# REDUCTION OF CINNAMIC ACID TO CINNAMALDEHYDE AND ALCOHOL

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The reduction of the carboxylgroup of phenylacrylic acids to form aldehydes and primary alcohols has long been inferred in the formation of lignin. In spite of the fact, that the stepwise reduction of ferulic acid (3-methoxy-4-hydroxycinnamic acid) to coniferylaldehyde and alcohol during the formation of lignin has been demonstrated *in vivo* (Higuchi and Brown, 1963), there exists no evidence for the enzymatic mechanism of such a conversion. The reduction of the phenylpropanoic acids to the analogous alcohols would be expected to require activation of the carboxylgroup and indirect evidence has accumulated that cinnamoylcoenzym A-thiolesters may be involved in this reaction in higher plants (e.g. Gross and Zenk, 1966). This report concernes the occurence of two distinct enzymes from a non lignin producing fungus, *Neurospora crassa*, which catalyzes the reduction of a number of cinnamic acids to cinnamaldehydes and further on to the corresponding cinnamalcohols. *N. crassa* has been known previously to reduce salicylic acid to saligenin *in vivo* (Bachmann *et al.*, 1960).

## Results and Discussion

From mycelia of *N. crassa* two enzymes could be extracted and partially purified; one previously unknown which catalyzes the reduction of aromatic acids to aromatic aldehydes and an

other one which catalyzes the reversible reduction of aromatic aldehydes to aromatic alcohols.

1. Aryl-aldehyde oxidoreductase:

Purification of this enzyme was carried out in 6 steps involving protamin and ammonium sulphate precipitation, calcium phosphate gel adsorbtion, chromatography on TEAE-cellulose (at pH 7,6 resp. 8,4) and on hydroxylapatite. The enzyme was purified in this way about 3oo fold with a 5o % yield, but still contained minor impurities. Its molecular weight was tentatively estimated as 120,000 using a calibrated sephadex G-2oo column (Andrews, 1964). This enzyme is not of constitutive nature, it is induced by salicylic acid during the growth of this fungus. As shown in Table I, the activity of the enzyme is completely dependent on NADPH and ATP; replacement of NADPH by NADH or replacement of ATP by CTP, GTP, or UTP did not yield an active system. A reduced thiol is necessary for full activity.

The enzyme has its pH optimum at pH 8,0, its temperature optimum at 35° and is stable for at least 2 months if kept at 0° in the presence of dithiothreitol. It has a broad substrate specificity towards aromatic acids of the $C_6$-$C_1$, $C_6$-$C_2$ and $C_6$-$C_3$ type. Among the cinnamic acids which are reduced (however with largely differing activities) are: cinnamic, o-, m-, p-coumaric, p-methoxycinnamic, caffeic, ferulic, isoferulic, 3,4-di-methoxy-cinnamic, 3,4-methylenedioxycinnamic and sinapic acid.

The stoichiometry of the reduction was determined by enzymatic and chromatographic analysis of the reaction products formed in the incubation mixture.

The enzyme catalyzes the following reaction:

$$R\text{-}COOH + ATP + NADPH + H^+ \longrightarrow R\text{-}CHO + ADP + PO_4^{-3} + NADP^+ + H_2O$$

Table I: Requirements for arylaldehyde oxidoreductase

| Reaction mixture | Rate of cinnamaldehyde formed ($m\mu moles \cdot min^{-1} mg^{-1}$) | Relative activity |
|---|---|---|
| complete | 374 | 1oo |
| omit $Mg^{++}$ | 261 | 7o |
| omit Dithiothreitol | 132 | 35 |
| omit NADPH | 0 | 0 |
| omit NADPH add NADH | 0 | 0 |
| omit ATP | 0 | 0 |
| heat denatured enzyme | 0 | 0 |

N. crassa (wild type, strain SY7A) was grown in shake cultures for 6o hours at 21° in 1 ltr of mineral salt medium (Beadle and Tatum, 1945) supplemented with 1 % saccharose, 0,2 % Difco-yeast extract and $10^{-2}$ M salicylic acid as inducer. The mycelium was filtered out and frozen at -15°. The fungal cells were disrupted sonically by means of a Branson S 125 Sonifier, and the extract was assayed for enzyme activity. The assay mixture for arylaldehyde oxidoreductase included in a total volume of 2 ml: tris-HCl, pH 8,o, 2oo µmoles; $MgCl_2$, 2o µmoles; dithiothreitol, 4 µmoles; potassium cinnamate, 4 µmoles; NADPH, 1 µmole; ATP, 4 µmoles and enzyme (spec.act. 45o mU/mg) as indicated. Temperature 3o°. The reaction was followed by measuring the decrease of the absorbtion of $NADPH_2$ at 34o mµ. This assay was used only with relatively pure enzyme preparations. With samples of less purity an assay involving $^{14}C$-labelled-substrate was used (Gross and Zenk, in preparation).

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For this enzyme we propose the systematic name: aryl-aldehyde: NADP oxidoreductase (ADP) (EC group 1.2.1). No evidence for activated intermediates has been obtained in this reaction; the reduction occures, at a depressed rate, even in the presence of 1 M hydroxylamine at pH 8,0. Aromatic acylphosphates and thiol-esters in the presence of NADPH are not reduced by this enzyme.

The stereochemistry of the transfer of hydrogen from NADPH is shown in Table II using p-methoxycinnamate as substrate, which gives the more stable p-methoxycinnamaldehyde as reaction product.

NADP-(-4-$^{3}$H) was prepared with a specific activity of 2,26·10$^{5}$ dpm/µmole (San Pietro, 1955) and the tritium transfere experiment conducted as described by Cornforth et al., 1966 .

Table II

| Cofactor | Specific activity of p-methoxy-cinnamaldehyde dpm/µmole |
|---|---|
| α-NADP$^{3}$H | 590 |
| ß-NADP$^{3}$H | 25 200 |

This results show clearly that the aryl-aldehyde oxidoreductase is a "B-type" enzyme as it uses the ß-hydrogen atom at carbon 4 of NADPH.

2. Aryl-alcohol oxidoreductase:

This enzyme, which catalyzes the reversible reduction of aromatic aldehydes to alcohols, has been purified by a similar sequence of steps as shown for the aldehyde oxidoreductase. The assay mixture for the alcohol dehydrogenase consisted in a total volume of 2 ml of: potassium phosphate, pH 6,8, 200 µmoles; cinnamaldehyde, 4 µmoles; NADPH, 0,5 µmoles and protein. Temperature 30°; again the consumption of NADPH during the reaction was measured. Due to the high activity of this enzyme, this assay could also be used with crude extracts by applying appropriate controls. The purification achieved was about 500 fold with a 38 % yield. Its molecular weight was tentatively estimated as 75,000. It is a constitutive enzyme of N. crassa. This enzyme is also dependend on NADPH. With NADH as cofactor, the rate of reaction is decreased to only 1 % of the rate achieved with NADPH. Maximum rates of reaction were observed in the case of aldehyde reduction at pH 6.8. In the reverse reaction pH 8.8 was optimal. The temperature optimum

is 55°. The equilibrium constant for cinnamalcohol as substrate was determined as $K = \frac{(\text{aldehyde})(\text{NADPH})(H^+)}{(\text{alcohol})(\text{NADP})} = 1.3 \times 10^{-10}$. The enzyme shows a broad substrate specificity. Of a series of aliphatic and aromatic aldehydes being attacked, cinnamaldehyde was the best substrate found. N-ethylmaleinimide and p-diazobenzene sulfonic acid at 1 mM concentration showed 0 and 100 % inhibition respectively. Substrate specificity studies and inhibition experiments as well as different other properties of this enzyme indicate that this enzyme belongs to the EC 1.1.1.1 category but that it differs from any known alcohol dehydrogenase (e.g. Katagiri et al., 1967; Culp and McMahon, 1968).

These two enzymes linked to each other would acount for the production of cinnamalcohols from cinnamic acids during lignin biosynthesis. However whether these enzymes actually do occure in higher plants is not yet known. Possibly a different way for the production of cinnamalcohols from the corresponding acids involving cinnamoyl-CoA-thiolesters or other metabolically-active bound cinnamoyl esters (El-Basyouni and Neish, 1966) takes place.

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