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CHARACTERIZATION OF PHOSPHOLIPASE C FROM A MARINE PLANKTONIC ALGA *(MONOCHRYSIS LUTHERI)*

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SUMMARY

1. A soluble phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) was obtained from photoauxotrophically grown cells of the marine planktonic chrysomonad *Monochrysis lutheri*. The enzyme catalyzed irreversible hydrolysis of [^{14}C]choline-labelled lecithin to [^{14}C]phosphorylcholine at a rate of approx. 65 mμmoles per min per mg protein in crude extracts.

2. Enzyme activity showed a sharp pH optimum near 5 and a temperature optimum near 60°. Heat treatment for 10 min at 80° inactivated the enzyme preparations.

3. EDTA and α,α'-dipyridyl had no effect on the activity even at unusually high concentrations. *o*-Phenanthroline caused a concentration-dependent inhibition, which was neither reversed by Zn^{2+}, Cu^{2+}, Fe^{2+}, dithiothreitol, nor prevented by prior addition of EDTA or prior complexing with Fe^{2+}.

4. A number of divalent metal ions tested (Fe^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, Cd^{2+}) had no effect on the activity, while Zn^{2+}, Cu^{2+}, Hg^{2+} were inhibitory in increasing order.

5. Among sulfhydryl reagents tested, *p*-chloromercuriphenyl sulfonate and dithiothreitol caused concentration-dependent inhibitions. The enzyme was completely inactivated by 1 mM lauryl sulfate.

6. The activity towards lysolecithin was only 10–12% of that for lecithin.

7. The results indicate a stable, pH-sensitive, heat-stimulated, lecithinase C. with no obvious divalent-metal ion requirement and with sulfhydryl and disulfide groups involved in the expression of activity.

INTRODUCTION

The first observation of an algal phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) was recently made in a marine phytoplankter belonging to the Chrysophyceae, *Monochrysis lutheri* Droop[1]. A study of its properties was undertaken in order to characterize the enzyme and to compare it with analogous

Abbreviations: EDTA, ethylenediamine tetraacetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

enzymes from bacteria implicated in food poisoning[2]. The present communication reports the effects of pH, substrate concentration, heat, metal ions, chelators, sulfhydryl and other reagents on the activity of the enzyme in crude extracts.

MATERIALS AND METHODS

Algal culture

Millport strain No. 60 of *M. lutheri* was obtained from Biological Station, Nanaimo (B.C.), and was photoauxotrophically mass cultured under conditions previously reported[3]. Acetone powders were prepared from the vacuum-dried (with P_2O_5) algal cells by a previously described method[4]. A 10-l mass culture yielded 1.7 g dry cells, of which 500 mg afforded 356–360 mg acetone powder.

Enzyme extracts

Algal acetone powder (25–50 mg) was gently shaken with Na_2HPO_4–NaH_2PO_4 buffer (0.05 M, pH 7.5) (2 ml) at 0–4° for 16 h, the suspension was centrifuged (approx. 22 000 × *g*) for 1 h, and the clear supernate was adjusted to a fixed volume (2 ml) with the same buffer. Extracts prepared from 25 mg acetone powder normally contained 1.9–2.0 mg protein per ml. Protein was determined by the method of LOWRY *et al.*[5], using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and rabbit-muscle aldolase (Sigma) as standards.

Preparation of ^{14}C-labelled substrates

Lecithin, labelled with ^{14}C in the choline moiety, was prepared by administering [*Me*-^{14}C]choline (New England Nuclear Corp., Boston, Mass.) to live rainbow trout[6]; it was purified from the total phospholipid by chromatography on aluminium oxide and silicic acid[7] and the purity was confirmed by thin-layer chromatography with chloroform–methanol–water (65:25:4, by vol.)[8]. The composition of lecithin, determined by the methods described by HANAHAN, DITTMER AND WARASHIMA[9], was as follows: P, 3.8% and P/choline/fatty acid molar ratio of 1:0.96:1.94. This lecithin was further washed with EDTA by the procedure of MAGEE *et al.*[10] when used as substrate in the experiments on the effect of metal ions on enzyme activity. Lysolecithin, labelled with ^{14}C in the choline moiety, was prepared by the action of *Crotalus adamanteus* snake venom (Sigma) on [^{14}C]lecithin, followed by silicic acid chromatographic isolation[11] and its purity was confirmed by thin-layer chromatography. The specific activity of both substrates was 13 500 counts/min per μmole. Before use, aqueous suspensions of substrate were prepared by vortex-mixing for 2 min.

Enzyme activity determinations

Phospholipase-C activity was determined by estimating the amount of [^{14}C]-phosphorylcholine released from ^{14}C-labelled lecithin or lysolecithin, using a modification of the radioactive tracer method of BILINSKI AND JONAS[6].

Unless otherwise stated, incubations were conducted in 0.1 M buffer solution at 35° using 0.2 mg enzyme-extract protein in a total volume of 1.0 ml. Incubations were terminated by adding serum albumin and trichloroacetic acid, and extracting with diethyl ether as previously described[6]. Aliquots of the aqueous phase were subjected to acid hydrolysis under the following conditions: (A) 1 M HCl at 100° for 20

min; (B) 2 M HCl at 125° for 24 h in sealed tubes. After hydrolysis, free choline was isolated as reineckate and its radioactivity was determined by plate counting[6]. The increase in radioactivity of choline reineckate from hydrolysis Condition B over that from A was taken to represent phosphorylcholine, as this compound is not hydrolysed to any significant extent by the mild Condition A[12] but is completely cleaved under the Condition B[13,14]. The validity of the hydrolytic procedure was confirmed with isolated [^{14}C]phosphorylcholine. Paper chromatography of the water-soluble products of hydrolysis had previously shown over 95% conversion of lecithin to phosphorylcholine from the activity of *M. lutheri*[1].

RESULTS

As previously observed with the acetone powder[1], the algal extracts showed almost exclusive hydrolysis of lecithin to phosphorylcholine; no significant formation of free choline or glycerylphosphorylcholine was detected. The hydrolysis reaction showed linear rate (zero order kinetics) up to at least 20 min incubation with the

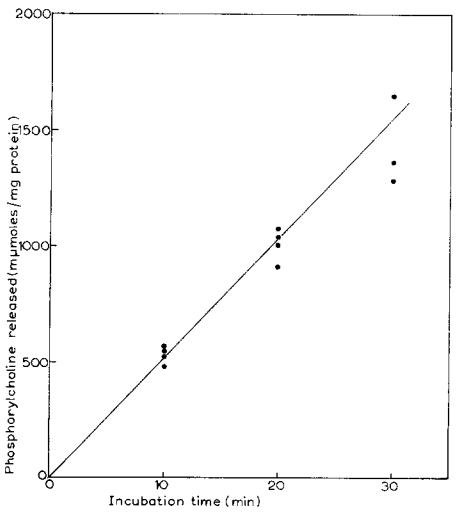


Fig. 1. Effect of incubation time on phospholipase-C activity. Incubation in 0.1 M sodium citrate buffer (pH 5.2) with 0.65 mM [^{14}C]lecithin.

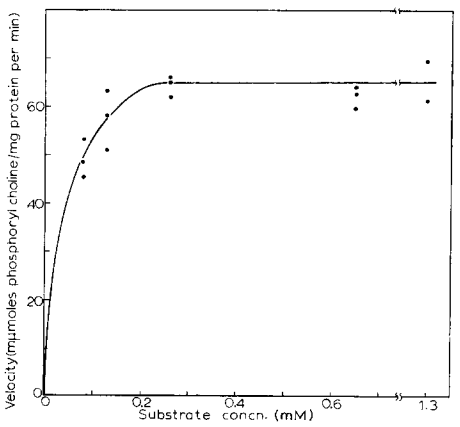
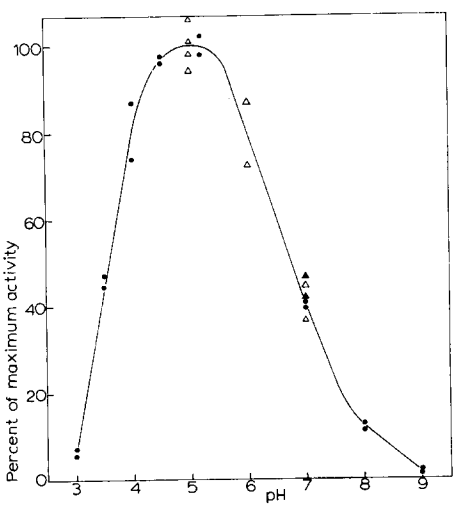


Fig. 2. Effect of substrate concentration on phospholipase-C activity. Incubation in 0.1 M sodium citrate buffer (pH 5.2) for 5 min with [^{14}C]lecithin.

appropriate extract concentration (Fig. 1). Determinations made with 0.2 mg extract protein per ml of incubation mixture gave lecithin conversion rates of the order of 65 mμmoles per min per mg protein at saturating substrate concentrations (Fig. 2). Studies of the effect of substrate concentration at pH 5.2 indicated a low K_m value estimated to be in the range of 100–10 μM (Fig. 2). The hydrolysis reaction appeared to be irreversible since complete conversion of lecithin to phosphorylcholine was obtained in all cases of sufficiently long incubation.

Studies of the effect of pH on the activity indicated a narrow maximum near



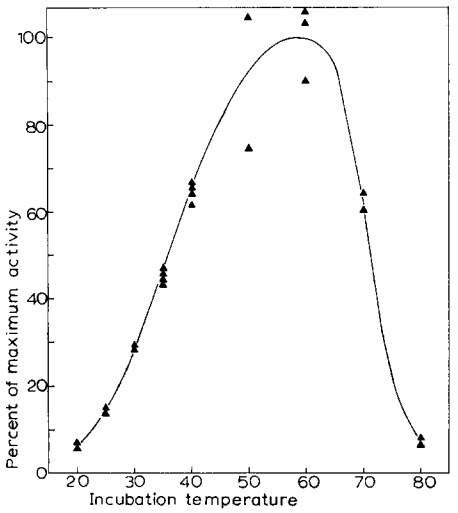


Fig. 3. Effect of pH on phospholipase-C activity. Incubation for 20 min with 0.65 mM [^{14}C]-lecithin. The following buffers were used at a final concentration of 0.1 M. ●, sodium citrate (pH 3.0–5.2); △, potassium MES (pH 5.0–7.0); ▲, sodium potassium phosphate (pH 7.0); ○, potassium Tricine (pH 7.0–9.0). All pH values refer to the incubation temperature (35°).

Fig. 4. Effect of incubation temperature on phospholipase-C activity. Incubation in 0.1 M sodium potassium phosphate buffer (pH 7.0–7.1 at 20–80°) for 20 min with 0.65 mM [^{14}C]lecithin at the temperatures shown.

pH 5 (Fig. 3). The pH dependence of the activity appears to be independent of the nature of the buffer used, since 4 buffers examined (including the zwitter-ionic buffers 2-(*N*-morpholino)ethanesulfonic acid (MES) and *N*-tris(hydroxymethyl)methylglycine (Tricine) of Good *et al.*[15]) gave results in close agreement (Fig. 3).

The enzyme reaction showed strong heat activation reaching a maximum near 60° and declining rapidly with higher temperatures (Fig. 4). An examination of the heat stability of the enzyme by a 10-min treatment at 80° in buffers of pH 3.5–8.0 before incubation with substrate showed over 95% loss of activity.

Neither of the chelators EDTA or α,α'-dipyridyl had any significant effect on the enzyme activity; this was confirmed by using unusually high concentrations of these reagents and prolonged periods of interaction with them (Table I). *o*-Phenanthroline, however, caused significant inhibition (15%) at 5 mM concentration which was increased to 40% on doubling the concentration; moreover, this degree of inhibition appeared to be unaffected by pH range 5–7 and by prolongation of the period of interaction (10–60 min). The *o*-phenanthroline (10 mM) inhibition was not reversed by Zn^{2+} (2.5–20 mM), Cu^{2+} (2.5 mM), Fe^{2+} (2.5 mM), dithiothreitol (1 mM), nor was it prevented by prior treatment of enzyme with EDTA (10 mM) or by prior complexing of the reagent with Fe^{2+} (3.3 mM). Surprisingly, the subsequent addition of Zn^{2+} enhanced this inhibition, which effect was found to increase with the metal ion concentration. None of the divalent metal ions tested alone gave any stimulation of the enzyme activity. Ca^{2+}, Mg^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+} caused little change, while comparable concentrations of Zn^{2+}, Cu^{2+}, and Hg^{2+} effected inhibitions of the order of 30, 80 and 100% respectively (Table II).

TABLE I

EFFECT OF VARIOUS REAGENTS ON PHOSPHOLIPASE-C ACTIVITY

Enzyme extract was preincubated with each reagent in sodium potassium phosphate buffer (pH 7.0) for 60 min (45 min at room temperature and 15 min at 35°). Incubations for activity determinations were effected in the same buffer for 20 min with 0.65 mM [^{14}C]lecithin.

Reagent	*Concn. (mM)*	*Enzyme activity (percentage of control)*
EDTA	1–50	98 ± 5*
EDTA	100	92, 84**
o-Phenanthroline	5	85, 86
o-Phenanthroline	10	59, 60
α, α'-Dipyridyl	10	98, 100
Sodium laurylsulfate	1	0, 0
p-Chloromercuriphenyl sulfonate	1	79, 72
p-Chloromercuriphenyl sulfonate	10	27, 33
Dithiobis-(2-nitrobenzoic acid)	1	77, 99
Dithiobis-(2-nitrobenzoic acid)	10	88, 108
Iodoacetate	1	89, 103
Iodoacetamide	1	111, 121
Iodoacetamide	10	92
N-Ethylmaleimide	1	104
Dithiothreitol	1	97, 97
Dithiothreitol	10	63, 63
Dithiothreitol	50	64, 46

* Mean value and standard deviation from 9 determinations. Preincubation for 15–120 min with 1 mM EDTA.

** Results from duplicate determinations.

Among other reagents tested, lauryl- (dodecyl-)sulfate caused complete inhibition at low concentration (Table I). The alkylating agents iodoacetate, iodoacetamide, *N*-ethylmaleimide, as well as the disulfide–sulfhydryl exchange reagent 5,5′-dithiobis-(2-nitrobenzoic acid)[16] did not show any significant effect. The organo-

TABLE II

EFFECT OF DIVALENT METAL IONS ON PHOSPHOLIPASE-C ACTIVITY

Enzyme extract was preincubated in sodium acetate buffer (pH 5.0) at 35° for 5 min with the chloride. Incubations for activity determinations were effected in the same buffer for 10 min with 0.25 mM [^{14}C]lecithin washed with EDTA (see MATERIALS AND METHODS).

Metal ion	*Concn. (mM)*	*Enzyme activity (percentage of control)*
Ca^{2+}	2.5	93, 93
Mg^{2+}	2.5	107
Cd^{2+}	2.5	106, 90
Co^{2+}	2.5	102
Fe^{2+}	2.5	95, 98
Mn^{2+}	2.5	105
Zn^{2+}	2.5	74, 69
Zn^{2+}	5.0	47, 65
Cu^{2+}	2.5	22, 22
Hg^{2+}	2.5	3, 0

mercurial *p*-chloromercuriphenyl sulfonate produced approx. 25% inhibition at 1 mM concentration which was increased to 70% at 10 mM (Table I). The sulfhydryl-groups-protecting agent dithiothreitol[17] was without effect at 1 mM concentration but caused approx. 40–50% inhibition at 10–50 mM (Table I).

Only one other phospholipid was examined for substrate specificity of the enzyme owing to limitations of the determination method. The tests with lysolecithin at pH 5 and 7 gave only 10–12% hydrolytic formation of phosphorylcholine relative to that from lecithin, indicating a high degree of specificity of the enzyme for the latter substrate.

DISCUSSION

Owing to paucity of algal material, it was not possible to purify the enzyme. However, since the crude extracts used showed consistently high and reproducible activity, the results of the present study may be reasonably considered to give valid indications of certain properties of the algal phospholipase C.

The absence of any inhibition from EDTA and dipyridyl as well as of any stimulation from all the metal ions tested indicate that the enzyme does not require a readily dissociable divalent metal ion for activity, but the possibility of a firmly bound non-exchangeable metal component is not precluded (*cf.* a mitochondrial pyruvate carboxylase with tightly bound Mn unaffected by EDTA[18]). The nature of the inhibition obtained with *o*-phenanthroline is difficult to explain and is unlike that observed by OTTOLENGHI[19] with phospholipase C from *Bacillus cereus*. The inhibition does not appear to be of the type recently observed with rabbit muscle aldolase[20] where the reagent was found to reversibly inactivate a non-metallo enzyme by oxidation of sulfhydryl groups to disulfides in the presence of air. Using similar test methods, we have failed to obtain reversal of, or protection from, the *o*-phenanthroline inhibition under all the conditions examined.

The inhibitions obtained from the metal ions Zn^{2+}, Cu^{2+}, Hg^{2+}, and from *p*-chloromercuriphenyl sulfonate suggest the involvement of sulfhydryl groups in the expression of activity[21]. The absence of any effect from the alkylating agents tested and from dithiobis-(2-nitrobenzoic acid) suggests that these sulfhydryl groups may be masked or poorly reactive[22]. This is also indicated by the remarkable stability of the enzyme preparation during storage without any sulfhydryl-group protection and by no stimulation of activity from the sulfhydryl-protecting agent dithiothreitol. The concentration-dependent inhibition obtained from this reagent may be interpreted in terms of reductive cleavage of disulfide bridges in the enzyme protein probably resulting in loss of molecular conformation required for activity; such cleavage of protein by the reagent has been previously reported[23].

The strong inhibitory effect of laurylsulfate on the algal phospholipase may be related to the electrokinetic requirements postulated for the activity of a bacterial phospholipase C by BANGHAM AND DAWSON[24], who attributed the inhibition from amphipathic anions to neutralisation of the net positive charge on the substrate required for enzyme action.

Comparison of the lecithinase C from *M. lutheri* with analogous enzymes from bacteria shows that the algal enzyme exhibits a much lower pH optimum (pH 5.0) than most of the bacterial phospholipases, which have a pH optimum near neutrali-

ty[25–29], and in this respect it more nearly resembles the enzyme from *Clostridium bifermentans* (optimum pH 6)[30,31]. The algal lecithinase does not have the heat stability of phospholipases C from *C. perfringens*[25] and *B. cereus*[27,32] which are only partially inactivated by heat treatment at 100° for 10 min. In contrast to the divalent cation independence shown by the algal lecithinase, Ca^{2+} requirement has been reported for the various bacterial phospholipases C[26–28,33,34,31]. It appears however from more recent studies that Ca^{2+} requirement might not be absolute[24,19], and that marked differences in metal-ion requirement might exist between bacterial phospholipases C such as the complete inactivation of the *C. perfringens* enzyme by chelating agents which have no significant effect on the *B. cereus* enzyme[35].

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