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Functional characterization of an extracellular keratinolytic protease, Ker AP from *Pseudomonas aeruginosa* KS-1: A putative aminopeptidase with PA domain

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ABSTRACT

A keratin degrading protease, Ker AP sharing peptide homology with putative aminopeptidase from *Pseudomonas aeruginosa* was cloned and expressed as an extracellular protein using pEZZ18-*Escherichia coli* HB101. It was a serine hydrolase with pH and temperature optima of pH 10 and 60 °C. It had a $t_{1/2}$ of 20.50 min at 70 °C. It hydrolyzed various complex proteins such as fibrin, hemoglobin, feather and casein. Ker AP possessed fibrin(ogen)olytic activity along with plasminogen activating activity. In addition, it also cleaved tetra-peptides more efficiently than single amino acid pNA esters. In silico analysis was done to understand this endopeptidase character of this putative aminopeptidase. Domain mapping revealed that it had an additional protease associated domain along with the aminopeptidase domain. Modeling and docking studies revealed that PA domain provided scaffold for binding of larger protein substrates facilitating its endopeptidase character. Glu341, Ser423 and His296 were functionally validated to be probable catalytic triad for its endopeptidase activity.

1. Introduction

Keratinases are special proteases which attack 'hard-to-degrade', insoluble keratin substrates. They are robust enzymes displaying a great diversity in their biochemical characteristics. They stand apart from the conventional proteases due to their broad substrate specificity toward a variety of insoluble, 'proteinase-K resistant' proteins including feather, wool, nail, hair, etc. [1]. Owing to this ability, keratinases find immense applications in various environmental and biotechnological sectors where conventional proteases lag behind. In the environment, they play an indispensable role in the cleanup of the ever accumulation recalcitrant waste such as feather and hair. They are also extensively used in the industrial sectors including feed, food, cosmetics, etc. [2]. Besides these outstanding applications, the protease dominant sectors such as leather, detergent and textile are also looking out for keratinases as better substitutes over conventional proteases for processes such as dehairing of leather, collar washings, removal of scurf and improving the dyeing capacity of wool [3–5]. To meet up with the growing requirements of various sectors, we still need to search for more promising keratinases with better substrate specificity. In this respect, we had previously isolated and purified two extracellular keratinases elaborated by *Pseudomonas aeruginosa* KS-1. Of these, one of the keratinase, Ker P has been described in detail [6,7]. The second keratinase has also been purified and biochemically characterized [8]. Its MS/MS analysis revealed it to have homology with putative aminopeptidase from *P. aeruginosa* POA-1 however its biochemical characterization revealed it to possess endopeptidase function. Here, functional characterization of this keratinase termed Ker AP is described and also attempt has been made to understand the basis of the endopeptidase character of this putative aminopeptidase.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains used in this study include *P. aeruginosa* KS-1 isolated from garden soil. It has been deposited in Microbial type culture collection, Chandigarh, India under the accession number MTCC 10775. *Escherichia coli* HB101 and pEZZ18 purchased from GE Healthcare, India were used as an expression host and vector. Recombinant *E. coli* harboring pEZZ18-KP1 was grown in LB (Luria–Bertani) medium supplemented with 100 μg/ml ampicillin.

2.2. Keratinase assay

The assay mixture containing 1 ml of appropriately diluted enzyme, 4 ml of glycine–NaOH buffer (50 mM, pH 9.0), and 20 mg of feather powder was incubated at 60 °C for 1 h. The reaction was terminated by addition of 4 ml of 5% (w/v) TCA (trichloroacetic acid) and the tubes were left undisturbed at room temperature

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(25 ± 1 °C) for 1 h to allow settling of the precipitate. Feather and insoluble residues were removed by filtration through glass wool. The filtrate thus obtained was centrifuged at 8000 rpm for 10 min and proteolytic products in the supernatant were determined by reading the absorbance at 280 nm against appropriate blank. The enzyme and substrate controls were also prepared. Enzyme control was prepared by addition of 1 ml of appropriately diluted enzyme sample, 1 ml of TCA and 3 ml glycine–NaOH buffer. Substrate control was prepared by addition of 5 ml buffer and 20 mg feather. One unit of keratinase is defined as the amount of enzyme required to bring an increase in absorbance (A_{280}) of 0.01 under standard assay conditions.

2.3. LC-M/MS analysis of keratinase Ker AP

Keratinase Ker AP was purified from *P. aeruginosa* fermentation broth as described previously in Sharma and Gupta [8]. The purified keratinase was identified by using mass spectroscopy, i.e. LC–MS/MS. Purified protein band was cut from the SDS PAGE. In Gel trypsin digestion of the purified protein was carried out using InGel Silver kit (G Biosciences, USA) following the manufacturer's protocol. The digested peptides were sent for LC–MS/MS analysis using Nano LC MS (Thermofinnnigan LCQ Deca) at The Centre of Genomics application (TCGA), Okhla, New Delhi, India.

2.4. Cloning and sequence analysis of keratinase Ker AP

The sequence of the putative aminopeptidase was taken from the *P. aeruginosa* PAO-1 genome database. Primers Ker AP F (5′ GC*GAATTC*ATGAGCAACAAGAAC 3′) and Ker AP R (5′ CG*GGATCC*ATTACTTGATGAAGT 3′) were designed with *EcoRI* and *BamHI* site in the forward and reverse primer respectively. PCR was performed using genomic DNA of *P. aeruginosa* KS-1 as template. The amplicon was cloned into the pGEMT-Easy vector (Promega) and recombinant clones were confirmed by DNA sequencing at the Central Instrumentation Facility, UDSC. The sequence has been submitted to NCBI GenBank. Further, Ker AP gene was sub-cloned from pGEMT-Easy into pEZZ18 vector. Positive clones were confirmed by restriction digestion.

2.5. Extracellular expression of keratinase Ker AP

Recombinant *E. coli* harboring pEZZ-Ker AP was sub-cultured (2%, v/v inoculum) from an overnight grown culture into LB-ampicillin (100 μg/ml) medium and incubated at 37 °C, 300 rpm for 24 h. Culture broth was centrifuged at 8000 rpm for 10 min and extracellular expression was checked in the supernatant by qualitative and quantitative estimation of keratinase activity on synthetic substrate of keratinase, i.e. N-Suc-Ala-Ala-Pro-Phe-*p*NA (AAPF) followed by SDS PAGE analysis. *E. coli* HB101 containing only pEZZ18 vector (without insert) was taken as control.

2.6. Purification of the recombinant keratinase

Extracellular keratinase was produced in LB-ampicillin medium at 37 °C, 300 rpm for 24 h and culture broth was centrifuged at 7000 rpm, 4 °C for 10 min. Cell free supernatant was concentrated with 50% (v/v) chilled acetone and subjected to purification by IgG sepharose chromatography. IgG sepharose 6 Fast flow matrix was purchased from GE Healthcare, India. It was equilibrated with three bed volumes each of 0.5 M acetic acid, pH 3.4 and TST (50 mM Tris–buffer, pH 7.6, 150 mM NaCl and 0.05% Tween 20). Acetone precipitate was resuspended in TST buffer and it was loaded onto the column. Next, the column was washed with 10 bed volumes of TST and 2 bed volumes of 5 mM ammonium acetate (pH 3.4). The samples were eluted with 0.5 M acetic acid, pH 3.4 and the column was re-equilibrated with TST. The unbound fraction of IgG column was loaded on a Q-Sepharose column (Sigma–Aldrich, USA) equilibrated with 10 mM Tris–HCl buffer (pH 8). The column was washed with the same buffer and a 15-ml fraction was collected at a flow rate of 2 ml/min. Bound protein was eluted in a linear salt gradient (0.1–1 M NaCl). 2-ml fractions were collected for each salt concentration till protein became zero. Protein elution was monitored by measuring absorbance at 280 nm and keratinolytic activity was determined as described by Sharma and Gupta [6]. The purity of the recombinant enzymes was checked by SDS-PAGE analysis.

2.7. Biochemical characterization of recombinant keratinase Ker AP

2.7.1. pH and temperature kinetics

The enzyme activity of keratinase Ker AP was assayed at 60 °C in the pH range of 4.0–12.0 using buffers (50 mM) of varying pH (citrate phosphate buffer (pH 4.0–6.0), phosphate buffer (pH 7.0), Tris–HCl buffer (pH 8.0–9.0), glycine–NaOH buffer (pH 10.0), phosphate hydroxide buffer (pH 11.0–12.0)). Activity was expressed as percentage relative activity with respect to maximum activity, which was considered as 100%. pH stability was determined by incubating the enzyme in buffers of varying pH (4.0–12.0) for 1 h at room temperature (25 ± 1 °C) and thereafter the residual activity was determined at optimum pH and temperature.

Effect of temperature on keratinase activity was determined by incubating the reaction mixture at different temperatures ranging from 30 to 70 °C under standard assay conditions. Activity was expressed as percentage relative activity with respect to temperature optima which was considered as 100%. The temperature stability was determined by incubating the enzyme samples at 60 °C and 70 °C for different time intervals, the residual activity was determined at optimum pH and temperature.

2.7.2. Effect of metal ions

The effect of various metal ions, viz. Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} on keratinase activity was studied by incubating the enzyme with various metal ions at a final concentration of 10 mM at room temperature (25 ± 1 °C) for 1 h and then determining the residual activity at pH 10, 60 °C. Residual activity expressed in terms of percentage activity against control without the metal ions was taken as 100%.

2.7.3. Effect of inhibitors

The effect of various inhibitors, viz. phenyl methyl sulfonyl fluoride (PMSF), ethylenediamine tetraacetate (EDTA), bromoacetic acid, iodoacetic acid, β-mercaptoethanol, dithiothreitol (DTT), 5,5′ dithio-bis(2-nitrobenzoic acid) (DTNB), phosphorhamidon, bestatin (Sigma–Aldrich, USA; ICN Chemicals, USA) on keratinase activity was determined by incubating Ker AP with the inhibitors at a final concentration of 10 mM for 10 min at room temperature (25 ± 1 °C). The residual activity was measured at pH 10, 60 °C. Residual activity was expressed in terms of percentage activity against control without the inhibitor which was taken as 100%.

In case of inhibition by EDTA, reversal of keratinase activity was checked by addition of metal ions, viz. Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, and Zn^{2+}. 5 mM of various metal ions was added to EDTA inhibited enzyme for 1 h followed by keratinase assay using standard assay protocol.

2.7.4. Substrate specificity of keratinase Ker AP

Activity of keratinase Ker AP was checked on a variety of complex, soluble and insoluble protein substrates as well as synthetic amidolytic substrates as described earlier by Sharma and Gupta [8]. Complex substrates used were feather keratin, casein, hemoglobin,