Toxicon, Vol. 21, No. 1, pp. 163–165, 1983.
Printed in Great Britain.

0041–0101/83/010163–03 $03.00/0

SHORT COMMUNICATIONS

PHOSPHOLIPASE A_2 ACTIVITY OF LONG-CHAIN CARDIOTOXINS IN THE VENOM OF THE BANDED KRAIT (*BUNGARUS FASCIATUS*)

WEN-CHANG CHANG,[1, 2] MUH-LIN LEE[1] and TUNG-BIN LO[1, 2]

[1]Institute of Biological Chemistry, Academia Sinica and
[2]Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan, Republic of China

(*Accepted for publication* 19 *May* 1982)

W.-C. CHANG, M.-L. LEE and T.-B. LO. Phospholipase A_2 activity of long-chain cardiotoxins in the venom of the banded krait (*Bungarus fasciatus*). *Toxicon* **21**, 163–165, 1983.—Re-investigation of the long-chain cardiotoxins from *Bungarus fasciatus* venom reveals that they are weak phospholipases of the A_2 type. The specific activities (units/mg) toward egg lecithin are 0.42, 1.65, and 0.29 for the long-chain cardiotoxins V-2, V-3 and VI, respectively.

THREE toxic components (V-2, V-3 and VI) isolated from the venom of *Bungarus fasciatus* (Miami Serpentarium Laboratories, U.S.A.) (LU and LO, 1974) have been characterized as "cardiotoxic" as they cause contracture in a chicken biventer cervical muscle assay (LIN SHIAU *et al.*, 1975). Later, their amino acid sequences were elucidated (LU and LO, 1978, 1981). It was surprising that they showed about 30% sequence homology with cobra phospholipase A_2 (phosphatidate 2-acylhydrolase, EC 3.1.1.4) but did not exhibit phospholipase A_2 activity. Moreover, both their circular dichroic spectra and chemical properties, such as molecular weights and amino acid compositions, were similar to that of phospholipase A_2 and totally unrelated to typical cobra cardiotoxins (LU and LO, 1981). These facts suggested that these toxins might belong to the phospholipase A_2 class. In the previous assays for phospholipase A_2 activity the samples were tested at nanogram levels and the results were negative. Recently the assays were repeated at higher doses and it soon became clear that these toxins exhibited phospholipase A activity at high doses (Table 1). It is clear from Table 1 that all three cardiotoxins show significant activity, although it is low as compared with cobra phospholipase A_2. Our cardiotoxin preparations have been purified and shown to be homogeneous by disc electrophoresis (pH 4.3, 7.5% gels). No extra bands were observed at doses up to 400 μg. Moreover, by extracting the protein from gel slices after electrophoresis, we could demonstrate phospholipase A activity exactly at the position corresponding to that of the protein band stained with amido black. This was true for all three cardiotoxins. It seems reasonable to conclude that the enzyme activity is intrinsic to these protein toxins and did not originate from other contaminating phospholipases A which, if present, should have been removed during extensive purification procedures and electrophoresis. Furthermore, it has to be pointed out that no other fractions from the venom of *Bungarus fasciatus* were active in the enzyme assay and it was impossible for our preparations to be contaminated with more active phospholipases A.

In order to confirm the enzyme activity on the one hand and to elucidate the site of

hydrolysis on the other, the hydrolysis products produced by the action of V-3 on egg lecithin were subjected to nuclear magnetic resonance studies as previously described. (CHANG and LO, 1975). The NMR spectra of lecithin and the lysolecithin and fatty acids obtained by the action of V-3 are shown in Fig. 1. It can be seen that the signals around $\delta = 5.36$ ppm (or $\tau = 4.64$ ppm), which are due to protons on a double bond in the lecithin molecule (CHAPMAN and MORRISON, 1966), are missing in the spectrum of lysolecithin, but are present in the spectrum of the released fatty acids. This means that unsaturated fatty acyl groups have been completely hydrolyzed to the free fatty acids. It is well established that the unsaturated fatty acyl groups are esterified mainly at the 2-position of the glycerol moiety (HANAHAN *et al.*, 1960). Therefore, our results indicate that V-3 attacks the ester bond at the 2-position and is a phospholipase A_2.

It has been reported that the histidine and aspartic acid residues at positions 47 and 48 in the amino acid sequence of snake venom phospholipases A_2 are involved in the active

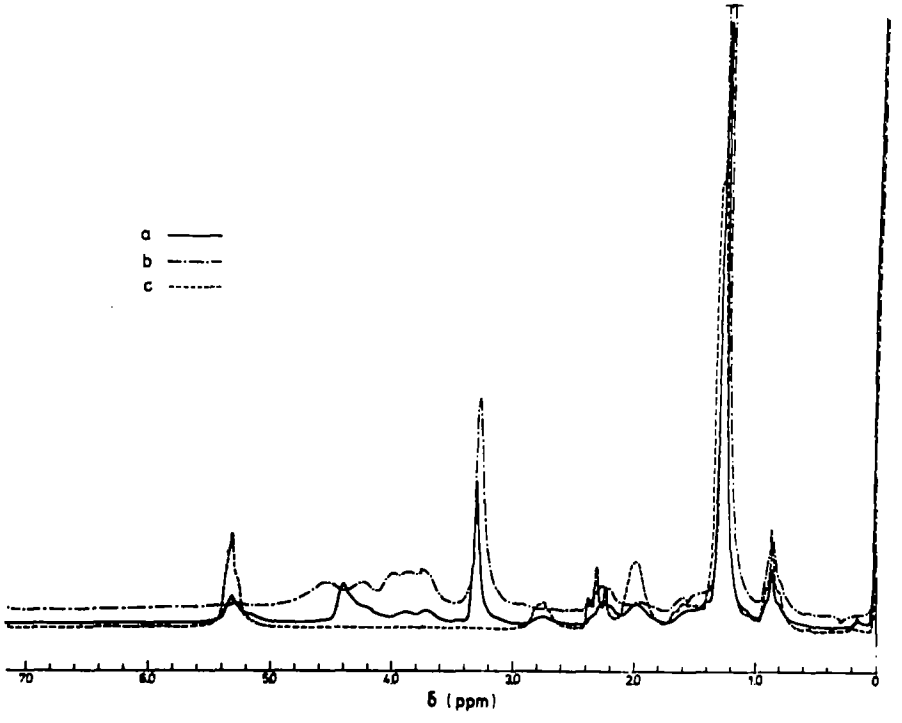

FIG. 1. NUCLEAR MAGNETIC RESONANCE SPECTRA OF(a) EGG LECITHIN, (b) LYSOLECITHIN AND (c) FATTY ACIDS OBTAINED FROM EGG LECITHIN BY THE ACTION OF V-3.

The solvent used was $CDCl_3$. Chemical shifts are expressed on the δ scale. The spectra were obtained on a JEOL FT-100 NMR spectrometer. The lysolecithin and fatty acids used in this study were prepared by incubating 0.5 mg of V-3 in 100 μl of 25 mM $CaCl_2$ with 1 g of egg lecithin in 10 ml of diethyl ether for 16 hr at 37°C. The precipitated lysolecithin was recovered by centrifugation and purified by repeated precipitation from methanolic solution with ether. The fatty acids remained in the ether phase and were extracted into 0.1 M Na_2CO_3. On acidification they were extracted with ether. These hydrolysis products (overall yield more than 70%) gave single spots in thin-layer chromatography (CHANG and LO, 1975).

TABLE 1. PHOSPHOLIPASE A ACTIVITY OF LONG-CHAIN CARDIOTOXINS

Cardiotoxin*	Dose per assay	Specific activity (unit/mg)†
V-2	84 μg	0.41; 0.43
V-3	30 μg	1.6; 1.7
VI	130 μg	0.28; 0.30
Phospholipase A_2 (*Naja naja atra*)‡	0.2 μg	450; 470

*The designations for these long-chain cardiotoxins are those of the previous report (LU and LO, 1981).
†One unit is the enzyme activity which will release 1 μmole of fatty acid in 1 min.
‡This typical enzyme preparation is included for comparison.
The enzyme activity was measured by the titrimetric method in an automatic recording pH-stat. The substrate solution was similar to that of STRONG *et al.* (1976), consisting of the following: 5 mM egg-yolk lecithin; 5 mM sodium deoxycholate; 25 mM $CaCl_2$; 0.1 M NaCl; 0.1 mM EDTA. The pH was adjusted to 7.5 before use. For each assay, 4 ml of the substrate solution was used and the rate of consumption of 2 mM NaOH was measured at 37°C.

site (VILJOEN *et al.*, 1977; ROBERTS *et al.*, 1977). For the long-chain cardiotoxins reported in the present paper, the histidine and aspartic acid residues are transposed to positions 39 and 40, respectively (LU and LO, 1981), and this might be the reason for the drastic decrease in enzyme activity.

REFERENCES

CHANG, W.C. and LO, T.B. (1975) The differentiation of phospholipases A_1 and A_2 by nuclear magnetic resonance spectroscopy. *J. Chin. biochem. Soc.* **4**, 43.

CHAPMAN, D. and MORRISON, A. (1966) Physical studies of phospholipids, IV. High resolution nuclear magnetic resonance spectra of phospholipids and related substances. *J. biol. Chem.* **241**, 5044.

HANAHAN, D.J., BROCKERHOFF, H. and BARRON, E.J. (1960) The site of attack of phospholipase (lecithinase) A on lecithin: a re-evaluation. Position of fatty acids on lecithins and triglycerides. *J. biol. Chem.* **235**, 1917.

LIN SHIAU, S.Y., HUANG, M.C. and LEE, C.Y. (1975) A study of cardiotoxin principles from the venom of *Bungarus fasciatus*. *Toxicon* **13**, 189.

LU, H.S. and LO, T.B. (1974) Chromatographic separation of *Bungarus fasciatus* venom and preliminary characterization of its components. *J. Chin. Biochem. Soc.* **3**, 57.

LU, H.S. and LO, T.B. (1978) Complete amino acid sequence of a new type of cardiotoxin of *Bungarus fasciatus* venom. *Int. J. Peptide Protein Res.* **12**, 181.

LU, H.S. and LO, T.B. (1981) Complete amino acid sequence of two cardiotoxin-like analogs from *Bungarus fasciatus* (banded krait) snake venom. *Toxicon* **19**, 103.

ROBERTS, M.F., DEEMS, R.A., MINCEY, T.C. and DENNIS, E.A. (1977) Chemical modification of the histidine residue in phospholipase A_2 (*Naja naja naja*). A case of half-site reactivity. *J. biol. Chem.* **252**, 2405.

STRONG, P.N., GOERKE, J., OBERG, S.G. and KELLY, R.B. (1976) β-Bungarotoxin, a pre-synaptic toxin with enzymatic activity. *Proc. natn Acad. Sci. U.S.A.* **73**, 178.

VILJOEN, C.C., VISSER, L. and BOTES, D.P. (1977) Histidine and lysine residues and the activity of phospholipase A_2 from the venom of *Bitis gabonica*. *Biochim. biophys. Acta* **483**, 107.