Phytochemistry, 1979, Vol. 18, pp. 1959–1962. © Pergamon Press Ltd. Printed in England. 0031-9422/79/1201-1959 $02.00/0

PEA SEED TRIOSE PHOSPHATE ISOMERASE

J. DAVID TOMLINSON and JOHN F. TURNER
Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006, Australia

(*Received* 11 *April* 1979)

Key Word Index—*Pisum sativum*; Leguminosae; pea; triose phosphate isomerase; P-enolpyruvate; 2-P-glycerate; 3-P-glycerate; 2-P-glycolate; Pi.

Abstract—Triose phosphate isomerase was purified *ca* 250-fold from pea seed extracts. The K_m for D-glyceraldehyde-3-P was 0.44 mM and for dihydroxyacetone-P, 0.88 mM. P-Enolpyruvate, 2-P-glycerate, 3-P-glycerate and 2-P-glycolate were strongly inhibitory. Pi and arsenate also inhibited pea seed triose phosphate isomerase.

INTRODUCTION

Triose phosphate isomerase (D-glyceraldehyde-3-phosphate ketol isomerase, EC 5.3.1.1) catalyses the reversible isomerization of D-glyceraldehyde-3-P and dihydroxyacetone-P. The enzyme is involved in glycolysis, gluconeogenesis and the photosynthetic process.

Triose phosphate isomerase activity was first characterized by Meyerhof and Kiessling [1] in calf muscle extracts and subsequently the enzyme from several animal tissues and yeast was purified and crystallized [2–5]. Triose phosphate isomerase activity was demonstrated in extracts from a variety of higher plant tissues by Turner *et al.* [6]. The enzyme from pea seeds was partially purified and was inhibited by Pi and a number of other anions including SO_4^{2-} and Cl^- [6]. Anderson [7] found different isoenzyme forms of triose phosphate isomerase in the chloroplasts and cytoplasm of pea leaves, and reported inhibition by several metabolites.

In the present investigation pea seed triose phosphate isomerase was purified and a preparation of high specific activity obtained. The enzyme was subject to strong competitive inhibition by P-enolpyruvate, the P-glycerates and 2-P-glycolate.

RESULTS

Effect of concentration of substrates

Preliminary experiments showed that pea seed triose phosphate isomerase utilized D-glyceraldehyde-3-P as substrate and was inactive with L-glyceraldehyde-3-P. Addition of L-glyceraldehyde-3-P did not affect the kinetics with D-glyceraldehyde-3-P. Purified pea seed triose phosphate isomerase showed hyperbolic kinetics with D-glyceraldehyde-3-P (added as the racemic DL mixture) and with dihydroxyacetone-P. The K_m for D-glyceraldehyde-3-P was 0.44 mM. Lineweaver–Burk plots gave an apparent K_m for dihydroxyacetone-P and this was corrected for the inhibitory effect of arsenate (K_i = 3.0 mM) used in the enzyme assay system with dihydroxyacetone-P as substrate [4, 8]. The corrected K_m for dihydroxyacetone-P was 0.88 mM.

Effect of pH

The enzyme showed a broad optimum from pH 6.8 to 8.8 when assayed in a series of Tris–acetate and acetate buffers. To allow for the sharp pH optimum of α-glycerophosphate dehydrogenase [9], ten times the usual amount of this enzyme was used in the assay system in these experiments.

Inhibition by anions

It was shown previously that chlorides and sulphates of monovalent cations inhibited partially purified pea seed triose phosphate isomerase [6]. Similar results were obtained with the purified pea seed enzyme (Table 1). There was increasing inhibition in the halide series fluoride, chloride, bromide and iodide suggesting that the extent of inhibition may be related to ionic size. Sulphate gave greater inhibition than the halides. Pi and arsenate were stronger inhibitors than sulphate.

Effect of P-*enolpyruvate*

The inhibition of pea seed triose phosphate isomerase by increasing concentrations of P-enolpyruvate is shown in Fig. 1: an inhibition of 50% was obtained with 0.2 μM

Table 1. Inhibition of triose phosphate isomerase by anions

Anion added	Final concn (mM) 1	5	10	25
F^-	0	0	2	8
Cl^-	0	3	7	14
Br^-	0	5	10	22
I^-	2	6	13	28
SO_4^{2-}	7	25	44	63
Pi	11	45	97	—
Arsenate	12	48	98	—

The reaction mixtures were of the composition described for the standard assay with Na^+ salts of anions added as shown. Pi and arsenate (pH 7.2) were added as mixed Na^+/K^+ acid salts. Results are expressed as % inhibition.

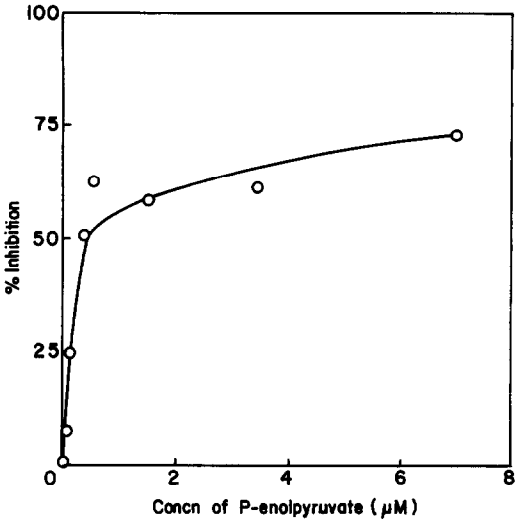

Fig. 1. Effect of P-enolpyruvate on the activity of pea seed triose phosphate isomerase. The reaction mixtures were of the composition described for the standard assay with the addition of P-enolpyruvate as shown.

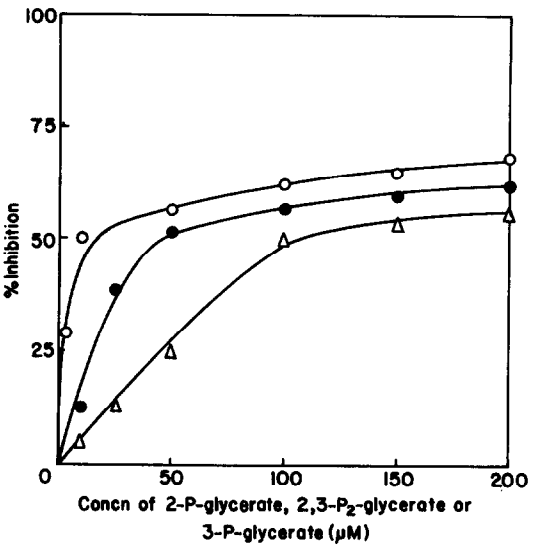

Fig. 2. Effect of 2-P-glycerate, 2,3-P_2-glycerate and 3-P-glycerate on the activity of pea seed triose phosphate isomerase. The reaction mixtures were of the composition described for the standard assay with the addition of 2-P-glycerate, 2,3-P_2-glycerate or 3-P-glycerate as shown. ○, 2-P-Glycerate; ●, 2,3-P_2-glycerate; △, 3-P-glycerate.

P-enolpyruvate. With a P-enolpyruvate concentration of 6.7 μM, the inhibition was *ca* 70%. Inverse and Dixon plots [10] at concentrations of P-enolpyruvate less than 0.4 μM showed that the inhibition was competitive with a K_i *ca* 0.1 μM. Enzyme activity was not inhibited by the addition of P-enolpyruvate preparations which had been hydrolysed in M HCl at 100° for 40 min. It was shown that the association between P-enolpyruvate and pea seed triose phosphate isomerase was reversible. A concentration (15 mM) of P-enolpyruvate which would completely inhibit enzyme activity was added to a pea seed triose phosphate isomerase preparation. After dialysis for 4 hr against 0.05 M Tris–acetate buffer, pH 8.0, 97% of the original enzyme activity was recovered.

P-Enolpyruvate protected pea seed triose phosphate isomerase from heat inactivation. When enzyme preparations were incubated with 1 mM P-enolpyruvate at 67° for 5 min and then diluted and dialysed to remove P-enolpyruvate, over 96% of the enzyme activity remained. In controls heated in the absence of P-enolpyruvate the enzyme was completely inactivated within 5 min.

Triose phosphate isomerases from animal tissues and yeast are inactivated by low concentrations of haloacetol phosphates [11–14]. Incubation of pea seed triose phosphate isomerase with 0.4 μM chloroacetol-P (1-hydroxy, 3-chloro, 2-propanone-P) for 10 min at 30° led to 90% loss of enzyme activity. The addition of P-enolpyruvate (final concn 1.2 μM) partially protected pea seed triose phosphate isomerase from inactivation and reduced the loss of enzyme activity from 90 to 60%.

Effect of 2-P-*glycerate*, 2,3-P_2-*glycerate and* 3-P-*glycerate*

Relatively low concentrations of 2-P-glycerate, 2,3-P_2-glycerate and 3-P-glycerate inhibited pea seed triose phosphate isomerase (Fig. 2). The concentrations of 2-P-glycerate, 2,3-P_2-glycerate and 3-P-glycerate required for 50% inhibition of enzyme activity were 10, 40 and 100 μM, respectively. The apparent K_i values (from Dixon plots of concentrations giving less than 50% inhibition) were, for 2-P-glycerate and 3-P-glycerate, *ca* 6 and 50 μM, respectively. Inhibition by the P-glycerates was not due to the formation of P-enolpyruvate in the reaction mixtures. Phosphoglycerate mutase and enolase could not be detected in the triose phosphate isomerase enzyme preparation. The addition of enolase or enolase *plus* phosphoglycerate mutase to enzyme assay mixtures containing 1 μM 2-P-glycerate or 3-P-glycerate, respectively, resulted in a rapid reduction of reaction velocity.

Effect of 2-P-*glycolate*

Pea seed triose phosphate isomerase was strongly inhibited by 2-P-glycolate (K_i = 0.6 μM). 2-P-Glycolate also protected enzyme preparations from inactivation by heat and chloroacetol-P in experiments similar to those previously described for P-enolpyruvate. Rabbit muscle triose phosphate isomerase was competitively inhibited by μmolar concentrations of 2-P-glycolate [15, 16]. Both chloroplast and cytoplasmic pea leaf triose phosphate isomerases were inhibited by 2-P-glycolate, the K_i values being 15 and 4 μM, respectively [7].

Inhibition by other metabolites

Pea seed triose phosphate isomerase was inhibited by a number of other metabolites. Under the standard assay conditions and with a final metabolite concentration of 5 mM, the following inhibitions were obtained: 6-P-gluconate (9%), glucose-1-P (10%), glucose-6-P (25%), fructose-6-P (44%), fructose-1,6-P_2 (69%), citrate (25%), succinate (9%) and ATP (39%).

DISCUSSION

Triose phosphate isomerase is one of the most active enzymes known and the molecular activity of purified preparations is at least 500000 mol of glyceraldehyde-3-P transformed per min per mol of enzyme [17]. The

specific activity of the pea seed triose phosphate isomerase obtained in the present investigation (11 975 units/mg protein) is comparable with the highest values for crystalline preparations from other sources. Putman *et al.* [18] reported a value of 11 200 units/mg for the chicken muscle enzyme under assay conditions similar to those used in the present study and the triose phosphate isomerase from yeast has a specific activity of 10 000 units/mg [4]. The specific activities of other of triose homogeneous or crystalline preparations phosphate isomerase from mammalian liver [4, 19] or muscle [20, 21] have been lower and in the range 2400–7800 units/mg.

The Michaelis–Menten constant of purified pea seed triose phosphate isomerase for D-glyceraldehyde-3-P was 0.44 mM which is close to the value of 0.36 mM previously reported for the partially purified enzyme [6] and also to the values of 0.32–0.47 mM found for the enzyme from animal tissues [2, 4, 8, 18, 19]. The K_m for D-glyceraldehyde-3-P of the brewer's yeast enzyme is higher (1.27 mM) [4]. Anderson [7] found that the K_m for D-glyceraldehyde-3-P was 0.42 mM for pea leaf chloroplast triose phosphate isomerase and 0.2 mM for the cytoplasmic enzyme. The K_m of pea seed triose phosphate isomerase for dihydroxyacetone-P was 0.88 mM and this may be compared to values of 0.58–0.97 mM for triose phosphate isomerase from animal tissues [4, 8, 18, 19]. The K_m for dihydroxyacetone-P of the yeast enzyme was again higher (1.23 mM) [4]. The values found for the pea leaf chloroplast and cytoplasmic enzymes were 1.1 and 0.39 mM, respectively [7].

A feature of plant triose phosphate isomerase is the competitive inhibition given by P-enolpyruvate and the P-glycerates. The pea seed enzyme was particularly sensitive to inhibition by P-enolpyruvate (K_i = 0.1 μM), and 2-P-glycerate (K_i = 6 μM) and 3-P-glycerate (K_i = 50 μM) were also strong inhibitors. P-Enolpyruvate also protected the enzyme from inactivation by heat and chloroacetol-P. Both chloroplast and cytoplasmic pea leaf triose phosphate isomerases were inhibited by P-enolpyruvate and the P-glycerates [7], although the enzymes were less sensitive than pea seed triose phosphate isomerase.

Triose phosphate isomerase activity is very high in those plant tissues which have been examined [6]. In pea seed extracts triose phosphate isomerase activity is greatly in excess of the maximum rate of glycolysis observed in similar preparations [6, 22]. The high enzyme activity could suggest that there should be rapid equilibration between the triose phosphates *in vivo*. Although the triose phosphate isomerase step is considered to be an equilibrium reaction in plant tissues [23], it is possible that there may be disequilibrium between the two triose phosphates *in vivo*. Gibbs and Kandler [24] found asymmetric distribution of labelling of sugars produced in *Chlorella* and leaves during photosynthesis in $^{14}CO_2$. Non-equilibration of glyceraldehyde-3-P and dihydroxyacetone-P because of concerted inhibition of triose phosphate isomerase by photosynthetic intermediates may provide an explanation for this observation [7]. It can be calculated from the data of Barker *et al.* [25] that the overall concentration of P-enolpyruvate in fresh pea seeds is *ca* 0.1 mM, a level which would substantially inhibit triose phosphate isomerase. Similarly, the concentrations of 2-P-glycerate and 3-P-glycerate in peas may be *ca* 0.03 and 0.4 mM, respectively, levels which could be strongly inhibitory. The concentration of Pi in peas is 11–14 mM [26] and this may also lead to significant inhibition of triose phosphate isomerase. The distribution of labelled carbon in glycogen synthesized from glycerol in rat liver has provided evidence for the non-equilibration of triose phosphate pools in this tissue [27, 28]. Veech *et al.* [29] also concluded that there was an apparent deviation of the triose phosphate isomerase system from equilibrium in the rat liver.

There was evidence for the presence of isoenzymes of triose phosphate isomerase in the purified pea seed preparation. The enzyme activity profile on elution from Sephadex G-200 was not symmetrical and there were indications of two components. The inhibition curves for P-enolpyruvate (Fig. 1) and 2-P-glycerate and 3-P-glycerate (Fig. 2) were biphasic suggesting that one component was more sensitive to inhibition than the other. Similar results were obtained with 2-P-glycolate.

EXPERIMENTAL

Preparation of D-*glyceraldehyde*-3-P. Fructose-1,6-P_2 was incubated with aldolase in the presence of a large excess of acetaldehyde [30]. The D-glyceraldehyde-3-P in the mixture was separated by gradient elution on a Bio-Rad AGl-X8 formate column (1 × 15 cm) using 50 ml H_2O in the mixing vessel and 50 ml 0.5 M NH_4 formate–0.2 M formic acid in the reservoir.

Preparation of L-*glyceraldehyde*-3-P. D-Glyceraldehyde-3-P in DL-glyceraldehyde-3-P was converted to 3-P-glycerate by incubation with glyceraldehyde-3-phosphate dehydrogenase in the presence of arsenate. The L-glyceraldehyde-3-P which remained was separated from the reaction mixture by the method described for the D-isomer.

Purification of pea seed triose phosphate isomerase. Pea seeds (*Pisum sativum* L. cv Progress No. 9) were finely ground and the powder extracted with Et_2O [31]. The defatted powder was further extracted with 5 vol. of Me_2CO at −20° and dried at room temp. All subsequent procedures were carried out at 2° unless otherwise stated. Me_2CO extracted powder (1 kg) was rolled on a mechanical roller for 2.5 hr in 4 l. of 50 mM Tris–HCl buffer (pH 8) containing 10 mM EDTA. After centrifuging at 23 000 *g* for 30 min, solid $(NH_4)_2SO_4$ was added to 50% satn, the pH adjusted to 7.6 with NH_4OH, and the mixture again centrifuged at 23 000 *g*. $(NH_4)_2SO_4$ was added to 65% satn, the mixture centrifuged as before and the ppt. dissolved in the minimum vol. of 10 mM Tris–HCl (pH 7.5) containing 1 mM EDTA (buffer A). After dialysis against buffer A and centrifuging, the prepn was placed on DEAE-cellulose column (3.5 × 55 cm) previously equilibrated with buffer A, pH 8.5. Triose phosphate isomerase was eluted with a 0–0.2 M KCl gradient in buffer A, pH 8.5. Tubes containing activity were pooled and treated with $(NH_4)_2SO_4$. The fraction precipitating between 45 and 70% satn was taken up in a minimum vol. of buffer A, pH 7.5, dialysed and concentrated by dialysis against 30% (w/v) polyethylene glycol in the same buffer until the vol. was 8 ml. The concentrate was then placed on a Sephadex G-200 column (1.6 × 66 cm) equilibrated with buffer A, pH 7.5, and eluted with the same buffer. Fractions containing enzyme activity were combined and used for the expts described in this communication. The sp. act. after Sephadex G-200 chromatography was 11 975 units/mg protein (Table 2). This represented *ca* 250-fold purification from the crude extract. One unit of activity is defined as 1 μmol dihydroxyacetone-P produced per min under the standard assay conditions.

Table 2. Purification of pea seed triose phosphate isomerase

Stage	Total activity (units × 10^{-3})	Specific activity (units/mg protein)	Purification
Crude extract	813	48	—
First $(NH_4)_2SO_4$ treatment	548	417	8.7
DEAE-cellulose chromatography	183	2460	51.3
Second $(NH_4)_2SO_4$ treatment	149	2995	62.4
Sephadex G-200 chromatography	96	11975	249

Assay of triose phosphate isomerase activity. Enzyme activity was normally assayed by coupling the production of dihydroxyacetone-P with the oxidation of NADH through α-glycerophosphate dehydrogenase [6]. The standard assay mixtures (total vol. 3 ml) contained 60 μmol Tris–acetate buffer, pH 7.2, 0.2 μmol NADH, 10 μg dialysed α-glycerophosphate dehydrogenase and 0.1 ml (containing *ca* 10 ng protein) of the enzyme prepn diluted with 5 mM Tris–acetate buffer, pH 7.5. After equilibration at 30° for 8 min, the reaction was started by the addition of DL-glyceraldehyde-3-P containing 4 μmol D-glyceraldehyde-3-P and the decrease in *A* at 340 nm followed. For the determination of triose phosphate isomerase activity with dihydroxyacetone-P as substrate, reaction mixtures contained in a final vol. of 3 ml, 60 μmol Tris–acetate buffer (pH 7.2), 0.24 μmol NAD^+, 6 μmol Na arsenate, 0.1 mg dialysed glyceraldehyde-3-phosphate dehydrogenase and pea seed triose phosphate isomerase prepn equivalent to 50 ng protein. The reaction was initiated by the addition of 8 μmol dihydroxyacetone-P and the increase in *A* at 340 nm followed. No change in reaction velocity was obtained with a 10-fold increase in the amount of either coupling enzyme. Aldolase, glyceraldehyde-3-phosphate dehydrogenase, α-glycerophosphate dehydrogenase, phosphatase and NADH oxidase were not detected in the purified pea seed triose phosphate isomerase. The enzyme prepn lost 5% of activity per week during storage at 2°.

Acknowledgements—One of us (J. D. T.) was the holder of a Commonwealth Research Studentship. This investigation was supported by the University of Sydney Research Grant.

REFERENCES

1. Meyerhof, O. and Kiessling, W. (1935) *Biochem. Z.* **279**, 40.
2. Meyer-Arendt, E., Beisenherz, G. and Bucher, Th. (1953) *Naturwissenschaften* **40**, 59.
3. Czok, R. and Bucher, Th. (1960) *Adv. Protein Chem.* **15**, 315.
4. Krietsch, W. K. G., Pentchev, P. G., Klingenburg, H., Hofstatter, T. and Bucher, Th. (1970) *Eur. J. Biochem.* **14**, 289.
5. McVittie, J. D., Esnouf, M. P. and Peacocke, A. (1972) *Eur. J. Biochem.* **29**, 67.
6. Turner, D. H., Blanch, E. S., Gibbs, M. and Turner, J. F. (1965) *Plant Physiol.* **40**, 1146.
7. Anderson, L. E. (1971) *Biochim. Biophys. Acta* **235**, 237.
8. Burton, P. M. and Waley, S. G. (1968) *Biochim. Biophys. Acta* **151**, 714.
9. Young, H. L. and Pace, N. (1958) *Arch. Biochem. Biophys.* **75**, 125.
10. Dixon, M. (1953) *Biochem. J.* **55**, 170.
11. Hartman, F. C. (1968) *Biochem. Biophys. Res. Commun.* **33**, 888.
12. Coulson, A. F. W., Knowles, J. R. and Offord, R. E. (1970) *Chem. Commun.* **1**, 7.
13. Coulson, A. F. W., Knowles, J. R., Priddle, J. D. and Offord, R. E. (1970) *Nature* **227**, 180.
14. Norton, I. L. and Hartman, F. C. (1972) *Biochemistry* **11**, 4435.
15. Wolfenden, R. (1969) *Nature* **223**, 704.
16. Wolfenden, R. (1970) *Biochemistry* **9**, 3404.
17. Burton, P. M. and Waley, S. G. (1966) *Biochem. J.* **100**, 702.
18. Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleton, B. and Knowles, J. R. (1972) *Biochem. J.* **129**, 301.
19. Lee, E. W., Barriso, J. A., Pepe, M. and Snyder, R. (1971) *Biochim. Biophys. Acta* **242**, 261.
20. Norton, I. L., Pfuderer, P., Stringer, C. D. and Hartman, F. C. (1970) *Biochemistry* **9**, 4952.
21. Krietsch, W. K. G., Pentchev, P. G. and Klingenburg, H. (1971) *Eur. J. Biochem.* **23**, 77.
22. Hatch, M. D. and Turner, J. F. (1958) *Biochem. J.* **69**, 495.
23. Turner, J. F. and Turner, D. H. (1979) *The Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds.) Vol. 2, (in press). Academic Press, New York.
24. Gibbs, M. and Kandler, O. (1957) *Proc. Natl. Acad. Sci. U.S.A.* **43**, 446.
25. Barker, J., Khan, M. A. A. and Solomos, T. (1967) *New Phytol.* **66**, 577.
26. Rowan, K. S. and Turner, D. H. (1957) *Aust. J. Biol. Sci.* **10**, 414.
27. Schambye, P., Wood, H. G. and Popjak, G. (1954) *J. Biol. Chem.* **206**, 875.
28. Rose, I. A., Kellermeyer, R., Stjernholm, R. and Wood, H. G. (1962) *J. Biol. Chem.* **237**, 3325.
29. Veech, R. L., Raijman, L., Dalziel, K. and Krebs, H. A. (1969) *Biochem. J.* **115**, 837.
30. Horecker, B. L. and Smyrniotis, P. Z. (1955) *J. Biol. Chem.* **212**, 811.
31. Turner, J. F. (1957) *Biochem. J.* **67**, 450.