journal of aqeic Vol:70 N1 2019

Enzymatic unhairing: Permeability assay of bovine skin epidermis with fungal enzyme extracts

Galarza Betina[1], Garro Maria Laura[1], Greco Carlos[1], Hours Roque[2]
[1]Research Center for Leather Technology (CITEC), National Institute of Industrial Technology; Scientific Research Commission of Province Buenos Aires (INTI-Cueros; CICPBA), Camino Centenario e/505 y 508, (1897) Gonnet, Argentina, +54(221)484-1876; betinagal@hotmail.com, mariagarro12@gmail.com
[2]Research and Development Center for Industrial Fermentations (CINDEFI, CONICET, UNLP); School of Science, National University of La Plata, 47 y 115, (B1900ASH), La Plata, Argentina, +54(221)483-3794; hours@biotec.org.ar

Abstract

The aim of enzymatic unhairing is to allow the separation of the outer sheath of hair root from the dermal connective tissue. This is considered an ecofriendly process. It's not expensive since if the enzymes are applied on the epidermis thereby surface extracts could be saved. The purpose of this work was to analyze the changes in pilosebaceus unit and basal lamina by unhairing enzyme action. Various keratinolytic fungi, previously isolated and selected from different soil samples were used: *Neurospora crassa*, *Verticillum albo-atrum*, *Trichophyton ajelloi*, *Chrysosporium sp*, *Aspergillus sydowii*, *Paecilomyces lilacinus* and *Acremonium murorum*. Fungal isolates were cultivated in solid state conditions using hair waste obtained from the hair-saving unhairing process as substrate. Enzyme extracts were characterized according to their keratinolytic-proteolytic activity and protein content. Bovine skins were placed in plastic plates where the epidermis contacted with different solutions: fungal enzyme extracts with commercial tensioactives (0.1%, 0.5%), biocide 0.2% w/w (wet skin) for 4 h (soaking) and 24 h (unhairing) at 25°C and 40 rpm. Samples of skin with and without enzymatic treatment were fixed in 4% formaldehyde, dried on increasing concentration of ethanol and analyzed with scanning electron microscopy. It was observed that the enzymes altered the epidermis, caused detachment of corneocytes and the emptying of hair follicles. Characteristic patterns of hair cuticle and collagen fibres were preserved in skin control with buffer. *Trichophyton ajelloi* caused the strongest depilatory effect . It was concluded that the enzyme penetration through epidermis could be employed for the application of reagents during the handcrafted tanning of leather.

Keywords: unhairing, keratinolytic fungal enzymes, permeability, epidermis

1. Introduction

The epidermis is a keratinized stratified squamous ephithelium derived from ectoderm and is the outermost layer of the skin. The cells of this layer are categorized into two major groups: keratinocytes and nonkeratinocytes. The epidermis layers can be classified from the basement membrane to the outer surface as follows: stratum basale (basal lamina), stratum spinosum (spinous or prickle layer), stratum granulosum (granular layer), stratum lucidum (clear layer) and stratum corneum (horny layer) (Monteiro-Riviere 2006).
In bovine skin the main barrier for substances is located in the upper layer, stratum corneum. To allow enzymes reach the pilosebaceous unit and basal lamina without collagen damage, they should diffuse through the epidermis involving transport across a series of resistances (Gupta 2012). These are crucial elements for the design of an unhairing system. Trabitzsch (1966) refers the technical and operational conditions for the loosening of hair. He indicates that even though corium (dermis) is thicker than epidermis (grain layer), enzymes diffuse faster through dermis (Cantera et al. 2004).
Stratum corneum keratin acts as a barrier, in this layer there is a continuous sheet of cells rich in protein (corneocytes) embedded in an intercellular matrix of lipids. These cells are highly organized and stacked one upon another to form vertical interlocking columns with a flattened tetrakaidecahedron shape that provide a minimum surface:volume ratio. The transepidermal water loss is a function of the

integrity and permeability of this layer because of this special arrangement, typical of hairy skin. Also in recent years follicular penetration processes have been considered. More recent studies strongly suggest that skin appendages play an important role in penetration processes of substances (Knorr et al. 2009).

For enzyme diffusion, it is necessary to generate channels through this barrier to allow the enzymes to reach the pilosebaceous unit. Ideal enzyme unhairing will be achieved when proteolytic activity is directed towards basal lamina and pilosebaceous unit with minimal effect on collagen producing a leather with good quality.

Changes in epidermis with the generation of more accessible routes for enzyme diffusion would let unhairing substances reach the basal lamina, minimizing in this way undesirable activity on collagen.

The objective of this research was to analyze the penetration of fungal enzymes, tensioactives and biocide through bovine epidermis to reach pilosebaceous unit and basal lamina evaluating morphological changes and depilatory effect by scanning electron microscopy.

2. Material and methods

Fungal strain isolates: cultures and characterization

Strains were isolated from alkaline-calcareous, neutral and alkaline-sodium soils (Elíades et al. 2010). Then fungal strains were screened in order to determine their ability to produce keratinolytic enzymes using Keratin Azure (Sigma K-8500) as substrate (Scott et al. 2004; Cantera et al. 2003).

Neurospora crassa, *Verticillum albo-atrum*, *Trichophyton ajelloi*, *Chrysosporium sp*, *Aspergillus sydowii*, *Paecilomyces lilacinus*, *Acremonium murorum* were the selected fungal strains.

Fungal crude enzyme extracts (CE) were obtained by solid state culture in hair waste substrate from the hair-saving unhairing process as described by Galarza et al. (2004). They were characterized according to their keratinolytic and proteolytic activities and protein content in presence of biocide and tensioactives.

Tensioactives used were: Isogras AN: ethoxylated fatty alcohol, anionic, used in soaking; Baymol AZ: non-ionic tensioactive, free of nonylphenol and nonylphenol-ethoxylates, used in unhairing step.

Biocide´s solution used was: (Thiocyanomethylthio)benzothiazole (TCMTB) 27%, N,N-Dimethylethylamine (penetrating/emulsify) 10%, cresol 63% (FUBA Chem).

Fungal crude enzyme extracts (CE) from selected fungi were tested in a permeability assay in the soaking and unhairing steps.

Assessment of protein content and proteolytic activity

Protein concentration was determined by Bradford's method (Bradford 1976).

Assay of proteolytic and keratinolytic activities

Azocaseinolytic activity was determined using azocasein as substrate (sulphamide azocasein, Sigma Chem.Co., St. Louis, MO). Reaction mixture containing 100 µl of crude extract and 250 µl of the 1% (w/v) substrate solution in 0.1 M Tris-HCl buffer pH 9, Isográs AN 0.1% 50 µl and Baymol 0.,5% µl was incubated for 30 min a 37°C, stopped by addition of 1 ml trichloroacetic acid (TCA) 10% (w/v) and centrifuged (3000xg, 15 min). One ml of NaOH was added to 0.9 ml of the supernatant, agitated and measured at 440 nm (triplicate). Blank was performed using enzyme solution inactivated for heating at 100°C, 5 min (Liggieri et al 2004). Azocasein activity unit (Uazoc) is defined as the amount of enzyme that, under the test conditions, causes an increase of 0.1 Abs440nm per minute.

Keratinolytic activity was assayed by using hair waste as substrate (washing with tensioactives, dried at 45°C, ground, autoclaved at 121°C and retained with 850 µm sieve, USA Standard ASTM E 11-61).

Reaction mixture containing 150 µl of CE and the 1%(w/v) substrate in buffer Tris-HCl 0.1 M, pH 9 (Yamamura et al. 2002), Isogras AN 0.1% 50 µl , Baymol AZ 0.5% 50 µl and biocide TCMTB (relation biocide/enzyme (w/w): 100 µg biocide/1 µg CE protein) was incubated at 37°C in agitation (100 rpm) for 60 min. Reaction was stopped by addition of 1 ml of trichloroacetic acid (TCA) 10% (w/v), centrifuged (5000xg 15 min) and the supernatant was measured at 280 nm (triplicate). Reaction blanks were performed by incubation 60 min: substrate, tensioactives and buffer. After that CE, biocide and TCA 10% (w/v) were added and the procedure was the same as before. Keratinolytic activity unit

(Uk) was defined as the amount of enzyme that, under test condition, causes an increase of 0.01 Abs280nm per minute.

Permeability assay of bovine skin epidermis with fungal enzyme extracts

Fresh bovine skin from the butt was placed in plastic plates of 26 cm length and 14cm width with 8 wells of 5 cm diameter and 20 ml volume (Fig. 1). These plates allow the contact between epidermis and substances without spilling. The skin was held up horizontally with wooden boards of 20 cm length and 2 cm width tightened with fixing nuts and placed in an orbital shaker. The wells with circular shape allowed watertight compartments to test different solutions (Figs.2 and 3) (Garro 2012).
Soaking step: Each well was filled with 8 ml of different CE, Isogras AN 0.1% w/v and biocide (0.2% w/w wet skin) (duplicate). Incubation was done during 4 h at 25°C with agitation (40 rpm).
Unhairing step: Baymol AZ 0.5% was added in each well after soaking. Incubation was done during 48 h at 25°C, agitation 40 rpm.
Control wells: I.- 8 ml buffer Tris-HCl 0.1 M pH 9 and biocide (0.2% w/w wet skin); II.- 8 ml buffer Tris-HCl 0.1 M pH 9, Isogras 0.1% p/v, Baymol 0.5% and biocide (0.2% w/w wet skin); III.- fresh skin without incubation.
After treatments, samples of skin from each well were fixed 2 h and postfixed for 24 h in 4% formaldehyde, dehydrated in 30%, 50% and 70% ethanol with three changes of 15 min each. The day of microscopic analysis one change of 100% ethanol was made. After this, samples were treated by critical point drying, mounted and metalized prior to the examination by scanning electron microscope (FEI-Quanta 200, LIMF Laboratorio de Investigaciones de Metalurgia Física, Facultad de Ingeniería, UNLP, Argentina)

Fig.1. - Permeability assay: plastic plates holding bovine skin

Fig. 2.- Plastic plates.

Fig. 3.- Wooden boards.

3. Results and discussion

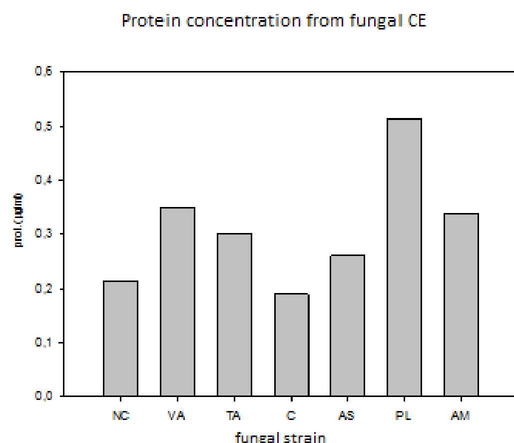

Fig. 4. Protein concentration in different fungal CE

Paecilomyces lilacinus CE showed the highest protein concentration (0.514 µg/ml) whereas the lowest value was found with Chrysosporium CE (0.190 µg/ml) (Fig.4). Both strains have been reported as proteolytic genera; even so Chrysosporium have reported keratinophilic activity (Wilson et al. 2007; Khan et al.2003).

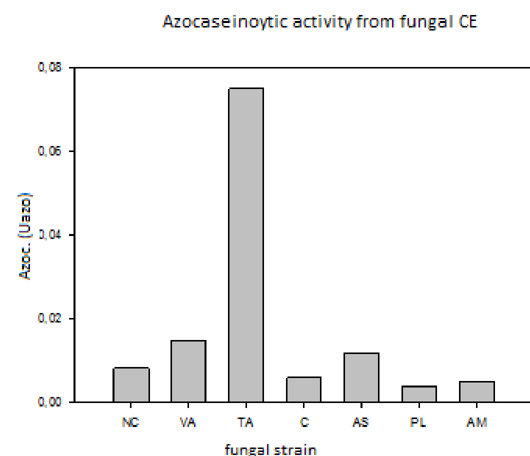

Fig.5. Azocaseinolytic activity in different fungal CE

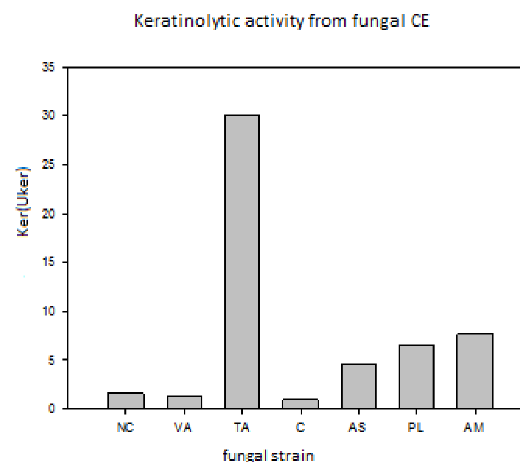

Fig.6. Keratinolytic activity in different fungal CE

Trichophyton ajelloi produced a CE with the highest enzymatic activities (proteolytic and keratinolytic): 0.075 Uazo and 30.1 Uker (Fig.6). This in accordance with "dermatophyte" taxonomic classification. Although this fungal strain is not pathogenic towards human or animals, keratinases are expressed. "Sulphytolisis" is the mechanism whereby filamentous fungi break down insoluble keratin by keratinases (Kunert 2000).

References

Neurospora crassa (NC)

Verticillium albo-atrum (VA)

Trichophyton ajelloi (TA)

Chrysosporium sp [C]

Aspergillus sydowii (AS)

Paecilomyces lilacinus (PL)

Acremonium murorum (AM)

SEM observation

Morphological changes were observed by scanning electron microscopy. Control skin with buffer and biocide had hair cuticle preserved (Fig.7). CE from Neurospora crassa, Chrysosporium sp, Aspergillus sydowii and Paecilomyces lilacinus (Fig.9) did not cause any effect in skin epidermis. In Verticillum albo-atrum and Acremonium murorum (Fig.8 and 10) a detachment of corneocytes have been found. CE action of Trichophyton ajelloi displayed empty hair follicles, stratum corneum with normal characteristics and collagen fibers with typical pattern (Fig.11.1-11.2).

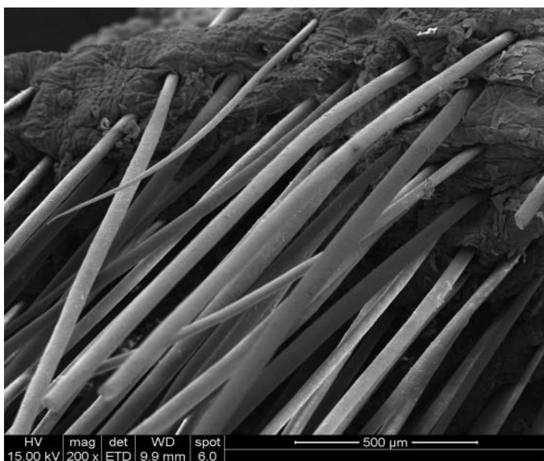

Fig.7 Control: buffer and biocide: hair cuticle preserved 200 x

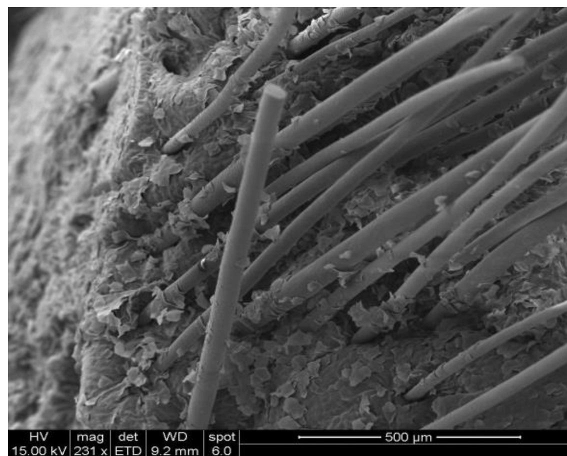

Fig.10 *Acremonium murorum* (AM): detached corneocytes 231x

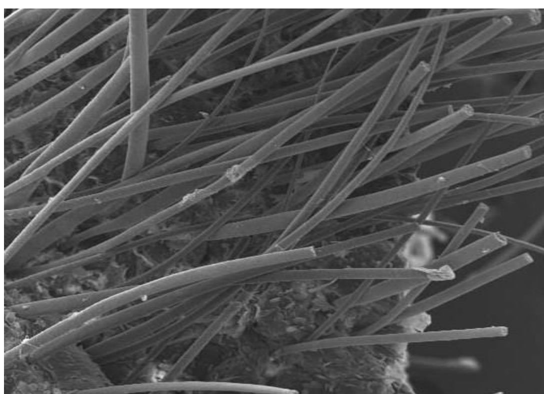

Fig.8.Verticillum albo-atrum(VA): corneocytes detached 400x

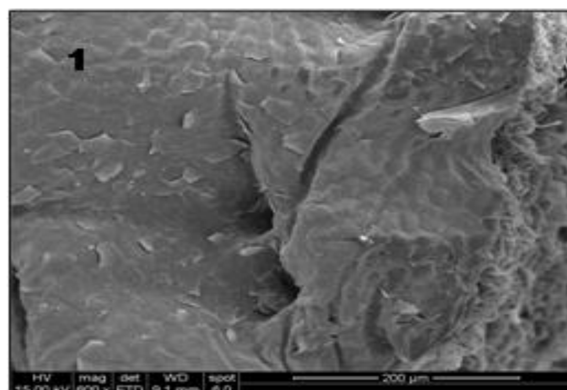

Fig.11.1 Trichophyton ajelloi (TA) (1) Stratum corneum with normal characteristics. Empty hair follicles 600x.

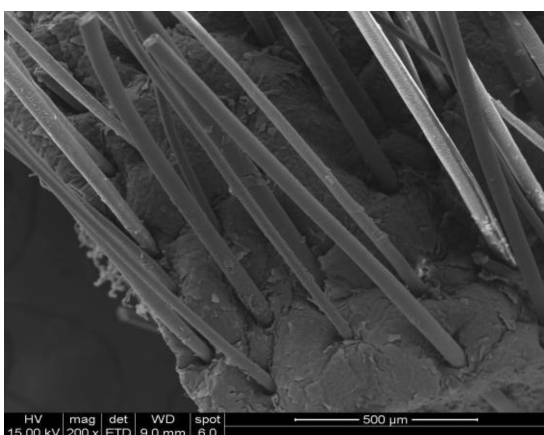

Fig. 9. Paecilomyces lilacinus (PL): hair and epidermis with normal characteristics 200 x

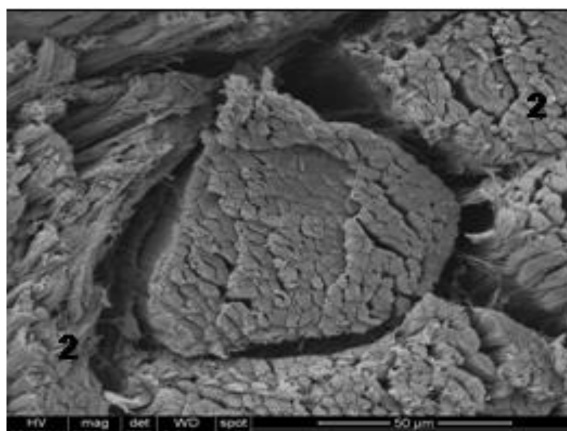

Fig.11.2 Trichophyton ajelloi (TA) (2): collagen fibers with characteristic pattern 2000 x

4. Conclusions

Correspondence of enzyme activity and depilatory effect was found in Trichophyton ajelloi CE, although the action was lower compared with assays when the skin was immersed (Galarza et al. 2016).

Finally, it was concluded that the enzyme penetration trough epidermis could provide benefits for the application of reagents during the handcraft tanning of leather.

sustainability
chemical products for leather

TRUMPLER LANGRO
FINISHING BY TRUMPLER

5. References

Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, 1976, 72, 248-254.

Cantera CS, Garro ML, Goya L, Barbeito C, Galarza B, Hair saving unhairing process: part 6, stratum corneum as a diffusion barrier: chemical-mechanical injury of epidermis, Journal of the Society of Leather Technologists and Chemists, 2004, 51, 121-131.

Cantera CS, Goya L, Galarza B, Garro ML, López LMI, Hair saving unhairing process. Part 5. Characterization of enzymatic preparations applied in soaking and unhairing processes, Journal of the Society of Leather Technologists and Chemists, 2003, 87, 69-77.

Elíades L, Cabello M, Voget C, Galarza B, Saparrat M, Screening for alkaline keratinolytic activity in fungi isolated from soils of the biosphere reserve “Parque Costero del Sur”, World Journal of Microbiology and Biotechnology, 2010, 26 (11), 2105-2111.

Galarza B, Garro ML, Martegani J, Hours R, Characterization and evaluation of a fungal enzymatic pool with unhairing activity, Journal of the Society of Leather Technologists and Chemists, 2016, 100, 257-262.

Galarza BC, Goya L, Cantera C, Reinoso H, Fungal biotransformation of bovine hair. Part I: isolation of fungus with keratinolytic activity, Journal of the Society of Leather Technologists and Chemists, 2004, 88, 3, 93-98.

Garro ML, Tesis, Depilado enzimático conservador del pelo: injuria química y mecánica de la epidermis para incrementar los procesos difusivos, 2012, http://sedici.unlp.edu.ar/handle/10915/27451).

Gupta RC, Veterinary Toxicology Basic and Clinical Principles, 2° edition, Academic Press, 2012, 338.

Khan A, Williams K, Molloy M, Nevalainnen H, Purification and characterization of a serine protease and chitinases from Paecilomyces lilacinus and detection of chitinase activity on 2D gels, Protein Expression and Purification, 2003, 32, 2, 210-220.

Knorr F, Lademann J, Follicular transport route: research progress and future perspectives, European Journal of Pharmaceutics and Biopharmaceutics, 2009, 71, 173-180.

Kunert J, Physiology of keratinophilic fungi. Revista Iberoamericana de Micologia, RKS Kushwaha & J. Guarro, Bilbao, 2000, 10, 77- 85.

Liggieri C, Arribère MC, Trejo S, Canals F, Avilés F, Priolo N, 2004. Purification and biochemical characterization of Asclepain c I from the latex of Asclepias curassavica, The Protein Journal, 2004, 23, 403-411.

Monteiro-Riviere N, Integument, Dellmann´s 6°edition, Textbook of Veterinary Histology, 2006, chapter 16, 320-349.

Scott J, Untereiner W, Determination of keratin degradation by fungi using keratin azure, Medical Micology, 2004, 42, 239-246.

Trabitzsch H, Concerning the possibility of wmploying enzyme in the beamhouse, Journal of the Society of Leather Technologists and Chemists, 1966, 50, 382-389.

Wilson A, Dodson HI, Janaway RC, Pollardà AM, Tobin DJ, Selective biodegradation in hair shafts derived from archaeological, forensic and experimental contexts. British Journal of Dermatology, 2007, 157, 450–457.

Yamamura S, Morita Y, Hasan Q, Yokohama K, Tamiya E, Keratin degradation: a cooperative action of two enzymes from Stenotrophomonas sp, Biochemical and Biophysical Research Communications, 2002, 294,1138-1143.