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PROTEIN STRUCTURE AND ENZYMATIC ACTIVITY

II. PURIFICATION AND PROPERTIES OF A CRYSTALLINE GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM *CANDIDA UTILIS*

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SUMMARY

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$ oxidoreductase, EC 1.1.1.49) has been purified about 500-fold and crystallized from the yeast *Candida utilis*. The K_m values for NADP$^+$ and glucose 6-phosphate were determined to be $6.7 \cdot 10^{-5}$ M and $2.3 \cdot 10^{-4}$ M, respectively. The enzyme, whose amino acid composition has been determined, has a molecular weight of about 104 000 as determined by Sephadex gel filtration. The enzymatic activity is inhibited competitively by pyridoxal 5'-phosphate and by various nucleotides.

INTRODUCTION

Glucose-6-phosphate dehydrogenase has been detected first in erythrocytes in 1931 by WARBURG AND CHRISTIAN[1], who named the enzyme Zwischenferment. Whereas early investigators believed the enzyme to be very instable, recently crystalline preparations have been obtained by various groups. JULIAN *et al.*[2] crystallized the enzyme after a 10-step purification from mammary gland, and NOLTMANN *et al.*[3] obtained a 2500-fold purification and crystallization from brewer's yeast in a 11-step operation. We have recently described a crystalline preparation from the yeast *Candida utilis*[4]. During the preparation of that enzyme it was observed that the glucose-6-phosphate dehydrogenase activity could not be precipitated in total by $(NH_4)_2SO_4$ within a reasonable range of salt concentration. The fraction precipitating between $s = 0.85$ and 0.95 has been crystallized by the procedure described earlier[4]; this communication describes the further purification and crystallization of the fraction precipitating between $s = 0.63$ and 0.75. By use of a Manton–Gaulin homogenizer (H. G. Schröder Nachf., D 2304 Lübeck-Schlutup, Germany) kg quantities of yeast can be processed, and 100 mg quantities of crystalline enzyme are obtained after five simple and well-reproducible purification steps.

MATERIALS AND METHODS

Materials

Cells of *Candida utilis*, grown industrially on waste lyes from the cellulose production by Zellstoff-Fabrik Waldhof (Mannheim, Germany) were harvested by centrifugation and could be stored as a frozen paste at $-15°$ without loss of glucose-6-phosphate dehydrogenase.

All chemicals used were analytical grade from E. Merck (Darmstadt). Protamine sulfate was purchased from Calbiochem (Los Angeles); all other biochemicals were from Boehringer (Mannheim).

Methods

Glucose-6-phosphate dehydrogenase was assayed in the following standard test. A cuvette (volume, 1 ml; light path, 1 cm; temperature, 25°) contained the following mixture: 93 mM glycine–NaOH (pH 9.1), 9.3 mM $MgCl_2$, 0.93 mM EDTA, 1.0 mM glucose 6-phosphate and 0.4 mM $NADP^+$. The reaction was started by the addition of enzyme. The increase of absorbance was recorded at 366 nm for 2 min. As long as the preparations contained 6-phosphogluconate dehydrogenase, the activities were corrected for the value measurable with 6-phosphogluconate as the substrate.

Our crystallized glucose-6-phosphate dehydrogenase has been assayed for contaminating activities by the following procedures:

(a) Hexokinase: 72 mM triethanolamine (pH 7.6), 0.72 mM EDTA, 20 mM glucose, 5 mM $MgCl_2$, 2.5 mM ATP, 1 mM $NADP^+$ and 30 μg enzyme protein.

(b) Phosphoglucomutase: 82 mM triethanolamine (pH 7.6), 0.82 mM EDTA, 5 mM $MgCl_2$, 5 mM glucose 1-phosphate, 0.5 mM $NADP^+$ and 30 μg enzyme protein.

(c) Hexose phosphate isomerase: 86 mM triethanolamine (pH 7.6), 0.86 mM EDTA, 0.7 mM $NADP^+$, 0.5 mM fructose 6-phosphate, 5 mM $MgCl_2$ and 30 μg enzyme protein.

(d) Glutathione reductase: 80 mM triethanolamine (pH 7.6), 0.8 mM EDTA, 0.5 mg serum albumin, 6 mg GSH, 0.6 mM $NADP^+$ and 30 μg enzyme protein.

1 unit of glucose-6-phosphate dehydrogenase was defined as that amount of enzyme which under the conditions of the standard test caused a reduction of 1 μmole $NADP^+$ per min.

Protein determinations were performed by the biuret method[5] or in highly purified fractions by the ultraviolet-absorption method of WARBURG AND CHRISTIAN[6].

The amino acid analyzer, model Unichrom manufactured by Beckman Instruments (Munich), was used for amino acid determinations.

Hydrolysis of proteins was performed for 21 h at 110° in glass tubes sealed under vacuum; the rotatory evaporator model Frequentax of E. Schütt jun. (Goettingen), was used for the subsequent removal of HCl from the hydrolyzed samples.

Cysteine was determined as cysteic acid after treatment of the protein with performic acid and subsequent hydrolysis[7] and tryptophan by the spectrophotometric method of BARMAN AND KOSHLAND[8].

Molecular weight determinations were achieved by filtering the unknown sample over a column of Sephadex G-100 (2.5 cm × 43 cm) previously calibrated with the protein standards shown in Fig. 3.

All procedures were performed at 2°.

RESULTS

Preparation of crystalline glucose-6-phosphate dehydrogenase

Step I: Extraction of yeast. Crude extracts were obtained by treatment of a 40% suspension of wet cells in 0.02 M triethanolamine (pH 7.6)–1 mM EDTA–0.5 mM 2-mercaptoethanol in a Manton–Gaulin homogenizer. After 3 min of recycling at 560 kg/cm^2, the viscous extract was collected. Since this breakage of cells is accompanied by heat development, the yeast suspension had been cooled to 0° before the start of the experiment, and the container part of the homogenizer was perfused with a −15° solution during the operation. Even so the extract would warm up to 20°; after re-cooling it was centrifuged and the precipitate discarded.

Step II: Removal of nucleic acids. Under mechanical stirring, a 2% solution of protamine sulfate (amount equivalent to 7% of the total protein present in the crude extract) was added dropwise. After an additional 5 min, the precipitate was centrifuged off and discarded.

Step III: $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to give a 63% saturation (380 g/l), and the precipitate was discarded. Further $(NH_4)_2SO_4$ was added to $s = 0.75$ (76.3 g/l) and the precipitating protein collected by centrifugation. The precipitate was dissolved in 0.1 M triethanolamin (pH 7.6); this solution may be kept frozen for several months without loss of activity. A considerable amount of glucose-6-phosphate dehydrogenase activity will stay in the supernatant after this step. This part of the enzyme will precipitate at 95% saturation; its crystallization has been described[4].

Step IV: CM-Sephadex column. The protein precipitated at 75% $(NH_4)_2SO_4$ saturation is exhaustively dialyzed against 0.01 M malonate (pH 6.7)–1 mM EDTA–0.5 mM mercaptoethanol and then added to a column of CM-Sephadex C-50 in the Na^+ form (column 2.5 cm × 40 cm), equilibrated with the same buffer. The column is washed with 2 vol. of the same buffer, followed by 2 vol. each of 0.1 M and 0.2 M NaCl, respectively, in 0.01 M malonate (pH 6.7). Subsequently the glucose-6-phosphate dehydrogenase was eluted using 0.4 M NaCl in the same buffer.

Step V: DEAE-Sephadex column. The pooled fractions containing the enzyme were dialyzed against 0.01 M imidazole·HCl (pH 7.0) and subsequently put on a column (2.5 cm × 40 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. Inactive proteins were washed out by passing through several column volumes of starting buffer and 0.05 M imidazole (pH 7.0), after which the enzyme was eluted using 0.15 M imidazole (pH 7.0).

Step VI: Concentration and crystallization. All fractions containing glucose-6-phosphate dehydrogenase were pooled and concentrated under vacuum in an Ultrahülse (purchased from Membranfilter-Gesellschaft, Goettingen) until a protein concentration of 10–20 mg/ml was reached. Solid $(NH_4)_2SO_4$ was added under magnetic stirring at 0° up to a saturation of 75% (4.8 g/10 ml); the addition of salt was done very slowly, particularly after the first "schlieren" had appeared. Recrystallizations can be done by collecting the crystals by centrifugation, by dissolving them in 0.1 M triethanolamine (pH 7.6) and by bringing the $(NH_4)_2SO_4$ saturation to 0.75 in the manner described above. This step has given us yields of 75–95% of glucose-6-phosphate dehydrogenase activity with no remarkable increase in specific activity. Crystals obtained after this additional step show, however, sharper contours.

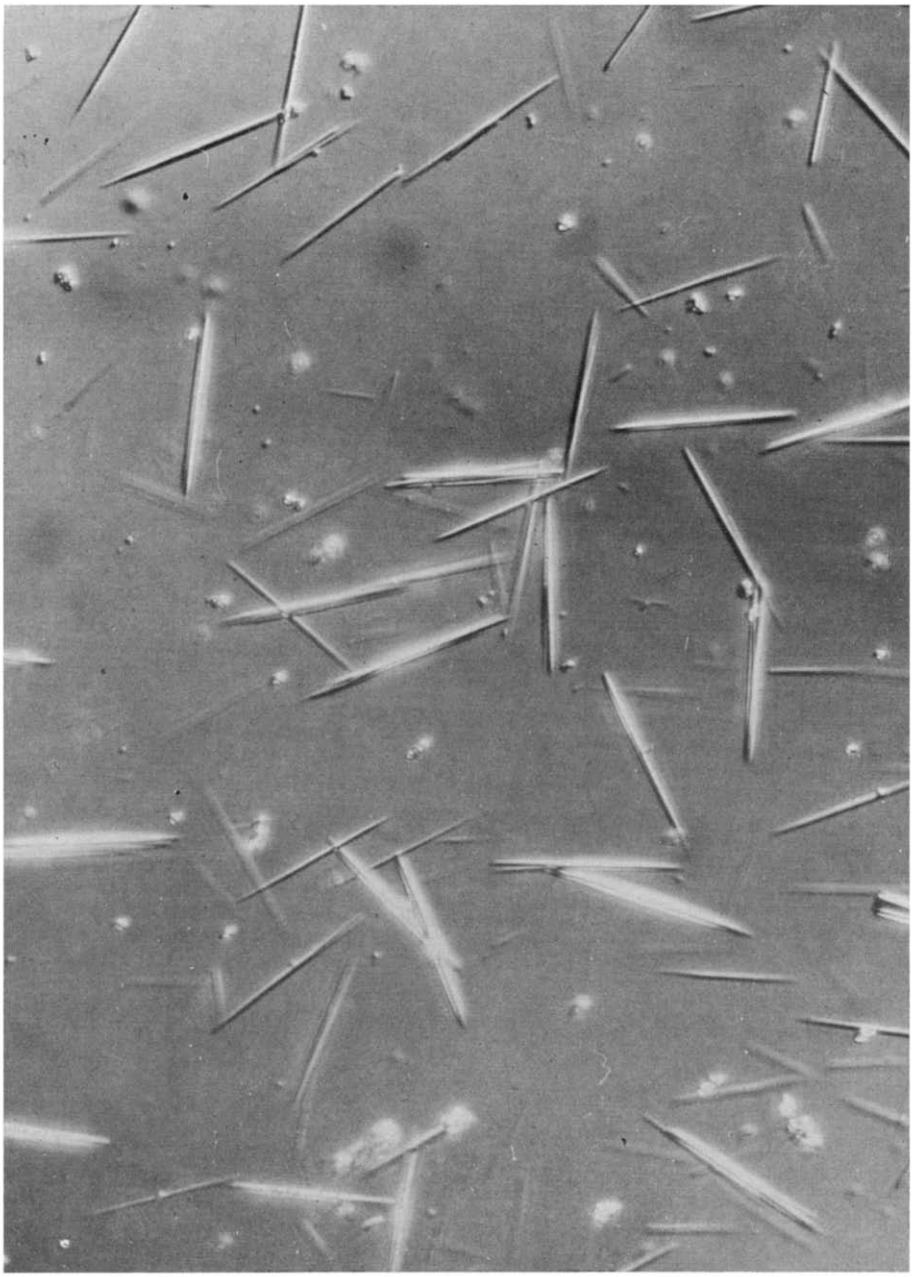


Fig. 1. Crystals of glucose-6-phosphate dehydrogenase (Magnification 200 ×).

Fig. 1 shows a microphotogram of the enzyme preparation; Table I gives the analytical data for a typical run.

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE

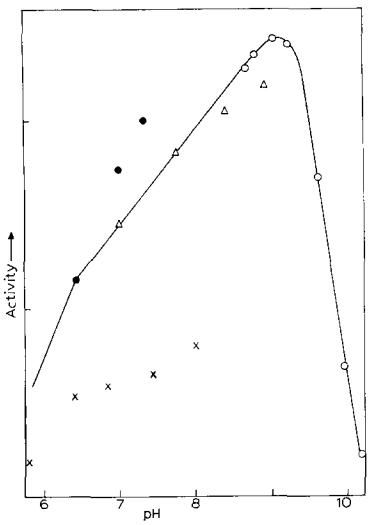
Starting material: 1630 g wet cells of *Candida utilis*.

Step	*Procedure*	*Volume (ml)*	*Total units*	*Total protein*	*Specific activity*	*Yield (%)*
I	Crude extract	2480	59 400	90.3 g	0.66	100
II	Protamine treatment	2500	57 500			97
III	$(NH_4)_2SO_4$ supernatant after $s = 0.63$	2650	54 880	9.54 g	5.8	92
IIIa	$(NH_4)_2SO_4$ precipitate $s = 0.63$–0.75	166	31 872	4.95 g	6.4	54
IIIb	$(NH_4)_2SO_4$ supernatant after $s = 0.75$	2735*	22 720*			38*
IV	CM-Sephadex (pH 6.7) eluate 0.4 M NaCl	19.8	20 910	430 mg	48.6	35
V	DEAE-Sephadex (pH 7.0) eluate 0.15 M imidazole	50.5	20 840	73 mg	285	35
VI	First crystallization	8	19 170	61 mg	314	32

* Fraction removed for separate purification procedure.

Physicochemical constants and other properties of the enzyme

The Michaelis constants for glucose 6-phosphate and $NADP^+$ have been determined by the method of Lineweaver and Burk and gave the following results: K_m for glucose 6-phosphate $= 2.3 \cdot 10^{-4}$ M; K_m for $NADP^+ = 6.7 \cdot 10^{-5}$ M. In these experiments, all the other reactants, with exception of the varied substrate, were in the concentrations given for standard test under *Methods*.



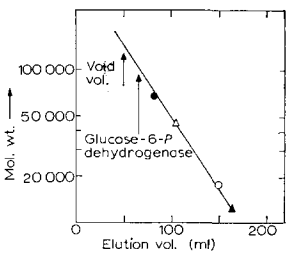


Fig. 2. pH optimum of glucose-6-phosphate dehydrogenase. The following buffers (all 0.1 M) were used instead of glycine–NaOH: ×, potassium phosphate; ●, imidazole–HCl; △, Tris–HCl; ○, glycine–NaOH. The other conditions were as described for standard test under METHODS.

Fig. 3. Determination of the molecular weight of glucose-6-phosphate dehydrogenase by gel filtration. The following protein standards were included in this experiment: ●, hemoglobin (mol. wt. 68 000); △, egg albumin (mol. wt. 45 000); ○, myoglobin (mol. wt. 17 800); ▲, cytochrome *c* (mol. wt. 12 400).

TABLE II

AMINO ACID COMPOSITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

The numbers given were calculated on the assumption of a molecular weight of 104 000. Amino acids marked with an asterisk were determined by special procedures.

Lysine	89	Glutamic acid	106	Methionine	15
Histidine	12	Proline	49	Isoleucine	47
Arginine	35	Glycine	83	Leucine	81
Aspartic acid	134	Alanine	67	Tyrosine	32
Threonine	44	Cysteine	14*	Phenylalanine	42
Serine	67	Valine	57	Tryptophan	10*

From the data presented in Fig. 2, the pH optimum of the enzyme was found to be 9.1 with a steep decrease in activity on the alkaline side of this value. An inhibition of the enzyme by phosphate ions, which had been noticed by THEORELL[9] in 1935, can be seen from our data.

The molecular weight of our enzyme was determined by gel filtration over Sephadex G-100 and was found to be 104 000 from comparing its elution volume with that of known protein standards; Fig. 3 shows the experimental data.

Table II gives the amino acid composition as compiled from hydrolysates obtained from different batches of the enzyme.

In order to check the purity of our crystallized glucose-6-phosphate dehydrogenase, this protein has been assayed for various enzymatic activities as shown in Table III.

Our enzyme is strongly inhibited by pyridoxal 5'-phosphate in low concentrations; Fig. 4 shows the data obtained with two different concentrations of this inhibitor. It could be shown that pyridoxal 5'-phosphate binds as a Schiff base to an active lysine residue; details of this inhibitory mechanism will be published elsewhere[10].

Moreover it was found that several nucleotides are strongly inhibitory to the glucose-6-phosphate dehydrogenase.

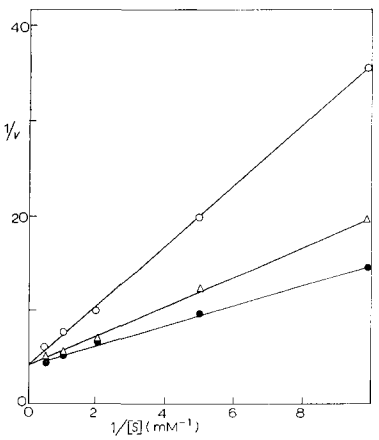


Fig. 4. Inhibition of glucose-6-phosphate dehydrogenase by pyridoxal 5'-phosphate. ●, no pyridoxal 5'-phosphate added; △, $5 \cdot 10^{-6}$ M pyridoxal 5'-phosphate; ○, $2 \cdot 10^{-5}$ M pyridoxal 5'-phosphate. [*S*] = [glucose 6-phosphate].

TABLE III

CHECK FOR CONTAMINATING ACTIVITIES IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Details on the determinations are given under METHODS.

Enzyme	*Relative activity (%)*
Glucose-6-phosphate dehydrogenase	100
6-Phosphogluconate dehydrogenase	0
Hexokinase	0.08
Hexose-6-phosphate isomerase	0
Phosphoglucose mutase	0
Glutathione reductase	0

DISCUSSION

Comparison of the physical and chemical data of the enzyme described here to the data of the enzyme isolated earlier[4] has not revealed any important differences. However, we have obtained other evidence for the existence of two different enzyme proteins[11]: antibodies formed in the rabbit against either of the two enzymes will inhibit the activity only of that enzyme which had been used as the antigen; fingerprints prepared by tryptic digestions of the two dehydrogenases following carboxymethylation show quite a different pattern. The two activity peaks appearing during the salting-out procedure are independent of the method used before for the removal of nucleic acids. Moreover, the two enzymes can be clearly distinguished by their pattern in polyacrylamide disc gel electrophoresis, and the Bengal red-catalyzed photooxidation appears to be different with the two enzymes[12].

The inactivation of glucose-6-phosphate dehydrogenase by pyridoxal 5'-phosphate provides a good method of labeling the active site of the enzyme by reduction of the Schiff base using ^{3}H-labeled sodium borohydride. Studies about the primary structure of this substrate binding center are in progress[13].

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