# Approach Towards Safe and Efficient Enzymatic Unhairing of Bovine Hides

by

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## Abstract

Unhairing is an essential step in leather manufacturing. The traditional hair-burning technology brings huge sulfide pollution and large COD, BOD in the effluent. Hair-saving enzymatic unhairing is a popular method for displacing sulfide unhairing. However, some of the problems associated with enzymatic unhairing are need for greater control over process parameters and possibility of grain damage to the pelt. To overcome these challenges, an attempt has been made to utilize bivalent metal ions for safe and efficient enzymatic unhairing on bovine hides. The effect of metal ions such as Ca(II), Mg(II), Mn(II), and Zn(II) ions on the activity of AS1.398 protease for hydrolyzing collagen and casein was investigated. The findings were corroborated with the bovine hide unhairing and the resultant leathers were of appreciable quality. The outcomes of this study could add to the knowledge of enzymatic unhairing leading to cleaner leather manufacturing in a safe and efficient way.

## Introduction

Sodium sulfide and lime are commonly used in traditional hair-burning processes. The wastewater produced in this step contains significant high levels of COD, BOD and sulfide ions contaminations.[1] Hydrogen sulfide, a highly toxic gas, may be generated in the following beamhouse procedures. Use of lime leads to the generation of huge amount of solid sludge discharged as well. Enzymatic unhairing has been proposed as an important alternative cleaner method.[2] Since it is a hair-saving process and sulfide free, enzymatic unhairing does not destroy the hair, thus there will be huge reduction in the BOD, COD, and sulfide ions of effluent water. In addition, enzymatic unhairing process also opens up the collagen fibers, consequently bating process usually could be skipped. A number of proteases from bacterial, fungal organisms have been studied for enzymatic unhairing.[3-5] However, the enzymes used for industrial applications cause some damage to the dermal collagen fiber, leading to grain damage. Moreover, the enzymatic unhairing process is difficult to be handled by the beamhouse workers.

Most commercial enzyme preparations for unhairing have multiple catalytic components, such as for cleaving casein, collagen and elastin.[6] It is well known that natural collagen molecules could not be cleaved by any enzymes except collagenase, which is a member of the family of zinc containing matrix metalloproteinases (MMPs).[7] For instance, collagenase type I (MMP-1) cleaves fibrillary collagens type I, II and III into characteristic 1/4 fragment and 3/4 fragment.

The enzymatic unhairing mechanism has revealed that the collagenase components play a critical role in the grain damage and sueding of upper leathers.[8] Therefore, the inhibition or reduction of the collagenase components in enzymatic unhairing process may be a predominant proposal to the achievement of a safer and more efficient enzymatic unhairing. In our previously study, cattle hide enzymaticly unhaired at 20-25°C is much safer than at higher temperatures,[9] while its unhairing duration lasted approximately 10-20 h, which is much longer than that of at higher temperatures (3-4h).

Microbial collagenases are multi-domain zinc-containing proteins consisting of four segments. Collagenase could be inhibited by certain kind of materials.[10-12] The main feature of collagenase inhibitors is the inclusion of zinc binding ligands with their zinc ions play a critical role in cleaving of collagen. However, it has also reported that excess metal ions could change the conformation of the enzymes thus causing its activity to be inhibited.[13]In this study, the influence of metal ions such as Ca(II), Mg(II), Mn(II) and Zn(II) to commercial AS1.398 protease on hydrolyzing of collagen fiber and casein were investigated. And based on these results, Mg(II), Mn(II) and Zn(II) ions were introduced into the enzymatic unhairing process to inhibit collagenase. The role of metal ions in enzymatic unhairing process was investigated.

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## Materials and Methods

### Materials

AS1.398 protease preparations were purchased from Luliang Biotech Company (Yunnan, China) without further purification process. Collagenase type I was purchased from Sigma. Bovine hide were purchased from local tannery. $CaCl_2$, $ZnCl_2$, $MnCl_2.4H_2O$ and $MgCl_2.6H_2O$ were purchased from KeLong Chemicals (Chengdu, China). Folin-phenol reagent was prepared in the lab. Ninhydrin, hydroxyproline, isoleucine, Bovine serum albumin (BSA), Tris-base were biological pure, and other chemicals were analytical grade and used as received.

### Enzymatic Activity Assays

*Collagen fiber as substrate*: A reaction mixture consisting of 3.8 mL of 50 mM Tris-HCl buffer (pH 7.5) and 10 mg of insoluble collagen fibers was incubated at 40 °C for 5 min, after which 200μL of 0.5 mg/mL AS1.398 protease solution was added with gentle shaking. The reaction kept at 40 °C for 60 min. The reaction was then terminated by adding 1 mL of 0.1M acetic acid. The collagenase activity was tested as the following: 0.5 mL of the supernatant of the reaction solution was mixed with 1 mL of 1% ninhydrin solution with vigorous mixture. Then the samples were incubated at boiling water for 15 min, after cooling to ambient temperature, it was calibrated to 25 mL by using double distilled water. The optimal density was measured by a UV-vis spectrophotometer (Ruili Analytical Instrument, China) at wavelength of 570 nm to determination the concentration of the amino acid based on the previously established calibration curve from standard isoleucine solution. The activity unit was expressed as μmol of equivalent isoleucine released per min per mg protein.

*Casein as substrate:* The hydrolytic activity of AS1.398 protease to substrate casein was determined at 680 nm according to the conventional casein-Folin phenol assay.[14] The enzymatic reaction was carried out by incubating the mixture of enzyme and substrate (pH 7.2) at 40 °C for 10 min. One unit (U) of enzyme activity was expressed as the amount of enzyme which released 1 μmol of tyrosine per min under assay conditions.

All the experiments were performed at least triple. The values used in calculations were the average of the experimental data.

### Effect of Metal Ions to Collagen Hydrolytic Activity

10 mg of insoluble collagen fibers was incubated in the reaction mixture consisting of 3.8 mL of 50 mM Tris-HCl buffer (pH 7.5) and a set concentration of metal ion at 40 °C for 5 min, after which 200 μL of 0.5 mg/mL AS1.398 protease solution was added. The hydrolytic ability of the enzyme under metal ion environment was determined according to the above mentioned enzymatic activity assay. The concentration of metal ions in the reaction was investigated from 0 mM to 10 mM.

The relative activity was used to evaluate the influence of metal ions. It is calculated by Eq. (1):

$$Relative\ activity\ (\%) = \frac{activity\ of\ experimental}{activity\ of\ control} \times 100\% \quad (1)$$

### The Procedure of Enzymatic Unhairing with Metal Ions

The soaked and fleshed bovine hide was cut into 20 cm ×25 cm pieces symmetrically by the backbone, with the longer side parallel to the backbone. The enzyme unhairing process was carried out in drums under 40 °C with 150% float at pH 8.0, adjusted by sodium carbonate. Then a weighed amount of metallic salt (to ensure the concentration of metal ions at 10 mM) was added and ran at 6 rpm for 30 min. After that, AS1.398 protease was used with the dosage of 200 U per gram hide. The drum ran for 60 min with 6 rpm and then it was stopped and then run 5 min every 30 min. The unhairing extend of the samples was observed during the process and the float was taken out periodically for total protein and hydroxyproline (Hyp) determination. The unhaired pelt was then processed by liming, deliming, pickling and chrome tanning according to the conventional process. The flowchart of the process performed is shown in Figure 1.

### Determination of the Total Protein Content

The supernatant of the unhairing solution was diluted to 20 times, and in which total protein content was measured at 750 nm by the Lowry assay,[15] using bovine serum albumin (BSA) as the protein standard.

### Determination of the Hydroxyproline Content

Hydroxyproline content in the supernatant of the unhairing solution was determined at 558 nm by the Chloramine-T method.[16] The hydrolysis degree of collagen is positive related to the Hyp content in the float.

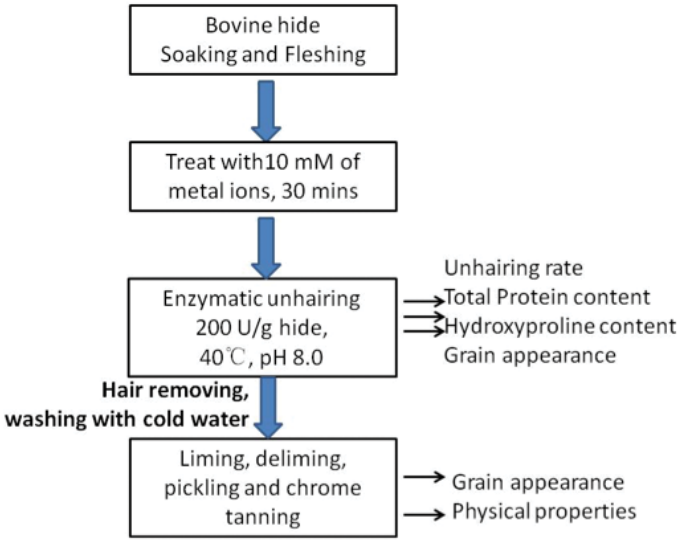


Figure 1. Flow chart of the enzymatic unhairing with metal ions on bovine hide.

**Physical Properties Testing**

Leather samples were conditioned at 20 °C and 65% relative humidity for 48 h, and then their physical properties such as tensile strength, elongation at break and tear strength were determined according to QB/T2726-2005 standard method.

## Results and Discussion

**Effect of Metal Ions to AS1.398 Hydrolyzing of Collagen Fibers**

Different concentrations of Ca(II), Mg(II), Mn(II) and Zn(II) ions were added into the enzymatic hydrolysis system to evaluate its influence for hydrolyzing collagen fibers. The results are shown in Figure 2.

It could be observed that different metal ions displayed different effect for AS1.398 protease hydrolyzing of collagen fibers. When the concentration of the metal ions is below 5 mM, Ca(II), Mg(II) and Mn(II) ions showed the active ability to collagenase component in AS1.398, while Zn(II) ion exhibits inhibition ability to collagenase component in AS1.398. When the concentration of metal ions is higher than 5 mM, Mg(II) and Mn(II) ions begin showing inhibition effect to collagenase component. However, Ca(II) ion still maintained active ability to collagenase component. When the concentration of the metal ions is at 10 mM, it showed 90% (Mn(II)), 65% (Mg(II)), 45% (Zn(II)), and 93% (Ca(II)) collagenase activity compared to the metal ion free system. It means that the presence of Mn(II), Mg(II), and Zn(II) ions at 10 mM causes AS1.398 protease hydrolysis of collagen being inhibited to 10%, 35% and 55% respectively. The catalytic domain of collagenases contains two zinc ions. However, excess metal ions may cause steric hindrance to the active site, this in turn leads to the decrease of catalytic activity.[8] But interestingly, usually three calcium ions are found and play a role in stabilizing its structure,[17] which may be the reason for higher activity of collagenase in the presence of Ca(II) ion than the other metal ions.

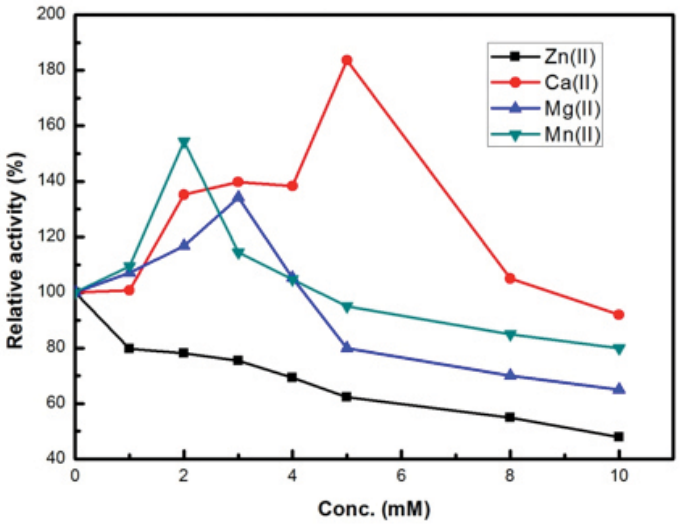


Figure 2. The effect of metal ions on the collagenase activity of AS1.398

**Effect of Metal Ions to AS1.398 Hydrolyzing of Casein**

The effect of metal ions to AS1.398 protease hydrolyzing casein was investigated by adding 10 mM of them into the enzyme solution. The activity of the mixture solution to hydrolysis of casein was determined periodically. The results are shown in Figure 3. It could be observed that the activity of AS1.398 protease to casein decreases gradually after dissolved in solution. The activity decreases 15% after incubating for 10 h. At the first hour of the incubation with the three metal ions, all the metal ions showed the inhibition effect to hydrolyze of casein. Incubation with 10 mM of Zn(II) ion for one hour, the activity of AS1.398 to casein decreased sharply to 82.8%. However, while extending of the incubation time, the inhibition effect of metal ions was gradually lessened. After 2 h, the relative activity reduction trend of the control is similar to that of with metal ions. It indicated that metal ions have a very limited inhibition effect to AS1.398 for hydrolysis of casein.

**Application on Enzymatic Unhairing Process**

The enzymatic unhairing on bovine hide by AS1.398 (200 U/g hide) was carried out under 40 °C with 150% float at pH 8.0 containing 10 mM of metal ion. EDTA is a well-known inhibitor to collagenase due to its significant chelating ability to metal ions. Therefore, as a comparison, 10 mM of sodium EDTA was added in AS1.398 protease unhairing. Unhaired by AS1.398 protease with metal ion free was used as the control. During each unhairing experiment, the float was periodically taken out

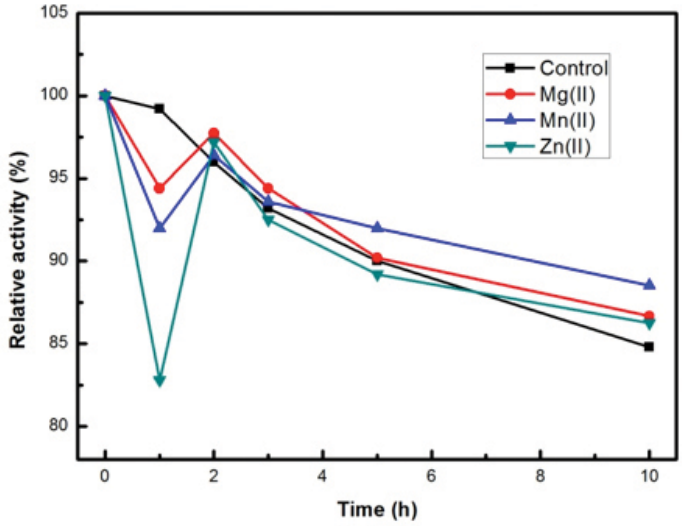


Figure 3. The effect of 10 mM of metal ions on the casein activity of AS1.398

for testing the total protein content and hydroxyproline (Hyp) content. The grain appearance and the unhairing degree were evaluated at the same time. The results are shown in Table I and Figure 4. It showed that metal ions have little influence on the unhairing rate in comparison to the control one. The hair could be removed easily after 3-4 h enzymatic unhairing with Mg(II) and Mn(II) ions, and for Zn(II) ion, the hair could be removed completely in 5 h, which is quite short than normal enzymatic unhairing under room temperature.[9] However, after 10 h, only 60% of hair could be removed for unhairing with EDTA. The grain of the enzymatic unhaired pelt with metal ions is clean and no damage could be observed. It illustrated that the metal ions added in enzymatic unhairing could make the procedure safe

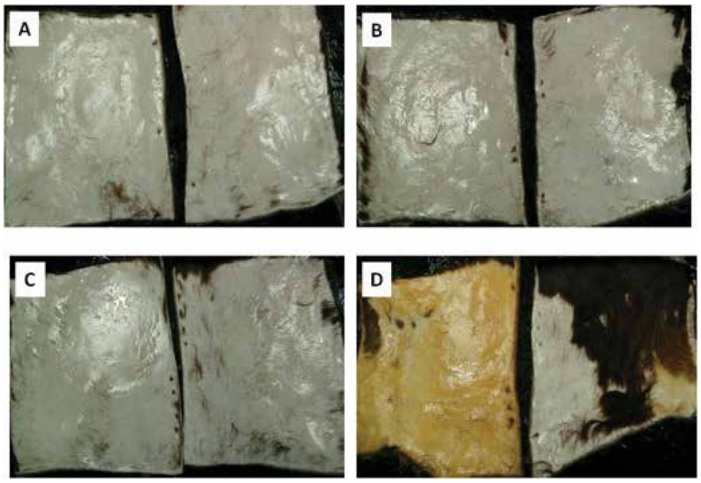


Figure 4. The digital camera images of bovine pelts unhaired by AS1.398 at 40°C for 4 hours. A~D Left: metal ions free or EDTA free enzymatic unhairing samples; A~D Right with 10 mM of metal ions or EDTA enzymatic unhairing samples: (A: Mg(II), B: Mn(II), C: Zn(II), D: EDTA).

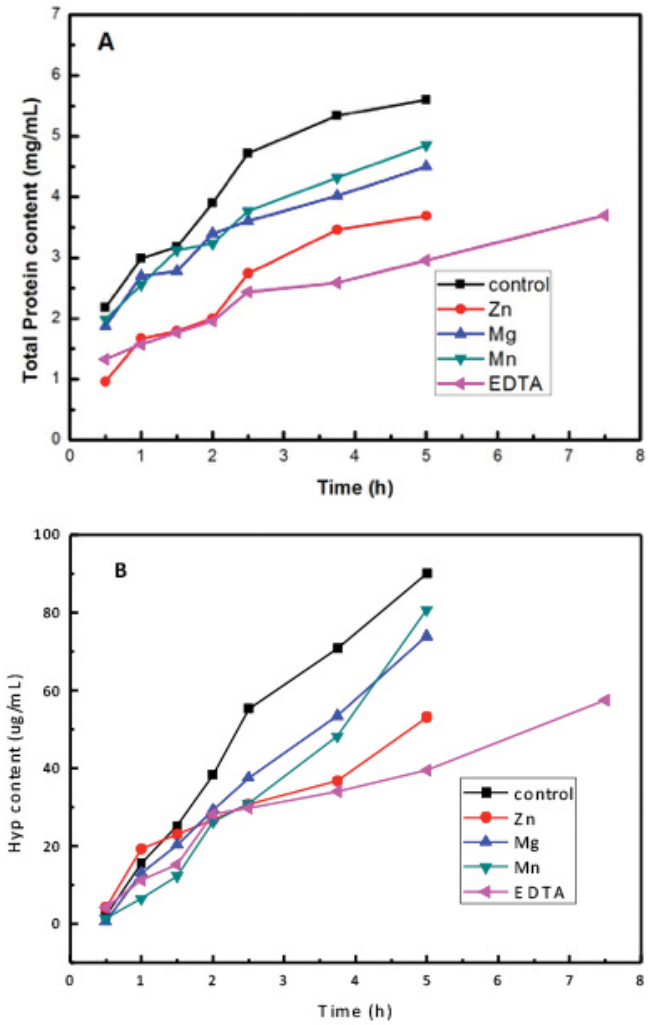


Figure 5. Hyp and Total protein content in the float during enzymatic unhairing

## Table I

**The unhairing extend during AS1.398 protease unhairing with collagenase inhibitors.**

| Unhairing Samples | Time /h | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Enzyme+Mg(II) | × | ×× | ××× | ×××× | ×××× |
| Enzyme+Mn(II) | × | × | ×× | ××× | ×××× |
| Enzyme+Zn(II) | × | × | ×× | ××× | ××× |
| Enzyme+EDTA | × | × | × | × | ×× |
| Enzyme control | × | × | ××× | ×××× | ×××× |

(×: the hair root is tight; ××: the hair root begins loosen; ×××: the hair root can be easily removed from the skin, ××××: completely unhaired)

**Table II**
**Physical properties of chrome tanned samples.**

| Unhairing Samples | Tensile strength (N/mm²) | Elongation (%) | Tear strength (N/mm) |
|---|---|---|---|
| Enzyme + Mg(II) | 6.7 ± 0.4 | 54.9 ± 1.2 | 31.9 ± 0.9 |
| Enzyme + Mn(II) | 9.8 ± 0.6 | 63.1 ± 0.8 | 26.1 ± 1.1 |
| Enzyme + Zn(II) | 7.6 ± 0.5 | 69.9 ± 1.5 | 35.6 ± 1.2 |
| Enzyme control | 7.6 ± 0.3 | 65.2 ± 1.5 | 28.2 ± 0.5 |
| Sulfide | 12.2 ± 0.3 | 62.6 ± 0.5 | 51.7 ± 0.8 |

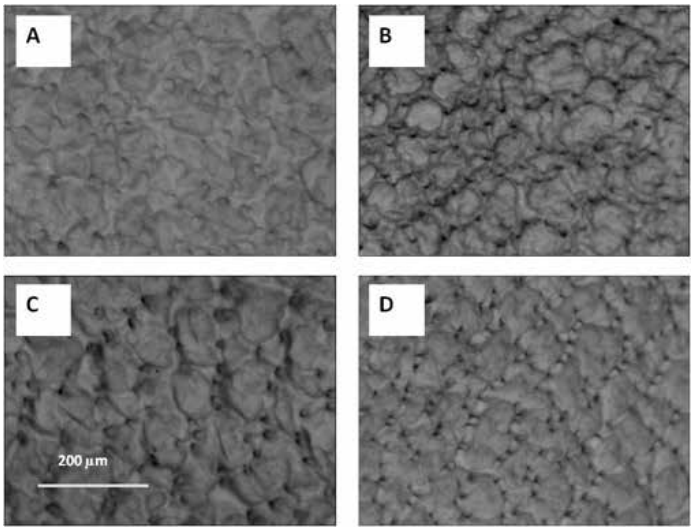


Figure 6. The stereomicroscope images of chrome tanned samples. A: metal ion free enzymatic unhairing at 40°C for 5 h, B: enzymatic unhairing with 10 mM of Mg(II) at 40°C for 5 h, C: enzymatic unhairing with 10 mM of Mn(II) at 40°C for 5 h, D: enzymatic unhairing with 10 mM of Zn(II) at 40°C for 5 h.

and efficient. Comparison to Zn(II) ion, a fast unhairing rate for Mg(II) and Mn(II) ions may be due to they have less inhibition effect to collagenase according to the previous investigation.

The Hyp content can evaluate the hydrolysis extent of collagen fiber while the total protein content in the unhairing float is an indicator of the degree of unhairing process. The results are shown in Figure 5. The total protein content and Hyp content in the float increases with the increase of the enzymatic unhairing duration. Compared to the control experiment, enzymatic unhairing with 10 mM of metal ion will slow the hydrolysis of collagen and total protein releasing. Zn(II) ion showed the biggest influence to the hydrolysis of total protein and collagen during enzymatic unhairing compared to the other two ions. We also observed that the unhairing float with Zn(II) ion is cleaner than the others. It indicates that Zn(II) ion may an ideal additive for safe and efficient enzymatic unhairing.

The enzymatic unhaired pelts were then treated by the procedures from normal liming to chrome tanning. The grain of the chrome tanned crust was observed by a stereomicroscope and the images are shown in Figure 6. The mechanical properties of chrome tanned crust were tested by QB/T2726-2005. Here, the chrome tanned crust by traditional beamhouse (sulfide unhairing) sample was used as a control. The results are shown in Table II. It could be observed that the grain of enzymatic unhaired by metal ion free was partly damaged and the hair pores were not clear (Figure 6a). However, the grains and hair pores of enzymatic unhaired by 10 mM of Mg(II), Mn(II) and Zn(II) ions were smooth and clear. It indicates that metal ions make the enzymatic unhairing safe. Table II shows that the tensile strength and tear strength of the samples by enzymatic unhairing is less than that of the samples by sulfide unhairing. There is not too much difference in physical properties for the sample enzymatic unhaired with or without metal ions.

## Conclusions

The addition of Mg(II), Mn(II) and Zn(II) ions shows safe and efficient unhairing effect in AS1.398 protease based unhairing process. The presence of Mn(II), Mg(II), and Zn(II) ions at 10 mM causes AS1.398 protease hydrolysis of collagen being inhibited to 10%, 35% and 55% respectively while showed little effect to hydrolysis of casein. Compared to metal ion free enzymatic unhairing, the addition of certain bivalent metal ions resulted in the same unhairing rate with less damage to the grain and the hair pores.

## Acknowledgement

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