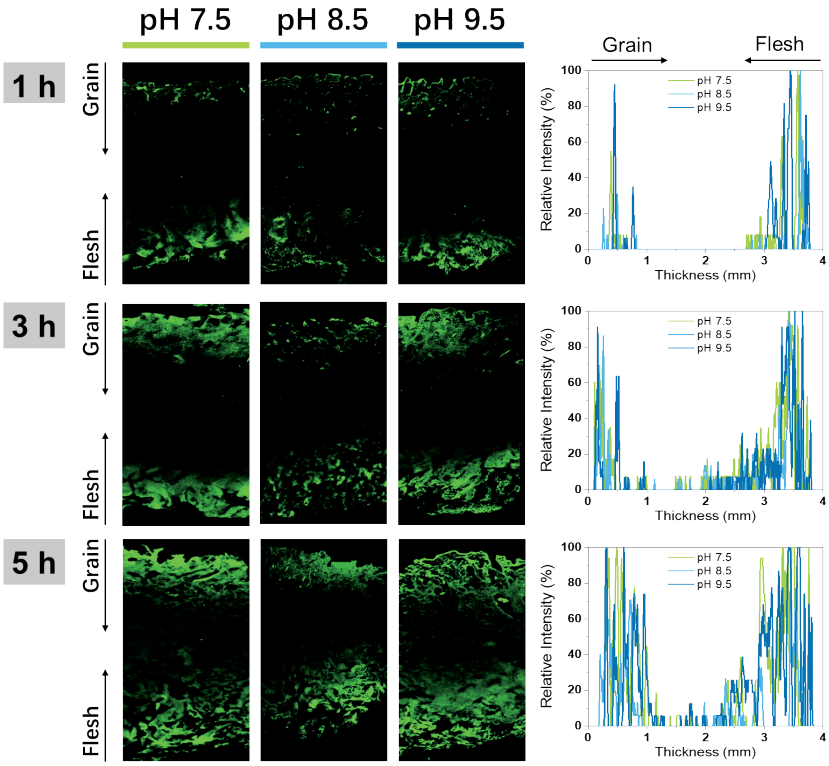


Figure 8. Permeation behavior of FITC-P-PTN110-T at various pH values

The pI of FITC-P-NB115-T was found to be 9.6. At pH 7.5, both protease and collagen carried a large number of positive charges, resulting in strong electrostatic repulsion. However, at pH 8.5 and 9.5, between the pI of the protease and collagen, the protease was positively charged while the collagen was negatively charged, resulting in electrostatic attraction. Therefore, the protease has better permeability at pH 7.5.

The three proteases have comparable molecular weights and particle sizes but different pI values. The apparent difference in their permeation behavior into pelts at different pH values is mainly due to the difference in affinity between protease and collagen due to their different charge states. As a result, the oppositely charged protease protein and the collagen fiber create a strong electrostatic attraction, which causes the protease to tend to remain on the surface

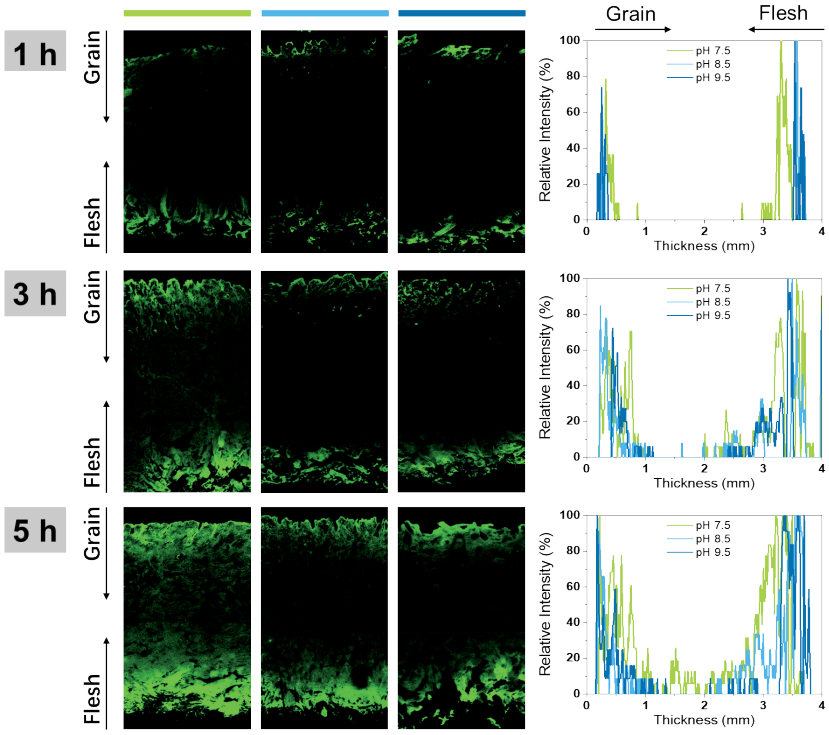


Figure 9. Permeation behavior of FITC-P-NB115-T at various pH values

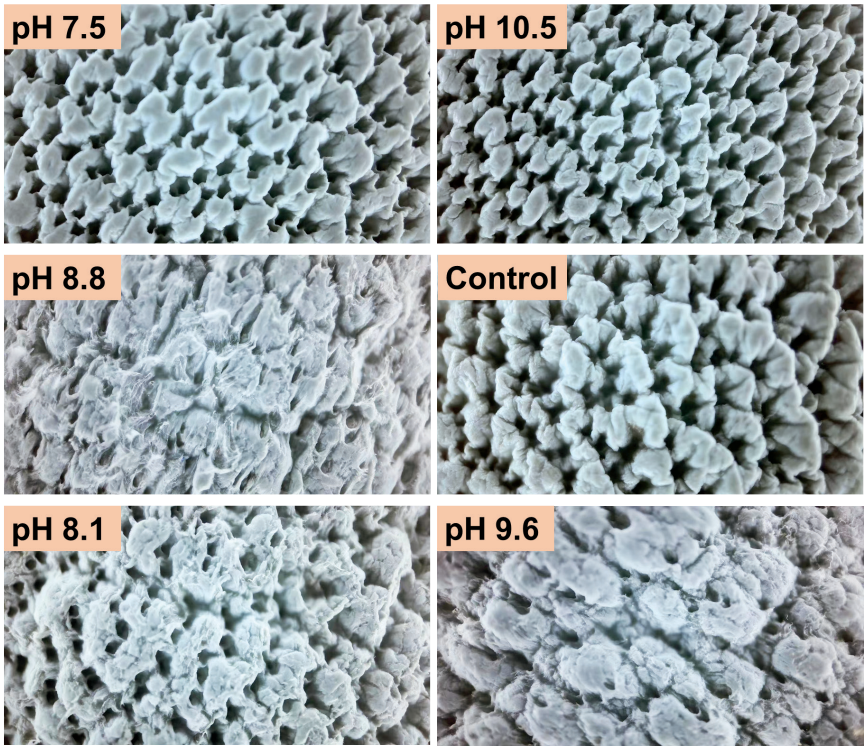


Figure 10. Grain surface of the crust leather after bated at different pH values

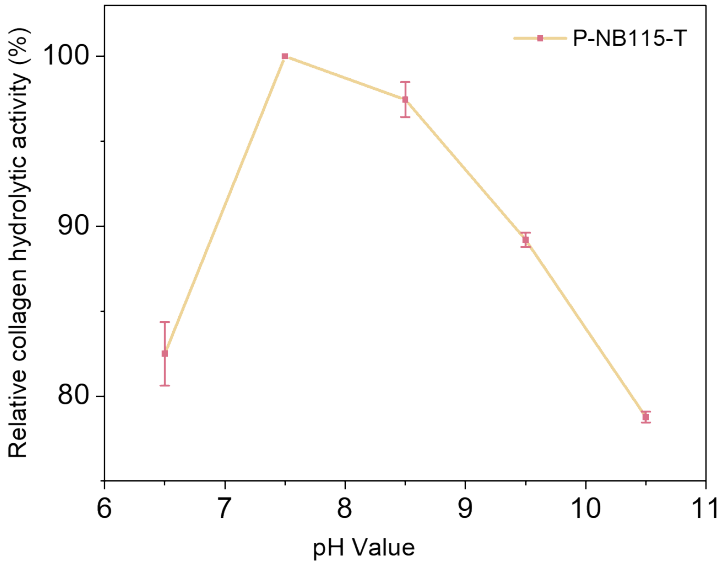


Figure 11. Relative collagen hydrolysis activity of P-NB115-T at different pH values

of the pelts, impeding its penetration. Conversely, if the proteins are similarly charged, the electrostatic repulsion reduces the attraction and facilitates the permeation of the proteases.

Effect of pH on the surface of the pelts during enzymatic bating

Bating experiments were carried out with unlabeled protease P-NB115-T to verify the results of the above permeation experiments using fluorescein-labeled proteases. The delimed pelts were bated for 6 hours at the same protease dosage and temperature under different pH conditions, followed by conventional pickling, Cr-tanning and post-tanning operations, the grain surface of the crust leather is shown in Figure 10.

The results in Figure 10 show that the grain surface of the crust leather after being bated at different pH values is significantly different. The grain surface of the pelt bated at pH 7.5 and 10.5 was intact with no damage, whereas the pelts bated at pH 8.1, 8.8 and 9.6 showed significant damage, particularly at pH 8.8. Figure 11 shows that the collagen hydrolysis activity of P-NB115-T decreases with the increasing of pH value in the range of 7.5 to 10.5. At the same

protease dosage, pH 7.5, with higher collagenolytic activity, resulted in weak hydrolysis of the grain surface collagen and left the grain intact; whereas pH 8.1, 8.8, and 9.6, with lower collagenolytic activity, resulted in apparent hydrolysis of the grain surface collagen and caused grain damage. Apparently, the hydrolysis of grain surface collagen was related to the permeation behavior of proteases in the pelts bating process.

At pH 7.5 or 10.5, P-NB115-T (pI 9.6) and delimed pelts (pI 8.09) were similarly charged (positively or negatively), and the electrostatic repulsion between the protease and the collagen promoted the permeation of the protease into the inner layer of the pelts and avoided the accumulation of the protease on the surface, resulting in weaker hydrolysis of the grain surface. At pH 8.8, the protease and delimed pelts are oppositely charged, the strong electrostatic attraction between the proteins facilitated the accumulation of protease on the grain surface and impeded its permeation into the inner layer, resulting in grain damage. At pH 8.1 and 9.6, corresponding to the pI of the collagen fiber and the protease, the electrostatic interaction is somewhere between the above two situations, and the protease permeation and the state of the grain surface are also somewhere between them. The results of the softness and organoleptic properties of the crust leather showed that the crust leather treated at pH 7.5 and 10.5 had better softness and uniformity than the samples treated at pH 8.8, which was attributed to the permeation of proteases into the inner layer and the uniformity of the bating process.

Charge regulation mechanism of protease permeation in delimed pelts

From the above results of the labeled and unlabeled protease permeation experiments, it can be concluded that the permeation of protease within the delimed pelt is significantly influenced by the electrostatic interactions between the collagen and protease proteins. And the interactions depend on the charge characteristics of the collagen and protease proteins, which are determined by both the pH of the solution and the pI of the proteins.

Various electrostatic interactions may occur between proteases and delimed pelts, such as (1) Strong electrostatic repulsion: as shown in Figure 12a, when the pH value of the solution is higher or lower than the pI values of both the protease and the delimed pelts, the protease and the delimed pelt are similarly charged, resulting in strong electrostatic repulsion. This repulsion is further enhanced by the mechanical force of the drum. Therefore, the protease molecules could easily permeate into the inner layers of the delimed pelt via the hair follicles and interfibrillar pathways, leaving only a small amount of protease on the surface layer, thereby avoiding the excessive degradation of grain collagen fibers. (2) Strong electrostatic attraction: as shown in Figure 12b, when the pH of the solution is between the pI of the protease and the delimed pelt, the protease and collagen carried opposite charges, resulting in strong electrostatic attraction; Therefore, the protease proteins tend to adhere and accumulate on the surface of the pelts, reducing the permeation rate and depth of the protease into the pelt, resulting in a damaged grain surface and inadequate opening-up of the inner layer. (3) Weak electrostatic interaction: when the pH value of the solution is close to the pI of either the protease or the collagen, as shown in Figure 12c, the electrostatic interaction between them is slight. Hence, the permeation performance of the protease into the pelt and the degree of action on the grain surface is intermediate between the two cases discussed above.

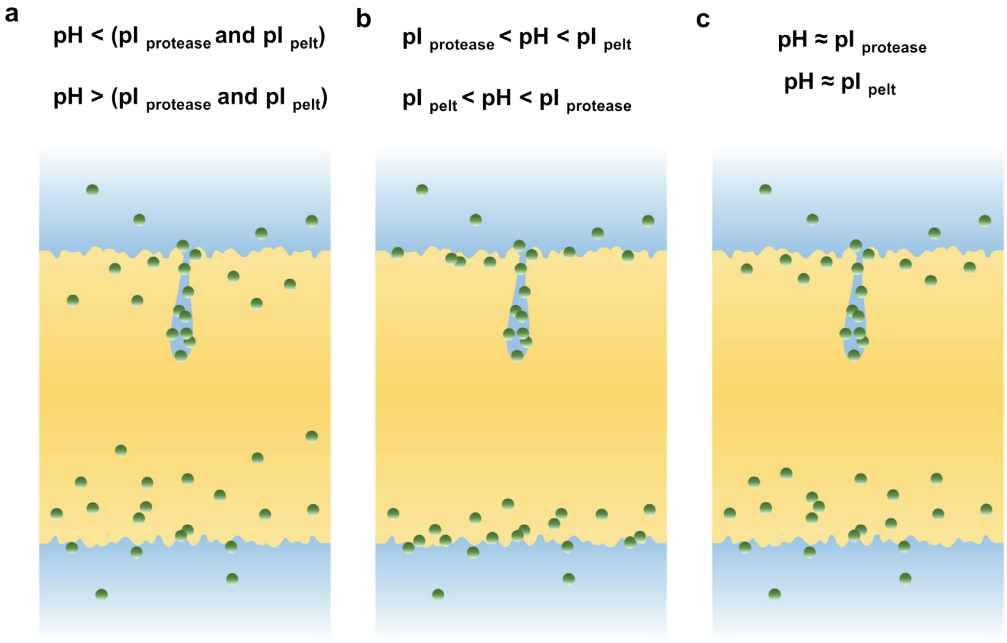


Figure 12. Schematic of the charge regulation of protease permeation into the pelts

Conclusion

The permeation behavior of typical proteases in the delimed pelts, the relative size of proteases and pelt pores, and the electrostatic interaction between protease molecules and collagen fibers were studied. The results indicated that, after dehairing, liming and deliming operations, which remove hair and interfibrillar substances, the enzyme can easily permeate into the pelts due to the formation of large cavities and interfibrillar gaps. The electrostatic interaction between protease and collagen proteins plays an important role in the permeation of protease into the delimed pelts. When the protease and collagen proteins are similarly charged, with electrostatic repulsion and weak affinity between the proteins, the proteases can easily permeate into the pelts; when the protease and collagen proteins are oppositely charged, with less electrostatic attraction, the permeation of protease into the pelts is difficult, which usually results in damage to the grain surface. Therefore, according to the electrostatic regulation mechanism of protease permeation in the pelt bating process, the permeation behavior of proteases can be regulated by selecting protease with appropriate pI and adjusting the pH value of the bating solution.

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