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NOTE

Production of Lipase and Phospholipase Enzymes from *Pseudomonas sp*. and Their Action on Phospholipids

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Abstract: One bacterial strain was isolated from soyphospholipid enriched media of pseudomonas group and cultured in peptone, yeast extract and beef extract containing media. The kinds of enzyme from the bacteria appeared to have both lipase and phospholipase activity and specificity as evident from their mode of action on triacylglyceride and phospholipid as well.

Key words: *Pseudomonas*, lipase, phospholipase

Lipolytic enzymes have an increasing use in the food and detergent industries and recently their role in producing various structured and/or modified lipids has been emphasized (1, 2). Lipases are enzymes that hydrolyse both triacylglycerides and phospholipids (3), while phospholipases only hydrolyse phospholipids (4). A great deal of work has been carried out for the isolation of both kinds of lipases from various sources particularly from microbial sources (5-10). Various report show that phospholipases are produced from animals, plants and microorganisms and a number of phospholipases of different kinds have been purified and characterized from *Pseudomonas sp*. (11, 12). Other report shows that it can utilize peptone and yeast as nitrogen sources (13).

This report presents a preliminary investigation on the kinds of lipases produced from the *Pseudomonas putida* SB 3 obtained from soil enriched with soyphospholipid.

All solvents used were of AR grade and purchased from E-MERCK India Ltd., Mumbai, India. Refined Soybean oil with no acetone insoluble was obtained at laboratory by successive extraction with acetone of commercial oil collected from local market (Kolkata, India) and finally storing the oil at 5℃ for 10 days and filtered through a Buckner filter. Purity of the samples was tested by TLC analysis. Soyphospholipid was extracted from crude soybean oil by water degumming method and was purified by repeated Chloroform-methanol extraction.

Soil samples were collected from crop fields and gardens. There were altogether 15 samples of soil of which 10 were selected for the experiment on modification of soyphospholipid by *enrichment test* (14).

Morphological and physiological characterization of isolates was examined at different time for Gram reaction and cell morphology (identification was done at National Institute of Cholera and Enteric Diseases, Kolkata). Other biochemical tests were performed following directions of the latest edition of Bergey's man-

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ual (15).

Cultures were incubated in the sterilized nutrient media (consisting of 1g beef extract, 2g yeast extract, 5g peptone, and 0.5g sodium chloride in 1000mL of distilled water) with shaking at 37℃ for 3 - 15 days. The pH of the medium was kept nearly at 7.0. To the resulting culture broth 0.1% (w/v) of deoiled phospholipid was added as substrate and incubated at 37℃ under continuous shaking. Samples from the cultures as well as from sterile controls were collected and analyzed by thin layer chromatography.

The hydrolytic activity of enzymes was determined by cupric-acetate method (16). To monitor the enzyme reaction, 1 mL of the culture supernatant was taken in a screw capped test tube, which was solubilized in 1mL of standard phosphate buffer, pH 7.0, followed by the addition of four drops groundnut oil (GNO). The test-tube was then shaken vigorously at 162 strokes/min in a shaker at 37℃ for 30 min. At the end of the reaction, 6(N) HCl and 5 mL of isooctane was added to stop the reaction and to extract the free fatty acids (FFA). Then 1 mL of clear isooctane in the upper layer was carefully drawn into a test-tube and its free fatty acid content was determined from the standard curve by the above method (1U of enzyme activity = μmol of FFA produced/min.)

Of all the bacteria isolates, a particular gram negative, rod shaped strain, SB 3 was found to produce the enzyme to a considerable extent.

Preparation of the acetone powdered form of the lipase & phospholipase was done by taking 60 mL of the culture medium (72 h) mixed with 60 mL acetone and keeping it for 1h at 0℃ in a refrigerator. This was then centrifuged in a cold centrifuge at 4℃ at 15,000g for 20 min. The clear liquid at the top was decanted and the whole residual mass was dried in a vacuum at 40℃ for 1h. The powder form of enzyme was stored at -30℃.

Esterification activity of enzymes was measured (17) with butyric acid (0.16 mol) and butanol (0.33 mol) in n-heptane. The experiment with each lipase was set up with 50 mL stoppered conical flask containing 3 mL of stock solution with the appropriate quantity of the lipase. Butanol did not react with butyric acid in the absence of the lipase within 2 h employed for measuring the activity.

Assay of phospholipase enzymes was done by the action of isolated enzymes on phospholipid. As phospholipids can be hydrolysed by both lipase and phospholipase enzymes and to ascertain therefore the phospholipase activity in the enzyme mixture, the detail analysis of the hydrolysed product should be determined. The deoiled phospholipid (about 0.4g) was taken in a conical flask, 1 mL phosphate buffer along with 1 mL supernatant from the culture was added and the mixture was shaken for 30 min at 37℃. The reaction was terminated by the addition of 1 ml of 1 (M) HCl and then the culture treated phospholipids were extracted with chloroform/methanol (3:1, v/v) mixture. The extracted mixture was spotted on a TLC plate. First the plate was developed with Hexane and Ether (80:20, v/v) to separate the neutral lipids. The neutral lipids (DG, MG, and FFA) were visualized by iodine vapour and again extracted back from TLC plate with petroleum ether solvent (40 - 60℃). The phospholipid fraction was again spotted on TLC plate and the plate was developed with chloroform/methanol/water (30:10:1 by vol). The spots were visualized by iodine vapour and extracted back by petroleum ether (40 - 60℃).

Gas Liquid Chromatogram gave the fatty acid composition of the products after preparing FAME of corresponding fatty acids (18).

Among the eight strains that were found to synthesize lipase, strain SB 3 shows the highest lipase activity of about 0.54 U/ml as determined by the cupric acetate method (**Table 1**) and gram staining and morphological test of that strain is given in (**Table 2**).

Table 1 Specific Activity of Bacterial Strains Isolated from Soyphospholipid Enriched Soil.

Strain	Specific activity of Lipase (U/ml)
SB 1	0.12
SB 2	0.11
SB 3	0.54
SB 4	0.03
SB 5	0.51
SB 6	0.23
SB 7	0.33
SB 8	0.04

(1U of enzyme activity = 1 μmol of FFA produced/ min)

Table 2 Identification of Bacterial Strains.

Characteristics	Strain SB 3
Gram	Negative
Shape	Rod
Number of flagella	＞1
Fluorescent, diffusible pigments	＋
Non-diffusible no fluorescent pigments	－
Poly-β-hydroxybutyrate accumulation	－
Growth at 41℃	－
Growth at 4℃	d
Oxidase reaction	＋
Denitrification	－
Gelatin hydrolysis	－
Starch hydrolysis	－
Utilisation of : Glucose	＋
Trehalose	－
2-Ketogluconate	＋
meso-inositol	－
Geraniol	－
l-Valine	＋
β-Alanine	＋
l-Arginine	＋
Nitrate used as a nitrogen source	＋

Table 3 Comparison of Hydrolytic Activities of Lipases Obtained from SB 3 between Triacylglycerol and Phospholipid.

Sample	Hydrolysis of triacylglycerol	Hydrolysis of phospholipid
with culture medium containing no phospholipid	0.31 ± 0.01	0.49 ± 0.11
with culture medium containing phospholipid	0.54 ± 0.09	0.73 ± 0.18

The higher hydrolytic activity of the strain towards phospholipid (**Table 3**) led to think about the presence of phospholipase enzyme which could be assessed by activities of the said enzyme on phospholipid itself. The effect of phospholipid in the culture medium in 1% level could also be understandable from the results shown in the **Table 3**.

When the phospholipid was hydrolysed with the enzyme isolated from the medium without any phospholipid, the fatty acid composition of the lysophospholipid obtained was almost similar to that obtained from the control system. This fact implies that the kind of lipase present in this enzyme preparation is either of random in nature or it is a mixture of lipase and phospholipase although have not been identified.

The kinds of enzymes collected from culture medium (of 72 and 96 h) with and without phospholipid were reacted with deoiled phospholipid in presence of phosphate buffer at pH 7 in order to ascertain the nature of hydrolyzed products. The resultant masses were extracted with chloroform and methanol, dried over sodium-sulphate and finally spotted on TLC plates. The plates were developed with Hexane: Diethyl ether (60:40, v/v) and the spots were visualized by iodine vapour. In order to characterize the kinds of lipase obtained from no phospholipid medium, soyphospholipid was hydrolysed and the hydrolysis product were separated by TLC when two spots -- one of lysophospholipid and one of free fatty acid along with original phospholipid were visualized. Similarly kinds of lipases obtained from phospholipid containing medium when used to hydrolyse soyphospholipid, four spots - two of diglycerides, one of lysophospholipid and one of free fatty acid along with the original phospholipid were visualized by TLC separation. This observation led to think about the presence of different types of lipases in the enzyme preparation. Although not purified, but it appears that there may be two varieties of phospholipases viz. phospholipase A2 and phospholipase C and / D are present in the enzyme obtained from the phospholipid containing medium. The formation of diacylglyceride indicates the cleavage of phosphate bond from the phospholipid molecule. The fatty acid composition of two diacylglycerides produced from the isolated enzyme is shown in **Table 4**. The two different diacylglycerides, both of similar composition, was found in case of enzyme obtained from phospholipid containing medium and the fact can only be justified by the acyl migration of the fatty acid molecule through glycerol backbone.

The esterification activity units are based on measurement of initial rates, which may also vary with tem-

Table 4 Fatty Acid Composition of the Diacylglycerides Produced from the Hydrolysis of Phospholipid by Enzymes Obtained from Phospholipid Containing medium.

Sample	$C_{16:0}$	$C_{18:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Original soyaphospholipid	23.1	2.4	20.7	52.2	1.7
Diacylglyceride obtained by enzyme hydrolysis of phospholipid (No. 1)	31.6	3.6	25.6	36.1	0.9
Diacylglyceride obtained by enzyme hydrolysis of phospholipid (No. 2)	32.9	3.2	24.9	34.6	0.5

Table 5 Comparison of the Esterification Activities between the Lipases Obtained from SB 3.

Lipase	Esterification activity in 1 μmol of butyric acid consumed per min per mg lipase
Lipase obtained from the no phospholipid medium	0.36 ± 0.9
Lipase obtained from the medium containing phospholipid	0.67 ± 0.11

perature and lipase source. The esterification of butanol with butyric acid was studied at 30, 40, 50 and 60℃. At 30 and 40℃, the extent of conversion was not satisfactory for accurate measurement of titre volumes. At 60℃, the reaction did not exhibit any regularity in esterification behavior. Hence, only data obtained at 50℃ is shown in **Table 5**. The results were highly reproducible. Although, the water activity of the reaction system was not determined. These results again prove the production of lipase enzymes with higher esterification activity in presence of phospholipid in the culture medium.

The production of lipase by *Pseudomonas species* is not uncommon. But simultaneous production of lipase and phospholipase by regulating the culture medium is prima facie new. This could be a valuable and potential finding to isolate phospholipase enzymes along with lipase from *Pseudomonas species*.

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