A Rapid Purification Procedure for Glyceraldehyde 3-Phosphate Dehydrogenase from Bakers' Yeast

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SUMMARY

A new procedure utilizing column chromatography has been devised for the purification of yeast glyceraldehyde 3-phosphate dehydrogenase. It yields enzyme of the highest specific activity in a shorter period of time and with a better yield than the traditional procedure. The isozyme isolated is the most abundant and most acidic of the set of isozymes previously identified.

The traditional Krebs procedure (1) for preparing yeast glyceraldehyde 3-phosphate dehydrogenase has many advantages, but it also has some serious disadvantages. The procedure requires long periods of waiting during the first few steps of making the crude extract and is not reproducible in the quality of the enzyme obtained. Lazarus et al. (2) have shown that proteolytic enzymes survive the toluene plasmolysis step in the purification of yeast hexokinase and affect the stability, specific activity, and even the number of forms of the enzyme. We find levels of proteolytic activity in the classic Krebs procedure for glyceraldehyde-3-P dehydrogenase comparable to those reported by Lazarus.

Since new resins and methods are available today, the purification of yeast glyceraldehyde 3-phosphate dehydrogenase has been re-examined and a scheme for rapid purification has been devised. This scheme requires 3 days and consistently gives enzyme of superior specific activity: 200 μmoles per min per mg in the back reaction (NADH + 1,3-diphosphoglycerate) and 100 μmoles per min per mg in the forward reaction (NAD + glyceraldehyde-3-P + arsenate). One pound of pressed yeast yields 150 to 200 mg of pure enzyme. The yeast enzyme has been shown to consist of a mixture of isozymes (3), and the species isolated in this procedure is the most acidic of the set.

MATERIALS AND METHODS

The assay was performed essentially as previously described (1). The substrate concentrations were as follows: nicotinamide adenine dinucleotide = 10^-3 M, p-glyceraldehyde-3-phosphate = 10^-3 M, Na₂HAsO₄ = 10^-2 M. The assay buffer was 0.05 M sodium pyrophosphate, pH 8.5 at 25°, containing 5 mM EDTA.

The reaction was initiated by addition of enzyme to 1 ml of assay mix in a cuvette thermostatted at 25°. Absorbance was followed at 340 nm on the Gilford 2000 recording spectrophotometer, and the increase from 15 to 45 s was used to calculate the specific activity in micromoles per min per mg.

The back reaction assay was performed according to the procedure of Kirschner and Voigt (4).

Protein concentration was determined spectrophotometrically at 280 nm using 0.03 as the absorbance of a 1.0 mg per ml solution. The molecular weight of the enzyme was taken to be 145,000.

NAD was purchased from Boehringer-Mannheim; glyceraldehyde-3-phosphate diethyl acetal barium salt was obtained from Sigma and converted to the free aldehyde as recommended by Sigma. Dithiothreitol was obtained from Calbiochem, ultrapure ammonium sulfate from Schwarz/Mann, and Alumina F20 from Alcoa. p-Nitrophenyl acetate was obtained from Calbiochem.

Bakers' yeast (Saccharomyces cerevisiae) was generously supplied by the Red Star Yeast Co., Oakland, California.

Purification Procedure

All steps are carried out at 5° unless otherwise stated.

Step 1. Extraction—One pound of fresh, pressed bakers' yeast is ground in small portions in a mortar using Alcoa F-20 alumina as an abrasive. The broken cells are stirred for 1 hour with 1500 ml of 0.1 M Tris-Cl, pH 9, containing 1 mM EDTA and 1 mM dithiothreitol. It may necessary be to add a little 1 M Tris from time to time to keep the pH above 8. The alumina is allowed to settle out and after decantation, the remainder of the suspension is centrifuged at 9000 rpm for 30 min.

Step 2. Ammonium Sulfate Fractionation at 50% Saturation—To the turbid brown supernatant, 313 g of ammonium sulfate per liter are added slowly with stirring. The pH is kept above 8 by small additions of concentrated ammonium hydroxide. After stirring for 1 hour, this mixture is centrifuged at 9000 rpm for 30 min.

Step 3. Ammonium Sulfate Fractionation at 80% Saturation—To the clear yellow supernatant fluid is added another 250 g per liter of ammonium sulfate, followed by stirring for another hour. After centrifugation at 9000 rpm for 1 hour, the somewhat turbid supernatant is discarded. The precipitate is redissolved in about


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100 ml of the pH 9 extraction buffer (see "Step 1") and the pH adjusted above pH 8 with 1 M Tris base.

**Step 4. Desalting by Sephadex G-50 Chromatography**—The enzyme solution (about 100 ml) is applied to a Sephadex G-50 column (40 x 7 cm) equilibrated with 0.01 M Tris Cl, pH 8.3, containing 1 mM EDTA and 0.3 mM dithiothreitol. Some yellow material as well as the salt is separated from the enzyme in this step.

**Step 5. DEAE-Sephadex Chromatography**—The desalted enzyme is pooled and adsorbed onto a column (30 x 7 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer used on the Sephadex G-50 column ("Step 4"). The enzyme is eluted in this same buffer system with a 0 to 0.15 M NaCl gradient of 3 liters total volume (1.5 liters in each reservoir). Loading the column should be accomplished during the first day, and the column can then be allowed to run overnight. Three peaks of activity are resolved (Fig. 1) but only the last, or most acidic, is pooled for further purification. The enzyme is now concentrated by ammonium sulfate precipitation. Ammonium sulfate (72 g per 100 ml of solution) is added and stirred for 1 hour. After centrifugation for 1 hour at 9000 rpm the precipitate is redissolved in 30 to 40 ml of the extraction buffer ("Step 1") and the pH adjusted above 8 with 1 M Tris base. The protein concentration of this solution should be high—10 to 20 mg per ml.

**Step 6. Crystallization**—The enzyme is crystallized by adding saturated ammonium sulfate solution (adjusted to pH 8.3 with NH₄OH) dropwise with stirring until a faint turbidity develops. The enzyme is then left to crystallize overnight. Usually a second crystallization is sufficient to bring the specific activity to 100 units per mg.

As can be seen in Table I, crystallization is not very efficient in terms of yield. Quite a bit of enzyme remains in the supernatant from the first crystallization. This enzyme can be brought down by addition of a little more saturated ammonium sulfate, and can then be recrystallized to give enzyme as good as the first crop. The yield can be nearly doubled in this way.

The enzyme is stored as the crystalline suspension. For use it

![Fig. 1. DEAE-Sephadex chromatography. The chromatography was performed as described in Step 5 of the purification procedure. The fractions from 1550 to 1900 ml were pooled for further purification.](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Units per mg</th>
<th>Total Units</th>
<th>mg per mg</th>
<th>Total mg</th>
<th>Units per mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1.650</td>
<td>64.3</td>
<td>106,000</td>
<td>32.1</td>
<td>53,000</td>
<td>2</td>
</tr>
<tr>
<td>50% supernatant</td>
<td>1.725</td>
<td>64.3</td>
<td>111,000</td>
<td>8.0</td>
<td>15,800</td>
<td>8</td>
</tr>
<tr>
<td>80% precipitate</td>
<td>745</td>
<td>121</td>
<td>90,000</td>
<td>7.6</td>
<td>5,630</td>
<td>16</td>
</tr>
<tr>
<td>DEAE-pool</td>
<td>400</td>
<td>87.5</td>
<td>35,000</td>
<td>1.6</td>
<td>636</td>
<td>55</td>
</tr>
<tr>
<td>First crystals</td>
<td>80</td>
<td>375</td>
<td>30,000</td>
<td>4.7</td>
<td>5/6</td>
<td>80</td>
</tr>
<tr>
<td>Second crystals</td>
<td>20</td>
<td>950</td>
<td>10,000</td>
<td>9.5</td>
<td>190</td>
<td>100</td>
</tr>
</tbody>
</table>

*Units are expressed as micromoles of NADH per mg per min.*

*In calculating a yield, it must be remembered that the isozyme of interest constitutes only about 60% of the total units originally extracted.

![Fig. 2. Isoelectric focusing. The 110 ml LKB electrofocusing column was used, and the sample was focused in a pH 5 to 8 / gradient for 3 days at 4°C with a final voltage of 800 volts as described in the LKB manual. Mercaptoethanol (10 mM) was also included in the column to preserve the enzyme.](http://www.jbc.org/)

![Fig. 3. Burst titrations using p-nitrophenyl acetate. The enzyme was incubated with dithiothreitol for 30 min at room temperature, and then passed through a small Sephadex G-25 column equilibrated with the desired buffer—0.05 M Tris Cl, 0.01 M EDTA either pH 8.5 or 7.75. The enzyme concentration was about 0.5 mg per ml in all cases. The enzyme (0.5 ml) was placed in a cuvette at 25°C, and, using rapid mixing technique, 10 μl of 5 x 10⁻² M nitrophenyl acetate in acetonitrile was added (final concentration = 10⁻³ M). Absorbance was recorded at 400 nm for several minutes. Controls were performed both without enzyme and with enzyme inactivated by reaction with 4 moles of iodoacetate. The plots shown have been corrected for spontaneous hydrolysis of nitrophenyl acetate by buffer and non-specific sites on the enzyme and also for steady state hydrolysis at the active site, so that only the burst itself is represented. An extinction coefficient of 1.8 x 10⁻³ was used for the yellow nitrophenolate anion, and a pKa of 7.14 was used in accounting for unionised p-nitrophenol. In A the burst is shown with a linear scale, and in B the plots have been converted to semi-log form.](http://www.jbc.org/)
is centrifuged, redissolved, and usually desalted on a small Sephadex G-25 or G-50 column.

RESULTS AND DISCUSSION

A summary of the purification yields and specific activities is given in Table I.

Polyacrylamide gel electrophoresis (5) shows that the enzyme runs as a single band indistinguishable from the enzyme obtained by the Krebs procedure. Occasionally, a very minor fast moving band can be seen on heavily loaded gels. This contaminant can be removed by a further crystallization.

Electrofocusing in polyacrylamide gels (6) was performed in the presence of 8 M urea. The enzyme gave one band in this test as well. This system dissociates the enzyme to monomers and is capable of resolving very small differences (down to 1 charge unit or better); thus, the enzyme appears to be composed of identical subunits.

Fig. 2 shows the pattern obtained when the enzyme is electrofocused in the absence of urea on the 110-ml LKB isoelectric focusing column. A single peak of activity and optical density is found.

In order to characterize the enzyme with respect to the number of functional active sites, we have measured the number of moles of p-nitrophenol released in the initial burst when p-nitrophenyl acetate is hydrolyzed. This hydrolysis is thought to occur at the same sulfhydryl group (cysteine 149) involved in the normal covalent catalysis, and thus should be a good measure of the active sites available for the oxidative phosphorylation of glycer-aldehyde 3-phosphate (7). As shown in Fig. 3, there are four active sites which appear to function with the same rate constant in the hydrolysis of p-nitrophenyl acetate.

The enzyme obtained by this rapid purification has been shown to be homogeneous, to be composed of identical subunits, and to have excellent specific activity associated with four functional sites. It is the most abundant and most acidic of the observed isozymes, and is indistinguishable electrophoretically from the enzyme obtained by repeated crystallization in the Krebs procedure. Its specific activity is slightly higher than that of the enzyme obtained in the traditional procedure, possibly due to the increased speed and efficiency of the method. Moreover, this new procedure allows one to prepare the enzyme quickly and reproducibly when it is needed and eliminates the necessity of storing a large quantity of the enzyme for long periods of time.

REFERENCES

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