BLUE SEPHAROSE*: A REUSABLE AFFINITY CHROMATOGRAPHY MEDIUM FOR PURIFICATION OF ALCOHOL DEHYDROGENASE

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SUMMARY: The separation of alcohol dehydrogenase from crude cottonseed extracts is accomplished in a single step by affinity chromatography. Blue Sepharose 6B effectively binds the enzyme, and elution is effected with NAD. The enzyme thus purified has a specific activity forty times higher than the original extract, and yields two closely-spaced bands with gel electrophoresis. The column is reusable for many separations without loss of efficiency.

Blue Sepharose 6B, a dye-coupled cross-linked agarose, has been shown to be an effective binding matrix for kinases and dehydrogenases. Selective elution may be accomplished by careful selection of cofactor concentrations and pH. Blue Sepharose 6B has been used to separate alcohol dehydrogenase (E.C.1.1.1.1) and other enzymes from yeast. Elution buffer for alcohol dehydrogenase contained 5 to 10 m*M* NAD, recovering 55% of the activity. The specific activity of the purified enzyme was 31X that of the crude extract (1).

We have applied the technique of purification by Blue Sepharose to crude cottonseed extracts. This paper reports the details of our application and the results obtained with its use on the alcohol dehydrogenase of cotton seeds.

METHODS

Cotton seeds (*Gossypium hirsutum* L.) were dehulled in a disc mill. Hulls and fines were removed by air classification, and the remaining

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kernels were stored desiccated at -50°. The kernels were ground in a knife mill for 15-30 seconds and brushed through a 60-mesh screen. This fine meal was homogenized and washed exhaustively with -20° acetone to remove lipid material. Following a final wash with -20° ether, the meal was dried at room temperature. Soluble proteins were extracted from 0.5 g meal with 7.5 ml cold water and centrifuged at 7700 x g for 20 min. at 5°.

Approximately 0.5 g dry Blue Sepharose, hydrated overnight in distilled water, yielded a packed column 0.8 x 12 cm. The column was equilibrated with starting buffer consisting of 20 m*M* Tris HCl, pH 6.4, 5 m*M* $MgCl_2$, 0.4 m*M* EDTA, and 1 m*M* 2-mercaptoethanol. The column, all solutions, and fractions were maintained at 5°.

The water extract, containing 3.2 mg protein and 0.8 international units of alcohol dehydrogenase per ml, was diluted 1:1 with starting buffer. After 4 ml of the diluted extract was loaded onto the column, the starting buffer was allowed to flow overnight at 0.12 ml/min to remove unbound sample material.

Elution of alcohol dehydrogenase was attempted with various concentrations of NAD in starting buffer. Flow rate was 0.12 ml/min; successive 0.6 ml fractions were collected until alcohol dehydrogenase elution was complete. Following elution, the column was washed with 5 ml 2 *M* NaCl in starting buffer, and equilibrated with 25 ml starting buffer for reuse.

Alcohol dehydrogenase activity was determined spectrophotometrically by measuring the increase in absorbance at 340 nm due to reduction of NAD with ethanol substrate (2). Enzyme activities are expressed in international units. Protein content was estimated by a Lowry procedure, modified for use in the presence of interfering materials such as Tris (3).

Electrophoresis of crude extracts and alcohol dehydrogenase-active fractions was accomplished using 2.5-27% linear gradient polyacrylamide gel slabs in 0.065 *M* Tris-borate buffer, pH 9.0. Protein bands in the gels were detected with Coomassie brilliant blue R-250 (4); alcohol dehydrogenase activity in the gels was indicated by nitro-blue tetrazolium (5).

RESULTS AND DISCUSSION

The Blue Sepharose column failed to bind alcohol dehydrogenase from crude unbuffered water extracts (pH 6.5). Binding was essentially complete, however, when the extract was diluted 1:1 with starting buffer. Although the point of column overload was not precisely determined, the maximum capacity of a column prepared from 0.5 g dry Blue Sepharose appears to be approximately 3 units of alcohol dehydrogenase, or twice the amount used in these studies.

Initial attempts at elution were successful using 5 m*M* NAD

Table 1. Recovery and Purification of Alcohol Dehydrogenase with Blue Sepharose 6B

Elution Starting Buffer plus	Recovery enzyme units	mg protein	specific activity	per cent recovered	purification
1 m*M* NAD	0.75	0.075	9.99	51%	43X
2.5 m*M* NAD	0.61	0.064	9.52	48%	40X
5 m*M* NAD	0.74	0.070	10.68	50%	45X
0-1 m*M* NAD gradient	0.51	0.050	10.14	30%	42X
0-5 m*M* NAD gradient	0.75	0.073	10.25	46%	43X

Sample loaded onto column contained 1.5 $\pm$ 0.2 units of enzyme activity and 6.4 $\pm$ 0.7 mg protein in 4.0 ml, specific activity 0.239 $\pm$ 0.003.

in starting buffer. Purification and recovery were comparable when the enzyme was eluted with 1, 2.5, and 5.0 m*M* NAD, or with a linear gradient of 0-5 m*M* NAD (Table 1). However, recovery was lowered when the enzyme was eluted with a linear gradient of 0-1 m*M*.

Elution patterns for alcohol dehydrogenase and protein are shown in Figure 1. Monitoring the absorbance at 280 nm indicated relative NAD concentration, since the NAD has some absorbance at this wavelength. The NAD peak obscures the peak which the protein would have given if it were, in fact, large enough to detect. Testing the fractions for alcohol dehydrogenase activity and for protein content revealed that the enzyme followed the leading edge of the NAD. For the 1 m*M* NAD elution, both enzyme activity and protein content are sharply peaked near fraction 7. The 0 to 5 m*M* NAD elutions all gave patterns similar to the 1 m*M* NAD elution. However, the elution pattern by the 0-1 m*M* NAD gradient peaks near fraction 13, and is not as sharply defined as the elution pattern by 1 m*M* NAD. Table I shows a corresponding difference in recovery with the 0 to 1 m*M* NAD gradient.

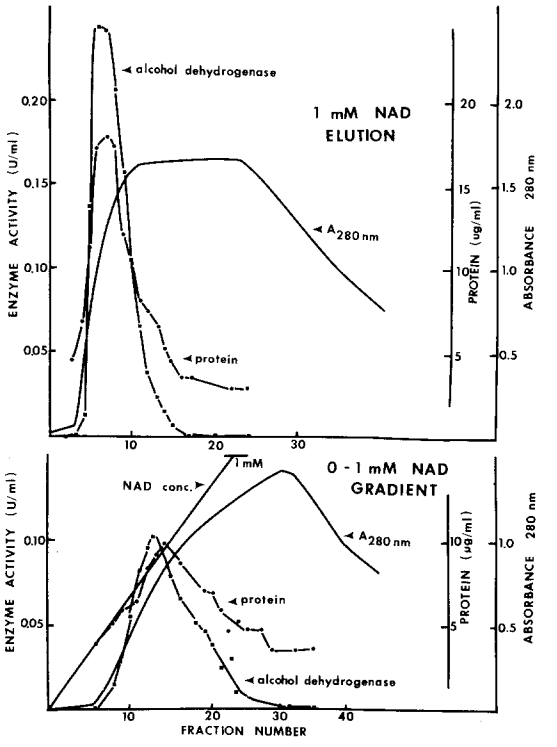

Figure 1. Elution patterns using 1 m*M* NAD and 0-1 m*M* NAD gradient for elution of alcohol dehydrogenase from Blue Sepharose. NAD conc. in lower figure refers to that added to the column; all other quantities were measured in effluent.

Specific activity of the combined active fractions (5 to 6 ml) was 10.1 $\pm$ 0.4 units per mg protein, representing 40 to 45X purification and about 50% recovery.

The addition of NAD to the starting buffer lowered the effluent pH to approximately 5. Because of the possibility of elution by pH change, a sample of the starting buffer was adjusted to pH 5 with HCl and passed through the column. No alcohol dehydrogenase activity was detected in the effluent solution, indicating that pH change alone is insufficient to remove alcohol dehydrogenase from the matrix of Blue Sepharose. The alcohol dehydrogenase was subsequently eluted with NAD, producing the typical elution pattern and yield.

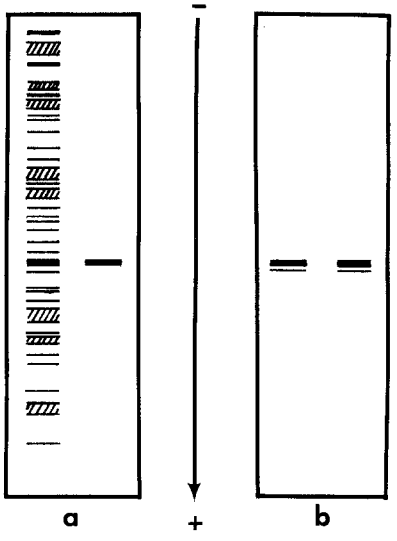

Figure 2. a. Schematic representation of polyacrylamide gel stained with Coomassie Blue following electrophoresis.

b. Schematic representation of polyacrylamide gel stained with nitro blue tetrazolium for alcohol dehydrogenase activity.

In each gel, left sample is water extract, right sample is pooled active fractions from column.

Electrophoresis of the water extract revealed the presence of many protein species, whereas the alcohol dehydrogenase-active fractions from the column show a single faint protein band midway in the gel (Figure 2a). In another gel, alcohol dehydrogenase activity was indicated by a large band and below it, a weaker band (Figure 2b). These were the only alcohol dehydrogenase-active bands in both the water extract and the purified fractions, indicating qualitative purification. The weaker band was probably not detected with the protein stain because the Coomassie blue is less sensitive to minute amounts of enzyme protein than the chromogenic enzyme reaction.

Affinity chromatography is a relatively new technique for enzyme purification. The commonly used columns for dehydrogenase are NAD derivatives bound to Sepharose matrices. However, in most cases there

has been a failure to bind the ligand uniformly and tightly to the matrix resulting in low recovery and in single-use columns (6).

The technique demonstrated here, of binding to a less selective matrix followed by selective elution with a cofactor, avoids many of the problems associated with NAD-Sepharose. A major advantage is the dry storage of the affinity gel, as noted by Easterday (1). More importantly, the column may be quickly prepared for reuse without repacking and without loss of efficiency. The Blue Sepharose 6B column used for this study has been used more than 40 times over a period of 3 months with no detectable changes in its characteristics.

ACKNOWLEDGEMENT

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