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PHOSPHATIDE ACYL-HYDROLASE AND TRIGLYCERIDE ACYL-HYDROLASE ACTIVITIES IN THE DEVELOPING SEEDS OF *CASSIA* SPECIES

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Variations in the activities of phosphatide acyl-hydrolase and triglyceride acyl-hydrolase with mature as well as developing seeds of *Cassia absus, Cassia fistula* and *Cassia occidentalis* were studied. Both the mature and developing seeds showed the presence of two acid lipase with pH optima 3.0 and 4.0 and a phospholipase with pH optimum 5.6 during maturation the lipase activities decreased from 7.6 μU and 6.3 μH per 20 seeds per hour in *C.absus* and *C.occidentalis* (both at 12 weeks after flowering WAF), to 3.3 μU per 20 seeds per hour (both at 12 WAF). In *C.fistula* the reduction was from 5.64 μU to 3.84 μU per 20 seeds per hour between 4-24 WAF. The activities of phospholipase were increased in the maturing seeds of the three species. The relative activities of the two enzymes studied in the various solvent systems were found to be similar.

Key words: Phospholipase, Phosphatide acyl-hydrolase, Triglyceride acyl-hydrolase.

INTRODUCTION

The role of triglyceride acyl-hydrolase (lipase Ec 3.1.1.3 [1-6] and phosphatide acyl-hydrolase (phospholipase A; Ec 3.1.1.4) in the resting seeds has extensively been studied [7-9]. From these studies it has been inferred that the lipase activity varies with changes in the seed environment. Similar studies, on the variation of lipase activities in the whole seedlings of the germinated seeds as well as their distribution in the primary roots of germinating seeds, have been also reported [10]. The activities of these enzymes in the maturing/developing seeds, however, seems to have attracted little or no attention at all.

Since these activities are of considerable consequence in the maturing/developing seeds, it was of interest to study them as well. As a result, therefore, the activities of phosphatide acyl-hydrolase and triglyceride acyl-hydrolase, with both mature and developing seeds of *Cassia absus, Cassia fistula* and *Cassia occidentalis* were studied and are reported here.

MATERIALS AND METHODS

Plant materials. The seeds of *Cassia absus, Cassia fistula* and *Cassia occidentalis* were harvested in the fields of PCSIR Laboratories, Lahore. The pods of *C.absus* and *C.occidentalis* were collected at 2 WAF till 12 WAF at interval of 2 weeks. The pods of *C.fistula* were collected at 4 WAF till 24 WAF, at intervals of 4 weeks. This difference in maturing period is due to certain genetic factors which are responsible for plant growth. The seeds were removed and stored in bags in a refrigerator till further analysis.

Isolation of lipase and phospholipase. The mature seeds (100 seeds) of the three *Cassias* species were hemogenised separately in 10 ml of 0.1 M citrate disodium hydrogen phosphate buffer (pH 5.0) for 5 minutes and then stirred further for one hour. The pH was checked by pH meter (HI 8414 STOCKIATIC). The mixture was centrifuged at 4000 rpm for 15 minutes and the supernatent (containing the enzyme) was removed. The enzyme extracted was measured and frozen to avoid the loss of activity. The seeds of the three species were collected between 2 – 12 WAF (4-24 WAF for *C.fistula*). The lipase and phospholipase in the seeds of each maturity stage were isolated and assayed as given for mature seeds. The lipase of matured seeds of the three species gave maximum activity at pH 4.0 and phospholipase at pH 5.6, so all the studies were made at pH 4.0 for lipase activity and at pH 5.6 for phospholipase activity.

Lipase assay in aqueous system. To the enzyme extract (1 ml), was added 5 ml of 0.1 M citrate disodium hydrogen phosphate buffer of pH 5, 1 ml of 10 % triolein emulsion, 1 ml of calcium chloride (final concentration was 0.1 M). The final volume of the mixture was made upto 10 ml with distilled water. The mixture was incubated at 40° for one hour in Gallen-Kamp shaking water bath. After incubation period the reaction was stopped by adding 5 ml chloroform; hexane (1:1) and the organic layer containing free fatty acid was separated. The absorbance was measured by Hitachi Model spectrophotometer at 440 nm and the

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concentration of free fatty acid was deduced from the standard curves. The appropriate controles were made for blank in the same manner by using 1 ml of boiled enzyme. Lipase activity was calculated as micro unit (μU) per 20 seeds per hour by following the method of K.C. Guven [12].

The effect of pH on the lipase activity of *Cassia* seeds was determined by incubating the above reaction mixture at various pH (2.6 to 6.0) using 0.1 M citrate disodium hydrogen phosphate buffer for 1 hour and the lipase activity was determined as described above.

Lipase assay in organic solvents. The assay method was followed as reported by Blain [13]. The enzyme powder from 20 seeds (with 5 % moisture content) was placed into 25 ml stoppered flask, to which was added 25 μL water and 2.5 ml of triolein (10 % w/v) in di-isopropyl ether. The mixture was then incubated in Gallen-Kamp shaking water bath at 50° for two hours. After the reaction, the mixture was cooled under tap water and an additional 2.5 ml of the solvent was thoroughly mixed. The mixture was then centrifuged at 2000 rpm for 2 minutes and 2.5 ml of the supernatent was pipetted into 10 ml ethanol in a 25 ml titration flask and titrated with 0.1 N NaOH using thymolphthalein blue as indicator. Enzyme blank was obtained by taking 2.5 ml of di-isopropyl ether instead of the substrate solution. The enzyme activity was calculated in term of micro moles free fatty acids liberated per hour.

Lipase activity was determined as described above by using triolein (10 % w/v) in ethyl methyl ketone, cyclohexane, cyclo-hexanol and *n*-heptane.

Phospholipase assay in aqueous systems. Into a stoppered flask was added 1 ml of enzyme extract of *C.absus,* 0.1 ml of calcium chloride 0.1 M, 1 ml of 0.1 M citrate disodium hydrogen phosphate buffer of pH 5.0 and 1 ml of lecithin (BDE Polle, England; purified by acetone precipitation) solution (2 mg) and 1 ml ether.

The reaction mixture was incubated at 37° for one hour in a Gallen-Kamp shaking water bath. The flask was cooled, the fatty acids were extracted with 3 ml chloroform: hexane (1:1) and the final volume was made upto 5 ml. The appropriate controls were made for blank in the same manner by using 1 ml of boiled enzyme. The organic layer was separated and the concentration of the free fatty acids as well as the phospholipase activity in micro unit (μU) per 20 seeds per hour was determined by the following method of K.C. G.uven [12].

The effect of various solvents and pH between 2.6 to 7.6 on phospholipase activity was carried out similarly as described under lipase except that the assay method and calculation were those for phospholipase [14].

Phospholipase specificity. The product from enzymatic hydrolysis were extracted with diethyl ether and methanol solution which was removed under reduced pressure by means of a rotary film evaporator under nitrogen. The dry residue was dissolved in chloroform and the resulting solution was filtered and made upto known volume with the requisite amount of chloroform: methanol (2:1) to get a solution containing free fatty acids, lysolecithin and unchanged lecithin which was then stored in refrigerator at 5°. Portions of this solution containing more than 400 μg of lipid were taken to dryness under nitrogen in the rotary film evaporator. As rapidly as possible the dry residue was dissolved in a small volume of chloroform and was then fractionated on column of silicic acid which was prewashed with chloroform: methanol (1:1) and then with chloroform alone. The fatty acids were eluted with the requisite volume of chloroform, unchanged lecithin with chloroform: methanol (3:2) and lysolecithin with methanol alone. The fatty acids of all these fractions, i.e., free fatty acids, lysolecithin and unchanged lecithin obtained from the silicic acid column were converted to their corresponding methyl esters according to the method described by Morrison and Smith [15]. The methyl esters of the three fractions were analysed by GC analysis according to the method of Akhtar [11].

RESULTS AND DISCUSSION

Lipase and phospholipase in Cassia seeds during maturation

Variation in lipase activity. Seeds of *C.absus* and *C.occidentalis* were collected between 2-12 WAF (at intervals of 2 weeks) and those of *C.fistula* were collected between 4-24 WAF (at intervals of 4 weeks). Lipase was extracted with 0.1 M buffer solution of citrate disodium hydrogen phosphate (pH 5.0) from the matured as well as the developing seeds of the three species at each stage of maturity and was analysed as described before.

Effect of pH lipase activity in mature seeds. Figures, 1-3, show the effect of different pH on the lipase activity of matured seeds of the three species. The optimal pH was determined with 0.1 M citrate disodium hydrogen phosphate buffer adjusted between 2.6 to 6.0 pH. Two optimum peaks were obtained showing a lipase at pH 3.0 with lipolytic activities as 2.8 μU, 3.2 μU and 2.8 μU per 20 seeds per hour respectively, in *C.absus, C. fistula* and *C.occidentalis* and the other pH 4.0 with activities as 3.3 μU, 3.84 μU and 3.82 μU per 20 seeds per hour respectively, in the three species. As the lipase activity was mixiumum at pH 4.0 further studies were made at this pH.

Variation in lipase activity at pH 4.0 during maturation. Variations in the lipase activities obtained from

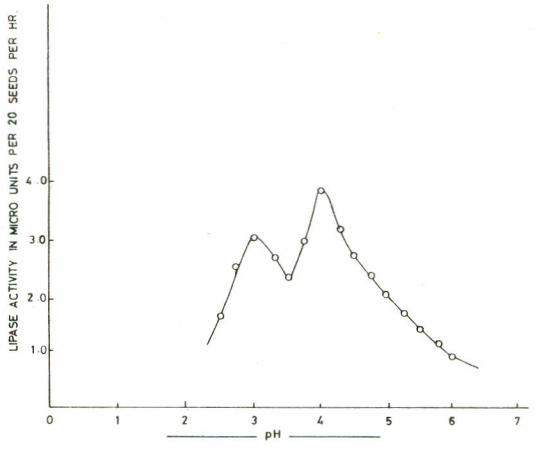

Fig. 1. Effect of pH on the lipase activity of mature seeds of *Cassia fistula*. The reaction mixture, consisting of 1 ml of the enzyme extract 5 ml of 0.1 M citrate buffer, 1 ml 10 % triolein emulsion and 1 ml of 0.1 M calcium chloride solution was incubated at 40° for one hour.

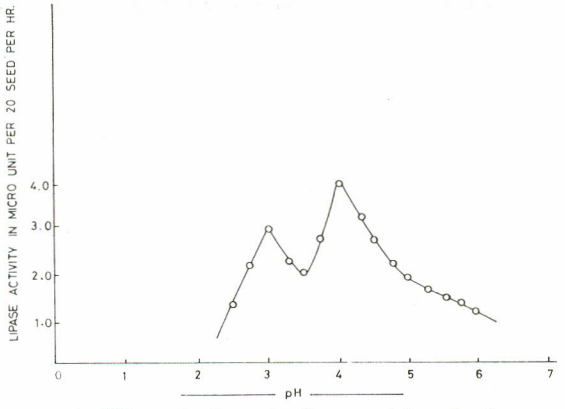

Fig. 2. Effect of pH on the lipase activity of mature seeds of *Cassia occidentalis*. The reaction mixture, consisting of 1 ml of the enzyme extract, 5 ml of 0.1 M citrate buffer, 1 ml 10 % triolein emulsion and 1 ml of 0.1 M calcium chloride solution was incubated at 40° for one hour.

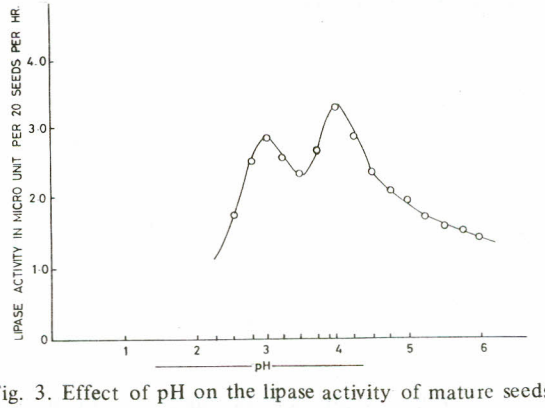

Fig. 3. Effect of pH on the lipase activity of mature seeds of *Cassia absus*. The reaction mixture consisting of 1 ml of the enzyme extract, 5 ml of 0.1 M citrate buffer, 1 ml 10 % triolein emulsion and 1 ml of 0.1 M calcium chloride solution was incubated at 40° for one hour.

different stages of maturity at pH 4.0 are shown in Table 1. Maximum activities were obtained after 2 WAF which were 7.76 μU and 6.13 μU per 20 seeds per hour in *C.absus* and *C.occidentalis* and 5.64 μU per 20 seeds per hour in *C. fistula* after 4 WAF. As the seeds matured, the lipase activities decreased to 3.3 μU and 3.82 μU per 20 seeds per hour in *C.absus* and *C.occidentalis* at 12 WAF and 3.84 μU per 20 seeds per hour in *C.fistula* at 24 WAF.

Table 1. Lipase activity in the seeds of *Cassia absus, Cassia fistula* and *Cassia occidentalis* at various stages of maturity.

Weeks* after flowering	Lipolytic activity (μU) per 20 seeds per hour in *C. absus*	Lipolytic activity (μU) per 20 seeds per hour in *C. fistula*	Lipolytic activity (μU) per 20 seeds per hour in *C. occidentalis*
2	7.76	5.64	6.13
4	6.98	5.38	5.78
6	6.11	5.08	5.27
8	5.39	4.68	4.96
10	4.45	4.14	4.34
12	3.30	3.84	3.82

*For *C. fistula* samples, weeks after flowering were 4, 8, 12, 16, 20 and 24.

The reaction mixture, consisting of 1 ml triolein (10 %) emulsion, 5 ml 0.1 M citrate buffer (pH 4.0), 0.1 ml, 0.1 M calcium chloride and 0.1 ml the enzyme extract was incubated at 40° for one hour.

Effect of solvents on lipase activity of mature seeds. The lipase activities of mature seeds of the three species in various organic solvents are shown in Table 2. Maximum activities were observed in di-isopropyl ether which were 10.2 μ Moles, 7.9 and 8.2 μ Moles free fatty acids released per seed per hour in *C.absus, C.fistula* and *C.occidentalis* respectively. The lipase activities in *C.absus, C.fistula* and *C.occidentalis* were 7.6 μ Moles, 6.1 μ Moles and 6.6 μ Moles free fatty acids, released per seed per hour respectively in ethyl methyl ketone; 4.9 μ Moles, 4.6 μ Moles and 4.4 μ Moles, 4.6 μ Moles and 4.4 μ Moles free fatty acids released per seed per hour respectively in cyclohexane; 4.6 μ Moles, 4.4 μ Moles and 4.2 μ Moles free fatty acids released per seed per hour respectively in cyclohexanol and 4.4 μ Moles, 3.8 μ Moles and 3.2 μ Moles free fatty acids released per seed per hour respectively in *n*-heptane.

Variation in phospholipase activity. Phospholipase activities were also determined in the seeds of *C.absus, C.fistula* and *C.occidentalis* at various stages of seed maturity under the conditions described before.

Table 2. Lipase activity in the mature seeds of *Cassia absus, Cassia fistula* and *Cassia occidentalis* in defferent solvent systems.

Solvet system	Lipolytic activity (μM) free fatty acids released per seed per hour in *C. absus*	Lipolytic activity (μM) free fatty acids released per seed per hour in *C. fistula*	Lipolytic activity (μM) free fatty acids released per seed per hour in *C. occidentalis*
Di-isopropyl ether	10.20	7.90	8.20
Ethyl methyl ketone	7.60	6.10	6.60
Cyclohexane	4.90	4.60	4.40
Cyclohexanol	4.60	4.40	4.20
n-Heptane	4.70	3.80	3.20

The reaction mixture consisted of enzyme powder from 20 seeds (with 5 % moisture content) in 5 ml of triolein solution (10 %) in different solvents. The reaction was carried out at 50° for two hours.

Effect of pH on phospholipase activity in mature seeds. Effect of pH on phospholipase activity in mature seeds of the three species has been determined. To determine optimal pH 0.1 M citrate disodium hydrogen phosphate buffer was used between pH 2.6 to 7.6 as described for lipase activity. As shown in Fig. 4-6 phospholipase was found to be most active at pH 5.6 under the assay conditions described. The phospholipase activities at this pH were 5.86 μU, 7.2 μU and 5.83 μU per 20 seeds per hour in *C.absus, C.fistula* and *C.occidentalis* respectively.

Variation in phospholipase activity at pH 5.6 during maturity. As shown in Table 3 the phospholipase showed low activities in immature seeds of *C.absus* and *C.occidentalis* at 2 WAF which were 3.26 μU and 3.65 μU per 20 seeds per hour and 4.48 μU in *C.fistula* at 4 WAF. The activities increased during maturity and were found to be maximum, i.e., 5.86 μU and 5.83 μU in *C.absus* and *C. occidentalis* at 12 WAF while the activity in *C.fistula* was 2.24 μU per 20 seeds per hour at 24 WAF.

Effect of solvents *on phospholipase activity of mature seeds.* The phospholipase activities were also determined in various solvent systems and shown in Table 4. The activities in *C.absus, C.fistula* and *C.occidentalis* were 14.8 μ Moles, 24.2 μ Moles and 14.6 μ Moles free fatty acids released per seed per hour in ethyl methyl ketone respectively; in diisopropyl ether the activities were 0.1 μ Moles,

Table 3. Phospholipase activity in the seeds of *Cassia absus, Cassia fistula* and *Cassia occidentalis* at various stages of maturity.

Weeks* after flowering	Phospholipase activity (μU) per 20 seeds per hour in *C. absus*	Phospholipase activity (μU) per 20 seeds per hour in *C. fistula*	Phospholipase activity (μU) per 20 seeds per hour in *C. occidentalis*
2	3.26	4.48	3.65
4	3.97	4.92	4.17
6	4.47	5.32	4.64
8	4.99	6.14	5.07
10	5.22	6.99	5.28
12	5.86	7.20	5.83

*For *C. fistula* sample weeks after flowering were 4, 8, 12, 16, 20 and 24.

The reaction mixture, consisting of 0.1 ml lecithin .3 μM, 0.6 ml of 0.1 M citrate buffer, 0.1 ml ether, 0.1 ml, 0.1 M calcium chloride and 0.1 ml, the enzyme extract, was incubated at 37° for one hour.

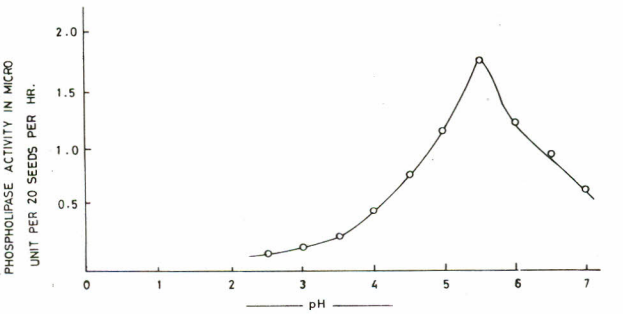

Fig. 4. Effect of pH on phospholipase activity of the mature seeds of *Cassia absus.* The reaction mixture consisting of 0.1 ml lecithin (3 μM), 0.60 ml of 0.1 M citrate buffer, 0.1 ml ether 0.1 ml (0.1 M) calcium chloride and 0.1 ml of the enzyme extract was incubated at 37° for one hour.

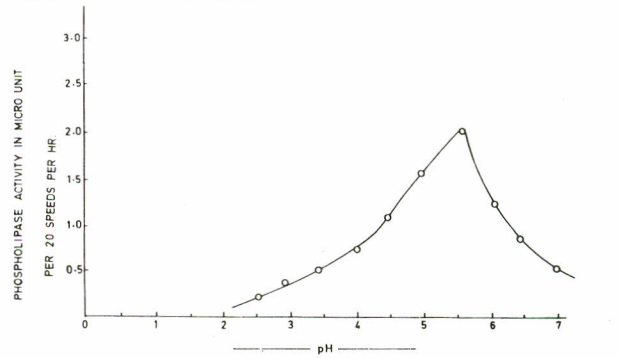

Fig. 5. Effect of pH on phospholipase activity of the mature seeds of *Cassia fistula.* The reaction mixture consisting of 0.1 ml lecithin (3 μM), 0.60 ml of 0.1 M citrate buffer 0.1 ml ether, 0.1 ml (0.1 M) calcium chloride and 0.1 ml of the enzyme extract was incubated at 37° for one hour.

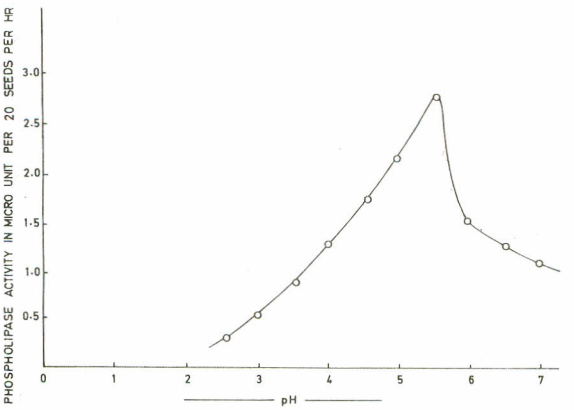

Fig. 6. Effect of pH on phospholipase activity of the mature seeds of *Cassia occidentalis*. The reaction mixture consisting of 0.1 ml lecithin (3 μM), 0.60 ml of 0.1 M citrate buffer, 0.1 ml ether, 0.1 ml (0.1 M) calcium chloride and 0.1 ml of the enzyme extract was incubated at 37° for one hour.

Table 4. Phospholipase activity in the mature seeds of *Cassia absus, Cassia fistula* and *Cassia occidentalis* in different solvent systems.

Solvent system	Phospholipase activity (μM) free fatty acids released per seed per hour in *C. absus*	Phospholipase activity (μM) free fatty acids released per seeds per hour in *C. fistula*	Phospholipase activity (μM) free fatty acids released per seeds per hour in *C. occidentalis*
Ethyl methyl ketone	14.80	24.20	14.60
Di-isopropyl ether	9.10	12.60	8.90
Cyclohexane	7.30	7.50	7.40
Cyclohexanol	6.00	6.30	6.20
n-Heptane	5.40	5.60	5.10

The reaction mixture, consisted of enzyme powder from 20 seeds (with 5 % moisture content) in 2 ml lecithin solution (10 %) in different solvents. The reaction was carried out at 50° for two hours.

12.6 μ Moles and 8.9 μ Moles free fatty acids released per seed per hour; in cyclohexane the activities were 7.3 μ Moles, 7.5 μ Moles and 7.4 μ Moles free fatty acids released per seed per hour; in cyclohexanol the activities were 6.0 μ Moles, 6.4 μ Moles and 6.2 μ Moles free fatty acids released per seed per hour and in *n*-heptane the activities were 5.4 μ Moles, 5.6 μ Moles and 5.1 μ Moles free fatty acids released per seed per hour respectively.

Specificity of phospholipase. The fatty acid composition of the three fractions, i.e., free fatty acids, lysolecithin and unchanged lecithin obtained from the hydrolysis product was determined Table 5 (Results for minor fatty acids are not given). As shown in Table 5, the quantity of

Table 5. The composition of the individual fatty acids (molar percentage of the total) in the fatty acids, lysolecithin and unchanged lecithin after 1 hour hydrolysis with enzyme.

Fractions	$C_{16:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:2}$
Fatty acids	16.4	10.5	47.6	22.2
Lysolecithin	40.9	26.0	20.4	10.5
Lecithin	32.9	12.6	31.8	14.0

$C_{16:0}$ found in lecithin was 32.9 % whereas the level determined in free fatty acids and lysolecithin fractions obtained after hydrolysis of lecithin were 16.4 % and 40.9 % respectively. The contents of $C_{18:0}$ were 26.0 % in lecithin and 10.5 % in free fatty acids while in lecithin the amount of $C_{18:0}$ was 12.6 %. $C_{18:1}$ showed increased level in free fatty acids 47.6 % as compared to lysolecithin 20.4 % and lecithin 31.8 %. The proportions of $C_{18:2}$ found in free fatty acids, lysolecithin and lecithin were 22.2 %, 10.5 % and 14.0 % respectively. All the three species showed similar results for lecithin, lysolecithin and free fatty acids.

Lipase was extracted with 0.1 M buffer of citrate disodium hydrogen phosphate (pH 5.0) from the mature seeds of *C.absus, C.fistula* and *C.occidentalis*. The activities were determined at various pH (2.6-6.0) which showed two pH optima, i.e., pH 3.0 and pH 4.0. More than one pH optima have also been reported in castor beans, coconuts and rice bran [16-18]. The lipase activities were determined at all the stages of maturity, at pH 4.0 and showed a reduction with maturity of the three *Cassia* species. Studies were also conducted to examine the effect of various organic solvents on lipase activities of the matured seeds of the three species. Maximum lipase activity was obtained in diisopropyl ether.

The effect of maturity on phospholipase activity was also examined in the mature and developing seeds of *C.absus, C.fistula* and *C.occidentalis*. The phospholipase activities were lower in the immature seeds of the three species but increased as the seeds matured. The effect of pH showed only one pH optima 5.6 in the mature seeds of the three species as compared to the lipase activities which showed two pH optima. The phospholipase activities were determined at all the stages of maturity at pH 5.6. The

phospholipase activities were also examined in different organic solvents. The activity of phospholipase unlike the lipase was higher in ethyl methyl ketone.

Specificity of the phospholipids activity, which could be due to any of the enzymes, phospholipase A, phospholipase B, phospholipase C and phospholipase D has already been determined. Egg lecithin was, therefore, hydrolysed by the enzyme extract from *C.absus, C.fistula* and *C.occidentalis.* The reaction product was separated into fractions which were free fatty acids, lysolecithin and unchanged lecithin. The fatty acid composition of each fraction was determined by GC analysis. The free fatty acid composition showed large quantities of unsaturated fatty acids which indicated that they were derived from the β-position of lecithin. The fatty acids of lysolecithin on the other hand contained large amount of saturated fatty acids showing that the acids are derived from the α-position of lecithin. It is concluded from the investigations that the phospholipase extracted from the three species attacks the ester bond located at β-position of lecithin which is specific for phospholipase A.

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