International Journal of Biological Macromolecules 87 (2016) 256–262

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

A novel alkaline keratinase from *Bacillus subtilis* DP1 with potential utility in cosmetic formulation

Gaurav Sanghvi[a,*], Harshit Patel[a], Devendra Vaishnav[a], Tejas Oza[a], Gaurav Dave[b], Prashant Kunjadia[c], Navin Sheth[a]

[a] Department of Pharmaceutical Sciences, Saurashtra Univeristy, Rajkot-360005, India
[b] Department of Biochemistry, Saurashtra University, Rajkot-360005, India
[c] B. N. Patel Institute of Paramedical and Sciences, Bhalej Road, Anand, India

ARTICLE INFO

Article history:
Received 16 October 2015
Received in revised form 23 February 2016
Accepted 25 February 2016
Available online 3 March 2016

Keywords:
Bacillus subtilis DP1
Alkaline keratinase
Dehairing cream
CD spectra

ABSTRACT

The *Bacillus subtilis* DP1 was isolated from poultry farm soil at Anand district, India. The highest enzyme production (379.65 U/ml) was obtained at pH 10.0, a temperature of 37 °C and a growth period of 72 h. The extracellular keratinase was purified by gel filtration chromatography with 27.98 purification fold. Purity was also confirmed by High-Performance Liquid Chromatography (HPLC) analysis, where a major peak having retention time of 2.5 min was obtained on C18 column using photo diode array detector. Purified keratinase was stable in a broad range of pH (8–12) and temperature (20–50 °C) with optimum at pH 10.0 and 37 °C. The metallic ions, Ca^{2+} and Mg^{2+} enhance keratinase activity. Secondary structure from Circular Dichroism (CD) spectra implies that purified keratinase is largely β-pleated sheet rich protein. For preparation of dehairing cream formulation, compatibility studies of excipients were carried out. Fourier transform infrared spectroscopy (FTIR) spectra of sodium stearate, calcium carbonate and sodium lauryl sulphate shows no reactivity of functional groups and hence mixture was compatible for formulation of keratinase dehairing cream. Prepared biological depilatory was able to remove hair more efficiently compared to marketed formulations.

1. Introduction

Keratins are insoluble fibrous proteins with cross-linked disulfide bridges, hydrogen bonds, and hydrophobic interactions. Keratins are chiefly found in nature constituting hair, wool, feather, nails and other epithelial covers of vertebrates. Due to tightly packed supercoiled polypeptide chains it is resistant to proteolysis by common proteases such as trypsin, pepsin, and papain [1]. Despite the recalcitrant character of keratins, diverse microbes like bacteria and fungi are identified and reported to utilize keratin as substrate by producing keratinolytic enzymes [2].

Keratinases have a broad spectrum of industrial applications and can be used in different biotechnological applications for environment-friendly processing of keratinous wastes. Keratinases (E.C. no. 3.4.99.11), a group of proteinase enzymes, are important for hydrolyzing feather, hair, wool, collagen and casein to clean obstructions in the sewage system during waste water treatment [3]. Insoluble feather keratins can be converted after enzymatic hydrolysis to feedstuffs, fertilizers, and glues or used for the production of rare amino acids serine, cysteine, and proline [1,2]. Enzyme proteases covers approximately 40% of worldwide sales and industrial demand are increasing with explicit interest in microbial proteases. Highly active preparations of proteases with appropriate specificity and stability of pH, temperature, surfactants, and organic solvents continue to stimulate the search for new enzymes from different microbial origin.

For new enzymes, microorganisms represents attractive sources, as they can be cultured in large quantities by established methods in a relatively short time, producing an abundant and regular supply of the enzymes. Among different microbial sources, representatives of the bacterial genus *Bacillus* are recognized as good protease producers and, used largely on commercial scale, as protease producer by submerged fermentations [4,5]. Several strains of *Bacillus licheniformis* and *Bacillus subtilis* are described as keratinolytic [6,7], and other species such as *B. pumilus* also produce keratinases [8]. *Bacillus* keratinases are of interest because of their effectiveness in degradation of insoluble keratinous substrates [9]. Research on keratinolytic microorganisms was mainly focused on biotechnological applications involving the hydrolysis of

* Corresponding author. Current address: Max Planck Insitute of Developmental Biology, Tubingen, Germany.
E-mail address: gv.sanghvi@gmail.com (G. Sanghvi).

http://dx.doi.org/10.1016/j.ijbiomac.2016.02.067
0141-8130/

keratinous byproducts. However, promising new applications related to drug delivery and hydrolysis of prion proteins was described in some findings [10,11].

The present investigation cites the novel approach of keratinase inclusion in formulation of hair removal cream. Hair removal cream is a chemical depilatory but in the present investigation formulated hair removal cream is a biological depilatory prepared using purified keratinase as substituent of chemical agents. Although hair removal creams vary between different manufacturers, they use the chemical thioglycolate mixed with sodium hydroxide or calcium hydroxide to melt the hair from skin epidermis. Therefore, the current study holds importance as it concerns with replacement of chemically toxic substance with purified alkaline keratinase isolated from *B. subtilis* DP1 species.

2. Materials and methods

2.1. Isolation of microorganisms

Feathers and feather wastes were collected from chicken poultry farms near Rajkot and Anand districts, Gujarat, India. Impurities were filtered through a sieve (diameter 3–4 mm). For isolation of bacteria, feather and feather wastes were flooded with autoclaved distilled water and suspension was streaked on feather meal agar. Plates were incubated at 37 °C for 5 days in incubator. Colonies giving clear zones were chosen for further work. Isolated purified culture was identified by 16S rDNA sequencing.

2.2. Production media and culture conditions

2.2.1. Inoculum preparation

Inoculum was prepared by adding loopful culture in 100 ml of nutrient broth media in 250 ml flask. Media was incubated for 24 h at 37 °C in rotatory shaker. 5 ml of activated culture was transfered in production media containing trace salts and feather as substrate. The production media contains NaCl 0.5 g, K_2HPO_4 0.4 g, KH_2PO_4 0.4 g and feather 20 g/l. All the chemicals were purchased from Himedia Labs. Pvt., Ltd. (Mumbai, India).

2.2.2. Optimisation of process parameters

Different substrates human hair, rabbit hair, duck feather, feather white, feather coffee (2%) were checked for the production of keratinase. The effect of pH on keratinase production was determined at values ranging from 8–12. Temperature was varied 25–50 °C for enzyme production.

2.3. Enzyme characterization

2.3.1. Keratinase enzyme assay

Keratinase activity was determined by incubating mixture of ground chicken feather (20 mg) in 3.8 ml of 1 M glycine NaOH buffer (pH 10.0) to which 0.2 ml of extracted enzyme was added. The mixture was incubated at 37 °C for 1 h. After incubation, assay mixture was cooled in ice for 10 min and remaining feathers were removed through a millipore cellulose filter (0.45 mm) under vacuum. One unit of keratinase activity was defined as the amount of enzyme that increases absorbance by 0.1 under the assay conditions used.

2.3.2. Protein assay

Protein concentration was measured by Lowry's method using BSA as standard [12]. In spectroscopy, protein was measured in terms of absorbance at 280 nm. Specific activity was expressed as the enzymatic activity per mg of protein.

2.4. Statistical analysis

All data were analyzed using Microsoft Excel. Values are expressed as mean standard error of results from three independent experiments.

2.4.1. Enzyme activity parameters

Five different concentrations of grounded chicken feathers (20, 40, 60, 80 and 100 mg) were used to determine effect of substrate concentration on enzyme activity. The effect of temperature on enzyme activity was determined within range of 25–50 °C. The effect of pH on enzyme activity was evaluated by incubating enzyme for 60 min with chicken feather in 1 M Glycine NaOH buffer at pH 8–12. The stability of enzyme was also characterized.

2.4.2. Purification of enzyme and purity check by High performance liquid chromatography (HPLC)

Crude filtrate was subjected to ammonium sulphate precipitation and 20%, 40%, 60% and 80% fractions were collected. Maximum activity was observed in 80% fraction thus it was further subjected to gel filtration chromatography using sephadex G-75 resin. The gradient fraction showing maximum activity was collected and checked for purity on a waters nova pack C18 reverse phase (RP) HPLC column (3.0 mm × 300 mm). Proteins were eluted with a linear gradient from 5% to 70% (v/v) acetonitrile containing 0.1% (v/v) Trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min and elution was monitored at 280 nm.

2.4.3. Secondary structure prediction by Circular dichroism (CD) spectroscopy

Prediction of secondary structure was done by CD spectroscopy. Sample preparation for CD was easy as isolated keratinase enzyme was extracellular in nature. In brief, the sample eluted from sephadex gel filtration chromatography was collected. It was checked for purity on SDS PAGE and zymography was performed. The sample was further lyophilized before delivering it for CD analysis at CSMCRI (Central salt and marine research institute, Bhavanagar, India). CD of Keratinase was performed on JASCO J-185CD Spectrometer (Japan).

2.5. Hair removal cream

2.5.1. Excipient compability study

Compatibility of excipients used in formulation was analyzed by Fourier transformed infrared chromatography (FTIR) in region of 400–4000/cm. Spectra was recorded for pure excipient and physical mixtures of excipients. The pH of 1% aqueous solutions of ideal paste was checked.

2.5.2. Formulation

Biological depilatory was prepared by mixing sodium stearate (1%) and calcium carbonate (2.4%) in dried mortar and triturated well with little addition of water (approximately 59%) till it conglomerate together. For easy efficient solubility and viscosity PEG 400 (4%) was added to mixture. Glycerol (4.5%) was added for smoothness and easy applicability. Once the viscous and consistent cream is prepared keratinase (5%) was added to mixture till it completely mixed with cream. Rose oil (0.5%) was incorporated in cream for fragrance.

Efficiency of the formulated cream was checked keeping commercially available cream veet as control. Veet was purchased from cosmetic shops at Rajkot, India. The rabbit's ear was mark with rectangles of approximately 2 cm in which cream was applied. The commercial as well as formulated cream was applied and kept for