AFFINITY CHROMATOGRAPHY OF A REGULATORY ENZYME BASED ON SPECIFIC INTERACTION WITH THE EFFECTOR*

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Summary

A crude extract of yeast was chromatographed on a column of Sepharose containing covalently bound L-tyrosine. The tyrosine-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthetase was retarded by the column relative to the bulk of the protein and was purified about 100-fold. In contrast, the phenylalanine-sensitive enzyme was not retarded. No retardation was found on an unsubstituted Sepharose column. The purification presumably resulted from the specific interaction between the effector-binding site of the enzyme and the effector attached to Sepharose.

It has been shown (Cuatrecasas et al., 1968) that in suitable cases, enzymes can be very efficiently purified by affinity chromatography on columns of agarose containing covalently bound inhibitors. The enzymes studied in previous work were all purified according to their affinity towards inhibitors of the substrate analog type. However it has been pointed out (Cuatrecasas et al., 1968) that the method should in principle apply to any protein with a specific binding site. A recent example is the purification of avidin on biocytin-agarose columns (Cuatrecasas and Wilcheck, 1968). Since regulatory enzymes possess effector binding sites, it was of interest to determine whether the method could be applied to such enzymes using agarose to which effectors had been covalently attached. We now report a successful example of

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chromatography based on this principle.

The enzyme, 3-deoxy-D-arabino-heptulsonate-7-phosphate (DAHP) synthetase, is known to be involved in the regulation of aromatic amino acid biosynthesis (Smith et al., 1962; Gibson and Pittard, 1968). In Escherichia coli, there are three isozymes of DAHP synthetase, each of which is sensitive to inhibition by one of the three end-products, i.e. tyrosine, phenylalanine and tryptophan (Smith et al., 1962; Doy, 1967). In the present study we have used extracts of Saccharomyces cerevisiae which, according to previous work, contains the tyr-sensitive and the phe-sensitive DAHP synthetases but no try-sensitive isozyme (Meuris, 1967; Lingens et al., 1966). This communication describes the purification of the tyr-sensitive DAHP synthetase by chromatography on a tyrosine agarose column.

Methods

Cell-free extracts were obtained by homogenizing baker's yeast with glass beads in sodium phosphate buffer 0.01 M, pH 6.5. The extract was treated with protamine sulfate (0.2 mg/mg protein) and after centrifugation the supernatant was dialysed. The dialysed extract was quickly frozen and kept at -15° until used. DAHP synthetase activity was stable under these conditions for three months. We have found that both of the yeast DAHP synthetases are inhibited by EDTA. The inhibition is reversed by Co^{2+} or Mn^{2+} and to a smaller extent by Zn^{2+}. Cobaltous sulfate (10^{-4}M) was therefore added to all buffers together with 10^{-4}M phenylmethylsulfonyl fluoride (PMSF), a proteolytic inhibitor (Fahrney and Gold, 1963). Details of these procedures will be published elsewhere (Takahashi and Chan, in preparation).

DAHP synthetase was assayed using the modified conditions of Jensen and Nester (1966). For practical convenience, a unit of activity is defined as the amount of enzyme which gives an absorbancy of 1.0 at 549 mμ in ten min. at 37°. This unit is equivalent to 0.02 μmole DAHP formed/min./ml. Assays were conducted in the range of 0.5 - 5.0 units over which absorbancy at 549mμ was proportional to the amount of enzyme added. The tyr-sensitive enzyme

was assayed in the presence of 2mM phenylalanine. Similarly, the phe-sensitive activity was determined in the presence of 1mM tyrosine. Protein concentraction was determined by the trichloroacetic acid turbidity method of Bücher (1947).

Sepharose 4B (a commercially available form of agarose) was activated with cyanogen bromide according to the procedure of Cuatrecasas et al. (1968). Coupling was conducted at pH 11 by adding 1 mmole L-tyrosine to each ml of the pre-treated Sepharose and the mixture was stirred for 16 hrs. The amount of L-tyrosine bound to Sepharose (3.2 μmole/ml) was determined by hydrolysis of a measured volume of the tyrosine-Sepharose derivative in 6N HCl for 24 hrs. followed by amino acid analysis according to Spackman et al. (1958).

Results and Discussion

L-tyrosine was coupled to Sepharose as described in the Methods section and the resulting tyrosine-Sepharose derivative was used in the chromatography of a crude yeast extract [Fig. 1 (a)]. While the peak of phe-sensitive DAHP synthetase coincides with the protein peak, the tyr-sensitive isozyme was appreciably retarded by the column and contained barely detectable amounts of protein. The presence of Sepharose-bound tyrosine was necessary for this effect, as shown in the control experiment in which the same extract was chromatographed on unsubstituted Sepharose [Fig. 1 (b)]. In this case, both of the DAHP synthetase activities emerged unretarded and in the same position as the protein peak.

The two peaks of DAHP synthetase showed very different sensitivity towards inhibition by the effectors tyrosine and phenylalanine (Table I). In the unfractionated crude extract phenylalanine and tyrosine each inhibited approximately half of the total DAHP synthetase activity. Fraction I was inhibited almost completely by phenylalanine but not significantly by tyrosine. Fraction II was however, strongly inhibited by tyrosine and to a much lesser degree by phenylalanine. In view of the good chromatographic separation shown in Fig. 1(a), the major part of the inhibition of Fraction II by pheny-

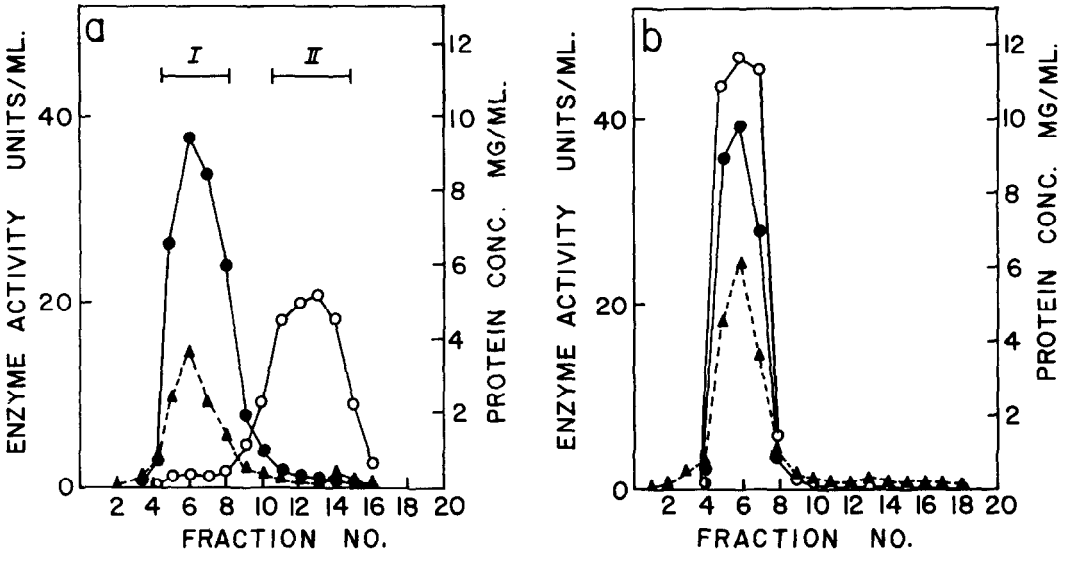

Fig. 1. Chromatography of crude yeast extract on (a) tyrosine-Sepharose and (b) unsubstituted Sepharose. The columns (0.6 cm x 15.5 cm) were equilibrated with 0.2M sodium phosphate buffer pH 6.5 containing $CoSO_4$ (0.1mM) and PMSF (0.1mM). The sample consisted of 2 ml crude extract also in the same buffer. The column was kept at 4° and one ml fractions were collected. (○——○), Tyr-sensitive DAHP synthetase; (●——●), Phe-sensitive DAHP synthetase; (▲---▲) protein concentration.

Table I

Sensitivity of the Enzyme Fractions to Inhibition

	Per Cent Inhibition		
	phe	tyr	phe + tyr
Fraction I	95	2	95
Fraction II	22	89	95
Unfractionated extract	43	48	93

lalanine is presumably an intrinsic property of the tyr-sensitive enzyme from yeast as has been previously reported (Meuris, 1967) and not due to contamination by the phe-sensitive enzyme. This is also supported by the fact that for

Fraction II the presence of both inhibitors resulted in only slightly more inhibition than obtained with tyrosine alone. Previous attempts to separate the two DAHP synthetases of S. cerevisiae by ammonium sulfate precipitation (Lingens et al., 1967; Doy, 1968) and ion-exchange chromatography (Takahashi and Chan, unpublished observations) resulted in much loss of activity together with loss of sensitivity towards inhibition by effectors. The present method thus offers a mild method for separating the DAHP synthetase isozymes.

The protein concentration in the peak containing the tyr-sensitive enzyme was too low to be accurately measured by the method used (<0.1 mg/ml). Since the crude extract contained 9.5 mg protein/ml, a purification of at least 100-fold was thus achieved in a single step. The recovery of activity was 95% for the phe-sensitive enzyme but only 67% for the tyr-sensitive enzyme. The loss of activity in the latter case probably resulted from denaturation at the very low protein concentration in the second peak.

The affinity chromatography described in this communication differs from those previously reported since in this instance, the enzyme is retarded but not absorbed by the column. It is therefore not possible to concentrate the enzyme with this column. However, the scale of operation can be increased many times since only a small column is required to obtain good separation of the enzyme from the main protein peak. The tyrosine-Sepharose can be used repeatedly with no loss of effectiveness provided that it is washed after each use with 0.5% sodium dodecyl sulfate solution containing 1M urea. In previous cases of affinity chromatography, it has been necessary to use relatively severe conditions (e.g. pH 3) for eluting the enzyme from the column. This may be an undesirable feature in some cases since many enzymes, especially regulatory enzymes, are not stable to such treatment. The weak binding between enzyme and the Sepharose derivative in the present system may have an advantage since only mild conditions are used.

The apparently low affinity observed between DAHP synthetase and the tyrosine-Sepharose is not due to an intrinsically weak binding of tyrosine to

the enzyme since a tyrosine concentration of only about 5 X 10^{-5}M is sufficient to cause a 50% inhibition of the enzyme activity (Lingens et al., 1967). One possible reason is the steric hindrance caused by proximity of the coupled tyrosine moiety to the Sepharose matrix (Cuatrecasas et al., 1968). Furthermore, tyrosine is probably coupled to Sepharose through its amino group with consequent loss of the ionic character of this group (Axen et al., 1967). If the charged amino group participates in the binding process, the loss of this charge may also be a reason for the low affinity. To increase the affinity, we are testing tyrosine derivatives which, when coupled to Sepharose, will not have these structural limitations.

The results in this paper suggest that affinity chromatography may be applicable to the purification of regulatory enzymes many of which are otherwise difficult to purify. Yeast chorismate mutase and prephenate dehydrogenase are also inhibited by L-tyrosine (Lingens et al., 1967) and we intend to examine if these enzymes also interact with tyrosine-Sepharose.

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