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Purification, partial characterization, crystallization and structural determination of AHP-LAAO, a novel L-amino-acid oxidase with cell apoptosis-inducing activity from *Agkistrodon halys pallas* venom

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A snake-venom protein named AHP-LAAO has been purified from *Agkistrodon halys pallas* venom using four-stage chromatography. AHP-LAAO is a novel member of the snake-venom L-amino-acid oxidase family. Its amino-acid sequence shows high homology to other members of this family. For L-leucine, the values of k_{cat} and K_M are 31.1 s^{-1} and 0.25 m*M*, respectively. The molecular weight of AHP-LAAO is about 60.7 kDa as determined by MALDI–TOF mass spectrometry. AHP-LAAO can also induce apoptosis of cultured Hela cells. Two sets of diffraction data with similar resolution limits (about 2.5 Å) were collected independently at MacCHESS (Cornell High Energy Synchrotron Source, USA) and IHEP (Institute of High Energy Physics, Beijing, China). The crystals belong to space group $I2_13$, with unit-cell parameter a = 169.31 Å, corresponding to one molecule in the asymmetric unit and a volume-to-weight ratio of 3.33 $Å^3$ Da^{-1}. The final structural model is similar to that of L-amino-acid oxidase from *Calloselasma rhodostoma* venom.

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PDB Reference: AHP-LAAO, 1reo, r1reosf.

1. Introduction

As a kind of naturally existing protein library, snake venoms have been widely investigated for the purposes of curing snakebites and other clinical uses. Snake-venom proteins affect the human body in many different ways, causing haemorrhage, haemolysis, oedema and other effects on blood circulation and nervous systems. Araki *et al.* (1993) first reported that haemorrhagic snake venoms could induce apoptosis in vascular endothelial cells, although the active component was not identified, which provoked many researchers to search for the apoptosis inducers in snake venoms. Soon afterwards, two groups (Suhr & Kim, 1996; Torii *et al.*, 1997) independently reported that snake-venom L-amino-acid oxidases (LAAOs) possess cell apoptosis-inducing activity. Moreover, LAAOs display apoptosis-inducing effects on many types of cell line, including vascular endothelial cells (Torii *et al.*, 1997), human embryonic kidney cells (293T; Torii *et al.*, 2000), human promyelocytic leukaemia cells (HL-60; Torii *et al.*, 1997), human monocytic cells (MM6; Ali *et al.*, 2000), mouse lymphocytic leukaemia cells (L1210) and human T-cell leukaemia cells (MOLT-4; Suhr & Kim, 1996). Only one LAAO crystal structure, that from *Calloselasma rhodostoma* venom, has been reported so far (Pawelek *et al.*, 2000). There are still many questions to be answered in order to understand the structure–function relationships of LAAO-induced apoptosis; for example, whether the cell apoptosis-inducing activity of LAAO can be completely explained by the reaction product H_2O_2 and if not, which part(s) may be involved in the interaction of LAAO with cells and what function(s) the carbohydrate of LAAO takes. In this report, we describe the purification, partial characterization and cell apoptosis-inducing activity of LAAO from *Agkistrodon halys pallas* venom. The crystallization and structural determination of AHP-LAAO provides a foundation for further investigations.

2. Materials and methods

2.1. Materials

Dried crude venom was obtained from Tunxi Snakebite Institute (Anhui Province, China). DEAE-Sepharose Fast Flow, Sephacryl S-100 High Resolution, Q Sepharose High Performance and Phenyl Sepharose High Performance were products of Amersham Biosciences (England). *o*-Dianisidine was purchased from Sigma (USA), the Cell Proliferation Assay Kit from Promega (USA) and the BCA (bicinchoninic acid) protein-assay kit from Pierce (USA). Other reagents and chemicals were of analytical grade.

2.2. Purification and assays for biochemical characterization

AHP-LAAO was isolated and purified by a four-step chromatography procedure at 277 K (see Table 1 and Fig. 1) and then desalted and concentrated for further characterization and crystallization.

The molecular weight of AHP-LAAO was determined by MALDI–TOF (matrix-assisted laser desorption–ionization time-of-flight) mass spectrometry. The N-terminal amino-acid residue sequence was determined by the conventional Edman degradation method using an ABI 491A sequencer. The complete coding sequence of AHP-LAAO was obtained by RT-PCR (reverse-transcription polymerase chain reaction).

The activity of the L-amino-acid oxidase was measured spectrophotometrically using a modification of the method of Ali *et al.* (2000). Briefly, 1 µg of AHP-LAAO was added to 2 ml of reaction solution mixed with 1 m*M* L-leucine or D-leucine, 0.4 m*M* *o*-dianisidine and horseradish peroxidase (50 µg ml^{-1}) in 0.1 *M* Tris–HCl pH 8.5; the absorbance at 436 nm was then recorded using a V550 spectrophotometer (Jasco, Japan). The assay was performed at room temperature (about 298 K). One unit of enzyme activity was defined as the production of 1 µmol H_2O_2 per minute. The values of K_M and k_{cat} were determined by measuring the LAAO activity at a series of concentrations of L-leucine between 50 µ*M* and 2 m*M* and the optimum pH was determined by the detection of LAAO activity in different pH buffers in the range 7.6–10.8.

2.3. Assays for cell apoptosis-inducing activity

HeLa cells were used to detect the apoptosis-inducing activity of AHP-LAAO. The effect of AHP-LAAO on cell proliferation was investigated using a cell-proliferation assay kit according to the method of Wang *et al.* (2003). In addition, DAPI (4′,6-diamidino-2-phenylindole) staining and DNA-fragmentation assays were performed on cells treated with AHP-LAAO.

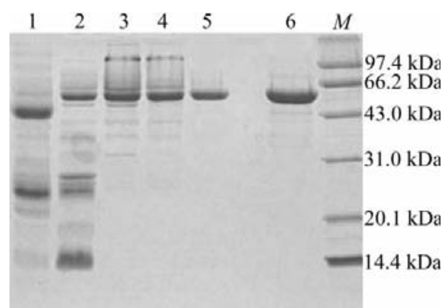

Figure 1
SDS–PAGE of AHP-LAAO. Lane 1, crude venom. Samples are shown after chromatography with DEAE (lane 2), S-100 (lane 3), Q Sepharose (lane 4) and phenyl Sepharose (lanes 5 and 6; non-reducing and reducing conditions, respectively). *M*, markers for molecular-weight estimation.

Table 1
Purification of L-amino-acid oxidase from *A. halys pallas* venom.

Fraction	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude venom	6000	14.38	0.002	100	1
DEAE Sepharose	695	10.03	0.014	69.7	8.6
S-100	152	9.83	0.065	68.4	39.6
Q Sepharose	91.5	8.87	0.097	61.7	65.6
Phenyl Sepharose	45.5	7.62	0.167	53.0	131.8

Table 2
Diffraction data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Data set	1	2
Synchrotron-radiation source	MacCHESS	IHEP
Space group	$I2_13$	$I2_13$
Unit-cell parameter (Å)	168.94	169.31
No. observations	197152	247448
No. independent reflections	27864	34867
Resolution limits (Å)	30–2.50 (2.54–2.50)	50–2.31 (2.39–2.31)
R_{merge}† (%)	8.4 (35.3)	5.4 (38.3)
Completeness (%)	96.6 (89.0)	98.2 (99.8)
Mosaicity (°)	0.15	0.05
$I/\sigma(I)$	30.0 (3.0)	5.8 (1.7)
Average *B* factor (Å^2)	53.8	48.3
R factor (%)	20.58	19.4
R_{free} (%)	24.32	21.6

† $R_{merge} = \sum_h \sum_j |\langle I(h)\rangle - I(h)_j| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the *j*th observed reflection intensity and $\langle I(h)\rangle$ is the mean intensity of reflection *h*.

2.4. Crystallization

The crystallization of AHP-LAAO was performed by the conventional hanging-drop vapour-diffusion technique. 2 µl AHP-LAAO solution (at a concentration of 44 mg ml^{-1} in double-distilled water as estimated using a BCA protein-assay kit, Pierce) was mixed with 1 µl reservoir solution (0.1 *M* sodium citrate pH 5.6 containing 2 *M* ammonium sulfate and 10 m*M* $NiSO_4$) and then equilibrated against 500 µl reservoir solution. Crystals appeared within 3–7 d and had grown to dimensions of approximately 0.4 × 0.4 × 0.4 mm one month later at room temperature (Fig. 2).

2.5. X-ray diffraction data collection and structure determination

A set of X-ray diffraction data was collected at the Cornell High Energy Synchrotron Source (MacCHESS) beamline F1. The wavelength of the incident X-rays was set to 0.9160 Å. The crystals diffracted poorly (to a resolution worse than 5 Å) at cryotemperature (about 100 K), so the data set was instead collected at room temperature. A total of 65 diffraction images were recorded using a Quantum 4 CCD detector (ADSC, USA) from two crystals at a crystal-to-detector distance of 200 mm. An oscillation angle of 1° and an exposure time of 2 s per frame were used. The diffraction data were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data-collection and processing statistics are listed in Table 2.

The structure was solved by the molecular-replacement method using the program *CNS* (Brünger *et al.*, 1998). The structural model of a homologous LAAO from *Calloselasma rhodostoma* venom (PDB code 1f8r) was chosen as the search model. Crystallographic refinement was carried out using the program *CNS*. Each cycle of refinement was followed by manual rebuilding using the program *O* (Jones *et al.*, 1991). The presence of difference density in the active site indicated that a molecule of

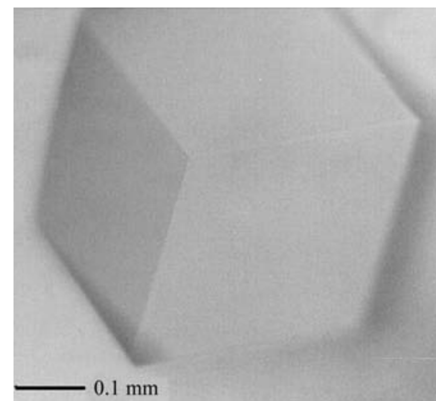

Figure 2
Photomicrograph of a crystal of AHP-LAAO.

citrate was bound. In the final model, the crystallographic R factor and free R factor fell to 20.58 and 24.32%, respectively.

At the time of data collection at MacCHESS beamline F1, beamline 3W1A of Beijing Synchrotron Radiation Facility at the Institute of High Energy Physics (IHEP; Beijing, China) was opened for protein crystallography. To evaluate the potential of IHEP for structure biology, another set of diffraction data from a crystal of AHP-LAAO was collected using a MAR345 imaging plate (MAR Research, Germany) with an incident X-ray wavelength of 0.8883 Å. A total of 60 imaging frames were recorded at room temperature from one crystal with a crystal-to-detector distance of 350 mm and an oscillation angle of 1° per frame in a dose-dependent exposure mode. The diffraction data were processed with the program *AUTOMAR* v.1.2.2 (Bartels & Klein, 2002). Data-collection and processing statistics are again listed in Table 2. The data collected at IHEP were used for independent refinement of the AHP-LAAO model, leading to an R factor and free R factor of 19.4 and 21.6%, respectively.

3. Results and discussion

Using four-stage chromatography, approximately 45 mg of AHP-LAAO could be purified from 6 g of crude *A. halys pallas* venom powder. AHP-LAAO is a novel member of the snake-venom LAAO family. The amino-acid sequence deduced from the cDNA shows a high homology to other members of this family of toxins. The second residue at the N-terminus of AHP-LAAO is an asparagine and differs from the other snake-venom LAAOs found so far. This difference may have implications for the enzyme reaction because of the important role of the N-terminal residues in substrate binding (Pawelek *et al.*, 2000).

AHP-LAAO appears as a single band on SDS–PAGE with a similar molecular weight under both reducing and non-reducing conditions (see Fig. 1), showing that there is no inter-chain disulfide bond in the protein. The molecular weight of AHP-LAAO is about 60.7 kDa (as determined by MALDI–TOF mass spectrometry). The crystal of AHP-LAAO belongs to space group $I2_13$, with unit-cell parameter 169.31 Å. The ratio of cell volume to molecular weight (V_M value; Matthews, 1968) is estimated to be about 3.33 $Å^3\,Da^{-1}$, indicating the presence of only one molecule in the asymmetric unit.

L-Leucine could be oxidized by AHP-LAAO, but D-leucine could not. The optimum pH value has been estimated to be 8.8 (see Fig. 3). For L-leucine, the values of k_{cat} and K_M for AHP-LAAO (31.1 s^{-1} and 0.25 mM, respectively) appear to differ from the corresponding values for the homologous LAAO from *C. rhodostoma* venom (k_{cat} = 3.3 s^{-1} and K_M = 0.63 mM; Ponnudurai *et al.*, 1994), indicating that AHP-LAAO possesses a high affinity for L-amino-acid substrates. AHP-LAAO seems to be an inducing factor for HeLa cell apoptosis, since it inhibits the proliferation of HeLa cells at the relatively low concentration of 0.5 µg ml^{-1} (Fig. 4) and induces DNA fragmentation and nuclear morphological changes in AHP-LAAO-treated HeLa cells (see insets in Fig. 4). Obviously, the functional mechanism and corresponding structural basis of snake-venom LAAO activity need to be investigated in detail.

Diffraction data sets from AHP-LAAO crystals were independently collected and processed using two synchrotron sources, MacCHESS and IHEP. The two sets of crystals diffracted to similar resolution limits (Fig. 5). The final structures refined against

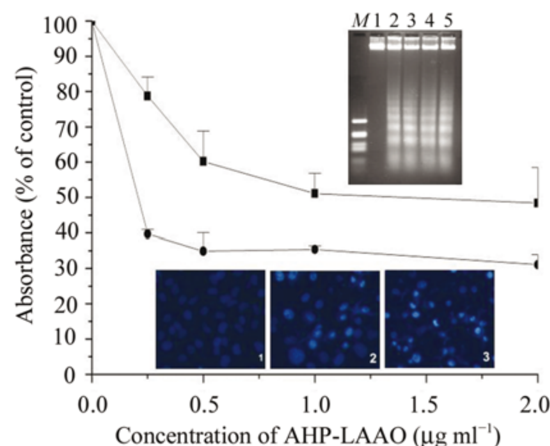

Figure 4
Effect of AHP-LAAO on the proliferation of HeLa cells. The cells were treated with a series of concentrations of AHP-LAAO for 12 h (squares) and 24 h (circles). Cell proliferation was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] method in a 96-well plate. The results are presented as the percentage of proliferation (*i.e.* the ratio of light absorbance of the sample to that of control) and are expressed as mean value ± standard deviation from three experimental repeats. Upper inset: DNA fragmentation in AHP-LAAO-treated HeLa cells. The cells were treated with AHP-LAAO at the concentrations indicated (0, 1, 2, 4 and 8 µg ml^{-1}) for 12 h. After harvesting the cells, the nuclear DNA fragmentation was analyzed by 2% agarose gel electrophoresis. Lane M contains DNA markers [pGEM-7Zf(+) *Hae*III digest]. Lower inset: nuclear morphological changes in AHP-LAAO-treated HeLa cells. The cells were treated with AHP-LAAO for 12 h and then stained with DAPI. (1) Control. (2) and (3) Cells treated with AHP-LAAO at a concentration of 0.25 and 0.5 µg ml^{-1}, respectively. The original photomicrograph was taken with an amplification of 400×.

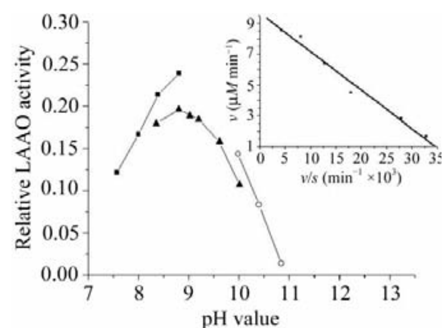

Figure 3
The relative oxidation activity of AHP-LAAO in buffers of different pH. Buffers were adjusted with bis-Tris propane (squares), CHES (triangles) and CAPS (circles). Inset: plot of v *versus* v/s used to calculate K_M and v_{max}. v is the reaction rate and s is the substrate concentration.

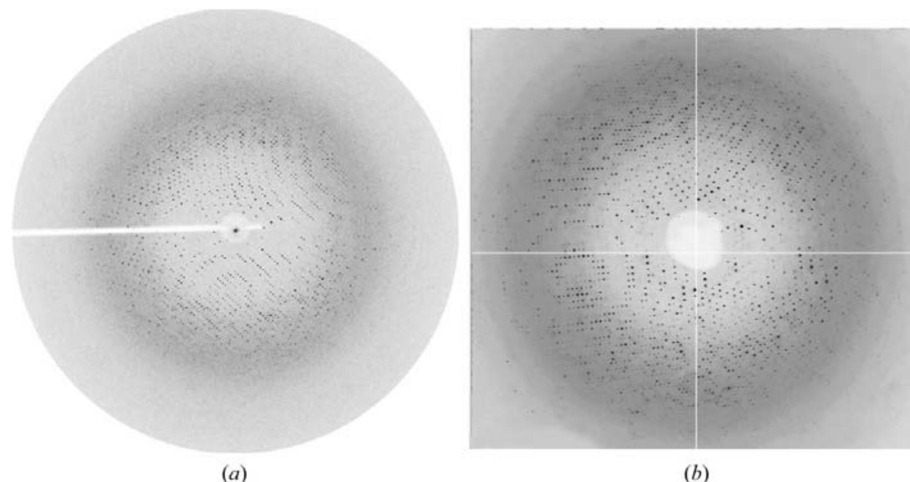

Figure 5
Diffraction patterns of AHP-LAAO crystals recorded at IHEP (*a*) and at MacCHESS (*b*).

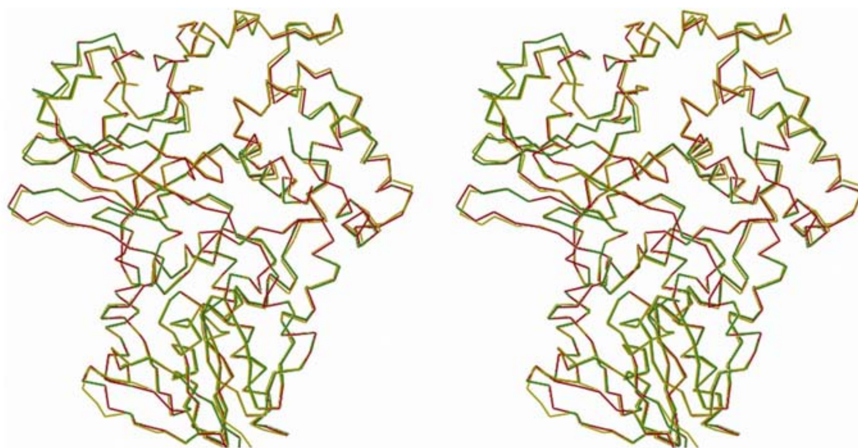

Figure 6
Superimposition of the LAAO models. Superimposition of the C^{α} coordinates of the models of AHP-LAAO refined against data sets collected at IHEP (red) and at MacCHESS (green) and LAAO from *C. rhodostoma* venom (yellow).

the two data sets are almost equivalent. The r.m.s. deviation between all atoms in the two models is only 0.186 Å (shown in red and green in Fig. 6). The C^{α} superimposition of AHP-LAAO and the homologous LAAO from *C. rhodostoma* venom leads to an r.m.s. deviation of 0.504 Å (shown in red and yellow in Fig. 6). Further structural analysis and related investigation need to be performed in order to expatiate the mechanism of the enzyme reaction.

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