Partial characterisaton of 1,3,6,8-tetrahydroxynaphthalene reductase from *Verticillium dahliae brm -1*

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Abstract

1,3,6,8-tetrahydroxynaphthalene reductase which isolated from *Veritcillium dahliae brm-1*, was purified and partially characterised. The result showed that optimum pH and temperature of the enzyme were pH 7 and 30^{0}C respectively. The enzyme was stable upto 40^{0}C, above that temperature enzyme activity slowly declined and inactivated above 60^{0}C. The probable primary sequence of the N-terminus of the enzyme was Ala-Lys-Ile-Tye-Asp-Asp-Arg-Leu-Thr-Gly respectively. Met and Arg are the other option for the first and 4th amino acids respectively.

1,3dihdroxynaphthalene is a new substrate for the enzyme and the substrate specificity of the enzyme for 1,3,6,8-tetrydroxynaphthalene (T4HN), 1,3,8-trihydroxynaphthalene (T3HN) and 1,3-dihydroxynaphthalene (1,3-DHN) is as follows.

T4HN>T3HN>1,3-DHN

Emodin appear to be inhibitor for the enzyme whereas 1,3-dihydoxy-6,8-dimethoxynaphthalene possibly acts as a competitive inhibitor as well as a weaker substrate for the enzyme.

Key word : Melanin biosynthesis, 1,3,6,8-tetrahydroxynaphthalene reductase, *Verticillium dahliae brm-1*, Characterisation

1. Introduction

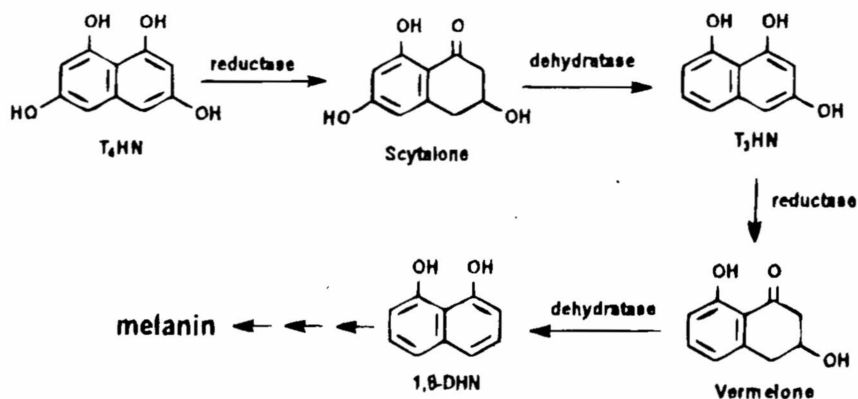
Fungal melanin, which synthesized from 1,8-dihydroxynpathalene via pentaketide pathway are important for the virulence of certain plant pathogenic fungi such as *Philaophora largerbergii, Pyricularia oryzae, Verticllium dahliae, Colletotrichum lagernarium etc*[1].

Melanin on the appressorial cell wall of these fungi play an indispensable role providing strength and rigidity for the penetration of pathogen into host tissue.

Several compounds eg. Tricyclazle, pyraquilon, PP-389, Capropamide which inhibits melanin synthesis are used at non fungi-toxic concentration to prevent the direct penetration of the pathogen into host tissue. Thus fungal diseases can be potentially controlled[1,2].

The biosynthesis of DHN melanin is well established. 1,3,6,8-Tetrahydroxynaphhalene (T4HN), scytalone, 1,3,8-trihydroxynapthalene (T3HN), vermelone and 1,8-dihydroxynaphthale are the major intermediates of the pathway. As shown in fig.1 reduction of T4HN to scytalone and dehydration of scytalone to T3HN accomplished by reductase and dehydratase enzymes respectively. Subsequent reduction and dehydration step give rise to vermelone and 1,8-DHN respectively and so formed 1,8-DHN polymerized to melanin by air oxidation[1,3].

Fig. 1 - Biosynthesis of 1,8-Dihydroxynaphthalene (1,8-DHN) melanin



Wheeler[1] showed that above reductase reaction, which required NADP as a cofactor were inhibited by melanin inhibitors such as tricyclazole, pyroquilon PP-389 where as Nakasako *et al.*[2] showed that dehydratase reaction was inhibted by carpropamide. Dehydratase of *Pyricularia oryzae* has been purified and characterized[4]. However, little is known about this reductase enzyme. Hence we aim to study the properties of this enzyme. Such a study would provide a better insight to understand the binding characteristics of melanin inhibitors as well as other synthetic inhibitors to the enzyme. The mutant *V. dahilae brm-1* which lacks the dehydratase activity necessary for melanin synthesis was selected as the most suitable organism for our study. Due to genetic lesion of dehydatase, this strain is incapable of coveting scytalone or vermelone into corresponding napthol intermediates, and so the reduction products accumulate. Thus the enzyme may be assayed simply and directly by following the transformation of T4HN to scytalone or T3HN to vermelone without the formation of other latter metabolites such as 1,8-

dihydroxynapthalene[1]. In a previous paper, we reported the purification of 1,3,6,8-tetrhydroxynaphthalene reductase from *Verticillium dahliae brm*[5]*-1.* Here we present the parial characterisation of the above purified enzyme.

2. Experimental

Materials and Methods

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

Culture Conditions

Stock slopes 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°C.

Enzyme Purification ;

Mycelia were harvested at 7th day after the inoculation (when enzyme active is reached to optimum level) of the culture. Crude extract were prepared. Thus, prepared enzyme fraction was purified by ammonium sulphate fractionation, ion exchange and gel filtration chromatography as reported earlier[5].

Standard Enzyme assay procedure

Before assaying for the reductase activity, the enzyme preparations were dialysed for four hours against potasim phosphate buffer (100mM, pH 6.9), contating EDTA (1mM) and dithiotheritol (1mM) at 4°C.

The enzyme solution (100µl,.~4.8 mg/ml) was diluted with potasium phosphate buffer (100mM, pH 6.9, 300µl) containing EDTA (1mM) and DTT (1mM) and incubatedwith NADPH (17µg) under nitrogen for 15 min. Tetrahydroxynaphhalene (4µg) in ethanol (2µl) was then added to the reaction mixture and the incubation was continued under nitrogen for a further 20 min at 30°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, 25 x 0.46 cm), equilibrated and run isocratically in acetonitrile : water : acetic acid (20 : 78 : 2v/v) at a flow rate of 1

ml/min. The metabolites were detected at λ 254 nm with the sensitivity set to 0.02 AUF. An authentic sample of scytalone, isolated from cuture[6] of *p. largerbergii* was used ad the reference.

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

Properties of the emzyme :

Kinetics of the product formation

The assay mixtures were incubated for 3, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. under the standard conditions. The products were analysed by HPLC and the peak area of produced scytalone was measured.

Optimum pH

The enzyme was assayed at different pH ranging from 5.8-9.0

0.1 M Citrate (pH 5-6), 0.1M phosphate (pH 6-7.5), 0.1M Tris-HCl (pH 8-9) were used as the buffers.

Optimum temperature

The enzyme was assyed at different temperature ranging from18-48°C in a thermostated water bath, under the standard assay condition. The products were then analysed by HPLC and the amount of scytalone product were quantified.

Thermal Stability

The enzyme was pre-incubated for 30 min. at 30°C, 40°C, 50°C, 60°C and 90°C in a thermostated water bath. Each sample was assayed under the standard assay conditions and snalysed by HPLC. The amount of scytalone produced was quantified.

Substrate Specificity

Reductase preparation (5ml, 1.28 mg/ml) in potassium phosphate (100 mM, pH 6.9) containing EDTA (1 mM) and dithiotheritol (1mM) was incubated with NADPH (35mg) for 15 min under nitrogen. 1,3-dihydroxynaphthalene (20mg) in acetone (500l) was added to the mixture and the incubation was continued overnight under nitrogen in the dark. The assay mixture was acidified with 2M phosphoric acid, saturated with brine and extracted into ethly accetate (2x5ml). The ethyl acetate layer was concentrated and analysed by t.l.c. (ethyl acetate : petroleum ether 6:4). The product was seen at Rf. 0.35. Isolation and purification of the product were accomplished by preparative t.l.c. using the same solvent system. The

pure product (1.2mg yeild, 6.6%) was analysed by ^{1}H NMR Spectroscopy and mass spectrometry. ^{S1}H in CD_2Cl_2 2.68 (1H, ddd, H-2az, J=1.67, 7.3,1.1 Hz), 2.95 (2H,m, H-2eq and H-4ax), 3.25 (1H, ddd, J=16.3,4,1.46 Hz, H-4eq), 4.46 (1H, septet, J=4.$_0$ Hz, H-3), 7.30 (2H, t, J=8.5Hz, Ar-H), 7.54 (1H,t, J=8.5 Hz, Ar-H), 7.89 (1H,d, J=8, Ar-H); m/e 162 (20%, M^+), 147 (17.4%, M^+ -CH3), 118 (19.9%), 91 (34.4%), 86(25%), 57(32.3%), 43(100%) $C_{10}H_{10}C_2$ requires M, 162

Inhibition studies

Preliminary Inhibition Studies

The enzyme (400μl, 1.3mg/ml) in pH 7 phosphate buffer containing EDTA (1 mM) and dithiotheritol (1mM) was used for each assay and pre-incubated with NADPH (14μg) for 15 min. under nitrogen. Each compound (400 nmole) which was to be investigated as an inhibitor (1,3-dihydroxy-6,8-dimethoxy naphthalene, resorcinol and emodin) was added to the reaction mixture and the pre-incubated under nitrogen for 10 min. the substrate 1,3,6,8-tetrahydroxynaphthalene (3μg) in ehtanol (2μl) was added and incubated for further 20 min. under nitrogen. The metabolites were extracted and analysed by HPLC under the standard assay conditions. Blank experiment was also performed in the same way without adding the inhibitor.

Inhibition level of 1,3-dihydroxy-6,8-dimethoxy naphthalene

The enzyme (400μl, 1.3mg/ml) in pH 7 phosphate buffer containing EDTA (1mM) and dithiotheritol (1mM) was used for each assay. Enzyme was pre-incubated with NADPH (14μg) for 15 min. under nitrogen. Then different dose levels 1,3-dihydroxy-6,8-dimethoxynaphthalene eg. 4.5, 45, 113, 227, 454 and 1363 nmole was added to the reaction mixtures respectively and incubated for 10 min under nitrogen. Afterwards the substrate 1, 3, 6, 8-tetrahydroxynaphthalene (3μg) in ethanol (2μl) was added to all the reaction mixtures and incubated overnight under nitrogen. The metabolites were extracted and analysed by HPLC under the standard assay condition . Blank experiment was also performed in the same manner without adding the inhibitor.

Inhibition level of Emodin

The experiment was performed similar to the experiment with 1,3-dihydroxy-6,8-dimethoxynaphthalene. The following dose level of emodin were used for the experiment. 10, 40, 220, 330, 400 nmoles emodin.

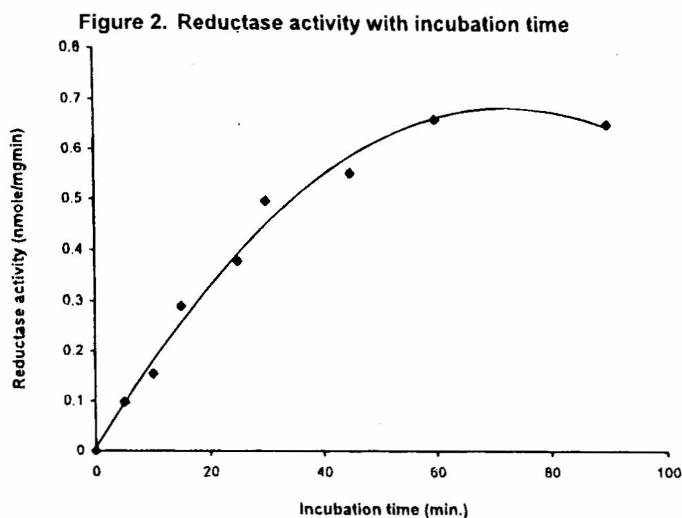
After the incubation metabolites were extracted and analysed by HPLC under the standard assay conditions. Blank experiment was also performed in the same manner without adding the inhibitor.

3. Results and Discussion

Kinetics of the product formation

The formation of the product was continued over 60 min. and linear for an initially period of 25min.

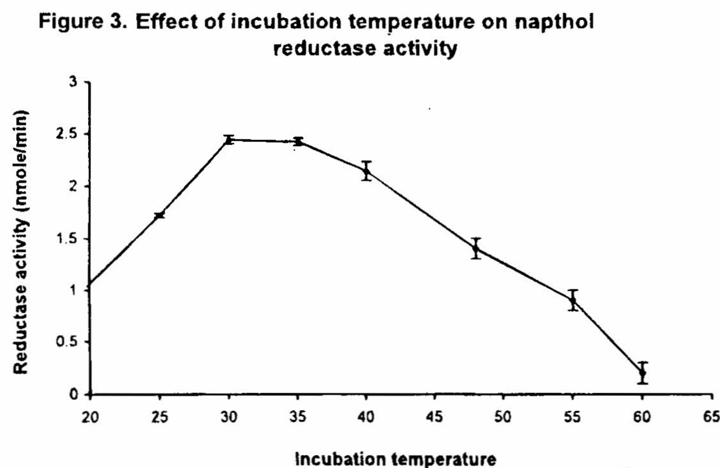
Figure 2. Reductase activity with incubation time

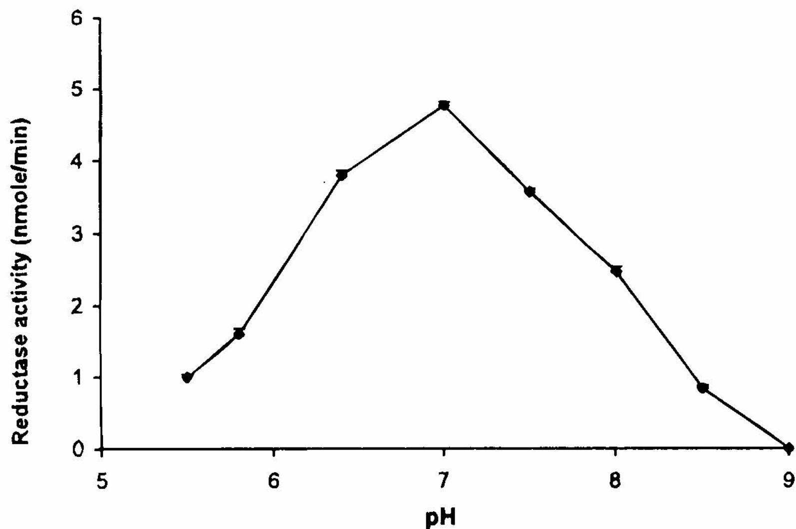


Optimum assay temperature and the pH

Optimal assay temperature for the enzyme lies between 30 -35°C and the optimum pH is found to be around pH 7. Interestingly these values are quite comparable with that reported for 1,3,8-trihydroxynapthalene reductase of *Magnapotha grisea*[7].

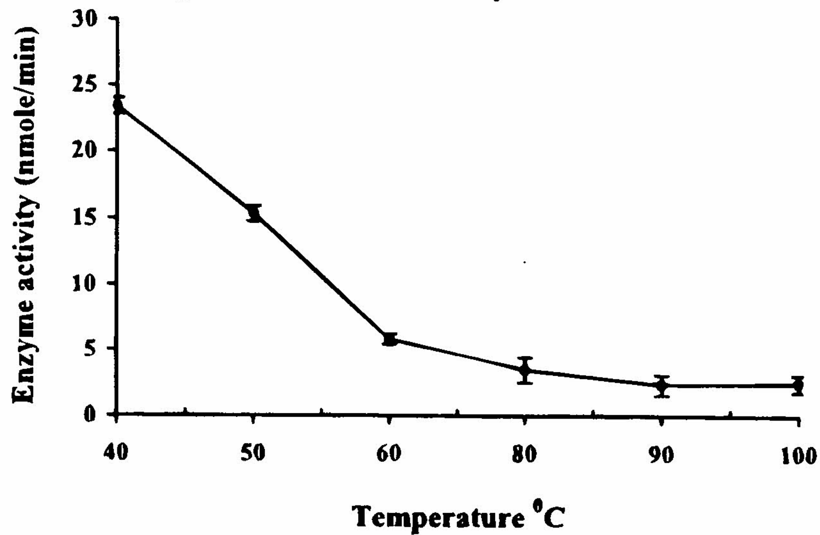
Figure 3. Effect of incubation temperature on napthol reductase activity





Thermal Stability

Enzyme was stable up to 40°C, beyond that temperature enzyme activity slowly decilned and completely inactivated above 60°C.



Substrate Specificity

1,-3dihydoxy-6,8-dimethoxynaphthalene, 1,3-dihydroxynaphthalene, resorcinol and emodin were investigated as possible alternative substrates of the enzyme. Since 1,3-dihydroxynaphthalen is a photosensitive compound this putative substrate was assayed in the darkness. The result obtained indicated that 1,3-dihydroxy-6,8-dimethoxynaphthalene, resorcinol and emodin were not metabolized by reductase preparation suggesting that these three compounds were not subsrtate for reductse. By contrast, when 1,3-dihydroxynaphthalene was included in the reaction mixture, a metabolic product was observed by t.l.c. at Rf. 0.35. NMR and Mass spectra. of the isolated product showed that it is 3,4-dihydro-3-hydroxy-1(2H)-naphthalenone which is an deoxy analogue of scytalone and vermelone.

Fig. 6 -^{1}H NMR of 3,4-dihydro-3-hydroxy-1-(2H)-naphthalenone

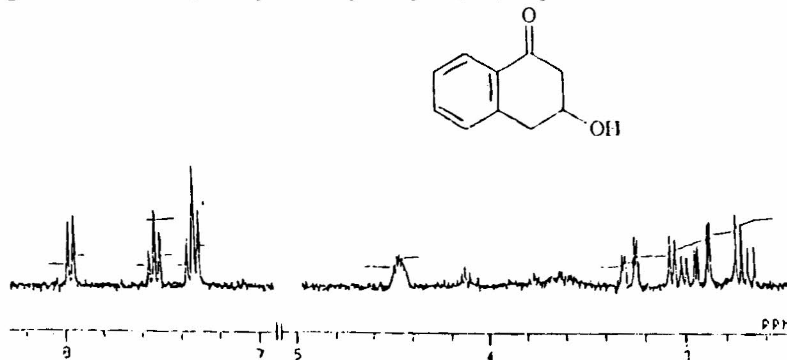
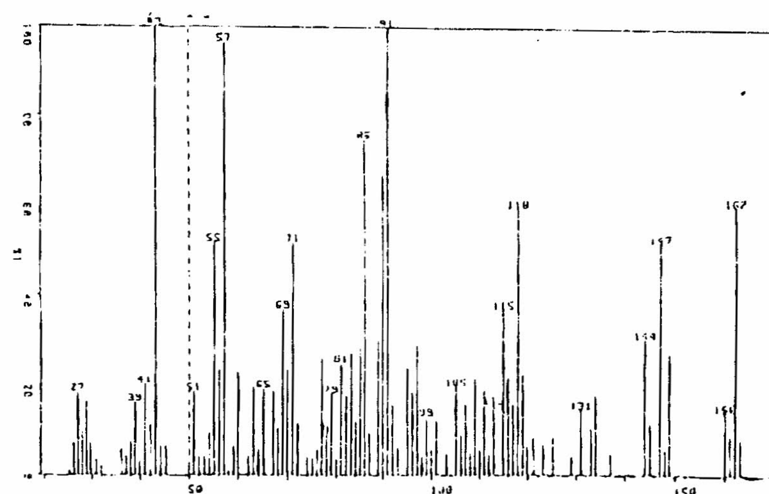


Fig. 7 - Mass spectra of 3,4-dihydro-3-hydroxy-1-(2H)-naphthalenone



Inhibition studies

Since 1,3-dihydoxy-6,8-dimethoxynaphthalene, reorcinol and emodin were not substrates for the reductase the possibility that they act as inhibitors was investigated. Preliminary incubations of reductase with 400 nmole of each inhibitor compound and 15 nmole of 1,3,6,8-tetrahydroxynaphthalene showed that 1,-3dihydoxy-6,8-dimethoxynaphthalene and emodin are inhibitors whereas resorcinol is not. For further investigations, the enzyme was incubated with different dose levels of each inhibitor.

Inhibition with 1,3-dihydoxy-6,8-dimehoxynphathalene

Incubation of the enzyme with 4.5 to 1363 nmole range of 1,3-dihydoxy-6,8-dimethoxynaphthalene showed that up to 227 nmole level 1,-3dihydoxy-6,8-dimethoxynaphthalene inhibits the reductase activity. However, above this dose level, enzyme activity again increased unexpectedly (Fig.8). This observation might be due to, the fact either 1,3-dihydoxy-6,8-dimethoxynaphthalene act as a weaker substrate and gives a metabolic product which has a similar retention time to that of scytalone or during the work up of the assay mixture, probably 1,3-dihydoxy-6,8-dimethoxynaphthalene decomposes into another compound which has a retention time same as scytalone. Further studies on this reveal that though 1,3-dihydoxy-6,8-dimethoxynaphthalene can decomposes to another compound. the retention time of that particular compound is not similar to scytlone. However, a very small peak having a same retention time as scytlone, was seen in the overnight incubation of 1,3-dihydoxy-6,8-dimethoxynaphthalene (1363 nmole) with the reductase in the absence of 1,3,6,8-tetrahydroxynaphthalne. This suggests that 1,3-dihydoxy-6,8-dimethoxynaphthalene act as a competitive inhibitor as well as a weaker substrate for the enzyme giving a metabolic product which has a same retention time as scytalone.

Inhibition with Emodin

Inhibition experiment with emodin showed that even at 11nmole level emodin completely inhibits the reduction of 1,3,6,8-tetrahydroxynaphthalene to scytalone. These results suggest emodin may be an irreversible inhibitor for the enzyme although further investigation are required to establish this fairly conclusively.

Primary sequence

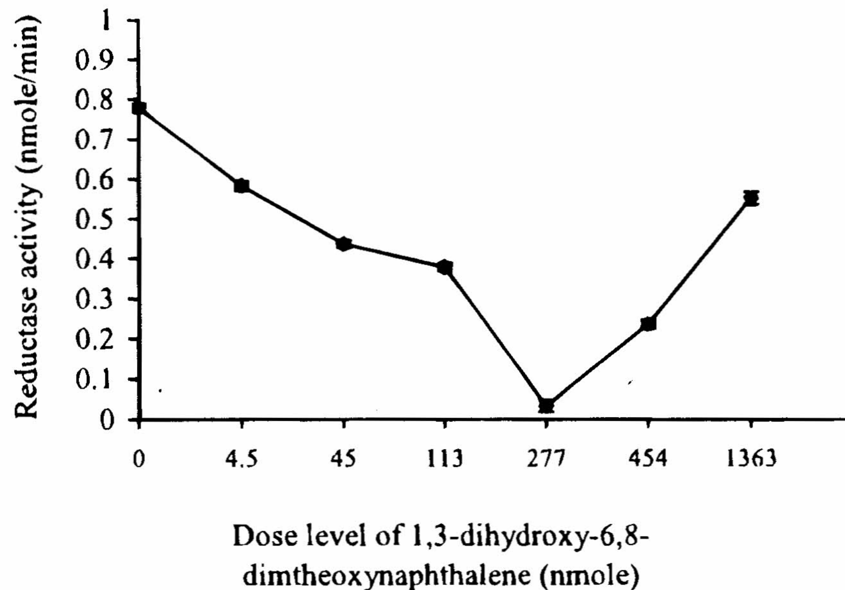
Primary sequence of the purified reductase was obtained on an applied biosystems 477A Protein Sequencer using Edmann degredation method. The experimental rsults showed that probable N-terminal primary sequence is as follows.

Ala-Lys-Ile-Tyr-Asp-Asp-Arg-Leu-Tyr-Gly
or or
Met Arg

The chromatogram of the 1st degradation cycle revealed that either Ala or Met is the possible amino acid in the sequence. Again the chromatogram of the fourth cycle showed that Tyr or Arg are the possibilities for the 4th amino acid.

During the sequencing process, partial proteolytic cleavage close N-termnus can take place releasing several N-termnal sub units. This could be reason for observing more than one residue in the first and fourth cycle.

Figure 8. Inhibition of reductase activity by 1.3-dihydroxy-6,8-dimethoxynaphthalene



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