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PHOSPHORYLATION REGULATION IN TWO SPECIES OF COLD-HARDY GOLDENROD GALL INSECTS

BY

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A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

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Ottawa, Ontario

June 1999

The undersigned hereby recommend to
the Faculty of Graduate Studies
and Research acceptance of this thesis

"PHOSPHORYLATION REGULATION IN TWO SPECIES OF COLD-HARDY GOLDENROD GALL INSECTS"

Submitted by Thomas D. Pfister

in partial fulfillment of the requirements for
the degree Master of Science

Thesis Supervisor

Chair, Department of Chemistry

Carleton University
1999

Abstract

The larvae of two cold-hardy insects, the freeze avoiding gall moth *Epiblema scudderiana* and freeze tolerant gall fly *Eurosta solidaginis*, endure prolonged exposures to subzero temperatures while overwintering. Both rely on a reduction in metabolic rate and the production of polyols for survival. The present study analyzed the role of several signal transduction enzymes in cold hardiness. Changes in cAMP-dependent protein kinase (PKA), protein phosphatases-1 (PP1), 2A, 2C and protein tyrosine phosphatase activities were monitored over the course of the winter season and also in insects exposed to -4C, -20C or anoxic conditions. The catalytic subunit of PKA (PKAc) and PP1 were purified to homogeneity from both species. Effects of low temperature on the enzymes included increased affinity of PKAc for the Kemptide substrate, and increased inhibition by okadaic acid of PP1. Differential regulation of kinases and phosphatases by low temperature appears to be key to regulating polyol production and, hence, cold survival.

ACKNOWLEDGMENTS

First and foremost I thank my thesis supervisor, Dr. Ken Storey for his assistance and guidance. I will always remember his words of advice "You must learn to think like an enzyme". I would especially like to thank Justin MacDonald, Kyra Cowan and Bill Willmore for teaching me many of the techniques required to complete this thesis. I also thank the rest of my friends in the Storey lab for making my time spent there an enjoyable experience and Jan Storey for her excellent suggestions and help in editing this thesis. Finally, I wish to thank Karen, Chris and my parents for their support and encouragement.

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LIST OF ABBREVIATIONS

ATP, ADP, AMP - adenosine tri-, di-, mono- phosphate

cAMP - cyclic 3', 5' adenosine monophosphate

ANOVA - analysis of variance

BSA - bovine serum albumin

DE-52 – diethylyamino cellulose

dpm - disintegrations per minute

E_a - activation energy

EDTA - ethylenediamine tetraacetate

EGTA - ethyleneglycol (B-aminoethyl ether) tetraacetate

FP - freezing point

gwm - gram wet mass

H7 - Isoquinolinesulonyl protein kinase inhibitor peptide H7

H89 - Isoquinolinesulonyl protein kinase inhibitor peptide H89

I_{50} - concentration of inhibitor that reduces V_{max} by one-half

K_a - equilibrium association constant

K_m - Michaelis-Menten constant; concentration of substrate that produces half-maximal enzyme velocity

KPi - mono- and di-potassium phosphate salt

MB - Membrane Bound

M.W. - molecular weight

MgATP - magnesium ATP complex

PEP 1 - PTP synthetic peptide 1: END(pY)INASL

PEP 2 - PTP synthetic peptide 2: DADE(pY)LIPQQG

pI - isoelectric point

Pi - inorganic phosphate

PKA - protein kinase A (cyclic AMP dependent Protein Kinase)

PKAc - catalytic subunit of PKA

PKAi - protein kinase A inhibitor peptide (5-24)

PMSF- phenylmethylsulfonyl fluoride

PP1 - protein phosphatase 1

PP2A - protein phosphatase 2A

PP2C - protein phosphatase 2C

PTP - protein tyrosine phosphatase

Q_{10} - factor by which a 10°C change in temperature reduces the rate of a chemical reaction.

SCP- supercooling point

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TCA – trichloroacetic acid

TPCK- tosyl-L-phenylalanine chloromethyl ketone

V_{max} - maximal enzyme velocity

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Chapter 1

General Introduction

Hypotheses: The following hypotheses were tested and examined in this thesis. Protein kinase A (PKA) and Protein Phosphatase-1 (PP1) are involved in mediating cryoprotectant production in overwintering *Epiblema scudderiana* and *Eurosta solidaginis*. Analysis of the responses of these enzymes to cold and anoxia exposures will help to obtain a better understanding of their roles in winter hardiness. Protein phosphatase 2A and 2C as well as protein tyrosine phosphatase also play integral roles in insect responses to low temperature and anoxia stresses.

Introduction

Many insects live in climates where they experience harsh subzero temperatures during the winter. To endure they use one of several survival strategies. Some insects can avoid cold by migrating to warmer climates but most survive, in part, by selecting a microhabitat in which they are partially insulated from the cold. Temperatures underground or in logs or stumps usually remain above the minimum temperatures of the ambient air and rarely fall more than a few degrees below 0°C. The insulation factor is even greater under a cover of snow (Wellington 1950; Baust 1976). Other insects are exposed to the cold with little or no insulation and have evolved biochemical and physiological adaptations to help them survive. Generally, one of two adaptive strategies are used: freeze tolerance or freeze avoidance (Duman *et al.*, 1991). Although extremely rare, different populations of the same species may use different strategies, or the same population of insects may use different strategies from year to year (Duman *et al.*, 1991).

Freeze Avoidance

Freeze avoiding animals prevent ice from forming within thier bodies by employing mechanisms that strongly suppress the freezing point (FP) and supercooling point (SCP) of body fluids (Storey and Storey, 1988). All water solutions have some capacity to supercool below their equilibrium freezing point. In fact, pure water under carefully controlled conditions can be cooled as low as -40°C before crystallizing. (Duman *et al*, 1991) In most cases, however, the presence of surfaces, solutes or impurities triggers crystallization at a temperature between the equilibrium FP of body fluids (usually about -0.5°C for summer insects) and -40°C. Freeze avoiding animals exploit this tendency of solutions to supercool with adaptations that enhance supercooling capacity and often push the crystallization temperature down to near -40°C. The main mechanism underlying this is the addition of antifreeze to body fluids. These are of two types: antifreeze proteins (AFPs) and low molecular weight sugar or sugar alcohol antifreezes (Duman, 1991).

AFPs act by adsorbing to the surface of small ice crystals and inhibiting their further growth. The action of low molecular weight antifreezes depends on the colligative properties of high concentrations of these molecules in solution. For example, the addition of one mole of a solute such as glycerol depresses the FP by -1.86°C. Furthermore, in a nucleator-free solution, the molar effect of low molecular weight antifreezes on SCP is often two to three times greater (MacKenzie, 1977; Block and Young, 1979). For solutes to be useful as antifreezes, they must be small and non-toxic at high concentrations. Inorganic ions cannot be used because they inhibit neuronal functions at high concentrations. Glycerol is the solute most commonly used as a

colligative antifreeze by overwintering insects but sorbitol and other polyhydric alcohols, as well as sugars such as trehalose and sucrose, are also used by some species. Using multiple antifreezes provides protection without requiring excessively high accumulation of any one solute which may be "metabolically difficult" (Morrissey and Baust, 1976; Ring, 1980). Another advantage of using multiple antifreezes is that it allows the cell to fuel different pathways when the antifreezes are catabolized in the spring.

Additional factors also contribute to freeze avoidance. Some species dehydrate substantially during cold-hardening. This has the effect of concentrating solutes, but must clearly be used in conjunction with synthesis of the appropriate antifreezes, since dehydration alone is not selective and would also concentrate undesirable solutes. Adaptations that remove or mask potential ice nucleators are also used. Non-specific nucleation by food particles and bacteria in the gut is reduced by clearing the gut prior to overwintering. The possibility of nucleation due to contact with environmental ice is also reduced by many species by wrapping themselves in a waterproof silk cocoon.

Freeze Tolerance

Many insects are freeze tolerant, enduring the freezing of body fluids at temperatures ranging from just a few degrees below 0°C for some species (Duman and Patterson, 1978) to as low as -87°C for the carabid beetle *Pterostichus brevicornis* (Miller, 1982). Freeze tolerant insects allow ice to build up in their bodies but limit the sites of ice formation to the extracellular spaces only. The liquid state of the cytoplasm is always preserved because intracellular ice formation can cause lethal damage by destroying subcellular structures and compartmentalization (Duman, 1991). A number of

mechanisms are employed to control ice formation in extracellular space and to ensure that intracellular water does not freeze. One of these is the specific synthesis and addition of ice nucleating proteins into extracellular spaces where they stimulate freezing at temperatures just below the equilibrium FP of body fluids. This allows a slow and controlled freezing of extracellular water. However, as ice crystals grow solutes are excluded from the ice lattice and concentrated in the remaining unfrozen extracellular water. This creates an osmotic gradient across the cell membrane which dehydrates the cells while increasing intracellular solute concentrations; the net effect of this is to lower the intracellular FP and SCP. The cell membrane also plays an important role in that it prevents seed crystals from entering the cell. Membrane lipid bilayer structure of the shrunken cell is stabilized by the actions of some of the low molecular weight cryoprotectants such as the sugar, trehalose, or the amino acid, proline. Freeze tolerant insects also accumulate high concentrations of sugars or sugar alcohols, just as freeze avoiding insects do, but in this instance use them to protect just the liquid state of the cytoplasm and the structural integrity of the proteins within (Shikama and Yamazaki, 1961). Glycerol is again the most common cryoprotectant but sorbitol, erythritol, ribitol, threitol and sucrose are also used as well as certain free amino acids, in particular proline, glutamine and glycine (Blum 1985); multiple cryoprotectant systems are common (Baust 1981). Some freeze tolerant insects also show evidence of AFPs in their hemolymph which seems contradictory. However, it is believed that the role of AFPs in this case is to inhibit recrystallization, a process by which small ice crystals regroup into larger ones over time. Since the formation of very large crystals could cause severe mechanical damage to cells, it appears that AFPs are used regulate crystal size. The free energy of

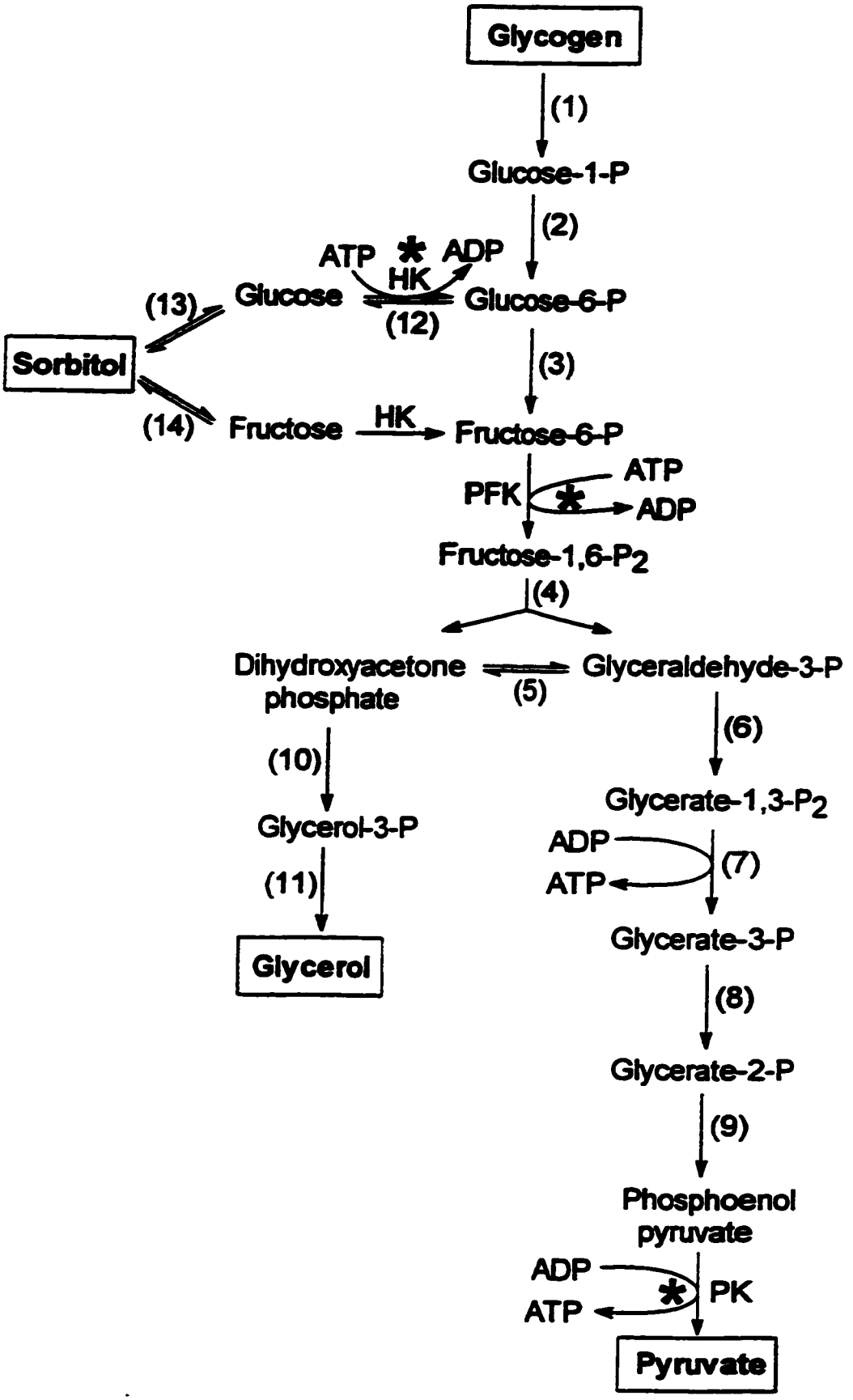
recrystallization is much lower than crystal growth so only very low concentrations of AFPs are required. Also important for the survival of freeze tolerant species is metabolic rate depression; many species overwinter in a diapause state.

Cryoprotectant (Glycerol and Sorbitol) Production

The two goldenrod gall insects studied in this thesis both accumulate low molecular weight polyhydric alcohols to help protect them from winter cold. The goldenrod gall moth *Epiblema scudderiana*, a freeze-avoiding species, accumulates high concentrations of glycerol whereas the goldenrod gall fly *Eurosta solidaginis* accumulates a mixture of 3-carbon (glycerol) and 6-carbon (sorbitol) polyols. Both are derived from glycogen reserves via pathways outlined in Figure 1.1. Glycogen phosphorylase catalyzes the rate-limiting step for glycogen degradation (van de Werve and Jeanrenaud, 1987). In insects, a primary response to cold is to increase glycogen phosphorylase activity in fat body to initiate the conversion of glycogen to glycerol. Sorbitol synthesis is initiated later (in those insects which use sorbitol in addition to glycerol). Glycogen phosphorylase is regulated by phosphorylase kinase, which phosphorylates the enzyme to convert it to the active a form, and phosphorylase phosphatase, which dephosphorylates the active enzyme to return it to the inactive b form. Phosphorylase phosphatase is now known to be synonymous with Protein Phosphatase 1(PP1). The mechanism of cold activation of glycogen phosphorylase has been postulated to be due to differential effects of cold on phosphorylase kinase and phosphorylase phosphatase (Hayakawa, 1985). Thus, with falling temperatures over a range of 30°C to 0°C phosphorylase kinase showed a normal reduction in activity

Figure 1.1 Glycerol and Sorbitol Production from Glycogen.

The figure shows how glycolysis and the synthesis and catabolism of glycerol and sorbitol are overlapping. The numbers indicate key enzymes involved in these pathways (1): glycogen phosphorylase, (2): phosphoglucomutase, (3): Phosphoglucoisomerase, (4): aldolase, (5): triosephosphate 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, HK: hexokinase, PFK: phosphofructokinase, PK: pyruvate kinase.


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

with a Q_{10} of about 2 but phosphorylase phosphatase (PP1) activity was much more greatly reduced at low temperatures. Hence, kinase activity prevailed at low temperature, leading to a net activation of glycogen phosphorylase (Hayakawa, 1985).

Glycerol synthesis follows the glycolytic pathway down to the level of dihydroxyacetone phosphate (DHAP). It then diverges and DHAP is converted to glycerol in two steps, involving dehydrogenase and phosphatase reactions. Sorbitol synthesis diverts carbon out of glycolysis at the level of glucose-6-phosphate (G6P) and also converts it to sorbitol using dehydrogenase and phosphatase reactions steps (Figure 1.1).

Metabolic Depression

Freeze tolerance and freeze avoidance are often associated with a state of metabolic rate depression (Storey and Storey, 1990). Many insects spend the winter in a state of diapause to conserve energy (Danks, 1987). Overall metabolic rate can fall to <5% of the normal in overwintering insects as measured by oxygen consumption (Danks, 1987). The need to conserve energy may partially dictate which overwintering strategy is used since frozen insects have been shown to use less energy than supercooled insects at the same temperature (Duman, 1991).

Enzyme Regulation

Metabolic depression and freeze tolerance/avoidance requires regulation of numerous enzymes in order to adapt to seasonal temperature changes and put in place the specific adjustments needed for cold hardiness and the transition into the dormant state.

Enzyme activities can be regulated in many ways. For example, enzyme activity can be altered by simply increasing or decreasing the amount of enzyme. This is done by regulating transcription, translation or degradation of mRNA or protein. The activity of a fixed quantity of enzyme can also be regulated by modifying the enzyme, either reversibly or irreversibly. One effective and widespread way of regulating enzymes reversibly is via covalent phosphorylation or dephosphorylation. This process is controlled by kinases and phosphatases, respectively (Figure 1.2).

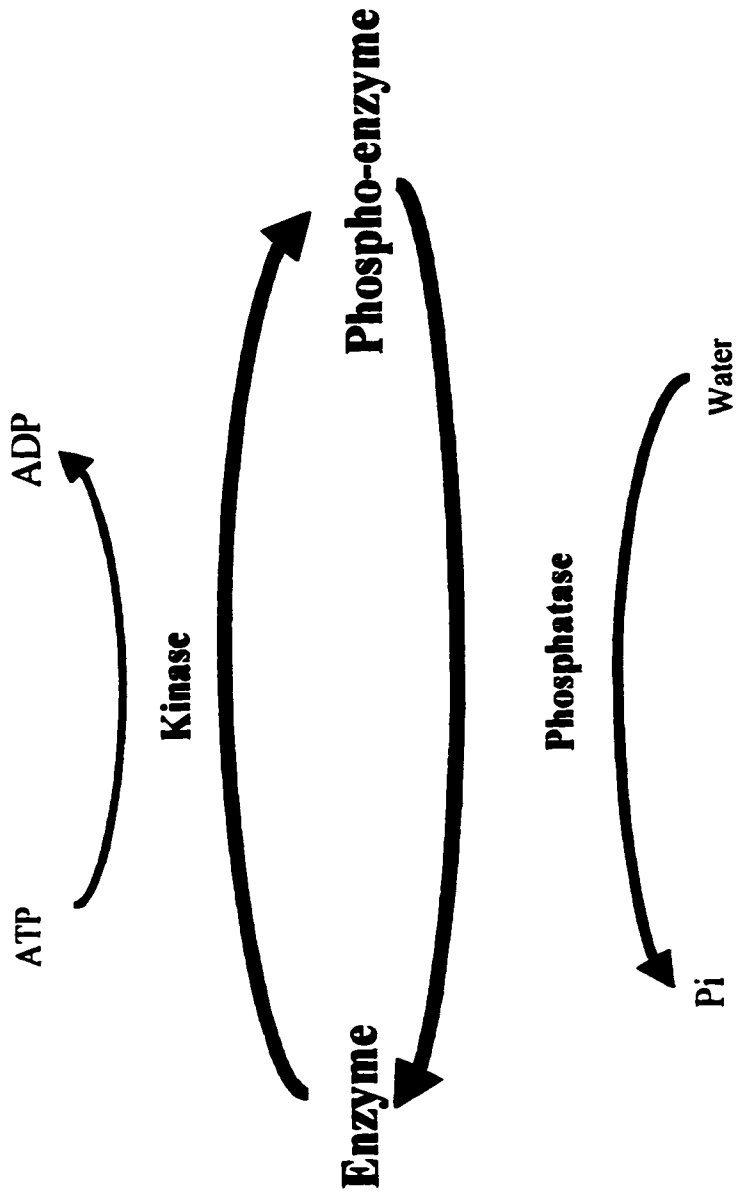
Regulation of enzymes at different temperatures may vary due to changes in enzyme conformation, altering the affinities for substrate and may affect the efficiency of activators and inhibitors (Storey, 1992). Serine, threonine and tyrosine residues are most commonly the sites of phosphorylation. The addition of a phosphate group introduces two extra negative charges which typically leads to a conformational change in the protein which, in turn, affects substrate or activator/inhibitor binding and changes the efficiency of catalysis by the enzyme. Since kinases and phosphatases often have numerous proteins and/or enzymes as substrates, they may control several enzymes or metabolic pathways at once, integrating and coordinating responses to a metabolic signal.

Several enzymes involved in phosphorylation and dephosphorylation are studied in this thesis.

Protein Kinases

The activation of a protein kinase or cascade of kinases is often initiated by the binding of a hormone to a membrane receptor (Shenolikar, 1988). In the case of protein kinase A hormone binding to the receptor activates the enzyme adenylate cyclase which

Figure 1.2 Enzyme Regulation by Reversible Phosphorylation.


Phospho-enzyme
ADP
Kinase
Phosphatase
Water
ATP
Enzyme
Pi

increases the concentration of cAMP. This second messenger then binds to the regulatory subunits of cyclic-AMP dependent protein kinase (PKA) causing the release of the active, catalytic (C) subunits (Figure 1.3 and 1.4) (Taylor and Radzio-Andzelm, 1994). PKA is one of the most widely distributed kinases in higher eukaryotic cells. The classic role of PKA is in the phosphorylation of glycogen phosphorylase kinase which, in turn, phosphorylates glycogen phosphorylase b to convert it to the active phosphorylase a. Hence, activation of phosphorylase kinase ultimately leads to the activation of glycogenolysis. PKA has also been implicated in many other roles including the mediation of cell growth, gene transcription and translation and metabolic regulation (Scott, 1993).

Protein Phosphatases

Like kinases, protein phosphatases have been shown to play a regulatory role in a number of signal transduction pathways (Cohen, 1989). The role of phosphatases is to reverse the covalent modification of kinases. As with the kinases, protein phosphatases are separated into two major classes: those which dephosphorylate tyrosine residues and those which act on serine/threonine residues. Some dual specificity phosphatases have also been reported, e.g. MAP kinase phosphatase-1 (Denu and Dixon, 1995).

The four major ser/thr phosphatases have been assigned to two different classes based on their susceptibility to inhibition by two small heat stable proteins (inhibitor 1 and 2) and their ability to dephosphorylate the α or β subunit of phosphorylase kinase (Shenolikar 1994). Type 1 phosphatases (such as PP1) preferentially dephosphorylate the ß subunit of phosphorylase kinase and are sensitive to both inhibitor proteins. Type 2

Figure 1.3 Regulation of Glycogen Phosphorylase by Protein Kinase A and Protein Phosphatase 1. Glycogen phosphorylase activity limits the rate of glycogen break down and subsequent production of polyols (shown in Fig. 1.1)

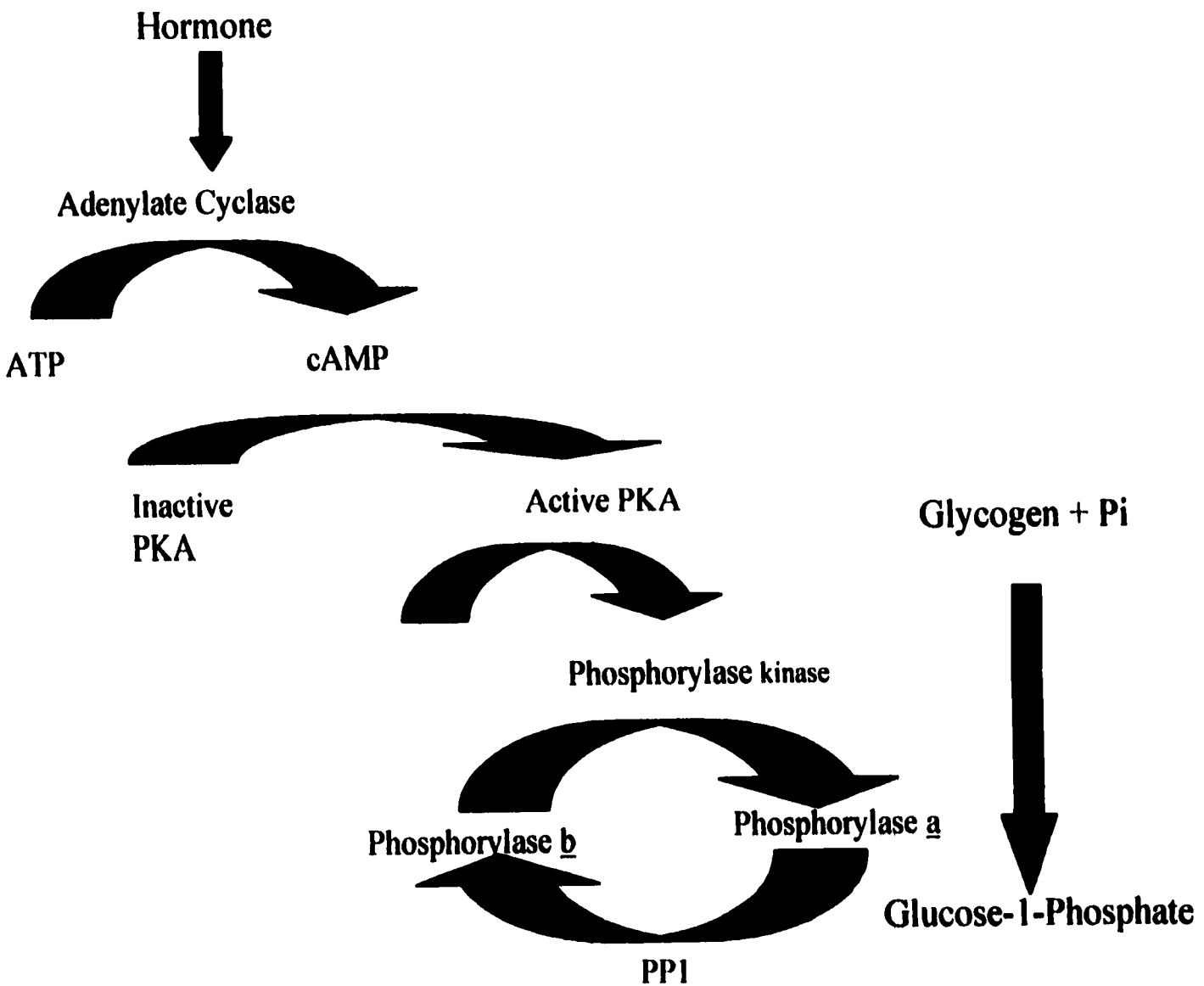
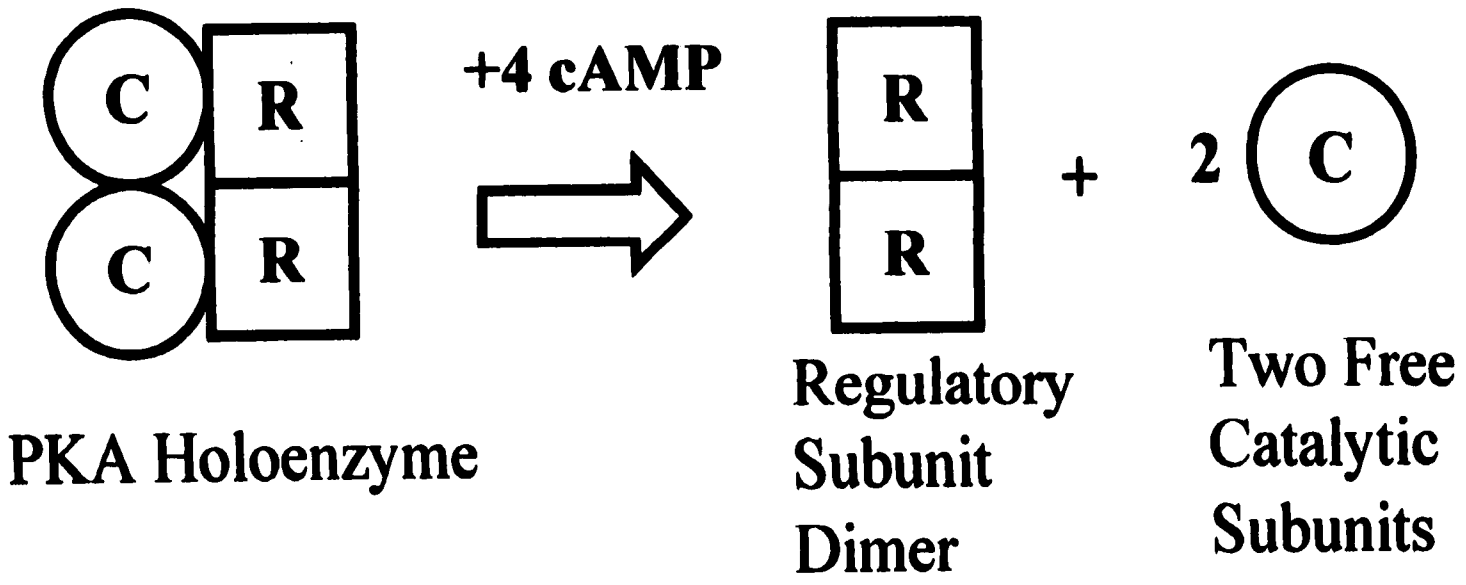

Hormone
Adenylate Cyclase
ATP
cAMP
Inactive PKA
Active PKA
Phosphorylase kinase
Phosphorylase b
Phosphorylase a
PP1
Glycogen + Pi
Glucose-1-Phosphate

Figure 1.4 Activation of Protein Kinase A (PKA) by cAMP.


C
R
C
R
+4 cAMP
R
R
+
2
C
PKA Holoenzyme
Regulatory Subunit Dimer
Two Free Catalytic Subunits

phosphatases preferentially dephosphorylate the α subunit and are insensitive to the inhibitor proteins. The three type 2 phosphatases (PP2A, PP2B, PP2C) are differentiated on the basis of ion/cofactor requirements and responses to okadaic acid, a marine sponge toxin (MacKintosh and MacKintosh, 1994). A concentration of 1 nM will completely inhibit PP2A whereas PP1 and PP2C require much higher concentrations. Protein phosphatases 1, 2A and 2B catalytic subunits also occur in association with various regulatory subunits, forming dimers or trimers whereas PP2C occurs only as a monomer. While these four major phosphatases account for virtually all measurable phosphatase activity *in vitro*, many new phosphatases (PPV, PPY, PPZ1, and PPZ2) have been discovered with properties intermediate to the four major ones described above (Cohen, 1997).

Summary of Objectives

The first objective of this thesis is to examine and compare kinase and phosphatase activities in freeze-avoiding *Epiblema scudderiana* and freeze-tolerant *Eurosta solidaginis* during overwintering and in laboratory cold- and anoxia-exposed insects. The second aim of this thesis is to examine and compare the characteristics of purified PKA and PP1 from both insects. Data collected from these experiments will yield a clearer understanding of how freeze avoidance and freeze tolerance survival mechanisms are regulated.

Chapter 2

Enzyme Activity Profiles in Overwintering *Epiblema scudderiana* and in Response to Subzero Temperature Exposure and Anoxia Stress

Introduction

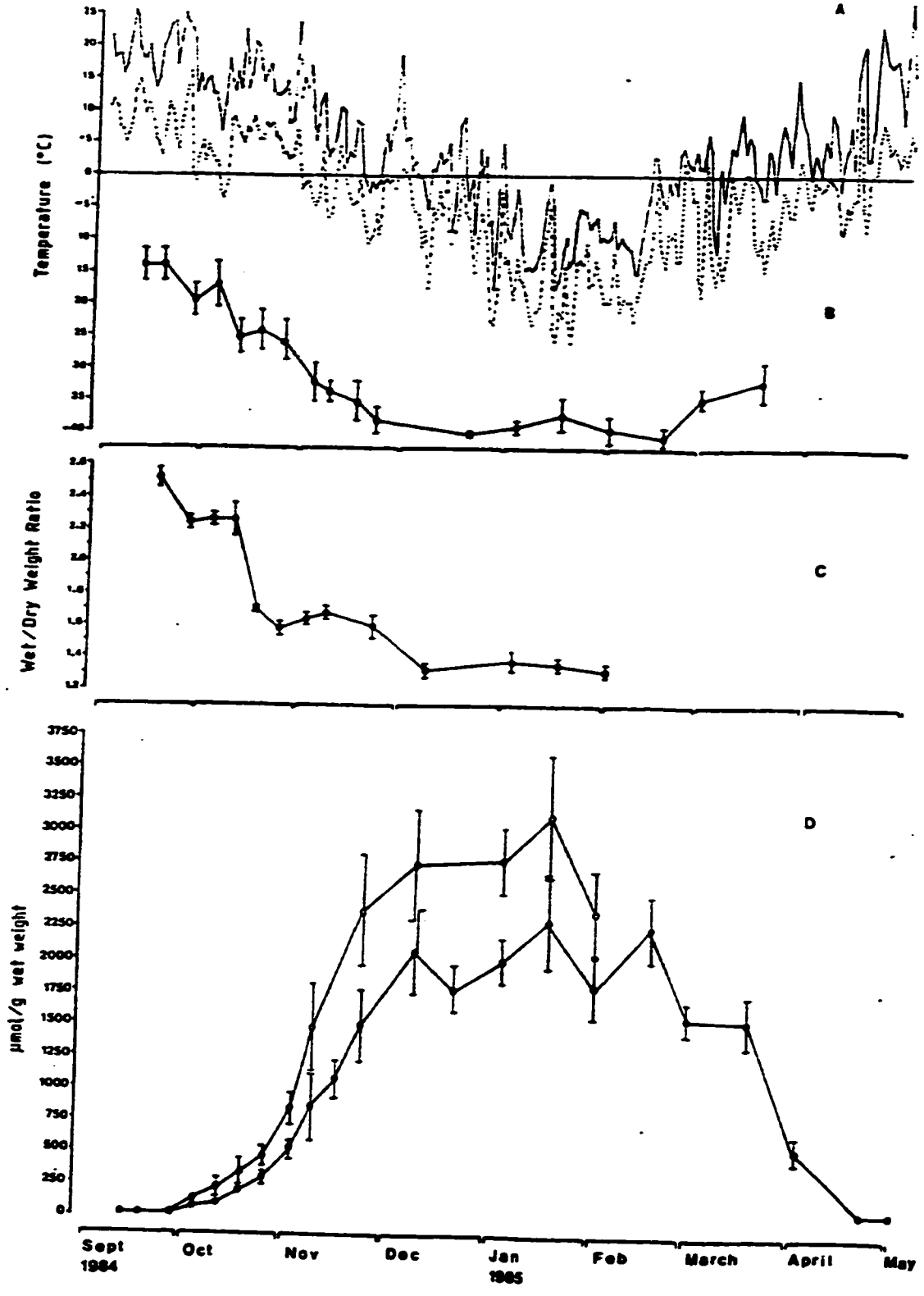
The larvae of the goldenrod gall moth *Epiblema scudderiana* overwinter in elliptical galls on the woody stems of goldenrod. The galls have little insulation value (Layne, 1991) and hence larvae must endure the coldest winter air temperatures. They do so by supercooling and are an excellent example of a freeze avoiding insect. Key to supercooling is the accumulation of glycerol as a colligative antifreeze with levels rising to over 2 moles/gram wet mass (over 2 molar), which represents about 18.7% of the mass of the insect. By midwinter, populations in the Ottawa area show SCPs reduced to about -38°C compared to -12°C in the absence of glycerol (Figure 2.1; Rickards *et al.*, 1987). Previous studies have shown that the synthesis of glycerol is initiated when temperatures fall to about 5°C with maximal rates occurring over the range 0 to -10°C (Kelleher et al., 1987). Transfer from 15°C (no glycerol synthesis) to -4°C was used to study the metabolic events associated with the rapid activation of glycerol biosynthesis in *Epiblema scudderiana*. Under this regimen significant changes in glycolytic intermediates and glycerol precursors were seen within 2 hours and glycerol production rapidly reached high rates (e.g. 10 $\mu mol.g^{-1}.h^{-1}$ between 2 and 12 hours of cold exposure) (Churchill and Storey, 1989a). The pathway of glycerol production is shown in Figure 1.1.

cAMP -Dependent Protein Kinase A (PKA)

The PKA holoenzyme is a catalytically inactive tetramer, composed of two regulatory subunits (R) and two catalytic subunits (C). The concentration of cAMP is what determines the ratio of inactive to active PKA in the cell. Upon binding of four

2.1 Overwintering Profile of Glycerol Contents in *Epiblema scudderiana*.

The diagram shows glycerol levels compared to the average ambient temperature from September to April in *E. scudderiana* (Rickards et al, 1987). A) Daily temperature minima and maxima, B) Supercooling point, C) Wet vs dry weight ratio of the insect, D) Glycerol content relative to wet mass (open circles) and dry mass, (solid circles).


A
Temperature (°C)
B
C
Wet/Dry Weight Ratio
D
µmol/g wet weight
Sept 1984
Oct
Nov
Dec
Jan 1985
Feb
March
April
May

molecules cAMP to the regulatory subunits, the holoenzyme dissociates into an R_2 dimer and two free catalytic subunits which are active (Figure 1.4) (Knighton, 1991). The regulatory subunits are thought to inhibit catalysis by competing with physiological substrates (Corbin et al., 1978). To date three forms of the catalytic subunit Cα, Cβ and Cγ and four different R subunits, $R_I\alpha$, $R_I\beta$, $R_{II}\alpha$, and $R_{II}\beta$ have been found in mammals. Different R subunits have not been found in the same tetramer but a tetramer may contain a heterodimer of catalytic subunits; therefore, it is possible to have at least 24 different forms of PKA holoenzyme (Woodford *et al.*, 1996). While R_I appears to be entirely cytosolic, R_{II} can be attached to various cellular structures via cyclic AMP dependent kinase (cAK) anchoring proteins (Scott and McCartney, 1994; Bregman *et al.*, 1989). This may provide a means of targeting PKA with the R_{II} subunit to specific cellular locations or influence local substrate availability. The general consensus sequence recognized as a substrate by PKA is Arg-Arg-X-Ser-[Thr]-Y, where X is any small amino acid and Y is any large hydrophobic group (Knighton, 1991).

Protein Phophatases 1 (PP1)

Protein Phosphatase–1 (PP1) is the major phosphatase involved in the activation of glycogen synthesis and inactivation of glycolysis *in vivo*(Cohen, 1991) The enzyme is known to dephosphorylate glycogen phosphorylase and glycogen synthase resulting in their respective inactivation and activation (Ingebritsen and Cohen, 1983). Regulation of PP1 occurs via association with high molecular weight "targeting" subunits and inhibitor peptides 1 or 2. PP1 in the cytosol usually exists in an inactive form complexed to the

heat stable inhibitor-2. Phosphorylation of I-2 by other kinases releases the catalytic subunit. Inhibitor-1 must be phosphorylated by PKA to bind to and inhibit PP1.

Protein Phosphatase 2 A (PP2A)

The catalytic subunit of PP2A can complex with a number of regulatory subunits including A, B and B'. Its natural substrates include p53, rsk, and members of the MAP kinase family. It has also been shown to dephosphorylate glycogen phosphorylase and glycogen synthase (Ingebritsen and Cohen, 1983).

Protein Phosphatase 2B (PP2B)

Also called calcineurin, PP2B is usually found in high concentrations in neural tissue. It requires calcium ions for activity and the presence of calmodulin has been shown to increase its activity by ten-fold. Since the amount of neural tissue in whole insects were is extremely low, PP2B activity was not surveyed in this study.

Protein Phosphatase 2 C (PP2C)

Protein phosphates 2C activity is dependent on the presence of magnesium ions. It is the major enzyme responsible for dephosphorylating HMG-Coenzyme-A reductase (Ingebristen and Cohen, 1983). It is also involved in an osmosensing mitogen activated protein (MAP) kinase cascade, comprising the High Osmolarity Glycerol Response-1 (HOG1) kinase (Shiozaki and Russell, 1995).

Protein Tyrosine Phosphatases (PTP)

Over 30 types of protein tyrosine phosphatases have been identified (Ide *et al.*, 1994). All share a common catalytic domain and appear to go through a phospho-cysteine intermediate (Walton and Dixon, 1993). Protein tyrosine phosphatases can be membrane bound or exist free in the cytosol. The membrane bound PTPs usually serve to dephosphorylate receptor proteins. The soluble form has been shown to play a role in cell cycle progression. Currently, there is still relatively little known about PTPs.

Studies have analyzed glycerol production in *Epiblema scudderiana* and the kinetic and regulatory properties of the enzymes of the biosynthetic pathway. Maximal activities of biosynthetic enzymes rise during the autumn months and fall again in late winter or early spring; enzymes of glycerol catabolism respond oppositely (Joanisse and Storey, 1994a). However, despite elevated maximal activities of biosynthetic enzymes, glycerol synthesis itself is only initiated by cold exposure which suggests that the signal transducing enzymes involved in activating biosynthesis may be cold sensitive and/or are activated by signals that respond to cold. This study examines the activities of several signal transducing enzymes that could potentially be involved in regulating the phosphorylation states of enzymes involved in freeze avoidance. An overwintering time course is examined, as well as the effects of laboratory subzero cooling and recovery. Furthermore, the responses to anoxia exposure were evaluated in *Epiblema scudderiana* would probably not experience anoxia as part of normal winter life. However this stress was evaluated for comparative purposes, with *Eurosta solidaginis* (see chapter 3) which endures anoxia/ischemia stress as the result of the winter freezing of body fluids.

Materials and Methods

Chemicals

Radioactive [γ -^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear (Montreal, PQ). Okadaic acid was from CalBioChem (La Jolla, CA) whereas Kemptide (LKRRASLG) and the synthetic peptides for assaying PP2A and PP2C (RRA**p**TVAA) and PTP (Peptide 1 [Pep 1]: END**p**YINASL, Peptide 2: [Pep 2] DADE**p**YLIPQQG) were obtained from Queen's University (Kingston, ON). Other chemicals and chromatography materials were obtained from Fisher Scientific (Ottawa, ON) or Sigma Chemical Company (St-Louis, MO).

Animals

Goldenrod galls containing *Epiblema scudderiana* (Clemens) were collected during September of 1992, 1994 and 1996 in Ottawa, Ontario. The insects were kept outdoors (1992) until sampled or in an incubator (1994 and 1996) at 15°C for 2 weeks, in their galls. Control insects were incubated for 2 weeks at 15°C. The galls were then rapidly opened and living insects (assessed by visual inspection) were immediately placed into liquid nitrogen and then stored at –80°C until used. After the two week incubation period, a group of insects (1996) was cooled to –4°C for 24 h. Half of this group was then sampled as above, whereas the other half was returned to 15°C and sampled 48 h later.

Another group of insects (collected in autumn 1996) was acclimated at 4°C for two weeks and then transferred to –20°C. Some larvae were sampled after 24 h whereas the rest were returned to recover at 15°C and sampled after 2, 4, 8, 12 or 24 h. Yet another group of insects (1994) was given anoxia exposure for 24 h at 15°C by placing

them in a sealed bucket through which 100% nitrogen gas was passed. Half of the insects were sampled at the end of the stress and the other half was allowed to recover at 15°C for 48 h before sampling. Insects held outdoors were sampled about once a month between September and April.

Preparation of ^{32}P-Labeled Substrate for PP1 assay

Radioactive ^{32}P-labeled phosphorylase a was prepared according to Cohen *et al.* (1988) with some modifications. Briefly, 30 mg phosphorylase b was incubated for 90 min at 30°C with 3 mg/ml of phosphorylase kinase in a 1 ml mixture containing 100 mM Tris-HCl buffer, 100 mM β-glycerophosphate, pH 8.2, 0.1 mM $CaCl_2$, 10 mM magnesium acetate, 0.1 mM ATP, and 0.2 mM γ^{32}P-ATP. The reaction was then terminated by adding 0.9 ml 90 % saturated ammonium sulfate and set on ice for 30 min followed by centrifugation at 10 000 g for 10 min. The supernatant was discarded and the pellet was resuspended in TMS buffer (20 mM Tris-HCl, pH 7.0, 50 mM ß-mercaptoethanol, 100 mM NaCl). Excess ATP was removed by passing the solution through a 0.7 x 6 cm Sephadex G-25 spun column. The eluant was dialyzed overnight against TMG buffer containing 20 mM Tris-HCl, pH 7.0, 10% (v/v) glycerol, 15 mM β-mercaptoethanol, and 100 mM NaCl, at 4°C. Aliquots of 30 nmol phosphorylase a were combined with an equal volume of glycerol and stored at -20°C until use.

PP1 Assay

Whole frozen insects were rapidly weighed, then homogenized 1:3 (w/v) using a Prohomogenizer 200 and ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2

mM EGTA, 15 mM ß-mercaptoethanol) containing the protease inhibitors: 1 mM PMSF, 0.1 mM TPCK, 1µg/ml aprotinin and 5 mM benzamidine. Homogenates were centrifuged in a BioTek microfuge at 1,000 g for 3 min at 5°C. Supernatants were removed and assayed immediately to determine active PP-1. Measurement of PP-1 in concentrated extracts gives an estimate of activity at physiological levels of modulating proteins and other factors (Toth et al. 1988). Aliquots of extracts were also diluted 1:20 v/v in buffer A containing 2 mg/ml bovine serum albumin to stimulate dissociation of modulators and were then used to measure "total" PP-1 activity (Drake and Palmer, 1995). Preliminary tests showed that this dilution gave maximal PP-1 activity (data not shown).

Protein phosphatase 1 activity was measured at 23.5°C, essentially as described by Ingebritsen *et al.* (1983). Reaction mixture contained 10 µl supernatant or 20-fold diluted supernatant, 20µl assay buffer (50mM Tris-HCl, pH 7.0, 0.25 mM EGTA, and 25 mM β-mercaptoethanol) and 20 µl ^{32}P-labeled phosphorylase a which was added to start the reaction. The reaction was stopped after 15 min with the addition of 100 µl ice-cold stop solution (25% TCA, 10 mM H_3PO_4), held on ice for 5 min and then centrifuged at 13000 g for 5 min. Blanks were identical except that the TCA stop solution was added before the substrate. A 100 µl aliquot of supernatant was pipeted into 1.5 ml Eppendorf tubes and placed into scintillation vials. Vials were counted for 1 min in a Packard 1900 Tri-Carb liquid scintillation counter using Cerenkov methodology. One unit of activity is defined as the amount of enzyme that releases 1 pmol of phosphate in 1 min at 23.5°C.

Tissue extraction for PP2A/C assays

Whole frozen insects were rapidly weighed, then homogenized 1:10 (w/v) using a Prohomogenizer 200 and ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 15 mM ß-mercaptoethanol) containing the following protease inhibitors: 1 mM PMSF, 0.1 mM TPCK, 1µg/ml aprotinin and 5 mM benzamidine. Homogenates were centrifuged at 13 000 g for 20 min. Low molecular weight ions and metabolites were removed from the supernatant (0.4 ml) by passage through a "spun column": supernatant was layered onto a 5 ml column Sephadex G-25 that was equilibrated in Buffer A and centrifuged at room temperature for 1 min. The eluant was removed and then passed through a second, fresh spun column and held on ice until use.

PP2A assay

Protein phosphatase activity was assayed as previously described (MacDonald, 1998). Assay mixture for PP2A contained 150 µM peptide (RRApTVA), 0.2 mM EGTA, 0.02% ß-mercaptoethanol, and 50 mM imidazole, pH 7.2. PP2A activity was detected as the difference in activity in the absence versus presence (blank) of 2.5 nM okadaic acid. Reactions were started by adding 10 µl enzyme extract (final assay volume = 50 µl) and then incubated for 90 min. Reactions were terminated with the addition of 50 µl malachite green dye solution (10% ammonium molybdate and 2% malachite green dye, both in 4 N HCl mixed 1:3 v/v and diluted 2:3 v/v with distilled, deionized water, 0.05% Tween 20 and 0.05% Triton-X-100) (Ekman and Jager, 1993). Reactions were run in a 96 well half-area microplate and read with a MR 5000 microplate reader at 595 nm. One

unit of activity is defined as the amount of enzyme that releases 1 pmol of phosphate in 1 min at 23.5°C.

PP2C assay

Assay mixture and incubation time for measuring PP2C activity was identical to PP2A except that all assays contained 2.5 nM okadaic acid (MacDonald, 1998). PP2C activity was detected as the difference in activity in the presence versus absence of 10 mM $MgCl_2$.

PKA: Tissue extraction and assay

Whole frozen insects were rapidly weighed, then homogenized 1:10 (w/v), with a few crystals of PMSF, using a Prohomogenizer 200 and ice-cold PEM buffer (20 mM KPi, pH 6.8, 2 mM EDTA, 15 mM ß-mercaptoethanol). The homogenate was centrifuged at 13 000 g for 3 min at 5°C and the supernatant was removed and stored on ice until use.

The assay for cyclic AMP dependent protein kinase (PKA) activity measured the incorporation of ^{32}P from ^{32}P-ATP onto "Kemptide" (LRRASLG), an artificial phosphate-accepting substrate (Jiang and Corbin, 1991). For measuring total PKA activity, an aliquot of 10 µl supernatant was immediately added to an assay mixture (60 µl final volume) containing final concentrations of 0.15 mM Kemptide, 20mM KPi, 1 mM EDTA, 2.13 mM magnesium acetate, 150 mM KCl, 0.1 mM cAMP, 244.7 mM cold ATP, and 0.6 mCi/assay γ ^{32}P-ATP. The reaction was started with the addition of the $\gamma^{32}P$-ATP. To measure activity of the free catalytic subunit only, the cAMP was omitted. After a 15 min incubation, the reaction was stopped by pipeting 40 µl aliquots of reaction

mixture onto 2 x 2 cm P81 phosphocellulose paper squares as per the method of Roskoski (1983). The paper squares were then washed three times with 5 mM phosphoric acid for 5 min, followed by one wash with ddH_2O and placed in 1.5 ml Eppendorf tubes inside scintillation vials. Radioactivity was counted for 1 min in a Packard Tricarb 1900CA liquid scintillation counter. Blanks were immediately stopped upon addition of the γ ^{32}P-ATP. The percent active PKA was calculated from the ratio of activities in the absence versus presence of cAMP. One unit of activity is defined as the amount of enzyme that catalyzed the incorporation of 1 pmol ^{32}P-ATP onto the substrate per minute at 23.5°C.

Tissue extraction for Protein Tyrosine Phosphatase (PTP) assay

Whole frozen insects were rapidly weighed and then homogenized 1:10 (w/v) using a Prohomogenizer 200 set at low (2) and ice-cold buffer B (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose,10 mM ß-mercaptoethanol) containing the following protease inhibitors: 1 mM PMSF, 0.1 mM TPCK, 1µg/ml aprotonin and 5 mM benzamidine. Homogenates were centrifuged at 13 000 g for 25 min. Supernatant (0.15 ml), containing cytosolic PTPs, was layered onto a 5 ml column of Sephadex G-25 that was equilibrated in Buffer B and centrifuged for 1 min to remove phosphate and low molecular weight metabolites that might interfere with the assay. The pellet was resuspended in half the original volume of buffer B, with 1% (v/v) Triton-X-100 to solubilize membrane bound PTPs. The resuspended fraction was then centrifuged and the resulting supernatant passed through a G-25 column as for the cytosolic fraction.

PTP assay

Two peptide substrates for PTP were tested in an attempt to cover a broad range of PTP types: Pep 1 was END(pY)INASL (Daum *et al.*, 1993) and Pep 2 was DADE(pY)LIPQQG (Zhang *et al.*, 1993). Assay mixture for PTP contained 100 µM peptide substrate, 50 mM imidazole, pH 7.2, 0.02% ß-mercaptoethanol and 0.2 mM EGTA. Blanks were identical except that they did not contain the peptides. PTP activity was detected as the difference in activity in the absence versus presence of each peptide substrate. The reaction was started by adding 10 µl tissue extract (final assay volume was 50 µl), incubated for 90 min at room temperature, and then stopped by addition of 50 µl malachite green dye solution prepared as described for PP-2 assay. Reactions were run in a 96 well, half-area microplate and read with a MR 5000 microplate reader at 595 nm. One unit of activity is defined as the amount of enzyme which catalyzes the release of 1 pmol of phosphate per minute from the synthetic peptides.

Statistical Analysis

All enzyme data are reported as means ± SEM for n= at least 3 samples, each sample consisting of 1 or 2 insects. Statistical analysis used a one-way ANOVA followed by a Dunnett's Test.

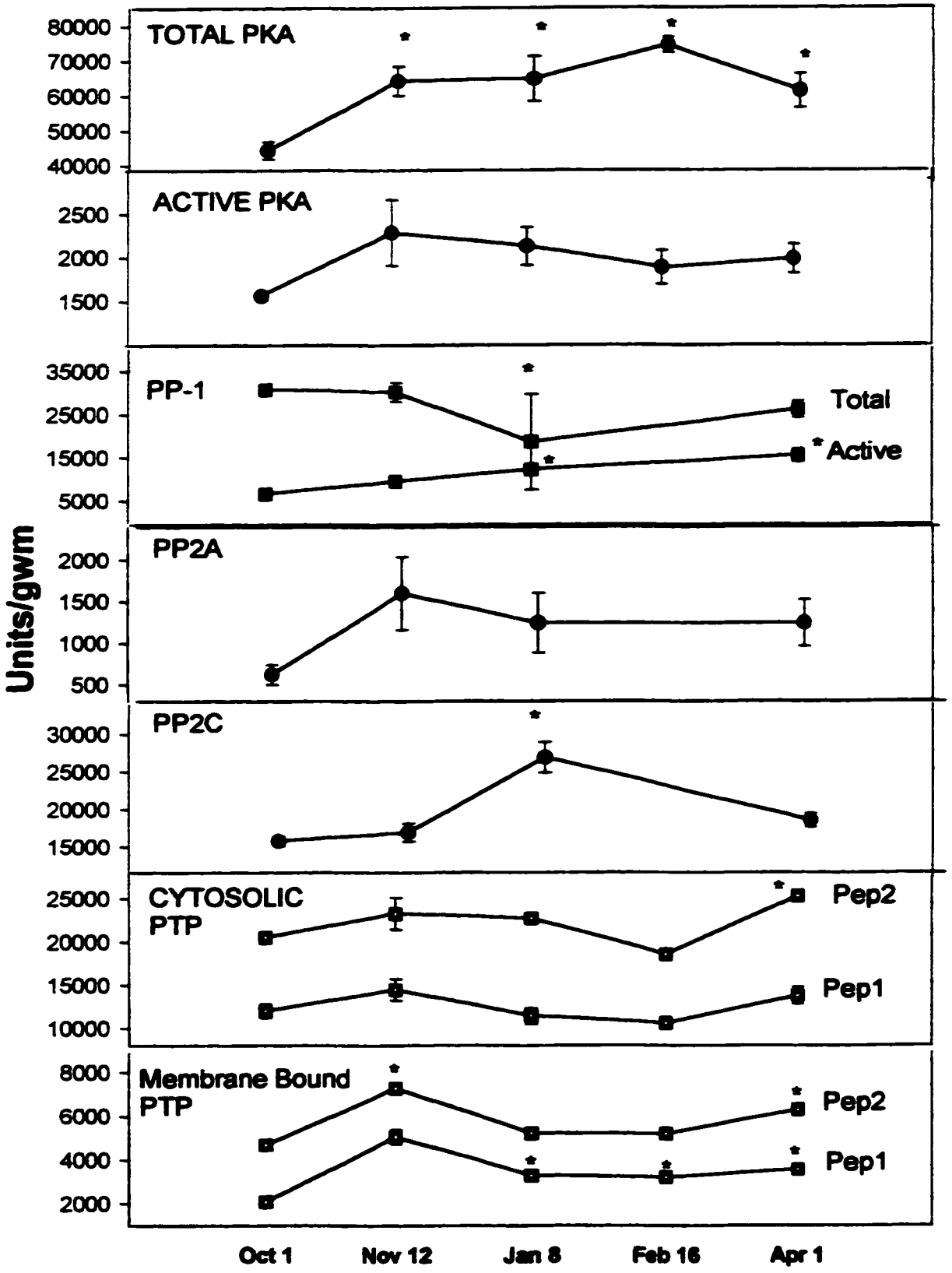
Results

Outdoor Overwintering animals

Glycerol levels in *Epiblema scudderiana* undergo major changes over a typical Ottawa (Ontario) winter (Figure 2.1; Rickards et al, 1987). Profiles of kinase and phosphatase activities over the winter season in *Epiblema scudderiana* are given in Figure 2.2. Compared with initial values measured in larvae at the beginning of October, total cyclic AMP dependent protein kinase (PKA) activity (as measured in the presence of 1 μM cAMP) increased over the winter months, rising by about 50% in November and January and reaching a maximum of 60% higher in February. Activity remained elevated in April. However, the amount of active free catalytic subunit PKA (PKAc) remained relatively constant throughout the winter. Total protein phosphatase-1 activity showed an opposite response to PKA and decreased significantly by 20 % in midwinter. However, the amount of active PP-1 actually rose slowly over the winter increasing in January by 1.2 fold and by 3 fold in April. Protein phosphatase 2A activity did not change significantly over the course of the winter but PP2C activity in the larvae increased by 70 % in January before returning to control values by April.

Tyrosine phosphatases (PTP) were assayed as a group with two different peptides: Peptide 1 (Pep1), END**p**YINASL and Peptide 2 (Pep2), DADE**p**YLIPQQG to increase the probability of detecting as many types of PTPs as possible. Since PTPs have been shown to be present in cells as both membrane bound receptor and free cytosolic enzymes, activities in both fractions were assayed. In the cytosolic fraction, PTP activities were generally unchanged over the winter except for a significant rise in PTP activity with Pep 2 as the substrate in April. The membrane bound PTPs acting on Pep 1

Figure 2.2 Protein Kinase and Phosphatase Activities in Overwintering *Epiblema scudderiana*. Results are mean activity (U/gram wet mass) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. For protein tyrosine phosphatases, data show activities with 2 different phosphopeptides (Pep1 and Pep2). *, significantly different from October 1 values using a one way ANOVA with a Dunnett's test, $P < 0.05$. Where error bars are not visible they are contained within the symbol.


TOTAL PKA
80000
70000
60000
50000
40000
ACTIVE PKA
2500
2000
1500
PP-1
35000
25000
15000
5000
Total
*Active
PP2A
2000
1500
1000
500
PP2C
30000
25000
20000
15000
CYTOSOLIC PTP
25000
20000
15000
10000
Pep2
Pep1
Membrane Bound PTP
8000
6000
4000
2000
Pep2
Pep1
Units/gwm
Oct 1
Nov 12
Jan 8
Feb 16
Apr 1

showed elevated activities for January, February and April, whereas for Pep 2, activities were significantly increased in November and April.

Figure 2.3 shows the percentages of PKA and PP1 that were in the active form at the different sampling times. The % PKAc was very low at all times, only 2.5-3.5 % of total activity, and no significant changes occurred over the time course. By contrast, the percentage of active PP-1 rose sharply between November and January, reaching a peak of 3.5 fold higher than September values in January and remained elevated through April.

Subzero Temperature Exposure Switch Time Course

The effects of 24 h subzero exposure at -4°C and subsequent 48 h recovery at 15°C were studied to examine cold-induced activation of kinases/phosphatases in *E. scudderiana* (Figure 2.4). Total PKA and PP1 activities remained constant (data not shown) for the entire experiment; hence, PKA and PP1 activities mentioned in this section refer to active enzyme only. The 24 h exposure at -4°C had no effect on active PKA, active PP1, PP2A and PP2C activities but tyrosine phosphatases increased significantly during cold exposure in all cases. Cytosolic PTP activity increased by 20 or 40 %, as assayed with Pep 1or Pep 2, respectively, whereas the membrane bound PTP activity increased by 10 % and 3.1-fold, respectively, during -4°C exposure. After 48 h returned to 15°C, all PTP activities had reverted to control levels. By contrast, activities of PKA, PP1 and PP2C changed only during the recovery phase. PKA activity decreased by 20 % whereas PP2C was strongly suppressed by 80%. PP1 activity, on the other hand, increased by 20 % during recovery.

Figure 2.3 Active PKA and PP1 as a Percentage of Total Activity in Overwintering *Epiblema scudderiana*. Results are means± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. *, significantly different from October 1 values using a one way ANOVA with a Dunnett's test, $P < 0.01$. Where error bars are not visible they are contained within the symbols.

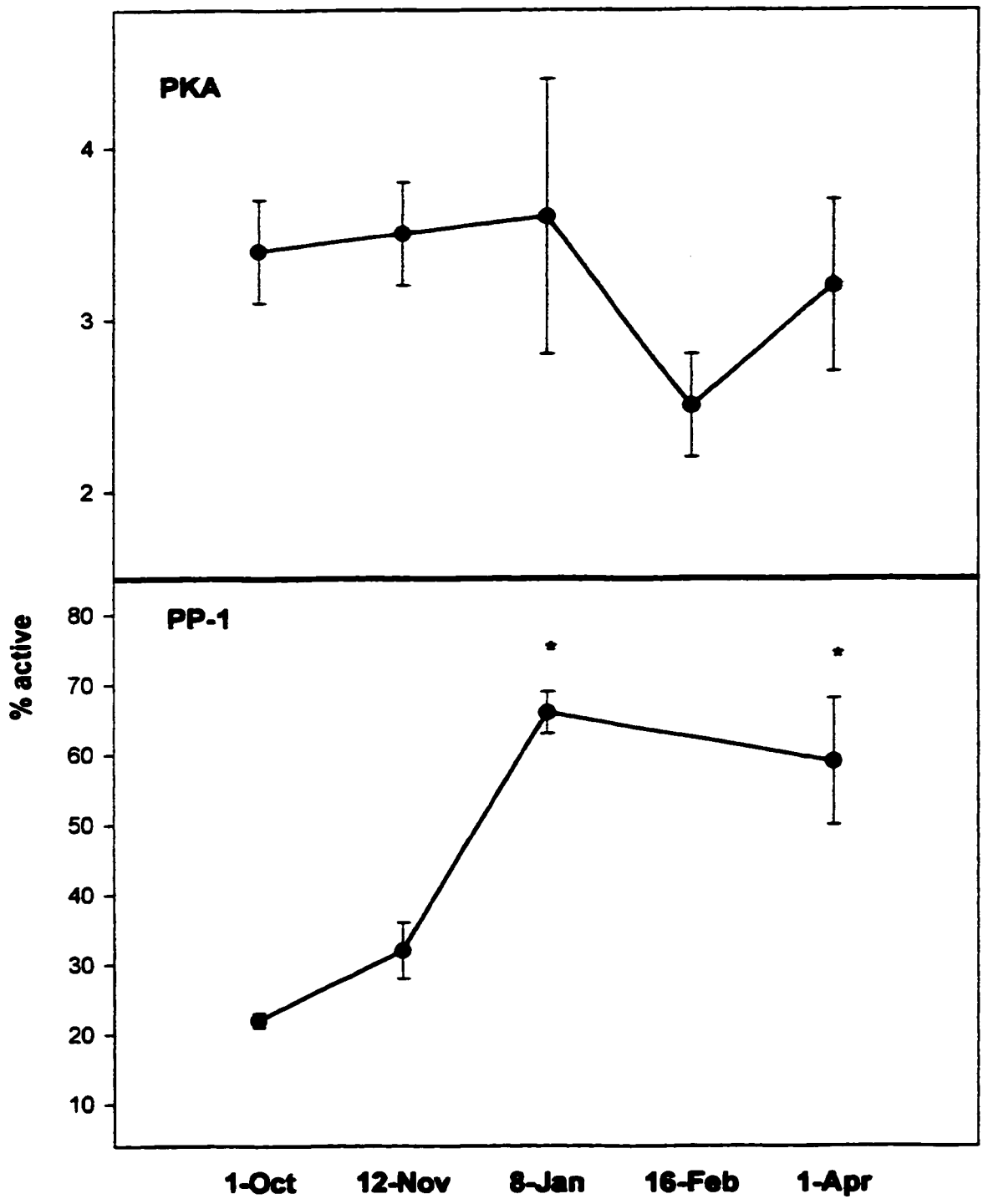
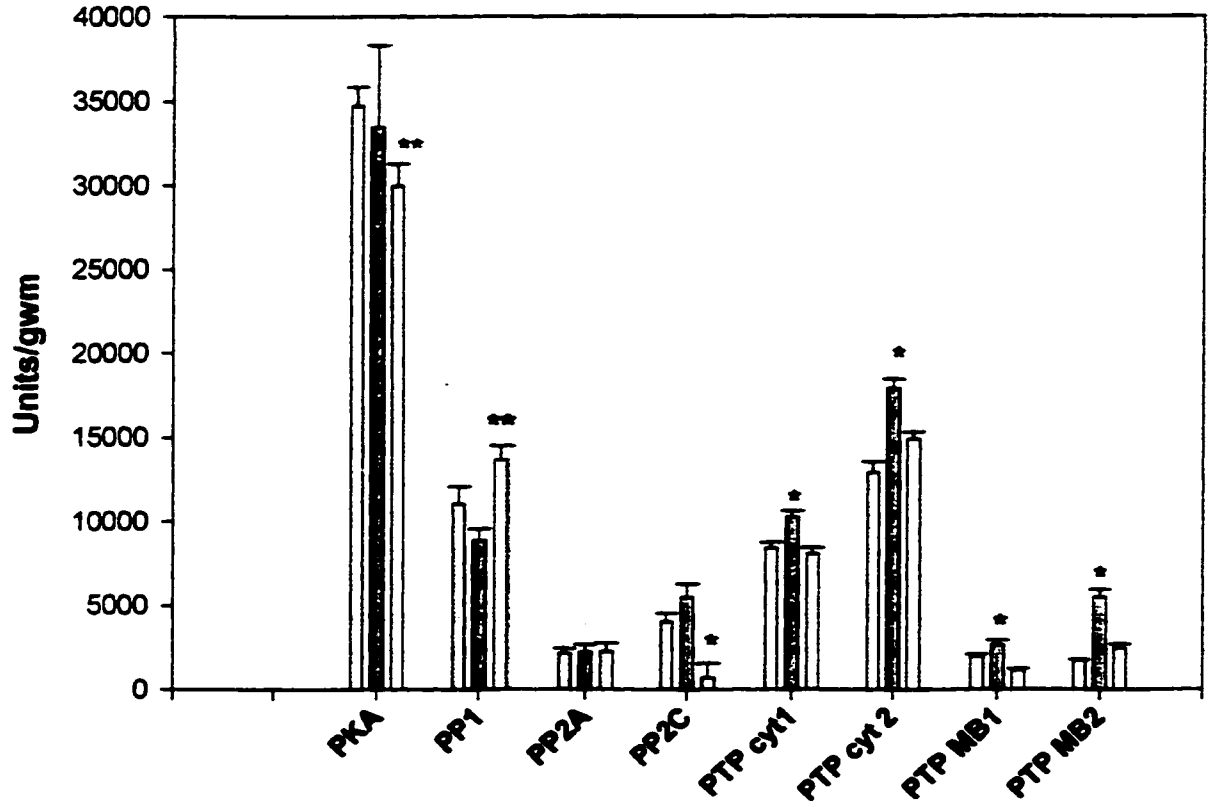

PKA
4
3
2
PP-1
% active
80
70
60
50
40
30
20
10
*
*
1-Oct
12-Nov
8-Jan
16-Feb
1-Apr

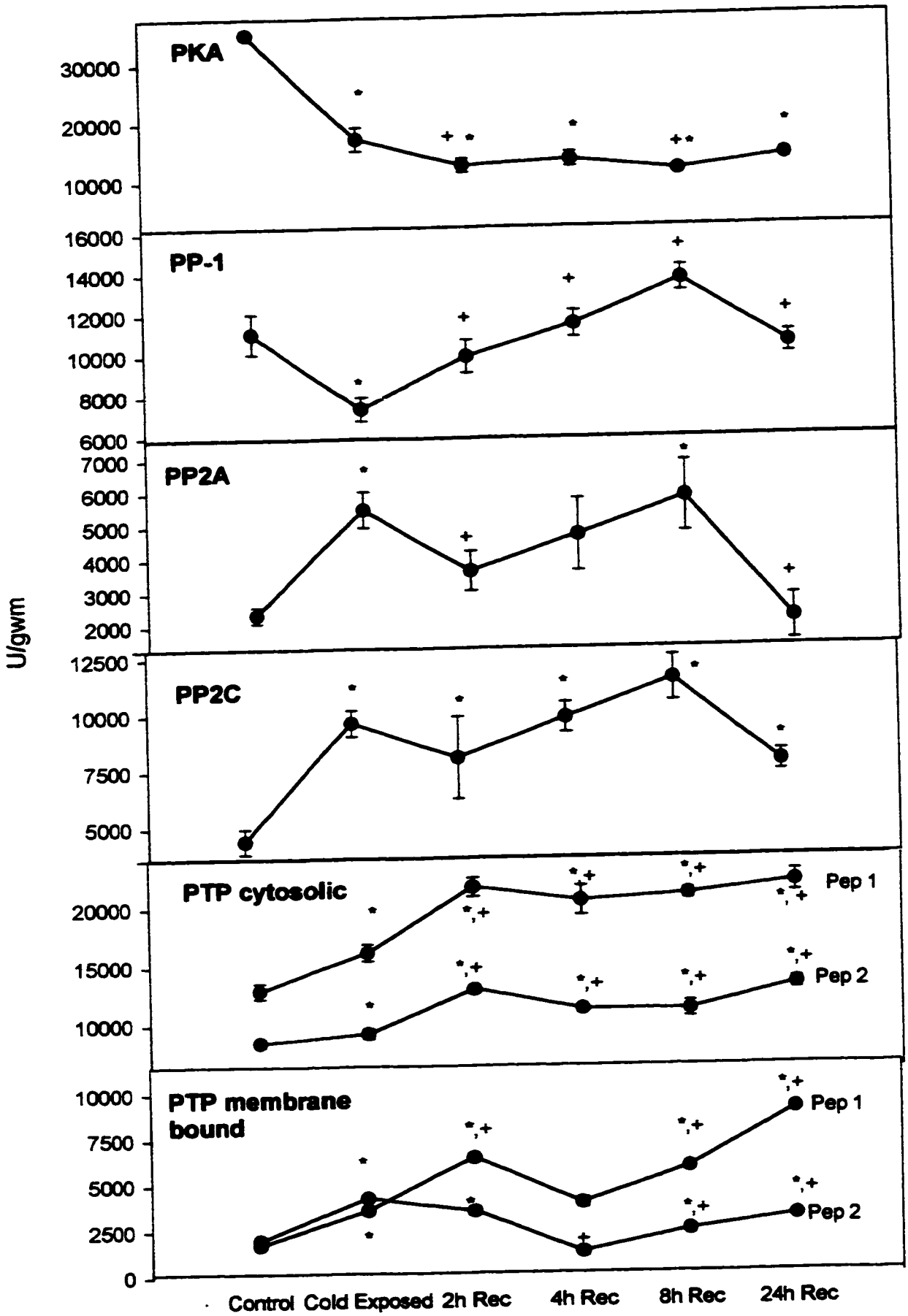
Figure 2.4 Protein Kinase and Phosphatase Activities over a cycle of Sub-Zero Exposure and Recovery in *Epiblema scudderiana*. Bars from left to right in each group are; Control (15°C), 24 hour at -4°C and 48 hour returned to 15°C. Results are mean (U/gwm) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. PKA and PP1 activities are "active" enzyme. *, significantly different from control values using a one way ANOVA with a Dunnett's test, $P < 0.01$, and **, $P < 0.05$. Where error bars are not visible they are contained within the symbol.


40000
35000
30000
25000
20000
15000
10000
5000
0
Units/gwm
PKA
PP1
PP2A
PP2C
PTP cyt1
PTP cyt 2
PTP MB1
PTP MB2

Recovery From -20°C Exposure

In another set of experiments, 15°C-acclimated insects were cooled to 4°C for two weeks to allow for a build up of glycerol and then larvae were exposed to -20°C for 24 h followed by a return to 15°C and sampling at multiple time points (2, 4, 8, 24 h). Figure 2.5 shows PKA and phosphatase activities at the end of -20°C exposure and over the time course after return to 15°C. Active protein kinase A activity was high in the cold-exposed larvae but decreased by 27 % within just 2 h back at 15°C; activity remained low with longer recovery times. Phosphatase-1 activity (active) fell to 60% of the control value when larvae were cold-exposed (in the 24 h at -20°C) but rapidly returned to control values when animals were rewarmed. Activity had increased significantly after 2 h recovery at 15°C and remained at values not different from controls over the rest of the time course. The activities of both of PP2A and PP2C in the larvae were elevated by cold exposure. PP2A increased by 3-fold after cold exposure (2 weeks at 4°C, then 24 h at -20°C) whereas PP2C rose by 2-fold. Upon return to 15°C, PP2A decreased by about one-third as an early (2 h) response, then rose again before dropping back to control levels after 24 h back at 15°C. PP2C activity followed a similar pattern but remained higher than control values even after 24 h recovery. Cytosolic PTP activity with Pep 1 and Pep 2 as substrates had increased by 30 and 10 %, respectively, after the -20°C exposure and rose further during recovery at 15°C. Values after 2 h return to 15°C were 1.7 fold and 1.3 fold higher than the cold exposed value, respectively, and remained high over the remainder of the time course. Membrane bound PTP also increased during -20°C exposure by about 50% for both Pep 1 and Pep 2. Activity using Pep 1 as the substrate

Figure 2.5 Protein Kinase and Phosphatase Activities for a Recovery from -20°C Exposure (Preceeded by a two week acclimation at 4°C) Time Course for *Epiblema scudderiana*. Results are mean (U/gwm) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. Acivities for PKA and PP1 are for the active form of the enzyme. *, significantly different from control values using a one way ANOVA with a Dunnett's test, $P < 0.01$ and +, different from -20°C exposed, $P < 0.01$.

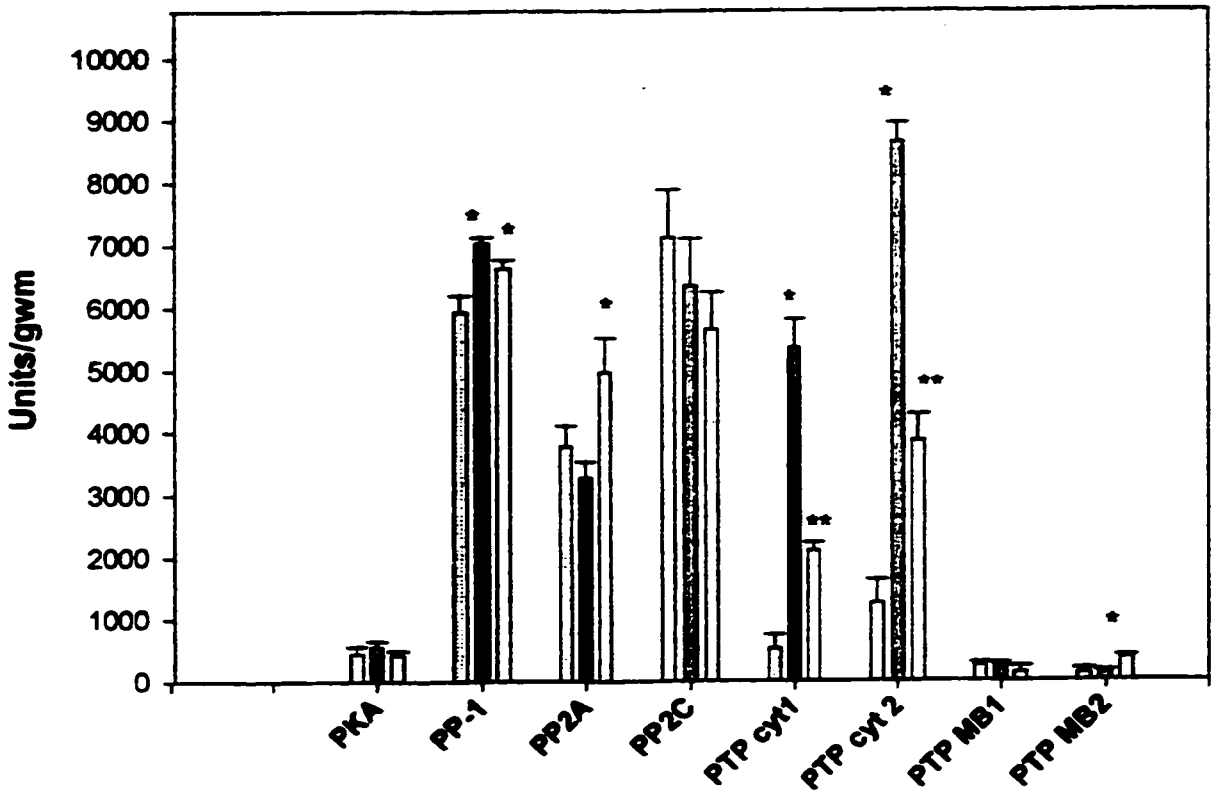

PKA
30000
20000
10000
PP-1
16000
14000
12000
10000
8000
6000
PP2A
7000
6000
5000
4000
3000
2000
U/gwm
PP2C
12500
10000
7500
5000
PTP cytosolic
Pep 1
Pep 2
20000
15000
10000
PTP membrane bound
Pep 1
Pep 2
10000
7500
5000
2500
0
Control
Cold Exposed
2h Rec
4h Rec
8h Rec
24h Rec

increased further after 2 h recovery and then rose again between 8 and 24 h of recovery, to a total 4-fold increase over controls. Activity with Pep 2 substrate decreased by 4 h of recovery to a value not significantly different from controls but then rose again with longer recovery.

Anoxia Exposure and Aerobic Recovery

The effects on *Epiblema scudderiana* larvae of a 24 h exposure to a nitrogen gas atmosphere at 15°C followed by 24 h aerobic recovery are shown in Figure 2.6. Even though *Epiblema scudderiana* probably does not experience anoxia in nature during the winter, this stress was studied to compare with *Eurosta solidaginis* (studied in chapter 3 of this thesis). PKA and PP1 activities mentioned in this section refer to active enzyme since total PKA and PP1 activities remained constant (data not shown). Anoxia and aerobic recovery had no effect on active protein kinase A activity in the larvae nor were PP2C or membrane bound PTP using Pep 1 substrate activities affected. PP1 activity increased by 20 % during anoxia and remained elevated, at 10 % higher than the control value after 24 h aerobic recovery. Anoxia exposure (24 h) had no significant effect on PP2A activity, but after 24 h recovery activity had increased 1.4 fold. Cytosolic PTP activity with Pep 1 and Pep 2 as substrates increased strongly during anoxia by 10.2 and 7.2 fold, respectively. However, after 24 h aerobic recovery PTP activities were much reduced again, although still 4.2 and 3.2 fold higher than controls. Membrane bound PTP activity with Pep 2 increased 3 fold during aerobic recovery.

Figure 2.6 Protein Kinase and Phosphatase Activities for an Anoxia-Recovery Cycle of *Epiblema scudderiana*. Bars from left to right in each group are; Aerobic control , 24 Anoxic and 24 hour aerobic recovery (All at 15°C). PKA and PP1activities are for "active" enzyme. Results are mean (U/gwm) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. *, significantly different from control values using a one way ANOVA with a Dunnett's test, $P < 0.01$.

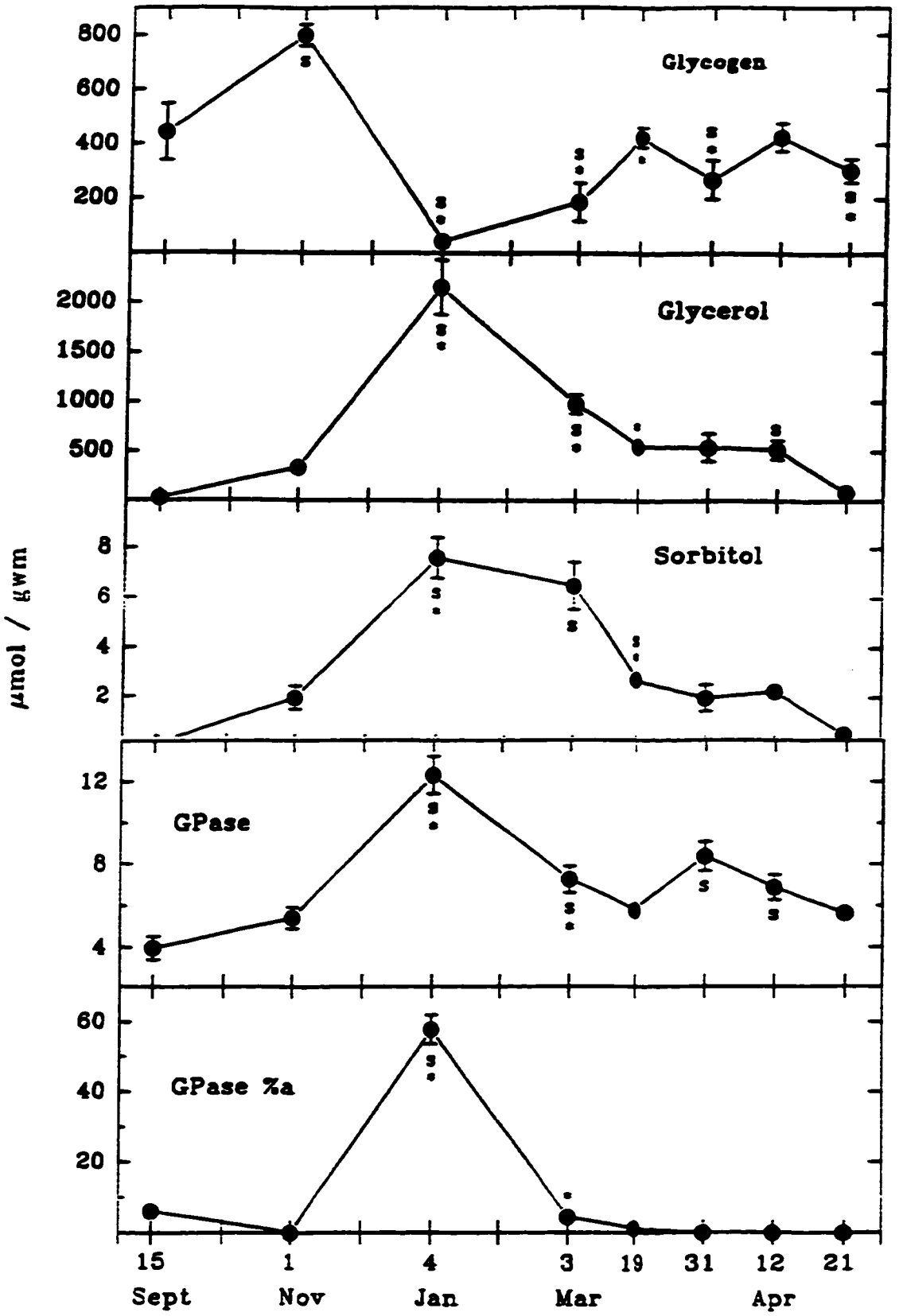

Units/gwm
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
PKA
PP-1
PP2A
PP2C
PTP cyt1
PTP cyt 2
PTP MB1
PTP MB2

Discussion

Many of the enzymes studied showed changes in activity over the course of the winter or when subjected to subzero exposure or anoxic conditions in the lab. Some of these changes appear to correlate with glycerol synthesis and/or degradation. In the autumn, the activities of various enzymes involved in glycerol biosynthesis increase in activity (Joanisse, 1994a); these include regulatory enzymes, such as glycogen phosphorylase, that are known to be controlled by reversible phosphorylation (Figure 2.7). Other enzymes, such as fructose-1,6-bisphosphatase, which is involved in glycerol catabolism, show changes in proportions of phosphorylated (active) and dephosphorylated (inactive) forms that would effectively minimize enzyme function over the winter but provide for an easy reactivation in the spring (Muise and Storey, 1997). Thus, it is likely that activities of kinases and phosphatases regulating these enzymes must also change with both a seasonal pattern and in response to cold exposure (since temperature is an important trigger of glycerol synthesis and catabolism). Changes in the activities of phosphatases may also be developmentally regulated since significant changes occurred in April which is a time when cold-hardiness is dissipating and larvae are preparing for the transition to the pupal stage. In particular, PTP activity with Pep2 as the substrate had increased in both membrane and cytosolic fractions at the April sampling time whereas midwinter changes in total PP1 and PP2C activities were reversed by April.

As previously stated, many insects respond to cold by increasing glycogen phosphorylase activity in fat bodies to initiate the conversion of glycogen to glycerol (or other polyols). Glycogen phosphorylase is regulated by phosphorylase kinase, which converts it to the active a form, and PP1, which returns it to the inactive b form (Figure

Figure 2.7 Polyol Levels in Correspondence with Gycogen Phosphorylase a Activity in Overwintering *Epiblema scudderiana* (Joanisse and Storey, 1994a). Results are mean ± SEM for n=4 samples. ^{s}, significantly different from sept 15 values. *, significantly diferent from previous sampling date. Where error bars are not visible they are contained within the symbol.


Glycogen
Glycerol
Sorbitol
GPase
GPase %a
µmol / gwm
800
600
400
200
2000
1500
1000
500
8
6
4
2
12
8
4
60
40
20
15 Sept
1 Nov
4 Jan
3 Mar
19
31
12 Apr
21

1.3). The classic role of PKA is to phosphorylate phosphorylase kinase which, in turn, phosphorylates phosphorylase b and converts it to the active phosphorylase a. Hence, PKA and PP1 effectively counteract each other in the activation versus inactivation of glycogenolysis. Increased active PP1 in January in overwintering *Epiblema scudderiana*, accompanied by a considerable drop in total PP1, resulted in a big increase in the percent active PP1 (Figure 2.2 & 2.3). While glycerol levels at this point are high, they have leveled off (Figure 2.1). This increase in PP1 activity likely corresponds to decreased glycerol synthesis *in vivo* and could suggest that PP1 is shutting down glycogen phosphorylase activity in the insect via an increase in enzyme dephosphorylation (Ingebritsen and Cohen, 1983). Whether phosphorylase kinase shows a corresponding decrease in activity has yet to be determined. Since active and percent active PP1 remain high in April, this may link the enzyme to developmental regulation, and/or regulating the clearance of glycerol. However, since the amount of active PKA remained constant throughout the winter it is unlikely that it is regulating phosphorylase kinase activity (activation). It therefore also appears that PKA is not inhibiting phosphatase activity and hence glycerol production, via the phosphorylation of Inhibitor 1, which is required for the association of Inhibitor 1 with PP1 (Nimmo and Cohen, 1982; Aitken et al, 1982).

Previous studies have indicated that PP2A can contribute a significant portion of the glycogen phosphorylase phosphatase activity in cells (Ingebritsen and Cohen, 1983). The enzyme can also dephosphorylate a number of other phosphoprotein targets. However, it would appear that in *E. scudderiana* under cold stress that PP2A is not involved in regulating phosphorylase activity (or any other enzyme) since it shows no significant change in activity over the winter time course.

Phosphatase 2C showed an increase in activity corresponding to the increase in glycerol in January. But since PP2C shows no affinity for glycogen phosphorylase in mammalian tissues (Ingebritsen and Cohen, 1983), and phosphatases are usually fairly conserved evolutionarily, it is likely that PP2C is involved in mediating some other element of freeze avoidance. One possibility is that PP2C is responding to high osmolality signals created by the 2 M glycerol which accumulated. PP2C has been shown to be involved in regulating the mitogen activated protein (MAP) kinase cascade comprising the High Osmolarity glycerol response-1 (HOG1) kinase (Shiozaki and Russell, 1995).

Since PTPs were studied as a group, the conclusions drawn from this study are very general. Cytosolic PTP only showed a significant change in activity for Pep2 in April indicating that some cytosolic PTPs may be involved in regulating events associated with the loss of cold hardiness (e.g. glycerol breakdown or others) or with the resumption of development, heading into the larval-pupal transition. Membrane bound PTPs with Pep2 hydrolyzing activity also only showed a significant change in activity in April. Protein tyrosine phosphatases acting on Pep1 also appear to play a role in regulating freeze avoidance.

In the 24 h -4°C cold exposed insects PKA, PP1, PP2A and PP2C were apparently not involved in regulating any response over this relatively short exposure (Figure 2.4). However, all PTPs did show increases and therefore they may be involved in mediating short term responses to -4°C exposure. This might include a stimulation of glycerol production which is known to occur with the switch from 15 to -4° C in *Epiblema scudderiana* (Churchill and Storey, 1989a), but since PTPs are not generally associated

with glycogen metabolism this may be unlikely. Other common events associated with a cold shock (at least in bacterial and yeast systems) are: 1) increased synthesis of cold shock proteins and 2) readjusting membrane fluidity by increasing the relative amounts of unsaturated phospholipids (typically via increased activities of desaturase enzymes; Thieringer *et al.*, 1998). Cold shock proteins appear to have primary actions either as RNA/DNA chaperones or as part of the ribosomal protein synthesis machinery so that the interruption of protein synthesis that is an immediate response to cold shock can be reversed. Whether similar metabolic adjustments are also initiated by cold shock in species such as *Epiblema scudderiana* that are seasonally adapted for low temperatures is still unknown, although a seasonal increase in lipid unsaturation occurs in this species when comparing September versus December lipid compositions. However, if they are, these functions (ribosomal protein synthesis, membrane fluidity adjustments) represent additional targets that could be regulated by cellular protein phosphatases and kinases.

During recovery from -4°C exposure, all PTP activities reverted to control values and significant changes were seen in other enzymes. An increase in PP1 activity and a decrease in PKA activity were seen during recovery possibly indicating a cooperative role of these enzymes in decreasing the overall phosphorylation state of enzymes involved in recovery from the short term (24 h) -4°C stress. This is consistent with what is expected and has been observed throughout this study. Their lack of response to the 24 h exposure at -4°C may seem troubling when compared with the results from the overwintering time course (Figure 2.2) and with the longer and deeper cold exposure (Figure 2.5). However, it may be that PP1 and PKA activities require a prolonged exposure to cold to show significant changes. This is supported by the study by Churchill and Storey (1989a)

which used a 10 day exposure at -4°C to stimulate glycerol synthesis. The fact that PP2C showed no change during the 24 h -4°C exposure may also strengthen the argument that it is involved in an osmosensing mechanism, since the short nature of this exposure would not allow for much glycerol build up and hence the osmolality of the cells would show very little change.

Exposure to –20°C preceded by a two week acclimation period at 4°C to allow for a build up of the cryoprotectant glycerol resulted in decreased PP1 which rises in recovery and high PKA activity which drops during recovery. Hence, PP1 and PKA again show opposite effects. Most of the trends seen in the two week acclimation period followed by -20°C exposure are comparable to those seen in the January, outdoor insects. The decrease in PP1 is likely facilitating a continued high activity of phosphorylase. The fact that PP1 levels return to control values in the first 2 hours of 15°C recovery does not mean that glycerol synthesis must stop because it has been shown to continue in repeated 10 or 12 day freeze thaw cycles (Churchill and Storey, 1989b). Therefore, it must follow that phosphorylase kinase must remain active and/or that the degradation of the phosphorylated phosphorylase is slow. Since the control data for PKA is from another experimental group from the same year its significance must be down- played, but it does appear that active PKA activity dropped in response to the two week acclimation period followed by -20°C exposure For this exposure the 2 week acclimation at 4°C would have allowed for an accumulation of glycerol, so an increase in PP2C activity is not surprising based on the assumption that it is indeed involved in an osomosensing mechanism. In most cases the fact that these enzymes do not return to control values with in the 24 hour 15°C recovery period could indicate that the enzymes are also involved in recovery or

more likely that the mechanism by which they are regulated takes more than 24 h in order to readjust all aspects of metabolism.

Increased activity of PP1 and cytosolic PTPs indicates that survival in anoxic conditions involves a number of dephosphorylation reactions. Such reactions may include the inactivation of a number of enzymes at key metabolic points such as glycogen phosphorylase in an attempt to decrease the metabolic rate of the insect to one that can be sustained over the long term by anaerobic ATP production alone. An increase in PP2A activity suggests its involvement in anoxia survival. Membrane bound PTP activity on peptide 2 is likely also involved in mediating recovery from the anoxic exposure. Again the fact that PP2C showed no change in anoxia or subsequent recovery may strengthen the argument that it is involved in an osmosensing mechanism.

The fact that whole animals were used means that this study may have missed minute enzyme fluctuations in specific parts of the larvae. Micro-dissection could be used to examine this theory. It could be very possible that some of the enzymes required for recovery from cold-exposure are also involved in developmental regulation, since this would allow the insect to maximize its metabolic requirements.

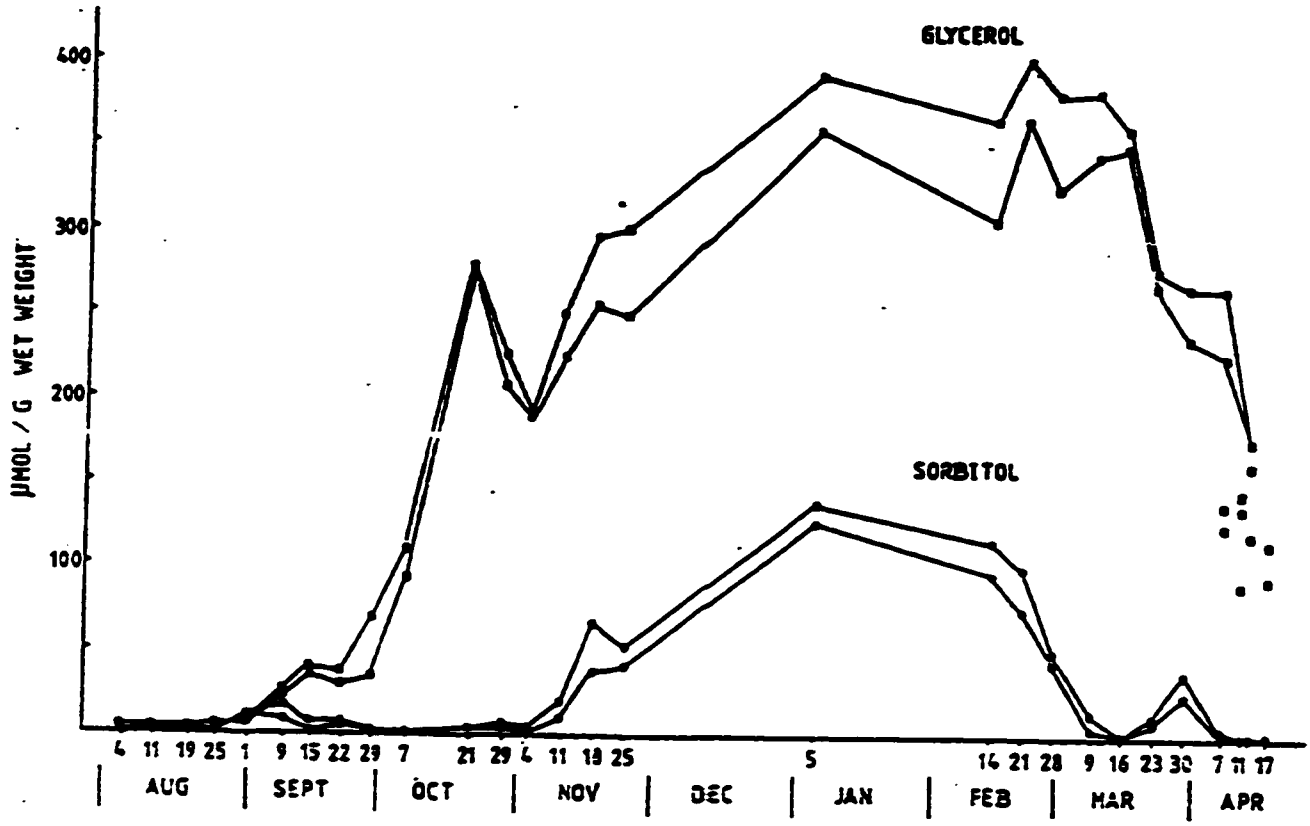
Chapter 3

Enzyme Activity Profiles in Overwintering *Eurosta solidaginis* and in Response to Subzero Temperature Exposure and Anoxia Stress

Introduction

The larvae of the goldenrod gall fly *Eurosta solidaginis* have been used extensively to study freeze tolerance in insects (Baust and Nishino, 1991; Storey and Storey, 1992). Freeze tolerance in this species, as in many others, results primarily from cryoprotectants and conservation of energy. The larvae are found in galls on goldenrod plants, but the galls offer almost no insulation (Layne, 1991). Supercooling capacity is limited with crystallization occurring at about -10°C. Glycerol and sorbitol are accumulated as colligative antifreezes. Glycerol levels reach about 0.5 to 0.6 M and sorbitol levels accumulate to ~0.2 M (Morrissey and Baust, 1976; Storey *et al.*, 1981). Figure 3.1 shows the pattern of polyol build-up and loss over a typical Ottawa winter (Storey and Storey, 1986). Glycerol and sorbitol make excellent cryoprotectants due to their high solubility and lack of toxic effects. Glycerol synthesis is triggered earlier than sorbitol at temperatures between 15°C and 5°C (Morrissey and Baust, 1976; Storey et al., 1981) whereas sorbitol is only produced at temperatures between 5°C and -10°C, just above the temperatures at which the larvae freeze (Storey et al., 1981). Glycerol provides a constant cryoprotection over the winter months, whereas sorbitol levels increase and decrease with fluctuations in ambient temperature (interconverted with glycogen pools). Many of the enzymes involved in polyol production have been identified and their regulatory properties explored (Storey, 1990; Joanisse and Storey, 1994b). Several of these are targets of reversible phosphorylation and are thus responsive to signals arising from stimuli such as temperature change that can be mediated via changes in the activities of protein kinases and phosphatases.

3.1 Overwintering Profile of Polyol Contents for *Eurosta solidaginis*. The diagram shows glycerol and sorbitol levels compared to the average ambient temperature from September to April in *E. solidaginis* (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

This study examines the activities of several enzymes which could potentially be involved in the regulation of phosphorylation states of the enzymes involved in freeze tolerance. An overwintering time course is examined, as well as laboratory freeze/thaw exposures and anoxia exposure and subsequent recovery. Some of the responses to freezing may originate from the oxygen limitation which accompanies freezing. Anoxia generally triggers a series of metabolic events, particularly a switch to glycolytic metabolism and frequently metabolic arrest, that can also be beneficial to freeze survival.

Materials and Methods

All materials and methods are identical to those described in Chapter 2, with the exception that larvae of *Eurosta solidaginis* were used as the experimental animals. This species was collected at the same time and in the same fields as *Epiblema scudderiana*. Assay times for PP2A and PP2C were 40 min instead of 90 min.

Results

Outdoor Overwintering animals

Profiles of protein kinase and protein phosphatase activities in *Eurosta solidaginis* over the course of a winter season are shown in Figure 3.2. Total cyclic AMP dependent protein kinase (PKA) activity increased over the autumn months and reached a maximum 1.3 fold increase in February which was maintained until April. However, despite the increase in total PKA, the amount of active PKA showed no significant change over the entire 6 month time period. Similarly, active PP1 did not change over most of the winter course but had risen by 50 % in April. Total PP-1 showed a trend opposite to PKA with activity reduced in midwinter, the February value being 70 % of the value in September. Protein phosphatase 2A activity showed no significant changes over most of the winter but activity increased in April to a value 80% higher than in September. By contrast, PP2C activities remained constant over the full season. Tyrosine phosphatases were assayed as a group with two different peptides. In the cytosolic fraction, PTPs capable of acting on Pep 2 had increased by 2-fold in February, compared with September values, but decreased again by April. No change in the activities of cytosolic PTPs acting on Pep 1 were detected. Activities of membrane bound PTPs, however, rose over the winter. A 2-fold increase in activity using Pep 1 was noted in January, whereas activities with Pep 2 were 50 % higher than September values in both November and December.

Figure 3.3 shows the percent active for both PKA and PP1 over the winter time course. The percentage of PKA present as the active catalytic subunit ranged from 22-29 % in the larvae but did not change significantly over the winter. However, the percent

Figure 3.2 Protein Kinase and Phosphatase Activities for Overwintering *Eurosta solidaginis*. Results are mean enzyme activity (U/gwm) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. *, significantly different from Oct 1 values using a one way ANOVA with a Dunnett's test, $P < 0.05$. Where error bars are not visible they are contained within the symbol.

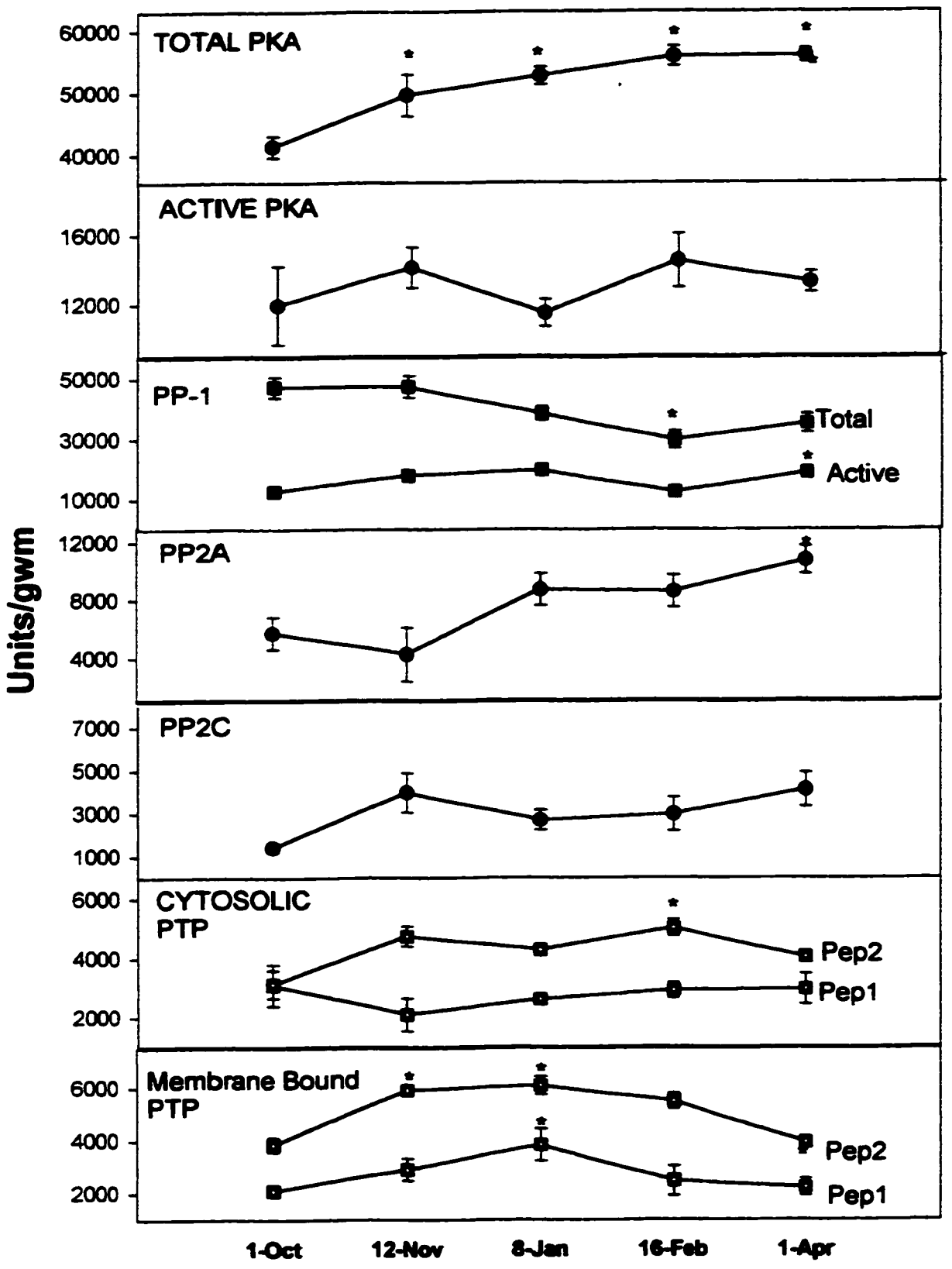
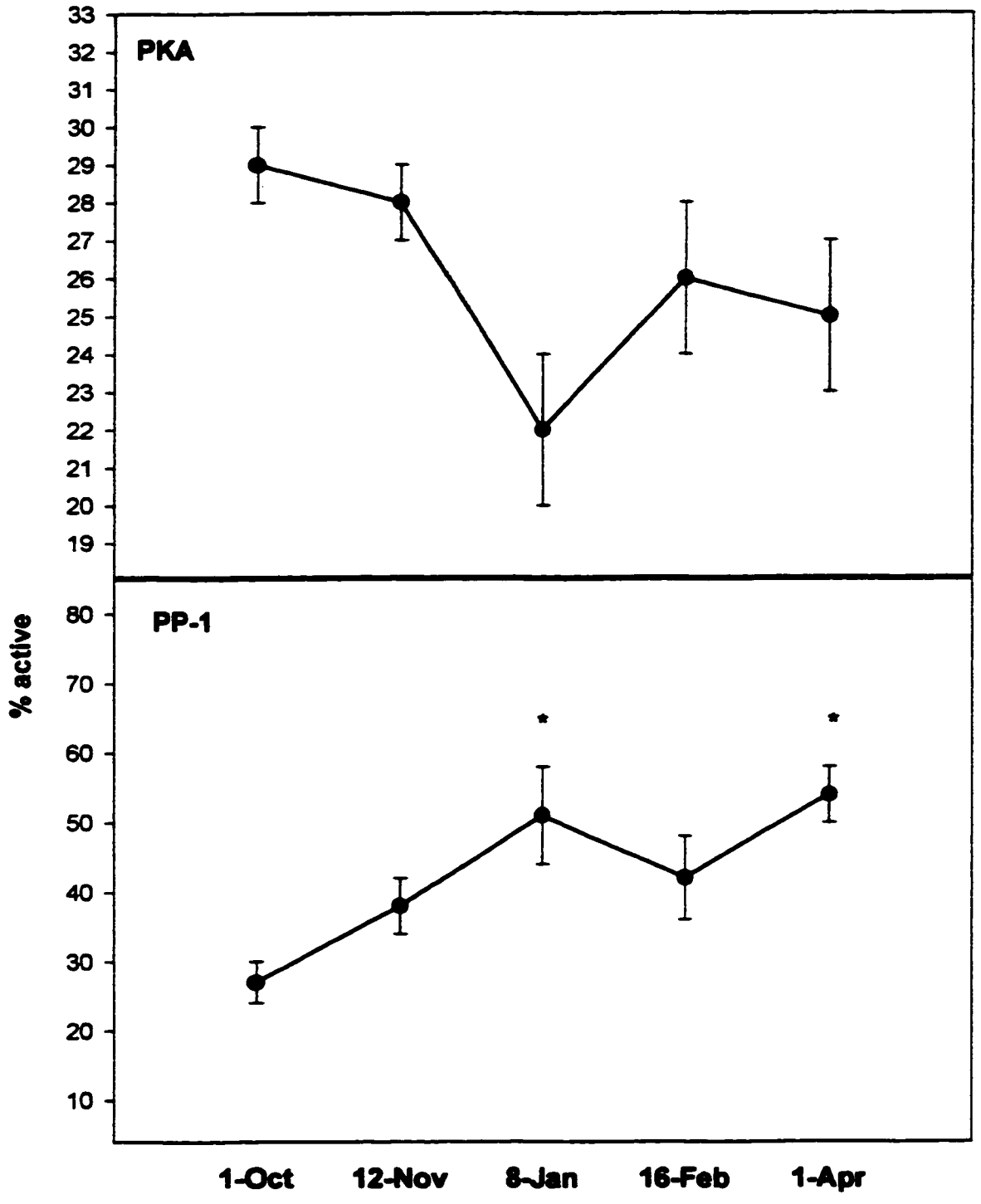

TOTAL PKA
60000
50000
40000
ACTIVE PKA
16000
12000
PP-1
50000
30000
10000
Total
Active
PP2A
12000
8000
4000
PP2C
7000
5000
3000
1000
CYTOSOLIC PTP
6000
4000
2000
Pep2
Pep1
Membrane Bound PTP
6000
4000
2000
Pep2
Pep1
Units/gwm
1-Oct
12-Nov
8-Jan
16-Feb
1-Apr

Figure 3.3 Percentage of Total PKA and PP-1 Activities Present in the active formin Overwintering *Eurosta solidaginis*. Results are means ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. *, significantly different from Oct 1 values using a one way ANOVA with a Dunnett's test, $P < 0.01$. Where error bars are not visible they are contained within the symbol.


PKA
PP-1
% active
33
32
31
30
29
28
27
26
25
24
23
22
21
20
19
80
70
60
50
40
30
20
10
*
*
1-Oct
12-Nov
8-Jan
16-Feb
1-Apr

active PP1 increased over the winter, rising from about 25 % in September to about 55 % in January and April.

Subzero (-4°C) Exposure and Recovery

The effects of subzero exposure at -4°C and subsequent recovery at 15°C on protein kinase and phosphatase activities in *Eurosta solidaginis* larvae are shown in Figure 3.4. Activities for PKA and PP1 mentioned in this section refer to active enzyme since total activities remained constant (data not shown). Activities of most enzymes were unaffected by the subzero exposure. However, PP1 decreased to 55 % of the control value after 24 h at -4°C whereas activities of cytosolic PTP increased 3 fold with Pep 1 as the substrate and 2.2 fold when measured with Pep 2. During recovery at 15°C, activities of cytosolic PTPs remained elevated and activity with Pep 1 actually increased further. PP1 activity recovered somewhat, rising to a value 70% of the control. PKA activity decreased during recovery to 80% of the control value, whereas the activity of membrane bound PTPs using Pep 2 substrate had increased by 3.3 fold over controls after 48 h recovery at 15°C.

Recovery From -20°C Exposure

Figure 3.5 shows the effects of cold exposure (2 weeks at 4°C) followed by 24 h at -20°C after acclimation at 4°C, and then recovery at 15°C on the maximal activities of PKA and phosphatases in the larvae. The -20°C exposure represents a freezing stress for *Eurosta solidaginis* and the main purpose of this study was to examine changes in enzyme activity occurring after thawing. Activities for PKA and PP1 mentioned in this

Figure 3.4 Protein Kinase and Phosphatase Activities over a cycle of Sub-Zero Exposure and Recovery for *Eurosta solidaginis*. Bars from left to right in each group are; Control (15°C), 24 hour at -4°C and 48 hour returned to 15°C. Results are mean enzyme activity (U/gwm) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. *, significantly different from control values using a one way ANOVA with a Dunnett's test, $P < 0.01$. Where error bars are not visible they are contained within the symbol.

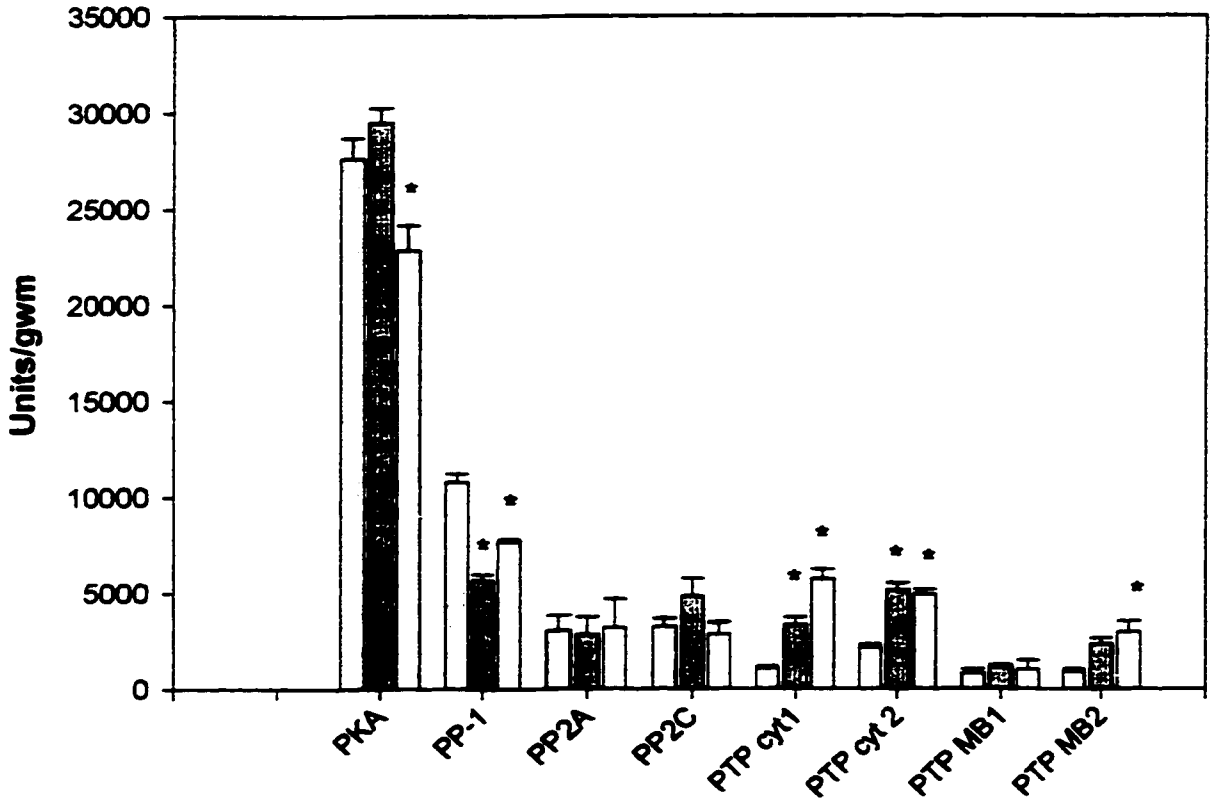
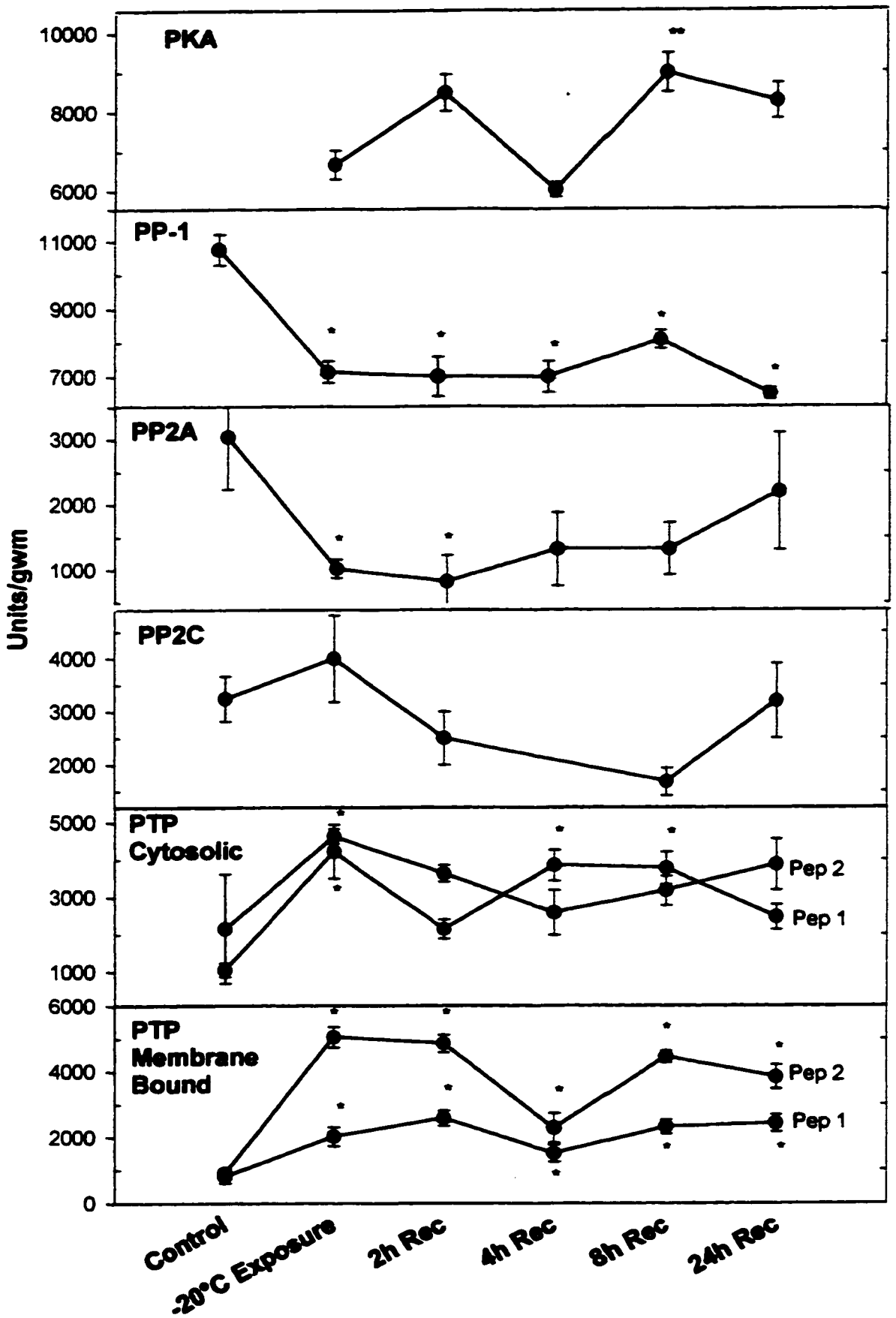

35000
30000
25000
20000
15000
10000
5000
0
Units/gwm
PKA
PP-1
PP2A
PP2C
PTP cyt1
PTP cyt 2
PTP MB1
PTP MB2

Figure 3.5 Protein Kinase and Phosphatase Activities for a Recovery from -20°C Exposure (Preceeded by a two Week Acclimation at 4°C) Time Course for *Eurosta solidaginis*. Results are mean (U/gwm) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. *, significantly different from control values using a one way ANOVA with a Dunnett's test, $P < 0.01$ and +, different from -20°C exposed, $P < 0.01$.

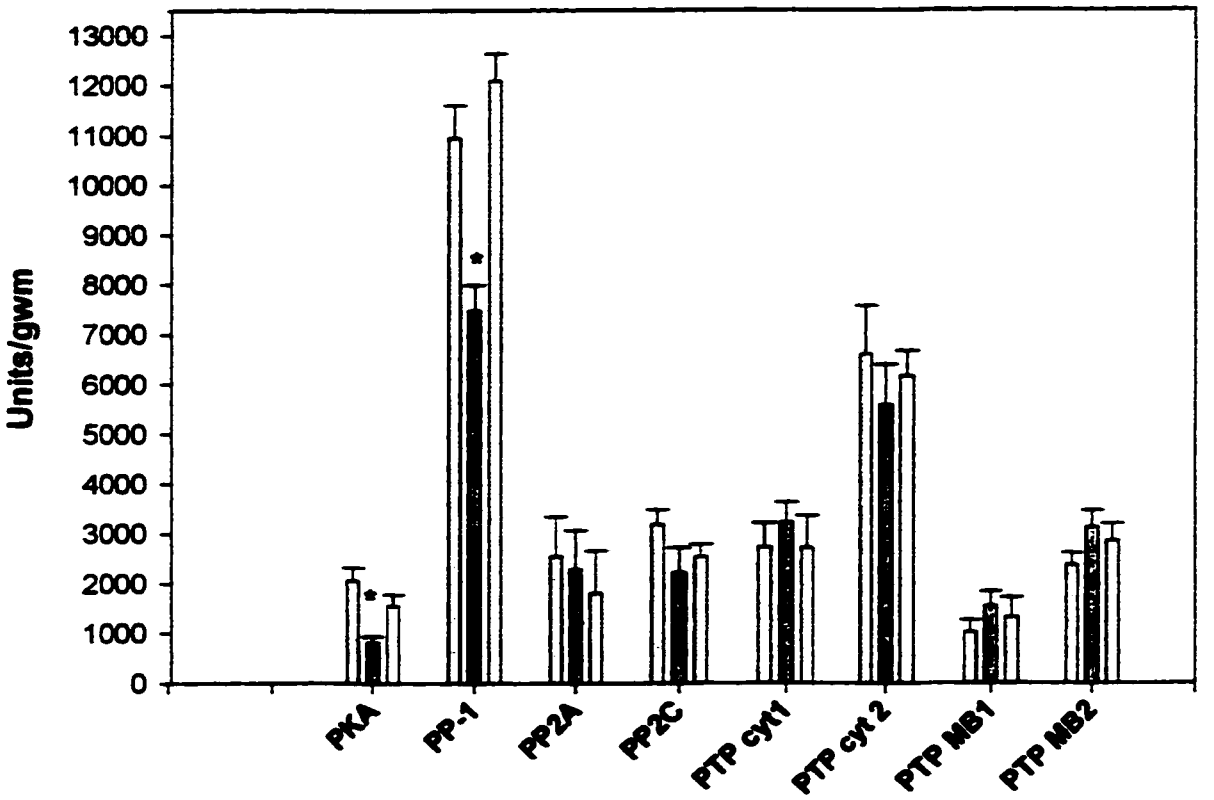

PKA
PP-1
PP2A
PP2C
PTP Cytosolic
PTP Membrane Bound
Pep 2
Pep 1
Pep 2
Pep 1
Units/gwm
10000
8000
6000
11000
9000
7000
3000
2000
1000
4000
3000
2000
5000
3000
1000
6000
4000
2000
0
Control
-20°C Exposure
2h Rec
4h Rec
8h Rec
24h Rec

section refer to active enzyme since total activities remained constant (data not shown). Cold exposure (2 weeks at 4°C followed by 24 h at -20°C) resulted in a strong decrease in PP1 activity which fell to 60% of the control value in the 24 h frozen larvae and remained suppressed over the subsequent 24 h course of recovery at 15°C. PP2A activity behaved similarly, decreasing to only 30% of the control value in the 24 h frozen sample, and again remained low during recovery (an apparent rise in PP2A activity after 24 h recovery was not significant due to high error values). PP2C activities did not change significantly during freezing or recovery. Protein tyrosine phosphatase activities were all elevated by freezing exposure and generally remained high over the 24 h recovery course. Cytosolic PTP activity with Pep 1 and Pep 2 increased 4 and 2.5 fold, respectively, after subzero exposure. Both were reduced somewhat after 2 h thawing but Pep 1 activity showed an increase again (over control values) at the 4 and 8 h recovery time points. Membrane bound PTP activity increased 5-fold when assayed with Pep 1 and 2-fold with Pep 2 during freezing and activities with both substrates remained elevated over the recovery course. Activities after 24 h of recovery were 3.8 and 2 fold higher than their respective control values. Protein kinase A activity was about 6500 U/gwm in frozen larvae and generally increased during the recovery period with a significant 30 % increase over the frozen value seen after 8 h recovery at 15°C.

Anoxia Exposure and Aerobic Recovery

The effects of anoxia exposure at 15°C on protein kinase and phosphatase activities in *Eurosta solidaginis* are shown in Fig. 3.6. Activities for PKA and PP1 again refer to active enzyme since total activities remained constant (data not shown). PKA was

Figure 3.6 Protein Kinase and Phosphatase Activities for an Anoxia-Recovery Cycle of *Eurosta solidaginis*. Bars from left to right in each group are; Aerobic control (15°C), 24 Anoxic (15°C) and 24 hour aerobic recovery (15°C). Results are mean (U/gwm) ± SEM, with at least n = 3 samples (1 or 2 larvae per sample) at each point. *, significantly different from control values using a one way ANOVA with a Dunnett's test, $P < 0.01$.


Units/gwm
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
PKA
PP-1
PP2A
PP2C
PTP cyt1
PTP cyt 2
PTP MB1
PTP MB2

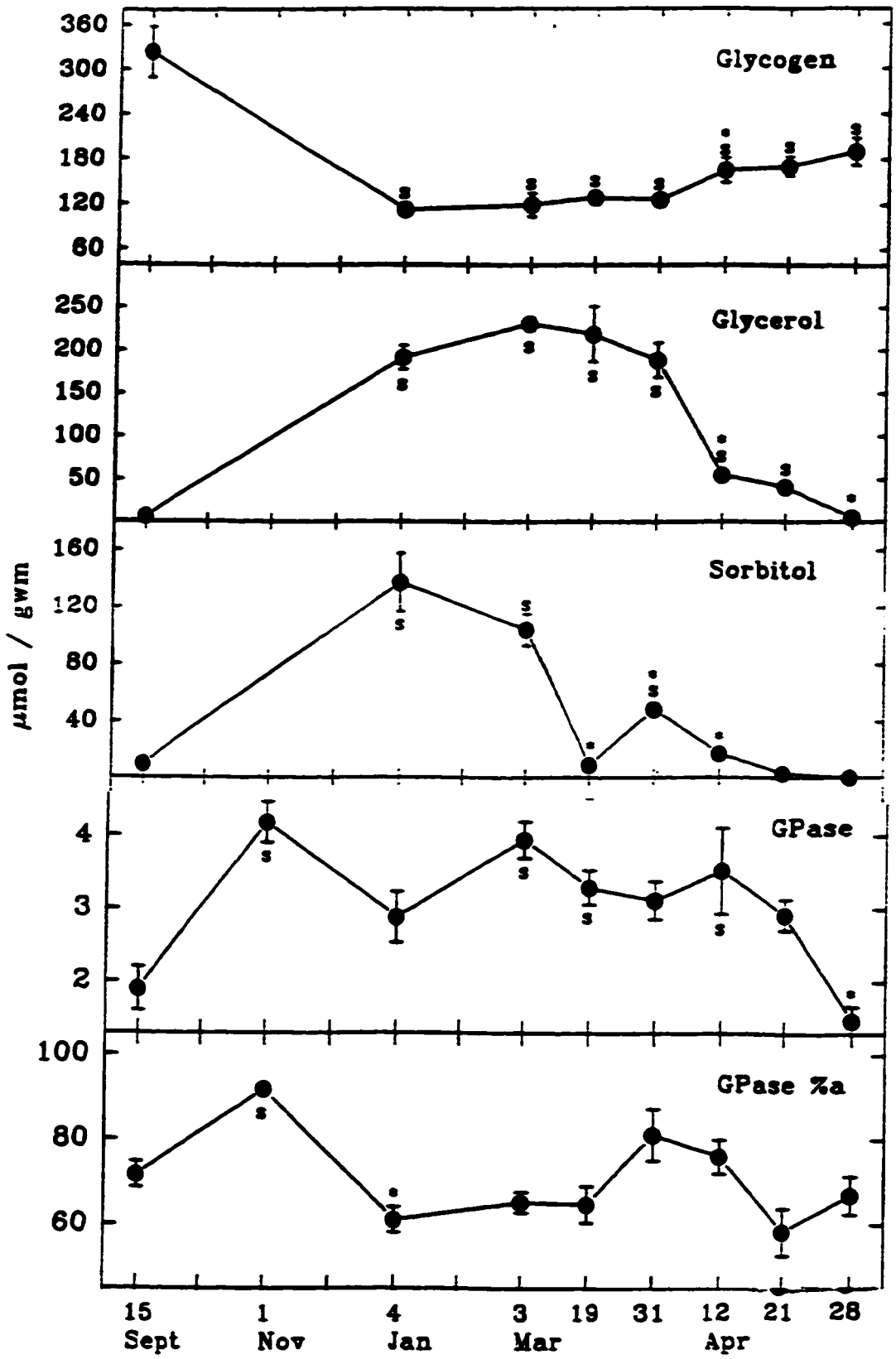
sharply reduced during anoxia, falling to 50 % of the control value but activity was restored after 24 h aerobic recovery. PP1 activity similarly decreased during anoxia to 80% of the control value but again was fully restored after the aerobic recovery period. PP2A and 2C and the PTPs were unaffected by anoxia and aerobic recovery

Discussion

Results seen for *Eurosta solidaginis* closely parallel those for *Epiblema scudderiana*. PKA activity tends to increase in cold-exposed insects, whereas PP1 decreases, which is consistent with the expected net stimulation of glycogen phosphorylase activity by cold exposure (figure 3.7). *Eurosta solidaginis* presents the added complexities that the role of sorbitol and the fact that the insect freezes at temperatures below -10°C must now also be considered. The fact that PP2C and certain PTP activities behave differently in *Eurosta solidaginis* than in *Epiblema scudderiana* may reflect their involvement in these additional roles.

Since no changes in the active forms of PKA and PP1 were observed in early winter or even January, this would seem to indicate that glycogen phosphorylase activity (and hence the production of glycerol and sorbitol) may be regulated solely by the inherent properties of PP1 which have been shown to be greatly reduced at low temperatures. In silkmoths, the activity of phosphorylase kinase showed a normal Q_{10} of about 2 with changing temperature, whereas the activity of phosphorylase phosphatase (PP1) was much more strongly suppressed at low temperature than would be expected (Hayakawa, 1985). So while it appears that the Vmax activities of PKA and PP1 don't change over the season, when assayed at 23.5°C, PP1 may have a higher Q_{10} value that suppresses its activity at low temperatures *in vivo*, thereby allowing for the net activation of glycogen phosphorylase in cold exposed insects. Such regulation may also provide a means to account for the two separate activations of glycogenolysis that are needed to produce first glycerol and then sorbitol. The effects of low temperature on purified PP1 are studied in Chapter 5.

Figure 3.7 Polyol Levels in Correspondence with Gycogen Phosphorylase a Activity in Overwintering *Eurosta solidaginis* (Joanisse and Storey, 1994b). Results are mean ± SEM for n=4 samples. ^{s}, significantly different from sept 15 values. *, significantly diferent from previous sampling date. Where error bars are not visible they are contained within the symbol.


Glycogen
Glycerol
Sorbitol
GPase
GPase %a
µmol / gwm
360
300
240
180
120
60
250
200
150
100
50
160
120
80
40
4
3
2
100
80
60
15 Sept
1 Nov
4 Jan
3 Mar
19
31
12 Apr
21
28

Unlike with *Epiblema scudderiana* no change in PP2C activity was seen either over the winter time course (Figure 3.2) or during cold exposures at -4 or -20°C (Figure 3.3 and 3.4). This likely indicates that PP2C is not acting in an osmosensing mechanism since its activity did not change despite the fact that accumulations of greater than 0.5–0.6 M glycerol and 0.2 M sorbitol occur (Morrissey and Baust, 1976). Furthermore, when the larvae are frozen, a large percentage (50-60%) of their body is water turned to ice, and hence the osmolality of remaining cellular fluids would rise 2-3 fold over a very short time. However, despite this, PP2C activities did not change. Thus, it may be possible that PP2C has a different role in freeze tolerant vs freeze avoiding insects. Further studies of this enzyme are still required to determine its exact role in cold tolerance.

The increase in membrane bound PTPs in November (Pep2) and January (Pep 1 and Pep 2) may implicate two separate groups of membrane bound PTPs in the regulation of freeze-survival mechanisms. As with *Epiblema scudderiana*, the PTPs may play a role in regulating cold shock responses (ribosomal protein synthesis and membrane fluidity adjustments).

In a short term subzero exposure PP1 activity decreased, which is consistent with the requirement for activating glycogen phosphorylase (figure 3.4). The fact that it did not increase after a 24 h recovery period at 15°C is consistent with the finding by Churchill and Storey (1989b), where glycerol production in another insect was continuous during several freeze thaw cycles. The decrease in PKA activity in the recovered sample is consistent with phosphorylase kinase no longer requiring activation and with decreased phosphorylation of PP1 inhibitor 1 thereby decreasing its inhibition.

This decrease may be due to the cessation of sorbitol production which would be activated by the -4°C exposure but turned off again when larvae were rewarmed to 15°C. Protein phosphatases 2A and 2C showed no change in activity and were thus apparently not involved in regulating any events over the course of the short term subzero exposure and recovery. This corresponds with the fact that neither of these enzymes was affected in the outdoor animals either. Cytosolic PTP activities acting on both pep1 and pep2 showed increases and remained elevated in the recovered samples. This differs from the pattern seen in the outdoor animals which also seemed to involve membrane bound PTPs in the stress response. So it would appear that cytosolic PTPs may be involved in short term mediation of cold exposure and membrane bound PTPs are required during a prolonged exposure. Membrane bound PTPs acting on Pep2 increased only during recovery; this may suggest that this group of enzymes has an active role to play in the recovery process.

A -20°C exposure preceded by a 2 week acclimation period at 4°C to allow for the accumulation of glycerol and sorbitol caused decreases in PP1 and PP2A activities and increases in all PTPs studied. As seen in the -4°C exposed insects, PP1 activity decreased and this probably facilitates the stimulation of glycogen phosphorylase activity to increase the production of glycerol and/or sorbitol. Since PP2A returned to control values within the first 4 h of recovery, this might link it to recovery. It has been shown that glycerol and sorbitol synthesis require longer periods (greater than 24 h for glycerol and 12-24 h for sorbitol) to subside (Churchill and Storey, 1989b: Storey and Storey, 1983). Again PP2C showed no changes in activity, consistent with all the other cold exposure studies in *Eurosta solidaginis*. It may be interesting to study the effects of a

higher acclimation temperature (above 5°C) so that the only cryoprotectant produced would be glycerol.

Anoxia exposure was studied to determine if it could mimic the ischemic event associated with the freezing of the insect's body fluids. Anoxia typically triggers a series of events, particularly a switch to glycolytic metabolism and frequently energy savings through metabolic arrest, that can also be beneficial to freezing survival. Hence, "normal" responses to anoxia may be utilized/modified to aid survival of the anoxia/ischemia stimulated by freezing. The only effects seen during anoxia exposure were decreases in both PKA and PP1 which returned to control values again after 48 h aerobic recovery. The reduced activities of both enzymes during anoxia may be part of an overall anoxia-induced metabolic rate depression. Since both PP1 and PKA activities decreased in anoxia whereas PP1 activity decreased during freezing and PKA increased, it would appear that the responses to the two stresses, anoxia versus freezing, are not the same. This suggests that the responses in the frozen insects may not be linked to energy conservation, and strengthens the argument for their role in regulating polyol production. Interestingly, PP1 activity in *Eurosta solidaginis* did not recover from the –20°C cold exposure over 24 h as it does in *Epiblema scudderiana,* which could be a difference between freeze-avoiding and freeze-tolerant species. The freeze tolerant species, perhaps not unexpectedly, would probably take longer to restore "normal" metabolism after an ischemic freezing episode than would a supercooled species.

As with *Epiblema scudderiana* (Chapter 2) cryoprotectant production in *Eurosta solidaginis* is likely regulated by a combination of increased PKA activity and decreased PP1 activity. Differential effects of low temperature on enzyme activity may also provide

a further means of regulating polyol production, and may also provide a means of differentially activating glycerol and sorbitol production. However, whereas differential temperature effects on these enzymes (and also on phosphorylase activity directly) may result in two separate activations of glycogen breakdown to support the synthesis of the two different polyols, controls on these enzymes cannot actually determine which polyol is synthesized. This determination must be made by regulating the other enzyme(s) that control the flow of glycolytic carbon into the specific pathways leading to either glycerol or sorbitol. The effects of cold temperature on PKA and PP1 are studied in chapters 4 and 5, respectively. Protein tyrosine phosphatases on the other hand show increases in activity, which will require further study to determine their exact role.

Chapter 4

Purification and Kinetic Characterization of Protein Kinase A Catalytic Subunit From *Epiblema scudderiana* and *Eurosta solidaginis*

Introduction

Protein kinase A has a very important role in the regulation of intermediary metabolism in cells. As the data in Chapters 2 and 3 show, PKA activity in *Epiblema scudderiana* and *Eurosta solidaginis* is modified on both a seasonal basis and in response to low temperature and/or freezing exposures. This suggests that the enzyme may be differentially regulated at high versus low temperatures. To further investigate the regulation of the enzyme, the catalytic subunit of PKA was purified from both insect species and its kinetic properties were analyzed at both high (23.5°C) and low (4°C) assay temperatures.

Temperature change affects enzyme function because it differentially affects the weak bond interactions that contribute to protein conformation, subunit interactions and substrate and effector binding. A decrease in temperature stabilizes hydrophilic bonds but destabilizes hydrophobic bonds (Hochachka and Somero, 1984). Thus, depending upon the relative contributions of hydrophobic and hydrophilic bonds to different processes, various parameters of PKA function (e.g. Km or I_{50} values as well as holoenzyme dissociation) may be significantly altered at high versus low temperatures and in ways that may be either positive or detrimental to enzyme function. Hence, the interaction of temperature with enzyme catalytic properties forms another mode by which enzyme activity can be regulated and another way in which enzyme function can be adapted to meet different cellular demands at high versus low environmental temperatures.

The presence of high concentrations of polyhydric alcohols, glycerol in *Epiblema scudderiana* and glycerol and sorbitol in *Eurosta solidaginis*, is one of the distinguishing features of these cold-hardy insect during the winter months. Polyols provide antifreeze

protection to the animals, maintaining the supercooled state in *Epiblema scudderiana* and preserving the liquid state of the cytoplasm during freezing in *Eurosta solidaginis*. However, polyols are also well known for their abilities to stabilize protein structure and counteract denaturing influences such as temperature extremes or dehydration. Hence, the polyols accumulated by these insects may also have important influences on the properties of cellular enzymes and affect their functions at low temperature. Therefore, for a comprehensive understanding of PKA function in these species and how it may change in summer versus winter seasons, it is necessary to evaluate the influences of both temperature and polyols on enzyme kinetic properties.

In this study a kinetic analysis of purified PKAc from *Epiblema scudderiana* and *Eurosta solidaginis* was undertaken at 5°C and 23.5°C to explore enzyme properties and the possible role of temperature in regulating PKAc. Kinetic studies included examining the effects cryoprotectants on the binding of substrate and holoenzyme dissociation in the presence of cAMP as well as the effects of various salts and known inhibitors of PKA.

Materials and Methods

Chemicals

The protein kinase A inhibitor peptide, PKAi (5-24), was obtained from Peninsula Labs and sucrose was from Fisher Scientific. Ampholines (pH 3.5-10) and all other chemicals were from Sigma Chemical Co. or from the suppliers designated in Chapter 2.

PKA Purification by Isoelectric Focusing (IEF)

PKA activity was assayed as described in Chapter 2. Supernatant from a 1:3 (w:v) homogenate of frozen larvae, collected in the autumn, was prepared as described in Chapter 2. The supernatant (6 ml) was loaded in the six middle fractions (not more than 1 ml per 4.6ml fraction) on an LKB 8100 IEF column containing carrier ampholytes (pH range 3.5-10.0) dispersed throughout a 0-30 % sucrose gradient (120 ml). The column was connected to a Hoffer power supply and run at 450 volts for 8-18 h (pH gradient and position of PKA peak did not change after 8 h) at 4°C. The column was then drained and 2 ml fractions were collected and assayed for activity; subsequently, the pH of each fraction was measured. Fractions that showed PKA activity in the presence of 1 μM cAMP in the assay (but not in its absence) represented the PKA holoenzyme and were pooled (6-10 ml). These were incubated for 1-2 h with 1 mM cAMP to dissociate the catalytic subunits and then run on a second IEF column as described above. Fractions were again collected and assayed for activity with and without 1 μM cAMP. Peak fractions from the second IEF that represented the catalytic subunit (activity the same with or without cAMP in the assay) were pooled and stored on ice or at 4°C until used for kinetic studies or concentrated and run on SDS-PAGE or reverse-phase HPLC.

Determination of Holoenzyme Molecular Weight with S-300 Gel Filtration

Fractions from the first IEF were pooled and concentrated against solid PEG 8000 to a volume of about 300 µl and then loaded onto a Sephacryl S-300 gel filtration column (1 cm x 45 cm) equilibrated with PEM buffer (as defined in chapter 2) containing 10 % (v:v) glycerol. Molecular weights of the holoenzyme were determined using S-300 gel filtration with known standards: phosphofructokinase (360 kDa); aldolase (150 kDa); hexokinase (100 kDa); ovalbumin (43 kDa); and cytochrome c (12.4 kDa). The standards were detected by protein determination (Bradford, 1976).

Reverse-Phase HPLC

Purity of the enzyme was assessed with a Zorbax RP-300 C-8 (2.1 x 150 mm) reverse phase column for a Beckman HPLC system with a UV/VIS detector at 225 nm. Peak fractions from Sephacryl S-300 were pooled and concentrated to a small volume (<100 µl) using Centricon-10 concentrators. Samples were loaded at 0% acetonitrile and 100% water containing 0.1% TFA and run for 10 min, followed by a linear gradient run to 100% acetonitrile and 10% water with 0.1% TFA over 70 min.

SDS-PAGE

Purified PKAc was concentrated using 10 kDa cut-off Centicons. SDS-PAGE was performed by the method of Laemmli (1970) with a 10% acrylamide separating gel and a 4.5% stacking gel. Gels were developed at 200 volts until the dye front just ran off the gel. Bio-Rad prestained Kaleidoscope standards were used to obtain a calibration curve: myosin (209 kDa), β-galactosidase (137 kDa), bovine serum albumin (84 kDa), carbonic anhydrase (44.3 kDa), soybean trypsin inhibitor (32.8 kDa), lysozyme (18.7 kDa), and aprotinin (7.2 kDa). The gel was then silver stained.

Kinetic Studies.

Kinetic studies of PKAc from both insects used pooled peak fractions collected from the second IEF column. All assays used 20 µl of pooled enzyme fractions and the optimal assay conditions outlined in Chapter 2. Kinetic studies were done at room temperature and at 4°C with assays incubated for 15 or 90 minutes, respectively. Enzyme activity was assayed with varying substrate concentrations of Kemptide or Mg-ATP (1:1 molar ratio using magnesium acetate, the concentration of Mg-ATP complex was determined using a computer program by Brooks, (1992b)) to determine K_m values with or without 1 M glycerol for *Epiblema scudderiana* or 1 M glycerol, 400 mM sorbitol, or a combination of 1 M glycerol + 400 mM sorbitol for *Eurosta solidaginis*. Inhibition studies determined I_{50} values for PKAi (5-24), and isoquinolinesulonyl protein kinase inhibitor peptides H7 and H89 and various salts: KCl, KBr, NaCl, NaBr, NaF, NH_4Cl, and $(NH_4)_2SO_4$. To determine the K_a cAMP for PKA holoenzyme (CCRR) dissociation, holoenzyme was obtained by Sephacryl S-300 gel filtration of crude supernatant. The enzyme was then incubated with varying concentrations of cAMP for 30 min followed by assay with and without cAMP to determine % active (i.e. % dissociation). Kinetic constants (K_m , I_{50}, and K_a) were calculated using a kinetics computer program (Brooks, 1992a).

Arrhenius Plots

Data for the Arrhenius plots (log v versus 1/temperature in Kelvin) were obtained by running blanks and assays at several temperatures between 2°C and 35°C using a temperature controlled water bath. All assays were run for 45 min. Fitting of lines and calculation of slopes was done by Sigma Plot version 3.0 (Jandel Scientific). Break point was determined and approximated to the nearest data point by visual inspection.

pH Curve

Optimal pH conditions were determined at room temperature and at 4°C, with and without polyols present. Stock solutions of assay buffer were adjusted to different pH values at room temperature using a pH meter and were rechecked immediately after the assay was run by blotting a 5µl sample onto narrow range pH paper. The pH in the 4°C assay was assumed to be the same as that measured at room temperature (KPi buffer).

Protein Determination

Protein was determined using the Coomassie blue dye binding assay and the Bio-Rad commercial reagent with bovine serum albumin as the standard (Bradford, 1976).

Statistical Analysis

All enzyme data are reported as mean ± SEM for at least n = 3. Statistical analysis used the Student's t-test.

Results

PKA Holoenzyme and Dissociation Studies

The PKA holoenzyme consists of two catalytic subunits bound to two regulatory subunits, which dissociate upon binding cAMP. Effects of temperature and the presence of polyols on the concentration of cAMP required to dissociate 50 % of the enzyme is summarized in Table 4.1. Using PKA holoenzyme from *Epiblema scudderiana* the K_a value for cAMP at 23.5°C was calculated to be 31.9 ± 4.3 μM without glycerol present. However, at 4°C the K_a value was much lower at 4.26 ± 0.94 μM, just 13 % of the value at 23.5°C. Glycerol had no effect on the K_a values.

A similar temperature effect on the cAMP was seen for the *Eurosta solidaginis* enzyme. Ka in the absence of polyols was 148 ± K_a 19 μM at 23.5°C but much lower (27.3 ± 2.2 μM) at 4°C, just 18 % of the value at 23.5°C. At 23.5°C the presence of added glycerol or glycerol + sorbitol did not affect K_a cAMP but incubations in the presence of 0.4 M sorbitol resulted in a 30 % higher K_a value than without polyols. At 4°C the addition of any of the polyols increased K_a; values were 2.1, 2.8 and 3.1 fold higher in the presence of 1 M glycerol, 0.4 M sorbitol and 1M glycerol + 0.4 M sorbitol, respectively, than in the absence of polyols

Isoforms

Figure 4.1a shows the results of isoelectric focusing of crude supernatant from *Epiblema scudderiana* larvae (autumn-collected) and Figure 4.2a shows the comparable result for *Eurosta solidaginis* extracts (autumn-collected). In both species, the PKA activity detected in crude extracts was primarily in the holoenzyme form; that is, assays

Table 4.1 Holoenzyme Dissociation: K_a values for cAMPof PKA from *Epiblema scudderiana* and *Eurosta solidaginis* at two temperatures.

Epiblema scudderiana	4°C	23.5°C
K_a cAMP (nM)	4.26 ± 0.94 x	31.9 ± 4.3
K_a cAMP with 1M Glycerol (nM)	4.24 ± 1.03 y	32.1 ± 7.6
Eurosta solidaginis	4°C	23.5°C
K_a cAMP (nM)	27.3 ± 2.2 x	148 ± 19
K_a cAMP with 1M Glycerol (nM)	59.4 ± 7.0 x,a	184 ± 14
K_a cAMP with 0.4M Sorbitol (nM)	76.5 ± 22.0 x,b	198 ± 8 b
K_a cAMP with Glyc & Sorb (nM)	83.4 ± 20.3 y,b	136 ± 7

Data are mean ± SEM for at least n = 3 trials. a, significantly different from K_a with no glycerol added at same temperature using the Student's t-test, P<0.025, b, P<0.01

x, significantly different from corresponding K_a value at 23.5°C, using a student t-test, P<0.025, y, P<0.01.

Figure 4.1 Isoelectric focusing of *Epibelma scudderiana* PKA Holoenzyme (A) and catalytic subunit after incubation with 1 mM cAMP (B) in a pH 3.5-10 gradient of ampholines. Solid circles show PKA activity in the presence of 1 μM cAMP in the final assay. Open circle represent PKA activity with no cAMP added in the final assay. The pH gradient is shown by open triangles. Values are relative to maximal peak.

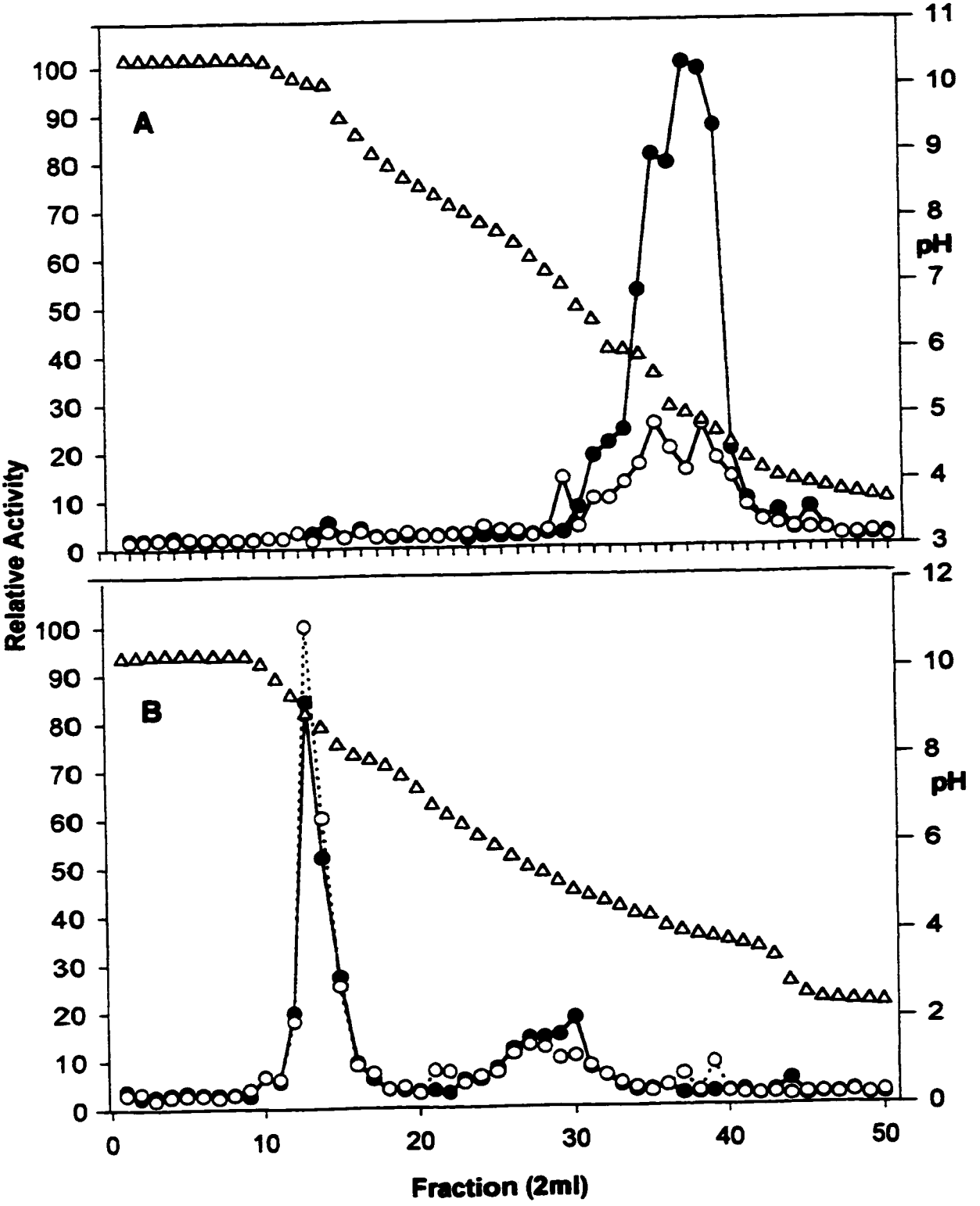
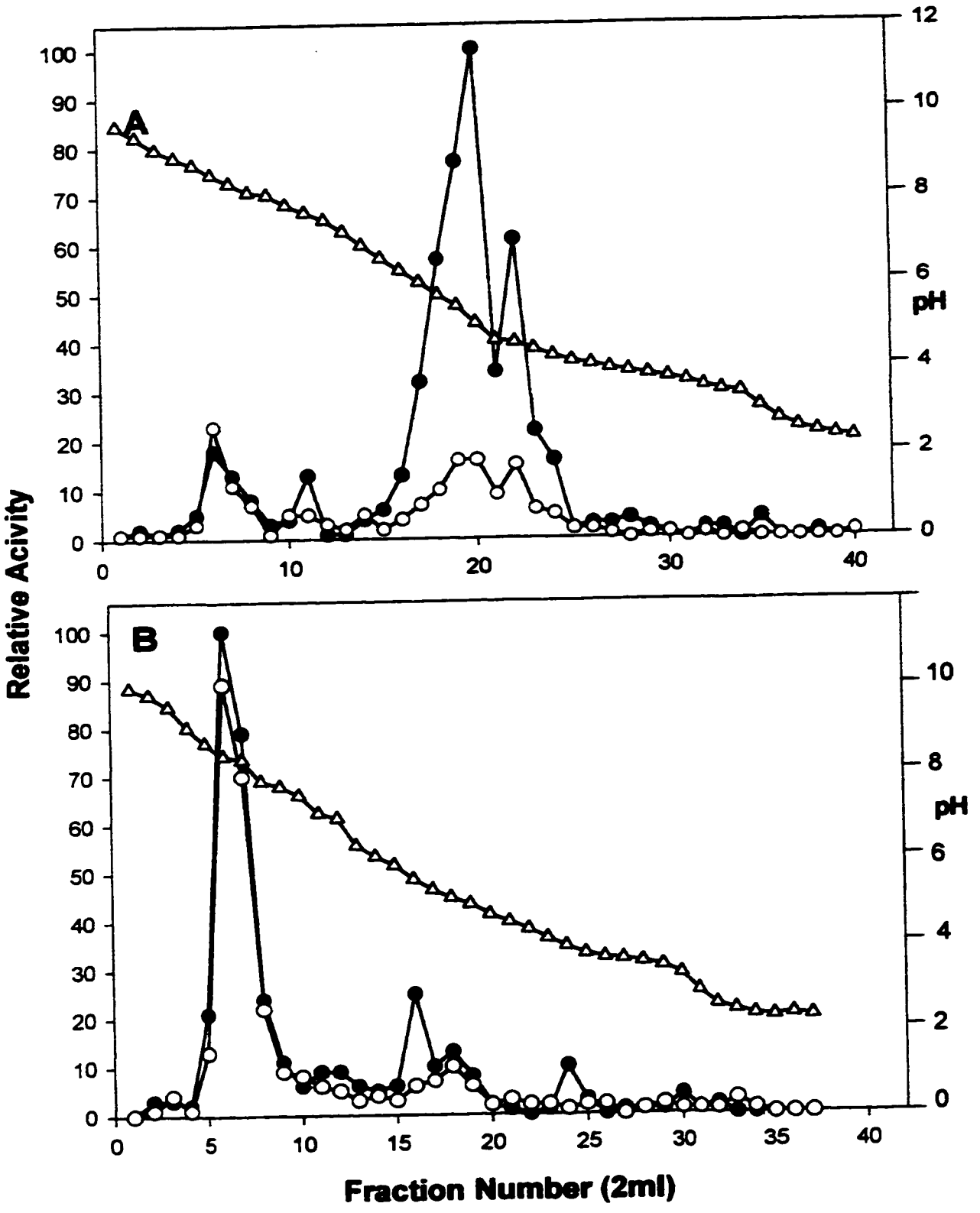

A
B
Relative Activity
pH
Fraction (2ml)
0
10
20
30
40
50
60
70
80
90
100
2
3
4
5
6
7
8
9
10
11
12

Figure 4.2 Isoelectric focusing of *Eurosta solidaginis* PKA Holoenzyme (A) and catalytic subunit after incubation with 1 mM cAMP (B) in a pH 3.5-10 gradient of ampholines. Solid circles show PKA activity in the presence of 1 μM cAMP in the final assay. Open circle represent PKA activity with no cAMP added in the final assay. The pH gradient is shown by open triangles. Values are relative to maximal peak.


A
B
Relative Acivity
pH
Fraction Number (2ml)
0
10
20
30
40
50
60
70
80
90
100
2
4
6
8
12
5
15
25
35

run ± 1 μM cAMP showed much greater activity in the presence of cAMP. In *Epiblema scudderiana* virtually no evidence of free catalytic subunit was seen in this first IEF run whereas in *Eurosta solidaginis* the majority of activity (>85%) was also found as the holoenzyme with only a small peak of catalytic subunit (activity the same with or without added cAMP in the assay) seen at a higher pH value. Both figures also showed two peaks of holoenyzme activity indicating that two isoforms of the enzyme were present in the larvae; these are probably composed of the two major forms of regulatory subunits that have been described in other systems. For *Epiblema scudderiana* the isoelectric points (pI) of the two peaks of PKA holoenzyme were pH 5.24 ± 0.13 (n=3) and pH 5.83 ± 0.14 (n=3) (Figure 4.1a). In *Eurosta solidaginis* the pI values for the two holoenzymes were pH 5.09 ± 0.33 and pH 5.49 ± 0.36 (n=3; Figure 4.2a). Fractions containing holoenzyme were combined and incubated with 1 mM cAMP to dissociate the enzyme and then the preparations were run on a second isoelectric focusing column to isolate the catalytic subunit. This time, catalytic subunit activity was primarily localized in single, sharp peaks at much higher pH values, 9.16 ± 0.30 (n=3) for *Epiblema scudderiana* (Figure 4.1b) and 8.93 ± 0.52 (n=3) for *Eurosta solidaginis* (Figure 4.2b). Only small peaks remained at the pH values where the holoenzyme had previously been found.

Purification of *Epiblema scudderiana* and *Eurosta solidaginis* PKAc

The dual isofocusing runs (Figure 4.1, 4.2) produced a highly purified catalytic subunit from each species that was used for further enzyme studies. Tables 4.2 and 4.3 summarize the purification; the enzymes from *Epiblema scudderiana* and *Eurosta*

Table 4.2 Purification of PKA Catalytic Subunit From *Epiblema scudderiana*

Results are from one purification: essentially equivalent results were obtained from subsequent preparations of purified enzyme. One unit (U) is defined as 1 pmol of phosphate transferred per minute at 23.5 °C.

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Supernatant	28 038	142	197	100	-
1st IEF	25 746	9.29	2 771	92	14
2nd IEF	21 986	0.296	74 277	78	377

Table 4.3 Purification of PKA Catalytic Subunit From *Eurosta solidaginis*. Results are from one purification: essentially equivalent results were obtained from subsequent preparations of purified enzyme. One unit (U) is defined as 1 pmol of phosphate transferred per minute at 23.5 °C.

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Supernatant	15 678	92	170	100	1
1st IEF	12 344	14.4	857	79	5
2nd IEF	7 612	0.102	74 627	49	438

solidaginis were purified 377- and 438-fold with yields of 78 and 49 %, respectively. The purification procedure was highly reproducible, with all trials yielding similar results. The final specific activities of purified PKAc assayed with Kemptide as the substrate were 74,277 U/mg for *Epiblema scudderiana* and 74,627 U/mg for *Eurosta solidaginis*. SDS-PAGE followed by silver staining of the purified enzymes showed single protein bands at 41 kDa for *Epiblema scudderiana* and 40 kDa for *Eurosta solidaginis* (Figures 4.3 and 4.4). The final specific activities were similar to those for the enzyme from other invertebrate sources (MacDonald, 1998). This compares with molecular weight of *Epiblema scudderiana* PKA holoenzyme determined to be 153 kDa by Sephacryl S-300 gel filtration and 181 kDa for *Eurosta solidaginis* holoenzyme (figure 4.5).

Reverse-phase HPLC of Insect and Porcine PKAc

Purity of the final PKAc preparations was also assessed using reverse-phase HPLC. The elution profiles showed that both insect preparations were highly pure with PKAc from *Epiblema scudderiana* eluting at 53.7 minutes and PKAc from *Eurosta solidaginis* eluting at 54.8 minutes (Figure 4.6). Commercial porcine PKAc from Sigma Chemical Co. was run for comparison and eluted at 53.7 minutes indicating that PKAc from both insects is very similar to the mammalian catalytic subunit.

Kinetic Characterization of Purified PKAc

Kinetic studies of purified PKAc from both insect species were completed at 23.5°C and 4°C to investigate temperature effects on enzyme function. Studies were also done in the presence versus absence of added polyols to assess possible influences of the

Figure 4.3. SDS-PAGE with silver staining of purified PKAc from *Epiblema scudderiana*. Lane 1, molecular weight (M.W.) markers ; lane 2, crude supernatant; lane 3, M.W. markers; lane 4, pooled fractions from 1st IEF; lane 5, M.W. markers; lane 6, Pooled fractions from 2nd IEF. The M.W. standards shown are: BSA (84 kDa), carbonic anhydrase (44.3 kDa), soybean trypsin inhibitor (32.8 kDa), and lysozyme (18.7 kDa).

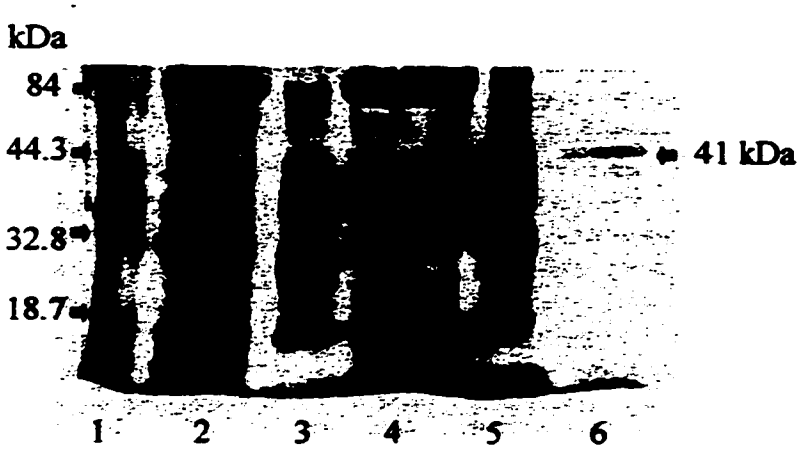

kDa
84
44.3
32.8
18.7
41 kDa
1
2
3
4
5
6

Figure 4.4. SDS-PAGE with silver staining of purified PKAc from *Eurosta solidaginis.* Lane 1, crude supernatant; lane 2, M.W. markers; lane 3, pooled fractions from 1st IEF; lane 5, M.W. markers; lane 7, Pooled fractions from 2nd IEF; lane 9, M.W. markers; lanes 4, 6, and 8 are empty. The M.W. standards shown are: BSA (84 kDa), carbonic anhydrase (44.3 kDa), soybean trypsin inhibitor (32.8 kDa), and lysozyme (18.7 kDa).

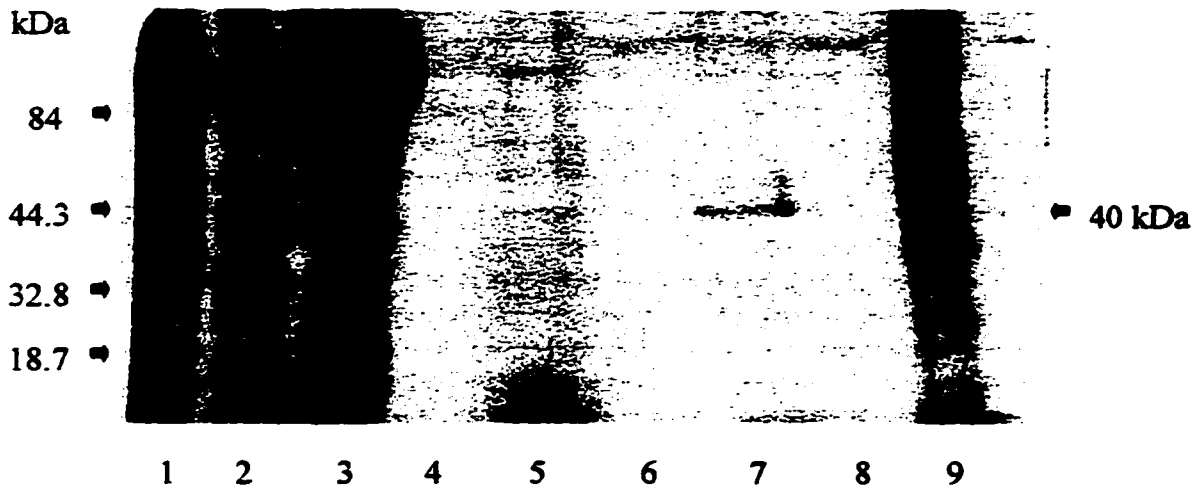

kDa
84
44.3
32.8
18.7
40 kDa
1
2
3
4
5
6
7
8
9

Figure 4.5 Standard Curve of Log Molecular Weight versus Elution Volume of Protein Standards for a Sephacryl S-300 Gel-Filtration Column. The log of the following molecular weight standards was plotted versus elution volume to determine the relative molecular weights of PKA holoenzyme isolated from *E. scudderiana* and *E. solidaginis.* The molecular weights of the standards were: 1) phosphofructokinase (360 000), 2) aldolase (150 000), 3) hexokinase (100 000), 4) ovalbumin (43 000), and 5) cytochrome c (12 400). Circle number 6 represents the position PKA holoenzyme from *E. scudderianna* was eluted and 7 represents *E. solidaginis holoenzyme.*

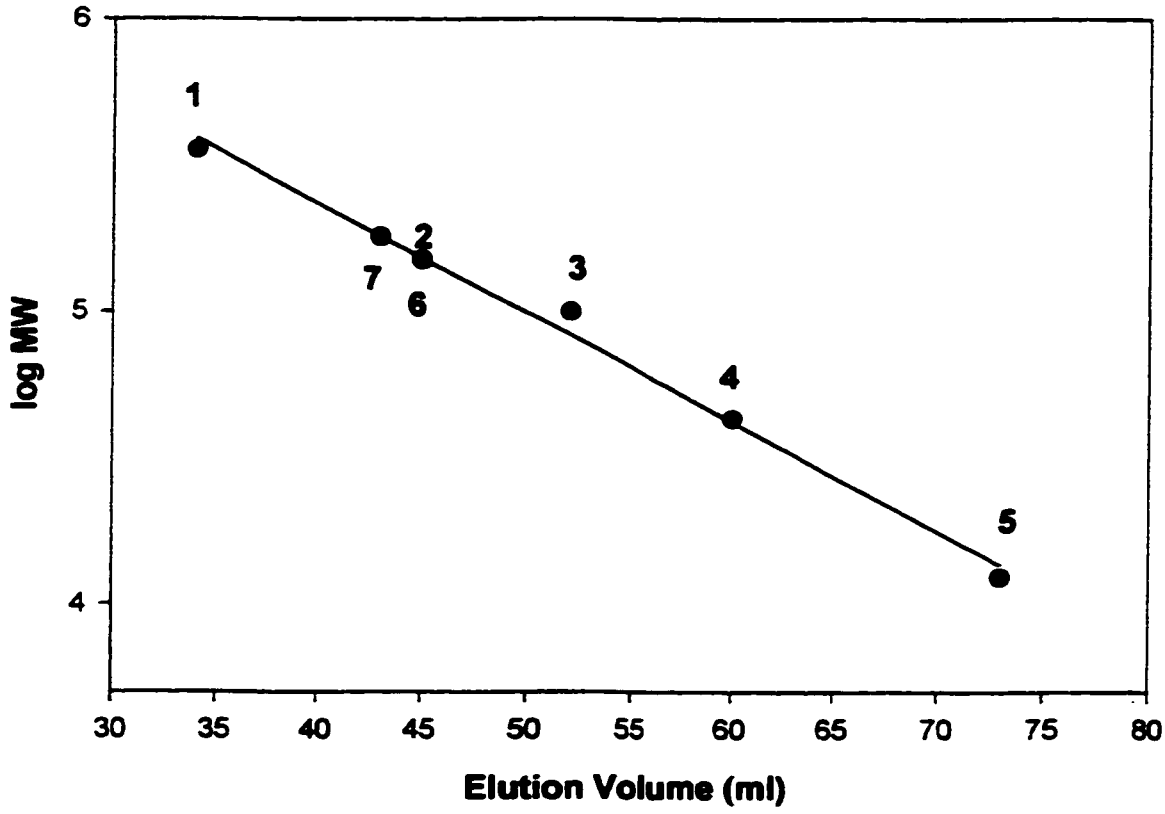
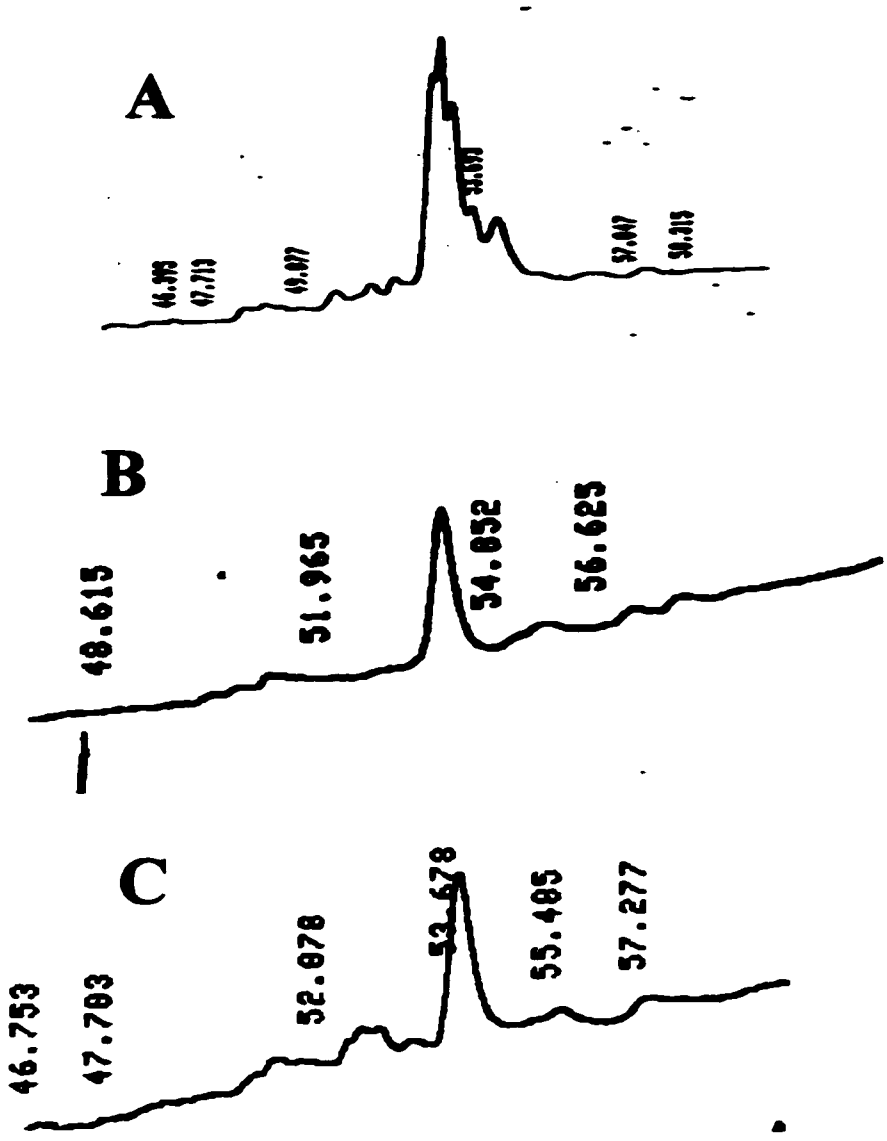

6
5
4
log MW
1
2
7
6
3
4
5
30
35
40
45
50
55
60
65
70
75
80
Elution Volume (ml)

Figure 4.6. Reverse-Phase HPLC Profiles of purified PKAc from (A) ***Epiblema scudderiana*****, (B)** ***Eurosta solidaginis*****, and (C) Porcine PKAc from Sigma Chemical Co.** Numbers represent elution times (min) from a 0-100% acetonitrile gradient started 10 min. after injection of the sample and reaching 100% acetonitrile at 80 min.


A
B
48.615
51.965
54.852
56.625
C
46.753
47.703
52.078
53.678
55.485
57.277

natural polyols on the enzymes. Substrate affinities of *Epiblema scudderiana* PKAc are shown in Table 4.4. Kemptide is a short peptide (LRRASLG) that represents the phosphorylation site in pyruvate kinase (Kemp et al., 1977) and is widely used as a substrate for studying PKAc. At 23.5°C the K_m for Kemptide of *Epiblema scudderiana* PKAc was 38.1 ± 4.9 μM which was 2.2-fold higher than the value at 4°C (17.3 ± 1.6 μM). The addition of glycerol (1 M) to the assay had no effect on Km Kemptide at 23.5°C, but lowered the Km value at 4°C by 41 %. The Km for Mg-ATP was about 60 μM at both temperatures and was not influenced by glycerol.

Inhibition studies of *Epiblema scudderiana* PKAc are shown in Table 4.5. The effects of three known peptide inhibitors of PKAc were tested and the enzyme from both insect species was sensitive to these compounds. Assays were performed with substrate concentrations as in Chapter 2 and I_{50} values show the concentration of inhibitor that decreased enzyme activity by one-half. Inhibition of *Epiblema scudderiana* PKAc by all three inhibitor peptides was unaffected by assay temperature and the I_{50} value for PKAi (5-24) was also unaffected by the addition of 1 M glycerol. *Epiblema scudderiana* PKAc was also inhibited by various salts. Sodium fluoride was the strongest inhibitor; inhibition was primarily due to the fluoride anion as other sodium salts showed I_{50} values up to 10-fold higher. Chloride and bromide salts showed similar levels of inhibition but sulfate was more inhibitory than chloride. Among cations, ammonium was the most inhibitory and potassium the least. Overall, the rank order of inhibition by salts at 23.5°C was: $NaF>(NH_4)_2SO_4>NaCl>NH_4Cl>KBr\sim NaBr\sim KCl$. At 4°C the order was slightly different: $NaF>(NH_4)_2SO_4>NH_4Cl>NaBr\sim KBr\sim NaCl>KCl$.

Table 4.4 Michaelis-Menton Constants for Protein Kinase A Catalytic subunit isolated from *Epiblema scudderiana.*

	4°C	23.5°C
Km Kemptide (μM)	17.3 ± 1.6 b	38.1 ± 4.9
Km Kemptide with 1M Glycerol (μM)	10.2 ± 1.0 a, b	31.4 ± 2.7
Km Mg-ATP (μM)	57.2 ± 5.2	61.1 ± 6.9
Km Mg-ATP with 1M Glycerol (μM)	58.9 ± 2.7	56.7 ± 2.7

Data are mean ± SEM for at least n = 3 trials. a, significantly different from Km with no glycerol added at same temperature, $P<0.025$. b, significantly different from value at 23.5°C, using the Student's t-test, $P<0.05$.

Table 4.5 Inhibition Constants for Protein Kinase A Catalytic subunit isolated from *Epiblema scudderiana.*

	4°C	23.5°C
I_{50} PKA_i (nM)	7.83 ± 1.67	7.98 ± 3.80
I_{50} PKA_i with 1M Glycerol (nM)	14.03 ± 3.29	21.0 ± 10.4
I_{50} H7 (nM)	9.25 ± 1.67	9.56 ± 0.27
I_{50} H89 (μM)	1.81 ± 0.59	0.87 ± 0.30
I_{50} KCl (mM)	441 ± 83	500 ± 22
I_{50} KBr (mM)	371 ± 18 a	480 ± 37
I_{50} NaCl (mM)	373 ± 43	284± 49
I_{50} NaBr (mM)	368 ± 43	482 ± 4
I_{50} NaF (mM)	36 ± 8	97 ± 31
I_{50} NH_4Cl (mM)	196 ± 49 a	348 ± 13
I_{50} $(NH_4)_2SO_4$ (mM)	137 ± 23	156 ± 23

Data are mean ± SEM for at least n = 3 trials. a, significantly different from value at 23.5°C, using a Student's t-test, $P<0.05$.

For *Eurosta solidaginis* the Km for Kemptide in the absence of glycerol was 3.67 ± 0.11 μM at 23.5°C and 60% higher (6.01 ± 0.66) at 4°C (Table 4.6). Effects of 1 M glycerol on the enzyme were also tested as well as sorbitol (which accumulates *in vivo* to ~40 % of the concentration of glycerol) and a glycerol/sorbitol mixture which represents the natural cryoprotectant situation in this species in midwinter. The addition of 1 M glycerol had no effect on the Km for Kemptide at 23.5°C but in the presence of 0.4 M sorbitol the Km rose by 2-fold whereas the mixture of 1 M glycerol and 0.4 M sorbitol decreased Km to 63 % of the value in the absence of polyols. At 4°C the influence of polyols was different and lowered Km in all cases. Addition of glycerol reduced Km Kemptide to 38 % of the value without polyols whereas with sorbitol or glycerol + sorbitol, Km was decreased to 69 % and 86 % of the value without polyols. The Km for Mg-ATP of purified *Eurosta solidaginis* PKAc was 30.7 ± 7.8 μM at 23.5°C. Neither temperature change nor addition of glycerol or sorbitol alone altered this value. However, in the presence of glycerol + sorbitol, the Km Mg-ATP at 4°C was 50 % higher than the value without polyols and 70 % higher than the corresponding value at 23.5°C.

Inhibition studies of PKAc purified from *Eurosta solidaginis* are shown in Table 4.7. Again, the I_{50} values for the peptide inhibitors PKAi (5-24), H7 and H89 were unaffected by temperature change, although enzyme sensitivity to both H7 and H89 inhibitors was substantially different when compared to *Epiblema scudderiana*. As also occurred for *Epiblema scudderiana* PKAc, NaF was by far the strongest salt inhibitor. Others salts showed I_{50} values that ranged from 125-350 mM, high values that suggest that inhibition by ions would be relatively unimportant for enzyme control *in vivo*. The

Table 4.6 Michaelis-Menton Constants for Protein Kinase A Catalytic Subunit Isolated from *Eurosta solidaginis*.

	4°C	23.5°C
Km Kemptide (μM)	6.01 ± 0.66 x	3.67 ± 0.11
Km Kemptide with 1M Glycerol (μM)	2.29 ± 0.41 b	2.76 ± 0.43
Km Kemptide with 0.4M Sorbitol (μM)	4.16 ± 0.62 y	7.48 ± 1.18 a
Km Kemptide with glyc & Sorb (μM)	5.20 ± 1.17 y	2.33 ± 0.38 a
Km Mg-ATP (μM)	30.7 ± 7.8	30.7 ± 4.1
Km Mg-ATP with 1M Glycerol (μM)	32.4 ± 4.8	28.7 ± 7.3
Km Mg-ATP with 0.4M Sorbitol (μM)	30.7 ± 3.1	36.0 ± 2.7
Km Mg-ATP with Glyc & Sorb (μM)	47.0 ± 4.0 x	26.25 ± 3.0

Data are mean ± SEM for at least n = 3 trials. a, significantly different from Km with no polyol added at same temperature, $P<0.05$, b, $P<0.01$. x, significantly different from value at 23.5°C, using the Student's t-test, $P<0.025$, y, $P<0.05$.

Table 4.7 Inhibition Constants for Protein Kinase A Catalytic subunit isolated from *Eurosta solidaginis*.

	4°C	23.5°C
I_{50} PKA_i 5-24 (nM)	8.32 ± 0.55	8.85± 0.34
I_{50} H7 (μM)	3.52 ± 0.55	5.59 ± 1.08
I_{50} H89 (nM)	111 ± 22	112 ± 6
I_{50} KCl (mM)	259 ± 29	346 ± 51
I_{50} KBr (mM)	248 ± 46	241 ± 21
I_{50} NaCl (mM)	261 ± 9	304 ± 43
I_{50} NaBr (mM)	188 ± 37	291 ± 35
I_{50} NaF (mM)	16 ± 4 a	55 ± 12
I_{50} NH_4Cl (mM)	341 ± 57	206 ± 12
I_{50} $(NH_4)_2SO_4$ (mM)	138 ± 19	127 ± 11

Data are mean ± SEM for at least n = 3 trials. a, significantly different from value at 23.5°C, using the Student's t-test, $P<0.05$.

relative order of inhibition by salts at 23.5°C was: NaF>$(NH_4)_2SO_4$>NH_4Cl>KBr>NaBr~ NaCl>KCl. At 4°C the order was: NaF>$(NH_4)_2SO_4$>NaBr>KBr>KCl>NaCl>NH_4Cl.

Arrhenius Plots of PKAc Purified from *E. scudderiana* and *E. solidaginis*

The effect of temperature on the maximum velocity (V_{max}) of purified PKAc from *Epiblema scudderiana*, in the presence and absence of 1 M glycerol, is shown as an Arrhenius plot in Figure 4.7. Activation energies were calculated using the slope of the plot of 1/temperature (Kelvin) versus log velocity and applying the Arrhenius equation.

$$E_a = -(\text{slope})(2.3\ R)$$

Both plots showed two distinct linear segments with a change in the slope occurring at 10°C. Activation energies were calculated for each linear segment. In the absence of glycerol, mean E_a values were 39.4 ± 3.2 kJ/mol for temperatures >10°C and 122 ± 15 kJ/mol for temperatures <10°C. In the presence of 1 M glycerol, the activation energies were 53.1 ± 4.3 kJ/mol for temperatures >10°C and much higher, 215 ± 4 kJ/mol, for temperatures <10°C.

The Arrhenius plots in Figure 4.8 show the comparable effects of temperature, in the presence and absence of polyols, on the V_{max} on PKAc purified from *Eurosta solidaginis*. All plots showed two linear segments. The plots without polyol added, with 1 M glycerol added and with a mixture of 1 M glycerol + 0.4 M sorbitol, showed breakpoints at 10°C whereas the plot for assays with 0.4 M sorbitol showed a break at 6°C. The activation energies for plots where no polyols were added were 37.3± 2.0 kJ/mol for temperatures above 10°C and 82.6 ± 5.7 kJ/mol for temperatures below 10°C.

Figure 4.7. Arrhenius Plot For *Epiblema scudderiana* PKAc activity versus temperature. Activity was measured as described in chapter 2. Open squares represent activity in the absence of glycerol and solid circles in the presence of glycerol. pH was set to 7.5 at 23.5°C and then allowed to self-adjust with changing temperature. Activity was assayed over a range from 2°C to 40°C. The assay mixtures were incubated for 15 min to allow for temperature eqilibration prior to starting the assay the radiolabeled substrate mixture. Data are means ± SEM for n = 3 samples. Error bars not visible are contained within the symbols.

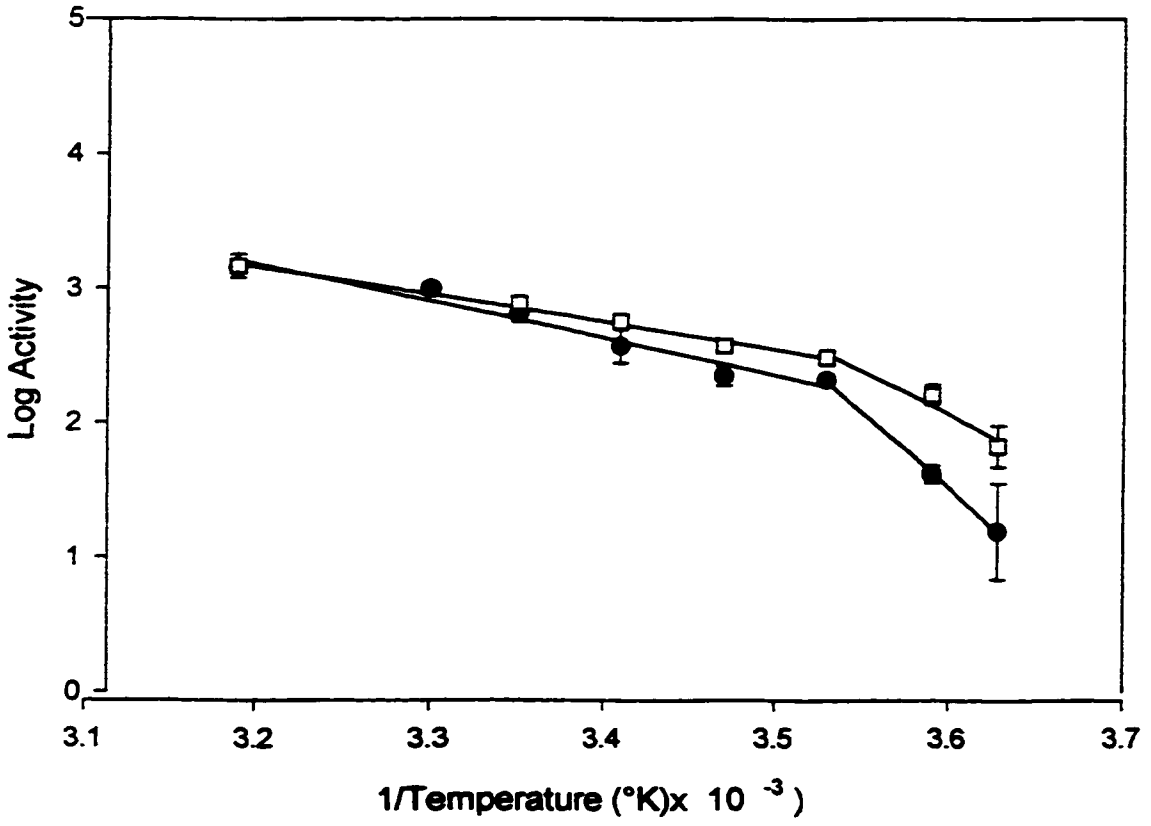
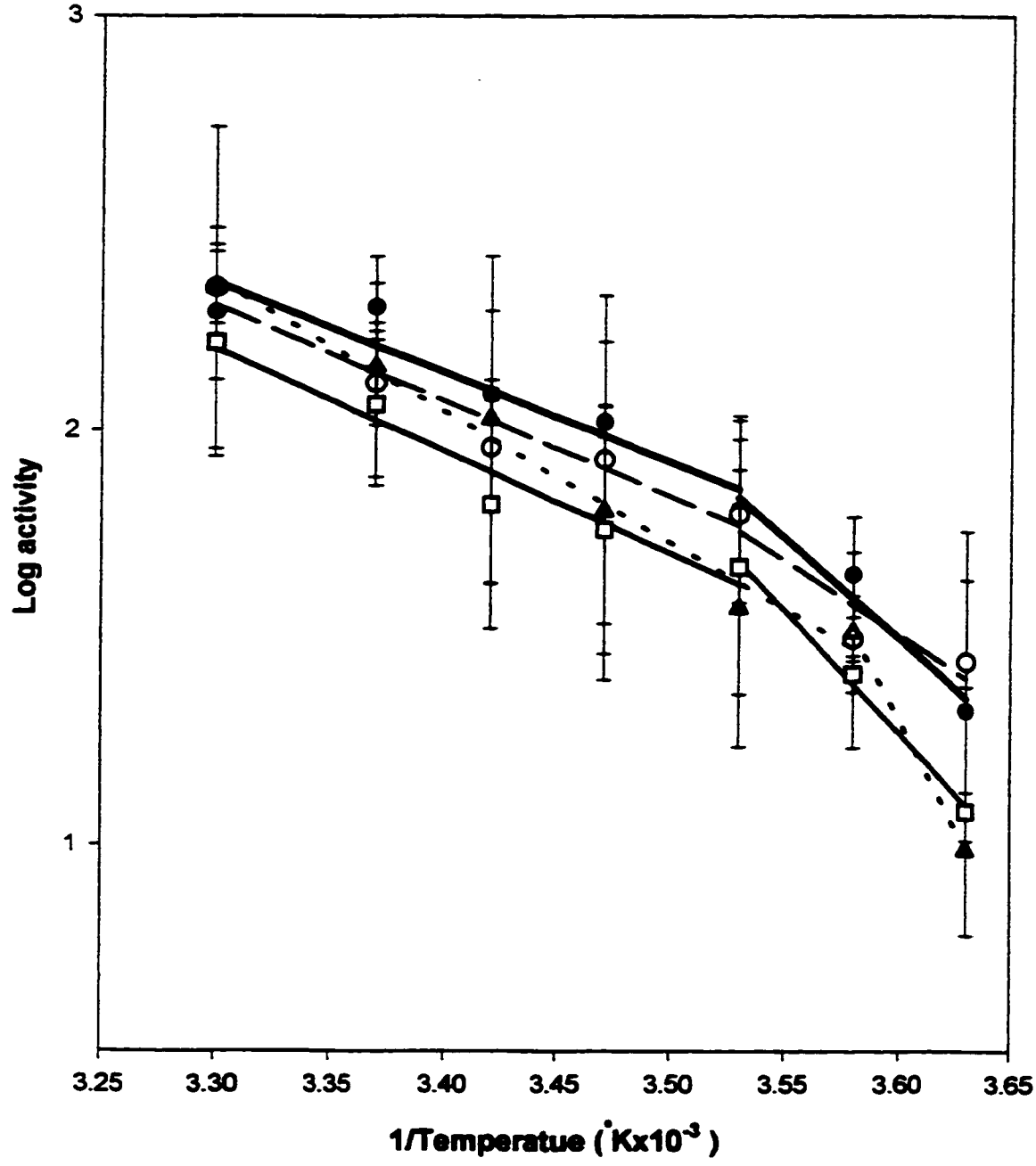

5
4
3
2
1
0
Log Activity
3.1
3.2
3.3
3.4
3.5
3.6
3.7
1/Temperature (°K)x 10 -3)

Figure 4.8. Arrhenius Plot For *Eurosta solidaginis* PKAc activity versus temperature. Activity was measured as described in chapter 2. Solid circles represent activity in the absence of polyols and open circles in the presence of 1 M glycerol, open triangles in the presence of 0.4 M sorbitol, and open squares in the presence of 1 M glycerol and 0.4 M sorbitol. pH was set to 7.5 at 23.5°C and then allowed to self adjust with changing temperature. Activity was assayed over a range from 2°C to 40°C. The assay mixtures were incubated for 15 min to allow for temperature equilibration prior to starting the assay the radio labeled substrate mixture. Data are means ± SEM for n = 3 samples. Error bars not visible are contained within the symbols.


3
2
1
Log activity
3.25
3.30
3.35
3.40
3.45
3.50
3.55
3.60
3.65
1/Temperatue (°Kx10-3)

In the presence of 1 M glycerol the corresponding activation energies were 43.9 ±5.0 kJ/mol and 66.1 ± 10.4 kJ/mol, respectively, and with both polyols the values were 47.0 ± 8.2 kJ/mol and 109 ± 38 kJ/mol. In the presence of 0.4 M sorbitol the activation energy rose from 60.1 ± 9.3 kJ/mol for temperatures >6°C to 193 ±17 kJ/mol at <6°.

pH Profiles of PKAc Purified from *E. scudderiana* and *E. solidaginis*

PKAc from *Epiblema scudderiana* showed optimal activity at pH 7.5 at 23.5°C with high activity between pH 7 and 8 (Figure 4.9). The addition of 1 M glycerol to assays had no effect on the profile. At 4°C the pH optimum in the absence of glycerol shifted to a higher value, pH 8 (although near-maximal activity was still maintained at pH 7.5) but in the presence of 1 M glycerol the pH optimum appeared to stay at pH 7.5.

The pH optimum of PKAc from *Eurosta solidaginis* was 7.0 at 23.5°C in the absence of polyols and stayed the same in the presence of 1 M glycerol and a mixture of 1 M glycerol + 0.4 M sorbitol (Figure 4.10). However, the addition of 0.4 M sorbitol shifted the pH optimum to pH 7.5. At 4°C the pH optimum was about pH 7.5 in all cases although near maximal activity was retained over a broad range from about pH 6.5 to pH 8.

Figure 4.9 pH curve of purified PKAc isolated from *Epiblema scudderiana*. Square symbols are at 23.5 °C and circles are at 4°C. Open symbols are without glycerol and solid symbols contain 1 M glycerol in the final assay mixture. Values are mean activity ± SEM (n = 3), relative to maximal activity at 23.5°C in the absence of glycerol. Where error bars are not visible they are contained within the symbols.

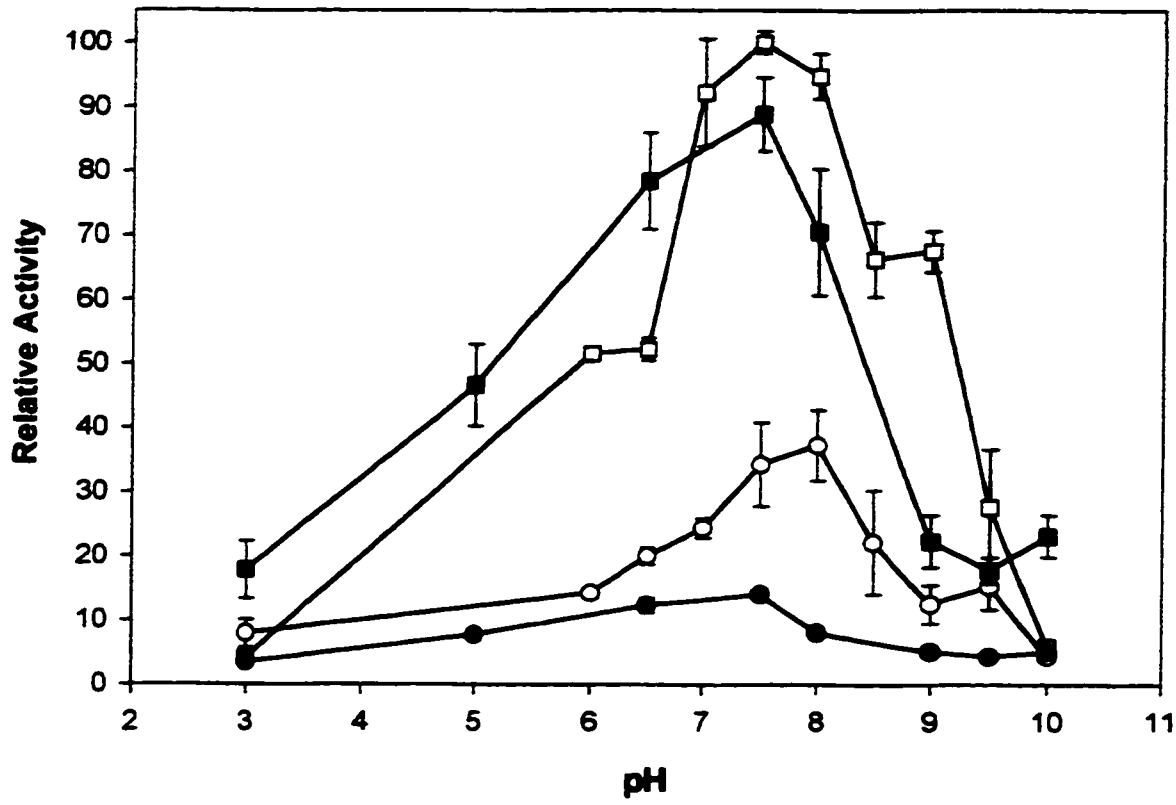
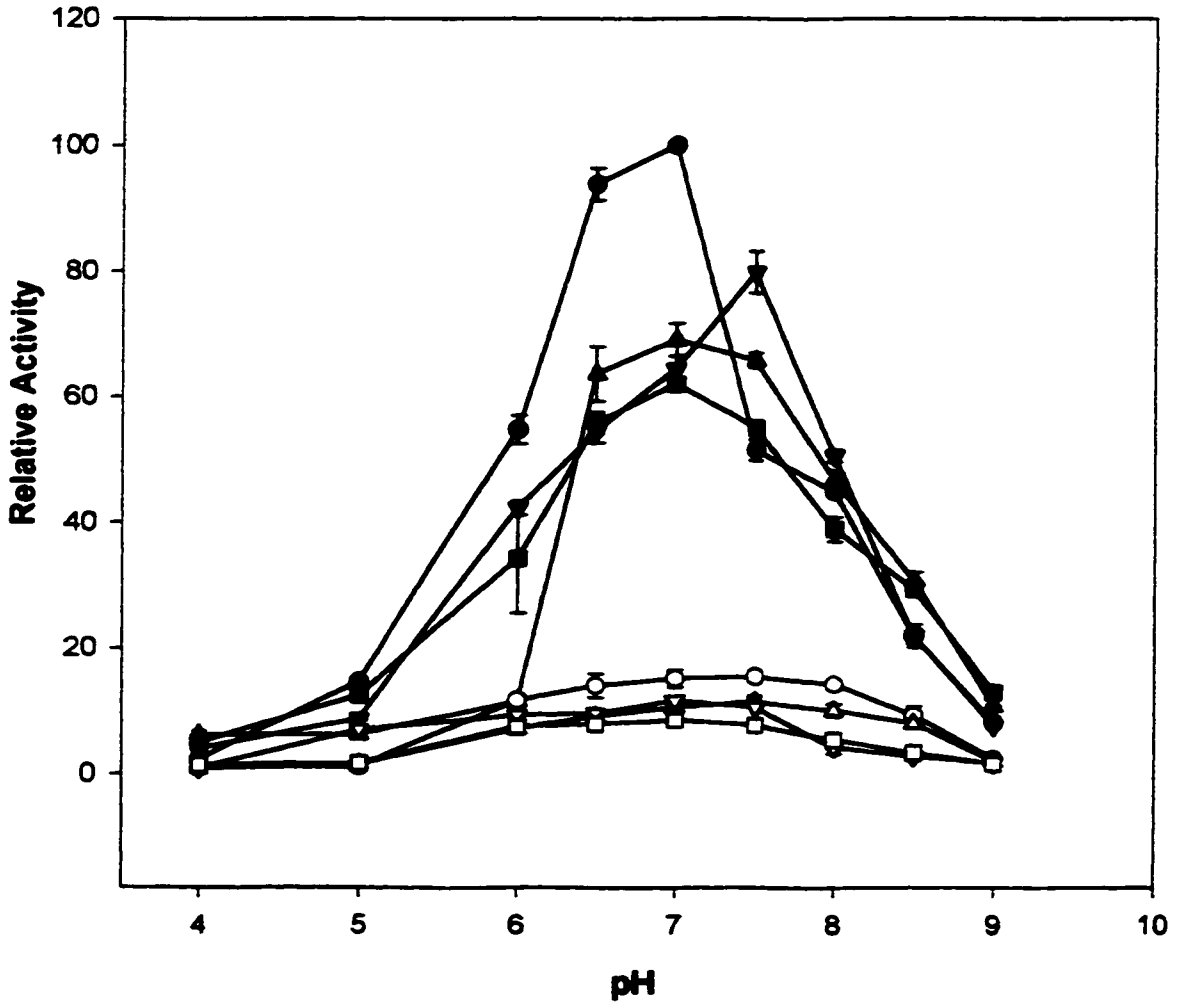

Relative Activity
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
11
pH

Figure 4.10 pH curve of purified PKAc isolated from *Eurosta solidaginis*. Solid symbols are at 23.5 °C and open symbols are at 4°C. Circles are without polyols, triangles up are in the presence of 1 M glycerol, triangles down are in the presence of 0.4 M sorbitol, and squares are in the presence of both 0.4 M sorbitol and 1 M glycerol. Values are ± SEM (n = 3), relative to maximal activity at 23.5°C in the absence of polyols. Where error bars are not visible they are contained within the symbols.


120
100
80
60
40
20
0
Relative Activity
4
5
6
7
8
9
10
pH

Discussion

Since cAMP dependent protein kinase (PKA) has been shown to be involved in the regulation of glycerol and sorbitol production (see Figure 1.3), key components in cold hardiness strategies of *Epiblema scudderiana* and *Eurosta solidaginis*, the regulation and function of the enzyme at high and low temperatures may be vital to the survival of these insects. Therefore, the present study was undertaken to determine whether enzyme kinetic or regulatory properties were influenced by cold temperature or by the presence of polyols. Furthermore, the study attempts to determine whether there are any differences in the kinetic properties between PKAc from freeze avoiding and freeze tolerant insects.

The purification scheme developed for the PKA catalytic subunit took advantage of the large difference in pI of the PKA holoenzyme (~ pH 5) and its catalytic subunit (~pH 8-9) in both insect species. Approximately 400-fold purification of the enzyme was obtained for both insects, resulting in final specific activities of ~74,000-75,000 U/mg protein at 23.5°C. In both cases the enzyme was judged to be homogeneous by SDS-PAGE. Reverse-phase HPLC also showed a single peak for the purified enzyme from both insects, with PKAc in both cases eluting at the same (*Epiblema*) or nearly the same time (*Eurosta*) as commercial porcine PKAc. This suggests that PKAc from both insects is very similar to the mammalian enzyme. Other authors have also shown that the catalytic subunit is highly conserved throughout the animal kingdom.

The molecular weight of PKAc, as determined by SDS-PAGE, was 41 kDa for *Epiblema scudderiana* and 40 kDa for *Eurosta solidaginis*. These are similar to values for the catalytic subunit from other sources, both mammalian (Taylor *et al.*, 1990; Olsen

and Uhler, 1989; Kinzel *et al.*, 1987) and insect (Haracska and Udvardy, 1992). Two major isoforms of PKAc, α and β, occur in mammals but are not easily separated by SDS-PAGE due small differences (<1 kDa) in their molecular weights (Gamm *et al.*, 1996; Olsen and Uhler, 1989). However, they are distinguishable by their relatively large difference in isoelectric points. For example, the two PKAc isoforms from bovine cardiac muscle show pI values of 7.5 for Cα and 8.0 for Cβ (Table 4.8; Kinzel *et al.*, 1987). Hence, the occurrence of just a single sharp peak of PKAc on IEF in each species (Figure 4.1b, 4.2b) suggests that only a single isoform of the catalytic subunit is present in each insect, at least during the overwintering (fall) larval stage.

The PKA holoenzyme is made up of two catalytic (C) and two regulatory (R) subunits, typically of about 40 kD and 50-55 kD, respectively, so that the molecular weight of the holoenzyme is about 180-190 kD (Foss *et al.*, 1994). S-300 gel filtration of the *Eurosta solidaginis* holoenzyme gave a molecular weight of 181 kDa, which agrees closely with the enzyme from other species. However, the value determined for *Epiblema scudderiana* PKA holoenzyme was smaller at 153 kDa which may suggest that the regulatory subunits are considerably smaller in this species (~35 kDa each) although further studies would be needed to confirm.

Mammalian Cα and Cβ subunits of PKA have been characterized and revealed relatively small differences in kinetic properties between the isoforms (Table 4.8; Gamm *et al.*, 1996). The kinetic properties of PKAc from the two insects showed similarities to each other and were also generally similar to the properties that have been reported for the catalytic subunit from other sources. The Km for ATP was about 60 μM in *Epiblema scudderiana* and about 50 % lower (30 μM) in *Eurosta solidaginis* but both values are

Table 4.8 Kinetic Properties of PKA Catalytic Subunits Cα and Cβ. Data is a summary of results from a study by D.M. Gamm, E.J. Baude and M.D. Uhler (1996).

	Cα	Cβ
Km Kemptide (μM)	14.0 ± 4.1	33.5 ± 12.7
Km Mg-ATP (μM)	41.7 ± 16.1	44.7 ± 9.9
I_{50} PKAi (5-24) (nM)	4.8 ± 0.8	12.7 ± 3.1
Ka (cAMP)* (nM)	63 ± 13	13 ± 8
pI	7.5	8.0

* Holoenzyme dissociation Constant is only for Holoenzymes containing an RII dimer.

within the range reported for other sources. The enzymes showed virtually identical I_{50} values for PKA_I and similar patterns of inhibition by inorganic salts, particularly strong inhibition by fluoride. However, the two enzymes differed strongly in their affinities for the substrate Kemptide with Km values 10-fold lower for the *Eurosta solidaginis* enzyme at 23.5°C. The difference was less pronounced (3-fold) at 4°C. Sensitivities to H7 and H89 peptide inhibitors also differed between the two enzymes.

Mammalian PKAc is known to occur in two major isoforms and a third has been found in some cases. Table 4.8 shows the kinetic properties of the alpha and beta isoforms of the catalytic subunit from a mammalian source. While there is no evidence to date that the same isoforms occur in insects, comparisons can be made to determine which of the isoforms the cold-hardy insect enzymes most resemble.

Kinetic constants of PKAc purified from *Epiblema scudderiana* are quite similar to those of the mammalian Cβ isoform. The K_m value of 38.1 ± 4.9 μM is essentially identical to that of mammalian Cβ (33.5 ± 12.7 μM). The I_{50} values for PKAi (5-24) also overlap; 7.98 ± 3.80 nM for *Epiblema scudderiana* and 12.7 ± 3.1 nM for mammalian Cβ (Gamm *et al.*, 1996). The Ka for holoenzyme dissociation of *Epiblema scudderiana* PKAc was 31.9 nM which falls between mammalian values Cα (63 nM) and Cβ (13 nM) when the holoenzyme is composed of RII regulatory subunits (Gamm *et al.*, 1996). Therefore, it appears that the insect regulatory subunits have a different Ka for cAMP or that the RI regulatory subunit is present in significant amounts in the insect preparation. Indeed, both R subunit types appear to be present in the insect since isofocusing showed two peaks of holoenzyme so the Ka cAMP value determined for both insect species would be a composite value for a mixture of the two holoenzyme types. The pI of 9.16

for *Epiblema scudderiana* PKAc was higher than that mammalian Cβ (pI = 8.0) (Kinzel *et al.*, 1987), which is consistent with other studies showing invertebrate PKAc to be more basic than its mammalian counterpart (Foster *et al*, 1984; Thalhofer *et al.*, 1988). Furthermore, high concentrations of Kemptide (> 1.5 mM) did not inhibit PKAc purified from *Epiblema scudderiana* (data not shown), again consistent with the mammalian Cβ subunit (Gamm *et al.*, 1996; Olsen and Uhler, 1989; Adams and Taylor, 1992).

Analysis of the kinetic data for PKAc purified from *Eurosta solidaginis* shows it to more closely resembled mammalian Cα. The Km for Kemptide of 3.67 ± 0.11 μM for *Eurosta solidaginis* was closer to the mammalian Cα value of 14.0 μM, than to the Cβ value of 33.5 μM. The I_{50} value for PKAi of 8.85 ± 0.34 nM was intermediate between mammalian Cα (4.8 μM) and Cβ (12.7 μM). The Ka for holoenzyme dissociation was 148 ± 18 nM which was also closer to the values for mammalian RII holoenzymes containing Cα (63 nM) versus Cβ (13 nM) catalytic subunits (Gamm *et al.*, 1996). However, again this parameter can not be conclusively evaluated as the presence of two peaks of holoenzyme on isofocusing indicates that PKA tetramers containing both RI and RII regulatory subunits were present in *Eurosta solidaginis* extracts (Figure 4.2a). The pI of 8.93 is more characteristic of Cβ than of Cα (Kinzel *et al.*, 1987), but this may again reflect generally high pI values seen for insect PKAc, compared with mammalian. The former seems more likely since PKAc from *Eurosta solidaginis* also displayed characteristics of mammalian Cβ, in that it did not show the substrate inhibition at high concentrations of Kemptide that is expected of Cα (>1.5 mM, Data not shown) (Gamm *et al.*, 1996; Olsen and Uhler, 1989; Adams and Taylor, 1992).

Comparison of kinetic constants at 23.5°C and 4°C obtained for PKAc from both insects suggest that temperature has a significant influence on enzyme activity. For example, the Ka for holoenzyme dissociation dropped dramatically for both insects at 4°C compared with 23.5°C; from 31.9 μM to 4.3μM in *Epiblema scudderiana* and from 148 μM to 27.3 μM in *Eurosta solidaginis*. This could have two consequences. First, if cellular levels of cAMP were similar at both temperatures, such a drop in Ka value would allow much more of the enzyme to be in the active, free catalytic form at lower temperatures. Second, when an activating signal is received (one that raises cAMP levels) the lower Ka at low temperature could result in a faster and greater increase in the percentage of holoenzyme dissociation; thus, response to a cAMP signal would be enhanced at low temperature. Since one of the best-known roles of PKA is in the phosphorylation of glycogen phosphorylase kinase which, in turn, phosphorylates glycogen phosphorylase b to convert it to the active phosphorylase a, an enhanced ability to activate PKA would ultimately facilitate the activation of glycogenolysis at low temperature to increase the production of polyols.

The K_m for Kemptide of *Epiblema scudderiana* PKAc also decreased at the lower assay temperature which could also facilitate increased phosphorylation of target proteins by PKAc at low temperature *in vivo*. *Eurosta solidaginis* PKAc showed relatively little influence of temperature on its K_m for Kemptide with values ranging between 2 and 7 μM in all cases; notably, however, the *Eurosta solidaginis* enzyme had a much higher affinity for Kemptide in all cases than did the *Epiblema scudderiana* enzyme. Temperature change had no effect on the Km for ATP of either enzyme nor was this parameter influenced very much by the addition of polyols to the assay. However, polyols

had an influence on Km for Kemptide and at 4°C polyols increased the affinity of both enzymes for the substrate. A comparison of probable physiological relevance can be made between kinetic properties at 23.5°C in the absence of polyols (ie. representing a summer or early autumn situation in the larvae) and properties 4°C in the presence of high polyols. For *Epiblema scudderiana* PKAc this comparison results in a 3-fold decrease in Km at the lower temperature which would greatly increase enzyme affinity for substrates in the cold. For *Eurosta solidaginis* this results in very little change in Km from the value of 3.67 μM at 23.5°C but improves enzyme affinity at low temperature compared to the situation without polyols.

Inhibition studies showed that the decrease in temperature had no significant effect on the I_{50} values for specific PKA peptide inhibitors and also relatively few effects on I_{50} values for salts, although there was a general trend for I_{50} values for salts to be lower at the lower assay temperature. Neither enzyme was particularly strongly inhibited by the salts tested although fluoride anion was by far the strongest inhibitor. Although there were only a few instances of differential effects of ions on PKAc at high versus low temperature, inhibition by cations and anions could become important in another way in the insects while they are overwintering. Thus, as one of its preparations for winter, the water content of *Epiblema scudderiana* larvae decreased by about one-half (Rickards et al., 1987), an effect that would result in the opposite elevation of the concentration of all ions in body fluids. *Eurosta solidaginis* larvae are susceptible to an even greater rise in ion concentrations during freezing when the conversion of about two-thirds of their body water into ice would lead to a 3-fold increase in the concentration of all dissolved solutes

in the cytoplasm. Under these situations, the inhibitory effects of ions on PKAc and other enzymes could prove important.

The biggest inhibition of PKAc is likely due to temperature. The activity at optimal pH dropped from its corresponding value at 23.5°C to 34% in *Epiblema scudderiana* and to 15% in *Eurosta solidaginis* at 4°C. The break seen at 10°C in the Arrhenius plots for PKAc from both insects suggests a change in the conformation of the enzyme that results in differentially stronger suppression of enzyme activity at lower assay temperatures and elevated activation energies at temperatures below 10°C. This change in conformation may be responsible for the observed increase in Km Kemptide of the *Eurosta solidaginis* enzyme at low assay temperatures but does not correlate with the decrease in Km of the *Epiblema scudderiana* enzyme at 4°C. A break in the Arrhenius plot at about 10°C has also been seen with several other enzymes from these cold hardy insects (Muise, 1993; Lautru, 1997).

Both insects show a general shift in pH optimum to a higher value at low temperature. This goes along with the general effect of low temperature on the intracellular pH of cold-blooded organisms which typically rises by 0.018 pH unit per 1°C decrease in temperature. In *Eurosta solidaginis*, for example the intracellular pH was 6.8 at 15° C and 7.1 at 0°C (Storey et al., 1984). By shifting the enzyme pH optimum to a higher pH value at the same time as cellular pH also rises, the enzyme keeps its relative activity much the same at high and low temperatures and retains the same relative response. Therefore, it appears unlikely that either insect uses the change in pH associated with the drop in temperature to regulate PKAc.

Chapter 5

Purification and Kinetic Studies of Protein Phosphatase-1 from Cold-hardy Goldenrod Insect Larvae *Epiblema scudderiana* and *Eurosta solidaginis*

Introduction

Protein phosphatase-1 (PP1) and its role in cryoprotectant production has been introduced in Chapters 1 and 2. Several holoenzyme forms of PP1 exist, consisting of a catalytic subunit complexed to various regulatory subunits. The glycogen bound enzyme ($PP1_G$) is complexed to a glycogen binding (G) subunit (Hubbard and Cohen, 1991), the ATP-dependent enzyme ($PP1_I$) which is located in the cytosol, is a complex of PP1 and inhibitor 2 (Ballou *et al.* 1985), the myofibril associated enzyme ($PP1_M$) consists of PP1 bound to myosin (Chen *et al.*, 1994), and the nuclear form ($PP1_N$) consists of PP1 bound to NIPP-1 (Nuclear inhibitor of PP1) protein (Beullens *et al.*,1993). These different holoenzymes serve to target PP1 to various subcellular locations.

As was shown in Figure 1.3, PP1 plays an important role in the regulation of phosphorylase a (inhibition), and thus in the production of cryoprotectants, in both *Epiblema scudderiana* and *Eurosta solidaginis.* As stated in Chapter 4, polyols are well known for their abilities to stabilize protein structure and counteract denaturing influences such as temperature extremes or dehydration and affect enzyme functions at low temperature. Therefore, for a comprehensive understanding of PP1 function in these species and how it may change in summer versus winter seasons, it is necessary to evaluate the influences of both temperature and polyols on enzyme kinetic properties.

In this study the kinetic characteristics of purified PP1 from *Epiblema scudderiana* and *Eurosta solidaginis* were undertaken at 4°C and 23.5°C to explore enzyme properties and the possible role of temperature regulation on PP1 in cold-hardiness. Kinetic studies included examining the effects of temperature, pH and okadaic acid, as well as the effects of various salts on PP1 activity.

Materials and Methods

Chemicals and PP-1 Assay

Microcystin-agarose was obtained from UBI (catalogue # 16-147). Synthetic peptide KR(pY)IRR was obtained from Queens University. All other materials are as previously described in chapter 2.

PP1 Purification

The catalytic subunit of PP1 was purified from *Epiblema scudderiana* and *Eurosta solidaginis* was purified in a manner similar to that used by MacDonald (1998). Supernatant of a 1:3 (w/v) homogenate of frozen larvae in buffer A was prepared as described in Chapter 2 and polyethylene glycol 8000 was added to 12% w/v. The sample was mixed on a rotary mixer for 30 min at 4°C, then centrifuged at 14 000 g for 10 min at 4°C. The supernatant was removed and the pellet, containing PP1, was resuspended in the original volume of buffer A. The resuspended pellet was then loaded onto a 1 cm x 4 cm DE-52 column equilibrated in buffer A. The column was washed and bound protein was eluted with 2 ml fractions of buffer A containing 500 mM KCl. The first 8 ml contained PP1 activity and these fractions were pooled and incubated for 1 h with 1ml of microcystin-agarose (Moorhead, 1995), equilibrated in buffer C (20 mM Tris-HCl, pH 7.4, 10 mM β-mercaptoethanol, 500 mM NaCl). The column was then allowed to drain and washed with 20 vol of buffer B. One column volume of buffer C containing 3M NaSCN was then added and the column flow was stopped, then another column volume of buffer C containing 3M NaSCN was added. The column was left to stand at 4°C for 30 min and then drained. Additional buffer C containing 3 M NaSCN was then added and 1 ml fractions were collected and stored on ice. A 250 µl sample of each 1 ml fraction was desalted by loading onto a 5 ml column of Sephadex G25 equilibrated in buffer A and centrifuging for 1 min at maximum speed in an IEC bench top centrifuge. Desalted fractions were then assayed for PP-1 activity for 30 min. Fractions with activity were

pooled (including spun column fractions) and dialyzed against two changes of buffer B overnight. Dialyzed sample was then concentrated with solid polyethylene glycol 8000 to a volume of 300 µl. The concentrated sample was loaded onto a Sephacryl S-200 gel filtration column equilibrated in buffer A containing 50 mM NaCl. The column was eluted with the same buffer and peak fractions from the lower molecular weight peak range were pooled, stored on ice and used for kinetic studies, since the higher molecular weight peak likely contained a mixture of phosphatase holoenzymes. The purified enzyme was stored at on ice or at 4°C and retained 70% of its activity after 2 days at 4°C, but lost all activity after 4 days.

Determination of purity and Molecular Weight

SDS-PAGE was used to determine purity and molecular weight as described in Chapter 4. Molecular weight was also determined by Sephacryl S-200 gel filtration (as above), with aldolase (150 kDa), hexokinase (100 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), egg albumin (29 kDa), and cytochrome c (13 kDa) as standards.

Kinetic Studies

Assays used 10 µl of pooled fractions from the higher molecular weight peak from the Sephacryl S-200 column for Km determination with the synthetic peptide and 10µl from the lower molecular weight peak from the Sephacryl S-200 column for all other kinetic studies. In some studies, enzyme activity was assayed with varying concentrations of synthetic peptides, RRA(pT)VA and KR (pY)IRR to determine K_m values. Assays with synthetic peptides were conducted like those for PP2A with incubation times of 60 min at 23.5°C and 90 min at 4°C followed by quantification of phosphate released by the molybdate dye reaction as described in Chapter 2. All other kinetic studies were done

using phosphorylase a as the substrate. Kinetic constants (K_m, I_{50}) were calculated using a kinetics program (Brooks, 1992). Reaction mixtures for all kinetic studies with phosphorylase a as the substrate contained 10 µl of enzyme containing fraction, 20µl assay buffer (50mM Tris-HCl, pH 7.0, 0.25 mM EGTA, and 25 mM β-mercaptoethanol) and 20 µl ^{32}P-labeled phosphorylase a (as prepared in Chapter 2) which was added to start the reaction. Inhibition studies determined I_{50} values for okadaic acid and various salts. Arrhenius plots were constructed for assays between 2°C and 30°C and pH curves (pH 5-10) were also obtained.

Results

Molecular weight determination of protein phosphatase 1

PP1 from *Epiblema scudderiana* and *Eurosta solidaginis* purified by precipitation with 12% PEG, DE-52, and microcystin was applied to a Sephacryl S-200 gel filtration column and eluted as two peaks (Figures 5.1 and 5.2). In each case, the higher molecular weight peak likely represents a mixture of phosphatase holoenzymes (m.w. > 100 kDa). The lower molecular weight peaks were due to PP1 catalytic subunit and had molecular weights of 66 kDa for the enzyme from both insects (Figure 5.3). Subsequent kinetic studies of the purified enzyme were done on the lower molecular weight fraction, representing the PP1 catalytic subunit.

However, both high and low molecular weight peaks were tested for activity with the synthetic peptides RRA(pT)VA and KR(pY)IRR (in addition to the phosphorylase a phosphatase activity shown in Figure 5.1). No activity was detected with the latter peptide in either peak (data not shown), whereas only the high molecular weight peak showed activity towards the former peptide. Michaelis-Menten constants for the artificial substrate RRA(pT)VA were determined to be 172 ± 15 μM and 191 ± 25 μM, respectively, at 23.5°C for the higher molecular weight peak of *Epiblema scudderiana* and *Eurosta solidaginis* PP1 activity (Table 5.1).

Purification of PP1 from *Epiblema scudderiana* and *Eurosta solidaginis*

The purification scheme for PP1 from *Epiblema scudderiana* and *Eurosta solidaginis* resulted in 129 fold purifications with yields of 6 and 15 % for the two insects, respectively (Tables 5.2 and 5.3). The scheme was highly reproducible with all trials

Figure 5.1 Elution Profile of PP1 from *Epiblema scudderiana* eluted from Sephacryl S-200 Gel-Filtration Column. Pooled, dialyzed eluate from microcystin column was applied to the column. Enzyme was eluted with 20 mM Tris, pH 7.0, 15 mM β mercaptoethanol, 50 mM NaCl and 30% (v/v) glycerol. Two ml fractions were collected.

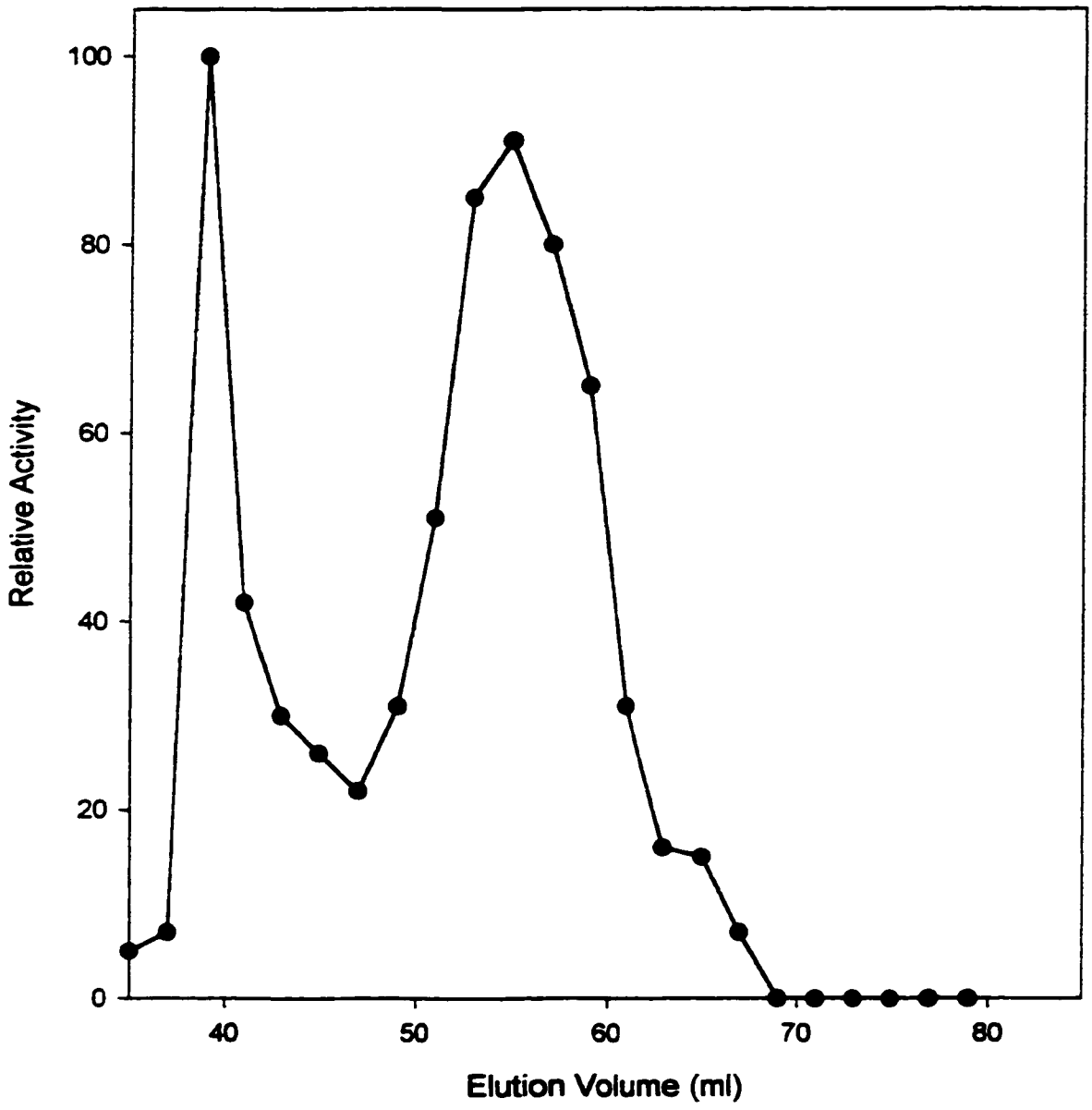

100
80
60
40
20
0
Relative Activity
40
50
60
70
80
Elution Volume (ml)

Figure 5.2 Elution Profile of PP1 from *Eurosta solidaginis* eluted from Sephacryl S-200 Gel-Filtration Column. Pooled, dialyzed eluate from microcystin column was applied to the column. Enzyme was eluted with 20 mM Tris, pH 7.0, 15 mM β mercaptoethanol, 50 mM NaCl and 30% (v/v) glycerol. Two ml fractions were collected.

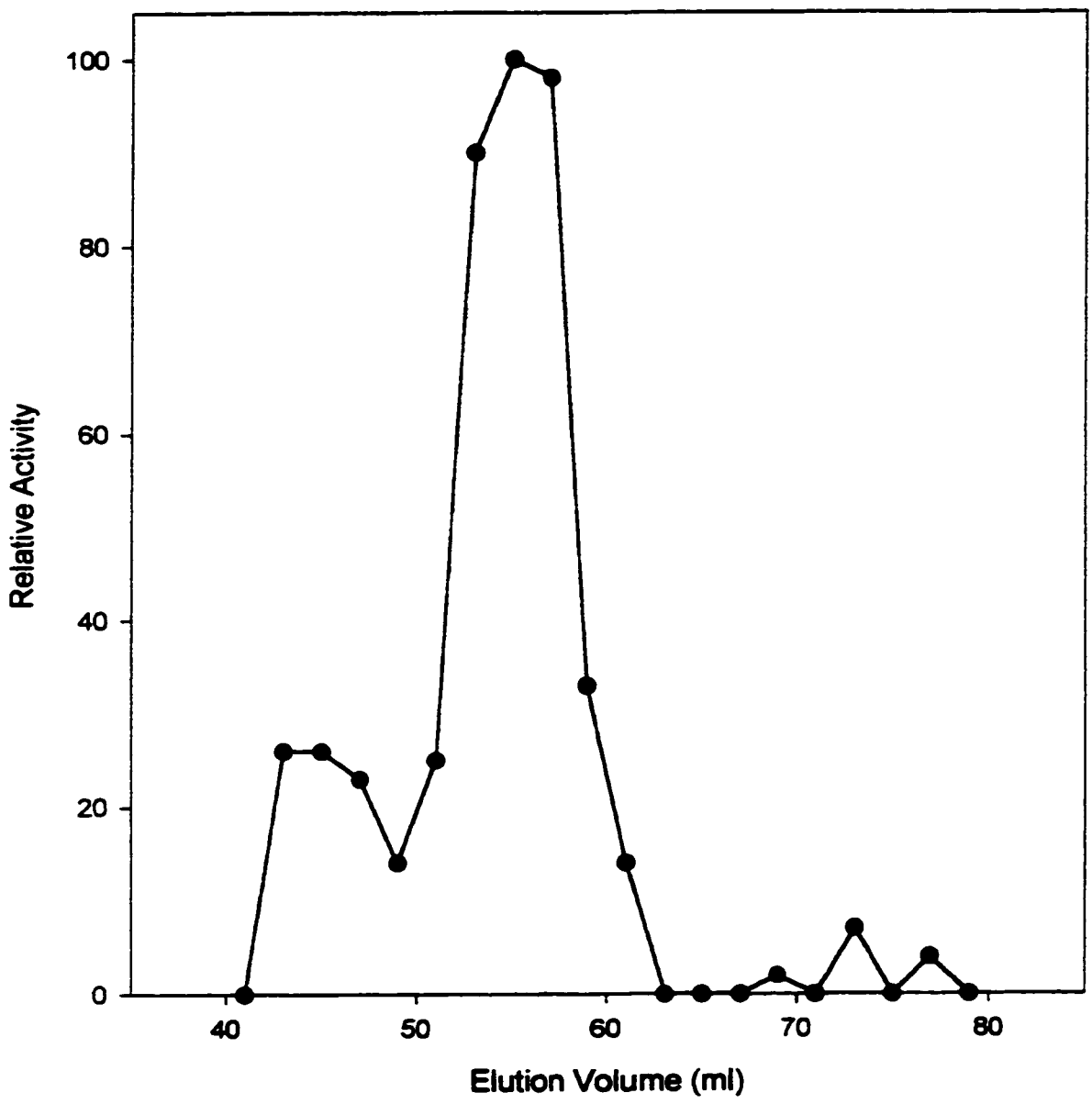

100
80
60
40
20
0
Relative Activity
40
50
60
70
80
Elution Volume (ml)

Figure 5.3 Standard Curve of Log Molecular Weight versus Elution Volume of Protein Standards for a Sephacryl S-200 Gel-Filtration Column. The log of the following molecular weight standards was plotted versus elution volume to determine the relative molecular weight of PP1 from *E. scudderiana* and *E. solidaginis*. The molecular weights were: 1) aldolase (150 000), 2) hexokinase (100 000), 3) bovine serum albumin (66 000), 4) ovalbumin (45 000), 5) egg albumin, and 6) cytochrome c (13 000). Circle number 7 represents the position PP1 from *E. scudderianna* and *E. solidaginis* eluted from the column.

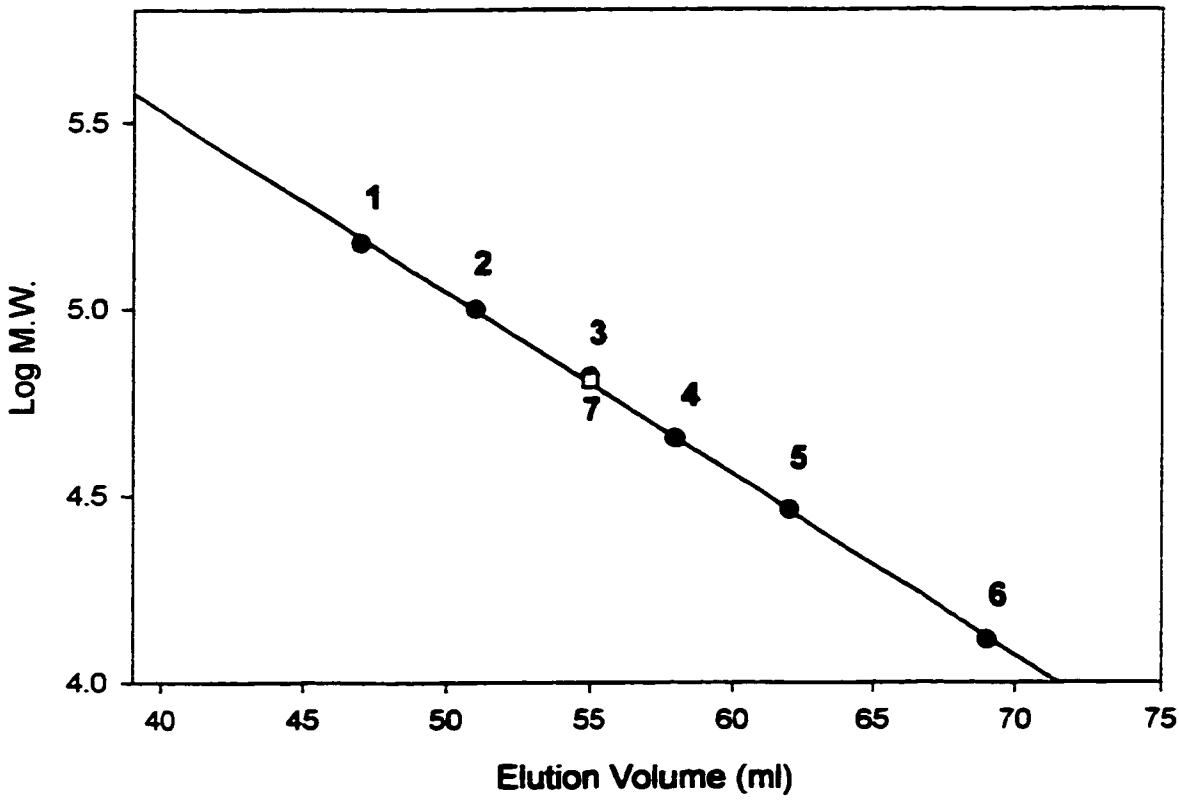

5.5
5.0
4.5
4.0
Log M.W.
40
45
50
55
60
65
70
75
Elution Volume (ml)
1
2
3
7
4
5
6

Table 5.1 Substrate Binding Constants (K_m) for the Hexapeptide RRA(pT)VA For *Epiblema scudderiana* and *Eurosta solidaginis*

	Without Okadaic Acid		With 2.5 nM Okadaic Acid	
	23.5°C	4°C	23.5°C	4°C
E. scudderiana (μM)	172 ± 15	207 ± 8	217 ± 9	188 ± 3 a
E. solidaginis (μM)	191 ± 25	175 ± 20	270 ± 36	237 ±29

Values are mean ± SEM for n =3 samples. a, significantly different from corresponding condition at 23.5°C using the Student's t-test, $P < 0.05$.

Table 5.2 Purification of PP1 From *Epiblema scudderiana*.

Results are from one purification: essentially equivalent results were obtained from subsequent preparations of purified enzyme. One unit (U) is defined as 1 pmol of phosphate released per minute at 23.5 °C.

	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Supernatant	155 681	237	656	100	1
12% PEG	125 906	161	782	81	1.2
DE-52	64 000	45	1422	41	2.2
Micro-cystin	16 320	0.334	48 862	10	74
S-200	9 845	0.116	87 123	6	132

Table 5.3 Purification of PP1 From *Eurosta solidaginis*.

Results are from one purification: essentially equivalent results were obtained from subsequent preparations of purified enzyme. One unit (U) is defined as 1 pmol of phosphate released per minute at 23.5 °C.

	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Supernatant	113 153	153	739	100	1
12% PEG	126 478	116	1 090	109	1.5
DE-52	58 033	25	2 321	51	3.2
Microcystin-Agarose	28 349	0.42	67 498	25	91
S-200	17 232	0.18	95 733	15	129

yielding similar results. The greatest fold-purification came from the microcystin-agarose step as this resin specifically binds phosphatases and is widely used for their purification (Moorhead *et al.*, 1995). The final specific activities of PP1 were assayed with phosphorylase a as the substrate and were 87,123 U/mg for *Epiblema scudderiana* and 95,733 U/mg for *Eurosta solidaginis* PP1. SDS-PAGE of purified PP1 showed single protein bands of 65 kDa for *Epiblema scudderiana* (Figure 5.4) and 66 kDa for *Eurosta solidaginis* (Figure 5.5) PP1.

Kinetic Properties of Purified Insect PP1

Kinetic studies were undertaken at 23.5°C and 4°C in order to assess the role of temperature in regulating PP1. Okadaic acid is a specific inhibitor of PP1; for the mammalian enzyme, I_{50} values are typically in the range of 10-15 nM (Cohen, 1991). At the warmer assay temperature (23.5°C) both *Epiblema scudderiana* and *Eurosta solidaginis* PP1 showed I_{50} values for okadaic acid within or close to this range, 11.0 ± 3.5 nM and 33.2 ± 9.1 nM, respectively (Tables 5.4, 5.5). However, in both cases, when assay temperature was lowered the enzymes became much more sensitive to the inhibitor with I_{50} values dropping to just 5 and 4 % at 4°C of the corresponding values at 23.5°C.

Table 5.4 also shows the inhibitory effects of various salts on PP1 purified from *Epiblema scudderiana*. Sodium fluoride was the strongest inhibitor of the enzyme, with I_{50} values of 8.4 and 16.2 mM at 23.5°C and 4°C, respectively; fluoride is a well-known inhibitor of phosphatases. Other salts had lesser effects with I_{50} values ranging from 35 to 75 mM. Sodium salts were generally more inhibitory that potassium. The relative order

Figure 5.4. Silver stained SDS-PAGE of purified PP1 from *Epiblema scudderiana*. Lane 1, molecular weight (M.W.) markers ; lane 2, pooled fractions from Sephacryl S-200 after PEG, DE-52 and Microcystin-agarose. The M.W. standards shown are: Myosin (204 kDa), β-galactosidase (121), BSA (78 kDa), carbonic anhydrase (39.5 kDa), soybean trypsin inhibitor (30.7 kDa), and lysozyme (19.7 kDa).

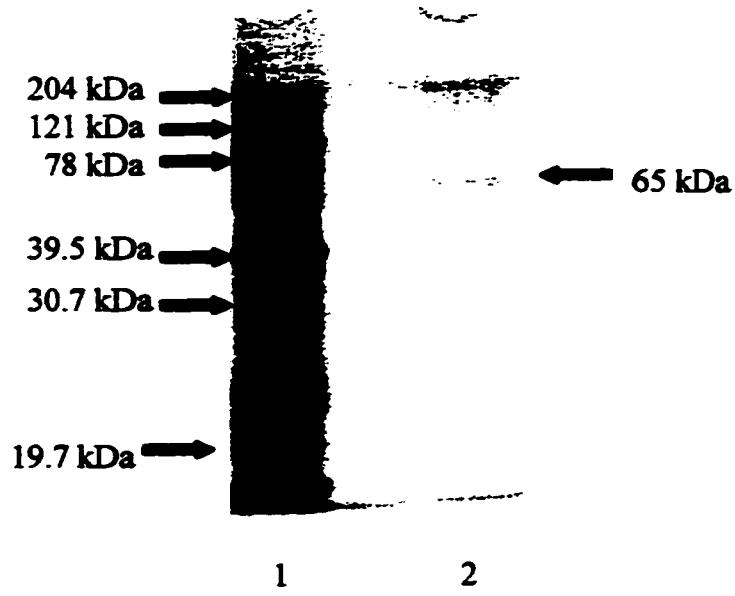

204 kDa
121 kDa
78 kDa
39.5 kDa
30.7 kDa
19.7 kDa
65 kDa
1
2

Figure 5.5. Silver stained SDS-PAGE of purified PP1 from *Eurosta solidaginis*. Lane 1, molecular weight (M.W.) markers ; lane 2, pooled fractions from Sephacryl S-200 after PEG, DE-52 and Microcystin-agarose. The M.W. standards shown are: Myosin (204 kDa), β-galactosidase (121), BSA (78 kDa), carbonic anhydrase (39.5 kDa), soybean trypsin inhibitor (30.7 kDa), and lysozyme
(19.7 kDa).

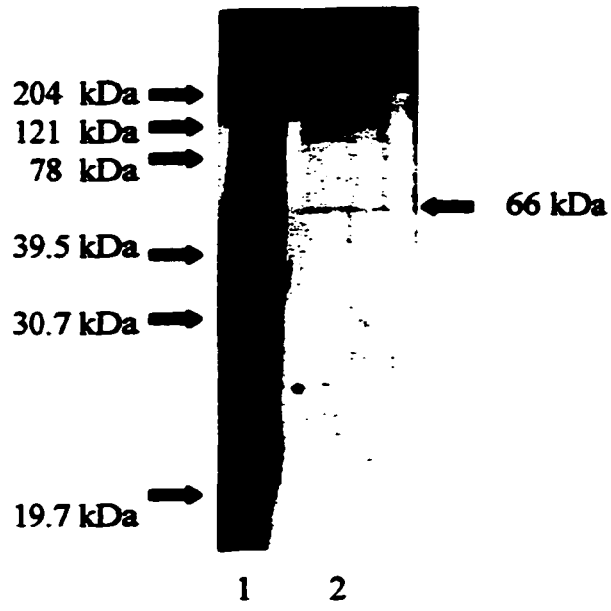

204 kDa
121 kDa
78 kDa
39.5 kDa
30.7 kDa
19.7 kDa
66 kDa
1
2

Table 5.4 Inhibition Constants For PP1 purified from *Epiblema scudderiana*

	I_{50} at 23.5°C	I_{50} at 4°C
Okadaic Acid (nM)	11.0 ± 3.5	0.58 ± 0.28 a
KCl (mM)	44.6 ± 1.3	74.8 ± 4.2 b
KBr (mM)	47 ± 11	52.6 ± 6.5
NaCl (mM)	31.7 ± 3.7	43.3 ± 4.3 y
NaBr (mM)	39.0 ± 0.9	58 ± 14
NH_4Cl (mM)	36.4 ± 1.6	38.6 ± 5.4 y
NaF (mM)	8.4 ± 0.7 x	16.2 (n=1) c, x

Values are means ± SEM for n=3 unless otherwise stated. a, significantly different from corresponding value at 23.5°C using a student's t-test, $P < 0.001$, b, $P<0.025$, c, $P < 0.05$. x, I_{50} for salt is significantly different from KCl at same temperature using a one way ANOVA with a Dunnett's test, $P< 0.01$, y, $P < 0.05$.

Table 5.5 Inhibition Constants For PP1 purified from *Eurosta solidaginis*

	I_{50} at 23.5°C	I_{50} at 4°C
Okadaic Acid (nM)	33.2 ± 9.1	1.26 ± 0.41 c
KCl (mM)	25.4 ± 1.2	46.9 ± 1.0 a
KBr (mM)	24.9 ± 1.9	35.7 ± 1.6 a
NaCl (mM)	24.4 ± 1.6	32.0 ± 7.7
NaBr (mM)	19.5 ± 1.9 y	52.3 ± 7.1 c
NH_4Cl (mM)	14.3 ± 0.7 x	56 ± 12 d
NaF (mM)	4.98 ± 0.06 x	22.7 + 3.0 b

Values are means ± SEM for n=3 samples. a, significantly different from corresponding value at 23.5°C using a student's t-test, $P < 0.001$, b, $P < 0.005$, c $P<0.025$, d, $P < 0.05$. x, I_{50} for salt is significantly different from KCl at same temperature using a one way ANOVA with a Dunnett's test, $P< 0.01$, y, $P < 0.05$.

of inhibition by salts at 23.5°C was: NaF>NaCl>NH_4Cl>NaBr>KCl>KBr whereas at 4°C the order was: NaF>NH_4Cl>NaCl>KBr>NaBr>KCl.

Comparable inhibition studies on PP1 purified from *Eurosta solidaginis* are shown in Table 5.5. Again, fluoride was the strongest inhibitor and inhibition by sodium fluoride was much stronger at 23.5°C (I_{50} = 5 mM) than at 5°C (I_{50} = 23 mM). I_{50} values for other salts were within a fairly tight range that was also lower (14-25 mM) at 23°C than at 4°C (32-56 mM). The relative order of inhibition by salts at 23.5°C was: NaF>NH_4Cl>NaBr>NaCl~KBr~KCl. At 4°C the order was: NaF>NaCl>KBr>KCl>NaBr >NH_4Cl.

pH Profile of PP1 Purified from *E. scudderiana* and *E. solidaginis*

Protein phosphatase-1 from *Epiblema scudderiana* showed optimal activity at pH 8 at 23.5°C (Figure 5.6). In the presence of 1M glycerol, however, the enzyme showed a broad optimum from pH 6.0-7.5. At 4°C the pH optimum in the absence of glycerol was at pH 6.8 and in the presence of 1 M glycerol the pH optimum shifted to a lower value (pH = 6). PP1 from *Eurosta solidaginis* showed a broad optimum between pH 6 and 7.5 at 23.5°C in the absence of polyols (Figure 5.7). This was largely unchanged by the addition of polyols although the optimum was more narrowly defined at about pH 7 in the presence of glycerol. At 4°C the pH optimum was shifted to pH 6.0 in the absence of polyols and 6.8 in the presence of 0.4 M sorbitol. In the presence of 1 M glycerol or a mixture of 1 M glycerol + 0.4 M sorbitol the pH optimum remained at 7.5.

Figure 5.6 pH curve of PP1 isolated from *Epiblema scudderiana*. Square symbols are at 23.5 °C and circles are at 4°C. Open symbols are without glycerol and solid symbols contain 1 M glycerol in the final assay mixture. Values are mean activity ± SEM, n = 3, relative to maximal activity at 23.5°C in the absence of glycerol. Where error bars are not visible they are contained within the symbols.

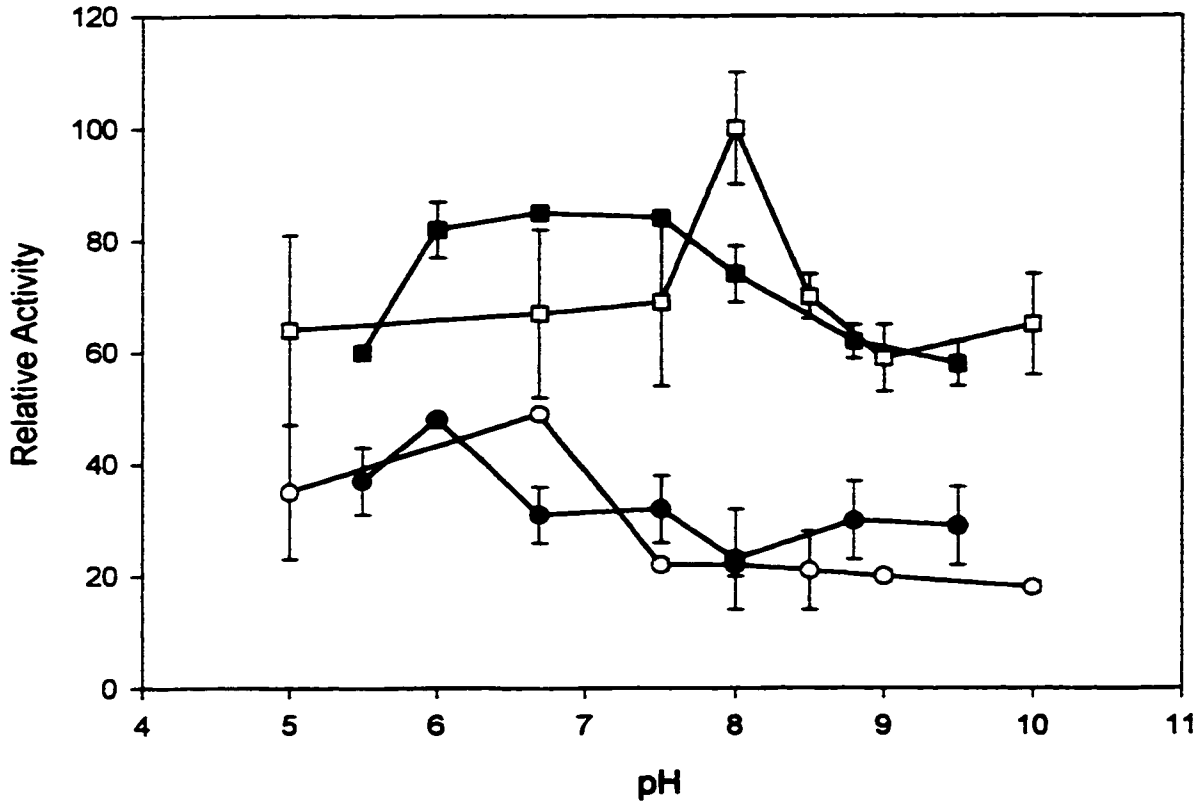
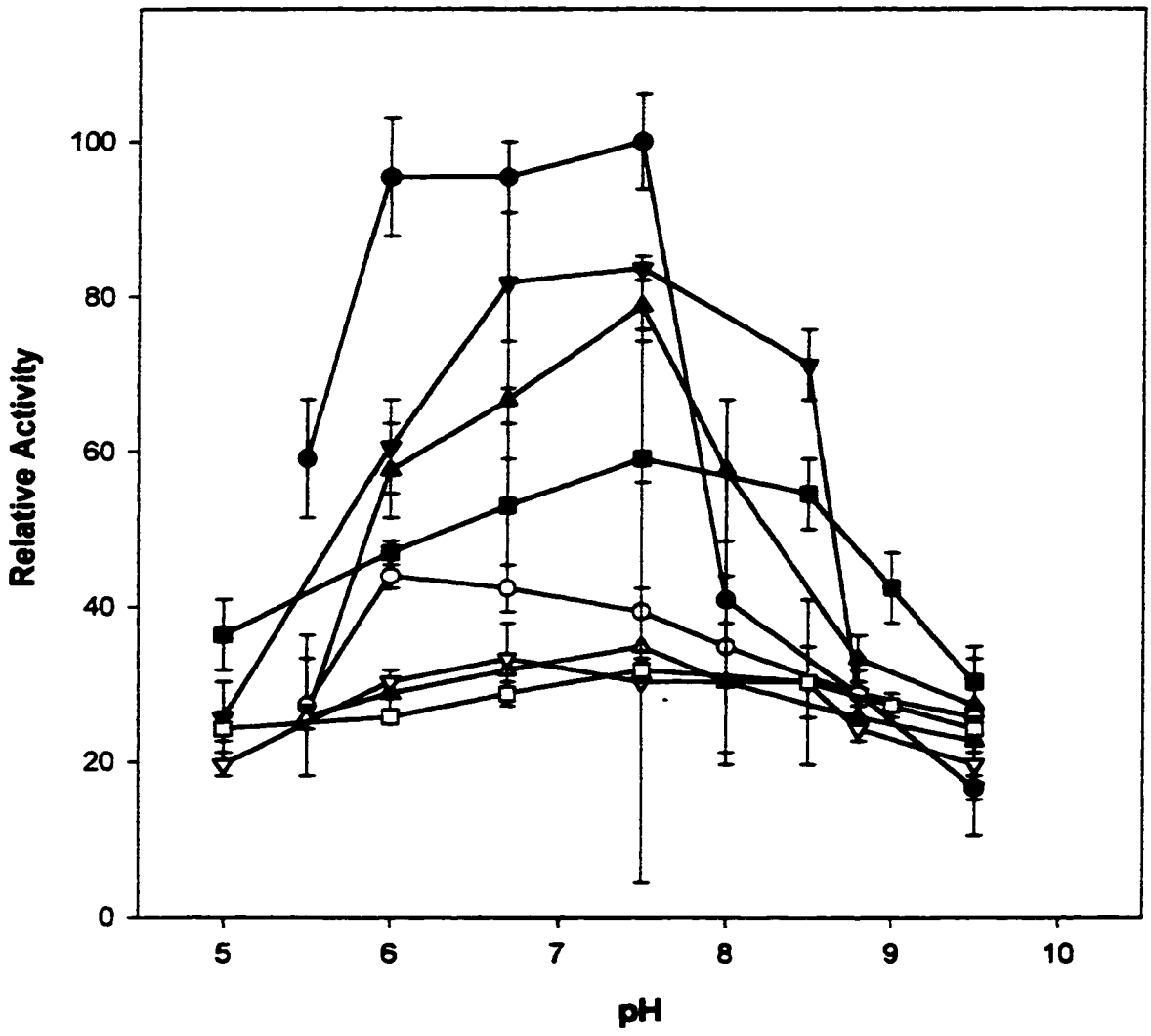

120
100
80
60
40
20
0
Relative Activity
4
5
6
7
8
9
10
11
pH

Figure 5.7 pH curve of PP1 isolated from *Eurosta solidaginis*.

Solid symbols are at 23.5 °C and open symbols are at 4°C. Circles are without polyols, triangles up are in the presence of 1 M glycerol, triangles down are in the presence of 0.4 M sorbitol, and squares are in the presence of both 0.4 M sorbitol and 1 M glycerol. Values are ± SEM, n = 3, relative to maximal activity at 23.5°C without polyols present. Where error bars are not visible they are contained within the symbols.


Relative Activity
100
80
60
40
20
0
5
6
7
8
9
10
pH

Arrhenius Plots of Purified PP1 from *E. scudderiana* and *E. solidaginis*

The effects of temperature on the assay of maximum velocity (V_{max}) of purified PP1 from *Epiblema scudderiana*, in the presence and absence of 1 M glycerol, are shown as Arrhenius plots in Figure 5.8. Activation energies were calculated by applying the Arrhenius equation on the slope of a plot of 1/temperature (°K) versus log activity.

$$E_a = -(\text{slope})(2.3\ R)$$

Both plots showed two distinct linear segments with a break occurring at 11°C. The activation energies (all n=3) in the absence of glycerol were 46.7 ± 2.5 kJ/mol for temperatures above 11°C and nearly 2-fold higher at 86.6 ± 5.4 kJ/mol for temperatures below 11°C. In the presence of 1 M glycerol the activation energies were 42.1 ± 0.4 kJ/mol for temperatures above 11°C and 96.8 ± 2.5 kJ for temperatures below 11°C.

The Arrhenius plots in Figure 5.9 show the effects of temperature, in the presence and absence of polyols, on the V_{max} of PP1 purified from *Eurosta solidaginis*. All plots were linear, with no breaks as determined by visual inspection. The calculated activation energies (all n=3) were 94.5± 7.3 kJ/mol in the absence of added polyols and 93.5 + 2.4, 110 ± 8, and 108 ± 6 kJ/mol for assays run in the presence of 1M glycerol, 0.4 M sorbitol, and 1M glycerol + 0.4 M sorbitol, respectively.

Figure 5.8. Arrhenius Plot For *Epiblema scudderiana* PP1 activity versus temperature. Activity was measured over a range from 2°C to 30 °C, as described in chapter 2. Open squares represent activity in the absence of glycerol and solid circles in the presence of glycerol. pH was set to 7.5 at 23.5°C and then allowed to self-adjust with changing temperature. The assay mixtures were incubated for 15 min to allow for temperature equilibration prior to starting the assay the radio labeled substrate mixture. Data are means ± SEM for n = 3 samples. Error bars not visible are contained within the symbols.

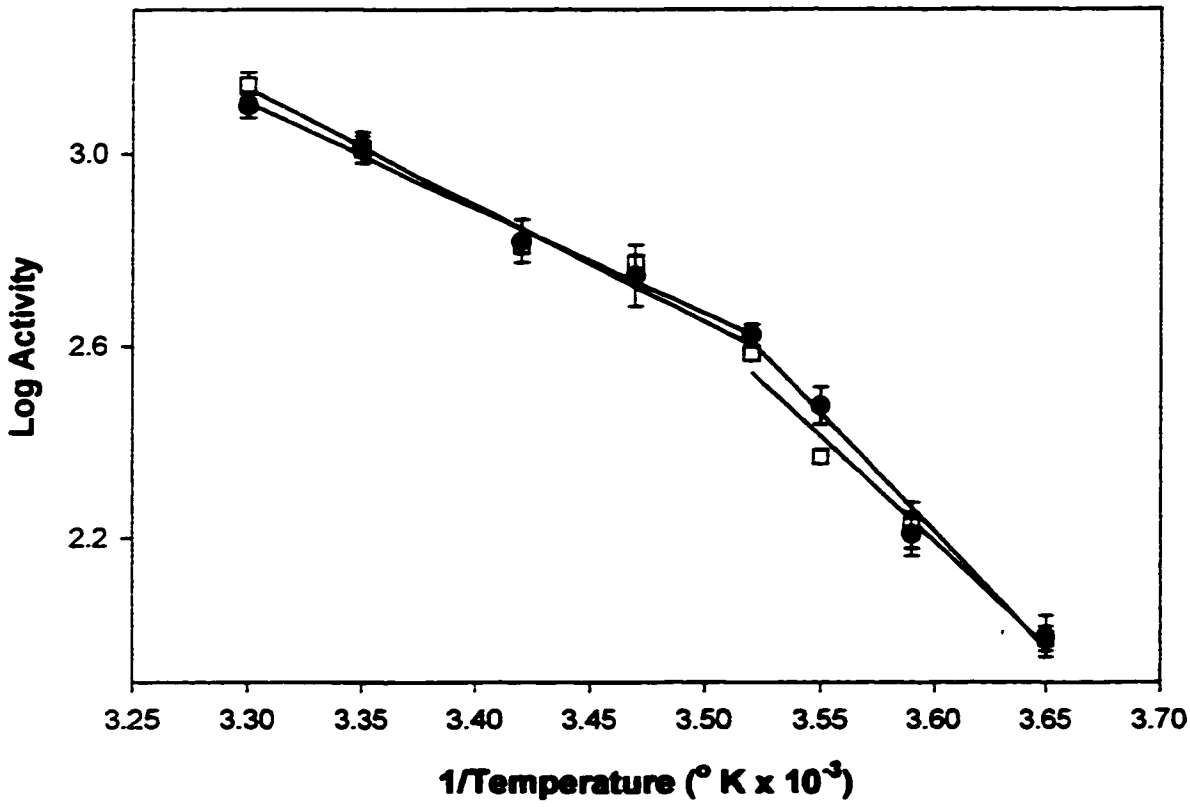
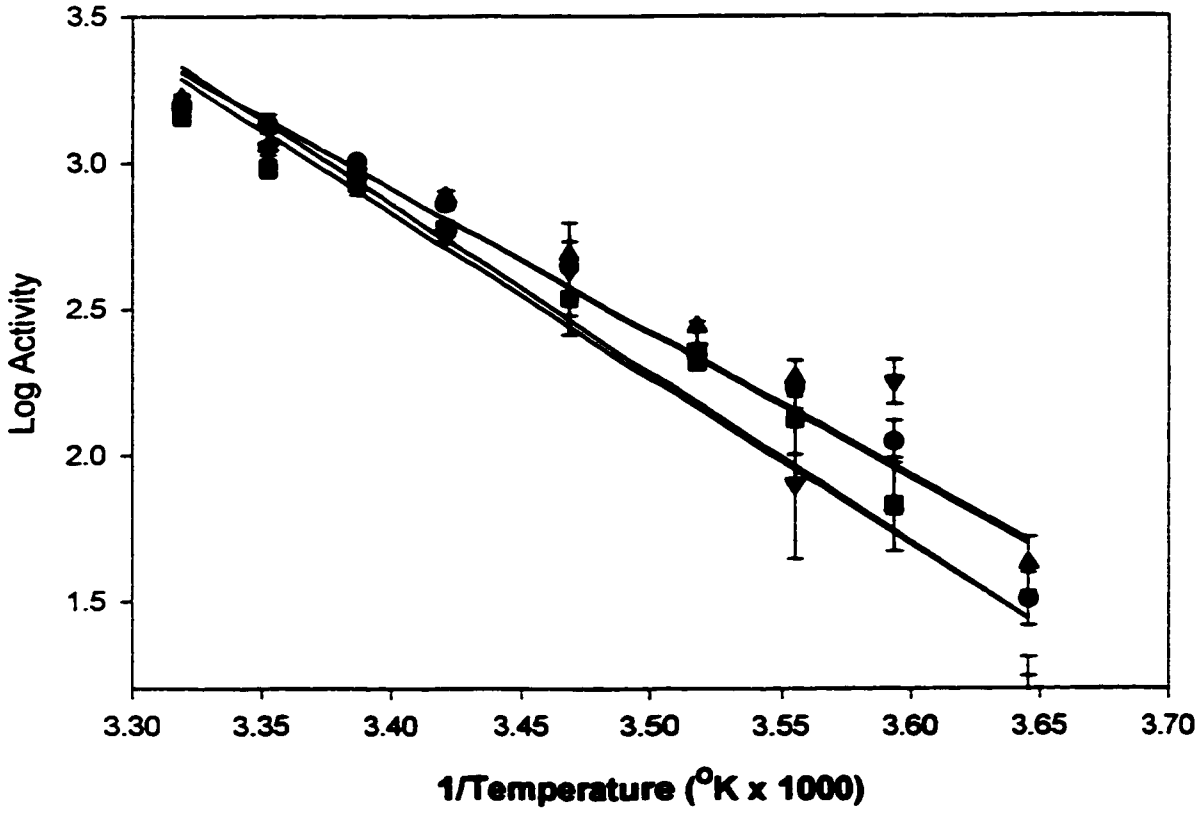

Log Activity
3.0
2.6
2.2
3.25
3.30
3.35
3.40
3.45
3.50
3.55
3.60
3.65
3.70
1/Temperature (° K x 10⁻³)

Figure 5.9. Arrhenius Plot For *Eurosta solidaginis* PP1 activity versus temperature. Activity was measured over a range of 2°C to 30°C, as described in chapter 2. Solid circles represent activity in the absence of polyols and open circles in the presence of 1 M glycerol, open triangles in the presence of 0.4 M sorbitol, and open squares in the presence of 1 M glycerol and 0.4 M sorbitol. pH was set to 7.5 at 23.5°C and then allowed to self-adjust with changing temperature. The assay mixtures were incubated for 15 min to allow for temperature equilibration prior to starting the assay the radio labeled substrate mixture. Data are means ± SEM for n = 3 samples. Error bars not visible are contained within the symbols.


3.5
3.0
2.5
2.0
1.5
Log Activity
3.30
3.35
3.40
3.45
3.50
3.55
3.60
3.65
3.70
1/Temperature (°K x 1000)

Discussion

Since *Epiblema scudderiana* and *Eurosta solidaginis* both rely on the production of cryoprotectants from glycogen for cold hardiness, this study examined a phosphatase capable of regulating glycogen phosphorylase a, which is the rate-limiting enzyme for cryoprotectant synthesis. The phosphatase purified from *Epiblema scudderiana* showed characteristics of protein phosphatase type-1 (PP1), particularly its activity towards glycogen phosphorylase a as a substrate and its sensitivity to inhibition by fluoride and okadaic acid. PP1 and PP2A are the only phosphatases that show activity with phosphorylase as a substrate *in vitro*, accounting for 88 and 12 %, respectively, of phosphorylase a dephosphorylation in rabbit skeletal muscle and 38 and 62 % in liver (Ingebritsen and Cohen, 1983). However, PP1 is believed to be the main or only enzyme carrying out this activity *in vivo*. Both of these enzymes are inhibited by okadaic acid, PP2A is very strongly inhibited (typically 1-2.5 nM added to an assay completely inhibits all PP2A) and PP1 less strongly. The I_{50} value for mammalian PP1 typically ranges between 10-15 nM (Cohen, 1991) and the I_{50} values for okadaic acid of 11 nM for *Epiblema scudderiana* and 33 nM for *Eurosta solidaginis* at 23.5°C are consistent with the insect enzymes being PP1. In addition, the assays were conducted in the absence of divalent cations which are an absolute requirements for activity by PP2C (Mg^{2+}) or PP2B (Ca^{2+} + calmodulin) so the insect enzyme was clearly neither of these phosphatases. Hence, the kinetic evidence supports the identification of the insect enzymes as PP1.

However, the molecular weight of the phosphatase purified from both insects was considerably greater than that reported for PP1 from other sources. S-200 gel filtration indicated a molecular weight of about 66 kDa for both enzymes whereas SDS-PAGE

gave values of 65 kDa for the *Epiblema scudderiana* enzyme and 66 kDa for the *Eurosta solidaginis* phosphatase. By contrast, PP1 from most vertebrate sources is generally reported to be about 35-38 kDa (Brandt, *et al.*, 1975; Mehrani and Storey, 1995). It has been reported that some freshly prepared fractions of PP1 holoenzyme have been larger than expected, possibly due to a larger catalytic subunit (Ingebritsen *et al.*, 1983), or a covalently modified subunit. Information on insect phosphatases is largely lacking, so it may be that the molecular weight of PP1 is simply larger in this animal group.

The insect enzymes also differed somewhat in substrate preference from what is typical for the mammalian enzyme. The purified insect PP1 taken from the lower molecular weight peak of phosphorylase a phosphatase activity eluted from the Sephacryl S-200 column (Figures 5.1, 5.2) showed no activity towards the artificial peptide KR(pY)IRR which has been used to assay PP1 activity in other species (Stefani *et al.*, 1993). The enzyme in this peak also showed no activity with the artificial peptide RRA(pT)VA, which is commonly used to assay PP2A. The higher molecular weight peak of phosphatase activity eluted on S-200, which likely contains a mixture of phosphatase holoenzymes, did show activity towards the RRA(pT)VA hexapeptide, but not the KR(pY)IRR hexapeptide. This higher molecular weight peak likely contains a least one holoenzyme form of PP1 since the activity in this peak was maintained in the presence of 2.5 nM okadaic acid which would have inhibited PP2A activity completely (Table 5.1).

Temperature change has significant effects on the PP1 from both insects and this could then be an important factor in the regulation of glycogen phosphorylase a that is needed in the production of cryoprotectants. A rapid increase in the percent active phosphorylase a upon cold exposure has been documented in many insects (Hayakawa

and Chino, 1982; Churchill and Storey, 1989a). The mechanism of this activation has been traced to the differential effects of low temperature on phosphorylase kinase versus phosphatase. In silkmoths, for example, Hayakawa (1985) found that over the range between 30 and 0°C, phosphorylase kinase underwent a normal reduction in activity with decreasing temperature (Q_{10} about 2) and the Km for phosphorylase b was conserved. Phosphorylase phosphatase, however, was rapidly inactivated at low temperature. As a result, kinase activity greatly exceeded phosphatase activity at low temperature and the net effect was an activation of glycogen phosphorylase.

A system that achieves comparable results may be at work in the gall insects. PP1 from both insects showed a strong decrease in I_{50} for okadaic acid at low temperatures. Although okadaic acid is not a natural inhibitor of PP1, this finding suggests that the enzyme may be more sensitive to inhibitors *in vivo* at low temperature, perhaps due to a conformational change in the enzyme protein. Although we don't know the effects of low temperature on phosphorylase kinase from the two insects, the results of Chapter 4 show that low temperature does not change the effects of inhibitors on PKAc (the enzyme which phosphorylates and activates phosphorylase kinase), does not alter the Km for ATP, and for *Epiblema scudderiana*, low temperature increased enzyme substrate affinity for Kemptide. Thus, the kinetic properties of kinases may be less susceptible to negative effects of low temperature than those of phosphatases.

The enzyme from *Epiblema scudderiana* showed a break in the Arrhenius plot at about 11 °C indicating a conformational change. Calculated activation energy doubled over the lower temperature range, so activity would be strongly suppressed by falling environmental temperatures below 11°C. Notably, this includes the range between +5°C

and -5°C where rates of glycerol synthesis are highest in *Epiblema scudderiana*. PP1 from *Eurosta solidaginis*, however, did not show any breaks in the Arrhenius relationship in the presence or absence of polyols over the full temperature range tested.

Although the phosphatases purified from *Epiblema scudderiana* and *Eurosta solidaginis* show characteristics of mammalian PP1, and probably are the insect PP1 enzymes (although with higher molecular weights), future work might reclassify them as one of the many new phophatases being identified such as PPV, PPY, PPZ1, and PPZ2, which all have properties intermediate to those of the four major ones described in Chapter 1 of this thesis (Cohen, 1997). However to make such a classification at this point may be premature, given that relatively little is known about insect phosphatases. Regardless of the classification of the phosphatase purified in this chapter, the present study shows that the phosphatase activities, purified from both insects by an identical method, were regulated by cold exposure, although the exact mechanism remains unclear. It also appears that the enzyme from the two species may be regulated differently, which may offer insight into the different strategies of glycogen phosphorylase control that would be needed for species that synthesize just 1 (glycerol) versus 2 (glycerol + sorbitol) cryoprotectants.

Chapter 6

General Discussion

Larvae of the goldenrod gall insects *Epiblema scudderiana* and *Eurosta solidaginis* endure harsh subzero temperatures for prolonged periods of time during the winter. *Epiblema scudderiana* uses the mechanism of freeze-avoidance to survive, whereas *Eurosta solidaginis* uses freeze tolerance. Both strategies involve depressing metabolic functions and accumulating high concentrations of polyhydric alcohols. In the former species glycerol levels reach about 2 M, sufficient to keep all water in the organism from freezing, whereas in the latter species a combination of glycerol and sorbitol reaching about 0.6 M prevents intracellular freezing when as much as 65 % of total body water is converted to extracellular ice. Biochemical mechanisms have evolved to control these functions, including the production and clearance of cryoprotectants.

The present study allowed us to form a clearer picture of the regulation of freeze avoidance and freeze tolerance, primarily with respect to the synthesis of polyols. Combining results from various enzyme time course surveys and enzyme kinetics of purified PKAc and PP1, as well as established data on glycogen phosphorylase and polyol levels in these insects, gives some insight into how cryoprotectant production is regulated. Differential changes in PP2A, PP2C, and PTP activities also showed that these too may have functions in regulating survival under such harsh conditions. In addition, the data in this study gives some insight into the differences in freeze avoiding and freeze tolerant insects.

The results reported in this thesis show both similarities and differences between the two insects in response to the stresses studied. In both species, PKA and PP1 showed general trends in

response to cold PKA activity increasing and PP1 decreasing. This finding is consistent with their known actions in regulating glycogen phosphorylase activity and supporting the synthesis of cryoprotectants. Protein tyrosine phosphatase activities in both the cytosolic and membrane bound fractions also increased with both peptides used during both the outdoor time course and the -20°C cold exposure in both insects.

However, differences between the two insects were seen in the responses of PP2A and PP2C to the stress used. *Epiblema scudderiana* showed an increase in PP2A activity with -4C exposure and increases in PP2C activity over the outdoor time course and -20°C cold exposure, whereas *Eurosta solidaginis* showed no increase in either PP2A or PP2C activity in response to cold exposure. In addition, membrane bound PTP activity increased in *Epiblema scudderiana* during -4C cold exposure but not in *Eurosta solidaginis*. This is could be due to the freeze avoiding *Epiblema scudderiana* still making metabolic adjustments, regulated via PP2's, at low subzero temperatures because they are still liquid. Whereas the freeze tolerant *Eurosta solidaginis* is largely shut down metabolically at subzero temperatures. Animals may shut down metabolic functions at subzero temperatures in anticipation of freezing, because once frozen a dormancy is basically imposed upon them .

Anoxia exposure was studied in *Eurosta solidaginis* to mimic the ischemic event associated with the freezing of the insect's body fluids. Anoxia exposure was also analyzed in *Epiblema scudderiana* for comparative purposes, since this insect does not freeze. Responses to anoxia in *Eurosta solidaginis* appeared to be linked to energy conservation and did not correspond to changes in enzyme activities observed in cold exposure. *Epiblema scudderiana* showed increases PP1

activity and in cytosolic PTP activities with both peptides in response to anoxia. These differences in response to anoxia stress may be implicated in the choice of cold survival strategy selected by each insect.

Purification and kinetic characterization of PKAc from both insects showed that temperature played a regulatory role in enzyme control. General increases in the affinity for Kemptide were seen at low temperatures, whereas the affinity for Mg-ATP and the effects of specific inhibitors and salts were largely unaffected by temperature. The argument for temperature regulation of PKAc was supported by a distinct break in the Arrhenius plots for PKAc from both insects at around 10°C. Since such a break in the Arrhenius plot is indicative of a conformational change, it would appear that the enzyme is changing shape in such a manner that increases the affinity of Kemptide without affecting the affinity for Mg-ATP or inhibitors.

Temperature also had a regulatory role in the control of PP1 from both insects. Most noticeable was a strong increase in inhibition by okadaic acid at low temperatures in both insects. As with PKAc, *Epiblema scudderiana* PP1 also showed a break in the Arrhenius plot, indicating a change in conformation, which could account for the large increase in okadaic acid inhibition. However, PP1 from *Eurosta solidaginis* maintained a constant slope in its Arrhenius plot and so the large increase in okadaic acid inhibition of this enzyme at low temperature cannot be accounted for by a conformational change in the enzyme. While okadaic acid is not found naturally in these insects, natural inhibitors may behave in a similar manner.

Based on the findings reported in Chapters 3 and 5 of this thesis, one key to regulating polyol production in *Eurosta solidaginis* (and potentially also in *Epiblema scudderiana*) may be due to PP1 being inhibited more strongly by cold than phosphorylase kinase. Studies on silkmoths have shown exactly such regulation (Hayakawa, 1985). While speculative, it may be this type of differential regulation by temperature of other enzyme pairs that is responsible for the stimulation of sorbitol production at lower temperatures than glycerol or other mechanisms required for cold-hardiness.

The present work has presented evidence for temperature regulation of PKAc and PP1 activities in cold hardy insects that can be involved in regulating the production of the cryoprotectants needed for winter survival. Low temperature stimulates PKA activity, which would then allow it to phosphorylate and activate phosphorylase kinase, which would in turn phosphorylate and activate glycogen phosphorylase. Low temperature could also inhibit PP1, thereby slowing the rate of dephosphorylation of glycogen phosphorylase and, hence, allowing it to remain active. Active glycogen phosphorylase can then initiate the conversion of glycogen into the desired cryoprotectant.

Future Studies

The enzyme activities (PKA, PP1, PP2A, PP2C, PTPs) measured in this study were chosen at several points during the winter season for which corresponding data for other enzymes and metabolites (Figures 2.7 and 3.7) was available. In future work it may be beneficial to take many samples over the periods when glycerol synthesis or breakdown is most active in order to get a more accurate picture of the roles of these enzymes in regulating these processes. This should be done

primarily for PKA and PP1, since they are known to be involved in the regulation of glycogen phosphorylase. Furthermore, PP2C deserves attention since this may reveal important differences between the two insects, based on the results of this work. Further kinetic studies on PP1 purified from both insects should also be performed. Of key interest will be its response to ATP, AMP, microcystin and divalent cations. It is expected that AMP and microcystin will be strong inhibitors of PP1 based on previous studies in other tissues (Mehrani and Storey, 1995; Moorhead *et al*, 1995). Response of PP1 to magnesium could help identify the specific isoform (Ballou *et al.*,1985).

Attempts to purify PKA and PP1 from mid-winter (January) insects may also be of interest if freeze avoidance and freeze tolerance in these insects is to be better understood. Such a study would determine if the kinetic characteristics of the enzymes changes, as may be the case if the insects are producing different isoforms than the fall insects which were used in this study. Purification of phosphorylase kinase (the target of PKA) and studies of its kinetic properties could also clarify the assumption (based on studies with silkmoth; Hayakawa, 1985) that PP1 was affected by cold to a greater extent than phosphorylase kinase.

Finally, both PKAc and PP1 are known to form complexes with regulatory subunits *in vivo*. While it is known that regulatory subunits are involved in targeting the enzymes to certain cellular locations, their behaviour at low temperature and their role in cold survival has never been tested. Subcellular fractionation and Microdisection combined with western blotting (with antibodies against catalytic and regulatory subunits) of insect samples taken at various points over the winter season could also be used to expand our knowledge of how these insects regulate their survival.

This however relies on the assumption that the insect protein possess enough similarity to their mammalian counterpart to be recognized by the available antibodies.

Conclusions

Thus it would appear that PKA and PP1 play a role regulating cryoprotectant production in both insects studied in this work. Furthermore temperature seems to be an important factor in regulating these enzymes, by affecting either the affinity for substrate (PKA) or inhibitors (PP1). This study has also given some insight into the differences in regulatory enzymes (PP2C and PTPs) between freeze avoidance and freeze tolerance mechanisms of cold survival.

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Publications List: Manuscripts in Preparation

-MacDonald, J.A., Brooks, S.P.J, Pfister T.D.,. and Storey, K.B. Phosphorylation Control in the Estivating Snail, *Otala lactea.*

-Pfister, T.D. and Storey, K.B. Survey of Enzymes Involved in Phospho-Regulation in Stress Response in *Epiblema scudderiana.*

-Pfister, T.D. and Storey, K.B. Survey of Enzymes Involved in Phospho-Regulation in Stress Response in *Eurosta solidaginis.*

-Pfister, T.D. and Storey, K.B. Purification and Characterisation of PKA Catalytic Subunit from two Species of Cold Hardy Insects.

-Pfister, T.D. and Storey, K.B. Purification and Characterisation of PP-1 from two Species of Cold Hardy Insects.