Purification and characterization of cytosolic aldolase from carrot storage root

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A single fructose-1,6-bisphosphate (FBP) aldolase has been detected in extracts from carrot storage roots (*Daucus carota* L.). The enzyme was purified 850-fold to electrophoretic homogeneity and a final specific activity of 26.3 μmol of FBP utilized/min per mg of protein. SDS/PAGE of the final preparation revealed a single protein-staining band of 40 kDa. The native molecular mass was determined by analytical gel filtration to be 159 kDa, indicating that the enzyme is a homotetramer. Denaturing isoelectric focusing revealed two predominant protein-staining bands, with pI values of 5.6 and 5.7. The enzyme is a class I aldolase, since EDTA or metal ions had no effect on its activity. The enzyme was relatively heat-stable, had an activation energy (E_a) of 68.3 kJ $\cdot\text{mol}^{-1}$, and had an absorption coefficient of $8.08 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm. K_m values for FBP and sedoheptulose 1,7-bisphosphate (SBP) were both determined to be 6 μM (pH optima 7.4). The specificity constant with FBP was 2.6 times that obtained with SBP. Ribose 5-phosphate, 6-phosphogluconate, MgAMP, glucose 1-phosphate and phosphoenolpyruvate (PEP) were inhibitors. PEP was a mixed-type inhibitor with respect to FBP ($K_i = 3.2 \text{ mM}$, $K'_i = 5.1 \text{ mM}$). No activators were found. Rabbit anti-(carrot aldolase) polyclonal antibodies immunoprecipitated the activity of both carrot root aldolase and spinach leaf cytosolic aldolase, but not that of spinach leaf plastid aldolase. Western-blot analysis also revealed cross-reactivity with cytosolic, but not plastid, spinach leaf aldolase, indicating that the single carrot root aldolase is cytosolic.

INTRODUCTION

Fructose-1,6-bisphosphate (FBP) aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) catalyses the reversible conversion of FBP into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Plant aldolases are similar to the enzyme isolated from animal sources. They have low catalytic-centre activities, and are homotetrameric with subunit molecular masses in the range 35–40 kDa [1–5]. Plastid and/or cytosolic isoenzymes of aldolase have been purified from green algae and a range of photosynthetic and non-photosynthetic higher-plant tissues [1–7]. In several instances, although preparations appear to be homogeneous, it was not determined whether a cytosolic or plastid aldolase had been purified [1,4]. To date, a tuber or storage-root aldolase has not been studied.

We have recently provided preliminary evidence indicating that the stimulation of respiration which accompanies aging of carrot storage-root slices may result, in part, from an elevated association of glycolytic enzymes (including aldolase) with the particulate fraction of the cell [8]. The micro-compartmentation of the glycolytic pathway which can result from this type of interaction could provide an effective means of glycolytic control by channeling of substrates between sequential enzymes and altering enzyme-kinetic properties owing to conformational changes occurring during binding [9]. Aldolase has been found to interact specifically with other functionally related glycolytic enzymes in animal systems [9]. Also, the enzyme has been implicated as being a key 'scaffolding' enzyme during the formation of a glycolytic complex on actin-containing filaments in mammalian muscle tissues [9]. In the present study we report the purification and characterization of cytosolic aldolase from carrot storage roots. It will be interesting to attempt to demonstrate specific interactions between the purified enzyme and other plant glycolytic enzymes.

EXPERIMENTAL

Chemicals and plant material

Biochemicals, coupling enzymes, SDS/PAGE molecular-mass standards, isoelectric-focusing standards, ampholytes (pH 4–6.5), bisacrylamide, alkaline phosphatase-tagged goat anti-rabbit IgG and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tris base and SDS were from Schwarz/Mann Biotech (Cambridge, MA, U.S.A.). Protein assay reagent was from Pierce Chemical Co. (Rockford, IL, U.S.A.) or Bio-Rad (Mississauga, Ont., Canada). Phosphocellulose P-11 was obtained from Whatman (Hillsboro, OR, U.S.A.). Ribi adjuvant (product code R730) was obtained from Ribi Immunochemical Research (Hamilton, MT, U.S.A.), and Schleicher and Schull nitrocellulose membranes (BA 85; 0.45 μm pore size) were from Mandel Scientific Co. (Rockwood, Ont., Canada). *Staphylococcus aureus* cells (Pansorbin) were from Calbiochem (San Diego, CA, U.S.A.) and poly(vinylidene difluoride) membranes (Immobilon transfer; 0.45 μm pore size) were from Millipore (Mississauga, Ont., Canada). Native molecular-mass standards, Q-Sepharose, Sephadex G-25, a Superose 6 HR 10/30 column, a Mono-Q HR 5/5 column and an f.p.l.c. system were obtained from Pharmacia (Baie D'Urfe, Que., Canada). All other reagents were of analytical grade, obtained from BDH Chemicals (Toronto, Ont., Canada). All buffers used in the present study were degassed and adjusted to their respective pH values at 25 °C. Carrots (*Daucus carota* L.) were purchased at a local market and used the same day.

Enzyme assays

The aldolase reaction was coupled with the triosephosphate isomerase and glycerol-3-phosphate dehydrogenase reactions and assayed at 25 °C by monitoring NADH utilization at 340 nm

Abbreviations used: FBP, fructose 1,6-bisphosphate; SBP, sedoheptulose 1,7-bisphosphate; DTT, dithiothreitol; PEG, poly(ethylene glycol); PEP, phosphoenolpyruvate.

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with a Varian DMS 200 spectrophotometer. Standard assay conditions for aldolase were: 50 mM-bis-tris-propane/NaOH buffer (pH 7.4), 0.5 mM-FBP, 0.1 mM-NADH, 10 units of triosephosphate isomerase and 1 unit of glycerol-3-phosphate dehydrogenase in a final volume of 1 ml. Assays were initiated by the addition of enzyme preparation and were corrected for NADH oxidase activity by omitting FBP from the reaction mixture. Coupling enzymes were desalted before use. One unit of enzyme activity is defined as the amount of enzyme resulting in the oxidation of 1 μ mol of NADH/min at 25 °C. In all cases the rate of reaction was proportional to concentration of enzyme assayed.

Kinetic studies

Apparent K_m and h (Hill coefficient) values were determined from the Michaelis-Menten and Hill equations respectively. Both equations were fitted to a non-linear least-squares regression computer kinetics program (S. Brooks, unpublished work). I_{50} values (inhibitor concentration producing 50% inhibition of enzyme activity) were determined by the method of Job *et al.* [10]. The pattern of inhibition by phosphoenolpyruvate (PEP) was evaluated by using a direct linear plot in accordance with Cornish-Bowden [11]. The competitive-inhibition constant (K_i) for PEP was determined from a Dixon plot, whereas the uncompetitive-inhibition constant (K'_i) for PEP was determined as described in [11]. All kinetic parameters are the means of duplicate determinations performed on two separate preparations of the purified enzyme, and are reproducible to within 10%.

Antibody production

After collection of preimmune sera, purified aldolase [650 μ g, dialysed exhaustively against phosphate-buffered saline (40 mM-sodium phosphate, pH 7.4, containing 150 mM-NaCl)] emulsified in Ribi adjuvant (total volume 1.0 ml) was injected (600 μ l intramuscularly, 400 μ l subcutaneously) into a 2 kg New Zealand rabbit. Booster injections (325 μ g) of the same protein emulsified in Ribi adjuvant were given at 3 and 5 weeks. At 1 week after the final injection, blood was collected by cardiac puncture. After incubation at 4 °C overnight, the clotted blood cells were removed by centrifugation at 1500 *g* for 10 min. The crude antiserum was frozen in liquid N_2 and stored at -80 °C in 0.04% (w/v) NaN_3 .

Affinity purification of antibodies

Rabbit anti-(carrot aldolase) IgG was affinity-purified as described in [12], except that the purified aldolase was dotted directly on to the nitrocellulose.

Immunotitration of aldolase activity

Immunoremoval of enzyme activity was tested by mixing 0.006 unit of aldolase with 25 mM-Hepes/NaOH (pH 7.5), containing 0.1 mg of BSA/ml, 10% (v/v) glycerol, 1 mM-DTT, and various amounts of rabbit anti-(carrot aldolase) immune serum, or preimmune serum, diluted into phosphate-buffered saline (total volume 0.1 ml). The mixture was incubated for 1 h at 30 °C, followed by addition of 30 μ l of *S. aureus* cells [a 10% (w/v) solution of *S. aureus* cells was centrifuged for 5 min at 16000 *g* in an Eppendorf micro-centrifuge, then resuspended in an equal volume of phosphate-buffered saline before use]. The mixture was incubated at 37 °C for 30 min, and then centrifuged for 5 min at 16000 *g*. The supernatant was assayed for residual aldolase activity. Each assay was corrected for contaminating NADH oxidase activity (present in the rabbit immune and pre-immune serum) by omitting FBP from the aldolase-reaction mixture.

Electrophoresis and Western blotting

Non-denaturing PAGE was performed with a Bio-Rad mini-gel apparatus by using the discontinuous system of Davis [13]. Final acrylamide monomer concentration in the 0.75 mm-thick slab gels was 7% (w/v) for the separating gel and 2.5% (w/v) for the stacking gel. Before pouring the stacking-gel solution, the separating gel was pre-electrophoresed for 2 h at 150 V constant voltage, with 250 mM-Tris/HCl (pH 8.8) as the electrode buffer. The stacking gel was polymerized with fluorescent light for 3–4 h. Gels were pre-cooled to 4 °C before use and were maintained at this temperature during electrophoresis. Samples containing 60% (v/v) glycerol were run at 150 V constant voltage for 2.5 h. Tris (25 mM)/glycine (190 mM) containing 1 mM-thioglycolate was used as the electrode buffer. Gels were either stained for protein with Coomassie Blue G-250 [14], or aldolase activity was located in the gel. To detect aldolase activity, a lane was sliced into 2 mm segments and each slice was incubated in 0.15 ml of 50 mM-bis-tris-propane/NaOH (pH 7.4) and 2 mM-FBP for 2 h at 37 °C. The incubation mixture was then added to 50 mM-bis-tris-propane (pH 7.4), containing 0.1 mM-NADH, 10 units of triosephosphate isomerase and 1 unit of glycerol-3-phosphate dehydrogenase (final volume 1 ml). Relative aldolase activity was determined by monitoring the decrease in A_{340} after a further 15 min incubation at 25 °C.

SDS/PAGE was performed with a Bio-Rad mini-gel apparatus and the discontinuous system of Doucet & Trifaró [15]. Final acrylamide monomer concentration in the 0.75 mm-thick slab gels was 4% (w/v) for the stacking gel and 12% (w/v) for the separating gel. The acrylamide/bisacrylamide ratio for both stacking and separating gels was 100:1 (by mass). All samples were preincubated in the presence of SDS sample buffer [70 mM-Tris/HCl, pH 6.7, containing 8 M-urea, 3% (w/v) SDS, 100 mM-DTT and 0.005% (w/v) Bromophenol Blue] for 2 min at 100 °C before being loaded on to the gels. Gels were run at a constant voltage of 155 V, applied for 1 h. For the determination of subunit molecular mass by SDS/PAGE, a plot of relative mobility versus log(molecular mass) was constructed with the following standard proteins: myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

Denaturing isoelectric focusing was performed in a vertical polyacrylamide mini-gel system by the method of Robertson *et al.* [16], except that the gel was 0.75 mm thick. The subunit pI was determined from a plot of pI versus R_f with the following standards: trypsin inhibitor from soybean (pI 4.55), β -lactoglobulin A from milk (pI 5.13), carbonic anhydrase B from bovine erythrocytes (pI 5.85) and carbonic anhydrase B from human erythrocytes (pI 6.57).

Western blotting and immunodetection of aldolase was performed as in [12] with affinity-purified rabbit anti-(carrot aldolase) IgG [diluted 20-fold with 50 mM-Tris/HCl (pH 7.5), containing 150 mM-NaCl, 0.05% (v/v) Tween 20 and 3% (w/v) skim-milk powder]. Immunological specificity was confirmed by performing Western blots in which rabbit preimmune serum was substituted for affinity-purified anti-aldolase IgG.

Amino acid analysis and N-terminal sequencing

Carboxymethylated carrot root aldolase was dialysed against deionized water for 2 days and freeze-dried. For amino acid analysis the protein was hydrolysed in 6 M-HCl at 110 °C for 24 h *in vacuo*. Analysis was carried out on a Beckman model 6300 automated amino acid analyser. N-Terminal sequencing was attempted with an Applied Biosystems model 470A gas-phase sequenator and 120 PTH amino acid analyser.

Determination of native molecular mass

This was done by f.p.l.c. on a Superose 6 HR 10/30 column, by using 100 μ l sample volumes and 20 mM-potassium phosphate (pH 7.1) containing 15 % (v/v) glycerol, 1 mM-DTT and 200 mM-KCl as column buffer. Fractions (0.4 ml) were collected with a flow rate of 0.2 ml/min and assayed for A_{280} or aldolase activity. The native molecular mass of aldolase was determined from a plot of K_D (partition coefficient) versus log(molecular mass) for the following protein standards: thyroglobulin (669 kDa), apoferritin (443 kDa), catalase (232 kDa), rabbit muscle aldolase (158 kDa) and ovalbumin (43 kDa).

Other methods

During protein purification, protein concentration was determined by using the method of Hill & Straka [17] and the Pierce Chemical Co. prepared bicinchoninic acid reagent. All other protein determinations were done with the dye-binding method of Bradford [18], unless specifically stated. Bovine γ -globulin was used as the standard in all cases. Carboxymethylation was performed as in [19]. Sugar determination was carried out by the method of Dubois *et al.* [20].

Buffers used in aldolase purification

Buffer A: 50 mM-Hepes/NaOH (pH 7.5) containing 1 mM-EDTA, 1 mM-DTT, 0.5 mM-MgCl₂, 10 mM-thiourea, 1 mM-phenylmethanesulphonyl fluoride, 3 mM-1,10-phenanthroline, 2 % (w/v) insoluble poly(vinylpyrrolidone), 100 mM-KCl and 10 % (v/v) glycerol. Buffer B: 15 mM-potassium phosphate (pH 7.1) containing 1 mM-EDTA, 2 mM-MgCl₂, 15 % (v/v) glycerol and 1 mM-DTT. Buffer C: 15 mM-bis-tris-propane/NaOH (pH 6.8) containing 1 mM-EDTA and 1 mM-DTT. Buffer D: 20 mM-bis-tris-propane/NaOH (pH 7.1) containing 0.5 mM-EDTA, 1 mM-DTT and 10 % (v/v) glycerol.

RESULTS

Purification of carrot root aldolase

Maximal activity of aldolase in carrot storage roots was about 0.5 unit/g wet wt. All purification procedures were carried out at 4 °C.

Crude extract. Diced carrot storage roots (1 kg) were homogenized in 1 vol. of buffer A with a Waring blender and a Polytron. The homogenate was filtered through cheesecloth and Miracloth, and the filtrate was designated the crude extract.

Acidification. The crude extract was adjusted to pH 5.25 with 7 M-acetic acid and gently stirred for 5 min. The extract was adjusted to pH 7.5 with 15 M-NH₃ and centrifuged at 14300 *g* for 15 min. Acidification of the crude extract eliminated pigmented material which interfered with subsequent purification steps.

Fractionation using poly(ethylene glycol) (PEG). PEG [average molecular mass 8 kDa; 50 % (w/v) dissolved in 50 mM-potassium phosphate (pH 7.0) containing 1 mM-EDTA] was added to the supernatant to give a final PEG concentration of 4 % (w/v). The solution was stirred for 30 min and centrifuged at 14300 *g* for 20 min. Pellets were discarded and the supernatant was adjusted to 30 % (w/v) by the slow addition of solid PEG, then stirred slowly for 45 min and centrifuged as above. Most of the protein present after the acidification step remained in the 4–30 %-PEG supernatant. The pellets were resuspended in 70 ml of buffer B and clarified by centrifugation at 37000 *g* for 15 min.

Q-Sepharose chromatography. The clear supernatant was absorbed at 2 ml/min on to a column of Q-Sepharose

(1.5 cm $\times$ 20 cm) which had been pre-equilibrated in buffer B. The column was connected to an f.p.l.c. system, washed with buffer B until the A_{280} decreased to about 0.1, and eluted with a linear 0–0.2 M-potassium phosphate gradient (250 ml) in buffer B (fraction size 8 ml). Only one peak of aldolase activity was resolved after application of the potassium phosphate gradient. If the gradient was run from 0 to 1.0 M-potassium phosphate, a second peak of aldolase activity was not detected. In subsequent preparations aldolase was eluted in a sharp peak after a step from 40 mM- to 110 mM-potassium phosphate. Peak activity fractions were pooled and concentrated to about 15 ml with an Amicon PM-30 ultrafilter. For desalting, the concentrated Q-Sepharose peak fractions were applied at 0.75 ml/min on to a Sephadex G-25 column (2.5 cm $\times$ 16 cm) previously equilibrated with buffer C.

Phosphocellulose chromatography. The desalted pooled fractions from Q-Sepharose (diluted to 6 mg/ml with buffer C) were absorbed at 0.5 ml/min on to a column of phosphocellulose P11 (2.5 cm $\times$ 5 cm), which was pre-equilibrated with buffer C. The column was washed with buffer C until the A_{280} decreased to baseline, then aldolase was eluted with buffer C containing 1 mM-FBP (fraction size 3 ml).

Mono-Q f.p.l.c. Pooled peak phosphocellulose fractions were absorbed at 0.5 ml/min on to a Mono-Q HR 5/5 column which had been previously equilibrated with buffer D. The column was washed until the A_{280} reached baseline, then eluted with a linear 0–0.3 M-KCl gradient (20 ml) in buffer D (fraction size 1.0 ml). The final step was done in two separate runs. Fig. 1 shows a typical elution profile for carrot root aldolase after Mono-Q f.p.l.c. Pooled peak activity fractions were concentrated to about 0.24 ml with an Amicon YM-30 ultrafilter, divided into 50 μ l portions, frozen in liquid N₂ and stored at –80 °C. The purified enzyme was stable for several months when stored frozen. As shown in Table 1, the enzyme was purified 850-fold to a final specific activity of 26.3 units/mg, and an overall recovery of about 22 %.

Physical and immunological properties

Gel electrophoresis. Denaturation, followed by SDS/PAGE of the final preparation, revealed a single protein-staining band of about 40 kDa which co-migrated with rabbit muscle aldolase (Fig. 2). Non-denaturing PAGE of the final preparation resulted in a single protein-staining band which directly co-migrated with aldolase activity (Fig. 3).

Isoelectric focusing in urea revealed two major protein-staining

Fig. 1. Mono-Q f.p.l.c. of carrot root aldolase

Details of the procedure are described in the text. ●, Aldolase activity; —, A_{280} ; ----, concn. of KCl.

Table 1. Purification of carrot root aldolase

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude	1560	15900	500	0.031	—	100
Acid step (pH 5.25)	1525	14100	500	0.035	1.13	100
PEG fractionation	70	578	235	0.41	13.1	47
Q-Sepharose	85	154	130	0.84	27.1	26
Phosphocellulose	9.5	7.8	113	14.5	468	23
Mono-Q*	0.24	4.1	108	26.3	848	22

* Concentrated pooled fractions from two separate runs.

Fig. 2. SDS/PAGE of various fractions obtained during the purification of carrot root aldolase

Lane 1 contains 22 µg of the Q-Sepharose fraction, lane 2 contains 14 µg of the phosphocellulose fraction, lanes 3 and 4 contain 1 and 2 µg of the Mono-Q fraction respectively, lane 5 contains 5 µg of rabbit muscle aldolase, and lane 6 contains 5 µg of molecular-mass markers. Protein staining was performed with Coomassie Blue G-250 [14]. TD, tracker dye front.

bands, with pI values of 5.6 and 5.7 (Fig. 4). Minor protein-staining bands were also present, which suggests that a degree of microheterogeneity exists in the purified aldolase. Carboxymethylation of the final preparation before focusing resulted in the same protein-staining pattern (results not shown). Sugar determination was carried out with up to 100 µg of the final preparation, and indicated that protein-bound carbohydrate was absent (results not shown).

Determination of native molecular mass. The native molecular mass of the enzyme as estimated by gel filtration of the final preparation on a f.p.l.c. Superose 6 HR 10/30 column was 159 ± 2.5 kDa (mean $\pm$ S.E.M., $n = 3$). Thus the native enzyme appears to be homotetrameric.

Heat-stability. The enzyme was relatively heat-stable, losing 0, 33 and 50 % of its original activity when incubated for 3 min at 50, 55 and 60 °C respectively. Incubation at 50 °C for 8 min resulted in only a 5 % loss in activity.

Activation energy. An Arrhenius plot was linear over the temperature range 2–32 °C (results not shown). The activation

Fig. 3. Non-denaturing PAGE of purified carrot root aldolase

(a) Protein staining was performed with Coomassie Blue G-250 [14]. (b) Distribution of aldolase activity; activity was determined as described in the Experimental section. The protein-stained lane contains 2 µg of protein, whereas the lane used to detect aldolase activity contains 6 µg of protein. O, origin; TD, tracker dye front.

Fig. 4. Isoelectric focusing in urea of purified carrot root aldolase

The single lane contains 5 µg of protein. The pI values shown were determined by using pH 4–6.5 ampholytes. Protein staining was performed with Coomassie Blue G-250 [14].

energy (E_a) for the enzyme was calculated from the Arrhenius equation to be $68.3 \text{ kJ} \cdot \text{mol}^{-1}$.

Absorption coefficient. The molar absorption coefficient of carrot root aldolase was determined to be $8.08 \times 10^{-4} \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm ($A_{1\text{cm}, 280}^{0.1\%}$ 0.508). This value was based on the bicinchoninic acid determination of protein concentration [17] and calculated by assuming a native molecular mass of 159 kDa.

Immunological characterization. The effect of anti-(carrot root aldolase) immune serum on the activities of carrot root aldolase and spinach leaf cytosolic and plastid aldolases is shown in Fig. 5. Increasing amounts of this serum immunoprecipitated up to 94 % of the activity of carrot root and cytosolic spinach leaf aldolase. No immunoremoval of spinach leaf plastid aldolase

Fig. 5. Effect of rabbit anti-(carrot root aldolase) immune serum on the activities of purified carrot root aldolase (●), spinach leaf cytosolic aldolase (○) and spinach leaf chloroplast aldolase (□)

Spinach leaf cytosolic and chloroplast aldolase isoenzymes were separated by ion-exchange chromatography [2]. All immunotitrations were performed with 0.006 unit of each aldolase preparation as described in the Experimental section.

Fig. 6. Western-blot analysis of carrot root and spinach leaf aldolases

Samples were subjected to SDS/PAGE and blot-transferred to nitrocellulose. Western analysis was performed with affinity-purified rabbit anti-(carrot root aldolase) IgG, and antigenic peptides were detected by using alkaline-phosphatase-linked secondary antibody as described in the Experimental section; phosphatase staining was for 3 min at 37 °C. Lane 1 contains 25 ng of homogeneous carrot root aldolase, lane 2 contains 0.80 µg of protein of partially purified spinach leaf cytosolic aldolase, and lane 3 contains 0.11 µg of protein of partially purified spinach leaf chloroplast aldolase. O, origin; TD, tracker dye front.

occurred. Pre-immune serum had no effect on any aldolase activity (results not shown).

Western blots probed with affinity-purified rabbit anti-(carrot aldolase) IgG revealed one immunoreactive protein, migrating with a molecular mass of 40 kDa when directed against purified carrot root aldolase or partially purified spinach leaf cytosolic aldolase (Fig. 6, lanes 1 and 2). No immunological cross-reactivity was detected when a Western blot of partially purified spinach leaf chloroplast aldolase was probed with the affinity-purified anti-(carrot aldolase) IgG (Fig. 6, lane 3). Furthermore, no immunoreactivity of any band was observed when rabbit pre-immune serum was substituted for the anti-(carrot aldolase) IgG (results not shown). These results are consistent with those of Fig. 5.

Table 2. Amino acid composition of carrot root aldolase compared with cytosolic and chloroplast spinach leaf aldolases

The amino acid composition of the spinach leaf aldolases is from [2]; nd, not determined.

Amino acid	Carrot root aldolase*	Spinach leaf aldolase	
		Cytosolic*	Chloroplast†
Asp + Asn	34	28	29
Thr	22	26	19
Ser	27	23	27
Glu + Gln	49	41	39
Pro	19	17	18
Gly	40	38	32
Ala	49	40	39
Half-Cys	4	6	6
Val	23	20	17
Met	4	2	5
Ile	12	14	13
Leu	38	36	32
Tyr	10	11	10
Phe	9	10	8
His	5	9	9
Lys	21	33	25
Arg	14	12	16
Trp	nd	nd	nd

* Expressed as residues/40 kDa.

† Expressed as residues/38 kDa.

Table 3. Kinetic constants for substrates and inhibitors of carrot root aldolase

Substrate	V_{max} (units/mg of protein)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	h
FBP	26.3	64.5	6	10.8	1.06
SBP	10.3	25.2	6	4.2	1.17

Inhibitor	I_{50}^* (mM)
PEP	2.6
6-Phosphogluconate	4.2
Ribose 5-phosphate	5.3
Glucose 1-phosphate	8.2
MgAMP	8.8

* Standard assay conditions were used, except that the concentration of FBP was 5 µM.

Amino acid analysis and N-terminal sequencing

N-Terminal analysis was attempted on the purified protein, but it was revealed that the enzyme was blocked at the N-terminus. The amino acid composition of carrot root aldolase, shown in Table 2, reveals that it is closer in composition to cytosolic spinach leaf aldolase than to the chloroplast form with respect to glycine, leucine and valine residues, but not to lysine or serine.

Kinetic properties

Effect of pH. The enzyme showed a fairly broad pH/activity profile, with a maximum occurring at about pH 7.4. All kinetic studies were subsequently carried out at pH 7.4.

Effect of cations. Aldolase activity was measured in the absence and presence of 1 mM-MgCl₂ or 1 mM-EDTA. No difference in activity was shown with or without MgCl₂ or EDTA (results not shown).

Substrate specificity. Table 3 shows the $V_{\max}$, k_{cat} , K_m , h and specificity constants (k_{cat}/K_m) obtained with FBP and SBP as substrates. In both cases Michaelis-Menten kinetics were observed. Although the K_m values were equal, the specificity constant with FBP was about 2.6 times that obtained with SBP. Notable are the relatively low catalytic-centre activities of 65 and 25 s⁻¹ for FBP and SBP respectively. Neither fructose 1-phosphate (20 mM) nor fructose 6-phosphate (5 mM) acted as substrates for the enzyme.

Metabolite effects. A wide variety of compounds were tested for effects on aldolase at approximate K_m concentrations of FBP (5 μ M). The following substances had no effects ($\pm 10\%$ control rate) on enzyme activity: DTT, dithioerythritol, P_i and dehydroascorbate (all 10 mM); EGTA, UDP-glucose, 3-phosphoglycerate, glycolate, 2-oxoglutarate, glucose 6-phosphate, malate, 2-phosphoglycerate, dimagnesium citrate, GSH, GSSG, NH₄Cl, glycine, glutamate, aspartate, alanine, cysteine, sodium pyrophosphate, glucose 1-phosphate, fructose 6-phosphate, fructose 1-phosphate, EDTA, glucose, sucrose, MgATP, MgADP (all 5 mM), Ca²⁺/bovine brain calmodulin (50 μ M/2 μ M), fructose 2,6-bisphosphate (50 μ M), acetyl-CoA (50 μ M) and trifluoperazine (50 μ M). Table 3 lists those substances that were found to inhibit the activity of the purified enzyme. Inhibition by PEP was determined to be mixed type with respect to FBP ($K_i = 3.2$ mM, $K'_i = 5.1$ mM). No activators were found.

DISCUSSION

A single FBP aldolase has been purified from carrot storage roots. The enzyme was purified about 850-fold to a final specific activity of 26.3 units/mg. Q-Sepharose anion-exchange f.p.l.c. was unable to resolve two forms of the enzyme. Previously, conventional ion-exchange chromatography without the use of the f.p.l.c. system has been able to resolve cytosolic and chloroplast isoenzymes of aldolase from a green alga and leaf extracts of spinach, corn, pea and wheat [2,6,7]. In the present study, Mono-Q f.p.l.c. was utilized for two reasons: first, a homogeneous preparation could be achieved, and second, it was thought that the increased resolution possible with this column would separate, if present, plastid and cytosolic isoenzymes of aldolase which could not be separated with Q-Sepharose. In all preparations, however, a sharp peak of protein and aldolase activity co-eluted from the Mono-Q column (Fig. 1).

SDS/PAGE of the final preparation revealed a single protein-staining band with a molecular mass of 40 kDa (Fig. 3). Identical results have been obtained with aldolase purified from rabbit muscle and spinach leaf or germinating mung-bean cytosol [1,2,5]. In contrast, spinach leaf plastid aldolase has a subunit molecular mass of about 38 kDa [2]. Non-denaturing PAGE revealed a single protein-staining band which co-migrated with aldolase activity. The apparent native molecular mass (159 kDa) implies that, like all other aldolases purified to date, the carrot root enzyme is homotetrameric.

The enzyme was found to be relatively heat-stable, which is similar to the reported heat-stability of chicken aldolase A₄ and spinach leaf cytosolic aldolase [2]. Conversely, spinach leaf plastid aldolase is almost completely inactivated by the same heat treatment [2]. In both maize and spinach, cytosolic aldolase has a blocked N-terminus [2,21], as was shown with carrot root aldolase, whereas spinach plastid aldolase has a free N-terminus

[2]. The amino acid composition of carrot root aldolase was similar to that of other plant cytosolic aldolases (Table 2) [2,4,21]. The above data are consistent with a cytosolic localization for the single carrot root aldolase purified in the present study.

Rabbit anti-(carrot root aldolase) immune serum immunoprecipitated carrot root aldolase and spinach leaf cytosolic aldolase activity up to 94%, but had no effect on spinach leaf plastid aldolase (Fig. 5). These data are supported by Western-blot analysis. Western blots revealed that only spinach leaf cytosolic aldolase and carrot root aldolase cross-reacted with the rabbit anti-(carrot root aldolase) IgG (Fig. 6, lanes 1 and 2). No immunological reactivity was seen with spinach leaf chloroplast aldolase. These immunological data indicate a high degree of structural similarity between the carrot and spinach leaf cytosolic aldolases, and further demonstrate a cytosolic origin for the carrot storage-root aldolase.

The purified enzyme showed a fairly broad pH/activity profile, with an activity peak at approx. 7.4. The enzyme is also a class I aldolase (non-metal-requiring) and showed an activation energy of 68.3 kJ·mol⁻¹. Denaturing isoelectric focusing suggests that two subunits of slightly different pI exist (5.6 and 5.7). These values are about 1 pH unit higher than previously published pI values for plant aldolases [4]. The microheterogeneity of carrot root aldolase was not dependent on oxidizable thiol groups or varying degrees of glycosylation. The presence of two closely focused bands may indicate that one band is phosphorylated. Recently it was demonstrated that aldolase from *Escherichia coli* is phosphorylated *in vivo* [22]. Further investigations are required to determine if carrot root aldolase is indeed phosphorylated.

Carrot root aldolase demonstrated an equal K_m for FBP and SBP, but had a 2.6-fold higher specificity constant with FBP as substrate (Table 3). Previous reports have shown that, as is the case with carrot root aldolase, higher plant cytosolic aldolases have an equal K_m for SBP and FBP, whereas plastid aldolases have a 2–4-fold lower K_m for SBP [4]. Although various workers have demonstrated aldolase activity with high concentrations of fructose 1-phosphate [4,6], we were unable to detect carrot root aldolase activity with up to 20 mM-fructose 1-phosphate. MgAMP, glucose 1-phosphate, ribose 5-phosphate and 6-phosphogluconate caused minor inhibition of the enzyme ($I_{50} \geq 4.2$ mM; Table 3). Owing to the central role of PEP in plant carbohydrate metabolism, the inhibition by this compound was investigated further. PEP was found to inhibit aldolase in a mixed-type manner, suggesting that the enzyme may possess an allosteric site. The significance of PEP inhibition of carrot root aldolase is unclear, as the K_i and K'_i values are at least 20-fold in excess of estimated PEP levels in plant tissues. PEP is a potent allosteric inhibitor of plant 6-phosphofructokinase [23]. However, the inhibition of aldolase by PEP has only been recently demonstrated for the cytosolic enzyme from germinating bean seeds [5]. Of interest are the findings that in certain animal tissues aldolase may possess regulatory (allosteric) properties, and may participate in regulating glycolytic flux [24–26]. More work is required to elucidate fully the potential role of aldolase in the regulation of plant glycolysis.

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