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THE PREPARATION OF AN ENZYME ASSOCIATED WITH AFLATOXIN BIOSYNTHESIS BY AFFINITY CHROMATOGRAPHY

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Summary: An affinity matrix for the purification of norsolorinic acid dehydrogenase, an enzyme involved in aflatoxin biosynthesis, was prepared by coupling norsolorinic acid to an agarose gel. This matrix was found to be ineffective in isolating active enzyme, and was therefore modified by methylation, using diazomethane. The methylated matrix produced a one-step purification of the enzyme from a crude homogenate, resulting in a 138-fold purification. The active isolate was found to contain one major and two minor bands upon non-denaturing electrophoresis, and all the norsolorinic acid dehydrogenase activity was associated with the major band. It was concluded that the matrix exhibited true affinity for the enzyme, and that affinity chromatography was a valuable approach to isolating other secondary metabolic enzymes involved in the biosynthesis of the aflatoxins.

Aflatoxins are biologically active, polyketide derived secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Knowledge of this biosynthetic pathway arises mainly from studies using nuclear magnetic resonance with stable isotopes (1) and mutants of *A. parasiticus*, which have the pathway blocked at specific points (2). Regulation of the pathway involves complex controls, which are strongly influenced by specific cellular events in the growth cycle (3,4).

To date very few enzymes involved in the biosynthesis of the aflatoxins have been isolated and fully characterized. Recently, however, an homogeneous protein has been obtained from *A. parasiticus* with methyl transferase activity (5). The presence of this enzyme has been known for some time (6) when it was found to convert sterigmatocystin to O-methylsterigmatocystin. The enzymes responsible for the earlier steps in the pathway have received less attention. Consequently we investigated the conversion of norsolorinic acid (NA) to averantin (AVN) by NA dehydrogenase (7,8) (Fig. 1).

In order to isolate this secondary metabolic enzyme with the minimum number of separation steps and the maximum of purification and activity, the technique of affinity chromatography has been utilized. The results of this work are presented here.

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MATERIALS AND METHODS

Nicotinamide cofactors, ω–aminohexylagarose and toluene sulphonyl fluoride were purchased from Sigma Chemical Co., USA. N-methyl-N-nitroso-p-toluene-sulphonamide (Diazald) was obtained from Aldrich Fine Chemicals, USA.

A versicolorin A accumulating mutant of *A. parasiticus* (1-11-105 Wh1, kindly donated by Dr. J. W. Bennet) was used as the source of crude enzyme.

A cell free extract was prepared by inoculating 100ml of sterile, chemically defined medium (8) in 250ml Erlenmeyer flasks with a 1ml spore suspension ($1x10^6$ spores), and shake incubated (150 rpm) at 28°C for 84 hr.

Powdered lyophilised mycelia were prepared as previously described (6,9) and stored desiccated at 4°C until required.

The powdered mycelia (5g) were suspended in 100ml cold 20mM phosphate buffer (pH 7.0) containing 10% v/v glycerol, 2mM monothioglycerol, 1mM EDTA and 2mM magnesium chloride (buffer A), containing 0.5mM toluene sulphonyl fluoride as a protease inhibitor. The homogenate was gently titurated for 30 min and centrifuged at 30 000 × g. The resulting supernatant was used as the crude enzyme preparation.

Enzyme activity was assayed by adding 1ml of enzyme preparation to 2ml buffer A containing 10mM NADPH. The reaction was initiated by adding 20µg NA dissolved in 20µl dioxane. The mixture was then incubated for 1 hr at 28°C. The reaction was stopped by shaking with 10 ml ethyl acetate and the AVN so produced was assayed for as described previously (8).

Protein concentrations of the active enzyme preparations were determined spectophotometricaly at 230 and 260 nm (10).

Preparation of the affinity matrix (11). A sample of Î-aminohexylagarose (AHA) (20 ml gel) was suspended in 40 ml 0.2M sodium borate buffer (pH 9.3). Twenty ml of a solution of p-nitrobenzoyl azide (0.1M) in dimethylformamide (DMF) was added to the gel suspension, and the mixture was gently stirred at 50°C for 1 hr and then at 25°C for 12 hr. Trinitrobenzenesulphonic acid was used to check for the completion of the acylation reaction (12). The gel was washed with cold distiled water containing 50% DMF. The p-nitrobenzamidoalkylagarose was suspended in a solution of 0.2 M sodium dithionite in 0.5 M sodium bicarbonate (pH 8.5) and the mixture was shaken for 2 hr at 40°C. The gel was filtered, washed with distiled water and suspended in an equal volume of ice-cold 0.5M hydrochloric acid. Sodium nitrite (0.1M) was added to the gel suspension and gently stirred for 7 min in an ice-bath. The gel was filtered and washed thoroughly with cold distiled water. It was suspended in an equal volume of cold phosphate buffer (0.2M, pH 7.0). NA (10mg) was dissolved in 20 ml phosphate buffer (0.2M, pH 7.0) : DMF (2:1, v/v). The NA solution was added to the diazonium agarose suspension, which was maintained on ice. The formation of a coloured gel (brick-red) began immediately, and coupling was complete in 30 min. The NA-agarose matrix was warmed to room temperature, filtered, and washed with distiled water.

The affinity gel was methylated by reacting it with diazomethane (prepared as per manufacturer's instructions). The affinity gel was suspended in 50% aqueous acetone (20 ml). Diazomethane in ether (20 ml) was gently added to the NA-agarose slurry and the methylation reaction proceeded at 25°C for 6 hr. The reaction was complete when the ether layer became colourless. Excess ether was decanted, and the gel was washed with water. The gel was then filtered and suspended in buffer A.

The NA-agarose was packed into a glass column (1.1 × 20 cm) and equilibrated with buffer A. The crude enzyme preparation (60 ml) was applied directly to the column, and unbound proteins were eluted with buffer A at a flow rate of 20 ml/hr, until the absorbance at 280 nm of the eluate reached a constant value of about 0.05 units. The enzyme was eluted with a linear gradient of 0 - 0.5 M KCl (40 ml) in buffer A, 4 ml fractions being collected. Each fraction was then assayed for norsolorinic acid dehydrogenase activity.

The active enzyme fraction from the NA-agarose column was analysed for homogeneity by 7.5% non-denaturing polyacrylamide gel electrophoresis (PAGE).

RESULTS AND DISCUSSION

The preparation of the initial affinity matrix depended on the diazotised agarose derivative coupling to the hydroxy-anthraquinone moiety of NA, at either positions 4,5 or 7 (Fig. 1) as directed by the hyroxyl (phenyl) groups. The reaction proceeded smoothly and was irreversible, as evidenced by the brick-red colour of the gel that could not be dislodged from the gel by extensive washing with buffers at various pH values.

The initial purification of the enzyme by this ligand proved disappointing, however, in that little or no NA

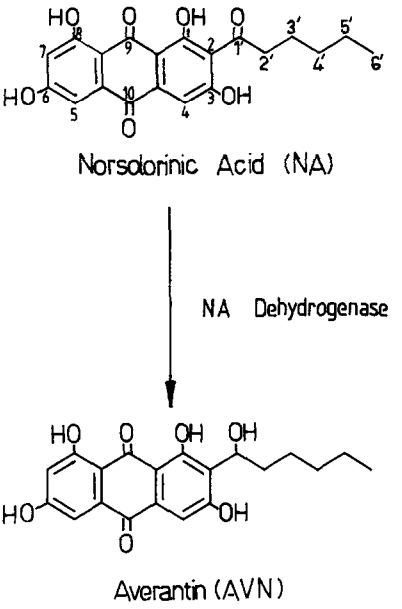

Figure 1. The conversion of norsolorinic acid to averantin by NA dehydrogenase.

dehydrogenase activity was recovered in the bound fraction. It was presumed that this was not due to a lack of specific binding between the enzyme and its immobilized substrate, since the points of attachment to the anthraquinone ring (either 5 or 7) would leave sufficient space for the enzyme to recognize the region of the molecule to which it normally binds (i.e. position 1') (Fig. 1).

A more likely reason for the loss of activity was the presence of phenol groups in the NA molecule, which seem to deactivate the enzyme when present in high concentrations. This effect has been noticed when assaying for enzyme activity using free substrate, and is particularly evident in purified enzyme preparations. It is presumed that this effect will be prevalent *in vitro* rather than *in vivo*, since in the latter case NA will tend to be associated with a membrane due to its hydrophobic character, and in addition, subsequent enzymes in the biosynthetic pathway will utilize NA, effectively preventing its accumulation. In order to test the potential adverse effects of phenolic groups, the affinity matrix was methylated by diazomethane, with conditions chosen to block only the more acidic groups at positions 5 and 7, leaving the less active hydroxyl group at position 1' available for specific enzyme recognition. That this treatment was effective was confirmed when the matrix changed colour from brick-red to orange.

When the crude enzyme preparation was applied to the methylated matrix, a highly active, purified enzyme solution was eluted in the bound fraction (Fig. 2). Non-denaturing PAGE of this fraction revealed one major

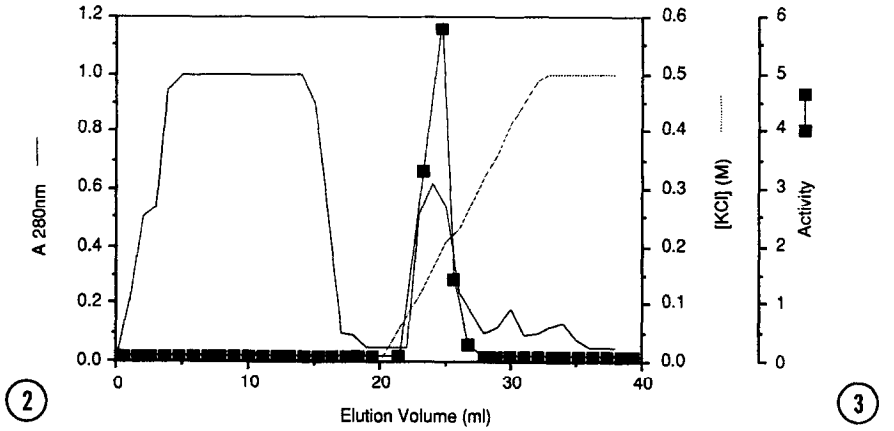

Figure 2. Elution profile for the affinity chromatographic separation of NA dehydrogenase on NA-Agarose. Crude enzyme (60 ml) was applied to 20 ml of the affinity matrix and washed with buffer A. Bound proteins were eluted by the addition of a linear gradient of KCl.
——— A 280 nm; ------- KCl; ■—■ NA dehydrogenase activity.

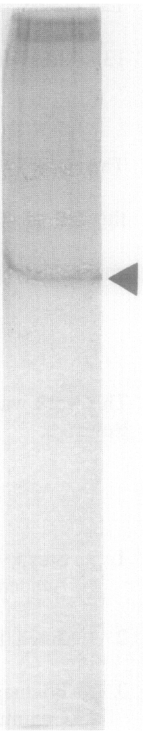

Figure 3. Electrophoretogram of NA dehydrogenase fraction after purification by affinity chromatography on NA-agarose.

and two minor protein constituents (Fig. 3). The major band, when excised and extracted from an unstained gel, contained all the NA dehydrogenase activity, whereas the two minor bands showed no activity towards NA.

Table 1. Purification of NA Dehydrogenase

Step	Vol. (ml)	Total Units [1]	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold
Crude Extract	60.0	190	360.0	0.53	1
NA-Agarose	6.0	95	1.3	73.0	138

[1] A Unit is defined as that amount of enzyme which will catalyse the conversion of 1 nmole norsolorinic acid to averantin per minute at pH 7.0 and 25°C.

The matrix described herein appears to display specific affinity towards NA dehydrogenase, and yielded a 138-fold purification of the enzyme in a single step from the crude preparation (Table 1).

The rapid purification of NA dehydrogenase from large amounts of contaminating fungal proteins has allowed the full characterization of this enzyme, and suggests that affinity chromatography should find wide applicability in the isolation of other secondary metabolic enzymes in the aflatoxin pathway.

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