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Studies on Phospholipase A from *Melia azadirachta* (Neem) Seeds Extract

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Abstract: The crude extract from dried *Melia azadirachta* seeds was prepared and it was noted that the seed contains sufficient amount of phospholipase A activity. Optimal activity of *Melia azadirachta* seeds was found at pH 6.0 and at temperature 35°C. The stability of enzyme was noted over the pH range 5-8. More than 30% activity of phospholipase A remained active up to 60 minutes at 60°C. The enzyme activity was markedly increased in the presence of calcium, manganese and zinc but it was decreased by the addition of EDTA.

Key words: Enzyme, phospholipase A, *Melia azadirachta*, seeds

Introduction

Phosphatidylcholine hydrolyzed to fatty acids and lysophosphatidylcholine by the action of snake venom phospholipase A was first demonstrated by Kyes (1903). In the early 1960, phospholipase A was further subdivided according to stearic and positional specificity such as Phospholipase A_1 and Phospholipase A_2. Phospholipase A_1 (3.1.1.32) hydrolyze the ester bond at position 1 of the Sn-3-phosphoglyceride (Phosphatidylcholine) molecule with the formation of 2-acyl-lysophosphoglyceride. On the other hand phospholipase A_2 (E.C. 3.1.1.4) hydrolysis Sn-3- phosphoglyceride into 1-acyl-lysophosphoglycerides (Hans & Jensen, 1974 and Lands, 1960). It is reported in the literature that Phospholipase A_1 is heat labile where as phospholipase A_2 is heat stable (Lands, 1965).

Various factors that influence the hydrolysis of lecithin by phospholipase A have been studied but a number of aspects of the reaction have not been clearly defined. Studies on the rate of different fatty acids released from the lecithin by the action of phospholipase A is often complicated and this depends on the nature of substrate. Lecithin from natural sources is posing problems to study the substrate specificity. These difficulties can be over come by the use of purified lecithin but still impure or crude lecithin is used as substrate for establishment of phospholipases activity at initial stage (Mongrand *et al*., 2000). Phospholipase A has been found in very few plants (Ghangro *et al*., 1992, Acker, 1985, Memon *et al*., 1985 and Gillard,1971) but it is frequently reported from animals (Valentin *et al*., 2000, Valentin *et al*., 1999, Dennis, 1997, Angulo, 2000 and Lindahl *et al*., 1995) and microorganisms (Steinbrueckner *et al*., 1995, Nishijima, 1974, Doi *et al*., 1972, Raybin *et al*., 1972, Scandella & Kornberg, 1971 and Proulx *et al*., 1967). Present study was undertaken to screen different plants for the presence of lipolytic enzymes and in this paper we describes some properties of crude phospholipase A, isolated from *Melia azadirachta* seeds. This type of work on phospholipase A from *Melia azadirachta* seeds has not been reported in literature so far.

Materials and Methods

Dry *Melia azadirachta* (Neem) seeds were collected during July - August from Sindh University New Campus, Jamshoro, Egg lecithin was purchased from Fluka Chemicals.

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

Preparation of Soluble Enzyme: The enzyme extract was prepared as described previously (Dahot & Khand, 1985 and Memon & Dahot, 1985).

Determination of Protein: Protein content of enzyme solution was determined according to the method of Lowery *et al*. (1951) using bovine serum albumin as standard.

Preparation of Substrate Emulsion: The substrate emulsion was prepared according to the previous reports (Dahot & Khand, 1985 and Memon & Dahot, 1985).

Assay of phospholipase activity: Phospholipase activity was determined according to the method described previously (Dahot & Khand, 1985 and Memon & Dahot, 1985) using egg lecithin emulsion as a substrate.

One unit of phospholipase activity is defined as the amount of enzyme required releasing one micro equivalent of free fatty acid per hour under standard reaction conditions.

Characterization of Phospholipase activity: The nature of phospholipase activity was observed from hydrolytic products of enzyme reaction mixture analyzed by thin layer chromatography using Silica gel G-60 (E.Merck). Chloroform -methanol-ammonium hydroxide-water (60:35:10:2.5 v/v) was used as a solvent. The separated components, unhydrolyzed phosphatidyl-choline and lysophosphatidyl-choline were developed with Zinzadze spraying reagent (Dittmer and Lester, 1964).

Results and Discussion

The results shown in Fig. 1 indicate that the rate of lecithin hydrolysis increases with respect to time of incubation up to 1 hour and then declines. The declination in enzyme activity after one hour reaction time may be suggested either due to product inhibition (Gillard,1971 and Gatt, 1968) or presence of other lipolytic enzymes in crude sample of *Melia azadirachta* seeds (Ghanghro *et al*., 1992) could not be ruled out. In subsequent experiments one hour time period was fixed.

The effect of pH on phospholipase activity of *Melia azadirachta* seeds was observed using pH 2-7 of universal buffer. Maximum phospholipase activity was found at pH 4.5 and 6 (Fig. 2). Reaction products were separated through thin layer chromatography and a greater accumulation of lysophosphatidyl- choline was observed at pH 6 due to the presence of phospholipase A. Whereas, complete deacylation was observed at pH 4.5 due to the presence of phospholipase

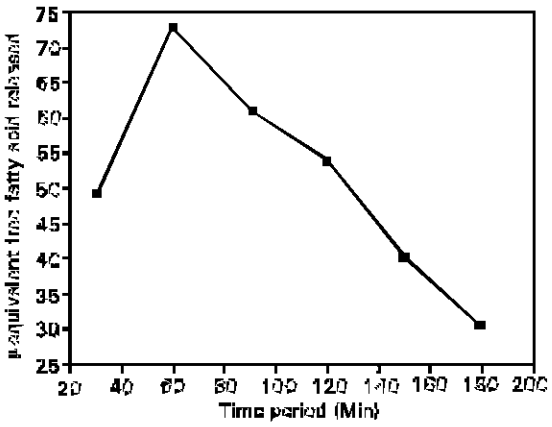

Fig. 1: Effect of time period on phospholipase activity of *M. azadirachta* seeds

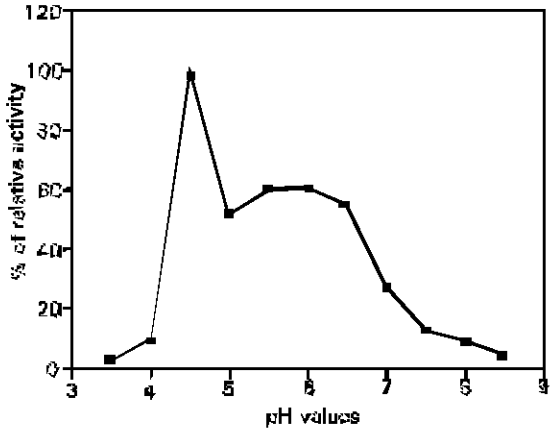

Fig. 2: Effect of pH on phospholipase activity of *Melia azadirachta* seeds

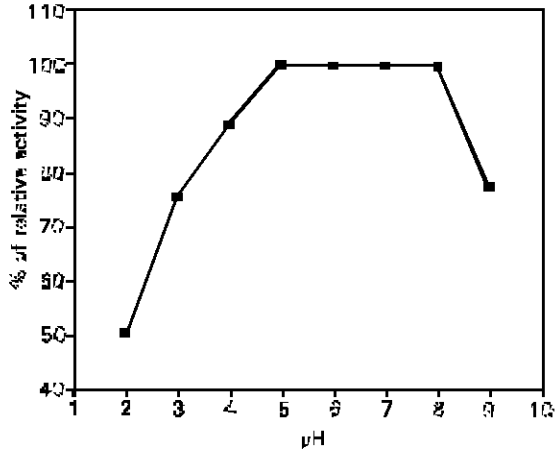

Fig. 3: pH stability of phospholipase A activity of *Melia azadirachta* seeds

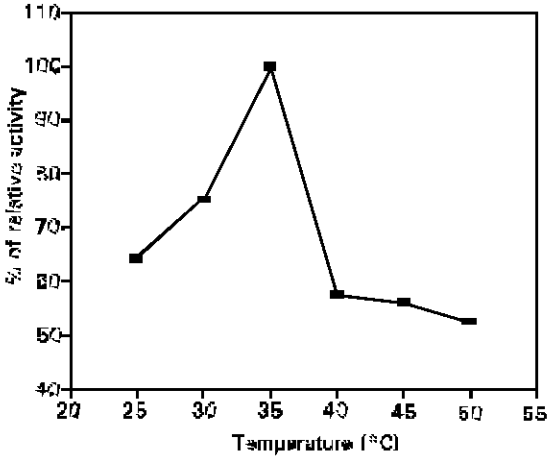

Fig. 4: Effect of temperature on Lipase A activity of *Melia azadirachta* seeds

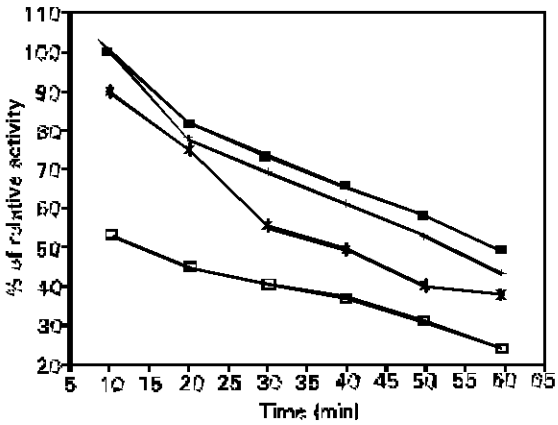

Fig. 5: Effect of heat stability on PLA activity of *Melia azadirachta* seeds

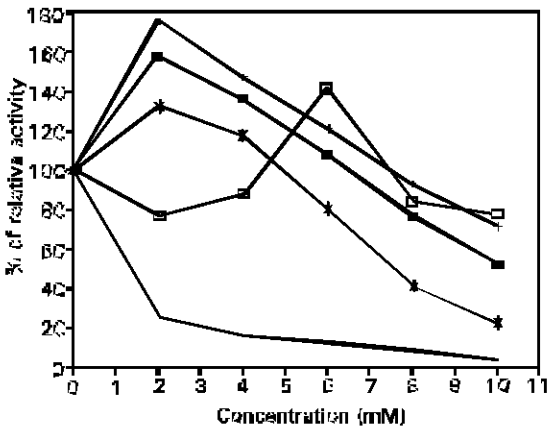

Fig. 6: Effect of reagent on Lipase A activity of *Melia azadirachta* seeds

B. Similar results were obtained with phospholipase of *Erythrina glabrescens* seeds (Dahot & Khand, 1985) and *Ricinus communis* seeds (Memon *et al*., 1985).

The effect of pH stability of phospholipase A activity from crude extract of *Melia azadirachta* seeds was determined by pre-incubating of enzyme solution with pH ranging from 2.0 to 9.0 (Universal Buffer) and substrate was added after 10 min. at desired temperature. The enzyme assay was carried out by standard method (Fig. 3). It was observed that phospholipase A activity is stable over the range of pH 5-8. The effect of temperature on *Melia azadirachta* seeds crude phospholipase A was studied (Fig. 4). Maximum enzyme activity at pH 6.0 was found at 35°C. The thermal stability of phospholipase A was observed at different temperatures ranging from 30-60°C by pre-incubating enzyme solution for 10,20,30,40,50 and 60 minutes at desired temperature. The residual activity was determined by standard method as reported in material and method section and results are summarized in Fig. 5. It was observed that more than 30% activity of phospholipase A remained active up to 60 minutes at 60°C. It is concluded that Phospholipase A is a thermostable enzyme.

Different concentration of common metal ions and detergent were investigated for their action on phospholipase A activity. Phospholipase A activity of *Melia azadirachta* seeds is stimulated with the increase of Ca^{2+}, Mn^{2+}, Zn^{2+} and SDS concentration up to 2mM, 2mM, 2mM and 6mM and then decreases with the increase of metal ion concentration (Fig. 6). EDTA shows strong inhibition effect on phospholipase A activity. The activation of crude enzyme at lower concentration of metal ions whilst inhibition at higher concentration (Fig. 6) suggest that the presence of these metal ions in crude enzyme could not be ruled out. These results are consistent with the findings of other workers in the isolation of enzymes from plant sources (William, 1951, Ball *et al*., 1937 and Dahot & Memon, 1987). The activation of enzyme activities with SDS may be suggested due to the solubilization of lecithin, which forms mixed micelles at molar ratio with maximal degree of hydrolysis. These results are in agreement with the observations reported for enzymes isolated from different sources (Dahot *et al*., 1995 and Yamakawa & Ohsaka, 1977).

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References

Acker, L., 1985. Phospholipase of cereals. Adv. Cereal Sci. Technol., 7: 85 - 104

Angulo,Y., T.O. Portugal, L. D. Possani and B. Lomonte, 2000. Isolation and characterization of myotoxin from Atropoides (Bothrops) nummifer snake venoum , a new Lys 49 phospholipase A_2 homologue. Intl. J. Biochem & Cell Biol., 32: 63-71

Ball,A.K., M.F.Matlack and I.M.Tudeer, 1937.The hydrolysis of glycerides by crude pancreatic lipase. J. Bol. Chem., 122:125-137

Dahot, M.Umar and F.D.Khand, 1985. Lipolytic enzymes of Erythrina glabrescens seeds. J. Pure & Appl. Sci., 4: 17 - 22

Dahot, M.Umar and A.R.Memon, 1987. Properties of *Moringa oleifera* seed lipase. Pak.J.Sci.Ind.Res., 30: 832-835.

Dahot M.Umar, A.B.Ghanghro and M. Yousuf Khan, 1995. Phospholipase C activity of castor bean. J. Pure & Appl. Sci., 14: 41-48

Dittmer,J.C. and R.L.Lester, 1964. A simple specific spray for the detection of phospholipids on thin layer chromatography J. Lipid Res., 5: 126 - 127

Doi,O., M. Ohki and S.Nojima, 1972. Two kinds of phospholipase A and lysophospholipase in Escherichia coli. Biochem. Biophys. Acta, 260: 244 - 258

Gatt,S., 1968. Purification and properties of phospholipase A_1 from rat 2 calf brain. Biochem.Biophys. Acta, 159: 304 - 316

Ghanghro,A.B., M.Umar Dahot and M.Yousuf Khan, 1992. Studies on phospholipase C from Melia azadirachta seeds. J.Sci.I.R.Iran, 3:1-4

Gillard,T., 1971. Enzymatic deacylation of lipids in plants. Eur. J. Boichem., 21: 90-98

Hans,B. and R.G.Jensen, 1974.Lipolytic Enzymes, Academic Press Inc. (London) LTD, U.K., pp:194-288

Kyes, P., 1903. Hemolysis of red blood cells with cobra venom in presence of lecithin. Berlin Klin. Wochen seher, 40: 956

Lands,W.E.M.,1965. Lipid metabolism. Annu.Rev.Biochem., 34:313-346

Lindahl,M., E.Backman, K.G.Henriksson, J.R.Gorospe and E.P. Hoffman, 1995.Phospholipase A2 activity in dystrophinopathies. Neuromuscular Disorder, 5: 193-199.

Lowry,O.H., N.J.Rosebrough, A.L.Farr and R.J.Randall, 1951. Protein measurementwith folin phenol reagent. J.Biol.Chem., 193: 265 - 275

Memon, A.R., M.Y.Khan, M.Umar Dahot, F.C.Nachnani and I.A.Ansari, 1985. Characterization of lipolytic enzyme present in castor bean. J.Pure & Appl.Sci., 4: 41 - 48

Memon,A.R. and M.Umar Dahot, 1985. Investigation of phospholipase B type activity in *Moringa oleifera* seeds. J.Chem.Soc.Pak., 7:7-15

Mongrand,S., C.Cassagne and J.J.Bessoule, 2000. Import of lysophosphatidylcoline into cloroplasts likely at the origin of Eukaryotic plastidal lipids. Plant Physiol., 122: 845-852

Nishijima,M., Y.Akamastu and S.Nojima, 1974. Purification and properties of membrane bound phospholipase A from Mycobacterium phelei. J.Biol.Chem., 249: 5658 - 5667

Proulx,P. and L.L.M.Van Deenen, 1967. Phospholipase activity of Escherichia coli. Biochem.Biophys.Acta, 144: 171 - 177

Raybin,D.M., Le.Roy, L.Bertsch and A.Kornberg, 1972. A phospholipase in Bacillus megaterium unique to spores and sporangia. Biochem., 11: 1754 -1763

Scandella,C.J. and A.Kornberg, 1971. A membrane bound phospholipase A1 purified from Escherichia coli. Biochem., 10: 4447-4456

Steinbrueckner,B.E., J.Aufenanger, A.Hartinger, N.E. Saris, P. Kinnunen and G.E.Hoffman, 1995. Phospholipase A activity in Pseudomonas aeruginosa. Zentralblatt Fur Bakteriologie Intl. J.Med. Microbiol. Virol. Parasitol and Inf. Dis., 282: 54 - 66

Valentin,E.,R.S.Koduri, J.C.Scimeca,G.Carle, M.H.Gelb, M.Lanzunski and G.Lambeau, 1999.Cloning and recombinant expression of a novel mouse secreted phospholipase A2. J.Biol.Chem., 274: 19152-19160.

Valentin,E., F.Ghomashchi, M.H.Gelb, M.Lanzunski and G.Lambeau, 2000. Noval human secreted phospholipase A2 with homology to the group III Bee venom enzyme. J. Biol.Chem., 275: 7492-7496

William,F.S.jr., 1951.A study of the relative specificity of lipases produced by Penicillum roqueforti and Aspergillus niger. Arch Biochem., 30: 165 - 179

Yamakawa, Y. and A.Ohsaka, 1977. Purification and some properties of phospholipase of C.peringens. J. Biochem., 81: 115 - 121