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Research Article

Characterization of Libyan Cobra (*Naja haje*) Venom using Fluorescence and UV-Visible Spectroscopy

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Abstract

Snake venoms act as a preparative to defend animals against predators and helps in immobilizing and digestion of prey. Venoms consist of a combination of enzymes and toxins, such as metalloproteases, phospholipase A2, L-amino acid oxidase and toxins, including cytotoxins and neurotoxins. In addition to their toxicity, venom components exhibit several pharmacological activities and can be used as templates for drug design. The Libyan cobra venom was studied using UV-visible and fluorescence spectroscopic techniques. The cobra protein main chain absorbs light in the region of 240-340nm. The aromatic sidechains of cobra venom contain tyrosine, tryptophan and phenylalanine which are responsible for the absorbance in this region. Cobra venom provides intrinsic fluorescence emissions due to excitation of tryptophan residues, with some contribution from phenylalanine and tyrosine emissions. In addition, di sulphide bridges contribute considerable absorption in this wavelength range. The main fluorescence obtained is due to tryptophan which has a wavelength of maximum absorption at 280 nm and an emission peak ranging from 310 to 350 nm. UV-visible absorption and fluorescence spectroscopic techniques are sensitive and rapid to study cobra venom in order to better comprehend the performance of this venom.

Keywords: Snakebite; Envenomation; Libyan Cobra; Protein Fluorescence

Introduction

Snake bites are crucial public health problem among many African countries including Libya, Tunisia, Egypt and Algeria [1,2]. Africa inhabits more than 400 snake species of which about 30 are extremely poisonous. These species which belong to four different families namely: *Viperidae, Colubridae, Atracta spididae* and *Elapidae*, were found to be responsible for most human fatalities, as reported by the World Health Organization (WHO) [3]. There are eight species of cobra snakes including *Naja mossambica, Naja haje, Naja nigricollis, Naja siamensis, Naja kaouthia, Naja melanoleuca, Naja sputatrix* and *Pseudechis australis*. The first seven species are cobras from the genus Naja and are found throughout Africa including Libya and Asia, while the king brown/mulga snake (*P. australis*) is indigenous to Australia (Cobras (Naja spp.)) [4-7].

Cobra and other snake venoms usually show absorption maxima between 275 and 280 nm which are caused by the absorbance of the two aromatic amino acids tryptophan (Trp) and tyrosine (Tyr) and, to a small extent, by the absorbance of cystine (i.e. of di sulphide bonds). The absorbances of Trp and Tyr depend on the micro-environment of their chromophores. They are slightly red-shifted when transferred from a polar to a non-polar environment [8-10]. Cobra venom contains three amino acids with intrinsic fluorescence properties, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) but only tryptophan and tyrosine respond experimentally because their quantum yields are high enough to give a significant fluorescence signal. The three residues could be used to follow protein folding because their fluorescence properties (quantum

yields) are sensitive to their environment which changes when the protein folds or unfolds [11-13]. The main aim of our study was to further characterize the Libyan cobra venom using spectroscopy techniques.

Materials and Methods

All experiments were conducted in Tris buffer (0.01M Tris, 0.1M NaCl at pH 7.4). Glass-distilled deionized water and analytical grade reagents were used throughout experiments. pH values of solutions were measured with a calibrated Jen way pH-meter model 3510 (Staffordshire, UK). All buffer solutions were filtered through Millipore filters (Millipore, UK) of 0.45 mm pore diameter.

Absorbance spectra

Absorbance spectra were measured with Analytic Jena Specord 200 Plus (1.4nm band width, scanning, dual beam, single cell holder spectrometer, London, UK) using quartz cells of 1.00 cm path length. UV-vis absorbance spectra were recorded in the 200-500 nm range and a spectral bandwidth of 3.0 nm. For the final spectrum baseline subtraction of the buffer solution was performed. The protein content of venom samples was determined by the spectrophotometric method of Markwell et al. [14]. Bovine serum albumin (BSA, Sigma) was used for standard assays.

Fluorescence spectra

Fluorescence emission and excitation spectra were measured using a Jasco FP-6200 spectrofluorometer (Tokyo, Japan) using fluorescence 4-sided quartz cuvettes of 1.00cm path length. The automatic shutter-on function was used to minimize photo bleaching of the sample. The selected wavelength chosen provided aggregate excitation of tryptophan and tyrosine residues. The emission spectrum was corrected for background fluorescence of the buffer.

Venoms

Libyan Cobra (*Naja haje*) venom was extracted by manual stimulation of the animal and obtained in liquid or semisolid form, respectively, from the Zoology Department, Faculty of Science, University of Tripoli (Libya) and stored at -20 °C until use. Venoms were added to 2ml of 0.01 M Tris, 0.1 M NaCl at pH 7.4.

Results and Discussion

Absorption spectra

Absorption spectra of cobra venom (Figure 1) exhibit absorption maxima between 275 and 280nm which result from the absorbance of the two aromatic amino acids tryptophan (Trp) and tyrosine (Tyr) and to a small extent by the absorbance of cystine (i.e. of di sulphide bonds). The absorbances of Trp and Tyr were measured in 0.01 M Tris, 0.1 M NaCl at pH 7.4 and hence the spectra are specific to this microenvironment. Recently, it has been reported that absorption spectra of proteins are not primarily characterized by the ultraviolet region (185-320 nm) of the electromagnetic spectrum. but the peptide aggregates revealed absorption beyond 350 nm, caused by monomeric proteins lacking aromatic amino acids, di sulphide bonds, and active site prosthetic groups which were expected to remain optically silent beyond 250 nm [15,16]. It was also reported that UV-vis absorption profiles for monomeric proteins rich in charged amino acids spanning 250-800 nm have opened a new label-free optical spectral window for probing biomolecular structure and interactions. By combining experimental and computational studies the authors suggested that the broad absorption profiles of these proteins arise from photo excited charge transfer (CT) transitions of spatially proximal charged amino acids such as lysine (Lys) and glutamate (Glu). The studies revealed that the tuned Lys-Glu dimer spectrum spans 150-650 nm exhibiting five specific types of CT excitations with diverse and large spatial charge separation length scales of 2-10Å. These include inter-/intra-residue peptide backbone to peptide backbone (BB-CT) excitations spanning 160-210 nm, inter-/intra-residue peptide backbone to side chain (BS-CT) excitations spanning 160-260 nm, and side chain to side chain (SS-CT) excitations, which show the broadest absorption range spanning 260-650 nm [17].

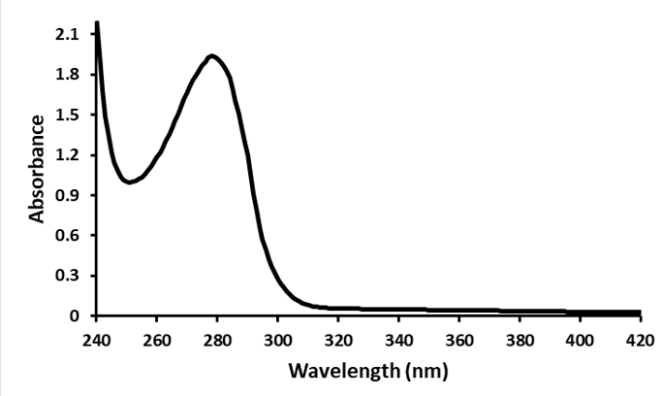

Figure 1: Ultraviolet-visible absorption spectrum of cobra venom (30 µg/ml) vs wavelength from 240-420 nm in 0.01 M Tris, 0.1 M NaCl at pH 7.4. The spectrum was corrected for small background fluorescence contributions from the buffer solution and was scaled to visualize the pure spectrum of the venom.

Fluorescence spectra

The fluorescence spectrum shows fluorescence intensity versus wavelength (Figure 2) of the Libyan cobra venom. The obtained fluorescence results from molecular rearrangements, excited-state reactions, ground state complex formation, collisional quenching and energy transfer. The fluorescence emission intensity as shown in Figure 2 exhibits a maximum emission at 350 nm suggesting that Trp residues activated by the excitation light moved from the buried hydrophobic environment into a relatively polar environment which is consistent with earlier reports [18].

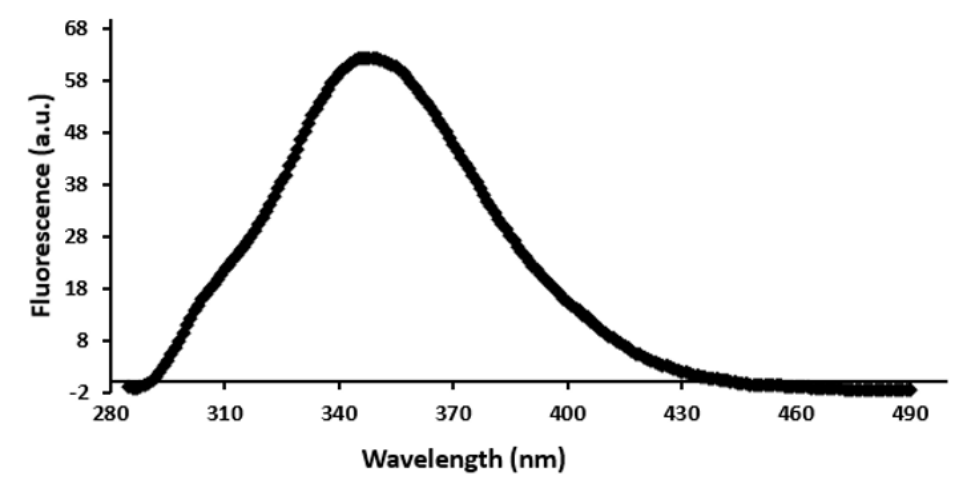

Figure 2: Plot of fluorescence emission of Libyan Cobra (*Naja haje*) venom (30 µg/ml) vs wavelength from 285-490 nm using excitation of λ280 nm in 0.01 M Tris, 0.1 M NaCl at pH 7.4. Spectra were corrected for small background fluorescence contributions from the buffer solution and were scaled to visualize the shift. (a.u.: arbitrary units)

Similar results report an increase of intrinsic tryptophan fluorescence and a blue shift of the maximum emission wavelength upon addition of Mg ATP to Ars A ATPase (catalytic subunit of the pump protein), indicating the movement of Trp-159 into a less polar environment [19]. The fluorescence of Phospholipases A2 if excited at 280 nm is mainly due to the presence of a single tryptophan residue (Trp3) which is located on the surface of the enzyme molecule exposed to the environment [20].

As indicated in the literature the solvent polarity and the local environment have profound effects on the spectral emission properties of fluorophores. The effect of solvent polarity is an important determinant of the Stokes shift, which was clearly observed in our experiments. Emission spectra are easily measured resulting in numerous publications on emission spectra of fluorophores in different solvents or bound to proteins, membranes, DNA or RNA. One general use of solvent effects is to determine the polarity of the probe binding site at the macromolecule. This is accomplished by comparison of emission spectra and/or quantum yields if the fluorophore bound to the macromolecule or dissolved in solvents of different polarity. The effects of the environment on fluorescence spectra and quantum yields are complex and are due to several factors including: solvent polarity, viscosity, rate of solvent relaxation, probe conformational changes, rigidity of the local environment, internal charge transfer, proton transfer, excited state reactions, probe–probe interactions or changes in radiative and non-radiative decay rates. These multiple effects may offer chances to probe the local environment surrounding a fluorophore. However, environmental effects are usually complex and even solvent polarity cannot be described using a single theory. The Lippert-Ma taga equation partially explains the effect of solvent polarity but does not account for other effects such as hydrogen bonding to the fluorophore or internal charge transfer that depends on solvent polarity [21,22].

Conclusion

The UV-visible absorption and fluorescence spectroscope approaches are sensitive and fast techniques to help the possibilities in searching for natural or synthetic inhibitors for cobra snake venoms for therapeutic purposes. In future studies to investigate interactions and type of venom components considering candidates for these interactions could be performed. We expect that Libyan cobra venom will play an important part in the advancement of understanding snake poisoning (in the near future).

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