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Purification and characterization of keratinase from recombinant *Pichia* and *Bacillus* strains

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ABSTRACT

The keratinase gene from *Bacillus licheniformis* MKU3 was cloned and successfully expressed in *Bacillus megaterium* MS941 as well as in *Pichia pastoris* X33. Compared with parent strain, the recombinant *B. megaterium* produced 3-fold increased level of keratinase while the recombinant *P. pastoris* strain had produced 2.9-fold increased level of keratinase. The keratinases from recombinant *P. pastoris* (pPZK3) and *B. megaterium* MS941 (pWAK3) were purified to 67.7- and 85.1-folds, respectively, through affinity chromatography. The purified keratinases had the specific activity of 365.7 and 1277.7 U/mg, respectively. Recombinant keratinase from *B. megaterium* was a monomeric protein with an apparent molecular mass of 30 kDa which was appropriately glycosylated in *P. pastoris* to have a molecular mass of 39 kDa. The keratinases from both recombinant strains had similar properties such as temperature and pH optimum for activity, and sensitivity to various metal ions, additives and inhibitors. There was considerable enzyme stability due to its glycosylation in yeast system. At pH 11 the glycosylated keratinase retained 95% of activity and 75% of its activity at 80 °C. The purified keratinase hydrolyzed a broad range of substrates and displayed effective degradation of keratin substrates. The K_m and V_{max} of the keratinase for the substrate *N*-succinyl-Ala-Ala-Pro-Phe-pNA was found to be 0.201 mM and 61.09 U/s, respectively. Stability in the presence of detergents, surfactants, metal ions and solvents make this keratinase suitable for industrial processes.

1. Introduction

Keratinases (EC 3.4.21/24/99.11) are serine or metalloproteases capable of degrading insoluble structure forming keratinous proteins. Keratinases accede to the bioconversion of keratinous wastes from leather and poultry industries into recoverable products such as feedstuffs, fertilizers, polymers and in the production of rare amino acids like serine, cysteine and proline [1]. *Bacillus* keratinases are of interest because of their effectiveness in degradation of insoluble keratinous substrates [2]. Particularly, *Bacillus licheniformis* has been used for several decades for the industrial production of α-amylase and various proteases. Several strains of *B. licheniformis* have paved way for commercialization of enzymes, particularly the use of Bioresource International's (BRI) Versazyme as feed additive for the improvement of broiler yield [3].

Keratinases from different microorganisms vary in their properties and they have been purified and characterized from different native strains using multistep procedures [4–7]. To overproduce the keratinase and to reduce the cost of extraction, mostly *Escherichia coli*, *Bacillus subtilis* and *Pichia pastoris* strains have been used as the host with appropriate inducible and constitutive systems for the overexpression and further purification of recombinant proteins [8–10]. Conventional ammonium sulfate precipitations followed by anion exchange chromatography have been still used for purification of histidine-tag keratinase from recombinant strains [11,12]. Recently several workers have reported *Bacillus megaterium* based system for the extracellular protein production and further affinity chromatographic method for the recovery of recombinant proteins [13,14].

In our previous study, keratinase gene of *B. licheniformis* MKU3 has been cloned, and expressed in *B. megaterium* under xylose inducible and α-amylase promoter and optimized the conditions for the production [15,16]. However, the stable maintenance of recombinant plasmid in the host requires selection via expensive antibiotics. Especially, in industrial production processes, this cost-intensive procedure is not desired and therefore, the stable integration of the complete expression construct into the chromosome of the host is essential. In this direction, several industrial yeasts have been developed as recombinant host systems for the commercial production of heterologous proteins [17]. One of the most commonly used systems is the methylotrophic yeast *P. pastoris*. Hence, in this study stable strain of *P. pastoris* with *ker* in its chromosome was developed to improve the keratinase production. Considering the potential use of this enzyme in biotechnological processes and the fact that keratin-degrading mechanism is still not well understood, we report here the purification and characterization of a histidine-tagged keratinase from recombinant *P. pastoris* and *B. megaterium* strains.

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2. Materials and methods

2.1. Strains, plasmids, enzymes and growth media

The strain *B. licheniformis* MKU3 maintained in our laboratory having high keratinolytic activity was used as source for keratinase gene [15]. *E. coli* Top10 was used for cloning, transformation and propagation of plasmids. The plasmid pPICZαA and *P. pastoris* strain X33 were used in cloning and analysis of recombinant protein expression (Invitrogen, CA, USA). Bacterial strains were grown in low salt Luria Bertani (LB)[1] medium (pH 7.5) and *Pichia* strains were grown in yeast peptone dextrose (YPD) medium. Whenever needed, the medium was supplemented with 25–100 μg/ml of zeocin. The recombinant *B. megaterium* MS941 (pWAK3) developed in our earlier study was used for the production of keratinase [16]. All enzymes for DNA manipulation were purchased from MBI Fermentas (Opelstrasse, Germany).

2.2. Cloning of ker gene in Pichia pastoris strain

The primers KpnKerpropF 5′-GGAGGTACCGCTCAACCGGCGAAA AATGTT-3′ and NotKerR 5′-CGAGCGGCCGCTTGAGCGGCAGCTTCGA CATTGAT-3′ were used to amplify DNA fragment of 1053 bp c™orresponding to prokeratinase without native signal peptide of *B. licheniformis* MKU3 with standard PCR conditions described in our earlier work [15]. After digestion of the PCR product, the DNA fragment was cloned into the KpnI and NotI sites of pPICZαA and transformed into *E. coli* Top10. All DNA isolation and gel extraction were performed using kit and according to the manufacturer's instruction (Qiagen, Hilden, Germany). The transformants were plated on low salt LB containing 25 μg/ml zeocin and positive colonies were selected to prepare plasmid for yeast transformation. The electrocompetent culture of *P. pastoris* strain X33 (mut$^+$) was transformed by introduction of the SacI digested, linearized recombinant plasmid into these cells using the EasySelect *Pichia* Expression system (Invitrogen, CA, USA) by electroporation using BTX (ECM399, Germany) according to manufacturer's instruction. Transformed cells were selected on YPD sorbitol (YPDS) agar plates containing zeocin (100 μg/ml). After 48 h of incubation at 28–30 °C, the colonies were screened for the integrants with cloned gene in its chromosome.

2.3. Expression of recombinant keratinase

Pichia pastoris transformants were inoculated into 25 ml of buffered complex medium (BMGY) containing (w/v); yeast extract—1%; peptone—2%; yeast nitrogen base (YNB)—1.34%; biotin—4×10^{-5}% and glycerol—1% with 100 mM phosphate buffer pH 6.0). The precultures were grown on an orbital shaker (250 rpm) at 30 °C to an OD_{600nm} of 2–6. The cells were harvested by centrifugation at 3000*g* for 5 min and resuspended in 50 ml of BMMY medium (the same as BMGY, except that glycerol was replaced by methanol 0.5% (v/v) in 500 ml of Erlenmeyer flasks. The cultures were grown at aerobic conditions. To maintain methanol induction of the *AOX1* promoter, 0.5% (v/v) methanol was fed every 24 h during the fermentation period. Culture aliquots (1 ml) were collected every 24 h and the cells were removed by centrifugation 8000*g* for 10 min. The optimum conditions used for the expression of keratinase in *B. megaterium* MS941 (pWAK3) were as reported earlier [16]. The recombinant *B. megaterium* strain was grown at 37 °C for 36 h in LB tetracycline medium.

2.4. Enzyme purification

Recombinant *B. megaterium* MS941 and *P. pastoris* strains were grown in optimized conditions. The cells were separated by centrifugation (8000*g* for 15 min at 4 °C) and the supernatant was concentrated by ultrafiltration (MW cutoff, 10 kDa, Amersham BioSciences, UK). Purification of recombinant keratinase was carried out at 22 °C in a Fast Protein Liquid Chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ). The culture supernatant containing His-tagged keratinase was passed through the charged HiTrap column (5 ml) and washed with 10 column volumes of start buffer (20 mM sodium phosphate with pH 7.4, 500 mM NaCl and 10 mM imidazole). The bound proteins were eluted by a linear gradient from 0 to 500 mM imidazole in elution buffer containing 20 mM sodium phosphate (pH 7.4) and 500 mM NaCl. Fractions (2 ml) were collected, dialyzed and assayed for keratinase activity.

2.5. Protein estimation

The protein content of the enzyme preparation was estimated by the method of Lowry et al. using bovine serum albumin as the standard [18].

2.6. Protease and keratinase assay

Protease activity was determined under standard conditions using azocasein (Sigma–Aldrich Chimie, St. Quentin Fallavier, France) hydrolysis [19]. Keratinase activity was determined by the method of Bressollier et al. using keratin azure (Sigma–Aldrich Chimie, St. Quentin Fallavier, France) as substrate [20]. Chicken feather, casein, BSA and gelatin 1% (w/v) were incubated in the above assay buffer with keratinase for 6 h with constant agitation. Peptide liberation was measured photometrically at OD_{280nm} in the supernatant after the addition of 20% (v/v) trichloroacetic acid. One unit (U) of enzyme activity was defined as the amount of enzyme required to cause an increase of 0.01 absorbance at 280 nm in 1 h [21]. The collagenolytic activity was determined at OD_{520nm} with 5 mg of azocoll (Sigma–Aldrich Chimie, St. Quentin Fallavier, France) per ml in 50 mM potassium phosphate buffer, pH 7.8 [22]. The synthetic substrate used in this study (Sigma–Aldrich Chimie, St. Quentin Fallavier, France) was prepared as stock solutions in DMSO: 20 mM *N*-succinyl-Ala-Ala-Pro-Phe-pNA (AAPF). The enzymatic reactions were carried out with purified keratinase in 88 mM Hepes buffer (pH 7.5) at 37 °C using different concentrations (100–600 μM) of AAPF. The hydrolysis of peptides was monitored spectrophotometrically at 410 nm for *p*-NA peptides. The hydrolysis was followed continuously and the initial velocities were determined. The values of Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) were calculated.

2.7. Electrophoretic methods

Active fractions were pooled, dialyzed and the purity of the enzyme was confirmed by Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) performed with 12% polyacrylamide gels as described by Laemmli [23]. Molecular weight markers (molecular weights 2.3–212 kDa; New England Biolabs, UK and 10–250 kDa rainbow marker; Amersham Biosciences, UK) were included, and the gels were stained with Coomassie brilliant blue R-250 and Schiff's reagent [24]. Purified keratinase 4–5 μg was deglycosylated using 1 U of Endoglycosidase H_f for 2 h at 37 °C according to the manufacturer's instructions

[1] *Abbreviations used:* LB, Luria Bertani; YPD, yeast peptone dextrose; BMGY, buffered complex medium; YPDS, YPD sorbitol; YNB, yeast nitrogen base; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetracetic acid; PMSF, phenylmethylsulfonyl fluoride; PCMB, *p*-chloromercuribenzoate; DTT, dithothreitol; β-ME, β-mercaptoethanol; DMSO, dimethyl sulfoxide.