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ISOLATION AND CHARACTERIZATION OF FRUCTOSE DIPHOSPHATE ALDOLASE FROM *PISUM SATIVUM**,**

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SUMMARY

1. Fructose diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) from peas (*Pisum sativum*) has been purified 580-fold by $(NH_4)_2SO_4$ fractionation and subsequent column chromatography on DEAE-Sephadex and Biogel P-300.

2. The specific activity of the purest enzyme fraction towards Fru-1,6-P_2 was 14.9 IUB units.

3. Fru-1-P is split at a rate of 6–12% of that of Fru-1,6-P_2; the Michaelis constants are 0.12 mM for Fru-1,6-P_2 and 12 mM for Fru-1-P.

4. After incubation with carboxypeptidase the enzyme shows 4% of its initial activity towards Fru-1,6-P_2, whereas the activity towards Fru-1-P remains unchanged.

5. The pH profile shows a broad maximum from pH 7.7 to 8.8.

6. The condensation of [^{14}C] formaldehyde with dihydroxyacetonephosphate observed for crude extracts is still catalyzed by a 300-fold purified enzyme fraction.

INTRODUCTION

Since the discovery of fructosediphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) in rabbit liver and in yeast in 1934 (ref. 1), very few attempts have been made to purify the enzyme from a higher plant. Apart from the work of STUMPF[2] in 1948, who describes a 90-fold purification of pea aldolase, there is only one other report, dealing with a slight purification of a cactus aldolase[3], apparently particulate-bound.

The present work was undertaken in order to prepare and characterize highly purified plants fructosediphosphate aldolase. The purification procedure, some

Abbreviation: B.U., Bücher units.

* Ninth communication on aldolases.

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physical, and some chemical characteristics are presented. These properties are compared with those of animal and microbial aldolases.

EXPERIMENTAL

Materials

All components of the enzyme test: glycerol-1-phosphate dehydrogenase and triosephosphate isomerase (available together in $(NH_4)_2SO_4$ suspensions), NADH, Fru-1,6-P_2 (sodium salt), Fru-1-P (cyclohexylammonium salt), and Tris buffer were obtained from Boehringer, Mannheim, Germany. All other reagents used were of analytical grade. For all experiments deionized, Pyrex-distilled water was used. Carboxypeptidase (A-DFP; 20 mg/ml, crystalline) was obtained from Sigma. Dialysis tubing (Visking, Chicago) was boiled in distilled water prior to use.

Methods

The aldolase activity was assayed at 25° by the method of RACKER[4] in an Eppendorf recording photometer at 366 mμ. Results are expressed in BÜCHER units (B.U.) per mg (see ref. 5) (*i.e.* the change of 0.1 absorbance unit per 100 sec per mg protein). The test mixture contained 0.25 ml 1.0 M Tris buffer (pH 7.8), 0.25 ml 3 mM NADH, 0.01–0.02 ml glycerol-1-phosphate dehydrogenase/triosephosphate isomerase (2 or 10 mg protein per ml), 0.05 ml 0.02 M Fru-1,6-P_2, 1.80–1.92 ml distilled water, and 0.02–0.10 ml aldolase probes.

Protein was determined by a modification of the Biuret method[5], by measuring the absorbance at 280 mμ, and by the method of LOWRY *et al.*[6]. Comparisons of biuret determinations and by absorption at 280 mμ, showed the protein to have an $E^{1\%}_{1\,cm}$ of 10.0.

$(NH_4)_2SO_4$ was pulverized for 40 min in a ball mill and was added to the protein solutions at a rate of 60 g/h. 30 min after reaching the final $(NH_4)_2SO_4$ concentration, the solution was centrifuged (90 min at 3000 × g, 0°). The sediment was then suspended in 40% satd. $(NH_4)_2SO_4$ (pH 8.6). Final fractions were kept at −30° under neutral, satd. $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ concentration was determined by a modification of the method of Malfatti (see ref. 7) and expressed as per cent saturation. The whole isolation procedure was carried out at 4°. All enzyme solutions contained 1 mM EDTA and 1 mM 2-mercaptoethanol.

For column chromatography, DEAE-Sephadex (A-50, medium, bead-form) and Biogel P-300 were used. The columns were packed at 4°. Sedimentation analyses were carried out in the Spinco Model E centrifuge (rotor AN-D, schlieren scanning, Rayleigh optics).

RESULTS

Preparation of aldolase

Dry pea seeds (Lincoln variety) were kept in glass-stoppered flasks at 4°. For each preparation 800 g of peas were washed in tap water until the water remained clear, rinsed twice with distilled water, and soaked for 20 h in distilled water.

The soaked peas were homogenized in 150-g portions in 390 ml of ice-cold 40% satd. $(NH_4)_2SO_4$ (pH 8.6) for 7 min in a Waring blendor. After filtration through

TABLE I

PURIFICATION OF PEA FRUCTOSEDIPHOSPHATE ALDOLASE

Fraction	*Vol. (ml)*	*B.U. per ml*	*B.U. (total units)*	*Protein (mg/ml)*	*B.U. per mg*	*Recovery (%)*	*Purification (-fold)*
Crude extract*		40.2		28.6	1.4		
1 pre-fractionated extract**	2760	65	180 000	19.4	3.4	100	2.4
2 supernatant at 43% $(NH_4)_2SO_4$	760	181	138 000	10.7	17.0	77	12
3 supernatant at 43% $(NH_4)_2SO_4$	865	141	122 000	5.4	26.0	68	18.5
4 sediment at 43–55% $(NH_4)_2SO_4$	72.5	760	55 000	20.5	37.0	30.5	26.5
5 first DEAE-Sephadex chromatography, Tubes 49–54	9.6	1670	15 900	5.5	305.0	1.4	218
6 second DEAE-Sephadex chromatography, Tube 19	4.0	1070	4280	2.2	486	0.38	347
7 Biogel P-300 chromatography, Tubes 31–33	9.0	170	1530	0.21	815	0.14	580

* Prepared for reference only.
** See Scheme I for preparative details.

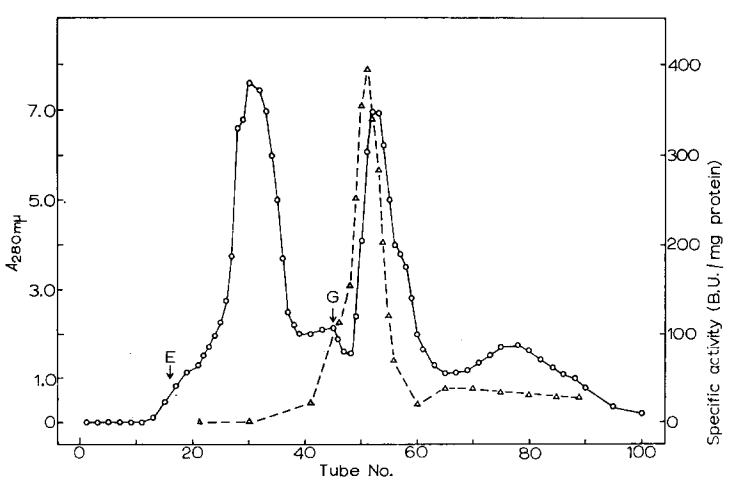


Fig. 1. Chromatography on DEAE-Sephadex of 1100 mg protein (Fraction 4) applied in 40 ml 0.2 M Tris buffer (pH 8.4). (Flow rate 7.5 ml/h, column 2.5 cm × 35 cm). At point E the elution was started with 0.3 M Tris buffer (pH 8.4). At point G a linear Cl^- gradient (0.096–0.248 M) was applied as 0.3 M Tris–HCl, buffered from pH 8.4 to 7.4. The enzyme was eluted between 0.096 and 0.110 M Cl^- (pH 8.4–8.3). ○—○, absorbance at 280 mμ; △— —△, specific activity (B.U. per mg protein).

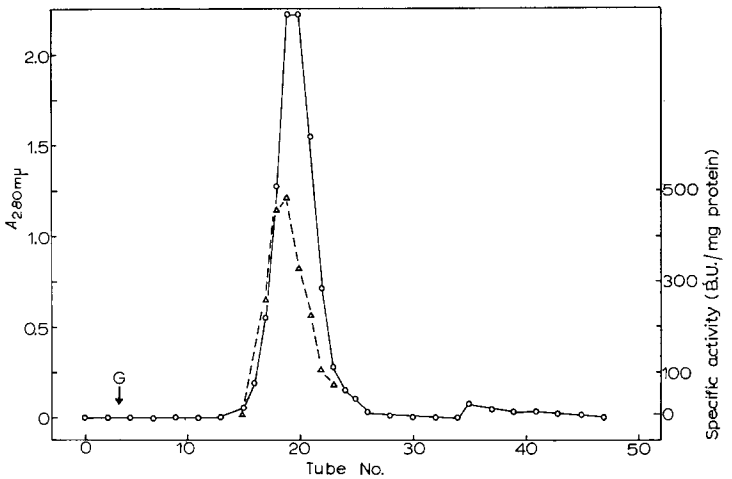


Fig. 2. Chromatography on DEAE-Sephadex of 52 mg protein (Fraction 5) applied in 9.6 ml 0.2 M Tris buffer (pH 8.4). (Flow rate 8 ml/h, column 1.5 cm × 25 cm). At point G a linear Cl^- gradient as in Fig. 1 was applied. The enzyme was eluted between 0.108 and 0.123 M Cl^- (pH 8.3–8.2). ○—○, absorbance at 280 mμ; △— —△, specific activity (B.U. per mg protein).

six layers of cheese-cloth and two layers of crude cotton (by a manually operated press), the crude extract was centrifuged for 40 min at 60 000 × *g* at 0°. The slightly opaque, yellowish supernatant (34% satd. $(NH_4)_2SO_4$) was brought up to 59% satn. by dispersing pulverized $(NH_4)_2SO_4$. The sediment, after centrifugation, was further fractionated according to Scheme I. The $(NH_4)_2SO_4$ fraction (see No. 4, Table I), was dissolved in 0.2 M Tris buffer (pH 8.4) and dialyzed overnight against 2 l of the same buffer. The external medium was changed after 3 h and after 6 h. A small amount of

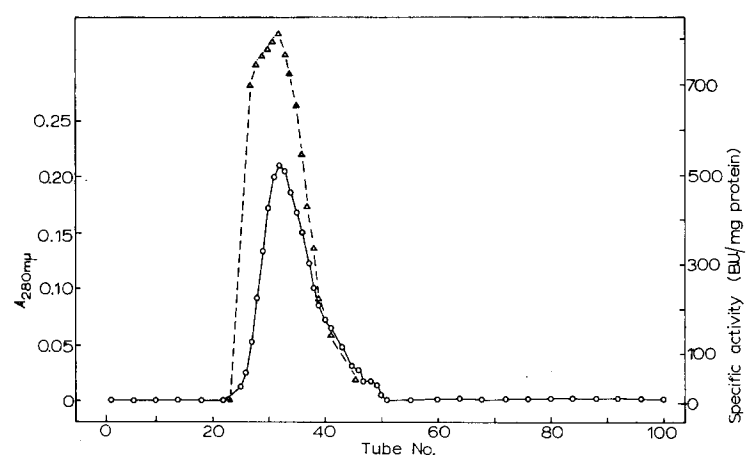


Fig. 3. Gel filtration on Biogel P-300 of 8.8 mg protein (Fraction 6) applied in 4.0 ml 0.3 M Tris buffer (pH 8.2). (Flow rate 8 ml/h, column 2.5 cm × 60 cm). The column was packed and eluted with 0.05 M Tris buffer (pH 7.2). ○—○, absorbance at 280 mμ; △— —△, specific activity (B.U. per mg protein).

precipitate (with no aldolase activity) was discarded. The dialyzed extract was then further purified in two successive column chromatographies on DEAE-Sephadex (Figs. 1 and 2). The purest fraction from the second column (Table I) was concentrated by vacuum dialysis[8] for 4 h against 0.05 M Tris buffer (pH 7.4) and chromatographed on Biogel P-300 (Fig. 3). Fraction 7 (Table I) was lyophilized after 8 h of dialysis against distilled water, and stored under vacuum at −30°.

Criteria of purity

The most highly purified fractions (Table I) exhibited a specific activity towards Fru-1,6-P_2 of 815 B.U. per mg protein (14.9 I.U.B units). In fractions from DEAE-Sephadex chromatography no triosephosphate isomerase activity could be demonstrated. A sedimentation analysis of a 300-fold purified fraction from gel filtration showed an s_{20} of 7.1 and a slight impurity with a lower sedimentation coefficient (Fig. 4). The aim of all isolation procedures was to reach the highest possible purity irrespective of yield. Therefore only the purest fractions were used.

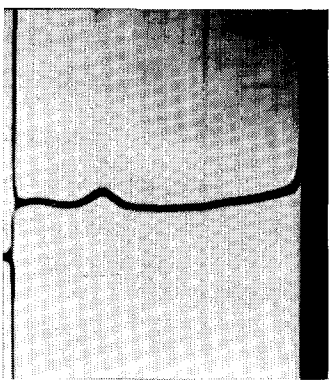


Fig. 4. Sedimentation of a 300-fold enriched aldolase fraction at 4°. (4° sector angle, schlieren scanning, 45° diaphragm angle, sedimentation velocity 56 100 rev./min). 4.0 mg protein were dissolved in 1.0 ml 0.01 M Tris buffer–0.2 M NaCl (pH 8.0). The photograph shown was taken 70 min after starting the run.

Chemical and kinetic studies

For the Fru-1,6-P_2 cleavage the pH dependence was determined in Tris buffer, in the range between pH 6.2 and 9.5. The individual pH values were obtained by mixing varying amounts of two stock solutions, both being 1.0 M in Tris buffer and which contained a mixture of the auxiliary enzymes: glycerol-1-phosphate dehydrogenase and triosephosphate isomerase in a concentration of 0.2 mg protein per ml. After addition of the remaining components, the pH was checked. The reaction was initiated by addition of aldolase. The final buffer concentration was twice that used for the normal enzyme assay, *i.e.* 0.2 M. Tris buffer could be used throughout the pH range of 6.2–9.5, although its buffering capacity at the two extremes is poor. The pH profile shows a broad maximum from pH 7.7 to 8.8.

The Michaelis constants were determined by a LINEWEAVEP–BURK plot[9] and amount to 0.12 mM for Fru-1,6-P_2 and 12 mM for Fru-1-P. To 1.0 ml of protein solution (0.7 mg protein per ml, 250 B.U. per mg protein in 0.01 M neutral Tris buffer)

10 μl of a carboxypeptidase suspension in 10% LiCl (100 μg protein per ml) were added. After 30 min of incubation with carboxypeptidase at 22°, the activity towards Fru-1,6-P_2 decreased to 4% of the initial value, whereas the activity towards Fru-1-P remained unchanged. The residual activity remained unchanged after 30 min. The simultaneous presence of 1 mM EDTA and 1 mM 2-mercaptoethanol preserves the activity of pure enzyme fractions which are otherwise unstable. $(NH_4)_2SO_4$ fractions of 35 B.U. per mg protein can, however, be stored under neutral satd. $(NH_4)_2SO_4$ at −30° over many months without loss of activity. Lyophilized material, purified on Biogel P-300 (Fraction 7; Table I) kept its activity for 2 to 4 weeks under vacuum at −30°.

Substrate specificity

Enzyme preparations at various stages of purity showed a lower activity (6–12%) toward Fru-1-P than toward Fru-1,6-P_2. [^{14}C]Formaldehyde was condensed with dihydroxyacetonephosphate in the presence of aldolase (420 B.U. per mg protein), whereas no condensation was found in the absence of the enzyme. The condensation product (probably erythrulose 1-phosphate) was traced radiochromatographically. Erythrulose 1-phosphate has been found by several others to be formed under similar conditions[10–13]. Actual reference material was not available.

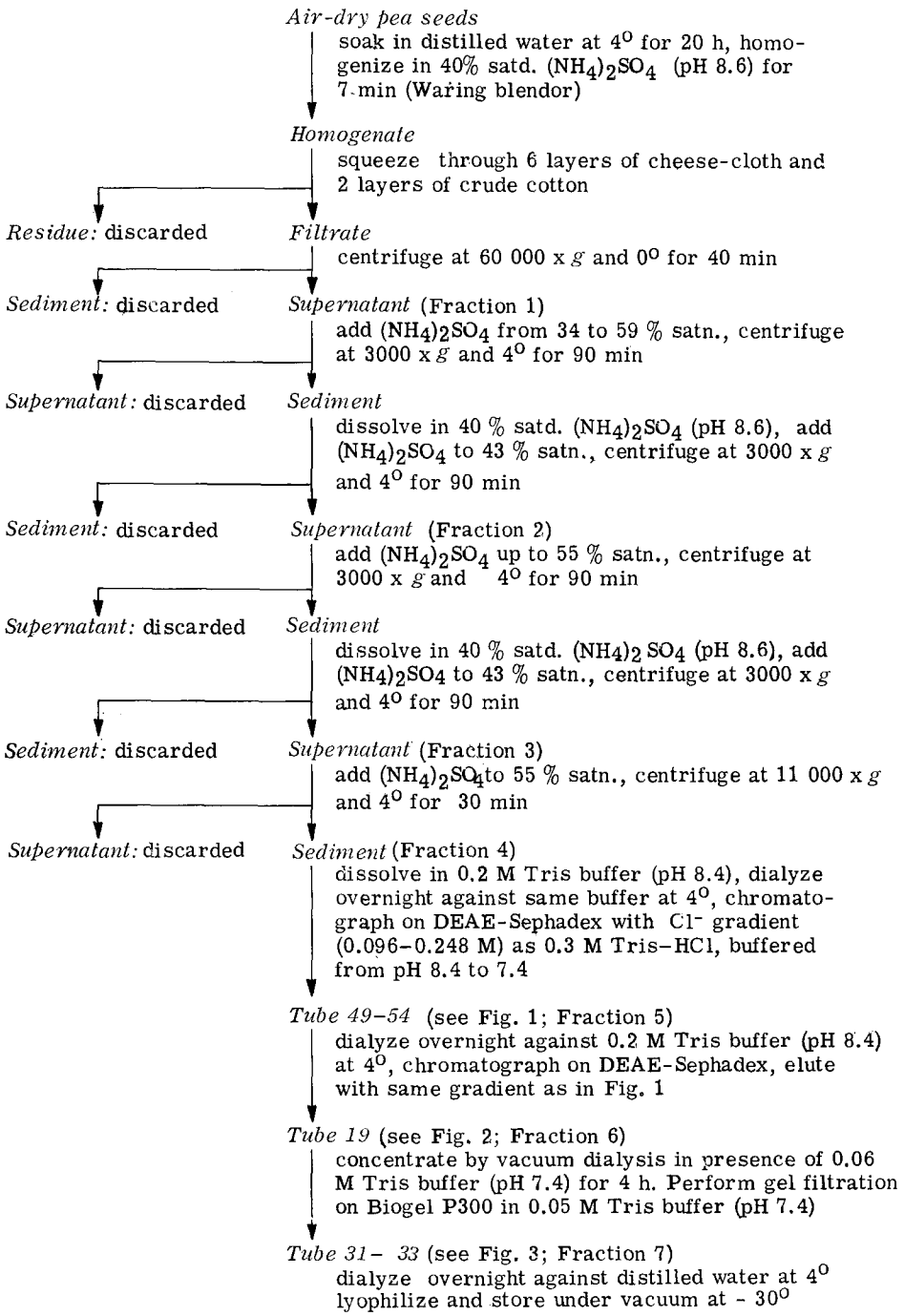
DISCUSSION

RUTTER[14] has classified aldolases on the basis of physical and chemical properties and found that they fall into phylogenic groupings: Class I from green algae, protozoa, higher plants and animals and Class II from bacteria, fungi and blue-green algae. Within Class I two aldolase types can be distinguished, as exemplified by the muscle and by the liver varieties, which differ especially in their substrate specificity, in their molecular activity towards Fru-1,6-P_2, and in their behavior after carboxypeptidase treatment[14–17]. From the following data the present enzyme can be grouped within Class I: The enzyme is not inhibited by EDTA (ref. 14), even when highly purified. The pH optimum obtained in Tris buffer (pH 7.7–8.8) is similar to that reported by RUTTER[14] (pH 6.9–8.8) using pea homogenate though he gave no indication of buffer. The residual activity after incubation with carboxypeptidase (4%) is in good agreement with the values for crude pea extracts (6%; see ref. 14) and for crystalline muscle aldolase[17]. In contrast to the muscle type, liver aldolase shows a residual activity of 50% (ref. 18). The activity of pea aldolase towards Fru-1-P is low and is not altered by carboxypeptidase treatment, both results being comparable to reports for the muscle enzyme[7,17,19]. In contradistinction to these low activities towards Fru-1-P, liver aldolase exhibits a ratio (Fru-1,6-P_2/Fru-1-P) of 1 (see refs. 18–21). In order to avoid the high substrate concentrations of Fru-1-P which would be necessary to obtain maximum reaction velocity (K_m = 12 mM), a Fru-1-P concentration of only a fraction of the Michaelis constant was chosen[7] and the maximum reaction rate calculated by the LINEWEAVER AND BURK equation[9].

The highest activity towards Fru-1,6-P_2 obtained in this preparation (815 B.U. per mg protein) is about half that of crystalline muscle aldolase (1800 B.U. per mg protein, see ref. 7) and about four times that of crystalline liver aldolase (220 B.U. per mg protein; see ref. 22). From sedimentation analyses the molecular weight was

SCHEME I

Flow sheet of aldolase isolation



calculated to be about 150 000. The purest fraction, therefore, has a molecular activity of 2235 with Fru-1,6-P_2 and 280 with Fru-1-P, compared with the corresponding values: 4740 and 210 for rabbit muscle aldolase[7], 570 and 520 for rabbit liver aldolase[23].

The s_{20} of 7.1 is in good agreement with the value obtained by sucrose density-gradient centrifugation of a crude pea homogenate (7.0; see ref. 14). Similar s_{20} values were also found for the liver[23] and muscle[24] enzymes.

The Michaelis constants with Fru-1,6-P_2 and Fru-1-P are also comparable to those of muscle[7], rather than liver aldolase[23].

Highly purified pea aldolase is more labile than pure aldolases from muscle or liver. HOUGH AND JONES found that crude fractions of pea aldolase containing phosphatase catalyze the condensation of D-erythrose and dihydroxyacetonephosphate (split from Fru-1,6-P_2) to form sedoheptulose[25] and the condensation of glycolaldehyde and dihydroxyacetonephosphate (split from Fru-1,6-P_2) to xylulose[26]. Erythrulose 1-phosphate can be obtained by incubation of [^{14}C]formaldehyde and dihydroxyacetonephosphate (split from Fru-1,6-P_2) with homogenates of Swiss chard (*Beta vulgaris*)[10]. Our own experiments, using a 300-fold enriched enzyme preparation, confirm this latter condensation, showing incidentally that this reaction is catalyzed by Fru-1,6-P_2-aldolase and not by an "aldolase-like" enzyme, a possibility suggested by STUMPF[27].

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