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Cloning, heterologous expression and characterization of two keratinases from *Stenotrophomonas maltophilia* BBE11-1

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ABSTRACT

The keratin-degrading strain *Stenotrophomonas maltophilia* BBE11-1 secretes two keratinolytic proteases, KerSMD and KerSMF. However, the genes encoding these proteases remain unknown. Here, we have isolated these two genes with a modified TAIL-PCR (thermal asymmetric interlaced PCR) method based on the N-terminal amino acid sequences of mature keratinases. These two keratinase genes encode serine proteases with PPC (bacterial pre-peptidase C-terminal) domain, which are successfully expressed with the help of *pelB* leader in *Escherichia coli* cells. Recombinant KerSMD (48 kDa) shows a better activity in feather degradation, higher thermostability and substrate specificity than KerSMF (40 kDa). KerSMD has a $t_{1/2}$ of 90 min at 50 °C and 64 min at 60 °C, and a better tolerance to surfactants SDS and triton X-100. The predicted model of KerSMD helps to explain the phenomenon of auto-catalytic C-terminal propeptide truncation, the special function of PPC domain, and the molecular weight of the C-terminal-processed mature keratinase KerSMD. This work not only provides a new way to overproduce keratinases but also helps to explore keratinases folding mechanism.

1. Introduction

The structure of keratin is strongly stabilized by several hydrogen bonds, hydrophobic interactions, and disulfide linkages to protect it from degradation by conventional proteases like trypsin, pepsin and papain [1]. Keratinase is a special protease, which has a wide substrate specificity and well-characterized ability for degrading keratinous proteins of feather, wool, and also prion proteins [2–4]. Therefore, it has a great potential application in waste treatment, leather industry, and pharmaceutical industry [5].

Various keratinases are discovered from keratin-degrading microbes, including *Bacillus licheniformis* PWD-1, *Bacillus licheniformis* RG1, and *Bacillus subtilis* S14 [6–8]. Keratinase genes from *Fervidobacterium pennivorans* and *Nocardiopsis* sp. TOA-1 were also cloned and expressed in various heterologous hosts [9,10]. Other excellent keratinases from *Streptomyces* and *Pseudomonas* have been explored [5]. Keratinolytic protease of *Chryseobacterium gleum* was covalently modified by attachment of polyethylene glycol to improve stability and reusability [11]. Although a number of keratinases were purified and the encoding genes were sequenced, there still exists novel keratinases in other microorganisms.

Many keratinolytic strains of *Stenotrophomonas* sp. have been isolated and their keratinases were purified and characterized [12,13]. However, there are few reports about keratinase genes from *S. maltophilia* and their explicit verification by heterologous expression. Since the potential pathogenic factor of *S. maltophilia*, the application of keratinase such as animal feedstock could be limited [1,14]. Therefore, cloning and heterologous expression keratinase gene can be important to enhance productivity, facilitate purification, and avoid potential risk due to application of the whole pathogenic organism [15].

Stenotrophomonas maltophilia BBE11-1 is different from other keratinolytic strains of *S. maltophilia*. The strain BBE11-1 could completely degrade feather within 48 h at 25 °C while feather degradation by *S. maltophilia* DHHJ took 72 h at the optimal temperature of 40 °C [12]. Besides, the strain BBE11-1 can directly use

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Table 1
Comparison with the properties of keratinases produced by keratinolytic *Stenotrophomonas maltophilia*.

Keratinase	Culture Source	MW (kDa)	Optimal pH	Optimal temp	K:C rate	Reference
L1	*S. maltophilia* DHHJ	35.2	7.8	40 °C	No data	[12]
PD-1[a]	*Stenotrophomonas* sp. D-1	40	8–9	40 °C	0.006[b], 0.15[c]	[13]
K1	*S. maltophilia* BBE11-1	48	9.0	50 °C	0.48	[15]
K2	*S. maltophilia* BBE11-1	36	9.0	50 °C	0.025	[15]

[a] Keratinase-like protease D-1.
[b] K:C rate of PD-1.
[c] K:C rate of PD-1 plus disulfide reductase-like protein (DR) from *Stenotrophomonas* sp. D-1.

wool waste for keratinase production [16]. The purified keratinases also showed excellent features for wool and feather treatment. The wild-type keratinases K1 and K2 from strain BBE11-1 display maximum activities at higher temperatures and at wider pH ranges than *S. maltophilia* DHHJ and *S. maltophilia* D-1 (Table 1), indicating the suitability of this keratinolytic strain for industrial applications [12,13]. The current work describes the isolation of two keratinase genes from *S. maltophilia* BBE11-1, as well as the heterologous expression and characterization of the recombinant keratinases.

2. Materials and methods

2.1. Strains and plasmids

Stenotrophomonas maltophilia BBE11-1 (CCTCC M2012495) was cultured at 25 °C for 48 h in feather media [15]. *Escherichia coli* JM109 and BL21 (DE3) were purchased from Novagen (Germany) and cultured in Luria-Bertani (LB) medium at 37 °C. The pMD18-T simple vector (Dalian TaKaRa, China) with T-cloning site was used to efficiently clone TAIL-PCR products. The vector pET-22b (Novagen, Germany) was used for expression of keratinase gene in *E. coli* BL21 (DE3).

2.2. DNA manipulations and cloning keratinase genes

Plasmids were extracted from *E. coli* using Plasmid Mini Kit (Shanghai Sangon, China) according to the manual. All restriction enzymes were Fast-Digest (Fermentas, Canada). DNA fragments from PCR or enzyme-digested products in agarose gel were recovered by Mini BEST Agarose Gel DNA Extraction kit (Dalian Takara, China). Genomic DNA was isolated from *S. maltophilia* BBE11-1 and used as the template DNA for PCR cloning. As shown in Table S1, corresponding primers were designed to clone entire genes using PrimeSTAR HS DNA polymerase (Dalian TaKaRa, China). A 50 μl reaction mixture of PCR was run with 100 ng genomic DNA templates, 1 μl of each 10 μM primers, 10 μl of 5× reaction buffer, 4 μl of 2.5 mM dNTPs and 0.5 μl of 2.5 U/μl PrimeSTAR HS DNA polymerase. The PCR products were purified, sequenced or linked to vectors.

2.2.1. TAIL-PCR method

Modified TAIL-PCR (thermal asymmetric interlaced PCR) was used for isolating keratinase genes [17]. The modified TAIL-PCR consisted of a two-step reaction. Specific primers (SP) were designed on the basis of the N-terminal sequences of keratinases translating to corresponding nucleotides (Table S1 and Fig. 1). The RAPD (Random amplified polymorphic DNA) primers, purchased from Shanghai Sangon, were 10 mers. TAIL-PCR was conducted in 30 μl reaction mixture containing 15 μl 2× BiosTaq PCR MasterMix (Hangzhou BioSci, China), 1 μl of 10 μM SP and RAPD primers, 100 ng genomic DNA template. The procedure of TAIL-PCR on the genome of *S. maltophilia* BBE11-1 is shown in Table 2. The yields of specific PCR products (objective DNA fragments) were higher than non-specific products [17]. So gel purification was done to separate objective DNA fragments, and integration to pMD-18T vector for sequencing was followed. All sequences were analyzed using NCBI online BLAST. Primary encoding sequences of keratinase genes were determined by splicing upstream and downstream sequences from TAIL-PCR results.

2.3. Expression and purification of keratinases

E. coli BL21 (DE3) harboring expression plasmid pET22b + KerSMD/F was used for recombinant keratinases production. When OD_{600} reached 0.6, culture temperature was changed from 37 °C to 20 °C to promote soluble protein production, and 0.1 mM isopropyl-β-D-thiogalactoside (IPTG, TaKaRa, China) was added to induce protein expression. After 3 days incubation, cells were collected at 8000 × *g* centrifugation and suspended again in 100 mM phosphate buffer (pH 7.0) for further ultrasonic treatment. Cell-free fermentation broth was also collected. Supernatant-containing keratinase was obtained by centrifugation at 10,000 × *g* for 15 min at 4 °C. HiTrap Phenyl FF 1 ml and HisTrap FF crude 1 ml (GE Healthcare, Sweden) were used for purifying recombinant keratinase by the machine AKTA purifier (GE Healthcare, Sweden).

2.4. SDS–PAGE and zymography

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10–12% separating polyacrylamide gel. Samples were prepared by mixing enzymes with 5× electrophoresis buffer (10 mM Tris-HCl buffer, pH 8.0; 2.5% SDS, w/v; 10% glycerol, v/v; 5% β-mercaptoethanol, v/v; 0.002% bromophenol blue, w/v), then heated at 100 °C for 5 min before electrophoresis. Zymography was carried out as same as SDS-PAGE except for the addition of 0.2% soluble keratin (Tokyo Chemical Industry, Japan) into the separating gels and unheated enzyme samples. After electrophoresis, the gel for zymogram analysis was incubated with 2.5% (v/v) Triton X-100 for 1 h and washed with 50 mM Tris-HCl buffer (pH 9.0) at 40 °C. All gels were stained with Coomassie Brilliant Blue R-250 and destained in acetate methanol solution. In zymograms, clear zones on a blue background of gels were the existences of keratinolytic enzymes. All chemical reagents were purchased from Shanghai Sangon.

2.5. N-terminal amino acid sequence analysis

The purified keratinases were subjected to 12% SDS-PAGE and then electrotransferred onto polyvinylidene difluoride membrane (Bio-Rad, America). Protein bands were stained with Coomassie blue and excised for N-terminal amino acid sequence analysis by Edman degradation at Shanghai Sangon.

2.6. Keratinolytic activity assay

Keratinolytic activity was measured with soluble keratin modified from Nam et al. [18]. Soluble keratin was purchased from Tokyo Chemical Industry (Japan). Keratinases at different concentrations were added to 200 μl 50 mM Gly-NaOH buffer (pH 9.0) and 100 μl soluble keratin (1%, w/v), and this mixture was incubated at 50 °C for 20 min. The incubation was stopped by addition of 200 μl 4% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 × *g* for 10 min. Then 200 μl supernatant was pipetted into another tube with 1 ml 4% (w/v) Na_2CO_3 and 200 μl Folin-Phenol reagent (Shanghai Sangon). The absorbance at 660 nm was measured. One unit (U) of keratinase activity was defined as an increase of 0.001 absorbance unit at 660 nm per min and per ml enzyme solution. All experiments were repeated three times. Protein concentration was determined using the bicinchoninic acid protein assay reagent kit (Sigma, America) with bovine serum albumin as a standard.

2.7. pH and thermostability analysis of keratinases

The relative activity of recombinant keratinases were tested at different pH values ranging from pH 4.0 to 12.0 in 100 mM of the following buffers, citrate phosphate (pH 4.0–6.0), sodium phosphate (pH 6.0–7.0), Tris-HCl (pH 7.0–9.0), glycine-NaOH (pH 9.0–11.0), and sodium hydroxide (pH 12.0). The optimal temperature for keratinase activity was measured between 30 and 70 °C. The keratinolytic temperature stability was measured by incubating keratinases at different temperature for different time with the Tris-HCl buffer (pH 7.0), then residual activity was detected at the optimal temperature 50 °C.

2.8. Substrate specificity of keratinases

The activities of these recombinant keratinases were checked on a variety of soluble and insoluble substrates. The protease activity on casein was measured as same as the assay of keratinase activity on soluble keratin, and these two soluble substrates were used at 1% (w/v) concentration. The measurements of keratinolytic activity on feather powder and keratin azure (Sigma-Aldrich, USA) were according to Yamamura et al. [13] and Bressollier et al. [19], respectively. The reaction temperature was 50 °C and the Gly-NaOH buffer (pH 9.0) was used for assays. In addition, the keratinase kerA from *Bacillus licheniformis* was used as a control [20]. Feathers were treated with recombinant keratinases, and changes on this substrate were observed by scanning electron microscope (PHILIPS, Netherlands).