

Hydrogen Peroxide produced by Two Amino Acid Oxidases Mediates Antibacterial Actions

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(Received May 31, 2004 / Accepted August 23, 2004)

The antibacterial actions of two amino acid oxidases, a D-amino acid oxidase from hog kidney and a L-amino acid oxidase from the venom of *Agkistrodon halys*, were investigated, demonstrating that both enzymes were able to inhibit the growth of both Gram-positive and Gram-negative bacteria, and that hydrogen peroxide, a product of their enzymatic reactions, was the antibacterial factor. However, hydrogen peroxide generated in the enzymatic reactions was not sufficient to explain the degree to which bacterial growth was inhibited. A fluorescence labeling assay showed that both of these two enzymes could bind to the surfaces of bacteria. To the best of our knowledge, this is the first report regarding the antibacterial activity of the D-amino acid oxidases.

Key words: amino acid oxidase, antibacterial activity, snake venom

Amino acid oxidases (AAOs) are flavoenzymes able to stereo-specifically catalyze the oxidative deamination of L-amino acids or D-amino acids to α -keto acids, along with the production of ammonia and hydrogen peroxide (H_2O_2). AAOs can be grouped into two classes, according to substrate specificity: L-amino acid oxidases (LAAOs) and D-amino acid oxidases (DAAOs). LAAOs exert a multiplicity of effects on biological organisms, functioning as platelet aggregation inducers or inhibitors (Li *et al.*, 1994; Sakurai *et al.*, 2001), apoptosis inducers (Suhr & Kim, 1996; Ali *et al.*, 2000), and protein factors possessing both hemorrhagic (Souza *et al.*, 1999) and antibacterial activity (Skarnes, 1970; Stiles *et al.*, 1991; Tempone *et al.*, 2001; Ehara *et al.*, 2002). These LAAO functions are believed to be at least partially predicated on the production of H_2O_2 during catalytic reactions (Skarnes, 1970; Stiles *et al.*, 1991; Li *et al.*, 1994; Ali *et al.*, 2000; Sakurai *et al.*, 2001; Tempone *et al.*, 2001; Ehara *et al.*, 2002;). However, D-amino acid oxidases have been reported to possess no antibacterial activity since Skarne's study (1970), although DAAOs produce the same products in their catalytic reactions as do LAAOs. Therefore, it seems possible that the carbohydrates in LAAOs might also be playing an important role in these processes (Geyer *et al.*, 2001), since the LAAOs found so far are all glycopro-

teins, whereas DAAOs are not. This short report describes the inhibitory effects of two amino acid oxidases (AHP-LAAO and hDAAO) on the growth of both Gram-positive and Gram-negative bacteria strains, in which H_2O_2 generated in the catalytic reactions is identified as the antibacterial factor. The role of LAAO carbohydrates in the inhibition of bacterial growth was also investigated.

Materials and Methods

Materials

Tryptone and yeast extract were Oxoid products (England). Catalase, PNGase F, and o-dianisidine were purchased from Sigma (USA). L-leucine, D-leucine, and D-serine were from ICN (USA). L-vinylglycine, FITC (fluorescein isothiocyanate) isomer 1, and hog D-amino acid oxidase (hDAAO) were from Fluka (USA). LAAO from the venom of *Agkistrodon halys* (AHP-LAAO) was purified according to the methods described by Zhang *et al.* (2004). Bacterial strains *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* were obtained from the Microbiology Lab of our school. Other materials were of analytical grade.

Detection of LAAO and DAAO activities in LB medium

The oxidative activities of AHP-LAAO and hDAAO were assessed using a modification to the method described by Zhang *et al.* (2004). In brief, either 4 μ g of AHP-LAAO or 20 μ g of hDAAO was added to 2 ml of LB (Luria-Ber-

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tani) medium (pH 7.2) containing 1mM o-dianisidine and 50 µg/ml horseradish peroxidase (for DAAO, 10mM D-leucine was added to LB medium as a substrate). Absorbance was recorded at 436 nm using a V550 spectrophotometer (JASCO, Japan). This assay was performed at 37°C. A series of different concentrations of H₂O₂ were used to construct a standard curve between the amount of H₂O₂ and the absorbance at 436 nm.

Assays for detection of antibacterial activity

The antibacterial actions of AHP-LAAO and hDAAO were assessed via a modification of the method described by Stiles *et al.* (1991). Twenty µl of bacterial suspension, cultured overnight, was added to several groups of tubes containing 2 ml of LB medium each. Variant concentrations of AHP-LAAO were added to different groups of tubes. After incubation at 37°C, at 280 rpm for 2.5 h, the absorbance of the bacterial suspension was recorded at 600 nm. The same assays were performed on hDAAO, with 10mM D-leucine added to LB medium as substrate. The half inhibitory dose was defined as the amount of LAAO or DAAO needed to bring the absorbance of bacteria suspension at 600 nm to a level half that of the control level.

FITC modification and bacteria binding activity of AHP-LAAO and DAAO

FITC modification of AHP-LAAO and DAAO was performed as follows: purified AHP-LAAO (2 mg/ml) or DAAO (2 mg/ml) was incubated in 0.1 M Na₂CO₃-NaHCO₃ pH 9.2 containing 0.25 mM FITC/DMSO (dimethyl sulfoxide) at room temperature for 12 h. Unchanged FITC was removed by passage through a Sephadex G10 gel filtration column. The modified enzymes were pooled and used in the bacteria-binding assay.

Twenty µl of bacteria suspension (cultured overnight) was added to tubes containing 2 ml of LB medium. The tubes were incubated at 37°C at 280 rpm for 2 h, and then modified AHP-LAAO or hDAAO was added to the medium. Bacteria was collected by centrifugation 1 h later, and washed three times with 1 ml of 1% NaCl. The bacteria were suspended in 200 µl of SDS-PAGE loading buffer, and 10 µl of bacteria was analyzed by SDS-PAGE, followed by FITC analysis using a fluorescence imager (Typhoon800, Amersham Biosciences, USA).

Results and Discussion

AHP-LAAO, a L-amino acid oxidase purified from the venom of *Agkistrodon halys* (Zhang *et al.*, 2004), significantly suppresses the growth of both the Gram-negative bacterium, *E. coli*, and the Gram-positive bacterium, *B. subtilis* (Fig. 1). The inhibitory effect is dose-dependent. The greater the amount of AHP-LAAO added to the LB medium, the more inhibition of bacterial growth is

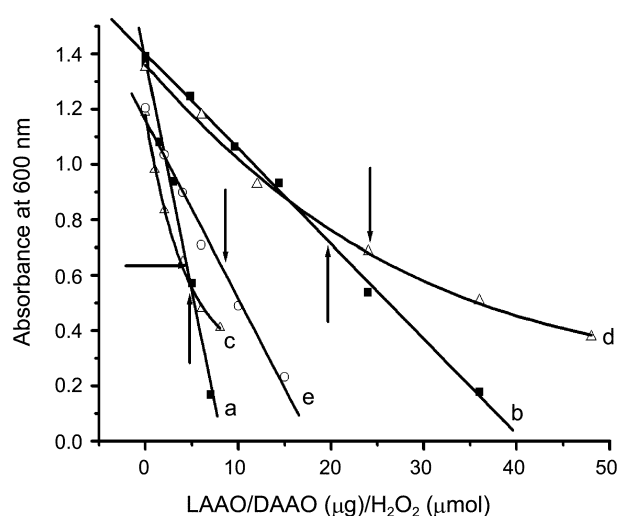


Fig. 1. Antibacterial actions of AHP-LAAO, hDAAO and H₂O₂ on *E. coli* and *B. subtilis*. The experimental procedures have been described in the materials and methods section. All values appearing in the curves are the means of three experimental iterations. Curves a, b, and e represent the inhibitory effects of AHP-LAAO, hDAAO, and H₂O₂ on *E. coli* strains, respectively. Curves c and d represent the inhibitory effects of AHP-LAAO and hDAAO on *B. subtilis* strains, respectively. Arrows near the curves indicate the half inhibitory doses.

observed (Fig. 1). Moreover, after being treated with L-vinylglycine, a suicide substrate which can irreversibly inhibit the oxidative activity of LAAOs (Marcotte and Walsh, 1976), AHP-LAAO loses its antibacterial activity completely (Fig. 2A). These observations may have implications such that the antibacterial action of LAAOs relates directly with oxidative activity. As mentioned above, H₂O₂ production may be relevant to the compounds antibacterial activity. Indeed, H₂O₂ exhibits an inhibitory effect similar to that of AHP-LAAO (Fig. 1). Furthermore, the inhibitory effect of AHP-LAAO was observed to be completely abolished upon the addition of catalase, a H₂O₂ scavenger which can clean out H₂O₂ generated during enzymatic reactions (Fig. 2A). Therefore, the antibacterial action of AHP-LAAO can be attributed largely to H₂O₂, a product of its catalytic reaction. This conclusion is consistent with observations of other LAAOs (Tempone *et al.*, 2001). On the other hand, the carbohydrates in LAAOs might still manifest as-yet-unknown functions in this process (Geyer *et al.*, 2001). Like other LAAOs so far discovered, AHP-LAAO is a glycoprotein (which can be observed in its crystal structure, deposited in RCSB PDB with the accession code 1REO). L-vinylglycine, a suicide substrate for LAAOs, interacts only with the catalytic cave of LAAOs, and not with the contained carbohydrates; therefore, the loss of antibacterial activity of AHP-LAAO when treated with L-vinylglycine indicates that the carbohydrates in AHP-LAAO are not directly relevant to its antibacterial activity. In a previous study, the recombinant apoxin 1 (a LAAO from *Crotalus atrox* venom) lost its

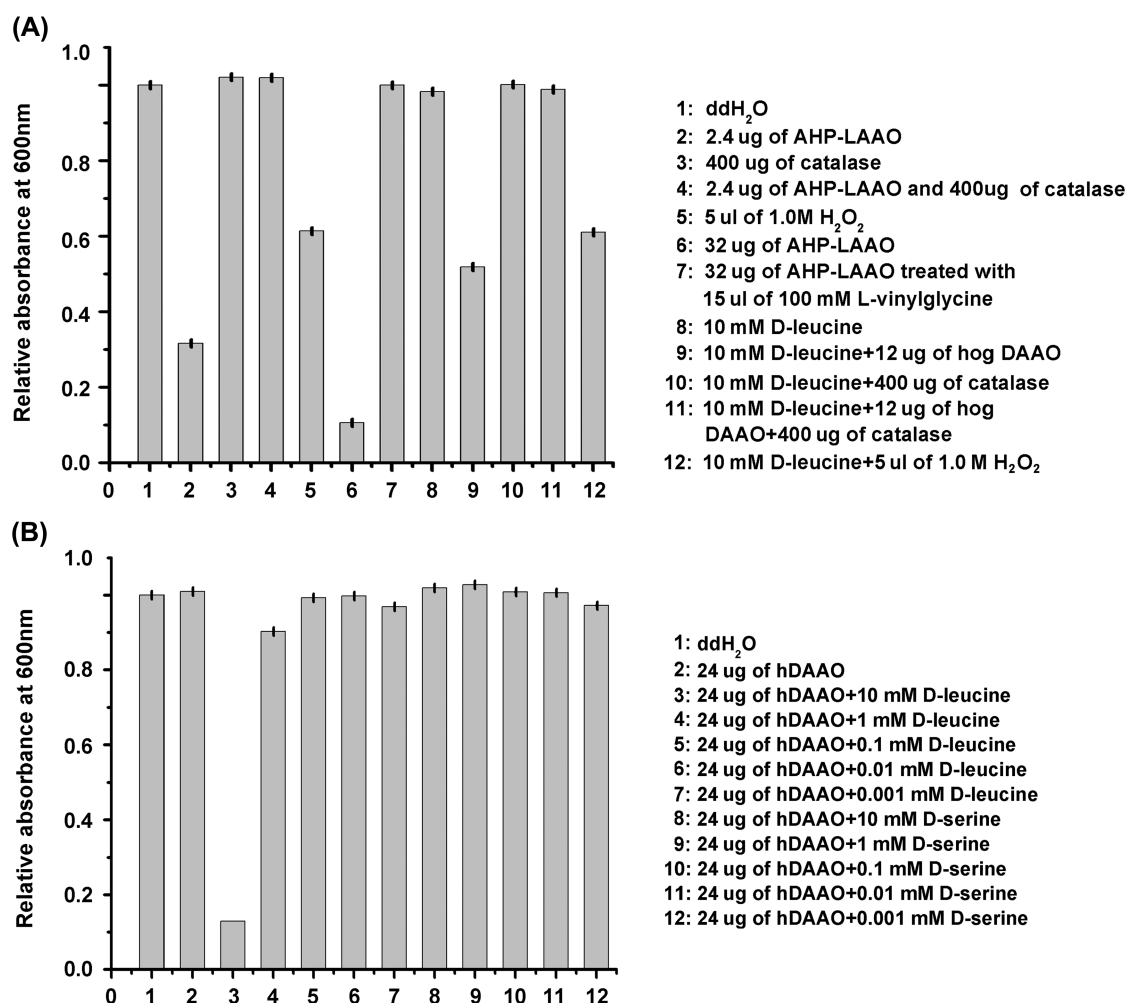


Fig. 2. Antibacterial effects of AHP-LAAO and hDAAO on *E. coli*. Forty μ l of *E. coli* suspension cultured overnight (at 37°C, 280 rpm) was added to a series of tubes containing 2 ml of LB medium. Two hundred μ l of other reagents and proteins dissolved in double distilled water (shown in the Fig.) were further added to the tubes. All tubes were incubated at 37°C, at 280rpm for 3 h. The absorbance was then measured at 600 nm. The experimental results are represented as the means of three iterations.

catalytic activity when its glycosylation was inhibited (Torii *et al.*, 2000). Similarly, when digested with PNGase F, the enzymatic activity of AHP-LAAO fell to about 75% of the levels exhibited by the native form (original data not shown), suggesting that carbohydrates may, indeed, be playing a role in the mediation of the enzymatic activity of LAAOs.

In a manner similar to that of AHP-LAAO, DAAO from hog kidney also exerts an antibacterial effect on the cultured bacteria strains (Fig. 1). The addition of the substrate D-leucine is crucial to its antibacterial action. In the absence of, or at low concentrations of D-leucine (e.g. lower than 1mM), no obvious inhibitory effects were observed (Fig. 2B). However, the addition of the substrate D-serine does not result in any change in the growth of *E. coli* (Fig. 2B); this would seem to indicate that D-serine is not a favorable substrate for hDAAO (D'Aniello *et al.*, 1993). Furthermore, the addition of catalase also com-

pletely abolishes the inhibitory effects of hDAAO (Fig. 2A), indicating that H₂O₂ is the operant antibacterial factor in hDAAOs inhibitory effects. In the investigation of Skarnes (1970), DAAO exhibited no antibacterial activity whatsoever. The reason for this might be that the concentrations of D-leucine added to LB medium were too low (10^{-4} ~ 10^{-6} M).

The antibacterial activities of both AHP-LAAO and hDAAO on *S. aureus* also evidenced positive results. Because *S. aureus* was not able to grow well in LB medium, we did not show it in Fig. 1.

Although H₂O₂ plays a predominant role in the antibacterial activities of AHP-LAAO and hDAAO, it should be noted that the amount of H₂O₂ produced by AHP-LAAO or hDAAO in LB medium is not sufficient to inhibit the growth of bacteria to the extent observed. The half inhibitory doses of AHP-LAAO and hDAAO on *E. coli* are about 2.0 and 10.0 μ g/ml, respectively. When incubated

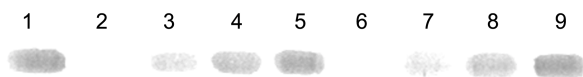


Fig. 3. Binding of AHP-LAAO or hDAAO with the cell surfaces of *E. coli* and *B. subtilis*. Lane1: 0.5 μ g AHP-LAAO modified with FITC; Lanes 2, 3, 4, and 5 are *E. coli* treated with unmodified AHP-LAAO, 10, 20, and 40 μ g AHP-LAAO modified with FITC, respectively. Lanes 6-9 are *B. subtilis* treated with unmodified AHP-LAAO, 10, 20, and 40 μ g AHP-LAAO modified with FITC, respectively. With regard to hDAAO, the same results were obtained, except that the amount of hDAAO used in this assay was a little larger than the amount of AHP-LAAO.

with 2 ml of LB medium for 2.5 h, the amounts of H_2O_2 generated in the oxidative reactions with 4 μ g of AHP-LAAO and 20 μ g of hDAAO were about 0.21 and 0.26 μ M, respectively, significantly less than the half inhibitory dose of H_2O_2 on *E. coli* (about 8.5 μ M, shown in Fig. 1). Thus, a certain mechanism must be operating, through which AHP-LAAO and hDAAO can inhibit bacterial growth with a small amount of protein. It has been reported that LAAOs are able to bind to the cellular surfaces of some cell lines (Suhr & Kim, 1996). Therefore, the bacterial surface-binding activities of AHP-LAAO and hDAAO were assessed via fluorescence detection of bacteria treated with AHP-LAAO or hDAAO, modified by FITC. Fig. 3 showed that AHP-LAAO and hDAAO could bind to the cell surfaces of *E. coli* and *B. subtilis*. Thus, by binding to bacterial surfaces and generating high concentrations of H_2O_2 in the local areas of those bacterial surfaces, AHP-LAAO and hDAAO are able to inhibit bacterial growth with only a small amount of protein.

Acknowledgments

Financial support for this project to LN and MT was provided by research grants from Chinese Nation Natural Science Foundation (grant No.s30121001, 30025012, 30130080), the "973" and "863" Plans of the Chinese Ministry of Science and Technology (grant No.s G1999075603 and 2002BA711A13) and the Chinese Academy of Sciences (grant No. KSCX1-SW-17). Mr. Wenrui Zhang has kindly supplied the bacterial strains.

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