PURIFICATION AND CHARACTERIZATION OF THE GLYCOLYTIC ENZYMES HEXOKINASE AND PYRUVATE KINASE FROM *EUROSTA SOLIDAGINIS*

by

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A thesis submitted to
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in partial fulfillment of the requirements for the degree of

Master of Science

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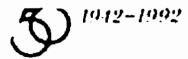

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Abstract

Larvae of the insect *Eurosta solidaginis* spend the winter in galls on goldenrod plants and survive low subzero temperatures by allowing the formation of extracellular ice within their bodies. Different mechanisms are used to allow survival of these extreme conditions: some ensure that the physical integrity of cells is maintained whereas other suppress metabolism so that only basal metabolic functions subsist. Glycolysis, which is at an intersection of several important metabolic pathways, must be subjected to fine regulation to allow the synthesis of the two cryoprotectants of the larvae, glycerol and sorbitol, during the autumn and as well as to facilitate metabolic depression during the winter.

In this study, hexokinase (EC 2.7.1.1) and pyruvate kinase (EC 2.7.1.40), two regulatory enzymes of glycolysis, were examined. Hexokinase was partially purified and kinetic studies were performed both at 6°C and 25°C and in the presence and the absence of the cryoprotectants of the insect, glycerol and sorbitol. The data indicate that besides a role in the regulation of glycolysis, hexokinase is probably involved in the control of the synthesis and catabolism of the two polyols.

Pyruvate kinase (PK) was purified to near homogeneity and kinetic and structural studies were performed on the purified preparations. Kinetic studies showed that the activity of PK was reduced at cold temperatures and in the presence of the two polyols, with an increase in inhibition by citrate and the loss of activation by fructose-1,6-bisphosphate. Fluorescence and heat denaturation studies underlined the importance of the cryoprotectants, especially sorbitol, for the stabilization of the enzyme.

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List of Abbreviations

ADP, ATP	adenosine di-, tri- phosphate
DHAP	dihydroxyacetone phosphate
E_a	activation energy
EDTA	ethylenediamine tetraacetate
EGTA	ethyleneglycol bis (B-aminoethyl ether) tetraacetate
$F1,6P_2$	fructose-1,6-bisphosphate
GuaHCl	guanidine hydrochloride
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
HK	hexokinase
I_{50}	concentration of inhibitor that lowers enzyme velocity by 50%
IEF	isoelectrofocusing
INP	ice-nucleating protein
K_a	concentration of activator that produces half of the maximal activation
K_m	Michaelis-Menten constant (substrate affinity constant)
KPi	potassium phosphate
LDH	lactate dehydrogenase
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
n_H	Hill coefficient

PEP	phosphoenolpyruvate
PFK	phosphofructokinase
PI	isoelectric point
PK	pyruvate kinase
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	tricarboxylic acid
THP	thermal hysteresis protein
V_{max}	maximal enzyme velocity

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CHAPTER ONE

GENERAL INTRODUCTION

All forms of life depend on chemical reactions. As every chemical reaction, they follow the thermodynamic laws, and their rate can vary greatly depending on temperature. Furthermore, the conformation of biochemicals, often essential for a proper function, will also be subjected to changes with temperature. To survive, it is therefore fundamental for organisms to cope with the variations of environmental temperature. Homeotherms (birds and mammmals) have chosen to free themselves from these variations by keeping a constant and optimal body temperature, using for that the heat produced during metabolic processes.

Poikilotherms, on the other hand, have their body temperature close to the external temperature. So one of their major problem is to keep all the reactions working even when temperature becomes unfavorable. In regions where temperature in winter can be as low as -30°C for days or even weeks, well-defined strategies to face cold are essential for survival.

Insect adaptations to cold

Most insects are poikilotherms, and for this reason, their body fluids should be frozen when they are exposed to very low environmental temperature. Two main strategies are used by insects to face temperatures that should be lethal. One of them is freeze tolerance, the second one is freeze avoidance. In this latter case, insects do not have the biochemical tools to deal with the formation of ice within their body. They need therefore to maintain body fluids in a liquid state. This is achieved by supercooling.

A supercooled state is metastable state where a solution remains liquid under its freezing point. Most animals can supercool to a certain extent, but survival at very low temperatures requires the implementation of specific mechanisms. These mechanisms

aim at (1) inhibiting the ice nucleation and (2) depressing the freezing point and supercooling point of the body fluids. The inhibition of ice nucleation is accomplished through the addition of antifreeze proteins to extracellular body fluids, the removal or masking of potential ice nucleating sites within the body and the shielding from environmental ice. The addition of low molecular weight cryoprotectants in body fluids and the decrease in total water content are used to achieved the second goal. Altogether, these mechanisms allow the supercooling point to be pushed down to -40°C or even -50°C (Storey and Storey, 1992).

The second of the strategies employed by insects to survive cold is freeze tolerance which is the ability to tolerate the formation of ice in the body tissues and fluids (Leather et al., 1993), and is the method adopted by the larvae of the insect *Eurosta solidaginis* (Fitch) (Diptera, Tephritidae) for overwintering. Up to 65% of the body fluids of these larvae can be frozen when temperatures below -10°C are reached. Freezing, however, cannot occur anywhere and anyhow, and insects need to prepare themselves for it. In the first place, freezing cannot take place within cells, or it will result in physical destruction of subcellular structures. Hence, the insect has to ensure that ice will form extracellularly. This is achieved through the production of ice nucleating proteins (INPs) in extracellular compartments and of low molecular solutes inside the cells: the INPs induce and control the formation of extracellular ice whereas low molecular weight solutes prevent intracellular ice formation by lowering the freezing point and increasing the content of unfreezable water. Thermal hysteresis proteins (THPs) also intervene to control the ice formation by inhibiting the recrystallization of ice particles and therefore the formation of large and potentially damaging crystals (Storey and Storey, 1992).

One of the consequences of freezing is the increase of solute concentrations in extracellular compartments. This produces an osmotic stress between the intracellular and extracellular solutions. Cells rapidly dehydrate and can collapse if dehydration is too severe. The answer of insects to this problem is the accumulation of high cellular

concentrations of low molecular weight cryoprotectants (polyhydric alcohols) that restrict the cell volume reduction. These solutes act also to stabilize the membrane bilayer and protect proteins from denaturation (Storey and Storey 1992).

Cryoprotectant system in *E. solidaginis*

Two polyhydric alcohols are used by the larvae of *E. solidaginis* for intracellular cryoprotection: glycerol and sorbitol (Baust 1981). Because of their high solubility in aqueous solutions, their absence of toxicity (they have few effects on metabolism even at very high concentrations) and the facility with which they cross cellular membranes, glycerol and sorbitol and more generally polyhydric alcohols are well adapted to cryoprotection purposes. The larvae can accumulate 0.5M to 0.6M glycerol and 0.2M sorbitol in haemolymph (Morrissey and Baust 1976). Figure 1-1 shows the levels of these two cryoprotectants over the winter time course (Storey and Storey 1986). Glycerol synthesis is triggered earlier than sorbitol, between 15°C and 5°C (Morrissey and Baust 1976, Storey et al. 1981a) well before the first exposure to freezing conditions. Glycerol provides a constant and permanent cryoprotection throughout the winter. Sorbitol, on the other hand, is produced in direct response to cold. Its synthesis begins below 5°C and continues down to temperatures as low as -10°C, the temperature at which freezing occurs (Storey et al. 1981). It provides a variable level of cryoprotection, the levels of this polyol following the increases or decreases of environmental temperature (Storey and Storey 1982 and 1983a).

Cryoprotectants synthesis and catabolism

Glycerol and sorbitol synthesis both begin at glycogen (see figure 1-2). Glycogen phosphorylase catalyzes its conversion into glucose-1-phosphate (G1P), and

phosphoglucosemutase the conversion of G1P into glucose-6-phosphate (G6P). Glycerol synthesis utilizes then the glycolytic pathway up to the synthesis of dihydroxyacetone phosphate (DHAP). It diverges at that point from glycolysis and DHAP is converted to glycerol in two steps.

For sorbitol synthesis, G6P is converted to glucose, the first substrate of glycolysis and then into sorbitol. During its catabolism, sorbitol is not reconverted to glucose but to fructose. The conversion of fructose to fructose-6-phosphate, catalyzed by hexokinase, is a key step which allows the return of sorbitol into the intermediary metabolism. It can finally be reconverted to glycogen or be used to produce energy by going through the end of glycolysis.

Glycolysis

Glycolysis is a series of 10 reactions starting from glucose and ending with pyruvate. It is the initial pathway of carbohydrates catabolism and is believed to be among the oldest of all the biochemical pathways. During this process, some of the potential energy stored in the hexose structure is released and used to synthesize ATP from ADP. Glycolysis has a central metabolic role, not only because it produces ATP, but also because it is closely coordinated with other major pathways of energy generation and utilisation, notably synthesis and breakdown of glycogen, gluconeogenesis, the pentose phosphate pathway and the citric acid cycle. Synthesis of cryoprotectants by insects is also intimately linked to glycolysis. The metabolic regulation of glycolysis is therefore of prime importance because it also controls a group of other pathways in a coordinated fashion.

For insects preparing themselves for overwintering, the flux of carbon through glycolysis will vary according to the stage of preparation. When glycerol is synthesized, most of the carbon entering the glycolysis will not be transformed into the last product ,

pyruvate. Instead, it will deviate from the glycolytic pathway to allow the massive synthesis of glycerol. Similarly, sorbitol synthesis requires the cessation of glycerol synthesis and the bringing into play of other mechanisms of regulation.

Regulation of metabolism

Regulation of metabolism is determined by enzyme control and regulation. There are two types of regulation by which control of enzyme activity can be exerted. The first one involves an alteration of enzyme activity with no change in the number of enzyme molecules. The second one implies a change in enzyme concentration. Alteration of enzyme activity can be induced by low molecular weight substances: first of all, variations of substrate concentration constitutes the simplest level of control. The second type of low molecular weight compounds altering enzyme activity, allosteric modifiers, includes inhibitors and activators. By binding to the enzyme in an area other than the active site, they induce a change in enzyme conformation and affect its catalytic efficiency. Some inhibitors can also compete with the substrate by binding at the active site of the enzyme. This kind of regulation can be affected by variations in temperature (Larner 1971, Storey 1992).

Enzyme activity can also be altered by covalent modification that can be reversible, for example protein phosphorylation and dephosphorylation, or irreversible (usually a hydrolytic modification).

The second important mechanism by which enzyme regulation can be exerted is the alteration of the number of enzyme molecules, achieved by control at transcription and translation levels and control of enzyme degradation (Larner 1971).

Regulation of glycolysis

As shown before, a fine regulation of glycolysis is a critical process for insect preparation to overwintering. Glycolysis regulation has been extensively studied and it is well known that it is primarily controlled by three enzymes, hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). The reactions catalyzed by these enzymes are irreversible and their activities can be regulated by the reversible binding of allosteric effectors or by covalent modification.

Hexokinase catalyzes the first step of glycolysis. It is allosterically regulated by the product of the reaction, glucose-6-phosphate. Because of its location, at the entry of glycolysis, HK could be thought of as the most important enzyme for the regulation of glycolytic pathway. This is not the case. Indeed, the product G6P is not only a glycolytic intermediate, it can also be converted into glycogen or it can be oxidized by the pentose phosphate pathway to form NADPH. The first irreversible reaction unique to the glycolytic pathway, called the committed step, is the phosphorylation of fructose-6-phosphate into fructose-1,6-bisphosphate. This is why PFK is the most important control element in glycolysis. PFK is inhibited by high levels of ATP, by citrate and activated by fructose-2,6-bisphosphate.

Finally PK, the enzyme catalyzing the third irreversible step in glycolysis, controls the outflow from this pathway. Fructose-1,6-bisphosphate, the product of the reaction catalyzed by PFK, activates PK. ATP, citrate and alanine have been shown to allosterically inhibit PK.

The role of PFK in the regulation of glycolysis during the synthesis of glycerol and sorbitol in the larvae of *Eurosta solidaginis* has already been investigated (Storey 1982). The maximal activity of PFK does not change in the larvae over the winter , showing that regulation of PFK is not due to a change in enzyme concentration but to an

alteration of the enzyme activity (Joanisse and Storey 1994). The aim of this study is to further explor the regulation of glycolysis in freeze tolerant insects, by studing the two other key enzymes involved in the control of glycolysis, hexokinase and pyruvate kinase in the freeze tolerant insect *Eurosta solidaginis*.

Figure 1-1 Concentrations of glycerol and sorbitol in the larvae of the gall fly, *Eurosta solidaginis*.

This figure shows the levels of the cryoprotectants in *E.solidaginis* measured during the autumn, winter and spring 1982-1983 (Storey and Storey 1986).

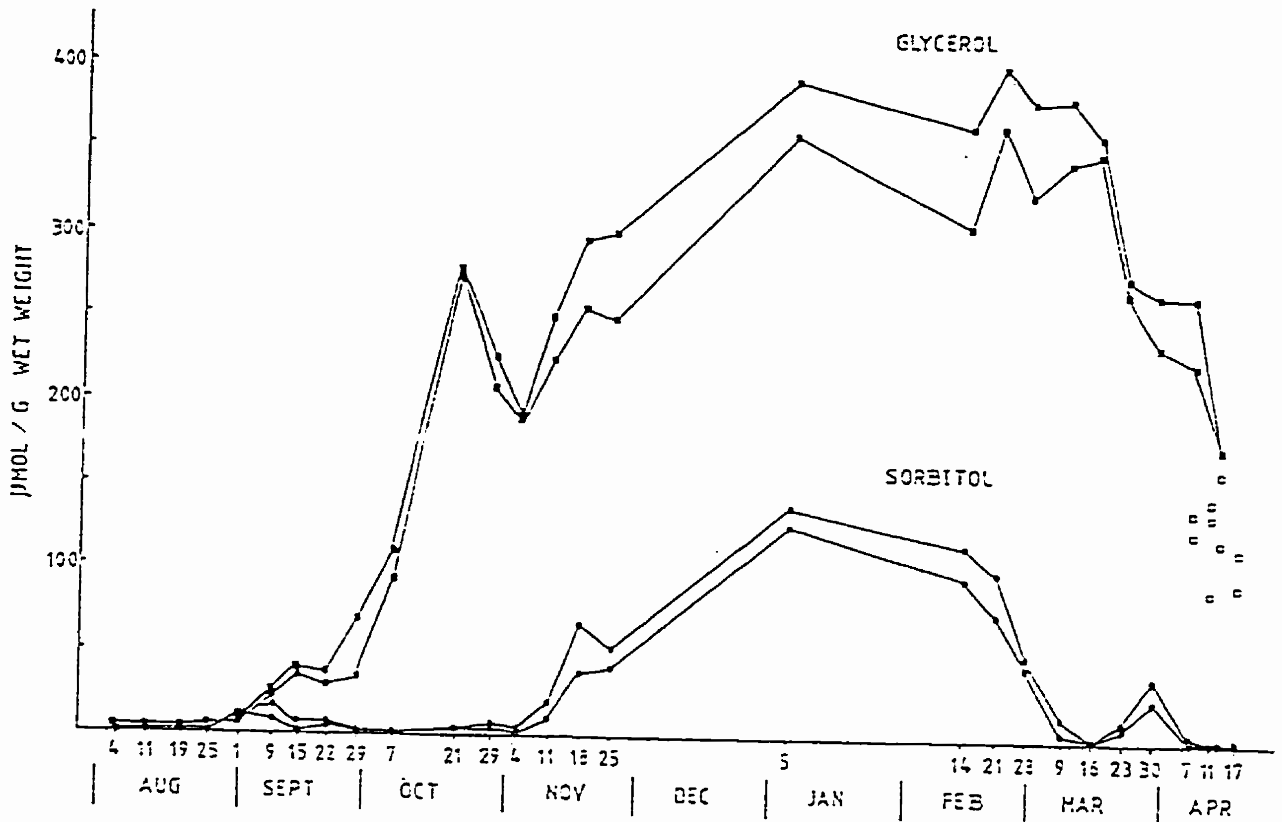
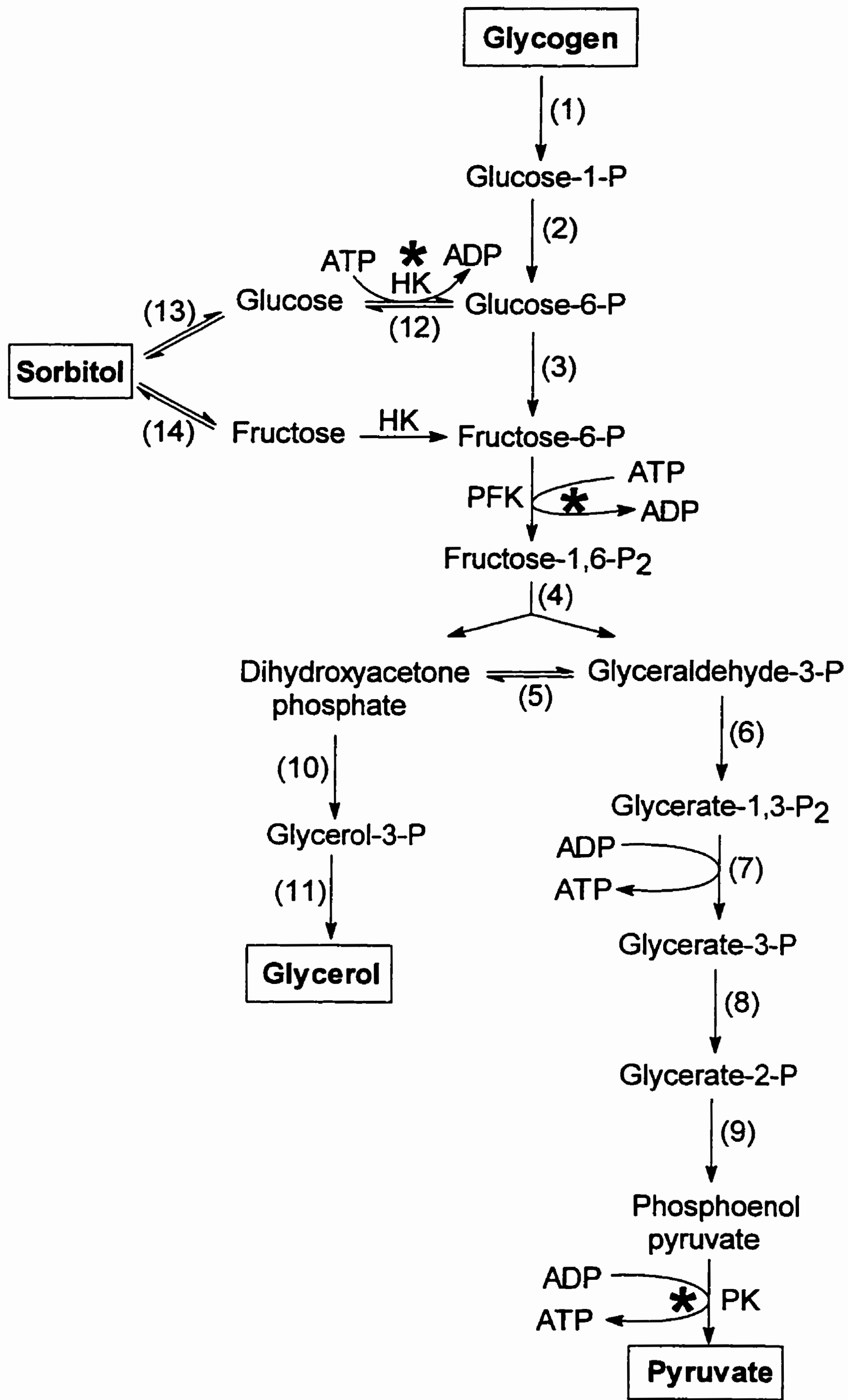

GLYCEROL
SORBITOL
µMOL / G WET WEIGHT
400
300
200
100
4 11 19 25 1 9 15 22 29 7 21 29 4 11 18 25 5 14 21 28 9 16 23 30 7 11 17
AUG
SEPT
OCT
NOV
DEC
JAN
FEB
MAR
APR

Figure 1-2 : Links between glycolysis and the metabolic pathways for glycerol and sorbitol.

This figure shows how glycolysis and the synthesis and the catabolism of glycerol and sorbitol are overlapping. The stars indicate the three key enzymes for the regulation regulation of glycolysis. HK: hexokinase, PFK: phosphofructokinase, PK: pyruvate kinase, (1): glycogen phosphorylase, (2): phosphoglucosemutase,
(3): phosphoglucoisomerase, (4): aldolase, (5): triose-phosphate isomerase,
(6): glyceraldehyde-3-phosphate dehydrogenase, (7): phosphoglycerate kinase,
(8): phosphoglycerate mutase, (9): enolase, (10): glycerol-3-phosphate dehydrogenase,
(11): glycerol-3-phosphatase, (12): Glucose-6-phosphatase, (13): polyol dehydrogenase,
(14): sorbitol dehydrogenase.


Glycogen
(1)
Glucose-1-P
(2)
ATP
*
ADP
HK
Glucose
Glucose-6-P
(12)
(13)
Sorbitol
(14)
(3)
Fructose
HK
Fructose-6-P
PFK
ATP
*
ADP
Fructose-1,6-P2
(4)
Dihydroxyacetone phosphate
Glyceraldehyde-3-P
(5)
(6)
(10)
Glycerol-3-P
(11)
Glycerol
Glycerate-1,3-P2
ADP
ATP
(7)
Glycerate-3-P
(8)
Glycerate-2-P
(9)
Phosphoenol pyruvate
ADP
ATP
*
PK
Pyruvate

CHAPTER TWO

HEXOKINASE

INTRODUCTION

Hexokinase (ATP : D-hexose 6 phosphotransferase, EC 2.7.1.1) catalyzes the phosphorylation of several hexose sugars, such as glucose:

Four different isozymes have been identified in mammals: hexokinases I, II, III and IV (or A, B, C and D), with distinct kinetic properties and tissue distribution. The first three isozymes are sometimes called "low K_m" hexokinases because their K_m values for glucose are in the range of 0.1 mM to 0.001 mM. Hexokinase IV, on the other hand, has a much higher K_m for glucose, in the millimolar range, and is often defined as the "high K_m" hexokinase and also called glucokinase (Wilson 1985, Stocchi et al. 1983, Middleton 1990). Glucokinase is largely confined to liver in vertebrates. Apart from glucose, other hexoses can be phosphorylated by the enzyme including fructose, mannose, 2-deoxy D-glucose, and glucosamine. However, the enzyme generally has less affinity for these than for glucose and their Km values are often much higher. For example, the Km values for pig red blood cell HK III are 0.0144 mM, 0.014 mM, 1.5 mM, 0.033 mM and 0.2 mM for D-glucose, D-mannose, D-fructose, 2-deoxy D-glucose and D-glucosamine, respectively (Stocchi et al. 1983).

Whereas hexokinases from yeast and a variety of vertebrates have been extensively studied (Wilson 1985, Garfinkel et al. 1987), relatively little is known in detail about the corresponding enzymes of invertebrate animals. In the lobster *Homarus americanus*, two isozymes have been found. One resembles the HK isozymes I and II of vertebrates. The second one, however, is apparently different from any animal HK previously described: it has a high affinity for mannose and fructose and a low affinity for glucose (Stetten and Goldsmith 1981). In the insect *Drosophila melanogaster*, however,

three isozymes (Hex A, Hex B and Hex C) have been identified, all having low K_m values for glucose (Lee, 1982).

HK isozymes in yeast exist as dimers, although they are readily dissociated to monomers of 40,000 to 50,000 Da (Middleton 1990). In mammals, most of the HK isozymes studied have molecular weights of about 100,000 Da, with the exception of glucokinase which is similar to the yeast HK with a mass of about 50,000 Da (Wilson 1985). For invertebrates, the molecular weight of HK also seems to be low. The three isozymes from *Drosophila melanogaster* have molecular weights in the range of 40,000 to 50,000 Da (Lee 1982), and HK from the brine shrimp, *Artemia,* has a molecular weight of about 40,000 Da (Rees et al. 1989). However, the enzyme from larvae of *Epiblema scudderiana*, a freeze avoiding insect, has a molecular weight of 94,000 Da (Muise, 1993).

Some types of hexokinase are capable of binding to the outer membrane of mitochondria. Appreciable amounts of HK I and II have been shown to be bound to mitochondria in tissues such as brain, heart, intestine, skeletal muscle, pancreas and spleen. HK III, on the other hand, is only found in the cytoplasm. The difference in binding capability can be explained by the ionic character of the bonds between HK and the mitochondria. There are two types of interactions: first, divalent cations can intercalate themselves between the enzyme and the membrane of the mitochondria creating a bridge between the negatively charged groups on HK and the mitochondria. The second type of bond is due to the attraction between oppositely charged groups on HK and the mitochondria. The pI values for the different types of HK rank in the following order: pI (type III) $<$ pI (type II) $<$ pI (type I) $<$ 7. Therefore, at physiological pH, HK III is the most negatively charged and, due to the repulsion of negative charges, will not be able to bind to the mitochondria. Binding to mitochondria is thought to be one way to control the flux of ADP and ATP between the mitochondria (where oxidative

phosphorylation occurs) and the cytoplasm (where most of the reactions using ATP occur) (Wilson 1985).

The most important effector that regulates HK activity is glucose-6-phosphate (G6P). This product of the HK reaction is a strong inhibitor of HK types I, II and III but not glucokinase which is largely insensitive to G6P inhibition. Inorganic phosphate (Pi), however, can reverse the effects of G6P and protect the enzyme against inhibition by HK. Glucose-1,6-bisphosphate is also a potent inhibitor of all isozymes of mammalian HK but again its action can be counterbalanced by Pi. Finally nucleotides such as ADP, AMP, and cAMP also inhibit HK. However, K_i values are generally much higher than the in vivo levels of these compounds, and their role in HK regulation is thought to be limited (Wilson 1985).

In the present study, hexokinase from *E. solidaginis* was purified and the kinetic properties of the enzyme were examined at 6°C and 25°C, in presence and in absence of the two cryoprotectants of the insect, glycerol and sorbitol, in order to assess the function and regulation of the enzyme in a freeze tolerant insect.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were purchased from Sigma Chemical Co. (St Louis, Mo, U.S.A.) or Boehringer Mannheim (Montreal P.Q., Canada).

Galls containing larvae of *Eurosta solidaginis* were obtained locally in fields around Ottawa in the fall. They were quickly opened and larvae were removed and frozen in liquid nitrogen. Frozen larvae were then stored at -70°C until use.

Purification

A sample of twenty frozen larvae were quickly weighed out. A few crystals of phenylmethylsulfonyl fluoride (PMSF) were added and the sample was homogenized 1:5 w/v in buffer A (10 mM imidazole-HCl, 15 mM 2-mercaptoethanol, 20% v/v glycerol, pH 7.0) using a Polytron PT-10 homogenizer. The homogenate was then centrifuged at 11,500 rpm for 20 minutes in a Hermle 2360K refrigerated centrifuge. The supernatant was removed and held on ice during the preparation of the isoelectrofocusing column (IEF column).

Isoelectrofocusing was carried out in an LKB 8101 column (110 ml) with pH 5-8 Ampholines in a sucrose density gradient according to the method of Vesterberg (1971). It was run overnight, for approximately 16 to 18 hours at a constant voltage of 300 V at 4°C. The column was then drained out in 2 ml fractions and these were assayed for activity. The pH gradient was also measured (first purification only). The most active fractions were pooled and concentrated against solid polyethylene glycol 8000. Concentrated enzyme preparation was then applied to a size exclusion column (Sephacryl-300) equilibrated in buffer B (10 mM imidazole, 15 mM 2-mercaptoethanol, 10% v/v glycerol , pH 8.0) and developed in the same buffer. Active fractions were pooled, concentrated against solid polyethylene glycol 8000 and the partially pure enzyme preparation was stored at -20°C until use.

Protein determination

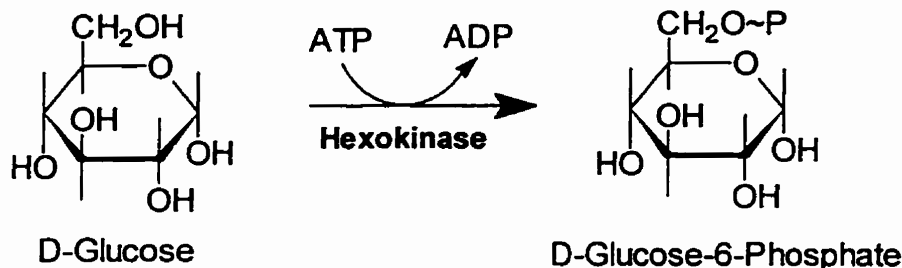
Protein concentration was determined using the Coomassie blue dye-binding method and the Bio-Rad assay kit with bovine serum albumin as the standard. Spectrophotometric quantification was performed at 595 nm using a Dynatech MR5000 Microplate reader in a final volume of 260 µl per microplate well.

SDS-PAGE

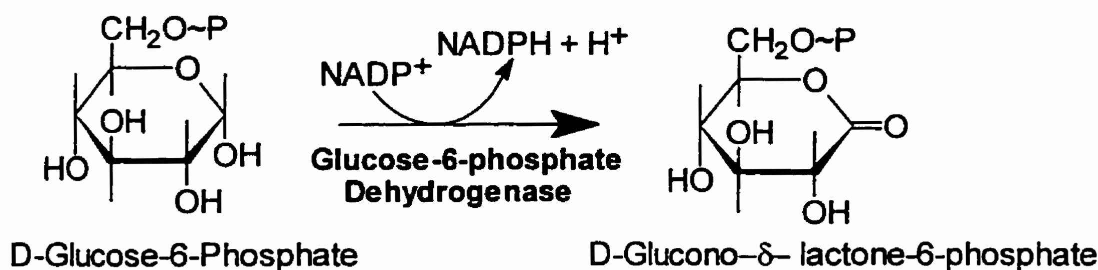
Subunit molecular weight was determined by sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS-PAGE) using a 8 % w/v acrylamide separating gel and a 5% w/v acrylamide stacking gel. The sample loading buffer (100 mM Tris-HCl, pH 6.8, 4% w/v SDS, 20% v/v glycerol and 0.2% v/v bromophenol blue) was mixed with 2-mercaptoethanol (final concentration 5%) and the resulting solution was mixed 1:1 v/v with the pure enzyme preparation. The samples were boiled for 2-3 minutes to denature the proteins and were then loaded on the gel parallel to prestained protein standards: myosin, 200,000 Da; β-galactosidase, 116,250 Da; phosphorylase b, 97,400 Da; serum albumin, 66,200 Da, ovalbumin, 45,000 Da; carbonic anhydrase, 31,000 Da; trypsin inhibitor, 21,500 Da; lysozyme, 14,400 Da; and aprotinin, 6,500 Da. The gel was run at 200V in Laemmli buffer (25 mM Tris-base, 250 mM glycine, 0.1% w/v SDS) for 45 minutes. It was then fixed for one hour in 30% v/v methanol and 10% v/v acetic acid, stained for two hours in 0.25 % Coomassie brilliant blue R, 50% v/v methanol and 7.5% v/v acetic acid and finally destained overnight in 25% v/v methanol and 10 % v/v acetic acid. The standard curve was obtained by plotting the logarithm of the molecular weight of standards against their relative migration (Rf) in the gel.

Measurement of activity

Hexokinase converts glucose into glucose-6-phosphate.



To measure the activity of this enzyme, the product of the reaction, glucose-6-phosphate, was used as a substrate for a coupling enzyme, glucose-6-phosphate dehydrogenase:



The conversion of $NADP^+$ into NADPH was monitored at 340 nm employing a Gilford 260 spectrophotometer for the purification and the Arrhenius plots and a Dynatech MR5000 Microplate reader for the kinetic studies.

The optimal assay conditions at 25°C were determined to be: 5 mM glucose, 2 mM ATP, 5 mM $MgCl_2$, 0.2 mM $NADP^+$ and one unit of the coupling enzyme glucose-6-phosphate dehydrogenase (G6PDH) in 50 mM Tris-base at pH 8.0. One unit of hexokinase activity is defined as the amount that utilizes 1 μmol glucose per minute at 25°C.

Arrhenius plots

Arrhenius plots were obtained by measuring the activity of hexokinase over a range of temperatures from 2°C to 36°C. The assay conditions were: 1 mM ATP-Mg^{2+}, 5 mM glucose, 10 mM $MgCl_2$, 0.2 mM $NADP^+$, 1 U G6PDH and 50 mM Tris, pH 8.0. Experiments were carried out either in the absence or the presence of 600 mM glycerol, 200 mM sorbitol, or both polyols. The spectrophotometer was attached to a circulating water bath (Brinkmann Lauda K-2/R) for thermal control of cuvette temperature which was recording using a Yellow Springs Instruments telethermometer. The logarithm of enzyme velocity was plotted against the reciprocal temperature (in Kelvin) and the calculated slope of the line was used to determine the energy of activation, E_a.

Kinetic studies

Kinetic studies measuring the K_m values for glucose and ATP and the activation constant for Mg^{2+} were performed on the purified preparations at both 6°C ± 0.5°C and 25°C ± 0.5°C. K_m values were also determined in the presence and absence of 600 mM glycerol or 200 mM sorbitol.

Data and statistics

All the data for kinetic studies and for Arrhenius plots were determined at least 3 times and analyzed using a non-linear least squares regression program (Brooks 1992). Results are presented as means ± S.E.M.. Statistical differences were determined through the Student's t-test (two tailed) employing the software package MYNOVA.

RESULTS

Purification

The purification of hexokinase from *Eurosta solidaginis* was carried out in two steps: a preparative isoelectrofocusing column followed by a size exclusion column. Results from a typical purification are summarized in Table 2-1 and Figures 2-1 and 2-2 show typical elution profiles for the IEF and Sephacryl S-300 columns, respectively. After isoelectrofocusing, 61% of the initial activity was recovered and the enzyme was purified by 3.29-fold. Concentration against polyethylene glycol, which was necessary to reduce the volume before loading onto the S-300 column resulted in the loss of some activity (yield fell to 46%) but did not affect specific activity. The second step, size exclusion chromatography, further increased the purity of the enzyme preparation and after a second concentration step, the final preparation had a specific activity of 5.37 U/mg. Overall, the total percentage of activity recovered was about 34 % and the final fold purification was 5.82. The SDS-PAGE gel shown in Figure 2-3 showed one dark band and three light ones and demonstrates partial purification of the enzyme. This partially purified enzyme preparation was used for kinetic studies.

Molecular Weight Determination

Hexokinase is a monomer. The molecular weight of the *E. solidaginis* enzyme was determined using two different techniques. First, using the standard curve of log MW versus the relative migration distance (Rf) for the protein standards run on SDS-PAGE (Figure 2-3 and 2-4), a molecular weight for HK of 89,600 Da was determined ($n = 1$). Secondly, from the standards run on the size exclusion column, the following relationship

was established

$$\log (MW) = -0.0246Ve + 6.345, \quad r^2 = 0.94 .$$

The three most active fractions containing HK were used for this determination and gave an mean molecular weight of 101,650 ± 2,700 Da (n = 2).

Isoelectric Point Determination

Two determinations of the isoelectric point were performed on a crude preparation, first using Ampholines with a wide pH range (3.5 to 10) and then Ampholines with a narrower pH range (5 to 8). In both cases, the pI value was found to be pI = 6.0 as illustrated in Figure 2-5. These results were in agreement with those obtained during the enzyme purification.

Kinetic studies

Kinetic data are summarized in Table 2-2 and Figures 2-6 and 2-7 present the graphs of velocity versus substrate concentration for glucose and ATP, respectively. A decrease in assay temperature from 25°C to 6°C resulted in a decrease in maximal velocity, whether polyols were present or not. The decrease was smaller in the presence of 200 mM sorbitol and 600 mM glycerol; thus, V_{max} at 6°C was about 43 % of V_{max} at 25°C in the presence of polyols but only about 27.5% in their absence.

The velocity versus substrate concentration curves were hyperbolic for both substrates. The substrate affinity constant for glucose was slightly lower (by 20 %) at 6°C (K_m = 0.126 ± 0.004 mM) than at 25°C (K_m = 0.154 ± 0.008 mM) in the absence of polyols. The addition of glycerol and sorbitol to the assay increased K_m glucose at both temperatures by 50-75 % but the K_m values in the presence of polyols were not different between temperatures.

The K_m for ATP-Mg^{2+} was 0.474 ± 0.019 mM at 25°C and did not change when assay temperature was lowered to 6°C. However, Km values at both temperatures were reduced significantly by about 25 % when glycerol and sorbitol were added.

The first attempt to measure the activation constant for Mg^{2+} was carried out in the presence of 2 mM ATP-Mg^{2+} in the assay solution. Since no activation was detectable under these conditions, the Mg^{2+} already present was removed and ATP alone was used. Therefore, the activation measured may not be the true activation of hexokinase by Mg^{2+} but a combination of this activation and the chelating effect of ATP. The activation constant was found to be 0.855 ± 0.019 mM at 25°C and was not affected by a decrease in assay temperature to 6°C.

Arrhenius plots

Arrhenius plots (Figure 2-8) were drawn by plotting the logarithm of enzyme velocity versus the reciprocal temperature (°K). Experiments were carried out in the presence versus absence of added polyols (200 mM sorbitol, 600 mM glycerol, or both 200 mM sorbitol + 600 mM glycerol). All plots exhibited linear relationships over the full range of temperatures tested and had similar slopes. The calculated activation energy for the enzyme without added polyols was E_a = 48.1 ± 3.0 kJ/mol. Table 2-3 summarizes the activation energies for the other experimental conditions; none of the additions significantly affected activation energy.

Table 2-1 Purification table for hexokinase from *E. solidaginis*

Step	Total Activity (U)	total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	3.96	4.29	0.923	---------	--------------
IEF column	2.41	0.7932	3.04	60.83	3.29
Concentration	1.83	0.5945	3.08	46.13	3.34
S-300	1.08	0.2782	3.88	27.13	4.20
Concentration	1.34	0.2496	5.37	33.72	5.82

Table 2-2 Effect of temperature and polyols on kinetic properties of *E. solidaginis* hexokinase.

Data are in mM and are presented as mean ± S.E.M. (at least 3 determinations) on the same purified preparation of the enzyme.

	Assay Temperature	
	25.0 ± 0.5°C	6.0 ± 0.5°C
K_m (Glucose)	0.154 ± 0.008	0.126 ± 0.004 [a]
K_m (Glucose) + 600 mM Glycerol + 200 mM Sorbitol	0.243 ± 0.009 [b]	0.225 ± 0.029 [b]
K_m (ATP)	0.474 ± 0.019	0.508 ± 0.032
K_m (ATP) + 600 mM Glycerol + 200 mM Sorbitol	0.363 ± 0.028 [b]	0.371 ± 0.036 [b]
K_a (Mg^{2+})	0.855 ± 0.019	0.866 ± 0.055

a = value significantly different from the value at 25°C by Student's t-test, $P<0.05$.

b = value significantly different from the value without sugars by Student's t-test, $P<0.05$.

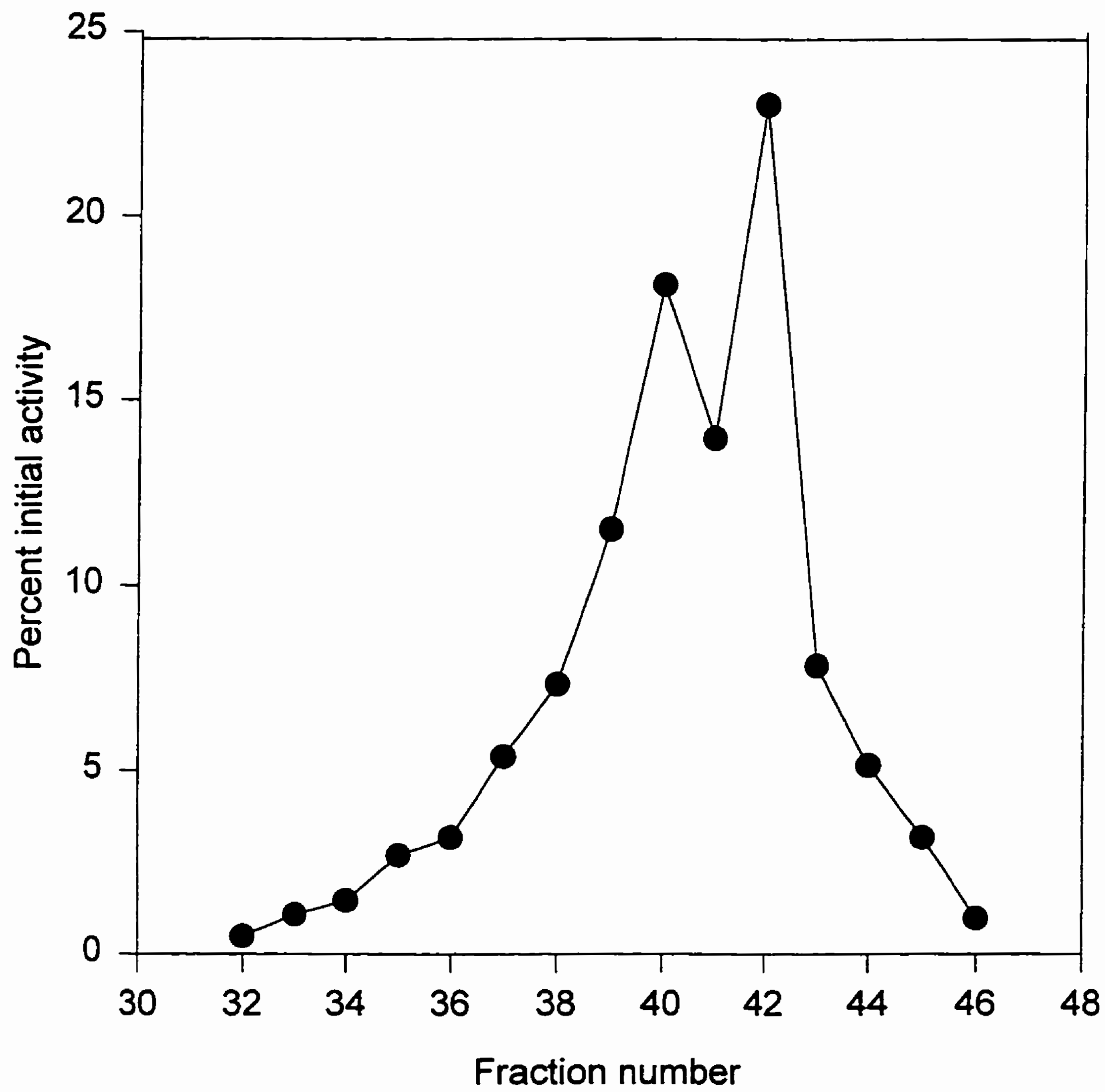
Table 2-3 Activation energies for *E. solidaginis* hexokinase

Data are presented as mean ± S.E.M., n = 4 on the same enzyme preparation

	E_a (kJ/mol)
Control	48.1 ± 3.0
Glycerol 0.6M	52.5 ± 7.1
Sorbitol 0.2M	51.8 ± 2.9
Glycerol 0.6M + sorbitol 0.2M	48.9 ± 3.3

Figure 2-1 Elution profile of *E. solidaginis* hexokinase from an isoelectrofocusing column.

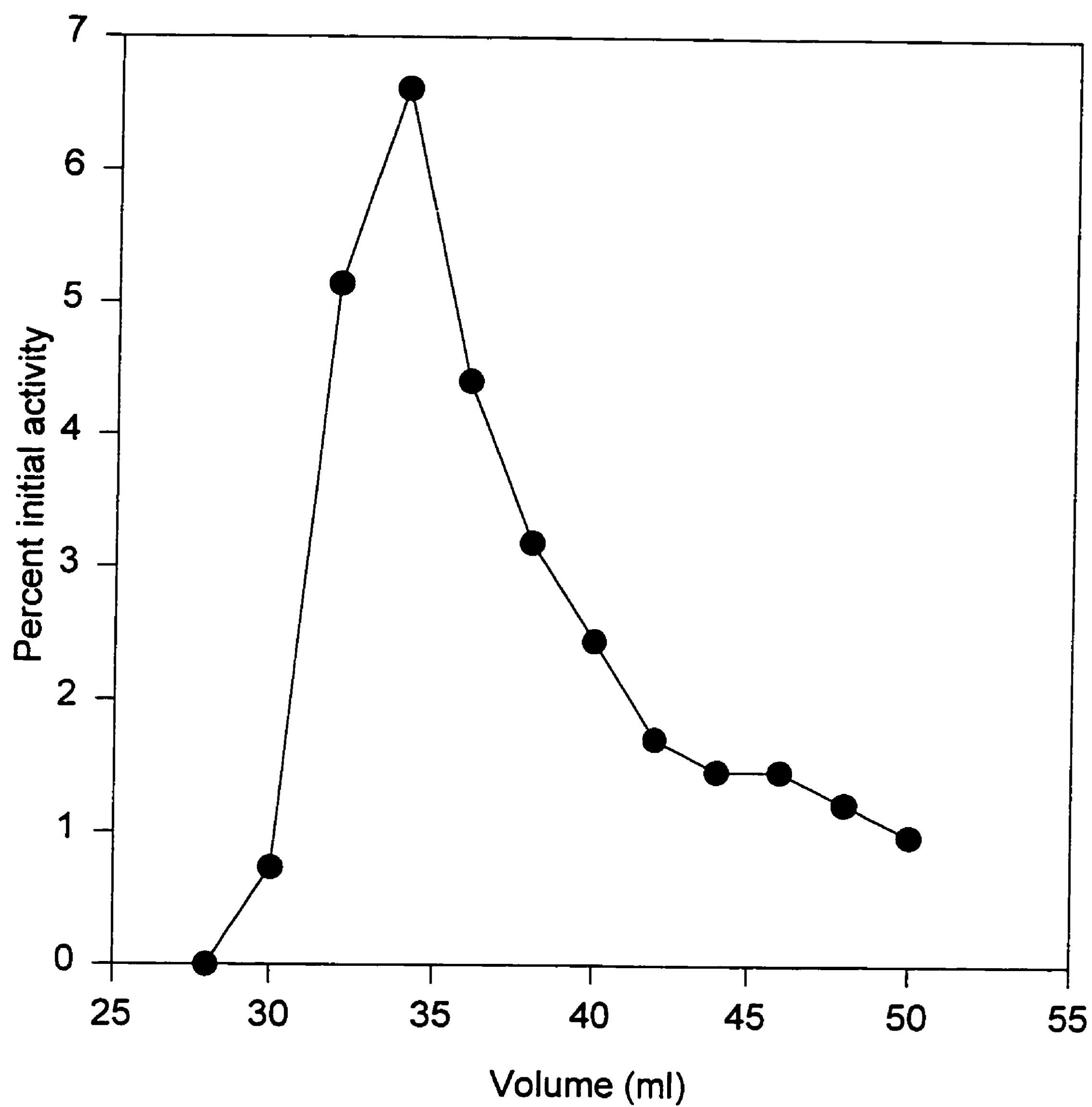
Activities are relative to the total activity in the crude supernatant applied to the column.



Percent initial activity
Fraction number
0
5
10
15
20
25
30
32
34
36
38
40
42
44
46
48

Figure 2-2 Elution profile of *E. solidaginis* hexokinase from a Sephacryl S-300 size exclusion column.

Activities are relative to the total activity of the crude supernatant.



Percent initial activity
Volume (ml)
0
1
2
3
4
5
6
7
25
30
35
40
45
50
55

Figure 2-3 SDS-PAGE gel of the purification of *E. solidaginis* hexokinase.

The SDS-PAGE gel shows the different steps of the purification of hexokinase. Standards are in lane 1. Lane 2 is the crude homogenate, lane 4 shows the enzyme after the IEF column and lanes 5 to 10 show the peak fractions obtained after the S-300 column. These fractions were then pooled and the enzyme was concentrated against polyethylene glycol.

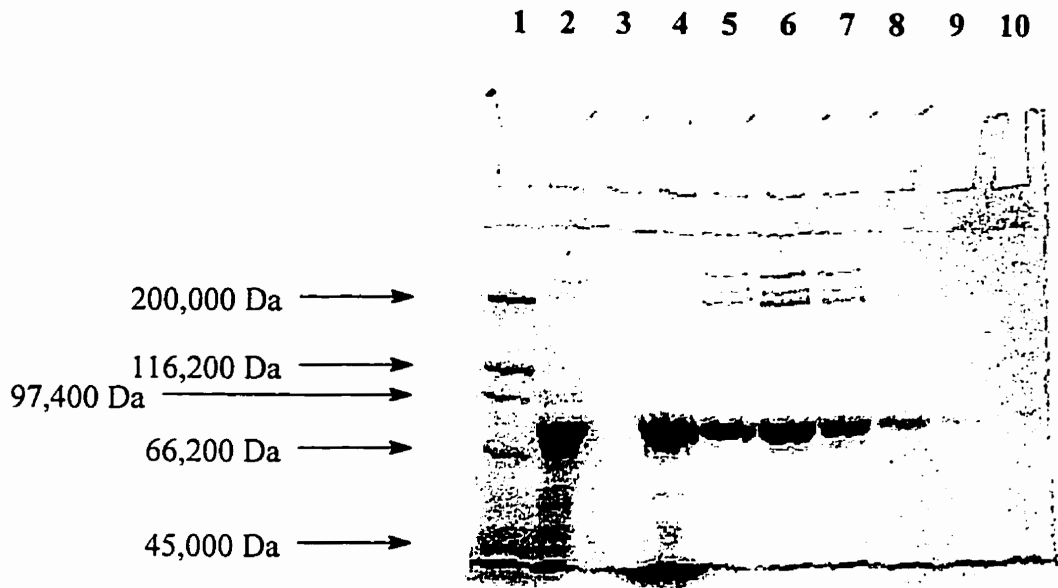
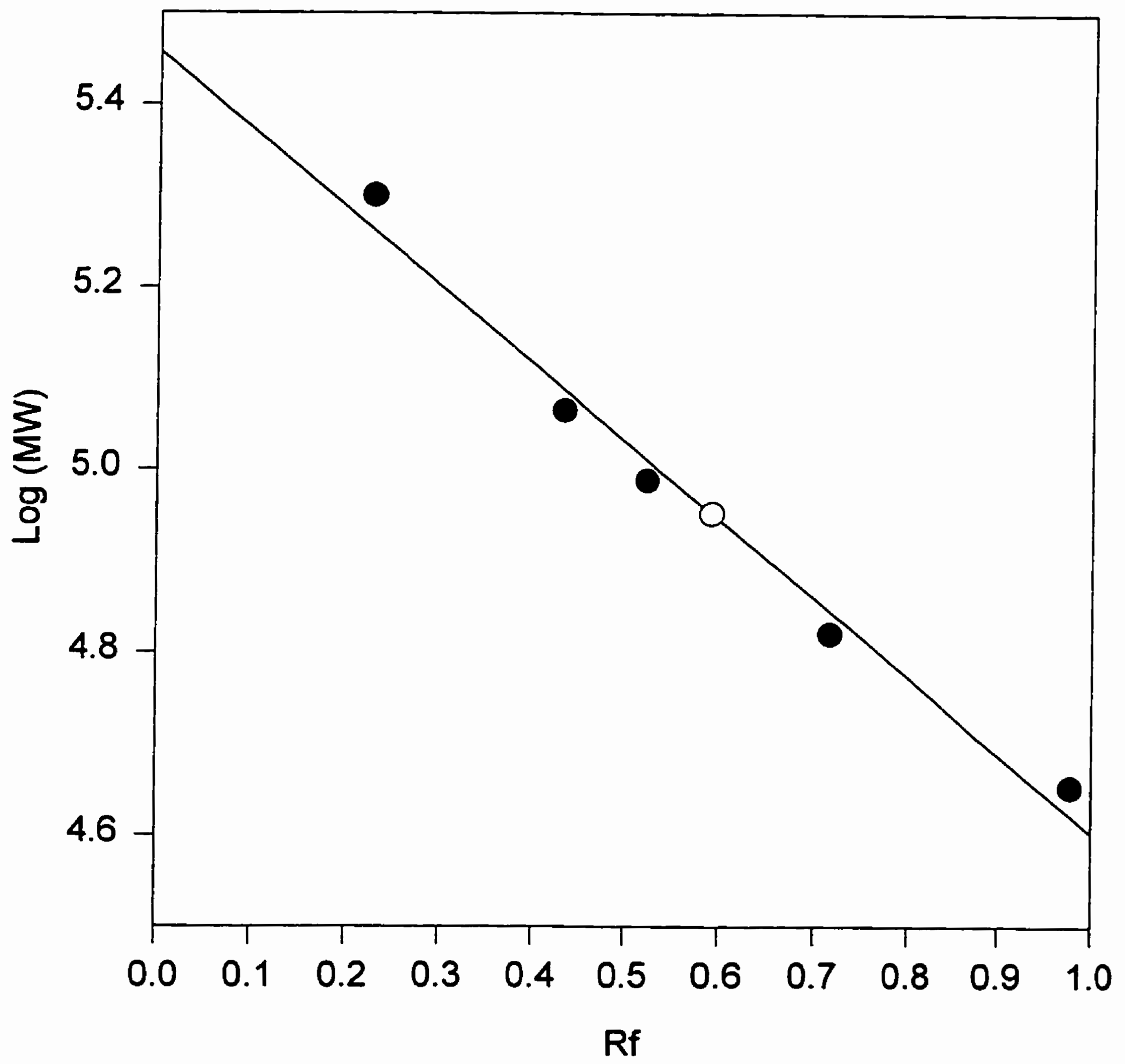

1 2 3 4 5 6 7 8 9 10
200,000 Da
116,200 Da
97,400 Da
66,200 Da
45,000 Da

Figure 2-4 Standard curve of logarithm of molecular weight versus relative migration front of protein standards on a SDS-PAGE gel.

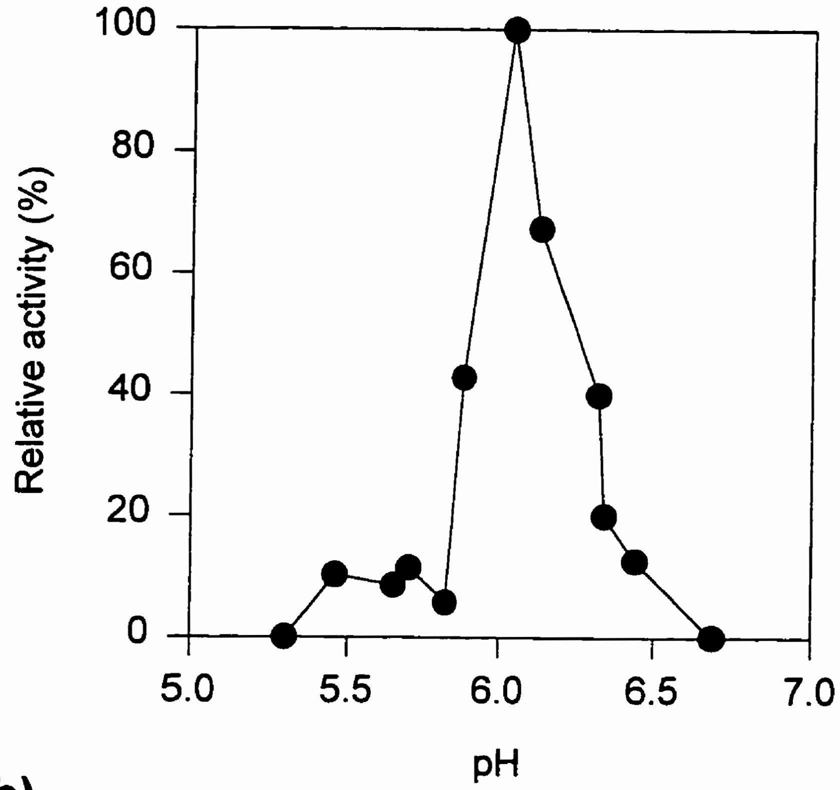
The logarithm of the molecular weight of the following standards were plotted against the relative migration front. The molecular weights were : 1) myosin (200,000 Da), 2) β-galactosidase (116,250 Da), 3) phosphorylase b (97,400 Da), 4) serum albumin (66,200 Da), 5) ovalbumin (45,000 Da) ($r^2 = 0.98$). The position of hexokinase is indicated by an open circle.



5.4
5.2
5.0
4.8
4.6
Log (MW)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf

Figure 2-5 Isoelectrofocusing profile of *E. solidaginis* hexokinase.

The pH range of ampholines was 3.5 to 10 for the upper profile (a) (crude preparation) and 5 to 8 for the lower one (b) (partially purified preparation). Enzyme activities are plotted relative to activity in the peak fraction.


a)
100
80
60
40
20
0
Relative activity (%)
5.0
5.5
6.0
6.5
7.0
pH

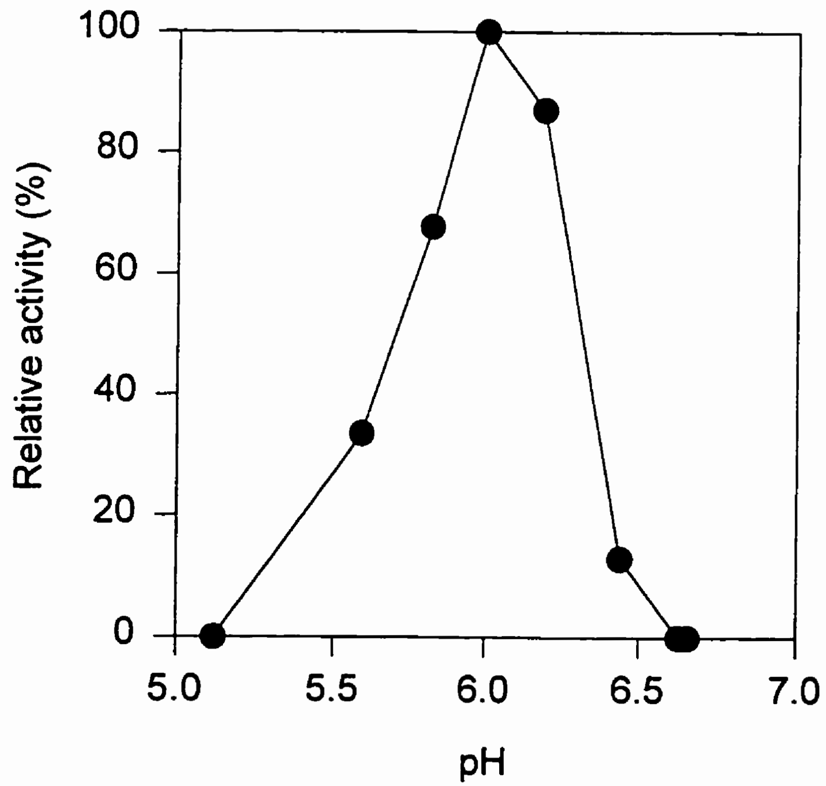
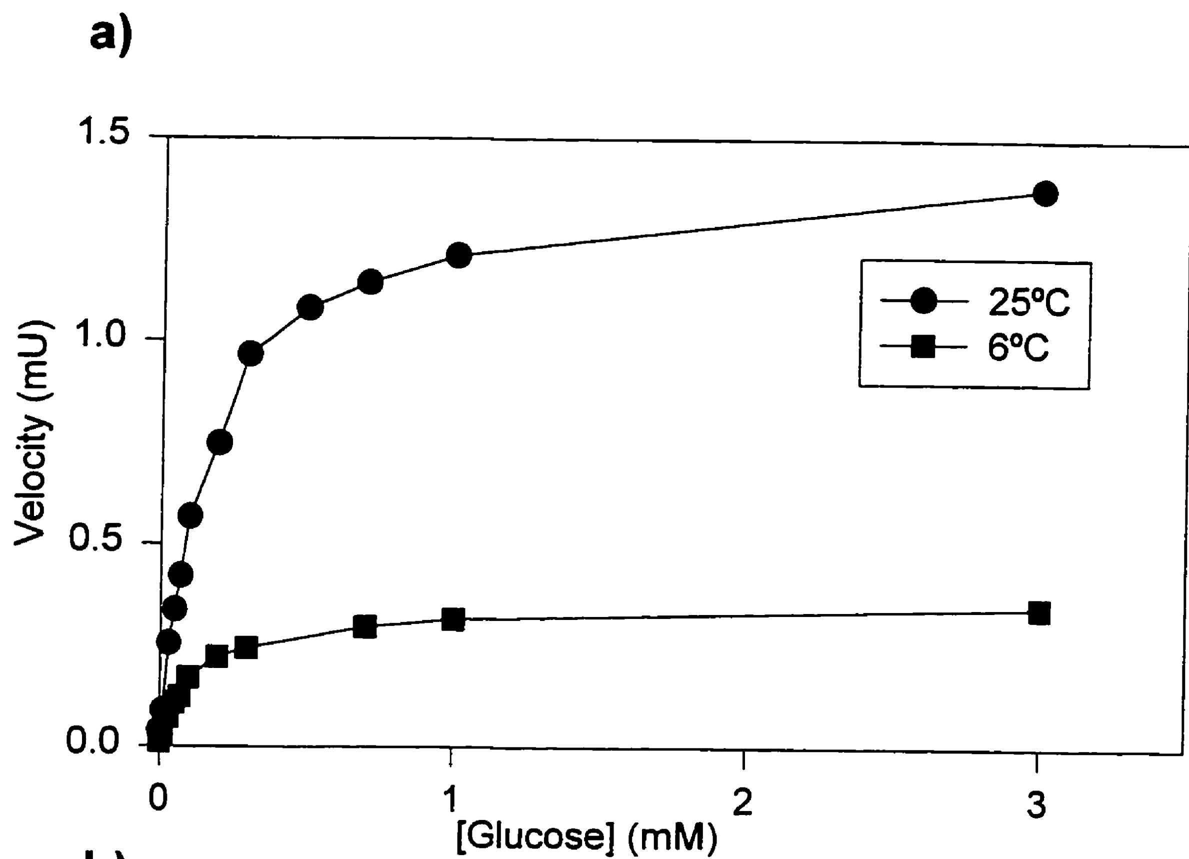

b)
100
80
60
40
20
0
Relative activity (%)
5.0
5.5
6.0
6.5
7.0
pH

Figure 2-6 Plots of velocity versus glucose concentration for *E. solidaginis* hexokinase at 6°C and 25°C.

The plots illustrate the effects of increasing glucose concentrations on enzyme velocity in the absence (a) and presence (b) of 600 mM glycerol and 200 mM sorbitol for assays at 6°C and 25°C. Circles represent hexokinase activity at 25°C and squares represent activity at 6°C. Activity was determined on purified enzyme preparations. Each point represents a single measurement.


a)
1.5
1.0
0.5
0.0
Velocity (mU)
25ºC
6ºC
0
1
2
3
[Glucose] (mM)

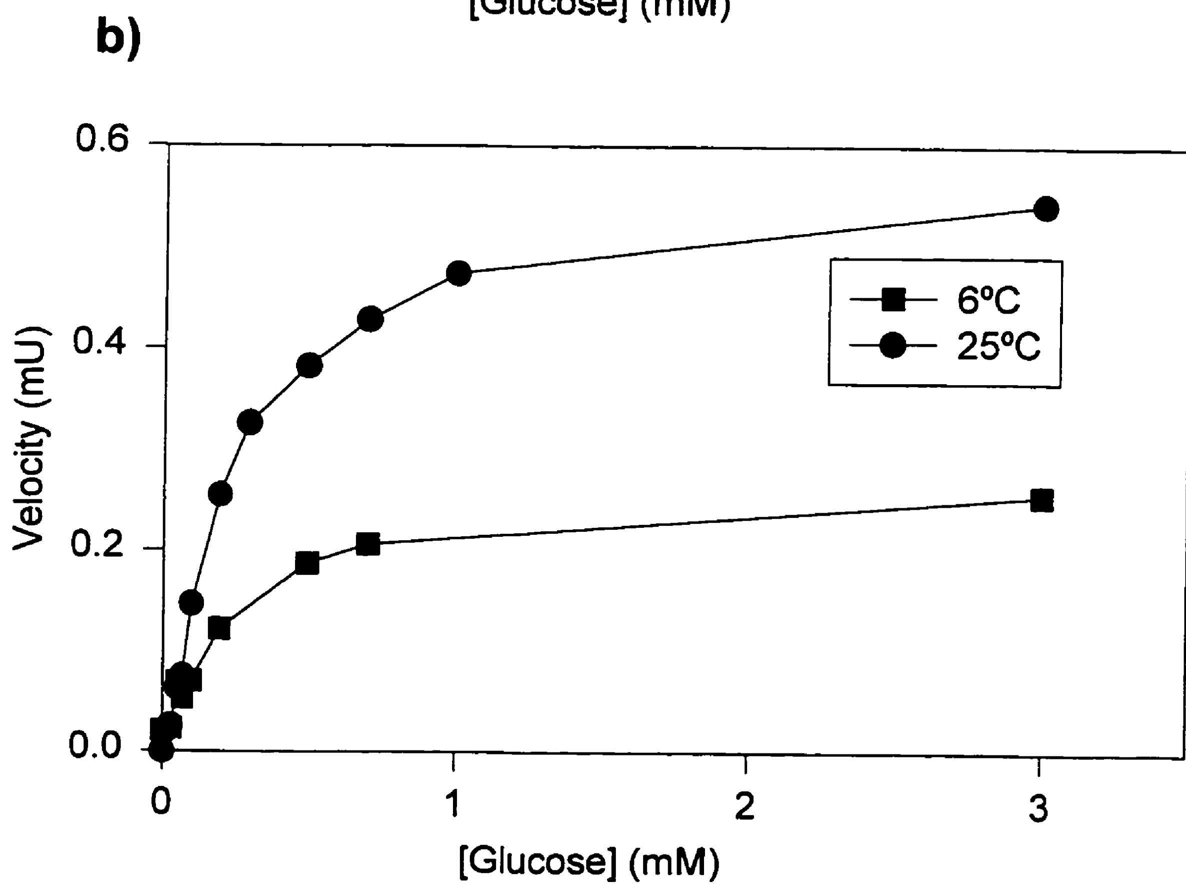
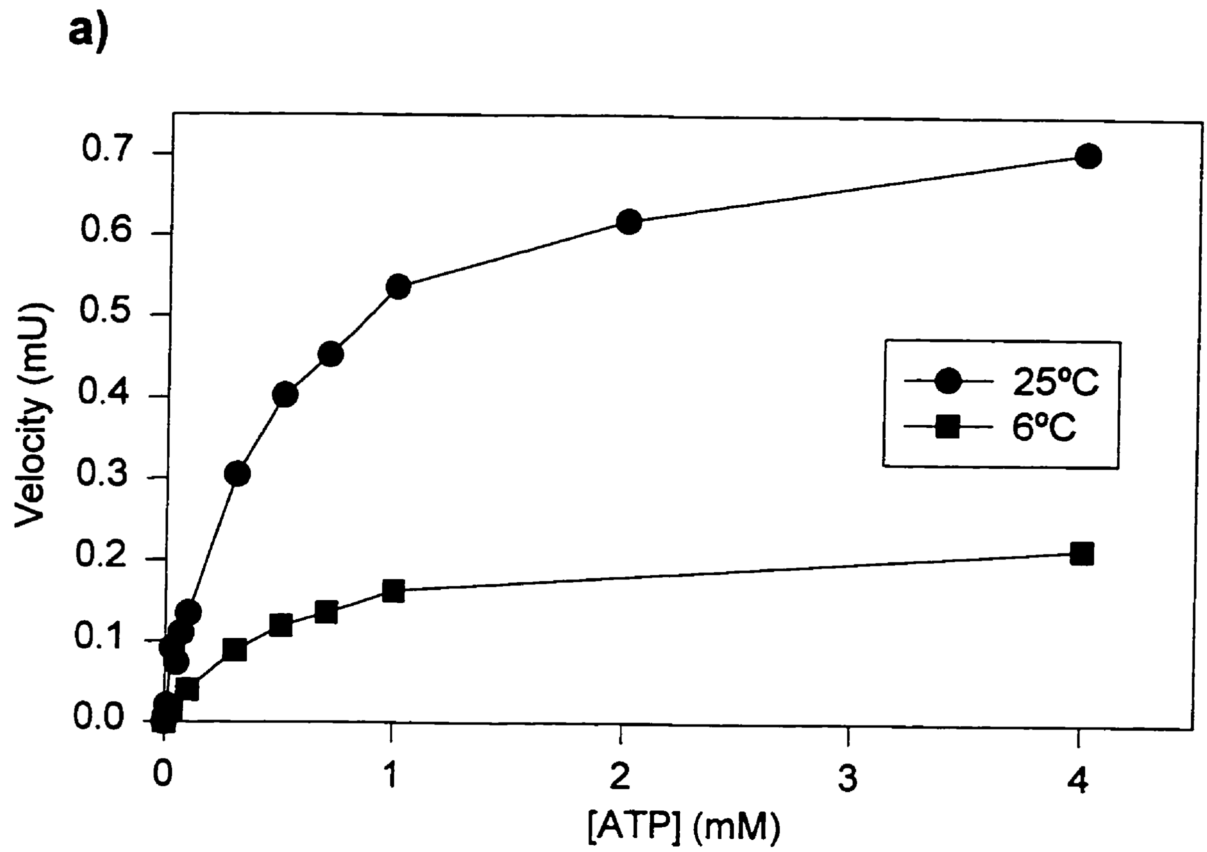

b)
0.6
0.4
0.2
0.0
Velocity (mU)
6ºC
25ºC
0
1
2
3
[Glucose] (mM)

Figure 2-7 Plots of velocity versus ATP concentration for *E. solidaginis* hexokinase at 6°C and 25°C.

The plots illustrate the effect of increasing ATP concentrations on enzyme velocity in the absence (a) and presence (b) of 600 mM glycerol + 200 mM sorbitol for assays at 6°C or 25°C. Circles represent hexokinase activity at 25°C and squares represent activity at 6°C. Activity was determined on purified enzyme preparations. Each point represents a single measurement.


a)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Velocity (mU)
0
1
2
3
4
[ATP] (mM)
25ºC
6ºC

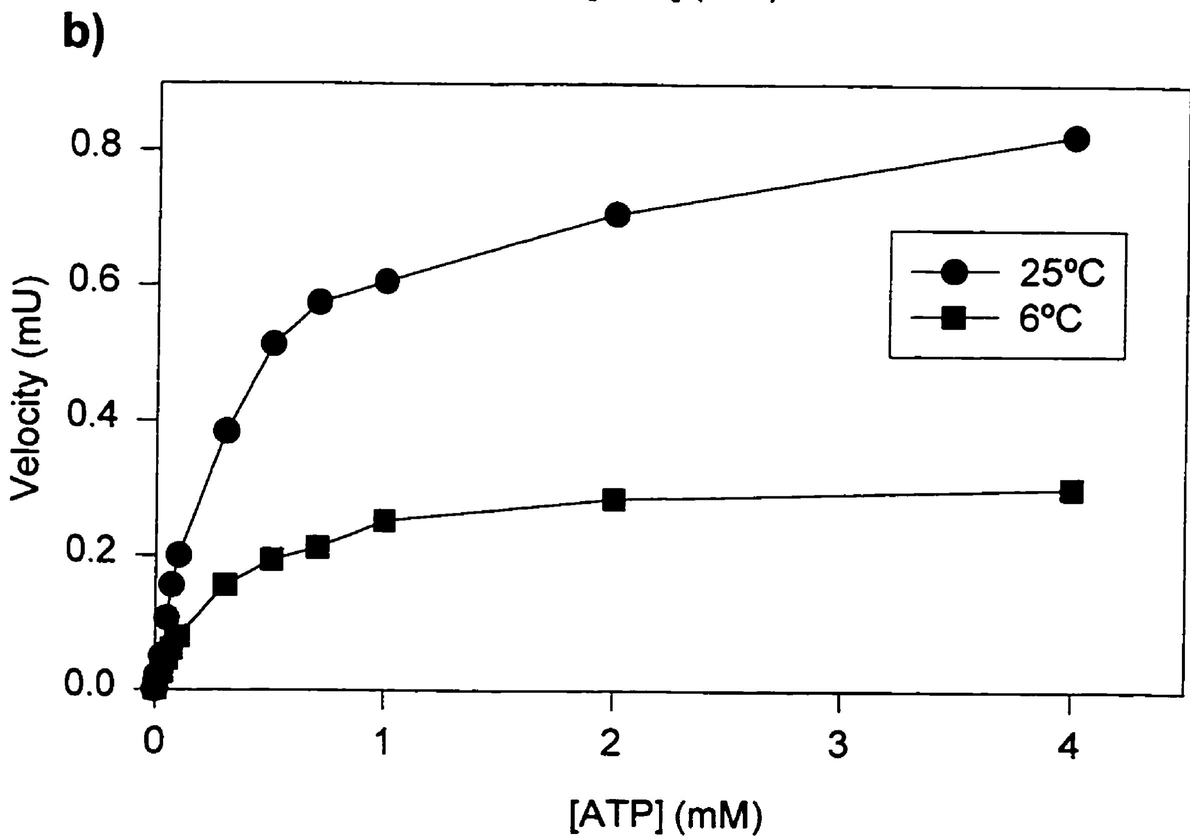
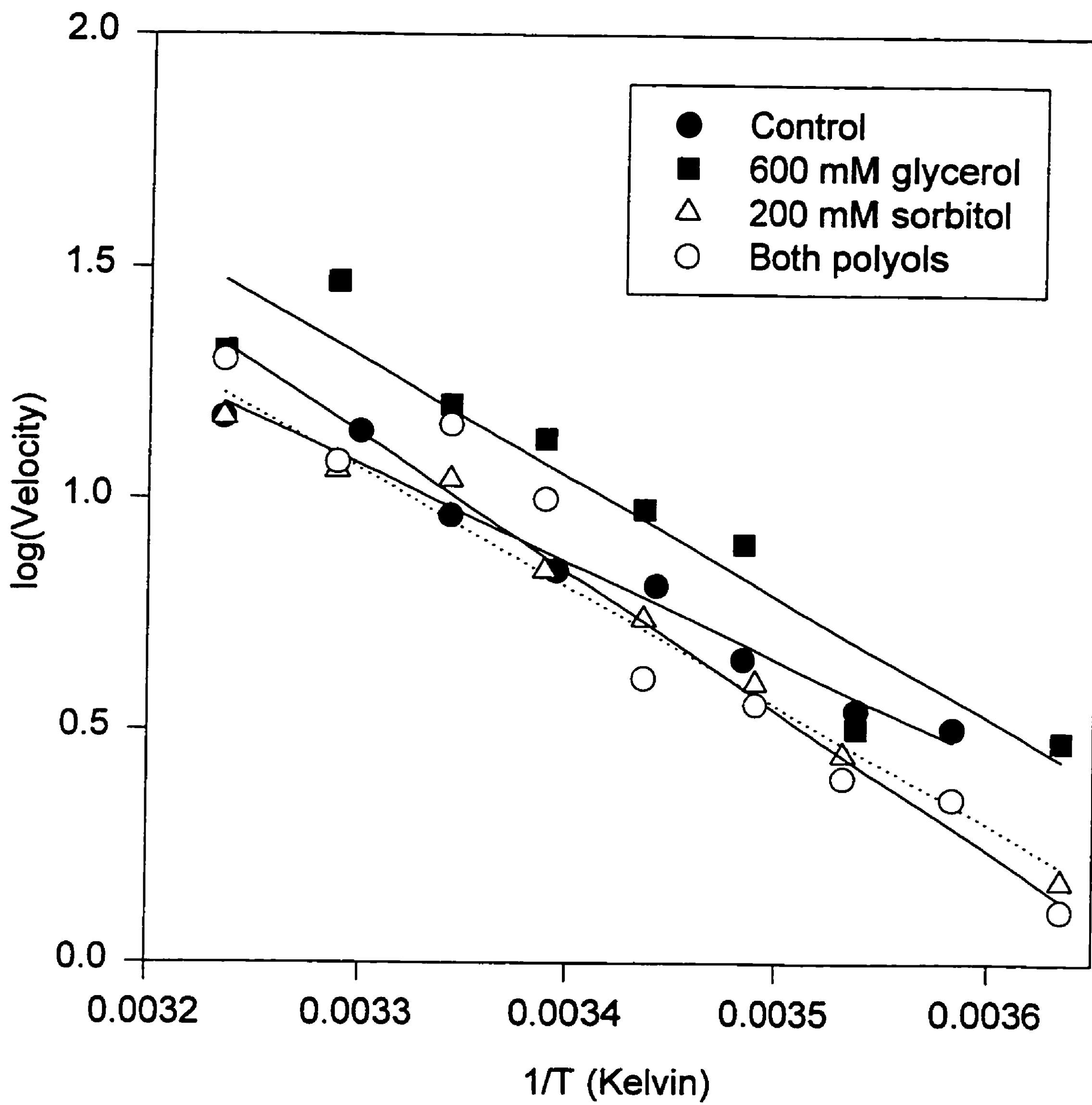

b)
0.8
0.6
0.4
0.2
0.0
Velocity (mU)
0
1
2
3
4
[ATP] (mM)
25ºC
6ºC

Figure 2-8 Arrhenius plots for *E. solidaginis* hexokinase.

The logarithm of the velocity versus the reciprocal temperature was plotted. Each point represents a single measurement. The slope of the plot (s) is related to the activation energy (Ea) by the equation :

$$s = -E_a/(2.303R)$$

Solid circles represent the Arrhenius plot for assays done in the absence of polyols, solid squares in the presence of 600 mM glycerol, open triangles in the presence of 200 mM sorbitol and open circles in the presence of the two polyols.



Control
600 mM glycerol
200 mM sorbitol
Both polyols
log(Velocity)
1/T (Kelvin)
2.0
1.5
1.0
0.5
0.0
0.0032
0.0033
0.0034
0.0035
0.0036

DISCUSSION

Because of its position at the entry of glycolysis and because it catalyzes an irreversible reaction, hexokinase can be considered as a potential regulation point of the glycolytic pathway. For *Eurosta solidaginis*, during the synthesis of the cryoprotectants, glycerol and sorbitol, the carbon flux comes from glycogen which is converted in two steps into glucose-6-phosphate. Therefore, hexokinase is not directly involved in the synthesis of the two polyols. However, glucose-1-phosphate and glucose-6-phosphate may be converted into glucose and it is then necessary to bring this carbon back into glycolysis. Furthermore, a high concentration of glucose may have an inhibitory effect on glycogenolysis and slow the synthesis of glycerol. Hence, the HK reaction has a role to play in the overall process of cryoprotectant synthesis and control at this locus must be maintained.

Sorbitol is produced from glucose-6-phosphate in two steps, a dephosphorylation to form glucose and then a NADPH-linked reduction to sorbitol. During active sorbitol accumulation, glucose levels can rise from control values of about 0.1 μmol/gww to over 30 μmol/gww in the larvae (Storey et al. 1981a). This huge increase in substrate concentration could promote the HK reaction and, with it, a wasteful consumption of ATP in a futile cycling of glucose back to glucose-6-P. Hence, to help ensure a unidirectional flow of glycolytic carbon into sorbitol production, HK activity should be inhibited when sorbitol synthesis is active. Oppositely, HK activity is required during sorbitol breakdown and reconversion into glycogen which may occur several times over the winter whenever environmental temperatures rise above about 5°C for a substantial length of time (Storey and Storey 1986). Sorbitol is converted to fructose and then fructose is phosphorylated by HK and re-enters the glycolytic pathway.

One mode of hexokinase regulation in the larvae is by the control of enzyme concentration over the winter months (Joanisse and Storey 1994). HK activity was

minimal during the autumn, the time of active sorbitol synthesis, but rose as winter progressed, increasing over 2-fold by March, a time when sorbitol catabolism is favoured. The object of the present study was to determine what other kinetic and regulatory mechanisms are involved in the control of hexokinase in *Eurosta solidaginis*.

Purification

Hexokinase from *Eurosta solidaginis* was partially purified to a final specific activity of 5.37 U/mg. Hexokinase has been purified to homogeneity in a number of species. Most hexokinases have a specific activity between about 60 U/mg (58 U/mg for human heart, Haritos and Rosemeyer 1985) and 200 U/mg (192.52 U/mg for human placenta HK, Magnani et al. 1988).

The two determinations of the molecular weight gave an approximate molecular weight for hexokinase between 89,600 Da and 101,650 Da. This value is similar to the molecular weight of the enzyme from the freeze avoiding insect *Epiblema scudderiana* (94,000 Da, Muise 1993) and agrees with values from the literature for HK from other insects: 100,000 Da for silkworm (Supowit and Harris, 1976), and 97,000 Da for locust flight muscle (Storey, 1980).

Isoelectric point

The isozyme composition of *Eurosta solidaginis* HK was previously assessed (Storey et al. 1981b). Only a single form of hexokinase was found in the larvae and it was the same isozyme at each of four acclimation temperatures: 24°C, 10°C, -10°C and -30°C. The results from isoelectrofocusing in the present study are in agreement with this, showing only one form of hexokinase with a pI of 6.0. This pI value is similar to the pI values found for HK I in rat brain (pI = 6.3, Chou and Wilson 1972), rat skeletal muscle

(pI = 5.7, Qadri and Easterby 1980), *Drosophila melanogaster* (pI = 6.1, Lee 1982) and for silkworm (pI = 5.9, Supowit and Harris 1976).

Kinetic studies

In September, when environmental temperatures are still high, the larvae of *Eurosta solidaginis* are still eating and they take in sugars which are stored as glycogen. The first step of the metabolic pathway leading from sugars to glycogen is their phosphorylation by hexokinase. High levels of HK activity are therefore required to ensure the proper synthesis of the glycogen stocks which will be needed later to make the two cryoprotectants, glycerol and sorbitol. Maximal levels of HK activity (about 2.7 U/gwm, Joanisse and Storey 1994) are indeed observed at the beginning of September. Furthermore, the present studies show that the affinity of HK for glucose is high (Km about 0.15 mM) and so the enzyme could function very efficiently in the phosphorylation of dietary sugars.

When the mean environmental temperature decreases to about 15°C in early autumn, glycerol synthesis starts in the larvae and is completed when temperatures of about 5°C are reached. During the synthesis of glycerol, the levels of glucose remain low and unchanged (less than 1.5 μmol/gwm, Joanisse and Storey 1994, Storey and Storey 1986). HK may have a role at this time in helping to preserve this low level of glucose in the larva and therefore to prevent the inhibition of glycogenolysis, and glycerol synthesis, that may be caused by high levels of glucose. Throughout the autumn the maximal activity of HK also decreases to about 0.5 U/gwm at the beginning of November (Joanisse and Storey 1994), a situation that would limit futile cycling of glucose when sorbitol synthesis begins (as outlined above).

When the temperature drops under 5°C, sorbitol synthesis is initiated and glucose levels rise to 15-30 mM (Storey et al. 1981a, Storey and Storey 1986) to push the polyol

dehydrogenase reaction. At that time, the activity of HK has to be minimal to prevent futile cycling of carbon and waste of ATP. The maximal activity of HK is at its lowest at the beginning of November (about 0.5 U/gwm) but the present results (Table 2-2) show that the K_m for glucose decreases at low temperature which means that the enzyme has a higher affinity for glucose at this time. However, the effect of added polyols counteracted the low temperature effect on K_m and raised K_m at 6°C by 80 %. The high glycerol content *in vivo* may act, therefore, to reduce the affinity of HK for glucose at low environmental temperatures and thereby promote the conversion of glucose into sorbitol. Furthermore, sorbitol as it accumulates could also inhibit the enzyme; as an hexose, sorbitol may compete with glucose at the active site, causing the increase in apparent K_m for glucose. In addition, during sorbitol synthesis, both glucose-6-phosphate and fructose-6-phosphate levels are high (Storey and Storey 1983b). Since glucose-6-phosphate is a known inhibitor of HK, this could also help to inhibit HK during the period of sorbitol synthesis.

When temperature rises in the spring and reaches 5-10°C, the catabolism of sorbitol begins while glycerol pools are still high and stable. The main pathway for sorbitol catabolism involves its conversion to fructose which is then transformed in fructose-6-phosphate by HK (Storey and Storey 1983b). Therefore, HK needs to be active and a rise in maximal HK activity is indeed observed at the beginning of the spring (Joanisse and Storey 1994). It would be interesting to determine how the Km for fructose varies with temperature and in the presence of glycerol and sorbitol. If high levels of polyols positively affected the affinity for fructose, this would create a favourable situation for sorbitol degradation since an enhancement of HK affinity for ATP (also needed for the phosphorylation of fructose by HK) in the presence of high polyols was observed (Table 2-2). Finally, since glucose-6-phosphate levels fall and stay low during sorbitol catabolism (Storey and Storey 1983b), this would also facilitate HK function by lowering inhibitor levels.

Arrhenius plots

The linearity of the Arrhenius plots in the range of temperature studied shows that no change in conformation affecting the catalytic capacity of hexokinase occurs at low temperature. The activation energy, with a value of 48.1 ± 3.0 kJ/mol is similar to the value obtained for rabbit brain HK (46 kJ/mol, Magnani et al. 1982) and for HK II of pig red blood cell (47.7 kJ/mol, Stocchi et al. 1983). The presence of polyols had no influence on the catalytic capacity of *E. solidaginis* hexokinase.

Conclusions

The kinetic studies performed on *E. solidaginis* hexokinase showed that control of this enzyme is not only at the level of enzyme concentration, but that the enzyme activity and kinetic parameters are also influenced by temperature and by the presence of the two cryoprotectants, glycerol and sorbitol.

CHAPTER THREE

PYRUVATE KINASE

INTRODUCTION

Pyruvate kinase (ATP : pyruvate phosphotransferase, EC 2.7.1.40) catalyzes the second of the two ATP forming reactions of the glycolytic pathway during the conversion of PEP to pyruvate :

This enzyme is a tetramer of identical or nearly identical subunits, each subunit having a relative molecular weight between 50 kDa and 70 kDa. In mammals, four different isozymes with different kinetic properties have been identified, reflecting the different metabolic requirements for enzyme function in different tissues. The M1 isozyme is found in skeletal muscle and brain and shows predominantly Michaelis Menten kinetics. The three other isozymes are allosteric enzymes: M2 (widely distributed throughout the body such as in kidney, adipose tissue, and lung), L (liver) and R (erythrocytes). They exhibit sigmoidal kinetics with respect to PEP and are allosterically activated by fructose-1,6-bisphosphate and inhibited by ATP, citrate, alanine and phenylalanine.

Pyruvate kinase, in general, has been recognized to be an important regulatory enzyme encountered universally in cells and tissues undergoing glycolysis, and its activity can influence the rate of both glycolysis and gluconeogenesis. The product, pyruvate, is involved in a number of metabolic pathways and thus, places the enzyme at a primary metabolic intersection.

In invertebrate species, both regulatory and non-regulatory PKs have been found. The PK from the body wall tissue of the flour beetle, *Tenebrio molitor*, resembles the M1 isozyme of mammals since it is not allosterically regulated and shows Michaelis Menten kinetics (Hoffmann, 1977). On the other hand, PK from the cricket *Acheta domesticus* L. (Hoffmann, 1975), the PK from the fat body of the male desert locus, *Schistocerca gregaria* (Bailey and Walker, 1969) and the PK from the fat body of the cockroach,

Periplaneta americana (Storey 1985) have been shown to be allosterically regulated. Like the mammalian allosteric PK, they are activated by F-1,6-P_2 and inhibited by ATP and exhibit sigmoidal kinetic with respect to PEP.

Regulation of PK activity also plays a key role in animals with anaerobic capacities. For invertebrates like the intertidal bivalve molluscs and benthic invertebrates, the exploitation of oxygen-poor or oxygen-free environments has necessitated major changes in their metabolic organization (Hoffmann 1977). PK, controlling the outflow from glycolysis, is at a critical position for the regulation of the aerobic/anoxic transition and its activity is greatly depressed when the animals change from an aerobic state to anoxic conditions. The enzyme in these animals is also strongly regulated by L-alanine, a product of anaerobic metabolism.

For *Eurosta solidaginis*, the control of PK during cryoprotectant biosynthesis is of great importance. An inactivation of PK will block the lower portion of glycolysis to facilitate the diversion of carbon out of glycolysis and into the reactions that result in the massive synthesis of glycerol and sorbitol. Inhibition of PK is also probably involved in the strong metabolic rate depression that characterizes the diapause state of the larvae. Finally, control at PK could be important in the switch to anaerobic metabolism that occurs when the larvae are frozen (Storey and Storey, 1985). The aim of this study was to establish whether an alteration of pyruvate kinase kinetic and structural properties takes place when the larvae prepare themselves to face cold and to determine how the enzyme is affected by thermal changes or by the presence of sugars.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were purchased from Sigma Chemical Co. (St Louis, Mo, U.S.A.) or Boehringer Mannheim (Montreal P.Q., Canada).

Galls containing larvae of *Eurosta solidaginis* were obtained locally in fields around Ottawa in the fall. They were quickly opened and larvae were removed and frozen in liquid nitrogen. Frozen larvae were then stored at -70°C until use.

Purification

The purification procedure was previously optimized for *Eurosta solidaginis* pyruvate kinase by Tolga Bilgen of our lab and this procedure was followed. A sample of 10-12 frozen larvae were quickly weighed out. A few crystals of phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, were added and the sample was homogenized in 1:5 w/v in buffer A (20 mM imidazole-HCl, 15 mM 2-mercaptoethanol, 50 mM sodium fluoride, 5 mM EDTA, 5 mM EGTA and 10% v/v glycerol, pH 6.0) using a Polytron PT-10 homogenizer. The homogenate was then centrifuged at 15,000 rpm (26,890 g) for 20 minutes in a Sorvall RC-5B refrigerated centrifuge. Supernatant was removed and desalted via centrifugation through a column of Sephadex G-25 previously equilibrated in buffer B (5 mM imidazole , 15 mM 2-mercaptoethanol, 10 % v/v glycerol , pH 6.0). The extract was then loaded onto a CM-cellulose 52 column (1.5 cm x ~4 cm) previously equilibrated in buffer B. The column was washed with 20 ml of buffer B and pyruvate kinase activity was eluted using a linear gradient of 0 to 500 mM KCl in buffer B. Fractions of 1 ml were collected and the most active fractions were pooled and

concentrated to approximately 1 ml by dialysis against solid polyethylene glycol 8000. The concentrated enzyme was then applied to a size-exclusion column (Sephacryl S-400) equilibrated in buffer C (20 mM imidazole-HCl, 15 mM 2-mercaptoethanol, 50 mM KCl, 10% v/v glycerol, pH 6.0) and developed in the same buffer. The eluant was collected in 2 ml fractions and the most active fractions were pooled and applied to a hydroxylapatite column equilibrated in buffer D (15 mM imidazole-HCl, 15 mM 2-mercaptoethanol, 10% v/v glycerol, 5 mM $MgCl_2$, pH 6.0). The column was washed with 10 to 20 ml of buffer D and pyruvate kinase activity was then eluted using a linear gradient of 0 to 500 mM KPi in buffer D. Active fractions were pooled and brought to 30 % v/v glycerol and stored at -20°C until use. For the denaturation experiments, the final concentration of glycerol used was 5% v/v in the enzyme preparation.

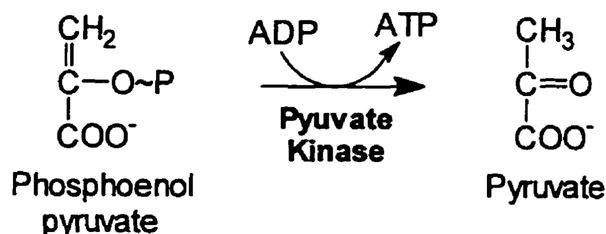
An SDS-PAGE was run as described in Chapter 2.

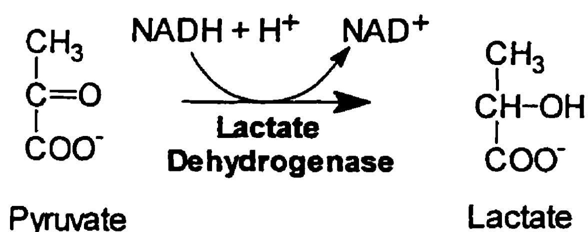
Isoelectrofocusing column

The isoelectric point (pI) of the enzyme was determined by running an isoelectrofocusing column, according to the method of Vesterberg (1971). A sample of 9 larvae was homogenized 1:5 v/v in buffer E (10 mM imidazole-HCl, 15 mM 2-mercaptoethanol and 20% v/v glycerol, pH 7.0) and centrifuged it for 20 minutes at 14,000 rpm in a Sorvall RC-5B refrigerated centrifuge. A 2 ml aliquot was applied to an LKB 8101 column (110 ml) with Ampholines of the pH range 3.5 to 10 in a sucrose density gradient. Isoelectrofocusing was carried out overnight for approximately 16 hours under a voltage of 450V at 4 °C. The column was then drained out in 2 ml fractions which were assayed for activity. The pH of the fractions was also measured.

Measurement of activity

The activity of pyruvate kinase was measured using a coupling enzyme, lactate dehydrogenase. This enzyme converts pyruvate (product of the reaction catalyzed by pyruvate kinase) into lactate using NADH which absorbs at 340 nm.





The optimal assay conditions at 22 °C for maximal activity were determined to be 0.5 mM PEP, 1 mM ADP, 5 mM $MgSO_4$, 100 mM KCl, 0.15 mM NADH and one unit of lactate dehydrogenase (LDH) in 20 mM imidazole-HCl buffer at pH 7.2. One unit of pyruvate kinase activity is defined as the amount that utilizes 1 μmol of PEP per minute at 22°C.

All the studies were carried out using a Gilford 260 spectrophotometer. For the Arrhenius plots and low temperature kinetics, a circulating water bath (Brinkmann Lauda K-2/R) was attached to the spectrophotometer for control of cuvette temperature. The temperature of the cuvette was checked using a Yellow Spring Instruments telethermometer.

Kinetic studies

Kinetic studies were performed at 22°C and at 5°C ± 0.5°C. The first constants determined were the substrate affinity constants (K_m) for PEP and ADP. K_m is defined as the concentration of substrate that produces half of maximal enzyme velocity. K_m values were determined by fitting data to the Hill equation for PEP and to the Michaelis-Menten equation for ADP.

L-alanine, citrate-$2Mg^{2+}$, ATP-Mg^{2+}, KCl and NaCl were tested as potential inhibitors. When a compound was found to be an inhibitor, its inhibition constant (I_{50}) was determined at optimal substrate concentration (1 mM ADP and 0.5 mM PEP). This constant was defined as the concentration of inhibitor which reduced reaction velocity to one half of its maximum value in absence of inhibitor.

Fructose-1,6-bisphosphate and L-aspartate were tested as potential activators. The activation constant (K_a) was defined as the concentration of activator which produced half maximal activation of the enzyme. K_a values were determined at 2 mM ADP and at suboptimal concentration of PEP (0.05 mM) using a modified Hill equation.

All data were determined at least 3 times, using a non-linear least squares regression program (Brooks 1992). Results are presented as means ± S.E.M.. Statistical tests were performed employing the software package MYNOVA using the Student's t-test (two tailed).

Arrhenius Plots

In order to obtain Arrhenius plots, pyruvate kinase activity was measured with substrate conditions of PEP (1 mM) and ADP (2 mM) over a range of temperature from 0°C to 35°C in absence or presence of effectors (glycerol (1 M) and sorbitol (0.5 M)). The logarithm of enzyme velocity was plotted against the reciprocal of the temperature

(in Kelvin) and the slope of the line was used to determine the activation energy (E_a) by the following equation:

$$\ln k = \ln A - E_a/(RT)$$

with k : constant rate of the reaction (proportional to the initial velocity)

A : constant of integration (lnA : intercept of Arrhenius plot with the y axis)

Each determination was done four times and statistical tests were performed employing the software package MYNOVA using the Student's t-test.

pH profile

The optimum pH was determined 4 times by measuring activities under optimal conditions of assay for pH values varying from 5.5 to 7.5 at 22°C. pH values were checked in each cuvette immediately after the assay.

Denaturation tests

Denaturation of pyruvate kinase in the presence or absence of various effectors was tested by heating the enzyme at 53°C ± 0.5°C and then monitoring changes in enzyme activity over time. The pure enzyme preparation was diluted 1:1 with 20 mM imidazole-HCl buffer (pH 7.2) or with solutions containing the effectors in the same buffer. This ensured that final volume and therefore the final protein concentration were constant. The effectors tested were glycerol (1 M), sorbitol (1 M), glucose (1 M), PEP (5 mM), KCl (1 M), urea (1 or 3 M) and a combination of urea and glycerol, or urea and sorbitol. The half life time ($t_{1/2}$) of the enzyme is the time after which the samples possessed only 50% of the original activity. It was determined for all the conditions studied. Each denaturation test was performed four times.

Fluorescence studies

Fluorescence measurements were carried out on a Perkin-Elmer LS-50 spectrofluorometer with a water jacketed cell holder for temperature control. Excitation maximum wavelength was 278 nm. Emission spectra were recorded from 300-500 nm.

The preparation of purified pyruvate kinase was concentrated to approximately 1 to 1.5 ml by dialysis against solid polyethylene glycol 8000. As imidazole buffer can not be used for fluorescence studies (it fluoresces in the same region as tryptophan), the buffer was changed by centrifugation of the enzyme sample through a column of G 25 previously equilibrated in buffer F (100 mM of potassium phosphate, 5% v/v of highly purified glycerol).

For temperature denaturation studies, measurements were performed in the absence or presence of 600 mM glycerol and 200 mM sorbitol (concentrations used for the kinetic studies) on 1.5 ml of purified enzyme in buffer E. Beginning from the lowest temperature, the enzyme preparation was equilibrated at each temperature for approximately 30 minutes followed by fluorescence scanning.

For chemical denaturation studies, 150 µl of purified enzyme in buffer F was incubated at 22°C in different concentrations of guanidinium chloride (total volume: 300 µl) for at least 30 minutes. The fluorescence emission was then recorded. Measurement of activity at optimal concentrations of substrates was then performed.

RESULTS

Enzyme Purification

Purification of *Eurosta solidaginis* pyruvate kinase was accomplished in three steps. From the cation exchanger, CM-cellulose, 50% of the activity originally present in the crude supernatant was recovered. Figure 3-1 shows the elution profile obtained in a typical purification; peak activity was eluted at about 150 mM KCl.

After concentration of the fractions collected from the CM-cellulose column, the enzyme preparation was applied to a Sephacryl S-400 size exclusion column. The elution profile is shown in Figure 3-2 and 46% of the original activity was recovered after this second step.

The third step in the purification scheme was affinity chromatography on hydroxylapatite. An overall recovery of 44% was obtained for pooled peak fractions from this column. The elution profile is shown in Figure 3-3; the peak fraction eluted at about 180 mM KPi.

The final purified enzyme preparation was purified 483.5 fold, with an overall yield of 44 % and a final specific activity of 783 U/mg protein.

SDS-PAGE of the final purified enzyme preparation revealed one major dark band and 3 light ones (Figure 3-4); hence, the final enzyme preparation was judged to be almost pure. A plot of log molecular weight versus Rf of the protein standards is shown in Figure 3-5 and was used to estimate a subunit molecular weight for PK of 53,140 Da. (n = 1).

Isoelectric Point Determination

The isoelectric point was determined using an isoelectrofocusing column and Ampholines with a pH range of 3.5-10. Figure 3-6 shows the IEF profile obtained with a single peak of pyruvate kinase activity. The pI value was 7.10 (n =1).

pH Profile

The pH profile shown in Figure 3-7 was obtained from a purified enzyme preparation. The relative activity remained greater than 80% of the maximum over the pH range from 6.59 ± 0.02 to 7.54 ± 0.01. The optimal pH was determined to be $pH_{opt} = 7.05 \pm 0.02$ (n = 4).

Substrate Affinity Constants

Kinetic studies were performed at 5°C and 22°C. Substrate affinity constants for the two substrates ADP and PEP are presented in Table 3-1. Figure 3-8 shows representative plots of velocity versus substrate concentration. The velocity versus PEP concentration curves were sigmoidal ($n_H = 1.3$ at 22°C and 1.60 at 5°C), but the ones for ADP were hyperbolic. A significant decrease of the maximal velocity was observed when assay temperature was lowered from 22°C to 5°C. At 5°C, the maximal velocity was approximately 20% of the maximal velocity at 22°C. K_m values for both PEP and ADP decreased significantly at 5°C falling to 31 and 75 %, respectively, of the corresponding values at 22°C.

Inhibitor Constants

Among all the possible inhibitors tested, L-alanine was the only one that was virtually without effect on pyruvate kinase activity; even at 100 mM alanine, 70% of PK activity still remained. Citrate-2Mg^{2+} and ATP-Mg^{2+} were effective inhibitors of PK and Figure 3-9a shows the velocity versus inhibitor curves for these compounds. Stock solutions of ATP and citrate both contained Mg^{2+} (1:1 and 1:2 molar ratios, respectively) because of their ability to chelate Mg^{2+} ions in the assay. Had they been tested alone, the resulting inhibition of PK could be much stronger due to combined effects of metabolite inhibition plus the Mg^{2+} chelation which would remove the Mg^{2+} necessary for PK catalysis. ATP-Mg^{2+} was the stronger inhibitor with an I_{50} of 7.7 ± 0.85 mM compared to I_{50} = 22.2 ± 0.95 mM for citrate-2Mg^{2+} at 22°C. The I_{50} value for ATP increased about 50 % when assays were at 5°C whereas the I_{50} for citrate decreased about 18%. (Table 3-2). The effect of temperature on the I_{50} value disappeared for ATP when polyols were added, but it was enhanced for citrate (about 40%).

The influence of polyols on ATP and citrate inhibition was also tested. The addition of 600 mM glycerol did not significantly change the I_{50} value for ATP at 22°C but lowered it by 30% at 5°C. In the presence of both glycerol and sorbitol, the I_{50} increased by 50 % at 22°C. Polyols also influenced citrate inhibition of PK. At 22°C the addition of 600 mM glycerol lowered the I_{50} value by 30 % and the I_{50} in the presence of glycerol + sorbitol was also significantly lower than the I_{50} without polyols. At 5°C, however, the addition of glycerol alone had no effect on I_{50} citrate but glycerol + sorbitol decreased the I_{50} value by 42 %.

Inhibition by salts, KCl and NaCl, was also tested (Table 3-2). KCl is normally present in the assay solution (100 mM) due to the requirement for K^+ ions by the enzyme. However, to test the true inhibition effect of this salt, its concentration was varied from 0 M to 2 M. At low concentrations of KCl (< 100 mM), an activation of

enzyme activity was observed, but when the amount of KCl was increased to very high concentrations, it acted as an inhibitor with an $I_{50} = 382 \pm 13$ mM. NaCl inhibition was tested in the absence of KCl in the assay medium. Pyruvate kinase activity was also inhibited by NaCl although at a lower concentration than for KCl, $I_{50} = 81 \pm 1.5$ mM. Neither KCl or NaCl inhibitions were significantly affected by the decrease of assay temperature from 22°C to 5°C.

Activator Constants

Two metabolites were tested as potential activators, L-aspartate and fructose-1,6-bisphosphate. L-aspartate did not active *Eurosta solidaginis* PK at either 22°C or 5°C. On the other hand, fructose-1,6-bisphosphate was a very good activator at 22°C, since µmolar concentrations of this metabolite were sufficient to activate the enzyme ($K_a = 0.36 \pm 0.01$ µM; Table 3-2) and since the velocity increased 3.3 fold when the enzyme was saturated with fructose-1,6-bisphosphate. However, fructose-1,6-bisphosphate failed to activate PK at 5°C.

The divalent cation Mg^{2+} was found to activate the enzyme. As for the substrate affinity constants, the K_a Mg^{2+} (Table 3-2) decreased when the assay temperature was lowered from 22°C to 5°C.

Arrhenius Plots

The effect of assay temperature on pyruvate kinase maximal activity was studied over a temperature range from 0°C to 35°C and analyzed via Arrhenius plots (Figure 3-10). All plots exhibited linear relationships between log(velocity) and reciprocal temperature (°K). The activation energy was calculated from the slope of the line by the formula:

$$E_a = -s \times R \times \ln 10$$

(where s is the slope and R is the gas constant, $R = 8.314$ $J.mol^{-1}.K^{-1}$).
In the absence of polyols, the activation energy for PK was 62 ± 3 kJ/mol. When assayed in the presence of 1 M glycerol, the activation energy did not change whereas in the presence of both 1 M glycerol and 0.5 M sorbitol it increased significantly by 14% (Table 3-3).

Thermal Denaturation Studies

Thermal decay curves were established by plotting relative activity versus time for incubations of the enzyme at 53°C (Figures 3-11 and 3-12). This temperature was chosen after initial tests because enzyme activity decayed relatively slowly at this temperature, with a half time of decay of 32 ± 3 min in the absence of any added effectors. In order to determine the influence of various metabolites on the thermal stability of PK, the enzyme was incubated in presence of polyols or sugars (1M glycerol, 1M sorbitol, 1M glucose), 1M KCl, substrate (5 mM PEP), or the denaturant, urea (1M or 3M; with or without 1M glycerol or 1M sorbitol). Half times for thermal denaturation were determined for all experimental incubation conditions and results are summarized in Table 3-4. Addition of 1 M glycerol had no effect on the thermal stability of PK but 1 M sorbitol greatly improved stability, extending the half time for denaturation to more than 400 min, but not preventing the ultimate denaturation of the enzyme. Glucose and KCl also improved the thermal stability of the enzyme, the half life times rising almost 3-fold. However, the best stabilizer was PEP; in the presence of 5 mM substrate, activity of the enzyme was virtually unchanged over 450 min of incubation (Figure 3-11).

Urea had strong denaturing effects on pyruvate kinase with $t_{1/2} = 4 \pm 0.2$ min in the presence of 1 M urea and $t_{1/2} = 2 \pm 0.2$ min for 3 M urea. The potential ability of glycerol or sorbitol to reverse the denaturing effects of urea was also tested. When 1 M glycerol was added along with urea (1 M or 3 M), it had no effect on the half time for

urea denaturation. However, sorbitol had a strong positive effect. Addition of 1 M sorbitol completely inhibited denaturation by 1 M urea, giving a half time for thermal denaturation ($t_{1/2}$ = 29 min ± 1 min) that was not significantly different from the control. However, at the higher urea concentration (3M), 1 M sorbitol had largely lost its stabilizing effect.

Fluorescence Studies

Fluorescence emission was used to monitor the unfolding of pyruvate kinase induced by guanidine hydrochloride. Two signals were recorded: the maximal emission wavelength and the fluorescence intensity at 320 nm. Figure 3-13 shows the effects of increasing guanidine hydrochloride concentration on the λ_{max} and the enzymatic activity of PK. Enzyme activity was largely unaffected by guanidine hydrochloride at concentrations up to 0.1 M but fell to 85-90 % of the control value by 0.5 M. In the presence of 1 M guanidine hydrochloride, however, activity was strongly suppressed with only about 10 % of the original remaining after the 30 min incubation time; activity was totally lost in the presence of 2 M denaturant. Guanidine hydrochloride had no significant effect on the λ_{max} at concentrations up to 1 M but λ_{max} had risen to 344.5 nm at 2 M and rose to 353 nm in the presence of 4 M guanidine hydrochloride.

Effects of guanidine hydrochloride on fluorescence intensity at a set wavelength (320 nm) are shown in Figure 3-14. The choice of the emission wavelength is dictated by the difference in fluorescence intensity between the native and the denatured states. In general, the greater the change in physical property when the protein unfolds, the better the signal is for following unfolding. Thus, the emission wavelength at which the fluorescence intensity is recorded is chosen in order to give a large difference in intensity between the native and denatured states. Figure 3-14 suggests that the mechanism of unfolding of pyruvate kinase is not a simple two-state mechanism, since fluorescence

intensity fluctuates considerably at low concentrations of guanidine hydrochloride (as also did λ_{max}) before showing a definite pattern of gradual decrease at concentrations of 1 M and above. The free energy of denaturation ΔG_D was nonetheless calculated assuming a 2-state mechanism since this gives a lower limit for the true ΔG_D value. The following equations were used :

$$f_D = (F_{320,N} - F_{320})/(F_{320,N} - F_{320,D}) \quad (2)$$

$$K_D = f_D/(1-f_D) \quad (3)$$

$$\Delta G_D = - RT\ln K_D \quad (4)$$

with F_{320} : fluorescence intensity at 320 nm

$F_{320,N}$: fluorescence intensity at 320 nm for the native state

$F_{320,D}$: fluorescence intensity at 320 nm for the denatured state

f_D : fraction of denatured enzyme

K_D : equilibrium constant for N ⇄ D

R : gas constant, R = 8.314 J/mol.K

T : temperature, T = 295 K

Figure 3-15 shows the plot of ΔG_D versus guanidine hydrochloride concentration. The free energy change in the absence of denaturant was linearly extrapolated and gave a value $\Delta G_D^{H_2O} = 5.11 \pm 0.09$ kJ/mol at 22°C. The dependence of ΔG_D on guanidine hydrochloride, m (slope of the line), was determined to be m = 3.48 ± 0.42 kJ.l/mol^2 at 22°C.

Fluorescence was also used to monitor the structural changes that occurred when the enzyme was subjected to heat denaturation. Figure 3-16 shows the variations in the maximal wavelength emission with increasing temperature in the presence and in absence of polyols (600 mM glycerol and 200 mM sorbitol). The general trend is an increase in λ_{max} with rising temperature (from about 340 nm to 344 nm) although a lesser effect

than that of guanidine hydrochloride. Figure 3-17 shows comparable temperature effects on fluorescence intensity at 320nm versus temperature. In the absence of added polyols, a large decrease in fluorescence intensity occurred between 30°C and 35°C, indicating a major change in the enzyme conformation. Glycerol and sorbitol protected pyruvate kinase from this sudden denaturation at 35°C and their presence instead resulted in a gradual decrease in fluorescence intensity between 35°C and 75°C.

Table 3-1 Effect of temperature on substrate affinity of *E. solidaginis*, pyruvate kinase

Data are in mM and are presented as mean ± S.E.M., with at least 3 determinations.

	22°C	**5°C**
K_m (PEP)	0.16 ± 0.006	0.05 ± 0.007 [a]
n_H	1.32 ± 0.07	1.60 ± 0.20
K_m (ADP)	0.24 ± 0.009	0.18 ± 0.002 [a]

a = value significantly different from the value at 22°C by Student's t-test at $p<0.005$

Table 3-2 Effect of temperature on activation and inhibition constants of *Eurosta solidaginis* pyruvate kinase.

Data are reported in mM and are presented as mean ± S.E.M., with at least 3 determinations.

	22 °C	5 °C
K_a (Mg^{2+})	1.97 ± 0.15	0.65 ± 0.07 [a]
K_a (F-1,6-P_2)	$4 \times 10^{-4} \pm 1 \times 10^{-5}$	∞
I_{50} (ATP,Mg^{2+})	7.67 ± 0.85	12.0 ± 0.83 [a]
I_{50} (ATP,Mg^{2+}) + 600 mM Glycerol	9.34 ± 0.16	8.68 ± 0.38
I_{50} (ATP,Mg^{2+}) + 600 mM Glycerol +200 mM Sorbitol	11.4 ± 0.13	10.2 ± 1.1
I_{50} (Citrate, $2Mg^{2+}$)	22.2 ± 0.9	18.3 ± 1.3
I_{50} (Citrate, $2Mg^{2+}$) + 600 mM Glycerol	15.2 ± 0.9	18.9 ± 0.6 [a]
I_{50} (Citrate, $2Mg^{2+}$) + 600 mM Glycerol +200 mM Sorbitol	19.0 ± 0.3	10.7 ± 1.1 [b]
I_{50} (KCl)	382 ± 13	356 ± 4
I_{50} (NaCl)	81 ±1	107 ± 12

a = value significantly different from the value at 22°C by Student's t-test at $p<0.05$ or less

b = value significantly different from the value at 22°C by Student's t-test at $p<0.005$ or less

Table 3-3 Activation energies for *E. solidaginis* pyruvate kinase

Data are presented as mean ± S.E.M., n = 4

	E_a (kJ/mol)
Control	62 ± 3
Glycerol 1M	67 ± 1
Glycerol 1 M + sorbitol 0.5 M	71 ± 1 [a]

a : significantly different from control by the Student's t-test at $p<0.05$

Table 3-4 Effect of thermal denaturation on activity of *E. solidaginis* pyruvate kinase.

Data are presented as mean ± S.E.M., n = 4

	$t_{1/2}$ (minutes)
Control (53°C)	32 min ± 3 min
1 M glycerol	28 min ± 3 min
1 M sorbitol	> 400 min
1 M glucose	85 min ± 8 min [a]
5 mM PEP	> 450 min
1M KCl	86 min ± 11 min [a]
1 M urea	4 min ± 0.2 min [b]
3 M urea	2 min ± 0.2 min [b]
1 M urea + 1 M glycerol	4 min ± 0.4 min [b]
1 M urea + 1 M sorbitol	29 min ± 2 min s [c]
3 M urea + 1 M glycerol	2 min ± 0.2 min
3 M urea + 1 M sorbitol	3 min ± 0.32 min [b,d]

a : values significantly different from control at $p < 0.005$

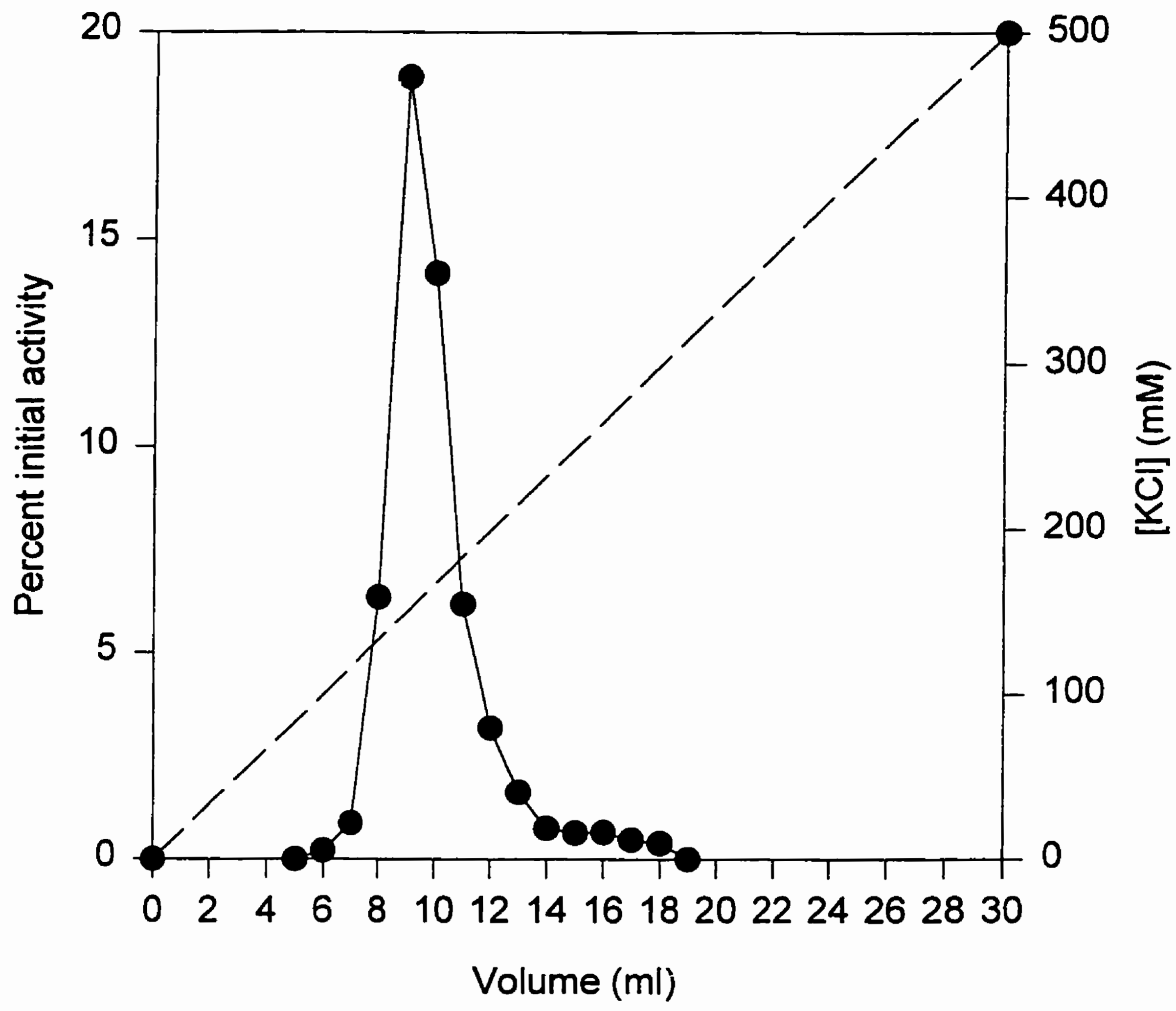
b : values significantly different from control at $p < 0.001$

c : value significantly different from 1 M urea at $p < 0.001$

d : value significantly different from 3 M urea at $p < 0.05$

Figure 3-1 Elution profile of *E. solidaginis* pyruvate kinase from a cation exchanger CM-cellulose 52 column.

The enzyme was eluted in a single peak with a linear gradient of KCl (0 to 500 mM; dashed line). The amount of activity in each fraction is shown as a percentage of the initial total amount of activity originally present in the supernatant.



Percent initial activity
[KCl] (mM)
Volume (ml)
0
5
10
15
20
0
100
200
300
400
500
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Figure 3-2 Elution profile of *E. solidaginis* pyruvate kinase from a Sephacryl S-400 size exclusion column.

Activities are relative to the crude supernatant total activity.

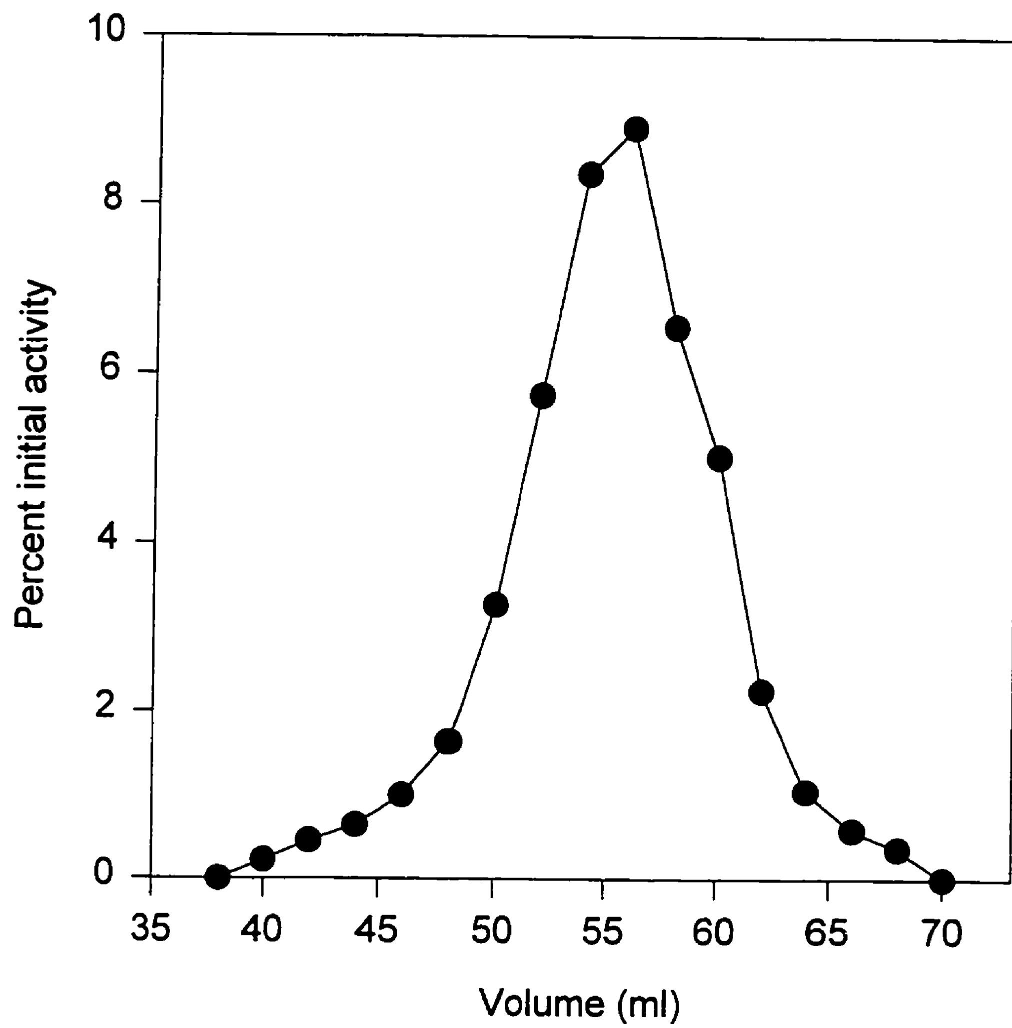
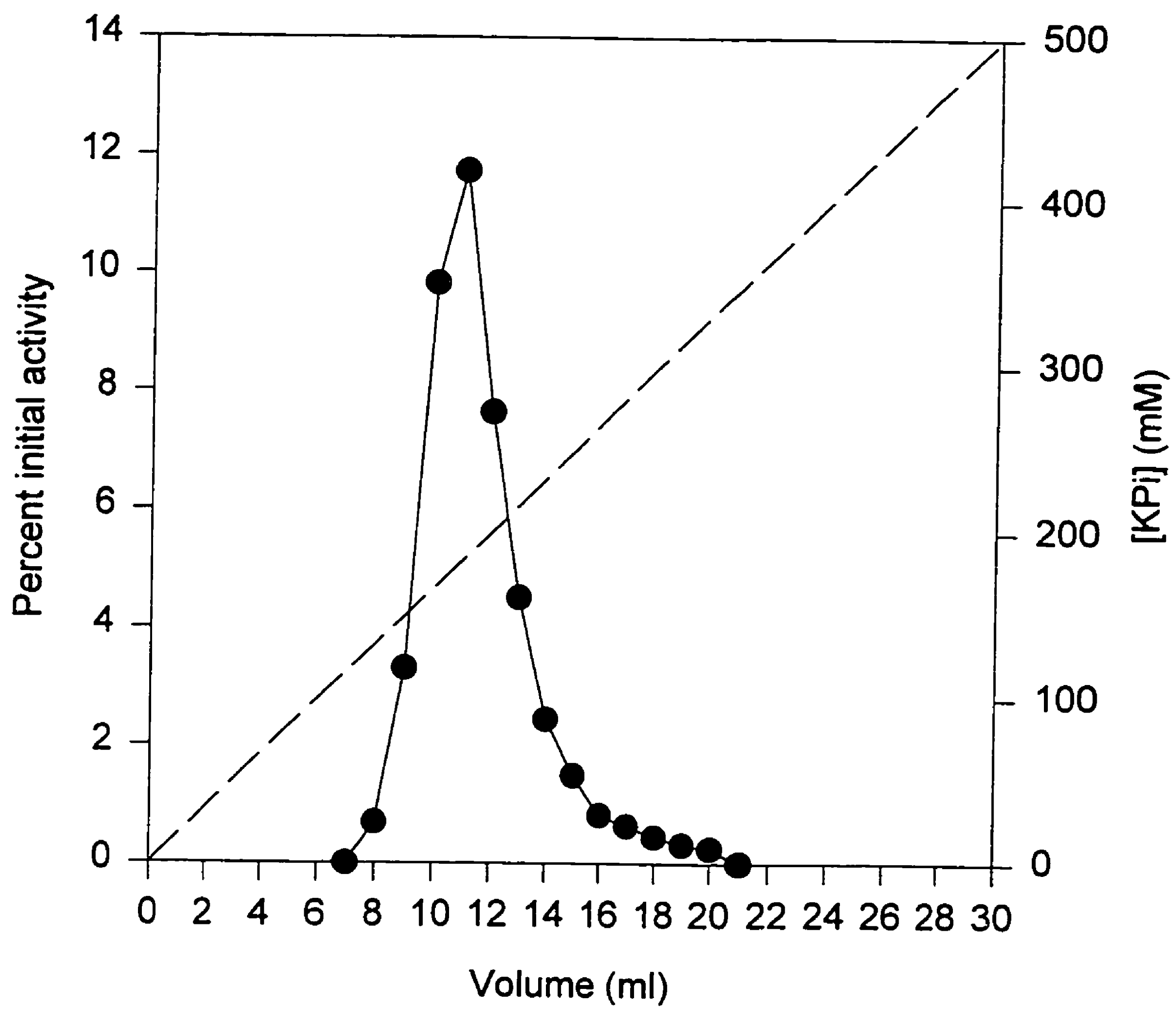

Percent initial activity
10
8
6
4
2
0
35
40
45
50
55
60
65
70
Volume (ml)

Figure 3-3 Elution profile of *E. solidaginis* pyruvate kinase from a hydroxylapatite affinity column.

The enzyme was eluted in a single peak with a linear gradient of KPi (0 to 500 mM; dashed line). Activities are relative to the crude supernatant total activity.



14
12
10
8
6
4
2
0
Percent initial activity
500
400
300
200
100
0
[KPi] (mM)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Volume (ml)

Figure 3-4 SDS-PAGE gel of purified *E. solidaginis* pyruvate kinase.

The standards are in lane 1 and lane 2 is the final enzyme preparation.

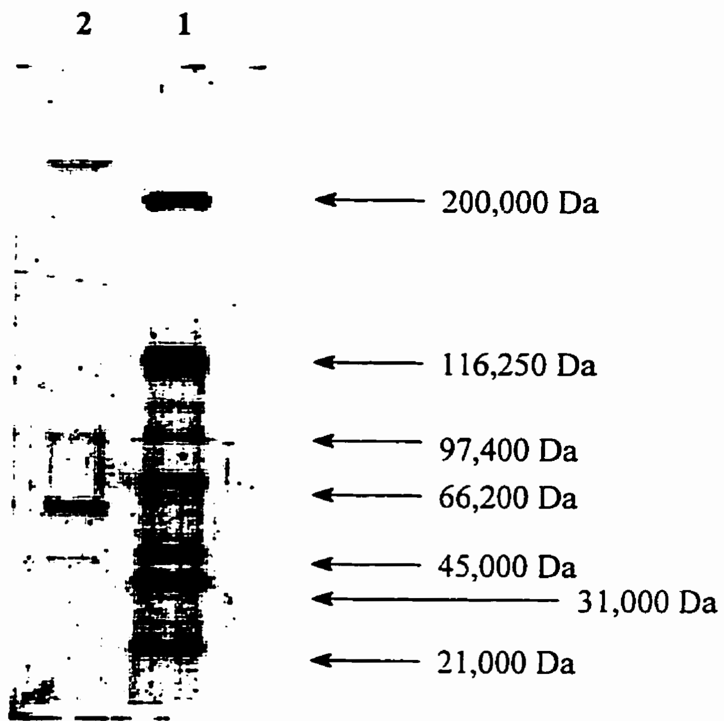

2
1
200,000 Da
116,250 Da
97,400 Da
66,200 Da
45,000 Da
31,000 Da
21,000 Da

Figure 3-5 Standard curve of logarithm of molecular weight versus relative migration of protein standards on a SDS-PAGE gel.

The logarithm of the molecular weight of the following standards were plotted against their relative migration in the gel ($r^2 = 0.93$). The molecular weights were: 1) myosin (200,000 Da), 2) β-galactosidase (116,250), 3) phosphorylase b (97,400 Da), 4) serum albumin (66,200 Da), 5) ovalbumin (45,000 Da), 6) carbonic anhydrase (31,000 Da), 7) trypsin inhibitor (21,000 Da), 8) lysozyme (14,400 Da), and 9) aprotinin (6,500 Da). The position of pyruvate kinase is indicated by an open circle.

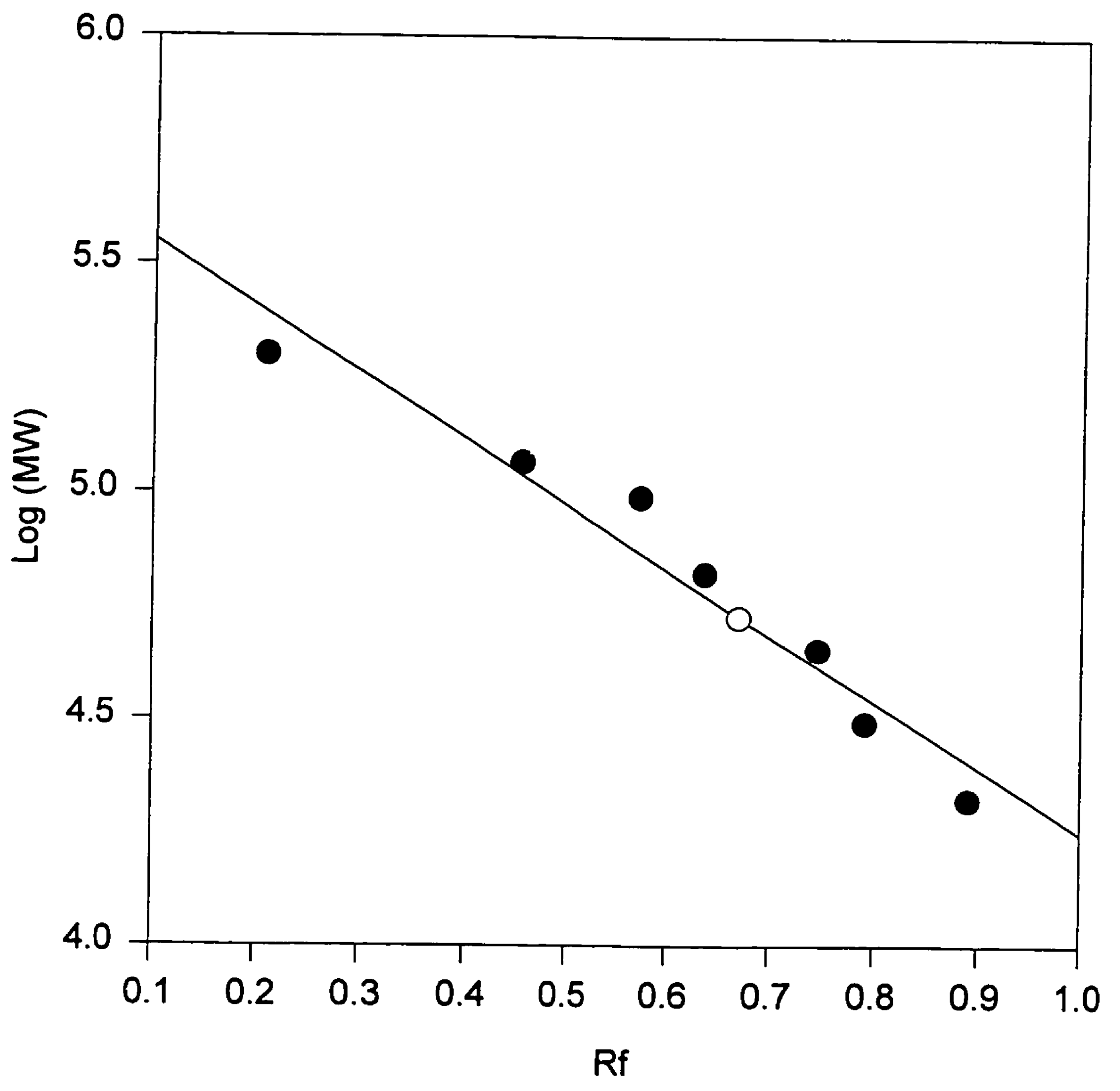
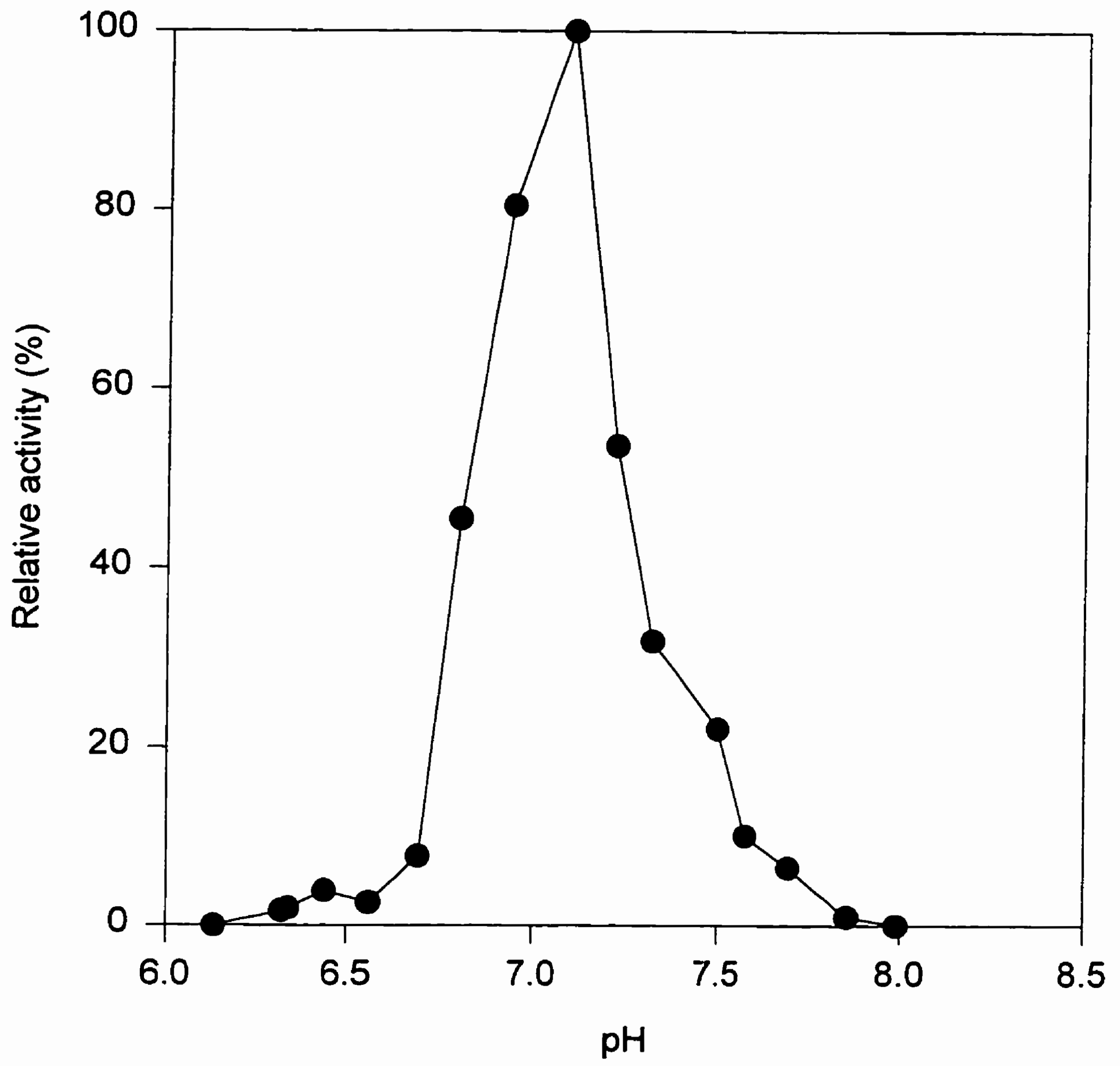

6.0
5.5
5.0
4.5
4.0
Log (MW)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf

Figure 3-6 Isoelectrofocusing profile of *E. solidaginis* pyruvate kinase.

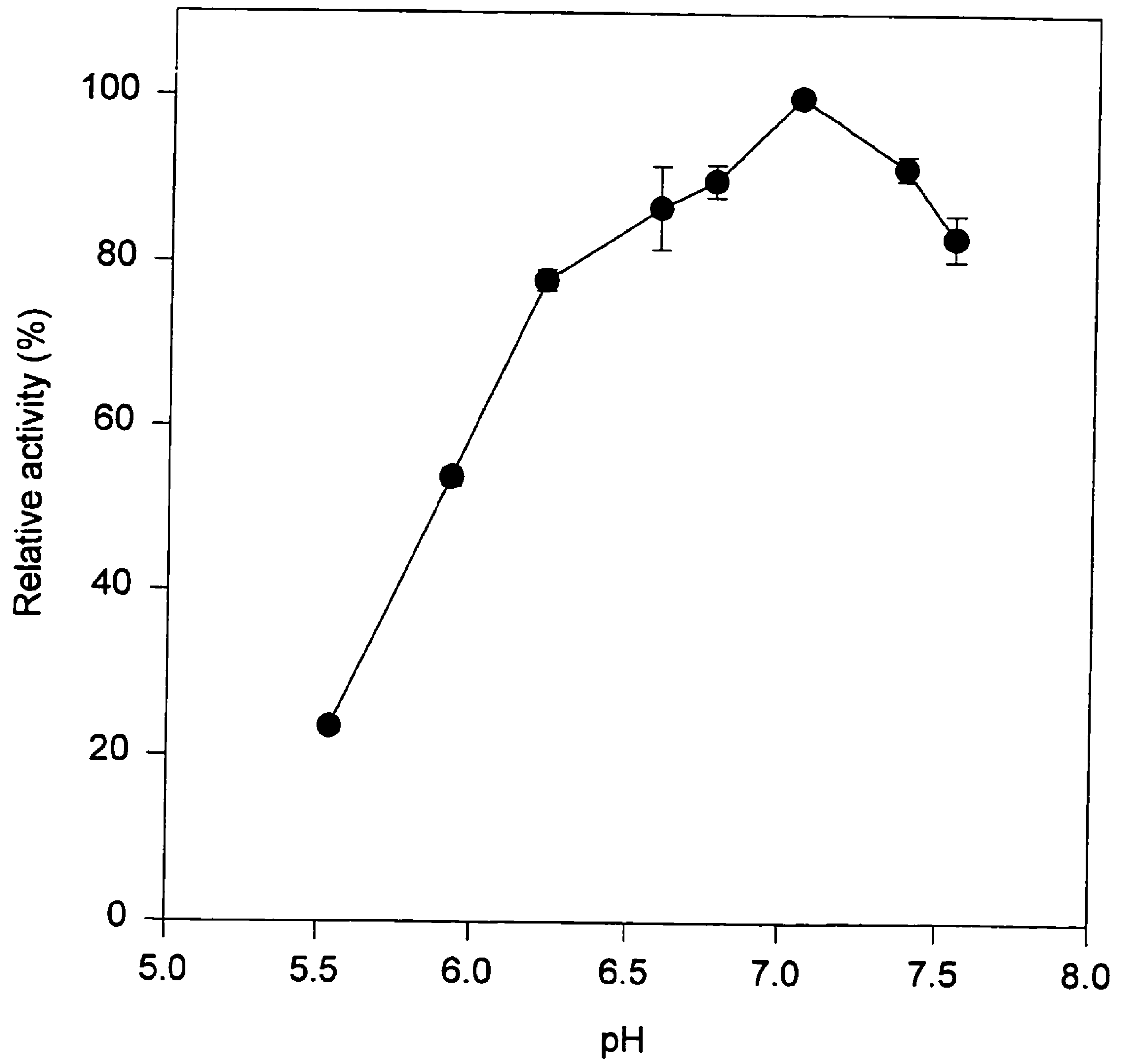
Enzyme activities are relative to the peak fraction and are plotted against the measured pH of each fraction.



Relative activity (%)
100
80
60
40
20
0
6.0
6.5
7.0
7.5
8.0
8.5
pH

Figure 3-7 pH profile of *E. solidaginis* pyruvate kinase at 22°C.

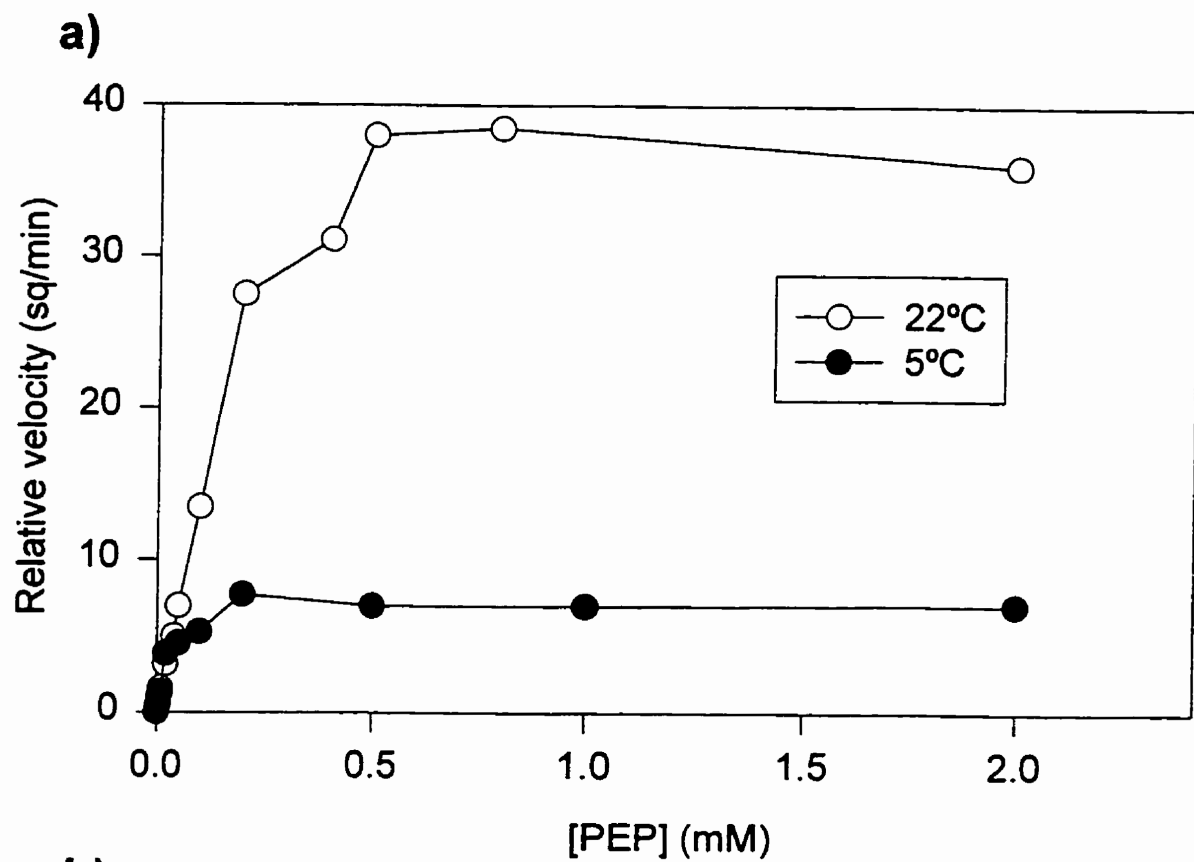
Four determinations were performed on the same enzyme preparation. All points are presented as mean ± S.E.M.. Where error bars are not shown, they are contained within the symbol. Activities are shown relative to the maximum activity.



Relative activity (%)
100
80
60
40
20
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

Figure 3-8 Plots of velocity versus substrate concentration for *E. solidaginis* pyruvate kinase at 5°C and 22°C.

The plots illustrate the changes in enzyme activity when concentrations of PEP (a) and ADP (b) were varied at 5°C or 22°C. Open circles represent pyruvate activity at 22°C and solid circles represent activity at 5°C. Activity was determined on a purified enzyme preparation. Each point represents a single measurement.


a)
Relative velocity (sq/min)
[PEP] (mM)
22ºC
5ºC
0
10
20
30
40
0.0
0.5
1.0
1.5
2.0

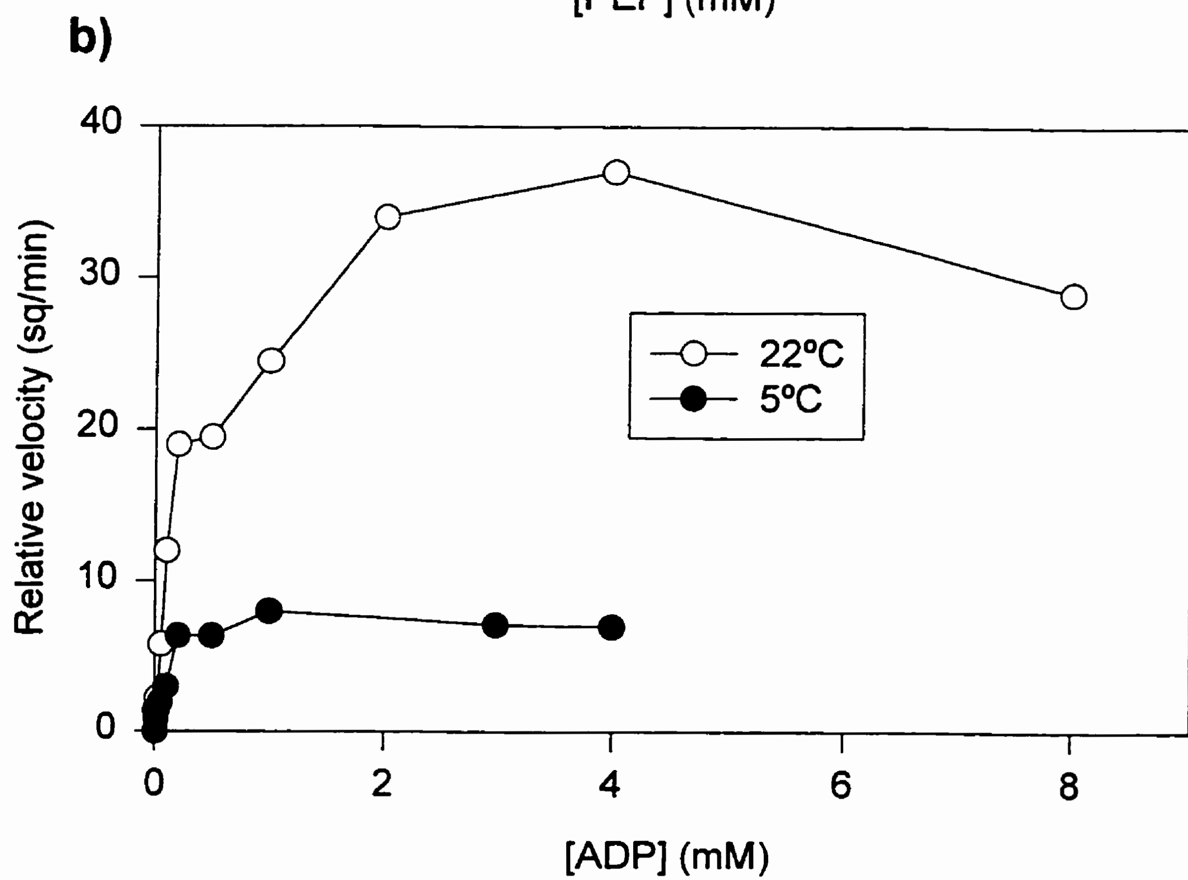
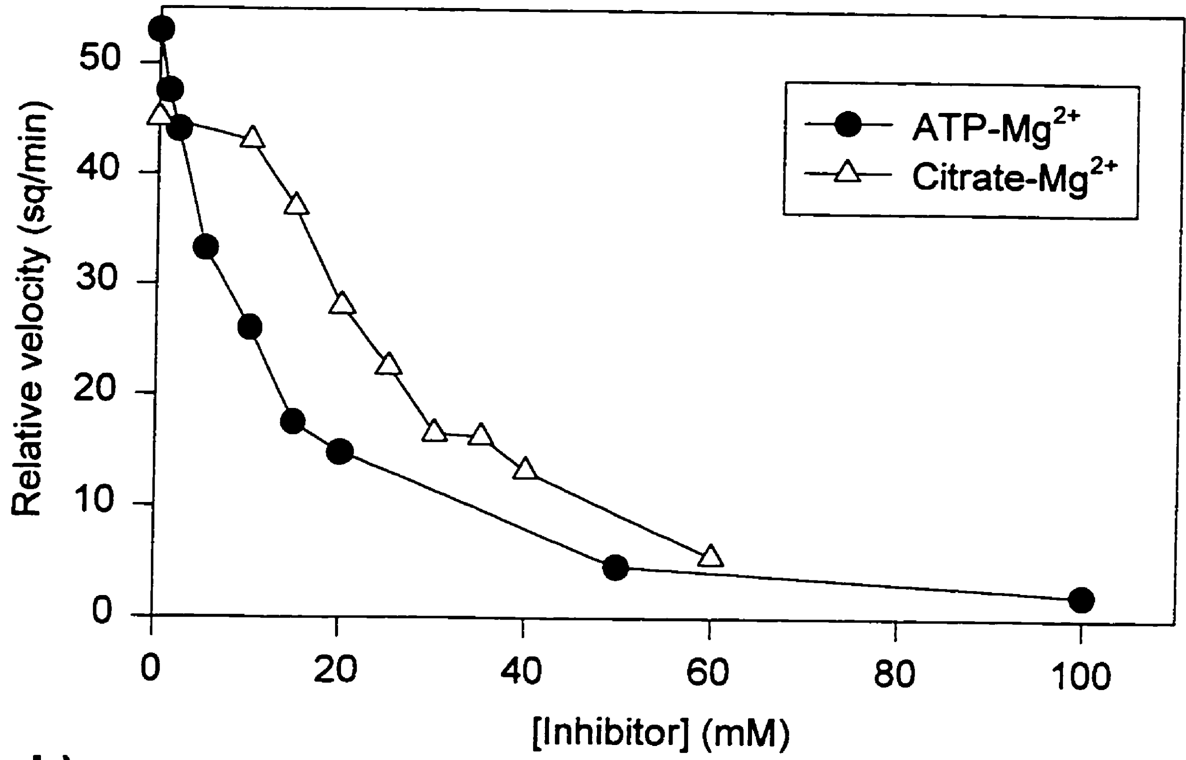

b)
Relative velocity (sq/min)
[ADP] (mM)
22ºC
5ºC
0
10
20
30
40
0
2
4
6
8

Figure 3-9 Plots of velocity versus various effectors concentration for *E. solidaginis* pyruvate kinase at 22°C.

The upper graph illustrates the effects of increasing concentrations of inhibitors (ATP-Mg^{2+} and citrate-2Mg^{2+}) on enzyme activity at 22°C. Solid circles represent ATP-Mg^{2+} inhibition and open triangles represent citrate-2Mg^{2+} inhibition. The lower graph illustrates the effect of increasing concentrations of fructose-1,6-bisphosphate on enzyme activity at 22°C. Each point represents a single measurement.


a)
Relative velocity (sq/min)
50
40
30
20
10
0
0
20
40
60
80
100
[Inhibitor] (mM)
ATP-Mg2+
Citrate-Mg2+

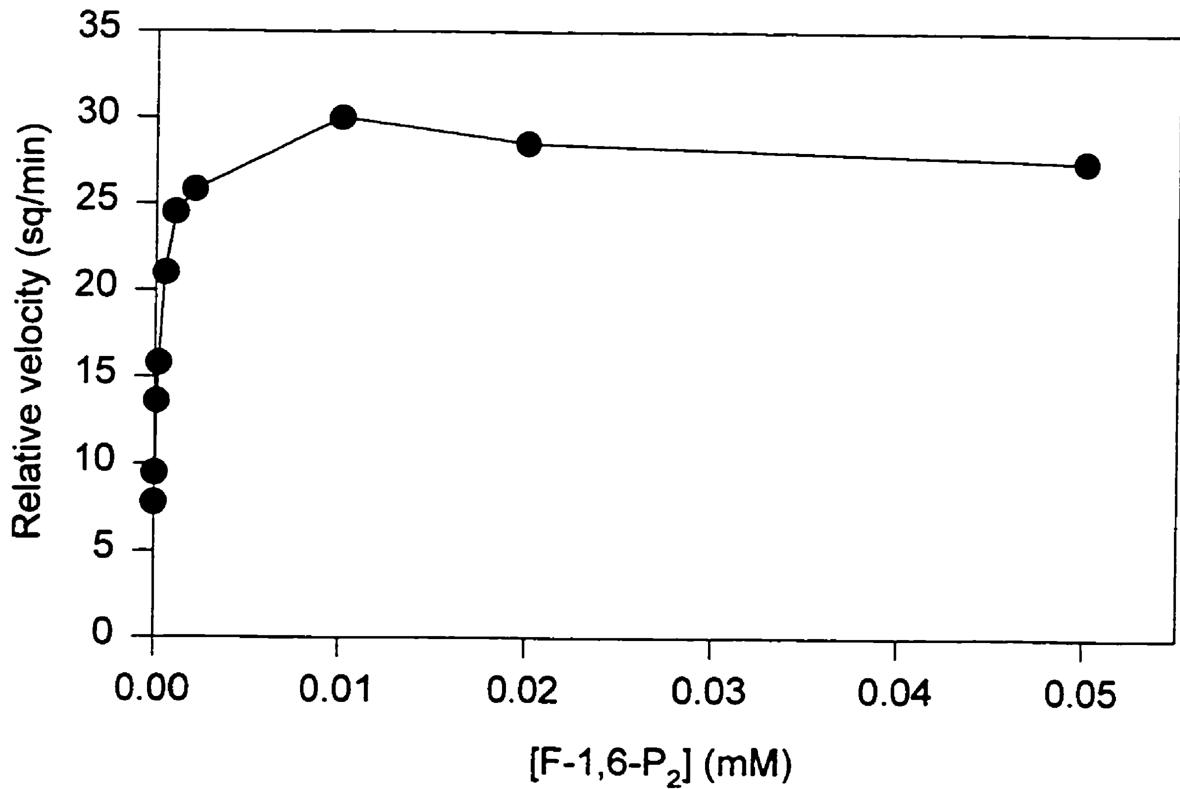
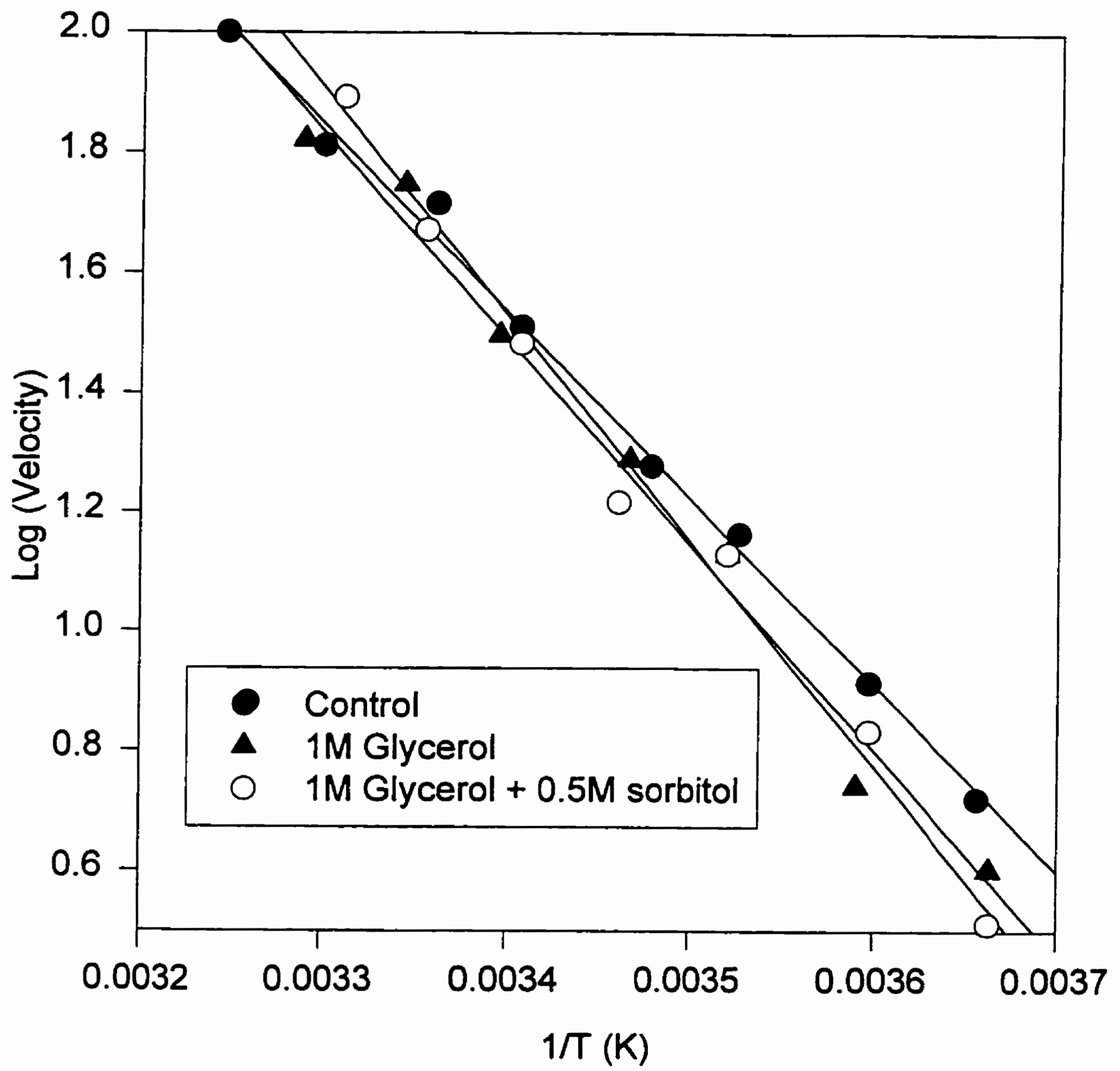

b)
Relative velocity (sq/min)
35
30
25
20
15
10
5
0
0.00
0.01
0.02
0.03
0.04
0.05
[F-1,6-P2] (mM)

Figure 3-10 Arrhenius plots for *E. solidaginis* pyruvate kinase.

The logarithm of the velocity versus the reciprocal temperature (°K) was plotted. Each point represents a single measurement. The slope of the plot is related to the activation energy (Ea) by the equation :

$$s = -E_a/(2.303R)$$

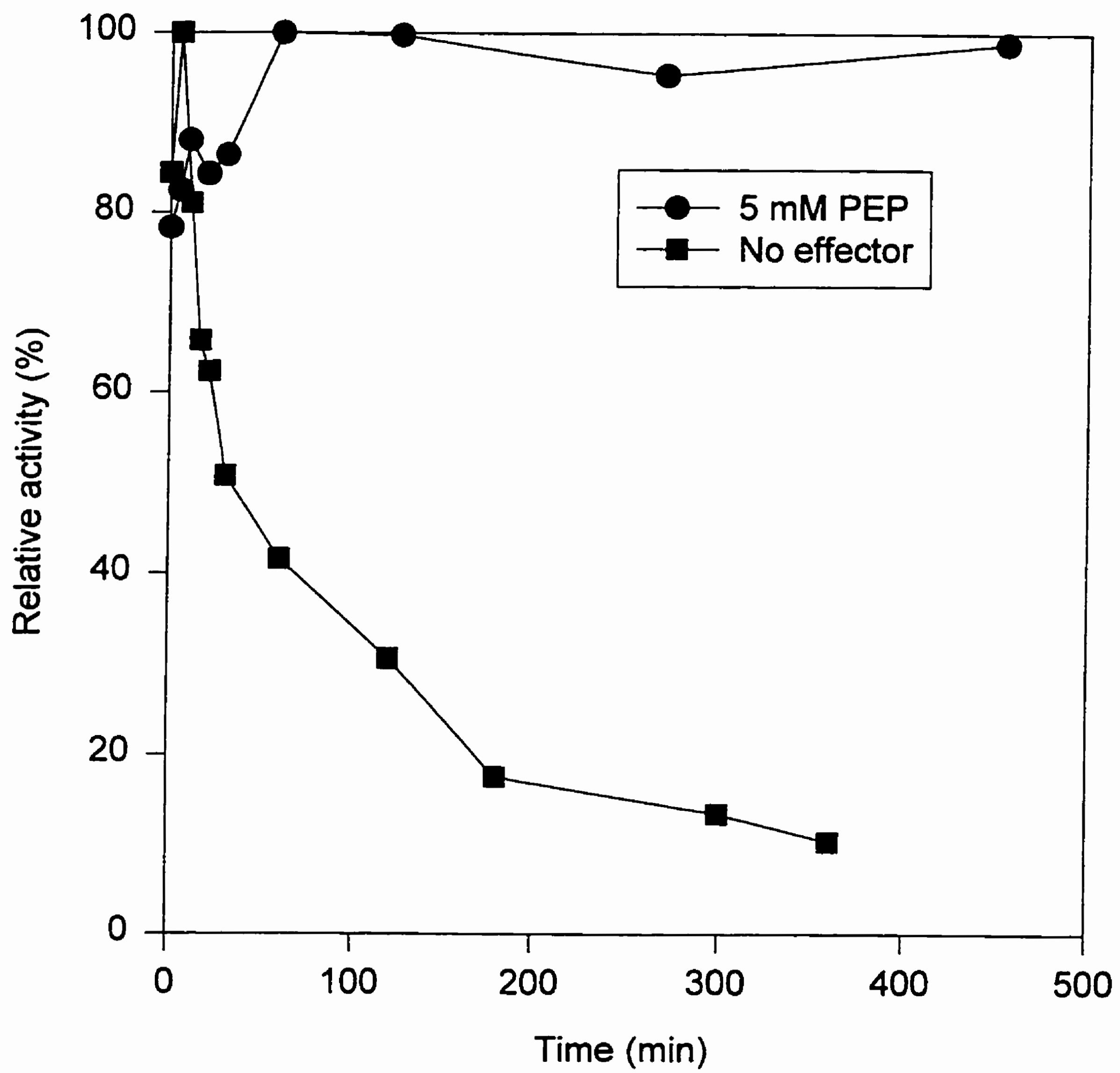
Solid circles represent values in the absence of polyols, solid triangles in the presence of 1M glycerol and open circles in the presence of 1M glycerol and 0.5M sorbitol.



2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
Log (Velocity)
0.0032
0.0033
0.0034
0.0035
0.0036
0.0037
1/T (K)
Control
1M Glycerol
1M Glycerol + 0.5M sorbitol

Figure 3-11 Thermal denaturation of *E. solidaginis* pyruvate kinase in the presence or absence of 5 mM PEP.

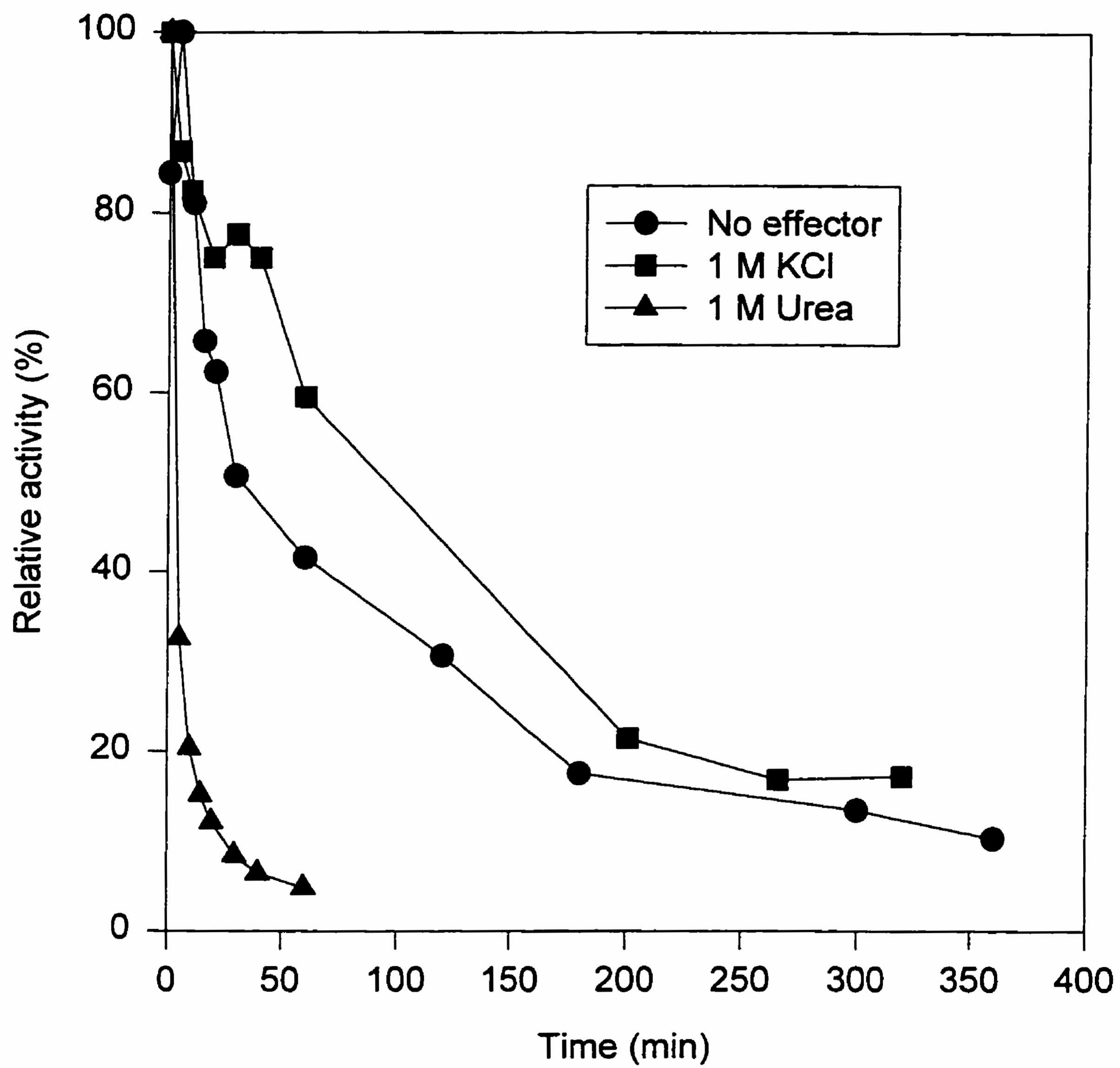
Experiments were carried out at 53°C. Solid squares represent the incubation in absence of any effector and solid circles the incubation in presence of 5 mM PEP. Each point represents a single measurement.



100
80
60
40
20
0
0
100
200
300
400
500
Relative activity (%)
Time (min)
5 mM PEP
No effector

Figure 3-12 Effects of KCl or urea on the thermal denaturation of *E. solidaginis* pyruvate kinase at 53°C.

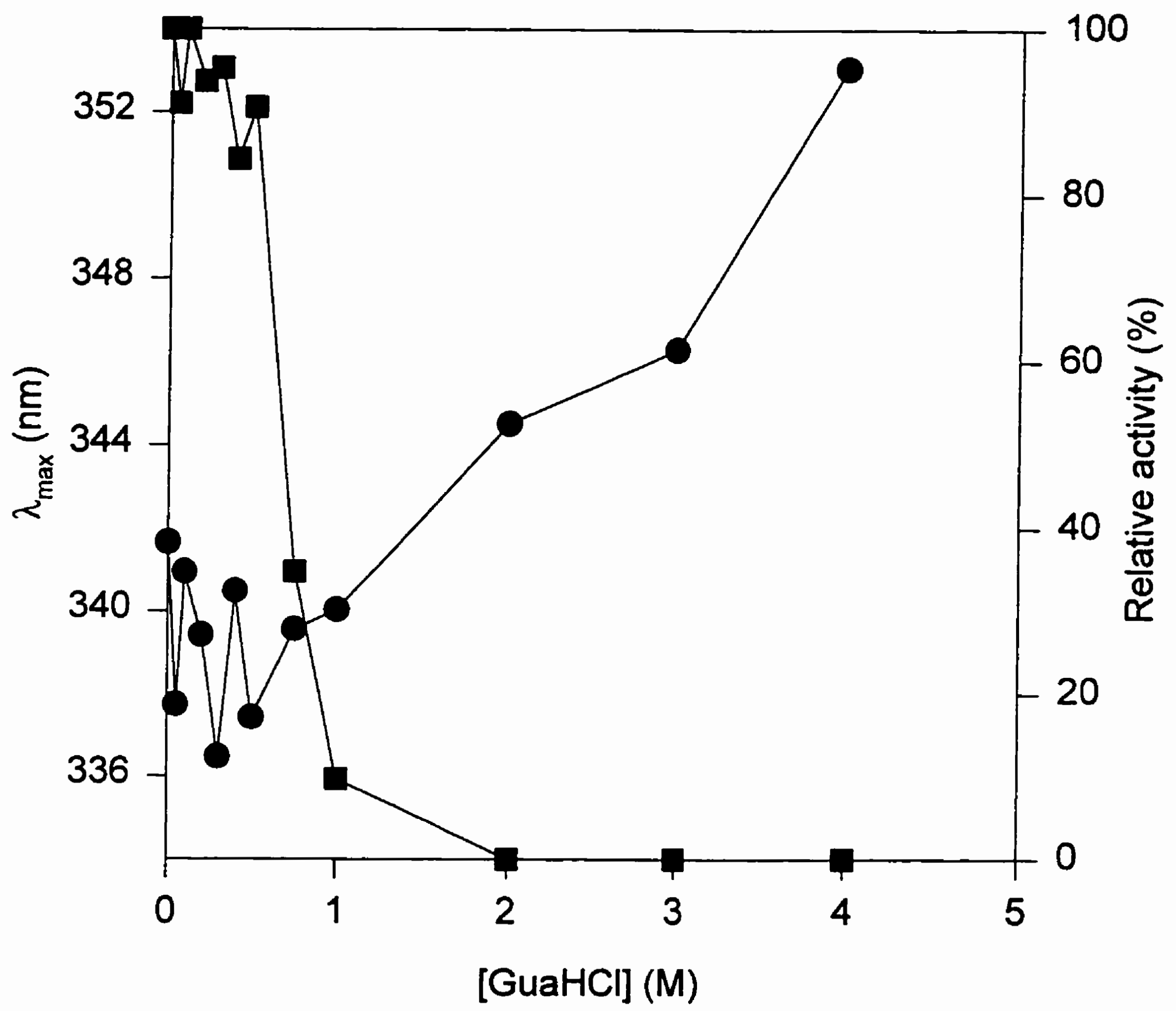
Solid circles represent the incubation in absence of any effector, solid squares the incubation in presence of 1M KCl and solid triangles the incubation in presence of 1 M urea. Each point represents a single measurement.



Relative activity (%)
Time (min)
No effector
1 M KCl
1 M Urea
0
20
40
60
80
100
0
50
100
150
200
250
300
350
400

Figure 3-13 Effect of guanidine hydrochloride on *E. solidaginis* pyruvate kinase.

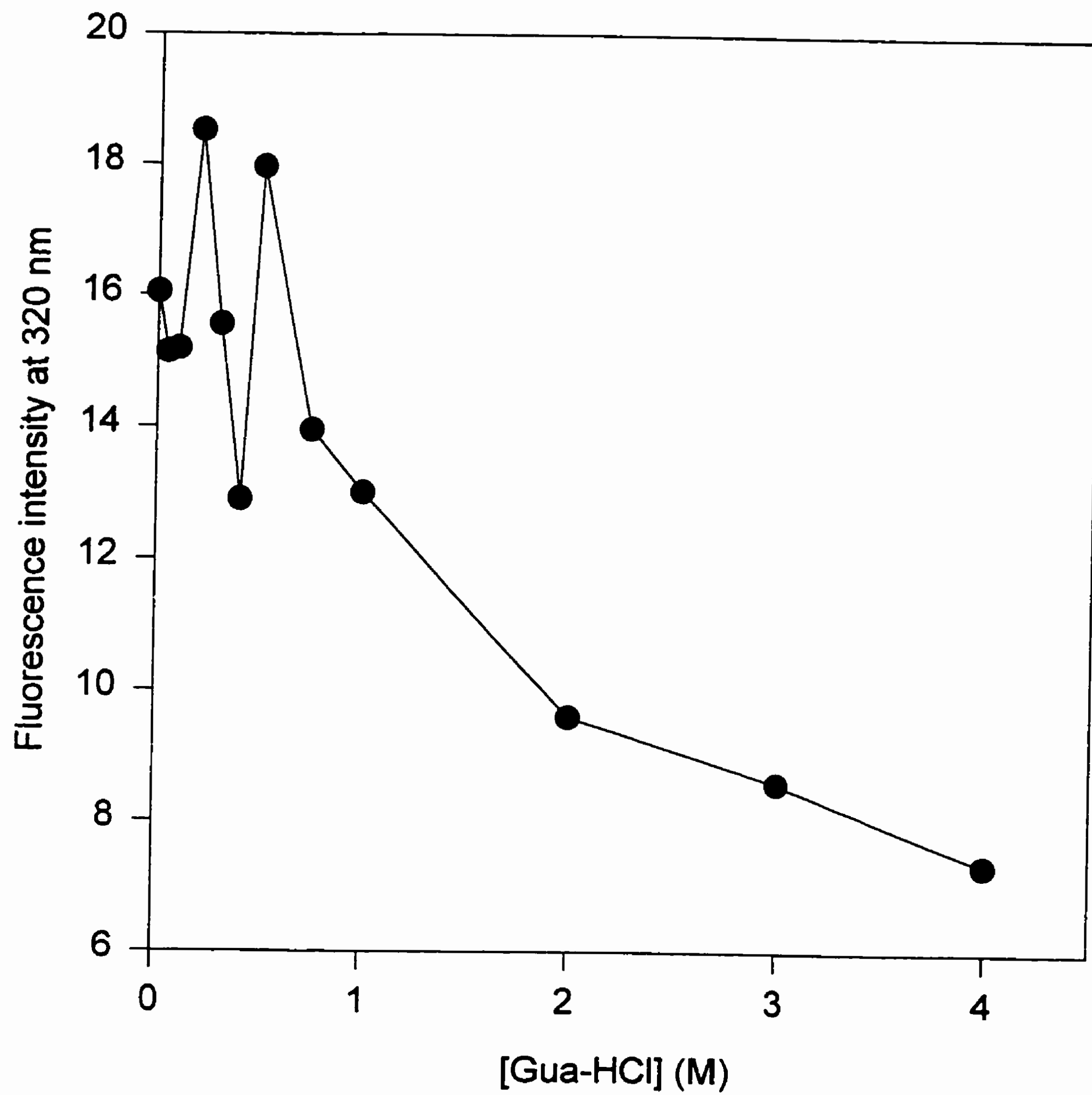
The plots illustrate the effect of increasing guanidine hydrochloride concentration on pyruvate kinase activity (squares) and maximal wavelength of fluorescence emission (circles). PK activity is reported relative to activity in the absence of added denaturant. Each point represents a single determination.



352
348
344
340
336
λ_{max} (nm)
100
80
60
40
20
0
Relative activity (%)
0
1
2
3
4
5
[GuaHCl] (M)

Figure 3-14 Effect of guanidine hydrochloride on the fluorescence intensity at 320 nm of *E. solidaginis* pyruvate kinase.

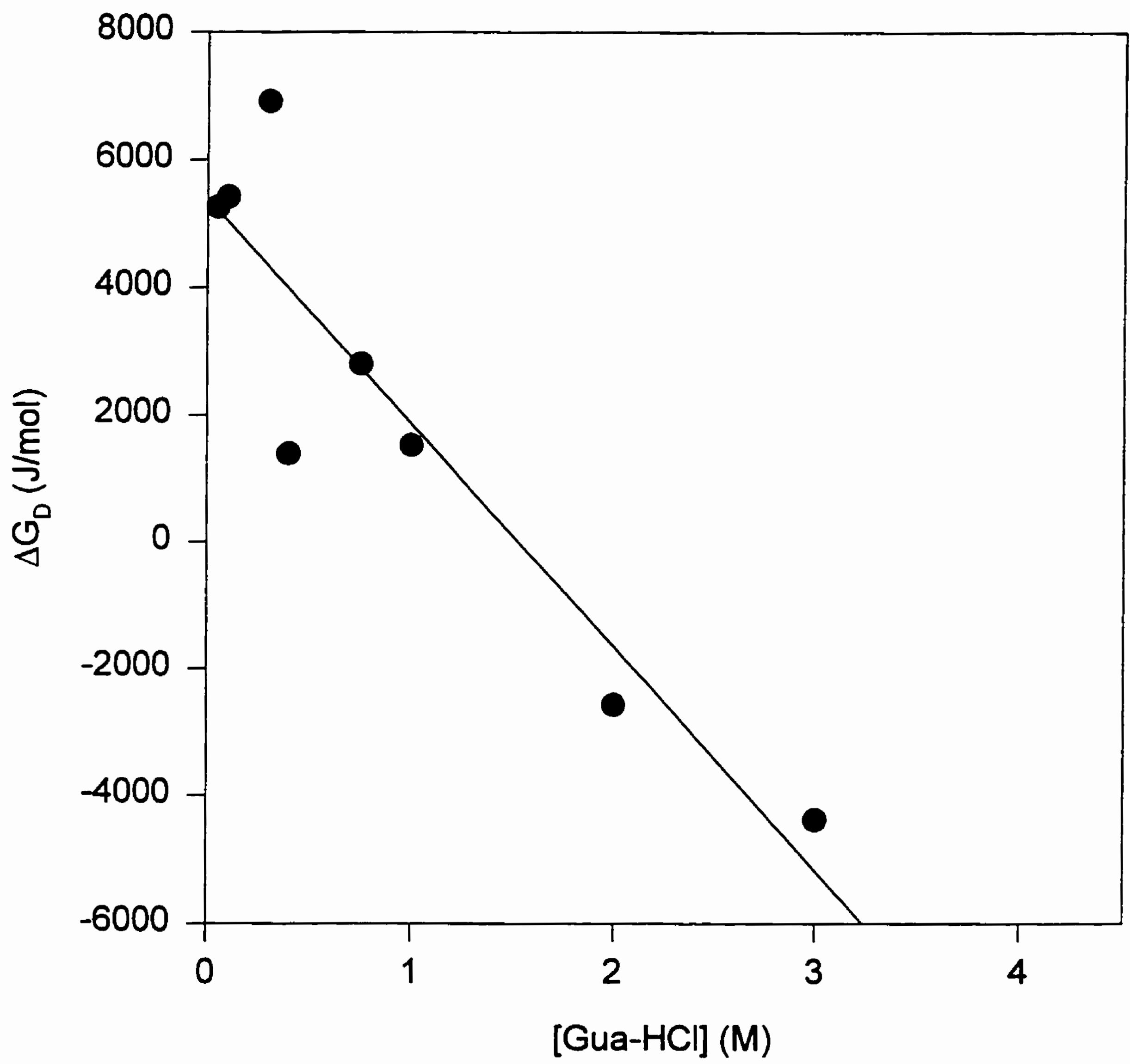
Each point represents a single determination.



20
18
16
14
12
10
8
6
Fluorescence intensity at 320 nm
0
1
2
3
4
[Gua-HCl] (M)

Figure 3-15 Determination of the free energy of denaturation of *E. solidaginis* pyruvate kinase.

The free energy of denaturation in aqueous solution was determined by plotting the free energy of denaturation for the different concentrations of guanidine hydrochloride and by extrapolating from the line obtained to the value for [GuaHCl] = 0 M. Each point represents a single measurement.

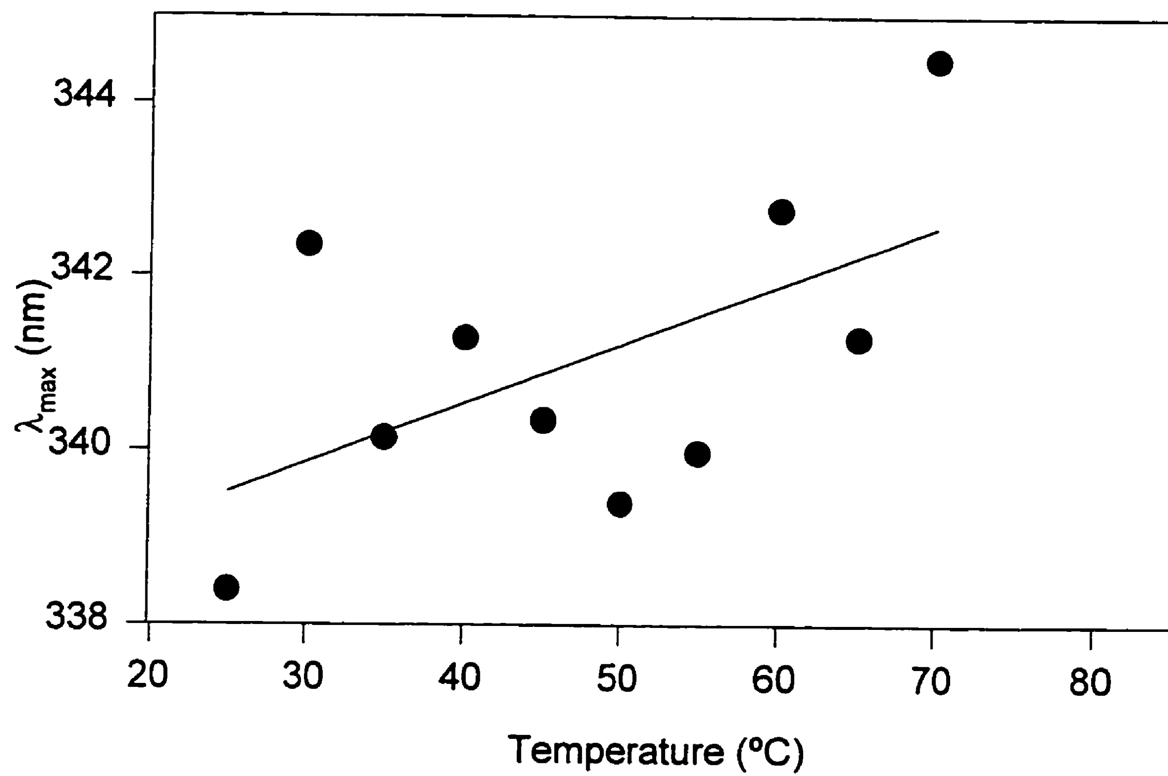


8000
6000
4000
2000
0
-2000
-4000
-6000
ΔG_D (J/mol)
0
1
2
3
4
[Gua-HCl] (M)

Figure 3-16 Effect of increasing temperatures on the maximal wavelength of fluorescence emission of *E. solidaginis* pyruvate kinase.

The upper graph (a) illustrates the variations in the maximal wavelength of the fluorescence emission spectrum with increasing temperature in the absence of polyols and the lower graph (b) in the presence of 200 mM sorbitol and 600 mM glycerol. Each point represents a single measurement.

a)



b)

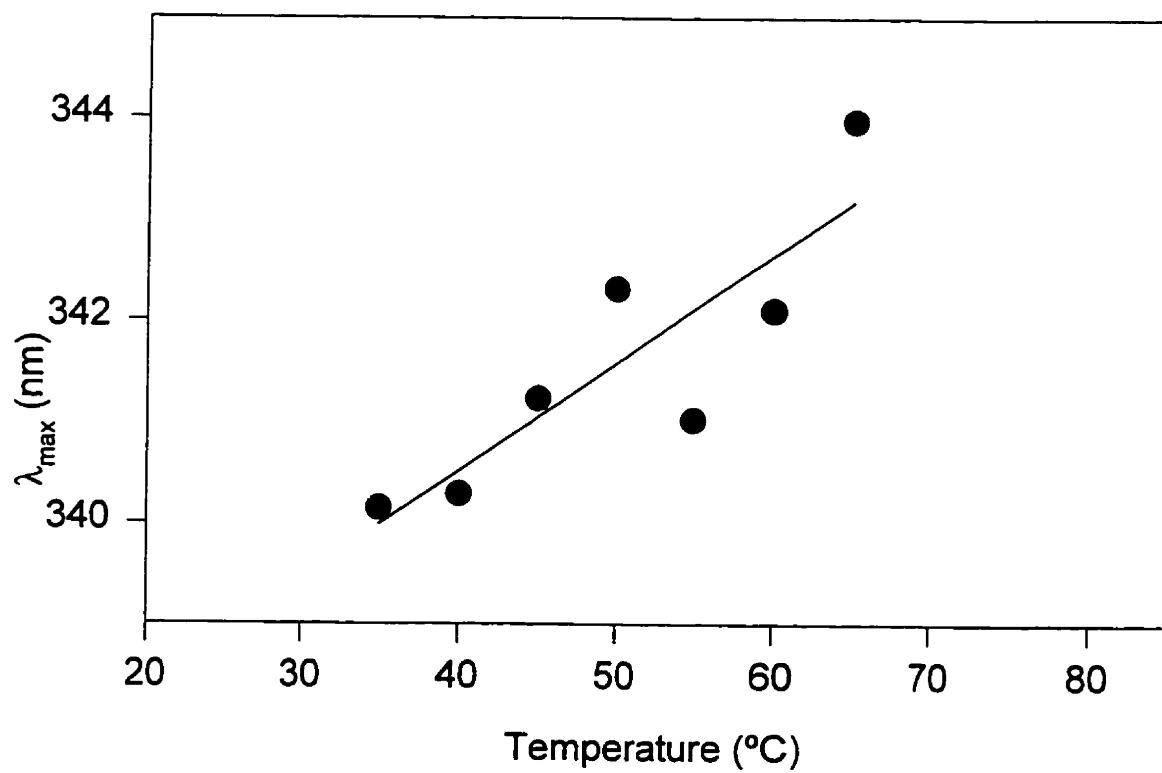
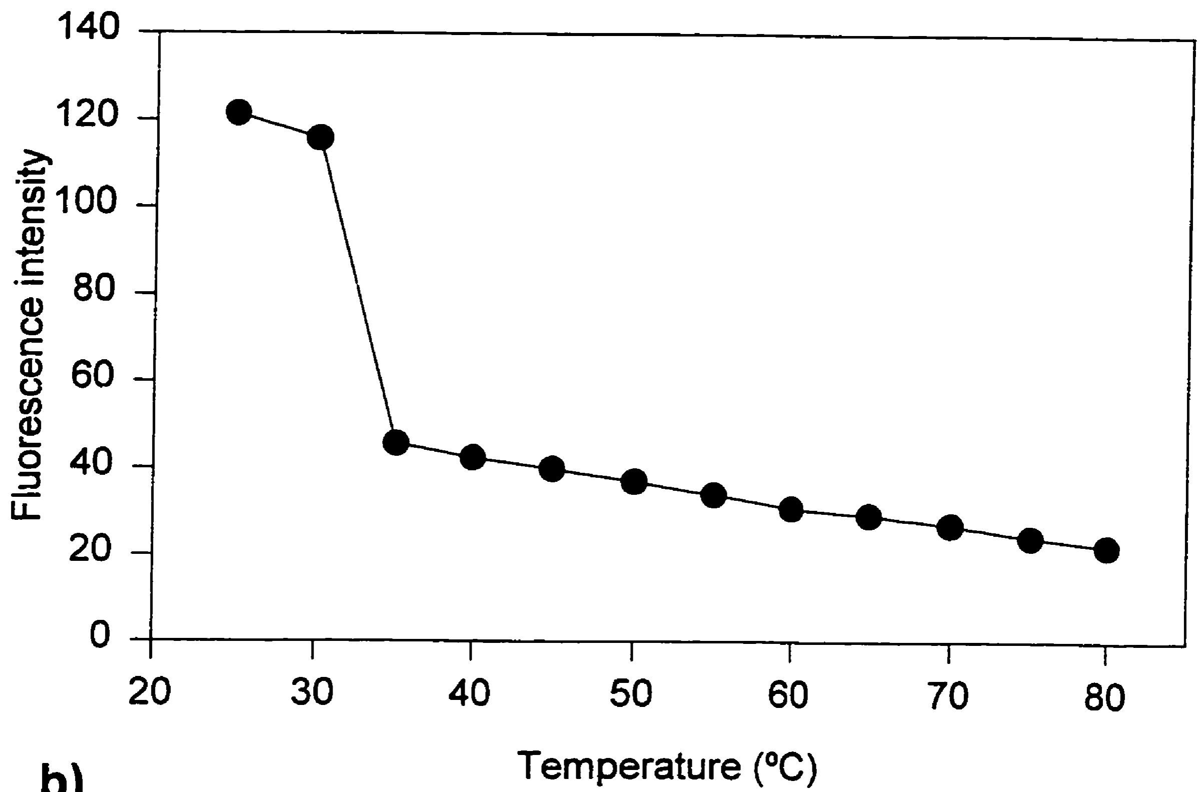
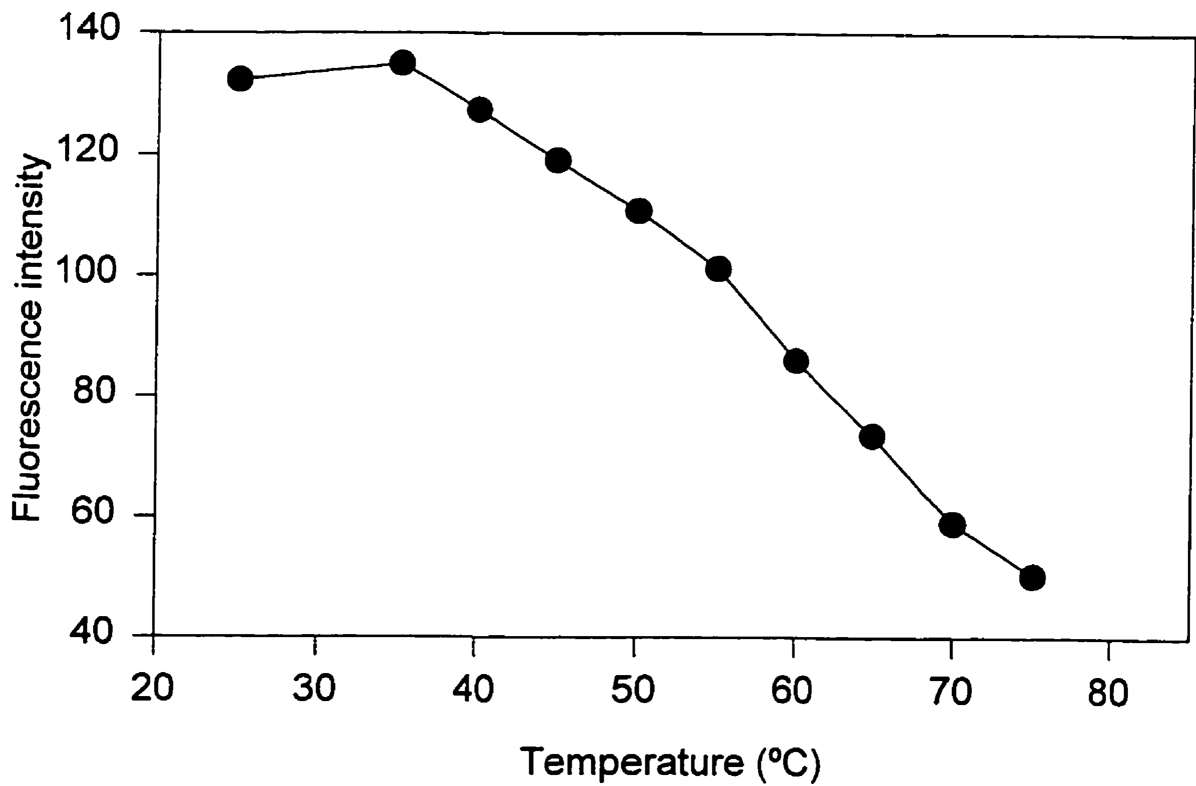


Figure 3-17 Effect of increasing temperatures on the fluorescence intensity at 320 nm of *E. solidaginis* pyruvate kinase.

The upper graph (a) illustrates the variations in fluorescence intensity with increasing temperature in the absence of polyols and the lower graph (b) in the presence of 200 mM sorbitol and 600 mM glycerol. Each point represents a single measurement.


a)
140
120
100
80
60
40
20
0
Fluorescence intensity
20
30
40
50
60
70
80
Temperature (ºC)


b)
140
120
100
80
60
40
Fluorescence intensity
20
30
40
50
60
70
80
Temperature (ºC)

DISCUSSION

To protect itself against cold *Eurosta solidaginis* produces large amounts of glycerol (600 mM) and sorbitol (200 mM). During the fall, mechanisms of metabolic regulation are used to modify the flux of carbon through the glycolytic pathway and to direct this flux towards the synthesis of polyols. Glycerol accumulates first, during the early autumn and while environmental temperatures are still quite high (ie. above about 10°C). Below about 5°C, however, sorbitol synthesis is activated. Since both polyols are produced from glycogen, differential metabolic controls are needed to regulate the production of each. Previous studies have shown that low temperature inhibition of phosphofructokinase is probably the major factor responsible for the cessation of glycerol synthesis and the diversion of carbon flux into sorbitol synthesis instead (Storey 1982). A block on glycolysis at phosphofructokinase halts carbon flow into the triose phosphates (from which glycerol is derived) and promotes the use of hexose phosphates instead for sorbitol synthesis. When glycerol is being synthesized it is reasonable to propose that an inhibitory block on the lower portion of glycolysis should be present to help divert triose phosphate carbon out of glycolysis and into the reactions of glycerol synthesis. Such a block could occur at PK. Indeed, changes in the levels of substrate (PEP) and product (pyruvate) of PK when larvae are abruptly chilled from 23°C to 13°C (stimulating glycerol synthesis) indicate that PK is inhibited by this temperature change (Storey and Storey 1983b). Coupled with an activation of glycogenolysis by the same temperature shift, this could set up carbohydrate flux to allow the massive synthesis of glycerol. Since PK concentration in the larvae, measured by enzyme maximal activity, hardly varies from early fall through to spring (Joanisse and Storey, 1994), the regulation of the activity of this enzyme must involve an alteration of the enzyme itself.

Purification

Pyruvate kinase from *Eurosta solidaginis* was purified 483.5-fold with a final specific activity of 783 U/mg. This specific activity is higher than the specific activity reported for other invertebrates: 181.1 U/mg for the foot muscle PK from the bivalve mollusc, *Cardium tuberculatum* (Chrispeels and Gäde 1985), 368 U/mg for muscle PK of the channeled whelk *Busycotypus canaliculatum* (Plaxton and Storey 1984) and 400-450 U/mg for flight muscle PK of the cockroach *Periplaneta americana* (Storey 1985).

The subunit molecular weight of *Eurosta solidaginis* pyruvate kinase obtained by SDS-PAGE gel was found to be 53,140 Da. This value is in the range of other values obtained for pyruvate kinases: 55 kDa for PK from the hepatopancreas of the shore crab, *Carcinus maenas* (Giles et al. 1976), 54 kDa for bovine L-type PK (Cardenas and Dyson 1973), and 52 kDa for the chicken L-type PK (Hall and Cottam 1978).

Isoelectric point

A previous study has shown that PK from *Eurosta solidaginis* is present in only one form, and the same single form occurred in larvae acclimated at 24°C, 10°C, -10°C or -30°C (Storey et al. 1981b). That study also found a pI value for the enzyme of 7.08 and the results obtained in the present study agree with this: isofocusing revealed only one form of PK with a pI of 7.10. The range of pI values found for pyruvate kinases from other sources is very wide, pI varying from 5.5 (pig kidney, M2-type PK, Berglund et al. 1977) to 8.5 (chicken breast M1-type PK, Cardenas et al. 1975), but some enzymes have pI values close to the one determined for *Eurosta solidaginis*. Human R-type PK, for example, has a pI value of 7.10 (Blume et al. 1971).

pH profile

At 22°C, the pH optimum for *Eurosta solidaginis* PK was found to be 7.10. However, the pH curve exhibits a broad optimum and at pH 6.8, the pH of the haemolymph of the larva at 18°C (Storey et al. 1986), the relative activity of PK is still very high (greater than 80% of optimal). Other invertebrates have a pH_{opt} for PK close to 7.10 including the desert locust *Schistocerca gregaria* (fat body PK, pH_{opt} = 7.2, Bailey and Walker 1969), the channeled whelk *Busycotypus canaliculatum* (muscle PK, pH_{opt} = 7.0, Plaxton and Storey 1984) and the cockroach *Periplaneta americana* (flight muscle PK, pH_{opt} = 7.0, Storey 1985).

Kinetic studies

Eurosta solidaginis pyruvate kinase is an allosteric enzyme that exhibits positive cooperativity in its kinetics with respect to PEP ($n_H = 1.3 \pm 0.7$). At high temperature and in the absence of any cryoprotectants, the situation in vivo for the larvae at the beginning of the autumn, PK is also allosterically activated by fructose-1,6-bisphosphate and inhibited by ATP and citrate, but not by alanine. In these properties the enzyme resembles fat body (the insect organ similar to liver) PK from the cockroach *Periplaneta americana* (Storey 1985) and the mammalian liver type of PK which exhibits sigmoidal PEP kinetics and allosteric regulation. The absence of alanine inhibition is interesting to note since alanine is a major physiological inhibitor of the enzyme in many animals such as the anoxia-tolerant mollusc channeled whelk *Busycotypus canaliculatum* (Plaxton and Storey 1984), the carp *Carassius carassius L.* (Johnston 1975) and the house cricket *Acheta domesticus L.* (Hoffmann 1975). In situations where alanine is a strong regulator of PK, the functions of its regulatory effects appear to be a) inhibit PK when free amino acids are available for gluconeogenesis, or b) inhibit PK to allow PEP to be directed into pathways

of anaerobic succinate formation (which are linked with ATP synthesis). Neither of these functions are probably relevant in the larvae, which may account for the lack of alanine effects on *E. solidaginis* PK.

When the environmental temperature decreased from 22 to 5°C, the enzyme V_{max} was reduced by 75-80%. However this is not particularly remarkable and is not a situation that larvae are likely to encounter (without the adequate preparation for the winter, cold temperatures would kill larvae). A more convincing indication of a suppression of PK at low temperature is given by the effects of fructose-1,6-bisphosphate. This metabolite, which was a very good activator at high temperature, completely lost its activating capacity when the assay temperature was reduced to 5°C. This could be very important as $F1,6P_2$ is a powerful activator and usually affects the enzyme by reducing its K_m for PEP. Since in vivo ADP and PEP levels are low, the loss of $F1,6P_2$ sensitivity may reduce significantly the efficiency of the enzyme in vivo at low temperature. Furthermore, the function of $F1,6P_2$ activation of PK is to provide feed-forward activation of the next important regulatory enzyme of glycolysis from the previous regulatory locus at PFK. High $F1,6P_2$ indicates that PFK has been activated and that glycolytic flux is increasing and so $F1,6P_2$ effects on PK provide the necessary activation to coordinate PK activity with that of PFK. By losing $F1,6P_2$ effects on PK at low temperature, PK activity becomes unlinked from PFK activity and the increased PFK activity that supports glycerol synthesis no longer automatically increases PK as well. Hence, one enzyme (PFK) can be activated while the other (PK) is inhibited. Finally, the behavior of the inhibitors ATP and citrate is also in full agreement with an inhibition of pyruvate kinase at cold temperature. Although in the absence of polyols the inhibitory effect of ATP (physiological levels are 2 mM, Storey et al. 1981a) decreases when the temperature is lowered, the differential effect of temperature on the inhibition constant disappears when polyols are present and the I_{50} ATP at 5°C is lower in the presence of glycerol than in its absence. This is favorable to the insect since the maintenance of PK

inhibition is important during the winter. Similarly, the I_{50} value for citrate (physiological level about 4-5 mM, Storey et al. 1981b) is also lower in the presence, than in the absence, of added polyols and this is especially true at 5°C. Thus as environmental temperature decreases in the autumn and polyols increase, PK becomes increasingly more sensitive to both ATP and citrate inhibition. Once again, this favors the suppression of pyruvate kinase activity and promotes the direction of carbon flux towards the synthesis of glycerol.

Once sorbitol has been synthesized, the metabolic rate of the larvae is very low, just enough to maintain basal metabolism. The inhibition of PK by citrate is stronger than it was when just glycerol was present (I_{50} divided by 1.8), suggesting that the flow of carbon going into the TCA cycle may be very reduced at low temperature. It is however necessary to maintain a certain flux of carbon through PK to produce some energy (ATP) and the enhanced affinity of PK for the two substrates (PEP and ADP) at low temperature as well as the lower K_a value for the divalent ion needed by the enzyme, Mg^{2+}, are probably used for this objective.

Like all other pyruvate kinases , PK from *E. solidaginis* needs K^+ to achieve maximal activity. High levels of K^+ are inhibitory, but since physiological K^+ in the cytoplasm is probably only around 100 mM, its effect on the enzyme are likely minimal. Like many other enzymes, PK is more sensitive to Na^+ inhibition than to K^+, but this correlates with the much lower intracellular Na^+ concentrations. Overall, ion effects on the enzyme are probably of limited regulatory significance.

Arrhenius plots

The linearity of the Arrhenius plots over the range of temperatures studied indicates that no change in conformation affecting the catalytic capacity of pyruvate kinase occurs at low temperature. The increase of the activation energy between the absence versus the presence of the two cryoprotectants glycerol and sorbitol, however, is a further indication (as was suggested above by the kinetic data) that PK activity in vivo may be strongly suppressed under the cellular conditions occurring during the winter.

Thermal denaturation studies

When an enzyme is heated, the weak bonds (hydrogen, ionic and hydrophobic bonds) that confer its three-dimensional structure are disrupted. The conformation of the enzyme changes, eventually affecting the active site to such an extent that catalytic activity is lost. Therefore, thermal denaturation experiments can give information on the stability of an enzyme. Pyruvate kinase showed quite good stability to heat since heating at 53°C was necessary to obtain a reasonable half life (about 32 min). A good stability of PK was indeed expected since *Eurosta solidaginis* is an ectothermic insect and has to face a wide range of environmental temperatures. Hence, its enzymes need to be able to function optimally over a wide range of temperatures.

Many organic solutes can stabilize protein structure if they are present at sufficiently high concentrations. They help to maintain a layer of water molecules around the protein and are called compatible solutes. It was therefore not surprising to find that sorbitol significantly stabilized the enzyme. What was unexpected, however, was the absence of a stabilizing effect of glycerol which is very commonly used as a protein stabilizer both commercially and in nature. For example, high concentrations of glycerol have been found in organisms living at high temperature, like the thermotolerant

eukariotic algae *Duneliella spp.* (Yancey et al. 1983) suggesting that glycerol can stabilize protein at high temperature as well as at low temperature. Perhaps the lack of a glycerol stabilizing effect on PK is due to the fact that sorbitol is a good stabilizer and that the presence of this solute is sufficient to provide the stabilization PK needs. On the other hand, glycerol may be better suited to stabilizing other proteins, and this may be a reason why *E. solidaginis* and other species have two polyols.

The substrate PEP was by far the best stabilizer. This is understandable since the measure of activity gives an indication of the active site stability. If PEP binds strongly to the enzyme, then the bonds between the enzyme and its substrate may prevent the denaturation of the active site.

Other molecules also stabilized PK. Glucose and KCl increased the half life by about 3-fold. Glucose is also accumulated by the larvae of *E. solidaginis* during the winter, although in a less extent than glycerol and sorbitol (Storey and Storey 1986). KCl, at low concentration, acts as a compatible solute and protects proteins from denaturation. At high concentration however, it becomes non-compatible and causes or helps the denaturation to occur. For *E. solidaginis* PK, 1M KCl still stabilized the enzyme.

Thermal denaturation studies carried out in presence of 1 M or 3 M urea showed that high concentrations of this non-compatible solute considerably affected the catalytic capacity of the enzyme. These high concentrations of urea can be used to test how sensitive PK is to the ionic character of its environment. The cellular dehydration that occurs due to extracellular ice formation when these insects freeze results in an increase of intracellular ion concentrations and this could be potentially disruptive for the weak bonds of proteins. However when such freeze-induced dehydration occurs, the two cryoprotectants sorbitol and glycerol are already present, so they were added to the assay solutions containing 1 or 3 M urea to see whether they would reverse the effect of strong denaturing conditions. Glycerol, which does not stabilize the enzyme on its own, was not able to counterbalance the effects of strong ionic conditions either. Sorbitol, on the other

hand, neutralized these effects to a certain extent (in the presence of 1 M urea) but lost its stabilizing power when the ionic character became too strong.

Fluorescence studies

Structural studies were performed using fluorescence spectroscopy. This is a useful technique because of its sensitivity (it can be used down to μmolar or lower concentrations) and because fluorescence signals are extremely sensitive to the microenvironment of a fluorophore.

Proteins contain three amino acid residues which may contribute to their fluorescence: tyrosine ($\lambda_{max,abs}$ = 274 nm), tryptophan ($\lambda_{max,abs}$ = 279.8 nm) and phenylalanine ($\lambda_{max,abs}$ = 257.44 nm). However, as protein fluorescence is generally excited at a wavelength near 280 nm and as tryptophan is the most highly fluorescent amino acid, fluorescence of most proteins is dominated by the tryptophan residues. This natural fluorophore is highly sensitive to the polarity of its surrounding environment and is therefore a valuable intrinsic fluorescence probe.

Among the fluorescence signals that can be monitored is the emission maximum wavelength λ_{max}. Its variations with the concentration of guanidine hydrochloride give valuable information on the average exposure of the tryptophan residues of the protein. Indeed, fluorescence λ_{max} of a protein is related to the polarity of the environment of the tryptophan residue and can range from 320 nm to 350 nm (Lakowicz 1983), with residues in apolar microenvironments having a blue emission. Unfolding of a protein almost always leads to a red shift in the emission λ_{max} of around 345 to 355 nm, that is a result of an increased exposure of tryptophan to water. This was indeed observed for *Eurosta solidaginis* PK: the emission λ_{max} shifted from about 340 nm to about 350 nm when the concentration of guanidine hydrochloride was increased from 0 M to 4 M, showing that the exposure to water of the tryptophan residues increased as the protein unfolded.

Other information relevant to protein stability can be obtained from the studies of denaturation with guanidine hydrochloride. The conformational stability of a protein can be defined as the difference between the molar free energy of the macromolecule in its denatured state and its native state :

$$\Delta G_D = G_n - G_d$$

In this definition of protein stability, both native and denatured states are involved. Three models have been suggested to determine $\Delta G_D^{H_2O}$ from the denaturation curves, assuming a 2-state mechanism for the unfolding process :

The simplest method consists of assuming that the linear relationship observed between ΔG_D and the denaturant concentration continues to zero concentration. In that case, the variations of the free energy of stabilization with denaturant concentration can be described by the following equation :

$$\Delta G_D = \Delta G_D^{H_2O} - m[\text{denaturant}] \quad (1)$$

and $\Delta G_D^{H_2O}$ is the intercept of the straight line with the y axis.

The second model has been established by Tanford. His model uses the free energy of transfer of a group (amino acid or peptide) from water to denaturant :

$$\Delta G_D = \Delta G_D^{H_2O} + \Sigma\alpha_i\, n_i \delta g_{tr,i} \quad (2)$$

where $\delta g_{tr,i}$ is the free energy of transfer of a group of type i from water to denaturant, n_i is the total number of groups of type i present in the protein and α_i is the average fractional change in the degree of exposure of group i when the protein unfolds. Because of the difficulty of evaluating α_i , this value is usually replaced by a single average value $\bar{\alpha}$. Thus equation (2) becomes :

$$\Delta G_D = \Delta G_D^{H_2O} + \bar{\alpha}\, \Sigma\, n_i \delta g_{tr,i} \quad (3)$$

The third model is called the denaturant binding model. It is based on the hypothesis that guanidinium chloride molecules bind to amino acid side chains (or to a peptide group) with a binding constant k which can be calculated using the equation :

$$\delta g_{tr} = RT\ln(1 + ka) \quad (4)$$

where a is the activity of the denaturant. The energy of stabilization is then calculated with the following equation, assuming that the number of binding sites for the denaturant (all identical and noninteracting) is greater on a denatured protein than on the native protein:

$$\Delta G_D = \Delta G_D^{H_2O} - \Delta n\, RT\ln(1 + ka) \quad (5)$$

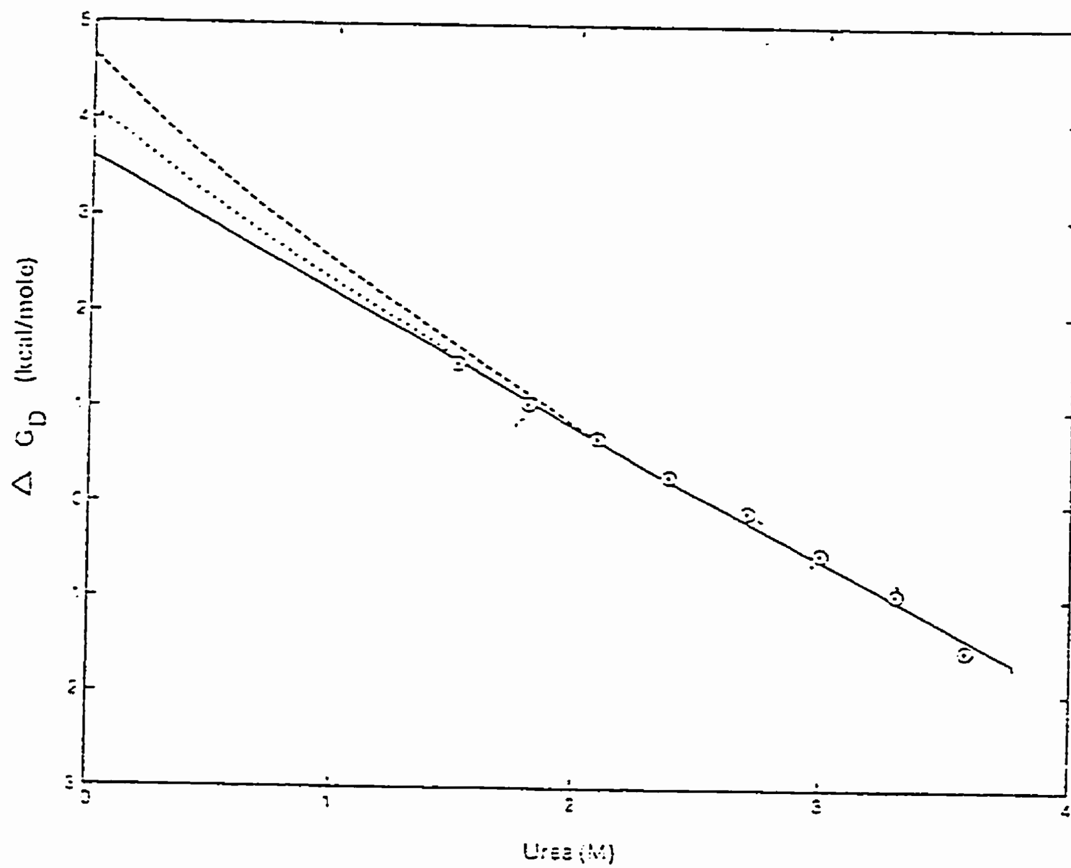
where Δn is the difference in the number of denaturant binding sites, k is the equilibrium constant for binding at each site, and a the activity of the denaturant (Pace, 1986).

The last two methods (Tanford's model and the denaturant binding model) lead to values of $\Delta G_D^{H_2O}$ that are generally too high estimates, whereas the linear extrapolation model usually leads to estimates that are too low, as illustrated by Figure 3-18 (Pace 1986). Since it is not clear which extrapolation leads to the best estimates (Pace 1986), the model that I used here was the linear extrapolation model.

Whereas the emission λ_{max} gives useful information on tryptophan exposure, it cannot be used for the determination of thermodynamic parameters such as $\Delta G_D^{H_2O}$ which are due to differences in the population of macrostates (the native and the denatured states in the case of $\Delta G_D^{H_2O}$). In fact, the emission λ_{max} will be representative of the population of the different states only if the quantum yield of the states is the same. If one state has a higher quantum yield than the other, then the former will dominate the emission spectrum and the apparent λ_{max} will be skewed toward this state (Eftink 1994). Therefore, another fluorescence signal has to be used when denaturation curves with guanidine hydrochloride are carried out in an attempt to determine the stability of the protein. In that case, a convenient fluorescent signal is the fluorescence intensity at some

Figure 3-18 : ΔG_D as a function of urea concentration.

This figure illustrate the differences between the three methods that can be used to calculate $\Delta G_D^{H_2O}$. The solid line represents the linear extrapolation model, the dotted line the denaturant binding model and the dashed line Tanford's model (Pace 1986).



Δ G_D (kcal/mole)
Urea (M)

pair of excitation and emission wavelengths. The fluctuations of λ_{max} at low concentrations of guanidine hydrochloride show that the unfolding of *Eurosta solidaginis* pyruvate kinase does not follow a simple two-state mechanism. This has already been reported for PK from *Neurospora* (O'Brien and Kapoor 1979), and this factor makes it difficult to precisely determine $\Delta G_D^{H_2O}$. However, analysis in terms of a two-state mechanism can still be useful, since the estimate of $\Delta G_D^{H_2O}$ obtained this way will at least serve as a lower limit for the true value of $\Delta G_D^{H_2O}$ (Pace, 1986).

For *Eurosta solidaginis* PK, the conformational stability was found to be $\Delta G_D^{H_2O} = 5.11 \pm 0.09$ kJ/mol, and the dependence of ΔG on denaturant concentration, $m = 3.48 \pm 0.42$ kJ.l/mol^2. The value of $\Delta G_D^{H_2O}$ is low compared to values obtained for other proteins (from 20 to 60 kJ/mol), but this was expected for the reasons explained above.

I also followed heat denaturation using fluorescence spectroscopy. Guanidine hydrochloride is often used as a protein denaturant because on time tested observation, no other denaturant gives a greater extent of unfolding. In most cases, the products of thermal denaturation are less completely unfolded than those of guanidine hydrochloride denaturation. Thermal denaturation generally leads to a product that still retains some ordered structure. For *E. solidaginis* PK, the variations of the wavelength from the native to the denatured states were only of about 5-6 nm whereas they were of about 10-12 nm for the denaturation with guanidine hydrochloride. The heat denaturation experiment performed in the presence of the cryoprotectants glycerol and sorbitol shows once again that these polyols have a stabilizing effect on PK, but that this effect is limited and cannot, if the temperature is increased too much, prevent denaturation from occurring.

Conclusion

Synthesis of glycerol during the fall requires a reorientation of the carbon flux passing through the glycolytic pathway. The suppression of pyruvate kinase activity, brought to the fore in this study, is part of the mechanism used by *E. solidaginis* to accomplish this. However, the survival of the insect during winter requires the maintenance of a low metabolic rate and therefore the maintenance of a small flux of carbon through PK. The increased affinity of the enzyme for its two substrates, ADP and PEP at low temperature and the remarkable stability of the enzyme are means to achieve this objective. Finally, the study also underlines the major role of the two cryoprotectants both in modulating the activity and stabilizing pyruvate kinase from *E. solidaginis*.

Future Studies

Several questions arose from the discussion that could lead to worthwhile future studies. First, regarding hexokinase, it would be interesting to determine K_m values for both substrates, ATP and glucose, at 6°C and 25°C in presence of only one polyol at a time (600 mM glycerol or 200 mM sorbitol) to determine whether only one or both polyols alter the K_m for those substrates. An analysis of fructose kinetics should also be undertaken following the same experimental conditions as for glucose (6°C and 25°C, with 600 mM glycerol and/or 200 mM sorbitol). This would allow a better understanding of the role of hexokinase in the catabolism of sorbitol during the spring. Inhibition studies with the reaction products of HK (glucose-6-phosphate, fructose-6-phosphate and ADP) would also be useful particularly to understand how enzyme activity is suppressed during active sorbitol synthesis when levels of both substrates (glucose, fructose) and products (glucose-6-phosphate) of the enzyme are very high in the larvae. Finally, structural studies comparable to those performed on PK (heat denaturation studies in the presence of various metabolites, guanidine hydrochloride denaturation studies) would give information on the stability of the enzyme and on its structure.

Concerning pyruvate kinase, to complete the heat denaturation studies, it would be interesting to study the effects of other metabolites such as the second substrate ADP, the products ATP and pyruvate or allosteric activators or inhibitors on enzyme stability. More work could also be done on the guanidine hydrochloride denaturation studies to try to identify the mechanism of unfolding of the enzyme.

Finally, it would be interesting to compare these enzymes from the freeze tolerant insect *Eurosta solidaginis* with the corresponding ones in the freeze avoiding insect *Epiblema scudderiana* which shares the same winter habitat in galls on goldenrod, and has the same need to control glycolysis with respect to metabolic rate depression, but synthesizes only glycerol as a cryoprotectant.

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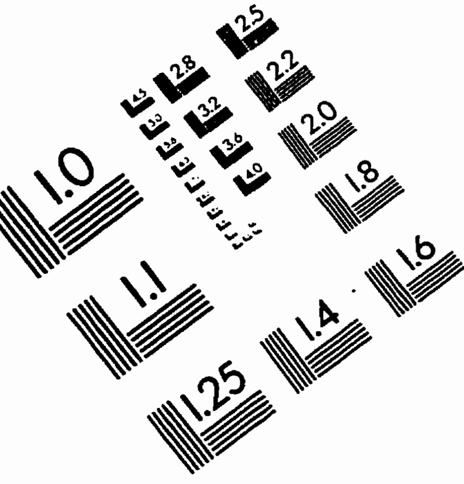

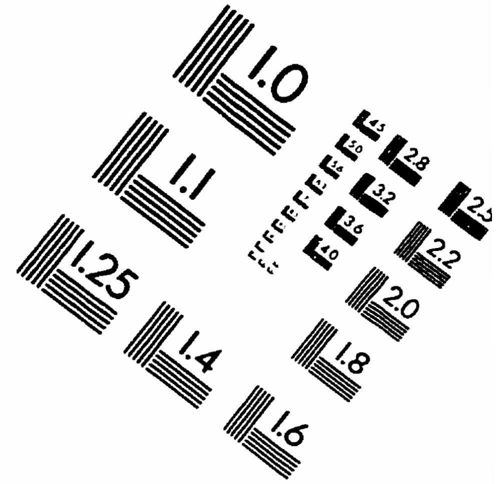

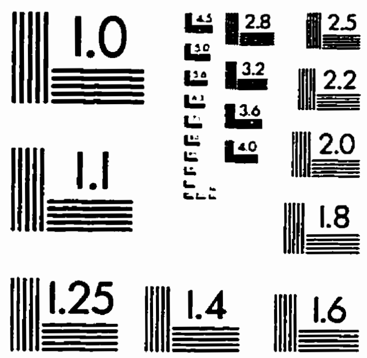

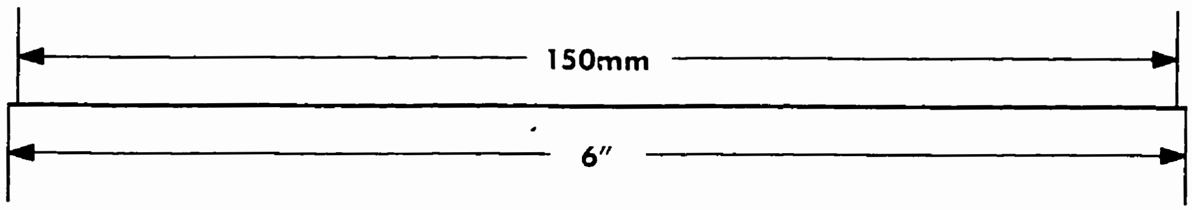

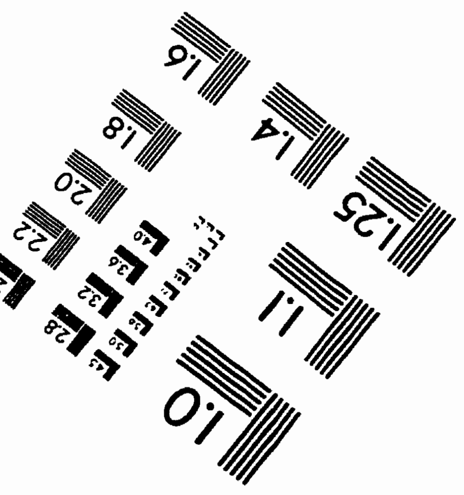
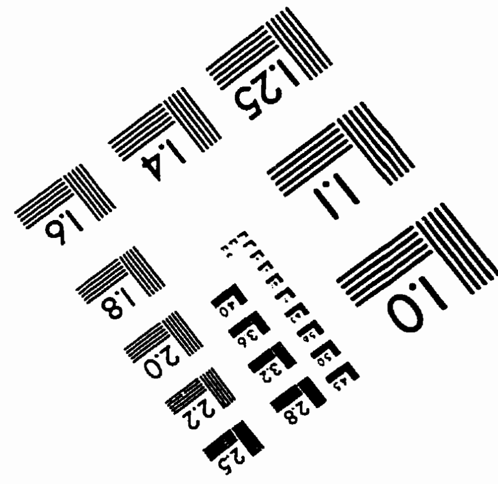

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