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Antibacterial action of a heat-stable form of L-amino acid oxidase isolated from king cobra (*Ophiophagus hannah*) venom

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ABSTRACT

The major L-amino acid oxidase (LAAO, EC 1.4.3.2) of king cobra (*Ophiophagus hannah*) venom is known to be an unusual form of snake venom LAAO as it possesses unique structural features and unusual thermal stability. The antibacterial effects of king cobra venom LAAO were tested against several strains of clinical isolates including *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Escherichia coli* using broth microdilution assay. For comparison, the antibacterial effects of several antibiotics (cefotaxime, kanamycin, tetracycline, vancomycin and penicillin) were also examined using the same conditions. King cobra venom LAAO was very effective in inhibiting the two Gram-positive bacteria (*S. aureus* and *S. epidermidis*) tested, with minimum inhibitory concentration (MIC) of 0.78 μg/mL (0.006 μM) and 1.56 μg/mL (0.012 μM) against *S. aureus* and *S. epidermidis*, respectively. The MICs are comparable to the MICs of the antibiotics tested, on a weight basis. However, the LAAO was only moderately effective against three Gram-negative bacteria tested (*P. aeruginosa*, *K. pneumoniae* and *E. coli*), with MIC ranges from 25 to 50 μg/mL (0.2–0.4 μM). Catalase at the concentration of 1 mg/mL abolished the antibacterial effect of LAAO, indicating that the antibacterial effect of the enzyme involves generation of hydrogen peroxide. Binding studies indicated that king cobra venom LAAO binds strongly to the Gram-positive *S. aureus* and *S. epidermidis*, but less strongly to the Gram-negative *E. coli* and *P. aeruginosa*, indicating that specific binding to bacteria is important for the potent antibacterial activity of the enzyme.

1. Introduction

L-amino acid oxidase (L-amino acid: O_2 oxidoreductase, EC 1.4.3.2, abbreviation LAAO) is a dimeric flavoenzyme containing non-covalently bound FAD or FMN as a prosthetic group. It catalyzes the oxidative deamination of an L-amino acid to produce the corresponding α-keto acid, hydrogen peroxide and ammonia via imino acid intermediate. Snake venoms are rich sources of LAAO (Du and Clemetson, 2002; Tan and Fung, 2009). The enzyme exhibits a wide range of biological activities including apoptosis-inducing, edema-inducing, inhibition or induction of platelet aggregation, antibacterial effect and antiviral activity (Tan and Fung, 2009). The role of LAAO in the pharmacological action of snake venom, however, is still not fully understood. Generally, the enzyme has a low lethal toxicity in mice.

Skarnes (1970) was the first to report the bactericidal activity of a LAAO isolated from *Crotalus adamanteus* venom. In the last 20 years, many authors have also reported the antibacterial activity of LAAOs from snake venoms (see Table 2 for a list) and other animals such as giant African snail and sea hares (Ehara et al., 2002; Yang et al., 2005; Nagashima et al., 2009). Antibacterial action of LAAOs appears to result from hydrogen peroxide generated by the oxidative action of the enzyme, as the effect is abolished in the presence of hydrogen peroxide scavengers such as catalase (Tan and Fung, 2009).

Several authors have reported isolation and characterization of L-amino acid oxidase from king cobra (*Ophiophagus hannah*) venom (Tan and Saifuddin, 1989, 1991; Li et al., 1994; Ahn et al., 1997; Jin et al., 2007). Tan and Saifuddin (1989) reported that king cobra venom LAAO exhibited unusual thermal stability. At pH 7.4, the enzyme retained 100% activity after incubation at 25 °C for 30 days. They also reported that unlike other snake venom LAAO, king cobra venom LAAO was stable at alkaline condition and was not inactivated by freezing. The enzyme also exhibited unique substrate specificity: it was very active against L-lysine, which was a poor substrate for other snake venom LAAOs. Structural studies showed that indeed king cobra venom LAAO is evolutionarily distant to other snake venom LAAOs (Jin et al., 2007). The substrate specificity and thermal stability of the enzyme are, however, similar to LAAO isolated from marine sources such as sea hare (Yang et al., 2005). In view of the unusual thermal stability and unique structural feature and substrate specificity of the LAAO, it would be interesting to investigate the antibacterial action of

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this enzyme. We also report here a comparative study on the potency of its antibacterial action with the commonly used antibiotics using standardized assay, as this aspect of antibacterial action of snake venom LAAOs have not been thoroughly investigated.

2. Materials and methods

2.1. Materials

King cobra (*O. hannah*) venom was provided by a local snake handler. The bacterial strain *Escherichia coli* American Type Culture Collection (ATCC) 25922 was purchased from American Type Cell Culture Institute, USA. Clinical isolates of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *E. coli* were obtained from Department of Medical Microbiology, University of Malaya, Kuala Lumpur, Malaysia. Bacterial culture medium was purchased from Oxoid Ltd, Cambridge, UK. Antibiotics were purchased from Duchefa Biochemie, Netherlands. Resource Q ion exchange column was purchased from GE Healthcare, Amersham Biosciences AB, Sweden. Bovine serum albumin (BSA), catalase and other analytical reagents were purchased from Sigma Aldrich Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade.

2.2. L-amino acid oxidase assay

L-amino acid oxidase activity was determined according to Bergmeyer (1983). To 925 μL of substrate (containing 0.34 mM L-leucine and 81 μg of *o*-dianisidine in 100 mM Tris–HCl buffer, pH 8.5), 50 μL of 0.0075% horseradish peroxidase and 25 μL of the sample were added to initialize the reaction. Increase in absorbance at 436 nm was recorded. One unit of enzyme activity was defined as the oxidation of 1 μmol of L-leucine per min. Molar absorption coefficient of the reaction product was $8.31\times10^{-3}\ M^{-1}\ cm^{-1}$.

2.3. Purification of king cobra venom LAAO

King cobra venom LAAO was isolated using a method modified from Tan and Saifuddin (1989). Lyophilized king cobra venom (20 mg in 200 μL) was subjected to Resource Q high performance liquid chromatography (HPLC) previously equilibrated with 20 mM Tris–HCl, pH 9.0. Elution was carried out with a linear gradient of NaCl in 20 mM Tris–HCl, at a flow rate of 1 mL/min. The gradient program was: 100% buffer A (20 mM Tris–HCl, pH 9.0) for 5 min, followed by a linear, 0–60% buffer B (20 mM Tris–HCl, pH 9.0, 0.5 M NaCl) for another 50 min and finally, 100% buffer B for 5 min. The UV absorbance of the eluate was monitored at 280 nm. Fractions exhibiting LAAO activity were pooled and stored at 4 °C.

2.4. Assessment of homogeneity and protein determination

The homogeneity of LAAO was assessed by 12.5% (w/v) SDS-PAGE (Laemmli, 1970). The gel was stained with Coomassie blue R-250. The molecular weight of the enzyme was determined using the Spectra™ broad range protein ladder (Fermentas Inc, Maryland, U.S.A.) as calibration standards (Mol mass 10 kDa to 260 kDa). Protein concentration was determined by the Bradford (1976) method using BSA as standard.

2.5. Broth microdilution assay and effect of catalase

Broth microdilution assay was carried out according to the protocol describe by the NCCLS, National Committee for Clinical Laboratory Standards (2000). Briefly, bacteria from frozen suspensions were cultured onto nutrient agar plates and passaged twice prior to susceptibility testing. The inoculum suspension was adjusted to the density of 0.5 McFarland (1 to 2×10^8 CFU/mL) and then diluted with 2× Mueller Hinton broth to a final concentration of 1×10^6 CFU/mL. Bacterial suspensions (50 μL) were incubated in 96-well plate in the presence of 50 μL of different concentrations of sterilized LAAO (ranging from 100 to 0.049 μg/mL) or antibiotics (ranging from 64 to 0.031 μg/mL) using 2-fold serial dilutions to yield the appropriate bacterial density of 5×10^5 CFU/mL. The plates were incubated at 37 °C for 24 h. After incubation, the MIC (minimum inhibitory concentration) end points were observed by visual inspection. The end point was reached when the culture medium was completely transparent or no precipitate was seen. Susceptibility of *E. coli* ATCC 25922 to tetracycline was chosen as quality control and its acceptable quality control limit is MIC of 0.5–2 μg/mL (NCCLS, National Committee for Clinical Laboratory Standards, 2000). The MIC was taken as the lowest concentration of antimicrobial agent that inhibited visible growth of the bacteria (n = 3). To evaluate the effect of catalase on the antibacterial action of the enzyme, the same assay was carried out in the presence of 1 mg/mL catalase.

2.6. Bacterial cell binding activity of the LAAO

Bacteria-binding activity of the king cobra LAAO was examined according to Kitani et al. (2008) with slight modifications. Two Gram-positive bacteria (*S. aureus* and *S. epidermidis)* and two Gram-negative bacteria *(E. coli* and *P. aeruginosa*) were chosen for this study. Bacteria were grown in nutrient broth at 37 °C. The cells were collected by centrifugation at 2000 ×*g* for 15 min, washed with phosphate buffered saline (PBS) and finally resuspended with 2× Mueller Hinton broth to form 1×10^9 CFU/mL. Bacterial suspensions (500 μL) were incubated with 500 μL of the LAAO (1 μg/mL) at 37 °C for 1, 3, 5 and 24 h. After the incubation time, the mixture was filtered using 0.20 μm filter, and the L-amino acid oxidase activity of the filtrate was measured.

2.7. Statistical analysis

Results for bacterial cell binding studies are presented as mean ± S.D. The significance of the differences of the means was determined by Student's *t*-test.

3. Results

3.1. Isolation of king cobra venom L-amino acid oxidase

Fig. 1 shows the isolation of king cobra venom LAAO using a one-step procedure. Seven major peaks were obtained, with only peak 7 exhibiting LAAO activity. The peak fractions were pooled and designated as purified king cobra venom LAAO. The purified enzyme was homogeneous as judged by SDS-PAGE, with a molecular mass of 65 kDa, in agreement with the previous report (Tan and Saifuddin, 1989). The specific activity of the purified LAAO was 437.7 μmol/min/mg.

3.2. The antibacterial action of king cobra venom LAAO

Table 1 shows the minimum inhibitory concentration (MIC) of king cobra venom LAAO against clinical isolates of Gram-positive bacteria (*S. aureus* and *S. epidermidis*) and Gram-negative bacteria (*K. pneumonia, P. aeruginosa,* and *E. coli*), as well as *E. coli* ATCC 25922. For comparison, the MICs of some common antibiotics against both Gram-negative bacteria (cefotaxime, kanamycin and tetracycline) and Gram-positive bacteria (cefotaxime, vancomycin and penicillin) were also determined. The MIC of tetracycline against the standard *E. coli* ATCC 25922 was 2 μg/mL, which is within the acceptable quality control limit of 0.5–2 μg/mL. For comparison, the MIC values were also expressed in μM.