ENZYMATIC DEHAIRING OF CATTLEHIDE WITH AN ALKALINE PROTEASE ISOLATED FROM *ASPERGILLUS TAMARII*[a,b]

by

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ABSTRACT

An enzymatic dehairing protocol based on the alkaline serine protease, isolated from the fungus *Aspergillus tamarii,* required 16h, and we observed concomitant grain damage. The use of sodium dodecyl sulfate (SDS) as a pretreatment to remove the lipids from the hide allowed a shortening of the dehairing time to 6 h without grain damage. We postulated that the SDS removed all of the sebaceous grease from the pores of the hair, facilitating the penetration of the enzyme through the grain layer. Using a lypophilic dye, Nile red, we showed that SDS did remove some of the grease from the grain side of the hide. By tagging the enzyme with a fluorescent label, we clearly showed, however, that the enzyme penetrated the hide only through its flesh side. The enzymatic dehairing process did not remove the fine hairs from the hide. Adding a common sharpening agent, sodium sulfide, to the liming step removed the fine hairs but also caused grain damage. Employing an auxiliary oxidative dehairing step, based on alkaline sodium percarbonate, did remove the fine hairs; under carefully controlled conditions, grain damage was not observed. Mechanical data were collected from leather prepared from enzymatically dehaired hide that had been limed, enzymatically dehaired hide that had been further oxidatively dehaired and hide that had been dehaired with sulfide and relimed. The tensile strength of the leather made from enzymatically dehaired and oxidatively treated hide and the leather prepared from the enzymatically dehaired and traditionally relimed hide was not significantly different from the control. The leather made from the enzymatically dehaired and oxidatively treated hide, however, was significantly stronger than the leather prepared from the enzymatically dehaired and traditionally relimed hide.

RESUMEN

Un protocolo de depilado enzimático basado en proteasa serina alcalina, aislada del hongo Aspergillus tamarii, exige 16 horas, y se observó daño concurrente en la flor. El uso de dodecilsulfato de sodio (SDS) como un pretratamiento para eliminar los lípidos de la piel permite que se reduzca el tiempo de depilado a 6 horas sin daños de flor. Hemos postulado que la SDS elimina todas las grasas sebáceas de los poros del pelo, facilitando la penetración de la enzima a través de la capa de flor. Por medio de un colorante lipofílico, Nilo Rojo, demostramos que SDS eliminó algunas de las grasa del lado flor de la piel. Marcando la enzima con una etiqueta fluorescente, demostramos claramente, sin embargo, que la enzima penetró en la piel sólo a través de su lado carne. El proceso de depilado enzimático no quita el pelo fino de la piel. Adicionando un agente común, sulfuro de sodio, en el pelambre elimina los pelos finos, pero también causó daños en la flor. El empleo de un auxiliar oxidante en el depilado, basado en un percarbonato de sodio alcalino removió los pelos finos y bajo

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condiciones cuidadosamente controladas, no se observó daño en la flor. Datos [de propiedades] mecánicas fueron recolectados de cueros depilados enzimáticamente que fueron luego apelambrados, de cueros enzimáticamente depilados y luego depilados mediante el auxiliar oxidante y de cueros que habían sido apelambrados con sulfuro y encalados. La resistencia a la tracción de la piel depilada enzimáticamente y oxidada y de la piel depilada enzimáticamente y encalada en forma tradicional, no fue significativamente diferente del control. El cuero depilado enzimáticamente y luego tratado con el auxiliar oxidante fue, sin embargo, significativamente más resistente que el cuero elaborado a partir del depilado enzimático y luego tradicionalmente encalado.

INTRODUCTION

Over the past five years, our research has focused on the development of a safe, economical, and ecofriendly dehairing protocol for use in either a tannery or meat packing plant. Though we have placed our main emphasis on the replacement of sulfide with oxidative chemicals, we have also studied the use of a bacterial alkaline protease as a dehairing agent for use in the beamhouse. The alkaline protease does not hydrolyze hard keratin (hair); instead it hydrolyzes non-fibril proteins. It has been postulated that the alkaline protease hydrolyzes the basement membrane proteins, releasing the hair from the hide.[1] Alkaline protease can hydrolyze collagen, but the rate of hydrolysis is much slower than the rate of hydrolysis of the basement membrane proteins. It is possible to over-process the hide by leaving it in the dehairing bath too long. Characteristics of over-processing are loose grain and poor mechanical properties.

We encountered two major drawbacks in the use of a bacterial protease: (a) the cost of the protease and (b) the failure of enzymatic dehairing to remove the 'fine' hairs from bovine hides.[1] An alkaline protease isolated from *Aspergillus tamarii* can remove hair from goat skins; the organism can be grown inexpensively on agricultural wastes.[2] and, like fungi in general, express larger quantities of enzymes than the corresponding bacterial enzyme expression systems.[3] We have been able to culture an *Aspergillus tamarii* isolate and optimize its production of the alkaline protease in sufficient quantities[4] for a systematic study of the enzymatic dehairing of bovine hides.

We attempted to use the same dehairing protocol developed by Gehring *et al.*[1] for the alkaline protease isolated from *Streptomyces griseus*. Although we did not get satisfactory dehairing with this protocol, we used those conditions as the starting point for the optimization of enzymatic dehairing using the fungal alkaline protease. The optimization of temperature, time, enzyme concentration, and pH is discussed, as is the application of surfactant to degrease the hide prior to dehairing. Although we were not able to remove the fine hairs from the hide enzymatically, we were able to remove them later using an oxidative dehairing step based on alkaline sodium percarbonate. Details of the oxidative dehairing step are presented along with the supporting mechanical testing data.

MATERIAL AND METHODS

Materials

All chemicals were of reagent grade and were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). The Alexa-Fluor 580 fluorescent dye and Nile Red dyes were obtained from Molecular Probes (Carlsbad, CA). Fresh steer hides were obtained from a local abattoir and were soaked, fleshed, and frozen before use. The hides were thawed at 4ºC before use.

Apparatus

All bench scale experiments were performed in 10-L 4-in-1 Dose Drums (Dose Maschinenbau GmbH, Lichtenau, Germany) with typical float volumes for the dehairing reactions of 200-350 mL. Physical measurements of stress, strain and toughness of crust leather were performed with an upgraded Instron Tensile Tester (Model # 1122) running TestWorks version 3.07 software (MTS Systems, Eden Prairie, MN).

A Nikon SMZ-2T stereo dissecting microscope (Nikon, Inc, Japan) was used to examine the grain on the crusted leather. Confocal microscopy was run on a model TCS NT/SP confocal microscope system (Leica Microsystems, Inc, Exton, PA), which includes a model IRBE microscope equipped with a 20 x lens. Samples were illuminated by the 488 nm line from an Argon laser and the fluorescence from the hide samples was collected in two separate channels, 500 - 540 nm and 580 - 620 nm, into sets of optical sections and extended image sets. All fluorescent microscopy was performed with a model MZ FLIII stereofluorescence microscope (Leica Microsystems, Inc, Bannockburn, IL) equipped with a 50 W Mercury lamphouse, filters for epifluorescence, and a DC200 charge-coupled device camera system. An Intralux 5000-1 halogen lamphouse and fiber optic illuminator (Volpi Manufacturing USA Co, Inc, Auburn, NY) was used for reflected white light.

Preparation of crude enzyme powder

Aspergillus tamarii was grown on wheat bran (50 g of wheat bran per 1-L Hoffkin's flask) that had been moistened to 70% water content. The substrate was inoculated with an inoculum containing approximately 10^7 spores/mL and the inoculated substrate was incubated for 72 h at 26-30ºC. After incubation, the substrate was extracted with 20 mM TRIS buffer, pH 8.0, containing 2 mM $CaCl_2$. The resulting mixture was filtered through cheesecloth and the crude enzyme powder was precipitated from the filtrate by the addition of cold acetone (4º) with stirring. The final mixture was stored overnight at 4ºC. The precipitate was collected by centrifugation at 10,000 X g for 20 min. The supernatant was decanted from the solid material and discarded. The solid crude enzyme was dissolved in a minimal amount of 20 mM TRIS buffer, pH 8.0, containing 2 mM $CaCl_2$ and dialyzed (Spectra /POR6 MWCO=1kDa) against the same buffer at 4ºC. The retentate was lyophilized and the crude enzyme powder was stored at -20ºC.

Characterization of the crude protein

The protein content of the crude powder was determined by the method of Lowry et al.[5] The enzyme activity in the crude enzyme powder was determined by a modified method of Anson[6] using 1% w/v casein containing 2 mM $CaCl_2$ in 0.02 M borate buffer, pH 8.0, and incubating the reaction mixture at 45ºC for 30 min. The unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μg of tyrosine under standard assay conditions.

The effect of pH on the protease activity was determined by using casein as a substrate under standard assay conditions. Protease activity was determined at various pH (6-13) using the appropriate buffer systems. The pH stability was determined by measuring the residual activity after 30 min of pre-incubation in the buffers (pH 6-13) at 30ºC. The effect of temperature on the activity of A. *tamarii* alkaline protease was determined using casein as the substrate and temperatures from 25 - 80ºC at pH 8. Likewise, the heat stability was determined after incubation for 30 min (25 - 80ºC) and then determining the residual enzyme activity.

Dehairing Studies

The preliminary dehairing reactions used approximately 6 cm (2.5 in) diameter hide pieces (cookies). The laboratory scale experiments used approximately 100 X 150 cm (6X6 in) hide pieces. All control hide were dehaired 4 h using 2% sodium sulfide, 2% lime and 1% soda ash (Na_2CO_3) in a 100% float.

The initial enzymatic dehairing experiments used the protocol of Gehring et al.[1] All of the hide pieces were pretreated with 2.5% bicarbonate/carbonate buffer (1.25% [w/v] each of sodium bicarbonate and sodium carbonate; pH 8.0) for 30 minutes. Enzyme solutions (0.1 to 6 mg/mL) were prepared in 0.2 M Tris-HCl buffer (pH 8.5) immediately prior to use. A 200% float of these solutions with 1% *N,N*-dimethyl-1-dodecanamine oxide was added to the drum, hide pieces were added respectively, and the extent of dehairing was examined at various time intervals (4, 6, 8, and 12 h). All enzymatic dehairing experiments were conducted at 37°C. This protocol was modified to include a pretreatment washing with a detergent before the bicarbonate/carbonate buffer pretreatment and elimination of the 1% *N,N*-dimethyl-1-dodecanamine oxide from the dehairing mixture.

To determine the effects of a detergent pretreatment on the effectiveness of the enzymatic dehairing, cattle hide pieces were soaked with either water (control) or washed with commercial detergents, either *N,N*-dimethyl-1-dodecanamine oxide or SDS (0.1%, 0.2%, 0.25%, and 0.5%). Additionally, the detergent wash time was optimized by varying the prewash time from 0.5 to 1.5 h. To remove the residual fine hairs from the hide an oxidative follow-up step was developed. The concentrations of the oxidative dehairing chemicals evaluated were 1% NaOH/1% sodium percarbonate, 2% NaOH/2% percarbonate, and 4% NaOH/4% percarbonate in a 200% float. The reactions were carried out at room temperature for 0.5, 0.75, 1, 2, and 4 h. The hides were washed with water (100%) after the oxidative follow-up step; using a tanning procedure that had been developed at this USDA facility, the hides were converted to upper shoe leather.

Mechanical testing was performed on panels that were adjacent to each other on the hide (shoulder), one panel of which (the control) was dehaired with the conventional sulfide dehairing (2% lime, 1% soda ash, 2% sodium sulfide), one panel of which was enzymatically dehaired and conventionally relimed (2% sulfide, 100%float), and one panel of which was enzymatically dehaired and oxidatively treated. The dehaired panels were converted to upper shoe leather and were mechanically tested. The dehaired hides were converted to upper shoe leather for mechanical testing using a protocol that had been developed at this USDA facility.[7]

Nile red labeling of bovine hide

Samples of green hides, 1 x 1" (2.5 X 2.5 cm) were soaked in 2.5% glutaraldehyde-0.1M imidazole buffer solution, pH 7.5, and were stained with Nile Red according to methods described by Greenspan and Fowler,[8] and then mounted in glass bottom microwell dishes (MatTek Corp., Ashland, MA).

Preparation of the fluorescently labeled enzyme

Crude enzyme (10.3 mg) was dissolved in 0.1M sodium bicarbonate buffer (pH 8.3, 1 mL). To this solution was added 0.100 mL of Alexa Fluor 568 carboxylic acid succinimidyl ester solution (10 mg/mL in dry DMSO) and the resulting solution was stirred at room temperature for 1 h. The labeled enzyme was purified on a 10 X 300 mm Bio-Gel P polyacrylamide gel column that had been equilibrated with PBS (pH 7.0). The eluted enzyme was divided into two equal portions (roughly 5 mg of labeled enzyme in each portion) and stored at -20ºC.

Absorption studies of the labeled enzyme by bovine hide

Two pieces of bovine hide (approx 8 X 8", 20 X 20 cm) were used for each labeling experiment; one piece of hide was washed with water (100% float) at room temperature for 10 min @ 6 rpm (control) while the second piece of hide was washed with SDS (100% float) at room temperature for 10 min @ 6rpm. The two hide pieces were added to a single drum with TRIS-HCl (100% float) and one of the portions of the labeled enzyme. The experiment was run at room temperature for 4 h @ 6 rpm. Both the control and

SDS-pretreated hides were sampled at 0.5, 1, 2, 3 and 4 h by removing a 1 X 1" (2.5 X 2.5 cm) piece of hide for microscopic examination. A second set of experiments was run following the exact same protocol except that the total enzyme concentration of 10 mg/mL (unlabeled enzyme + labeled enzyme) was used.

Mechanical properties of the finished leather
Physical testing of crust leather samples (cut perpendicular to the backbone) taken from the butt were performed using methods previously described by Liu and McClintick.9 Physical tests were performed on samples that had been conditioned for 48 h at 23°C (73ºF) and 50% relative humidity, using the standard test method described in ASTM D2209-95.

RESULTS AND DISCUSSION

The alkaline protease isolated from *A. tamarii* has a typical pH-activity profiled for a serine alkaline protease with an optimal pH of 8.5 – 9.0.[10] The optimal temperature was 50ºC, though there was little difference in enzyme activity between 40 and 50ºC. A temperature of 80ºC greatly reduced the enzymatic activity, but did not completely deactivate the enzyme.

The enzyme activity of the freshly isolated enzyme did not vary significantly from one batch to the next, nor did the enzymatic activity vary significantly over periods of time less than two months when the enzyme was stored at -20ºC. There was a loss in the efficacy of the enzyme if it was stored, at -20ºC, for periods longer than 2 months. For all of our studies we used freshly isolated enzyme. Therefore, for these enzymatic dehairing studies, instead of standardizing the enzyme concentration based on enzymatic activity, we used fresh enzyme every time and standardized the enzyme by concentration (mg/mL).

To determine the optimal enzyme concentration and the optimal dehairing time we ran a series of dehairing reactions using the protocol of Gehring et al. As expected, the lower concentrations and the shorter dehairing times were ineffective. What was not expected was that even the highest concentrations and 6 h reaction time were not sufficient to remove more than 30-50% of the hair. Concentrations of 5 or 6 mg/mL removed all of the hair in 16 h with the exception of the fine hairs. This time is too long for use in a commercial tannery and is much longer than the 4-h dehairing time Gehring et al. achieved using an alkaline protease isolated from *Streptomyces griseus*. Additionally, at these longer times, the enzyme also started to hydrolyze the collagen in the hide, adversely affecting the grain layer.

The dehairing protocol called for the use of *N,N*-dimethyl-1-dodecanamine oxide, as a surfactant, to be added to the enzymatic mixture. To determine if the surfactant was acting as an enzyme inhibitor we repeated the dehairing experiment without the surfactant and obtained the same results as with the surfactant. There was little, if any, difference in the dehairing time or effectiveness in removal of the fine hairs. The intended role of the surfactant was the removal of sebaceous grease and other lipids from the hide to facilitate the penetration of the enzyme. We thought that it would be more effective if the degreasing occurred before the enzymatic dehairing step. In this study, we used two surfactants: *N,N*-dimethyl-1-dodecanamine oxide and sodium dodecylsulfate (SDS). SDS can denature proteins (enzymes) and therefore we insured that there was not any residual SDS in the hide or drum before the enzymatic dehairing step. A water wash (100% float) immediately followed surfactant pre-wash. The final pretreatment was the stabilization of the hide pH with a carbonate/bicarbonate pretreatment. These two 'washes' were sufficient to remove any residual *N,N*-dimethyl-1-dodecanamine oxide or SDS before the addition of the enzyme. For a 12-h dehairing reaction we found that either SDS or *N,N*-dimethyl-1-dodecanamine oxide at concentrations of 0.1% to 0.25% facilitated the dehairing reaction; using 0.25% SDS in the pre-wash yield the best results by reducing the dehairing time to 6 h.

The enzyme should hydrolyze both the hair bulb proteins and the basement membrane (which increases the size of the hair pore), allowing the hair to slip out of the hair pore. We expected hair removal would be faster if the enzyme penetrated the hide from the grain side. To test the postulate we stained two pieces of hide, one piece pretreated with SDS and one piece not SDS-pretreated (control), with Nile red. We examined the Nile red-stained pieces of hides with a confocal microscope. The micrograph of the control hide showed sebaceous/lipid materials were present on the hair shaft and plugging the hair pores. The pretreated piece of hide still had residual sebaceous grease/lipid on its hair shafts and surface, though it appears that the hair pores were either completely open or partially open (Figure 1). We also used epifluorescence microscopy to examine a cross-section of the stained hide that had been pretreated with 0.25% SDS in water. The micrograph clearly showed a sebaceous gland filled with stained lipid material (Figure 2). The Nile red stain had managed to penetrate through the pore and stain the lipids; the pore itself appears not to contain any sebaceous grease. Nile red on the other hand is a much smaller molecule than the alkaline protease and it was not clear if the protease was capable of penetrating the hide through the pore.

To further test our hypothesis we labeled the alkaline protease with a fluorescent dye. Again we prepared two hide samples; one pretreated with SDS and one untreated sample (control). We performed two separate enzymatic dehairing studies, one study using only the labeled enzyme and one study using a mixture of both the labeled and unlabelled enzyme. Initially the control hide took up the labeled enzyme faster than the pretreated hide. For both treatments the labeled enzyme penetrated the hide from the flesh side.

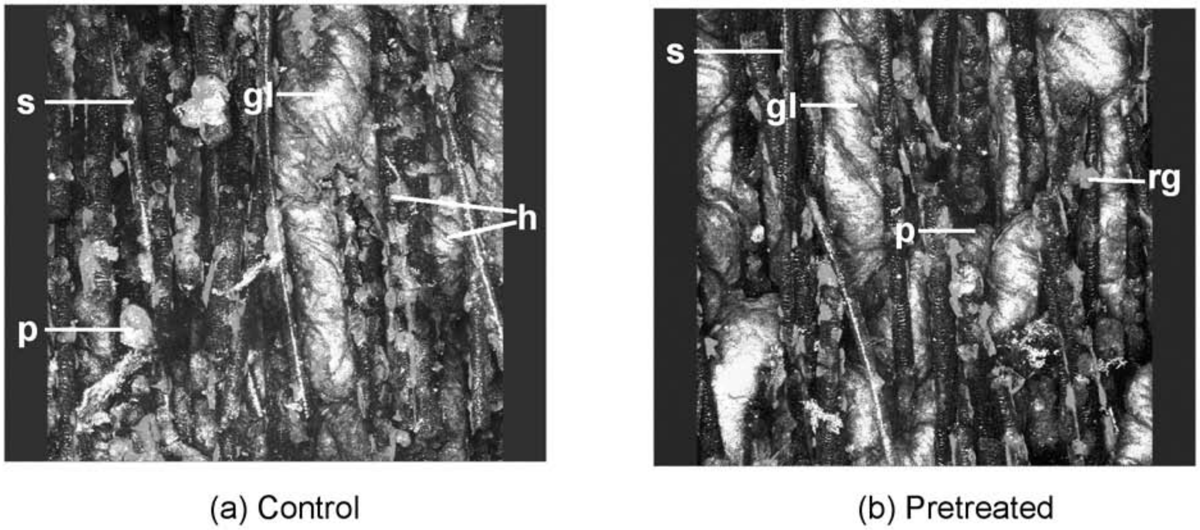

Figure 1: Confocal micrograph of the grain layer of hide. The control hide (1a) received only a water wash while the pretreated side (1b) was pretreated with 0.25% SDS in water. Both pieces of hide were stained with Nile red. The size of each image is approximately 5 x 5 mm. Abbreviations (1a) gl: grain layer; h: grease-coated hairs; p: hair pore filled with grease; s: hair shaft coated with grease; (1b) gl: grain layer; p: grease-free pore; rg: residual grease; s: hair shaft.

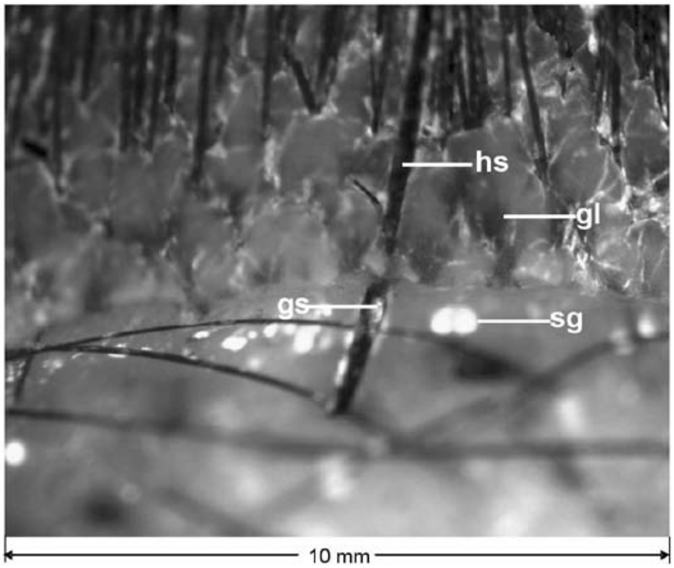

Figure 2: Epifluorescence micrograph of a cross-section of hide that was pretreated with 0.25% SDS in water. The lipids were stained with Nile red. Abbreviations — gl: grain layer; gs: grease on hair shaft; hs: hair shaft; sg: sebaceous gland filled with grease.

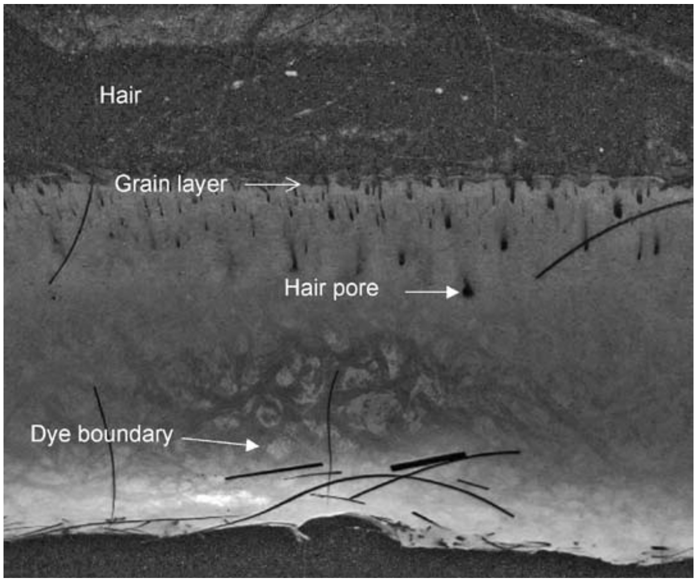

Figure 3: Epifluorescence micrograph of a cross section of hide that was pretreated with 0.25% SDS in water and then reacted with a yellow-fluorescing labeled enzyme. The width of the slice is ~6 mm and the thickness of the hide is ~5 mm.

There was no evidence that the labeled protein had penetrated the grain layer. After approximately 30 minutes, the labeled enzyme had penetrated the pretreated hide from the flesh side. After the initial penetration, it appeared that the labeled enzyme diffused faster in the pretreated hide than in the control hide. The labeled enzyme did not penetrate fully to the grain layer (Figure 3). Thus, it appears that the role SDS plays in the enzymatic dehairing is in the removal of the lipids from the flesh side of the hide, facilitating the uptake of the enzyme through the flesh side. The use of an alternate degreaser in the initial soaking of the hide followed by an efficient fleshing may give similar results, but this was not part of the current study.

The SDS pretreatment improved the efficiency of the enzymatic dehairing, but fine hairs remained. Is it feasible to remove the fine hairs in a subsequent liming step using a sharpening agent? We initially used a 100% float, 2% lime, and 0.5% Na_2S to lime enzymatically dehaired hides. After liming for 16 h the hides were examined for the effectiveness of hair removal and the integrity of the grain layer. There was no evidence of residual hairs; but there were areas of grain damage as well as areas where the grain layer had separated from the corium.

TABLE I
Summary of Mechanical Test Data

Dehairing Protocol	Tensile Strength (MPa)	Elongation (%)	Young's modulus (MPa)	Toughness Index
Sulfide Control n = 4	6.74 ± 1.10 (a, b)	33.06 ± 7.85	18.87 ± 0.56	0.366 ± 0.062
Enzymatic, relimed n = 6	4.86 ± 1.78 (a)	48.47 ± 12.07	15.14 ± 6.39	0.249 ± 0.143
Enzymatic, oxidative post-treatment n = 6	7.96 ± 1.51 (b)	40.07 ± 11.52	20.88 ± 8.65	0.462 ± 0.243

Letters in parenthesis are from Bonferroni statistical analysis. Means that have the same letter are not significantly different at the 95% confidence level.

A better alternative to a sulfide sharpening agent might be an oxidative dehairing agent. We have shown that oxidative dehairing using sodium percarbonate is an effective and environmentally friendly alternative for dehairing of cattle hide.[11] Additionally there is the potential of being able to eliminate the liming step, reducing the amount of water used in the wet end, as well as reducing the amount of solids in the waste stream. We performed oxidative trials on enzymatically dehaired hides and found that the concentrations of 4% NaOH/4% sodium percarbonate for 2 h yielded the best dehairing results. Grain damage occurred, however, if the temperature was greater than 35°C or if the oxidative dehairing time was greater than 2 h. Likewise, if the enzymatic dehairing time was greater than 8 h grain damage occurred. This damage was independent of the time or concentration of the oxidative chemicals. We chose to use a 4-h enzymatic dehairing for the preparation of leather pieces for mechanical testing.

We determined the mechanical properties on leather produced from matched hide pieces that had been dehaired by traditional sulfide dehairing (2% Na_2S, 2% lime, 1% soda ash, 100% float, 4 h) followed by a traditional relime (100% float, 2% lime), enzymatic dehairing (4 h) followed by traditional reliming, and enzymatic dehairing (4 h) followed by an oxidative dehairing. There was no observable grain damage on the hide pieces, though the hide piece that had been enzymatically dehaired and traditionally relimed had hair on greater than 50% of its surface. We converted the dehaired hide pieces to upper shoe leather. The summary of the mechanical test data is given in Table 1. The differences in each of the data sets were insignificant (at the 95% confidence level) for % elongation, Young's modulus and toughness index. Bonferroni analysis[12] of the test data showed only significant difference in the tensile strength of the leather. The tensile strength of the leather made from enzymatically dehaired, oxidatively post-treated hide was significantly stronger than either the leather prepared from the enzymatically dehaired or traditionally relimed hide. The tensile strength of the leather produced from the sulfide control was intermediate between the other two samples (Table 1). The other mechanical parameters (% elongation, Young's modulus and toughness index) had large standard deviations and were statistically identical.

CONCLUSIONS

We have demonstrated that dehairing may be conducted using an alkaline serine protease from *A. tamarii*. It was not possible to completely remove the hair from the hide using the enzymatic process alone; an additional oxidative dehairing step was required to remove the residual fine hair. As an enzyme-free, alkaline sodium percarbonate dehairing[11] is less expensive than an enzymatic dehairing, is thorough, does not induce any grain damage, and is a safer and environmentally more acceptable approach than conventional dehairing using sulfide, it appears to be a better approach than dehairing with an alkaline serine protease isolated from *Aspergillus tamarii*.

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