Removal of Calcium from Pelt During Bating Process: An Effective Approach for Non-ammonia Bating

by

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Abstract

Ammonium salts can improve the performance of bating by trypsin. But it is one of the main origins of ammonia nitrogen (NH_3-N) in tannery wastewater. In this study, the action mechanism of ammonium sulfate (AS) on trypsin bating was investigated. It was found that the activity of trypsin was actually not influenced by AS (1~30 g/L) or pH variation caused by the addition of AS. In fact, the activity of trypsin was partially inhibited by calcium remained in pelts, particularly those concentrated in the grain layer. The main function of AS in bating is to remove calcium so as to maintain the proteolysis activity of trypsin. Based on these findings, several calcium chelating agents, including sodium hexametaphosphate and the composite of sodium citrate and citric acid, were employed in the trypsin bating process. The extents of calcium removal from pelts by using these chelating agents were higher than AS, and the bating performances were better in terms of protein and hydroxyproline contents in bating liquor.

Introduction

Ammonia nitrogen (NH_3-N) is one of the most problematical pollutants in industrial effluents. Excessive discharge of NH_3-N would lead to eutrophication of aquatic ecosystems and impair the survival of aquatic animal.[1] Conventional tannery wastewater is commonly rich in NH_3-N, which results in the disproportion between carbon and nitrogen in wastewater, and hence increases the difficulty of biochemical treatment.[2] Our previous research has confirmed that deliming and bating processes are mainly responsible for NH_3-N pollution in tannery wastewater due to the use of ammonium salts.[3] The best approach to solve this problem is undoubtedly to discard ammonium salts at the origin. Up to now, many non-ammonia deliming methods have been developed, such as CO_2 deliming,[4] magnesium salts deliming,[5] weak acids deliming,[6] peracetic acid deliming,[7] and carbonate deliming.[8] In our earlier research, the combination of sodium hexametaphosphate (SHMP) and boric acid had been employed in deliming successfully. The addition of SHMP can remove calcium from limed pelt and theoretically favor the treatment of wastewater.[9] However, there are few reports concerning non-ammonia bating technology.

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Manuscript received October 18, 2012, accepted for publication January 7, 2013

The bating process is generally performed to remove non-collagenous proteins from pelt, open up collagen fiber network and make the pelt soft and smooth for the purpose of tanning.[10,11] Trypsin is regarded as the best bating enzyme due to its moderate proteolytic activity and safety,[12] and a mixture of trypsin and ammonium salts is commonly used in bating to obtain a better performance as reported in literature.[10,13] Although bating with trypsin and ammonium salts has been employed for many years, its scientific principle is still received much attention recently. Some researchers have focused on the action mechanism of trypsin on skins and discussed whether elastic fibres are removed after conventional bating process,[14,15] while some have shown interest in the development of better bating systems by replacing trypsin with other enzymes.[16,17] However, the action of ammonium salts on bating has not been clearly explained although it is usually considered that the main functions of ammonium salts are to activate trypsin to some extent and buffer the pH of bating bath. Therefore, we investigated the mechanism of ammonium sulphate (AS) on enhancing trypsin bating in this study. After figuring out the real mechanism, the strategy of eliminating NH_3-N from bating is suggested through replacing ammonium salts by other substances.

EXPERIMENTAL

Materials

Wet salted cattle hides were used as raw materials. The chemicals used for leather processing were of commercial grade. Conventional soaking and liming were performed at first. The limed pelts (thickness 5~6 mm) were split to obtain the upper layers (grain layer, thickness 2.3~2.5 mm). Then they were cut into 20 cm × 20 cm pieces and delimed by using 1.4% non-ammonia deliming agent and 0.4% non-ionic degreasing agent (based on lime weight) at 32°C for 2 h, where the non-ammonia deliming agent is a composite of boric acid, citric acid and sodium citrate in the weight ratio of 2 : 1 : 1. The final pH of the deliming float was approximately 8.3. After washing for 5 min, the delimed pelt pieces were prepared for the following bating trials. Trypsin used for bating was purchased from Luliang Chemical Co. Ltd (Yunnan, China), and no NH_3-N was detected in it. AS, SHMP, sodium citrate (SC) and citric acid (CA) used for bating and the other chemicals used for analysis were of analytical grade.

Trypsin Activity Assay

Determination of Trypsin Activity

The activity of trypsin was determined by UV Spectrophotometry according to the national standard.[18] The 3 mg/mL trypsin solution and 2% (w/v) casein solution were prepared using 50 mmol/L Tris-HCl buffer (pH 8.3) as solvent. Then 1 mL of trypsin solution and 1 mL of casein solution were mixed and incubated at 32°C for 10 min. The incubation was stopped by adding 2 mL of 0.4 mol/L trichloroacetic acid (TCA). The mixture was settled at 32°C for 20 min and successively filtered. The absorbance of the filtrate was measured at 275 nm by UV Spectrophotometer (UV2501PC, Shimadzu, Japan). Then the concentration of tyrosine was calculated. Blanks were also prepared in the same way except that TCA was added prior to casein solution. One unit of trypsin activity is defined as the amount of enzyme releasing 1 μg tyrosine per min at 32°C.

Effect of AS Concentration on Trypsin Activity

The trypsin solutions were prepared by dissolving 0.3 g trypsin and X g AS (X= 0, 0.1, 0.3, 0.5, 1, 3, respectively) in 100 mL Tris-HCl buffer (pH 8.3). It should be noted that the concentrations of trypsin and AS were set on the basis of practical bating system, similarly hereinafter. Then the activity of trypsin was measured as above.

Effect of pH on Trypsin Activity

Trypsin activity was determined over the pH range of 7.5~8.5 in 50 mmol/L Tris-HCl buffer with pH interval of 0.2. The other operations were the same as the description above.

Effect of SHMP, SC or CA Concentration on Trypsin Activity

For investigating effect of SHMP on trypsin activity, 0.3 g trypsin and X g SHMP (X= 0, 0.2, 0.5, 1, respectively) were dissolved in 100 mL Tris-HCl buffer (pH 8.3). For investigating effect of SC on trypsin activity, the trypsin solutions were prepared by dissolving 0.3 g trypsin and Y g SC (Y= 0, 0.1, 0.3, 0.5, 1, respectively) in 100 mL Tris-HCl buffer (pH 8.3). For investigating effect of CA on trypsin activity, 0.3 g trypsin and Z g CA (Z= 0, 0.02, 0.06, 0.1, 0.2, respectively) were dissolved in 100 mL Tris-HCl buffer (pH 8.3).Then the activity of trypsin was measured as mentioned above.

Bating Trials

Conventional Bating with AS

The delimed pelts (dimensions 20 cm × 20 cm) prepared as described in Materials section from matched areas were bated using 0.3% trypsin, X% AS (X= 0, 0.3, 1, 3, respectively) and 100% water at 32°C for 1 h. The percentages of offer were based on lime weight. At the same time, the bating floats were sampled for protein and hydroxyproline (Hyp) determinations.

Non-ammonia Bating with Calcium Chelating Agents

Four groups of bating trials were performed under the same conditions as mentioned above except that calcium chelating agents were used in two of the four groups instead of AS. The bating recipes are shown in Table I. The offer of the calcium chelating agents had been optimized in our preliminary experiments. Bating liquors were sampled for protein and hydroxyproline (Hyp) determinations. At the same time, the calcium contents of delimed and bated pelts were determined.

TABLE I
Bating recipe.*

Group	Bating agents (based on lime weight)
1 (control)	0.3% trypsin
2	0.3% trypsin + 1% AS
3	0.3% trypsin + 1% SHMP
4	0.3% trypsin + 1% SC + 0.05% CA

* The offers of bating agents are the optimized amounts based on our preliminary experiments.

Analytical Methods

Determination of Calcium Content of Delimed and Bated Pelts[9,19]

Delimed and bated pelt samples (approx. 10 g) were cut into small pieces and dried to constant weight (Wc) at 102°C. Then they were calcined at 700°C for 8 h in a muffle furnace. The ash obtained was mixed with 10mL of 2 mol/L HNO_3, then heated at 110°C for 5 h and filtrated. The concentration of calcium in filtrate was measured by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Optima 2100DV, PerkinElmer, USA). The calcium content in pelt and the calcium removal extent after bating were calculated as:

$$\text{calcium content} = \frac{\text{weight of calcium in pelt}}{\text{Wc}} \times 100\%$$

$$\text{calcium removal extent} = \frac{\text{calcium content of delimed pelt} - \text{calcium content of bated pelt}}{\text{calcium content of delimed pelt}} \times 100\%$$

In addition, a part of the pelts were lyophilized and finely split into three layers (thickness of each layer 0.7 mm) by splitter (C520L, Camoga S.p.A, Italy). The calcium content of each layer was also determined.

Energy Dispersive X-ray Spectroscopy Analysis

Samples from delimed and bated pelts were lyophilized and then their histological cross sections were obtained. The cross sections were observed by Scanning Electron Microscope (SEM, JSM-7500F, JEOL, Japan), and elemental mapping of the cross sections was performed by Energy Dispersive X-ray Spectroscopy (EDS, INCA X-MAX 50, Oxford Instruments, UK) to detect the distribution of calcium (Ca) and carbon (C) on the cross sections.

Determination of Protein Concentration in Bating Float

The bating liquor samples were centrifuged at 8000 rpm for 6 min. The protein concentration in supernatant was measured by the method of Lowry using bovine serum albumin as a standard.[20]

Determination of Hyp concentration in Bating Float

The bating liquor were filtered by using 100 mesh filter cloth, and their Hyp concentrations were determined following standard procedures.[21]

RESULTS AND DISCUSSION

Performance of Bating with AS

Table II is the effect of AS on bating performance. We can see that the protein and Hyp concentrations in bating float in the presence of AS are all higher than those in control. This means the addition of AS can improve the removal of non-collagenous protein and denatured collagen, and hence benefit the opening up of the fiber network. In fact, it was observed that the bated pelts were softer and smoother when AS was used. These facts demonstrate that AS does favor the trypsin bating process.

TABLE II
Effect of AS offer on bating performance.

AS offer (%)	Concentration in bating float (mg/L)		pH of bating float
	Protein	Hyp	
0 (control)	1133	29	8.24
0.3	1573	39	8.17
1	1796	53	8.11
3	1465	45	7.98

Investigation of the Action Mechanism of AS on Bating

To explore the role of AS in bating, the effect of AS on the activity of trypsin was first studied. Figure 1 shows that the activity of trypsin is relatively stable as AS concentration increases even up to 30 g/L. Therefore, the enhanced bating performance is not attributed to activation of trypsin by AS. Furthermore, the effect of pH variation on trypsin activity was also considered because the pH of the bating float slightly decreased when AS was used (see Table II). The effect of pH on trypsin activity is shown in Figure 2. The result indicates that the pH variation in the range of 7.5 to 8.5 hardly affects the activity of trypsin. This implies that the pH variation caused by AS is not the exact reason for the improved bating performance as well.

The results in figure 1 and 2 demonstrate that AS does not influence the trypsin activity directly. So the investigation focused on the interaction of AS with calcium. As we know,

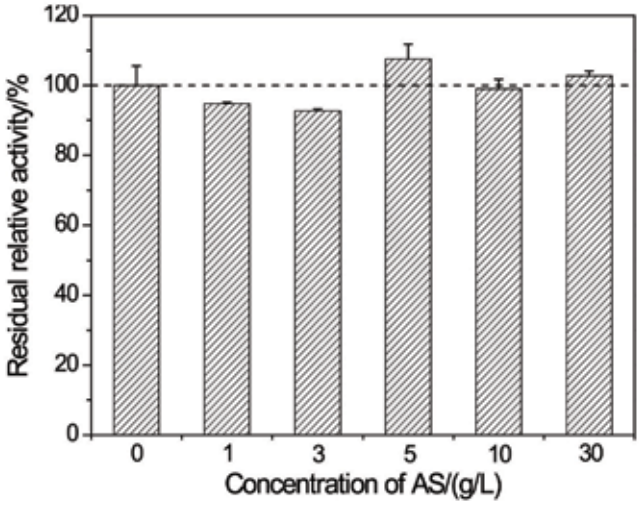

Figure 1. Effect of AS on trypsin activity.

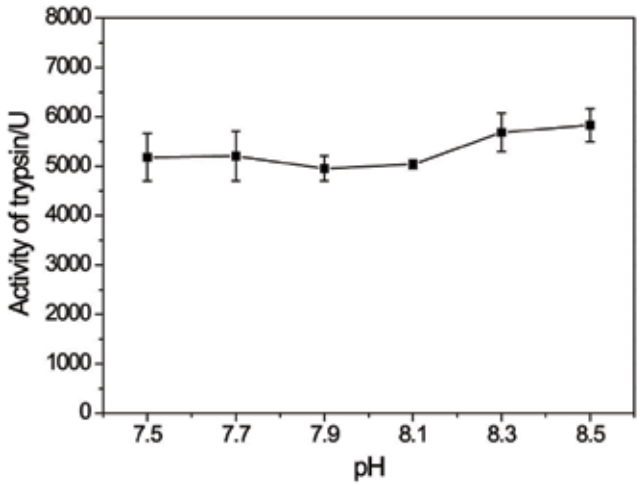

Figure 2. Effect of pH on activity of trypsin.

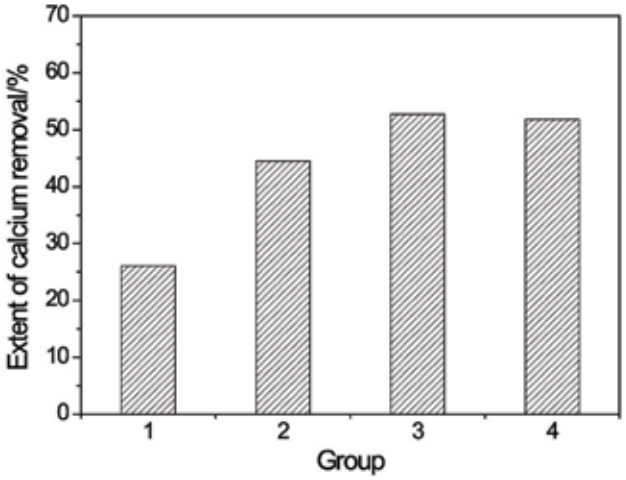

Figure 3. Extent of calcium removal from pelts using different bating systems.

calcium in the pelt cannot be completely removed during deliming. Our previous work has found that the grain layer of delimed pelt remained 0.2%~0.4% calcium content (based on dry weight of pelt) after deliming with AS or non-ammonia deliming agents.[9] So it is likely that calcium will be further removed in the following processes. AS is commonly used in deliming to reduce the pH of pelt and remove calcium from pelt. To prevent interference, non-ammonia deliming agent was used in deliming in this study. Then four groups of delimed pelts were bated according to the recipes in Table I, respectively. The results indicated that the use of AS in bating could largely improve the removal of calcium compared with the control (group 1 and 2 in Figure 3). Therefore, the relation between calcium removal and bating performance can be established. Fullmer et al reported in 1975 that the calcium-binding protein was resistant to proteolysis by trypsin in the presence of excess calcium.[22] This may explain the reason that the removal of Ca by AS can improve the bating performance. The EDS elemental mapping of C and Ca on the cross section of delimed pelt is shown in Figure 4(a). The mapping of C is clear and intensive since carbon is the richest element in pelt. By contrast, the signal of Ca is much weaker due to its extremely low content. But it is still obvious that Ca is mainly distributed on the grain layer. This fact is further proved by the quantitative determination of Ca content in each layer of the delimed pelt (Figure 5). This would probably hinder trypsin from acting on hide protein because grain layer is regarded as the place where bating occurs.[23] AS plays a significant role in removing Ca in the grain layer, as shown in Figure 5(b), and the total Ca content in pelt is reduced (Figure 3). This may be a key point for trypsin to achieve satisfactory bating effect. So our investigations suggest that the enhanced bating performance of trypsin in the presence of AS is mainly attributed to the good Ca removing ability of AS.

Non-ammonia Bating using Calcium Chelating Agent

The results in figures 4, 5 and 6 have demonstrated that the key role of AS in bating is to remove Ca from pelt. This suggests that chemicals that have similar ability to remove Ca from pelt may play the same role as AS. Therefore, several calcium chelating agents, viz. SHMP, and the composite of SC and CA (SC-CA) were chosen to substitute AS in bating. Figure 6 shows that SC and CA do not influence the activity of trypsin, whereas SHMP reduces approx. 20% of trypsin activity. From Figure 3 we can see that the extent of Ca removal from pelt using SHMP or SC-CA is higher than that bating with AS. EDS elemental mapping and layer distribution of Ca illustrate that these calcium chelating agents removed the remaining Ca on the grain layer of delimed pelts effectively (Figure 4 and 5). Based on these results, it could be inferred that the bating performances using calcium chelating agents

should be better than that using AS. This speculation was confirmed by analyses of protein and Hyp concentrations in bating floats using different bating systems, shown in Table III. Trypsin produced considerably stronger hydrolysis action for non-collagenous proteins in the presence of SHMP or SC-CA, which once again demonstrated that removal of Ca favors the bating action of trypsin. Moreover, the smoother and softer bated pelts were obtained by using the non-ammonia calcium chelating agents. Therefore, AS can be substituted by non-ammonia calcium chelating agents, such as SHMP and SC-CA, which benefits the elimination of ammonia nitrogen.

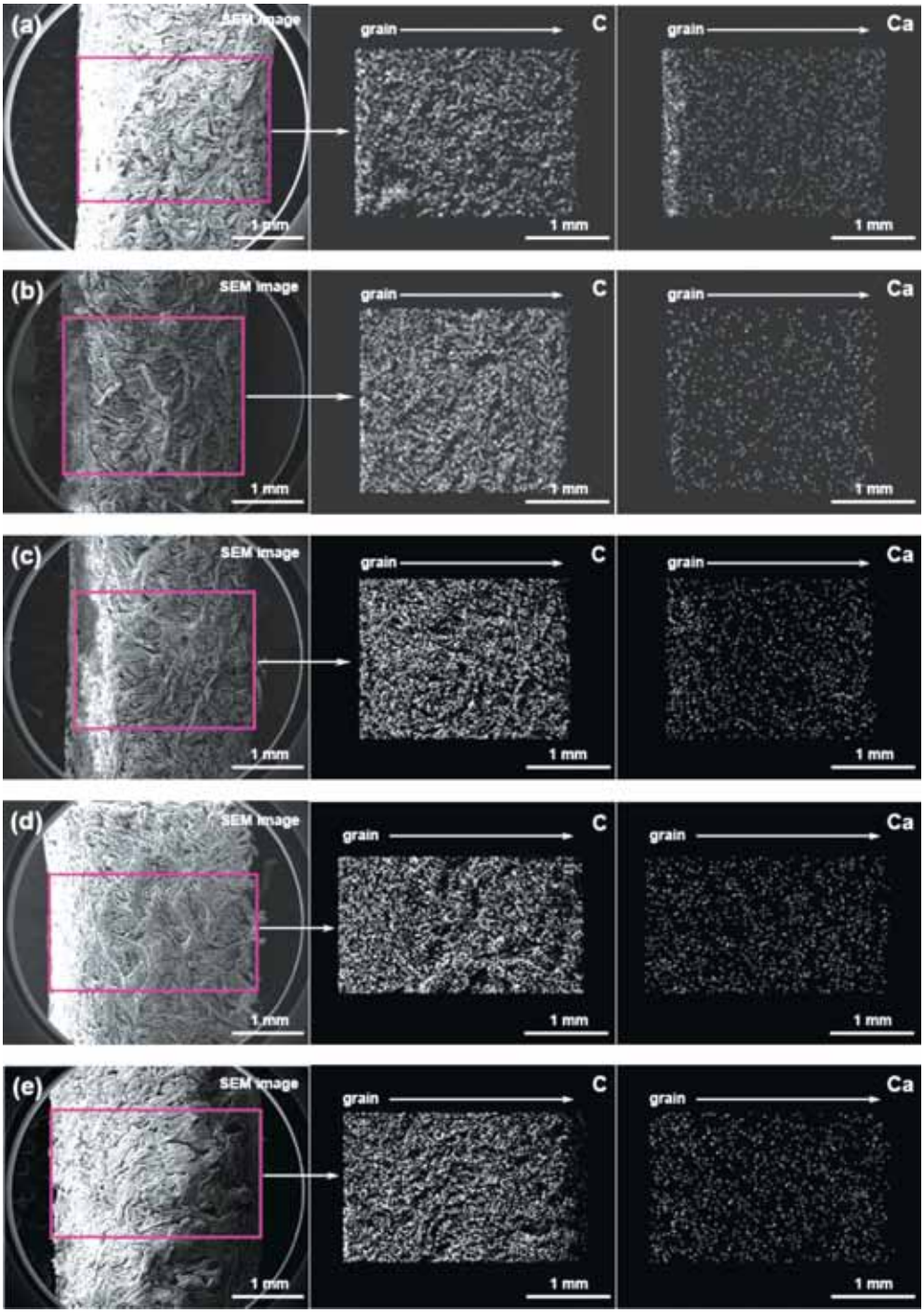

Figure 4. SEM image and EDS elemental mapping of C and Ca on cross sections of pelts: (a) delimed pelt; (b) bated pelt of group 1; (c) bated pelt of group 2; (d) bated pelt of group 3; (e) bated pelt of group 4.

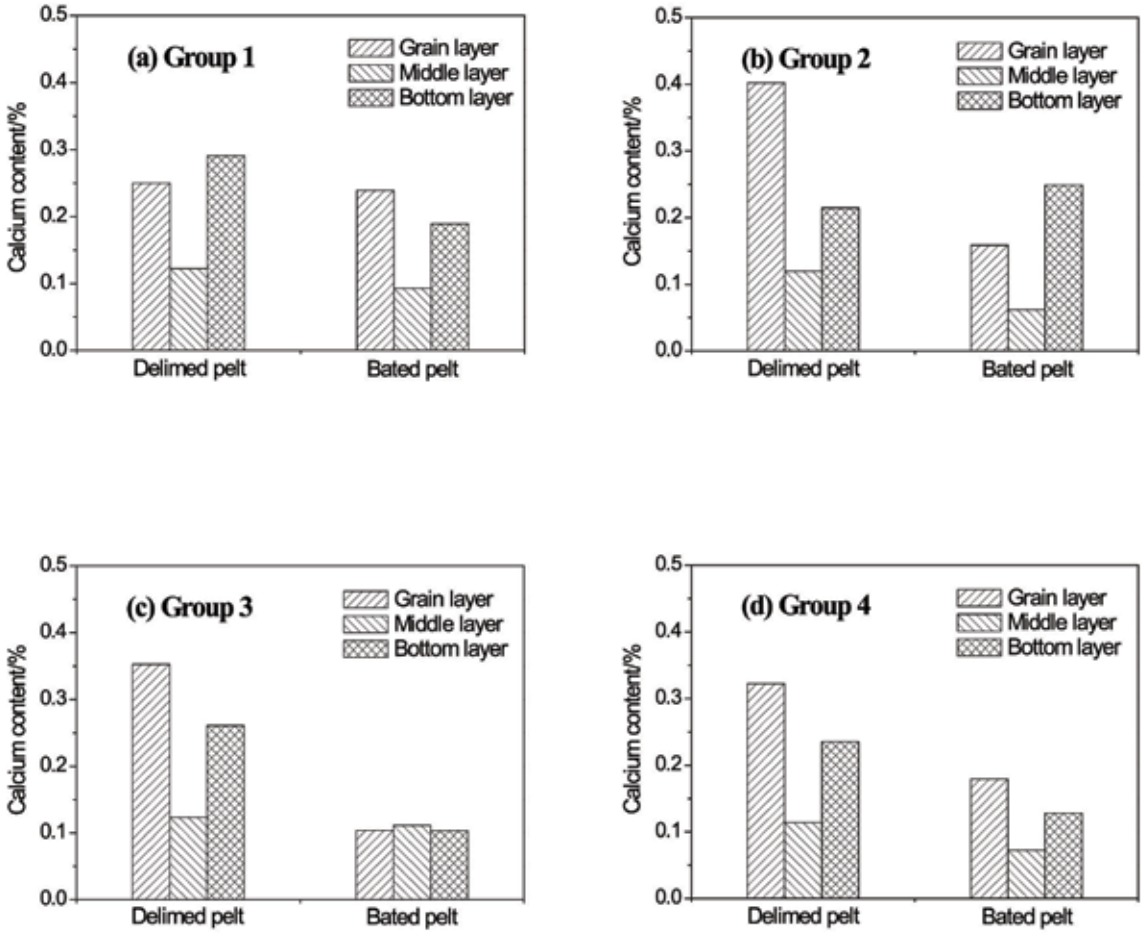

Figure 5. Layer distribution of calcium in delimed and bated pelts.

CONCLUSIONS

In the bating process, AS is effective in removing calcium from the pelt, particularly the calcium in the grain layer, rather than activating trypsin. The removal of calcium improves the proteolysis of hide proteins by trypsin and results in satisfactory bating effect. Calcium chelating agents, such as SHMP and SC-CA, perform even better than AS in removal of calcium from pelt. As a result, the non-ammonia calcium chelating agents can be considered as a potential substitute for ammonium salts.

ACKNOWLEDGEMENT

This project is financially supported by the National Key Technologies R&D Program (2011BAC06B11) and the Program for New Century Excellent Talents in University (NCET-11-0358).

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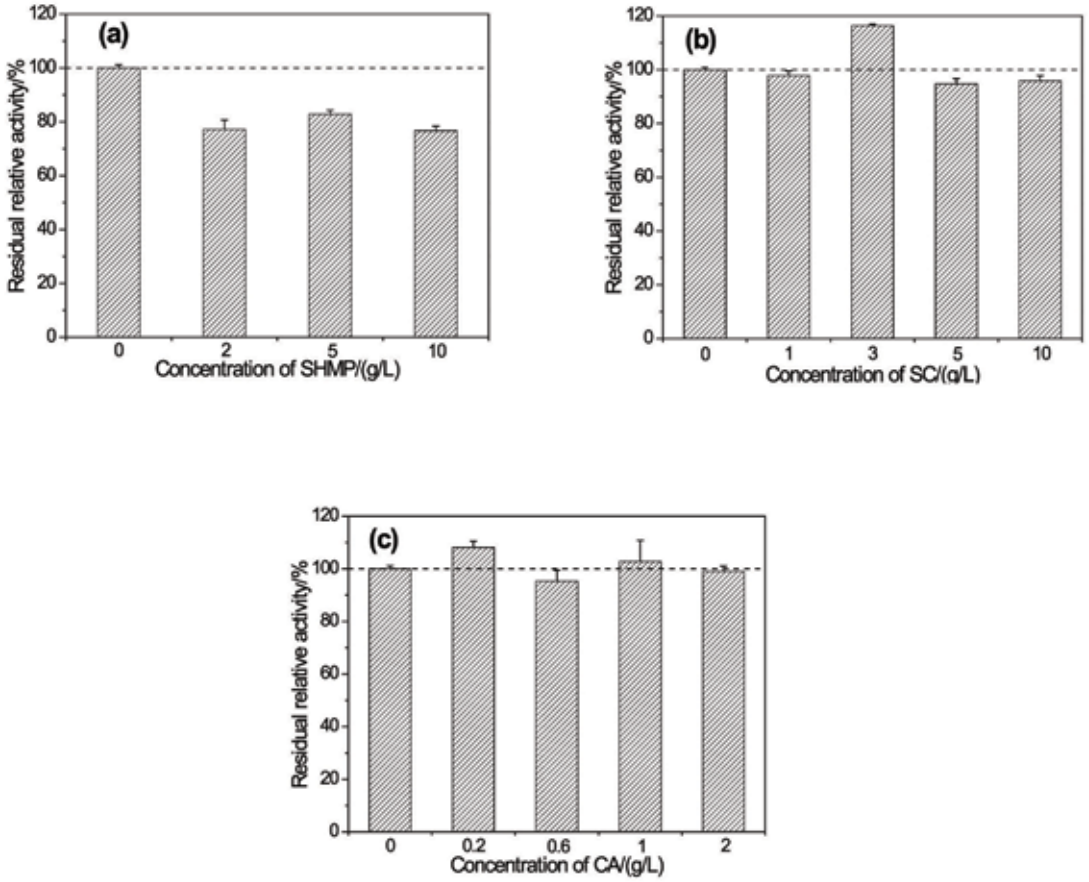

Figure 6. Effect of chemical agents on trypsin activity: (a) SHMP; (b) SC; (c) CA.

TABLE III
Comparison of bating performances using different bating systems.

Group	Calcium removing agent	Concentration in bating float (mg/L)		pH of bating float
		Protein	Hyp	
1	None	1213	39	8.48
2	AS	1314	33	8.12
3	SHMP	2340	35	8.15
4	SC-CA	2181	40	8.24

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