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Degradation of keratin by keratinase and disulfide reductase from *Bacillus* sp. MTS of Indonesian origin

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ABSTRACT

Bacillus sp. MTS isolated from Tangkuban Perahu crater Indonesia was found capable of degrading whole chicken feather effectively. The bacteria produced extracellular alkaline keratinase and disulfide reductase. When grown in feather media, *Bacillus* sp. MTS produced multi-fractions of both enzymes. The purified enzymes worked optimally at alkaline pHs, for keratinase at pH 8–12, and for disulfide reductase at pH 8–10. Optimum temperature for the extracellular keratinase was within 40–70 °C, while that for disulfide reductase was 35 °C. When the purified keratinase was mixed with purified disulfide reductase, enzyme activities on the natural keratin substrates (feather and wool) were greatly increased compared to activity of each enzyme alone, activity of proteinase K or activity of purified keratinase in the presence of reducing agents. The mutual action of the two enzymes on feather was examined by Scanning Electron Microscope.

1. Introduction

Keratin, a tough protein substance, is the chief constituent of epidermal layer of skin, hair, feather, nail, hoof, wool and certain shell. As much as 90% of feather is made up of keratin, the fibrous and insoluble structural protein. Mechanical stability of keratin and its resistance to biochemical degradation depend on tight packing of protein chains in the α-helix (α-keratin) or β-sheet (β-keratin) structure and linkage of the structures through disulfide bonds. Digestive enzymes, such as trypsin and pepsin, are not effective for keratin degradation (Bockle and Muller, 1997; Brandelli et al., 2010; Gupta and Ramnani, 2006; Onifade et al., 1998).

The majority of reports on keratinase enzymes focussed mainly on their proteolytic action. Nonetheless, reduction of disulfide bonds affects degradation of keratin significantly (Bockle and Muller, 1997; Cai et al., 2008; Prakash et al., 2010; Ramnani et al., 2005; Yamamura et al., 2002). Thiol formation by *Vibrio* strain kr2 grown in feather keratin media suggested disulfide reduction (Sangali and Brandelli, 2000). Reduction of disulfide bond was observed during the growth of *Streptomyces pactum* and *Bacillus megaterium* on feather (Bockle and Muller, 1997; Swerdlow and Setlow, 1983). *Stenotrophomonas* sp. strain D-1 produced two types of extracellular proteins, a proteolytic enzyme and a disulfide bond-reducing protein. The protease belongs to a serine enzyme and the disulfide bond-reducing protein was suggested as a disulfide reductase enzyme (Yamamura et al., 2002).

We had screened and isolated a feather degrading bacteria from Tangkuban Perahu crater West Java Indonesia and based on its morphology and biochemical reactions, the isolate was grouped as a *Bacillus* species and tentatively refered to as *Bacillus* sp. MTS. The aerobic mesophillic bacteria was very effective in degradation of whole chicken feather and this appeared to be related to activity of the extracellular keratinase and disulfide reductase enzymes. We had optimized conditions for enzymes production and characterized the crude enzymes (Rahayu et al., 2010).

In this study, we purified the extracellular keratin hydrolyzing enzyme (keratinase) and disulfide reductase from *Bacillus* sp. MTS grown in feather containing media, characterized the purified enzymes and analyzed the mutual actions of both enzymes in degradation of keratin substances.

2. Materials and methods

2.1. Growth conditions

The aerobic mesophilic *Bacillus* sp. MTS screened and isolated from Tangkuban Perahu crater West Java-Indonesia was used in these experiments. The agar medium for culture maintenance

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contained 0.6% crushed dried feather (powder), 0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.05% NaCl and 0.05% NH_4Cl (Macedo et al., 2005). For enzyme production, 250 ml medium containing 0.03% K_2HPO_4, 0.01% $MgCl_2.6H_2O$, 0.05% NaCl, 0.05% NH_4Cl and 1.0% chicken feather powder was used (Lin et al., 2001). pH was adjusted to 7.5 and incubation was carried out in a 1 l flask at 37 °C 100 rpm for 48 h. The culture was filtered and centrifuged at 4000 g 4 °C for 10 min to harvest the extracellular enzymes.

2.2. Protein and enzyme assay

Protein content was measured at 595 nm according to Bradford, using bovine serum albumin as the standard protein (Waterborg, 2002). The Bradford reagent consisted of 100 mg Coomassie Brilliant Blue G-250 in 50 ml ethanol 95%. As much as 100 ml of phosphoric acid 85% (b/v) was added and the mixture was made to 1 l by adding aquadest. This reagent was stored at refrigeration temperatures; 0.2 ml of sample was mixed with 4 ml of Bradford reagent, vortexed and kept for 5 min and the absorbance was measured at 595 nm.

Keratinase activity was determined according to Walter (Walter, 1984) using 1% feather powder in Tris/HCl (50 mM, pH 8.0) as the substrate. As much as 200 μl of enzyme sample was mixed with 800 μl of 0.5% w/v keratin substrate. Following incubation for 10 min at 37 °C, 500 μl of Trichloracetic acid (5%) was added and the mixture was kept at 37 °C for another 30 min, then centrifuged at 1000 g for 10 min. The supernatant was mixed with 1 ml of Na_2CO_3 and 200 μl of Follin reagent which had been previously diluted with deionized water (1:2). Incubation was further conducted at 30 min 37 °C to develop the color. After centrifugation at 1000 g for 10 min, the absorbance was read at 660 nm. A tyrosin standard curve was made for quantification. Similar procedure was repeated using varying tyrosin concentration instead of the enzyme samples. One unit of enzyme activity was defined as the amount of enzyme which liberate 1 μmol tyrosine in 1 min.

Disulfide reductase activity was measured as described by Serrano et al. Serrano et al. (1984) with a few modifications. As much as 100 μl of enzyme was incubated with 500 μl of Tris/HCl buffer (0.13 mM, pH 9.0) containing 0.05 mM oxidized glutathione (GSSH) and 0.05 mM EDTA at room temperature for 10 min. The reaction mixture was centrifuged at 1000 g 4 °C for 10 min and the reaction product was detected by addition of 100 μl 20 mM DTNB (dithiobis-nitro benzoic acids) and 1.35 ml Tris/HCl buffer (0.13 mM, pH 9.0) to the 50 μL of supernatant. Absorbance was measured at 405 nm after 2 min of stable color development.

2.3. SDS-PAGE and zymogram

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out using 10% of separating polyacrylamide gel according to Walker (Walker, 2002). Electrophoresis was conducted at 100 V and 50 A for 1.5 h and the gel was stained in silver nitrate solution (Dunn, 1994; Walker, 2002). Gelatin (0.5%, w/v) in Tris–HCl buffer (50 mM, pH 8.0) was mixed into the separating gels for zymogram analysis. After electrophoresis, the gel was washed with 2.5% (v/v) Triton X-100 for 60 min followed by overnight incubation at 55 °C in Tris–HCl buffer (50 mM, pH 8.5). The gel was stained with Coomassie Brilliant Blue R-250 for 30 min and destained in acetate ethanol solution. Low molecular weight (LMW) proteins containing β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) were used as the protein standard.

2.4. Enzyme purification

At the end of incubation, the bacterial culture was filtered and centrifuged at 4000 g 4 °C for 10 min to separate the cell and harvest the extracellular enzymes. The supernatant was precipitated by addition of 50% (w/v) ammonium sulphate and centrifuged at 4000 g 4 °C for 30 min. The enzyme precipitate was resuspended in 3 ml of Tris/HCl buffer (50 mM, pH 8.0) and further dialyzed for 5 h in the same buffer using 12 kDa cut off membrane. Enzyme solution was then applied onto Butyl Sepharose FF coloumn (10/20 mm) which was previously equilibrated with 30% ammonium sulphate in Tris/HCl buffer (50 mM, pH 8.0). The same buffer with 30%, 15% and 0% ammonium sulphate was used to wash the gel and elution was performed at 0.5 ml/min. Fractions of 3 ml were collected and assayed for keratinase and disulfide reductase and the fractions with highest activity was further applied to the Sephacryl S-200HR column (10/40 mm). A Tris/HCl buffer (50 mM, pH 8.0) solution was used to elute the column at 0.2 ml/min. The purified enzyme fractions were used for analysis of optimum pHs and temperatures and for further experiments.

2.5. Degradation of keratin by mutual activities of keratinase and disulfide reductase

In this experiment, we used purified keratinase and disulfide reductase fractions which showed highest stabilities and purification fold. The commercial enzyme Proteinase K which is known to have keratin degrading ability was used to compare keratinase activities of this enzyme with that of *Bacillus* sp. MTS. The protein content of enzymes used was 0.2 μg/ml. Three kinds of natural keratin were used as enzyme substrates, namely native chicken feather, prehydrolyzed chicken feather and natural wool.

100 μl of enzyme was mixed with 1 ml of substrate at 1% in 50 mM buffer Tris–HCl pH 8.0. In this case, the volume ratio of keratinase and disulfide reductase applied was 4:1. Incubation was conducted for 60 min at the optimum enzyme temperature: 35 °C for Disulfide reductase reaction or 50 °C for keratinase reaction or 37 °C for proteinase K. The product was measured according to Walter (Walter, 1984). We also tested the effect of reducing agents on keratinase ability to degrade the keratine substrates. Dithiothreitol (DTT), β-mercaptoetanol (BMT) and urea were used at concentrations of 0.1 mM (DTT), 0.2 mM (BMT) and 0.3 mM (urea). Initially, we incubated keratin with disulfide reductase or reducing agents (DTT and BMT) or urea in 50 mM Tris/HCl buffer (pH 10.0) at 35 °C for 10 min, then keratinase or proteinase K was added and the mixtures were further incubated at 50 °C for 60 min.

2.6. Scanning electron microscopy

For scanning electron microscope analysis, we used whole native feather, which was incubated with 50 μL of keratinase and 50 μL of disulfide reductase, or 0.1 mM dithiothreitol at 50 °C in Tris/HCl buffer (50 mM, pH 9.0) for 90 min. The reaction was stopped by addition of trichloroacetic acid and the feather was washed several times with the same buffer. Fixation was carried out with 2.5% (v/v) glutaraldehyde and 2% (v/v) formaldehyde for 48 h. The specimens were dehydrated several times with 70–100% acetone and dried at 50 °C for 10 min. The gold treated specimens were analyzed in JSM-5310LV SEM JEOL Japan.

3. Results

Results of purification of keratinase and disulfide reductase from *Bacillus* sp. MTS are summarized in Tables 1 and 2 and