

Triosephosphate Isomerase of Pea Seeds^{1, 2}

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Triosephosphate isomerase catalyzes the reversible transformation of D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. This enzyme, together with aldolase was shown by Meyerhof and Kiessling (9) to form the zymohexase system of rabbit muscle and yeast extracts (10).

Isomerase activity has been found in a number of animal tissues and yeast by Oesper and Meyerhof (11). Highly purified preparations have been obtained from rabbit muscle (7) and the enzyme may be crystallized from calf muscle extracts (1, 6). The properties and kinetics of the yeast and animal enzymes have been investigated (6, 7, 8, 11).

There are no reports of studies of isomerase from higher plants although results of previous work indicated that the enzyme was present in extracts of pea seeds (4, 13, 14, 15) and of pea roots (2). In the present investigation, a partially purified preparation of triosephosphate isomerase was obtained from pea seeds, and some properties of the enzyme were examined.

Materials and Methods

DL-Glyceraldehyde-3-Phosphate. This was prepared from DL-glyceraldehyde-3-phosphate diethylacetal, barium salt (Sigma Chemical Company) by heating 40 mg with 0.72 ml of 0.225 M H₂SO₄ in a boiling water bath for 3 minutes and cooling in ice. The precipitated barium sulfate was centrifuged and washed, and the supernatant fraction and washings were adjusted to pH 2.8 with 0.2 N NaOH and diluted to 8 ml. The solution was freshly prepared each week and stored at -15°.

At the maximum concentration of D-glyceraldehyde-3-phosphate used (final conc 0.27 mM), the sulfate concentration (< 0.35 mM) had no detectable inhibitory effect on triosephosphate isomerase activity.

Dihydroxyacetone Phosphate. Approximately 5 mg of the cyclohexylamine salt of dihydroxyacetone phosphate dimethyl ketal monohydrate (Sigma Chemical Company) was dissolved in 0.4 ml of water, and 0.1 g (wet wt) of Dowex 50 (H⁺) resin was added. The mixture was swirled for 30 seconds, filtered, and the resin washed with small amounts of water. The combined filtrates were maintained at 38° to 40° for 4 hours to hydrolyze the ketal, then adjusted to pH 4.5 with KHCO₃, diluted to 5 ml and stored at -15°.

Glyceraldehyde-3-Phosphate Dehydrogenase. This enzyme was obtained from C. F. Boehringer und Soehne, Mannheim, Germany.

α-Glycerophosphate Dehydrogenase. Ammonium sulfate suspensions of this enzyme were obtained from C. F. Boehringer und Soehne. Diluted aliquots were dialyzed, with rocking, against glass distilled water for 3 hours at 4° to remove the ammonium sulfate.

Buffers. Tris buffers (30 mM and 0.3 M with respect to Tris) were adjusted to the required pH with HCl. Tris-acetic acid buffers (5 mM and 30 mM with respect to both Tris and acetic acid) were adjusted to the required pH with NaOH.

Spectrophotometry. OD was measured in a Beckman model DU spectrophotometer. All assays were carried out at 23° in cuvettes of 1 cm light path.

Protein Estimation. The method of Warburg and Christian (16) was used.

Estimation of D-Glyceraldehyde-3-Phosphate. The reduction of NAD resulting from D-glyceraldehyde-3-phosphate oxidation in the presence of glyceraldehyde-3-phosphate dehydrogenase was determined spectrophotometrically (3). The increase in OD 340 mμ was measured in reaction mixtures of the following composition: 300 μmoles of Tris, pH 8.3, 17 μmoles of sodium arsenate, 12 μmoles of cysteine, 1.2 μmole of NAD, 0.1 unit of glyceraldehyde-3-phosphate dehydrogenase (1 unit = 1 μmole of substrate per min), approximately 0.4 μmole of DL-glyceraldehyde-3-phosphate, final volume 3.0 ml. The reaction was started by the addition of the substrate. No substrate was added to the control cuvette.

Assay of Glyceraldehyde-3-Phosphate Dehydrogenase Activity. For this assay, the reaction mixtures described for the estimation of D-glyceraldehyde-3-phosphate were used, except that glyceraldehyde-3-phosphate dehydrogenase was replaced by the pea seed triosephosphate isomerase preparation.

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Assay of α -Glycerophosphate Dehydrogenase Activity. With dihydroxyacetone phosphate as substrate, the method used was essentially that described below for the coupling system in the assay of triosephosphate isomerase. The decrease in OD 340 $m\mu$ was measured in the following reaction mixture: 60 μ moles of Tris-acetic acid buffer, pH 8.0, approximately 0.2 unit of α -glycerophosphate dehydrogenase (1 unit = 1 μ mole of substrate per min), 0.6 μ mole of NADH, 0.4 μ mole of dihydroxyacetone phosphate, final volume 3.0 ml.

Assay of Triosephosphate Isomerase Activity. The method described in this paper is essentially the same as the assays of Beisenherz (1) and Gibbs and Turner (3), and is based on the optical assay for aldolase (12). With D-glyceraldehyde-3-phosphate as substrate and α -glycerophosphate dehydrogenase as the coupling enzyme the oxidation of NADH by dihydroxyacetone phosphate was followed by measuring the decrease in OD 340 $m\mu$.

The standard reaction mixture contained, in 3 ml: 60 μ moles of Tris-acetic acid buffer, pH 7.2, 2.0 units of α -glycerophosphate dehydrogenase, 0.4 μ mole of NADH, 0.1 ml of pea seed triosephosphate isomerase preparation (containing approximately 0.004 mg of protein), and 0.8 μ mole of DL-glyceraldehyde-3-phosphate. The reaction was started by the addition of the substrate and the OD determined at intervals for 3 minutes. Dilutions of the triosephosphate isomerase preparation were such that OD changes of 0.02 to 0.06 per minute were obtained. Under these experimental conditions, there was less than 15% conversion of D-glyceraldehyde-3-phosphate in 2 minutes and the rate of reaction was approximately constant during this incubation period. Enzyme activity has been expressed as μ moles of D-glyceraldehyde-3-phosphate utilized per reaction mixture per minute.

A large excess of α -glycerophosphate dehydrogenase was added under all conditions studied, e.g. at various pH values, in the presence of inhibitors, etc.

Preparation of Triosephosphate Isomerase from Pca Seeds. A crude extract of defatted pea-seed powder was prepared as described by Turner (15), except that toluene was omitted. Subsequent operations were carried out at 0° to 4°. The crude extract was centrifuged at 1000 g for 10 minutes and the supernatant further centrifuged at 24,000 g for 15 minutes. Saturated $(\text{NH}_4)_2\text{SO}_4$, pH 7.0, was added to the clear supernatant, and the fraction precipitating between 40 and 60% saturation was dissolved in 8 ml of 5 mM Tris-acetic acid buffer, pH 8.0, and dialyzed, with rocking, against the same buffer for 3 hours. A small amount of protein precipitated during dialysis and was removed by centrifugation.

The clear enzyme extract could be stored at -15° for 4 weeks with little (5%) loss of activity. Heating for 4 minutes at 50° resulted in 85% loss of triosephosphate isomerase activity.

The preparation contained approximately 32 mg

of protein per ml and was used for all the studies reported. Cold 5 mM Tris-acetic acid buffer, pH 8.0, was used to dilute the enzyme before use.

Preparations of Triosephosphate Isomerase from Other Plant Tissues. Soaked seeds of pea, broad bean, wheat and maize were grown in damp sand in a glass house, until the shoots were 5 to 8 cm high. The shoots and roots were removed and approximately 15 g fresh weight of each tissue was ground in a mortar with 15 ml of 0.04 M NaHCO_3 , squeezed through muslin and centrifuged at 24,000 g for 15 minutes. The supernatant fluid was then fractionated with $(\text{NH}_4)_2\text{SO}_4$ and dialyzed as described for the pea seed preparation.

Seeds of broad bean, wheat and maize were soaked overnight, and 50 g samples were blended with 50 ml of 0.04 M NaHCO_3 . The homogenates were treated as described above.

Results

Triosephosphate Isomerase Activity. Results demonstrating triosephosphate isomerase activity are presented in figure 1. The reaction mixture contained 0.064 mg of pea-seed protein. After 24 min-

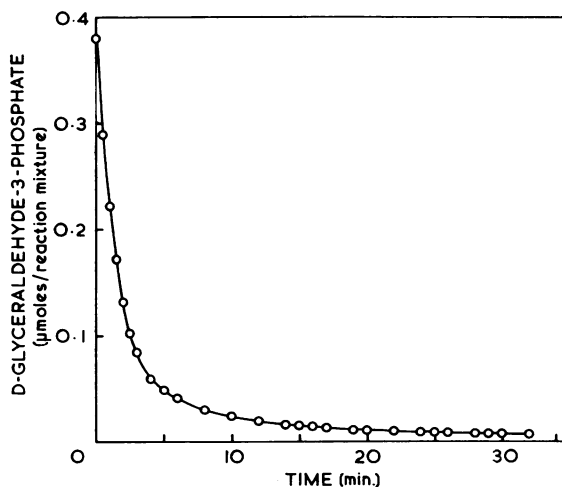


FIG. 1. Triosephosphate isomerase activity as measured by D-glyceraldehyde-3-phosphate loss. The reaction mixture (total vol. 3.0 ml) contained 60 μ moles of Tris-acetic acid buffer, pH 7.2, 2.0 units of α -glycerophosphate dehydrogenase, 0.6 μ mole of NADH, 0.2 ml of enzyme (containing 0.064 mg of protein), and 0.76 μ mole of DL-glyceraldehyde-3-phosphate. Temperature 23°.

utes 49% of the total DL-glyceraldehyde-3-phosphate had been transformed; in experiments of this type, 48 to 52% of added DL-glyceraldehyde-3-phosphate could be accounted for by the oxidation of NADH, as measured by the decrease in OD 340 $m\mu$. Similar reaction mixtures from which DL-glyceraldehyde-3-phosphate was omitted, or in which NADH was re-

placed by NAD showed no change in OD 340 $m\mu$ during the incubation period. In reaction mixtures containing 12.68 mg protein (i.e. 200 times the amount in fig 1) there was a very small decrease in OD 340 $m\mu$, when α -glycerophosphate dehydrogenase was omitted, with and without substrate.

Under standard conditions of assay for D-glyceraldehyde-3-phosphate dehydrogenase (i.e. in the presence of cysteine and arsenate) very slight activity of this enzyme was detected in reaction mixtures containing 0.024 mg protein of the pea seed preparation but no activity was detected when only 0.004 mg of protein was present.

It was therefore assumed throughout the study that, under the conditions of assay used, the pea seed triosephosphate isomerase preparation was specific for the D form of D-glyceraldehyde-3-phosphate as substrate and was free from detectable interfering enzyme activity.

Effect of pH on Triosephosphate Isomerase Activity. The activity of the enzyme in a series of 0.02 M Tris-acetic acid buffers, over the range pH 6.5 to pH 9.1 is shown in figure 2. There was a broad optimum from approximately pH 7.2 to pH

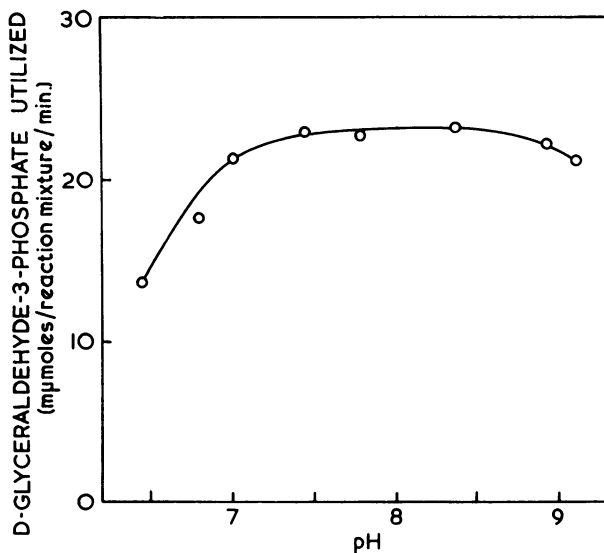


FIG. 2. Effect of pH on triosephosphate isomerase activity. Reaction mixtures were of the composition described for the assay of the enzyme. Reactions were studied in 0.02 M Tris-acetic acid buffers of the required pH. Temperature 23°.

8.9. In experiments in which the reaction was allowed to proceed for 15 minutes or longer, the activity of the enzyme in reaction mixtures at pH 7.0 was greater than at pH 8.0 and pH 9.0. This was probably due to the destruction of the substrate at the higher pH values, and to avoid this, pH 7.2 was chosen for the standard assay procedure.

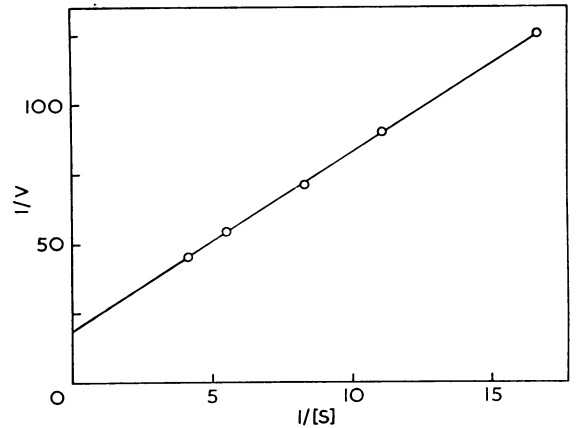


FIG. 3. Lineweaver-Burk plot of $1/V$ against $1/[S]$ for triosephosphate isomerase. V is the initial velocity (μ moles of D-glyceraldehyde-3-phosphate utilized/reaction mixture per min); $[S]$ is the substrate concentration (mmoles/liter). Reaction mixtures were of the composition described for the assay of triosephosphate isomerase activity, with the substrate concentration varied as shown. Temperature 23°.

In 0.01 M Tris-acetic acid buffer, the activity was the same as in 0.02 M buffer, but the activity in 0.05 M buffer was reduced by 10 to 15%. Activity of the enzyme in 0.02 M Tris (approximately 0.01 M with respect to HCl) pH 8.0 was only 80 to 85% of the activity in 0.02 M Tris-acetic acid buffer. It will be shown subsequently that chloride ions at this concentration inhibit the enzyme.

Effect of Substrate Concentration. The effect of concentration of D-glyceraldehyde-3-phosphate on the initial reaction velocity is shown in figure 3 in a Lineweaver-Burk (5) plot. The Michaelis-Menten constant (K_m) calculated from this plot was 3.6×10^{-4} M.

Effect of Anions. The inhibitory effect on triosephosphate isomerase activity of the addition of various salts is shown in table I. Both the sulfates and

Table I. Effect of Anions on Triosephosphate Isomerase Activity

Reaction mixtures were of the composition described for the assay of the enzyme, with the addition of salts as shown. Temperature 23°. The results are expressed as percent inhibition.

Addition	Final conc (M)		
	0.025	0.005	0.001
$(NH_4)_2SO_4$...	45	6
Na_2SO_4	...	43	6
K_2SO_4	...	43	7
Li_2SO_4	...	47	...
NH_4Cl	23	5	...
$NaCl$	17	3	...
KCl	14	4	...

chlorides of monovalent cations inhibited the pea-seed triosephosphate isomerase and the sulfates produced stronger inhibition of the enzyme than the chlorides. The nature of the monovalent cation had little effect. The salts of divalent cations also inhibited the enzyme. In separate experiments using reaction mixtures of the composition described for the assay of triosephosphate isomerase activity, the addition of sodium acetate (final conc 0.02 M) effected a 4% inhibition of activity, whereas 0.01 M magnesium acetate inhibited by 25%.

Effect of Inhibitors. The effect of several enzyme inhibitors on triosephosphate isomerase activity is shown in table II. Phosphate, arsenate and molybdate inhibited activity markedly, but fluoride had no effect. Iodoacetate effected little inhibition, although *p*-chloromercuribenzoate (0.1 mM) reduced activity by 77%. The enzyme was sensitive to Cu^{2+} ions, and to cysteine.

Table II. *Effect of Enzyme Inhibitors on Triosephosphate Isomerase Activity*

Reaction mixtures were of the composition described for the assay of the enzyme with the addition of inhibitors as shown. Temperature 23°.

Inhibitor	Final conc (mM)	Inhibition (%)
Sodium arsenate	5	44
Potassium dihydrogen phosphate	20	70
Ammonium molybdate	0.5	43
Ammonium fluoride	10	0
<i>p</i> -Chloromercuribenzoate	0.1	77
Iodoacetate	10	5
Cysteine	4	18
Cupric sulfate	0.25	69

Distribution in Plant Tissues. Triosephosphate isomerase activity was detected in extracts from all the plant tissues examined. Extracts were prepared from broadbean, wheat and maize seeds, the shoots and roots of pea, broadbean, wheat and maize seedlings, and mature leaves of pea plants.

Discussion

This investigation has established that active preparations of triosephosphate isomerase may be readily obtained from pea seeds.

Some of the properties of the triosephosphate isomerase from pea seeds were similar to those of the enzyme in extracts from animal tissues. Oesper and Meyerhof (11) state that the activity of the enzyme was the same at pH 7 as at pH 8, but decreased 50% at pH 6.3. The pea-seed enzyme showed a wide optimum from pH 7.2 to pH 8.9 and there was a decline in enzyme activity below pH 7.0. The Michaelis-Menten constant of the pea-seed triosephosphate isomerase was 3.6×10^{-4} M and this agrees well with

the figure of 3.9×10^{-4} M obtained for triosephosphate isomerase from calf muscle (6). Triosephosphate isomerase from rabbit muscle was inhibited 75% by 0.05 M phosphate (11) whereas the pea-seed enzyme was inhibited 60% by 0.02 M phosphate. Arsenate and cysteine inhibited the pea-seed enzyme; this was not due to any effect on glyceraldehyde-3-phosphate dehydrogenase as the diluted preparation showed no activity when assayed for this enzyme. The inhibition by *p*-chloromercuribenzoate indicated the presence of thiol groups essential for enzyme activity in the pea-seed triosephosphate isomerase.

An unexpected phenomenon was the pronounced inhibition of the pea-seed triosephosphate isomerase by relatively low concentrations of sulfate ions. Chloride ions also inhibited but a higher concentration was required. There are no reports of similar effects of these ions on the muscle or yeast isomerase enzymes. Inhibition of enzymes by sulfates and chlorides is unusual.

The enzyme was found in all plant tissues examined as would be expected from the important role of triosephosphate isomerase in the major pathways of carbohydrate metabolism including glycolysis, the pentose phosphate pathway and photosynthesis. The enzyme preparations from pea seeds were very active and the standard reaction mixture contained only 0.004 mg protein of the partially purified triosephosphate isomerase preparation. Insofar as glycolysis was concerned there was a great excess of triosephosphate isomerase in the pea seed extract and it can be calculated that the potential triosephosphate isomerase activity in these extracts is over 7000 times the maximum overall rate of glycolysis observed in similar extracts (4). The triosephosphate isomerase activity in several animal tissues and yeast was found always to be about 40 to 200 times in excess over aldolase (11).

The inhibition by P_i is interesting especially as the subsequent step in the glycolysis sequence (glyceraldehyde-3-phosphate dehydrogenase) has a requirement for P_i . This inhibition by phosphate could be of metabolic significance in glycolysis in minimizing the diversion of glyceraldehyde-3-phosphate formed by aldolase into dihydroxyacetone phosphate.

Summary

An active preparation of triosephosphate isomerase was obtained from pea seeds. All other plant tissue extracts examined showed triosephosphate isomerase activity.

The enzyme had a pH optimum of 7.2 to 8.9. With the experimental conditions used, the Michaelis-Menten constant for D-glyceraldehyde-3-phosphate was 3.6×10^{-4} M.

Sulfate ions, and to a lesser extent chloride ions, inhibited the enzyme. Inorganic phosphate, arsenate, *p*-chloromercuribenzoate and Cu^{2+} ions also reduced enzyme activity.

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