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TRIOSEPHOSPHATE ISOMERASE FROM SELECTED CHLOROPHYTA

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Abstract—The triosephosphate isomerase systems from two algal species, *Ankistrodesmus Braunii* and *Scenedesmus acuminatus*, have been isolated and partially purified by a series of solvent and salt fractionations. The enzymes from *A. Braunii* and *S. acuminatus* were electrophoretically homogeneous with uncorrected mobilities of $-1{\cdot}2 \times 10^{-5}$ cm^2 V^{-1} sec^{-1} and $-1{\cdot}01 \times 10^{-5}$ cm^2 V^{-1} sec^{-1} respectively. For both systems the pH optimum was sharp at pH 7·7 in $2{\cdot}5 \times 10^{-5}$ M Tris-HCl buffer. Kinetic analysis of the isomerase reactions gave Michaelis constants (K_m) of $4{\cdot}34 \times 10^{-4}$ M and $9{\cdot}7 \times 10^{-4}$ M GAP respectively for the enzymes from *A. Braunii* and *S. acuminatus*. The algal isomerase systems were inhibited by sulfate, phosphate, and to a lesser degree, chloride ions.

INTRODUCTION

UNICELLULAR green algae have been used extensively for fundamental investigations of plant metabolism. The pathway of carbohydrate degradation in green algae appears to be similar to that found in higher plants. Crude enzyme preparations from a number of laboratories indicate activity for enzymes associated with the glycolytic pathway, the hexose monophosphate system and the tricarboxylic acid cycle.[1]

Richter reported the presence of triosephosphate isomerase, aldolase and glucose-6-phosphate dehydrogenase in a crude protein extract from a species of Chlorella.[2] He later followed his original work with a comparative study of the enzymes of two algal species, *Anacystis nidulans* and *Chlorella pyrenoidosa*.[3]

Triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol isomerase, EC 5.3.1.1.) catalyzes the reversible isomerization between glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). Since the discovery of the enzyme in 1934,[4] this reaction has been extensively investigated in animal systems;[5–9] however, few studies have been reported on triosephosphate isomerase in plants.

[1] G. JACOBI, in *Physiology and Biochemistry of Algae* (edited by R. A. LEWIS), p. 125, Academic Press, New York (1962).
[2] G. RICHTER, *Z. Naturforsch.* **12b**, 662 (1957).
[3] G. RICHTER, *Naturwissenschaften* **46**, 604 (1959).
[4] O. MEYERHOF and L. V. BECK, *J. Biol. Chem.* **156**, 109 (1944).
[5] G. BEISENHERZ, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 391, Academic Press, New York (1955).
[6] CZOK and T. BUCHER, in *Advances in Protein Chemistry* (edited by C. B. ANFINSEN, JR., M. L. ANSON, K. BAILEY and J. T. EDSALL), Vol. 15, p. 381, Academic Press, New York (1960).
[7] S. V. RIEDER and I. A. ROSE, *J. Biol. Chem.* **234**, 1007 (1958).
[8] R. SNYDER and E. W. LEE, *Arch. Biochem. Biophys.* **117**, 587 (1966).
[9] P. M. BURTON and S. G. WALEY, *Biochem. J.* **100**, 702 (1966).

It was the purpose of this study to isolate, purify and investigate some of the properties of triosephosphate isomerase from two unicellular green algae, *Ankistrodesmus Braunii* and *Scenedesmus acuminatus* (Chlorococcales).

RESULTS AND DISCUSSION

Purification of the Enzymes

The purifications of the triosephosphate isomerase systems from *Ankistrodesmus Braunii* and *Scenedesmus acuminatus* are summarized in Tables 1 and 2. The purification procedures,

TABLE 1. PURIFICATION OF TRIOSEPHOSPHATE ISOMERASE FROM *A. Braunii*

Fraction number	Volume in ml	mg protein per ml	Units per ml	Specific activity	Total activity*
1. Crude extract	540·0	42·00	47·6	1·1	25704·0
2. 30% acetone	850·0	1·56	40·0	25·6	34000·0
3. Ammonium sulfate					
37% supernatant	830·0	0·12	7·6	63·4	6316·0
50% precipitate	33·0	0·58	142·8	246·2	5712·0
4. Ammonium sulfate solution—					
75% precipitate	7·9	0·36	166·6	461·7	1316·0
5. Lyophilized		0·33	66·6	200·0	526·0

* Overall yield through fraction 4 was 3·8%.

TABLE 2. PURIFICATION OF TRIOSEPHOSPHATE ISOMERASE FROM *S. acuminatus*

Fraction number	Volume in ml	mg protein per ml	Units per ml	Specific activity	Total activity*
1. Crude extract	256·0	103·50	57·2	0·55	15159·0
2. 30% acetone	425·0	2·64	28·4	10·75	16320·0
3. Ammonium sulfate					
37% supernatant	460·0	0·24	18·1	75·40	8347·0
50% precipitate	25·0	1·65	153·8	94·40	3825·0
4. Ammonium sulfate solution—					
75% precipitate	16·0	0·53	90·9	173·10	1454·0
5. Lyophilized		0·27	28·5	105·55	800·0

* Overall yield through fraction 4 was 8·9%.

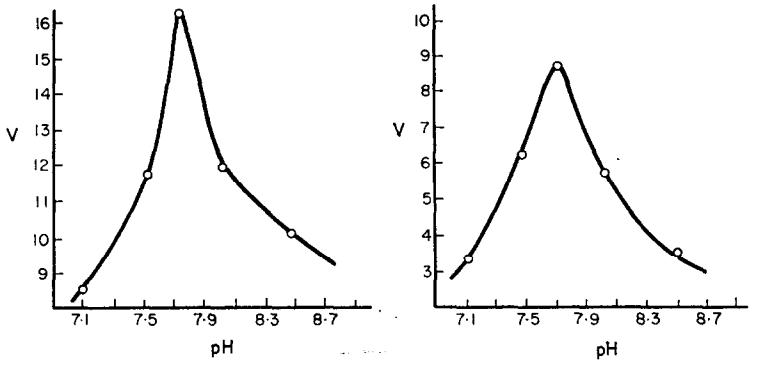
involving a series of acetone and ammonium sulfate fractionations, were similar to those outlined for the purification of calf muscle isomerase.[5]

Electrophoretic homogeneity of the lyophilized preparations was indicated for the isomerase from each species. The mobilities of the protein bands in barbital–sodium barbital buffer (0·05 ionic strength, pH 8·6) were $-1{\cdot}2\times10^{-5}$ cm^2 V^{-1} sec^{-1} and $-1{\cdot}01\times10^{-5}$ cm^2 V^{-1} sec^{-1} (uncorrected) respectively from *A. Braunii* and *S. acuminatus*.

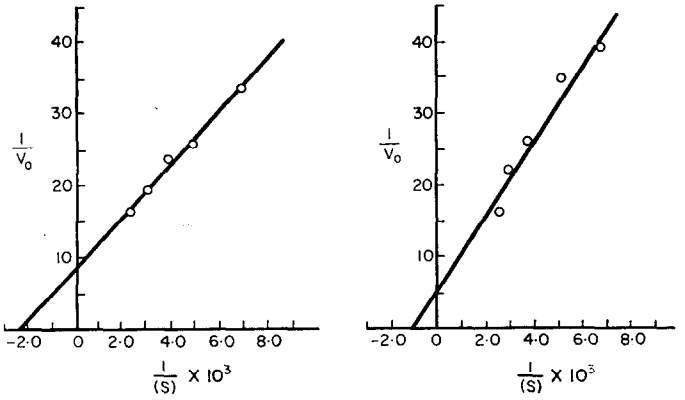
The level of purification attained for the isomerase systems from *A. Braunii* (460-fold) and *S. acuminatus* (313-fold) appears to be the highest, to date, obtained from any botanical species.

Effect of pH *on Enzyme Activity*

The activity of the isomerase in the coupled assay system, determined in 0·025 M Tris-HCl, attained a well defined maximum at pH 7·7 for the enzymes from both algal species (Fig. 1). This pH optimum differs from the broad range of pH 7·2–8·9 reported as optimum for the enzymes from calf muscle[5] and pea seed.[10]



FIGS. 1 AND 2. pH OPTIMA FOR TRIOSEPHOSPHATE ISOMERASE ACTIVITY FROM *Ankistrodesmus Braunii* (LEFT) AND *Scenedesmus acuminatus*.
Velocity is reported as micromoles of NADH oxidized per min × 10^{-2}.



FIGS. 3 AND 4. LINEWEAVER–BURKE PLOTS FOR THE ACTIVITY OF TRIOSEPHOSPHATE ISOMERASE FROM *Ankistrodesmus Braunii* (LEFT) AND *Scenedesmus acuminatus*.
Initial velocity is in micromoles NADH oxidized per min; (S) is the concentration of GAP in moles per l. × 10^{-4}. The intercepts, 8·54 and 5·6, and the slopes, 3·61 and 5·44 respectively, were calculated from the experimental data.

The buffer used in this study, Tris-HCl, differed from those cited in the literature for the reaction coupled with α-glycerophosphate dehydrogenase. Beisenherz,[5] Richter,[3] and Burton and Waley[9] used 0·02 M triethanolamine·HCl; Turner *et al.*[10] used 0·03 M Tris-HAc.

A large change in the pH of the Tris-HCl system was noted in enzyme assays at high concentrations of GAP (above $2{\cdot}7 \times 10^{-4}$ M). This pH change was minimized by increasing

[10] D. H. TURNER, E. S. BLANCH, M. GIBBS and J. F. TURNER, *Plant Physiol.* **40**, 1146 (1965).

the molarity of the Tris buffer to 0·05 M. The observed increase in activity of the enzyme at high substrate concentrations in 0·05 M Tris-HCl buffer appeared to be a function of the stabilized pH rather than the increased molarity of the buffer.

Effect of Substrate Concentration on Enzyme Activities

The determination of the effect of varying GAP concentrations on isomerase activity was carried out as described for the assay procedure using 0·05 M Tris-HCl buffer.

Initial velocities (V_0) are reported in micromoles of NADH* oxidized per minute. The Michaelis constants for the isomerase systems from *A. Braunii* and *S. acuminatus*, determined from Lineweaver–Burke plots[11], were $4{\cdot}34 \times 10^{-4}$ M GAP and $9{\cdot}7 \times 10^{-4}$ M GAP, respectively. These values are of the same magnitude as those reported for the isomerase systems from calf muscle ($3{\cdot}9 \times 10^{-4}$ M GAP)[5] and pea seed ($3{\cdot}6 \times 10^{-4}$ M GAP).[10]

Activation and Inhibition of Enzyme Activity

The inhibitory effects of various salts on the algal triosephosphate isomerase activity are summarized in Tables 3 and 4. Both sulfate and phosphate ions showed considerable

TABLE 3. ANION INHIBITION OF TRIOSEPHOSPHATE ISOMERASE FROM *A. Braunii*

Salt	Final concentration (M)		
	0·05	0·025	0·005
$(NH_4)_2SO_4$	81·7	70·0	41·7
Na_2SO_4	78·7	64·5	22·8
K_2HPO_4	78·7	58·4	29·5
NH_4Cl	33·0	3·0	

Reactions were run at 25°, pH 7·7, with $1{\cdot}08 \times 10^{-4}$ M GAP. Results are expressed as percent inhibition of control assay.

TABLE 4. ANION INHIBITION OF TRIOSEPHOSPHATE ISOMERASE FROM *S. acuminatus*

Salt	Final concentration (M)		
	0·05	0·025	0·005
$(NH_4)_2SO_4$	74·2	70·4	26·8
Na_2SO_4	72·6	63·8	19·8
K_2HPO_4	71·5	63·8	20·9
NH_4Cl	19·8	7·7	

Reaction conditions and results were as described in Table 3.

* Reduced form of nicotinamide adenine dinucleotide.

[11] H. LINEWEAVER and D. BURKE, *J. Am. Chem. Soc.* **56**, 658 (1934).

inhibition of the reaction. Inhibition to a lesser degree was observed with chloride ion. Calf muscle isomerase was inhibited 75 per cent by 0·05 M phosphate.[5] Pea seed isomerase was inhibited about 44 per cent by 0·005 M sulfate and 70 per cent by 0·02 M phosphate.[10] Inhibition of the algal isomerase reactions was observed in all purification procedures involving ammonium sulfate.

The inhibition was shown to affect the isomerase activity rather than the α-glycerophosphate dehydrogenase system by using the latter system in a separate reaction. DHAP, formed by the action of aldolase on fructose-1, 6-diphosphate, was transformed to L-α-glycerol phosphate by α-glycerophosphate dehydrogenase and NADH. The shift in the absorption spectrum of the coenzyme was the same in the presence and absence of the salts, indicating no inhibitory effect on the α-glycerophosphate system.

The active site of the triosephosphate isomerase from rabbit muscle is thought to involve the amino acid histidine.[9] Turner *et al.*[10] reported 77 per cent inhibition with 10^{-4} M *p*-chloromercuribenzoate, 18 per cent inhibition by 4×10^{-3} M cysteine and 5 per cent inhibition by iodoacetate for the pea seed isomerase. Czok and Bucher[6] reported that inactivated rabbit muscle isomerase was reactivated by incubation with reduced glutathione.

Inactivated algal isomerase was incubated up to 12 hr at 5° with 0·06 moles reduced glutathione and failed to show any activation. Similarly, no increase or inhibition of activity was noted when cysteine was included in the reaction mixture.

Snyder and Lee[8] studied the effect of a number of dicarboxylic acids on triosephosphate isomerase. Both horse liver isomerase and rabbit muscle isomerase were stimulated by dicarboxylic acids ranging from 2–10 carbons, including glutamic acid. This stimulation occurred only at high concentrations of substrate. The algal isomerase systems were not stimulated by the addition of 0·05 M glutamic acid; however, the substrate concentrations used in this study were considerably lower than those used by Snyder and Lee.

EXPERIMENTAL

Algal Culture and Harvest

Ankistrodesmus Braunii (culture number 245) and *Scenedesmus acuminatus* (culture number 415), bacteria free unialgal cultures, were obtained from Indiana University, Bloomington, Indiana, and maintained as stock cultures on agar slants of Bristol's medium supplemented with proteose.

Both algal species were mass cultured under sterile conditions in a modified aqueous inorganic nutrient medium (Table 5). Culture flasks, 5-gal pyrex carboys each containing 15 l. of nutrient solution, were placed on a culture rack at a controlled temperature of 20° ± 2°. The culture flasks were exposed to light at an intensity of about 38 lux emitted from fluorescent and tungsten bulbs mounted above and below the rack. The cultures were aerated with a continuous flow of air containing approximately 3% CO_2 by volume.

Aqueous unialgal starter cultures were initiated by inoculating 25 ml of autoclaved inorganic medium with algae from the stock agar slants. After two to three weeks the starter cultures were aseptically transferred to the culture flasks. Bacterial contamination was checked before inoculation and before harvest by plating on nutrient agar and subsequent incubation at 37°.

The Chlorococcales were harvested when it became visually apparent that growth had become an excess function of light intensity. *S. acuminatus* was harvested by three filtrations through Reeve Angel grade number 835 filter paper. The wet residue, consisting of whole algal cells, was scraped from the filter paper and frozen. *A. Braunii* was harvested in the same manner using Eaton Dikeman grade number 512 fluted filter paper.

Assay of Enzyme Activity

The assay procedure was the coupled reaction reported by Beisenherz[5] using GAP as substrate for the isomerase and α-glycerophosphate dehydrogenase (GPD) as the coupling enzyme. The reaction was followed spectrophotometrically by measuring the decrease of absorption at 340 nm.

The reaction was initiated by pipetting 0·1 ml of isomerase solution into 2·9 ml of the reaction mixture. The time required for a decrease in absorptivity of 0·1 unit was recorded. A unit of enzyme was defined in terms of the *time* (in secs) required for a change of 0·001 unit.

TABLE 5. COMPLETE INORGANIC MEDIUM FOR MASS CULTURE

Compound	Macronutrients Weight (g/l)	ppm
KNO_3	1·00	1000
$MgSO_4$	0·25	250
KH_2PO_4	0·25	250

Compound	Micronutrients Weight (g/l in stock solution)	0·1 ml/l gives ppm of the metal
H_3BO_3	2·860	0·050
$MnCl_2 \cdot 4H_2O$	1·810	0·050
$ZNSO_4 \cdot 7H_2O$	0·222	0·005
$CuSO_4 \cdot 5H_2O$	0·079	0·002
MoO_3	0·015	0·001
$Ca(NO_3)_2 \cdot 4H_2O$	59·000	1·000

Compound	Iron solution and acetate Weight (g/l)	1·0 ml/l gives ppm of Fe
$FeC_6H_5O_7 \cdot 3H_2O$	5·30*	1·000
$NaC_2H_3O_2 \cdot 3H_2O$	0·25	

* In stock solution.

Note: The three macronutrients, the iron and sodium acetate were autoclaved separately. The micronutrients were autoclaved together. The pH of the sterile medium was 6·5.

Protein concentration was determined by absorption at 276 nm using a bovine serum albumin standard.

Stock solution of NADH (Calbiochem, Los Angeles, California) (3·08 μmoles per ml) was prepared each week and kept at 0–5° when not in use.

α-Glycerophosphate dehydrogenase was obtained as a crystalline suspension in 2·0 M ammonium sulfate (Calbiochem). 0·025 ml of the suspension in 2·5 ml of Tris buffer (0·025 M, pH 7·5) was dialyzed against the same buffer for 8 hr. The dialyzed solution, free from sulfate, containing 0·105 to 0·16 mg protein per ml, was used for assays.

DL-glyceraldehyde-3-phosphate was prepared from the diethyl acetal monobarium salt (Sigma Chemical Company, St. Louis, Missouri), by treatment with Dowex 50 resin (H^+ form). Determination of GAP was carried out using the assay developed by Velick.[12]

A stock solution of glyceraldehyde-3-phosphate dehydrogenase was prepared by dissolving 0·1 ml of the crystalline suspension in a 0·9 ml of Tris buffer, pH 8·5.

Isolation and Purification of the Enzymes

Identical procedures were used in the isolation and purification of triosephosphate isomerase from *A. Braunii* and *S. acuminatus*. All purification steps were at solution temperatures of 0–10°. A stock solution containing $5{\cdot}4 \times 10^{-3}$ M EDTA, was used for dialysis and as a solvent for the enzyme and saturated ammonium sulfate solutions. Ammonium sulfate treated solutions, unless otherwise stated, were allowed to stand for 4 hr at 5° before centrifugation.

[12] S. F. VELICK, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 387, Academic Press, New York (1955).

The harvested algae were thawed and mixed into a wet cell paste with 0·3 M sodium pyrophosphate buffer (pH 7·1, containing $5{\cdot}4 \times 10^{-3}$ M EDTA). The cells were macerated in a chilled mortar with an equal mixture of fine washed sand and levigated alumina. An equal volume of buffer was added and the resulting mixture allowed to stand for 4 hr before centrifugation at 39,000 *g* for 1 hr. The supernatant solution was decanted and retained. The pellet was resuspended in buffer, macerated and centrifuged as before. The supernatant fractions from both centrifugations were combined (Fraction 1) and the pellet discarded.

Chilled acetone (−10°) was added dropwise over a period of 45 min to bring the concentration to 30%. The supernatant (Fraction 2) was dialyzed for 18 hr against cold running tap water.

Solid ammonium sulfate (enzyme grade) was added to the dialyzed supernatant to bring the solution to 37% saturation. The precipitate was collected by centrifugation and discarded. The supernatant was brought to 50% saturation with solid ammonium sulfate, and the precipitate collected by centrifugation. The supernatant fraction contained no triosephosphate isomerase activity and was discarded. The pellet (Fraction 3) was dissolved in a minimal amount of the EDTA solution and dialyzed against this solution for 4 hr.

The enzyme solution was taken to 20 % saturation by the addition of saturated ammonium sulfate solution. The resultant solution was allowed to stand for 5 days, then dialyzed against the EDTA solution. The protein precipitating during dialysis was removed by centrifugation and discarded. The supernatant fraction was then taken to 50% saturation with ammonium sulfate solution and allowed to stand for 72 hr. The precipitate was removed by centrifugation and discarded. Saturated ammonium sulfate solution was again added to bring the supernatant to 75% saturation. After standing with occasional stirring for 32 hr, the solution was centrifuged and the supernatant decanted and discarded. The pellet (Fraction 4), containing all of the isomerase activity, was dissolved in a minimal amount of cold EDTA solution and dialyzed against the EDTA solution for 4 hr.

The enzyme solution was concentrated to dryness by lyophilization. The lyophilized protein (Fraction 5) was dissolved in Tris buffer (0·025 M, pH 7·5) and dialyzed against the buffer before use.

Electrophoresis of the Purified Enzymes

Electrophoretic studies of triosephosphate isomerase from *A. Braunii* and *S. acuminatus* were carried out on a Buchler electrophoresis apparatus using cellulose acetate strips. The buffer system, barbital-sodium barbital (0·05 ionic strength, pH 8·6), was replaced every third run.

The cellulose acetate strips (Gelman Instrument Co., Ann Arbor, Michigan) were soaked for a minimum of 30 min in freshly prepared buffer, placed in the migration chamber and allowed to equilibrate. The protein solution (0·025 ml), containing about 1 mg protein per ml, was added transversely to the center of each equilibrated strip. Runs were carried out at 150 V for 3 hr. The strips were dried at room temperature, stained for 5–7 min in Ponceau S solution and cleared by three washings in 5% acetic acid.