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A catalase–peroxidase for oxidation of β-lactams to their (*R*)-sulfoxides

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ABSTRACT

In this communication we report for the first time a biocatalytic method for stereoselective oxidation of β-lactams, represented by penicillin-G, penicillin-V and cephalosporin-G to their (*R*)-sulfoxides. The method involves use of a bacterium, identified as *Bacillus pumilis* as biocatalyst. The enzyme responsible for oxidase activity has been purified and characterized as catalase–peroxidase (KatG). KatG of *B. pumilis* is a heme containing protein showing characteristic heme spectra with soret peak at 406 nm and visible peaks at 503 and 635 nm. The major properties that distinguish *B. pumilis* KatG from other bacterial KatGs are (i) it is a monomer and contains one heme per monomer, whereas KatGs of other bacteria are dimers or tetramers and have low heme content of about one per dimer or two per tetramer and (ii) its 12-residue, N-terminal sequence obtained by Edman degradation did not show significant similarity with any of known KatGs.

1. Introduction

β-Lactam is a well-known pharmacophore for antibiotics and is widely used in clinic for the treatment of bacterial infections (von Nussbaum et al., 2006). However, application of these compounds in medicinal chemistry is not limited to their traditional use as antibiotics only. Recent findings have shown that β-lactam derivatives inhibit mammalian serine and cystine proteases, in addition to bacterial transpeptidase. Since, these proteases have been implicated as important targets for the development of inhibitors as potential therapeutic agents, a number of β-lactam derivatives have been synthesized and evaluated for this activity (Doherty et al., 1990; Elliott and Sloane, 1996; Xing et al., 2008; Zhou et al., 2003). In addition, β-lactam derivatives have also been evaluated for the β-lactamase inhibition activity (Drawz and Bonomo, 2010). The activity of β-lactam derivatives correlates strongly with the oxidation state of the sulfur moiety in the lactam ring. Therefore, a systematic study of the biological activity of these molecules requires simultaneous evaluation of the activity of a relevant β-lactam derivative along with its (*S*)-sulfoxide, (*R*)-sulfoxide and sulfone (Aleksanyan et al., 2002). Whereas, (*S*)-sulfoxide and sulfone can be easily prepared by direct oxidation of the parent β-lactam with a variety of reagents, no method, chemical or biological has been reported in literature till date for the direct oxidation of β-lactams, i.e. without involving protection–deprotection steps, which stereoselectively produces (*R*)-sulfoxide. Currently, (*R*)-sulfoxides of β-lactams are prepared by multistep synthetic routes, which are tedious to accomplish.

The preferential formation of (*S*)-sulfoxide during oxidation of penicillins has been attributed to the directing effect of the carboxyamido group, most likely through the hydrogen bonding of the peroxide reagent with the amide proton prior to the delivery of reactive oxygen to sulfur atom of the sulfide (Nieuwenhuis, 1995). We envisaged that the directing influence of amide group is likely to be absent in enzyme catalyzed reactions, since the preliminary reaction of the peroxide occurs at the metal center (Fe^{III}). The delivery of reactive oxygen will then be dictated by the orientation of the substrate within the binding cavity of enzyme or it will occur from sterically less hindered side.

Here, we report isolation of a bacterium identified as *Bacillus pumilis*, which stereoselectively oxidized the parent β-lactams, penicillin G and V and cephalosporin G to their (*R*)-sulfoxides. The enzyme responsible for oxidase activity has been purified and characterized as catalase–peroxidase (KatG). The catalase–peroxidase of *B. pumilis* is similar to other KatG's of bacterial origin in terms of its spectral properties, but differs in terms of oligomeric structure and heme content. Also, 12-residue N-terminal sequence obtained by Edman degradation did not show significant similarity with any of known KatG in the database.

2. Methods

2.1. Source of chemicals and microorganisms

Penicillin G was from USB Corporation, Ohio, USA and cephalosporin G was a gift from Ranbaxy, India. Penicillin V,

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2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), Sephacryl S-200, bovine serum albumin (BSA), β-mercaptoethanol, sodium dodecyl sulfate (SDS), Q-Sepharose, molecular weight standards were from Sigma Chemical Company, USA. Q-Sepharose fast-flow and Phenyl Sepharose CL-B4 were from Pharmacia (Freiburg, Germany). Thin layer chromatography (TLC) was performed on Merck silica gel DC Alurolle Kieselgel 60F_{254} plates. All other chemicals and reagents were from E. Merck, India. Pure cultures of microorganisms were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh (http://mtcc.imtech.res.in). These were single colony isolates from soil and water samples from a variety of niches in India.

2.2. Growth conditions

Culture of *B. pumilis* was maintained as 25% glycerol stocks at −78 °C. A 100 mL Erlenmeyer flask was charged with 20 mL sterile medium comprising peptone (0.5%), beef extract (0.2%), yeast extract (0.1%) and NaCl (0.5%). After inoculation with bacterium, the flask was incubated with shaking at 200 rpm in an incubator-shaker at 37 °C for 8 h. Eight milliliters of this was used to inoculate a 2 L Erlenmeyer flask containing 400 mL of same media. The flask was then incubated at 37 °C with shaking at 200 rpm on an orbital shaker for 20 h. Cells were isolated by centrifugation at 7000 rpm for 15 min at 4 °C and washed with phosphate buffer pH 7.0 (2 × 200 mL). Wet cell mass obtained was about 12 g L^{-1} of medium.

2.3. Identification of the strain

Strain was identified as *B. pumilis* based on the physiological, morphological, biochemical characterization and 16 s *r*DNA sequencing by Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh. The culture has been deposited with MTCC and has been assigned accession number MTCC B6033. The methods for taxonomic characterization have been described in detail in Supplementary information.

2.4. Preparation of cell-free extract

B. pumilis was grown in several 2 L flasks, as described in Section 2.2. Fifty grams of cells (wet weight; equivalent to dry cell weight of about 11.5 g) were suspended in 150 mL of 10 mM phosphate buffer (pH 7.0) and disrupted by sonication for 30 min with pulse on and pulse off for 30 and 10 s, respectively. The cell free extract was obtained by centrifugation at 15,000 rpm for 45 min at 4 °C.

2.5. Ammonium sulfate fractionation

Solid ammonium sulfate was added in small portions to the cell free extract at 4 °C to 20% saturation. Stirring was continued for another 1 h and then the precipitate removed by centrifugation at 7000 rpm for 40 min. The supernatant was then brought to 90% saturation, maintaining the pH at 7.0 with 0.1 N sodium hydroxide. The precipitate of this step contained all activity. The precipitated proteins were collected, dissolved in 50 mL of 0.1 M phosphate buffer pH 7.0.

2.6. Hydrophobic interaction chromatography

Sodium sulfate (1 M) was added to the protein solution in phosphate buffer, pH 7.0. The particles were removed by centrifugation and clear solution was loaded onto a Phenyl Sepharose fast-flow column (Amersham Biosciences, 2.7 × 20 cm) pre-equilibrated with 10 mM phosphate buffer, pH 7.0 containing 1 M sodium sulfate. The column was washed with same buffer until no more unbound proteins eluted. A decreasing linear gradient of sodium sulfate of 1–0 M in 10 mM phosphate buffer pH 7.0 at a flow rate of 60 mL h^{-1} was used to elute the proteins. Fractions were analyzed for amount of protein by Bradford method (Bradford, 1976). Enzyme activity was assayed for both catalatic and peroxidatic activities. The active fractions (40 mL) were pooled and desalted in an ultrafiltration cell using a 30 kDa membrane.

2.7. Ion exchange chromatography

The desalted Phenyl Sepharose active fractions were loaded on a Q-Sepharose column pre-equilibrated with phosphate buffer (10 mM, pH 7.0) at a flow rate of 30 mL h^{-1}. The column was washed with same buffer until no more unbound proteins eluted. The bound proteins were eluted with a linear gradient of 0–1 M sodium chloride in the same buffer. The active fractions were pooled, desalted and concentrated in an ultra-filtration cell using a 30 kDa membrane to a concentration of 1 mL.

2.8. Gel filtration chromatography

The desalted and concentrated sample from Q-Sepharose chromatography step was loaded on a Sephacryl S-200 column pre-equilibrated with phosphate buffer (50 mM, pH 7.0) containing 0.15 M sodium chloride at a flow rate of 2.4 mL h^{-1}. The column was run with the same buffer; active fractions were collected, pooled and concentrated in an ultrafiltration cell using a 30 kDa membrane.

2.9. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis under non-denaturing conditions was performed in 8% (w/v) polyacrylamide gels according to Maurer (1968). SDS–PAGE was performed in 12% (w/v) gels. The molecular weight standards (Sigma, USA) used were phosphorylase b, rabbit muscle (97,000 Da), albumin, bovine serum (66,000 Da), ovalbumin, chicken egg white (45,000 Da), carbonic anhydrase, bovine erythrocyte (30,000 Da), trypsin inhibitor, soybean (20,100 Da), and α-lactalbumin, bovine milk (14,400 Da). Proteins were stained with Coomassie brilliant blue G-250.

2.10. Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS)

Purified catalase–peroxidase (10 μM) and fresh matrix (3, 5-dimethoxy-4-hydroxycinnamic acid, sinapinic acid, 10 mM) solutions were premixed in a small Eppendorf tube and applied directly to the sample support plate. The sample was allowed to air evaporate and irradiated by a nanosecond laser pulse and analyzed using MALDI-TOF Voyager DE-STR instrument.

2.11. N-terminal sequencing

Pure catalase–peroxidase was solubilized in Laemmli's sample buffer and separated by SDS–PAGE. The gel was washed once with water and equilibrated with 10 mM CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) buffer, pH 11.0 containing 0.037% (w/v) SDS and 10% methanol for 15 min. A polyvinylidine difluoride (PVDF) membrane of gel size was cut and wetted with methanol for 1 min, rinsed once with water and equilibrated with the above mentioned buffer for 15 min. Proteins were electrophoretically transferred onto PVDF membrane at 300–350 mA for 1 h at 4 °C. The extent of transfer was visualized by staining with 0.1% (w/v) amido black (in 1% acetic acid) for 5 min. Destaining was done in 50% (v/v) methanol. Transferred protein of interest was excised and washed once with 20% (v/v) methanol for 1 h at room