ARGININE DECARBOXYLASE FROM THE PATHOGENIC FUNGI, *Colletotrichum gleosporoides* : PURIFICATION AND PROPERTIES

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ABSTRACT

Arginine decarboxylase, a polyamine biosynthetic enzyme, was isolated from a phytopathogenic fungi, *Colletotrichum gleosporoides*, which causes Anthracnose in wide range of plants in many parts of the world.

The enzyme was purified 25 fold with 16.7 % recovery by elution through Sepharose 4B gel column and DEAE Cellulose ion exchange column. As determined by Sepharose 4B gel chromatography, the native molecular mass of the purified enzyme was ~ 265 kDa. SDS-PAGE of the purified enzyme showed two bands around 65 kDa and ~25 kDa, suggesting that possibly this enzyme could be a hexamer of above two sub units. Optimum pH and temperature for the enzyme was 5.2 and 40 °C respectively. Beyond 50 °C enzyme activity slowly declined and was almost deactivated by 80 °C. Approximate K_m of the enzyme for the substrate arginine was 67mM.

Key words : Anthracnose, *Colletotrichum gleosporoides*, Arginine decarboxylase , Polyamine metabolism

INTRODUCTION

The disease Anthracnose which is caused by the species of the genus *Colletotrichum* is widespread in many parts of the world and has significantly affected the economy of many agricultural countries (Bailey & Jeger 1986).

These fungi attack a wide range of plants all over the world, particularly in the tropical areas and also in subtropical and temperate areas in significant levels. The damage caused by the disease is more severe on perennial crops and other cash crops including mango, banana, citrus, avocado, tomato, bean, chilies, onions, coffee, tea, rubber, tobacco, cashew, sugar cane, soy bean and cotton. The pathogen attacks several parts of the same plant at several stages, e.g., leaf, blossom and pre- and postharvest

stages of the fruit. The losses caused by the disease occur as a direct reduction of the quality and quantity of the harvested yield. The damage is most noticeable and severe when *Colletotrichum* directly attacks the harvested portion of the crop, often the fruit. Mango, avocado, papaya, banana are the most affected fruit crops by *Colletotrichum* disease in most part of the world (Baker *et al.*, 1994).

Current fungicides have failed to control the disease completely due to increasing resistance of the fungi. Hence, designing an alternative controlling method was essential and urgent. As a consequence of this, polyamine metabolism which governs the growth of the fungus is receiving increasing attention recently with the aim of developing more effective controlling methods (Burtin *et al.*, 1998).

Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) are the rate limiting enzymes of polyamine biosynthesis. Recently, several attempts have been made to protect crop plants from the attack by pathogenic fungi through the use of specific inhibitors of two key enzymes. For e.g., inhibitors such as alpha - difluoromethyl arginine (α -DFMA) and alpha difluoro methyl ornithine (α -DFMO) irreversibly inhibit the action of arginine and ornithine decarboxylase respectively depleting the intracellular polyamine content. Consequently growth of pathogenic fungi was retarded *in vitro* while exerting no apparent effects on host physiology (Baker *et al.*, 1993).

However, reports indicate that the growth sensitivity of phytopathogenic fungi to α -DFMO and α -DFMA varies widely and cannot always be taken as a reliable indicator of their efficacy for disease control (Gramarnik *et al.*, 1994). Therefore, developing novel compounds that selectively inhibit polyamine biosynthesis and suppress pathogenic infection in plants is of importance.

Hence, an attempt was made to purify and characterize enzyme arginine decarboxylase of *Colletotrichum gleosporoides* with the aim of designing potent inhibitors for the enzyme, which could provide more effective mechanism to control Anthracnose.

For the first time, this paper reports the purification of arginine decarboxylase from *Colletotrichum gleosporoides* by ion exchange and gel filtration chromatography. Some properties of purified enzyme are also discussed.

MATERIALS AND METHODS

All the chemicals used were of analytical grade. All the culture media, glassware and other consumables were sterilized in an autoclave at 121 °C and 32 lbf/in² (2.2 bar) pressure for 30 minutes.

Culture conditions

Cultures were grown on Cook's 2 liquid medium (Chakravarthy, 1957) at 37 °C under static condition.

Kinetics of the enzyme activity with the culture growth

Cultures were grown as described above and the mycelia were harvested by filtration through a buchner funnel after 2, 3, 4, 5, 6, 7, 8, 9, 10 and 12 days of the inoculation. Cell free extracts were prepared by grinding the mycelia with sand in a

motor and pestle and the enzyme activity of the prepared extracts were assayed as given below.

Standard Enzyme assay procedure

The activity of arginine decarboxylase was determined by measuring the evolved CO₂ using the conventional Warburg manometer (Najar, 1957). Warburg flask containing 0.5 ml of water, 1.0 ml of enzyme, 1.0 ml of acetate buffer (50mM, pH 5.2) and pyridoxal phosphate (0.01%, 10 µl) was set up to the manometer. After 10 minutes equilibration period, 0.5 ml of arginine (1%) was added to initiate the reaction. Reaction mixture was incubated for 20 minutes at 37 °C. Enzyme activity was assayed by measuring the CO₂ evolved. Control experiment was also performed without the enzyme (Najar, 1957).

Enzyme Purification

All the purification steps were carried out at room temperature unless otherwise specified.

Preparation of cell free extracts for enzyme purification

Liquid cultures were grown in Cook's 2 medium for 9 days at 37 °C under static condition. Mycelia were harvested from a total volume of 5 liters culture medium and immersed in acetate buffer (50 mM, pH 5.2) containing 1 mM EDTA and 5 mM 2-mercaptoethanol. The cells were lysed by grinding with sand in a motor and pestle at 0 °C, and the crude extract thus obtained was centrifuged at 15,000 g for 30 minutes at 2 °C. The supernatant solution was fractionated with ammonium sulphate at 0 °C and the 0-25% pellet, which had the highest activity was obtained. Thus obtained enzyme preparations were stored in a freezer until further purification.

Purification by Sephadex 4B gel chromatography

The enzyme was purified by passing through a Sephadex 4B column (30 x 1.8 cm) and eluted with acetate buffer (50 mM, pH 5.2) containing 1mM EDTA and 5mM 2-mercaptoethanol at a flow rate of 20 ml/hr. Fractions (2 ml) were collected and enzyme activity of each fraction was assayed.

Purification by DEAE Cellulose chromatography

Active fractions eluted from above step were pooled and chromatographed on DEAE column (9 x 1.8 cm) eluting with potassium phosphate buffer (50mM, pH 7) containing 1mM EDTA and 5 mM 2-mercaptoethanol at a flow rate of 20 ml/hr. Fractions (2 ml) were collected and enzyme activity of each fraction was assayed.

The protein content of the individual fractions obtained was monitored by measuring the extinction at 280 nm. Quantitative estimation of protein after each step of purification was done by Bradford method (Scopes, 1994).

Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)***Sample preparation***

Polyacrylamide gel electrophoresis of the enzyme fraction, obtained from the final purification step was performed under denaturing condition following the discontinuous buffer system of Lamelli (Lamelli, 1970). The slab gel was made of a 4% stacking gel and a 10% separating gel. Constant currents of 20 mA and 40 mA in the stacking and separating phases were respectively applied. The gel was silver stained by Nondiamine staining method (Carl & Murray, 1990).

Estimation of the molecular mass of enzyme

The molecular mass of the purified enzyme was estimated by calibrating the sepharose 4B gel filtration column using urease 545 kDa, alcohol dehydrogenase (141 kDa), bovine serum albumin (66 kDa), pepsin (34 kDa) and trypsin (25 kDa) as the standards.

Properties of the enzyme

The following experiments were carried out to study the properties of the enzyme. The enzyme fraction which obtained after DEAE step was used.

Effect of pH on enzyme activity

The enzyme was assayed at different pH values ranging from 4.0 to 7.0 under the conditions given in standard enzyme assay procedure.

Effect of incubation temperature on the enzyme activity

The enzyme was assayed at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C and 55 °C in a thermostated water bath under the conditions given in standard enzyme assay procedure.

Thermal stability of the enzyme

The enzyme was pre-incubated at 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C in a thermostated water bath for 30 min. The samples were immediately cooled in ice water and then residual enzyme activity was assayed under the conditions given in standard enzyme assay procedure.

Determination of the K_m for enzyme

A concentration series of arginine ranging from 0.005 M to 0.4 M was prepared. From each concentration (1.00 ml), acetate buffer (50 mM, pH 5.2, 1.00 ml) and enzyme fraction (1.00 ml) were used for each assay. The enzyme activity was assayed under the conditions given in standard enzyme assay procedure. K_m was determined by plotting $1/\text{initial velocity}$ against $1/[\text{Substrate}]$.

RESULTS & DISCUSSION

Kinetics of the enzyme activity with the growth of *C. gleosporoides* cultures were studied to determine the ideal time to harvest the mycelia. As shown in Fig.1,

enzyme activity of the liquid cultures appeared as a sharp peak and it reached the maximum on day 9. This time, which provide maximum enzyme yield always correlated with a change in the color of the culture from off white to pale orange.

The crude enzyme extract, which was prepared at this stage, was fractionated with ammonium sulphate. The result showed that arginine decarboxylase of *C. gleosporoides* precipitates at 0-25% saturation of ammonium sulphate. Thus prepared enzyme fraction was purified by Sepharose 4B gel filtration chromatography. The enzyme was eluted as a narrow peak at fraction No. 6 (Fig. 2). The enzyme fraction which obtained from above gel filtration step was further purified by DEAE Cellulose chromatography. Enzyme was eluted at fraction no. 15 as a sharp peak before applying the NaCl gradient (Fig. 3).

The purity of the final enzyme fraction (obtained from DEAE Cellulose step) was screened by 10% SDS-PAGE (Fig. 4). The results of the enzyme purification are given in Table 1. Enzyme arginine decarboxylase was purified 25 fold with 16.7% recovery.

Table 1. Purification table of arginine decarboxylase from *C. gleosporoides*

Step	Total activity ($\mu\text{moles min}^{-1}$)	Total protein (mg)	Specific activity ($\mu\text{moles mg}^{-1}\text{min}^{-1}\text{protein}$)	% recovery	Purification fold
Crude	7.2	90	0.08	100	11
Ammonium sulphate	4.02	8.75	0.46	55.9	5.75
Sepharose 4B gel column	2.7	2.70	1.0	37.5	12.5
DEAE Cellulose ion exchange	1.2	0.6	2.0	16.7	25

Molecular mass of the purified enzyme as determined through Sepharose 4B gel filtration chromatography is around 265 kDa (Fig. 5). This value falls in the molecular mass range reported for arginine decarboxylases of *Lathyrus sativus* seed lings (Ramakrishna & Adiga, 1975) and soya bean hypocotyls (Nam *et al.*, 1997). SDS-PAGE (10%) of the purified enzyme showed two bands at 65 and 25 kDa. These results suggest that possibly this enzyme could be a hexamer of two subunits, resembling arginine decarboxylases of *Lathyrus sativus* seedlings (Ramakrishna & Adiga, 1975).

At this purification stage the properties of the enzyme were studied. Under this, effects of incubation temperature and pH, and the thermal stability of the enzyme were investigated. The purified enzyme was quite stable and showed an optimum at pH 5.2 (Fig. 6), which was comparable with that reported for arginine decarboxylase of *E. Coli*, *Panus triginus* and *Aeromonas shigelloides* (Boeker & Snell, 1968; Khan & Minocha, 1989). The enzyme showed high activity in the temperature range of 35 °C – 45 °C with a maximum at 40 °C (Fig. 7), which was very close to that reported for arginine decarboxylases of *Lathyrus sativus* seed lings (Ramakrishna & Adiga, 1975).

Beyond 50 °C enzyme activity is slowly declined and almost nil at 80 °C (Fig. 8) similar to that reported for Arginine decarboxylase of *E. Coli* (Boeker & Snell, 1968). As determined by Lineweaver-Burk plot approximate K_m value of arginine decarboxylase for substrate arginine was 67 mM (Fig. 9) which was quite comparable with that reported for arginine decarboxylases of soya bean hypocotyls (Nam *et al.* 1997).

Fig.1

Fig. 1 Variation of enzyme activity with the growth of *C. gleosporoides* cultures

Fig. 2

Fig. 2 Sephacrose 4B Gel filtration chromatogram of Arginine decarboxylase from *C. gleosporoides*

FIG. 3

Fig. 3 DEAE Cellulose chromatogram of the enzyme after Sepharose 4B step

Fig. 4 SDS-PAGE of the purified enzyme

Fig. 5

Fig. 5 Determination of the molecular mass of arginine decarboxylase from *C. gleosporoides*

Fig.6

Fig. 6 Variation of arginine decarboxylase activity of *C. gleosporoides* with pH

Fig. 7

Fig. 7 Variation of arginine decarboxylase activity of *C. gleosporoides* with incubation temperature

Fig. 8

Fig. 8 Thermal stability of arginine decarboxylase from *C. gleosporoides*

Fig. 9 Determination of K_m for arginine decarboxylase from *C. gleosporoides*

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