Hydrolyzing Proficiency of Keratinases in Feather Degradation

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Abstract The keratinase degrade highly rigid, cross linked structural polypeptides with different efficiency depending on the type of source. Two newly isolated strains of *Bacillus subtilis* (RSE163 and RSE165; NCBI Accession no JQ887983 and JQ887982) were found to be efficient keratinase producers with unusual catalytic activity result in different morphological changes in degradation pattern of feather, confirmed by their scanned electron micrographs. Maximum keratinolytic activity of both the strains *B. subtilis* RSE163 and RSE165 were found to be 366 ± 15.79 and 194 ± 7.26 U after 72 h of incubation. While the disulphide reductase activity of RSE163 and RSE165 estimated 0.24 ± 0.05 and 0.15 ± 0.03 U/ml of enzyme after 24 h of incubation. A total of 16 free amino acids of variable concentration were also analyzed in the cell free supernatant of hydrolyzed feather from two strains. Present study demonstrates the action of two different keratinases in feather degradation.

Keywords Sulphitolysis · Reductases · Amino acids · Propensity · β -Sheet

Introduction

Keratinases encompasses a distinctive group of proteolytic enzymes, show their complete involvement in hydrolysis of

insoluble keratin (feathers, hair, nails, horn and keratin-rich wastes) into simple polypeptides and amino acids [1]. Disulphide bonds between two cysteine molecules strengthen the structure of keratin [2]. At present, treatment of feather with microbial keratinase draws wide attention with several applications due to its substantiality as feasible source of dietary protein in food and feed supplements. Keratinases are likely to engender a prospective worldwide market similar to other proteases [3]. Different keratinases may carry out erratic activity towards different keratin substrates, due to extracellular secretion from microorganisms like bacteria, fungi and actinomycetes [4]. Among all, bacteria specifically *Bacillus* species are found to be most dynamic and frequent keratinase producing bacteria [5]. The keratin degradation mechanism includes sulfitolysis, proteolysis and followed by deamination, although pattern of degradation is not clear till date [6]. As the feather has β -pleated sheet structure consists of 20 amino acids, mainly of cysteine and its structural configuration comprises of a central carbon linked to functional groups (amine, $-\text{NH}_2$, and carboxylic acid, $-\text{COOH}$), the hydrogen atoms and the group R further twisted and cross linked by disulfide bridges to form a cable-like structures [7]. Enzymatic degradation of the feather entails the mutual characteristic actions of two enzymes disulphide reductase and keratinase [8]. Disulphide reductase breaks the disulphide bridges which are responsible for the rigidity of keratin found in feather and keratinase cleaves the hydrogen bonds (hydrolysis) of β -pleated sheet and releases different amino acids (deamination) [9]. We in our lab while working on the production of keratinases have tried to observe the function of keratinase in feather degradation from two newly isolated *Bacillus subtilis* RSE163 and RSE165 (NCBI Accession no JQ887983 and JQ887982) at regular interval. This manuscript presents the distinctive behavior of keratinases towards the degradation mechanism of feather.

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Materials and Methods

Culture Conditions

Two different strains of *B. subtilis* RSE163 and RSE165 previously isolated in the laboratory was cultured and maintained at -20°C in 50 % glycerol.

Assay of Keratinolytic Activity

The keratinolytic activity of both the strains was determined by using the cell free extract of feather media containing feather as substrate after 72 h of incubation at 37°C . The reaction mixture contained 100 μl of enzyme preparation and 4.9 ml glycine NaOH buffer (0.5 M pH 9) with 25 mg of feather as substrate, the mixture was incubated at 60°C for 1 h. The reaction was stopped with 4 ml 5 % (w/v) trichloroacetic acid. Absorbance of the filtrate was measured spectrophotometrically at 280 nm. An increase of a 0.01 absorbance was considered as one unit of keratinase per ml in 1 h at 280 nm under the assay conditions [10].

Assay of Disulfide Reductase Activity

To ensure the presence of sulphytolysis, the disulfide bond reducing activity was determined spectrophotometrically at 412 nm by measuring the yellow-colored sulfide formed upon reduction of 5, 5-dithiobis (2-nitrobenzoic acid). 10.00 ml reaction mix includes, the 2 ml of (50 mM) potassium phosphate, 3 ml of enzyme and 5 ml distilled water. 20 μl DTNB was added to 3 ml of this mixture. The absorbance of the developed color (within 2') was measured at 412 nm. One unit of disulfide bond-reducing activity was defined as the amount of enzyme that catalyzes the formation of 1 μM of sulfide per min.

Electron Microscopy

Degraded feather at different interval was removed and washed twice by distilled water and fixed with the help of fixative. The substrates were then dried and put on stubs and a thin layer of gold was coated by gold sputter coater and then observed under scanning electron microscope (model: Leo 435 VP) at AIIMS, New Delhi, India.

Amino Acid Analysis

Amino acids analysis of cell free supernatant of hydrolyzed substrate was also performed in triplicates from Agilent 1,100 high performance liquid chromatography (HPLC) using C-18 column with methanol, acetonitrile, sodium acetate, triethanol amine and tetra hydrofuran as mobile phase at medical research foundation Sankara Nethralay

Chennai. Samples was derivatized and analysed by with *S*-carboxymethyl-L-cysteine as the internal reference. OPA (*o*-phthalaldehyde 3-mercaptopropionic acid) was used for derivatives preparation of primary amino acids and there detection was done using diode array detector where as FMOC (9-fluorenylmethyl chloroformate) was used for derivatization of secondary amino acids which were detected fluorometrically [11].

Results and Discussion

Keratinolytic activity is very well documented among genera *Bacillus* [12]. Two different strains of *B. subtilis* previously isolated in the lab had shown maximum keratinase production about 366 ± 15.79 U from *B. subtilis* RSE163 and 194 ± 7.26 U from *B. subtilis* RSE165 after 72 h of incubation at 37°C in presence of feather as substrate. Other species of *Bacillus* such as *B. licheniformis*, *B. pumilus* and *B. cereus* are also potential keratinase producers showed maximum activity of 54.9, 438, 60.67 U respectively [13–15]. Keratinolytic activity also associated with strains like *Streptomyces* and *Fervidobacterium* [16, 17]. Efficiency of keratinase in keratin degradation totally contingent on its source, culture conditions, chemical composition and nature of substrate [18]. Initial step in the mechanism of keratin degradation exhibited by disulphide reductase (EC 1.8.1.8), by cleaving the disulphide bridges of β -sheet [19]. It is also act as a crucial enzyme in the cellular antioxidant system protects cell against free radicals [20]. Sulphydryl bridges play a major role in maintaining the suitable configuration of proteins [21]. The disulphide reductase activity from *B. subtilis* RSE163 and RSE165 estimated about 0.24 ± 0.05 and 0.15 ± 0.03 U/ml of enzyme after 24 h of incubation. Reduction of disulfide bonds changes the conformation of amino acids in β -sheet of keratin and rendered different hydrolytic sites for the keratinolytic attack [22]. Hydrolysis of hydrogen bonds of β -sheet presented in outer and inner quill keratins of feather resulted disparate physical and morphological changes in the degradation pattern of feather. This mechanism can be clearly understood from scanned electron micrographs of degraded feather (Fig. 1A, B), Keratinase of *B. subtilis* RSE165 after 48 h of incubation act on the hydrogen bonds of outer quill keratin directing its fragmentation (Fig. 1Ab, Ac). Further, it acts on inner quill keratin making the rachis hollow and transparent (Fig. 1Ad) after 72 h of incubation; the degradation extends with the breakage of outer quill (dense structure) and promotes the complete degradation of the feather. Whereas keratinase of *B. subtilis* RSE163 act simultaneously on the hydrogen bonds of both inner and outer quill keratin in 48 h (Fig. 1Bb) resulting in tapering of rachis (Fig. 1Bc), proceeds with the complete hydrolysis of feather. Action of keratinase causes deamination reaction leading to the

Fig. 1 Scanned electron micrographs images of feather degradation at different time interval **A** Degradation of feather by the strain RSE165 *Bacillus subtilis* *a* Intact feather as control *b* Partial degradation of feather barbs after 24 h of incubation. *c* Colonization of bacteria and fragmentation of rachis after 48 h of incubation *d* Degradation of inner quill keratin and leading to complete degradation of feather after 72 h of incubation **B** Degradation of feather by the strain RSE163 *Bacillus subtilis* *a* Intact feather as control *b* Partial degradation of feather barbs after 24 h of incubation. *c* Simultaneous degradation of both outer and inner quill keratin after 48 h of incubation. *d* Complete degradation of feather after 72 h of incubation

production of free amino acids. A total of 16 free amino acids of variable concentration were identified in cell free supernatant of hydrolyzed substrate from both the strains shown in supplementary Table 1, after 72 h of incubation. Every amino acid has their specific feature which is defined by its side chain [23]. Variation found in concentration of these free amino acids in cell free supernatant of hydrolyzed substrate is due to the low and high propensity of the side chain towards water, it may be classified as hydrophobic (low propensity of side

chain), polar/charged (high propensity and actively favorable) [24]. The charged amino acid residues include lysine (+), arginine (+), aspartate (−) and glutamate (−), polar amino acids include serine, threonine, asparagine, glutamine, histidine and tyrosine and hydrophobic amino acids include alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophane, cysteine and methionine [25]. Content of polar amino acids glutamic acid, serine and histidine were copious, about 33.5, 45.5 and 37.9 $\mu\text{M}/\text{ml}$ in hydrolyzed product from

B. subtilis RSE163 while it is only 14.2, 18.5, and 9.0 $\mu\text{M/ml}$ in hydrolyzed product from *B. subtilis* RSE165 where as hydrophobic amino acids valine was abundant in concentration of 83.4 $\mu\text{M/ml}$ from *B. subtilis* RSE165 in comparison to 47.7 $\mu\text{M/ml}$ from *B. subtilis* RSE163. Other amino acids like arginine, tyrosine, methionine, phenylalanine isoleucine, leucine, lysine, cysteine and aspartic acid were present in very less amount (7–33 $\mu\text{M/ml}$) in both hydrolyzed product (supplementary Table 1). As tyrosine, tryptophan, glutamine and serine have negative correlation with valine, isoleucine and leucine in β -sheet, therefore concentration of different amino acids in degraded product indicate the site of action of keratinase for hydrolyzation of the exposed sites in *B. subtilis* RSE163 and RSE165 strains respectively [26]. Results of amino acid profiling elucidated the clear evidences in support of the characteristic behavior of two different keratinases on hydrolysis of feather keratin by showing discrepancy in the concentration of the amino acids analyzed from the hydrolyzed feather from two strains. Similar trend in release of free amino acids also reported by Kumar et al. [27], during feather degradation by *B. altitudinis* among 19 amino acids phenylalanine, isoleucine, leucine and valine in concentration of 124.2, 17.1, 19.6 and 88.1 $\mu\text{M/ml}$ found to be essential. Presence of these amino acids in feather makes it as valuable dietary protein supplement for animal feedstuffs (flour and feather meal) after its hydrolysis [28]. Keratinolytic microorganisms and their enzymes may be a subject of substantial scientific interest due to their capability to hydrolyzed keratin into amino acids in balanced form without losing their original property [29].

Conclusion

This study confirmed the potentiality and dissimilar behaviour of keratinases of two different strains of *B. subtilis* RSE163 and RSE165 in terms of their degradation mechanism, keratinolytic, disulphide reductase activity and amino acid profiling. Taking into account the hydrolyzing proficiency of keratinase, it can be used to upgrade the waste management and nutritional value of feather meal.

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