The Preparation of Alcohol Dehydrogenase and Glyceraldehyde 3-Phosphate Dehydrogenase from Baker's Yeast

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(Received 2 March 1970)

A procedure has been developed for the preparation of alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase from the same sample of baker's yeast. The two enzymes were obtained in good yield in a pure crystalline form. The method minimizes the work involved in preparing the two enzymes and would be of particular advantage for preparing the enzymes in radioactive form from yeast grown in a radioactive medium.

Various procedures for the preparation of yeast alcohol dehydrogenase (EC 1.1.1.1) have been described (e.g. Racker, 1950; Hayes & Velick, 1954; Wallenfels & Sund, 1957; Keleti, 1958; Hvidt & Kågi, 1963). In all of these procedures the yeast was broken by some mechanical technique, usually after drying, and the crude yeast extract resulting from an extraction of the soluble proteins from the broken cells was subsequently submitted to fractionation with organic solvents and, in some cases, by heat denaturation.

We have been studying the amino acid sequence of both yeast alcohol dehydrogenase and yeast glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12). The usual method for preparing yeast glyceraldehyde 3-phosphate dehydrogenase (Krebs, 1955) involves disrupting the yeast cell wall by toluene plasmolysis and we have tried using this technique for the preparation of yeast alcohol dehydrogenase. This has proved successful and we have developed a method allowing the preparation of both enzymes from the same sample of yeast.

MATERIALS AND METHODS

All reagents were of A. R. grade or of the highest grade commercially available and were used without further purification.

Baker's yeast was obtained from the Distiller's Co. Ltd., Dagenham, Essex, U.K.

Double-distilled water which had been redistilled in a quartz still was used for all buffers and solutions in the preparation.

Polyacrylamide-gel electrophoresis was carried out by using the pH 8.5 system of Ornstein & Davies (1962). The gels were stained for protein with 1% Amido Black in 7%

(v/v) acetic acid and were destained electrophoretically as described by Ornstein & Davies (1962) or by applying the current transversely (Richards, Coll & Gratzner, 1965).

Electrophoresis on cellulose acetate strips was performed in a 'Microzone' Electrophoresis Apparatus (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) with 0.05 M-veronal adjusted to pH 8.6 with hydrochloric acid (Lebherz & Rutter, 1967) as buffer. The protein was stained with 0.2% Ponceau S in 5% trichloroacetic acid and destained with four washes of this solvent.

Staining for enzyme activity was carried out as described below. For yeast alcohol dehydrogenase the gel or strip was incubated at 37°C for 2 h in the following solution: tris (6g/l)–glycine (28.8g/l), 2.35 ml; 95% (v/v) ethanol (3.0 ml)–3 M-tris (3.3 ml) in 100 ml, 0.2 ml; NAD⁺ (30 mg/ml), 0.125 ml; phenazine methosulphate (5 mg/ml), 0.0125 ml; Nitro Blue Tetrazolium (10 mg/ml), 0.25 ml. The bands of alcohol dehydrogenase activity were shown by an insoluble precipitate of purple dye, and the reaction was stopped by transferring to 7% (v/v) acetic acid for two washes and then storing the gel or strip in this solution.

For yeast glyceraldehyde 3-phosphate dehydrogenase cellulose acetate strips were incubated in the solution described by Williams (1964), omitting the agar, and the enzyme was detected as a purple-staining band.

Enzyme assays were carried out by following the production of NADH at 340 nm. One unit of enzyme activity was defined as that amount necessary to catalyse the production of 1 μ mol of NADH/min under the conditions of assay. All assays were performed at room temperature (about 25°C) in 1 cm cuvettes and the reaction was started by the addition of enzyme. The following final concentrations were used: for yeast alcohol dehydrogenase (Racker, 1950), 0.1 M-sodium pyrophosphate, pH 8.6, 0.1 M-ethanol and 0.05 mM-NAD⁺, blank without ethanol; for yeast glyceraldehyde 3-phosphate dehydrogenase (Allison & Kaplan, 1964), 0.047 M-sodium pyrophosphate, pH 8.4, 5.0 mM-sodium arsenate, 0.25 mM-NAD⁺ and 4.0 mM-2-mercaptoethanol, blank without enzyme. The rates were directly dependent on enzyme concentration over the range 0.002–0.05 unit/3.0 ml of reaction mixture for yeast alcohol dehydrogenase and up

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to 0.05 unit/3.0 ml of reaction mixture for yeast glyceraldehyde 3-phosphate dehydrogenase.

Protein concentrations were determined spectrophotometrically, by using $E_{280}^{0.1\%}$ 0.792 for yeast alcohol dehydrogenase (Hayes & Velick, 1954) and $E_{280}^{0.1\%}$ 0.910 for yeast glyceraldehyde 3-phosphate dehydrogenase (Krebs, 1955). For the initial fractionation of the crude yeast extract a value of $E_{280}^{0.1\%}$ 1.0 was taken.

Spectrophotometry was carried out with a Gilford model 2000 recording spectrophotometer with a Unicam SP.500 monochromator.

Centrifugation was carried out with an MSE Major centrifuge (4 × 1250 ml swinging-bucket rotor) or with an MSE High Speed 18 centrifuge (6 × 100 ml angle head).

Analytical ultracentrifugation was carried out in a Beckman Spinco model E analytical ultracentrifuge fitted with schlieren optics.

RESULTS AND DISCUSSION

The yeast cells were broken by toluene plasmolysis (Krebs, 1955). A 10 lb portion of yeast was crumbled into 2400 ml of toluene in a preheated vessel in a 45°C water bath. The mixture was allowed to stand, with occasional stirring, until the yeast liquefied (about 90 min) and was then removed from the water bath and left at room temperature for a further 3 h. All subsequent steps were carried out at 4°C. Then 4800 ml of 1.0 mM-EDTA was added and after several hours of occasional stirring, the mixture was left to stand overnight. The lower (aqueous) layer was siphoned off and clarified by centrifugation (1000g for 1 h) to remove the coarser debris followed by filtration, with Hyflo Supercel as a filter aid, under gentle suction. The resulting clear yellow liquid was called the 'yeast extract'.

The yeast extract was fractionated by successive additions of ammonium sulphate, and the resulting precipitates were removed by filtration under gravity. A typical fractionation is shown in Table 1.

The precipitate corresponding to the alcohol dehydrogenase activity (0–60% saturation with

ammonium sulphate) was redissolved in 0.04 M-sodium phosphate–1.0 mM-EDTA, pH 7.6, and the solution was centrifuged (40 000g for 1 h) to remove some insoluble material. The solution was then brought to turbidity by the addition of a saturated solution of ammonium sulphate–1.0 mM-EDTA, apparent pH (glass electrode) 7.6, and left at 4°C to crystallize. Further ammonium sulphate was added at intervals, and then the 'first crystals' were centrifuged down (40 000g for 1 h), redissolved in the above buffer, and the enzyme was recrystallized twice by the same technique. Further recrystallization did not result in any further increase in the specific activity.

The precipitate corresponding to the glyceraldehyde 3-phosphate dehydrogenase activity (60–80% saturation with ammonium sulphate) was dissolved in 5.0 mM-tris hydrochloride–1.0 mM-EDTA, pH 7.6. The enzyme was crystallized by bringing the solution to turbidity by the addition of a saturated solution of ammonium sulphate–1.0 mM-EDTA, apparent pH (glass electrode) 8.2 (by adjusting the pH of the solution to 8.2 by the addition of 2 M-ammonia) and leaving the solution at 4°C to crystallize. The crystals were centrifuged down (40 000g for 1 h), redissolved in the same buffer and recrystallized by the same procedure twice more.

The details of the protein and activity of both enzymes during the steps of a preparation are shown in Table 2. Final specific activities ranged from 45 to 75 units/mg for yeast alcohol dehydrogenase and from 40 to 50 units/mg for yeast glyceraldehyde 3-phosphate dehydrogenase. Final E_{280}/E_{260} ratios were typically about 1.75 (highest 1.84) for the alcohol dehydrogenase and about 1.45 (highest 1.67) for the glyceraldehyde 3-phosphate dehydrogenase.

Yeast alcohol dehydrogenase gave a single band, which stained for both protein and enzymic activity, on electrophoresis in polyacrylamide gels. In the ultracentrifuge, it gave a major peak of about 7.5S and also a minor peak (up to about 10% of the total)

Table 1. *Fractionation of yeast extract*

For details of the assay see the text. Yields and purifications refer to extract.

Fraction	Protein (g)	10 ⁻³ × Activity (units)		Yield (%)		Purification	
		Alcohol dehydro- genase	Glyceraldehyde 3-phosphate dehydrogenase	Alcohol dehydro- genase	Glyceraldehyde 3-phosphate dehydrogenase	Alcohol dehydro- genase	Glyceraldehyde 3-phosphate dehydrogenase
Yeast extract	190	144	619	100	100	1.0	1.0
0–60% saturation with (NH ₄) ₂ SO ₄	35	122	80	85	13	4.6	0.7
60–80% saturation with (NH ₄) ₂ SO ₄	41	21	428	15	69	0.7	3.2
80% saturation with (NH ₄) ₂ SO ₄ : supernatant	107	1	113	0	18	0.0	0.3

Table 2. *Details of preparations from baker's yeast (10 lb)*

(a) Alcohol dehydrogenase

Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification
Yeast extract	185 000	140 000	0.76	100	1.0
0–60%–satn.-(NH ₄) ₂ SO ₄ fraction	33 900	119 000	3.51	85	4.6
First crystals	1740	62 800	36.20	45	47.8
Once recrystallized	864	53 300	62.40	38	82.3
Twice recrystallized	671	50 400	72.90	36	98.7

(b) Glyceraldehyde 3-phosphate dehydrogenase

Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification
Yeast extract	194 000	634 000	3.36	100	1.0
60–80%–satn.-(NH ₄) ₂ SO ₄ fraction	41 600	437 000	10.50	69	3.2
First crystals	15 100	298 000	19.80	47	6.1
Once recrystallized	3800	168 000	44.20	27	13.6
Twice recrystallized	2200	96 000	43.60	15	13.4

of about 2.0S. This latter was observed by Sund (1960) and has since been shown (Bühner & Sund, 1968, 1969) to be derived from the dissociation of inactive alcohol dehydrogenase tetramers into monomers. During the amino acid sequence analysis on this material no evidence has been found for any heterogeneity (Butler, 1967; Butler & Harris, 1968).

The recrystallized yeast glyceraldehyde 3-phosphate dehydrogenase also gave a single protein band when subjected to electrophoresis in polyacrylamide gels or on cellulose acetate strips, where it was shown to be enzymically active. The presence of electrophoretically separable forms of glyceraldehyde 3-phosphate dehydrogenase has been demonstrated in crude yeast extracts (Krebs, 1953; Lebherz & Rutter, 1967; Kirschner & Voigt, 1968), but crystallization appears to select for a single form (Krebs, Rafta & Junge, 1953). Resolution of the crystalline enzyme into subcomponents was, however, shown by these workers and by Kirschner & Voigt (1968). Although we have found electrophoretically distinct components in the presence of 8M-urea, a detailed study of the amino acid sequence (Jones & Harris, 1968; Jones, 1969) of yeast glyceraldehyde 3-phosphate dehydrogenase prepared as above has shown that the enzyme appears to consist of four essentially identical polypeptide chains, each of 331 amino acid residues. Only in the case of residue 328, which can be either isoleucine or valine, has any heterogeneity in the primary structure been positively identified (Jones, 1969).

From all of these criteria, both yeast enzymes appear to be pure proteins when prepared as described above. This particular method eliminates the somewhat inconvenient techniques previously used for the disruption of the yeast cells in the

preparation of alcohol dehydrogenase. It also allows the more inconvenient steps involving large volumes to be done only once for the two enzymes. Also, if it were desired to prepare labelled enzymes by growing the yeast cells on a radioactive medium, there would be considerable saving in preparing more than one enzyme from a single batch of yeast.

Both authors thank the Medical Research Council for Scholarships for Training in Research Methods throughout the time when this work was carried out and also their supervisor, Dr J. I. Harris, for his advice and encouragement.

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