Quantitative Determination of Enzymatic and Chemical Dehairing of Skins by an Electronic Force Sensor

by

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Abstract

The dehairing effect of 2 keratinolytic enzyme preparations from liquid cultures of *Bacillus cereus* IZ-06b and *B. cereus* IZ-06r and 3 commercial proteolytic enzyme formulations used in leather manufacturing for soaking, dehairing, and bating processes were quantified by a computerized force sensor. Measurements of the force needed to remove individual hairs from skin, showed that the keratinolytic enzymes did loosen hairs from sheepskins. These enzyme preparations may therefore be useful alternatives to present days' beamhouse chemicals, although the traditional dehairing procedure with lime and sulphide still exhibited the strongest effect in hair loosening.

Resumen

El efecto depilante de dos preparaciones queratinolíticas enzimáticas producidas por cultivos líquidos del *Bacillus cereus* IZ-06b y *B. cereus* IZ-06r, así cómo las de tres formulaciones proteolíticas comerciales empleadas en curtiembres para los procesos de remojo, depilado, y rendido fueron cuantificadas por medio de un medidor de fuerza computarizado. Determinaciones de la fuerza requerida para remover pelos individualmente de la piel demostraron que las enzimas queratinolíticas sí aflojaron los pelos de pieles ovinas. Estas preparaciones podrían ser alternativas útiles a los productos químicos del pelambre, aunque los procedimientos tradicionales con cal y sulfuro exhiben todavía los efectos depilantes más fuertes.

Introduction

Microbial keratinases are becoming important enzymes for the leather industry, where they can be used as environmentally friendly dehairing agents. Traditional dehairing processes using lime and sulphide solutions are some of the most pollutant operational steps in leather manufacturing. Alkaline proteases, including keratinase, collagenase, and elastase can be used to minimize the need for sulphide and reduce the organic waste load of the dehairing process.[1] Keratinolytic proteases selectively degrade the keratin tissue in the follicles in the hides and skins[2] and keratinolytic activity combined with mild collagenolytic and elastolytic activities can result in a proteolytic dehairing process, which is gentle towards the proteins forming the leather.[3]

Different protease formulations are presently available for different leather manufacturing steps, including soaking, dehairing and bating. The proteases in commercial soaking enzyme formulations (e.g. Buzyme 148, Buckman Laboratories, Belgium) remove blood, albumin, and mucous from green hides, and contribute to a uniform rehydration of brine cured and wet-salted hides. Unhairing enzyme (e.g. Buzyme 7705, Buckman Laboratories, Belgium) is used in the dehairing step of the liming process, before or after an initial treatment with lime. The dehairing process can be modified to work either as a hair save or hair burn process and can reduce BOD and COD in the effluent waste. Bating enzyme (e.g. Pellucit 1000, Pulcra Chemicals, Germany) is used after the deliming process for bating in order to break down non-structural proteins.

The dehairing effect of microbial proteolytic enzymes has been documented in several studies, mainly by qualitative comparisons of the ease by which hair is removed from enzymatically treated skins relative to skins not treated by enzymes, when hairs are pulled out by gentle scraping with fingers, or indirectly from analysis of color, smoothness, and silkiness of the pelt.[2,4-12] In this study, we have used direct measurements of the force needed to remove individual hairs

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from skin for quantitative comparisons of the dehairing activity of commercial proteases on sheep skin, as well as crude preparations of the proteolytic enzymes secreted by two keratinolytic strains of *Bacillus cereus* named IZ-06b and IZ-06r. These bacteria produce a mixture of keratinolytic, collagenolytic, and elastolytic activities when grown in batch culture[1]. The kinetics of hair loosening by the various enzymatic preparations were compared in experimental dehairing processes.

EXPERIMENTAL

Enzymatic preparations and production

Bacillus cereus IZ-06b and *B. cereus* IZ-06r, isolated from wool, were used for the production of crude proteolytic enzyme preparations with keratinolytic, collagenolytic, and elastolytic activities.[4] The cultures were grown in batch cultures in liquid media containing 0.5 g l^{-1} NaCl, 0.3 g l^{-1} K_2HPO_4, 0.4 g l^{-1} KH_2PO_4, 0.04 g l^{-1} MgCl, 5 g l^{-1} maltose, 3 g l^{-1} meat peptone, pH 7.4 in conical flasks shaken at 150 rev min^{-1} on an orbital shaker at 30°C. After 20-24 hours when the maximal keratinolytic activities in the batch cultures were attained[4] cell-free culture supernatants were harvested and used as crude enzymatic preparations for enzymatic dehairing of sheepskins.

Dehairing of sheepskins by commercially available enzyme formulations used in the leather industry was investigated at conditions recommended by the manufacturer. The effect of the soaking enzyme formulation, Buzyme 148 (Buckman Laboratories, Belgium) was investigated at pH 7 at a dosage level of 0.1%. The unhairing enzyme formulation (Buzyme 7705, Buckman Laboratories, Belgium) was investigated at pH 6 at dosage level of 0.2%. The bating enzyme formulation (Pellucit 1000 LVU g^{-1}, Pulcra Chemicals, Emery, USA) was investigated at pH 7 at a dosage level of 1%.

Enzymatic and chemical treatment of skins

The dehairing effects of the enzymatic preparations were tested using raw, dry salted (metis type) sheepskin. After soaking of sheepskins for 2 h in water, pieces of 25 cm^2 sheepskins were incubated for up to 48 h at 30°C in 15-30 ml *B. cereus* IZ-06b or *B. cereus* IZ-06r crude enzyme preparations depending on leather weight, or in solutions of soaking, dehairing, or bating enzymes prepared according to recommended dosage levels of the manufacturer as described above, and shaken at 110 rev min^{-1} in an orbital shaker. Microbial activity was inhibited by addition of 0.1% of the commercial bactericide Gemacide LP (Gemsan, Istanbul, Turkey). Sheepskins were also incubated in a solution of 3% lime and 2% sodium sulphide in order to compare the enzymatic dehairing to the traditional beamhouse dehairing process.

Force measurements

The force needed to remove individual hairs from sheepskins was determined by a computerized PS-2104 force sensor (Pasco, California). The sheepskins were mounted on a hook on the force sensor and 1-10 hairs were gently pulled out by a pair of tweezers while the force applied to the hairs was recorded at a frequency of 10 Hz. The force needed to remove the hairs was taken as the maximal force recorded at the time the hairs got loose. Every 2 hours, the sheepskins were removed from the incubation baths where they were enzymatically or chemically treated, mounted on the force sensor, and hairs were repeatedly pulled out for at least 10 times. The number of individual hairs removed each time was counted and compared to the maximal force that had been recorded by the force sensor each time hairs were pulled out. By this way, the action of the enzymes was recorded as a decrease in the force needed to remove individual hairs from the skins over time.

RESULTS

Figure 1 shows how sheepskins were mounted onto the PS-2104 force sensor and how individual hairs were pulled out from the skin by a pair of tweezers. Selection and removal of single hairs proved impractical and time consuming due to high densities of hairs on the skin surfaces, and the tweezers typically got hold on 1-10 hairs each time. A force between 0 and 2 N was needed to remove these numbers of hairs depending on the skin and how it had been treated. Figure 2 compares sets of force measurements on hair repeatedly removed from raw metis type sheepskin soaked for 2 h in water and after additional incubation in soaking enzyme for 48 h.

The maximal force needed to remove hairs increased non-linearly by the number of hairs removed (Figure 3). In particularly when more than 10 hairs were simultaneously removed, the force per hair decreased, probably because not all hairs got loose at the same time. The relationship between force and number of hairs removed followed a power function

$$F = a \cdot n^b \qquad (1)$$

where F is force, n is number of hairs removed, and a (force needed to remove 1 hair) and b are constants specific for a given piece of skin and treatment. The force needed to remove 1 hair was calculated from Eq. 1, using $n = 1$.

The effects of *B. cereus* IZ-06b and *B. cereus* IZ-06r crude enzyme preparations on the force needed to remove hairs from raw metis type sheepskin are shown in Figure 4 and compared to skins incubated in water. The force measurements have been normalized relative to the initial force needed in order to compensate for the variations in strengths of hair-binding to skin between different pieces of skin. Although incubation in water had an effect on the loosening of the hairs, the effect of

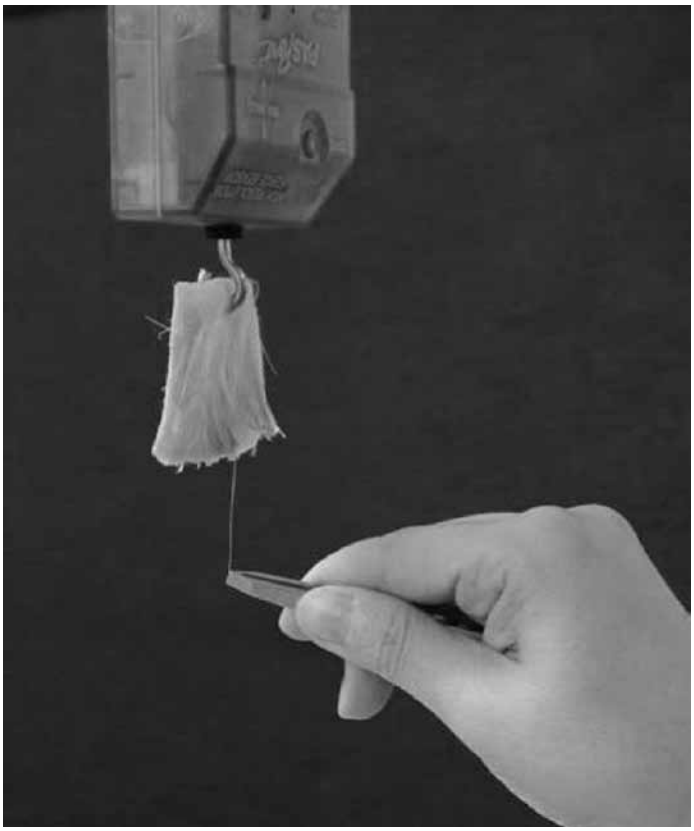

Figure 1. Determination of force needed to remove individual hairs from raw metis type sheepskin by PS-2104 force sensor.

both of the two crude enzyme preparations clearly exceeded the effect of the water.

The dehairing effects of different commercial enzymes and the traditional dehairing chemicals, lime and sodium sulphide were also compared by the Pasco PS-2104 force sensor (Figure 5). The lime and sodium sulphide solutions completely loosened the hairs from the skins in less than 2 h, while the soaking enzyme, Buzyme 148 was the only enzymatic preparation that also loosened the hairs completely. Neither the unhairing enzyme, Buzyme 7705 nor the bating enzyme, Pellucit 1000 LVU g^{-1} loosened hairs as efficiently as *B. cereus* IZ-06b or *B. cereus* IZ-06r crude enzyme preparations (Figure 4). The effect of the bating enzyme treatment was similar to the effect of treatment in only water (Figure 5).

DISCUSSION

The Pasco PS-2104 or similar force sensors provide a quantitative alternative to the qualitative methods that have usually been used to evaluate the effect of enzymes and chemicals in dehairing processes.[2,4-12] The force measurements are rapidly recorded, and the sensor gives reproducible results and works well at the desired range of forces (0-2 N) needed to remove hairs from sheepskin. Force measurements can

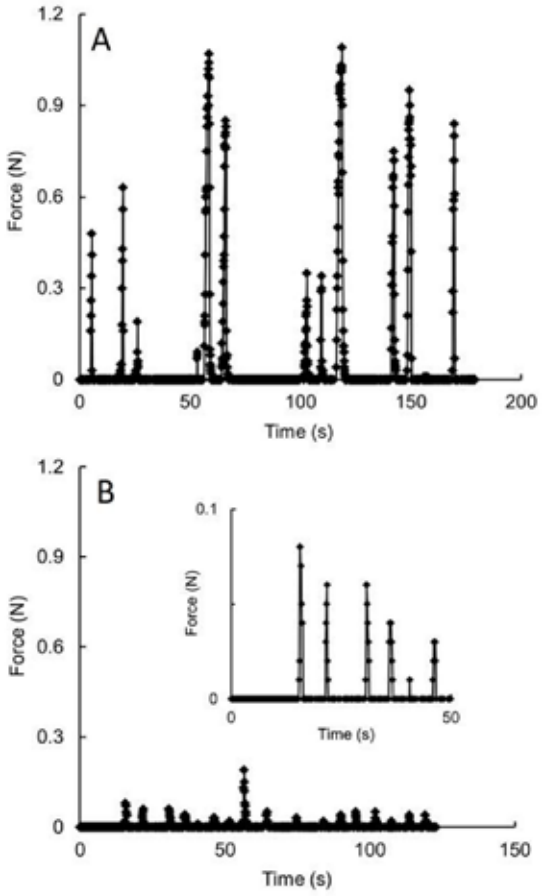

Figure 2. Examples of force measurements during removal of hairs from raw metis type sheepskin. A. Hairs repeatedly removed 11 times from skin soaked for 2 h in water. The maximum of each spike indicate the force needed to remove between 1 and 10 hairs from the skin. B. Hairs repeatedly removed 16 times from the same skin after additional incubation in soaking enzyme, Buzyme 148 for 48 h at 30°C. Inset shows force recordings during the first 5 hair removal trials on expanded scale.

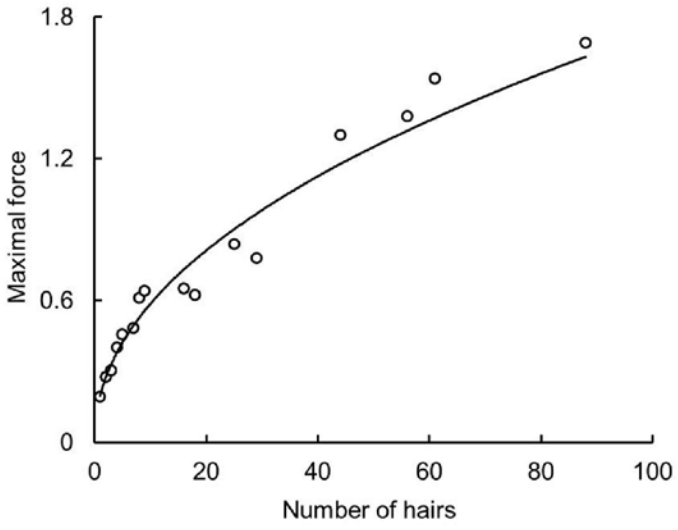

Figure 3. Relationship between maximal force recorded and number of hairs removed from metis type sheepskin after incubation for 4h at 30°C in water. Force estimated from best fit of Eq. 1 to data, $a = 0.20$ N and $b = 0.47$.

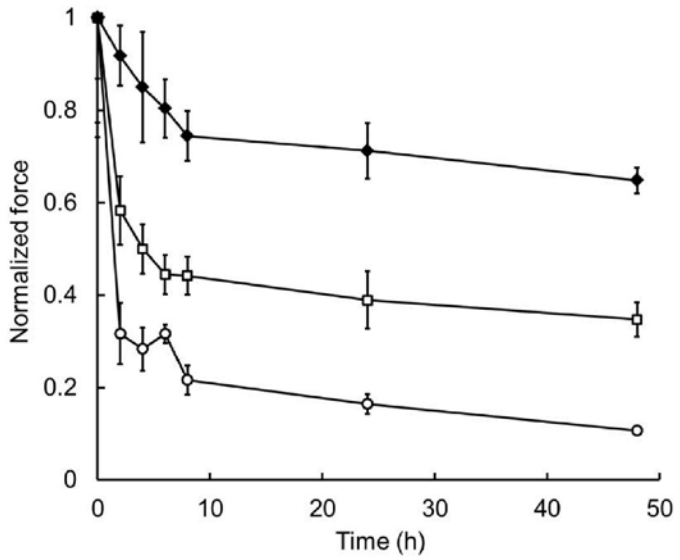

Figure 4. Changes in force needed to remove individual hairs from raw metis type sheepskin incubated at 30°C in *B. cereus* IZ-06b (□) or *B. cereus* IZ-06r (○) crude enzyme preparations or water (◆). Error bars indicate standard error of mean of 10 replicate hair removal trials.

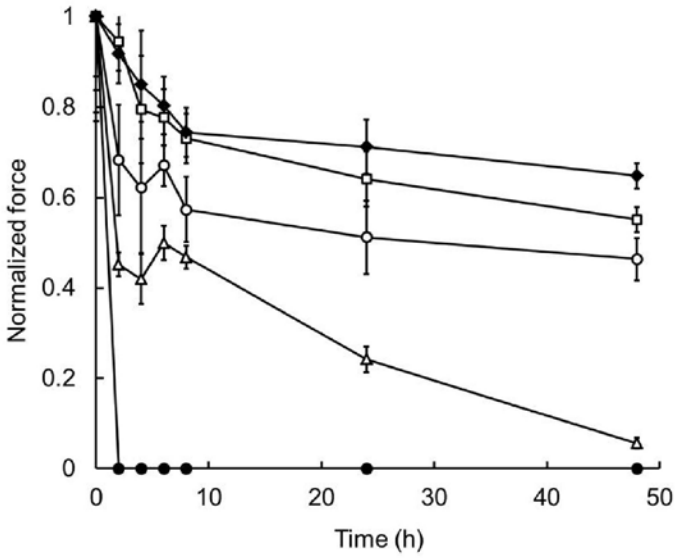

Figure 5. Changes in force needed to remove individual hairs from raw metis type sheepskin incubated at 30°C in 3% lime and 2% sodium sulphide (●), 0.1 % soaking enzyme Buzyme 148 (Δ), 0.2 % unhairing enzyme Buzyme 7705 (○), 1 % bating enzyme Pellucit 1000 LVU g^{-1} (□), or water (◆). Error bars indicate standard error of mean of 10 replicate hair removal trials.

therefore be used to determine the kinetics of enzymatic and chemical dehairing processes. In contrast to qualitative evaluation methods, the force measurements will also allow direct comparisons between different procedures and dehairing agents.

Hairs removed from skins one by one provide direct recordings of the force needed to remove individual hairs. However, catching only single hairs by the pair of tweezers was impractical and time consuming and hair removal trials were conducted more rapidly when simultaneous removal of up to 10 hairs was accepted. The force used to remove hairs was applied manually. No mechanical device was available for this purpose, and the manual removal of 1-10 hairs each time seems the fastest way to conduct the analysis at this moment. The power function (Eq.1) provides a suitable description of the relationship between recorded force maxima and number of hairs simultaneously removed (Fig. 3), from where the force used to remove individual hairs can be deducted. Errors on the force estimates stemming from the manual hair pulling procedures are minimized when hairs are pulled out repeatedly a number of times.

The keratinolytic enzyme preparations produced by both *B. cereus* IZ-06b and *B. cereus* IZ-06r reduced the force needed to remove hairs from the skins. This demonstrates the dehairing ability of the proteases secreted by these bacteria. The force needed to remove hairs decreased most rapidly during the first 8 hours, although the hair loosening process continued for more than 24 hours of incubation.

The two keratinolytic enzyme preparations from *B. cereus* IZ-06b and *B. cereus* IZ-06r were not as efficient dehairing agents as the traditional lime and sodium sulphide for the dehairing process but their efficiency were comparable to the commercial soaking and unhairing enzymes, and more efficient than only water. However, soaking the skins in only water also reduced the force needed to remove hairs, almost as efficiently as bating enzyme preparation, which is also not used for dehairing of skins. The hair loosening effects of the soaking and unhairing enzymes, as well as the *B. cereus* supernatants, must therefore be attributed to specific proteolytic activities in these preparations and supernatants.

CONCLUSIONS

A general method for quantitative measurements of mechanical force to assay the effect of proteases and other chemicals in hair removal processes has been developed and successfully employed. Quantitative measurements of the dehairing will provide an objective way to compare different enzymes, chemicals and procedures, something that is not possible by the qualitative observations that are normally used to assay the effect of enzymatic dehairing procedures. Force sensors can be useful also in fur and double-face production to control hair-loosening defects. In addition, force sensors can be used to control hair slip in raw hide and skin, and be useful supplement to the experience and empirical knowledge of the tanners.

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REFERENCES

1. Gupta, R., Beg, Q.K. and Lorenz, P.; Bacterial Alkaline Proteases: Molecular Approaches and Industrial Applications. *Appl Microbiol Biotechnol* **59**, 15–32, 2002.
2. Foroughi, F., Keshavarz, T. and Evans, C.S.; Specificities of Proteases for Use in Leather Manufacture. *J Chem Technol Biotechnol* **81**, 257–261, 2006.
3. Gupta, R. and Ramnani, P.; Microbial Keratinases and Their Prospective Applications: An Overview. *Appl Microbiol Biotechnol* **70**, 21–33, 2006.
4. Adigüzel, A.C., Bitlisli, B.O., Yasa, I. and Eriksen, N.T.; Sequential Secretion of Collagenolytic, Elastolytic, and Keratinolytic Proteases in Peptide Limited Cultures of Two *Bacillus cereus* Strains Isolated from Wool. *J Appl Microbiol* **107**, 226-234, 2009.
5. Anbu, P., Gopinath, S.C.B., Hilda, A., Lakshmi Priya, T. and Annadurai, G.; Purification of Keratinase from Poultry Farm Isolate-*Scopulariopsis Brevicaulis* and Statistical Optimization of Enzyme Activity. *Enzyme Microb Technol* **36**, 639–647, 2005.
6. Macedo, A.J., da Silva, W.O.B., Gava, R., Driemeier, D., Henriques, J.A.P. and Termignoni, C.; Novel Keratinase from *Bacillus subtilis* S14 Exhibiting Remarkable Dehairing Capabilities. *Appl Environ Microbiol* **71**, 594–596, 2005.
7. Nilegaonkar, S.S., Zambare, V.P., Kanekar, P.P., Dhakephalkar, P.K. and Sarnaik, S.S.; Production and Partial Characterization of Dehairing Protease from *Bacillus cereus* MCM B-326. *Biores Technol* **98**, 1238–1245, 2007.
8. Prakash, P., Jayalakshmi, S.K. and Sreeramulu, K.; Production of Keratinase by Free and Immobilized Cells of *Bacillus halodurans* Strain PPKS-2: Partial Characterization and Its Application in Feather Degradation and Dehairing of the Goat Skin. *Appl Biochem Biotechnol* **160**, 1909–1920, 2010.
9. Riffel, A., Ortolan, S. and Brandelli, A.; De-Hairing Activity of Extracellular Proteases Produced by Keratinolytic Bacteria. *J Chem Technol Biotechnol* **78**, 855-859, 2003.
10. Shrinivas, D. and Naik, G.R.; Characterization of Alkaline Thermostable Keratinolytic Protease from Thermoalkalophilic *Bacillus halodurans* JB 99 Exhibiting Dehairing Activity. *Int Biodeter Biodegrad* **65**, 29-35, 2011.
11. Tiwary, E. and Gupta, R.; Medium Optimization for a Novel 58 kDa Dimeric Keratinase from *Bacillus licheniformis* ER-15: Biochemical Characterization and Application in Feather Degradation and Dehairing of Hides. *Biores Technol* **101**, 6103–6110, 2010.
12. Zambare, V.P., Nilegaonkar, S.S. and Kanekar, P.P.; Production of an Alkaline Protease by *Bacillus cereus* MCM B-326 and Its Application as a Dehairing Agent. *World J Microbial Biotechnol* **23**, 1569-1574, 2007.