Received: November 8, 2004
Accepted: February 4, 2005
Published online: July 1, 2005

J. Venom. Anim. Toxins incl. Trop. Dis.
V.11, n.3, p.350-360, 2005.
Original paper - ISSN 1678-9199.

VIPOXIN SPECIFICITY STUDIED BY GAS CHROMATOGRAPHIC DETERMINATION OF ENZYMATIC REACTION PRODUCTS. INFLUENCE OF Ca^{2+}, Mg^{2+} AND Sr^{2+}

ATANASOV V. (1), BARDAROV V. (2), ALEKSIEV B. (3), MITEWA M. (1)

(1) Department of Analytical Chemistry, Faculty of Chemistry, University of Sofia, Sofia, Bulgaria; (2) Toxicochemical Laboratory, Department of Toxicology, Military Medical Academy, Sofia, Bulgaria; (3) Department of Organic Chemistry, University of Chemical Technology and Metallurgy, Sofia, Bulgaria.

ABSTRACT: Gas chromatographic procedure with mass spectrometric detection was applied to quantitatively determine the enzymatic specificity and activity of vipoxin (a neurotoxin from the *Vipera ammodytes meridionalis* venom) as well as the influence of Ca^{2+}, Mg^{2+} and Sr^{2+} on these properties.

KEY WORDS: vipoxin, phospholipase A_2, enzyme specificity, Ca^{2+}, Mg^{2+}, Sr^{2+} influence.

CORRESPONDENCE TO: M. MITEWA, University of Sofia, Faculty of Chemistry, Department of Analytical Chemistry, 1, J. Bourchier Avenue, 1126 Sofia, Bulgaria.
Fax: + 3592 96 25 438. Email: mmitewa@chem.uni-sofia.bg

INTRODUCTION

It is already well documented that vipoxin is the main toxic factor of the *Vipera ammodytes meridionalis* venom (2), showing both neurotoxic action (LD_{50} value of 400 µg/kg for white mice) and phospholipase activity (1). Detailed study on its chemical structure has proved the formation of an ionic complex consisting of basic and acidic subunits – toxic phospholipase A_2 and nontoxic inhibitor, respectively (12). Both subunits are built from 122 amino acid residues each and show high structural homology (62% amino acidic identity) (6, 12). After separation of the subunits, the phospholipase A_2 (both in solution and in lyophilized form) irreversibly looses its activity in a short period of time. When complexed with the inhibitor, however, the toxicity is retained for a long period combined with the phospholipase activity; the latter being lower compared with the freshly obtained free phospholipase A_2 (12).

The enzymatic specificity of the above-mentioned phospholipase A_2 is of immense interest because of the important role the different types of this enzyme play in numerous bioprocesses (10). Until the present moment, however, only its total enzymatic activity has been studied using pH-stat titration of the released fatty acids and isotopically labeled substrates; fluorescently marked ones are also used in spectrophotometric and chromatographic studies (3, 5, 8, 9, 13, 14). So far only the chromatographic techniques allow the determination of the individual fatty acids concentration. In the present paper a gas chromatographic method with mass spectrometric detection (GC-MS) is applied allowing the kinetic monitoring of the enzymatic specificity together with the total and specific phospholipase activity.

On the other hand, there are only scarce data about the influence of Ca^{2+} ions on the phospholipase A_2 activity, its physicochemical properties, and the high structures (4) of vipoxin, whereas the influence of metal ions on the enzymatic specificity has not been studied. Therefore the vipoxin specificity in presence of Ca^{2+} was investigated since it is expected to influence enzymatic specificity. For comparison purpose, the influence of Mg^{2+} and Sr^{2+} ions was also studied.

MATERIALS AND METHODS

The vipoxin used was isolated from air-dried venom of *Vipera ammodytes meridionalis* using the column chromatography procedure (11) followed by lyophilization and storage at 0-5ºC.

The soybean lecithin was purchased from “Serva”. All other chemicals and solvents used were AR grade (Merck) and the solvents used in the chromatographic experiments were GC grade (Merck).

Fatty acids and fatty acid methyl esters (FAMEs) reference substances were purchased from “Serva”.

Diazald®, used for preparation of diazomethane, was purchased from Sigma.

The enzyme reaction was carried out at optimum conditions followed by extraction of the released fatty acids and their quantitation after methylation.

The individual determination of FAMEs was made on a gas chromatograph Trace DSQ Thermo-Finnigan equipped with a split/splitless injector working in “splitless” mode, capillary column Supelcowax 10 (30m x 0.32mm x 0.4µm film, Supelco Inc.), at temperature program ranging from 70ºC to 230ºC, helium carrier gas with flow rate of 1.2 ml/min, and quadruple EI mass spectrometric detector operating in “SCAN” mode in the 50-350 m/z range.

Soybean lecithin, emulsified in 40% (v/v) methanol/deionized water free from Ca^{2+} ions, was used as substrate.

The enzymatic reaction was carried out at optimum conditions (12), 40 µl freshly prepared 0.1 mM vipoxin solution was added to 4.0 ml of 0.5 mg/ml neutral substrate solution containing a definite amount of Ca^{2+}. The mixture was incubated for 25 min at 25ºC and stopped by the addition of 1 ml 1M H_2SO_4. The fatty acids released were immediately extracted by three-fold extraction with portions of 2 ml each (n-propanol/hexane/heptane [4: 1: 1]). After the addition of 100 µl internal standard (100 µg/ml heptadecanoic acid) the solvent was evaporated under nitrogen flow and the dry residue was methylated with ether solution of diazomethane. The methylated esters obtained were dissolved in methanol and the aliquots were analyzed using the GC-MS technique.

RESULTS AND DISCUSSION

According to literature data (7, 15), it was established that the substrate of the enzymatic reaction, 1,2-diacyl-*sn*-glycerol-3-phosphocholine (natural lecithin), predominantly contains saturated fatty acids (palmitic and stearic) at the first position (A_1) and unsaturated fatty acids (linoleic and linolenic) at the second (A_2) (Table 1 and Figure 1).

The dependence of the vipoxin total enzymatic activity (R_{Vip}) on the Ca^{2+} concentration and on the enzymatic specificity ratio (R) in presence of Ca^{2+} is presented in Figures 2 and 3, respectively. It is evident that at 1.5 mM Ca^{2+} concentration maximum vipoxin total enzymatic activity is observed, in agreement with some literature data available (13). Also at this Ca^{2+} concentration the highest enzymatic specificity was recorded, which is approximately 1.3 times higher than the one found in absence of Ca^{2+}.

The influence of Mg^{2+} and Sr^{2+} on the enzymatic activity and specificity was also studied, and the experimental conditions were the same as those in which the maximum influence of Ca^{2+} ions was observed.

The data obtained are summarized in Figure 4, showing the values of the corresponding activities and specificities in absence and presence of Ca^{2+}, Mg^{2+} and Sr^{2+} ions. As it can be observed, the enzymatic activity is slightly increased in presence of Mg^{2+}, while Sr^{2+} ions decrease it. Quite different is their influence on the specificity - in presence of 1.5 mM Ca^{2+} it is increased 1.3 times, while both Mg^{2+} and Sr^{2+} cause its decrease. Positive effect of Ca^{2+} (strong) and Mg^{2+} (small) on the vipoxin phospholipase activity was confirmed together with the negative effect of Sr^{2+}.

Table 1: Relative contents of fatty acids obtained from lecithin basic hydrolysis and enzymatic reaction.

Fatty acid	Type	Relative content of substrate in the hydrolysate (%)	Relative content in the enzymatic hydrolysate (%)
Palmitic acid	Saturated	52.87	4.50
Stearic acid	Saturated	15.28	0.89
Oleic acid	Unsaturated	6.30	6.97
Linoleic acid	Unsaturated	22.64	81.77
Linolenic acid	Unsaturated	2.91	5.88

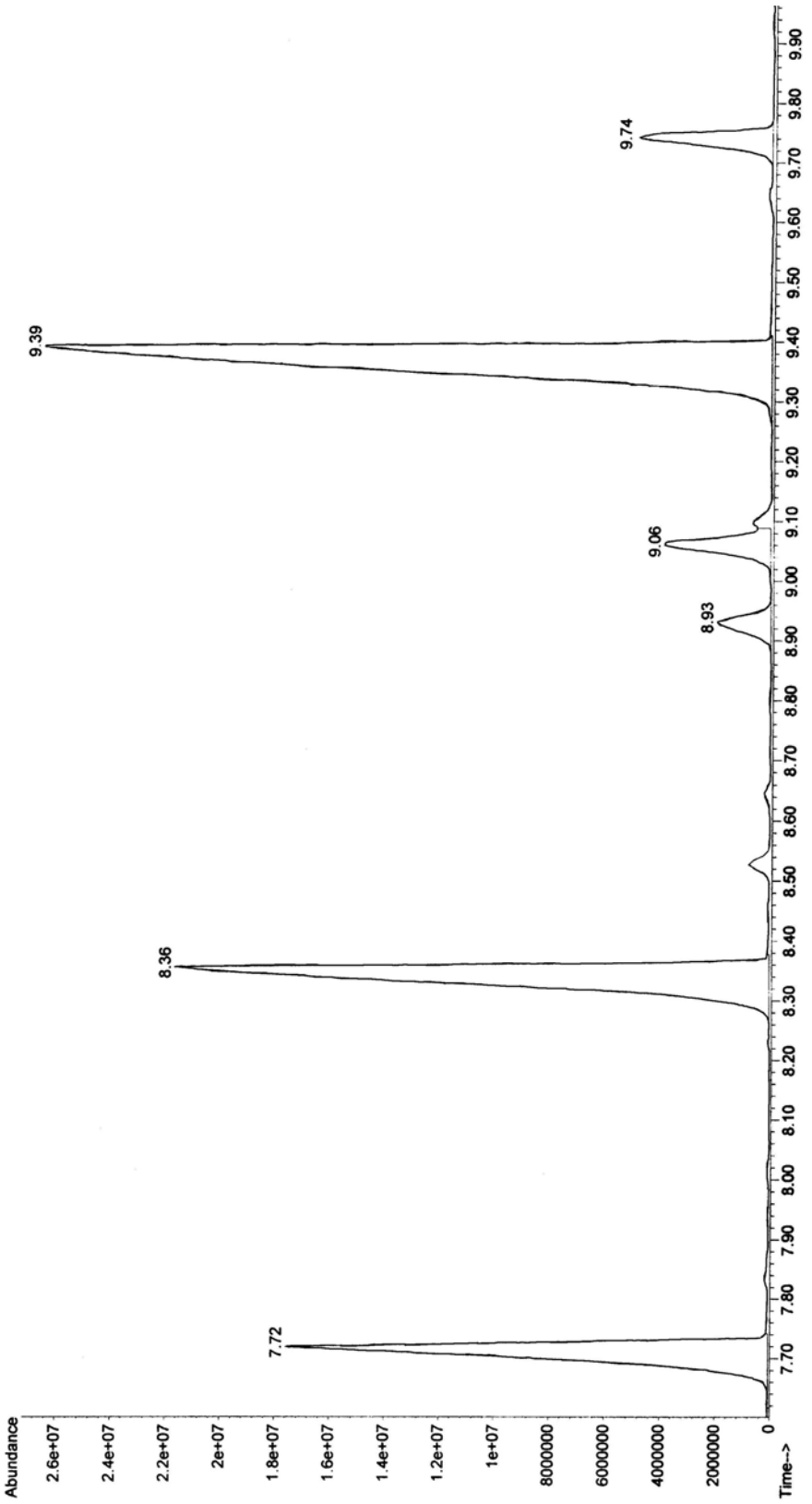

Figure 1a: Gas chromatograms of lecithin hydrolysate obtained in presence of 0.5 mol/l NaOH. (Incubation time: 25 min at the conditions described in the text). t_R=7.72 Palmitic acid (16:0); t_R=8.36 Internal Standard (Heptadecanoic acid, 17:0); t_R=8.93 Stearic acid (18:0); t_R=9.06 Oleic acid (18:1); t_R=9.39 Linoleic acid (18:1); t_R=9.74 Linolenic acid (18:3)

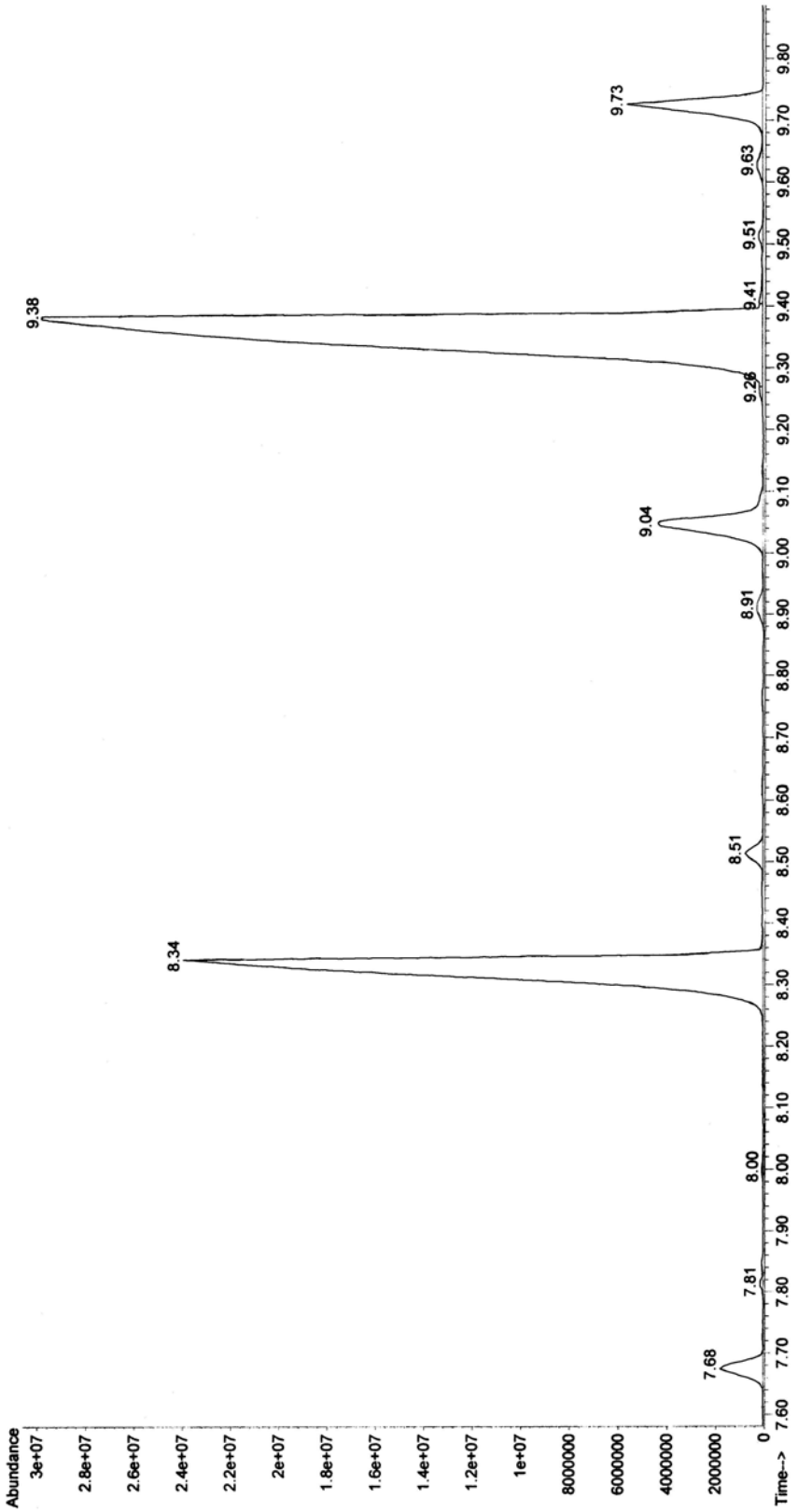

Figure 1b: Gas chromatograms of lecithin hydrolysate obtained in presence of vipoxin 1 mmol/l (b). (Incubation time: 25 min at the conditions described in the text). t_R=7.72 Palmitic acid (16:0); t_R=8.36 Internal Standard (Heptadecanoic acid, 17:0); t_R=8.93 Stearic acid (18:0); t_R=9.06 Oleic acid (18:1); t_R=9.39 Linoleic acid (18:1); t_R=9.74 Linolenic acid (18:3)

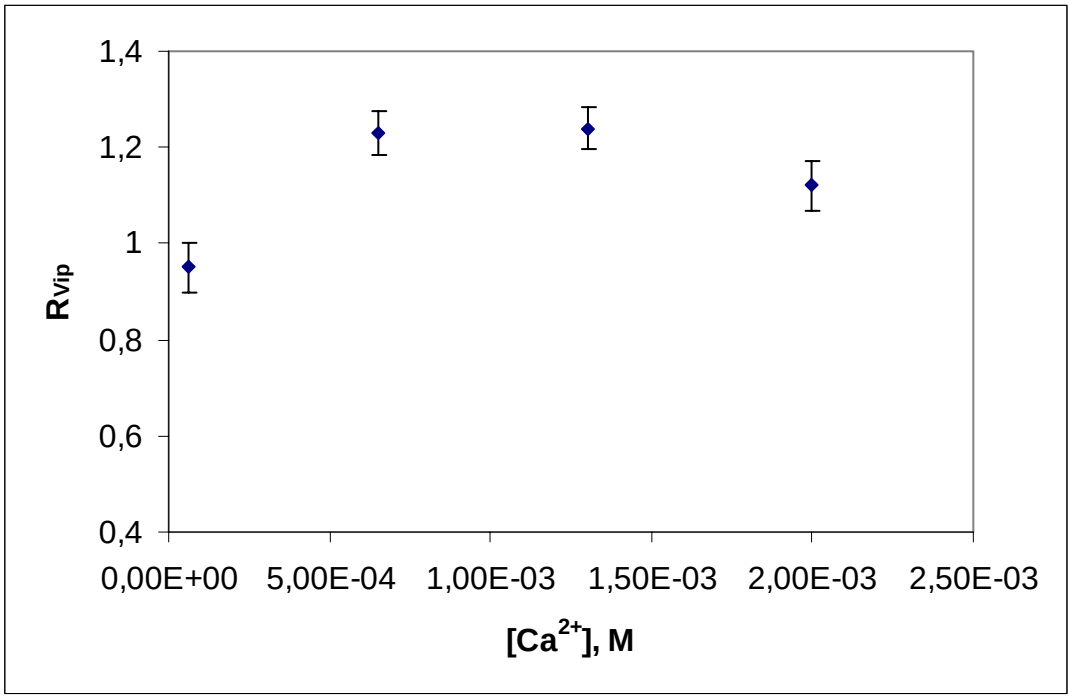

Figure 2: Dependence of the enzymatic activity on Ca^{2+} concentration. R_{vip} is the ratio of the amount of fatty acids released in presence of Ca^{2+} ions to the amount of the ones released in absence of Ca^{2+}. Incubation time: 25 min at the conditions described in the text.

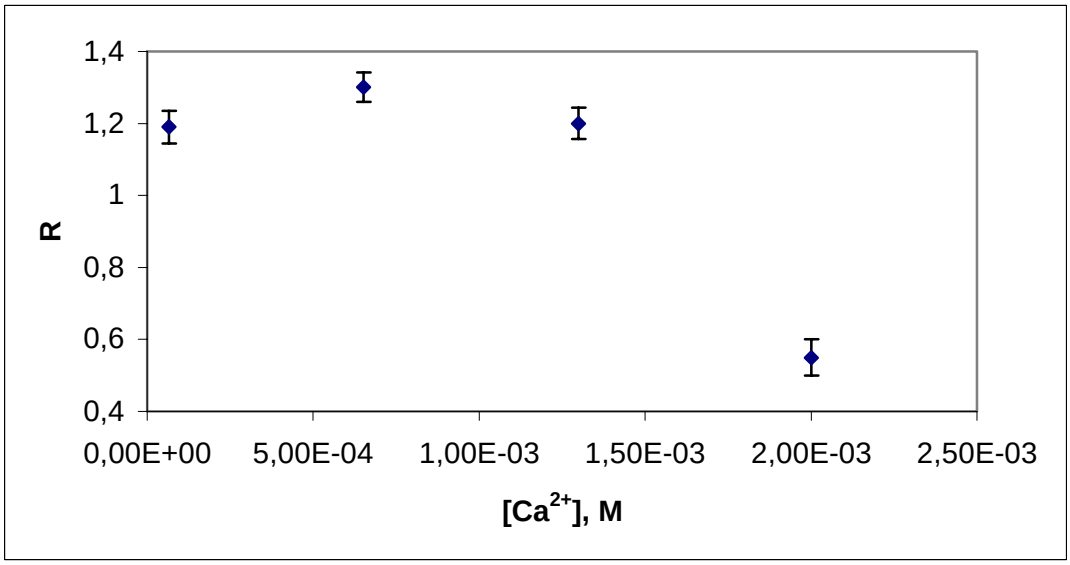

Figure 3: Dependence of the enzymatic specificity (R) on Ca^{2+} concentration. R is the A_2/A_1 ratio in absence and presence of the corresponding Ca^{2+} concentration. (A_1: saturated fatty acids; A_2: unsaturated fatty acids). Incubation time: 25 min at the conditions described in the text.

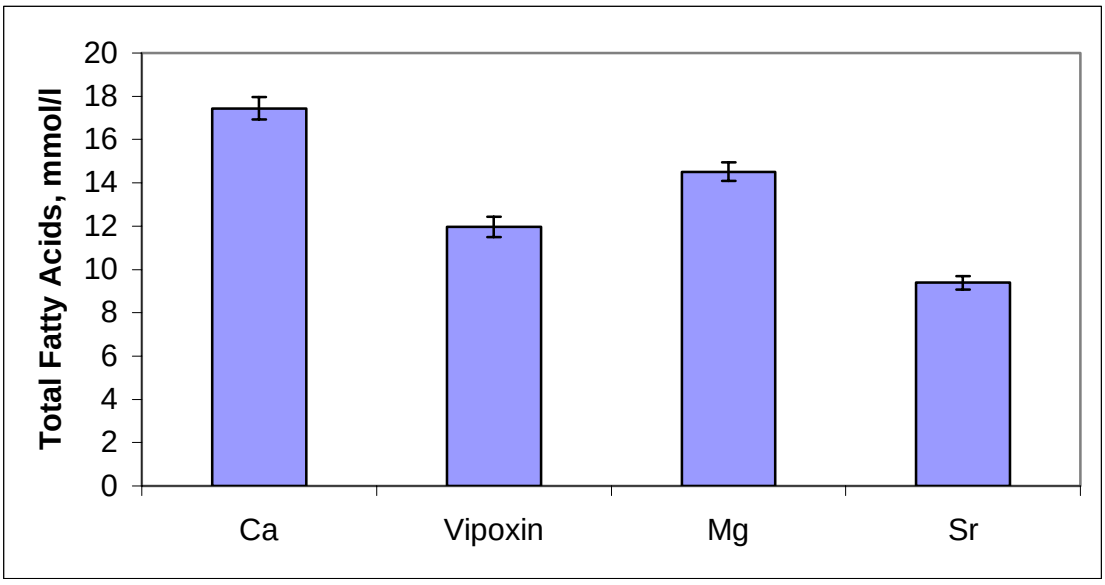

Figure 4a

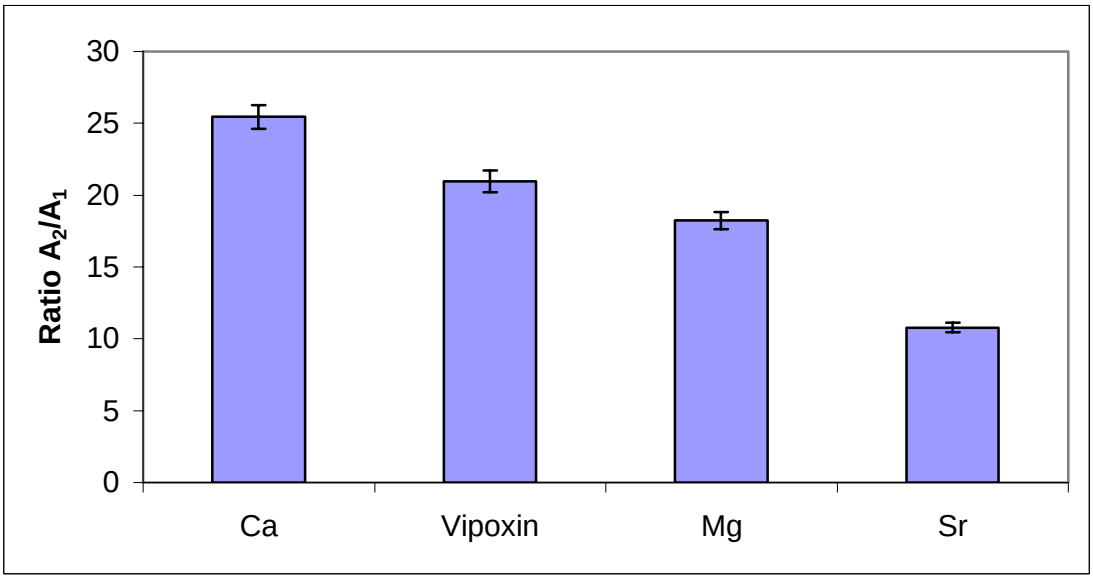

Figure 4b

Figure 4: Comparison of the vipoxin activity (a) and specificity (b) in absence and presence of different metal ions. The marked columns represent the corresponding values in absence of metal ions. Incubation time: 25 min at the conditions described in the text. $[M^{2+}]$ = 1.5 mmol/l

REFERENCES

1 ALEKSIEV B., TCHORBANOV B. Action on phosphatydylcholine of the toxic phospholipase A_2 from the venom of Bulgarian viper (*Vipera ammodytes meridionalis*). *Toxicon*, 1976, 14, 477-85.

2 BARDAROV V., ALEKSIEV B. Fractionation of Bulgarian viper (*Vipera ammodytes*) venoms. Relation of venom content and subspecies affiliation of the snakes. *Chromatographia*, 2002, 56, 345-9.

3 ELSBACH P., WEISS J. Utilization of labeled *Escherichia coli* as phospholipase substrate. In: DENNIS EA. Ed. *Methods in enzymology*. San Diego: Academic Press Inc., 1991: 24-31.

4 GEORGIEVA DN., BETZEL C., ALEKSIEV B., GENOV N. Spectroscopic investigation of the calcium binding sites in the neurotoxin vipoxin and its components – relation to the X-ray structure. *Spectrochim. Acta Part A*, 2000, 56, 2811-6.

5 KOUMANOV KS., MOMCHILOVA AB., QUINN PJ., WOLF C. Ceramides increase the activity of the secretory phospholipase A2 and alter its fatty acid specificity. *Biochem. J.*, 2002, 363, 45-51.

6 MANCHEVA I., KLEINSHMIDT T., ALEKSIEV B., BRAUNITZER G. The primary structure of phospholipase A_2 of vipoxin from the venom of Bulgarian viper (*Vipera ammodytes meridionalis*). *Biol. Chem. Hoppe-Seyler*, 1987, 368, 343-52.

7 NELSON LD., COX MM. The composition and architecture of membranes. In: RYAN M. Ed. *Lehninger principles of biochemistry*. New York: Worth Publishers, 2000: 369-89.

8 NUHN P., HEINE L., BENECKE R. Gaschromatographische Aktivitaetsbestimmung von Phospholipase A2. *Zentralbl. Pharm.*, 1985, 124, 77-81.

9 RICHIERI GV., KLEINFIELD AM. Continuous measurement of phospholipase A2 activity using the fluorescent probe ADIFAB. *Anal. Biochem.*, 1995, 229, 256-63.

10 SCOTT DL., WHITE SP., OTWINOWSKI Z., YUAN W., GELB MH., SIGLER PB. Interfacial catalysis: the mechanism of phospholipase A2. *Science*, 1990, 250, 1541-6.

11 TCHORBANOV B., ALEKSIEV B. A simple procedure for the isolation of the vipoxin – a neurotoxin with weak phospholipase activity from the venom of the Bulgarian viper (*Vipera ammodytes meridionalis*). *J. Appl. Biochem.*, 1981, 3, 558-61.

12 TCHORBANOV B., GRISHIN E., ALEKSIEV B., OVCHINNIKOV Y. A neurotoxin complex from the venom of the Bulgarian viper (*Vipera ammodytes meridionalis*). *Toxicon*, 1978, 16, 427-37.

13 WELLS AM. A kinetic study of the phospholipase A_2 (*Crotalus adamanteus*) catalyzed hydrolysis of 1,2-dibutyryl-sn-glycero-3-phosphorylcholine. *Biochemistry*, 1972, 11, 1030-41.

14 YON CH., HAN J. Analysis of trimethylsilyl derivatization products of phosphatidylethanol by gas chromatography-mass spectrometry. *Exp. Mol. Med.*, 2000, 32, 243-5.

15 ZUBAY GL., PARSON WW., VANCE DE. Biosynthesis of membrane lipids. In: SIEVERS EM. Ed. *Principles of biochemistry*. New York: William C. Brown, 1995: 438-41.