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Note

Purification of argininosuccinase by high-pressure immunoaffinity chromatography on monoclonal anti-argininosuccinase–silica

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Purification of enzymes is typically a long and tedious process. However, once a specific antibody directed against an enzyme is obtained, enzyme purification can be shortened by the use of immunological techniques. These techniques include the immobilization of antibodies against the desired enzyme on such supports as Sepharose and agarose for use in low-pressure affinity chromatography (LPAC). This method can greatly simplify enzyme purification and reduce the time required. However, LPAC may still require days of laboratory work before a pure enzyme is obtained. The time required for column work can be shortened considerably by using high-pressure affinity chromatography (HPAC) instead of LPAC. The use of HPAC requires that the antibody be immobilized on supports such as silica which can withstand the high pressures associated with HPAC. Josic *et al.*[1] immobilized an antibody against glycoprotein GP 105 (a membrane bound protein) on silica and demonstrated its effectiveness in purifying GP 105. Ohlson *et al.*[2] have immobilized polyclonal antibodies against transferrin and have shown quantitative recovery of pure transferrin. However, the elution conditions used by Josic *et al.*[1] included a detergent and pH 2.4 and those used by Ohlson *et al.*[2] included pH 1.3. These conditions are likely to denature many proteins. The purification of an active enzyme by this method has yet to be demonstrated. We describe here the synthesis of an antibody–silica HPAC column and its use to purify active argininosuccinase from a crude homogenate using conditions less harsh (pH 3, no detergent) than those previously employed[1,2].

MATERIALS AND METHODS

Chromatography

All high-pressure chromatography was at room temperature (20°C) using a Varian 5000 (ternary gradient) chromatograph outfitted with a Jasco UV–VIS detector, a Gilson 321/401 autosampler, and an Apple II Plus computer to serve as an integrator using the Chromatochart software (Interactive Microware, State College, PA, U.S.A.).

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Preparation of anti-argininosuccinase

Anti-(beef)argininosuccinase (subclass IgG_1) was obtained from hybridoma culture media (Dulbecco's Modified Eagle Medium, IgG_1 concentration approximately 10 μg/ml) provided by Dr. Merrill Benson and William Kuster, DRTC Hybridoma Core, Indiana University Medical Center, Indianapolis, IN, U.S.A. A volume of 20 ml of the above medium was mixed with 20 ml of a saturated solution of ammonium sulfate in 10 m*M* sodium phosphate, pH 7.0. The mixture was held at 5°C for 30 min and centrifuged (10 000 *g*, 10 min, Sovall RC-5 centrifuge). The precipitant was resuspended in 3 ml of 10 m*M* sodium phosphate, 1 *M* sodium sulfate, pH 7.0 (buffer A).

Further purification of the anti-argininosuccinase was accomplished by HPAC using a protein A–silica column[3]. 1.5 ml of the ammonium sulfate fractionated anti-argininosuccinase was applied to the column which was equilibrated in buffer A. During sample loading, the flow-rate was 0.1 ml/min. The column was washed for 5 min with buffer A at 1 ml/min, and then eluted with 10 m*M* sodium phosphate, 1 *M* sodium sulfate, pH 3.0 (buffer B) also at 1 ml/min. The effluent was monitored at 280 nm. Fractions of 1 ml were collected in tubes containing 0.1 ml of 0.5 *M* sodium phosphate, pH 12 which, after mixing, gave a final pH for each fraction of about 7.5. The final combined volume from processing two 1.5-ml portions of the ammonium sulfate fractionated antibody was 3.2 ml of 18 μg/ml antibody (assuming $E^{1\%}_{280\mathrm{nm}}$ = 14.0) at pH 7.52.

Synthesis of anti-argininosuccinase–silica

All reactions were conducted at room temperature (20°C) unless otherwise stated. An amount of 1.0 g of 3-glycidyloxypropyl silica (7 μm bead, 500 Å pore) was incubated with 3.2 ml of the above solution containing the purified anti-argininosuccinase while shaking for 36 h. The support was washed with buffer A, and excess coupling sites on the silica were quenched by incubating the support for 24 h with 3.0 ml of buffer A containing 0.5 *M* 2-aminoethanol. The support was then washed with buffer A and *ca.* 0.3 g was packed into a 30 × 4.6 mm I.D. stainless-steel column (Alltech, Deerfield, IL, U.S.A.).

Purification of argininosuccinase

The source of argininosuccinase was a 5% crude beef liver extract (approximately 30.9 μg of argininosuccinase per ml) provided by Dr. Philip Snodgrass, Veterans Administration Medical Center, Indianapolis, IN, U.S.A.).

The anti-argininosuccinase column was equilibrated with 10 m*M* sodium phosphate, 100 m*M* sodium sulfate, pH 7.0 (buffer C) at a flow-rate of 1 ml/min. Injected were 200 μl of a 1/10 dilution of the beef liver extract (buffer C was the diluent) on the column which was then washed for 5 min with buffer C. The column was eluted using 10 m*M* sodium phosphate, 100 m*M* sodium sulfate, pH 3.0 buffer (buffer D). The effluent was monitored at 280 nm and 1-ml fractions were collected in test tubes containing 0.1 ml of 0.5 *M* sodium phosphate, pH 12 to rapidly adjust the effluent to pH 7.5 as previously described.

Purity of fractions were determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, 5% acrylamide and 0.25% bis-acrylamide) using the method of Laemmi[4]. Argininosuccinase activity was measured using the method described by Havir *et al.*[5]

Storage

When not in use, the antibody–silica column was stored in buffer C at 4°C. No other special precautions or washed were taken.

Capacity of the anti-argininosuccinase column

One unit of argininosuccinase is defined as that which will form 1.0 μmole of L-arginine from L-argininosuccinate per minute at pH 7.5 and 37°C as determined by the formation of urea. A volume of 500 μl of buffer C containing 2 units of pure argininosuccinase (Sigma, St. Louis, MO, U.S.A.) was loaded onto the column under the same conditions used for the beef liver extract. The effluent was collected and assayed for argininosuccinase activity. A volume of 1000 μl containing 4 units of the pure argininosuccinase solution was also tested and gave similar results.

RESULTS AND DISCUSSION

Anti-argininosuccinase was purified on the Protein A–Silica column (data not shown); based on previous experience, the eluted fractions were considered to be pure anti-argininosuccinase[3]. This purified monoclonal antibody was then coupled to glycidyloxypropyl–silica, presumably by the scheme depicted in Fig. 1.

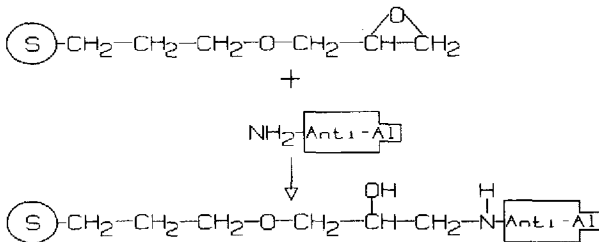

Fig. 1. Coupling of anti-argininosuccinase to silica. An amount of 1 g of 3-glycidyloxypropyl–silica reacted with 3.2 ml of a solution containing purified anti-argininosuccinase (Anti-AI) in 10 m*M* sodium phosphate, 1 *M* sodium sulfate, pH 7.5. Amino groups on the anti-argininosuccinase (*e.g.*, ε-amino groups on lysyl side chains) are thought to have reacted with the epoxide group on the silica ("S") to form a covalent bond and providing a seven atom spacer between the silica and the antibody.

The anti-argininosuccinase–silica column was next used to purify argininosuccinase from a crude liver extract containing 0.021 units of activity, as shown in Fig. 2. SDS-PAGE of the eluted fractions showed a single band (Fig. 3) with a relative mobility identical to that observed for pure argininosuccinase in other experiments (data not shown). In the eluted fractions 0.013 units of argininosuccinase activity were present.

When either 2 or 4 units of pure argininosuccinase was loaded on the column, most of the activity was recovered in the unretained fractions indicating that the column was saturated in both instances and elution in either case gave 0.12 units. Thus, the capacity of the column (containing 0.3 g of the support) corresponds to 0.4 units of argininosuccinase per gram silica.

After approximately a dozen runs over a period of 3 months (longest time tested), there was no evidence of deterioration in the antibody column's performance

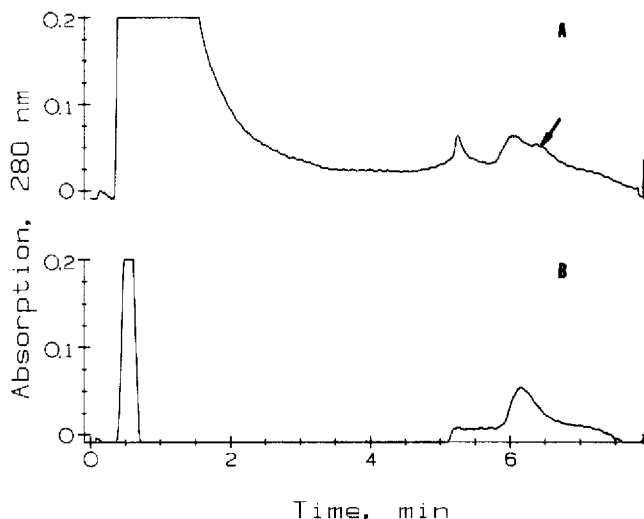

Fig. 2. Purification of argininosuccinase on anti-argininosuccinase–silica. (A) Purification of argininosuccinase on the anti-argininosuccinase–silica column. A volume of 200 μl of 1/10 diluted beef liver extract containing 0.021 units of argininosuccinase was loaded onto the column equilibrated in buffer C. Approximately 5 min later the column was eluted with buffer D. The amount of argininosuccinase retained by and eluted from the column was small (0.013 units) and the arrow indicates the largest change in the chromatogram as compared to the baseline illustrated in (B). Activity measurements confirm the elution of argininosuccinase at the position indicated; the remaining argininosuccinase activity was found in unretained fractions eluting before 3 min. The baseline (B) consisted of the same protocol used for (A) except the load consisted only of buffer C with no sample included.

using the elution regimen described. A Protein A column has been used with a similar protocol[3] in our lab for more than 1½ years without a noticeable decline in activity. Josic *et al.*[1], using somewhat harsher elution conditions has noted such deterioration. Ohlson *et al.*[2] used pH 1.3 for elution but unfortunately did not discuss the durability of their columns. Since pH 3 is adequate to ensure elution and columns remain stable over long periods using these conditions,the milder elution regimen described here is preferable.

The coupling of antibodies directly to high-performance liquid chromatographic supports for immunoaffinity purification of antigens appears to be promising. Here, using a column based upon a monoclonal antibody, argininosuccinase was purified from a crude liver extract to apparent homogeneity in a single purification step requiring less than 10 min. Argininosuccinase retains its biological activity under the conditions used; the monoclonal antibody on the column also appears stable even after repeated uses over several months. Some enzymes can be purified in an active state; argininosuccinase is such an enzyme. However, it should not be expected that all enzymes would remain active under the conditions used; for example, previously we found that arginase is inactivated by the pH 3 used for elution[3]. For such pH sensitive enzymes, it may be possible to develop an additional elution protocol tailored to the sensitivities of individual enzymes. High concentrations of some salts (*e.g.* potassium thiocyanate) have been effective in LPAC and may be applicable to HPAC.

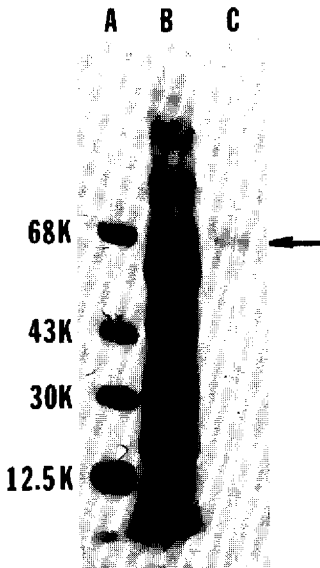

Fig. 3. SDS-PAGE of purified argininosuccinase from liver extract. Unretained and eluted fractions from a separation of crude beef liver extract under the conditions described for Fig. 2 were examined by SDS-PAGE including 2-mercaptoethanol in the sample buffer (reducing conditions). The gel was 5% acrylamide and 0.25% bis-acrylamide. In order from left to right, the lanes contained: (A) molecular weight standards (in descending order bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*); (B) the unretained fractions; and (C) material eluted at pH 3. Lane C shows a single band with a molecular weight slightly less than the bovine serum albumin standard (*i.e.*, 68 K). This is very near the reported molecular weight of argininosuccinase subunit reported by Schulze *et al.*[6] and the relative mobility (R_F) is the same as that observed for authenic argininosuccinase in other experiments. K = kilodaltons.

Direct coupling of antibodies to HPAC supports will probably be of most value in situations where repeated purification of a particular antigen is needed or for quantitative assays as suggested by Ohlson *et al.*[2]. Immunoaffinity purification of antigen–antibody complexes using protein A–silica[1,3] does not require the synthesis of new supports for each antibody–antigen pair and may be more practical in some cases.

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