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Purification of catecholase from Solanum melangema (brinjal)

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Catecholase was purified from cortex of Solanum melangena (brinjal) on natural affiant, lignin. The elution profile showed seven peaks withthe 6th peak having 4616-fold purity. The 6th pure fraction loaded on PAGE showed two protein bands on staining with Coomassie brilliant blue, one at the point of application and other neat he dye front. These bands exhibited catecholase activity, when stained with4-methyl catechol and proline, Basic fuschin and ethidium bromide showed positive tests, indicating that catecholase is a ribonucleoglycoprotein.

Keywords : brinjal, Solanum, melangena, polyphenol oxidase, 4-methyl catecholase, o-diphenol oxidase

Brinjal (Solanum melangena, L.) is a commonly grown vegetable in India and the most of the popular cultivars used are purple in color with white strips and oval shaped fruits. When fresh fruits and vegetables are damaged, they turn into brown color, due to polyphenol oxidase (PPO), which is of economic concern to most fruits/vegetables processors. PPO is a group of enzymes which are ubiquitous in nature and classified on the basis of substrate specificity i.e. cresolase [E.C. 1.14.18.1] and catecholase or orthodiphenol oxidase (EC1.10.3.2). Browning of fruits/vegetables occurs due to the oxidation of 0-diphenols to 0-quinone1. The matricesused for the purification of a number of enzymes, such as amylase are either natural affiants viz, starch, chitin, lectins etc. or the commercial Sepharose column coupled with the inhibitors of respective enzymes2,3. PPO from a number of sources is reported to be a glycoprotein4,5,6, hence, the lectin-bound matrics have also been used for its purification7,8. Covalent attachment of RNA to PPO is also reported from different sources viz., Aspergillus nidulans9, Vicia faba10, red delicious apple peel11, potato4, spinach beer5, and mammalian system6. In the present study, catecholase was estimated, using 4-methyl catechol as a substrate. The purification was carried out, using lignin isolated from coconut coir pith. The enzyme was characterized by polyacrylamide gel electrophoresis (PAGE). Specific staining methods were used for the detection of enzyme activity, protein, RNA and carbohydrates. SDS-PAGE was carried out for the determination of molecular weight of catecholase.

Materials and Methods

4-Methyl catechol was obtained from Fluka, Germany, casein from Difco, USA; ammonium sulfate, sodium phosphate (mono- and dibasic) and sodium citrate from S. D Fine Chemicals, India; and acrylamide, bis-acrylamide, ammonium per sulphate, ethidium bromide. Coomassie brilliant blue R-250, Tris, molecular weight markers and RNA from Signa, USA, TEMED, sodium dodecyl sulphate (SDS), basic fuschin, periodate and orcinol from BDH, England, Sephadex G-25, G-100 from Pharmacia,Triton X-100 fro Ubichem Ltd. Hampshire, UK. Ascorbic acid was procured from E. Merck, Germany. Methanol and acetone were obtained locally and distilled prior to use.

Locally available variety of brinjal having maximum catecholase activity was used. Brinjal was cleaned and peeled, exposed to air for 11 to 13 hr and homogenized in a Warring blender with chilled 100 mM sodium phosphae buffer (pH 6.8) ith 0.8% Triton X-100 (w/v), 0.1 mM of EDTA and 0.15 mM ascorbic acid. Activity of catecholase was obtained in the supernatant, when the extract was centrifuged at 20000 g for 20 min.

Protein content estimation and catecholase assay were carried out at each stage12.13. The oxidation of 4-methyl catechol was assayed indirectly by measuring the rate of disappearance of ascorbic acid at 265 nm at 250C. In a standard ascorbic acid reaction. 0.9 O.D. gives 100 units of enzyme activity13. Partial purification of enzyme was achieved with 60% saturation at 40C with solid (NH4)2SO4 precipitation, followed by dialyzed against 10mM sodium phosphate buffer (pH 6.8). The dialyzed sample was then loaded on the pre-equilibrated lignin column by 50 mM citrate phosphate buffer (pH 4.6).

Lignin was isolated by the method described earlier14 and was packed (25g) in column (40 cm x 2.7 cm) and the column was equilibrated with 50 mM citrate phosphate buffer (pH 4.6) till the effluents showed zero reading at A 260 nm. Enzyme containing 25 mg of protein with 292500 I U catecholase activity was loaded on lignin column. Flow rate was kept constant (15ml/35 min) and 15 ml fractions collected. Each fraction was asayed for catecholase activity, using 4-methyl catechol as a substrate. Protein was determined by the Lowry's method12. The 260/280 ratio was calculated for each fractions.

Elution profile showed seven activity peaks and is shown in Fig. 1. The pure peak fraction (peak 6), having the highest activity was loaded on Sephadex G-100 and subsequently concentrated against solid sucrose. Desalting was carried out by passing through Sephadex G-25. This sample was characterized by PAGE. For detection of RNA, the sample was treated with 20% sodium dodecyl sulphate (SDS) at 600C for 5-10 min and was also treated with salivary RNase to cleave protein-RNA bond.

The pure enzyme sample was characterized by PAGE using 15 ...l enzyme having 1000 to 2000 I U activity. Electrophoresis was carried out using Trisglycine buffer (pH 8.3) for 2 hr at 24 mA current. The size of gel tubes was 9 cm x 0.4 cm and the gel concentrations used were 7.5 and 5.5%15. Activity staining was carried out by the method described16, using 1.0 mM 4-methyl catechol and 0.1 mM L-Proline in 100 mM phosphate buffer (pH 6.8). Protein band was stained by Coomassie Brilliant Blue R-250. For detection of carbohydrates, i)when pure enzyme was incubated with concanavalin A (0.3 mg/ml) for 20 min at 370C, precipitation takes place which implies the presence of glyco part in the enzyme; ii) the gel were stained with Schiff's reagent17; and iii) total carbohydrate content was estimated by phenol-sulphuric acid method, using mannose as a standard18. 260/280 ratio of the fractions is greater than one, indicating the presence of RNA. Therefore, to confirm the presence of RNA, i) the gels were stained with ethidium bromide19; ii) the SDS-treated sample was heated upto 750C measured at 280 mm and 260 mm; iii) pure enzyme was treated with RNase (1 mg/ml) for 24 hr at 250C; iv) treatment with manganese sulphate (100 mM) and Streptomycin sulphate 5% (w/v) at pH5.5 and 9.5; v) treatment with 10% trichloroaceticacid; and vi) total RNA was also estimated by Bial's orcinol method, using yeast RNA as a standard20.

For determination of molecular weight of catechol oxidase, SDS-PAGE was carried out, using the method described previously21, The marker proteins used were ...-galactosidase (116000), phophorylase b (97400) bovine serum albumin (66000), furnarsase (48500), carbonic anhydrase (29000) and a-lactalbumin (14200). The gel concentration used was 7.5% (fig. 4).

Results and Discussion

The extensively dialyzed, (NH4)2SO4 precipitated fraction was loaded on lignin column. The enzyme was eluted with 50 mM citrate phosphate buffer (pH 4.6). The elution profile pattern showed seven peaks (Fig. 1). The fold purification data of enzyme is given in Table 1. A good retention and recovery of enzyme was observed on the legnin affinity column. Maximum protein was eluted up to 120 ml volume (peaks 1, 2 and 3). The highest purity of enzyme was leuted in the peak 6, having 4616-fold purification; the increase in activity in peak 6 may be due to removal of enzyme inhibitor. The three activity peaks (1,2 and 3) with maximum protein were pooled and concentrated against solid sucrose. The concentrated enzyme sample was loaded on Sephadex G-100 column, eluted as a single peak, having 93-fold purification (Fig. 2). Total activity recovered from the column was 109791 I U, as compated to 29250 I U

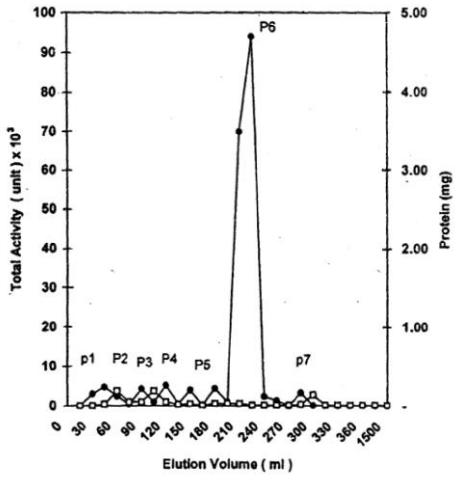

Fig. I-Affinity chromatography of brinjal cortex catecholase. ( ) represents protein ; and ( ) catecholase activity]

Table 1 - Purification of Brinjal contex catecholase

Step	Volume (ml)	Total Activity (Units)	Specific Protein (mg)	Fold activity (units/mg)	% Purification	% Recovery
Crude	140	25,7962	1244	207	1	100
60% saturation with $(NH_4)SO_4$	55	80465	336	239	1.0	31
Affinity Chromatography						
Peak 1,2 and 3 loaded on Sephadex G -100 column						
G-100 (Signle peak)	4,5	9560	0.500	19196	93	3.8
Peak 6	30	9556	0.010	955600	4616	3.7

INDIAN BIOCHEM, BIOPHYS., VOL. 40, DECEMBER 2003

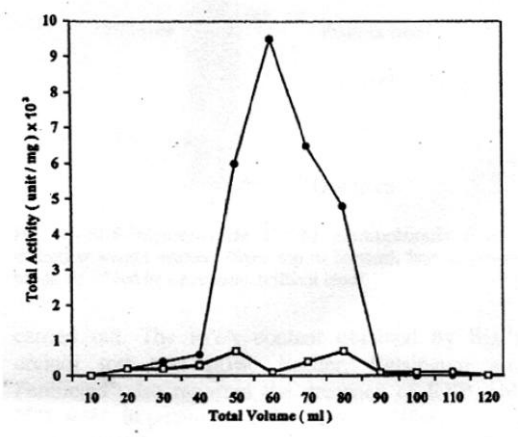

Fig. 2 - Pooled and concentrated peaks 1, 2, 3 loaded on sephadex G-100 column (Protein ( ) represents and ( ) represents cateholase activity).

activity loaded on the column. Reproducible results were obtained after rechromatography. Fractions with activity showed pale-yellow color. No heterogeneity was observed when each peak was individually loaded on Sephadex G-100. The enzyme was eluted immediately after the void volume, indicating higher molecular weights.

The pure enzyme sample from a 6th peak was subjected to PAGE; according to the method of Laemmli15. The staining showed two bands i.e. one at the point of application, which is due to aggregation of enzyme, and other near to dye front is of pure enzyme in both 7.5 and 5.5% acrylamide gel concentrations (Fig. 3). Similar results were reported for banana PPO22, After activity stainint, purple color band was developed within 1-2 min at the site of activity. Blue color protein bands were observed corresponding to the position of activity band.

To detect ribonucleoprotein (RNP) nature of the enzyme, each pure fraction was measured at 260 nm

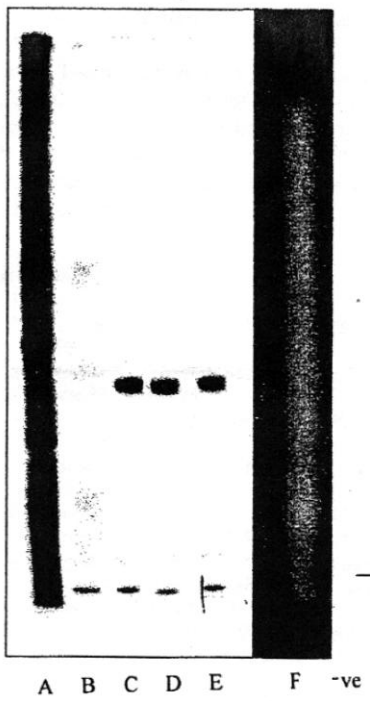

Fig. 3 - PAGE (5.5%) of pure cateholase preparation at various purification steps (The pure enzyme fraction passed through Sephadex G-100, followed by Sephadex G-25, Protein bands were visualized by Coomassie brilliant blue staining A: protein bands of crude sample; B: 60% $(NH_4)_2SO_4$ treatment; C: band of pure protein; D: catecholase activity visualized by 4-methy 1 catechol and proline; E: carbohydrate by Schiff's reagent; and F: RNA by ethidium bromide]

and 280 nm spectrophotometrically. The E 260/E 280 ratio was found to be greater than 1.1 for pure fractions. Hence to confirm the presence of RNP. qualitative analysis by electrophoresis (PAGE) and qualitative analysis by Bial's orcinol test19 were

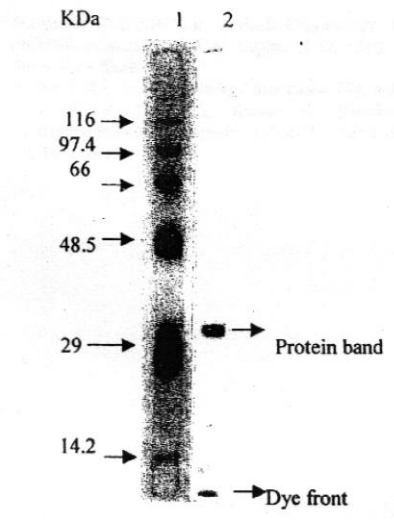

Fig. 4 -SDS-Polyacrylamide (7.5%) electrophoresis [Lane1, molecular weight markers (from top to bottom); lane 2, protein bands visualized by Coomassie brillinat blue]

carried out. The RNA content obtained by Bial's orcinol test was 50%. Earlier, Balsingum and Ferdinand4 also reported the presence of RNO with 50% RNA in potato by 260/280 ratio. When SDS or RNase-treated enzyme was subjected to PAGE to break the RNP nature of the enzyme, fluorescent pink colored blobs were observed on gel at the positions corresponding to that of activity bands. No separation of RNA from RNP was achieved by SDS - or RNase treatment and Precipitation of the protein with trichloroacetic acid left RNA insolution indicating that RNP complex of the enzyme is resistant to these treatments. No change in the E 260/E 280 ratio was observed, when the enzyme was heated up to 700C for 5 min; the ratio before heating and after heating up to 700C was 1.06 and 1.04, respectively. Effective change in ratio was not observed. {It is also confirmed that it is not DNA because DNA gives more diffraction after heating at 260 mm. RNA is covalently bound to the catecholase because it has eluted in the same fraction of pure enzyme. Manganese suplhate as well as Streptomycin sulphate failed to precipitate any RNA. According to Balsingam and Ferdinand4, it is not fully established that all the RNA present is actually bound to the protein; the presence of one or more species of RNA of similar milecular weight to the protein that are neither attacked by ribonuclease not precipitated by common RNA precipitants cannot be ruled out. These observations confirm that catecholase from brinjal pulp is associated with RNA.

The precipitation with concanavalin A and staining of the gels with periodic acid-Schiff's (PAS) reagent showed two pink colored bands, confirming the presence of carbohydrates. The positive carbohydrate test is not due to the presence of RNA, but due to glycosylation associated to catecholase, as RNA does not give positive test to periodate Schiff's reagent A23. The percentage of carbohydrates estimated by phenol-sulphuric acid method was 4%

The electrophoresis of SDS-PAGE showed the same pattern of bands after staining as that of native gel, i.e. one at the point of application and other near to dye front. The molecular mass of two bands is 120,000 and 30,000 Da respectively (Fig 4). The reported molecular mass of catecholase from different sources between 31,000 Da-158, 000 Da24,25. All above data confirms catecholase from brinjal cortex is ribonucleoglycoprotein.

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