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PURIFICATION OF A BACTERIAL ORGANOPHOSPHATE-HYDROLYSING PHOSPHATASE BY CIBACRON 3GA-SEPHAROSE AFFINITY CHROMATOGRAPHY

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Summary: A phosphatase catalysing the hydrolysis of organophosphorus pesticides was purified to homogeneity using Cibacron 3GA-Sepharose CL 6B affinity chromatography. The enzyme which is localized in the periplasm of the bacterium Alcaligenes NC_5 was extracted by treating with 0.2M $MgCl_2$, pH 8.4. The enzyme was adsorbed to the Cibacron-Sepharose at pH 7.0 and eluted with Tris-HCl buffer at pH 8.0, with 47 per cent recovery. The enzyme thus obtained was electrophoretically homogeneous. This simple affinity purification procedure enhances the potential for its use in large scale detoxification systems.

Introduction : Alcaligenes NC_5, a microbe isolated from soil treated with the organophosphorus pesticide, fensulfothion(O,O-diethyl O-(4-methyl sulfinyl phenyl) phosphorothioate) (1) was found to hydrolytically cleave fensulfothion and a number of structurally related pesticides by a periplasmically localized phosphatase (figure 1). This phosphatase (referred to as FNT phosphatase) is a key enzyme in the detoxification process of the organophosphates because it gives rise to products which are non-inhibitory to cholinesterase unlike the parent molecule which is a potent cholinesterase inhibitor.

There are few reports in literature on the purification of organophosphate-degrading enzymes from microorganisms. Hence attempts were made to isolate the FNT phosphatase from Alcaligenes NC_5. Development of affinity chromatography procedures will markedly cut down the tedious steps involved in purification by conventional procedures and enhance the prospects of the enzyme being used on a larger scale for detoxification.

Materials and Methods: Cibacron 3GA was from Ciba Geigy (India), Bombay, India. Tris(hydroxy methyl) amino methane, acrylamide, riboflavin, p-nitrophenyl phosphate(disodium salt) and Sepharose CL 6B were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Technical grade fenitrothion, O,O-dimethyl O-(3-methyl 4-nitrophenyl) phosphorothioate was a gift from

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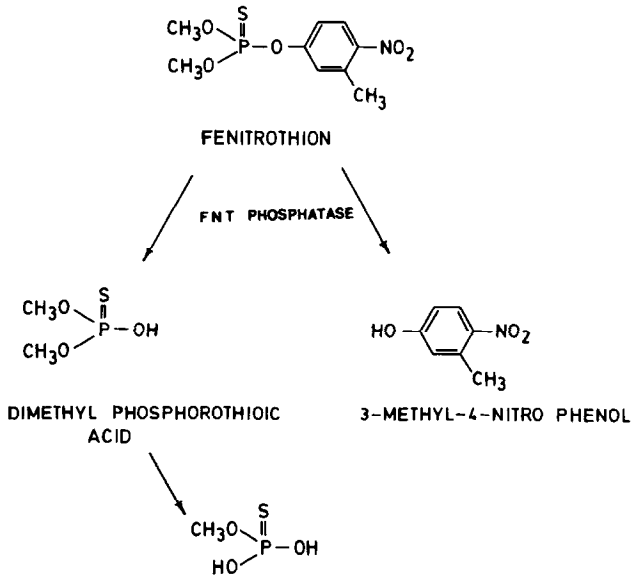

Figure 1: Pathway of degradation of the organophosphorus pesticide, fenitrothion by Alcaligenes NC_5.

Bayer(India) Ltd., Bombay,India. p-nitrophenol obtained from Robert Johnson, India was purified on silica gel thin layer chromatography and used. All other chemicals used were of analytical grade.

Preparation of Alcaligenes NC_5 cells for isolation of FNT phosphatase : Alcaligenes NC_5 cells grown for 24 hr in nutrient broth (peptone:0.5%,beef extract:0.3% in distilled water and pH adjusted to 7.0) were resuspended in mineral salts basal medium containing 4.49 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.20 mM $MgSO_4$, 0.68 mM $CaCl_2$, 1.87 mM NH_4Cl, 0.57 mM K_2SO_4, 0.05 mM $FeSO_4$, yeast extract 0.01%, 0.08 mM $ZnCl_2$, 0.04 mM $MnCl_2$, 0.004 mM $CoCl_2$, 0.008 mM H_3BO_3, 0.01 mM Na_2MoO_4 and 0.05 µM $CuCl_2$, pH 7.0. Fenitrothion was added to the basal medium at a concentration of 3.6 mM to serve as the source of carbon. The cell suspension was then kept on a rotary shaker at 30 C for 12 hr after which the cells were pelleted in a Sorvall RC2B centrifuge at 7000 x g for 15 min. The cells were washed under centrifugation twice with 0.05 M Tris- HCl buffer; the entire operation being carried out at 4 C. Cell pellet from 8 litres of culture was used for extraction of the enzyme.

Preparation of the calibration curve for p-nitrophenol: Varying concentrations of p-nitrophenol ranging from 1 to 50 µg in 0.01 M Tris-HCl buffer were prepared. Concentrations of p-nitrophenol ranginig between 1 µg/ml and 25 µg/ml showed linear absorbance as determined spectrophotometrically at at 410 mm.

Enzyme assay:The FNT phosphatase was assayed essentially by the method of Garen and Levinthal(2) by monitoring the formation of p-nitrophenol from p-nitrophenyl phosphate. Incubation was done at 30 C for 10 min.

Affinity chromatography on Cibacron 3GA-Sepharose:Cibacron 3GA cross-linked to Sepharose CL 6B by the triazine coupling method (3) was packed in a glass column (8.5 cm x 1.5 cm) and equilibrated with 25 ml of Tris-HCl buffer,pH 7.0. The crude enzyme was obtained by suspending the bacterial cells in 0.2 M $MgCl_2$, pH 8.4. The cell suspension(1 gram cells/ 5ml of $MgCl_2$ solution was stirred continuously for 15 min and then centrifuged at

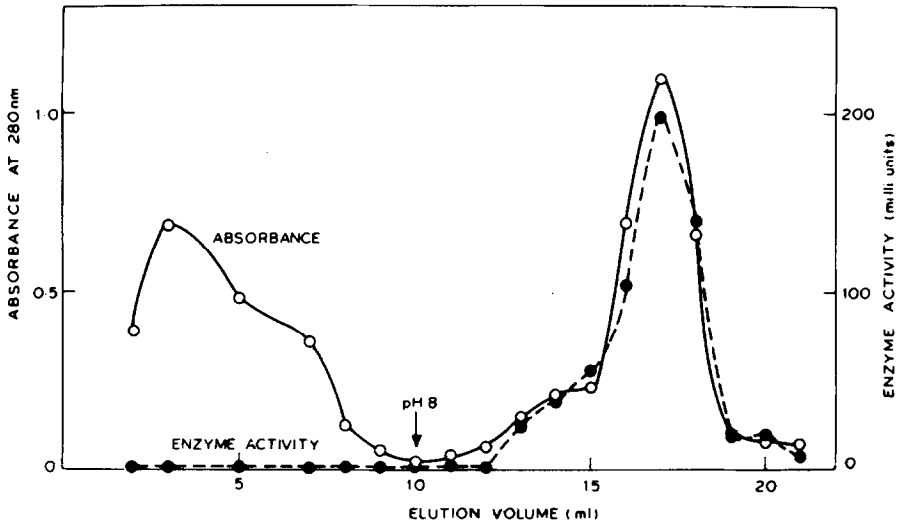

Figure 2: Elution profile of FNT phosphatase from Cibacron 3GA-Sepharose. The crude enzyme was adsorbed on the column and washed with 0.05 M Tris-HCl buffer, pH 7.0. Elution of the enzyme was done by using 0.05 M Tris-HCl buffer, pH 8.0. Absorbance of the fractions at 280 nm (-O-) and assay of the enzyme activity (-●-) was carried out.

9000 x g for 10 min. The supernatant was found to contain the phosphatase activity. The pH of the above supernatant was adjusted to 7.0 with 0.2 M HCl and loaded on the Cibacron-Sepharose column. The unadsorbed fraction was collected. The column was then washed with 10 ml of 0.05 M Tris-HCl buffer, pH 7.0. One ml fractions of these were collected. This was followed by elution with 0.05 M Tris-HCl buffer, pH 8.0 and one ml fractions were collected. Absorbance of the fractions at 280 nm as well as the activity of the phosphatase was determined. Homogeneity of the phosphatase was ascertained by polyacrylamide gel electrophoresis in 7.5 per cent gel using Tris-glycine buffer at pH 8.6 as the electrode tray buffer.

Results and Discussion: The FNT phosphatase from Alcaligenes NC_5 which degrades a number of organophosphorus pesticides could be extracted from the cells employing osmotic shock and high ionic strength. This suggests the periplasmic localization of the FNT phosphatase.

Earlier reports using Cibacron suggested the specific presence of dinucleotide-fold in the proteins binding to the Cibacron dye (5). While it is not known whether such a structure is present or not in the FNT phosphatase, there are also reports which prove that enzymes lacking a dinucleotide-fold like phosphogluconate dehydrogenase, fructose diphosphatase can bind to the dye (6). In these cases the binding could mostly be by hydrophobic interactions(6). A similar situation could be likely in the case of FNT phosphatase too.

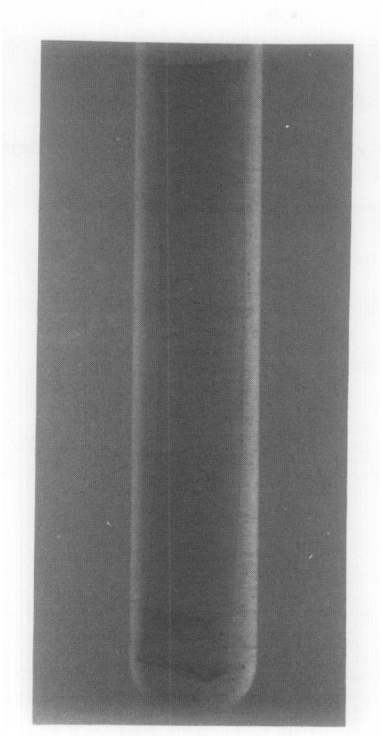

Figure 3: Disc electrophoresis of FNT phosphatase. Protein (15 µg) from the lyophilized active fraction (figure 2) was subjected to electrophoresis in 7.5 % polyacrylamide gel (7) and stained with Coomassie blue.

The elution profile of the enzyme from the column is depicted in Figure 2. The enzyme activity coincided with the protein peak. The lyophilized enzyme from the peak fractions on polyacrylamide gel electrophoresis gave a single band suggesting protein homogeneity (Fig. 3). A recovery of 46.63 per cent was achieved by the purification process (Table 1). Calculations based on the amount of protein loaded on the column and the

Table I : Affinity chromatography of FNT phosphatase using Cibacron-blue 3GA-Sepharose

Fraction	Protein (mg)	Total Activity (milli units)	Specific Activity (milli units/mg protein)	Recovery (%)
Crude ($0.2M\ MgCl_2$, pH 7)	10.80	3959.95	366.97	100
Active eluate (0.05M Tris-HCl, pH 8)	3.39	1846.52	544.70	46.63

enzyme recovered show that FNT phosphatase forms 31.38 per cent of the total 0.2 M $Mgcl_2$-extractable periplasmic proteins.

The Cibacron 3GA-Sepharose affinity chromatography of FNT phosphatase thus presents an easy procedure for the isolation of a key enzyme involved in the detoxification of organophosphorus pesticides. The simplicity of the procedure enhances its potential for application in industrial and large scale detoxification systems.

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