International Biodeterioration & Biodegradation 127 (2018) 124–129

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Biodegradation of human keratin by protease from the basidiomycete *Pleurotus pulmonarius*

Fabíola Dorneles Inácio[a,b], Alessandro Francisco Martins[c], Alex Graça Contato[b,*], Tatiane Brugnari[b], Rosane Marina Peralta[b], Cristina Giatti Marques de Souza[b]

[a] Federal Institute of Paraná, Campus Jacarezinho, Jacarezinho, Paraná, Brazil
[b] Laboratory of Biochemistry of Microorganisms, Department of Biochemistry, State University of Maringá, Maringá, Paraná, Brazil
[c] Federal Technological University of Paraná, Campus Apucarana, Paraná, Brazil

ARTICLE INFO

Keywords:
Basidiomycete
Hair
Keratinolytic activity
Protease
P. pulmonarius

ABSTRACT

Many microorganisms have been studied for their ability to produce keratinolytic enzymes, among which are basidiomycetes fungi. This paper describes, for the first time, the keratinolytic activity of a protease from *Pleurotus pulmonarius,* a white rot wood fungus, both *in vivo* and *in vitro*, using hair as substrate. The fungus was grown in solid medium containing hair and the production of a protease of 16 kDa with keratinolytic activity was confirmed by solid-state culture. *In vitro,* tests showed that the protease was able to act on different keratinous substrates. Regarding SEM-EDS, morphological changes were seen in the treated samples with *P. pulmonarius* in relation to the control, whereas in the FTIR, these alterations were visualized in the spectral regions of the 1044 cm^{-1} and 1200-400 cm^{-1}, indicating sulfitolysis, which occurs due to the keratinolytic activity of the protease from the fungus *P. pulmonaris* and consequent degradation of the capillary fibers.

1. Introduction

The structure of keratin is very rigid. The numerous disulphide bridges, crossed links, hydrophobic interactions and hydrogen bonds result in rigidity and insolubility, which makes keratin residues highly recalcitrant (Park and Son, 2007; Mazotto et al., 2013). The keratins accumulate in the environment through the waste produced by industries of processing of leather and poultry (Gupta and Ramnani, 2006). The characteristics of these residues make them non-specific substrates for hydrolysis by papain, trypsin or pepsin (Park and Son, 2007; Korniłłowicz-Kowalska and Bohacz, 2011), and therefore, the keratinases have aroused growing interest in the field of biotechnology in recent years (Tiwary and Gupta, 2010). These enzymes have a high capacity to degrade keratins and their importance in the control and treatment of environmental pollution has recently increased. The remains of feathers, horns, hooves and hair are difficult to degrade and their processing can release hazardous wastes and pollutants, which require specific treatment that involve several stages (Okoroma et al., 2012; Demarche et al., 2012; Saran et al., 2013).

In addition, the keratinases are used in the production of fertilisers and in pharmaceutical and food industries (Tiwary and Gupta, 2010). Furthermore, the use of such enzymes can increased the processing of feather meal and the production of amino acids or peptides from keratinous substrates (Anbu et al., 2005) because the current method of thermal processing of feather meal leads to the loss or reduction of the nutritional properties of amino acids (Tiwary and Gupta, 2010).

However, not all the keratins are equal. The structure of human hair and skin contains highly compacted keratin in the α-helix form (α-keratin), while in feathers the β-pleated sheet conformation (β-keratin) predominates. These types of keratin differ in their susceptibility to degradation, the latter being more readily degraded than the former (Anbu et al., 2005; Moreira et al., 2007; Korniłłowicz-Kowalska and Bohacz, 2011). Hair is composed of 85% of α-keratin, which it is a protein that is insoluble in water and mechanically and structurally resistant. The outermost part of hair is the cuticle, which comprises five to ten layers of overlapping cells lining the hair. Proteins make up 75% of the cuticle, of which 30% correspond to the amino acid cysteine (Mazotto et al., 2010).

The keratinases are used in leather processing. Although the production of keratinolytic enzymes by microorganisms is continuous, the amount produced is not sufficient to meet the demand needed by industry, especially the leather industry, which uses keratinases in

* Corresponding author. Laboratory of Biochemistry of Microorganisms, Department of Biochemistry, State University of Maringá. Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brazil.
E-mail addresses: fabiola.inacio@ifpr.edu.br (F.D. Inácio), afmartins50@yahoo.com.br (A.F. Martins), alexgraca.contato@gmail.com (A.G. Contato), tatiane.bru@gmail.com (T. Brugnari), rosanemperalta@gmail.com (R.M. Peralta), cgmsouza@hotmail.com (C.G.M.d. Souza).

https://doi.org/10.1016/j.ibiod.2017.11.010
Received 18 August 2017; Received in revised form 13 November 2017; Accepted 13 November 2017
Available online 29 November 2017

tanning processes (Sundararajan et al., 2011). Although bacteria, especially of the genus *Bacillus*, are the main producers of keratinases, fungi have also been studied because of the ability to produce these enzymes (Anbu et al., 2005; Korniłłowicz-Kowalska and Bohacz, 2011).

The species of bacteria that stand out in terms of their keratinolytic activity are *Bacillus cereus, Bacillus pumilis, Bacillus subtilis* and *Lactobacillus casei*, as well as non-spore-forming bacteria such *Stenotrophomonas and Steptomyces* species. Among fungi, the genus *Aspergillus, Chrysosporium, Fusarium, Microsporum, Myceliophthora, Paecilomyces, Penicillium, Rhizopus, Trichophyton* and *Thermoactinomyces*, are examples (Rieger et al., 2017; Anbu et al., 2005; B1yskal, 2009). Among keratinolytic microorganisms, those that commonly stand out are zoophilic dermatophytes and saprophytes (Korniłłowicz-Kowalska and Bohacz, 2011). However, most keratinolytic fungi do not use keratin as the only source of carbon and nitrogen (Blyskal, 2009), and because of their effective action on keratin many keratinases from fungi have been purified (Macedo et al., 2005).

Basidiomycetes are a group of fungi that play an important role in the ecosystem and they have a rich enzymatic apparatus. Certain basidiomycetes are known as “white rot wood fungi”, in other words, they are excellent wood degraders due to the production of oxidative enzymes (Chirinang and Intarapichet, 2009). Various types of proteases produced by saprophytic basidiomycetes have been studied, such as metalloproteases, aspartic proteases and serine proteases (Faraco et al., 2005; Fujimoto et al., 2004), which leads to the need for the individual study of each action mechanism of these enzymes, as well as their biotechnological potential (Said and Pietro, 2004).

This paper describes for the first time the *in vitro* and *in vivo* keratinolytic activity of *Pleurotus pulmonarius*, which is an edible basidiomycete belonging to the group of white rot wood fungi, using human hair as substrate.

2. Materials and methods

2.1. The microorganism

Pleurotus pulmonarius Fries (Quélet) CCB 20 was acquired from the Botanical Collection of Cultures, São Paulo, Brazil. The basidiomycete was maintained in a medium comprising wheat bran (2%), agar and mineral medium (Vogel, 1956) not supplemented with glucose, with periodical samplings.

2.2. Preparation of keratinous substrates

Dark fibers of human hair were used, which had not been chemically treated, to study the *in vitro* and *in vivo* degradation. First, the hair fibers were cut into fragments (10 cm) and then washed with distilled water and detergent. The material was dried in an oven with air circulation for 72 h at 60 °C and then stored in sealed bags at 25 °C until the time of use. In order to compare the *in vitro* degradation, feathers, beef skin and wool were also used, where these substrates were cut into 3 mm pieces and they were processed through the same procedure as described for cleaning the hair fibers (Mazotto et al., 2010).

2.3. Cultivation conditions

The cultures in solid medium were prepared by pouring melted agar containing mineral medium (Vogel, 1956) not supplemented with glucose and hair (1%) in Petri dishes (90 mm diameter). A plug (15 mm diameter) of active *P. pulmonarius* mycelium was inoculated in the centre of each plate and incubated at 28 °C. The tests were performed in triplicate and the fungus growth was measured daily until the complete colonisation of the plate (Moreira et al., 2007).

The solid-state cultures in Erlenmeyer flasks (250 mL) containing 4 g of damp hair with distilled water in order to adjust the humidity to 80%, were inoculated with 3 agar plug covered with active mycelium (15 mm diameter) in triplicate, and abiotic controls were prepared. The Erlenmeyer flasks were autoclaved at 121 °C for 15 min before inoculation. All the cultures remained in the dark for 25 days at 28 °C and were stopped by adding 20 mL of cold distilled water, followed by homogenisation through slight manual shaking and filtration through filter paper (Whatman N°. 1). The filtrates, denominated crude enzymatic extracts, were stored for determination of keratinolytic activity and the hairs were thoroughly washed after the procedure. The hair fibers were subsequently placed on a tray and dried in an oven at 40 °C to constant weight (Mazotto et al., 2013).

2.4. Enzymatic activity

The keratinolytic activity was determined according to Tiwary and Gupta (2010) with modifications. The capillary fibers (20 mg) were suspended in 4 mL of Tris-HCl buffer (0.1 M, pH 7.0) and 1 mL of crude enzymatic extract. The reaction occurred at 37 °C for 1 h and was ended by the addition of 4 mL of TCA (0.6 M). The mixture was then centrifuged and the solid residue of hair was discarded. The absorbance of the clear supernatant was analysed at 280 nm. The keratinolytic activity was expressed as a unit of enzyme corresponding to an increase of 0.1 in absorbance.

2.5. In vitro degradation of keratinous substrates

Samples of hair fibers, feather, wool and beef skin were suspended in 25 mL of Tris-HCl buffer added with crude enzymatic extract (22 U) and kept under agitation (150 rpm) in a shaker for 48 h at 40 °C. The controls were performed by boiling the crude enzymatic extract for 5 min before the experiment. After 48 h, the Erlenmeyer flasks content were filtered using Whatman N°. 1 filter paper and the remaining solid material was dried at 80 °C to constant weight. The degradation of the fibers was measured by calculating the difference between the mass before and after the experiment (Okoroma et al., 2012; Moreira et al., 2007).

2.6. Fourier transform infrared spectroscopy (FTIR)

After solid-state cultivation in Erlenmeyer flasks, the dry hair samples were analysed by Fourier transform infrared spectroscopy (FTIR). For this analysis, 2.0 mg of each sample (hair treated with *P. pulmonaris* and control hair) were compressed into a disc with 200 mg of KBr and the analysis of the spectra was performed using Opus software, version 6.5. The spectra were obtained in the absorbance mode with a typical resolution of 2 cm^{-1} and then normalised. The area of the bands in the FTIR spectra was determined according to the methodology described by Pandey and Pitman (2003).

2.7. Scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDS)

After solid-state cultivation in Erlenmeyer flasks, dry samples (hair treated with *P. pulmonaris* and control hair) were treated with gold to make them electrically conductive. The samples were characterised by scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDS) technique using a Shimadzu microscope, SS-550 model.

2.8. Electrophoresis

The crude enzymatic extract obtained from a 15-day old solid-sate culture of *P. pulmonarius* on hair fibers was concentrated with acetone. About 5 mg of protein were applied on a 10% polyacrylamide gel, following the Laemmli (1970) method, under non-denaturing conditions. Molecular weight markers were used to estimate the MW of the proteases of the crude extract. The markers used were bovine albumin