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

Mui Li Lee a, Nget Hong Tan a,⁎, Shin Yee Fung a, Shamala Devi Sekaran b

a Department of Molecular Medicine, Faculty of Medicine, CENAR, University of Malaya, Kuala Lumpur, Malaysia
b Department of Medical Microbiology, Faculty of Medicine, CENAR, University of Malaya, Kuala Lumpur, Malaysia

1. Introduction

L-amino acid oxidase (L-amino acid: O2 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 Clementson, 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 Safiuddin, 1989, 1991; Li et al., 1994; Ahn et al., 1997; Jin et al., 2007). Tan and Safiuddin (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
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 Biochemicals, Netherlands. Resource Q ion exchange column was purchased from GE Healthcare, Stockholm, 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 × 10⁻³ 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 SpectraMax™ 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 × 10⁸ CFU/mL) and then diluted with 2× Mueller Hinton broth to a final concentration of 1 × 10⁶ 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 × 10⁶ 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 × 10⁹ 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. pneumoniae, 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.
Our results show that king cobra venom LAAO is a potent antibacterial agent, effectively inhibiting both the growth of Gram-positive and Gram-negative bacteria examined. Its MICs against the Gram-positive bacteria *S. aureus* and *S. epidermidis* were 0.78 and 1.56 μg/mL, respectively. Its MICs against the Gram-negative bacteria *K. pneumonia*, *P. aeruginosa* and *E. coli*, range from 25 to 100 μg/mL. On a molar basis, its MICs range from 0.006 μM to 0.8 μM. The antibacterial action of LAAO against all bacteria tested was abolished in the presence of 1 mg/mL of catalase.

Under the same experimental conditions, the MICs of the 3 antibiotics against Gram-negative bacteria examined range from 0.06 μg/mL to >64 μg/mL, whereas the MICs of the 3 antibiotics against the two Gram-positive bacteria range from <0.031 μg/mL to 2 μg/mL.

### 3.3. The binding of king cobra venom LAAO to bacteria

Table 3 shows the bacteria-binding activity of the king cobra venom LAAO. At a bacteria concentration of 0.5 × 10⁹ cells/mL and LAAO concentration of 0.5 μg/mL (3.8 × 10⁻¹² mol/mL, molecular weight of the LAAO = 130,000), the enzyme binds effectively to all 4 bacteria tested. The binding was time-dependent and reached a plateau after 5 h incubation. At 24 h, approximately 40% of the LAAO in the incubation mixture was bound to the two Gram-positive bacteria *S. aureus* and *S. epidermidis*, and 25% was bound to the two Gram-negative bacteria, *E. coli* and *P. aeruginosa*.

### 4. Discussion

#### 4.1. Comparison of the antibacterial activity of king cobra venom L-aminoo acid oxidase with common antibiotics

Skears (1970) was the first to report that a LAAO isolated from *C. adamanteus* venom possessed bactericidal activity. Since then, many snake venom LAAOs have been demonstrated to exhibit antibacterial activity. However, most of the authors did not compare the antibacterial activity of the LAAOs with common antibiotics; hence it is difficult to assess quantitatively the potential of therapeutic application of snake venom LAAO as antibacterial. Stiles et al. (1991) did report that the *in vitro* antibacterial effects of LAAOs isolated from the Australian elapid *Pseudechis australis* were 17.5 to 70 times more effective, on a molar basis, than tetracycline. In the present report, using the standardized broth microdilution assay according to the protocol described by NCCLS, we have demonstrated that king cobra venom LAAO is indeed a potent antibacterial agent when compared to 5 common antibiotics. On a molar basis, the antibacterial activity of the enzyme against all bacteria tested is far more potent than most of the antibiotics examined. For example, the enzyme has a MIC of 0.006 μM against *S. aureus*. In comparison, the MICs for the antibiotics penicillin, vancomycin and cefotaxime against the same bacterium were <0.09 μM, 0.34 μM and 2.0 μM, respectively. Against *P. aeruginosa*, the MIC of the enzyme was 0.2 μM, also far more effective than cefotaxime (MIC = 35.1 μM), kanamycin (MIC = 109.8 μM) and tetracycline (MIC = 144.0 μM). On a weight basis, the MICs of the LAAO against the two Gram-positive bacteria were comparable to the antibiotics tested, except for penicillin, which was far more potent than the LAAO. On the same (weight) basis, however, the MICs of the LAAO against Gram-negative bacteria were generally higher than the antibiotics tested, except against the clinical isolates of *P. aeruginosa*, where the enzyme has a MIC (25 μg/mL), comparable to the three antibiotics tested (16–64 μg/mL).

Thus, in view of its highly potent and broad-spectrum antibacterial action and its exceptional thermal and alkaline stability (as compared to other snake venom LAAOs), king cobra venom LAAO may be a suitable LAAO to be developed into an antibacterial agent with therapeutic applications. Even though LAAO is known to possess some...
cytotoxic activity, it is also known that the enzyme generally has very low lethal toxicity in mice (Tan and Fung, 2009), Ciscotto et al. (2009) argued that there is a ‘bactericidal window’ where reactive oxygen species concentrations sufficient to abolish bacterial growth are not harmful to the host (human) cells, as microorganisms are generally several fold more sensitive to reactive oxygen species than human tissues (Sorg, 2004). However, because of the proteicaceous nature and the relatively high cost (venom sources or even cloned products) of snake venom LAAO, the therapeutic application of the enzyme as an antibacterial will always have its limitation. Nevertheless, the understanding of the mode of antibacterial action of LAAO (as an efficient hydrogen peroxide generator) may lead to the design of new drugs or therapeutic approaches.

4.2. Comparison of the antibacterial activity of king cobra venom \( \text{l-amino acid oxidase} \) with other snake venom \( \text{l-amino acid oxidases} \)

Table 2 lists the antibacterial actions of LAAO isolated from 14 venoms, mainly from crotalids, with two venoms each from the viperid and elapid snakes. The bacteria inhibited by these enzymes include the Gram-positive Candida albicans, Bacillus subtilis, B. megaterium, B. subtilis, S. aureus and S. mutans and Gram-negative bacteria Aeromonas sp., E. coli, P. aeruginosa, Salmonella typhimurium, and Xanthomonas axonopodis pv passiflorae.

Unfortunately, it is difficult to quantitatively compare the antibacterial potency of these LAAOs with the king cobra venom LAAO, mainly because different methods of antibacterial assay were used by different authors. Of these, only two reported MIC of the enzyme using the standard broth microdilution assay according to NCCLS: Costa et al. (2010) reported that LAAO from \( \text{B. marajoensis} \) has a MIC of 50 μg/mL against \( S. aureus \); whereas Zhong et al. (2009) reported that the MICs of \( D. russelli \) siamensis LAAO for \( S. aureus, P. aeruginosa \) and \( E. coli \) were 9 μg/mL, 144 μg/mL and 288 μg/mL, respectively. In comparison, king cobra venom LAAO was a much more potent antibacterial against these same organisms.

Our results show that king cobra venom LAAO was more potent against the two Gram-positive bacteria tested than the Gram-negative bacteria tested. This is in agreement with the selectivity of antibacterial action of LAAOs of \( B. marajoensis, D. russelli \) siamensis, \( C. durissus cascavellosa \) and \( T. mucrosquamosus \). On the other hand, LAAOs from \( B. pauloensis, V. lebetina, N. oxiana \) and \( P. australis \) were more active against Gram-negative than the Gram-positive bacteria, whereas LAAOs from \( A. halys, B. ps. alternatus \) and \( M. moqeni \) inhibited Gram-positive and Gram-negative bacteria almost equally (Table 2). These differences in the selectivity of the antibacterial action of these LAAOs are presumably due mainly to the differences in bacteria-binding activity of the enzymes (see Section 4.4 below).

4.3. Comparison of the antibacterial activity of king cobra venom LAAO with some LAAOs isolated from other animal sources

Table 2

<table>
<thead>
<tr>
<th>Venom source of LAAO</th>
<th>Antibacterial actions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Crotalus adamanteus} )</td>
<td>Almost equally active against ( G(-) ) P. aeruginosa and ( E. coli )</td>
<td>Skarnes (1970)</td>
</tr>
<tr>
<td>( \text{C. durissus cascavellosa} )</td>
<td>More active against ( G(-) ) S. aureus (most active. MIC=50 μg/mL)</td>
<td>Toyama et al. (2006)</td>
</tr>
<tr>
<td>( \text{A. halys} )</td>
<td>Almost equally active against ( G(-) )</td>
<td>Zhang et al. (2004)</td>
</tr>
<tr>
<td>( \text{Bothrops alternatus} )</td>
<td>Almost equally active against ( G(-) ) E. coli and ( G(+): B. subtilis )</td>
<td>Stabeli et al. (2004)</td>
</tr>
<tr>
<td>( \text{B. marajoensis} )</td>
<td>Active against ( G(+): S. aureus ) (most active. MIC=9.0 μg/mL)</td>
<td>Costa et al. (2010)</td>
</tr>
<tr>
<td>( \text{B. moqeni} )</td>
<td>Almost equally active against ( G(-) ) E. coli, Salmonella typhimurium, P. aeruginosa and ( G(+): S. aureus )</td>
<td>Stabeli et al. (2007)</td>
</tr>
<tr>
<td>( \text{B. pauloensis} )</td>
<td>More active against ( G(-) ) E. coli than ( G(+): S. aureus )</td>
<td>Rodrigues et al. (2009)</td>
</tr>
<tr>
<td>( \text{B. pirojai} )</td>
<td>Almost equally active against ( G(-) ) E. coli and ( P. aeruginosa )</td>
<td>Izidoro et al. (2006)</td>
</tr>
</tbody>
</table>
| \( \text{P. (Trimeresurus) jordianus} \) | Active against \( G(-) \) B. megaterium and \( S. aureus \) (MIC=0.078 μg/mL). Photobacterium damselae (MIC=0.16 μg/mL) and Vibrio parahaemolyticus (MIC=0.63 μg/mL), but were less active against the Gram-positivie \( B. subtilis \). Micrococcus luteus and \( S. aureus \) (MIC=5 μg/mL). Recently, Nagashima et al. (2009) isolated LAAOs from skin mucus of the great sculpin that possess potent antibacterial activity against both Gram-positive and Gram-negative bacteria, and its MIC against \( S. salmonicida \) JCM7874 was as low as 0.02 μg/mL. Thus, it appears that LAAOs isolated from the molluscs and marine animals are more potent as antibacterials than snake venom LAAO, including the king cobra venom LAAO. The LAAO from the giant snail seems to have similar selectivity in its antibacterial action as the king cobra venom LAAO, being more effective against the Gram-positive \( S. aureus \) than the Gram-negative \( E. coli \). On the other hand, the LAAO from the rockfish exhibited rather different selectivity: it strongly inhibited several Gram-negative bacteria but was much less effective against the Gram-positive \( S. aureus \). Again, these differences may be due to the difference in bacterial-binding activity of the enzyme (see Section 4.4 below).

4.4. Bacteria-binding activity of king cobra venom LAAO

The mechanism of antibacterial action of snake venom LAAO has been investigated by many authors, and it is well established that snake venom LAAO kills bacteria by the \( \text{H}_2\text{O}_2 \) generated as a result of the oxidation of \( \text{l-amino acids} \) in the media (Tan and Fung, 2009). Toyama et al. (2006) reported that the LAAO isolated from \( C. durissus cascavellosa \) venom induced bacterial membrane rupture and promoted extravasation of plasmatic content to the outside of the cells. They argued that the amount of hydrogen peroxide generated was sufficient to inhibit bacterial growth, and that the ability of the enzyme to bind to bacterial membranes is not important in its antibacterial activity. Investigation of the mechanism of antibacterial action of LAAOs isolated from other organisms, however, yielded a different picture. Using immunochemical analysis, Ebara et al. (2002) showed that Achatina (LAAO isolated from the giant snail, Achatina fulica ferussac) binds significantly to the bacteria \( S. aureus \) and \( E. coli \). The fact that the bacteria-binding activity was higher for \( S. aureus \) than for \( E. coli \) is...
consistent with the observed differences in MICs between the two bacteria. The results suggested that binding to bacteria is important for the antibacterial activity of the enzyme, as the concentration of H$_2$O$_2$ generated by the enzyme was not sufficient to kill bacteria. It was argued that binding of LAAO to the bacteria enables production of highly localized concentration of H$_2$O$_2$ in or near the binding sites that will be sufficiently potent to kill the bacteria. This also explains why a very small amount of LAAO could effectively inhibit bacteria growth, as the MIC of Achacin against _S. aureus_ was as low as 0.2 μg/mL.

Kitani et al. (2008) further confirmed that bacterial-binding activity plays an important role in the antibacterial activity of SSAP (LAAO isolated from the skin mucus of Rockfish _S. schlegelli_). They showed that the MICs of the SSAP for the Gram-negative bacteria _A. salmonicida_, _P. damselae_ and _V. parahaemolyticus_ were between 0.078 μg/mL to 0.63 μg/mL, much lower than that for the Gram-positive bacteria _S. aureus_ (MIC=5.00 μg/mL), yet the MICs of H$_2$O$_2$ against all these 4 bacteria were all determined to be 0.31 mM. The authors suggested that the differences in the MICs of SSAP against these bacteria were due to different bacteria-binding activity. The explanation was substantiated by Western blotting and measurement of residual LAAO activity of the bacteria-SSAP incubation mixture, which demonstrated the binding of SSAP to _P. damselae_ but not to _E. coli_. Unfortunately, due to the limited amount of SSAP available, they were not able to examine the bacteria-binding activity of other species of bacteria.

Our results on binding studies here (Table 3) firmly substantiated the findings of Ehara et al. (2002) and Kitani et al. (2008), and demonstrated that snake venom LAAO also exhibited similar bacteria-binding properties. The binding activity of the enzyme was higher against _S. aureus_ than against _E. coli_, and this is in agreement with the bacteria-binding activity of the Achacin, (the giant snail LAAO), as reported by Ehara et al. (2002). An interesting observation was that the binding activity of the enzyme against _S. aureus_ was approximately the same as the other Gram-positive bacteria _S. epidermidis_, and that the binding activity of the enzyme against the two Gram-negative bacteria, _E. coli_ and _P. aeruginosa_ was also comparable. The stronger bacteria-binding activity of king cobra venom LAAO against the Gram-positive bacteria than against the Gram-negative bacteria are consistent with the differences in the MICs reported in this study and may explain the higher antibacterial potency of king cobra venom LAAO against Gram-positive than Gram-negative bacteria tested. The molecular mechanism of bacteria-binding activity of LAAO has yet to be elucidated. The results so far suggest that different LAAO has different binding affinity towards different bacteria. In our studies, the incubation mixture contains 0.5 μg/mL of LAAO (3.8 × 10$^{12}$ mol) and 0.5 × 10$^{10}$ cells/mL. After 24 h incubation, approximately 40% of the LAAO (1.52 × 10$^{12}$ mol, or 9.15 × 10$^{11}$ molecules) was bound to _S. aureus_ or _S. epidermidis_. This implies that approximately 1800 molecules of LAAO were bound per bacterial cell. In the same way, only approximately 1100 molecules of LAAO were bound to each _E. coli_ or _P. aeruginosa_ cell.

It is noted that Kitani et al. (2008) reported that SSAP did not bind to _E. coli_. This either reflects difference in bacteria-binding activity of the LAAOs or the ratio of bacteria/LAAO used in their experiments may not be appropriate to detect the lesser binding activity for _E. coli_. Our results also showed that the bacteria-binding of LAAO is a time-dependent process, and reached saturation only after approximately 5 h of incubation at 37 °C. This is an important consideration in the design of assay of antibacterial activity of LAAO: that insufficient incubation time may lead to underestimation of the antibacterial activity of the enzyme.

**Acknowledgements**

This work was supported by a PPP research grant PS 185/2010A from the University of Malaya and CENAR.

**References**


