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Phospholipase C from *Bacillus cereus*. Action on Some Artificial Lecithins

CLIVE LITTLE

Institute of Medical Biology, University of Tromsø, P.O. Box 977, N-9001 Tromsø, Norway

The hydrolysis by phospholipase C from *B. cereus* of several lecithins of different fatty acyl chain length was examined. The enzyme showed significant activity towards monomolecularly dispersed short chain lecithins and the reaction obeyed normal Michaelis-Menten kinetics. Rate *vs.* substrate concentration curves obtained with dihexanoyl-, diheptanoyl- and dioctanoyllecithins showed marked discontinuities in the region of the known critical micelle concentrations for these substrates and distinctly higher rates were obtained just above these levels. Using these three lecithins at levels below their respective critical micelle concentrations, rate increases were noted if the reactions were allowed to proceed to a sufficiently great extent. The presence of deoxycholate in the reaction system had little or no effect on the rate of enzyme-catalysed hydrolysis of lecithins of fatty acyl chain length $\leq C_8$, but for fatty acyl chain lengths $\geq C_{10}$, significant rate increases occurred. The pH profile for the enzyme activity was also examined.

Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* catalyses the hydrolysis of appropriate phospholipids at the phosphate moiety. Recently, studies have been carried out on the structure of the enzyme and on its active site.[1–3] As a part of a further investigation into the enzyme and its mechanism of action it was decided to try to obtain some simple kinetic data on the reaction. Normal substrates for phospholipase C are insoluble phospholipid structures and the use of such substrates would greatly complicate kinetic studies. Several synthetic lecithins with fairly short fatty acyl chains can be prepared and these, under certain conditions, exist in a monomolecularly dispersed form and as such are more suitable for use in kinetic investigations. A further point of interest is that whereas these lecithins are water-soluble at low concentrations, most of them are water-insoluble at high concentrations and form micelles. With phospholipase A_2 [4] and pancreatic lipase,[5] enzyme activity towards micellar substrates is considerably greater than towards monomolecularly dispersed (monomeric) substrates. With pancreatic lysophospholipase, however, there seems to be no preference for micelles over monomers.[6] Regarding the relative behaviour of phospholipase C towards micelles and monomers, no reports have been published apart from a very brief reference to a micelle effect by Zwaal and Roelofsen.[7] The present paper examines this micelle effect in more detail. In addition, some simple kinetic studies are reported together with the effect of pH on the reaction rate with a water-soluble lecithin substrate.

EXPERIMENTAL

Phospholipase C was prepared from the culture supernatant of *Bacillus cereus* by the method of Little *et al.*[8] with the small modification described.[2] *sn*-Glycero-3-phosphorylcholine (GPC) was prepared as the $CdCl_2$ adduct by methanolysis of 90 % egg lecithin (BDH).[9] Free GPC was obtained by allowing an aqueous solution of the $CdCl_2$ adduct to percolate through a column of mixed bed ion exchange resin consisting of Amberlites IRC-50 and IR-45. The eluent from the column was lyophilized and stored over P_2O_5.

Fatty acid anhydrides other than butanoic anhydride were synthesized [10] and recrystallized from acetone using low temperatures

where necessary. Butanoic anhydride was purchased from Fluka and fatty acids were obtained from Sigma.

Substrates. The various lecithins, *O*-(1,2-diacyl-*sn*-glycero-3-phosphoryl)choline, (3-*sn*-phosphatidylcholine) were prepared using free GPC with the molten anhydride constituting the reaction medium.[11] Ratios of GPC:fatty acid salt:fatty acid anhydride of 1:2:10, by weight, were used. Potassium salts of fatty acids were used except for the synthesis of dibutanoyllecithin, where the tetraethylammonium salt was employed. The various lecithins were purified from the reaction mixtures by chromatography on silicic acid (Mallincrodt CC-7) using established procedures. Lecithins of fatty acyl chain length $\leqslant C_7$ were further purified by chromatography on neutral alumina (Biorad AG-7). All lecithins used appeared as single spots when analysed by thin layer chromatography on silica gel H.

Chromatograms were developed using chloroform-methanol-acetic acid-water (65:25:10:4, by vol.) and visualized both by iodine vapour and 2,7-dichlorofluorescein. In addition, when the lecithins were incubated with phospholipase C, the enzyme eventually released > 95 % (typically 97 – 98 %) of the theoretically available acid when the latter was titrated with NaOH to pH 7.5.

Enzyme assays were carried out at 22 – 23 °C on Radiometer pH stat equipment. Except where the pH effect was studied, the end point was pH 7.5. 0.02 M NaOH was used as titrant and prepared by diluting a standard solution of the base (Titrisol, Merck). Under the conditions used, the nonenzymic rates were negligible.

Lecithins were added to a solution of NaCl (0.15 M) and subject to 4 – 6 one minute bursts of sonication whilst being maintained on ice. The suspensions of didodecanoyl- and ditetradecanoyllecithin in 0.15 M NaCl were distinctly turbid even after prolonged sonication. When the effect of deoxycholate was studied, the detergent was added after sonication.

RESULTS

1. *Hydrolysis of lecithins in solution.* In order to carry out a simple investigation of the kinetics of phospholipase C action, the enzyme activity was measured against lecithins with fairly short fatty acyl chains at concentrations where pure solutions are believed to occur. Relatively high rates of hydrolysis occurred especially with dihexanoyllecithin and it was found that the reactions followed normal Michaelis Menten kinetics. Thus, the reaction was first order in enzyme concentration and showed a tendency towards saturation at high substrate levels (see, for example, Fig. 1). From the data in Fig. 1, K_m values for phospholipase C of 37 mM with dibutanoyllecithin as substrate and 0.4 mM with dihexanoyllecithin were calculated. Maximum velocities, expressed as maximum molecular activities were

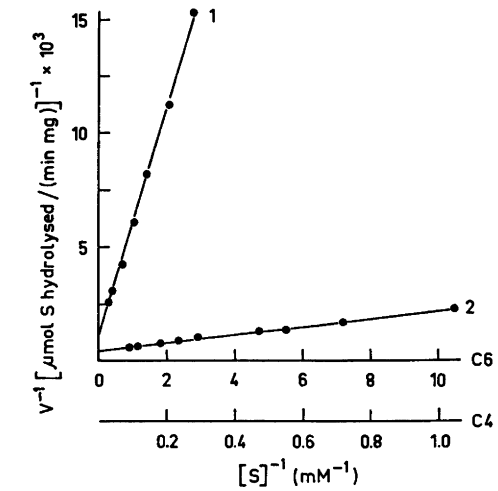

Fig. 1. Lineweaver-Burk plots showing the effect of substrate concentration on the rate of phospholipase C catalysed hydrolysis of dibutanoyllecithin (*1*) and dihexanoyllecithin (*2*). The lower of the [S]$^{-1}$ scales (0 – 1.0 mM^{-1}) refers to the reaction with dibutanoyllecithin.

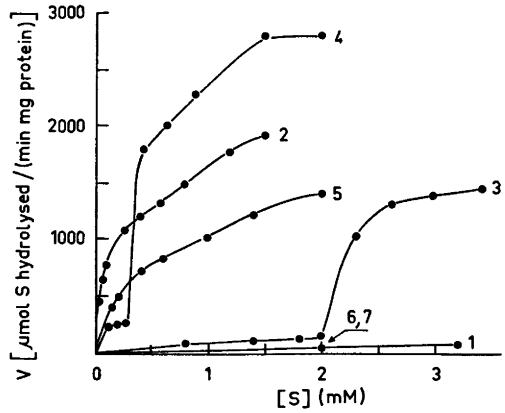

Fig. 2. Effect of substrate concentration on the rate of phospholipase C catalysed hydrolysis of different lecithins. *1*, dibutanoyllecithin; *2*, dihexanoyllecithin; *3*, diheptanoyllecithin; *4*, dioctanoyllecithin; *5*, didecanoyllecithin; *6*, didodecanoyllecithin; *7*, ditetradecanoyllecithin.

calculated to be 17 000 mol of dibutanoyllecithin hydrolysed/(min mol enzyme) and 50 000 mol of dihexanoyllecithin hydrolysed/(min mol enzyme).

2. *Hydrolysis of lecithin micelles.* In an attempt to study the possible micelle effect with phospholipase C, the activity towards a range of lecithins of different fatty acyl chain length was examined. The reaction rates were measured at different substrate concentrations up to about 3 mM (Fig. 2). In the absence of detergents, extremely low rates of reaction were obtained with didodecanoyl- and ditetradecanoyllecithins, whereas high rates occurred with dihexanoyl-, diheptanoyl-, dioctanoyl- and didecanoyllecithins. An interesting feature of Fig. 2 is the discontinuities obtained in the reaction rate *vs.* substrate concentration curves for diheptanoyl- and dioctanoyllecithins. The discontinuities occurred around 2 mM for the former substrate and around 0.3 mM for the latter, values which correspond well with the solubility limits for these lecithins.[6,4] Above these limits micelle formation occurs and it appears that phospholipase C from *B. cereus* has a significantly higher activity towards lecithin micelles than it does towards the corresponding monomers. Further support for this view was obtained by using dihexanoyllecithin as substrate at concentrations around its reported critical micelle concentration. Fig. 3 shows

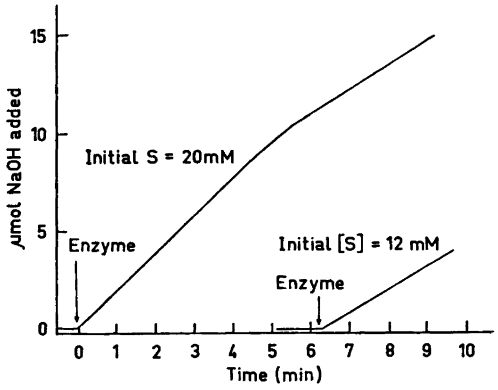

Fig. 3. Hydrolysis of dihexanoyllecithin. The figure shows the trace obtained from the pH stat recorder when enzyme was added to an initial volume of 5 ml of dihexanoyllecithin at pH 7.5. The initial substrate concentration was 20 mM or 12 mM as indicated in the figure.

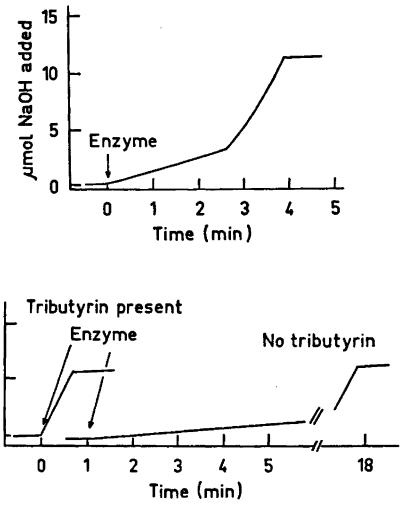

Fig. 4. Time course of hydrolysis of dioctanoyllecithin. Reactions were carried out in a volume of 40 ml at pH 7.5. pH stat recorder traces are shown. A: Hydrolysis of substrate at 0.28 mM initial concentration. B: Effect of tributyrin. The initial substrate concentration was 0.15 mM. A freshly sonicated dispersion of tributyrin to a final concentration of 1 mM was added to one sample just before addition of enzyme. The other sample of substrate received the same amount of enzyme but no tributyrin.

the results of an experiment in which the enzyme was incubated with 20 mM dihexanoyllecithin. It can be seen that initially a constant rate of reaction occurred. Later, the rate changed fairly suddenly and then the reaction continued at a constant but reduced rate. The remaining substrate concentration at which this rate change occurred was calculated after correcting for dilution as 13.3 mM, a value extremely close to the reported critical micelle concentration of 13.8 mM for this lecithin.[4] The reduced rate occurring after the rate change was equal to the initial rate obtained using a 12 mM solution of this lecithin. Consequently, it is unlikely that the rate change arose from enzyme inactivation or product inhibition. During the present work, another unusual rate effect was observed. Using concentrations of diheptanoyl- and dioctanoyllecithin below the critical micelle concentration resulted in relatively low initial rates. However, if the reaction was allowed to proceed further, marked increases in rate even-

Table 1. Effect of deoxycholate on the rate of hydrolysis of different lecithins. The activity of phospholipase C towards various lecithins in 0.15 M NaCl was measured in the presence and absence of 0.25 % (w/v) sodium deoxycholate at pH 7.5 and 22–23 °C. Substrate concentration, 2 mM.

Lecithin	Enzyme activity μmol S hydrolysed/(min mg enzyme)	
	−Deoxycholate	+Deoxycholate
Dihexanoyl-	1900	1350
Diheptanoyl-	150	150
Dioctanoyl-	2800	2200
Didecanoyl-	1400	2500
Didodecanoyl-	< 5	4800
Ditetradecanoyl-	55	5300

tually occurred and these elevated rates remained until the reaction stopped fairly abruptly at a point corresponding to total hydrolysis of the substrate (Fig. 4 A). The beginning of these rate increases coincided well with the onset of turbidity in the reaction mixture (results not shown). These rate increases could also be initiated if a sonicated suspension of tributyrin (1,2,3-tributanoylglycerol) was added to the reaction mixture. A further observation was that these elevated rates obtained below the critical micelle concentration were of a magnitude comparable with those obtained above the critical micelle concentration. These effects were most marked with deheptanoyl- and dioctanoyllecithins, but also occurred to a lesser extent with dihexanoyllecithin. No other lecithin used showed this behaviour. These rate increases occurring during the course of the reaction were abolished if 0.25 % deoxycholate was present in the reaction mixture. The presence of this detergent also had a marked effect on the initial rates obtained with some of the lecithins. Table 1 shows that with didodecanoyl- and ditetradecanoyllecithins, very large rate enhancements occurred when 0.25 % deoxycholate was present. A much smaller rate increase was obtained with didecanoyllecithin and the presence of the detergent had little or no effect on the rates occurring with lecithins of shorter fatty acyl chain length.

3. *Effect of pH on the rate of hydrolysis of a soluble lecithin.* Otnaess *et al.*[12] studied the effect of pH on the activity of phospholipase C towards lecithin micelles. A somewhat unusual pH profile occurred in which two peaks of enzyme activity, one around pH 6.5 and another around pH 8.5 were observed. To examine whether this double pH optimum also occurred with a monomolecularly dispersed substrate, the effect of pH on the rate of hydrolysis of a 5 mM solution of dihexanoyllecithin was examined. The results in Fig. 5 illustrate that in the pH range 5.5–10.0, the enzyme shows a single peak of activity around

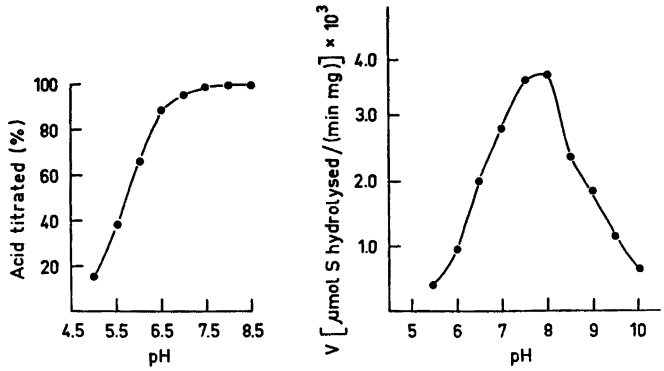

Fig. 5. Effect of pH on enzyme activity. The rate of hydrolysis of a 5 mM solution of dihexanoyllecithin was examined at different pH's by setting the end point on the pH stat to different pH values and measuring the rate of reaction. A: Titration efficiency at different pH values for phosphorylcholine in 0.15 mM NaCl. B: pH profile of phospholipase C activity. Activities are expressed as specific activities corrected for titration efficiency.

pH 7.5 – 8.0. This fits fairly well with the more alkaline of the two peaks reported by Otnaess *et al.*[12] With the soluble substrate there is clearly no evidence for an activity peak around pH 6.5.

DISCUSSION

It is apparent from the present results that phospholipase C from *B. cereus* shows relatively high rates of reaction with both monomolecularly dispersed and micellar substrates. Regarding the activity with monomolecularly dispersed substrates, the ratio of V_m/K_m for dihexanoyllecithin to the V_m/K_m value for dibutanoyllecithin is 303 under the reaction conditions used. Thus, the former lecithin is a clearly superior substrate to the latter. However, in the concentration range up to 2 mM, diheptanoyllecithin gave reaction rates which, although significantly greater than those obtained with dibutanoyllecithin, were considerably smaller than those shown with dihexanoyllecithin. It would therefore appear that there is no simple relationship between the length of the fatty acyl chain and the ability of a lecithin to serve as a substrate in the soluble form.

With three different lecithins, clearly defined discontinuities in the rate-substrate concentration curves were noted in the region of the reported respective critical micelle concentrations. The results strongly suggest that phospholipase C from *B. cereus* like pancreatic phospholipase A_2[4] and pancreatic lipase[5] shows a clearcut preference for a substrate in the micellar form over the same substrate in the monomolecularly dispersed or soluble form. In view of the apparent preference of the enzyme for micelles, the reaction rates obtained with solutions of dihexanoyllecithin are remarkably high. In fact, the enzyme shows a higher activity towards a 1.5 mM solution of this substrate than it does in the "standard" assay system for this enzyme which uses micelles of crude egg lecithin with detergent.[13,8]

Using solutions of dihexanoyl-, deheptanoyl- or dioctanoyllecithin, a pronounced rate increase was obtained during the reaction if the latter was allowed to proceed far enough. The reason for this behaviour is not completely clear, but three pieces of information may be pertinent: (a) the onset of the rate increase coincided with the onset of turbidity in the reaction mixture, (b) the stimulation could be induced by adding to the reaction system a sonicated dispersion of tributyrin, (c) the stimulation led to rates that were of a similar order to those occurring at lecithin levels above the critical micelle concentration. Turbidity in the present assay system arises from the aggregation of diglyceride produced during the hydrolysis and the present observations are compatible with the view that the monomolecularly dispersed lecithin molecules adsorb to the diglyceride aggregates to produce a type of micellar structure. The rate increase would then simply reflect the ability of micelles to serve as superior substrates. These stimulatory effects were not observed with dibutanoyllecithin, the least hydrophobic of the substrates used, which would be expected to have the smallest tendency to adsorb to diglyceride aggregates. The presence of deoxycholate in the reaction system abolished both the formation of turbidity and the rate increase. Other experiments involving deoxycholate provide further support for the view that the precise physical state of the substrate has a great effect on enzyme activity. This detergent at 0.25 % probably has little direct effect on the enzyme. Thus, with monomolecularly dispersed lecithins, the presence of 0.25 % deoxycholate had little or no effect on reaction rates. With micellar substrates the effect of the detergent on reaction rate was more complex. Deoxycholate (0.25 %) seemed to have a slight inhibitory effect on the rate of hydrolysis of dioctanoyllecithin, almost doubled the rate with didecanoyllecithin, increased the rate with ditetradecanoyllecithin some 100-fold and increased the rate with didodecanoyllecithin by around 1000-fold. With the latter two substrates, however, it was not possible to obtain optically clear dispersions in 0.15 M NaCl simply by ultrasonication. The enormous effects of the detergent may in these cases be due, in part at least, to the formation of superior dispersions. The pH profile of phospholipase C was also examined. Whereas Otnæss *et al.*[12] found two peaks of activity (at pH 6.5 and at pH 8.5) when using lecithin micelles, the present work with a soluble lecithin indicated a single peak of activity around pH 7.5 – 8.0. It is possible that the pH 6.5 peak reported by Otnæss *et al.*[12]

reflects some pH transition in the structure of the particular micelles used rather than an inherent property of the enzyme.

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REFERENCES

1. Little, C. and Otnæss, A. B. *Biochim. Biophys. Acta 391* (1975) 326.
2. Aurebekk, B. and Little, C. *Biochem. J. 161* (1977) 159.
3. Otnæss, A. B., Little, C., Sletton, K., Wallin, R., Johnsen, S. and Flengsrud, R. *Submitted for publication.*
4. De Haas, G. H., Bonsen, P. P. M., Pieterson, W. A. and Van Deenen, L. L. M. *Biochim. Biophys. Acta 239* (1971) 252.
5. Entressangles, B. and Desnuelle, P. *Biochim. Biophys. Acta 159* (1968) 285.
6. De Jong, J. G. N., Dijkman, R. and Van den Bosch, H. *Chem. Phys. Lipids 15* (1975) 125.
7. Zwaal, R. F. A. and Roelofsen, B. *Methods Enzymol. 32 Part B* (1974) 154.
8. Little, C., Aurebekk, B. and Otnæss, A. B. *FEBS Lett. 52* (1975) 175.
9. Chadha, J. S. *Chem. Phys. Lipids 4* (1970) 104.
10. Selinger, Z. and Lapidot, Y. *J. Lipid Res. 7* (1966) 174.
11. Cubero Robles, E. and Van den Berg, D. *Biochim. Biophys. Acta 187* (1969) 520.
12. Otnæss, A. B., Prydz, H., Bjørklid, E. and Berre, Å. *Eur. J. Biochem. 27* (1972) 238.
13. Zwaal, R. F. A., Roelofsen, B., Comfurius, P. and Van Deenen, L. L. M. *Biochim. Biophys. Acta 233* (1971) 474.

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