International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2020): 7.803

Purification and Properties of *ortho* Ring Cleavage Enzyme Catechol 1, 2 - dioxygenase from Genetic Modified Strain of *Pseudomonas aeruginosa*

Ashok Kr. Singh

SRTC PG College, Utraula, Balrampur - 271604, UP India
E Mail: *ashokarsingh[at]gmail.com*

Abstract: ***Pseudomonas aeruginosa utilize 4 - chlorobezoic acid as sole source of carbon and energy, degrading it through ortho ring cleavage pathway. They synthesized catechol 1, 2 - dioxygense enzyme which is unique its properties to transform catechol to cis, cis - muconic acid. The procedure for purification and homogeneous nature was describe by polyacrylamide gel electrophoresis. The molecular weight of enzyme was 55 kDa estimated by agrose gel electrophoresis. The subunit size was 25 kDa determined by SDS - PAGE.***

Keywords: 4 - cholorobenzoic acid, *Pseudomonas aeruginosa*, catechol 1, 2 - dioxygenase chlorocatechol, catechol, *cis, cis* - muconic acid

1. Introduction

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). The chlorinated catechols have been reported to be key intermediates in the biodegradation by aerobic organisms of various chloroaromatics such as cholorobenzoates and clorophenoxyacetate (Ghosal D et. al.1985). Catechol 1, 2 - dioxygenase a non heme, trivalent, iron - containing enzyme, catalyzes the cleavage of the aromatic ring of catechol to *cis, cis* - muconate with the incorporation of two atoms of molecular oxygen into the substrate. It represents the initial enzyme of the β - ketoadipate pathway, a metabolic sequence used by microorganisms for the degradation of aromatic compounds (Stainer R Y 1973 Bugg T 2003). The enzymes of β - ketoadipate pathway, including CO, are inducible in microorganisms. Comparative studies of the mechanisms of regulation in different bacterial genera indicate distinctive mechanisms of induction. Thus, regulation of enzyme β - ketoadipate pathway are different in *Acinetobacter* and the fluorescent group of *Pseudomonas*. These two groups of organisms of taxonomically separated on the basis of morphology and deoxyribonucleic acid content. Thus, organisms of the *Acinetobacter* genus are non motile with a DNA content ranging from 40 to 47% guanine plus cytosine and the motile rod shaped *Pseudomonas* species, which have a DNA content ranging from 58 to 69%. (Mandel M 1966). This report describe procedures for the purification of catechol 1, 2 - dioxygenase from *Pseudomonas aeruginosa* and some of its properties. We hope studies of this enzyme will provide information on the essential active site conformation for intradition cleavage of aromatic rings.

2. Materials and Methods

Culture

The culture of *Pseudomonas aeruginosa* was isolated from soil and incubated in a Erlenmeyer flask containing a selective medium gl^{-1}, $Na_2HPO4\ 2\ H_2O$, 7.8; $KH_2\ PO4$, 6.8; MgSO4, 0.2; NaNO3, 0.085; Ca (NO3) 2.4 H_2O, 0.05; ferrous ammonium citrate, 0.01 and 1 ml of trace elements (Pfennig 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 °C in a rotator shaker at 150 rpm.

Preparation of Cell Extract

The cells of *Pseudomonas aeruginosa* was harvested from over night culture by centrifugation at 7000 rpm for 30 min. The supernatant was discarded and the pellets were 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 recentrifuged at 12, 000 rpm for 20 min at 4 °C. 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 1 µM of *cis, cis* - muconic acid per min at 24 °C. 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 M 1976.

Licensed Under Creative Commons Attribution CC BY

2.1 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 were 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}. The absorbance at 280 nm was measured for each fraction by Beckman DU - 70 Spectrometer. The 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.

Polyacrylamide Gel Electrophoresis

PAGE was performed as describe bay Raymond S (1962) in 7% gel system as vertical gel electrophoresis. The gel buffer was 50 mM tris glycine (pH 7.5). The running and sample buffer was 50 mM tris glycine (pH 8.8). The sample containing 100 µg protein, 30% sucrose and 5 µl (0.05%) bromophenol blue in total volume of 0.1 ml sample applied to the slots. A constant current of 300 volts was applied for 2 hours. The gel was strained with 0.1% amino black in 7% acetic acid 30% methanol and detained in the same solvent.

Sodium Dodecyle Sulphate – Gel Electrophoresis

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 % β - mercaptoethanol and 10 % glycerol. The sample were kept at 100 °C 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 commissive 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 - β (94 kDa); bovine serum albumin (68 kDa); ovalbumin (43 kDa); carbonic anhydrase (29 kDa) and α - lactalbumin (14 kDa).

3. Results

Purification of Enzyme

The activity of enzyme was found to be maximum in the supernatant of a culture of 12 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 (Fig 1.)

Characterization of Enzyme

The purified fraction from the column chromatography were concentrated and subjected to gel filtration chromatography. The molecular mass of the protein was determined by Kav value calibrated with known molecular mass protein. The present investigation indicated the molecular mass of protein was 55 kDa. The single fraction obtained by gel filtration is represented the purify of enzyme (Fig.2).

Polyacrylamide Gel Electrophoresis

The homogeneity of enzyme was determined by polyacrylamide gel electrophoresis which give a very good resolution of charged protein molecules by separating them according to there sized and charge. The protein visualized two bands of the mixture of fractions I and II (lane M) and single band of each fraction I and II separately. (lane I and II). The separation of single band in the mixture of fraction I and II gel surface indicates that the enzyme was homogeneous in nature (Fig 3).

SDS - PAGE

The fraction of gel filtration was subjected to SDS - PAGE which showed a single band on the gel. This band was calibrated with standard protein and result indicated that molecular mass of fraction was 25 kDa (Fig.4). This single band appeared on the gel surface revealed a homogeneous preparation of 55 kDa protein (Fig.5).

4. Discussion

Pseudomonas aeruginosa was grown on 4 - chlorobenzoates induced only catechol I, 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 Hegeman (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 aeruginosa* utilized 4 - chlorobenzoic acid as a carbon source synthesized only catechol I, 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 fumerate which is the end product of the 4 - chlorobenzoic acid (Hollinger C,

Licensed Under Creative Commons Attribution CC BY
Paper ID: SR211007180528 DOI: 10.21275/SR211007180528

Zehnder A J B et al., 1996). The high production of enzyme may indicate that *Pseudomonas aeruginosa* 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, Knackmuss 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. (Schomburg D 2006). Thus catechol 1, 2 - dioxygenase of *Pseudomonas aerugirosa* 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.

5. Acknowledgement

This research was supported by Department of Botany, Banaras Hindu University, Varanasi, India. The author Acknowledged the Prof. R. S Upadhyay for his support and encouragement in preparation of this manuscript.

References

[1] Alexander M.1981 Biodegradation of chemicals of environmental concern. Science, 211: 132 - 138.
[2] Bradford M. M.1976. A rapid and sensitive method for the quantitation of protein utilizing the protein dye binding. Anal Biochem., 72: 248 - 254.
[3] 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.
[4] Dorn E. and Knackmuss H. J.1978. Chemical structure and biodegradability of halogenated aromatic compounds, substituent effects on 1, 2 - dioxygenation of catechol. J. Biochem., 174: 85 - 94.
[5] Feist C. F. and Hageman G. D.1969. Phenol and benzoate metabolism by *Pseudomonas putida*. J. Bacteriol., 100: 869 - 877.
[6] Ghosal D., You I. S., Chatterjee D. K. and Chakra warty A. M.1985. Genes specifying degradation of 3 - chlorobenzoic acid in plasmid pAC 27. Proc. Natl. Acad. Sci. USA, 82: 1638 - 1642.
[7] Hollinger C. and Zehnder A. J. B.1996. Anaerobic biodegradation of hydrocarbons. Current Opanion in Biotechnol., 7: 326 - 330.
[8] Ignazi G. S., Gagnin J., Bcguin 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* CH - 34. Arch. Microbiol., 166: 42 - 50.
[9] Klages U. and Lingens F.1979. Degradation of 4 - chlorobenzoic acid by *Nocardia* sp. FEMS Microbial. Lett., 6: 201 - 203.
[10] Laecmmli U. K.1970. Cleavage of structural proteins during the assembly of the head of bacteriophase T4. Nature, 227: 680 - 685.
[11] 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.
[12] Nakazawa T., Nakai C. and Nozaki M.1988. Purification and properties of catechol 1, 2 dioxygenses from *P. putida* mt - 2 in comparison with that from *P. arvilla* c - 1. Arch. Biochem. Biophys., 267: 701 - 713.
[13] Pfening N., Lippert K. D. and Das U.1986. Vitamin B - 12 Bedurfnis phototropher Schwefwl Bakterien. Arch. Mikrobiol., 155: 245 - 256.
[14] Whitaker J. R.1963. Determination of molecular weight of proteins by gel filtration on sephadex. Anal Chem., 35: 1950 - 1953.
[15] Raymond S.1962. A convenient apparatus for vertical gel electrophoresis Clin. Chem.8: 455 - 470.
[16] Schomburg D.2006. Catechol 1, 2 - dioxygenase class - 1 Springer Handbook of Enzyme 25 2nd edi. Springer Berlin Heiddberg pp.382 - 394
[17] Bugg T.2003 Dioxygenase enzyme catalytic mechanisms and chemical models. Tetrahedron 59 (36) 7075 - 7101.
[18] Stanier R Y. and L N. Ornston 1973. The β - Ketoadipate pathway. page 89 - 151. A. H Rose and DW Tempest. Advance in Microbial Physlogy Vol.9 Academic Press Newyork.
[19] Mandel M.1966 DNA based composition in the genus *Pseudomonas*. J Gen Microbial 13: 273 - 292.
[20] Meagher R. B. and L. M. Ornaston 1973. Relationship among enzymes of the β - Ketoadipate pathway. Property of cis, cis - muconate lactonizing enzyme and muconolatone isomer from *P. putida.* J Biochemistry 12: 3523 - 3530.

Licensed Under Creative Commons Attribution CC BY

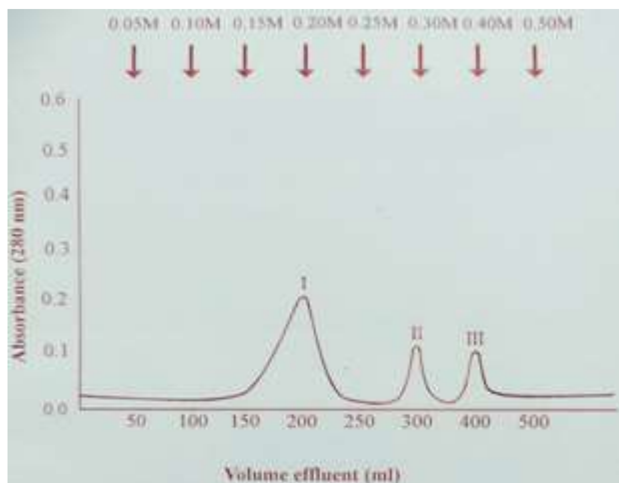

Figure 1: DEAE - Cellulose column chromatography of cell extract with stepwise increasing concentration of NaCl in elusion buffer. The Peaks I. II & III showed the enzyme activity.

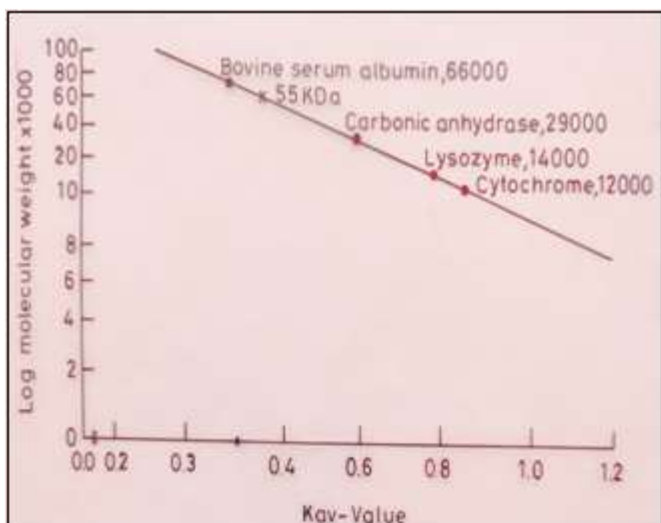

Figure 2: Molecular weight of the purified enzyme was determined by gel filtration chromatography (**.**) the represent known molecular weight of the standard proteins, (X) represent the molecular weight of the purified enzyme.

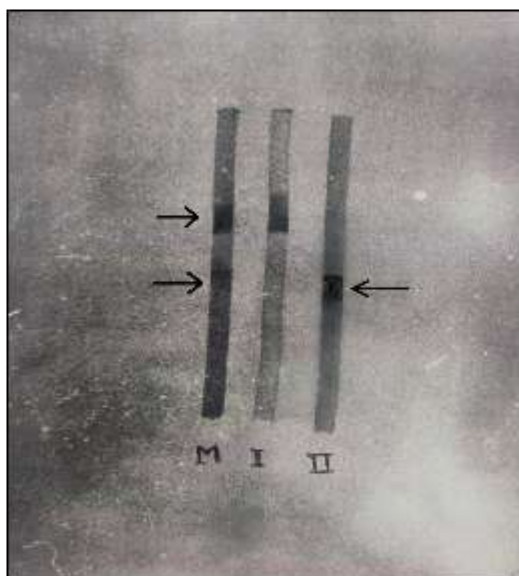

Figure 3: Polyacrylamide gel electrophoresis of the purified enzyme lane M - mixture of fractions I and II, lane l - purified enzyme of fraction I, lane II - purified enzyme of fraction II.

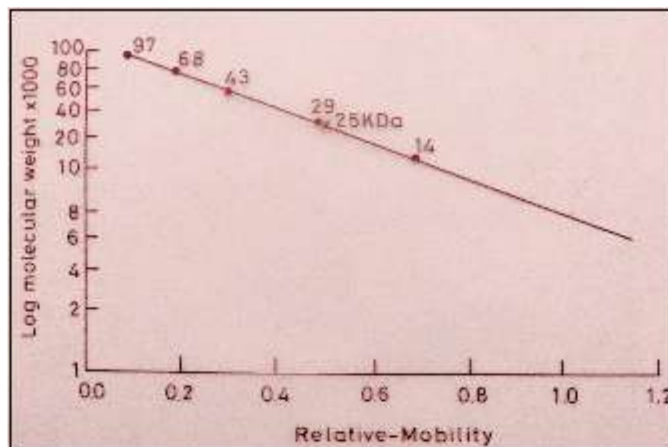

Figure 4: Molecular weight of purified enzyme was determined by SDS - PAGE. (**.**) represented the known molecular weight of standard protein (**X**) represented the molecular weight of purified enzyme.

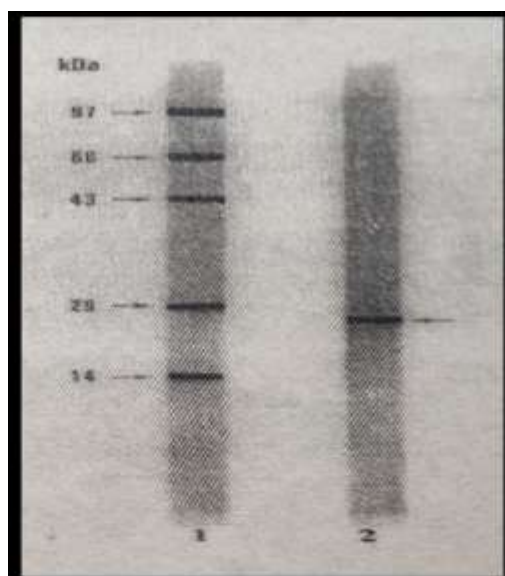

Figure 5: SDS - PAGE of the purified enzyme.
Lane I - represented the molecular weight of the marker protein: 97 kDa phosphorylase - β, 68 kDa bovine serum albumin, 43 kDa carbonic anhydrase, 14 KDa lactalbumin. Lane II - represented the single band of the purified enzyme on the gel surface.

Licensed Under Creative Commons Attribution CC BY