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Research Article

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In vitro Kinetics Study of *Cerastes Cerastes* Phospholipase A_2 using Olive Leaf Extract: A Fluorescence Approach

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Abstract

Understanding snake venom kinetics is crucial for developing risk evaluation strategies and determining the best dose and timing of antivenom required to bind all venom in snakebite patients. Polyphenolic compounds have shown to inhibit toxic effects induced by snake venom proteins. The interaction of polyphenols with Phospholipase A2 of Cerastes cerastes snake venom was investigated by fluorescence spectroscopy. The decrease in the fluorescence versus time was conducted at room temperature in 0.01 M Tris, 0.1 M NaCl at pH 7.4. The decrease in fluorescence was following a pattern of zero-order kinetics rate in which the fluorescence is decreasing linearly with time. This study is expected to offer additional information about the interactions of PLA2 with natural product that might lead to therapeutic drug.

Keywords: PLA_2; AOLE; fluorescence

Abbreviations PLA_2: Phospholipase A2; AOLE: Aqueous Olive Leaf Extract

Introduction

Snake venom causes considerable mortality and morbidity typically needing hospitalization and sometimes causing permanent disabilities, and in severe cases may lead to death [1, 2]. Delayed admittance to suitable medical facilities, short of antvenom, and incomplete treatments are believed the major contributors to the high mortality and morbidity [3, 4]. Snake venoms contain a combination of biologically active polypeptides and proteins (encompass around 90–95% of a venom load), beside with other non-protein components including lipids, carbohydrates, amines, in addition to the inorganic salts [5]. The proteins and polypeptides available in the snake venom could be enzymes such as phospholipase A2, (PLA_2), L-amino acid oxidases (LAAO)], serine proteases (SVSP), and metalloproteases (SVMP), and the non-enzymatic substances such as kunitz peptides (KUN), disintegrins (DIS)] and three-finger toxins (3FTx) [5]. The treatment of snake envenomation mainly involves of taken of specific antivenoms for the snake species or type concerned, and helpful care. Snake antivenoms are antibodies that are obtained from the plasma of animals such as horses or sheep [6]. The antibodies which are taken intravenously could be antibody fragments [F(ab')2 or Fab] or IgG immunoglobulin or that react and neutralize the free venom in the plasma of the patient [6]. The dosage and timing of the antivenom administration is still mainly experimental and usually based on *in vitro* studies in animals by neutralisation of the venom [6]. The study of snake venom kinetics provides significant information about the time course of the exposure to venom. The kinetic studies allow for an improved determination of an adequate dose and timing of antivenoms administration obtained from animals or herbs. Phospholipases A_2 (PLA_2s-E.C. 3.1.1.4) are a big class of extracellular and intracellular enzyme. It catalyzes the hydrolysis of sn-2 acyl bonds of sn-3-phoapholipids. The Intracellular PLA_2s are frequently membrane related and are concerned in phospholipid metabolism nevertheless extracellular PLA_2s are also available profusely in the venom of insects and snake [7]. PLA_2s are extremely stable protein due to the existence of disulfide bonds that help in many biochemical studies such as kinetic which is the purpose in this paper. In addition, this stability makes the enzyme easy for biochemical and structural characterization in addition to their pathological effects and other bioactivities [8, 9]. Phospholipases A2 are the major venom constituent of snakes classified to genus Bothrops and display a wide range of biological effects including myotoxicity, antiplatelet activities, cardiotoxicity, anticoagulant, hemolysis, and neurotoxicity [10-12]. Phospholipases A_2 contain 121 amino acids residues and its molecular mass was calculated to be 13566.7 Da [13, 14]. Tryptophan (Trp) which is present in phospholipase A_2 exhibits fluorescence emission properties that are dependent on the polarity of the local environment around the Trp side chain [15]. Aqueous olive leaver extract (AOLE) of *Olea europaea L.* (*Oleaceae*) contains large number of phenolic compounds which classified into five recognized groups; flavones (luteolin-7-glucoside, diosmetin-7-glucoside, apigenin-7-glucoside,

diosmetin and luteolin); flavan-3-ols (catechin), flavonols (rutin); and substituted phenols (hydroxytyrosol, tyrosol, vanillin, caffeic acid and vanillic acid). The oleuropein is the richest phenolic compound in olive leaves, followed by luteolin-7-glucosides, hydroxytyrosol, verbascoside and apigenin-7-glucosides [16-18]. The main aim of this paper is to study the kinetics properties of Phospholipases A_2 which could interact with AOLE polyphenolic components at the molecular level to understand phospholipase A_2 –inhibitor interactions.

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 determined with a calibrated Jenway 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 on a Jenway UV-visible spectrophotometer, model 6505 (London, UK) using quartz cells of 1.00 cm path length. The UV-Vis absorbance spectra were recorded in the 200 - 500 nm range, and spectral bandwidth of 3.0 nm. For the final spectrum of each solution analyzed baseline subtraction of the buffer solution was performed. The protein content of venoms samples was determined by the spectrophotometric method of Markwell., *et al* [19]. 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 1.00 cm 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 kinetic spectrum was corrected for background fluorescence of the buffer. The plot of fluorescence kinetic emission of snake venom (*Cerastes cerastes*, 24.6 µg/ml) vs time (sec) from 0- 900 seconds using excitation of λ_{280} nm was performed in 0.01 M Tris, 0.1 M NaCl at pH 7.4.

Preparation of aqueous Olea europaea leave extracts

Leaves of olive trees (*Olea europaea*) were collected from the Novellien zone, Tripoli Centre, Tripoli, Libya during October 2018. The leaves (5g) were cleaned and washed with distilled water and dried at a room temperature of 25°C for about 20 minutes. Dried leaves were grinded in a homogenizer (HO4A Edmund Buhler GmbH, UK) along with 15 ml of distilled water. The resulting aqueous solution was filtered using a Millipore filter (0.45 µm, GHD Acrodisc GF, UK).

Venoms

Snake (Cerastes cerastes) venom was extracted by manual stimulation and were obtained in liquid and semisolid forms, respectively, from the Zoology Department, Faculty of Science, University of Tripoli (Libya) and stored at -20°C until use. Venoms were added to 2 ml of 0.01 M Tris, 0.1M NaCl at pH 7.4.

Results and Discussion

Olive leaves contain many powerful antioxidant polyphenolic compounds which are able to decrease the fluorescence due to a structural change in the 3D configuration of the phospholipase A2 molecule, leading to exposure of formally buried tryptophan residues. It has been reported that low molecular phenolic compounds can affect both the secondary and tertiary structure of the proteins as established by FTIR and circular dichroism and hence can led to a significant quenching of tryptophan of the phospholipase A2. In addition, tryptophan quenching by polyphenols could result by many process such as molecular rearrangements, excited-state reactions, ground state complex formation, energy transfer, and coalitional quenching [22-24]. Fluorescence quenching could be differentiated by two main mechanisms; dynamic and static and both of them could lead to a decrease in the fluorescence [23].Recently, we proves that the fluorescence intensity of the two amino acids intrinsic fluorophores species, tyrosine (Tyr) and tryptophan (Trp), decreased on the addition of the AOLE. In addition, the possible correlation of the decrease in the fluorescence intensity of phospholipase A2 on addition of 50 µl of AOLE to various molecular interactions processes [25].

The fluorescence of phospholipase A_2 was investigated and the achieved kinetic fluorescence spectrum showed that phospholipase A_2 excited at 280 nm, a decrease in fluorescence was observed in the presence of 50 µl of AOLE (figure 1). The interaction between phospholipase A_2 and AOLE constituents was confirmed by a decrease in fluorescence intensity with increasing time. This significant fluorescence change proposes a strong association between phospholipase A2 and the AOLE constituents. Figure 1 also showed that the fluorescence intensity of PLA_2 decreased in the presence of 50 µl AOLE, indicating that the microenvironments of amino acid fluorophores were affected by presence of the polyphenols of the AOLE. In order to explicate the response behavior in fluorescence is related to the AOLE, a set of control experiments were performed using only buffer instead of AOLE. No change in the fluorescence intensity was seen indicting the changes is attributed to the AOLE constituents. The decrease in the fluorescence intensity may also indicate that the polyphenols act as a quencher and their accessibility is dissimilar depending on the regions of interaction with PLA_2 and this consistent with the Cotrim proposal [26]. The emission of tryptophan (W68) at 350 nm of the PLA_2 obtained in Figure 1 before addition of AOLE (zero time) demonstrates that this residue under specific condition is easier exposed to the buffer and this is consistent with the literature [26].

PLA_2 is tetramer and was called as A, B, C and D. In the solution the two dimers formed from monomers of A and B are in the asymmetric unit display diverse conformation states owing to glycosylation by tetra ethylene glycol (TTEG) and have active site like region. Nevertheless, the second dimer formed by molecules C and D have an empty active site region. In all monomers the microenvironments formed by amino acids have eight tyrosine's, which distributed on hydrophobic region as well as on the hydrophilic surface and just one tryptophan in between the dimmers interface [27]. Furthermore upon addition of AOLE lead to possible slight conformational changes induced by the polyphenols and this affect the microenvironments of the tryptophan and tyrosine and a hence a decrease in the fluorescence seen. This assumption was confirmed by many published studies using fluorescence resonance energy transfer (FRET) and molecular docking [27-31]. The decrease in the fluorescence was following a zero order (with rate constant, k=-0.011 obituary fluorescence unit/sec) because the rate is independent of substrate concentration, and is equal to constant k. The formation of product proceeds at a rate

which is linear with time. The addition of more substrate does not serve to increase the rate.

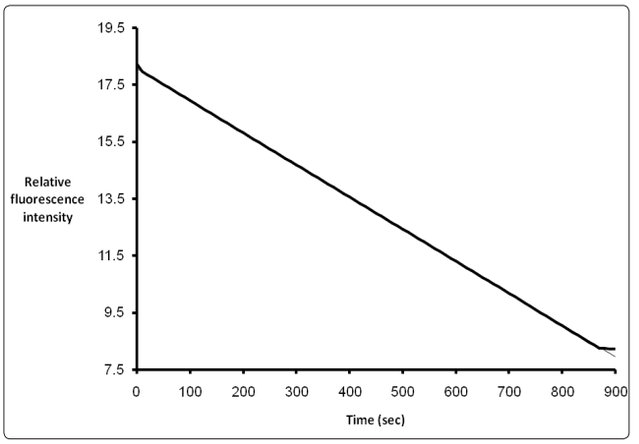

Figure 1: Fluorescence perturbation of snake venom by addition of AOLE. Plot of fluorescence emission of snake venom (*Cerastes cerastes*) (24.6 µg/ml) *vs* time (sec) from 0- 900 seconds using excitation of λ280 nm in 0.01 M Tris, 0.1 M NaCl at pH 7.4. Kinetic spectrum was corrected for small background fluorescence contributions from the buffer solution and was scaled to visualize the kinetic effect.

Similar kinetic study was performed by Tanweer *et. al* using Bee venom PLA_2 and they found that tryptophan and tyrosine residues have fluorescence due to the hydrophobic environment [32]. When the enzyme was treated with a molar equivalent of oleoyl imidazolide at pH 8.0 the fluorescence emission decreased piercingly and then declined progressively, undergoing a small, but detectable red shift. The time course of the slow phase corresponded to a half-life of calculated five minutes which is in sensible agreement with the kinetics of activation [32]. The logic interpretation of these results is that the highly hydrophobic reagent like in our case polyphenols bind to the PLA_2 very rapidly, disturbing the surroundings of one of the tryptophan residues, and then experiences a comparatively slow reaction in which the oleoyl group is transferred to an acceptor residue; this leads to further perturbation of the tryptophan environment and eventually lead to a decrease in the fluorescence.

Conclusion

In this paper, the kinetic interaction of polyphenols of AOLE with PLA2 has been studied using fluorescence spectroscopy. The decrease in the fluorescence intensity *versus* time could be related to the interaction between polyphenols of AOLE constituents. The decrease in the fluorescence was following a zero order pattern. This study of binding is of great significance to understand chemical-biological interactions for future drug design, biochemistry, and pharmacology studies. Additionally, this study is expected to provide more information about the interactions of PLA_2 with natural product in vision of a function as a therapeutic drug.

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