Indian J.Sci.Res. 08 (2): 137-140, 2018 ISSN: 0976-2876 (Print)
ISSN: 2250-0138 (Online)

PURIFICATION AND CHARACTERIZATION OF CATECHOL 1, 2- DIOXYGENASE FROM *Pseudomonas putida* STRAIN PS-I

ASHOK KUMAR SINGH[a] AND ALOK KUMAR SINGH[b1]

[a]Department of Boatny S. R. T. C. P.G. College, Utraula, Balrampur, Uttar Pradesh, India
[b]Department of Botany, Harish Chandra PG College, Varanasi, Uttar Pradesh, India

ABSTRACT

***Pseudomonas putida* Strain PS-I utilized 4-chlorobenzoic acid as a sole source of carbon and energy, degrading it through the *ortho* ring cleavage pathway. They synthesized the enzyme catechol 1, 2- dioxygenase which is unique in its properties to transform chlorocatechol of 4-chlorobenzoic acid in to chloro *cis- cis* muconic acid , while catechol 1, 2- dioxygenase is only efficient degrader of catechol in to *cis-cis* muconic acid. The enzyme was purified to homogeneity by ion exchange and gel filtration chromatography from culture supernatant. The purified native had an estimated molecular mass 55 kDa and it is composed of two sub units each of 25 kDa determined by SDS-PAGE.**

KEYWORDS: 4-chlorobenzoic Acid; Catechol 1, 2-dioxygenase; *Pseudomonas putida* Strain PS-I

Many xenobiotics introduced for industrial and agricultural crop production are halogenated organic compounds. These constitute one of the largest groups of environmental pollutants. Microorganisms, by their rapid growth and indispensable enzyme system, are capable of degradation of such pollutants and resulting in elimination of a wide range of the xenobiotics chemicals from the environment [Alexander M, 1981]. Chlorinated catechols have been reported to be key intermediates in the biodegradation by aerobic organisms of various chloroaromatics such as cholorobenzoates and chlorophenoxyacetate [Ghosal D. et al., 1985]. Cleavage of the aromatic ring in these catechols occurs by deoxygenating prior to de chlorination and hence the first attack on these compounds results in production of various intermediates compounds which are dehalogenated at the later step with removal of chlorine atom in form of chloride [Klages U. et al., 1979 and Mellon J E et al, 1996]. Complete degradation of chlorinated catechols depends upon plasmid gene. These genes can be easily propagated between strains by increasing the diversity of Pseudomonas species capable of degrading these compounds. *Pseudomonas putida* strain PS-I utilized 4- chlorobenzoic acid as a carbon as energy source. The chlorocatechols is known to be intermediates in the duration of these compounds. We are interested to purify and characterize the catechol 1, 2-dioxygenase from *Pseudomonas putida* strain PS-I.

MATERIALS AND METHODS

Bacterial Strain and Growth Medium

The culture of *Pseudomonas putida* strain PS- I was isolated from soil and incubated in a Erlenmeyer flask containing a selective medium gl^{-1}, Na_2HPO_4. 2 H_20, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; $NaNO_3$, 0.085; $Ca(N0_3)_2$. 4 H_20, 0.05; ferrous ammonium citrate, 0.01 and 1 ml of trace elements [Pfening N., 1986]. The 5 mM of 4- chlorobenzoic acid was added in the medium as a sole source of carbon and energy. The flasks were incubated at 30 ^{0}C in a rotator shaker at 150 rpm.

Preparation of Cell Extract

The cells of *Pseudomonas putida* strain PS-I was harvested from overnight culture by centrifugation at 7000 rpm for 30 min. The supernatant was discarded and the pellet was washed several times in 0.05 M chilled phosphate buffer (pH 7.0). The cell suspension in the buffer was subjected to ultrasonication for 4 min and was re centrifuged at 12,000 rpm for 20 min at 4 ºC. The supernatant was used for the enzymatic estimation.

Enzyme Assay

The catechol 1, 2-dioxygenase assay is based on the measurement of the rate of formation of *cis, cis*-muconic acid at 260 nm [Nakazawa T. et al., 1988]. The assay mixture consisted of 4 µM EDTA, 100 µl catechol (10 mM), 2.7 ml phosphate buffer (0.05 M, pH 7.0) and the reaction was initiated by adding 100 µl of enzyme extract. One unit of enzyme activity is defined as the amount of the enzyme which catalyzes the formation of 1µM of *cis, cis*-muconic acid per min at 24 ºC. The conversion of 1 µM of *cis, cis*- muconic acid causes an increase in absorbance of 5.66 units at 260 nm. Protein was estimated by the method of Bradford [1976].

[1]Corresponding author

Enzyme Purification

DEAE –Cellulose Column Chromatography

The purified supernatant was directly passed through activated diethyl amino ethyl cellulose column (2x 40 cm size) equilibrated with tris buffer (pH 8.0). A few ml buffers were used to wash down the enzyme sample from column sides and then a continuous buffer reservoir was attached to the column with an opened outlet at 30 ml $h^{-1.}$ flow rate. The column was run using the same buffer until all the retained protein came out. Tris buffer with different increasing concentration of NaCl was poured in to the column serially and step wise elution was done. Five ml fractions was collected in test tube and analyzed by measuring optical density at 280 nm.

Gel Filtration Chromatography

The active fraction from column chromatography was fractioned in Sephadex G-100 column (2 x 28 cm size). It was washed prior to equilibration with tris buffer (0.5 M, pH 7.2). Five ml fractions were collected at the elution rate of 20 ml $h^{-1.}$ Absorbance at 280 nm was measured for each fraction by Beckman DU-70 Spectrometer. Active fractions were fractionated separately and molecular mass was determined by method of Whitaker J R [1963] using known molecular mass protein as: bovine serum albumen (66 kDa), carbonic anhydrase (29 kDa), lysozyme (14 kDa) and cytochrome-c (12.5 kDa).The void and bed volume of the column was measured through blue dextran.

SDS-PAGE

The molecular mass of the enzyme was determined by SDS-PAGE according to Laemmli U K [1970] using 10 % gel in 0.1 % SDS and 0.5 M tris glycine buffer (pH 8.3). Protein samples were mixed in 2 % SDS, 5 % ß-mercaptoethanol and 10 % glycerol. The sample were kept at 100 ºC for 5 min. The proteins were loaded on gel surface and electrophoresis was performed initially using 2 mA current for 20 min and then by increasing the current to 3 mA till the end. The gels were fixed and stained with 0.5 % of Commassie brilliant blue- 250 dissolved in methanol, acetic acid and distilled water in the ration 20:10:65. The destaining was performed in the same solvent system. The protein band that appeared on the gel was calibrated with known molecular mass proteins as standard : phosphorylase - ß (94 kDa); bovine serum albumin (68 kDa); ovalbumin (43 kDa); carbonic anhydrase (29 kDa) and α-lactalbumin (14 kDa).

RESULTS

Purification of Enzyme

The activity of enzyme was found to be maximum in the supernatant of a culture of 14 hours growth. This is purified by column chromatography and was eluted with linear gradient. The passage of the crude enzyme sample through DEAE cellulose column resulted in the resolution of three peaks of the enzyme activity. The first peaks were larger than the other . The catechol 1, 2- dioxygenase was estimated in all peaks fractions.

Characterization of Enzyme

The purified fraction from the column chromatography were concentrated and subjected to gel filtration chromatography. The molecular mass of the fractions was determined by ka*v* value calibrated with known molecular mass protein. The present investigation indicated that the molecular mass of the fraction was 55 KDa (Fig. 1). The single fraction obtained by gel filtration chromatography is represented in the purity of the enzyme. The fraction of the gel filtration was subjected to sodium dodecyl sulphate polyacrylamide electrophoresis. The SDS-PAGE pattern of purified enzyme showed a single band on the surface visualized after staining the gel with Coommassie brilliant blue- R 250 (Fig. 2). This band was calibrated with standard protein and the result indicated that the molecular mass of the fraction was 25 kDa (Fig. 3). The single band appeared on the gel surface revealed a homogeneous preparation of 55 kDa protein. Further, this band was calibrated with standard protein and the result indicated that the molecular mass of the fraction was 25 kDa.

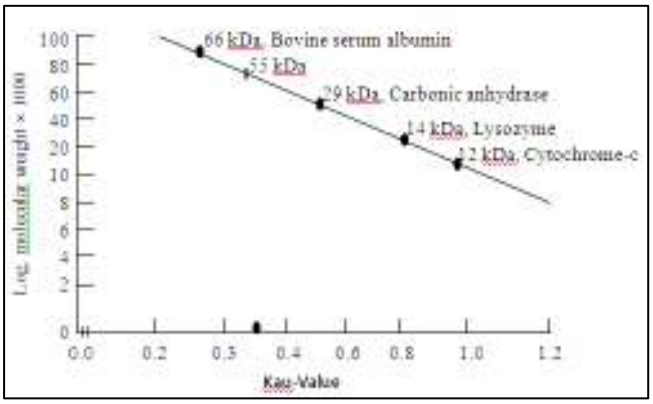

Figure 1: Molecular weight of the purified enzyme was determined by gel filtration chromatography. (●) represent the known molecular weight of the standard

proteins, (×) represent the known molecular weight of the purified enzyme.

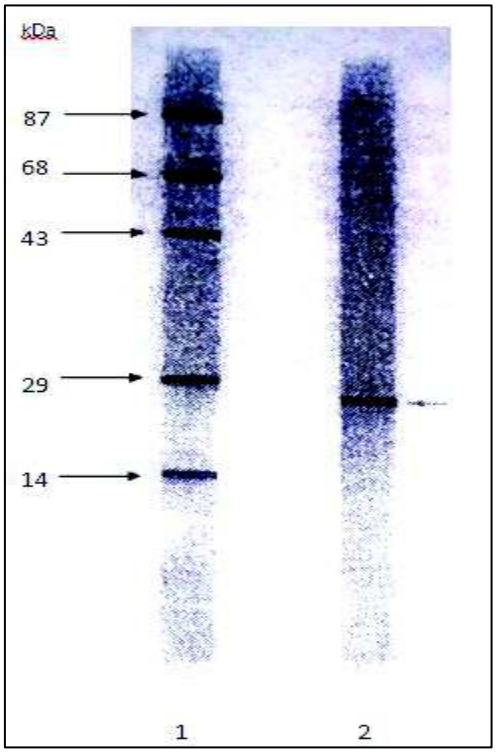

Figure 2: Sodium dodecyle sulphate-polyacryl amid gel electrophoresis of the purified enzyme, Lane 1, represented the molecular weight of marker protein; 97 k Da, phosphorylase-β; 68 kDa , bovine serum albumin; 43kDa, carbonic anhydrase; 14 kDa, lactalbumin, Lane 2 , represented the single band of the purified enzyme on gel surface.

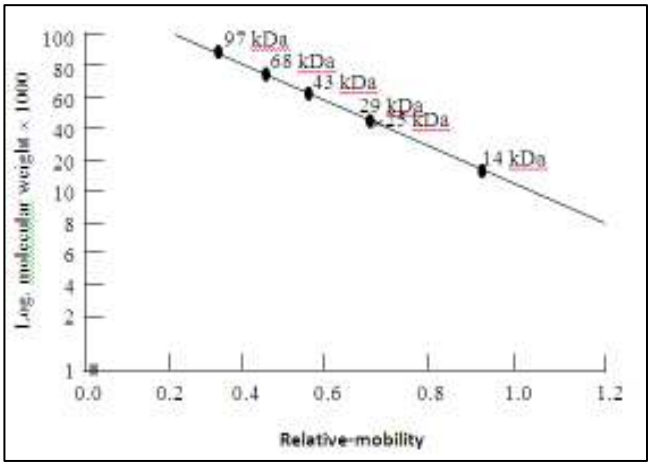

Figure 3: Molecular weight of the purified enzyme determined by SDS-PAGE. (●) represent the known molecular weight of the marker protein

DISCUSSION

Pseudomonas putida strain PS-I was grown on 4-chlorobenzoates induced only catechol 1,2-dioxygenase. This enzyme cleaved the benzene ring at *ortho* position. In some respect this seemed similar to other catechol and chlorocatechol 1,2-dioxygenase. Fiest and Hegmen [1969] describe that the catechol 1,2-dioxygenase is responsible for the degradation of *ortho* position of catechol. The pattern of induction of this enzyme differs from species to species. This enzyme has been reported to be active almost exclusively on catechol and to a lesser extent on chlorocatechol. The chlorocatechol 1,2- dioxygenase display a border specificity and to attack a variety of chlorinated catechols [Broderick J B, Halloram T V., 1991]. *Pseudomonas putida* strain PS-I when utilized 4-chlorobenzoic acid as a carbon source synthesized only catechol 1, 2-dioxygenase which degrades the 4-chlorocatechol in to chloro *cis-cis* muconic acid. In a later step dehalogenation occurred due to removal of chlorine atom as chloride and chloro *cis-cis* muconic acid is transformed in to cis-*cis* muconic acid. Further, this is converted in to acetate and fume rate which is the end product of the 4-chlorobenzoic acid [Hollinger C , Zehnder A J B et all., 1996]. Such high production of enzyme may indicate that *Pseudomonas putida* strain PS- I has a potential value in commercial production of the enzyme. This strain is capable of utilizing a wide range of chemicals present in the environment. The carbon sources play a significant role for the production of dioxygenase. Several workers reported that the *Alcaligens eutrophus* CH-34 can produce at least two different dioxygenase depending to the carbon source [Dorn E, Knack muss H J, 1978].

The enzyme was purified to homogeneity by ion exchange and gel filtration chromatography from cell free culture supernatant. The purified native form had an estimated molecular mass of 55 kDa and it is composed of two sub units each of 25 kDa determined by SDS-PAGE. Ignazi et al. [1996] have estimated the molecular mass of catechol 1,2 -dioxygenase by gel filteration and SDS-PAGE in the culture filtrates of *Alcaligens eutrophus* CH-34 was 76 kDa and 36 kDa respectively. The comparison of kinetic parameter of several catechol and chlorocatechol 1,2-dioxygenase confirmed that the catechol 1,2 dioxygenase interacted poorly with halogenated aromatic compounds, while chlorocatechol 1,2-dioxygenase was much more efficient in degradation of chlorocatechol . [Ignazi et al, 996]. Thus catechol 1,2-dioxygenase of *Pseudomonas putida* strain PS-I has much more potential for degradation

of chlorocatechol than catechol. This is first time report that the catechol 1,2- dioxygenase binds a greater variety of aromatic legends with a high apparent affinity. The ability of this enzyme to bind and transform the halogenated compounds, specially 4-chlorocatechol, make it a unique enzyme.

ACKNOWLEDGEMENT

This research was supported by Centre of Advance Study in Botany, Banaras Hindu University, Varanasi , India.

REFERENCES

Alexander M., 1981. Biodegradation of chemicals of environmental concern. Science, **211**: 132-138.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of protein utilizing the protein dye binding. Anal Biochem., **72**: 248-254.

Broderick J.B. and Halloram T.V., 1991. Overproduction, purification and characterization of chlorocatechol deoxygenate a non heam dioxygenase with broad substrate tolerance. J. Biochem., **30**: 7349-7357.

Dorn E. and Knack muss H.J., 1978 b. Chemical structure and biodegradability of halogenated aromatic compounds, substituent effects on 1, 2 dioxygenation of catechol. J. Biochem., **174**: 85-94.

Fiest C.F. and Hageman G.D., 1969. Phenol and benzoate metabolism by *Pseudomonas putida.* J. Bacteriol., **100**: 869-877.

Ghosal D., You I.S., Chatterjee D.K. and Chakra arty A.M., 1985. Genes specifying degradation of 3-chlorobenzoic acid in plasmid pAC 27 and pJP4. Proc. Natl. Acad. Sci. USA, **82**: 1638-1642.

Hollinger C. and Zehnder A.J.B., 1996. Anaerobic biodegradation of hydrocarbons. Current Opanion in Biotechnol., **7**: 326-330.

Ignazin G.S., Gagnin J., Beguin C., Barrelle C.B.M., Markowicz Y. and Toussaint J.P.A., 1996. Characterization of a chromosomally encoded catechol 1, 2 dioxygenase from *Alcaligenes eutrophus* C H- 34. Arch. Microbiol., **166**: 42-50.

Klages U. and Lingens F., 1979. Degradation of 4-chlorobenzoic acid by *Nocardia* sp. FEMS Microbial. Lett., **6**: 201-203.

Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophase T_4. Nature, **227**: 680-685.

Mellon J.E. and Cotty P.J., 1996. Purification and characterization of an elastinolytic proteinase from *Aspergillus flavus* culture filtrates. Appl. Microbiol. Biotechnol., **46**: 138-142.

Nakazawa T., Nakai C. and Nozaki M., 1988. Purification and properties of catechol 1,2 dioxygenses (pyrocatechase) from *P. putida* mt-2 in comparison with that from *P. arvilla* c-1. Arch. Biochem. Biophys., **267**: 701-713.

Pfening N., Lippert K.D. and Das U., 1986. Vitamin B-12 Bedurfnis phototropher Schwefwl Bakterien. Arch. Mikrobiol., **155**: 245-256.

Whitaker J.R., 1963. Determination of molecular weight of proteins by gel filtration on sephadex. Anal Chem., **35**:1950-1953.