

PURIFICATION OF ISOLEUCYL TRANSFER RIBONUCLEIC ACID SYNTHETASE BY AFFINITY CHROMATOGRAPHY ON BLUE DEXTRAN-SEPHAROSE

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1. Introduction

The aminoacyl-tRNA synthetases are a family of twenty enzymes that function in the first step of protein biosynthesis by esterifying the twenty amino acids to their cognate tRNAs [1]. Purification procedures for these enzymes are generally long and laborious. Recently several affinity chromatographic methods have been reported. Several of the aminoacyl-tRNA synthetases have been purified utilizing columns of purified, immobilized tRNA [2–5]. A method for purification of isoleucyl-tRNA synthetase (IRS) involves the use of L-isoleucinyl 5'-adenylate, an aminoacyl-AMP analog, as the insoluble ligand [6]. Unfortunately, the preparation of these column materials is generally laborious, often expensive, and may yield chromatographic media of limited stability suitable only for small scale preparations.

Stellwagen [7] has reported that blue dextran linked to Sepharose may be used as an affinity chromatographic medium for many proteins which bind dinucleotides and ATP. Because the material is easily synthesized and stable, we have studied its potential as an affinity chromatographic medium for the purification of aminoacyl-tRNA synthetases. We now report the purification of IRS of *Escherichia coli* (EC 6.1.1.5) to near-homogeneity from crude cellular extracts by chromatography on two sequential blue dextran-Sepharose columns.

Abbreviations: IRS, ILE-RS, Isoleucyl-tRNA Synthetase; Val-RS, Valyl-tRNA Synthetase; Leu-RS, Leucyl-tRNA Synthetase; Gly-RS, Glycyl-tRNA Synthetase

2. Materials and methods

Cyanogen bromide and 1-methyl-2-pyrrolidinone were obtained from Aldrich, L-[³H]isoleucine from ICN, Sepharose 4B from Sigma and blue dextran from Pharmacia. *E. coli* B was obtained from Grain Processing, Muscatine, Iowa. *E. coli* B tRNA was obtained from Schwarz-Mann. ATP was purchased from Calbiochem.

Homogeneous IRS (used in initial characterizations of the chromatographic medium) was prepared by the method of Eldred and Schimmel [8]. Partially purified valyl- and leucyl-tRNA synthetases were prepared by the same method through the DEAE-cellulose chromatography step.

Aminoacyl-tRNA synthetases were assayed by the esterification of their cognate ³H-labeled amino acids to tRNA in 0.05 M Tris-HCl at 30°C according to the method of Muench and Berg [9]. Protein concentration was determined by the method of Lowry as modified by Ross and Schatz [10] throughout most of the purification procedure. At the final step, concentrations of IRS were determined spectrophotometrically at 280 nm using a molar extinction coefficient of 1.909×10^5 [11]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was conducted by the method of Weber and Osborn [12].

Blue dextran-Sepharose was prepared by the procedure of Ryan and Vestling [13]. The fines were removed and the blue dextran-Sepharose was stored in 0.02% sodium azide at 4°C. After each use, the resin was washed with 4 vol. 6 M guanidine-HCl (practical grade, filtered before use) and then extensively with water before being once again stored in 0.02% sodium

azide at 4°C. Using these procedures, very little loss of blue chromophore was observed after the initial washing procedure. Six months of continuous use is a reasonable minimum expected lifetime if the resin is always cleaned immediately after use.

3. Results and discussion

In preliminary experiments we characterized the elution of purified IRS from 1.0 ml blue dextran–Sephacel minicolumns by various buffers [7]. ATP at less than 5 mM eluted all of the applied IRS activity while 100 mM NaCl was required to have the same effect. This result suggested that the interaction was affinity chromatography. Furthermore, Mg^{2+} (5 mM) enhanced the binding of IRS to the medium, while the presence of isoleucine (0.1 mM) in the elution buffer caused IRS activity to elute at a lower concentration of ATP.

In the isolation of IRS, all operations were carried out at 4°C. The buffer used was 5 mM sodium phosphate, 10 mM β -mercaptoethanol, pH 7.00, measured at room temperature, with additions as noted. ATP solutions were prepared and neutralized immediately before use.

To a semi-micro blender (Eberbach, 360 ml) were added 10 g frozen *E. coli* B, 20 ml buffer, and 20 g glass beads (Virtis 16-220). After the cells thawed, the mixture was blended at medium speed (setting 4 on Waring Commercial blender model 5012). Temperature was maintained below 10°C. After 21 min of blending 40 ml buffer was added and blending continued for 5 s. The glass beads were allowed to settle and the supernatant decanted. The beads were again washed with 40 ml of the same buffer and the supernatants were pooled. The extract was centrifuged at $35\,000 \times g$ for 30 min. The clear supernatant was decanted, diluted with an equal volume of buffer, and loaded directly onto a blue dextran–Sephacel column (2.5 \times 24 cm) equilibrated with buffer. After loading, the column was washed sequentially with the following solutions (all in buffer with the indicated addition): 800 ml 25 mM NaCl, 300 ml 5 mM Mg^{2+} (acetate salt), 500 ml 4 mM ATP/9 mM Mg^{2+} , 300 ml buffer without Mg^{2+} . As can be seen in fig.1, these washes removed much of the bound protein without eluting an appreciable amount of IRS activity. Removal of Mg^{2+} by the

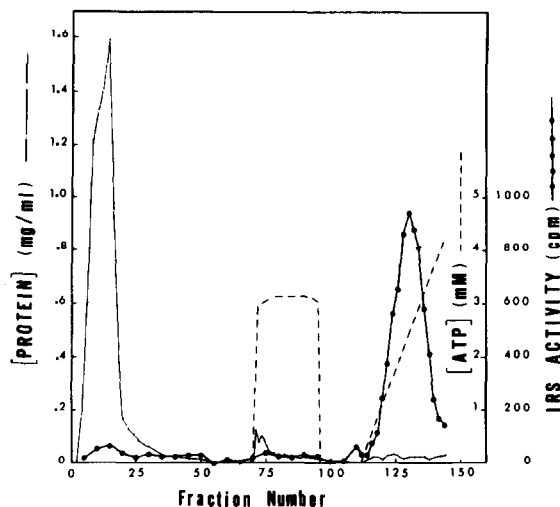


Fig.1. Chromatography of IRS on blue dextran–Sephacel. Extract from 10 g *E. coli* was applied to a blue dextran–Sephacel column (2.5 \times 24 cm) equilibrated with 5 mM sodium phosphate buffer at pH 7.00, 10 mM β -mercaptoethanol. Washes and gradient were as described in the text. Flow rate was maintained at 175 ml/h by gravity. Fraction size was 20 ml. Aliquots were diluted 1:5 for assays of enzyme activity. Where appropriate, ATP concentration was estimated by its absorbance at 259 nm using a molar extinction coefficient of 1.54×10^4 [14].

final wash was necessary to allow subsequent elution of IRS by a linear gradient of ATP (0–5 mM, 800 ml total volume).

The fractions from the entire activity peak were pooled. Sufficient neutralized 1 M Mg^{2+} was added to make the $[Mg^{2+}]$ 5 mM in excess of $[ATP]$. Addition of Mg^{2+} at this point facilitated reabsorption of the enzyme by blue dextran–Sephacel without dialysis to remove the ATP. This pool was then applied to a second blue dextran–Sephacel column (2.5 \times 12 cm) which had been equilibrated with buffer containing 5 mM Mg^{2+} . After loading, the column was washed with 300 ml buffer with 0.1 mM Ile and no Mg^{2+} , and then a linear gradient from 0–5 mM ATP (300 ml/chamber, $[Ile]$ constant at 0.1 mM) was run. The presence of Ile in the buffer caused IRS to elute earlier in the ATP gradient, thereby resolving it from previously copurifying proteins. The activity peak (figure not shown) was pooled and concentrated in an Amicon ultrafiltration cell fitted with XM-50

Table 1
Summary of purification procedure

Fraction	Total units ^a	Total protein (mg)	Yield (%)	Specific activity (units/mg protein)
Crude extract	2680	682	100	3.9
ATP eluate	1280	15.2	48	84
Ile + ATP eluate	1050	1.9	39	553

^a One unit of enzymatic activity will catalyze the aminoacylation of one nanomole tRNA in 10 min at 30°C in 0.05 M Tris-HCl, pH 7.8.

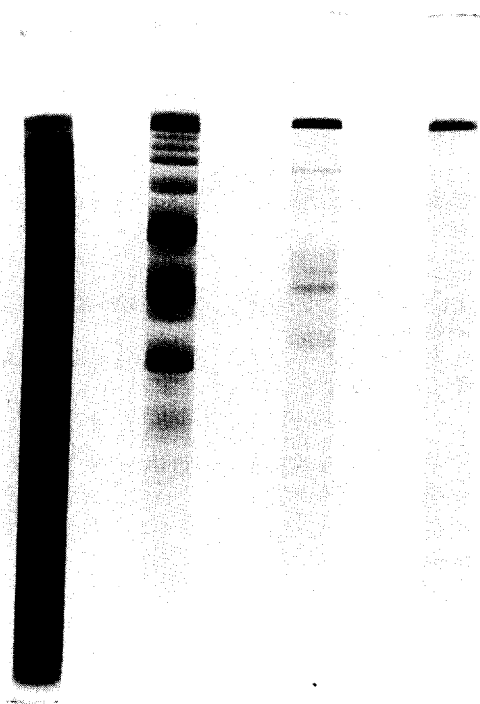


Fig.2. Electrophoretic pattern of IRS at each stage of purification. Samples from each stage of the preparative procedure was subjected to electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecylsulfate [12]. The gels were cut at the bromphenol blue front. The samples applied to the gels, with the amount of IRS activity they represent indicated parenthetically are (from left to right): Crude extract (3 units), first blue dextran-Sepharose pool (30 units), second blue dextran-Sepharose pool (15 units), DEAE pool (15 units).

membrane. A summary of this purification procedure is presented in table 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed on samples taken from each step of the purification procedure (fig.2). By this criterion, IRS still had minor amounts of contaminating protein after the second blue dextran-Sepharose column. These were completely removed by chromatography on DEAE-cellulose (conditions as in [8]) without significant increase in specific activity.

In order to test the general applicability of blue dextran-Sepharose to purification of aminoacyl-tRNA synthetases, we cochromatographed a mixture of

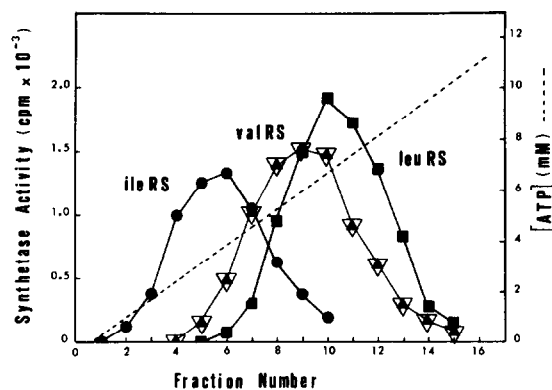


Fig.3. Cochromatography of the branched-chain aminoacyl-tRNA synthetases on blue dextran-Sepharose. A solution containing approximately 15 units of each enzyme was dialyzed against 5 mM sodium phosphate buffer at pH 7.00, 10 mM β -mercaptoethanol, and then loaded onto a blue dextran-Sepharose column (0.7 x 8 cm) equilibrated with the same buffer. A linear ATP gradient (0–20 mM in buffer; 100 ml total volume) was run and 3 ml fraction were collected. The fractions were then assayed for all three enzymatic activities.

partially purified branched chain aminoacyl-tRNA synthetases on a blue dextran–Sephadex column. As can be seen in fig.3, the enzymes eluted in an ATP gradient in the order: Ile-RS, Val-RS, Leu-RS. Under similar conditions, Gly-RS from crude *E. coli* extract was not bound by the column. However, if 5 mM Mg^{2+} was included in the buffer, Gly-RS was retained and could be eluted by a decreasing Mg^{2+} gradient.

Stellwagen [7] has proposed that affinity interaction with blue dextran–Sephadex is a characteristic of proteins possessing the dinucleotide fold [15] as part of their tertiary structure. If the criterion proves valid, our data suggests that many, if not all, of the aminoacyl-tRNA synthetases possess the dinucleotide fold.

Given the number of parameters available to the researcher in the study of the interaction of aminoacyl-tRNA synthetases and blue dextran–Sephadex ionic strength, ATP, and the selective inclusion of magnesium and/or cognate amino acid – we are confident that blue dextran–Sephadex will prove to be generally applicable to the purification of aminoacyl-tRNA synthetases. Studies of potential applications of this medium are continuing in our laboratory.

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