SHORT COMMUNICATIONS

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Ligand-induced association–dissociation as a means for enzyme purification

In recent years a number of enzymes have been shown to undergo changes in molecular weight in the presence of specific ligands, *i.e.* substrates, inhibitors and activators[1–7]. Since a number of gels are available for chromatography of proteins based on molecular weight, it seemed to us that a simple improvement in protein purification might be devised based on ligand-induced changes in molecular weight. In one step the protein is chromatographed on a molecular sieve gel, appearing in a peak with proteins of molecular weight M_1. The fractions containing the protein under study are then rechromatographed in the presence of ligands which induce association or dissociation. The molecular weight of the enzyme will then be fM_1 where f is some integer or fraction, *e.g.* 2 or 0.5. The probability that two proteins which have the same initial molecular weight in the absence of effectors will associate or dissociate to the same extent with the same specific effectors is extremely low.

To take a specific example, CTP synthetase (UTP:ammonia ligase (ADP), EC 6.3.4.2) can be purified by a procedure which is involved and only moderately reproducible[8,9]. The DEAE-cellulose step was found to be the source of the poor reproducibility in repeated purifications from different batches of *Escherichia coli* B. In the course of studies on this enzyme it was found that a mixture of the substrates UTP, ATP and Mg^{2+} induced a polymerization of a 105 000 dimer to a 210 000 tetramer[9]. Accordingly, this property was tested for an improvement in the purification of the protein.

To reduce the large amounts of contaminated proteins from the initial *E. coli*

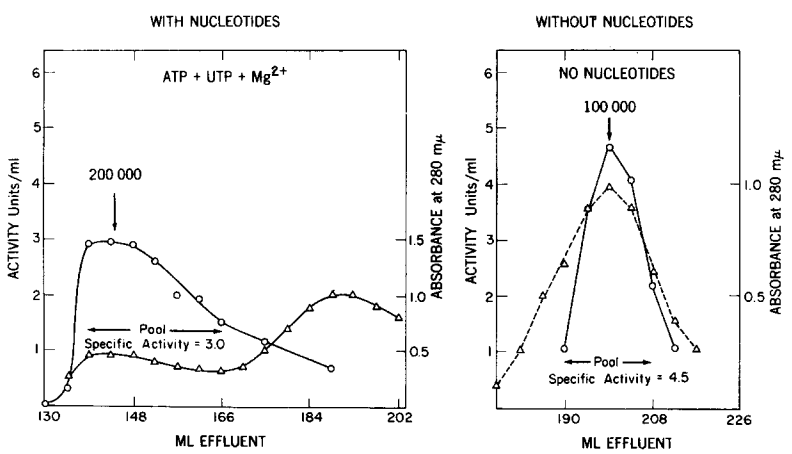

Fig. 1. Peak fractions on the columns with and without effectors. 150 units of CTP synthetase from the Sephadex G-100 step were applied to the Biogel A-0.5 m column equilibrated with nucleotides and then to a Biogel A-0.5 m with no nucleotides. Experimental details are given in Table I. △—△, absorbance at 280 mμ; ○—○, activity,

TABLE I

PURIFICATION OF CTP SYNTHETASE

Procedure: The procedure through the third $(NH_4)_2SO_4$ precipitation was identical to that described by LONG AND PARDEE[8]. The enzyme was desalted on a Sephadex G-50 column (6 cm × 40 cm). From the $(NH_4)_2SO_4$ steps the buffer used throughout the purification contained 0.02 M sodium phosphate buffer, pH 7.4, 1 mM EDTA, 4 mM glutamine and 70 mM β-mercaptoethanol. The desalted enzyme was applied to a DEAE-Sephadex A-50 column (6 cm × 30 cm) and eluted with a gradient of 0–0.19 M $(NH_4)_2SO_4$ in the above buffer (1200 × 1200 ml). The enzyme elutes at 0.08–0.09 M $(NH_4)_2SO_4$. The pooled fractions were precipitated with 55% saturated $(NH_4)_2SO_4$ and desalted on a Sephadex G-100 column (4.2 cm × 75 cm) run at 60 ml/h. The pooled enzyme was applied to a Biogel A-0.5 m column (2 cm × 85 cm) equilibrated with 0.75 mM ATP, 0.75 mM UTP and 0.01 M $MgCl_2$ in the above buffer. The appropriate fractions were precipitated with 55% saturated $(NH_4)_2SO_4$ and applied to a Biogel A-0.5 m column (2 cm × 85 cm) equilibrated with the above buffer, not containing nucleotides. The pooled fractions were precipitated using 60% saturated $(NH_4)_2SO_4$ and applied to a Sephadex G-200 column (2.5 cm × 90 cm) from which a pure enzyme was obtained.

Step	*Activity (units)*	*Specific activity (μmoles CTP/min per mg)*	*Yield of pooled fractions (%)*
Streptomycin sulfate	390	—	100
Third $(NH_4)_2SO_4$	260	0.05	67
DEAE-Sephadex	160	0.30	41
Sephadex G-100	150	1.30	38
Biogel A-0.5 m + ATP + UTP + $MgCl_2$	150	2.00	38
Biogel A-0.5 m, no nucleotides	112	4.60	29
Sephadex G-200	100	5.80–6.10	26

preparation, the first four steps were similar to the procedure described previously[8] (*cf.* Table I). The enzyme was then desalted on a Sephadex G-50 column and chromatographed once on a DEAE-Sephadex A-50 column[8]. The pooled fractions were concentrated by 55% saturated $(NH_4)_2SO_4$ precipitation and applied to Sephadex G-100 in the absence of added effectors (Table I). Under these conditions the protein had a molecular weight of 105 000 and the activity eluted at a point which was consistent with this value. The peak activity fractions were then applied to a Biogel A-0.5 m column which had been equilibrated with ATP, UTP and $MgCl_2$. Under these conditions aggregation to the 210 000 tetramer occurs[9]. The activity was found at an elution volume consistent with such a molecular weight (Fig. 1). The pooled fractions (Table I) were concentrated and applied on an identical Biogel column with no nucleotides. Under these conditions the protein was chromatographed as a 105 000 molecular weight species (Fig. 1). Essentially pure protein was obtained and a final purification was achieved by application to a Sephadex G-200 column (Table I). The method was found to be easier and more reproducible than previous procedures. Five preparations gave the same yields, within 5%, as described in Table I.

The advantages and disadvantages of this approach can be extrapolated from this example. The steps prior to the use of Biogel columns were aimed at reducing the amount of protein, thus avoiding the use of a large column which would consume large amounts of expensive nucleotide effectors. $(NH_4)_2SO_4$ fractionation steps are easier in early stages than column procedures because of the large amounts of protein

impurities. The use of effectors at later stages when columns can be much smaller will reduce the cost factor drastically. However, when the effector molecules are inexpensive they may be utilized earlier in the purification procedure. The purification that is achieved by this procedure will depend on the resolution in the chromatogram, the number of effectors which must be added to achieve the polymerization or depolymerization, and the uniqueness of the effectors.

Although this procedure will usually be used to improve cumbersome procedures or to purify partially purified enzymes further, it may also be used on proteins in which only a few rudimentary facts are known. Since peak fractions in molecular sieve columns can be determined accurately, changes in molecular weight can be tested using molecular sieves. Substrates and inhibitors determined in kinetic tests can be tried as effectors for molecular weight changes even if purified enzyme is not available. In view of the fairly large number of enzymes which have already been shown to change their state of aggregation under the influence of effectors[1–7], this procedure may have general usefulness.

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