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RESEARCH PAPER

Latex peptidases of *Calotropis procera* for dehairing of leather as an alternative to environmentally toxic sodium sulfide treatment

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Abstract Dehairing of crude leather is a critical stage performed at the beginning of its processing to obtain industrially useful pieces. Tanneries traditionally apply a chemical process based on sodium sulfide. Since this chemical reactive is environmentally toxic and inefficiently recycled, innovative protocols for reducing or eliminating its use in leather depilation are welcomed. Therefore, latex peptidases from *Calotropis procera* (CpLP) and *Cryptostegia grandiflora* (CgLP) were assayed for this purpose. Enzyme activity on substrates representative of skin such as hide powder azure (U_{HPA}), elastin (U_E), azocollagen (U_{AZOCOL}), keratin (U_K), and epidermis (U_{EP}) was determined, while depilation activity was assayed on cow hide. Only CpLP was active against keratin (13.4 U_K) and only CgLP was active against elastin (0.12 U_E). CpLP (93.0 U_{HPA}, 403.6 U_{AZOCOL}, 36.3 U_{EP}) showed higher activity against the other substrates than CgLP (47.6 U_{HPA}, 261.5 U_{AZOCOL}, 8.5 U_{EP}). In pilot assays, CpLP (0.05% w/v with sodium sulfite 0.6% w/v as activator) released hairs from cow hide pieces. Macroscopic and microscopic analyses of the hide revealed that the dehairing process was complete and the leather structure was preserved. The proteolytic system of *C. procera* is a suitable bioresources to be exploited by tanneries.

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Introduction

Crude leather was the raw material for one of the most ancient types of clothing in human use and helped to protect the earliest civilizations against the largest climate extremes. Currently, manufactured leather goods are highly socially prestigious worldwide. Leather processing involves a set of sequential steps from raw hide to the finished leather, and enzymes are used extensively in many of these process steps as environmentally benign alternatives, either replacing the chemicals completely or at least partly substituting for them [1]. The raw material is processed to achieve a series of desired industrial qualities, namely structural stability, resistance, adequate elasticity, lack of spots, and depilation, among others [2]. Depilation is the initial phase of leather processing to eliminate hair. Sodium sulfide (SS) is universally applied for this purpose. This chemical not only gives rise to unfavorable consequences on environment but also affects theefficacy of effluent treatment plants [3]. Therefore, finding a cleaner alternative to lime-sulfide dehairing constitutes an efficient strategy for reducing the negative impact of tanneries on the environment.

Enzymes are the most successful biomolecules in industrial exploitation regardless of the process involved [4–6]. In addition to their peculiar kinetic properties, that accelerate processes, enzymes are suitable for reusability [7] and they are environmentally friendly. All these features make them attractive tools for industries. Consequently, the use of enzymes as active tools to replace chemicals exhibiting hazardous environmental toxicity in manufacturing

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practices is universally desired. Indeed this strategy has been proposed in an increasing number of studies wherein enzymatic dehairing with microbial enzymes is suggested as it is environmental friendly compared to the chemical process [8–11]. Ideal enzyme dehairing will be achieved when the proteolytic activity is directed, primarily, towards the active sites at the basement membrane and the cells of the outer root sheath and follicle bulb under conditions in which the action on collagen can be minimized, the activity on elastic tissue can be controlled, and leathers can be produced with the required qualities [12].

Plant latex is a natural source of enzymes and peptidases are abundantly found in numerous latex fluids [13–16]. These enzymes have been widely studied and their usefulness in different industrial processes has been highlighted [17, 18]. Five closely related cysteine proteases were purified and characterized from the latex of *Calotropis procera* (Apocynaceae) and an extensive analysis of their biochemical and functional properties was performed [19–21]. Further, cysteine proteases have also been isolated and characterized from latex of *Cryptostegia grandiflora*, another species of the Apocynaceae family [21]. These enzymes can be readily obtained within a water-soluble latex proteolytic fraction according to the protocol established earlier [22]. In this study, the ability of latex peptidases of *C. procera* and *C. grandiflora* to digest keratin, collagen, and elastin was examined as a preliminary screening with the aim of looking for advantageous potentialities for depilation of crude leathers. Accordingly, the selected proteolytic source was further tested and the microscopic characteristics of the leathers obtained after enzymatic treatment were compared to those obtained by the traditional (chemical) method.

In the present study, plant proteolytic extracts were characterized and tested as an environmental friendly alternative for the dehairing of cow skins.

Materials and methods

Proteolytic preparations

The proteolytic extracts were obtained from the latex of *Calotropis procera* and *Cryptostegia grandiflora*. The latex was obtained as reported formerly, and after processing by centrifugation and dialysis, the freeze-dried proteolytic fractions were obtained [22–24]. The following nomenclature was applied to identify the proteolytic samples: *C. procera* is denoted as CpLP and *C. grandiflora* as CgLP. Before testing the samples, their protein profiles and proteolytic activities were characterized by electrophoresis according to Laemmli [25] and enzymatic assays as reported previously [21].

Proteolytic assays on non-keratinous substrate

Casein substrate

The reaction mixture containing 1.1 mL of casein solution (1% p/v in buffer of Tris–HCl 0.1 M, pH 8.0, with cysteine 10 mM) and 0.1 mL of sample was incubated for 10 min at 37 °C. The reaction was stopped by adding 1.8 mL of trichloroacetic acid (TCA) at 5% p/v and the absorbance at 280 nm of supernatant was determined [26]. The unit of activity (Ucas) was defined as the amount of enzyme that, under test conditions, caused a change in 1 unit of A280 per minute.

Hide powder azure (HPA) and Azocollagen substrates

Ten milligrams of HPA (Sigma-H6268, Hide–Remazol Brilliant Blue R) was weighed in a test tube and then 3.8 mL of Tris/HCl 100 mM buffer (pH 8.0) and 200 µL of sample were added. The reaction was carried out at 37 °C with orbital stirring ($\omega = 75$ rpm) during 10 min. Subsequently, the dispersion was centrifuged and the absorbance was measured at 595 nm [27]. The control was prepared by adding the substrate and buffer without the enzymatic preparation. Each determination was carried out in triplicate. The HPA activity unit (U_{HPA}) was defined as the amount of enzyme that, under test conditions, caused a change of 0.001 unit of A595 per minute. For azocollagen substrate (the determination was performed following the same protocol as for HPA except that the absorbance was measured at 520 nm. The Azocollagen activity unit (U_{AZOCOL}) was defined as the amount of enzyme that, under assay conditions, caused a change of 0.001 unit of A520 per minute.

Table 1 Proteolytic activity of latex proteases

Enzyme	Keratin azure ($U_{K/mg}$)	Epidermis ($U_{EP/mg}$)	HPA ($U_{HPA/mg}$)	Azocoll ($U_{AZOCOL/mg}$)	Elastin red ($U_{E/mg}$)
CpLP	13.4	36.3	93.0	403.6	nd
CgLP	nd	8.5	47.6	261.5	0.12

CpLP: latex peptidases from *Calotropis procera*, CgLP latex peptidases from *Cryptostegia grandiflora*. Activity on keratin (U_{KA}), activity on epidermis (U_{EPI}), activity on HPA (U_{HPA}), activity on azocollagen (U_{AZOCOL}) and activity on elastin (U_E), nd: not detectable activity. All values are expressed per mg of lyophilized enzyme preparation. Data shown are mean of three independent measurements

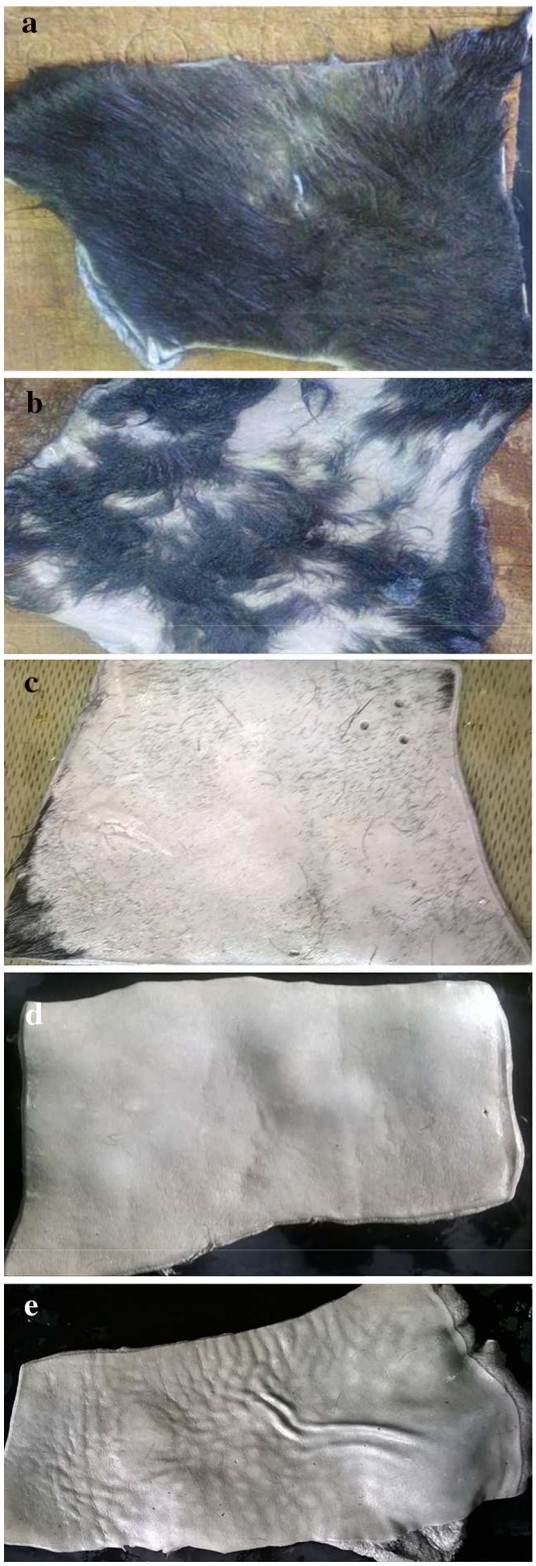


Fig. 1 Depilation effect on bovine hide. **a** Control with 0.6% (w/v) sodium sulfite without adding enzyme, **b** 0.025% (w/v) CpPL without sodium sulfite, **c** 0.025% (w/v) CpPL with 0.6% (w/v) sodium sulfite, **d** 0.05% (w/v) CpPL with 0.6% (w/v) sodium sulfite, **e** 0.10% (w/v) CpPL with 0.6% w/v sodium sulfite

Elastin substrate

Ten milligrams of elastin (Sigma-E0502, Elastin–Congo Red) was weighed in a test tube and then 3.5 mL of Tris/HCl 100 mM buffer (pH 8.0) and 500 µL of sample were added. The reaction was carried out at 37 °C with orbital stirring ($\omega = 75$ rpm) during 90 min. Subsequently, the dispersion was centrifuged and the absorbance measured at 495 nm. Blank determinations were performed. Each determination was carried out in triplicate. The elastin activity unit (U_E) was defined as the amount of enzyme that caused a change of 0.001 unit of A495 per minute [28].

Proteolytic assays on keratinous substrate

Keratin substrate

A degradation test on keratin [29] was performed using keratin (Sigma K-8500, keratin azure) by means of a procedure similar to that carried out in the case of the HPA substrate. The keratinolytic activity unit (U_{KA}) was defined as the amount of enzyme that, under test conditions, originated a change of 0.001 A595 units per minute.

Epidermis substrate

This substrate was obtained by means of the procedure indicated by Cantera et al. [30]. To assess the activity, 40 mg of substrate was weighed into a test tube. Then, 3.8 mL of Tris/HCl 100 mM buffer (pH 8.0) and 200 µL of solution of the enzymatic product were added. The reaction was carried out at 37 °C with orbital stirring ($\omega = 75$ rpm) for 60 min. The reaction stopped with the addition of TCA 5% p/v and was then centrifuged and the absorbance was measured at 280 nm. The assessment was carried out in triplicate, and a blank test was also run. The unit of activity (UEPI) was defined as the amount of enzyme leading to a change of 0.001 unit of A280 per minute under the test conditions.

Dehairing experiments

The experiments were carried out in a pilot scale using bench reactors (drums) with typical float volumes for dehairing reactions of 500 mL and controlled rotation speed and temperature. Wet-salted bovine skins (0.1 kg) were initially soaked in water (0.5 L) containing bactericide (1.5 g/L) and detergent (3.0 g/L) at 30 °C and 23 rpm for 24 h. Then the baths were drained and different groups of dehairing experiments were carried out. Six tests were done to evaluate the enzyme concentrations and the

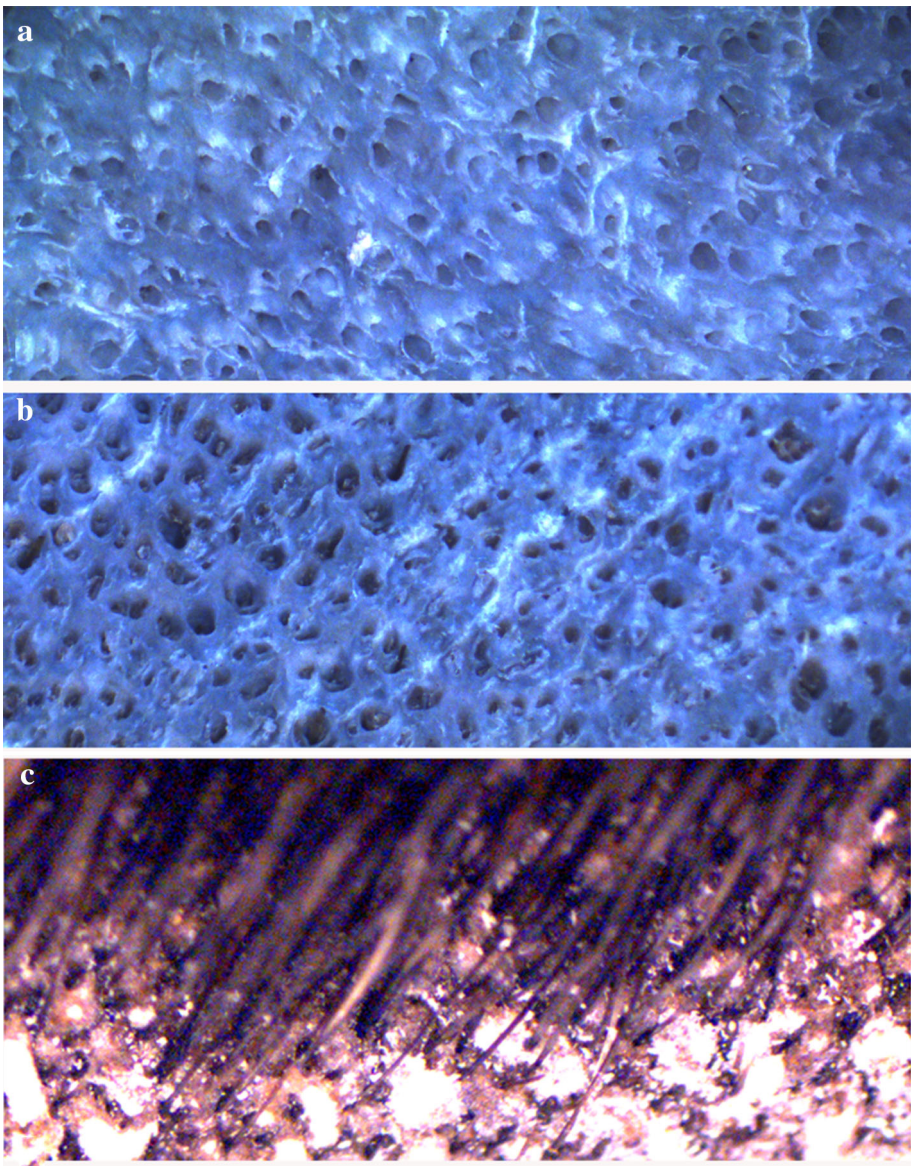


Fig. 2 Magnifying glass analysis of wet blue hide surface **a** ×15, enzymatic dehairing (×15); **b** ×15, SS dehairing; **c** ×15, blank

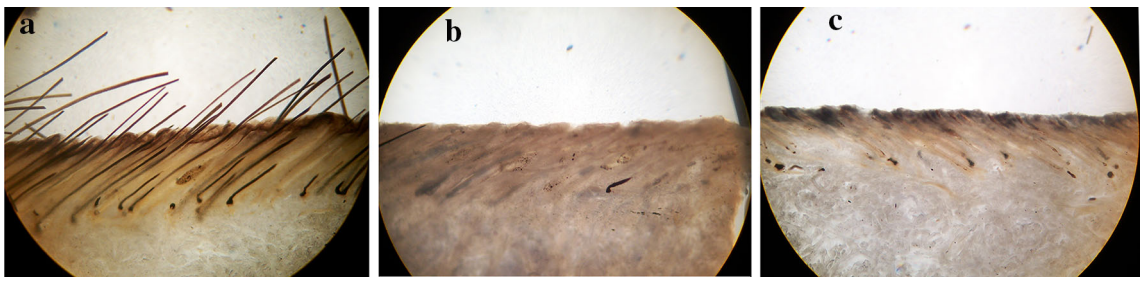
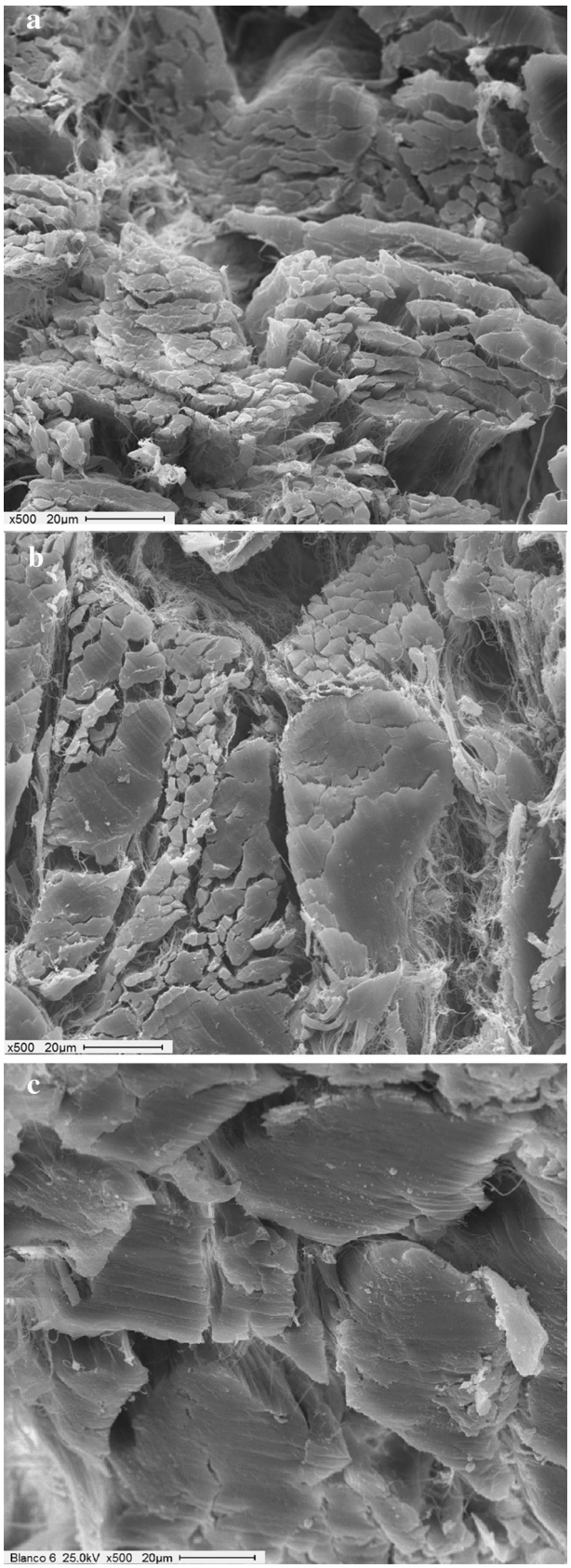


Fig. 3 Microscopic analysis of cross-section of wet blue hide **a** ×40, blank, **b** ×40, enzymatic dehairing; **c** ×40, chemical dehairing

addition of chemicals [0.1, 0.05, and 0.025% (w/v) enzyme, with and without 0.6% (w/v) sodium sulfite]. Conventional dehairing (control) with lime 0.15% (w/v) and SS 0.06% (w/v), and the soaking solution with 0.6% (w/v) sodium sulfite (blank). All experiments were carried out at 25 °C and 23 rpm for 24 h in drums. After unhairing, samples obtained from both processes were finished as wet blue according to conventional procedures.



◀**Fig. 4** Scanning electron micrographs (SEM), cross-section of wet blue hide **a** ×500, enzymatic dehairing; **b** ×500, chemical dehairing; **c** blank

Microscopy analysis

To examine the structure of the leather samples from the experimental treatment, control and blank groups, each leather was cut into samples of uniform thickness without any pretreatment. All specimens were coated with gold. The micrographs for the grain surface and cross-section were obtained by operating a scanning electron microscope (SEM, Philips 505) with an accelerating voltage of 25 kV.

Results and discussion

Proteases from latex of *C. procera* and *C. grandiflora*, two different plant species belonging to Apocynaceae, were characterized by SDS–PAGE and zymography to check the integrity of the enzyme preparation, the results were in accordance with the data previously reported for the latex of these plant species [31]. Each extract showed a characteristic protein pattern and was able to hydrolyze casein from gel under the assayed conditions. Further, the activity was assayed using casein as a substrate to determine total proteolytic activity. The results showed that specific activity for CpLP (1.4 Ucas/mg of protein) was two-fold higher than for CgPL (0.7 Ucas/mg of protein).

In the hides, the collagen exists in association with the non-collagenous constituents which are partially or completely removed in various tanning operations. During dehairing, the hair along with the epidermis, basement membrane, non-collagenous proteins, and other cementing substances are removed from the skin [32]. The action of keratinase is important for eliminating hair from the hide. In addition, high collagenase activity is not desirable because these enzymes can damage the hide (leather) grain and the physical–mechanical characteristics of the hides [9].

To select the most suitable enzymatic preparation for use in the dehairing process, CpLP and CgLP were assayed towards specific proteins (substrates) of animal hide. Keratin azure and epidermis substrate were used as representative substrates of keratin, Elastin-Congo Red as elastin, and HPA and Azocoll as representative substrates of collagen. The values of enzymatic activity expressed in units of activity against each substrate are summarized in Table 1. The activity of CpLP and CgLP on representative substrates of keratin, whose hydrolysis is crucial to the process of depilation revealed that only CpLP showed a

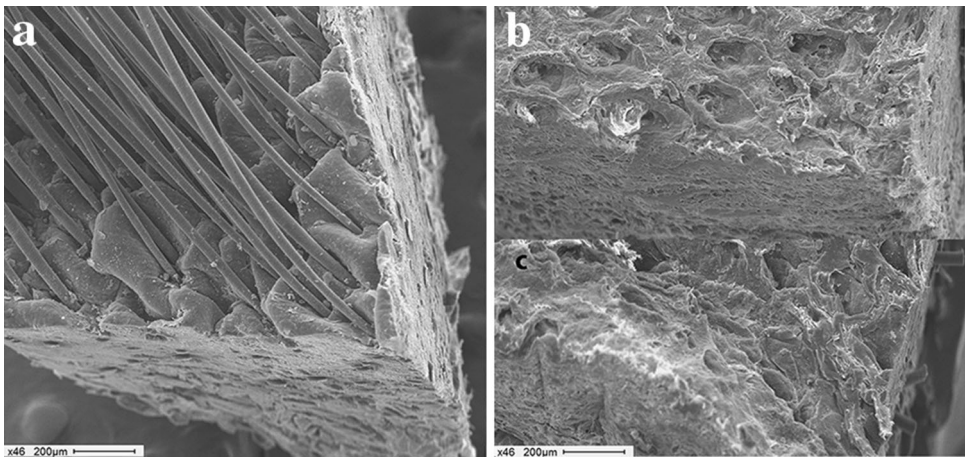


Fig. 5 Scanning electron micrographs (SEM), superficial view of wet blue hide **a** ×46, blank, **b** ×46, enzymatic dehairing; **c** ×46, chemical dehairing

significant activity against keratin. Moreover, CpLP showed higher activity against HPA and Azocoll than CgLP, while only CgLP showed a significant activity against elastin. CpLP was active against keratinous substrates (13.4 U_K, 36.3 U_{EP}) and collagen (93.0 U_{HPA}, 403.6 U_{AZOCOL}). Based on these results, CpLP showed the highest ratio of keratinolytic/collagenolytic activities, and thus the enzymatic preparation CpLP was selected to carry out the dehairing process at pilot scale.

Several experiments were carried out to achieve the optimal conditions for the enzymatic depilation of bovine hides by the CpLP protease preparation at pilot scale (pre-commercial small scale evaluation). Tests were performed using different concentrations of the enzyme alone or the enzyme with the addition of sodium sulfite. Unlike SS (Na_2S), sodium sulfite (Na_2SO_3) is a nontoxic reducing agent that acts as an activator of cysteine peptidases and is also capable of cleaving the disulfide bonds of the keratin substrate (sulfitolysis), making it more accessible for digestion. Thus, the sulfitolysis reaction may play an important role in the chemical injury of the epidermis [12, 33].

The optimal conditions for enzymatic dehairing were 0.05% w/v enzyme with 0.6% w/v sodium sulfite for 24 h at 30 °C. The test with sodium sulfite without enzyme (Fig. 1a) reveals that by itself the sodium sulfite is not able to depilate the skin of cow. Figure 1d shows the full depilation achieved with CpLP enzymes under the conditions described above on cowhide leather. Images in Fig. 1b, c show incomplete depilation action, while Fig. 1e shows an excessive enzymatic effect that alters the cowhide structure.

Several enzymatic dehairing with microbial enzymes has been reported [11, 34], but using concentrations higher than 0.05% (w/v). For example, the proteolytic enzyme isolated from *Aspergillus tamarii*, was able to depilate goat skins at pH 9–11 and temperatures 30–37 °C with enzyme concentration of 1% w/v and incubation periods of 18–24 h [35], and the thermostable serine alkaline protease from *Bacillus pumilus* was assayed at 1% w/v exhibiting promising result in the dehairing of goat skin [36]. The synergistic action of the different enzymes present in the enzymatic preparation of *C. procera*, added to the sodium sulfite effect that would facilitate the penetration and action of the proteolytic system, could explain this advantage. It should be noted that without the addition of sodium sulfite, the experiments demonstrate that to obtain the same efficacy, at least twice the amount of enzyme had to be employed (data not shown).

The process was evaluated through the different microscopy techniques. The wet blue hide obtained by enzymatic dehairing using CpLP was compared with lime-sulfide dehairing (conventional chemical process). Figure 2 shows the surface appearance observed through the magnifying glass for each treatment; good color uniformity and grain smoothness were detected for both enzymatic and lime-sulfide treatment. When optical microscopy was used (Fig. 3), it could be observed that the hair pores on pelts with enzymatic (Fig. 3b) or chemical processing (Fig. 3c) did not show residual hair, indicating hair removal from the root. The SEM analysis of the cross-section of the leather with enzymatic or chemical treatment (Fig. 4) revealed similar images: open collagen bundles without apparent damage can be seen for both treatments. Moreover, the surfaces of the wet blue hides were observed by SEM (Fig. 5). The micrographs confirmed the dehairing action by enzymatic and chemical processing, since the hair and epidermis were completely removed from treated skins, showing a clear surface with no grain damage and clean hair pores. In future experiments the immobilization of the enzyme will be tested aiming at the purpose of it reusing and improving its selectivity.

Conclusion

In the context of the leather industry, alternative methods of depilation are being sought. Enzymatic dehairing with plant proteases represents a biotechnological alternative to the conventional process of the tanning industry at a much lower ecological cost. CpLP, a plant enzymatic preparation with 0.6% sodium sulfite efficiently dehaired cow hide in tests on a pilot scale without damaging the collagen layer, and is, therefore, potentially useful for the replacement of SS in the leather industry.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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