ISOLATION OF PHOSPHOLIPASE A ISOENZYMES FROM NAJA NAJA VENOM AND THEIR ACTION ON MEMBRANE-BOUND ENZYMES

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Cobra (Naja naja) venom or partially purified phospholipase A from that source have been widely used for the differential extraction of membrane-bound enzymes, e.g., NADH dehydrogenase (Ringler et al., 1963), choline dehydrogenase (Rendina and Singer, 1959), α-glycerophosphate dehydrogenase (Ringler and Singer, 1958), D-lactate cytochrome reductase (Gregolin and Singer, 1963), and β-hydroxybutyrate dehydrogenase (Fleischer et al., 1966). Cobra venom and purified phospholipase A at low concentrations selectively interrupt electron transport in the NADH-CoQ region of the respiratory chain by the removal of essential lipids and the formation of inhibitory phosphatides (Fleischer et al., 1964; Machinist and Singer, 1965). By abolishing permeability barriers to charged compounds cobra venom provides a means of assaying the full activity of mitochondrial enzymes (Arrigoni and Singer, 1962).

On the basis of an apparent lack of correlation of activity on purified substrates and the capacity to solubilize membrane-bound enzymes during attempts at purification of phospholipase A on DEAE cellulose, it has been suggested that phospholipase itself might not be the component of cobra venom responsible for its manifold effects on mitochondria (King, 1966). The resolution of this question and of numerous problems concerning the role of phospholipids in energy transduction called for highly purified preparations of the enzyme.

Although crystalline preparations of the enzyme from various species of venom have been reported (De, 1944; Suzuki et al., 1958; Slotta and Fraenkel-Conrat, 1938), the preparations are either impure (e.g. crotoxin) or not reproducible.

Phospholipase A activity was measured by following the initial rate of pH change with an expanded range, recording pH meter at 25°. The method is suitable for kinetic studies since no lag is observed and it uses commercially available substrates. The

reaction mixture (pH 8.0 to 8.1) contained in 2 ml volume 0.02 M $CaCl_2$, 0.7 mM EDTA, 0.5 mM TRIS, 0.055 M NaCl, 0.5 % (w/v) aldehyde-free Triton X-100, and 3 to 5 mg chromatographically pure egg lecithin or its catalytically hydrogenated form (General Biochemicals), added in 0.05 to 0.10 ml ethanol. The mixture was magnetically stirred under a stream of N_2 and the reaction was started by adding sufficient enzyme to give 0.05 to 0.1 pH unit change in 1 to 5 minutes. The rate was linear and proportional to the enzyme concentration; saturated lecithin gave about 1/6th the rate of the unsaturated form.

The starting material for purification was the preparation of Cremona and Kearney, 1964, from N. naja venom which is obtained by heat inactivation and chromatography on Sephadex G-75. Isoelectric focusing (Vesterberg and Svensson, 1966) of this material in the LKB electrofocusing column at pH 3 to 10 or 4 to 6 (2°, 48 hours, 700 V) gave three coincident protein and activity peaks with isoelectric points at pH 4.7 (I), 4.95 (II), and 5.5 (III). Peak II is the predominant component of the venom in terms of phospholipase A activity. The material corresponding to each of these peaks was combined from two runs (representing 100 mg of starting material), freed from sucrose on Sephadex G-25, concentrated and Ampholine carrier partially removed by ultrafiltration and each was further fractionated by electrofocusing in a narrow pH range (0.8 to 1.0 pH unit), under the conditions given in the legend of Fig. 1. The material from Peak I (Fig. 1a) gave a single major enzymatic component (I.E. point = pH 4.64). Peak II (Fig. 1b) is resolved into 3 components with I.E. points at 4.94 (IIA), 4.90 (IIB), and 5.02 (IIC). Peak III is resolved in the narrow pH range into 2 major phospholipase components with I.E. points at pH 5.51 (IIIA) and 5.56 (IIIB).

The same 6 principal forms of phospholipase A may be separated by electrophorizing the Sephadex G-75 passed enzyme directly in narrow range gradients: a pH 5.4 to 6.3 gradient yields components IIIA and B (other components pass into electrode compartment), while at pH 4.4 to 5.4 components I, IIA, IIB, and IIC are obtained well separated from each other.

Discontinuous polyacrylamide electrophoresis by the technique of Reisfeld and Small, 1966, but without reduction and urea gave the results shown in Fig. 2. Peak I was further resolved into 2 proteins, both active in phospholipase A assays on elution. The components of Peak II (IIA and IIC), although well resolved from each other in isoelectric focusing, were indistinguishable in analytical acrylamide electrophoresis. A mixture of the components of Peak III (IIIA and IIIB) gave the expected 2 principal bands on acrylamide plus two minor ones, all of which were catalytically active.

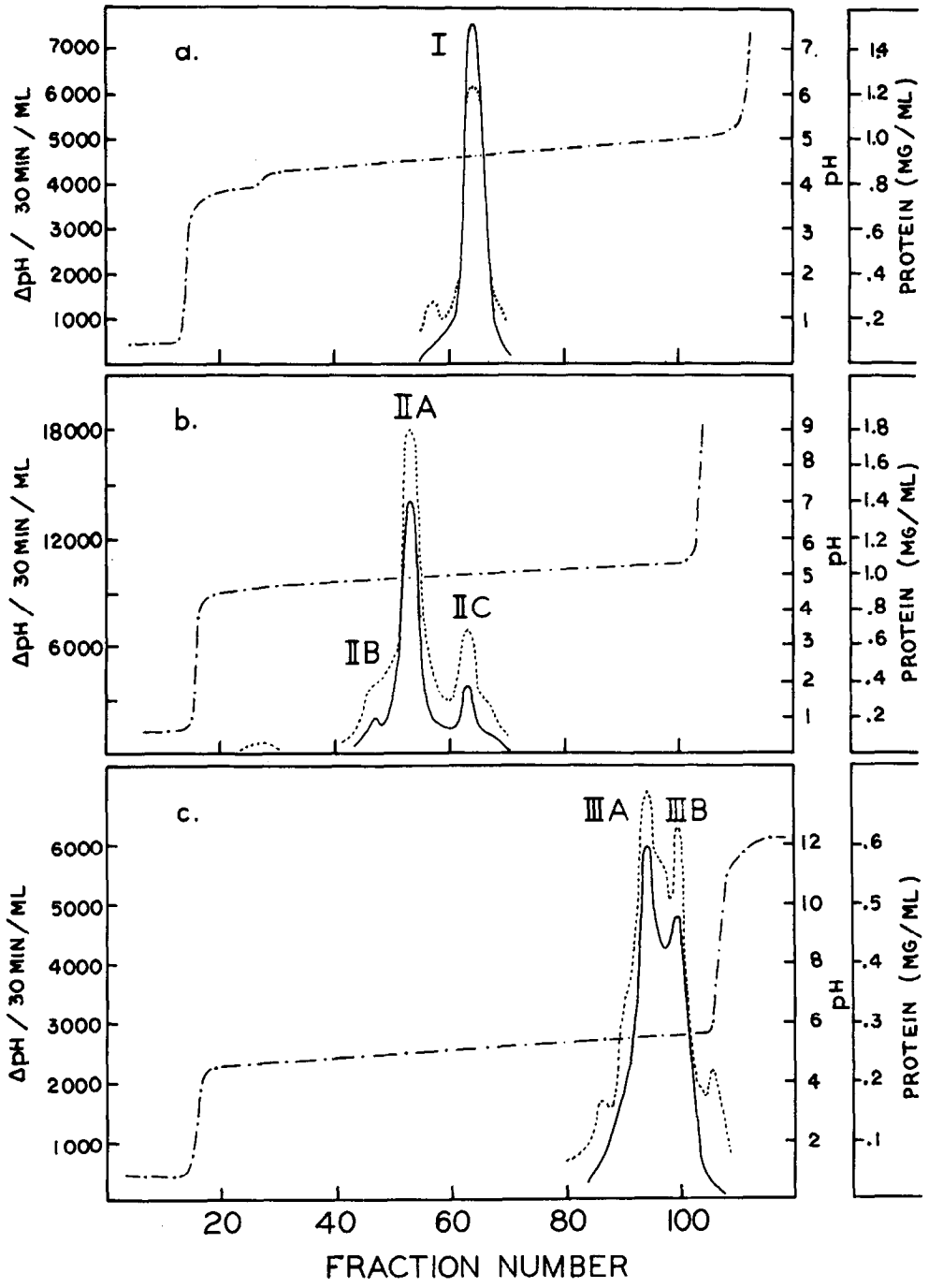

Fig. 1. Separation of different molecular forms of phospholipase A by electrofocusing. Conditions were as in text, except as follows: 1a, pH gradient = 4.2 to 5.2, 227 watt hours, 1000 V for first 40 hours, 1500 V for last 25 hours; 1b, pH gradient = 4.7 to 5.5, 175 watt hours, voltage as above; 1c, pH gradient = 4.8 to 5.8, 526 watt hours, 1000 V for first 60 hours, 1500 V for last 36 hours. Solid line, phospholipase A activity; dotted line, protein; —— ·—— = pH of fraction.

The same 5 major phospholipase components were detected on acrylamide electrophoresis of crude cobra venom or the mixture eluted from Sephadex G-75.

Provisional estimates of the molecular weights of the various forms of phospholipase A was obtained by the elution profile from Sephadex G-100 columns with horse heart cytochrome c, trypsin, and horse liver alcohol dehydrogenase as marker enzymes. Peak I, which is resolved on acrylamide as consisting of 2 constituents of the enzyme, gave also 2 components on Sephadex G-100 with molecular weights of 8,500 and 10,800, respectively, while IIA gave a molecular weight of 20,000 to 22,000. A mixture of IIIA + IIIB gave 3 distinct peaks on Sephadex with molecular weights of 11,250, 14,400, and 15,900. The same multiple components are revealed on careful chromatography of untreated N. naja venom on Sephadex G-100 and hence, are not preparative artifacts. However, refinements of these techniques allow the detection of as many as 9 or 10 catalytically active components.

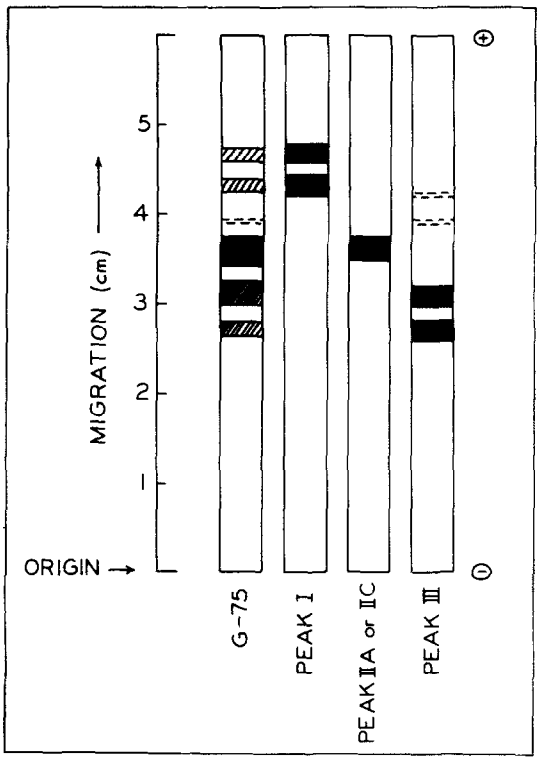

Fig. 2. Polyacrylamide electrophoresis of enzyme fractions. The bands represent amido black stain; G-75 represents the starting material (heated venom, chromatographed on Sephadex G-75 as per Cremona and Kearney, 1964).

In view of the differences in molecular weight, there is some question whether these are isoenzymes in the traditional sense. On ovolecithin or its catalytically hydrogenated form they show little difference in activity per mg of protein and their pH-activity curves are similar. Marked differences in specificity are revealed, however, when their action on membrane systems is examined.

The question whether phospholipase A, rather than some other enzyme in N. naja venom is responsible for the solubilization of respiratory chain-linked flavoproteins was examined with the peak tubes from narrow range electrofocusing runs (Fig. 1), since these appeared to be free from impurities as judged by the coincidence of protein and activity distribution, by acrylamide electrophoresis and gel filtration. The order of effectiveness in liberating soluble choline dehydrogenase from rat liver mitochondrial acetone powder was: IIIA + B >> IIA >> I. For the solubilization of NADH dehydrogenase from ETP the order was III A + B > IIA > IIC = I. For α-glycerophosphate dehydrogenase solubilization from pig brain mitochondria the order was: III A + B ≥ IIA > IIC > I. The order of effectiveness in producing inhibitors of NADH oxidase (Salach et al., 1967) was: IIA > IIIA + B > IIC = I. It seems clear, therefore, that phospholipases A are the enzymes solely responsible for the solubilization of mitochondrial flavoproteins by snake venom and for the interruption of electron transport in the NADH oxidase chain. Contrary conclusions (King, 1966) may be traced to the facts: 1) that DEAE cellulose is unsuitable for the separation of the various forms of phospholipase A, 2) that the form of the enzyme recovered from DEAE, while responsible for most of the activity of the venom on pure phospholipids, accounts for only a minor part of its activity in solubilizing NADH dehydrogenase, and 3) that there is no simple linear relation between enzyme concentration and solubilizing activity.

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