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CRYSTALLIZATION AND PROPERTIES OF PEA GLUCOSEPHOSPHATE ISOMERASE*

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SUMMARY

Glucosephosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) of pea was isolated in the crystalline state in 14.5% yield from pea extract after 1500-fold purification. The molecular weight of the enzyme was estimated as 110 000 by ultracentrifugal analysis. The K_m and molecular activity of the enzyme were determined as $2.7 \cdot 10^{-4}$ M (Glc-6-*P*) and 165 000 in Tris–HCl buffer (pH 8.5) at 37°. The isomerization reaction was equilibrated in 65% Glc-6-*P* and 35% Fru-6-*P* at pH 7.5 at 37°. 6-Phosphogluconate competitively inhibited the reaction and its K_i was $1.3 \cdot 10^{-5}$ M. The pH optimum of the reaction was found to be 8.5 in Tris–HCl buffer and 9.5 in glycine–NaCl–NaOH buffer. The maximal reaction rate was observed at 50° and the activation energy was calculated as 8100 cal. Sulfhydryl groups are not thought to be involved in its active center because the reaction was not inhibited by *p*-chloromercuribenzoate and monoiodoacetate. The enzyme was able to isomerize glucose into fructose in the presence of arsenate.

INTRODUCTION

In 1933, glucosephosphate isomerase was first demonstrated by LOHMANN[1] in extracts of yeast, brain, heart, kidney, liver and skeletal muscle. TANKÓ[2] and later, SOMERS AND COSBY[3] found this enzyme in pea meal.

SLEIN[4] effected a 7-fold purification of a crude extract of the enzyme obtained from rabbit muscle. Since then the enzyme has been purified from a number of tissues[5–8]. In 1960, the crystallization of glucosephosphate isomerase from bakers' yeast was reported by KLOTZSCH AND BERGMEYER[9]. More recently, the isolation procedures used to obtain the crystalline enzyme from rabbit skeletal muscle and

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brewers' yeast were described by NOLTMANN[10,11] and by NAKAGAWA AND NOLTMANN[12], respectively. However the crystallization of the enzyme from higher plants has not been reported.

This article deals with the procedure for isolation and crystallisation of the enzyme from pea and the basic properties of the crystalline enzyme.

MATERIALS AND METHODS

The sodium salt of glucose 6-phosphate and of 6-phosphogluconate, and the barium salt of fructose 6-phosphate were obtained from Boehringer and Manheim Co. Tris(hydroxymethyl)aminomethane was obtained from Sigma Chemical Co. DEAE-Sephadex (A-50) was obtained from Pharmacia Fine Chemicals.

Glucosephosphate isomerase activity was assayed by SLEIN's[4] method, measuring Fru-6-*P* formed from Glc-6-*P*. In some cases, the carbazole and sulfuric acid method[13] was also used.

Appropriately diluted enzyme solution was incubated in a medium containing 16 mM Glc-6-*P* and 40 mM Tris–HCl buffer (pH 8.5) at 37° for 3 min in a total volume of 0.25 ml. The reaction was terminated by addition of 2 ml of 8 M HCl and 0.5 ml of 0.1% resorcinol in 95% ethanol. The mixture was heated for 10 min at 80°. After cooling, the resulting pink color was measured in a Klett–Summerson colorimeter with filter No. 54.

One unit of the activity was defined as the formation of 1 μmole of Fru-6-*P* per min, under the above conditions.

Protein was determined by the method of LOWRY *et al.*[14] using bovine serum albumin as a standard.

The ultracentrifugal studies were carried out using a Hitachi ultracentrifuge Model UCA-1 type at 54 800 rev./min at 22.8°. The protein concentration was 6.5 mg/ml in 0.1 M NaCl solution.

Ultraviolet absorption spectra were measured using a Hitachi Perkin-Elmer UV-VIS spectrophotometer. The protein concentration was 0.8 mg/ml in 0.01 M Tris–HCl buffer (pH 7.5).

TABLE I

PURIFICATION OF GLUCOSEPHOSPHATE ISOMERASE

Purification steps	*Volume (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Recovery (%)*
1. Crude extract (from 2 kg of dry immature pea)	5860	157500	194300	0.81	100
2. $MgCl_2$ supernatant	5700	146000	139600	1.05	92.7
3. Zinc acetate supernatant	5660	131000	67900	1.93	83.3
4. First ammonium sulfate precipitate (0.45–0.6 salt saturation)	900	108000	14300	7.56	68.5
5. Bentonite supernatant	700	83200	2150	37.0	52.3
6. Isopropyl alcohol precipitate (48–63%)	123	60600	269	225	38.5
7. Second ammonium sulfate precipitate	60.5	48300	145	333	30.7
8. DEAE-Sephadex column eluate	43	34500	27.3	1260	21.5
9. Crystalline enzyme	2.1	22800	15.5	1470	14.5

RESULTS

Crystallization of glucosephosphate isomerase

The purification steps are summarized in Table I. The following operations were carried out at 0–4° unless otherwise indicated.

Step 1. Extraction. Dry immature peas were soaked in water at 25° overnight to soften them. The peas were then homogenized in a Waring Blendor with 1.5 vol. (per weight of the soaked peas) of a cold buffer mixture of 0.1 M Tris and 0.01 M EDTA (adjusted to pH 7.5 with HCl) for 5 min. The homogenate was squeezed through gauze and centrifuged at 14 000 × *g* for 7 min to remove cell debris and some particulate matter (crude extract).

Step 2. $MgCl_2$ treatment. To the crude extract a 0.05 vol. of 1 M $MgCl_2$ was added. The resulting precipitate was removed by centrifugation at 14 000 × *g* for 7 min.

Step 3. Zinc acetate treatment. To the supernatant a 0.05 vol. of 1 M zinc acetate was added, with stirring, and the pH was adjusted to 7.5 with 1 M Tris. After 30 min continual stirring, the precipitate was centrifuged off. The supernatant was dialyzed against distilled water for 3 h.

Step 4. First ammonium sulfate fractionation. To the dialyzed solution saturated ammonium sulfate solution (pH 7.5) was added to give 45% saturation. After 30 min stirring, the precipitate was centrifuged off at 14 000 × *g* for 10 min. To the supernatant solution saturated ammonium sulfate solution (pH 7.5) was added to give 60% saturation and the resulting suspension was kept overnight. The precipitate was collected by centrifugation and dissolved in 0.05 M Tris–HCl buffer containing 0.005 M EDTA (pH 7.5) to give a protein content of about 10 mg/ml. The precipitate, suspended in 60% saturated ammonium sulfate solution (pH 7.5), could be stored at −10° for several months without loss of the activity.

Step 5. Bentonite treatment. 20 g of bentonite per g of protein were added to the Step 4 solution, with stirring. After 30 min stirring, the adsorbent was removed by centrifugation. The enzyme activity remained in the solution.

Step 6. Isopropyl alcohol precipitation. Isopropyl alcohol chilled in a freezer (−10°) was added, slowly, to the above solution at −7° to a final isopropyl alcohol concentration of 48% (v/v) and the precipitate formed was discarded after centrifugation at −7°. To the supernatant solution isopropyl alcohol was added to increase the alcohol concentration to 63%. The precipitate was collected by centrifugation at −7° and suspended in 0.05 M Tris–HCl containing 0.005 M EDTA (pH 7.5). Insoluble material was removed by centrifugation.

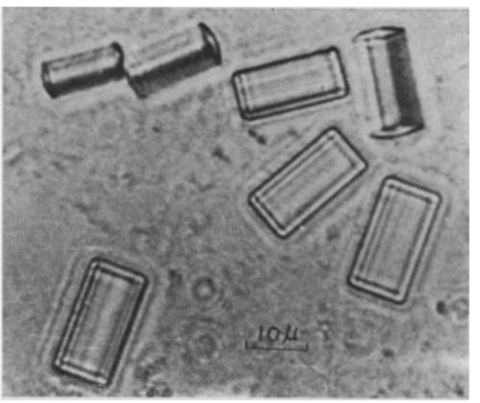
Step 7. Second ammonium sulfate fractionation. Saturated ammonium sulfate solution (pH 7.5) was added to the Step 6 solution to give a 65% saturated solution. The suspension was stored overnight. After centrifugation, the precipitate was dissolved in 0.01 M Tris–HCl containing 0.001 M EDTA (pH 7.5).

Step 8. Chromatography on DEAE-Sephadex. The Step 7 solution was dialyzed against 0.01 M Tris–HCl buffer containing 0.001 M EDTA (pH 7.5) for 4–5 h. The solution was slowly flowed onto a preequilibrated DEAE-Sephadex (A-50) column (equilibrated with the same buffer, 40 mg of protein per ml (wet volume) of the Sephadex). The column was eluted with the same buffer and then with the buffer containing 0.05 M NaCl. The enzyme was eluted with the buffer containing 0.1 M

NaCl. The enzyme was concentrated from the eluate by the addition of saturated ammonium sulfate solution to 70% saturation and resulting precipitate was collected by centrifugation. The precipitate was dissolved in 0.01 M Tris–HCl buffer containing 0.001 M EDTA (pH 7.5) to a solution of protein content about 8 mg/ml.

Step 9. Crystallization. The crystallization of the enzyme was carried out by the addition of saturated ammonium sulfate solution (pH 7.5) to the above solution. If the solution became opaque below 40% saturation, the precipitate was removed by centrifugation. The salt saturation was slowly increased up to 60% saturation to complete the crystallization. The crystalline glucosephosphate isomerase thus obtained is shown in Fig. 1.

The same crystalline enzyme was obtained from fresh or deep frozen immature peas using this method. When the enzyme suspension was stored at 4°, 50% activity was lost after five months.



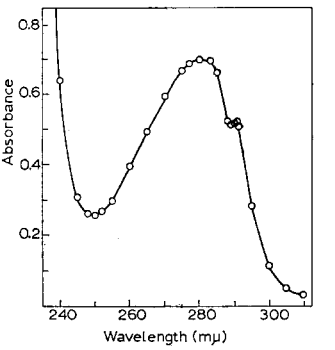


Fig. 1. Photomicrograph of the crystalline glucosephosphate isomerase.

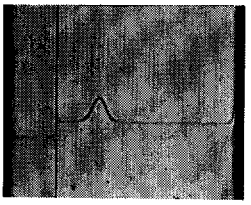
Fig. 2. Ultraviolet absorption spectrum of the glucosephosphate isomerase.

Properties of the crystalline enzyme

Absorption spectrum. The absorption spectrum of the enzyme showed a maximum at 280 mμ with a slight shoulder at 290 mμ and a specific extinction coefficient $E^{1\%}_{1\,cm}$ (280 mμ) of 8.75 (Fig. 2).

Molecular weight. The centrifugation patterns (Fig. 3) indicate that the crystalline enzyme is pure and homogeneous. Its sedimentation coefficient was 6.8 S at 20°. By the Archibald method[15] the molecular weight was found to be 110 000, assuming a specific volume of 0.720 (ref. 15). The molecular weight of yeast glucosephosphate isomerase had been reported as 145 000 (ref. 16).

pH-Stability. The enzyme solution, adjusted to the chosen pH with 0.2 M Tris–HCl buffer, was incubated at 50°. The residual enzyme activity was determined after the pH had been readjusted to 8.5. As indicated in Fig. 4, maximal stability was obtained at pH 7.0 and the enzyme was unstable at acid pH. Similar observations were made on the enzyme isolated from human erythrocyte[17], green gram[7], muscle[11] and yeast[12].



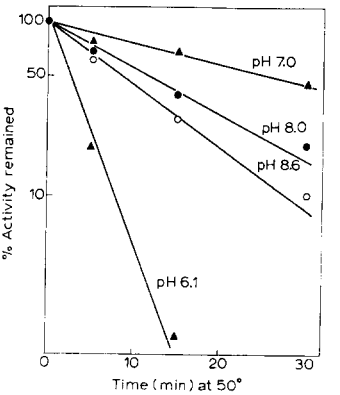
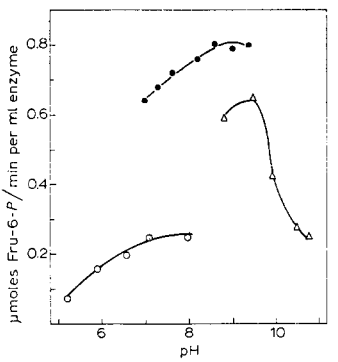


Fig. 3. Ultracentrifugal pattern of the glucosephosphate isomerase. Centrifuged for 29 min. See text.

Fig. 4. pH-Stability of the glucosephosphate isomerase.

pH-Optimum. As shown in Fig. 5, the enzyme has a broad pH-activity range with a maximum at pH 8.5 in Tris–HCl buffer and at pH 9.5 in glycine–NaCl–NaOH buffer. In Tris–maleate–NaCl buffer the enzyme had a lower activity owing to inhibition by maleate, as reported in yeast enzyme[16].

K_m for Glc-6-P. The effect of Glc-6-*P* concentration on the rate of the reaction is presented in Fig. 6. The Michaelis constant (K_m) was found to be $2.7 \cdot 10^{-4}$ M for Glc-6-*P* and the maximal velocity 1500 units/mg, using the Lineweaver–Burk method[18].



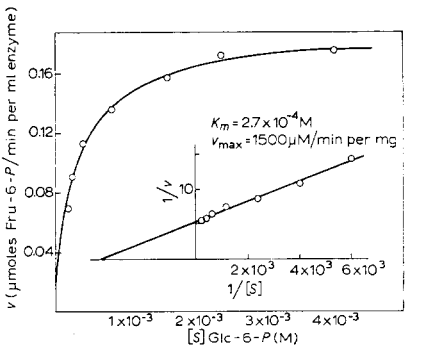
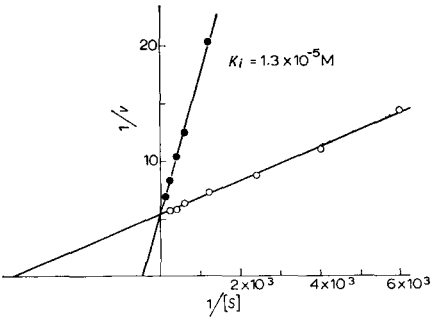


Fig. 5. pH–Activity curves of the glucosephosphate isomerase. Reaction mixtures were in the condition described for the assay of the enzyme, except in 0.16 M of indicated buffers. ○—○, Tris-maleate–NaOH; ●—●, Tris–HCl; △—△, glycine–NaCl–NaOH.

Fig. 6. Glc-6-*P* concentration dependence of the glucosephosphate isomerase activity. Conditions were the same in the activity assay, except at pH 7.5.

Inhibitors. As shown in Fig. 7, 6-phosphogluconate competitively inhibits the pea enzyme. The K_i value for 6-phosphogluconate was determined as $1.3 \cdot 10^{-5}$ M. A similar inhibition has been observed with the enzyme present in yeast[16], mammalian[19], bacteria[20] and potato[21].



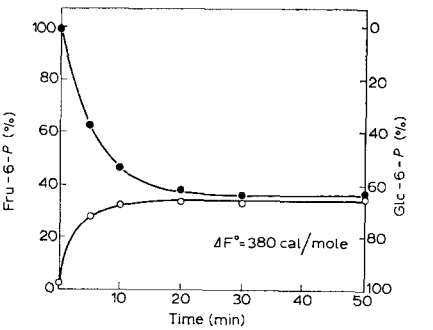


Fig. 7. Lineweaver–Burk plot of the activity with and without 6-phosphogluconate. Conditions were the same in Fig. 6. ○—○, Glc-6-*P*; ●—●, Glc-6-*P* + $1 \cdot 10^{-4}$ M 6-phosphogluconate.

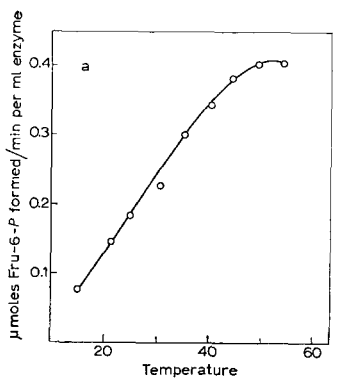
Fig. 8. Interconversion of Glc-6-*P* and Fru-6-*P* by glucosephosphate isomerase. The enzyme was incubated in 2.4 mM Glc-6-*P* or Fru-6-*P* and 40 mM Tris–HCl at pH 7.5 at 37°. ○—○, started with Glc-6-*P*; ●—●, started with Fru-6-*P*.

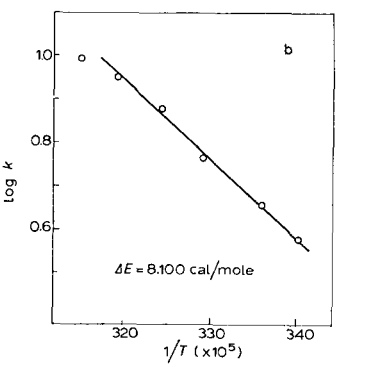
In the presence of sulfhydryl reagents, *p*-chloromercuribenzoate ($1 \cdot 10^{-4}$ M), and monoiodoacetate ($1 \cdot 10^{-4}$ M) the enzyme was not inactivated. The enzyme appeared, therefore, to have no –SH group in its active center, as with the enzymes obtained from yeast[12], muscle, human erythrocyte[12] and green gram[2].

Equilibrium. Fig. 8 shows the equilibrium obtained using either substrate, Glc-6-*P* and Fru-6-*P*. The equilibrium concentration was at 35% of Fru-6-*P* and 65% Glc-6-*P*. ΔF was calculated as 380 cal/mole at 37°, pH 7.5.

The effect of temperature on the reaction rate. The effect of temperature on the reaction rate was determined using Glc-6-*P* as substrate (Fig. 9a). The reaction rate was maximal at 50°. A plot of the log of reaction rate against the reciprocal of absolute temperature is shown in Fig. 9b and from the slope of the curve the activation energy was calculated as 8100 cal. The activation energies of the bean[7] and serum[21] enzymes have been reported as 4800 and 11 000 cal/mole, respectively.

Glucose isomerization activity. An isomerization activity for the conversion of free glucose to fructose, in the presence of arsenate, has been found in some microbial preparations[22–24] and the activity has been supposed to be due to the action of a





Figs. 9a and b. Temperature dependence of the glucosephosphate isomerase activity. Conditions were the same in the activity assay, except at pH 7.5.

TABLE II

RELATIVE ACTIVITY OF GLUCOSE 6-PHOSPHATE AND GLUCOSE ISOMERIZATIONS

Purification steps	*Glucose 6-phosphate isomerizing activity**	*Glucose isomerizing activity***
4. First ammonium sulfate precipitate (0.45–0.6 salt saturation)	51	
9. Crystalline enzyme	52	

* 0.016 M glucose 6-phosphate, 0.04 M Tris–HCl (pH 7.5) in the reaction mixture, 37°.
** 1.6 M glucose, 0.8 M arsenate–HCl (pH 7.5) in the reaction mixture, 37°.

glucosephosphate isomerase. The same isomerization ability was found in this pea enzyme preparation. As shown in Table II, the relative activities of the Glc-6-*P* and glucose isomerizations in the pea preparations are constant during the purification steps. The isomerization of glucose, in the presence of arsenate, was therefore considered to be an action of the glucosephosphate isomerase.

DISCUSSION

In the purification procedures of yeast and muscle isomerase, fractionation with ethanol and acetone was effectively employed[11,12]. With the pea enzyme, however, such a fractionation procedure caused a substantial loss of activity, but, isopropyl alcohol could be used to advantage. When attempting ammonium sulfate fractionation, addition of the solid salt caused a considerable loss of enzyme activity, even when the pH of the suspension was carefully maintained at 7.5 by the addition of aq. NH_4OH solution during the procedure. Almost all activity was recovered, however, by the addition of saturated ammonium sulfate at pH 7.5. Removal of protein impurities by bentonite adsorption was effectively employed in the pea enzyme preparation, as with other sources of the enzyme[11,12].

The crystalline enzyme showed a high molecular activity of 165 000 at 37°. The specific activity of the pea preparation (1470 units/mg protein) appears to be higher than that of the crystalline yeast (390 at 25° (ref. 9), 675 at 30° (ref. 12)) and muscle enzyme (544 at 30° (ref. 11)).

The enzyme from various sources has been found to be competitively inhibited by 6-phosphogluconate. Recently, DAVIS, KULL AND GANDER[25] reported that the yeast enzyme was not inhibited by 6-phosphogluconate at high concentration of Glc-6-*P*, but was inhibited at low concentration. In our pea preparations, 6-phosphogluconate was a potent competitive inhibitor when the Glc-6-*P* concentration was less than $8 \cdot 10^{-3}$ M.

Only limited data are available concerning with the properties of pure glucosephosphate isomerase. NOLTMANN AND BRUNS[16] have reported some properties of the yeast preparation, which was homogeneous by the criterion of ultracentrifugation. Comparing the properties of the pea enzyme with those of the yeast enzyme, the pea enzyme has a lower molecular weight but appears to be similar in pH optimum,

K_m for Glc-6-*P* and the lack of an –SH group at its active center. From its differing behaviour in the purification procedure, however, the pea enzyme exhibits slightly different characteristics to the muscle[11] and yeast[12] enzymes in its fractionation and crystallization with ammonium sulfate or organic solvents and the treatment with zinc acetate.

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