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EFFECT OF SOME DIVALENT METAL IONS ON ENZYMATIC ACTIVITY OF SECRETED PHOSPHOLIPASE A_2 ($sPLA_2$) ISOLATED FROM BULGARIAN *VIPERA AMMODYTES MERIDIONALIS*

Vasil Atanasov[1,2], Silviya Stoykova[1,2], Hristo Kolev[1], Mariana Mitewa[1], Svetla Petrova[3] and Ivayla Pantcheva[1]
[1]Sofia University, Faculty of Chemistry and Pharmacy, Sofia, Bulgaria
[2]Military Medical Academy, Emergency Toxicology Clinic, Sofia, Bulgaria
[3]Sofia University, Faculty of Biology, Sofia, Bulgaria
Correspondence to: Vasil Atanasov and Ivayla Pantcheva
E-mail: VAtanasov@chem.uni-sofia.bg, IPancheva@chem.uni-sofia.bg

ABSTRACT

The effect of Ca^{2+} and some divalent metal ions related to it (Mg, Sr, Ba, Cd) on the hydrolysis of natural and artificial substrates catalyzed by vipoxin secreted phospholipase A_2 subunit ($sPLA_2$) was evaluated. The results showed that the hydrolysis of natural glycerophospholipids proceeds at a highest rate when the enzyme is activated by calcium ions. The catalytic activity of $sPLA_2$ decreased in the presence of other metal ions possibly due to their lower coordination ability to the head group of lipids. Oppositely, the hydrolysis of artificial substrates was facilitated by metal ions with ionic radii larger than Ca^{2+}, such as Sr^{2+} and Ba^{2+}, suggesting that not only the interaction with metal cations, but also the origin of the head group should be considered. The fluorescence assay revealed that conformational changes occur during the coordination of metal ions into the catalytic site of $sPLA_2$ prior to subsequent hydrolysis. It could be concluded that the rate-limiting step in the catalytic cycle (e.g. the chemical interaction) is not completely adequate to evaluate all factors affecting the hydrolytic activity of vipoxin $sPLA_2$.

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Keywords: vipoxin, $sPLA_2$, divalent metal ions

Introduction

Snake venoms are rich sources of physiologically active compounds such as neurotoxins, myotoxins, hemotoxins, cardiotoxins, etc., that determine the total toxicity of the venom (8). The most toxic components of *Crotalinae*- and *Viperinae*-snake venoms are secreted phospholipase A_2 enzymes ($sPLA_2$, phosphatide *sn*-2 acylhydrolase, EC 3.1.1.4), which catalyze specifically the hydrolysis of the 2-acylester bond (*sn*-2) of 1,2-diacyl-3-*sn*-phosphoglycerides in a calcium-dependent manner, releasing both free fatty acids and lisophospholipids (13).

The neurotoxin vipoxin is the main and most toxic component isolated from the venom of the Bulgarian long-nose snake *Vipera ammodytes meridionalis*. It is a heterodimeric postsynaptic ionic complex composed of two protein subunits: a basic, and toxic His-48-containing $sPLA_2$; and an acidic, enzymatically inactive and non-toxic Gln-48-containing component (vipoxin acidic component, VAC). Both subunits display a high degree of sequence homology (62 %) and each vipoxin subunit binds one Ca^{2+} ion, according to spectroscopic studies (6).

The vipoxin $sPLA_2$ subunit belongs to the calcium-dependent Group IIA phospholipases where calcium is obligatory for binding substrates to the catalytic site of the enzyme (16, 17). The studies on various $sPLA_2$s show that the metal ion is essential for the electrophilic catalysis, polarizing the carbonyl group of the substrate *sn*-2-ester bond (10, 12, 16, 18). The metal ion in phospholipases serves as a mediator between the enzyme and substrate, binding O-atoms originating from the phosphate group and fatty acid ester at *sn*-2 position (11).

Crystallographic studies of vipoxin, however, show that its basic $sPLA_2$ and acidic subunits are significantly different from other dimeric $sPLA_2$ (9). The region referred to be the calcium-binding loop is also structurally different from other $sPLA_2$ where Ca^{2+} ion is usually a part of highly conserved Asp-49-containing active site (Ca^{2+} binding loop) (5, 11). The coordination of Ca^{2+} to the Asp-49-containing site of vipoxin $sPLA_2$ is restricted due to various intermolecular and ionic interactions with VAC. It is assumed that the self-stabilization of both vipoxin subunits occurs by neutralization of Asp-49 negative charge *via* intersubunit electrostatic interactions (6).

The absence of calcium in the crystal structure of vipoxin (9) raises the question of what the role of Ca^{2+} for $sPLA_2$ activity is. Moreover, in the absence of calcium, vipoxin still shows enzymatic activity, although it is greatly reduced.

In this paper we report our results on the catalytic properties of $sPLA_2$ in the presence of Ca^{2+} ions and the effect of its replacement with other divalent metal ions, using natural phospholipids and artificial substrates.

Materials and Methods

Materials

The crude air-dried venom from Bulgarian long-nose viper *Vipera ammodytes meridionalis* was purchased from the Thracian Herpetological Society (Stara Zagora, Bulgaria). The neurotoxin vipoxin was isolated from the crude venom as earlier described (15). The toxic subunit of vipoxin ($sPLA_2$) was separated using a fast protein liquid chromatography

(FPLC) procedure (1). The $sPLA_2$-containing fraction was directly used after dialysis against deionized water. The protein content was determined using the bicinchonic acid assay (14).

The natural substrates 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Sigma Chemicals Co. (USA). The artificial substrate 4-nitro-3-(octanoyloxy)benzoic acid (NOBA) was synthesized as described in the literature (4). The methyl ester of NOBA (NOBAMe) was prepared using a diethyl ether solution of freshly prepared diazomethane and was analyzed by thin-layer chromatography (TLC) and Fourier transform infrared spectroscopy (FT-IR) spectroscopy. All other chemicals, solvents and HPLC-grade solvents used in the present study were purchased from Sigma Chemicals Co. (USA) or Merck (Germany). In all experiments, deionized water was used (18.2 MΩ·cm).

$sPLA_2$ activity assay using natural substrates

HPLC analysis of the reaction products, using DPPC and POPC as substrates, was performed as described elsewhere (2) with some minor modifications.

A substrate solution (DPPC or POPC in methanol, 100 μL 1 mg/mL) was mixed with a buffer solution (670 μL 50 mmol/L Tris-HCl, pH 8.0), metal(II) salt (50 μL 100 mmol/L), NaCl (100 μL 1 mol/L), and internal standard: margarinic acid ($C_{17:0}$) (50 μL, 0.5 mmol/L in MeCN), in a final volume of 970 μL. After sonication for 1 min at 37 ºC, 30 μL of enzyme solution (320 μg/mL) were added to the reaction mixture and it was incubated for 10 min at 37 ºC. The further procedures were performed in accordance to Atanasov et al. (2).

The products of derivatization reaction (*p*-bromophenacyl esters of the fatty acids) were separated on C8 reverse-phase column (125 × 4.5 mm, 5 μm size particle, Alltech, USA) with an isocratic elution with 80 % (v/v) MeCN/water as a mobile phase. The analyses were performed on a Prominence UFLC quaternary pump (Shimadzu, Japan) combined with Spectra Sys UV200 UV-detector (Thermo electron, USA) at a flow-rate of 1.0 mL/min and detection at 257 nm.

The hydrolysis of POPC and HPLC analysis of fatty acids in the presence of disodium ethylenediaminetetraacetate (Na_2EDTA) was performed as described above with a Tris-HCl buffer containing additionally 10 μL of 100 mmol/L Na_2EDTA.

$sPLA_2$ activity assay using NOBA and NOBAMe

The assay was performed according to Holzer and Mackessy (7). The stock reaction solution was prepared by mixing NOBA or NOBAMe, respectively (200 μL of 3 mmol/L solution in MeCN), NaCl (500 μL of 1 mol/L solution) and Tris-HCl buffer (4300 μL 50 mmol/L, pH 8.0). The tested metal ions (30 μL 100 mmol/L) were added to 250 μL of the above reaction mixture in a 96-well plate and the reaction was initiated by the addition of 20 μL of PLA_2 (210 μg/mL). The reaction was quenched after 3 min of incubation by addition of 25 μL of 2.5 % (v/v) Triton X-100 and the absorbance at 425 nm was measured using a Dynex multiwell plate reader (Dynex Technologies, USA).

To ensure the abstraction of any calcium ions from the native enzyme *in situ*, disodium ethylenediaminetetraacetate (Na_2EDTA) was used as a complexation agent. The stock solution was prepared as described before with a final 1 mmol/L concentration of the chelating agent. The final molar ratio Na_2EDTA – metal(II) ions during the enzymatic assay was kept at 1:10.

Intrinsic fluorescent studies

To the reaction mixture containing $sPLA_2$ (50 μL 200 μg/mL) and disodium ethyleneglycoltetraacetate (Na_2EGTA) (10 μL 100 mmol/L) a solution of corresponding divalent metal ions (50 μL 100 mmol/L) was added. The final volume was adjusted to 500 μL with Tris-HCl buffer (50 mmol/L, pH 8.0). The sample without metal(II) ions served as a control.

Intrinsic tryptophan $sPLA_2$ fluorescence was performed on a Cary Eclipse Fluorescence spectrophotometer (Varian, USA, 1 cm cuvette), excitation beam at 295 nm, slit width of 10 nm and emission slit width of 2.5 nm. The fluorescence emission spectra were acquired in the range from 300 nm to 450 nm.

Results and Discussion

In the present paper the influence of different divalent metal ions on the enzymatic properties of $sPLA_2$ isolated from vipoxin is reported. The hydrolysis of two substrate types, natural and artificial, was studied. The natural substrates are glycerophospholipids that differ by the type of fatty acid chains at *sn*-2 position. DPPC contains *sn*-1 and *sn*-2 saturated palmitic acid residues, while POPC is an ester of saturated palmitic and unsaturated oleic acid at *sn*-1 and *sn*-2 positions, respectively. The artificial substrate NOBA used in this study is designed to mimic the properties of natural phospholipids. In NOBAMe the carboxylic group is modified (blocked) to prevent its interaction with the enzyme. In all performed experiments, the spontaneous substrate hydrolysis (if any) served as a blank. According to the published reports and experimental protocols (2), the total activity of pure phospholipase A_2 subunit achieved in the presence of Ca^{2+} ions is defined as 100 %.

Generally, the substrate hydrolysis proceeds in three steps and the formation of several complex species should be taken into account in evaluating activity the of $sPLA_2$:

$sPLA_2 + M^{2+} \rightarrow sPLA_2\text{-}M^{2+}$ (complex **1**)

$sPLA_2\text{-}M^{2+}$ + substrate $\rightarrow$ $sPLA_2\text{-}M^{2+}$-substrate (complex **2**)

complex **2** $\rightarrow$ complex **1** + hydrolysis products (fatty acids),

where M^{2+} = Ca, Mg, Sr, Ba, Cd.

The results from kinetic studies using pancreatic $sPLA_2$ had shown that the rate-limiting step of the catalytic turnover cycle is the chemical step (formation of complex **2**), in which the nature of the lipid head group and its interaction with Ca^{2+} ions plays a critical role (10, 18).

Enzyme hydrolysis of natural substrates

First, the effect of divalent metal ions on $sPLA_2$ enzymatic activity was studied using DPPC as a substrate. DPPC contains saturated fatty acid residues at both the *sn*-1 and the *sn*-2

position and for that reason the total activity of $sPLA_2$ can only be measured ($A_1 + A_2$) (**Fig. 1**).

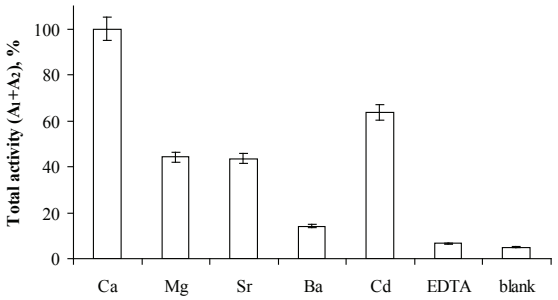

Fig. 1. DPPC hydrolysis catalyzed by $sPLA_2$.

To ensure the absence of metal ions, their abstraction was attained by addition of Na_2EDTA, as the purified $sPLA_2$ subunit most probably contains some Ca^{2+} ions which might have been left after its chromatographic separation and subsequent dialysis (1). The activity of the enzyme treated with chelating agent displays values comparable with those observed for the blank sample.

When a divalent metal ion is added to the pure $sPLA_2$ subunit, the catalytic activity varies depending on the nature of the cation. The vipoxin's phospholipase A_2 showed maximum activity in the presence of Ca^{2+} (5 mmol/L). On the other hand, the replacement of Ca^{2+} with divalent metal ions studied (applied at the same molar concentration, 5 mmol/L) led to a significant decrease in the enzymatic activity, especially in the case of Ba^{2+} ions (*c.a.* 7-fold). The addition of Mg^{2+} and Sr^{2+} ions resulted in a decrease in the catalytic activity up to *c.a.* 50 %, while Cd^{2+} ions retain $sPLA_2$ activity in *c.a.* 65 % as compared to that in the presence of Ca^{2+}.

From the study on DPPC hydrolysis achieved by vipoxin $sPLA_2$, it could be concluded that Cd^{2+} ions activate the enzyme catalytic properties to a higher extent as compared to the effect of other ions with smaller or larger metal ion radii than calcium.

Next, POPC was selected as a natural substrate, since during its hydrolysis the selective A_2 mode of action of $sPLA_2$ can be distinguished in contrast to the experiments using DPPC. In **Fig. 2a**, the total ($A_1 + A_2$) enzymatic activity of $sPLA_2$ is presented. **Fig. 2b** summarizes the data on specific substrate hydrolysis (A_2) compared to a substrate hydrolysis at position *sn*-1 (A_1).

Addition of Na_2EDTA to the enzyme caused the same effect as in the case of DPPC. The presence of Ca^{2+}, Mg^{2+} and Cd^{2+} ions activates both total and specific POPC hydrolysis, with Ca^{2+} being most effective. At the same time, the total activity in the presence of Sr^{2+} and Ba^{2+} cations reaches only *c.a.* 35 % of that observed with Ca^{2+} and these ions do not selectively affect substrate hydrolysis. From the results obtained, it can be concluded that Cd^{2+} ions activate $sPLA_2$ during POPC hydrolysis in a manner similar to that when DPPC was used as a substrate.

The total and specific A_2 activity of $sPLA_2$ achieved by divalent metal ions was evaluated successfully in the case of POPC. For that reason, we extended our study on reactivation of enzyme properties in the presence of a chelating agent. In this experiment the Ca^{2+} ions (possibly coordinated to the native enzyme) were removed by Na_2EDTA. Next, in the same reaction mixture, the enzymatic activity was evaluated by adding an excess of the corresponding metal(II) ion.

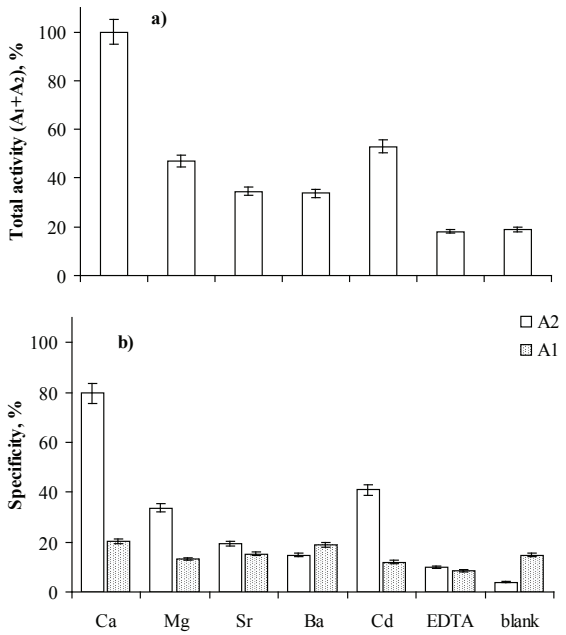

Fig. 2. POPC hydrolysis catalyzed by $sPLA_2$: total ($A_1 + A_2$) activity (**a**), and A_1 and A_2 specificity (**b**).

The results are presented in **Fig. 3a** (total activity, $A_1 + A_2$) and **Fig. 3b** (specific A_2 activity). The data on the total activity of $sPLA_2$ showed that in the case of removing any Ca^{2+}, the addition of the same ions recovers the enzyme activity (set as 100 %). Compared to the other metal ions studied, Mg^{2+} ions possess the highest effect (*c.a.* 60 %). It is interesting to note that Cd^{2+} does not recover the $A_1 + A_2$ activity of $sPLA_2$ as it was observed in our previous experiments (**Fig. 1** and **Fig. 2**).

It can be concluded that Ca^{2+} ions are essential for the selective A_2 mode of action of $sPLA_2$ in the presence of Na_2EDTA. Except Ba^{2+}, the addition of other divalent metal ions also leads to selective A_2 mode of action, although to a lesser degree as compared to calcium. Barium(II) ions exert a negligible effect on phospholipase A_2 activation, when DPPC and POPC were used as substrates.

The results on both DPPC and POPC natural substrates revealed that the effect of Ca^{2+} ions on $sPLA_2$ enzymatic activity is of great importance and the presence of these cations is responsible for the A_2 selectivity of substrate hydrolysis. Since the rate-limiting step is the chemical interaction between the substrate head group and the metal(II) ion (already coordinated to the catalytic site of $sPLA_2$, complex **1**) (10, 18), the formation of complex **2** appears to be essential for the hydrolysis to proceed. Thus, the coordination ability and the ionic radii of calcium seem to be most important for the metal ion to play a mediator function. Cadmium has a similar ionic radius and complexation properties to those of Ca^{2+}, so that the decreased activity of $sPLA_2$ in this case could be a consequence of small

conformational changes in the protein molecule upon formation of complex **1**. The other divalent metal ions studied, Mg^{2+} and Sr^{2+}, Ba^{2+}, smaller and larger than Ca^{2+}, did not promote the binding of natural substrates at a high rate, resulting in the detection of a small amount of hydrolytic products.

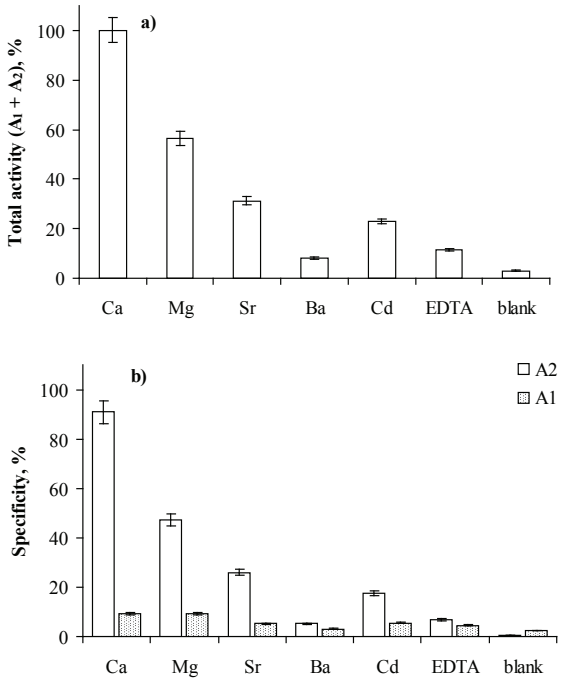

Fig. 3. POPC hydrolysis catalyzed by $sPLA_2$ in the presence of Na_2EDTA: total ($A_1 + A_2$) activity (**a**); and specific A_1 and A_2 activity (**b**).

Enzyme hydrolysis of artificial substrates

The experiments using natural substrates represent a complex system in which many factors should be taken into account as laborious and time-consuming procedures, etc.

Artificial substrates are often used in enzymatic studies due to their advantages, such as experimental simplicity, kinetic mode of measurements and reaction sensitivity. NOBA resembles natural glycerophospholipids with respect to: i) the coordination ability of the carboxylic group – similar to that of the phosphate group function; ii) the structural distances between the COOH-moiety and the acyl chain, which resemble those observed in natural substrates. On the contrary, NOBAMe contains a modified carboxylic group which does not function as a free moiety.

The ability of vipoxin $sPLA_2$ to hydrolyse NOBA in the presence of different divalent metal ions is shown in **Fig. 4**. The enzymatic activity was evaluated without or with addition of Na_2EDTA in the reaction system similarly to the POPC experiments. The hydrolytic activity achieved with calcium in the absence of chelating agent was set as 100 %.

The results showed that activation of $sPLA_2$ by Ca^{2+} proceeds at a comparable level in the presence or absence of Na_2EDTA. In the absence of chelator, the phospholipase A_2 enzymatic activity measured in the presence of Ca^{2+} and Sr^{2+} was comparable, while Ba^{2+} enhanced the release of caprylic acid by *c.a.* 30 %. Magnesium and cadmium did not affect significantly the hydrolytic reaction. When chelating agent was added to pure $sPLA_2$, the catalytic activity decreased in the following order: $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$, Cd^{2+}.

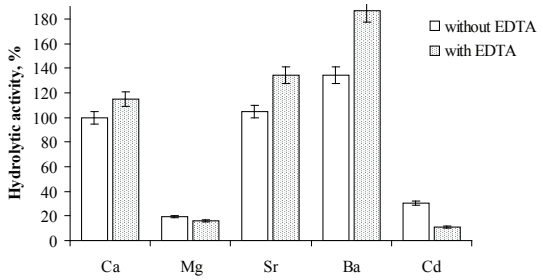

Fig. 4. NOBA hydrolysis.

As Rogers et al. (10) and Yu et al. (18) stated, the origin of the lipid head group is important for the chemical step of the catalytic cycle. In the case of NOBA, the function of the phosphate group is realized by the carboxylic moiety and that is at least a reason for the differences in the effect of metal ions on enzyme activity as compared to the natural substrates. It seems that metal ions with large ionic radii preferably form complex species **2**. Our preliminary study on the Michaelis–Menten kinetics of NOBA hydrolysis (Lineweaver–Burk double reciprocal plot) showed that metal ions do not affect the Michaelis constant value (K_m) but influence only the maximum enzyme reaction rate (V_{max}) (data not shown).

To confirm that the investigated metal(II) ions bind the NOBA substrate through its carboxylic group, we modified NOBA by preparing the corresponding methyl ester (NOBAMe). The enzymatic reaction using NOBAMe as a substrate did not proceed in the presence of any of the studied metal(II) ions. These results confirm that the NOBA carboxylic group function is essential for metal ion coordination and for the subsequent hydrolysis to proceed.

Intrinsic fluorescence study

It should be considered that, during the phospholipase A_2 reaction, the enzyme can also undergo conformational changes due to the coordination of divalent metal ions (complex **1**). To verify this hypothesis, a series of intrinsic fluorescence experiments were carried out (**Fig. 5**).

It is well known that tryptophan- and tyrosine-containing proteins (as vipoxin's $sPLA_2$ subunit) possess intrinsic fluorescence. These residues can be used to follow protein folding because their fluorescent properties (quantum yield) are sensitive to the environment, which changes when the protein folds/unfolds. For selective excitation of tryptophan (Trp) residues only, 295 nm wavelength was used in the present study.

In a hydrophobic environment (buried within the core of the protein), Trp has a high quantum yield and, therefore, a high fluorescence intensity. In contrast, in a hydrophilic environment (exposed to solvent) its quantum yield decreases, leading to low fluorescence intensity. On the other hand, not only the intensity, but also the maximum emission wavelength of Trp differs depending on its environment.

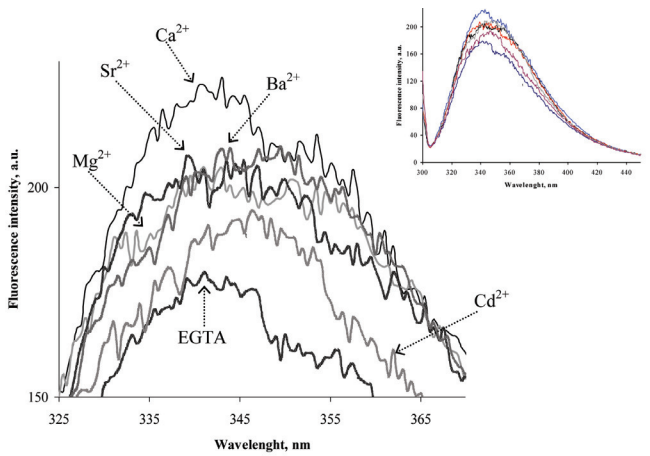

Fig. 5. Fluorescence emission spectra of $sPLA_2$ in the presence of chelating agent and after addition of divalent metal ions.

First, $sPLA_2$ was treated with Na_2EGTA to abstract any Ca^{2+}; next, the corresponding metal ion was added in the same reaction mixture. As it can be seen, the spectrum of $sPLA_2$ treated with chelator (inactive enzyme form, unfolded protein) and that recorded after subsequent addition of Ca^{2+} (active form, native protein) showed two maxima at 340 nm and 355 nm. According to Burstein et al. (3), it can be proposed that two groups of Trp residues can be distinguished: the first one is partially exposed on the surface of the protein ($\lambda_{max} \approx$ 340 nm) and the second group is fully solvent exposed ($\lambda_{max} \approx$ 350 nm). The increased intensity of the spectrum obtained after treatment of $sPLA_2$ with a chelating agent and calcium clearly demonstrates that the protein folds and recovers its native form. The enzyme emitted at the same wavelengths upon treatment with a multidentate ligand and Mg^{2+} but with lower fluorescence intensity than in the presence of Ca^{2+}. Most likely magnesium partially recovers the 3D structure of the protein molecule.

The spectra of $sPLA_2$ in the presence of Sr^{2+}, Ba^{2+} and Cd^{2+} consisted of one maximum at 348 nm, indicating that coordination of these metal ions leads to conformational changes in the enzyme (3). The increased emission intensity of $sPLA_2$ compared to that of the enzyme treated with chelating agent only suggests that the folding of the protein takes place to a higher degree with Sr^{2+} and Ba^{2+} than with Cd^{2+} ions.

As the qualitative fluorescence studies showed, $sPLA_2$ conformational alterations due to the presence of divalent metal ions (complex **1**) should be also taken into account. Subsequently, these changes might reflect the formation (and/or dissociation) of species **2** and the release of hydrolysis products, using natural or artificial substrates. In order to evaluate the role of metal ions on the enzymatic activity of vipoxin secreted phospholipase A_2 in more details, extensive future research should be performed.

Conclusion

In the present study, the effect of Ca^{2+} and some metal ions related to it (Mg^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}) on the catalytic activity and specificity of vipoxin secreted phospholipase ($sPLA_2$) subunit was assayed. Calcium ions were found to be unequivocally essential for the total $A_1 + A_2$ (DPPC, POPC) and specific A_2 (POPC) enzyme activity. It was demonstrated that the coordination of the carboxylic group and the size of the metal ionic radius are important factors for the hydrolysis of the artifical NOBA substrate. The fluorescence study showed that protein conformational changes occurring upon binding of different divalent metal ions might be responsible for the ambiguous effects observed during $sPLA_2$-catalyzed hydrolysis of natural and artificial substrates.

Acknowledgements

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