



Purification and Characterization of Aldolase from the Cold Hardy Insect *Epiblema scudderiana*: Enzyme Role in Glycerol Biosynthesis

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Aldolase was purified to homogeneity from larvae of the freeze avoiding gall moth, *Epiblema scudderiana* to a final specific activity 16.5 U/mg protein. The tetrameric enzyme had a native molecular weight of 160 ± 11 kDa and a subunit molecular weight of 37.8 ± 1.0 kDa. Aldolase in both 15°C and -4°C acclimated larvae occurred in a single enzyme form with an isoelectric point of 5.0–5.1. The pH optimum of the enzyme was 6.0 when assayed in imidazole buffer at 22°C and increased to 6.8 at 5°C . The Arrhenius plot was linear between 5 and 40°C with an activation energy of 68.9 ± 2.31 kJmol $^{-1}$. K_m values for fructose 1,6-bisphosphate increased 2-fold when assay temperature was decreased from 22 to 5°C . No allosteric activators of the enzyme were found but α -glycerophosphate, inorganic phosphate, and glycerol were effective inhibitors under the saturating substrate concentrations that exist for aldolase during glycerol biosynthesis. Inhibitory effects of α -glycerophosphate and inorganic phosphate increased when assayed at 5°C but the opposite was true of inhibition by glycerol. Inhibitory controls on aldolase may function to help bring about the cessation of cryoprotectant synthesis as well as maintain the cryoprotectant glycerol pool over the winter months.

Cryoprotectant biosynthesis Insect cold-hardiness Glycerol metabolism

INTRODUCTION

Many terrestrial insects endure deep subzero temperatures while wintering in seasonally cold regions of the earth. Survival at temperatures below the freezing point of body fluids is dependent on one of two strategies—freeze avoidance, the ability to stabilize a supercooled liquid state, or freeze tolerance, the ability to endure extracellular ice formation within the body (Zachariassen, 1985; Storey and Storey, 1992). An important adaptation supporting both strategies is the accumulation of high concentrations of low molecular weight cryoprotectants, most often polyhydric alcohols. Glycerol concentrations in many freeze avoiding insects can reach 2 M or more, representing about 20% of the fresh weight of the animal over the winter months. For freeze avoiding insects, high glycerol concentrations

act colligatively to depress the freezing point of body fluids and promote supercooling to very low temperatures. Such high levels of glycerol in the body fluids of gall moth larvae, *Epiblema scudderiana* (Clemens) (Lepidoptera: Olethreutidae) help to lower the supercooling point to -38°C , a temperature well below the mean environmental temperatures typically encountered by the species in winter (Rickards *et al.*, 1987).

Aspects of the enzymatic control of polyol biosynthesis by cold-hardy insects have been investigated in several species (for review Storey and Storey, 1991). A primary focus has been on the regulation of fat body glycogen phosphorylase that controls glycogenolysis to provide substrate for polyol synthesis. Other studies have assessed the induction of specific enzymes of polyol metabolism during development or cold-hardening, the role of the hexose monophosphate shunt in providing reducing equivalents for the conversion of sugars to polyols, and the differential control of enzymes that is needed in species that produce dual cryoprotectants. Less attention has been given to what enzymatic controls are necessary in order to stabilize the cryoprotectant pool over the winter and what regulatory mechanisms

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Abbreviations used: F1,6P₂, fructose-1,6-bisphosphate; α GP, α -glycerophosphate; P_i, inorganic phosphate; SDS, sodium dodecyl sulfate.

are involved in promoting cryoprotectant catabolism in the spring. The present study analyzes the regulation of the aldolase reaction, the enzyme that interconverts fructose 1,6-bisphosphate (F1,6P₂) and the triose phosphates, glyceraldehyde-3-phosphate and dihydroxyacetonephosphate. Control of this reaction appears to contribute to both promoting glycerol biosynthesis at low temperature and maintaining the glycerol pool over the winter months.

MATERIALS AND METHODS

Animals and chemicals

Galls containing caterpillars of the gall moth *E. scudderiana* were collected in the autumn of 1989. Galls were acclimated in the lab for 3 weeks at either 15 or -4°C . These temperatures were chosen because at 15°C no glycerol biosynthesis occurs whereas -4°C stimulates rapid glycerol production by *E. scudderiana* (Churchill and Storey, 1989). After acclimation galls were opened, larvae were removed and frozen in liquid nitrogen, and then transferred to -80°C for storage. Biochemicals were purchased from Boehringer Mannheim Corp., Montreal, P.Q. or Sigma Chemical Co., St Louis Mo and were of the highest quality available. Sephacryl S-300 was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Enzyme purification

For enzyme purification larvae acclimated to -4°C were used. Frozen larvae (c. 1 g or 20 larvae) were homogenized 1:4 (w/v) in homogenizing buffer containing 50 mM imidazole-HCl (pH 7.2) and 15 mM 2-mercaptoethanol (buffer A) using an Ultra Turrax Homogenizer. A few crystals of solid phenylmethylsulfonyl fluoride, a protease inhibitor, were added immediately prior to homogenization. The homogenate was then centrifuged in a Sorvall RCSB refrigerated centrifuge at 27,000 g for 25 min at 5°C . The supernatant was removed and layered onto a column of phosphocellulose (1.5 \times 5 cm) equilibrated in buffer A. The column was washed with 2 \times 10 ml of buffer A and then aldolase was eluted in a single peak with a linear salt gradient from 0 to 1 M KCl in buffer A. One ml fractions were collected and assayed; peak fractions were pooled and dialyzed for 2 h against buffer A.

The dialyzed enzyme was applied to a second phosphocellulose column also equilibrated in buffer A. The column was washed as above and then aldolase was eluted in a single peak using a linear gradient from 0 to 1.0 mM F1,6P₂ in buffer A. Peak fractions were pooled, dialyzed as above, and then used as the aldolase source for further study.

Enzyme and protein assay

Aldolase activity was monitored by following NADH oxidation at 340 nm using a Gilford 240 recording spectrophotometer with a water-jacketed cell

holder attached to a Lauda K2/R waterbath for temperature control of the cuvettes; enzyme kinetics were assessed at 22 and 5°C . Optimal assay conditions were 50 mM imidazole-HCl buffer (pH 7.2), 0.1 mM F1,6P₂, 0.15 mM NADH, 1 U triose phosphate isomerase and 2 U glycerol-3-phosphate dehydrogenase (Storey, 1980). Protein was measured by the method of Bradford (1976) using the BioRad Laboratories prepared reagent and bovine gamma globulin as the standard.

Kinetic studies

Substrate affinity constants (K_m values) were determined using a nonlinear least squares regression computer program (Brooks, 1992). I_{50} values (the concentration of inhibitor that decreases enzyme velocity by 50%) were determined using plots of $V_{\max}/V_{\text{inhibitor}}$ vs [inhibitor]. K_i values for P₁, α GP, and glycerol were determined from plots of $1/V$ vs [inhibitor] at different F1,6P₂ concentrations. An Arrhenius plot was constructed by measuring enzyme activities under optimal assay conditions at intervals over the 5 – 40°C range. All kinetic parameters are means \pm SEM for $n = 3$ separate preparations of purified aldolase.

Molecular weight determination

The native molecular weight of aldolase was determined using a Sephacryl S-300 gel filtration column (45 \times 1 cm) equilibrated in 50 mM KH₂PO₄, 15 mM 2-mercaptoethanol, 0.1% w/v NaN₃ and 10% v/v glycerol, pH 7.2. Fractions of 0.5 ml were collected and assayed. Standards were monitored by activity assays at 340 nm for rabbit muscle phosphofructokinase (360,000), rabbit muscle aldolase (160,000), rabbit liver fructose 1,6-bisphosphatase (140,000) or by absorbance at 280 nm for bovine hemoglobin (64,500) and bovine heart cytochrome c (13,370). The molecular weight of aldolase was determined from a plot of K_a vs log mol. wt for the protein standards.

SDS-PAGE

Samples were run on a 10% w/v acrylamide lower gel and a 3.5% w/v stacking gel for 5 h under a constant current of 50 mA. The running buffer was a 24.8 mM Tris, 192 mM glycine, pH 8.8 containing 1% w/v SDS. Protein samples were mixed 1:1 v/v with this buffer in Eppendorf tubes and incubated in a boiling water bath for 90 s prior to loading onto the gel. The subunit molecular weight standards for the gel were glycogen phosphorylase *b* (97,400), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), bovine erythrocyte carbonic anhydrase (29,000), and bovine α -lactalbumin (14,200). The gel was fixed overnight in 10% v/v acetic acid + 25% v/v methanol, stained for 2 h in 0.25% Coomassie brilliant blue R + 50% methanol + 7.5% acetic acid, and then destained overnight in 25% methanol + 10% acetic acid. The subunit molecular weight was determined from a plot of R_f value vs log mol. wt of the protein standards.

Isoelectrofocusing

Column isoelectric focusing was performed by the method of Vesterberg (1971) using 27,000 g supernatants as the enzyme source. A pH gradient of 3.5 to 10 LKB ampholines was run in a sucrose density gradient using a LKB Products 8101 (110 ml) column. The column was developed at 500 V for 14 h and then 1 ml fractions were collected and assayed for activity.

RESULTS

Aldolase purification and molecular weight determination

Aldolase was purified by chromatography on two phosphocellulose columns. Elution from the first column was with a KCl gradient with peak enzyme activity eluting at 450 mM KCl [Fig. 1(a)]. Peak fractions from this column were then pooled and applied to a second column and eluted this time with a gradient of F1,6P₂. The peak of activity eluted at 300 μM F1,6P₂ [Fig. 1(b)]. The columns gave a net purification of 298-fold and resulted in a final specific activity of 16.5 U/mg protein. SDS-PAGE of the purified enzyme showed a single

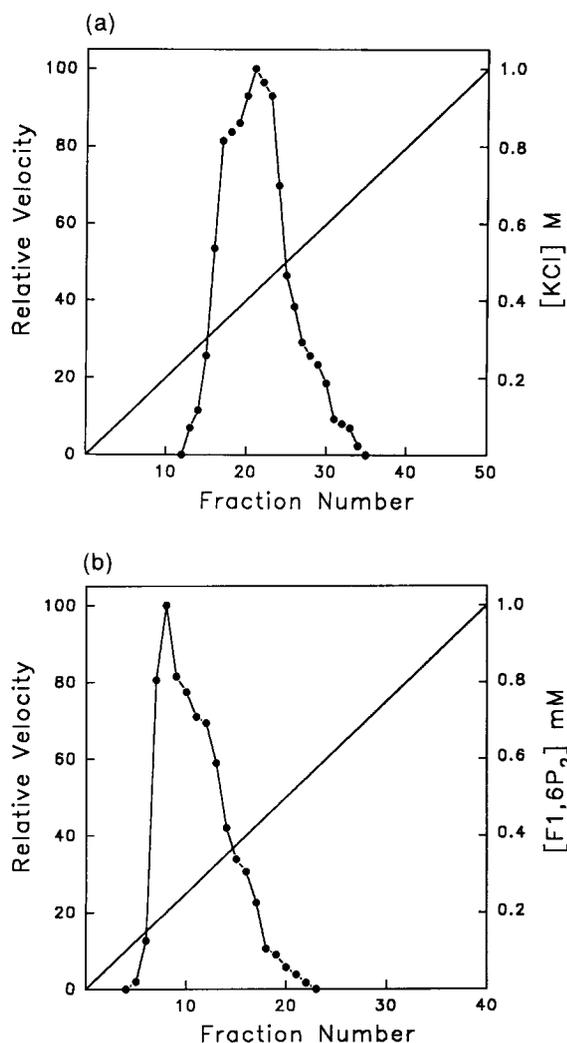


FIGURE 1. Purification of *E. scudderiana* aldolase using sequential phosphocellulose columns with gradient elution by (A) 0-1 M KCl, and (B) 0-1 mM F1,6P₂.

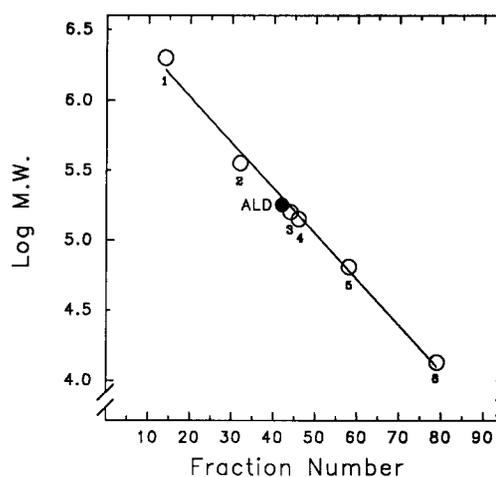


FIGURE 2. Native molecular weight of *E. scudderiana* aldolase determined by Sephacryl S-300 gel filtration with standards: 1, blue dextran; 2, phosphofructokinase; 3, rabbit muscle aldolase; 4, fructose-1,6-bisphosphatase; 5, bovine hemoglobin; and 6, bovine heart cytochrome c.

band indicating a homogeneous preparation. The subunit molecular weight determined from SDS-PAGE was $37,800 \pm 1000$ ($n = 3$). The native molecular weight of *E. scudderiana* aldolase, determined by Sephacryl S-300 gel filtration, was $160,000 \pm 11,000$ Da ($N = 3$) (Fig. 2).

Substrate affinity

In *E. scudderiana* larvae at 22°C glycerol synthesis is inactive (autumn) and glycerol catabolism is promoted (spring) whereas at 5°C glycerol synthesis occurs at high rates (autumn) and glycerol pools are subsequently maintained throughout the winter as long as temperature remains low (Storey and Storey, 1992). Thus, aldolase kinetics were assessed at both high (22°C) and low (5°C) temperature to determine how the enzyme would function under different metabolic conditions in the larvae *in vivo*. The relationship between aldolase activity and F1,6P₂ substrate concentration was hyperbolic at both assay temperatures. K_m values for F1,6P₂ were 1.13 ± 0.01 μM at 22°C and 2-fold higher, 2.45 ± 0.01 μM at 5°C.

Allosteric inhibitors

Various metabolites were tested for allosteric effects on aldolase using a suboptimal (physiological) concentration of F1,6P₂ (5 μM); these included Mg²⁺, citrate, Mg citrate (1:1), ATP, Mg ATP (1:1), phosphoenolpyruvate, α-glycerophosphate (αGP), inorganic phosphate (P_i), fructose-6-phosphate, glucose-6-phosphate, fructose-2,6-bisphosphate, and glycerol. No enzyme activators were found. Table 1 shows I_{50} values for those compounds that were effective inhibitors at subsaturating (5 μM) levels of F1,6P₂. The enzyme was inhibited by both ATP and citrate and in both cases inhibition decreased (I_{50} increased) when the compounds were added as 1:1 mixtures with MgSO₄. α-Glycerophosphate and inorganic phosphate also in-

TABLE 1. I_{50} values for inhibitors of *E. scudderiana* aldolase measured at a suboptimal (5 μ M) F1,6P₂ concentration

Inhibitor	I_{50} value (mM)	
	22°C	5°C
ATP	2.6 ± 0.13	7.3 ± 0.18*
Mg ATP	7.9 ± 0.23	7.5 ± 0.54
Citrate	5.0 ± 0.29	5.6 ± 0.28
Mg citrate	9.7 ± 0.40	8.1 ± 0.17
MgSO ₄	14.8 ± 1.52	19.3 ± 0.58
α -Glycerophosphate	4.6 ± 0.23	2.3 ± 0.20*
Inorganic phosphate	7.3 ± 0.61	3.5 ± 0.03*

Data are means \pm SEM for $n = 3-5$ separate enzyme preparations. Mg ATP and Mg citrate stock solutions contained MgSO₄ in a 1:1 molar ratio. *Significantly different from the corresponding value at 22°C by the Student's t -test, $P < 0.05$.

hibited the enzyme. Inhibition by ATP decreased significantly at 5°C (I_{50} increased), as compared with 22°C, but the inhibitory effects of α GP and P_i increased at low temperature, the I_{50} for each dropping by 2-fold at 5°C.

At a saturating concentration of F1,6P₂ (0.1 mM), inhibitors were much less effective; several compounds no longer inhibited the enzyme under these conditions and I_{50} values for ATP, α GP and P_i increased to high levels (Table 2). Low assay temperature had the same qualitative effects under saturating F1,6P₂ concentration as were seen in Table 1: I_{50} values for P_i and α GP decreased significantly whereas at 5°C inhibition by ATP was abolished. The sugar phosphates (F6P, G6P, PEP, F2,6P₂) did not inhibit the enzyme in the presence of saturating amounts of F1,6P₂, as also occurred at sub-saturating F1,6P₂.

Further analysis of inhibitor effects on aldolase showed that inhibition was of the mixed competitive type. K_i values are shown in Table 3. Low temperature led to significant decreases in the K_i values for both inorganic phosphate and α -glycerophosphate; K_i for P_i dropped by 7-fold at 5°C compared with 22°C whereas K_i α GP decreased by 25% at the lower temperature. Glycerol effects on the enzyme were also assessed; very high concentrations of the polyol were inhibitory and the K_i increased by 4-fold at 5°C.

pH optima, isoelectric points and Arrhenius plots

Figure 3 shows the effect of pH on aldolase activity at both high and low temperature. Aldolase was relatively

TABLE 2. I_{50} values for inhibitors of *E. scudderiana* aldolase measured at optimal (0.1 mM) F1,6P₂ concentration

Inhibitor	I_{50} value (mM)	
	22°C	5°C
ATP	19.2 ± 0.14	—
Inorganic phosphate	70.0 ± 2.8	21.5 ± 1.31*
α -Glycerophosphate	18.1 ± 0.44	12.2 ± 0.88*

Data are means \pm SEM for $n = 3-5$ separate enzyme preparations. *Significantly different from the corresponding value at 22°C by the Student's t -test, $P < 0.001$.

TABLE 3. Inhibition of *E. scudderiana* aldolase

Inhibitor	K_i at 22°C	K_i at 5°C
Inorganic phosphate, mM	5.35 ± 0.14	0.72 ± 0.04*
α -Glycerophosphate, mM	3.00 ± 0.26	2.20 ± 0.07*
Glycerol, M	0.35 ± 0.02	1.45 ± 0.06*

Data are means \pm SEM for $n = 3-5$ enzyme preparations.

*Significantly different from the corresponding value at 22°C by the Student's t -test, $P < 0.001$.

insensitive to pH change over a broad range between about pH 5.5 and 7 at 22°C and showed a similarly broad optimum at 5°C. The pH optimum shifted with the change in assay temperature from a peak at pH 6.0 at 22°C to an optimum at pH 6.8 at 5°C.

Isoelectric focusing of crude extracts from both warm (15°C) and cold (-4°C) acclimated larvae showed single peaks of aldolase activity in both cases (Fig. 4). Isoelectric points (pI) were 5.0 and 5.1 (each a mean of two runs) for the two groups of larvae, respectively, and were not significantly different from each other.

Figure 5 shows the effect of assay temperature on *E. scudderiana* aldolase activity presented as an Arrhenius plot. The relationship was linear over the range from 5 to 40°C with a calculated activation energy of 68.9 ± 2.31 kJ mol⁻¹. Calculated Q_{10} for the 0-10°C interval was 2.94 ± 0.10 .

DISCUSSION

E. scudderiana aldolase was purified 300-fold to a homogeneous preparation with a final specific activity of 16.5 U/mg. This compares favorably with the purification of the enzyme from other sources and final specific activities of 10.9 U/mg in the dipteran, *Ceratitis capitata*, 13 U/mg in locust flight muscle, and 16 U/mg for rabbit skeletal muscle (Fernandez-Sousa *et al.*, 1978;

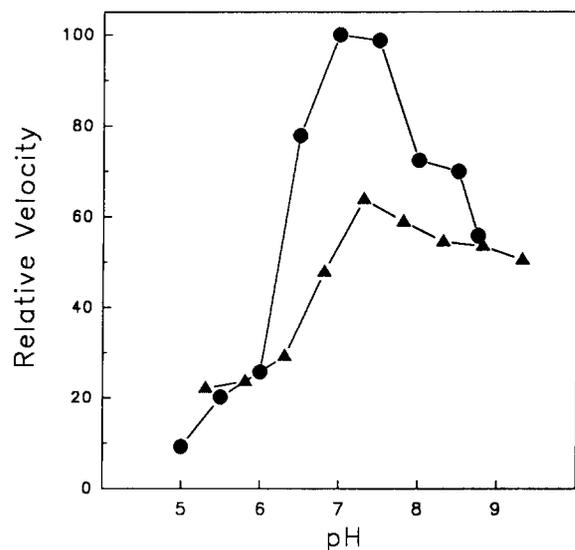


FIGURE 3. Effect of pH on *E. scudderiana* aldolase activity at 22°C (circles) and 5°C (triangles). Data are means of $n = 3$ trials of separate preparations of purified enzyme; SEM bars are contained within the symbols.

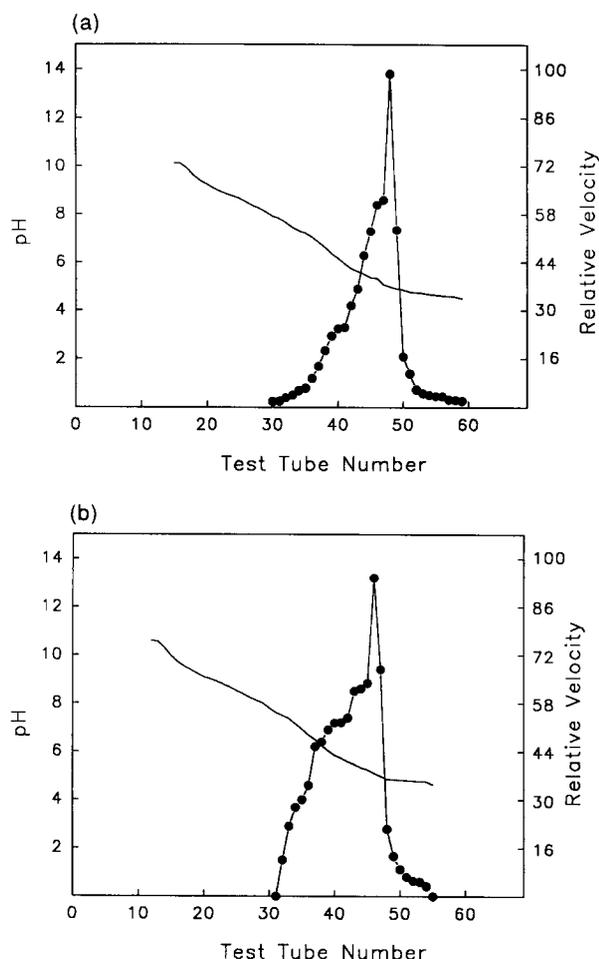


FIGURE 4. Isoelectric focusing of aldolase from autumn *E. scudderiana* larvae acclimated to (A) 15°C or (B) -4°C. Data are means of $n = 2$ runs. Circles show aldolase activity; line shows measured pH values.

Grazi and Trombetta, 1979; Storey, 1980). The native molecular weight of *E. scudderiana* aldolase was 160 ± 11 kDa, comparable to values of 155–162 kDa for Class 1 aldolases from both insect and mammalian sources (Levenbook *et al.*, 1973; Bauer *et al.*, 1978;

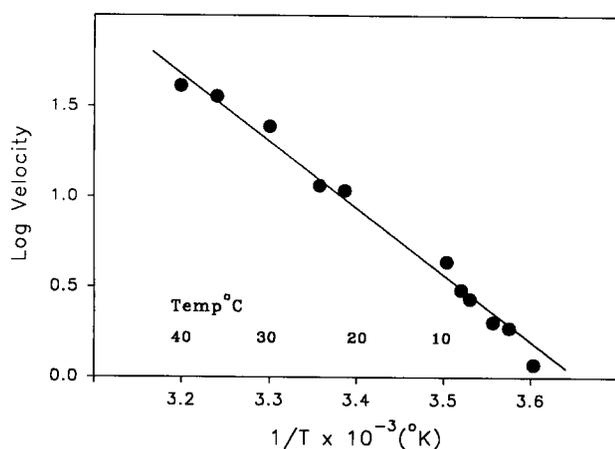


FIGURE 5. Arrhenius plot for *E. scudderiana* aldolase. Data are means of determinations on $n = 4$ preparations; SEM bars are contained within the symbols used.

Chappel *et al.*, 1978; Storey, 1980). The subunit molecular weight of *E. scudderiana* aldolase (37.8 ± 1 kDa) was also similar to the range of 40–42 kDa reported for the enzyme from other sources and indicated that the enzyme was a tetramer like other animal aldolases (Levenbook *et al.*, 1973; Bauer *et al.*, 1978; Chappel *et al.*, 1978; Storey, 1980). Isoelectrofocusing showed a single peak of aldolase in all cases with pI values of 5.0–5.1 that did not change with acclimation of the larvae to two different temperatures (Fig. 4). Hence, the enzyme does not occur in isozymic forms; this was also supported by the single peaks of enzyme activity eluted from both phosphocellulose columns. Aldolase from other insects also occurs in a single enzymatic form with pI values in the range 4.9–6.0 (Levenbook *et al.*, 1973; Fernandez-Sousa *et al.*, 1978; Storey, 1980).

Contrary to other Class 1 aldolases, however, *E. scudderiana* aldolase showed a rather low pH optimum of about 6.0 at 22°C, compared with values of 6.8–8.4 for the enzyme from other sources (Bauer *et al.*, 1978; Fernandez-Sousa *et al.*, 1978; Storey, 1980). The pH optimum increased to 6.8 when assay temperature decreased. This is a common feature of enzymes from ectothermic animals. Intracellular pH in ectotherms typically rises by *c.* 0.018 pH unit per 1°C decrease in temperature in a phenomenon called alaphastat regulation that maintains a constant charge state on the ionizable groups on proteins (Somero, 1981) and the pH optima of enzymes frequently track this.

E. scudderiana aldolase showed a hyperbolic relationship between velocity and substrate concentration that fitted the Michaelis–Menten equation. Like aldolase from other sources (Buczylko *et al.*, 1980; Storey, 1980), the enzyme showed a very high affinity for F1,6P₂ substrate (K_m 1.13 μ M at 22°C, 2.45 μ M at 5°C) that could make the enzyme highly sensitive to changes in substrate availability. Measured levels of F1,6P₂ *in vivo* were very high compared with this K_m value. F1,6P₂ levels were 0.14 μ mol/g wet wt in control larvae at 16°C and rose to over 0.40 μ mol/gww during active glycerol synthesis when larvae were switched to -4°C (Churchill and Storey, 1989); assuming 60% body water content (Rickards *et al.*, 1987), F1,6P₂ concentrations should be at least 230–670 μ M. It appears, then, that aldolase should be functioning under saturating substrate conditions *in vivo*. In line with this, our survey of metabolite effects on *E. scudderiana* found no allosteric activators of the enzyme, as also appears to be the case for other aldolase enzymes (Fernandez-Sousa *et al.*, 1978; Buczylko *et al.*, 1980; Storey, 1980).

However, temperature and temperature-modulator interactions may serve to modify aldolase activity in the larvae in a way which favours glycerol synthesis at low temperatures and glycerol catabolism at high temperatures. The Arrhenius plot was linear between 5 and 40°C and the activation energy, 68.9 kJ mol⁻¹ was similar to that reported for *C. capitata*, 75 kJ mol⁻¹ (Fernandez-Sousa *et al.*, 1978). This indicated no major effects of temperature on enzyme conformation or aggregation

state over this temperature range. However, a negative influence of low temperature was seen in the 2-fold rise in K_m for F1,6P₂ with the decrease in assay temperature from 22 to 5°C. Whether this change in K_m would have physiological relevance is questionable, however, given the high *in vivo* F1,6P₂ concentrations discussed above.

Several metabolites acted as allosteric inhibitors of *E. scudderiana* aldolase. Effects of ATP, citrate, α -GP and P_i occurred at concentrations that were near-physiological when tested at suboptimal (5 μ M) levels of F1,6P₂. Negative modulation by ATP and ATP-Mg²⁺ has also been reported for aldolase from other animal sources (Spolter *et al.*, 1964; Storey, 1980). ATP inhibition was greatly reduced at 5°C, and along with a transient drop in larval ATP levels that occurs during the period of active glycerol synthesis at low temperature (Churchill and Storey, 1989), this could facilitate flux through aldolase during glycerol production from glycogen. However, *E. scudderiana* aldolase was unique in that the enzyme did not exhibit inhibition by AMP and ADP, as is seen with aldolase from other animals (Spolter *et al.*, 1964; Storey, 1980). Magnesium ion (Mg²⁺) reduced the inhibitory effect of ATP and citrate as can be seen in Table 1, but alone the inhibitory capacity of the ion occurred at concentrations above physiological levels for this insect. Inhibitor effects by glycerol on aldolase were also temperature sensitive with inhibition decreasing at low assay temperature. This is a not unexpected result since glycerol accumulates to about 2 M in these larvae and hence, enzymes involved in glycerol biosynthesis should be relatively insensitive to the polyol (K_i was 1.45 M at 5°C) under the low temperature conditions that stimulate rapid glycerol production.

Inorganic phosphate and α -glycerophosphate were the most potent inhibitors of larval aldolase and K_i values for both compounds were well within physiological levels (Table 1). Inhibition by both of these compounds increased at low temperature. However, α GP levels in the larvae dropped from about 4 μ mol/g wet wt in control larvae at 16°C to 1 μ mol/g when glycerol accumulation was initiated by low temperature exposure (Churchill and Storey, 1989). This would serve to reduce α GP inhibition of aldolase during active glycerol biosynthesis. As glycerol levels rise towards their maximum, however, α GP levels again rise (Churchill and Storey, 1989; Storey and Storey, 1992) and, thus, α GP inhibition of aldolase could be one factor that helps to shut down glycerol synthesis. α GP is also a strong inhibitor of 6-phosphofructose-2-kinase (PFK-2) which synthesizes F2,6P₂; F2,6P₂ is a potent activator of 6-phosphofructose-1-kinase (PFK-1), the key regulatory enzyme of glycolysis (Storey and Storey, 1992). The fall in α GP when glycerol production is initiated also then facilitates flux through PFK-1 during cryoprotectant synthesis and correspondingly, the rise in α GP levels as cryoprotectant

synthesis comes to an end would result in feedback inhibition of PFK-2, and hence PFK-1. Through α GP inhibitory effects targeting several enzyme loci, a coordinated cessation of cryoprotectant synthesis can be achieved.

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