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Purification of NADP dependent, 1, 3, 6, 8 - tetrahydroxynaphthalene reductase from *Verticillium dahliae brm* - 1 (ATCC 44571)

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Abstract

NADPH dependent 1,3,6,8- tetrahydroxynaphthalene Reductase, one of the key enzymes of 1,8-dihydroxy melanin (DHN melanin) biosynthesis was isolated from *Verticillium dahliae brm-1* (ATCC 44571) and purified to near homogeneity (as shown by SDS-PAGE), using ammonium sulphate fractionation, ion exchange chromatography on DEAE Cellulose, FPLC Mono Q anion exchange and Superose - 12 gel filtration chromatography. Fold purification and yield of the enzyme were found to be 1423 and 0.47% respectively. Specific activity of the final enzyme fraction was 79.7 (nmole/ mg.min) Approximate molecular weight of the purified enzyme was determined as~24 kDa by Superose-12 gel filtration chromatography.

A satisfactory HPLC based enzyme assay was also developed for the purification of enzyme.

Keywords : Melanin biosynthesis, *Verticillium dahliae brm-1*, Tetrahydroxynaphthalene Reductase, purification.

1. Introduction

Melanin synthesised via the pentaketide pathway (Fig. 1) from 1,8-dihydroxynaphthalene (DHN) is an integral part of the cell walls of a number of plant pathogenic fungi[1,2] such as *Verticillium dahliae, Pyricularia oryzae, Colletotrichum lindemuthianum* and *Thielaviopsis basicola.*

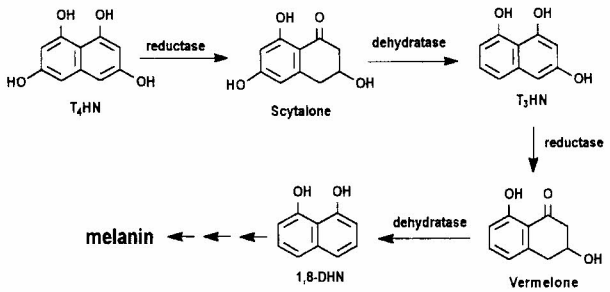


Figure 1. Pentaketide pathway of 1,8-Dihydroxynaphthalene (DHN) melanin biosynthesis

Melanized cells of these fungi are directly involved in pathogenesis. For example, studies with inhibitors of melanin synthesis and melanin-deficient strains of fungi have shown that melanin formation provide the required strength and rigidity to the appressoria of the pathogen for a successful penetration into the host tissue [3,4,5,6,7].

Known inhibitors of melanin synthesis such as Tricyclazole, fthalide, pyriquilon and carpropamide are used at nonfungitoxic concentrations to control rice blast disease caused by fungus *Pyricularia oryzae* by blocking DHN melanin synthesis in appressorial cell walls [8,9,10,11].

The biosynthesis of DHN melanin is well documented and 1,3,6,8 Tetrahydroxynaphthalene (T4HN), (+)-scytalone, 1,3,8-Trihydroxynaphthalene (T3HN) (-)-vermelone and 1,8-Dihydroxy naphthalene (1,8-DHN) have been found as the major intermediates of the biosynthetic pathway (Fig.1). The pentaketide intermediate, assembled from C_3 acetate units cyclised to form symmetrical T4HN, presumably by a specific polyketide synthase. Reduction of T4HN to scytalone and dehydration of scytalone to T3HN accomplished by reductase and dehydratase enzymes respectively. Subsequent reduction and dehydration steps give rise to vermelone and 1,8-Dihydroxy naphthalene (1,8-DHN) respectively [12,13,14,15,16] and so formed, 1,8-DHN polymerised to fungal melanin by air oxidation.

Wheeler showed [13] that above reductase reactions, which required NADPH as a co factor were inhibited by fungal melanin inhibitors such as tricyclazole, pyriquilon, PP-389 whereas Nakasako et al.[11] showed that dehydratase reactions was inhibited by carpropamide.

Scytalone dehydratase of *Pyricularia oryzae* has been purified [17] and no work has been reported on purification of reductase or dehydratase from *Verticillium dahliae.* However, the reductase activity has been demonstrated in cell-free homogenates of *Verticillium dahliae*[13, 18] and these homogenates effectively reduce 1,3,6,8-tetrahydroxynaphthalene (T4HN) and 1,3,8-trihydroxy naphthalene (T3HN) into scytalone and vermelone respectively.

Our aim was to purify 1,3,6,8-terahydroxynaphthalene reductase from *Verticillium dahliae.* Once the purified enzyme is available, and it will be able to study in detail, the binding characteristics of the melanin inhibitors as well as other synthetic inhibitors to the enzyme.

In this work mutant *V.dahliae brm-1* (ATCC 44571) which lacks the dehydratase activity necessary for melanin biosynthesis was selected as the most suitable organism to isolate the enzyme reductase. Due to the genetic lesion of dehydratase, this strain is incapable of converting scytalone or vermelone into their corresponding napthol intermediates, and so the reduction products accumulate [1,12,14]. Thus, the enzyme may be assayed simply and directly by following the transformation of T4HN to scytalone or T3HN to vermelone without formation of other later metabolites such as 1,8-Dihydroxynaphthalene or melanin.

For the first time, this paper reports the purification of tetrahydroxynaphthalene reductase from mutant *V.dahliae brm-1* (ATCC 44571) using ion exchange and gel filtration chromatography.

2. Materials and Methods

V.dahliae brm-1 (ATCC 44571) was kindly provided by ICI plant protection. Berkshire, U.K. All biochemicals were obtained from Sigma Chemicals Co., St. Louis, Mo, USA. Sterilisation of all the materials prior and subsequent use was carried out in autoclave at 15 p.s.i for 30 minutes. Centrifugations were performed on a Sorvall RC 5C high speed centrifuge. The shaken cultures were grown on an orbital shaker Innova, manufactured by New Brunswick Scientific Co., USA.

Culture conditions

Stock slope were maintained on Potato Dextrose Agar (Oxoid). Liquid cultures were grown on modified Brandt's sucrose nitrate medium shaking at 200 rpm in the dark at 26 ^{0}C.

Kinetics of the enzyme activity with the culture growth

Cultures were grown as described above the mycelia were harvested, after 3, 4, 5, 6, 6. 5, 7, 7.5 days of the inoculation. Cell free extracts were prepared and assayed for the enzyme activity as given below. In each assay, enzyme activity was quantified by the amount of scytalone (ng) produced per milligram of total protein and per minute.

Standard Enzyme assay procedure

Before assaying for the reductase activity, the enzyme preparations were dialysed for four hours against potassium phosphate buffer (100mM, pH 6.9), containing EDTA (1mM) and dithiotheritol (lmM) at 4^{0}C.

The enzyme solution (100µl,. 4.8mg/ml) was diluted with potassium phosphate buffer (100mM,pH 6.9, 300µl) containing EDTA (1mM) and DTT (1mM) and incubated with NADPH (17µg) under nitrogen for 15min. Tetrahydroxynaphhalene (4µg) in ethanol (2µl) was then added to the reaction mixture and the incubation was continued under nitrogen for a further 45 min at 30^{0}C. Similarly, a control experiment was performed by incubating the substrate in the absence of enzyme. The assay mixture was then acidified to pH 5 with phosphoric acid (2M), saturated with brine and extracted into ethyl acetate (2x500µl). The ethyl acetate layer was concentrated and analysed by HPLC.

Samples (25µl) were applied to a C_{18} reverse phase column (Anachem spherisorb, 25x0.46 cm), equilibrated and run isocratically in acetonitrile: water: acetic acid (20:78:2v/v) at a flow rate of 1ml/min. The metabolites were detected at λ 254 nm with the sensitivity set to 0.02 AUF. An authentic sample of scytalone, isolated from cultures[19] of *P.largerbergii* was used as the reference.

1,3,6,8-Tetrahydroxynaphthalene was synthesized as the method described in *Vivani et al*[20]. Standard curve for known concentration of scytalone against the peak area was constructed to quantify the amount of scytalone produced in each assay.

Enzyme Purification

Unless otherwise stated, all the operations were carried out at 0 to 4°C. Quantitative estimation of protein was done by Bradford method[21] using Bovine serum albumin as the standard.

Preparation of cell-free extracts

V.dahliae brm-1 cultures were grown in 20 liters of modified Brandt's sucrose nitrate medium as described above. Mycelia (280g), grown for 6-7 days were harvested suspended in potassium phosphate buffer (100mM, pH 6.8) containing EDTA (1mM) and dithiotheritol (1mM) mixed with glass beads lysed in a carbon dioxide cooled ball-mill (Braun cell homogeniser) for 4 min. Crude extract was centrifuged at 38,000g for 20 min. at 4°C to remove partculate materials and glass beads. The supernatants were collected.

Removing nucleic acids

Streptomycene sulphate (1%, w/v) was added to the crude extract, stirred on ice for 30min. The solution was centrifuged at 38,000g for 20 min at 4°C.

Ammonium sulphate fractionation

The 35-90% pellet was obtained by fractionating supernatant solution with ammonium sulphate and centrifuging at 40,000g for 20 min at 4°C. The protein pellets were resuspended in potassium phosphate buffer (100mM, pH 6.8) containing EDTA (1mM), dithiotheritol (1mM) and glycerol (10%, v/v) and stored at-80°C, until required for purification and other analysis.

Ion exchange chromatography on DEAE Cellulose

As the preliminary step bulk protein pellet was purified by ion exchange chromatography on Whatmann DE 52 as given below. The enzyme, eluted between 0.2-0.8 M NaC1 was collected.

The protein solution (160mg in 257 ml) which was dialysed against the phosphate buffer (50mM, pH, 7.5) containing EDTA (1mM), dithiotheritol (1mM), glycerol (20%, v/v) and NaCl (0.2M) for 4 hours at 4°C was added to the ion exchanger and stirred slowly for 15 min with a glass rod. The slurry was allowed to settle down for approximately 30 min and the supernatant (A) decanted. Further 60 ml of buffer was added to the ion exchanger and again stirred for 15 min. Once, the slurry has settled, the supernatant was decanted and combined with the supernatant (A).

Secondly, ion exchanger was stirred with above buffer (80ml) in 0.8 M sodium chloride as in the previous step. Once, the slurry had settled, the supernatant (B) was decanted. The procedure repeated with another volume (60ml) of above buffer in 0.8M sodium chloride. The supernatant was decanted and added to the supernatant (B).

Purification by FPLC

MonoQ anion exchange chromatography

The enzyme fraction eluted from above ion exchange step(0.2-0.8M NaC1 fraction) was dialysed against the Bis-Tris propane (50mM, pH 6.9) containing EDTA (1mM) and dithiotheritol (1mM) and fractionated by Mono Q anion exchange chromatography. For each run, enzyme fraction (10ml, 1.5 mg/ml) was applied to the column at a flow rate of 1ml/min. Above Bis-Tris buffer was used as the buffer A and the same buffer with 1M sodium chloride was used as the buffer B. Linear gradient was run from 0 to 40% of buffer B over 40 ml and increased to 100% over another 5ml. Fractions (1ml) were collected and assayed for reductase activity. The enzyme was eluted at salt concentration of 0.33-0.35 M in three fractions (fraction 32-34). The highest activity was found in fraction 33.

Superose 12 gel filtration chromatography

Active fractions obtained from the above steps were pooled and concentrated to 5ml using Amicon ultrafiltration kit. Further concentration was achieved by dialysing against a solution of polyethylene glycol (70%, w/v). Then concentrated protein solution (4.4 mg in 1.0ml) was then applied in 200µl aliquots to a Superose - 12 (pharmacia) gel filtration column, equilibrated and eluted with Tris-HCI (50mM, pH 6.9) containing EDTA

(lmM), dithiotheritol (lmM) and sodium chloride (150mM) at a flow rate of 0.25ml/min. Fractions (lml) were collected and assayed for reductase activity. Fractions no. 15 16 and 17 showed the highest reductase activity.

Purity assessment by SDS-PAGE gel electrophoresis

Fractions 15,16 and 17 of Superose 12 column which contained highest reductase activity were analyzed by SDS-PAGE gel electrophoresis. Aliquots (200µl) of each fractions was concentrated to 90µl and proteins were precipitated by trichloro acetic acid (72%w/v, 10µl).

Bio-rad Mini Gel protean II apparatus was used to run the SDS-PAGE (10%) gel. SDS molecular weight markers (Sigma) were used as the gel markers. Following buffers were used for SDS-PAGE gel.

Gel buffer : Tris-HCI (3M, pH 8.45) containing SDS (0.3%w/v)

Cathode buffer : Tris-HCI (100mM) containing glycine (100mM) and SDS (0.1%, w/v)

Anode buffer : Tris-HCl (200mM, pH (8.9)

The gel was run at 300m A over 2.5 hours. Initial 30 min, was run at 35V and the last two hours at 75 V. After running, the gel was stained with Coomassie Blue and then silver stained for further investigation.

Estimation of the molecular weight of napthol reductase

The Superose - 12 gel filtration column was pre-equilibrated and run in Tris-HCl (50mM, pH 6.9) containing EDTA (lmM) and sodium chloride (150mM) at a flow rate of 0.25ml/mm under the same condition as given in above Superose-12 gel step. Following standard proteins, were used to calibrate the Superose 12 column.

Bovine serum albumin (66 kDa), egg albumin (45 KDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) carbonic anhydrase (29 kDa) and α-lacto albumin (14 kDa) were used to calibrate the superose-12 gel column. A mixture of each protein (100µg) in Tris-HCl (50mM, pH 6.9, 200µl) containing EDTA (lmM), dithiotheritol (lmM) and sodium chloride (150mM) was loaded to the column. The retention time of each protein was measured. Curve for the log M.W. versus retention time was plotted and molecular weight of NADPH dependent napthol reductase was determined.

3. Results and Discussion :

Kinetics of the reductase activity with the growth of *V.dahliae brm-1* cultures were measured to determine the ideal time to harvest the mycelia in order to get the optimum enzyme yield. Enzyme activity was appeared as a sharp peak as shown in fig. 2 and it reached to maximum on day 6-7. This time, which provides maximum enzyme yield always, correlates with a change in the color of the culture from off-white to pale-pink.

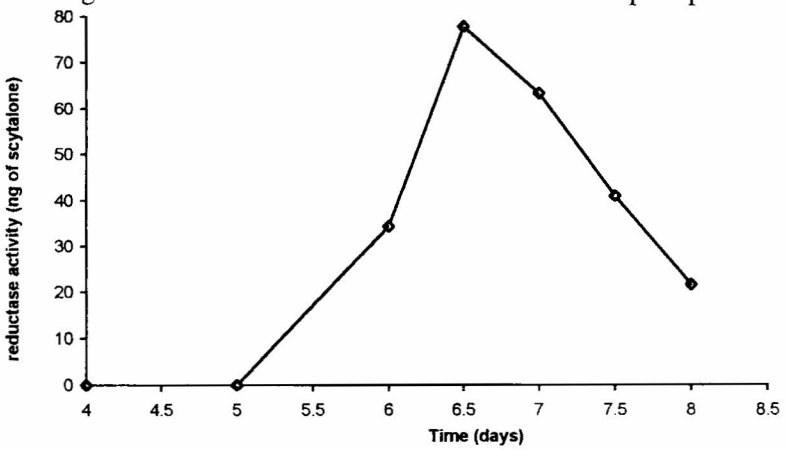


Figure 2. Kinetics of the reductase activity with the growth of *Verticillium dahliae brm-1* cultures

Conversion of T4HN to scytalone was analyzed under isocratic conditions (acetonitrile: water : acetic acid - 20 : 78:2) using C_{18} reverse phase column. Retention time of scytalone was 5.9 and that of T4HN was 12.5 min respectively (Fig.3)

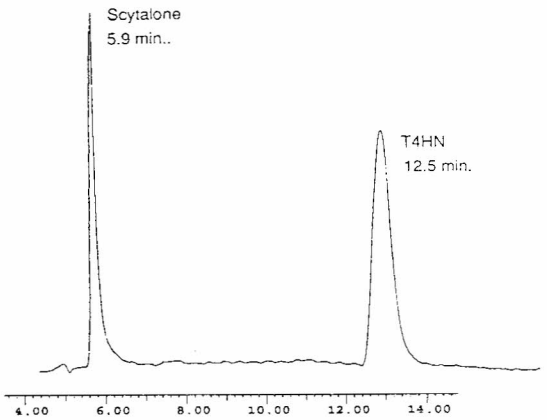


Figure 3. HPLC separation of 1,3,6,8-tetrahydroxynaphthalene (T4HN) and Scytalone in acetonitrile: water: acetic acid 20: 78:2 (v/v)

Scytalone isolated from ***P.largerbergii*** used as the reference. Under these conditions scytalone gave a sharp well-defined peak which can be easily detected even upto 100ng.

Optimum conditions for batchwise adsorption and desorption technique were established by performing a trial experiment with a small column of DE 52. In the FPLC purification on a Mono Q anion exchange column, the enzyme eluted at 0.33-0.35 M concentration of sodium chloride as a narrow peak which was limited to only three fractions (fraction 32-34). Most of the activity was found in fraction 33 (Fig. 4). Further purifica-

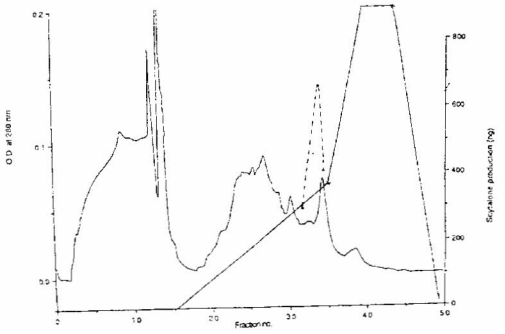
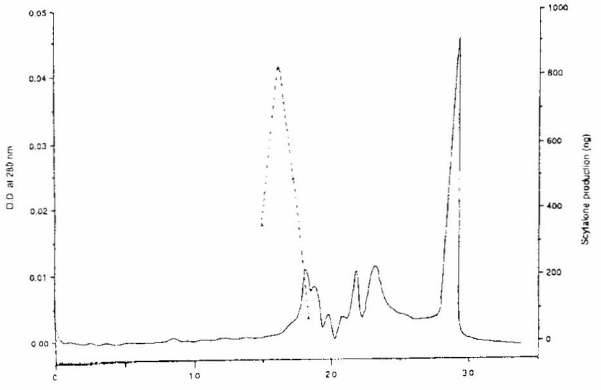


Figure 4. Fractionation of DEAE Cellulose fraction on Mono Q anion exchange column

--------reductase activity —X— NaCl concentration ——— protein elution profile

tion was achieved by applying concentrated Mono Q enzyme fraction onto a Superose - 12 gel filtration column. In a trial experiment enzyme was eluted with Tris-HCl (50mM, pH 6.9) at a flow rate of 0.5ml/min and the enzyme activity was found over 9 fractions providing poor resolution. Hence, the methodology was improved to enhance the resolution by using 150mM sodium chloride with Tris-HCl (50mM, pH 6.9) buffer containing EDTA (lmM) and DTT (lmM) and running the column at flow rate of 0.25ml/min instead of normal rate 0.5ml/min. The activity was limited to three fractions (fraction no.15-17, fig 5). The highest activity was in fraction no. 16.



These active fractions were concentrated and analyzed by SDS-PAGE gel electrophoresis. After staining with Coomassie Blue all three fraction produced a single band of approximate Mr. 24,000 and for further investigations the gel was silver stained. All three fractions showed the major band around Mr. 24,000 and this band was more intense in fraction no. 16. In addition to this major fraction nc, 15 showed a very faint band around Mr 36,000. Fraction 16 showed two faint band around Mr. 36,000 and Mr. 16,000 and fraction 17 showed a faint band around Mr. 16,000. But these bands are too faint to be visualized on a gel photograph (Fig. 6).

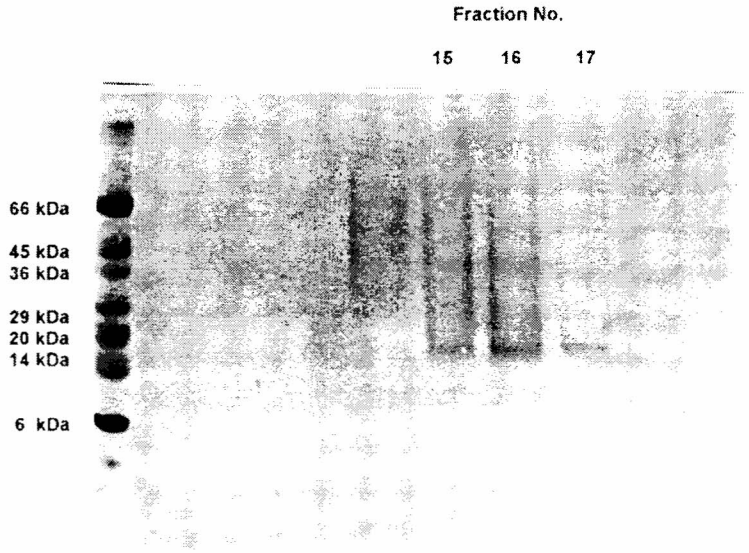


Figure 6. SDS-PAGE (10%) of reductase enzyme after fractionating through Superose-12 column

Specific activity, percentage recovery and progress of purification of the reductase at each step given in Table 1.

Table. 1 Purification Table

Step	Volume (ml)	Total Protein (mg)	Specific activity (nmole/mg min.)	Recovery %	Purification fold
Ammonium sulphate fraction	256	1690	0.056	100	1
Batch	163	528	0.14	78	2.5
Mono Q	125	4.4	10.5	48.8	187.5
Superose -12	15	0.03x	79.7	0.47	1423

M. W. of the purified enzyme as determined through Superose 12 gel filtration is also around 24 kDa (Fig. 7). Interestingly, this value is almost the same as that determined through SDS-PAGE showing that possibly this enzyme could be a monomer.

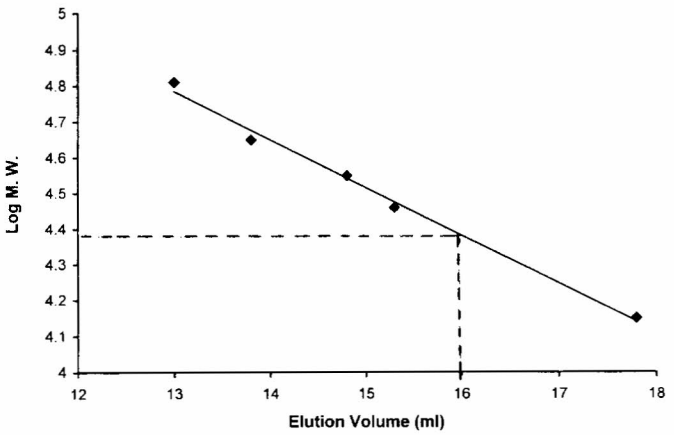


Figure 7. Estimation of the molecular mass of native reductase by Superose - 12 gel chromatography

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