PARTIAL PURIFICATION OF PHOSPHOLIPASE C FROM OXYSTELMA ESCULANTUM

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SUMMARY: Phospholipase C from Oxystelma esculantum was purified by acetone precipitation and column chromatography on Sephadex G-100. It was then separated into two fractions (Phospholipase C I and II). Phospholipase C was homogeneous on polyacrylamide disc gel electrophoresis. The optimum pH of Phospholipase C I was 5.5 and the temperature 30°C. The nature of phospholipase C I was that of a metalloenzyme activated in presence of $CoCl_2$, $ZnCl_2$ and $CaCl_2$ whereas it was completely inhibited by EDTA due to chelation with metal ion. It was heatlabile.

Key Words: Oxystelma esculantum, tuber, phospholipase C.

INTRODUCTION

Recently much attention has been focused on phospholipase C due to its versatile applications as a powerful tool for studying structure and function of membrane lipid (1). As a tool in membrane research, this enzyme has been used in the field of blood banking (2,3) and as a therapeutic agent in prevention of post traumatic intra-vascular coagulation (4,5).

Phospholipase C is one of four phospholipases (A, B, C and D) which is capable to catalyze phosphoglyceride (Lecithin) into phosphorylcholine and diacylglycerol. Very little work has been carried out for phospholipase C from plant sources. However, so far phospholipase C activity has been found in spinach leaves, carrot roots (6), rice grain (7), rice bran (8), celery, cauliflowers and daffodils (9), *Lilium Longiflorum* (10,11) and *Melia azadirachta* seeds (12).

Several workers (8,11,13,14) have demonstrated the presence of two different phospholipases C of the plant origin, depending upon the specificity of substrate hydrolyzing phosphatidylcholine and phosphatidylinositol. Few reports are available on phospholipase C purification from plant origin. Recently Helsper *et. al.* (13) reported that purified phospholipase C of *Lilium longiflorum* is strongly stimulated by Ca^{2+}, although Mg^{2+} and Co^{2+} replaced Ca^{2+} while Cu^{2+} strongly and EDTA completely inhibited enzyme activity. Takano *et. al.* (8) reported that purified phospholipase C of rice bran is effectively stimulated with Co^{2+} but strongly inhibited by Cd^{2+} and EDTA.

In the previous presentation we reported some kinetic properties of crude phospholipase C of *Oxystelma esculantum* root tuber (15). We describe here in the present report the purification and some properties of *Oxystelma esculantum* root tuber phospholipase C (Table 1).

MATERIALS AND METHODS

Materials

Oxystelma esculantum root tuber (Kondhir) was collected in the months of July-September after the monsoon rains from Diplo, District Tharparkar, Sindh, Pakistan. All reagents of analytical grade were used without further purification.

Methods

Enzyme preparation: The enzyme solution was prepared according to the previous report (16).

Substrate preparation: The substrate emulsion of phosphatidylcholine was prepared as reported earlier (16).

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Determination of phospholipase C activity: Phospholipase C activity was measured by egg lecithin emulsion method as described previously (15).

Phospholipase C activity unit was defined as the amount of enzyme required to release one mg of inorganic phosphate from lecithin per hour under the specified conditions of the assay.

Purification of phospholipase C: To 200 ml crude enzyme solution, two volumes of chilled acetone was added and kept over night at 4°C. The precipitates were collected by centrifugation at 5000 x g and were dissolved in a small volume of 0.1 M Tris-HCl buffer pH 7.5. The sample was dialyzed overnight against the same buffer.

Sephadex G-100 column chromatography: Dialyzed enzyme sample was applied on Sephadex G-100 column (2.5 x 26 cm) equilibrated with 0.1M Tris-HCl buffer pH 7.5. The enzyme sample was eluted with the same buffer containing 1mM $ZnCl_{2+}$ and 0.4M NaCl. The flow rate was adjusted 30ml per hour with a fraction volume of 4.0 ml. The fraction protein was monitored at 280 nm and the enzyme activity was determined in alternate fraction (Figure 1).

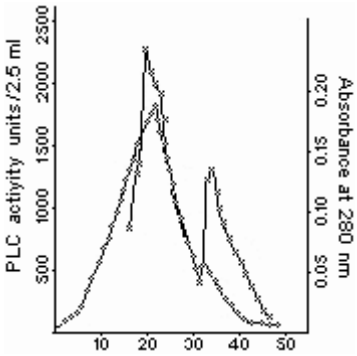
Polyacrylamide disc gel electrophoresis: The homogeneity of the pooled fractions was confirmed by polyacrylamide disc gel electrophoresis by the method of Davis (17). 50µl of enzymatic protein sample was loaded with sample gel and a constant current supply of 4 mA per gel rod (13 x 0.6 cm) was applied for 3 hours. After the electrophoresis run, gels were stained with 1% Coomassie brilliant blue R-250 and were destained with acetic acid: methanol: water (7.5: 5.0: 87.5 v/v/v) till the appearance of blue band against a clear background.

Determination of protein: The absorbance at 280nm was measured to monitor the protein during the chromatographic separation. The protein content was measured by the method of Lowry *et. al.* (18), using bovine serum albumin as a standard.

Table 1: Purification of phospholipase C from *Oxystelma esculantum* R. Br Root tuber.

Purification steps	Total Protein mg	Total activity Units	Specific activity Units/mg	% Recovery
Crude	140.00	145600	1040.00	100.00
Dialyzed	31.20	54960	1761.53	37.74
Sephadex G-100 F-I	23.80	34166	1435.54	23.46
F-II	2.76	15702	5689.13	10.78

Figure 1: Elution profile in Sephadex G-100 gel fileration of extracellular phospholipase C of *Oxystelma esculantum* 0-0-absorbance at 280 nm; x-x-x PLC activity.



RESULTS AND DISCUSSION

The *Oxystelma esculantum* crude enzyme was precipitated with cold acetone to a final concentration of 45%. The precipitates were dissolved in small volume of 0.1M Tris-HCl buffer pH 7.5 and dialyzed. The dialyzed enzyme sample was passed through Sephadex G-100 column (2.5 x 26 cm) with 0.1M Tris-HCl buffer pH 7.5. Figure 1 shows the elution pattern of enzyme and the two distinct peaks of

Figure 2: Electrophoresis of the partially purified enzyme on polyacrylamide gel (C) Crude enzyme 1. Partially purified phospholipase C.

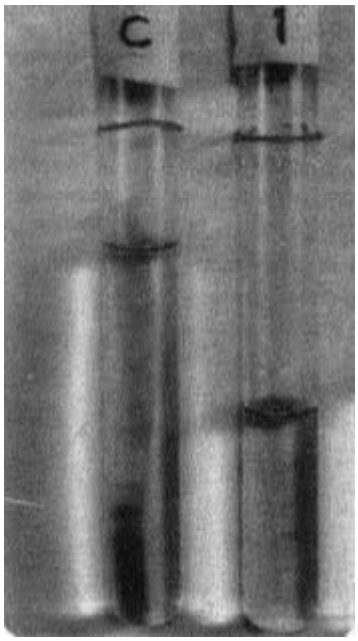
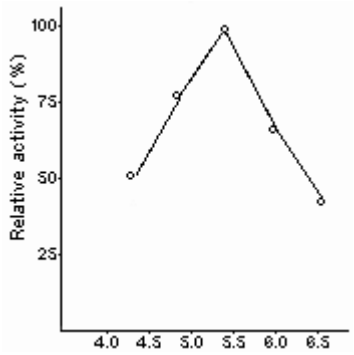


Figure 3: Effect of PH on purified portion I of Phospholipase C of *Oxystelma esculantum*.



phospholipase C activity were obtained (phospholipase C I and II).

The homogeneity of phospholipase C I and II was checked on polyacrylamide disc gel electrophoresis and a single band was observed in phospholipase C I as shown in Figure 2.

The pH profile of *Oxystelma esculantum* phospholipase C I was determined and optimum activity was found at pH 5.5 as shown in Figure 3. The *Oxystelma esculantum* phospholipase C-I similar behavior with respect to pH to spinach plastids phospholipase C (6) and rice grain phospholipase C (7). The optimum pH (5.5) of *Oxystelma esculantum* phospholipase C-I is slightly higher than Lilium longiflorum particulate phospholipase C pH 5.2 whereas it is less than the cytosolic phospholipase C pH 6.0 (14).

The enzyme activity of *Oxystelma esculantum* phospholipase C-I was assayed at different temperatures 25 to 50°C at pH 5.5. It is clear from Figure 4 that maximum

Figure 4: Effect of temperature on purified portion I of PLC from *Oxystelma esculantum*.

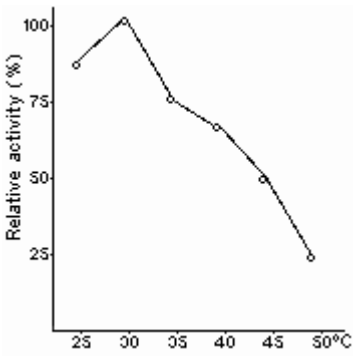
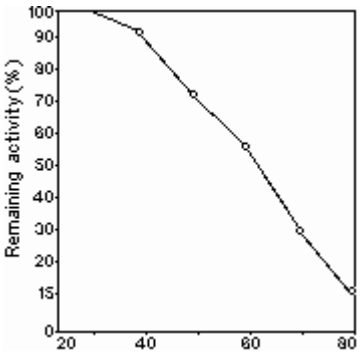


Figure 5: Showing the effect of heat stability on purified phospholipase C-1 of *Oxystelma esculantum*.



enzyme activity observed at 30°C. This result is consistent with the findings of Helsper *et. al.* (13), in case of cytosolic phospholipase C (30°C) but it is lower than the particulate phospholipase C 35°C of *Lilium longiflorum*.

The thermal stability of *Oxystelma esculantum* phospholipase C was investigated by determining the residual activity (at 30°C for 1 hour) after preincubating the enzyme solution at various temperatures (30 to 70°C) for 10 minutes. It is observed from Figure 5 that enzyme is fairly stable up to 40°C but lost 70% of activity by heating at 70°C for 10 minutes. This is in agreement with the finding of Chrastil *et. al.* (7) in case of rice grain phospholipase C. It is suggested that the enzyme is heat labile.

Table 2: Showing the effect of various reagents in given concentration on partially purified phospholipase C-I activity of *Oxystelma esculantum*. The influence effect of various reagents was tested by incubation of the reaction mixture at 30°C for 1 hour.

Reagents	Concentration mM	PLC activity Units/2.5ml	% Relative* activity	% Activation (inhibition)
Control	-	1248	100	-
Sodium deoxycholate	1	2148	172.10	72.10
Co^{2+}	5	1386	111.00	11.00
Zn^{2+}	10	1872	150.00	50.00
Ca^{2+}	5	1456	116.60	16.60
EDTA	1	0	0	-
EDTA + Zn^{2+}		1874	150.16	50.16

*Expressed as % of the activity with no addition.

The effect of various metal ions and reagents on *Oxystelma esculantum* phospholipase C-I was determined and the results are presented in Table 2. Sodium deoxycholate (72%), Zn^{2+} (50%), Ca^{2+} (16.6%) and Co^{2+} (11%) enhance enzyme activity while EDTA showed complete inhibition. The inhibition with EDTA could be due to the chelation with Zn^{2+} ion of metalloenzyme and reversed by the addition of Zn^{2+}. The notion is that the enzyme is a zinc metalloenzyme possessing two ions per molecule, one of which is tightly bound and the other loosely associated. This enzyme could easily function as rate limiting parameter following addition of Zn^{2+}/Co^{2+}. Similar findings have been reported by Clive Little *et. al.* (20), suggesting that inactivation by EDTA and ophenanthroline was fully reversed by the addition of Zn^{2+} or Co^{2+}.

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