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STUDIES ON PHOSPHOLIPASE C FROM MELIA AZADIRACHTA SEEDS

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Abstract

The activity of phospholipase C in crude enzymatic preparation of *Melia azadiracht* seeds (Neem seeds) was studied by the use of lecithin as a substrate in aqueous medium. The enzyme activity was found optimum at pH 2.5 and temperature 35°C. The phospholipase C was found heat labile, being inactivated 88% within 10 minutes at 90°C.

Introduction

Diacylphosphoglyceride hydrolyzes to diacylglycerol and phosphorylcholine was first demonstrated by Kates in carrot roots extract by phospholipase C [1]. Phospholipase C activity was subsequently found in many plants, rice grains [2], rice bran [3], celery, cauliflower, daffodils [4], *Lilium longiflorum* [5,6] and *Oxystelma esculantum* root tuber [7].

The use of phospholipase, particularly phospholipase C is expanding in a quite interesting way. The extracellular phospholipase C from various sources are widely used in the elucidation of structure and function of cell membrane [8], in the blood banking [9,10] and a potential therapeutic value in certain cases of intravascular coagulation [11,12]. The potential applications of phospholipase C inspired us to search new and cheaper sources. The presence of phospholipase C enzyme in plant seeds has not been established and it was decided to measure the activity of this enzyme in a variety of plant seeds using lecithin (diacylphosphoglyceride) as a substrate in aqueous system which we had earlier found suitable for the measurement of root tuber phospholipase C.

In this paper, we are describing the presence of phospholipase C in *Melia azadirachta* seeds (Neem seeds) and report some of its properties from crude enzyme.

Keywords: Phospholipase C, Melia azadirachta seeds

Materials and Methods

Materials: The seeds of *Melia azadirachta* (Neem seeds) were collected during July and August in dry state from the plants located at Sindh University, New Campus, Jamshoro. Egg lecithin was purchased from Fluka Chemicals. All the reagents used were of analytical grade.

Methods: Enzyme powder preparation: The *Melia azadirachta* seeds were crushed with a pestle and mortar, after removing their seed coat. The crushed seeds were defatted with diethylether. The defatted residue was further crushed in ice cold acetone. Acetone was removed by filtration through Whatman filter paper No. 1 and the residue was dried at low temperature.

Preparation of soluble enzyme: The enzyme extract was prepared as described previously [13]. 10.0g of acetone dried powder (crude enzyme) was crushed to a fine powder and dissolved in 30 ml of ice cold D.water using pestle mortar. The solution was centrifuged at 5000 rpm for 15 minutes, supernatant was transferred to 100 ml volumetric flask. This procedure was repeated twice and total volume was made up to 100 ml.

Determination of protein: Protein content of enzyme solution was determined according to the method of Lowry et al [14], using bovine serum albumin as a standard and found to be 2.40 mg/ml.

Preparation of substrate emulsion: The substrate emulsion was prepared according to the previous reports

[15,16].

To 10.0g egg lecithin was added 80 ml D.water and homogenised using homogenizer (Universal Lab.Aid Type 309 Poland) at maximum speed for 10 minutes with succesive intervals of one minute to prevent over heating. Total volume was made up to 100 ml with D.water and pH was adjusted at 6.8.

Assay of phospholipase C activity: In 25 ml conical flasks 2.5ml of enzyme solution was taken in duplicate and 2.5 ml of substrate emulsion was added in each flask. Appropriate controls were also taken for blank enzyme and blank substrate. The conical flasks were covered with aluminium foil and incubated for 2 hours in shaking incubator (Gallenkamp) adjusted at 120 rev/ min at 35°C. The reaction was stopped by adding 2.5 ml of chloroform-methanol (2:1 v/v) in each flask and centrifuged. Aqueous and non-aqueous portions were separated. Inorganic phosphate was determined from aqueous portion by Yamakawa et at. [17] and Chen et.al with slight modifications.

1.0 ml aqueous portion was deproteinized by adding 1.0ml trichloroacetic acid. After vigorously shaking, it was centrifuged at 4000 rpm for 10 minutes.

A 0.2 ml portion of supernatant was taken into a pyrex tube and 0.5 ml of 70% perchloric acid was added to the tube. The mixture was heated at 100°C for 30 minutes. The volume of hydrolysate was made up to 4.0 ml with D.water.

A 2.8 ml of ascorbic acid-ammonium molybdate mixture (1.0 ml of 10% ascorbic acid +6.0 ml of 0.42% ammonium molybdate in 1N sulphuric acid) was added to 1.2 ml of hydrolysatre in pyrex tube (or 1.2 ml of D.water for blank). The reaction mixture was incubated for 20 minutes at 45°C. The colour intensity was measured at 820nm. KH_2PO_4 was used as a standard inorganic phosphate.

One unit of phospholipase C was defined as the amount of enzyme liberating one microgram of inorganic phosphate per two hours from lecithin under the specified conditions of assay.

Diacylglycerol was visualised by thin layer chromatography on silica gel G-60 from non-aqueous portion. The solvent system was used ahexane: Diethylether: Formic acid (80:20:2 v/v/v)[19]. The separated components such as phosphatidylcholine, diacylgycerol, monoacylglycerol and free fatty acids were visualised with iodine vapours.

Results and Discussion

Fig-1 shows the time course of the hydrolysis of phosphatidylcholine by crude phospholipase C from *Melia azadirachta* enzyme extract. Initially the rate of hydrolysis increases with respect to time of incubation up to 2 hours and then remains nearly constant. This result is in agreement with the findings of other workers in the case of lipolytic enzymes of *Erythrina qlabrescens* [20] and castor bean [21].

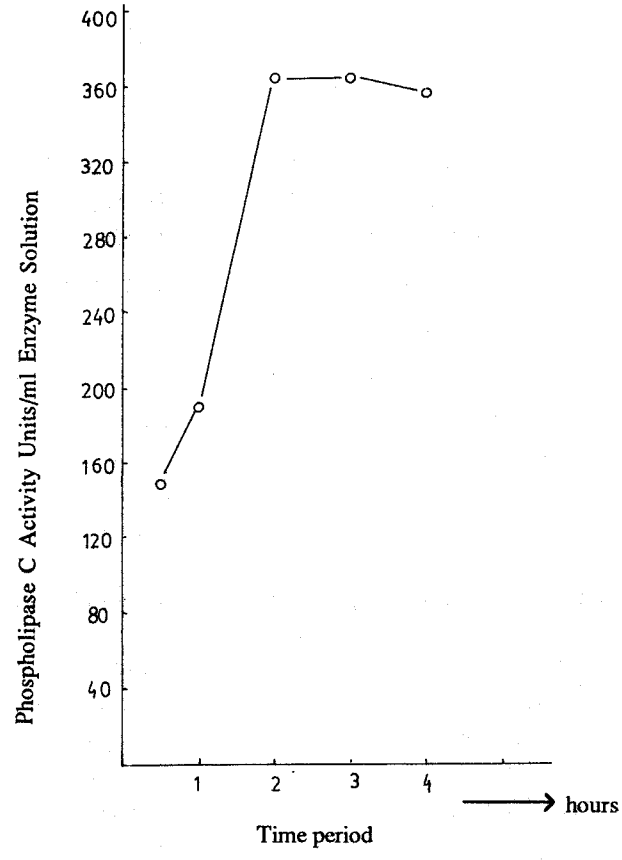

Figure 1- Effect of time period on *Melia azadirachta* Phospholipase C activity. Reaction mixture contained 2.5 ml of enzyme solution and 2.5 ml 10% Ovolecithin emulsion were incubated at 35°C.

The effect of substrate concentration on the phospholipase C activity of **Melia azadirachta** seeds was investigated using different concentrations of phosphatidylcholine as a substrate from 2.5 to 15.0% The optimum substrate concentration was found to be 10% as shown in Fig-2. Declination in rate of hydrolysis beyond 10.0% lecithin may be suggested due to physiochemical properties of the emulsion towards

enzymatic hydrolysis of phospholipid [22]. In subsequent experiments 10.0% lecithin was used as substrate.

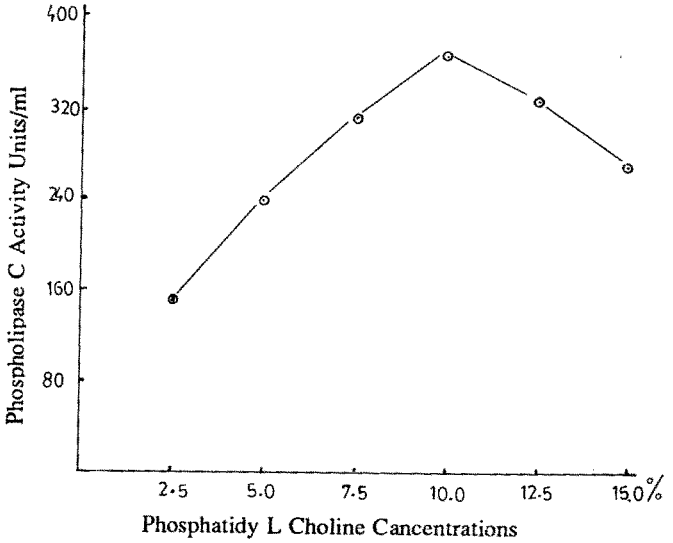

Figure 2- Effect of Substrate concentration on *Melia azadirachta* Phospholipase C activity when reaction mixture was incubated at 35°C for 2 hours.

The pH profile of *Melia azadirachta* seeds phospholipase C is shown in Fig-3. The results indicate that maximum rate of reaction of phospholipase C was obtained at pH 2.5 and then it decreased slowly. The pH optima of *Melia azadirachta* seeds phospholipase C (2.5) is not in agreement with the phospholipase C isolated from plant origin. The pH optima of most tplant phospholipase C are in the acidic range, lying between 3.5-6.0 [7, 23].

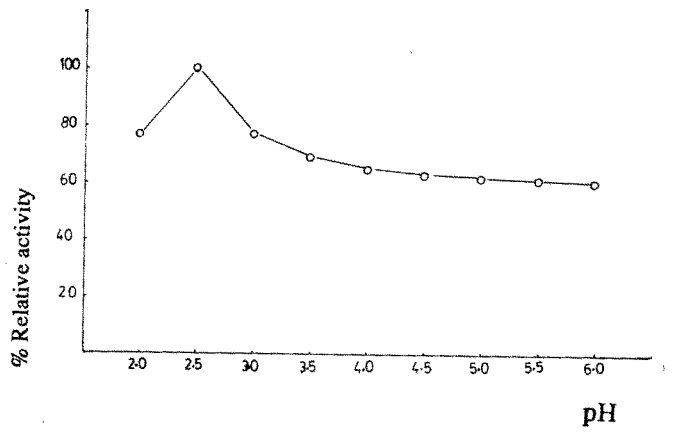

Figure 3- Effect of pH on the activity of *Melia azadirachta* seed Phospholipase C.

The effect of various temperatures on the enzyme activity with lecithin as a substrate is illustrated in Fig-4. The optimum temperature for enzyme reaction was found to be 35°C. Similar observations have been

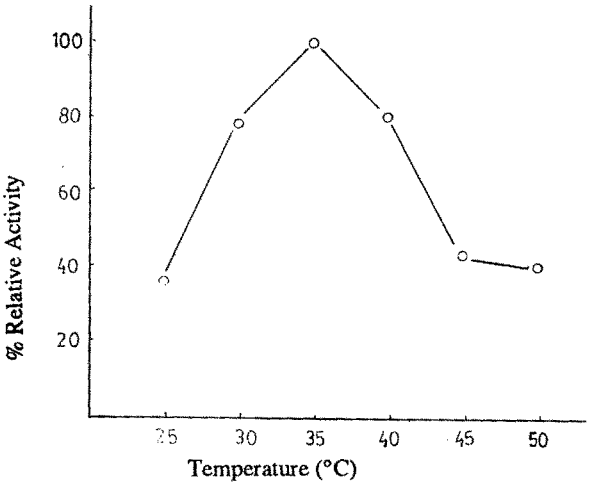

Figure 4- Effect of temperature on *Melia azadirachta* seed Phospholipase C activity.

reported by Helsper et al. [23] in case of *Lilium longiflorum* cytosolic phospholipase C.

Stability of ***Melia azadirachta*** seeds crude phospholipase C was studied by heating the ambient volume of enzyme solution at various temperatures from 40-100°C for 10 minutes. The activities remaining after heat treatment were assayed by standard method. The results shown in Fig-5, suggest that the enzyme is fairly stable up to 60°C and completely loses its activity when heated at 100°C for 10 minutes. This observation is in confirmity with the finding of other workers in the case of phopholipase C from *Oxystelma esculantum* [7] and rice bran [4].

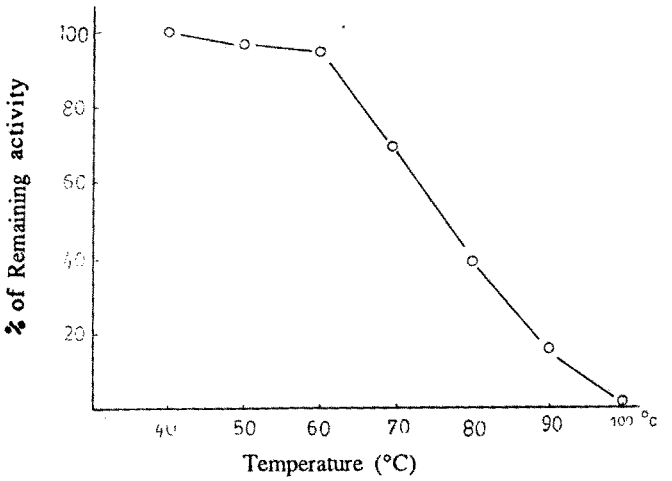

Figure 5- Effect of heat treatment on *Melia azadirachta* seed phospholipase C activity.

The effect of various metal ions and other reagents on phospholipase C activity of ***Melia azadirachta*** seeds were also studied. The presence of Ca^{2+}, Co^{2+}, mercaptoethanol and dithiotheritol enhance the enzyme activity where as Mn^{2+}, Zn^{2+},Hg^{2+},Ag^{2+}, EDTA and o-phenanthroline inhibited enzyme activity as shown in Table-1. Inhibition of phospholipase C with metal chelating agents such as EDTA and o-phenanthroline, may be suggested to the presence of metal containing active site of the phospholipase C from ***Melia azadirachta*** . The existence of metal dependent phospholipase C from various sources have been reported by different workers [7, 24-26].

Acknowledgements

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Table 1-

Effect of metal ions and other reagents on crude enzyme of *Melia azadirachta* phospholipase C activity.

Metal ion 5x $10^{-3}{}_{M}$	Activity units/ml	% Relative* activity	% Activation /(inhibition)
Control	400	100	-
Ca2+	405	101.2	1.2
Co2+	432	108	08
Mercaptoethanol	465	116.25	16.25
Dithiotheritol	455	113.75	13.75
Mn2+	200	50	(50)
Zn2+	150	37.5	(62.5)
Hg2+	175	43.75	(56.25)
Ag2+	345	86.25	(13.75)
E.D.T.A	240	60	(40)
o-phenanthroline	54	13.5	(86.5)

*Expressed as % of the activity with no addition.

References

1. M. Kates; *Can. J. Biochem.* **35** , 575(1955)
2. J. Chrastil and F.W. Parrish; *J. Agric. Food. Chem.* **35**, 624 (1987)
3. K. Takano; I. Kamoi and T. Obara; Nakagu Shuho (Tokyo) **28**, 262 (1984)
4. R.F. Irvine; A.J. Letcher and R.M.C. Dawson; *Biochem. J.* **176**,475 (1980)
5. J.P.F.S. Helsper; P.F.M.DeGroot; H.F. Linsken and J.F. Jackson; *Phytochemistry* **25**,2193 (1986)
6. J.P.F.S. Helsper; P.F.M. DeGroot; H..F.Linsken and J.F. Jackson; *Phytochemisty* **25** 2055 (1986)
7. A.N. Memon; Ph.D. Thesis, , University of Sindh, Paksitan (1990)
8. E.L. Kurg and C. Kent; *Arch. Biochem. Biophys.* **231**,400 (1984)
9. C. Little and M.G. Rumsby; *Biochem.J.* **188**,39 (1980)
10. C. Little; *Biochem. Soc. Trans.* **9**,448 (1981)
11. K.E. Giercksky; A.B. Othaess; E. Bjorklid and H. Prydz; Thrombsis and Haemostasis **36**,641 (1976)
12. K.E. Giercksky; and E. Bjorklid; *Scand. J. Haematol.* **17**,305 (1976)
13. M.U. Dahot; M.Yakoub Khan and M. Hanif Noomrio; *Proc. Natl. Chem. Conf.* **1**,364 (1989)
14. O.H. Lowry; N.J. Rosebrough; A.L. Farr and R. Randall; *J. Biol. Chem.* **193**,265 (1951)
15. A.R. Memon and M.U. Dahot; *J. Chem. Soc .Pak.* **7**,7 (1985)
16. M.U. Dahot and A.R. Memon; *J.Nat. Sci. Maths.* **27**,171 (1987)
17. Y. Yamakawa and A. Ohsaka; *J. Biochem.* **81**,115 (1977)
18. P.S. Chen jr.; T.Y. Toribara and H. Warner; *Anal. Chem.* **281** 156 (1956)
19. T.A. Clayton; T.A. McMurry and W.R. Morrison; *J. Chromat.* **47**277 (1970)
20. M.U. Dahot and F.D. Khand; *J. Pure & Appl. Sci.* **4**, 17 (1985)
21. M. Yakoub Khan; M.Sc. Thesis, University of Sindh, Pakistan (1981)
22. B. Orlowska; J. Meduski and W.D . Uspienskaya; Bull. Acad. Polon. Sci. Classe II**6**, 463 (1958)
23. J.P.F.G. Helsper; J.W. M. Haemskerk and J.H Veerkamp; Physiol. Plantarum **71**, 120 (1987)
24. S. Sonoki and H. Ikezawa; *Biochem. Biophys. Acta* **403**, 412 (1975)
25. A.C. Ottolenghi; *Biochem. Biophys. Acta* **106**, 510 (1965)
26. O. Doi and S. Nojima; *Biochem. Biophys. Acta* **248**, 234 (1971)