Investigation of Phospholipase B Type Activity in Moringa oleifera Seeds

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Summary: Lipolytic enzyme activity was demonstrated in Moringa oleifera seeds by the use of lecithin as a substrate in aqueous and non aqueous system of assay. Comparatively high lipolytic activity in aqueous system suggested presence of a souble enzyme. Under both assay system the complete deacylation of ovolecithin resulted in water soluble glyceryl phosphorylcholine and the free fatty acid and no accumulation of monoacylphosphatidyl choline was observed.

Introduction

Much controversy in the lipolytic activity of phospholipase B enzyme has been reported in literature. In this connection enzymes from different sources have been explored and more research is being carried out in the settlement, whether the complete deacylation of diacylphosphoglyceride into free fatty acids and water soluble phospho-di-esters is caused by the one enzyme phospholipase B, [1] or by the two enzyme system [2]. However other workers have recently produced an evidence which strongly support the presence of diacylphosphohydrolases in E.coli [3], in potato [4], in intestinal mucosa [5] and in A.niger [6] respectively.

In the present study, an attempt is made to provide more evidence in support of the existance of Phospholipase B, by its presence in Moringa oliefera seeds. Also no enzymatic studies on Moringa oleifera seeds have been reported in the literature.

The name phospholipase B enzyme which deacylated both fatty acid ester linkages simultaneously from sn 3-phosphoglyceride has been reserved by Kates [7]. The complete deacylation of diacylphosphoglycerides could be achieved by the combined action of phospholipase.A and lysophospholipases, with former being rate limiting [2]. However other possibility leading to breakdown of phosphoglyceride by lipase [8,9], cannot be ignored since crude enzyme preparation used in this work showed the presence of lipase activity also.

Several methods for the assay system of phosphlipases have been proposed, but comparative values of lipolytic activity are difficult to evaluate because of the variation in parameters [10] employed such as assay conditions, preparation and stabilization of emulsion. Among the several methods currently in common use are aqueous assay system for soluble phospholipase and non aqueous assay system for insoluble or membrane bound phospholipase (11,12,13). Since the Morigna oleifera seed powder was used as the source of enzyme, the possibility of membrane bound or insoluble phospholipase can not be ignored. This will be useful to compare the results in aqueous and nonaqueous system of assay [14,15].

Experimental

The seeds of Moringa oleifera were collected during June-August in dry state from the plants in local areas from University of Sind employees colony.

Enzyme Preparation

(a) Enzyme powder preparation

The seeds of Moringa oleifera were defatted with diethyl ether according to the methods as reported by A.R. Memon [6], and the defatted residue was stored in vaccum desiccator until it was used.

(b) Soluble enzyme preparation

10 grams of defatted residue was crushed with glass powder in 30 ml ice cold distilled water and centrifuged. The supernatant was transferred to a volumetric flask of 100 ml capacity and this procedure was repeated twice and total volume of 100 ml was made up with distilled water to get the soluble enzyme.

Substrate Preparation

Egg lecithin purchased from Merck chemicals was dissolved (10%) in solvent di-isopropylether (dried with molecular sieve 4A) for non aqueous assay system. Water emulsion was prepared to give the required concentration (10% w/v) by using homogenizer (Universal Lab. Type 309 Poland) at maximum speed for ten minutes with successive intervals.

Phospholipase Assay Methods

1. Aqueous assay method:

In 50 ml conical flask 150 mg of enzyme powder preparation was taken in duplicate and 2.5 ml of freshly prepared substrate emulsion was added to each flask. Appropriate controls were also taken for blank enzyme and blank substrate. The conical flasks were covered with aluminium foil and incubated for one hour at 120 rev m^{-1} using shaking incubator (Gellenkamp) temperature adjusted at 37°C.

2. Aqueous assay system for soluble enzyme

In 50 ml conical flask 2.5 ml of soluble enzyme was taken in duplicate and 2.5 ml of substrate emulsion freshly prepared was added to each flask. Appropriate controls were also taken for blank enzyme and blank substrate. The conical flasks were covered with aluminum foil and incubated for one hour at 120 rev m^{-1} using shaking incubator, temperature adjusted at 37°C.

3. Non aqueous assay method

In 50 ml conical flask 150 mg of enzyme powder preparation was taken in duplicate and 2.5 ml of freshly prepared substrate and 0.1 ml distilled water, was added to each flask. Appropriate controls were also taken for blank enzyme and blank substrate. The flasks were covered with aluminium foil and incubated for one hour at 120 rev m^{-1} using shaking incubator, temperature adjusted at 40°C.

The reaction was stopped by adding chloroform:methanol (2:1 v/v) and free fatty acids liberated were estimated by titrating against 0.05N sodium hydroxide in methanol using thymol blue as an indicator [16]. The hydrolytic products were separated by extracting the aliquots after titration with chloroform:methanol (2:1 v/v) successively and the total extracts were collected and made up to 10 ml in volumetric flask with chloroform methanol mixture.

Characterization of Phospholipase Activity:

The nature of phospholipase activity was demonstrated from hydrolytic pro-

ducts using thin layer chromatography on silica gel G-60 and the solvent system used was according to the methods reported by McMurray and Morrison [17], chloroform-methanol-ammonia solution-water (120-70-10-5, v/v/v/v) and the separated components phosphatidyl choline, lysophosphatidyl choline developed with spraying zinzadze reagent [18] were observed to be disappeared in comparison with controls. The phospholipase activity were estimated by titration of liberated free fatty acids using suitable controls and the unit of activity was defined as the amount of free acids released in micro equivalents per 100 mg of enzyme per hour.

The various parameters like time, temperature, enzyme concentration substrate concentration and PH effect, studies were carried out by the use of above standard assay procedure.

Effect of Heat Treatment

It has been common practice to attempt to differentiate more than one activity in crude preparation by heating the enzyme in buffer or water for several minutes at different temperatures following which the remaining enzyme activities are examined. The effect of heat treatment on this preparation of Moringa oleifera powder was therefore studied in an attempt to determine the type of activity present.

The experiments were carried out by heating the soluble enzyme preparation for ten minutes at various temperatures between 50-98°C. The activity of this material was examined in the usual manner using the aqueous assay system for the soluble enzymes. The reaction products extracted were separated by thin layer chromatography using silica gel G-60 as described previously.

Effect of Sodium Deoxycholate

The phospholipase activity was demonstrated by incubating 150 mg of enzyme powder preparation with 0.13M evolecithin water emulsion as substrate with different concentrations (50 mg to 250 mg) of sodium deoxycholate and incubated in shaking incubator adjusted at 120 rev m^{-1} for 1 hour at 37°C the nature of phospholipase activity was demonstrated by thin layer chromatography.

Effect of Free Futty Acid

The effect of free fatty acids on the nature of the phospholipase activity was determined by incubating 150 mg of enzyme powder preparation with 0.13M evolecithin water emulsion as substrate with different concentration (25 meq to 150 meq) of palmitic acid incubated in shaking incubator at 120 rev m^{-1} for one hour at 37°C and the effect of fatty acid on lipolytic chromatography.

Results and Discussion

Time Curve

The time curve studies were demonstrated using aqueous and non aqueous assay system for the phospholipase activity of Moringa oleifera seed enzyme preparation. The results observed displayed a normal behaviour with the increase in time, the activity was also increased and declined after 3 hours suggesting deactivation of the enzyme. However 18 hours incubation showed only 24% decline in the activity. The product effect could not be suggested since the amount of free fatty acid released was not sufficient to show the inhibitory effect as observed in other experiments (Fig.1 and 2).

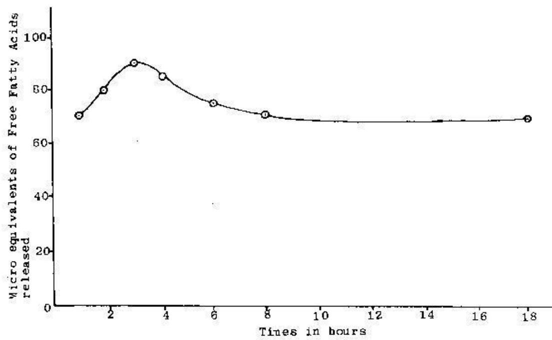

Fig.1: Graph showing effect of time on the amount of hydrolysis when 150 mg powedered enzyme preparation from Moringa oleifera seeds and 0.13M ovolecithin in emulsion form were incubated for different time period at 120 revm^{-1} at 37°C in snaking incubator.

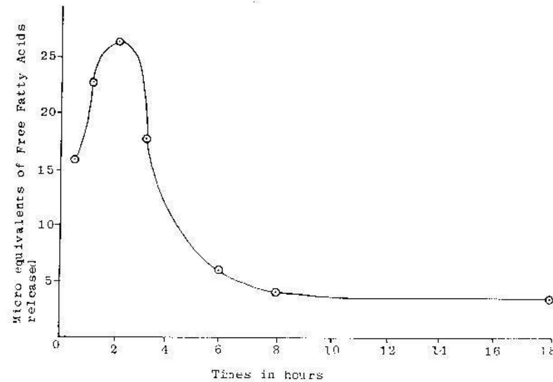

Fig.2: Graph showing effect of time on the amount of hydrolysis in non aqueous assay system when 150 mg powedered enzyme preparation from Moringa oleifera seeds and 0.13M ovoleci-thin were incubated for different time period at 120 rev m^{-1} on 40°C in shaking incubator.

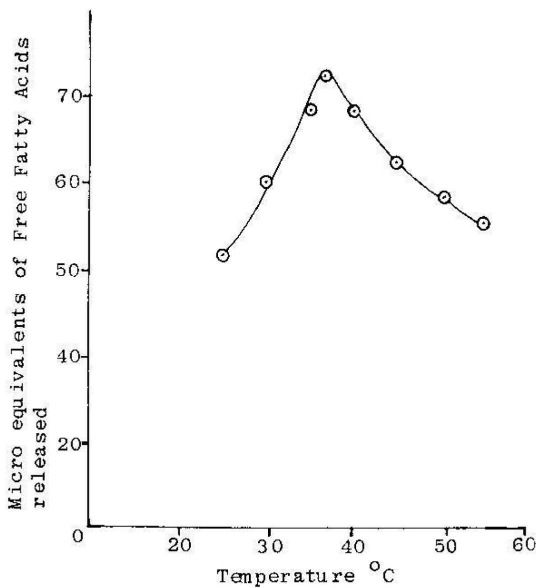

Fig.3: Graph showing effect of temperature on the amount of hydrolysis when 150 mg of Moringa oleifera seed powdered enzyme preparation and 0.13M ovo-llecithin in emulsion were incubated for different temperatures (25°C to 55°C) at 120 rev m^{-1} for one hour in shaking incubator.

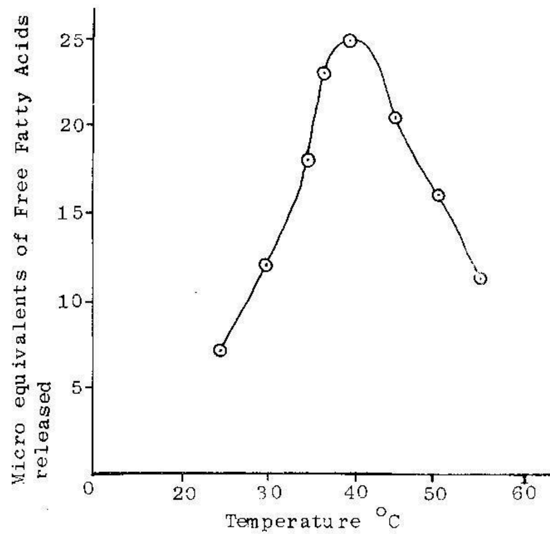

Fig.4: Graph showing effect of temperature on the amount of hydrolysis in non-aqueous assay system when 150 mg of Moringa oleifera seed powedered enzyme preparation and 0.13M ovolecithin were incubated for different tempe-ratures (25°C to 55°C) at 120 rev m^{-1} for one hour in shaking incubator.

Effect of Temperature:

The study of phospholipase activity was carried out at various temperatures between 25-55°C. The optimum temperature was found to be 37°C in aqueous and 40°C in non aqueous assay system. The results are shown in Fig (3) and (4).

Optimum pH:

The activity of Moringa oleifera enzyme preparation at various pH levels was assayed at 37°C. The results are shown in Fig. (5). The optimum pH was 5.2.

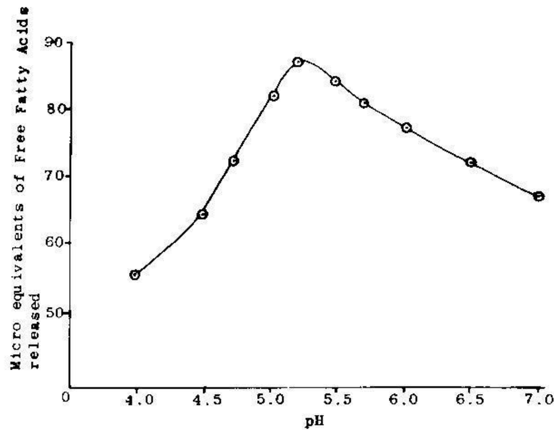

Fig.5: Graph showing effect of pH on the amount of hydrolysis when 150 mg of Moringa oleifera seeds powedered enzyme preparation and 0.13M ovolecithin in emulsion form were incubated for different pH values at 37°C for one hour at 120 rev m^{-1} in shaking incubtor.

Effect of Substrate Concentration:

The effect of substrate concentration on the of rate hydrolysis was followed by using 150 mg of Moringa oleifera seed enzyme powder preparation by incubating at 37°C using the aqueous assay system. The Lineweaver-Burk plot [19] shows a linear relation between 1/V and 1/S in the range of 0.0125 to 0.15M substrate concentration.

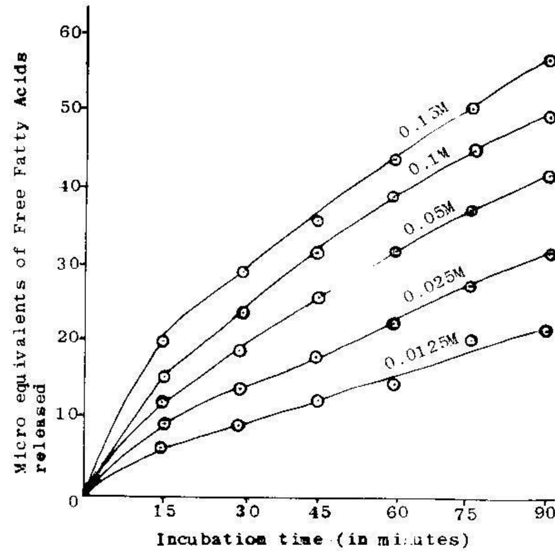

Fig.6: Showing rate of hydrolysis of ovolecithin by Moringa oleifera seed powder enzyme preparation using different amount of concentration of the substrate. The standard assay consistts of 150 mg of enzyme preparation poweder for different periods of time at 37°C.

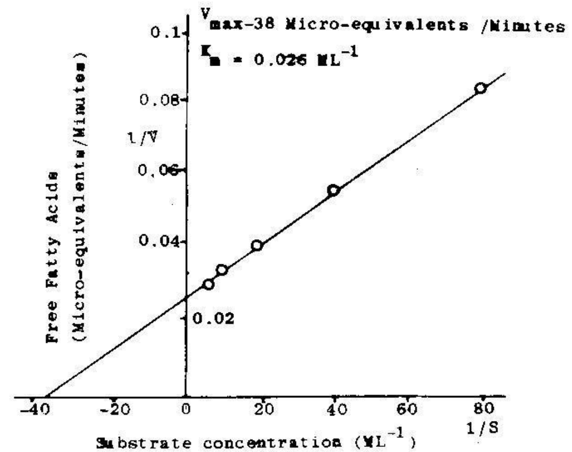

Fig.7: Linewearer-Burk Plot of the hydrolysis of ovolecithin by 150 mg enzyme preparation powder of Moringa oleifera seeds.

The results are shown in fig. (6) and from these rate curves and from the results for 45 minutes period at other concentrations, a Lineweaver-Burk plot was produced, this is shown in fig. (7) Km Value of 0.026M L^{-1}

and v_{max} 38 micro equivalents per minutes were calculated.

Effect of the Enzyme Preparation on Hydrolysis

The hydrolytic activity of the soluble and powder preparation of the enzyme was compared by using aqueous and nonaqueous assay system, and it was observed that in aqueous system of assay the hydrolytic activity was significantly similar in both preparations, while in non-aqueous system only 33% of the activity was observed. The comparatively less activity in the non-aqueous assay method could be ascribed with the accessibility of the enzyme substrate system [20], because the substrate taken was dissolved in non-aqueous solvent whereas the enzyme was water soluble.

Effect of Heat Treatment

Heat treatment has been previously used to demonstrate the presence of more than one enzyme by use of the effect of different heat inactivation. For instance lysophospholipases are more readily inactivated than phospholipase A_2 [11,5] phospholipase A_1 [21] in this case complete deacylation observed would be due to combined action of phospholipase A_1 and phospholipase A_2 and accumulation of lysophosphatidyl choline would have occured following heat treatment. This was not observed and no overall change in pattern of activity was in fact found. However some action is necessary before ascribing the observed activity to a phospholipase B type enzyme, and not all phospholipases are heat labile. For instance the enzyme activity associated with viper palestine venom is reported not to be inactivated [22]. The fact that no change in the pattern of the observed complete deacylation occurs on heat treatment does suggest that this activity is catalysed by single enzyme activity.

As reported previously the action of lipases in hydrolysing phosphoglyceride [8,9] in these experiments could also be suggested here, because the crude preparation of Moringa oleifera used in these experiments did give the lipase action. However the effect of heat treatment on the crude enzyme completely destoryed the lipase activity therefore this heat inactivation also confirms that the deacylation of phosphoglyceride possibly occurs by the phospholipase B and and not by lipase present (Table 1).

Effect of Sodium Deoxycholate:

Sodium deoxycholate is reported to be inhibitory towards the action of lysophospholipase [23,24] and to be stimulatory to phospholipase A_2 [25]. In the light of these observation the effect of sodium deoxycholate at different concentrations (50 to 250 mg) was studied with the enzyme of Moringa oleifera using aqueous assay system. Maximum 59.3% inhibitory effect was noted when 250 mg of sodium deoxycholate was used. Simultaneously no accumulation of lysophosphatidyl choline was noticed when the hydrolytic products separated on thin layer chromatography were studied. This suggested the existence of an inhibitory effect in the deacylation by the phospholipase B and also this finding therefore suggested the absence of lysophospholipase (Table 2).

Effect of Fatty Acid:

In order to study the effect of fatty acid, different amounts of palmitic acid in range 25 to 150 milli equivalents were incubated by the usual procedure with enzyme and substrate in aqoeus media. The low tirate value than the controls noted 74% inhibition at 150 milli equivalents of palmitic acid concentration. Some inhibitory effect by palmitic acid to the rat brain lysophospholipase was noted by Zelina Leibovitz and Shimon Gatt [26]. However the

Table 1: Results showing the relative percentage activity after heat treatment. Soluble enzyme preparation was heated for 10 minutes at different temperatures in presence of distilled water; 2.5 ml of soluble enzyme preparation of *Moringa oleifera* was incubated with 2.5 Ml of 0.13M ovolecithin emulsion at 37°C for 1 hour.

Temperature (°C) at which soluble enzyme was heated.	Percentage of total fatty acids released. % hydrolysis	Relative % activity
Control	21.23	100
50	19.69	92.74
60	17.85	84.07
70	16.92	79.22
80	14.46	68.11
90	13.23	62.31
90	13.23	62.31
98	11.38	53.60

Hydrolysis percentage is assumed both fatty acids are released.

Table 2: Effect of Sodium deoxycholate

Results showing the inhibition brought about by addition of sodium deoxycholate on the hydrolysis of ovolecithin. 2.5 ml of 0.13M ovolecithin emulsion was incubated with 150 mg of enzyme preparation powder of *Moringa oleifera* seeds in presence of different amounts of sodium deoxycholate for 1 hour at 37°C.

Sodium deoxycholate in mg.	% hydrolysis	% inhibition
Control	21.84	0.0
50	21.23	2.79
100	12.92	40.84
150	11.53	47.19
200	9.84	54.94
250	9.23	59.39

% hydrolysis is the percentage of total available fatty acids released assuming both acids potentially hydrolysable.

Table 3 : Effect of fatty acid (palmitic acid)

Results showing the inhibition brought about by addition of palmitic acid on the hydrolysis of ovolecithin. 2.5 ml of 0.13M ovolecithin emulsion was incubated with 150 mg of enzyme preparation powder of Moringa oleifera seeds in presence of different amounts of palmitic acid for 1 hour at 37°C.

Palmitic acid added in milli equivalent	Percentage of hydrolysis	Percentage of inhibition
C	21.84	00
25	20.00	8.42
50	15.23	30.26
75	13.55	37.95
100	10.00	54.21
125	6.61	69.73
150	5.69	73.94

% hydrolysis is the percentage of total available fatty acids released assuming both acids potentially hydrolysable.

chromatogram of reaction products separated by thin layer chromatography suggested that only phospholipase B type activity is inhibited by palmitic acid since no accumulation in lysophospholipid was observed on the same chromatogrsms (Table 3).

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