Purification of Yeast α-Isopropylmalate Isomerase

HIGH IONIC STRENGTH HYDROPHOBIC CHROMATOGRAPHY*

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α-Isopropylmalate isomerase, the second enzyme specific for leucine biosynthesis, can be purified from extracts of yeast utilizing a chromatographic procedure that allows separation of proteins in the presence of high concentrations of (NH₄)₂SO₄. The purification procedure utilizes the stabilizing effect of glycerol and (NH₄)₂SO₄ on the isomerase and their opposing effects on protein retention on valine-Sepharose and leucine-Sepharose. The method effectively separates the isomerase from fumarase, a stable internal marker protein that was co-purified in early steps. High ionic strength hydrophobic chromatography, based on differential retention as a function of the length of the hydrophobic sidearm and ionic strength, yields approximately 200-fold purified α-isopropylmalate isomerase and may be of general utility in purifying unstable enzymes requiring high ionic strength.

Affinity chromatography as developed by Cuatrecasas (2) has led to many novel procedures of protein purification, in most cases based on a specific interaction between protein and a ligand that has been covalently attached to a Sepharose. Subsequent developments have demonstrated the utility of Sepharoses substituted with nonpolar ligands, whereby chromatographic separations are based on hydrophobic interactions between protein and the substituted Sepharoses (3-6). The procedure has been termed hydrophobic chromatography. Recent studies have shown that interaction of protein with the substituted Sepharoses, especially Sepharoses with any one of the branched-chain amino acids attached, may be enhanced by salts of the lyotropic, or Hofmeister, series (7-11). The salts contained 10 mM L-malate.

Enzyme Assays—α-IPM isomerase was assayed by the method of Gross et al. (12) using dimethylcitraconate as substrate. Fumarase was assayed by a similar procedure except that the reaction mixture contained 10 mM L-malate.

Units—One unit of α-IPM isomerase activity is defined as that amount of enzyme that catalyzes the disappearance of 1 μmol of dimethylcitraconate per min at 30°C under standard assay conditions.

Protection Determination—Protein concentration was determined by the method of Lowry et al. (13). Crystalline bovine serum albumin served as the standard.

Media—The basal medium used in growth of yeast strain S288cα was that of Halvorson (14) and was modified as previously described (15). The trace element solution and vitamin solution were those of Lucas et al. (16).

Preparation of Yeast Strain S288cα Cells—When yeast strain S288cα was employed, cells were inoculated into 1 liter of minimal medium in a 2-liter fluted Erlenmeyer flask and incubated in New Brunswick incubator-shaker at 30°C. Inocula were grown in the same medium the day before. Growth was measured turbidimetrically by using a Klett-Summerson colorimeter with a blue No. 42 filter. Late

*The abbreviation used is: α-IPM, α-isopropylmalate.
log phase cells were harvested on a Büchner funnel and washed with 500 ml of cold 0.05 m potassium phosphate buffer, pH 6.8. Cells were treated with 1% nitrogen and stored in liquid nitrogen.

Preparation of Columns—Sepharose 4B was modified by covalently coupling leucine or valine to the matrix via the amino group through the use of the cyanogen bromide activation procedure of Cuatrecasas (2).

The degree of substitution of the leucine and valine-Sepharoses was determined by acid hydrolysis of the modified Sepharose and analysis of the supernatant fluid in a Technicon AutoAnalyzer. Washed valine- or leucine-Sepharose was dried at 110° for 24 hours and desiccated over P₂O₅ to a constant weight in a glass ampoule. The ampoules were sealed after adding 6 N HCl and the samples were heated for 24 hours at 110°. The contents of the ampoules were filtered and the filtrate analyzed. Valine-Sepharose contained 14 μmol of valine/g of Sepharose, dry weight. Leucine-Sepharose contained 15 μmol of leucine/g of Sepharose, dry weight.

Preparation of Enzyme Extract—The yeast employed as the enzyme source was bakers' yeast available from Anheuser-Busch, Inc. In preparing protein for chromatographic experiments, 100 g of yeast were frozen in liquid nitrogen, thawed, and suspended in 100 ml of 0.10 m potassium phosphate buffer, pH 6.8, 1 mm β-IPM. All of the procedures were performed at 0-5°. Thirty-five-milliliter portions were then disrupted by sonic oscillation with a Branson S75 Sonifier at a power setting of 5 amperes using one 30-s treatment. Such a treatment released virtually all of the isomerase yet only a fraction of the fumarase and protein that could be obtained with a longer treatment and in effect constituted a purification of severalfold (Fig. 1). These results agree with the findings of Ryan et al. (20) who found that the isomerase was released much more readily than α-IPM synthase, the first enzyme specific for leucine biosynthesis, and the mitochondrial markers cytochrome oxidase and citrate synthase. Since S.288c yeast was more resistant to breakage, four 30-s sonic oscillation treatments were used.

(NH₄)₂SO₄ fractionations were performed by the slow addition of solid (NH₄)₂SO₄ with stirring. The suspensions were allowed to stand for 20 min after which they were centrifuged in a Sorvall RC-2 centrifuge for a period of 10 min at 27,000 x g. The 0 to 50% saturation (NH₄)₂SO₄ fraction thus obtained was discarded and the 50 to 65% fraction resuspended in 2.0 ml of the column equilibration buffer desired. This protein preparation was loaded onto a valine- or leucine-Sepharose column. Protein resolution was enhanced by applying the protein gently onto the surface of the substituted agarose from a pipette tip extended beneath the column buffer. Five-milliliter fractions were collected and assayed immediately.

Chromatography on Leucine-Sepharose Under Stabilizing Conditions in Presence of Glycerol and (NH₄)₂SO₄—Early experiments with α-IPM isomerase revealed the stabilizing effects of glycerol and (NH₄)₂SO₄ on the enzyme, both of which increased the half-lives of preparations prepared with 0.05 m potassium phosphate, pH 6.8, buffer alone. Marked stabilization occurred with 0.05 m potassium phosphate buffer, pH 6.8, 30% (v/v) glycerol, and 1.24 m (NH₄)₂SO₄, the equivalent to 30% saturation, brought to 1 liter aqueous solution at 0°. Such preparations possessed half-lives of months. It seemed likely that affinity chromatography with the stabilizing buffer would be useful in purifying the isomerase since the enzyme could be isolated by a slightly binding site that might interact with the isobutyl group of leucine attached to Sepharose 4B.

Thus, the initial experiment (Fig. 2) employing leucine-Sepharose and the phosphate-glycerol-(NH₄)₂SO₄ buffer was performed where protein, α-IPM isomerase, and fumarase, a chosen stable internal marker protein, were monitored. Chromo-

**RESULTS**

High Ionic Strength Hydrophobic Chromatography

Preparation of Enzyme Extract—The yeast employed as the enzyme source was bakers' yeast available from Anheuser-Busch, Inc. In preparing protein for chromatographic experiments, 100 g of yeast were frozen in liquid nitrogen, thawed, and suspended in 100 ml of 0.10 m potassium phosphate buffer.

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G. J. Doellgast, personal communication.
matography of a 50 to 65% (NH₄)₂SO₄ fraction resuspended in the column equilibration buffer resulted in a retardation of the protein and a further retardation of the two enzyme activities, isomerase and fumarase, which eluted together. A turbid fraction containing a small amount of protein was excluded. The presence of 2 mM leucine in the elution buffer did not alter this profile. The absence of a leucine effect was expected since the presence of 10 mM of any of the branched-chain amino acids, separately or in concert, in the reaction mixture did not influence the activity of the isomerase or fumarase.

**Chromatography on Valine-Sepharose in Presence of Glycerol and (NH₄)₂SO₄**—On valine-Sepharose (Fig. 3), employing the same glycerol-(NH₄)₂SO₄ conditions, the isomerase and fumarase were only slightly separated from the bulk of the protein. The length of the hydrophobic group of the amino acid substituent seemed influential in producing this result.

**Chromatography on Leucine-Sepharose in Presence of Glycerol**—The effect of glycerol and (NH₄)₂SO₄ on the chromatographic behavior of isomerase and fumarase was clearly demonstrated by the elution profiles produced when only one of these agents was present. Leucine-Sepharose equilibrated with a potassium phosphate buffer 30% (v/v) in glycerol resulted in the protein, isomerase, and fumarase being eluted together (Fig. 4). Glycerol proved ineffective in allowing retention on leucine-Sepharose. The trailing minor peak of isomerase activity is presumed to have been caused by residual (NH₄)₂SO₄ in the 50 to 65% protein fraction resuspended in the phosphate-glycerol buffer, (NH₄)₂SO₄ which thereby produced the effect depicted in Fig. 2.

**Chromatography on Leucine-Sepharose in Presence of (NH₄)SO₄**—On leucine-Sepharose columns equilibrated with a potassium phosphate-1.24 M (NH₄)₂SO₄ buffer, virtually all protein and both enzymes were completely retained (Fig. 5). Prolonged washing with the original buffer failed to release protein or enzyme activity. A shift to a potassium phosphate 30% glycerol buffer at Fraction 88, however, released the retained protein, isomerase, and fumarase. Thus, glycerol and (NH₄)₂SO₄ exerted opposing effects on protein retention and produced an intermediate effect when both were present. Chromatography on unsubstituted Sepharose 4B under conditions identical with those in Fig. 5 produced no retardation of either the isomerase or the fumarase.

**Ionic Strength Dependence of Interaction of Protein with Leucine-Sepharose**—Interaction of the isomerase and fumarase with leucine-Sepharose was clearly ionic strength-dependent. The capacity, defined as recoverable enzyme units after a phosphate-glycerol buffer shift, increased markedly when ionic strength was increased to about 3.5 (Fig. 6). In contrast to the behavior of the isomerase, the fumarase interacted with leucine-Sepharose at a lower ionic strength. Furthermore, the stability of the isomerase on the columns and in the eluate increased markedly with increased ionic strength.

Enzyme that was not adsorbed to the leucine-Sepharose still interacted with the matrix and was retarded. The retardation, expressed in terms of V/V₀, was also ionic strength dependent (Fig. 7). Fumarase again was retarded at a lower ionic strength than was the isomerase. The enzymes could be separated on leucine-Sepharose only at higher salt concentrations as de-
picted in the inset of Fig. 7. The affinity of the hydrophobic matrix for fumarase was generally stronger than its affinity for isomerase.

Gradient Elution on Leucine-Sepharose—When a 50 to 65% (NH₄)₂SO₄ fraction was adsorbed on leucine-Sepharose from the phosphate-1.24 M (NH₄)₂SO₄ buffer described, elution with a buffer containing a gradient of increasing glycerol and decreasing (NH₄)₂SO₄ concentration reproduced the effects demonstrated in earlier experiments (Fig. 8). Proteins were distributed in response to the gradient. The fumarase again interacted more tightly with the matrix.

Chromatography on Valine-Sepharose in Presence of (NH₄)₂SO₄—On valine-Sepharose equilibrated with a potassium phosphate-1.24 M (NH₄)₂SO₄ buffer, some protein was retarded while some protein was completely retained and could be released only upon a phosphate-glycerol buffer shift at Fraction 210 (Fig. 9). The isomerase was retarded on valine-Sepharose under these conditions and could be separated from the bulk of the protein. A fraction of the fumarase was not adsorbed, the result of a capacity effect. The fumarase that was eluted could be rechromatographed and adsorbed. All of the fumarase applied was adsorbed on more highly substituted valine-Sepharose, one containing 16 µmol of valine/g of Sepharose, dry weight (Fig. 10).

Enzyme Purification

Purification procedures that retained α-IPM isomerase in a favorable environment of high ionic strength yet allowed chromatographic separations were devised. An early procedure involved chromatography on leucine-Sepharose as illustrated in Fig. 2. Fractions with maximal activity were concentrated by ultrafiltration with an Amicon XM50 membrane and the concentrate dialyzed to remove glycerol in order to allow (NH₄)₂SO₄ fractionation. Because fumarase was precipitated at a slightly higher (NH₄)₂SO₄ concentration, several precipitations yielded electrophoretically pure isomerase, but owing to the dialysis step, at a purification of only 13-fold. The more efficient procedure based on differential retention as a function of sidearm length and ionic strength yielded 200-fold purified isomerase (Table I).

Chromatography on Valine-Sepharose—The 50 to 65% (NH₄)₂SO₄ fraction obtained as described above was applied onto a valine-Sepharose column (2 x 21.5 cm) equilibrated with 0.05 M potassium phosphate, pH 6.8, 1.24 M (NH₄)₂SO₄. Elution with this buffer resolved the isomerase from excluded and adsorbed proteins (Fig. 11). A shift to a buffer of low ionic strength again released adsorbed proteins.

Chromatography on Leucine-Sepharose and Concentration of Pooled fractions—Valine-Sepharose fractions containing most of the isomerase activity were applied onto a leucine-Sepharose column (2 x 21 cm) in the potassium phosphate-1.24 M (NH₄)₂SO₄ buffer and the effluent monitored, verifying that all of the isomerase was retained. A gradient of a total volume of 1000 ml was employed as described under “Experimental Procedure.”

Fig. 6. Dependence of the capacity of leucine-Sepharose on ionic strength of (NH₄)₂SO₄ in the eluting buffer. Capacity is defined as units recovered after a shift to a buffer containing potassium phosphate and 30% glycerol. Protein was adsorbed as in Fig. 5. Columns were prepared as described under “Experimental Procedure.”

- - - - α-IPM isomerase; O - - O, fumarase.

Fig. 7. Retardation on leucine-Sepharose as a function of ionic strength of (NH₄)₂SO₄ in the eluting buffer. Retardation is defined as $V/V_v$, the ratio of the elution volume of the enzyme activity to the void volume. The inset represents the separation (the arithmetic difference of the $V/V_v$ values of the two enzymes) as a function of ionic strength of (NH₄)₂SO₄.

○-○, α-IPM isomerase; O- - O, fumarase.

Fig. 8. Elution of a 50 to 65% (NH₄)₂SO₄ fraction adsorbed on a leucine-Sepharose column (2 x 21 cm) with a gradient of increasing glycerol and decreasing (NH₄)₂SO₄ concentration. Total gradient volume was 600 ml. The protein was adsorbed in a buffer containing 1.24 M (NH₄)₂SO₄ and the leucine-Sepharose washed with 65 ml of this buffer. The gradient was applied at Fraction 14. Δ-Δ, protein; ○-○, α-IPM isomerase; O- - O, fumarase.
FIG. 9. Chromatography on valine-Sepharose in the presence of a potassium phosphate buffer containing 1.24 M (NH₄)₂SO₄. The arrow at Fraction 210 indicates the point at which the buffer was changed to one consisting of potassium phosphate, 30% glycerol. Δ—Δ, protein; ●—●, α-IPM isomerase; ○—○, fumarase.

FIG. 10. Chromatography of a 50 to 65% (NH₄)₂SO₄ fraction obtained from yeast strain S288cα on more highly substituted valine-Sepharose. The eluting buffer was potassium phosphate containing 1.24 M (NH₄)₂SO₄. The arrow indicates the point at which the buffer was changed to one consisting of potassium phosphate, 30% glycerol. Column dimensions were 2 x 11.5 cm. Δ—Δ, protein; ●—●, α-IPM isomerase; ○—○, fumarase.

FIG. 11. Purification of a 50 to 65% (NH₄)₂SO₄ fraction on valine-Sepharose equilibrated with a potassium phosphate buffer containing 1.24 M (NH₄)₂SO₄. The arrow indicates the point at which the buffer was changed to one consisting of potassium phosphate, 30% glycerol. Δ—Δ, protein; ●—●, α-IPM isomerase; ○—○, fumarase.

FIG. 12. Chromatography of fractions obtained from the valine-Sepharose step described in Fig. 10 on leucine-Sepharose. Protein was adsorbed in the potassium phosphate-1.24 M (NH₄)₂SO₄ buffer and eluted with a buffer of increasing glycerol and decreasing (NH₄)₂SO₄ concentrations. Δ—Δ, protein; ●—●, α-IPM isomerase; ○—○, fumarase.

Supernatant fluid discarded. This precipitate was resuspended in 0.05 M potassium phosphate, pH 6.8, 54% saturated with (NH₄)₂SO₄, centrifuged, and the precipitate discarded. The resultant supernatant fluid was made 62% saturated with the addition of solid (NH₄)₂SO₄, centrifuged, and the precipitate was washed again with 0.05 M potassium phosphate, pH 6.8, 62% saturated with (NH₄)₂SO₄. The final precipitate was resuspended in stabilizing buffer, 0.05 M potassium phosphate, pH 6.8, 1.24 M (NH₄)₂SO₄, 30% (v/v) glycerol and stored at −20°C. In these fractionation procedures, 1 hour of equilibration was allowed before the precipitates were removed by centrifugation.

Variability in Fold Purification—The specific activity, fold purification, and yield varied and depended on the amount of time allowed for each step. The range of fold purification was 70 to 200 for bakers' yeast α-IPM isomerase. The range of the specific activity for the final preparation was 2.3 to 6.2. Thus, the indication is that active and inactive enzyme were not separated during the purification procedure. The enzyme from yeast strain S288cα could be purified to an extent falling in the lower portion of this range, suggesting that the enzyme is somewhat less stable in this strain.

Electrophoresis of Enzyme—Polyacrylamide disc gel electrophoresis of 10 μg of purified bakers' yeast α-IPM isomerase yielded a single band (Fig. 13A). The necessary dialysis of the protein to remove stabilizing salt destroyed most of the enzyme activity. However, using a simple gel slicing procedure, remaining traces of activity were correlated with the protein band. A diffuse band was commonly observed (Fig. 13B) that...
appeared as two bands with higher protein concentration (Fig. 13C). Purified enzyme from yeast strain S288cα consistently yielded two bands (Fig. 13C). Sodium dodecyl sulfate disc gel electrophoresis of 10 μg of purified S288cα α-IPM isomerase yielded one band (Fig. 14E).

DISCUSSION

The purification procedure for α-IPM isomerase described here has utilized conditions that preserve its activity and effect its interaction with a hydrophobic matrix, high ionic strength. The sulfate ion employed allows both stabilization and hydrophobic interaction. The effect of sulfate and other lyotropic series salts on the behavior of proteins in solution has long been studied and is believed to be mediated by the structure of water and therefore entropy effects (21, 22), or by a more direct effect of these salts on the protein itself (23-25). However, the effect of such salts on the intermolecular and intramolecular interactions of proteins is not completely understood. High ionic strength hydrophobic chromatography, besides being useful as a preparative tool, may allow the study of many purified proteins that require high ionic strength.

Many enzymes have been shown to respond to or require high salt concentrations. Multiple RNA polymerases have been found to be released from sea urchin embryos, rat liver nuclei (26), and yeast (27) upon extraction in buffers of high salt concentrations, 20% (v/v) glycerol and 0.8 M (NH₄)₂SO₄ in the case of yeast. Some of the polymerases, which are believed to differ in their location in the cell, display an (NH₄)₂SO₄ dependence for optimal activity. Rat liver carbamoylphosphate synthetase has been chromatographed on Sephadex G-200 in a buffer containing 20% (v/v) glycerol and 0.5 M (NH₄)₂SO₄ and the purified protein stored in 20% (v/v) glycerol.

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Sodium dodecyl sulfate-treated酵母isomerase appears as one band upon disc gel electrophoresis. The presence of one subunit species or two very similar subunits cannot as yet be distinguished from conformational states. Especially interesting is the effect of salts on the structure of succinate-ubiquinone reductase, which can be dissociated by chaotropic salts and reconstituted by removal of these salts or addition of anti-chaotropic ions such as SO₄²⁻, HPO₄²⁻, or F⁻ (33). The integrity of the complex is believed to be maintained by hydrophobic associations, interactions strengthened by anti-chaotropic salts in aqueous media. The effect of anti-chaotropic salts on the stability of α-IPM isomerase from Salmonella typhimurium and yeast has been studied and appears general.

Sodium dodecyl sulfate-treated yeast isomerase appears as one band upon disc gel electrophoresis. The presence of one subunit species or two very similar subunits cannot as yet be distinguished. However, based on genetic evidence in S. typhimurium, the isomerase is believed to be coded for by two genes, leuC and leuD (12, 34). Since frequent unlinked suppressor mutations, termed supQ (35, 36), of a total leuD deletion are possible, it is conceivable that the role of the leuD gene is one other than catalytic function in vitro. If so, the genetics of the isomerase in S. typhimurium would be similar to that in yeast where many genes have been demonstrated to be involved in determining isomerase activity (15). If one type of subunit is present, the role of these other genes becomes an interesting problem for further investigation.

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Fig. 13. Disc gel electrophoresis of purified enzyme preparations. Material from the last step of Table I was used. A, B, and C represent baker’s yeast enzyme from the same purification experiment applied in amounts of 10, 20, and 50 μg of protein, respectively. D represents 10 μg of protein from yeast strain S288cα. E represents 10 μg of sodium dodecyl sulfate-treated protein from yeast strain S288cα. Gels were stained with Amido black 10B. Coomassie blue R was used in the case of the sodium dodecyl sulfate gel.
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