Novel Keratinase from *Bacillus subtilis* S14 Exhibiting Remarkable Dehairing Capabilities

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We report here a novel keratinase from *Bacillus subtilis* that has the potential to replace sodium sulfide, the major pollutant from tanneries, and may completely replace it. Its unique nonactivity upon collagen enhances its industrial potential.

Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides (4, 6, 9, 22, 23). Tanneries are constantly concerned about the obnoxious odor and pollution caused by the extremely toxic sodium sulfide used in the dehairing process step (24). Deaths due to this toxic chemical process have even been reported (2, 8). Worldwide, it is estimated that 315 million bovine leathers are produced per year. Considering a waste treatment cost of $0.30 per m² of leather produced (A. Klein, personal communication), more than $1 million is spent per day to treat the waste from tanneries around the world. We report here a novel keratinase from *Bacillus subtilis* that has the potential to replace sodium sulfide in the dehairing process.

**Microorganism isolation.** Bovine hair, skins wastes, and soil samples were suspended and cultivated in a feather-broth medium (composition in grams per liter: delipidated feather meal [the sole carbon and nitrogen source], 10.0; NaCl, 0.5; K₂HPO₄, 0.3; and KH₂PO₄, 0.4 [pH 7.5]).

The best keratinase-producing organism was identified as a *B. subtilis* strain (named strain S14) after classification based on homology (99%) of its 16S fragment with sequences from *B. subtilis* strains and the characterization of the enzyme does not destroy or modify skin structure. As the dermis remains totally intact, this microorganism produces an enzyme with a high biotechnological potential.

**Dehairing assay.** A fresh fleshed bovine hide was washed with a commercial detergent solution and cut into 15- by 5-cm pieces. Two hundred grams of skin (usually two pieces) was processed in a drum flask at 4 rpm with crude extract or water (control) in a proportion of 1.0 ml of liquid per g of skin. When necessary, pH was adjusted with lime. At the end of the process, the skin pieces were gently scraped with fingers to remove loose hairs. This procedure was necessary because rubbing in this laboratory-scale process was not as vigorous as in industrial drums. Total skin depilation was observed in the pH range from 7 to 10, with 4.8 U/g of skin. A complete depilation was reached in 9 h at pH 9.0, 24°C, with 4.8 U/g of skin.

Samples of bovine skin were kept in contact with the crude extract, and the skin fragments were fixed as described by Prophet et al. (21) and examined by a light microscope. Comparing treated skins with untreated controls, we observed that only skin epidermis and adnexa, including hair bulbs, were digested, showing a histological autolytic-like appearance (Fig. 1A and B), as can be expected for proper skin depilation. In contrast, the collagen structure retained the same morphological aspect as the control (Fig. 1C and D), proving that the enzyme does not destroy or modify skin structure. As the dermis remains totally intact, this microorganism produces an enzyme with a high biotechnological potential.

**Enzyme characterization.** The crude extract keratinase was purified by cation-exchange chromatography. Enzyme purity was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13), two-dimensional electrophoresis (10, 19), and capillary electrophoresis (12). The N-terminal sequence (determined by the Edman procedure) is AQSVPYG ISOIKAPALHSQGYTGS———VAVINS (dashes indicate a gap in the sequence). Searches in sequence databases showed similarity between this sequence and sequences of several subtilisins.

Calf skin collagen (1 mg/ml; Sigma, St. Louis, Mo.) was incubated with 15 U (units calculated upon azokeratin substrate) of *B. subtilis* S14 crude extract/ml, purified keratinase, subtilisin (Calbiochem, La Jolla, Calif.), and, as a control, collagenase (Sigma) (20 mM Tris-HCl buffer, pH 8.5, 24 h, 28°C). After incubation, no difference between negative con-
lished leather (9, 16, 23, 26). This enzyme opens the possibility to a direct introduction of enzymatic dehairing without changes in the time of the traditional tanning process (5, 17). Also, attaining dehairing at pH 8.0 is advantageous because it avoids high-pH effluents like those that occur in sulfide-using processes. Additionally, the use of this novel keratinase has the advantage of being a “hair-saving dehairing” process, allowing separation of the hair and avoiding the huge semigelatinous content and high level of organic matter in the wastewater caused by the sulfide “hair-destroying dehairing” methods (25). Other microorganisms able to produce dehairing enzymes, such as Aspergillus flavus (16, 22), Rhizopus oryzae (20), and another Bacillus sp. strain (23), need the use of the unpractical painting method. Also, dehairing with other enzymatic preparations still needs sodium sulfide (4, 6).

For the first time, a new keratinase that is able to complete dehairing, using the worldwide dip method, but without sodium sulfide, has been described. The processing time, as well as the pH activity range and the avoidance of collagen damage, are properties that make this new enzyme an exceptional candidate for dehairing, as it fulfills the industry tanning requirements. Further studies are currently being done to evaluate this potential.

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