CONDUCTIMETRIC ASSAY OF PHOSPHOLIPIDS AND PHOSPHOLIPASE A

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1. Introduction

Direct measurement of chemical change during enzyme-catalysed hydrolysis is, in principle, an attractive method for qualitative and quantitative analysis of phosphatidyl phospholipids. Phospholipase type A_2 (EC 3.1.1.4) of high activity and broad specificity is available from bee venom, the source used in this work, and from mammalian pancreas. We have developed a rapid conductimetric assay which has a detection level below 10 μg of phospholipid (1–2 μg P_i) and below 10 ng of purified enzyme.

The reaction:

lecithin → lysolecithin + fatty acid anion $^- + H^+$

has been followed by titration [1] or by turbidity change in protein-rich solution [2], but neither of these methods has been adapted for routine estimation of phospholipids. The conductimetric method [3, 4] requires minimum sample preparation and the apparatus is simple and readily adapted for routine large-scale determinations.

Bee venom enzyme has highest activity with substrates in true solution [1], and for naturally occurring phospholipids this is obtained in dilute aqueous solutions of organic solvents; however, the intrinsic activity of the enzyme is decreased by organic solvents and optimum rates are given in 12–20% n-propanol : water mixtures. In addition activity is increased 5–10-fold by 0.1 mM Ca^{2+} but abolished by excess EDTA [1].

2. Materials and methods

Phospholipids were purified from egg yolk and from soya bean extract by the methods of Ansell and Hawthorne [5]. Purified bee venom phospholipase A_2 [1] was a gift from Dr. Shipolini, dihexanoyl lecithin was a gift from Dr. Cottrel (both of University College London), bee venom was from Sigma Chemical Co., Ltd. Enzymes were dialysed against distilled water and stored at 0°. The apparatus [3, 4] consists of an open type of conductivity cell, an LKB conductolyser, or a conductivity meter with linear response [6], and a recorder set to give full-scale deflection for a 5–10% conductance change.

Triethanolamine buffer, 100 mM, pH 8.0 was prepared from the free base by addition of isobutyric acid. The final reaction solution contained 10 mM buffer in 20% v/v n-propanol solution and was either calcium-free or contained 0.1 mM Ca^{2+} (chloride or gluconate). Organic solvent extracts of phospholipids were dried in a stream of nitrogen, taken up with 1 ml of reaction solution and transferred to the cell. Purified phospholipids were dissolved in 50% v/v n-propanol solution and small volumes added to reaction solution in the cell. Solutions were incubated at 25° and enzyme added by microsyringe; conductance changes were recorded and a correction for enzyme addition determined, where necessary, by further addition of enzyme at the end of reaction.

3. Calibration and results

The conductance change due to hydrolysis of neutral phospholipids may be exactly reproduced by addition of fatty acid to buffer solution; for charged phospholipids there is an additional effect (less than 10%) due to increased mobility of the parent molecule

Fatty acid anions may bind positively charged com-

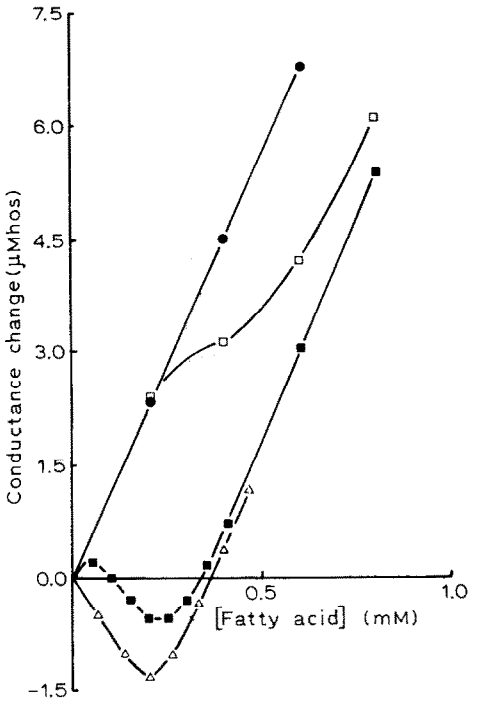

Fig. 1. Conductance change for addition of fatty acids to standard substrate solution (10 mM triethanolamine/isobutyrate buffer pH 8.2 in 20% v/v n-propanol/water) containing 0.1 mM $CaCl_2$ at 25°. •, Oleic acid; □, myristic acid; ■, palmitic acid; △, stearic acid.

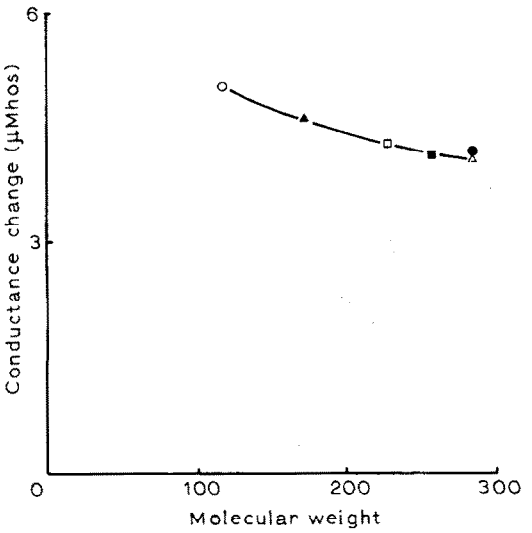

Fig. 2. Conductance change as a function of molecular weight for the addition of fatty acid (final conc. 0.4 mM) to calcium-free substrate solution. ○, Hexanoic acid; ▲, decanoic acid; □, myristic acid; ■, palmitic acid; △, stearic acid; •, oleic acid.

pounds, particularly where additional stability is conferred by side-chain interactions, and binding is affected in a complex way by organic solvents. Briefly, addition of solvent decreases side-chain interactions but increases coulombic interactions and weakens Ca^{2+} binding, although it may potentiate buffer ion binding. Of the totally water-miscible solvents tested, n-propanol was most effective in decreasing Ca^{2+} binding, and of the positively charged buffers tested, triethanolamine showed no measurable interaction with fatty acid anions in aqueous n-propanol solutions (cf. Tris and imidazole). Negatively charged buffers, which would not interact, give far smaller conductance changes [4].

Calibration in calcium-free reaction solution is limited only by solubility, and saturated acids up to, and including, behenate and probably all unsaturated acids can be determined. Calibration of reaction solution containing 0.1 mM Ca^{2+} demonstrates binding by higher saturated acid anions (fig. 1). The conductance changes obtained in the absence of Ca^{2+} binding, for the common fatty acid substituents of phospholipids show a small variation (ca. ± 3%) (fig. 2), and this defines the limit of accuracy of the determination for uncharacterised samples.

Most accurate results are obtained in calcium-free solution, but the faster reaction rates in the presence of calcium are generally preferred. Fig. 3 shows time courses for hydrolysis of purified soya bean lecithin and demonstrates linearity of the assay over the range 50–500 μg of substrate; range may be extended below 10 μg by use of 1 mM buffer. Similar results have been obtained with purified egg lecithin, a phospholipid extract from tissue culture cells, and a sample of phosphatidyl serine. Of all the samples investigated, only crude soya bean lecithin appeared to release higher saturated acids during hydrolysis (shown by a falling portion, or an inflexion in the time course (fig.4), but the amount, less than 5% of the total, could not be accurately determined by comparison assays in calcium-containing and calcium-free solution.

Fig. 5 shows the effect of enzyme concentration on initial hydrolysis rate with both soya bean leci-

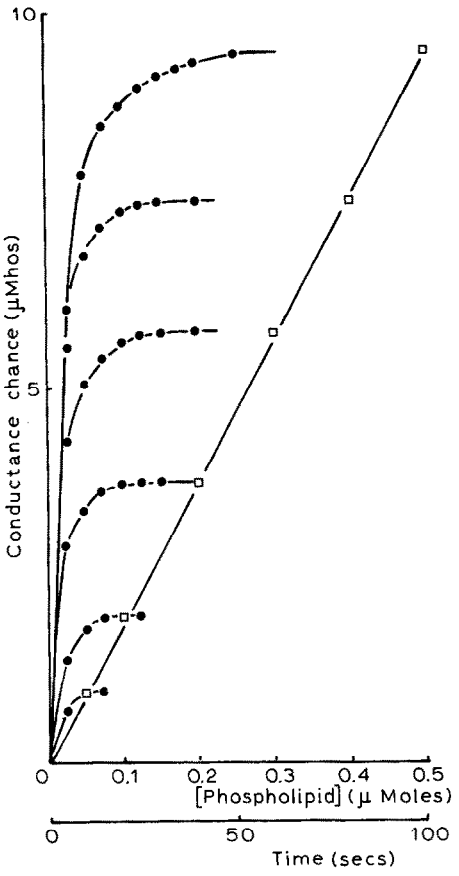

Fig. 3. Hydrolysis of purified soya bean lecithin by 10 μg dry weight of bee venom phospholipase A, in standard substrate solution containing 0.1 mM $CaCl_2$ at 25°. •, Reaction curves; □, conductance change for total hydrolysis.

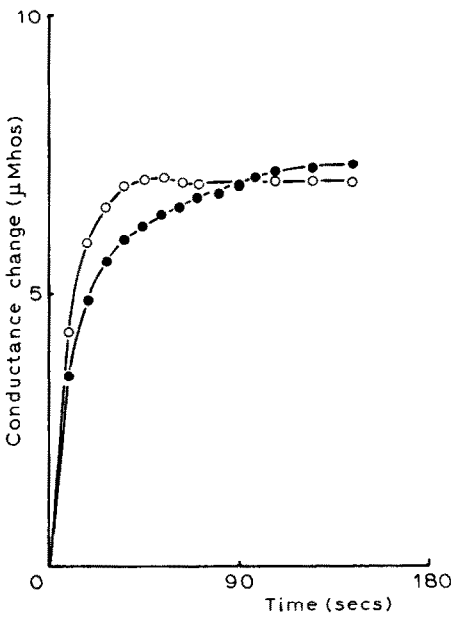

Fig. 4. Hydrolysis of soya bean lecithin by 2 μg dry weight of bee venom phospholipase A using standard conditions: ○ in the presence, and • in the absence, of 0.1 mM $CaCl_2$.

thin and with dihexanoyl lecithin in pure aqueous solution. The specific activity of the enzyme depends upon the substrate concentration; but for 1 mM substrate the specific activity was 3 mmoles × min^{-1} × mg^{-1} for soya bean lecithin and 1.5 mmoles × min^{-1} × mg^{-1} for dihexanoyl-lecithin.

4. Discussion

The conductimetric method provides a rapid and sensitive assay for phospholipase A (either A_1 or A_2) activity and, in addition, is a good kinetic method for use at low salt concentration. If stability of an enzyme sample in n-propanol solution is not established the assay with dihexanoyl lecithin is preferred. Many of the kinetic properties of the bee venom enzyme are not completely elucidated and are under study. Those relevant to the assay system are: the large negative temperature coefficient for reaction in n-propanol solutions, an initial lag phase for some purified substrates (e.g. dimyristoyl lecithin) and the exact nature of the requirement for Ca^{2+}.

This method provides a more specific and convenient assay for phospholipids than does phosphate determination and is of comparable accuracy for unknown samples, especially as lyso-compounds do not interfere.

Use might be made of the differences in calcium binding, among the higher saturated acids, to determine the concentration of an individual acid or group of acids by two hydrolyses under selected conditions.

The enzyme from pancreas requires high salt concentration for full activity [7] and may be less suitable for this type of assay than the bee venom enzyme. Finally there is no evidence of hydrolytic enzymes in

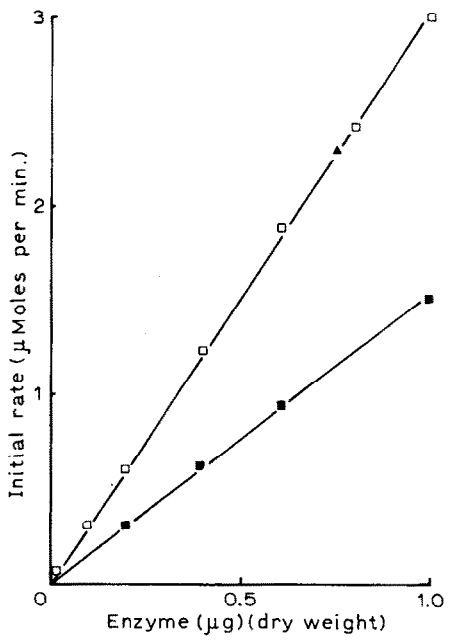

Fig. 5. Initial rates of hydrolysis as a function of enzyme concentration. □, for 1 mM purified soya bean lecithin with purified bee venom enzyme in 1 ml of standard substrate solution containing 0.1 mM $CaCl_2$ at 25°, and ▲ using crude bee venom (5 μg, dry weight). ■ for 1 mM dihexanoyl lecithin with purified bee venom enzyme in 1 ml of 10 mM triethanolamine/isobutyrate buffer pH 8.2, 0.1 mM $CaCl_2$ in water at 25°.

crude bee venom which might interfere with the determination and consequently no advantage in the use of highly purified enzyme.

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