PURIFICATION AND CHARACTERISATION OF CELL WALL ACID PHOSPHATASES OF ROOTS OF WHITE CLOVER (*Trifolium repens* L.)

A thesis presented in partial fulfilment of the requirements
for the degree of

Doctor of Philosophy
in Plant Biology

at Massey University
Palmerston North
New Zealand

CELIA ZHANG

1999

Abstract

Plants of white clover (*Trifolium repens* L., cultivar Huia, genotype PgH_2) were either grown in half-strength Hoaglands solution (P-containing media) or subjected to phosphate starvation by omitting the sole source phosphate (KH_2PO_4) from Hoaglands solution media for a period of five weeks. The phosphate content of the first fully expanded leaf was determined in plants from both treatments. After 2 weeks, the P content in leaves from plants grown in P-deprived media was significantly lower ($p < 0.001$) than the P-supplied plants, and continued to decrease over the 5-week time course. Ionically bound acid phosphatases were extracted with 1 M NaCl from the cell walls of roots. In roots of plants maintained in P-deprived media, acid phosphatase activity increased over the 5-week time course, while the activity in roots of plants grown in P-containing media did not change. After four weeks in P-deprived media, the cell wall ionically-bound acid phosphatase fraction was subjected to hydrophobic column chromatography and two distinct acid phosphatases (designated Apase I and Apase II) identified. There is a temporal difference in induction of Apase I and Apase II. After one week of P-deprivation, the activity of Apase II reached its maximum and did not increase further in following weeks. The activity of Apase I was only half that of Apase II after one week of P-deprivation, but increased continually to be significantly higher than the activity of Apase II by the end of week 4. Apase I and II were further purified using gel filtration column chromatography, and each enzyme subsequently separated further into two isoforms by ion-exchange chromatography. Both isoforms of Apase I (Ia and Ib) exist as active monomers of 52 kD as determined by SDS-PAGE and by gel filtration. For Apase II, both isoforms (IIa and IIb) also exist as active monomers of 112 kD as determined by SDS-PAGE and 92 kD by gel filtration. Both Ia and Ib are glycosylated as determined by recognition by a *Galanthus nivalis* (GNA) lectin (which recognises terminal mannose or oligomannose N-linked glycan chains) or by a monoclonal antibody YZ1/2.23 (which recognises xylose/fucose-containing complex-type glycan chains). Apase Ia was recognised by both sugar probes, while Apase Ib was recognised by YZ1/2.23 only. Apase IIa was not recognized by either of sugar probes, while Apase IIb is a glycoprotein as determined by recognition by YZ1/2.23.

Using ρNPP as substrate, the pH optima for Apase Ia, Ib, IIa and IIb are 5.8, 6.2, 5.8 and 6.8, respectively. Isoelectric focusing determined that Apase Ia split into two bands with pI values of 7.0 and 7.3, Apase Ib showed a major band with a pI of 6.7, Apase IIa showed a single band with a pI of 4.4 and Apase IIb split into two closely located bands with pI values of 5.2 and 5.3. The activity of all four isoforms was severely inhibited by Cu^{2+}, Zn^{2+} and molybdate. Fe^{3+} is also an inhibitor but not as potent as the other three metal ions. Co^{2+} and Al^{3+} displayed greater inhibition of Apase I when compared with Apase II. Tartrate and EDTA had no effect on the activity of all four isoforms, but inorganic phosphate is a strong inhibitor of all the four isoforms. Each of the four isoforms showed a broad range of substrate specificity, with ATP and PPi the preferred substrates, and PEP and 3-PGA the least preferred substrates. All four isoforms showed no hydrolysis activity toward phytic acid. A short sequence containing 5 amino acid residues was obtained from Apase Ib, but no significant sequence identity with any existing protein sequence was found.

Acknowledgments

I gratefully acknowledge my chief supervisor, Dr Michael T. McManus, for his excellent supervision, understanding and encouragement throughout the course of this project.

I would like to thank my co-supervisor, Associate Professor David W. Fountain, for his friendship and encouragement throughout my study.

Before mentioning anybody else I wish to thank Ms Lyn Watson, our research technician, for her invaluable help.

My thanks also go to Robert Henroid and Lan bin Guo for their contributions toward statistical analysis of some data in this thesis.

I wish to thank Dr Donald Hunter and Lyn Watson for the initial establishment of the protocol for growing white clover plants in liquid media.

My appreciation also goes to the staff in the Plant Growth Unit, Massey University for their efforts in looking after white clover plants for me.

Thanks to the fellow members of lab C 5.19: Deming, Trish, Sang Dong, Greg, Simone and Don for their kindness and friendship.

I am very grateful to the New Zealand Pastoral and Agricultural Research Institute for providing research grant as well as three year's stipend and New Zealand Society of Plant Physiologists for providing generous travel and accommodation assistance grant to make it possible for me to go to a conference in Australia.

My final but very special thanks go to my husband, Jing Zhen, and my son, Gary, for being so supportive especially during my writing days.

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Abbreviations

AAA	lectin from Aleuria aurantia
ADP	adenosine diphosphate
Apase	acid phosphatase
APS	ammonium persulphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
^{0}C	degree Celsius
ca.	approximately
Con A	concanavalin A
CTP	cytidine triphosphate
CyDTA	trans-1, 2-cyclohexanediamine-N, N, N' N'-tetra-acetic acid
Da	Dalton (unit of molecular weight)
dATP	2'-deoxyadenosine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDGP	extracellular dermal glycoprotein
EDTA	ethylendiaminetetraacetic acid
FPLC	Fast Protein Liquid Chromatography
g	gram
GNA	lectin from *Galanthus nivalis*
HPLC	High Pressure Liquid Chromatography
hr	hour
IAA	indole-3-acetic acid
IEF	isoelectric focusing
KBPAP	Red kidney bean purple acid phosphatase
kD	kiloDalton (unit of molecular weight)

Km	Michaelis constant
L	Litre
LL	lentil lectin
M	molar, moles per litre
mA	milliampere
MES	4-morpholine ethanesulphoric acid
mg	milligram
min	minute
mL	millilitre
MW	molecular weight
NBT	nitro blue tetrazolium chloride
NCBI	database, the National Center for Biotechnology Information, USA
nm	nanometre
NMR	nuclear magnetic resonance
P	phosphorus
P-NMR	phosphorus-nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline + Tween 20
3-PGA	3-phosphoglyceric acid
PEP	Phospho(enol)pyruvate
Pi	inorganic phosphate
pI	isoelectric point
PNA	lectin from *Arachis hypogaea*
ρNPP	ρ-nitrophenyl phosphate
PPi	inorganic pyrophosphate
PVDF	polyvinylidene dilfluoride
RNA	ribonucleic acid
SBA	soybean agglutinin
SDS	sodium dodecyl sulphate
*st*A	*solanum tubersum* agglutinin

TEMED	N, N, N',N'-tetramethylethylendiamin
Tris	tris (hydroxymethyl) methylamine
Tween 20	polyxyethylenesorbitan monolaurate
µg	microgram
V	Volt (m^2 kg S^{-3} A^{-1})
V_0	void volume
Ve	elution volume
v/v	volume per volume
Vmax	maximal velocity
Vmax/Km	specificity constant
W	Watt (m^2 kg S^{-3})
w/v	weight per volume
WGA	wheat germ agglutinin
YZ1/2.23	a monoclonal antibody

Chapter One: Introduction

Overview

One of the most important, yet least available mineral nutrients for plant growth is phosphorus (P). The phosphate anion is the preferential form for absorption by plant roots from soil. Many studies have been performed in an effort to better understand the acquisition and utilisation of phosphorus by plants. Acid phosphatases (Apases) comprise a group of enzymes which catalyse the hydrolysis of phosphate monoesters. There is much evidence which indicates that the activity of plant acid phosphatase (Apase) increases under P-deficient conditions. The extracellular Apases of roots are proposed to be involved in hydrolysing and mobilising P from organic phosphate in the soil thereby increasing the availability of free phosphate which can be taken up by plants. White clover (*Trifolium repens* L.) is one of the most important forage legumes distributed world wide. This thesis examines the response of white clover plants to P-deprivation, in particular the identification and characterisation of ionically-bound (high salt extractable) cell wall Apases synthesised in the roots in response to P-deprivation.

1.1 Phosphorus and plants

Phosphorus (P) is an essential element in the growth and development of plants. It is one of 9 macronutrients and comprises up to 0.2% (g/g) of plant dry matter (Rains 1976). The importance of P in plant life can be attributed to a variety of factors including: (a) its function as an important constituent of macromolecules such as nucleic acids and phospholipids, (b) its role in energy transduction via the production of ATP, (c) its role in metabolism of carbohydrates, lipids, proteins and nucleic acids, and (d) its role in the regulation of enzyme activity.

1.1.1 Phosphate pools in the cell

In many enzyme reactions, P is either a substrate or a product. For example, phosphorylated derivatives are important in plant metabolism. The first-formed stable product of C_3 photosynthetic carbon dioxide fixation is 3-phosphoglyceric acid. Phosphorylated sugars are key intermediates in carbohydrate metabolism, such as glucose 6-phosphate and fructose 1,6-diphosphate.

Phosphorus is also involved in metabolic regulation by altering the activity of specific target proteins or enzymes through covalent attachment [phosphorylation or removal of phosphate molecules (dephosphorylation)]. Protein phosphorylation / dephosphorylation is thought to be one of the major regulatory mechanism of enzymes in plant cells. Protein phosphorylation-dephosphorylation cycles are catalysed by protein kinases and protein phosphatases respectively, resulting in either inactivation or activation of the target proteins for switching cellular pathways in response to changes in the environment (Hardie 1996).

Compartmentation of P is therefore essential for the regulation of metabolic pathways. The terms "pool" and "compartment" are often used to describe where different portions of a compound are metabolically isolated from one another, whether or not this is due to their physical separation in cell organelles (Bieleski 1973). For example, in the vacuole, $H_2PO_4^-$ is the dominant form and the four main organic-P fractions, DNA, RNA lipid-P and ester-P (which can be vacuole-located), can be regarded as four ester pools (Bieleski 1973). NMR (^{31}P) studies have revealed that plant cells selectively distribute P between cytoplasmic (metabolic) and vacuolar (storage) pools (Theodorou and Plaxton 1993). When plants have adequate phosphorus supply, 85 to 95% of the total P is located in the vacuole (Marschner 1995). Under P deficiency, the vacuole acts as a reservoir of P to maintain the cytoplasmic pool. Mimura (1995) isolated protoplasts and vacuoles from barley leaves grown under conditions of P deficiency and measured changes in the subcellular distribution of P. It was reported that P concentrations in the cytoplasm were always constant and were essentially independent of the supply of P. However, the vacuolar level of P gradually decreased to maintain this constant cytoplasmic P concentration.

1.1.2 Phosphorus deficiency symptoms of plants

The major symptoms of phosphorus deficiency are a retarded rate of growth of both shoots and roots (Wallace 1951). Shoots become short and thin, and growth is spindly. The leaves are smaller and turn dark-bluish-green. Spots develop on leaves or stems and are usually purple (Wallace 1951). Phosphorus deficient plants tend to be delayed in flowering (Wallace 1951). In fruit trees, P deficiency impairs fruit setting. The rate of development of fruits is also retarded and often only small fruits of poor quality are harvested (Mengel and Kirkby 1982). Phosphorus deficiency of white clover also leads to deformation of growth. The usual prostrate growth form of the plant becomes restricted and stems and petioles are thin. The colour of the leaves changes to dark green. The older leaves yellow prematurely and become necrotic (Wallace 1951).

1.2 Phosphorus availability in the environment

Despite its importance to plant growth and metabolism, P is the least available nutrient in soil (Bieleski 1973). Soils often contain large amounts of both organic and bound (insoluble) mineral phosphorus, however the level of P in the soil solution rarely exceeds 1.5 μM, and is often below the level of many micronutrients (Bieleski 1973). For plants, the orthophosphate anion ($H_2PO_4^-$) is the preferentially absorbed form of P (Schachtman et al 1998). Considered simply from the viewpoint of plant nutrition, mineral phosphorus in soil can be classified into three fractions (Mengel and Kirkby 1982):

1) Phosphate in the soil solution

2) Phosphate in the labile pool

3) Phosphate of the non-labile fraction

As phosphate ions are removed from solution by root absorption, P in solution is replenished from the labile pool. The labile phosphate fraction consists mainly of phosphate absorbed to the surface of clay minerals, such as Fe and Al phosphate. This fraction is in equilibrium with the free phosphate in the soil solution. Because of the

very low solubility of P compounds in soil and the tendency of equilibrium to favour the solid phase, the amount of P in the soil solution at any one time is very low (Holford 1997). The non-labile phosphate fraction consists of soil phosphate minerals. These are mainly minerals of phosphorus combined with calcium, aluminum and iron, either in crystalline form or amorphous phosphate mineral form. Some phosphate in the labile pool can be absorbed and precipitated in mineral particles becoming immobile, non-labile phosphate. Under anaerobic conditions, immobile phosphates can become soluble due to hydrolysis of Al- and Fe-phosphates by anaerobes (Mengel and Kirkby 1982).

Apart from inorganic P compounds, the organic P compounds in the soil may be another P source for plants to use. The organic P compounds are mostly esters of orthophosphoric acid. Inorganic phosphate is released from organic matter by means of a phosphatase reaction. The phosphatase enzyme is produced by the roots of higher plants as well as by numerous microorganisms.

1.3 Phosphorus uptake by plants

Growing plant roots absorb phosphate ions from the soil solution. Evidence suggests that P is taken up as the monovalent form, $H_2PO_4^-$ when soil pH is below 7 (Schachtman et al 1998). Most of the phosphate ions entering the root are taken up by the root hairs or outmost cell layers (Schachtman et al 1998). Phosphate ions move across the cortex either through apoplastic (cell walls and intercellular space) or symplastic (cytoplasm interconnected via plasmodesmata) pathways, then cross the endodermis and enter the xylem (Figure 1.1). Phosphate ions are conducted up to the aerial parts of plants through xylem (Mehra and Farago 1994). There is a requirement for energised transport of phosphate ions across the plasma membrane from the soil to the plant as has been demonstrated by the effects of metabolic inhibitors, which rapidly reduce phosphate uptake (Schachtman et al 1998). The movement of phosphate across the plasma membrane is accomplished by the action of membrane-spanning proteins (phosphate transporters) which co-transport phosphate ions together with H^+ or Na^+ (Mimura 1995;

Martinez and Persson 1998). The genes encoding these phosphate transporter proteins have been recently identified in roots of *Arabidopsis thaliana* (Muchhal et al 1996), in seedlings of *Arabidopsis thaliana* (Smith et al 1997), in potato (Leggewie et al 1997), in tomato (Liu et al 1998 a), and in *Medicago truncatula* (Liu et al 1998 b).

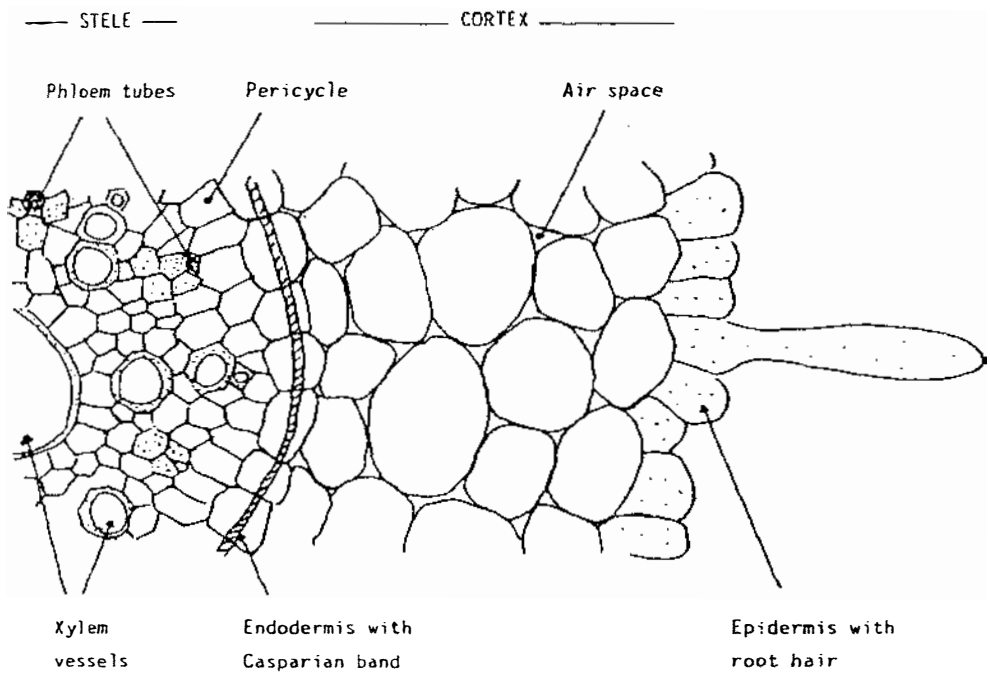

Figure 1.1

Transverse section of a typical root, showing the range of root tissues that Pi crosses before entering the xylem (From Farago 1986).

1.4 Acid phosphatases of plants

Enzymes which catalyse hydrolysis reactions are known as hydrolases and if the compounds acted upon (substrates) are esters, they are known as esterases. If the action is specific to phosphate esters, they are known as phosphoesterases or phosphatases. Phosphatases may also be termed alkaline phosphatases or acid phosphatases according to the pH at which they are most effective. If an enzyme catalyses only monoester hydrolysis it can be known as a phosphomonoesterase. If the action is specific to diesters, it may be known as a phosphodiesterase. Nucleases are a special class of phosphodiesterases which liberate mononucleotides from nucleic acids. Pyrophosphatases are enzymes which split pyrophosphates into orthophosphates (Corbridge 1978). Acid phosphatases (orthophosphoric monoester phospho-hydrolases) (EC3.1.3.2 Webb 1984) catalyse the hydrolysis of a wide range of orthophosphate monoesters with a pH optimum below pH 7.0 (Duff et al 1989), as described in Figure 1.2

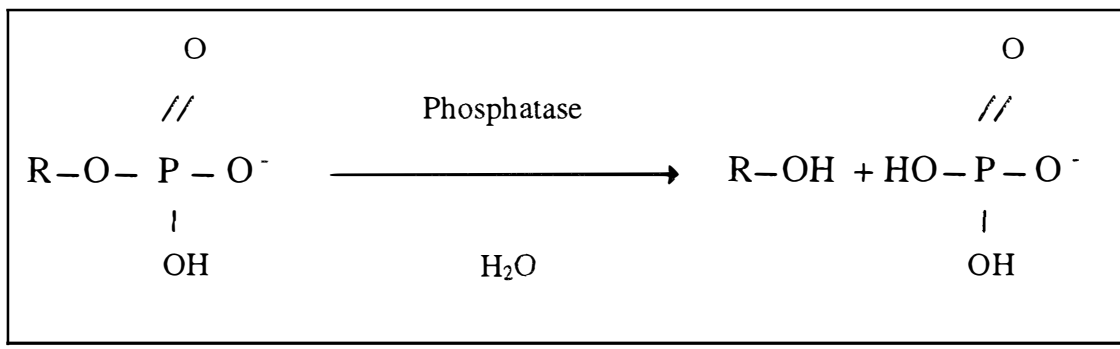

Figure 1.2 Hydrolysis reaction of acid phosphatases

1.4.1 Localisation of plant acid phosphatases

Acid phosphatases (Apases) have been found in all plant species and tissues that have been studied thus far. Acid phosphatases have been found in leaves (De Leo and Sacher 1970; Besford 1979 a; Jonsson 1981; Barrett-Lennard and Greenway 1982; McLachlan et al 1987; Tanaka et al 1990); in soybean root nodules (Penheiter et al 1997), in seeds

(Lorenc-Kubis and Morawiecka 1980; Park and Van Etten 1986; Ullah and Gibson 1988; Pasqualini et al 1996; Biswas et al 1996), storage tubers (Sugawara et al 1981; Kruzel and Morawiecka 1982; Gellatly et al 1994), in roots (Ridge and Rovira 1971; Panara et al 1990; Ozawa et al 1995; Li and Tadano 1996; Ascencio 1997), in bulbs (Guo and Pesacreta 1997) and in cells derived from various tissues in culture (Ueki and Sato 1971; Suzuki and Sato 1976; Kaneko et al 1990; Duff et al 1991 a; LeBansky et al 1992; Theodorou and Plaxton 1996; Kaneko et al 1998). Apases are broadly considered either extracellular or intracellular in terms of location (Duff et al 1994).

Extracellular Apases appear to be ubiquitous in roots and plant cell culture (Duff et al 1994). Evidence for the existence of Apases in cell walls of higher plants have been reported by many researchers (Suzuki and Sato 1976; Crasnier et al 1980; Sugawara et al 1981; Dracup et al 1984; Crasnier and Giordani 1985; Kaneko et al 1990). For example, phosphatase activity from roots of subterranean clover can be eluted using 0.5 M KCl (Dracup et al 1984). Crasnier and Giordani (1985) solubilised 65% of Apase activity from the cell wall of sycamore cells in culture by three washings with 1 M NaCl, but the enzyme remaining bound could not be solubilised by further treatment with higher concentration of salt (up to 5 M). The Apases which can be solubilised by salt are believed to be electrostatically bound to the cell walls, and are termed ionically-bound (Brett and Waldron 1996). The Apases which remain strongly bound to the cell wall after high salt washes are considered to be covalently bond (Dracup et al 1984; Crasnier and Giordani 1985).

A purple acid phosphatase (KBPAP) from red kidney bean has been localised in the cell walls by an activity-based histochemical method involving a lead-precipitate, using ATP as substrate. The free phosphate released by the KBPAP is precipitated as insoluble lead phosphate using lead nitrate, and subsequent electron-microscope studies indicated that the lead precipitate was present only in the cell wall of the cotyledon cells. KBPAP is a cationic protein that strongly binds to the cell wall and requires high salt (0.5M NaCl) for desorption (Cashikar et al 1997). Apase activity has also been located in the cell walls of sycamore cells in culture. Here activity is located principally on external side of the cell wall (Crasnier and Giordani 1985).

Extracellular Apase activity has also been studied with intact roots of wheat (Ridge and Rovira 1971), maize (Clark 1975) and subterranean clover (McLachlan 1980). This root-surface activity is considered to be secreted or comprisng Apase located on the external surfaces of cell walls. For example, in the case of maize roots, the roots were gently rinsed with deonised water before being submerged in the phosphatase assay solution. Root surface Apase activity has also been demonstrated by histochemical staining of whole roots (Boutin et al 1981; Hunter et al 1999). In these experiments, high activity was observed in newly formed root tissue.

Extracellular Apases may be secreted by roots or suspension cells into the surrounding rhizosphere or culture media. The secretion of Apases by tomato roots and lupin roots has been reported by Li & Tadano (1996). In these experiments, tomato or lupin seedlings were grown in 56-L plastic vessels containing nutrient solution which was routinely collected and measured for Apase activity. The secretion of Apases has also been observed from suspension culture cells, such as maize endosperm cultures (Miernyk 1992), soybean suspension cultures (LeBansky et al 1992), *Brassica nigra* suspension cells (Lefebvre et al 1990) and *Brassica napus* L. microspore-derived cultures (Kocsis et al 1996).

Intracellular plant Apases have been mainly localised in the cell vacuole (Nishimura and Beevers 1978; Duff et al 1991 b). Using histochemical and subcellular fractionation methods, a phosphoenol pyruvate (PEP) phosphatase has been purified from *B. nigra* (black mustard) leaf petiole cell suspension cultures and localised in the vacuole (Duff et al 1991 b). Vacuolar acid phosphatases have also been localised in endosperm tissue of 4-day-old castor bean seedlings (Nishimura and Beevers 1978) and in cultured buckwheat cells where 87.5% of protoplasmic acid phosphatase activity was localised in the vacuole (Vögeli-Lange et al 1989).

Intracellular Apases are not only confined to the vacuole, but also exist in the other intracellular locations. The existence of two membrane-bound acid phosphatases in yam tubers was reported by Kamenan and Diopoh (1983). Pfeiffer (1996) reported that acid phosphatases from *Zea mays* coleoptile tissue is associated with the Golgi membranes and Olmos and Hellin (1997) localised acid phosphatases to multivesicular bodies associated with the Golgi bodies of a salt-adapted cell line of *Pisum sativum.* In the

endosperm of date palm, Apase activity has been associated with protein bodies (DeMason et al 1989).

Cytoplasmic acid phosphatase have been purified (or partially purified) and characterised from wheat leaves (Barrett-Lennard and Greenway 1982), barley root (Panara et al 1990) and barley coleoptiles (Pasqualini et al 1997). These enzymes are termed 'soluble' or 'cytosolic' since they are extracted with lower molarity buffers and are contained in the supernatant after centrifugation of the homogeneous slurry. However, their location within the cell (vacuolar/cytoplasmic) has yet to be determined.

1.4.2 Functions of acid phophatases as determined by substrate specificity

Phosphatases are known to play a crucial role in phosphate turnover in plants. However, the exact role of acid phosphatases in plants has been elusive because of insufficient knowledge of their substrate specificities *in vivo*.

Plant Apases have been classified into two distinct categories based on their substrate specificities (Duff et al 1994). One category comprise enzymes which demonstrate clear, but not absolute, substrate specificity, and represent specialised Apases which often exhibit distinct metabolic functions. The second category comprises the non-specific Apases that display no substrate preference. The activity of these non-specific Apases can be induced by various developmental processes including seed germination (Biswas et al 1979; Tamura et al 1982; Biswas et al 1996; Pasqualini et al 1996); seedling development (Bhargava and Sachar 1987; De-Kundu and Banerjee 1990; Haraguchi et al 1990) and stress conditions such as phosphate deficiency (Dracup et al 1984; Kummerová 1986; Goldstein 1992; Tadano et al 1993; Fernandez and Ascencio 1994).

1.4.2.1 Specialised acid phosphatases

Phytase:

Phytase (myo-inositol hexaphosphoric acid phosphohydrolase; EC 3.1.3.8) hydrolyses the phosphate linkage of myo-inositol hexaphosphate to release P and myo-inositol. Unlike some other specialised Apases, phytase are relatively non-specific enzymes that are able to hydrolyse a variety of natural and synthetic P-esters (Duff et al 1994). Gibson and Ullah (1988) have purified and characterised a phytase from soybean seeds, and shown that the purified enzyme possesses a higher affinity for phytic acid when compared with other substrates. The activity of phytase changed during seed germination, and reached a peak from Day 8 to Day 12. In seeds, phytases are believed to be important in mobilising phytic acid, a phosphate reserve, for the growing seedling (Gibson and Ullah 1988).

Phytase has been shown not only to exist in seeds but in other organs and tissues (Hübel and Beck 1996; Li et al 1997 a). Hübel and Beck (1996) purified three phytase isoforms from the roots of 8-day-old maize seedlings. These workers localised both acid phosphatase and phytase activity in maize roots using externally supplied sodium phytate or ρ-nitrophenyl phosphate (ρNPP). The phosphatase activity was found in both the pericycle and the endodermis, while phytase activity was strictly confined to the endodermis of the primary root. The root phytase is proposed to be operative in the utilisation of soil-borne phytate, which usually represents 50% of the organic phosphate compound in soils (Hübel and Beck 1996). Li et al (1997 a) reported the secretion of phytase from the roots of 16 plant species grown with low or adequate supply of P, and also suggested that these secretary phytases may assist in utilisation of soil organic P. Support for a role for phytase in utilisation organic P in soil has come from a number of studies which show that plants are capable of utilising supplied inositol phosphate when grown in hydroponic media or sand culture (Islam et al 1979; Tarafdar and Claassen 1988; Adams and Pate 1992). Adams and Pate (1992) demonstrated inositol phosphate was at least equal to KH_2PO_4 as a source of phosphate for the growth of lupins in sand.

3-PGA Phosphatases

Randall and co-workers (1971) reported the presence of 3-phosphoglycerate (3-PGA) phosphatase in 52 diverse plant taxa. 3-PGA phosphatase has a preference for 3-PGA as a substrate but it is not the only substrate hydrolysed *in vitro.* A 3-PGA phosphatase from sugarcane leaves has been purified 2530-fold (Randall and Tolbert 1971 a) and shown to catalyse the hydrolysis of P from 3-PGA. This may provide an alternate metabolic route from glycerate to serine in place of photorespiration and the glycolate pathway in leaves (Randall and Tolbert 1971 a). A particulate form of 3-phosphoglycetate phosphatase has been localised in starch grains of spinach leaves with a postulated function of regulating glycan synthesis through the control of ADP-glucose pyrophosphorylase activity by the enzyme's effector, 3-P-glycerate (Randall and Tolbert 1971 b).

Phosphoenolpyruvate (PEP) phosphatase

PEP phosphatase displays a clear, but not absolute, substrate specificity for PEP (Duff et al 1989; Duff et al 1991 b). Duff et al (1989) reported the purification and characterisation of a PEP phosphatase from *B. nigra* leaf petiole suspension cells. The PEP phosphatase possessed at least a 6-fold greater affinity for PEP than for any other of 10 non-synthetic substrates assayed. PEP is a key intermediate of plant glycolysis which plays a central role in plant metabolism. During periods of prolonged P deprivation, PEP phosphatase may endow plants with a capability for the continued conversion of PEP to pyruvate despite the fact that pyruvate kinase activity may become limited by the availability of its substrate, ADP. This biochemical adaptation would provide P starved plants with the capacity to utilise P provided by ATP and ADP degradation for the most essential metabolic functions, without impairing the conversion of PEP to pyruvate (Duff et al 1989).

1.4.2.2 Non-specific acid phosphatases

Non-specific acid phosphatases catalyse the dephosphorylation *in vitro* of diverse range of substrates and a large number have been reported in the literature (Duff et al 1994; Kawarasaki et al 1996; Cashikar et al 1997; Guo and Pesacreta 1997; Olczak et al 1997; Pasqualini et al 1997; Ferreira et al 1998 b). The non-specific nature of many of these has been identified by virtue of a direct comparison with the synthetic substrate, ρNPP (Hasegawa et al 1976; Sugawara et al 1981; Basha 1984; Biswas and Cundiff 1991; Pasqualini et al 1997). However, a more accurate comparison is to use the specificity constant (Km/Vmax). Using this parameter of comparison, non-specific acid phosphatases have been identified in red kidney bean (Beck et al 1986), maize (Miernyk 1992), yellow lupin seeds (Olczak et al 1997) and soybean seeds (Ferreira et al 1998 b). In terms of function, these enzymes are proposed to predominate in cell walls and play a role in the hydrolysis of a wide variety of phosphate esters (Duff et al 1994).

1.4.3 Functions of acid phosphatases in regulating specific physiological process

Acid phosphatase in seeds

Many acid phosphatases as distinct enzymes from phytase have been identified and purified from dry seeds (Lorenc-Kubis and Morawiecka 1980; Basha 1984; Teno et al 1987; Cashikar et al 1997; Ferreira et al 1998 b); germinating seeds (Tamura et al 1982; Teno et al 1987; Biswas and Cundiff 1991; Biswas et al 1996) and developing seedlings (DeMason et al 1989; De-Kundu and Banerjee 1990; Haraguchi et al 1990; Pasqualini et al 1996). Phosphate plays an extremely important role in a variety of reactions in germinating seeds, where it appears primarily in an organic form, so that hydrolytic enzymes such as acid phosphatases are needed to release free inorganic orthophosphate. Firenzuoli et al (1969) measured acid phosphatase activity, free phosphate, and total phosphate in different parts of germinating *Pinus pinea* seeds during the first 20 days of germination. They reported that total phosphate remained constant, whereas both acid phosphatase activity and free phosphate increased. It was proposed that acid phosphatase acted on reserve material to release free phosphate from storage tissue to

supply P to the developing seedling (Firenzuoli 1969). An increase of acid phosphatase activity during seed germination has also been observed in *Vigna sinensis* (Biswas and Cundiff 1991), and in developing seedlings of *Vigna radiata* (De-Kundu and Banerjee 1990), *Phoenix dactylifera* (DeMason et al 1989), and *Vigna mungo* (Haraguchi et al 1990). Together, these results suggest that acid phosphatases, in concert with phytases, play an important role in mobilisation of food reserves during seed germination and early seedling development.

Acid phosphatases in roots

Apase activity has been found in roots of a large number of plant species (Chang and Bandurski 1964; Ridge and Rovira 1971; Panara et al 1990; Ozawa et al 1995). In terms of the intracellular forms, these enzymes may be involved in the regulation of the cytosolic Pi levels (Pasqualini et al 1997). In terms of the extracelluar phosphatases, Chang and Bandurski (1964) demonstrated that various organic phosphates were hydrolysed when they were added to a nutrient solution by the roots of intact seedling corn plants. Further, organic phosphorus compounds when added as the sole phosphorus could support the growth of clover (*Trifolium alexandrinum* L.), barley, oats and wheat that the amount of P hydrolysed closely correlated to the measured phosphatase activity (Tarafdar and Claassen 1988). Both experiments support the notion that the function of acid phosphatase activity in roots is to access external sources of P. However, perhaps the best support for such a role for root acid phosphatase at least in terms of the extracellular isoforms, come from extensive studies on the regulation of acid phosphatase activity and P supply.

1.4.4 Regulation of acid phosphatases

Regulation of acid phosphatases by Pi

Many studies have now shown an induction in acid phosphatase activity in response to P-deprivation in different tissue of plants or in cell cultures (Barrett-Lennard and

Greenway 1982, Nakazato et al 1997, Fernandez and Ascencio 1994, Tadano et al 1993, Li et al 1997 b and Ozawa et al 1995). Recently Hunter et al (1999) reported the influence of phosphate status on white clover where plants were grown in either P-containing liquid media, or in media without phosphate, and water-soluble and ionically-bound (1 M salt-extractable) cell wall protein extracts were made from leaf and root tissue. Acid phosphatase activity was highest in all extracts from P-deprived plants with the biggest difference in the root cell wall and the smallest fold-increase was observed in the leaf water-soluble extract. These results suggest that the induced acid phosphatase activity is principally the extracellular acid phosphatases. These results are in agreement with research findings of others with other species including subterranean clover (Dracup et al 1984) and barley (Lee 1988).

When plants are under phosphate stress, the secretion of acid phosphatase from roots increases which is considered to be another important response to phosphorus deficiency (Li et al 1997 b). The secreted acid phosphatases can hydrolyse organic phosphorus in soils, such as nucleotides, phospholipids and sugar phosphates into inorganic phosphate for uptake therefore avoiding phosphorus stress (Barrett-Lennard et al 1993; Li et al 1997 b). Ozawa et al (1995) studied the acid phosphatase secreted by lupin roots under phosphorus deficient conditions and observed the activity of acid phosphatases increased 20-fold when compared with that of control plants. Based on activity staining of acid phosphatase on IEF gels, many isoforms were present in the roots but only one of these was secreted into the rhizosphere. However, no difference in the growth between plants subjected to P-deprivation and the control plants were observed suggesting that lupin plants quickly respond to phosphorus deficiency by the secretion of acid phosphatase before their growth or metabolism was markedly disturbed (Ozawa et al 1995).

Plant suspension cell cultures show similar phosphate-starvation-inducible responses to those of plant roots. Increased activity of acid phosphatases associated with the cell wall and increased secretion of acid phosphatases in response to P-starvation has been reported by many workers (Ueki and Sato 1971; Ueki and Sato 1977; Goldstein et al 1988; Goldstein et al 1989; Lefebvre et al 1990). These results, taken together with the whole plant studies described previously (Boutin et al 1981; Hunter et al 1999) support the proposal that these cell-wall-associated acid phosphatases cleave extracellular

organic phosphates to inorganic phosphates which are readily absorbed by plants (Nakazato et al 1997).

Such studies on the regulation of acid phosphatases have led to the proposal of a second major function of these enzymes, and that is one of a salvage mechanism which convert leaked esterified P to inorganic P which is reabsorbed by plant cells (Lefebvre et al 1990). This function may be an activity of extracellular Apase from roots, and from cell-wall associated phosphatases that are induced by P-deficiency in leaf cell walls (Hunter et al 1999).

Regulation of acid phosphatases by other factors

Phosphorus starvation is not the only stress condition that influences the activity of acid phosphatases. Salt stress, drought and aging have also been shown to influence the activity of the enzyme (Barrett-Lennard and Greenway 1982; McLachlan 1984; Pan 1987; Szabó-Nagy and Erdei 1995). McLachlan (1984) examined the effect of drought, aging and phosphorus status on leaf acid phosphatase activity in wheat. Phosphatase activity increased with plant age, with drought and with phosphorus deficiency. Incremental salt stress brought about a clear enhancement of the activity of acid phosphatase in hydroponically grown spinach leaves (Pan 1987). It has also been reported that elevated atmospheric CO_2 concentrations increased wheat root phosphatase activity when growth was limited by phosphorus (Barrett et al 1998). Some plant hormones can influence the activity of acid phosphatases (De Leo and Sacher 1970; Hooley 1984; Pfeiffer 1996; Pfeiffer 1998). The effect of indole-3-acetic acid (IAA) on extracellular acid phosphatase activity in coleoptiles and mesocotyls from maize seedlings has been studied by Pfeiffer (1996). Segments from maize coleoptiles and mesocotyls were washed with 20 mM MES-buffer containing different concentrations of IAA prior to the measurement of acid phosphatase activity. IAA stimulated extracellular acid phosphatase activity in coleoptiles but not in mesocotyls. A half-maximal increase of phosphatase activity in coleoptiles was found with 5 µM IAA, and a concentration of 30 µM increased the extracellular acid phosphatase activity of coleoptiles by 146% when compared with control (non-treated tissue) (Pfeiffer 1996).

Abscisic acid has also been reported to accelerate the onset and enhance the magnitude of the increase in acid phosphatase activity which accompanies leaf senescence (De Leo and Sacher 1970). In the presence of gibberellic acid (GA_3), aleurone layers and isolated aleurone protoplasts of *Avena fatua* accumulate specific isozymes of acid phosphatase. GA_3 stimulates the enzyme secretion across the plasma membrane and release through, and from, the aleurone cell wall (Hooley 1984). Increased acid phosphatase activity was also found to be correlated with the formation of male flowers on female plants of *Morus nigra* L. after being treated with phthalimides which may act like gibberellins (Lal and Jaiswal 1988).

1.5 Properties of acid phosphatases

1.5.1 Subunit composition and molecular weight of acid phosphatases

Acid phosphatases from plant sources, reported so far, vary in molecular weight and in subunit composition. Monomeric and dimeric structures seem to be a rather general phenomenon among plant acid phosphatases with reported subunit molecular weight values ranging from 27,000 to 100,000 Da.

Monomeric forms of the enzyme have been reported in sycamore cells (Crasnier et al 1980), aleurone particles of rice grain (Yamagata et al 1980), *Poa pratensis* seeds (Lorenc-Kubis and Morawiecka 1980), soybean hypocotyls (Lin et al 1980), needles of *Pinus silvestris* L. (Jonsson 1981), potato tubers (Kruzel and Morawiecka 1982), *Asclepias curassavica* latex (Giordani et al 1986), cotyledons of germinating soybean seeds (Ullah and Gibson 1988), wheat germ (Waymack and Van Etten 1991), maize endosperm cultures (Miernyk 1992), soybean leaves (Staswick et al 1994), roots of lupin (Li and Tadano 1996), tomato roots (Li and Tadano 1996), barley coleoptiles (Pasqualini et al 1997), pea plumules (Guo et al 1998) and tobacco cell culture (Kaneko et al 1998).

Some Apases exist as either homodimers (with two identical subunits) or heterodimers (consisting of two different molecular weight subunits). Some examples of homodimeric Apases are: tomato leaf Apase (Tanaka et al 1990), red kidney bean Apase (Beck et al 1986), sweet potato tuber Apase (Sugiura et al 1981), soybean cell culture Apase (Ferté et al 1993), barley root cytoplasmic Apase (Panara et al 1990), purple Apase secreted by soybean suspension cultures (LeBansky et al 1992), duckweed Apase (Nakazato et al 1997), a homodimer from bulb of *Allium cepa* L. (Guo and Pesacreta 1997). Some examples of heterodimeric Apases that have been reported are: a potato tuber Apase which has significant phosphotyrosine phosphatase activity and consists of 57 kD and 55 kD subunits (Gellatly et al 1994); a yellow lupine seed Apase made up of 50 kD and 44 kD subunits (Olczak et al 1997); an Apase from soybean root nodule made up of 31-kD and 28-kD subunits (Penheiter et al 1997); a heterodimer Apase from sunflower seeds is believed to have two equivalent peptide subunits but with different carbohydrate moieties (Park and Van Etten 1986) and a rye germ Apase which has a native molecular weight 90 kD which consists of 30-kD and 60-kD subunits (Ferens and Morawiecka 1985).

A few of plant acid phosphatases are reported to have multiple subunits. The molecular weight of an Apase from cotton seedlings is 200 kD when determined by molecular sieving on Sephadex G-200, but SDS gel electrophoresis yields only one subunit with a molecular weight of 55 kD. These results suggested that the acid phosphatase is a tetramer, made up of four identical subunits (Bhargava and Sachar 1987). An acid phosphatase from peanut seeds was found to be composed of six identical 42 kD subunits. The apparent molecular weight of hexamer was estimated to be approximately 240 kD by gel filtration chromatography (Basha 1984).

1.5.2 Multiple forms of acid phosphatase

It is well recognised that a large number of enzymes exist in multiple forms. Multiple forms of acid phosphatase have been identified in many plant tissues (Hasegawa et al 1976; Mizuta and Suda 1980; Sugawara et al 1981; Park and Van Etten 1986; Haraguchi et al 1990; De-Kundu and Banerjee 1990; Panara et al 1990; Biswas and

Cundiff 1991; Miernyk 1992; Pasqualini et al 1996; Ferreira et al 1998 b). The terms isozyme and isoform have both been used to describe this phenomenon. It seems most authors use isozymes for those which are different proteins but with similar enzymatic activity and are encoded for by distinct genes. Isoforms are the same proteins with similar enzymatic activity, but differ in some way due to post-translational modifications. However, Apases for the most part have yet to be classified correctly as isozymes or isoforms.

An acid phosphatase purified from sunflower seed has been shown to be a mixture of two isoforms as distinguished by SDS-PAGE. Both isoforms are homodimers comprising 56 kD subunits and 52 kD subunits respectively. Because these isoforms are glycoproteins, the difference in molecular weights between the two enzyme forms is believed to be simply due to the carbohydrate portion since the amino acid composition of each is identical (Park and Van Etten 1986). The use of ion-exchange chromatography to purify soybean root nodule acid phosphatases revealed that there are three isozymes (I, II and III) although amino acid sequencing or composition analysis has yet to confirm this. Fraction I and III each comprised approximately 25% of the total Apase activity and fraction II comprised approximately 50% (Penheiter et al 1997). Acid phosphatase activity in axes of germinating mung beans (*Vigna radiata*) was fractionated into two isozymes (AP-I and AP-II) with a ion-exchange DEAE cellulose column. The molecular weights of AP-I and AP-II are 101 and 118 kD respectively, as determined by gel filtration through a Sephadex G-200 column (De-Kundu and Banerjee 1990). A time-course study of acid phosphatase activity in the cotyledons of germinating *Vigna mungo* seeds demonstrated that isozymes are differentially regulated during seedling development. Extracts from the day-6 cotyledons of *Vigna mungo* contained three forms of acid phosphatases (a, b and c), but extracts from cotyledons of dry seeds (day 0) showed only two forms (a and b) (Tamura et al 1982).

The expression of multiple forms of acid phosphatases are also regulated by environmental factors (Pan 1987; Barrett-Lennard and Greenway 1982). Multiple forms of acid phosphatase separated as high (300 kD), intermediate (100 kD) and low (35 kD) molecular weight were observed in spinach leaves. Comparisons of these multiple acid phosphatases after salt-stress suggested that an incremental salt stress caused increased

activity which was contributed solely by the 300 kD isozyme (Pan 1987). Specific isozymes have been shown to be induced in response to P-deprivation in wheat leaves (Barrett-Lennard and Greenway 1982; McLachlan 1984; McLachlan et al 1987) and in tobacco cell cultures (Ueki and Sato 1977). Soluble phosphatases from wheat leaves were separated into two fractions by CM-cellulose chromatography. Phosphatase activity in the fraction absorbed by CM-cellulose chromatography increased with P deficiency and was found only in 'low P' but not 'high P' plants. Phosphatase activity in the fraction not absorbed by CM-cellulose increased with both P deficiency and water deficit (Barrett-Lennard and Greenway 1982). The occurrence of multiple forms of acid phosphatases in plants is an ubiquitous phenomenon and regulated by developmental and physiological as well as environmental factors. However, it should be noted that the term "isozyme" cannot be used unequivocally until these proteins are characterised further at the gene levels.

1.5.3 Glycosylation of acid phosphatases

Many plant acid phosphatases have been reported to be glycoproteins including an Apase isolated from *Poa pratensis* seeds (Lorenc-Kubis and Morawiecka 1980), a cell wall Apase from sycamore cell cultures (Crasnier et al 1980), an Apase from tomato leaves (Tanaka et al 1990), a secreted Apase from maize endosperm cultures (Miernyk 1992), an Apase from potato tuber (Gellatly et al 1994), a secreted purple Apase from soybean suspension cultures (LeBansky et al 1992), an Apase from soybean leaf (Staswick et al 1994), the 63 kD soluble Apase from barley coleoptiles (Pasqualini et al 1997), an Apase from the bulb of *Allium cepa* L. (Guo and Pesacreta 1997) and an Apase from soybean root nodule (Penheiter et al 1997).

Concanavalin A (Con A) affinity column and periodic acid-Schiff techniques are commonly used for glycoprotein identification. Con A is a lectin isolated from jack bean (*Canavalia ensiformis*) meal and is an ideal general ligand for use with a variety of polysaccharides and glycoproteins (Lowe and Dean 1974). For example, the Apase Ia_1 from *Vigna mungo* seedlings was considered a glycoprotein because of its absorption to a Con A-agarose column. This was supported by treatment with

trifluoromethanesulfonic acid for 30 min at 0^0C, after which the molecular weight of Ia_1 was reduced from 53 kD to 51 kD, suggesting the removal of a 2 kD carbohydrate moiety (Haraguchi et al 1990). A Sycamore cell wall Apase was shown to be glycoprotein by periodic acid-Schiff reagent following acrylamide gel electrophoresis (Crasnier et al 1980).

Con A affinity chromatography is very effective in the purification of some glycosylated Apases and has been used in the purification of the enzyme from a large number of plant tissues. For example, an Apase from yellow lupin seeds was purified 20-fold with a Con A-Sepharose column (Olczak et al 1997), and maize endosperm secretary Apase was bound to Con A-Sephorose column and then eluted off with α-methyl-D-mannoside. This indicates that it is a glycoprotein with (at least) a high mannose-type glycan side chain (Miernyk 1992).

To obtain some information about the structural features of the oligosaccharide chains, the interactions between an Apase from yellow lupin seeds and various lectins were studied by Olczak et al (1997). The enzyme strongly interacted with GNA and AAA, indicating the presence of terminal mannose sugars and the fucosylation of the N-glycosidic oligosaccharide chains (Olczak et al 1997).

A more detailed analysis of oligosaccharide chains attached to red kidney bean purple acid phosphatase has been undertaken using a combination of selective enzymic degradation and matrix-assisted laser desorption/ionization mass spectrometry (Stahl et al 1994). These studies revealed that the dimeric purple acid phosphatase contains five oligosaccharide chains on each subunit and each glycan was shown to have a complex-type xylose-containing structure with four of the five having an additional fucose.

1.5.4 Activators and inhibitors of acid phosphatases

In order to better understand the mechanism of action of Apases, various agents have been tested for their effects on the activity of the enzymes, including various metal ions, anions and chelating agents.

Inorganic phosphate (Pi), a product of phosphatase hydrolysis whatever the substrate, is a potent competitive inhibitor (Giordani et al 1986; Biswas and Cundiff 1991; Duff et al 1991 b; Li and Tadano 1996; Cashikar et al 1997; Nakazato et al 1997; Olczak et al 1997). It is proposed that Pi inhibition could play a physiological role in sustaining a stable level of phosphate in the cell through a feedback regulation of phosphatase activity.

Molybdate is a potent inhibitor to almost all Apases tested so far (Ching et al 1987; Biswas and Cundiff 1991; Guo and Roux 1995; Kawarasaki et al 1996; Olczak et al 1997; Pasqualini et al 1997; Penheiter et al 1997; Ferreira et al 1998 b). Zinc (Zn^{2+}) is an activator for a cell wall Apase from potato tuber (Tu et al 1988), but more often is either an inhibitor of the enzyme (Uehara et al 1974; Yamagata et al 1980; Sugawara et al 1981; Guo and Roux 1995; Kawarasaki et al 1996; Olczak et al 1997), or has no obvious effect (Ching et al 1987; Li and Tadano 1996; Penheiter et al 1997;). Copper (Cu^{2+}), iron (Fe^{3+}) and fluoride (F^-) are reported to be inhibitory to some Apases (Uehara et al 1974; Yamagata et al 1980; Sugawara et al 1981; Biswas and Cundiff 1991; Guo and Roux 1995; Kawarasaki et al 1996; Olczak et al 1997).

Fewer activators of the enzyme have been reported when compared with inhibitors, but a few reports detail the activation of Apase by divalent cations. In addition to the activation of a cell wall Apase from potato (Tu et al 1988) by Zn^{2+}, the metal ion has also been shown to increase the activity of an Apase isolated from tomato leaves 7-fold, while the addition of Mg^{2+} and Mn^{2+} increased the activity 5-fold (Tanaka et al 1990). The cell wall acid phosphatase (1 M NaCl extractable) from potato tuber showed a 2 or 3-fold activity stimulation by Mg^{2+} and a slight stimulation by Ca^{2+} (Sugawara et al 1981).

The discovery of metals bound to some Apases has triggered studies on the roles that metals play in Apase activity (Uehara et al 1974; Beck et al 1986; Sträter et al 1992; Klabunde et al 1996; Grote et al 1998). Violet-colored Apase of sweet potato contains both manganese and magnesium (Uehara et al 1974). To determine whether or not these metals are essential components of the enzyme, the chelating agents 2 mM EDTA, CyDTA (*trans*-1,2-cyclohexanediamine-N, N, N', N'-tetraacetic acid), α,α'-dipyridyl and *o*-phenanthroline were tested. No significant inactivation of the enzyme was

observed with EDTA or CyDTA. However the enzyme was inactivated by prior incubation with α,α'-dipyridyl and *o*-phenanthroline. The restoration of the activity of the *o*-phenanthroline treated enzyme was carried out with the addition of various metal ions. Zinc resulted in complete restoration of the activity. Manganese and cobalt were also very effective. Magnesium and calcium were completely ineffective. This result indicates that manganese is an essential component of sweet potato acid phosphatase (Uehara et al 1974). A manganese-containing acid phosphatase has been isolated and crystallised from sweet potato tubers (Sugiura et al 1981). The Mn ion present in the enzyme plays an essential role in the catalytic hydrolysis reaction. It is speculated that this enzyme uses the Mn(III) ion to induce effective binding of phosphate substrate and maintain structural stability of the enzyme in an acidic environment. The dimeric kidney bean purple acid phosphatase contains Fe(III) and Zn(II) in the active sites (Klabunde et al 1996). Metal-assisted catalysis has been proposed for the enzyme, featuring a binding of the substrate to Zn(II) and nucleophilic attack by an Fe(III)-bound hydroxide ion. Although many plant acid phosphatases have yet to be clarified in terms of existence of metal ions in their structures (Hasegawa et al 1976; Suzuki and Sato 1976; Crasnier et al 1980; Sugawara et al 1981; Bhargava and Sachar 1987; Haraguchi et al 1990; Kaneko et al 1990; Duff et al 1991 b; Kaneko et al 1998), the metal chelating agent EDTA has been widely used in study of the requirement of metal ions of acid phosphatase activity (Uehara et al 1974; Yamagata et al 1980; Ching et al 1987; De-Kundu and Banerjee 1990; Ferté et al 1993; Guo and Roux 1995; Nakazato et al 1997). EDTA demonstrated differential effects on Apases from different plant tissues. EDTA showed a 2.5-fold stimulating effect on a tyrosine phosphatase from pea nuclei (Guo and Roux 1995), a 40% inhibition of the activity of soybean cell wall Apase (Ferté et al 1993), but had no effect on the Apase from duckweed (Nakazato et al 1997), and violet-colored Apase from sweet potato (Uehara et al 1974).

1.6 Amino acid sequencing and the identification of acid phosphatase genes

Although many acid phosphatases have been identified, purified and characterised in various plant tissues, very few studies have been undertaken to determine amino acid sequences of the enzyme. Partial N-terminal amino acid sequence of purified potato phosphatase was reported by Polya and Wettenhall (1992). The sequence containing 27 amino acid residues and subsequent database analysis revealed a sequence homology with storage glycoproteins from soybean leaf and stem tissues. A N-terminal sequence containing 23 amino acid residues of the phosphatase from pea plumules was determined by Guo et al (1998), and had 75% identity with the N-terminus of a sequence translated from a cDNA of castor bean. Short sequences of 15, 16 and 14 amino acid residues of Apases from wheat germ, soybean suspension cells and tomato leaves were determined but no homologous sequences were found when aligned with the protein sequence database (Tanaka et al 1990; LeBansky et al 1992; Kawarasaki et al 1996).

A few genes of acid phosphatase have now been cloned including a phytase gene from maize seedlings (Maugenest et al 1997) and soluble inorganic pyrophosphatase from barley germinating grains (Visser et al 1998). Other acid phosphatases appearing on the database include Apase from tomato (Accession number: P27061) and soybean (Accession number: CAA11075). It is anticipated that the identification and sequencing of more Apases of plant origin will identify molecular families of the enzyme which may provide important clues toward function, in the same way as has been observed in bacterial and other eukaryote species (Thaller et al 1998).

1.7 White Clover

White clover (*Trifolium repens* L.) is the plant used in this thesis for studying acid phosphatases induced in response to phosphate deprivation. White clover belongs to the genus *Trifolium* which consists of 250 to 300 species. This genus is classified taxonomically in the family Leguminosae (Heywood 1971). White clover leaves are

characteristically trifoliate except the first unifoliate leaf. The main stem is short, with only 2 or 3 terminal nodes without stolons. The prostrate stolon is the basic structural unit of the plant. It consists of a series of internodes separated by nodes. Each node bears a trifololate leaf with an erect petiole, two root primordia and an axillary bud which is capable of growing into a lateral stolon. Production of lateral stolons leads to the vegetative spread of a plant. When the root primordium at a node is in contact with a moist substratum it grows out into nodal roots, providing a degree of nutritional independence to each lateral stolon (Thomas 1987). A lateral stolon with nodal roots can be severed from the parental plant after which it develops into an independent individual. It is ideal therefore to use stolon cuttings as means of producing clonal material for experiments.

1.7.1 White clover in New Zealand agriculture

White clover is one of the most nutritious and widely distributed forage legumes of the world. In New Zealand pastures, white clover is largely grown in combination with ryegrass (Bowling and Dunlop 1978). White clover benefits pastoral agriculture in the following ways (Gibson 1966):

(1) As a forage legume: It provides a highly nutritive feed as pasture, hay, and silage for livestock and poultry.

(2) By fixing atmospheric nitrogen.

(3) As a cover crop: The growth of stolons provides a ground cover that promotes soil stabilisation and reduces erosion.

The New Zealand agricultural industry is based to a considerable extent on a ryegrass-clover system which requires substantial inputs of phosphate to achieve optimum production. Each year, approximately two million tons of phosphorus fertilizer are applied to New Zealand agricultural land and a considerable proportion of this is lost via stock transfer, soil erosion and water runoff (Ward et al 1985). The application of

phosphorus fertilizer is not only costly to farmers, but is also contaminative to the environment. When phosphorus fertilizer reaches waterways (rivers and lakes) through water runoff and land erosion, growth of aquatic plants is promoted and excessive growth results in water quality degradation.

1.7.2 White clover and phosphate uptake and utilisation

Although white clover is a basic constituent of most productive pastures (Spencer and Hely 1982), it has been found to be a poor competitor for P, K, S, especially when P, K, and S are limiting (Dunlop and Hart 1987). The root morphology of white clover contributes to its poor competitive ability. Its thicker and less profusely branched root system explores the soil less thoroughly than the fine, extensively branched root systems of grasses (Dunlop and Hart 1987). Although white clover roots don't compete very well with the grass roots, it has been reported that white clover stolons are capable of absorbing phosphate from soil surface (Hay and Dunlop 1982). The position of stolons in pastures allows them to absorb phosphate without competition with grass roots. Because of the importance of white clover in pastures, detailed investigations of white clover germplasm in terms of P-utilisation and plant growth have been undertaken (Dunlop et al 1990; Caradus et al 1992). In terms of molecular studies on P acquisition by white clover, Bowling and Dunlop (1978) demonstrated that phosphate uptake is correlated with the membrane electrical potential difference of the cortical cells of the white clover roots and suggested the existence of an electrogenic phosphate pump in the membrane. Further, phosphate absorption by higher plants grown with adequate phosphate involves cotransport with protons via a H^+-$H_2PO_4^-$ symport in roots of *T. repens* operating with a stoichiometry of 1:1 (Dunlop 1989). Dunlop and Gardiner (1993) also demonstrated a difference in the characteristics of phosphate absorption between phosphate-adequate and phosphate-deficient plants in terms of the stoichiometry of proton-phosphate cotransport.

Under phosphate starvation white clover has been shown to display increased acid phosphatase activity in both leaf and root tissues (Caradus and Snaydon 1987; Hunter et al 1999). Caradus and Snaydon (1987) measured white clover root exocellular Apase

activity by suspending the intact root systems in the assay solution and reported that the root exocellular Apase activity increased with decreasing P in the growth media. Hunter et al (1999) examined both soluble and cell wall acid phosphatase activity in leaf and root tissues of white clover grown in P-deprived condition. No significant enhancement of activity was observed in isozymes from soluble extracts of leaf tissue, but the activity of two cell wall isoforms from leaf tissue increased. In the soluble root extract, a major staining lower mobility isozyme and a minor staining higher mobility isozyme are both enhanced. In the root cell wall extract, both of the two isozymes are clearly enhanced (Hunter et al 1999).

While constitutive Apase activity has been demonstrated in high salt extractions of plant cell walls (Hall and Butt 1968; Suzuki and Sato 1973; Hasegawa et al 1976; Igaue et al 1976; Crasnier et al 1980; Sugawara et al 1981; Dracup et al 1984; Tu et al 1988; Kaneko et al 1990; Ferté et al 1993), there have been fewer studies in which the induction of this group of Apases in response to P-deficiency in cell wall extracts have been characterised (Ueki and Sato 1977; Hunter and McManus 1999; Hunter et al 1999). Previous to the studies published by Hunter and McManus (1999) and Hunter et al (1999), there has been no report in which the induction of ionically-bound (high salt extractable) Apase by P-deficiency/deprivation in roots of whole plants has been studied. The main aim of this thesis, therefore, is to characterise this group of Apases which have been shown to respond to P-deficiency in cell walls of white clover roots.

1.8 Thesis aims

(1)To identify the response of a single genotype of white clover to phosphorus starvation by measuring levels of phosphorus in leaves and acid phosphatase activity in roots.

(2)To identify acid phosphatases in the roots of white clover grown under P-deprived conditions and to compare these with those which occur in roots of white clover grown in P-supplied conditions.

(3)To isolate and characterise acid phosphatases from the roots of white clover grown under P-deprived conditions particularly in terms of substrate specificity and other characteristics to determine a potential role for the enzyme(s).

1.9 Terminology used in this thesis

A survey of the quite extensive literature concerning the purification and characterisation of Apases reveals different terminologies used to describe these enzymes. The terms (intracellular / soluble; extracelullar / cell wall / secreted) reflect cellular location, although very few studies on the precise cellular location have been reported. The soluble enzymes are those extracted with lower molarity buffers (or 25 mM 2-mercaptoethanol) and are considered to include the cytosolic fraction (cytoplasmic/vacuolar). However, if whole roots are used in the extraction, the preparation may also contain the secreted (root surface) isoforms which are not tightly bound to the wall.

Cell wall acid phosphatases (the extracellular proteins) can contain the secreted, the ionically-bound and the covalently bound forms and are generally denoted by the extraction method used. Secreted forms can be detected in the surrounding medium and are not generally considered to be bound to the wall. The ionically-bound forms are those that can be eluted from the cell wall pellet using high concentrations (0.5 M, 1.0 M) of salt (NaCl, KCl) and the covalently-bound forms are those remaining after exhaustive salt washing.

For the purposes of this thesis, the ionically-bound (high salt extractable) acid phosphatase are being studied and are referred to as cell wall Apases. The covalently-bound enzymes are referred to as cell-wall-bound and the secreted forms remain as secreted.

Chapter Two: Material and Methods

2.1 Plant material

White clover (*Trifolium repens* L., cultivar Huia, genotype: PgH_2) was obtained from the New Zealand Pastoral Agricultural Research Institute Inc. (AgResearch Grasslands) at Palmerston North, New Zealand.

2.2 Growth of white clover plants

2.2.1 Stock plants

Stock plants of white clover were grown in trays of horticultural-grade sand and watered daily and supplemented with Hoaglands solution (Hoagland and Arnon 1950) once a week. Plants were maintained in a heated glasshouse in the Plant Growth Unit, Massey University, Palmerston North. The minimum temperature of the glasshouse was 15 ^{0}C and the ventilation temperature was set at 25 ^{0}C.

2.2.2 Growth of white clover in liquid media

Half-strength Hoaglands solution was used as the liquid medium in all experiments.

White clover apical cuttings with 3-4 leaf nodes were excised from stock plants below a node from which nodal roots had been well established (usually node 4) (Figure 2.1). These cuttings were grown for 2 weeks in half strength Hoaglands liquid medium contained within 1 L plastic beakers which were wrapped with black plastic to exclude light. The beaker was covered with a lid made of black plastic with a hole in the center. The white clover shoots were supported by a U-shaped metal wire sitting on top of the beaker and the roots were placed through the hole of the lid and submerged in the liquid media.

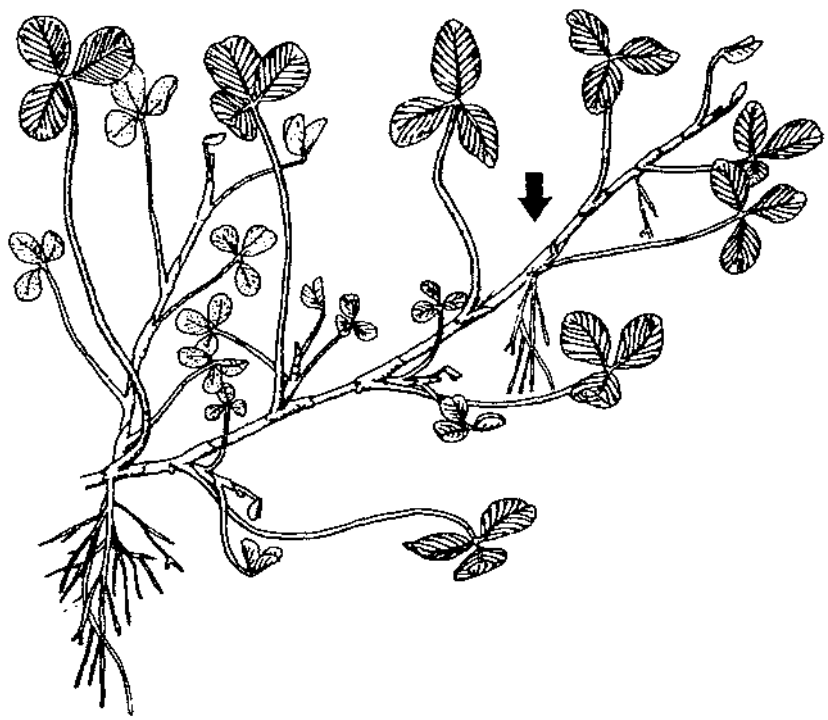

Figure 2.1

Stolon growth of white clover (From Thomas 1987). The arrow denotes the site of excision, basal to the first major rooted node, to obtain apical cuttings for use in experiments.

In order to minimiSe bacterial and fungal contamination, both plant cuttings and the plant growth containers were washed with 5% (v/v) industrial bleach (35 g/L sodium hypochlorite, Franklins Limited, Australia) and rinsed with milli-Q water before the excised apical plant cuttings were transferred to the liquid medium.

After two or three weeks growth in phosphate-supplied half-strength Hoaglands solution, white clover plants were then divided into populations of plants maintained either in P-deprived Hoaglands medium (-P) or in P-supplied medium (+P) for the appropriate time interval. To harvest root tissue, whole plants were removed from the media, the roots were then severed *ca.* 1 cm below the crown, washed in water, blotted dry between tissue paper, weighed, wrapped in tin foil, frozen in liquid nitrogen and stored at -80 ^{0}C until further use.

To harvest leaf tissue, the first fully expanded leaf (usually subtending from node 3) was collected, weighed and placed individually into labeled eppendorf tubes, then frozen in liquid nitrogen and stored at -80 ^{0}C until required.

2.3 Chemicals

Unless otherwise stated, the chemical reagents used were analytical grade, obtained from BDH Laboratory Supplies (Poole, BH15 1 TD, England), Sigma Chemicals Company (St. Louis, Mo., USA), Bio-Rad Laboratories (Richmond, CA, USA) and Pharmacia Biotech (Uppsala, Sweden). The laboratory supply of purified water used for making solutions was produced by reverse-osmosis (RO), followed by passage through a microfiltration system containing ion exchange, solvent exchange, organic and inorganic removal cartridges (Milli-Q, Millipore Corp., Bedford, Massachusetts, USA).

2.4 Measurement of leaf phosphate

Reagents:

- Concentrated phosphate assay reagent: 16 mM ammonium molybdate, 2.25 mM H_2SO_4, 0.15 mM antimony potassium tartrate (oxide) (Dilute concentrated phosphate assay reagent: Dissolve 0.87 g of ascorbic acid in 25 mL dH_2O, add 6.25 mL of stock solution and dilute to 50 mL with dH_2O. This reagent was prepared fresh just before use.)

- Phosphate standard solution (0.2 mM KH_2PO_4)

- 5 M H_2SO_4

2.4.1 Procedure for Pi standard curve

To make a series of phosphate standards comprising final concentrations of 0, 40, 80, 120, 160 and 200 μM, aliquots 0, 10, 20, 30, 40 and 50 μL respectively of the phosphate standard solution were pipetted into microtitre plate wells (Nunc, A/S, Roskilde, Denmark) in triplicate and then corresponding volumes of H_2O were added to bring each well to 50 μL.

2.4.2 Preparation of samples

Frozen leaf tissue (30-32 mg fresh weight) was ground to a fine powder and extracted with 100 μL of 5 M H_2SO_4 by continually grinding for a further 1 min. The extract, comprising a small mass of pulp in the bottom of the eppendorf tube was diluted by adding 1 mL H_2O into each tube. The tubes were vortexed at room temperature for 30 sec and then centrifuged at 13,000 x g for 3 min. A 10 μL aliquot of the supernatant was pipetted into microtitre plate wells in triplicate and adjusted to 50 μL by adding 40 μL H_2O in each well.

2.4.3 Measurement of phosphate levels

To each sample or standard, 200 µL of diluted phosphate assay reagent was added and the blue color that subsequently developed was measured at 620 nm using an Anthos HTII plate reader (Anthos labtec Instruments, Salzbury, Austria). A linear relationship was obtained between the absorbance at 620 nm and concentrations of Pi over the concentration range of 0 to 200 µM (Figure 2.2). Phosphate levels (expressed as % P g^{-1} fresh weight) in leaf extract were calculated from this phosphate standard curve.

2.5 Measurement of protein concentration

Reagents:

- Coomassie Brilliant Blue G-250 concentrate dye reagent (Bio-Rad Protein Assay Concentrate)
- BSA (Bovine serum albumin, Fraction V standard grade, Serva Feinbiochemica, Heidelberg, Germany)

Protein concentration was estimated by a microassay, based on the method of Bradford (Bradford 1976), using a commercially available dye reagent (Bio-Rad Protein Assay Concentrate).

2.5.1 Standard curve for Bio-Rad Protein Assay

A BSA solution containing 0, 2, 4, 6, 8 and 10 µg protein in a volume of 160 µL was pipetted in triplicate into microtitre plate wells. To each well, 40 µL of Bio-Rad protein assay dye reagent was added, the protein and reagent were mixed thoroughly and after standing for 5 min, the absorbance at 595 nm was measured using an Anthos HTII plate reader (Figure 2.3).

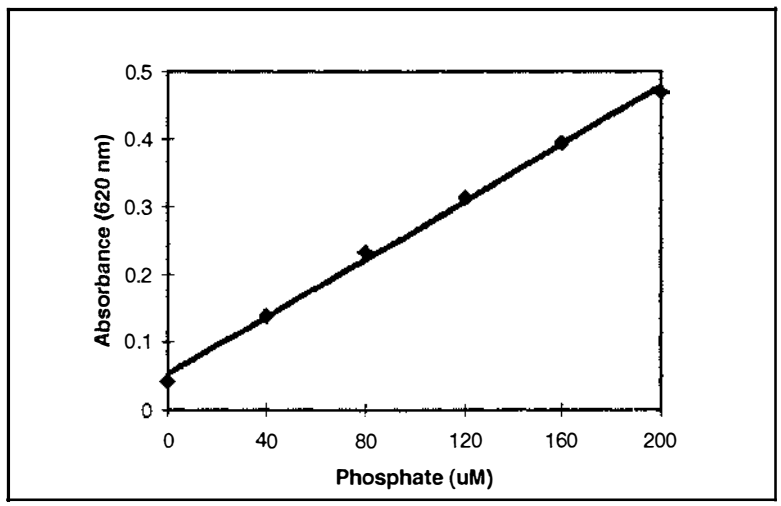

Figure 2.2
Phosphate standard curve for leaf phosphate content determination.

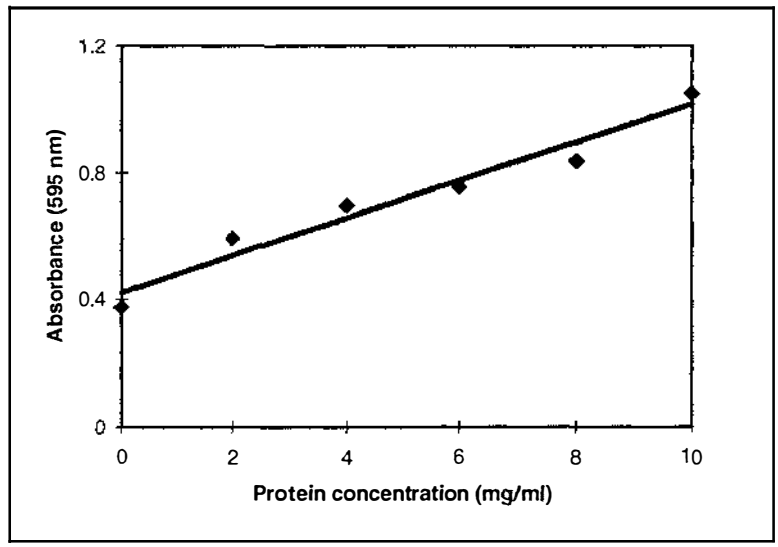

Figure 2.3
An example of a protein (BSA) standard curve for the Bio-Rad protein microassay procedure.

2.5.2 Quantitation of protein content in root tissue extracts

Extracts were diluted 1:10 with water before protein measurement. Aliquots (10 µL) of the diluted extract were pipetted into microtitre plate wells in triplicate, and made up to 160 µL with water. To each well, 40 µL Bio-Rad dye reagent was added and mixed well. The absorbance was read at 595 nm using an Anthos HTII plate reader. The protein content in the sample was determined by comparison of the absorbance against the BSA standard curve.

2.6 Acid phosphatase activity assays

Reagents:

- 50 mM Citrate buffer (Lillie 1948)
- 50 mM Maleic acid-Tris-NaOH buffer (Gomori 1955)
- ρ-Nitrophenyl phosphate (ρNPP) tablets (Sigma)
- Adenosine 5'-Triphosphate (ATP), disodium salt (Sigma)
- Phospho (enol) pyruvate (PEP), trisodium salt (Sigma)
- O-phospho-L-tyrosine (Sigma)
- D (-) 3-phosphoglyceric acid (3-PGA), disodium salt (Sigma)
- O-phospho-L-serine (Sigma)
- Phytic acid, dodecasodium salt (Sigma)
- Sodium pyrophosphate (PPi) (Sigma)
- Phosphate reagent (see section 2.4)

- 1M NaOH

2.6.1 Phosphatase activity assay A:

For routine assays of acid phosphatase, the hydrolysis of ρNPP to ρNP was carried out, unless otherwise specified, in 50 mM citrate buffer pH 5.6. In each assay, 2-20 µL of the appropriate was made up to 50 µL with buffer and incubated at 37 ^{0}C for 5 min before the addition of 200 µL substrate solution (8 mM ρNPP in 50 mM citrate buffer) also equilibrated at 37 ^{0}C. After the appropriate time interval (5-10 min), 50 µL of reaction mixture was removed and added to 50 µL NaOH in the wells of the microplate to terminate the enzymatic reaction. The absorbance of liberated ρNP was read at 405 nm using the Anthos HTII plate reader. The amount of liberated ρNP by acid phosphatase activity was determined by comparison of the absorbance against the ρNP standard curve (Figure 2.4). The absorption of the substrate solution without the addition of the enzyme was measured at 405 nm as a blank.

2.6.2 Modifications to phosphatase assay A

To determine the pH optima of each isoform, 8 mM ρNPP was made up with either 50 mM citrate buffer to provide a pH range of 3.0 to 6.2, or 50 mM maleic acid-Tris-NaOH buffer to provide a pH range of 5.2 to 8.6. The activity of each isoform was measured as in Assay A.

To determine the effects of inorganic phosphate on the activity of each isoform, a series of 1, 2.5, 5, 10 and 20 mM sodium phosphate ($Na_2HPO_4.2H_2O$, BDH Laboratory Supplies) was added to 8 mM ρNPP substrate in maleic acid-Tris-NaOH of the optimal pH of each isoform, and the activity was measured as described in Assay A.

To determine the effects of metal ions, tartrate and EDTA on activity, 5 mM final concentration of each was added to 8 mM ρNPP substrate in maleic acid-Tris-NaOH buffer at the optimal pH for each isoform and the activity was measured as described in Assay A.

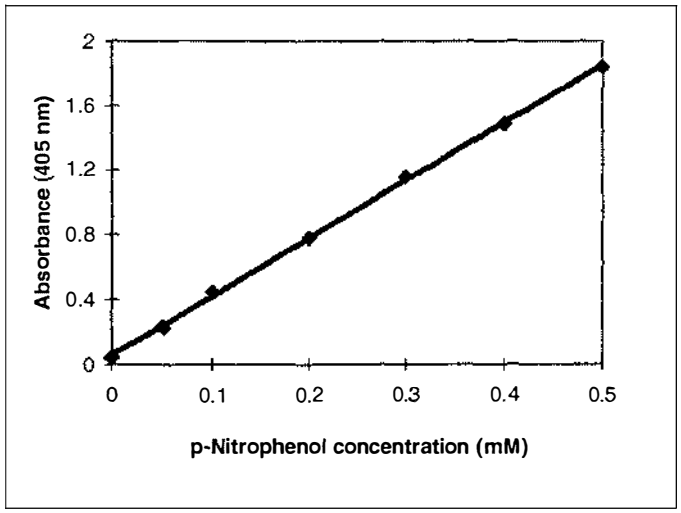

Figure 2.4

Nitrophenol (ρNP) standard curve for the determination of the amount of ρNP liberated by hydrolysis of acid phosphatases.

2.6.3 Phosphatase activity assay B:

For substrates other than ρNPP, the release of orthophosphate from phosphorylated substrates was determined by measuring the release of inorganic phosphate. The substrates assayed were: ATP, PPi, PEP, 3-PGA, phospho-tyrosine, phsopho-serine and phytic acid. The buffer used was 50 mM maleic acid-Tris-NaOH at the optimal pH determined for each isoform preparation. An aliquot (5 µL) of enzyme preparation was added to 245 µL of substrate solution pre-equilibrated at 37 ^{0}C. After 5 min, aliquots (50 µL) reaction mixture were pipetted into 200 µL phosphate reagent (see section 2.4) for inorganic phosphate determination. The absorbance of developed blue color was measured at 620 mm. The amount of Pi already present in each substrate was determined by the identical incubation at 37 ^{0}C without the addition of enzyme. In order to calculate the amount of liberated Pi from the substrates, a standard curve of Pi was established by plotting a range of Pi concentrations (0 to1.0 mM) versus absorbance at 620 nm (Figure 2.5).

2.6.4 Determination of kinetic constants

To determine the Michaelis constant (Km) and maximal velocity (Vmax) for ρNPP for each isoform, Assay A was used. For substrates rather than ρNPP, Assay B was used. A range of substrate concentrations (0.05-10 mM) for each substrate were examined in initial trials and a preliminary value of Km was then estimated (as *ca.* 50% of the Vmax). Eight substrate concentrations ranging from 0.2-fold to 5.0-fold this preliminary value were then used in kinetic parameter study. The Km and Vmax of acid phosphatase isoforms were determined graphically from Lineweaver-Burk reciprocal plots using the computer kinetics program, ENZYPLOT (Walker 1997).

2.7 Extraction of ionically bound cell wall proteins

Reagent:

- 25 mM 2-mercaptoethanol

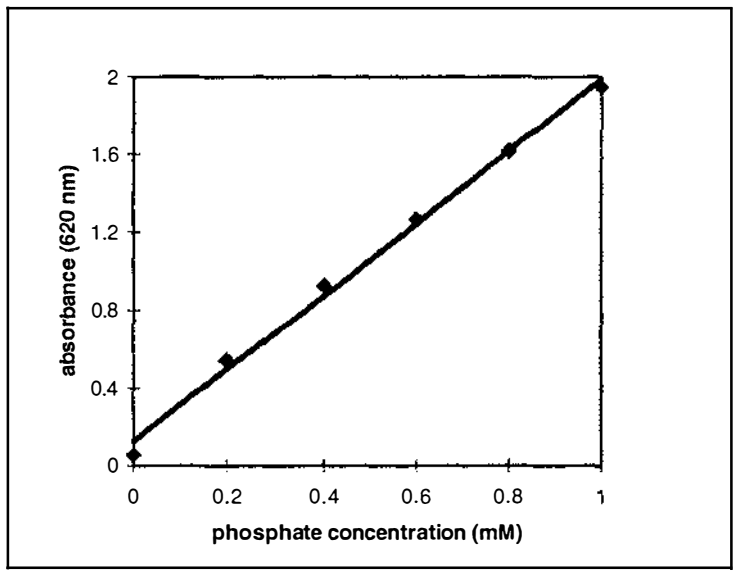

Figure 2.5

Phosphate standard curve for the calculation of Pi released from a range of substrates used for the determination of Km and Vmax.

- 1 M NaCl

Frozen (-80 ^{0}C) white clover root tissue (typically 300 to 900 g) was homogenised with 3 volumes of root weight of 25 mM 2-mercaptoethanol in a Warring blender (Warring product division, Dymanics Corporation of America, New Hartford, Connecticut, USA). The homogenate was incubated on ice for half an hour, then centrifuged at 12,000 x g for 10 min at 4 ^{0}C, the supernatant removed and the pellet extracted with 25 mM 2-mercaptoethanol twice more. The pellet was then resuspended with water and centrifuged. This water wash step was repeated up to 10 times. The acid phosphatase activity of all supernatants was measured using assay A (Figure 2.6). The activity measured in the three 2-mercaptoethanol supernatants was high after which activity decreased in the water wash supernatants, until it was non-detectable in the supernatants of last two water washes. The supernatants of the 2-mercaptoethanol and water extracts contain the soluble acid phosphatases and was not studied further as part of this thesis. After the final water wash, the pellet was suspended with 1 volume of 1M NaCl at 37 ^{0}C for 1 hour, and then centrifuged at 12,000 x g for 20 min. The supernatant was decanted and stored at 4 ^{0}C. The pellet was suspended with the same volume of 1M NaCl again and incubated at 4 ^{0}C for 16 hrs. The suspension was then centrifuged and the supernatant was decanted carefully to avoid disturbance. The supernatant fractions of 1 M NaCl extracts showed increased activity (Figure 2.6) and contained the ionically-bound (high salt extractable) cell wall acid phosphatases. The pooled supernatant fractions of the first and second of 1 M NaCl extractions (designated the cell wall extract) was then concentrated to 10 to 50 mL with a Filtron stirred cell (150 mL, Filtron Technology Corporation, Northborough, MA, USA).

2.8 Purification of acid phosphatases

Reagents:

- 2 M $(NH_4)_2SO_4$, pH 5.6
- 20 mM KH_2PO_4, pH 7.0

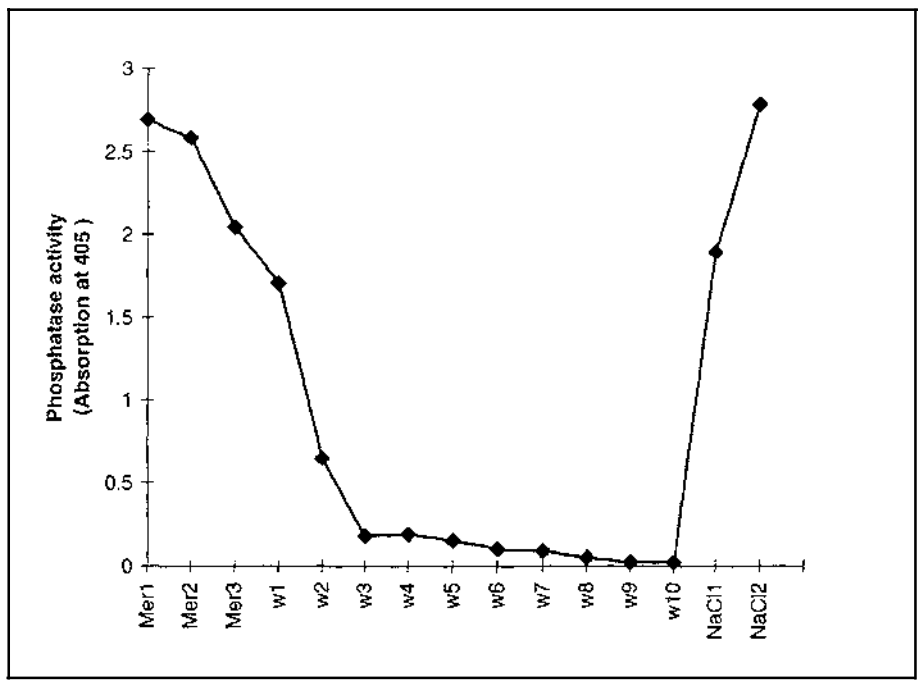

Figure 2.6

Phosphatase activity of the supernatants of 25 mM 2-mercaptoethanol extracts of white clover roots (Mer1, Mer2 and Mer3), water extracts (W1 to W10) and 1 M NaCl (cell wall) extracts (NaCl 1, 2).

- 50 mM Tris (hydroxymethyl) methylamine, pH 7.5, containing 150 mM NaCl
- 50 mM sodium acetate, pH 4.5
- 50 mM sodium acetate, pH 4.5, containing 1 M NaCl

A three-step fast protein liquid chromatography (FPLC) procedure was used for purification of acid phosphatases.

2.8.1 Step 1: hydrophobic chromatography

The crude (1 M NaCl) extract (section 2.7, typically 8 mg protein) was loaded onto a Phenyl Superose column (HR 5/5, Pharmacia Biotech) which had been pre-equilibrated with 2 M $(NH_4)_2SO_4$ (buffer A). The column was eluted in a linear gradient of 100% buffer A : 0% buffer B (20 mM KH_2PO_4) to 0% buffer A : 100% buffer B at a flow rate of 0.5 mL. min^{-1} and 3.3 mL fractions collected. Another hydrophobic column, Phenyl Sepharose (26/10, Pharmacia Biotech), was used for larger scale (900 g roots) purification procedures. For this, the column was pre-equilibrated with 2 M $(NH_4)_2SO_4$ and proteins eluted as described previously at a flow rate of 3 $mL.min^{-1}$. The acid phosphatase activity in each fraction was measured (using 10 µL of fraction) as described in section 2.6.1, Assay A. Routinely, 6 to 8 separate column separations were performed using Phenyl Superose for 300 grams of roots, and one column separation of Phenyl Sepharose for 900 grams of roots performed.

2.8.2 Step 2: gel filtration chromatography

Fractions with acid phosphatase activity were purified further with a Superose-12 gel filtration column (HR 10/30, Pharmacia Biotech). The column was equilibrated with 50 mM Tris, pH 7.5, containing 150 mM NaCl at a flow rate of 0.2 $mL.min^{-1}$. Fractions (1-2 mg protein) containing acid phosphatase activity after Phenyl Superose column chromatography were concentrated to 200 µL with Microcon concentrators (Amicon

Inc. Beverly, MA, USA) before loading onto the Superose-12 column. The column was eluted with the same buffer (50 mM Tris, pH 7.5, containing 150 mM NaCl) at a flow rate of 0.4 $mL.min^{-1}$. Fractions (0.8 mL) were collected and the acid phosphatase activity measured in each fraction as described in section 2.6.1, Assay A.

2.8.3 Step 3: ion exchange chromatography

Fractions from 4 to 6 separate column separations, using the Superose-12 column, containing acid phosphatase activity were concentrated to 0.5 mL with a Centriprep-10 concentrator (Amicon Inc. Beverly MA, USA) and then dialyzed against 50 mM sodium acetate, pH 4.5. The dialysed preparations were then concentrated again to 0.5 mL and loaded separately onto a cationic exchanger, Mono-S column (HR 5/5 Pharmacia Biotech) which had been pre-equilibrated with 50 mM sodium acetate, pH 4.5 (buffer A). The column was eluted within an increasing salt gradient of 100% buffer A : 0% buffer B (50 M sodium acetate, pH 4.5, containing 1 M NaCl) to 0% buffer A : 100% buffer B. Fractions (1 mL) were collected and measured for acid phosphatase activity. Fractions containing acid phosphatase activity were concentrated to 200 µL with a Microcon concentrator and used for further characterisation and enzyme kinetic experiments.

2.9 Molecular weight determination by gel filtration

Reagents:

- Elution buffer: 50 mM Tris, pH 7.5, containing 150 mM NaCl
- Molecular weight marker kit (MW-GF-200, Sigma chemicals)

  Blue dextran 2000 (MW: 2,000,000)

  β-amylase (MW: 200,000)

  Alcohol dehydrogenase (MW: 150,000)

Albumin bovine serum (MW: 66,000)

Carbonic anhydrase (MW: 29,000)

Cytochrome C (MW: 12,400)

2.9.1 Void volume (V_0) determination

The void volume (V_0) was determined first with blue dextran 2000 (MW: 2,000,000). To do this, the Superose-12 gel filtration column was equilibrated with 50 mM Tris, pH 7.5, containing 150 mM NaCl buffer for at least 16 hrs. Blue dextran (200 µg) was dissolved in 100 µL 50 mM Tris, pH 7.5, containing 150 mM NaCl buffer, applied to Superose-12 column and eluted with the same buffer at a flow rate of 0.4 $mL.min^{-1}$. Fractions (0.4 mL) were collected and the elution of blue dextran monitored by readings at 280 nm. The V_0 was determined by measuring the volume of eluate from the point of sample application to the center of the blue dextran peak.

2.9.2 Elution volume (Ve) determination for protein standards

Standard proteins were first dissolved in equilibration buffer (50 mM Tris, pH 7.5, containing 150 mM NaCl)

Albumin bovine serum	10 $mg.mL^{-1}$
Alcohol dehydrogenase	5 $mg.\ mL^{-1}$
β-Amylase	4 $mg.\ mL^{-1}$
Carbonic anhydrase	3 $mg.\ mL^{-1}$
Cytochrome C	2 $mg.\ mL^{-1}$

Each protein (10 µL) was applied separately to the Superose-12 column and fractions (0.4 mL) were collected at a flow rate of 0.4 $mL.min^{-1}$. The elution of the standard proteins was monitored at 280 nm and the elution volumes (Ve) determined by

measuring the volume of eluate collected from the point of sample application to the centre of the eluate peak.

A standard curve was obtained by plotting Ve/V_0 of standard proteins versus the $\log_{10}$ molecular weight.

2.9.3 Molecular weight determination of acid phosphatases

Acid phosphatase preparations were chromatographed through the Superose-12 column as described in section 2.9.2. The elution volume was determined using the activity assay (section 2.6.1) and the molecular weight determined from the standard curve constructed with proteins of known molecular weight (Section 2.9.2).

2.10 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins on the basis of molecular weight and was originally described by Laemmli (1970).

2.10.1 SDS-PAGE mini-gel

Reagents:

- 40% (w/v) acrylamide stock solution (Bio-Rad)
- 2 x resolving gel buffer: 0.75 M Tris-HCl, pH 8.8, containing 0.2% (w/v) SDS
- 2 x stacking buffer: 0.25 M Tris, pH 6.8, containing 0.2% (w/v) SDS
- APS (ammonium persulphate) 10% (w/v)
- TEMED (N,N,N'N-tetramethylethylendiamin)

- Running buffer: 3.0 g Tris, 14.4 g glycine, 1.0 g SDS, water to 1 L
- SDS gel loading buffer: 60 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 5% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue, stored at -20 ^{0}C
- Low molecular weight range of prestained SDS-PAGE protein standards (Bio-Rad).

Phosphorylase B	MW: 104,000
Bovine serum albumin	MW: 82,000
Ovalbumin	MW: 49,000
Carbonic anhydrase	MW: 33,400
Soybean trypsin inhibitor	MW: 28,600
Lysozyme	MW: 19,400

- High molecular weight range of prestained SDS-PAGE protein standard (Bio-Rad).

Myosin	MW: 205,000
β-Galactosidase	MW: 116,000
Bovine serum albumin	MW: 85,000
Ovalbumin	MW: 47,000

Table 2.1 Acrylamide gel solutions for a 12% acrylamide resolving gel and 4% acrylamide stacking gel for SDS-PAGE for use with the mini protein apparatus

order of addition	components	resolving gel sln (mL)	stacking gel sln (mL)
1	water	2	4
2	2x resolving buffer	5	
3	2x stacking buffer		5
4	acrylamide stock	3	1
5	10 % APS	0.1	0.1
6	TEMED	0.01	0.01

A 12% acrylamide resolving gel solution was prepared by mixing the components in the order outlined in Table 2.1. The resolving gel solution was then poured into a slot (0.15 cm thick) sandwiched between two glass plates until the gel level was 1 cm below the top of the shorter glass plate. Isobutanol was then layered onto the gel surface to prevent atmospheric oxidation of the gel surface. After 30 min polymerisation, the layer of isobutanol was discarded, and stacking gel solution poured onto the top of resolving gel and the sample well-formation comb was inserted. After another 30 min, the stacking gel was polymerised.

Prior to electrophoresis, protein samples were prepared in two ways. The samples were either mixed with an equal volume of gel loading buffer containing 2-mercaptoethanol (5% v/v) and boiled for 3 min, or mixed with the gel loading buffer without 2-mercaptoethanol, and the heating treatment omitted. Routinely, 5 µL of prestained molecular weight standard (Bio-Rad) was loaded and electrophoresis was conducted at 200 V for 50 to 60 min.

2.10.2 Gradient (8 to 15%) SDS-PAGE

Reagents:

- 40% (w/v) acrylamide stock solution (Bio-Rad)
- Sucrose
- 4 x resolving buffer: 1.5 M Tris, pH 8.8, containing 0.4% (w/v) SDS
- 2 x stacking buffer (section 2.10.1)
- APS: 10% (w/v)
- TEMED
- Low molecular weight range of prestained SDS-PAGE standards (Bio-Rad)

Table 2.2 Components of resolving and stacking gel solutions used in the SDS-PAGE gradient gel

Reagents	Heavy gel solution (15%)	Light gel solution (8%) mL	Stacking gel solution mL
sucrose	3 g		
water	7.5 mL	11	8
4x resolving buffer	5 mL	5	
2x stacking buffer			10
acrylamide stock	7.5 mL	4	2
APS	0.067 mL	0.067	0.2
TEMED	0.005 mL	0.005	0.02

Two resolving gel solutions were mixed according to the formulations described in Table 2.2 and each placed into separate chambers in a gradient former. The mixture was then poured into the glass plate assembly through a peristaltic pump to within 2 cm of the top of the shorter glass plate and topped with a layer of water. The gel was polymerised for at least 16 hrs at 4 ^{0}C, the layer of water discarded and the stacking gel solution (Table 2.2) was poured on top of separation gel. After insertion of the comb, the stacking gel was stood to polymerise for 1 hr and 30 min. Samples were prepared for loading as described in section 2.10.1 and electrophoresis was conducted at 1000 V, 30 mA and 30 W for 5 hour and 30 min.

2.10.3 Staining of SDS-PAGE gel

Reagents:

- Coomassie blue staining solution: 0.1% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid.
- Destaining solution: 30% (v/v) ethanol
- Activity staining solution:

0.01% (w/v) fast garnet, sulphate salt (Sigma Chemicals), 0.02% (w/v) β-naphthyl acid phosphate, monosodium salt (Sigma Chemicals), 0.1% (w/v) $MgCl_2.6H_2O$ in 200 mL 50 mM citrate buffer, pH 5.6

For Coomassie blue staining, each gel after the conclusion of electrophoresis was soaked in Coomassie blue staining solution for 30 min with gently rocking. The gel was then rinsed with destaining solution and maintained in the final destaining solution until background non-stained regions had cleared.

For acid phosphatase activity staining, the gel after the conclusion of electrophoresis was immersed in 50 mM citrate buffer, pH 5.6, for 10 min, rinsed with water and incubated in activity staining solution until activity bands reached the appropriate

intensity. The reaction was terminated by washing the gel with Milli-Q water several times.

2.10.4 Preservation of SDS-PAGE gel

The SDS-PAGE gels were usually photographed immediately and then air dried by being sandwiched between two sheets of GelAir cellophane (Bio-Rad) which were tightly pressed on a plastic frame.

2.10.5 Molecular weight determination by SDS-PAGE

Reagents:

- Low molecular weight range of prestained SDS-PAGE protein standards (Bio-Rad) (section 2.10.1)
- High molecular weight range of prestained SDS-PAGE protein standard (Bio-Rad) (section 2.10.1)

Prestained SDS-PAGE protein standards (5 μL for mini-gels, 15 μL for gradient gels) were separated using SDS-PAGE as described in Sections 2.10.1 and 2.10.2. After electrophoresis and protein staining, the distance each standard protein had migrated from the origin was measured and a calibration curve established by plotting migration distances of standard proteins versus log of their molecular weight. The molecular weight of proteins of interest were determined by locating the point on the calibration curve which corresponded to the migration distance of the enzyme and then calculate the molecular weight from logarithmic scale.

2.11 Isoelectric focusing

2.11.1 Isoelectric focusing electrophoresis

Reagents:

- 1 M H_3PO_4 (anode electrode solution)
- 1 M NaOH (cathode electrode solution)
- Fixing solution: 11.6% (w/v) trichloroacetic acid, 0.34% (w/v) sulphosalicylic acid
- Destaining solution: 25% (v/v) ethanol, 8% (v/v) acetic acid
- Coomassie blue staining solution: 0.12% (w/v) Coomassie blue R-250 made up with destaining solution
- Preserving solution: 10% (v/v) glycerol in destaining solution.
- Activity staining solution (section 2.10.3)
- Isoelectric focusing calibration kit, pH 3 - 10 (Pharmacia Biotech)

Isoelectric focusing was carried out with precast polyacrylamide gels, (Ampholine PAGplate, Pharmacia Biotech) using a Multiphor II Electrophoresis Unit (LKB 2117, Pharmacia Biotech). The whole system was cooled by immersing tubing carrying tap water in ice, before passing through the cooling plate. After paraffin oil was spread on the cooling plate, the gel was positioned on the cooling plate with care to avoid trapping air bubbles. Electrode strips were applied to the long edges of the gel after pre-soaking in electrode solutions. Care was taken to blot excess electrode solution from each strip.

Prior to IEF, samples were desalted using a Microcon concentrator (Pall Filtron Corporation, MA, USA) before loading. Isoelectric focusing calibration proteins were applied approximately 1 cm from the cathode and samples (3 to 5 µg) were applied approximately 1 cm from the anode. Isoelectric focusing was conducted at 1500 V, 50 mA and 30 W for 1 hr and 30 min. If half of an Ampholine PAGplate was used, the current and power settings were reduced to 25 mA and 15 W.

After isoelectric focusing, the gel was routinely cut into half. One half of the gel was immediately put in fixing solution for 1 hr and 40 min and then rinsed with destaining solution. The gel was stained in Coomassie blue staining solution for 30 min and then

transferred to destaining solution. Once sufficient destaining had occurred, the gel was kept in preserving solution. The second half of the gel was soaked in 50 mM citrate buffer for 10 min, washed with water and then placed in activity staining solution and incubated at 37 ^{0}C until acid phosphatase activity bands reached appropriate intensity. The reaction was terminated by rinsing the gel with water several times.

2.11.2 Determination of isoelectric points

Reagent:

- Activity staining solution (section 2.10.3)
- Broad pI calibration kit (Pharmacia Biotech)
- Coomassie blue staining solution (section 2.11.1)

A set of broad pI protein standards (20 μL) was isoelectric focused with each isoform preparation using the procedure outlined in Section 2.11.1. After isoelectric focusing, standard proteins were stained with Coomassie blue and the distances moved of each pI marker protein from the cathode measured. A pI gradient calibration curve was established by plotting the distance of each pI marker from the cathode against their pI. For each protein of unknown pI, the distance migrated from the cathode was measured and its pI was determined by locating the point on the pI gradient calibration curve.

2.12 Western blotting and immunodetection

Reagents:

- Polyvinylidene Difluoride (PVDF) transfer membrane (NEN^{tm} Life Science Products, Boston, MA, USA)
- Transfer buffer: 74 mM Tris, 190 mM glycine, 10% (v/v) methanol.
- PBS (phosphate buffered saline): 50 mM sodium phosphate buffer, pH 7.4,

containing 250 mM NaCl

- PBST (phosphate buffered saline + Tween 20): 0.5% (v/v) Tween 20 in PBS
- I-block (Tropix, Massachusetts, USA): 0.2% I-block powder in PBST
- Primary antibodies:

1) 0.1% (v/v) GNA-Digoxigenin-labeled (Boehringer Mannheim Biochemica) in PBST

2) 0.005% YZ1/2.23 (McManus et al 1988) in PBST

- Secondary antibodies:

1) Anti-DIG-alkaline phosphatase conjugate (Sigma): 0.1% (v/v) in PBST

2) Anti-rat-alkaline phosphatase conjugate (Sigma): 0.01% (v/v) in PBST

- 150 mM Tris-HCl, pH 9.7
- Phosphatase substrate solution

150 mM Tris-HCl, pH 9.7 containing 0.01% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 0.02% (w/v) nitroblue tetrazolium chloride (NBT), 1% Dimethyl Sulphoxide (DMSO), 8 mM $MgCl_2$

2.12.1 Protein Blotting

After isoelectric focusing, the IEF gel was placed onto a glass plate wetted with a little transfer buffer and the PVDF membrane, soaked firstly in 100% methanol and then rinsed with transfer buffer, and cut to the exact size of the gel, was laid on top of the gel. Next was placed two layers of wet Whatman chromatography paper (soaked in transfer buffer) then 3 layers of dry Whatman chromatography paper, each cut to the exact gel size. A stack of paper tissue was then put on top of the dry Whatman chromatography paper, and a weight (1 kg) placed on top to keep membrane and the gel in good contact. The protein transfer was carried out for 2 hrs at room temperature.

2.12.2 Immunodetection

Following protein transfer, unoccupied protein binding sites on the membrane were blocked by incubation in 0.2% I-block solution for 60 min. The membrane was then rinsed in PBST and incubated with DIG-labeled GNA, or YZ1/2.23 for 60 min at 37 ^{0}C and then washed 3 times (5 min each) in PBST. The membrane was then incubated in secondary antibody solution (anti-DIG alkaline phosphatase conjugate or anti-rat-alkaline phosphate conjugate) for 60 min at room temperature, and washed in PBST 3 times (10 min each) followed by 2 washes of 5 min in 150 mM Tris-HCl pH 9.7.

To visualise antibody binding, the membrane was immersed in phosphatase substrate solution and incubated in the dark. The development was carefully monitored and terminated by washing the membrane with Milli-Q water.

2.13 Amino acid sequence determination

On the conclusion of SDS-PAGE or isoelectric focusing, the Coomassie blue stained protein bands of interest were cut out, placed in a vial filled with water and sent to Ms Catriona Knight, Department of Biochemistry, University of Auckland, New Zealand, for amino acid sequencing. Amino acid sequence obtained was compared against the NCBI database (The National Center for Biotechnology Information, USA).

2.14 Statistical analyses

One-way analyses of variance were conducted using SAS 6.2 (SAS Inst. Inc.) for windows. Significant values were those below an alpha of 0.05.

Chapter Three: Results

3.1 White clover growth in phosphate deprived conditions

3.1.1 Visual changes in white clover plants maintained in -P conditions

White clover apical cuttings, with 3-4 leaves, were grown hydroponically in half-strength Hoaglands solution for 2 weeks. Plants were then divided into three groups. One group of plants was transferred to half-strength Hoaglands medium without phosphate (-P medium); the second group was transferred to -P medium a week later; and the third group remained in complete half-strength Hoaglands solution (+P).

The availability of phosphorus affected the growth of white clover. White clover showed no visual signs during the first two weeks in -P medium but started to show symptoms from the third week. After 5 weeks, white clover plants grown in -P medium were observed to possess thinner stems and petioles when compared with plants grown in +P medium. Leaves were observed to first develop purplish or bronze spots on their surfaces, and later turned yellow. These colour spots were observed on the older leaves only. A red-purple tint appeared on phosphorus deficient white clover stems. Many leaves of the basal section of stolons had died by the end of week 5 and the plants appeared poorly supplied with foliage, although no final fresh weight measurements were made (Figure 3.1).

3.1.2 Inorganic phosphate (Pi) content in leaves

Phosphate levels were measured in the first fully expanded leaf excised from plants grown in +P medium and plants grown in -P medium. Measurements were carried out at seven day intervals for up to 5 weeks (Figure 3.2). Phosphate content in leaves harvested from plants grown in +P medium remained at a similar level, *ca.* 0.05% g^{-1} fresh weight of leaf tissue over the 5-week period. Phosphate levels in leaves of plants grown in -P medium were significantly lower than control values (*ca.* 0.05% g^{-1} fresh

+P -P -P

Figure 3.1

White clover plants grown in half-strength Hoaglands solution with Pi (KH_2PO_4; +P), without Pi for 4 weeks (-P, middle) and without Pi for 5 weeks (-P, right).

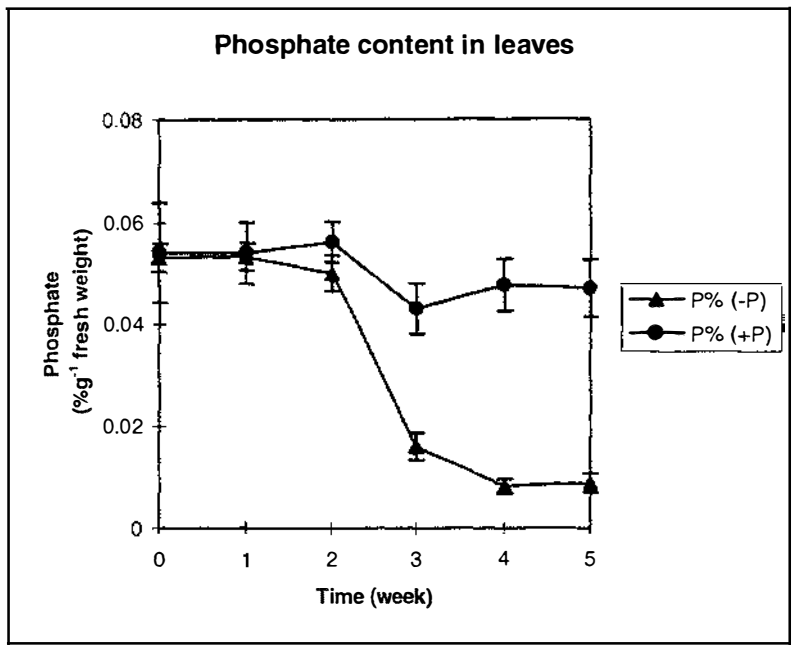

Figure 3.2
Comparison of phosphate content in the first mature leaf from plants grown in -P and +P media over a 5-week period. Values are means ± s.e., n = 19.

weight of leaf tissue) from week 2 onwards ($P < 0.001$). By week 5, the phosphate level in leaves of plants grown in -P medium decreased to *ca.* 0.008% g^{-1} fresh weight of leaf tissue.

3.1.3 Comparison of cell wall (1M NaCl extractable) acid phosphatase activity of roots of white clover grown in +P and -P media

Acid phosphatase activity was assayed in each cell wall extract from roots at 7 day intervals from plants grown in either -P media or +P media over a 5-week period. Activity from plants grown in -P media reveals that there was an increase in acid phosphatase activity over the 5-week period (Figure 3.3). At the end of week one, the acid phosphatase activity of -P plants was already significantly higher than the plants grown in +P media ($P < 0.01$) with a change in activity from 0.1447 (±0.05) μmol min^{-1} g^{-1} root fresh weight at week 0 to 0.2859 (±0.04) μmol min^{-1} g^{-1} root fresh weight in the -P roots at week one. The acid phosphatase activity of plants grown in +P medium remained at similar levels, at *ca.* 0.144 μmol min^{-1} g^{-1} root fresh weight over the 5-week time course.

3.1.4 Selection of root material for the extraction and characterisation of acid phosphatases

Although plants grown in -P medium for five weeks still demonstrated an increase in Apase activity, they also suffered more severe phosphate deficiency symptoms. In particular, leaf necrosis were most marked at the basal end of the stolon (Figure 3.1). By comparison, plants at week 4 in -P media demonstrated a significant induction of Apase activity but did not show such marked leaf necrosis (Figure 3.1). Consequently root tissue was always harvested from plants after 4 weeks in -P media, and used in further experiments.

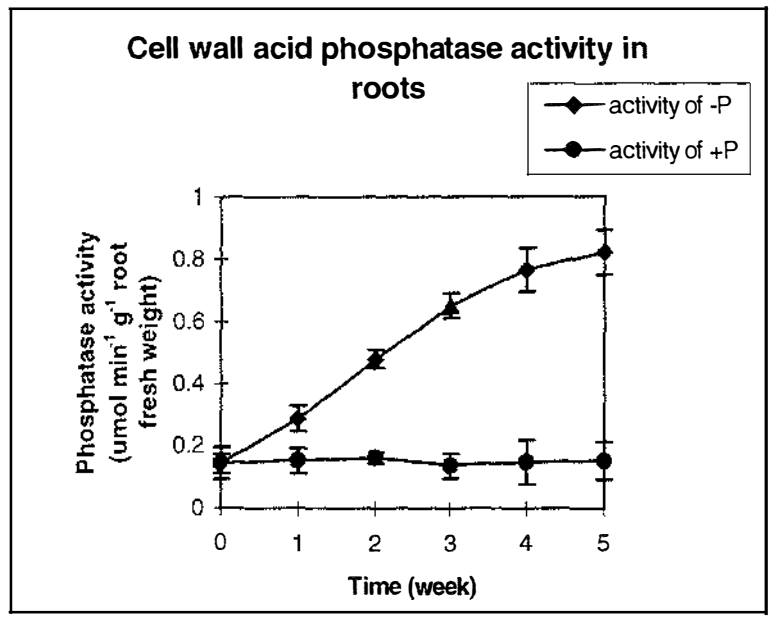

Figure 3.3

Comparison of cell wall acid phosphatase activities of roots from plants grown in -P and +P media over a 5-week period. Values are means ± s.e., n = 5.

3.2 Separation and partial purification of four isoforms of acid phosphatase

A three-step fast protein liquid chromatography (FPLC) procedure was used to partially purify the acid phosphatases contained in the cell wall fraction from roots of white clover plants grown in -P media for 4 weeks.

3.2.1 Hydrophobic chromatography

A Phenyl-Superose column (hydrophobic chromatography) was used first in the FPLC procedure. The Phenyl Superose column was equilibrated with buffer A, (2M $(NH_4)_2SO_4$, pH 5.6) and then the 1 M NaCl cell wall extract loaded (Figure 3.4). Some of the protein did not bind to the column (eluted in the void volume), but all phosphatase activity was retained and eluted within a linear gradient of 100% buffer A: 0% buffer B (20 mM KH_2PO_4, pH 7.0) to 0% buffer A : 100% buffer B. Two peaks of acid phosphatase activity, designated phosphatase I (Apase I) and phosphatase II (Apase II) determined by their order of elution, were separated from the cell wall extract on the Phenyl Superose column (Figure 3.4, Apase I and Apase II indicated by arrows). Apase I was eluted off the column at 58-65% buffer B and Apase II at 73-80% buffer B. Apase I was purified 5.4 fold with a specific activity of 31.4 μmol min^{-1} mg^{-1} when using ρNPP as substrate after this chromatography step. The recovery of the enzyme activity was 82.3%. There was only a 2.1-fold increase in the enzyme specific activity for Apase II with a 27.5% recovery (Table 3.1).

Another hydrophobic column, Phenyl-Sepharose was used for larger scale purification of acid phosphatases. The column was also equilibrated with buffer A, and bound proteins eluted at a gradient of 100% buffer A : 0% buffer B to 0% buffer A : 100% B.

Apase activity bound to the Phenyl Sepharose column more tightly than to the Phenyl Superose column. Apase I eluted off the column at 87-92% buffer B, while Apase II eluted off the column 7-10 min after the elution gradient reached 100% buffer B (Figure 3.5; Apase I and Apase II indicated by arrows).

3.2.2 Gel filtration chromatography

Apase I and Apase II, separated using Phenyl Superose or Phenyl Sepharose, were further purified with a Superose-12 gel filtration column. The column was equilibrated with 50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl and pooled fractions from the Phenyl Superose or Phenyl Sepharose columns containing either Apase I or Apase II were loaded separately. The activity of Apase I was detected in the eluent on the left shoulder of the first protein peak (A_{280}) at an elution volume of 13 mL (Figure 3.6, Separation after Phenyl Superose, Apase I indicated by an arrow). Apase II was eluted off the column earlier than Apase I at an elution volume of 12.2 mL (Figure 3.7, Separation after Phenyl Superose, Apase II indicated by an arrow)[1]. At the conclusion of gel filtration chromatography, Apase I was purified 26.4 fold with a recovery of 26.4%. Apase II was purified 4.5-fold with a recovery of 15.8%. The specific activity of Apase I was 153.4 μmol min^{-1} mg^{-1} protein and Apase II was 26.4 μmol min^{-1} mg^{-1} protein (Table 3.1).

3.2.3 Ion exchange chromatography

The final separation step was performed by ion-exchange chromatography using a Mono-S cationic exchanger. Apase I and Apase II, after gel filtration, were first equilibrated with 50 mM sodium acetate, pH 4.5 (buffer A) and then loaded separately onto the Mono-S column, pre-equilibrated with the same buffer. Apase I and Apase II were eluted within an increasing salt gradient from 100% buffer A : 0% buffer B (50 mM sodium acetate, pH 4.5 containing 1M NaCl) to 0% buffer A : 100% buffer B. Apase I was resolved into two activity peaks, designated Apase Ia and Apase Ib (Figure 3.8). Apase II was also resolved into two activity peaks, designated as Apase IIa and Apase IIb (Figure 3.9). Apase Ia was eluted at 200-205 mM NaCl, Apase Ib at 250-255 mM NaCl. The fraction containing Apase IIa was eluted at 210-215 mM NaCl and Apase IIb at 300-305 mM NaCl. The final specific activities of Apase Ia, Apase Ib, Apase IIa and Apase IIb were 705.2, 338.9, 78.1 and 539.0 μmol min^{-1} mg^{-1} protein

[1] Both Apase I and II eluted at the identical position from the gel filtration column regardless of whether they had been chromatographed previously on the Phenyl Superose or Phenyl Sepharose column.

respectively, and the purification fold of each of four isoforms was 121.3 (Apase Ia), 58.3 (Apase Ib), 13.4 (Apase IIa) and 92.7 (Apase IIb). The recovery of Apase Ia was 13%, Apase Ib was 8.4%, Apase IIa was 3.1% and Apase IIb was 13.9% (Table 3.1). After the 3-step FPLC purification, Apase Ia was chosen to be subjected to SDS-PAGE for purity examination and amino acid sequencing since Ia was the isoform which had been purified the highest fold and showed the highest specific activity when compared with the other three isoforms.

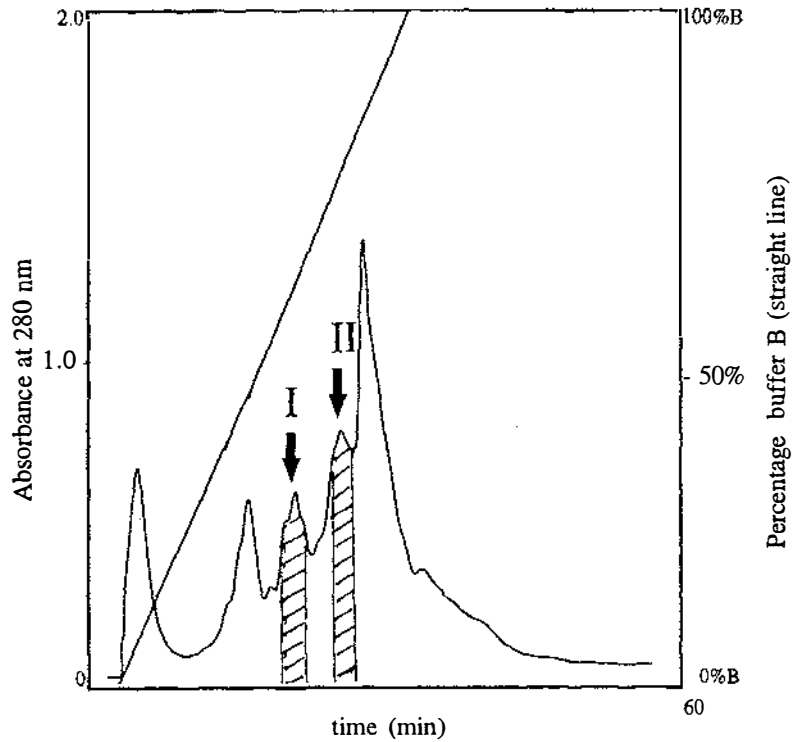

Figure 3.4

Separation of the cell wall extract by hydrophobic column chromatography using Phenyl Superose. The arrows indicate Apase I (I) and Apase II (II).

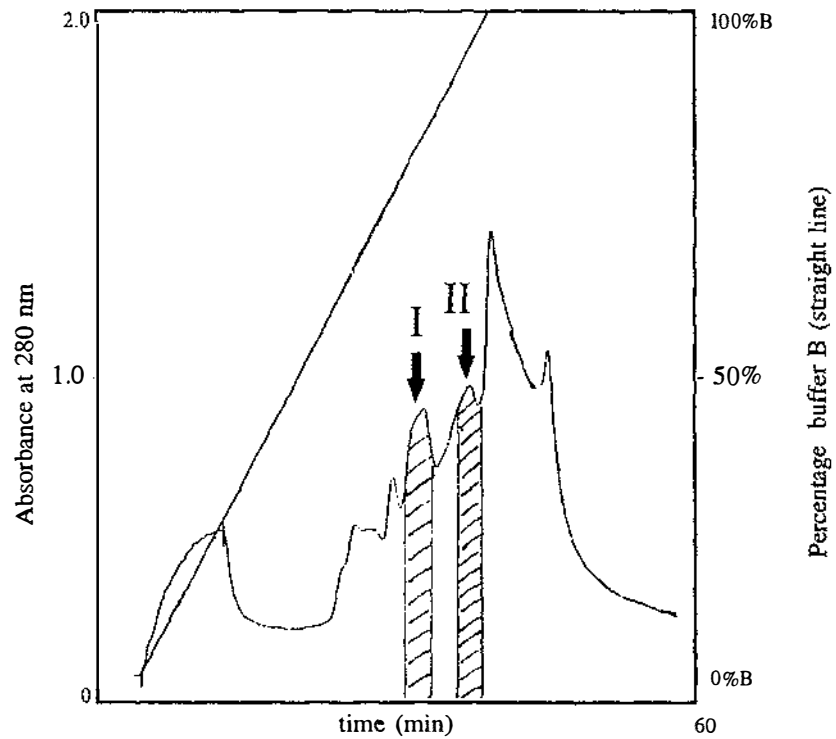

Figure 3.5

Separation of the cell wall extract by hydrophobic column chromatography using Phenyl Sepharose. The arrows indicate Apase I (I) and Apase II (II).

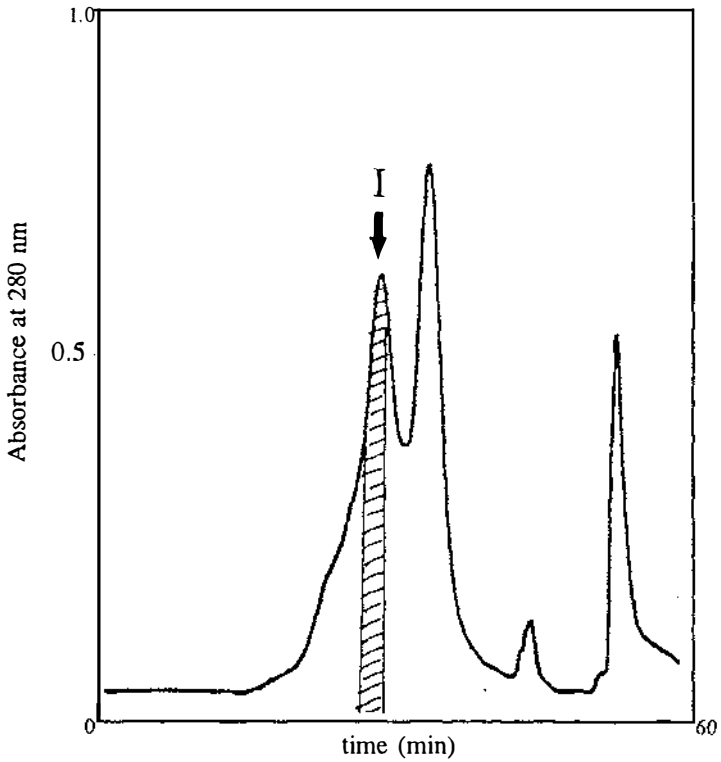

Figure 3.6
Purification of the Apase I preparation through a Superose-12 gel filtration column after Phenyl Superose (Figure 3.4). The arrow and shaded area indicate Apase I activity.

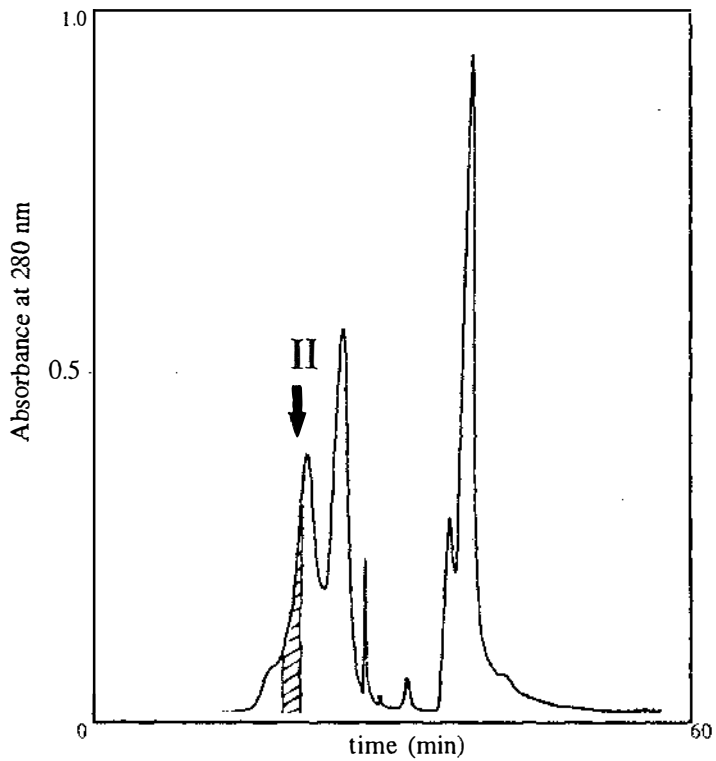

Figure 3.7
Purification of the Apase II preparation through a Superose-12 gel filltration column after Phenyl Superose (Figure 3.4). The arrow and shaded area indicate Apase II activity.

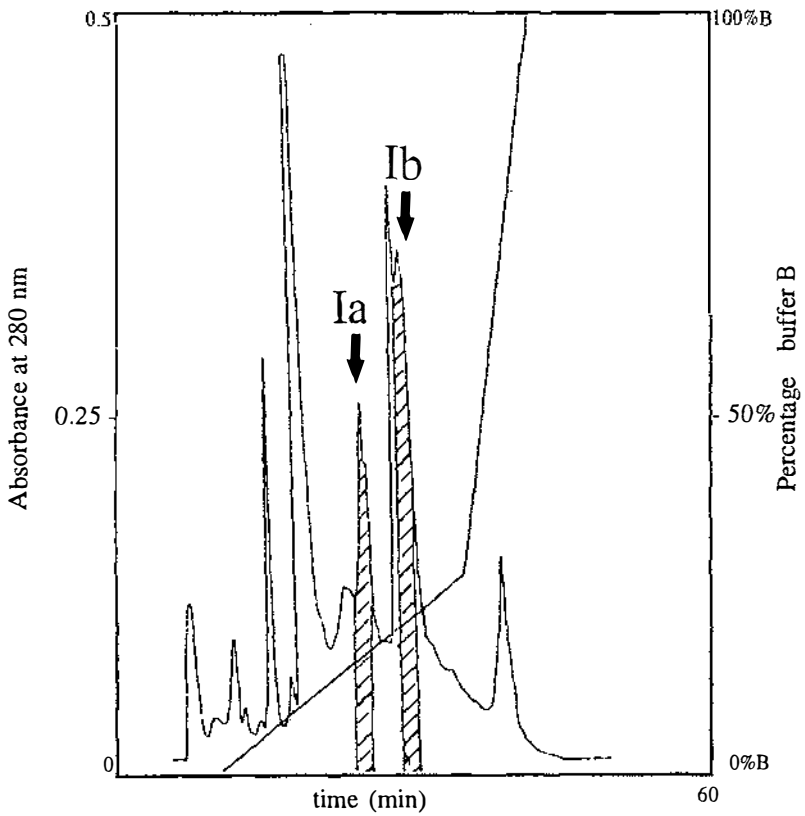

Figure 3.8
Separation of the Apase I preparation using a Mono-S ion exchange column after gel filtration column chromatography (Figure 3.6) The arrows and shaded areas indicate activities of Apase Ia & Ib.

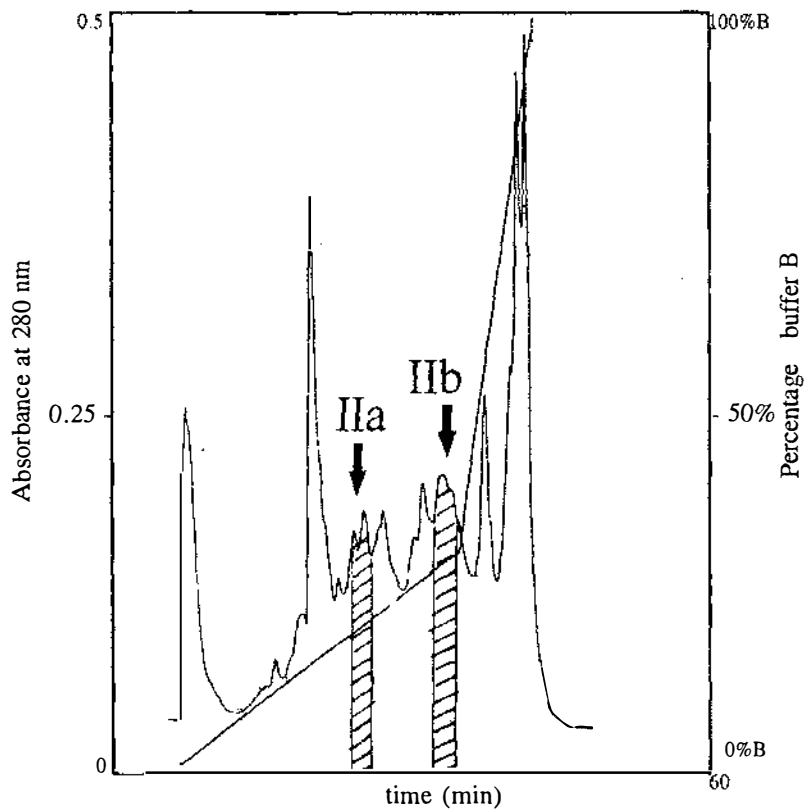

Figure 3.9
Separation of the Apase II preparation using a Mono-S ion exchange column after gel filtration column chromatography (Figure 3.7). The arrows and shaded areas indicate activities of Apase II a and IIb.

Table 3.1

Summary of purification of acid phosphatases from the cell wall fraction of roots of white clover maintained in -P medium.

Purification step	Apase	Activity unit	Protein	Specific activity	Purification	Recovery
		µmol min^{-1}	mg	units mg^{-1}	fold	%
Crude extract		372.12	64	5.8	1	100
Phenyl-	I	206.4	6.57	31.4	5.4	82.3
Superose	II	102.3	8.51	12.0	2.1	27.5
	I	98.2	0.64	153.4	26.4	26.4
Superose-12	II	58.9	2.23	26.4	4.54	15.8
	Ia	48.6	0.07	705.2	121.3	13
Mono-S	Ib	31.2	0.09	338.9	58.3	8.4
	IIa	11.6	0.15	78.1	13.4	3.1
	IIb	51.8	0.1	539.0	92.7	13.9

3.3 SDS-PAGE and amino acid sequencing of Apase Ia

SDS-PAGE of the Apase Ia fraction from ion-exchange chromatography showed a major (52 kD) Coomassie blue stained protein band (Figure 3.10) which corresponded to the activity stained band (data not shown). This 52 kD Coomassie blue stained protein band was excised and submitted for amino acid sequencing. Two sequences from trypsin-generated peptides were obtained and compared with existing protein sequences in the NCBI database. One peptide sequence containing 19 amino acid residues was found to have some identity with a stress-related protein, EDGP (extracellular dermal glycoprotein) purified from a carrot suspension cell culture (Satoh et al 1992) (Figure 3.11) and another peptide sequence (D-F-I-S-G-C-T-D-S-M-A-F) did not match any existing protein sequences in the NCBI database. These results suggest the presence of (at least) more than one protein in the Apase Ia fraction.

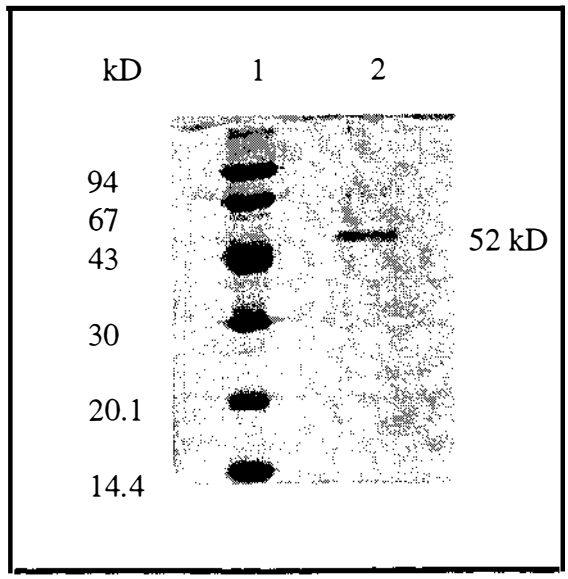

Figure 3.10

SDS-PAGE and Coomassie blue staining of the Apase Ia fraction after Mono-S ion-exchange chromatography (Figure 3.8). Lane 1 is standard proteins. Lane 2 is the Apase Ia fraction.

EDGP	389	TSIVIGGHQLEDNLVQFDL	408
WCLO		TAVVIGGHQLEDNLLEFDL	

Figure 3.11

Alignment of the amino acid sequence of the 52 kD protein from cell walls of white clover roots with that of EDGP of carrot roots (Satoh et al 1992).

EDGP: extracellular dermal glycoprotein

WCLO: white clover 52 kD protein

3.4 Induction of Apase I and II in response to P-deprivation

The identification of two distinct Apases in roots of white clover grown in -P media raises the question as to whether one or both of these enzymes are induced by P-deprivation.

3.4.1 Determination of acid phosphatase activity in extracts of cell walls from roots *of plants maintained in +P*

After white clover had been well established in +P media, the plants were divided into two groups. One group was moved to P-deprived medium and the other remained in P-supplied medium. The roots of a fraction (1/4) of both +P plants and -P plants were harvested at weekly intervals for four weeks. When the cell wall extracts of roots from +P plants were separated using hydrophobic column chromatography, activity was resolved into two activity peaks (Figure 3.12), regardless of at which week plants were harvested. The elution of each Apase activity was identical in terms of % buffer B to that of P-deprived plants from which Apase I and Apase II were separated using Phenyl Superose column (Figure 3.13). However the activity of peak 1 and peak 2 from plants grown in +P media was much lower than that of Apase I and Apase II from P-deprived plants. The activity of peak 1 was 16.9 $\mu mol.mL^{-1}.min^{-1}$ and peak 2 was 16.6 $\mu mol.mL^{-1}.min^{-1}$ at week 0 (Figure 3.12), and remained at that level throughout the time course of the experiment. This compares with 70 and 45 $\mu mol.mL^{-1}.min^{-1}$ respectively for peak 1 (Apase I) and peak 2 (Apase II) of P-deprived plants (Figure 3.13, week 4).

3.4.2 Changes in Apase I and Apase II activity during a 4-week time course in -P media

Extracts were made from equal fresh weight of root tissue harvested at weekly intervals over a 4-week period from plants maintained in -P medium, and the activity of Apase I and Apase II measured after separation using hydrophobic chromatography with the Phenyl-Superose column. Apase I and Apase II responded differentially to phosphate

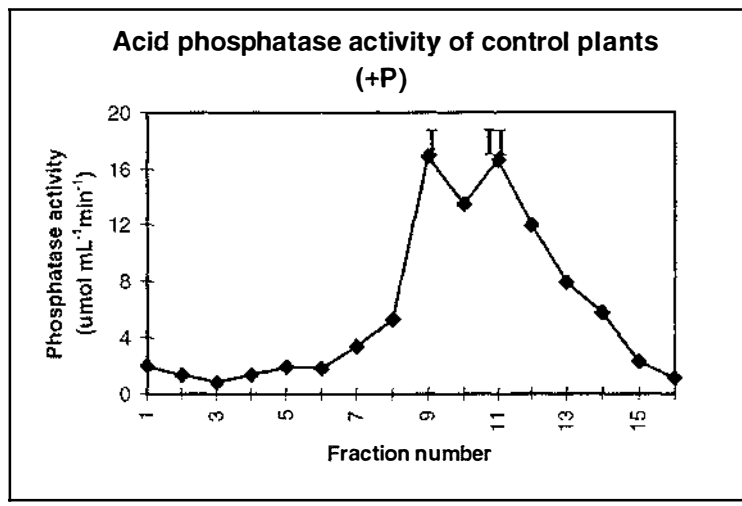

Figure 3.12

Acid phosphatase activity in the cell wall extract of roots of plants grown in +P media. The activity was measured after Phenyl Superose chromatography and the two peaks of acid phosphatase identified are labelled I and II. Values are the means of three measurements.

deprivation (Figure 3.13). After one week of phosphate-deprivation, the activity of Apase II increased to 44.8 $\mu mol.mL^{-1}.min^{-1}$ from 16.6 $\mu mol.mL^{-1}.min^{-1}$ of the control (see 3.4.1). The activity of Apase I increased slightly to 19.5 $\mu mol.mL^{-1}.min^{-1}$. The activity ratio of Apase I to Apase II was 0.43. The activity of Apase I continued to increase during the 4 weeks of P-starvation such that it was higher than Apase II by week 3, and reached 70 $\mu mol.mL^{-1}.min^{-1}$ by the end of week 4. At this time point the ratio of the activity of Apase I to Apase II was 1.55. The activity of Apase II did not increase after the first week of phosphate starvation, and at week 4 in -P media, the activity of Apase II had decreased slightly from 55 $\mu mol.mL^{-1}.min^{-1}$ at week 3 to 45 $\mu mol.mL^{-1}.min^{-1}$ at week 4.

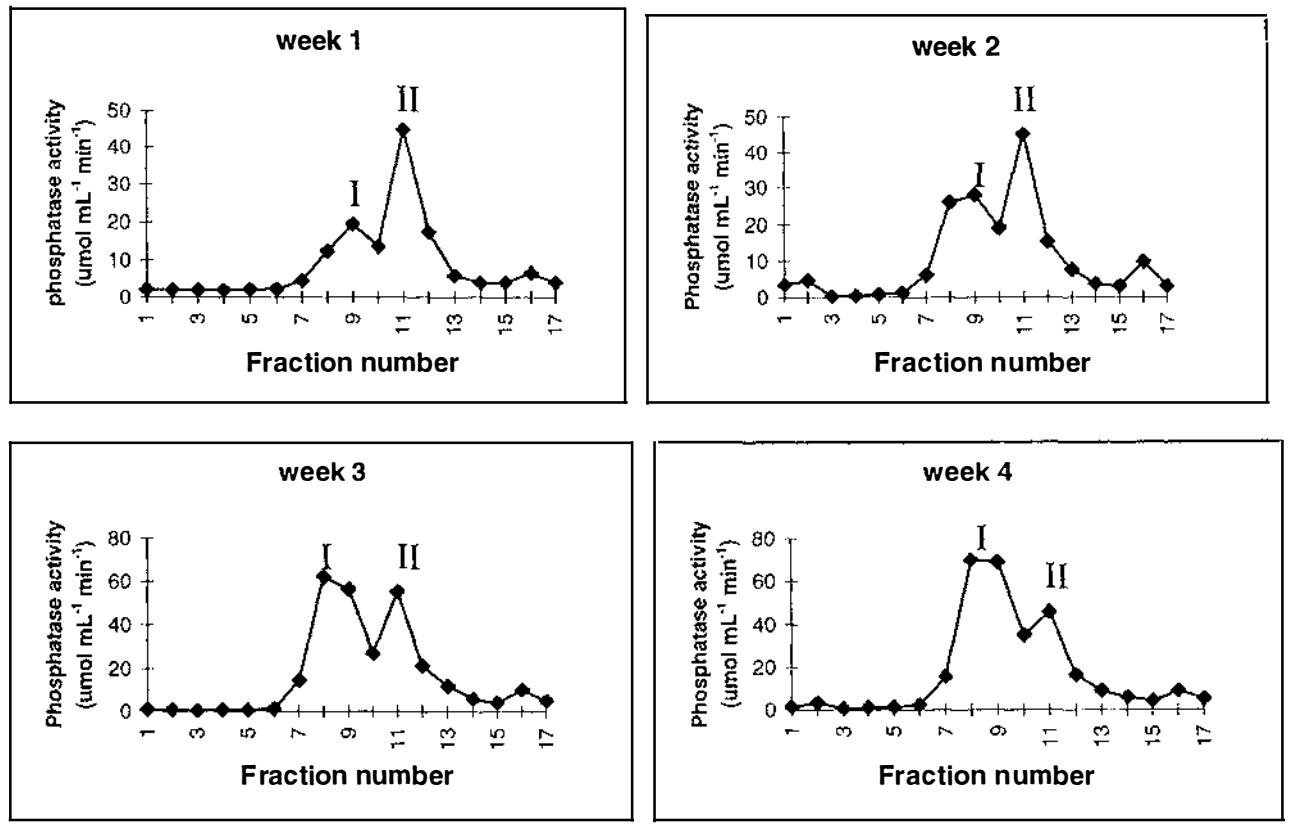

Figure 3.13

Changes in the activity of Apase I (I) and Apase II (II) measured at 7-day intervals over a 4-week time course in cell wall extracts from roots of plants maintained in -P media. Apase I and II were separated using hydrophobic column chromatography with Phenyl Superose. Values are means of three measurements.

3.5 Characterisation of acid phosphatase isoforms from the cell wall fraction of white clover roots

Amino acid sequencing of Apase Ia revealed at least one protein contaminant in the enzyme preparation after FPLC (see Section 3.3). This suggests that Apase Ia has not been purified to homogeneity. However, the 3 FPLC steps have resolved each Apase into two distinct isoforms, and so each can be characterised further. The first part of this characterisation involves the use of gel electrophoresis for molecular weight determinations, which can also act as an assessment of purity for the other Apase preparation.

3.5.1 Molecular weight determination

3.5.1.1 Determination of molecular weight of Apase Ia and Ib by 8-15% gradient SDS-PAGE

To determine the molecular weight of each Apase isoform identified after Mono-S ion exchange chromatography, SDS-PAGE in combination with an in-gel activity staining methods was used so that the protein band(s) responsible for the phosphatase activity could be identified. Non-denatured preparations of Apase Ia and Ib (no 2-mercaptoethanol, no heating treatment) were fractionated through a 8-15% polyacrylamide gradient gel containing 0.1% (w/v) SDS. After electrophoresis, one half of the gel containing Apase Ia and Ib was stained with Coomassie blue and the second half containing both Apase Ia and Ib was incubated in an acid phosphatase substrate solution, β-naphthyl acid phosphate; Fast Garnet (Figure 3.14). In the Coomassie blue stained gel, the Apase Ia preparation was fractionated into two very closely located, equally strongly stained bands at *ca.* 49 kD. Apase Ib showed the same separation pattern as Apase Ia. In the phosphatase activity stained gel, both Apase Ia and Ib preparations revealed a single band of activity at *ca.* 49 kD, which corresponded to the lower molecular weight protein of the two revealed by Coomassie blue staining.

To calculate the molecular weight of Apase Ia and Ib with greater accuracy, the mobility of prestained low range SDS-PAGE protein standards was plotted against their

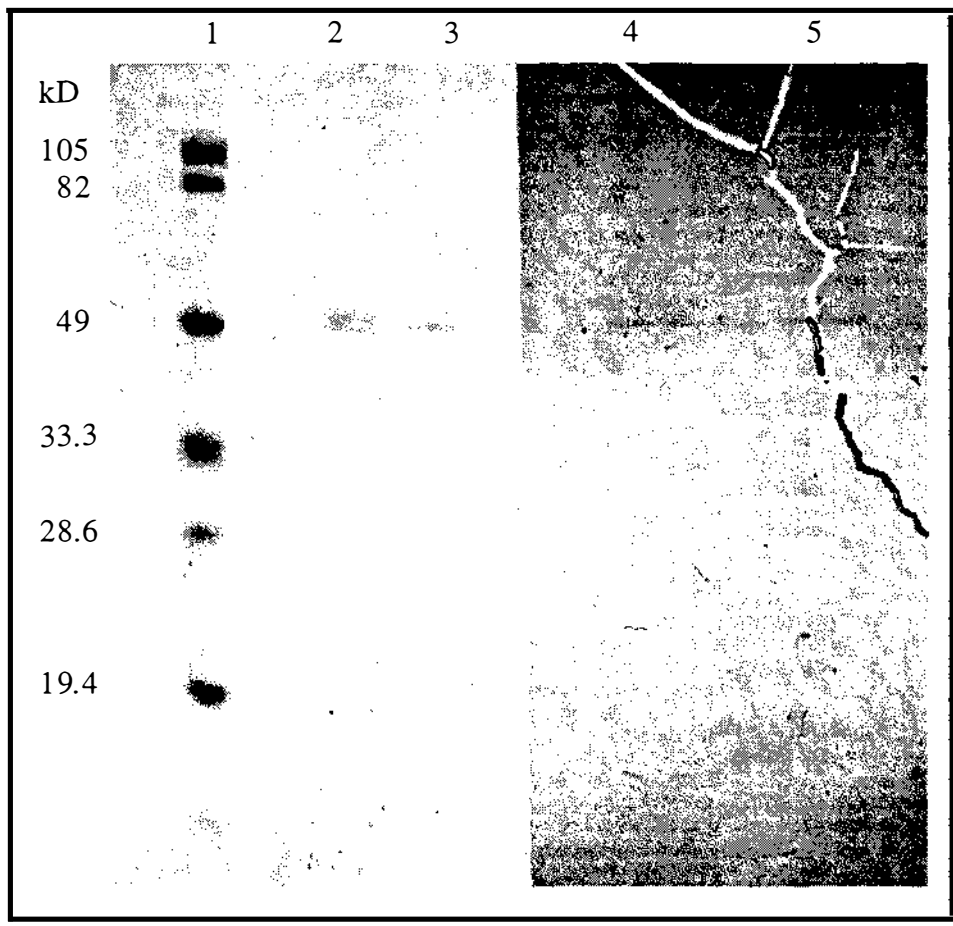

Figure 3.14

Molecular weight determination of Apase Ia and Ib by an 8 to 15% polyacrylamide gradient SDS-PAGE. Lane 1 are standard proteins. Lane 2 and 3 are separated Apase Ia and Ib preparations after Coomassie blue staining. Lane 4 and 5 are separated Apase Ia and Ib preparations after activity staining with β-naphthyl acid phosphate and Fast garnet. The arrow indicates the position of Apase Ia and Ib as determined by activity staining.

molecular weight (Figure 3.15). The molecular weight of both Apase Ia and Ib was calculated to be 52 kD according to their mobilities in the activity gel.

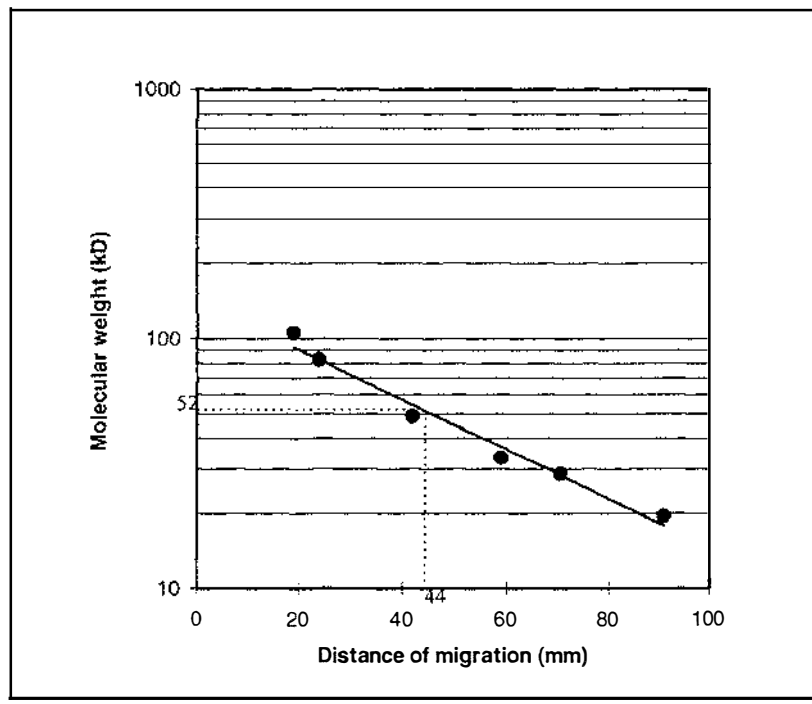

Figure 3.15

Molecular weight determination of Apase Ia and Ib by standard proteins separated using gradient SDS-PAGE (Figure 3.14). The solid line is a plot of log (molecular weight) against the migration distances of prestained protein standards. The dashed lines represent the migration distance of both Apase Ia and Ib and their calculated molecular weight in kD.

3.5.1.2 Determination of molecular weight of Apase IIa and IIb by mini-SDS-PAGE

Non-denatured preparations (no 2-mercaptoethanol, no heating) of Apase IIa and IIb were applied to a SDS-mini-gel and yielded a protein band of *ca.* 116 kD as revealed by Coomassie blue staining. Both Apase IIa and IIb revealed a single activity band (Figure 3.16). The molecular weight of Apase IIa and IIb was calculated to be 112 kD by comparing their electrophoretic mobility in the activity gel with that of the protein standards (Figure 3.17).

3.5.1.3 Subunit analysis of four isoforms

Subunit analysis was performed by first reducing each Apase preparation with 2-mercaptoethanol and heating and then subjecting each isoform to SDS-PAGE. On conclusion of SDS-PAGE, each gel was incubated in Apase activity staining solution. The activity band of each isoform appeared at the identical molecular weight as the equivalent non-reduced sample (Figure 3.18). Because of the identical separation, no calculation of molecular weight was undertaken.

3.5.1.4 Determination of Apase I and Apase II molecular weights by gel filtration

Pooled fractions containing Apase I and Apase II after Phenyl Superose column chromatography were used to determine the apparent native molecular weight of each Apase using Superose-12 gel filtration column chromatography and the elution volume of Apase I was 13 mL and Apase II was 12.2 mL (Figures 3.6; 3.7). The void volume was determined by blue dextran elution (V_0 = 7.6 mL) The column was calibrated with the following standards: β-amylase (Ve = 10.9 mL), alcohol dehydrogenase (Ve = 11.6 mL), albumin bovine serum (Ve = 12.3 mL), carbonic anhydrase (Ve = 14 mL), cytochrome C (Ve = 15 mL) and based on the elution pattern of molecular weight standards separated on the same Superose-12 column, the apparent molecular weight of Apase I was calculated as 52 kD, and Apase II was calculated as 92 kD (Figure 3.19).

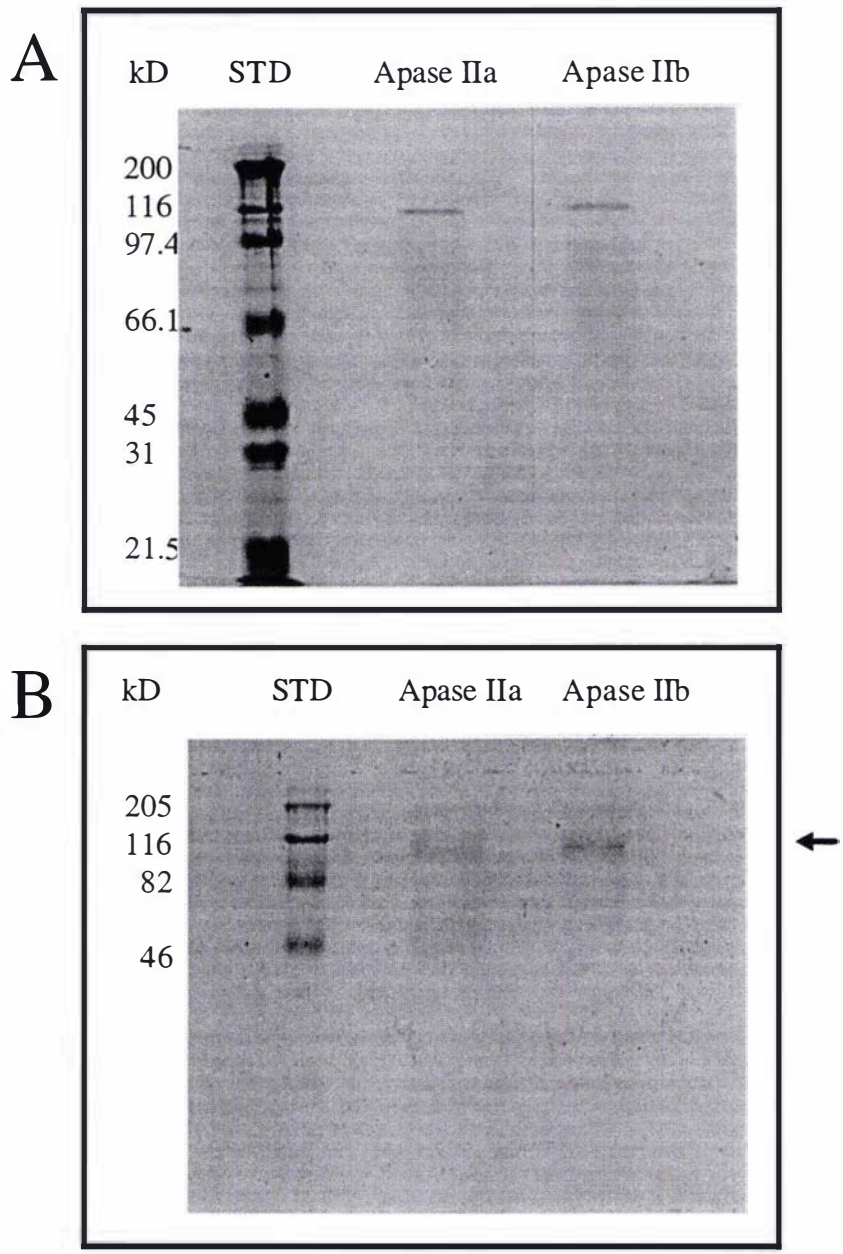

Figure 3.16

Molecular weight determination of Apase IIa and IIb by mini-SDS-PAGE. A: separated Apase IIa and IIb fractions after Coomassie blue staining; B: separated ApaseIIa and IIb fractions after activity staining with β-naphthyl acid phosphate and Fast Garnet. The arrow indicates the position of Apase IIa and IIb as determined by activity staining. STD: standard proteins.

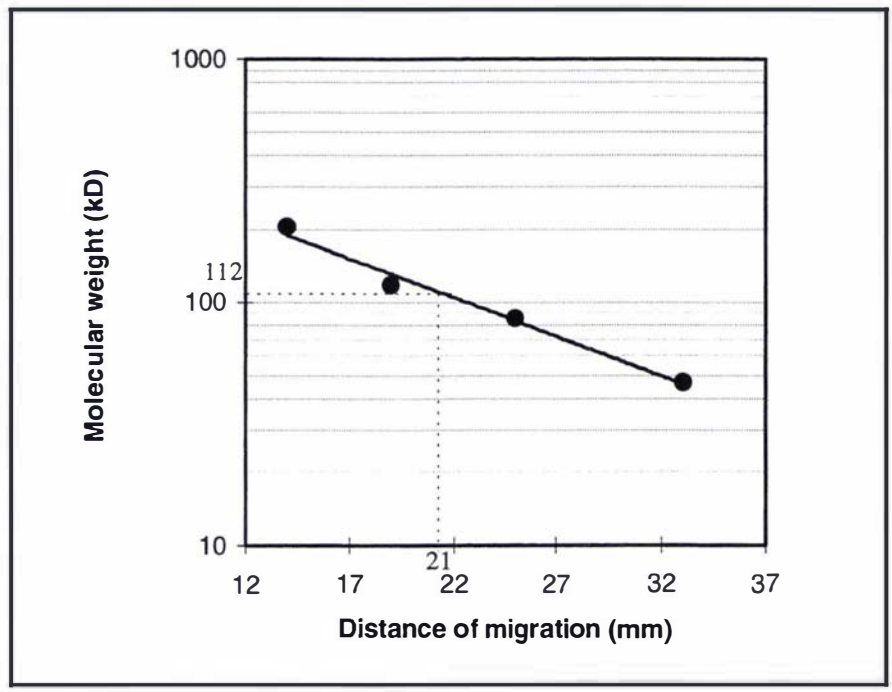

Figure 3.17

Molecular weight determination of Apase IIa and IIb by standard proteins separated using mini-SDS-PAGE (Figure 3.16). The solid line is a plot of log (molecular weight) against the migration distances of protein standards. The dashed lines represent the migration distance of both of Apase IIa and IIb and their calculated molecular weight in kD.

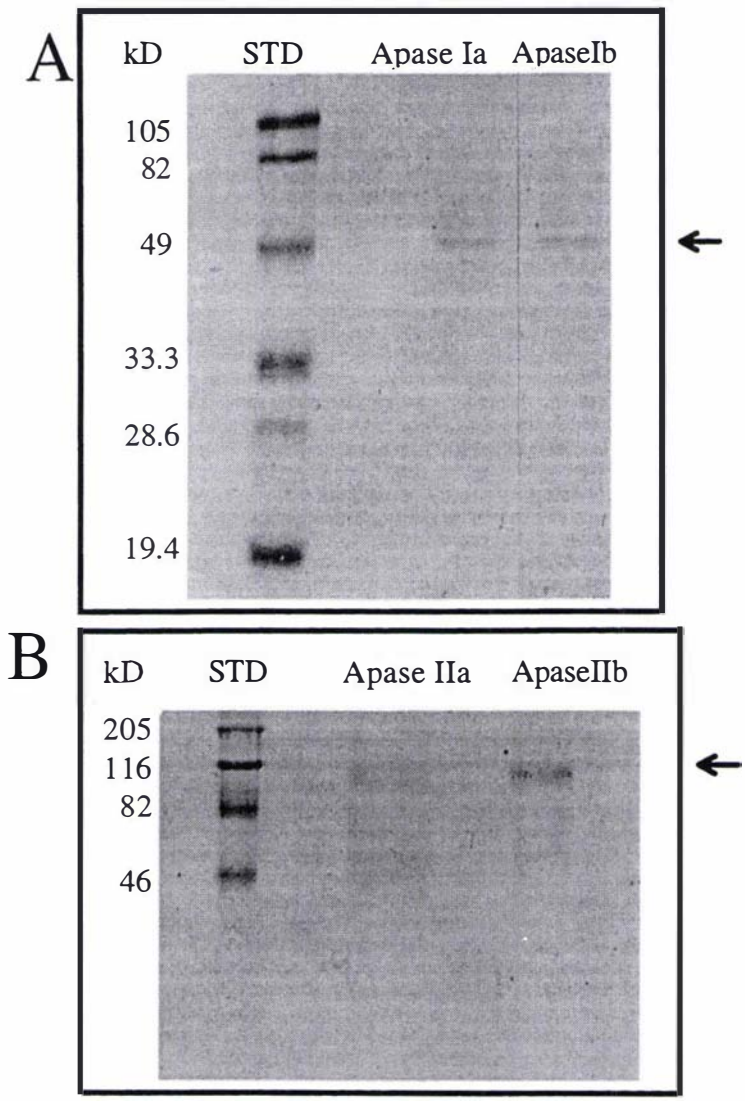

Figure 3.18

SDS-PAGE of Apase I (A) and Apase II (B) after reduction with 2-mercaptoethanol. Each band is visualised after activity staining with β-naphthyl acid phosphate and Fast Garnet. The arrows indicate the position of each Apase.

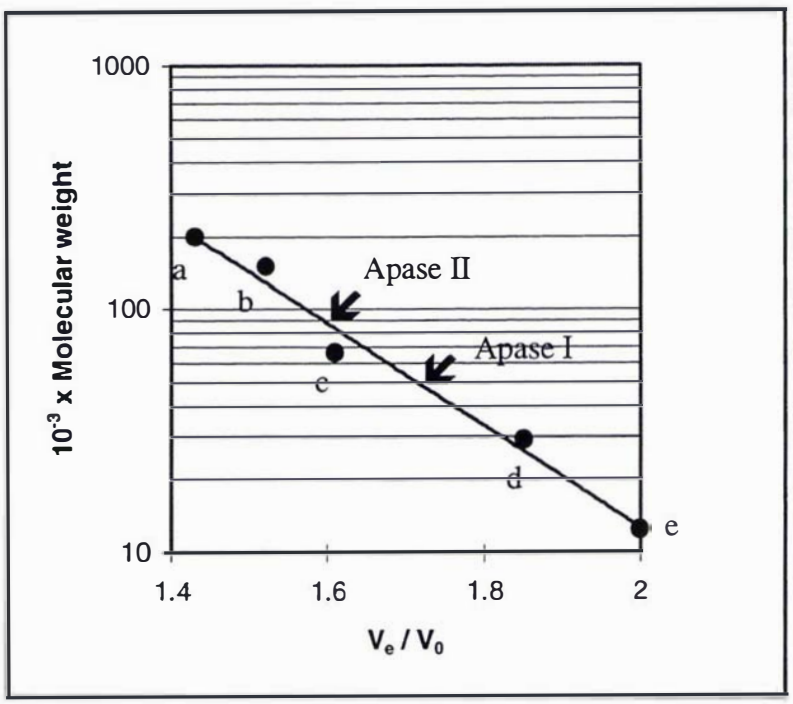

Figure 3.19

Molecular weight determination of Apase I and Apase II by gel filtration chromatography. The straight line is a plot of log (molecular weight) against the ratio of the elution volumes of protein standards (a-e) to the void volume. The arrows indicate the relative elution points of Apase I and II.

3.5.2 pH optimum determination of each Apase isoform

The effect of pH on the activity of the four Apase isoforms identified and separated by ion-exchange column chromatography was examined. A series of different pH values was obtained using 50 mM citrate buffer (pH range 3.0 to 6.2) and 50 mM maleic acid-Tris-NaOH buffer (pH range 5.2 to 8.6). Enzyme reactions were conducted at 37 ^{0}C with 8 mM ρNPP as substrate for 5 min. The four pH-dependent phosphatase activity profiles obtained showed that all four isoforms were active over a broad pH range with Apase activity of Apase Ia maximal at pH 5.8 (Figure 3.20) and the pH optima of Apase Ib, IIa and IIb were pH 6.2, 5.8 and 6.8 respectively (Figure 3.21 to 3.23). Phosphatase IIb exhibited the broadest pH optimum, with greater than 80% maximal activity from pH 6.1 - 7.2.

3.5.3 Isoelectric point (pI) determination

The isoelectric point of each of the four acid phosphatase isoforms was determined by activity staining after separation on a precast IEF polyacrylamide gel. A broad range of isoelectric focusing pI standards were focused with each Apase preparation and visualised with Coomassie blue staining (Figure 3.24). Each acid phosphatase isoform was visualised by activity staining with β-naphthyl acid phosphate and Fast Garnet, and the pI value for each isoform was determined by comparing the distances of their isoelectric focusing points from cathode (-) with those of standard proteins (Figure 3.25). Apase Ia split into two bands with equal staining intensity with isoelectric points of 7.0 and 7.3. Apase Ib showed a major band with a pI of 6.7 plus some minor bands, two of which co-migrated with the Apase Ia bands. Phosphatase IIa showed a single band with a pI of 4.4. Phosphatase IIb was again split into two closely located bands with pI values of 5.2 and 5.3.

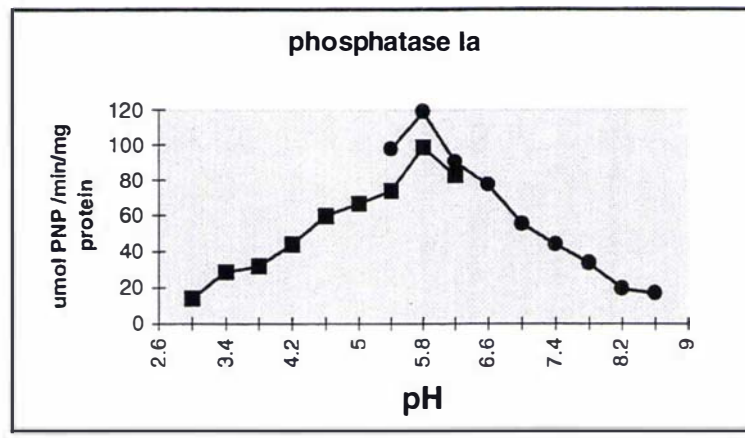

Figure 3.20

Effect of pH on the activity of Apase Ia using 50 mM citrate buffer, pH 3.0 to 6.2 (series 1 ■); 50 mM maleic acid-Tris-NaOH buffer, pH 5.2 to 8.6 (series 2 ●). Values are means of three measurements.

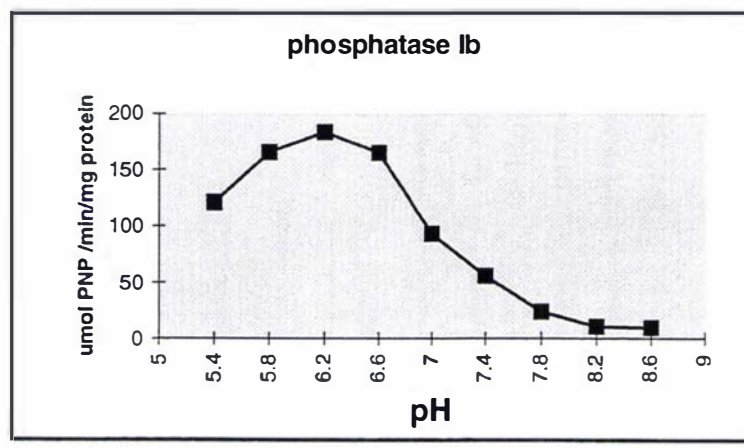

Figure 3.21

Effect of pH on the activity of Apase Ib using 50 mM maleic acid-Tris-NaOH buffer (pH 5.2 to 8.6 ■). Values are means of three measurements.

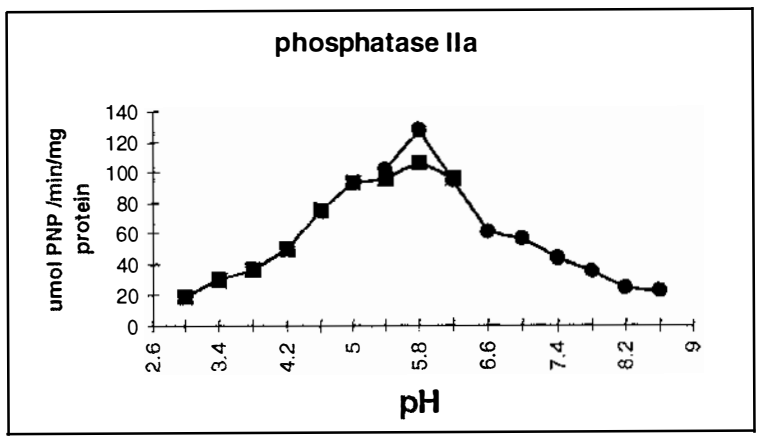

Figure 3.22

Effect of pH on the activity of Apase IIa using 50 mM citrate buffer pH 3.0 to 6.2 (series 1 ■); 50 mM maleic acid-Tris-NaOH buffer pH 5.2 to 8.6 (series 2 ●). Values are means of three measurements.

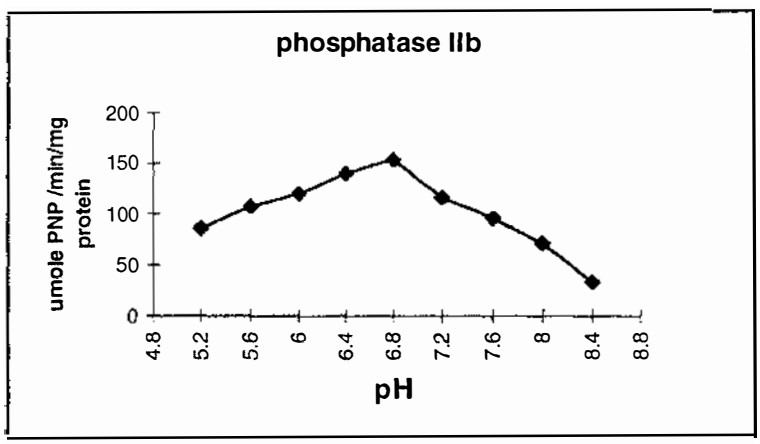

Figure 3.23

Effect of pH on the activity of Apase IIb using 50 mM maleic acid-Tris-NaOH buffer pH 5.2 to 8.6. (◆) Values are means of three measurements.

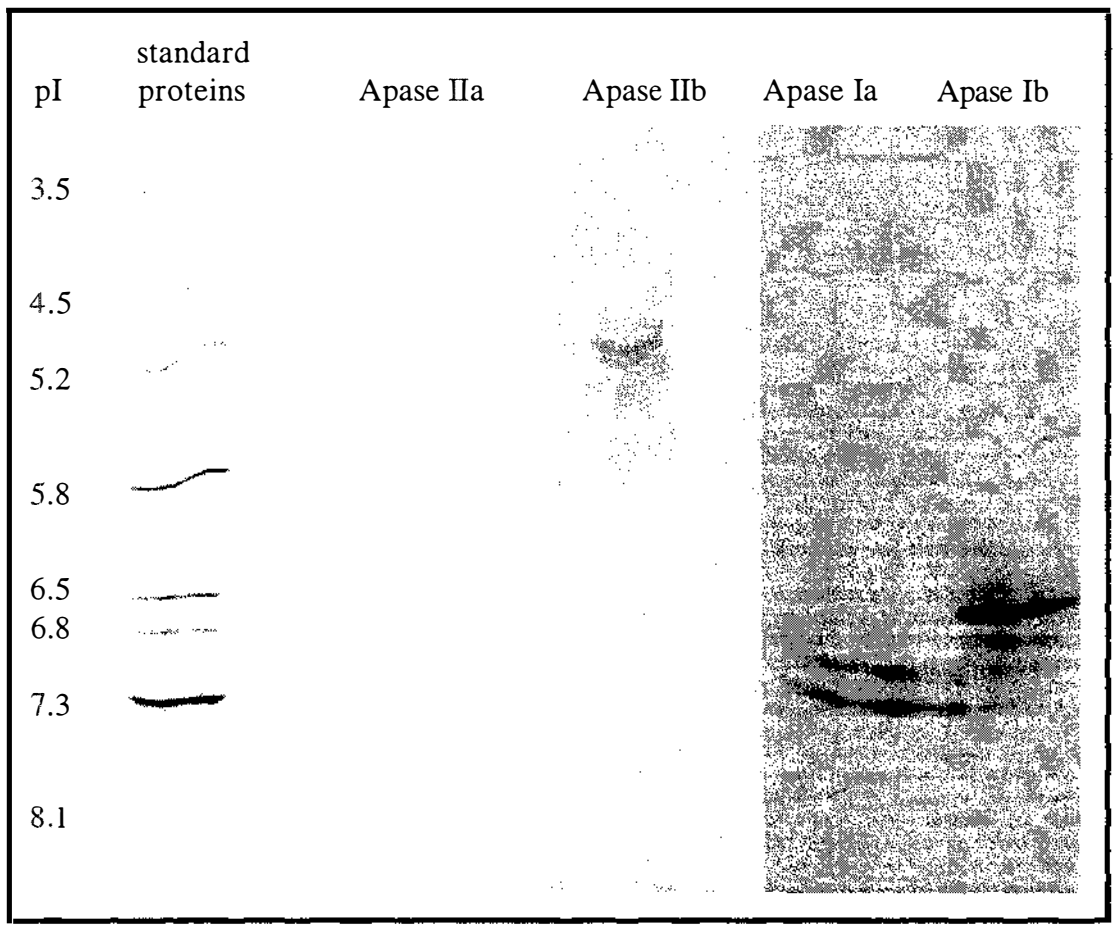

Figure 3.24

Isoelectric focusing of the four Apase isoforms. Each isoform was visualised by activity staining. A broad pI range of standard proteins stained with Coomassie blue was used for estimation of pI values of each isoform.

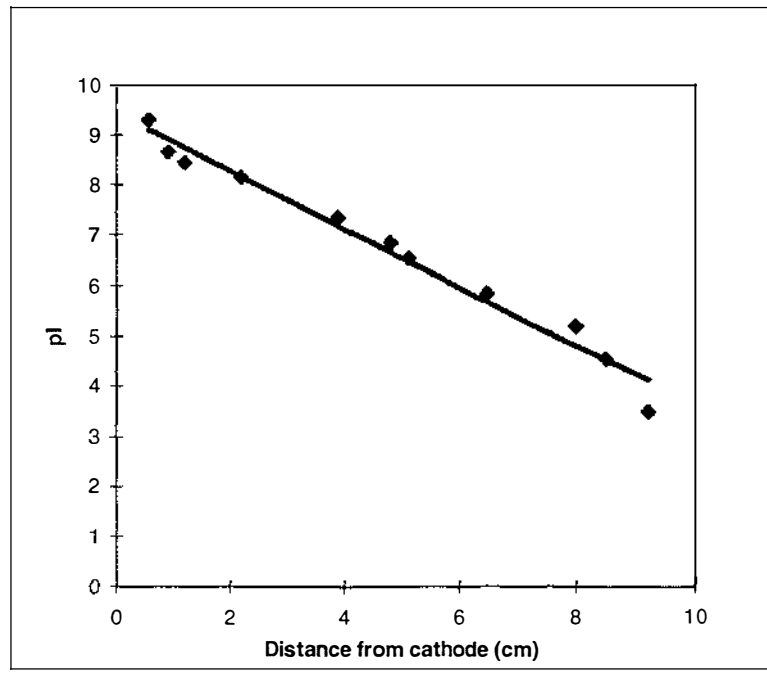

Figure 3.25

Determination of pI profile using the broad pI calibration kit on IEF gel. The straight line is a plot of the migration distances against pI values of standard proteins. The standard proteins used are:

Trypsinogen pI-9.30

Lentil lectin-basic pI-8.65

Lentil lectin-middle pI-8.45

Lentil lectin-acidic pI-8.15

Myoglobin-basic pI-7.35

Myoglobin-acidic pI-6.85

Human carbonic anhydrase B pI-6.55

Bovine carbonic anhydrase B pI-5.85

β-lactoglobulin A pI-5.20

Soybean trypsin inhibitor pI-4.55

Amyloglucosidase pI-3.50

3.5.4 Thermal stability

To examine the thermal stability of each isoform, each enzyme preparation was heated at 45 ^{0}C, 55 ^{0}C, 65 ^{0}C, 75 ^{0}C and 85 ^{0}C for 15 min and the remaining activity measured at 37 ^{0}C. The activity of each isoform measured at 37 ^{0}C without heating is defined as 100%.

Apase I and Apase II showed very different thermal stability, although there were no differences between Apase Ia and Ib or Apase IIa and IIb. Apase I is relative heat sensitive when compared with Apase II. The remaining activity of Apase I was 72-74% of the control after incubation at 45 ^{0}C. The activity was reduced to 25% for both isoforms after 65 ^{0}C treatment. The activity was completely abolished after incubation at 75 ^{0}C. Apase II is more heat stable than Apase I. Apase II did not lose any activity after being heated at both 45 ^{0}C and 55 ^{0}C. There was still 66% of the original activity of both isoforms after incubation at 65 ^{0}C. Activity was not completely lost until incubation at 85 ^{0}C (Table 3.2).

Table 3.2

Remaining activity after each isoform was incubated at 45 ^{0}C to 85 ^{0}C for 15 min. The activity of each isoform preincubated at 37 ^{0}C is defined as the control value (100%). The remaining activity was measured at 37 ^{0}C after heat treatment and is expressed as a percentage of the control. Values are means of three measurements.

	45^{0}C	55^{0}C	65^{0}C	75^{0}C	85^{0}C
Apase Ia	72%	65%	25%	0%	0%
Apase Ib	74%	64%	25%	0%	0%
Apase IIa	100%	100%	66%	16%	0%
Apase IIb	100%	100%	66%	15%	0%

3.5.5 Inhibitory effects of inorganic phosphate (Pi) on isoform activity

A range of Pi concentrations from 1 mM to 25 mM were tested for the inhibitory effect on each of the four acid phosphatase isoforms (Figure 3.26). Pi was found to be a potent inhibitor. The activity of all isoforms was reduced or abolished completely by the range of concentrations tested. The two isoforms of Apase I were more sensitive to Pi than the two isoforms of Apase II. Apase I lost significantly more activity (about 40%) than Apase II (25%) ($p < 0.01$) in the presence of 1 mM Pi. The activity of Apase I was abolished by 20 mM Pi, while Apase II still showed 20% activity. Apase II completely lost its activity when subjected to 25 mM Pi.

3.5.6 Effects of metal cations, tartrate and EDTA on acid phosphatase activity

Various metal ions that are known to have effects on acid phosphatase activity were tested in this study. Metal ions (5 mM) was added to each isoform preparation and acid phosphatase activity measured using 8 mM ρNPP as substrate (Table 3.3). The rate of ρNPP hydrolysis without addition of any metal ions was taken as the control value (100%), and activity values obtained in the presence of each metal ion are expressed as a percentage of the control. With the exception of Ba^{2+}, no metal ions tested in this study stimulated the enzyme activity significantly. Ba^{2+} stimulated Apase Ib by 40%. The most notable inhibitors were Cu^{2+}, Zn^{2+} and MoO_4, of which Zn^{2+} was the most potent inhibitor for all isoforms tested. Zn^{2+} completely inhibited the activity of Apase Ib, IIa and IIb, and reduced the activity of Apase Ia to a trace level. Fe^{3+} caused about a 50% reduction in the activity of all four isoforms. The effects of Co^{2+} and Al^{3+} on the activity of Apase I and II are significantly different ($P < 0.001$). Co^{2+} and Al^{3+} reduced the activity of Apase Ia and Ib [61%, 35.5% (Co^{2+}) and 65.1%, 42.7% (Al^{3+})] more than that of Apase IIa and IIb [89.1%, 104.3% (Co^{2+}) and 79% and 95.8% (Al^{3+})]. Tartrate and EDTA had no measurable effect on the activity of four isoforms.

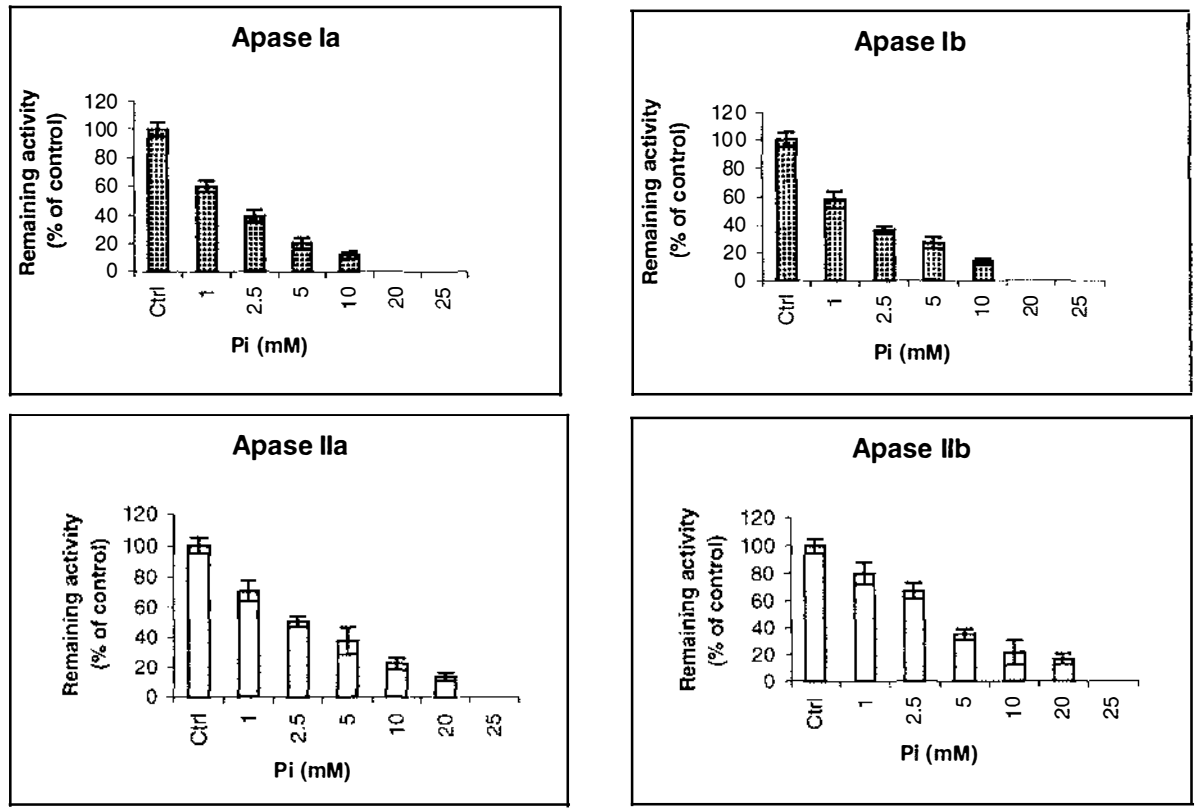

Figure 3.26

The inhibitory effects of a range of Pi concentrations on the activities of Apase Ia, Ib, IIa and IIb. Error bars represent standard errors of the mean of 3 measurements. Ctrl: activity measured without addition of Pi in the assay mixture.

Table 3.3

Effects of metal cations, tartrate and EDTA on the activity of each Apase Isoform. The activity determined in the presence of 5 mM effectors (metal cations, tartrate and EDTA) is expressed as a percentage of the control which has no added effectors.

	Apase Ia	Apase Ib	Apase IIa	Apase IIb
control	100	100	100	100
$CaCl_2$	59.9 ± 3.0	85.7 ± 4.1	77.0 ± 4.1	97.9 ± 2.7
$CoCl_2$	61.0 ± 2.5	35.5 ± 2.6	89.1 ± 3.2	104.3 ± 6.4
$CuCl_2$	1.5 ± 0.2	0.9 ± 0.1	7.5 ± 0.15	9.9 ± 1.4
$FeCl_3$	60.3 ± 2.8	54.4 ± 2.9	43.3 ± 1.8	32.1 ± 2.3
$BaCl_2$	91.4 ±6.9	139.9 ± 9.9	107.5 ± 7.6	92.2 ± 5.2
$AlCl_3$	65.1 ± 1.9	42.7 ± 3.8	79.0 ± 3.7	95.8 ± 3.1
$ZnCl_2$	0.2 ± 0.02	nd	nd	nd
MoO_4	0.6 ± 0.05	nd	5.0 ± 0.4	3.9 ± 1.6
KCl	96.9 ± 7.6	110.1 ± 8.6	91.6 ± 4.5	90.5 ± 9.1
LiCl	86.6 ± 6.8	107.7 ± 8.8	127.4 ± 8.5	93.6 ± 4.7
NaCl	84.7 ± 2.1	105.6 ± 11.1	113.2 ± 5.8	86.8 ± 2.1
$MgCl_2$	79.3 ± 8.7	85.2 ± 7.7	113.6 ± 10.8	102.1 ± 9.5
Tartrate	108.9 ± 1.2	105.8 ± 0.9	106 ± 0.5	107 ± 1.1
EDTA	93.4 ± 6.4	87.2 ± 3.3	114.7 ± 5.5	86.5 ± 7.8

nd = not detectable

3.5.7 Determination of N-linked glycosylation of each Apase isoform

The identification of N-linked oligosaccharide chains attached to each acid phosphatase isoform was carried out by western blotting and incubation with sugar-structure-specific probes after isoelectric focusing.

Each acid phosphatase isoform preparation was applied to the IEF gel in duplicate. After IEF, one half of the gel was stained to reveal acid phosphatase activity while proteins in the other half of the gel were blotted onto PVDF membrane. After blotting, the membrane was probed either with a *Galanthus nivalis* (GNA) lectin which detects either oligomannose or terminal mannose N-linked glycan chains or with a monoclonal antibody, YZ1/2.23, which recognises specifically xylose/fucose-containing complex-type oligosaccharides. The protein bands corresponding to the activity bands of each Apase isoform were examined for recognition by the GNA lectin or YZ1/2.23.

Apase Ia was recognised by both GNA and YZ1/2.23 (Figures 3.27 and 3.28), while Apase Ib was recognised by YZ1/2.23, but not by GNA (Figures 3.29 and 3.30). The GNA lectin did not recognise Apase IIa (Figure 3.31) and there was no or very faint recognition of Apase IIa by YZ1/2.23 (Figure 3.32). Apase IIb was recognised by YZ1/2.23 (Figure 3.34) but not by the GNA lectin (Figure 3.33).

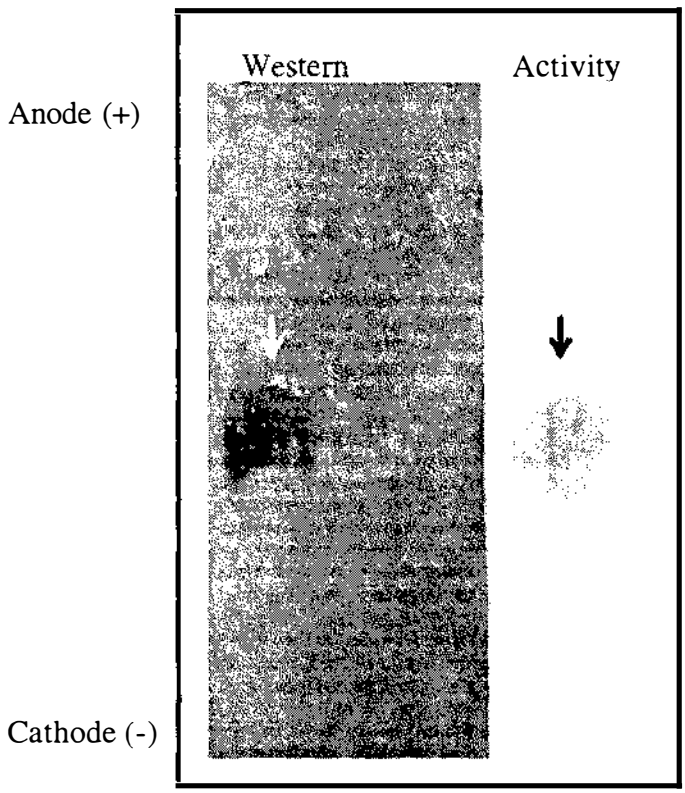

Figure 3.27

Activity staining and Western blotting with the GNA lectin of Apase Ia after IEF.

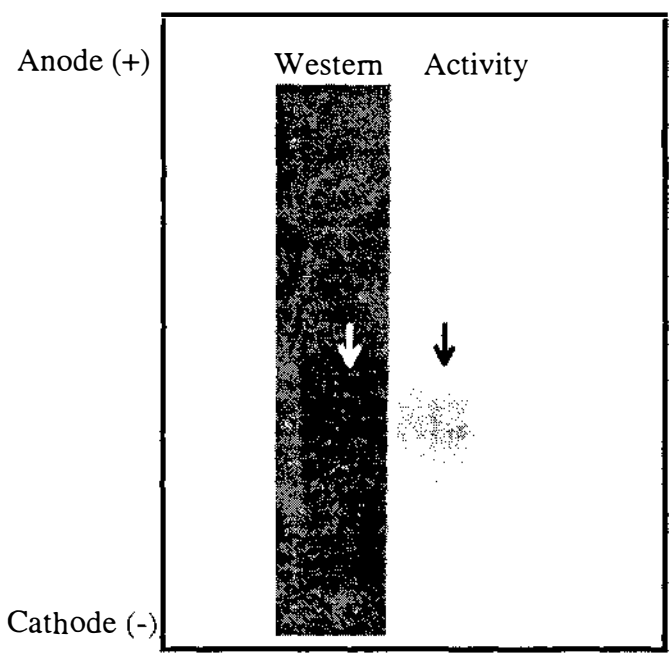

Figure 3.28

Activity staining and Western blotting with antibody YZ1/2.23 of Apase Ia after IEF.

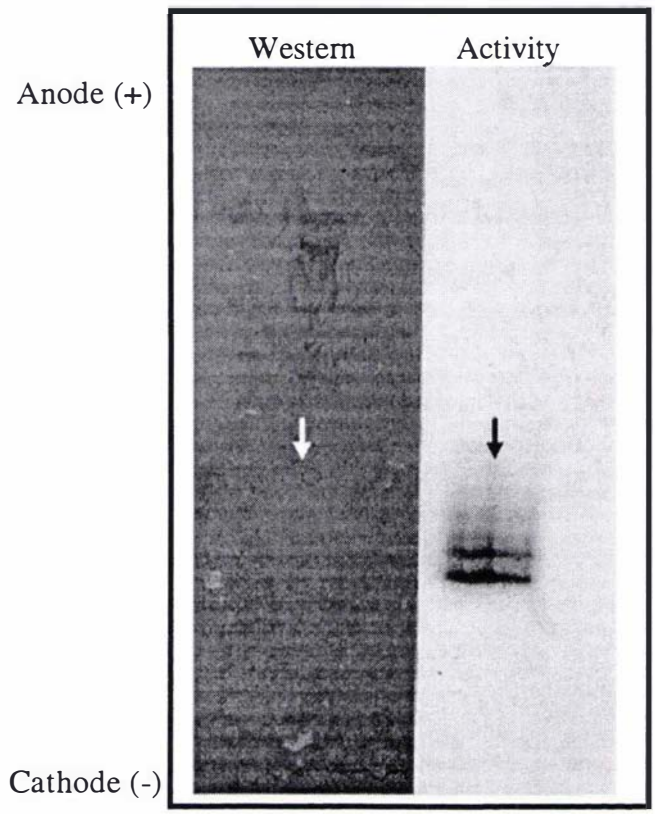

Figure 3.29

Activity staining and Western blotting with the GNA lectin of Apase Ib after IEF.

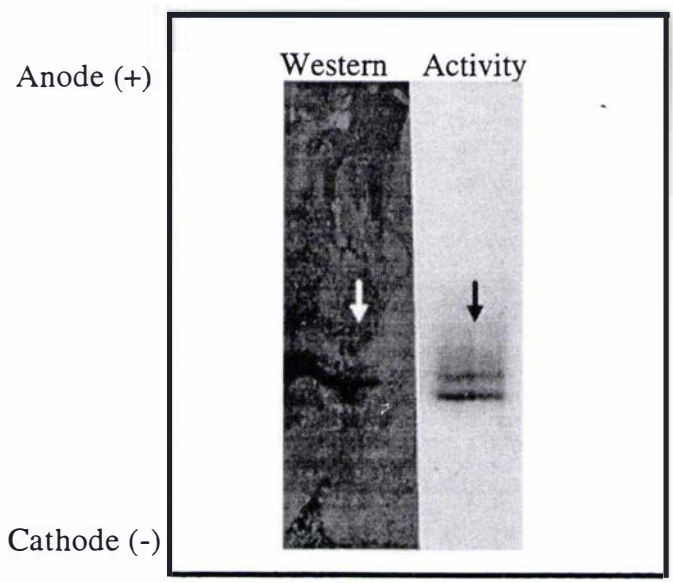

Figure 3.30

Activity staining and Western blotting with antibody YZ1/2.23 of Apase Ib after IEF.

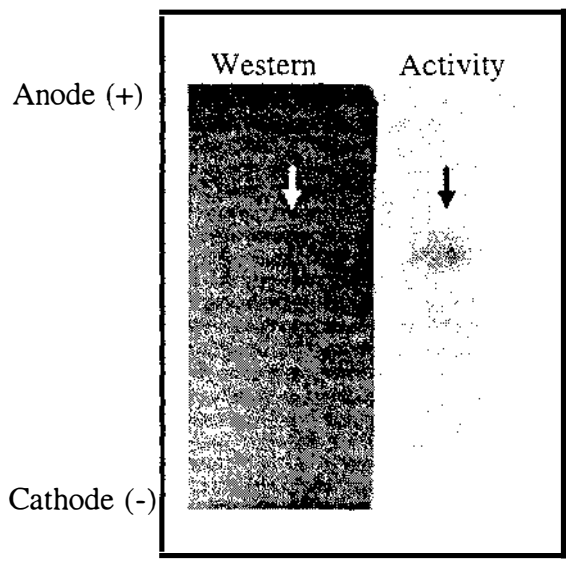

Figure 3.31

Activity staining and Western blotting with the GNA lectin of Apase IIa after IEF.

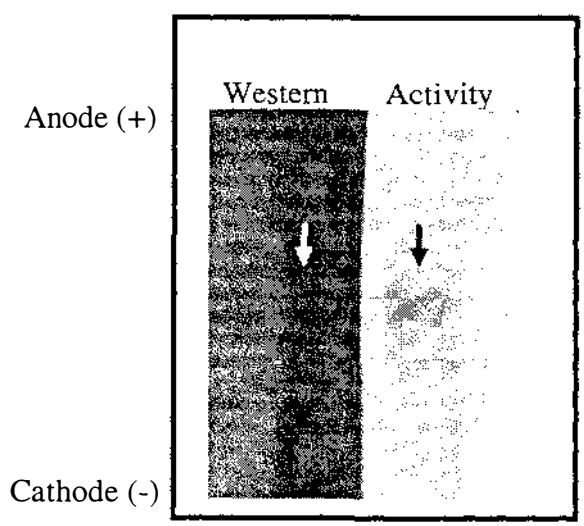

Figure 3.32

Activity staining and Western blotting with antibody YZ1/2.23 of Apase IIa after IEF.

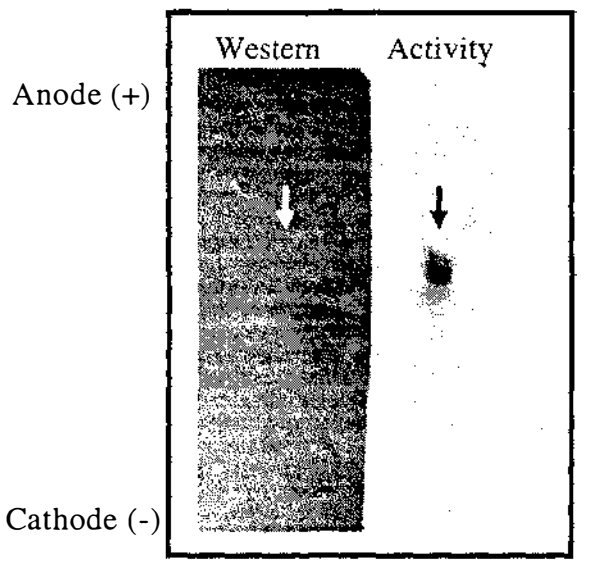

Figure 3.33

Activity staining and Western blotting with the GNA lectin of Apase IIb after IEF.

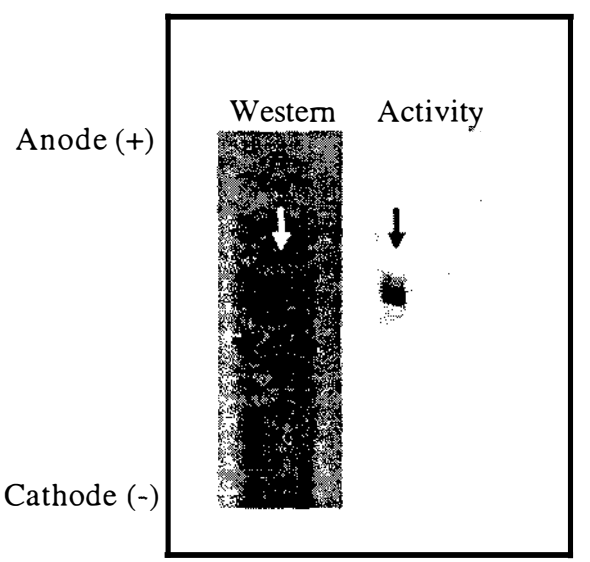

Figure 3.34

Activity staining and Western blotting with antibody YZ1/2.23 of Apase IIb after IEF.

3.5.8 Substrate specificity

3.5.8.1 Initial reaction velocity determination with seven substrates

In order to satisfy the requirement that the initial rate with the lowest substrate concentration used for the Km determination was linear, the reaction rate was measured at 2, 4 and 6 min intervals for each substrate at 0.05 mM (Figure 3.35). The Apase reaction rates for all 7 substrates tested were still linear at 6 min at the lowest substrate concentration tested.

3.5.8.2 Kinetic parameters: Km, Vmax and Vmax/Km

Reciprocal (Lineweaver-Burk) plots were used for the determination of Km and Vmax values. A Lineweaver-Burk plot of Apase Ia with ρNPP as substrate is shown as Figure 3.36. All calculations of the enzyme kinetic parameters were done using the program, ENZYPLOT, and the Km, Vmax values along with specific constants (Vmax/Km) for 7 substrates are shown in Table 3.4. The acid phosphatases extracted from the cell wall roots of white clover appeared to have a rather broad substrate specificity. The highest affinity was observed for ATP for all four isoforms with observed Km values for Apase Ia, Ib, IIa and IIb of 0.37, 0.28, 0.16 and 0.27 mM respectively. ATP was the best substrate for all four isoforms in terms of the specificity constant Vmax/Km. Pyrophosphate (PPi) was the second best substrate for Apase Ia, Ib and IIa, with *o*-phospho-L-serine the second best substrate for Apase IIb. 3-PGA and PEP were relatively poor substrates for all four isoforms. Phytic acid was not hydrolysed by any of acid phosphatases of white clover (data not shown).

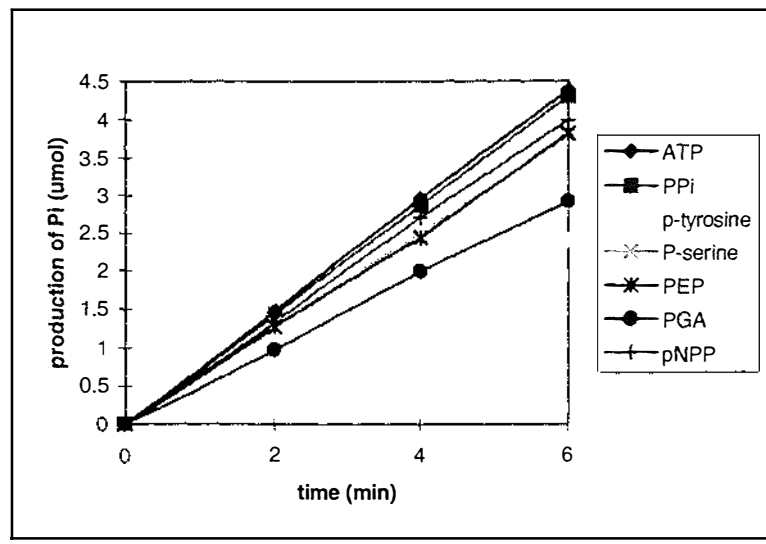

Figure 3.35

Acid phosphatase reaction rates with 7 substrates assayed at a concentration of 0.05 mM. Values are means of three measurements.

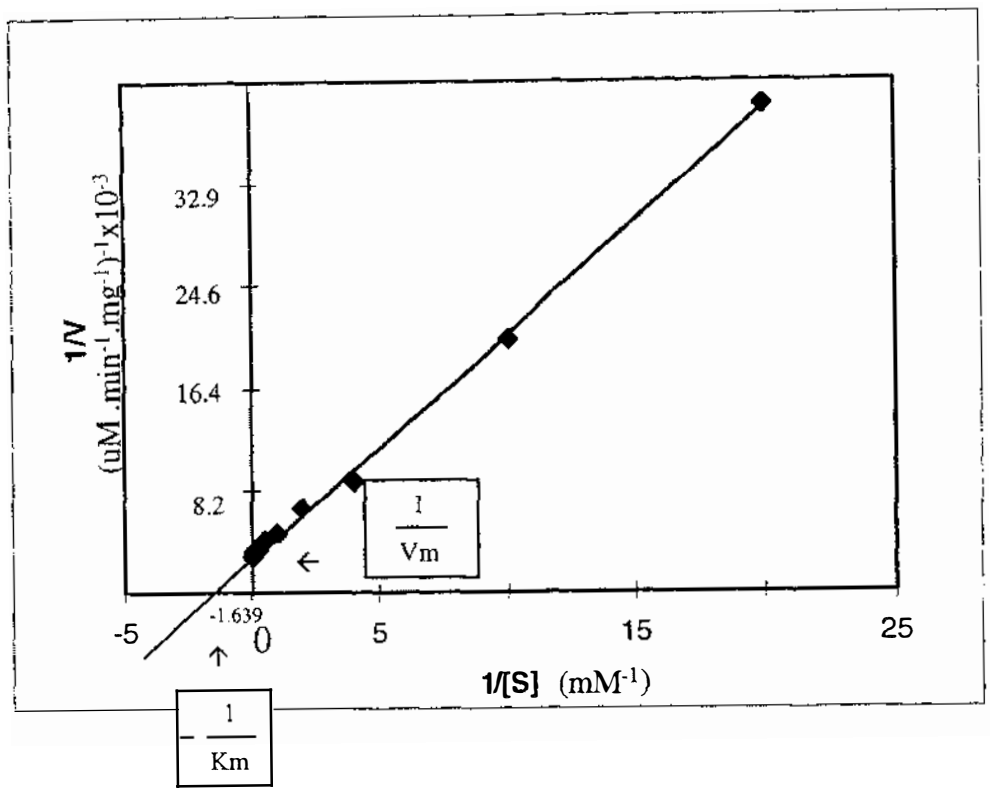

Figure 3.36

Lineweaver-Burk plot of Apase Ia with ρNPP as substrate.

Table 3.4

Substrate specificities of the four Apase isoforms.

		Vmax	Km	Vmax/Km
		μmol $min^{-1}mg^{-1}$	mM	
Apase Ia				
	ATP	152.8	0.37	412.97
	PPi	164.0	0.43	381.39
	ρNPP	222.3	0.61	364.42
	o-phspho-L-serine	188.3	0.56	336.25
	PEP	244.5	1.05	232.85
	3-PGA	106.2	0.65	163.38
	o-phospho-L-tyrosine	131.2	0.91	144.17
Apase Ib				
	ATP	53.52	0.28	191.14
	PPi	61.57	0.35	176.20
	o-phospho-L-tyrosine	88.65	0.53	167.26
	o-phspho-L-serine	82.23	0.57	144.26
	ρNPP	56.21	0.45	124.91
	PEP	93.06	0.83	112.12
	3-PGA	44.6	0.54	82.60

Table 3.4 (Cont)

		Vmax	Km	Vmax/Km
		μmol $min^{-1}mg^{-1}$	mM	
Apase IIa				
	ATP	84.57	0.16	528.56
	PPi	184.0	0.38	484.21
	3-PGA	138.01	0.53	260.39
	ρNPP	79.72	0.31	257.18
	o-phospho-L-tyrosine	108.25	0.58	186.64
	PEP	90.00	0.51	176.47
	o-phospho-L-serine	45.61	0.33	138.21
Apase IIb				
	ATP	120.01	0.27	444.48
	o-phospho-L-serine	172.66	0.41	421.14
	PPi	143.29	0.50	286.58
	ρNPP	26.52	0.30	88.40
	3-PGA	44.48	0.61	72.91
	o-phospho-L-tyrosine	37.83	0.56	67.55
	PEP	51.74	1.04	49.75

3.6 Further amino acid sequencing of Apase I

The first attempt of amino acid sequencing (Section 3.3) revealed that the single 52 kD protein band of Apase Ia fraction may contain more than one protein. Since SDS-PAGE did not separate those proteins, IEF was used to further fractionate Apase Ia and Ib after ion-exchange chromatography. Isoelectric focusing revealed that the Apase Ia preparation comprised five major protein bands visualised with Coomassie blue staining (Figure 3.37, a, b, c, d, e) and the Apase Ib preparation showed three major protein bands after Coomassie blue staining (Figure 3.38 b, c, d). However none of the major protein bands corresponded to the acid phosphatase activity bands (data not shown). The Coomassie blue staining bands which did correspond to the activity bands were very faint for both Apase Ia and Ib. Nevertheless a Coomassie stained band which co-migrated with Apase Ib (pI 6.7, Figure 3.38, band a) was cut out and trypsin digested (data not shown). Several peptides were applied to the gas-phase sequencer but only a short sequence (D-L-G-L-T) was obtained. This amino acid sequence was aligned with protein sequences in the NCBI database, but no significant sequence identity with any existing protein sequence was found.

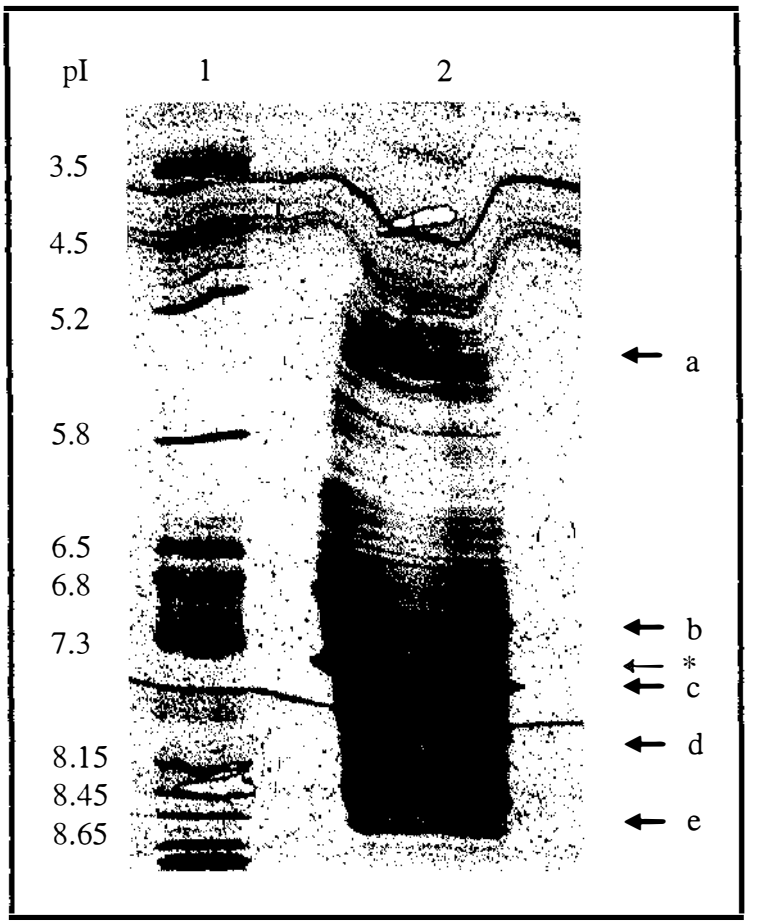

Figure 37

The separation of the Apase Ia preparation by IEF gel.

The proteins were visualised with Coomassie blue stain.

Lane 1 is standard proteins and Lane 2 is the Apase Ia preparation.

The letters a, b, c, d and e indicate five major protein bands separated from Apase Ia preparation. Apase Ia, identified by activity staining (not shown) is indicated here by the asterisk.

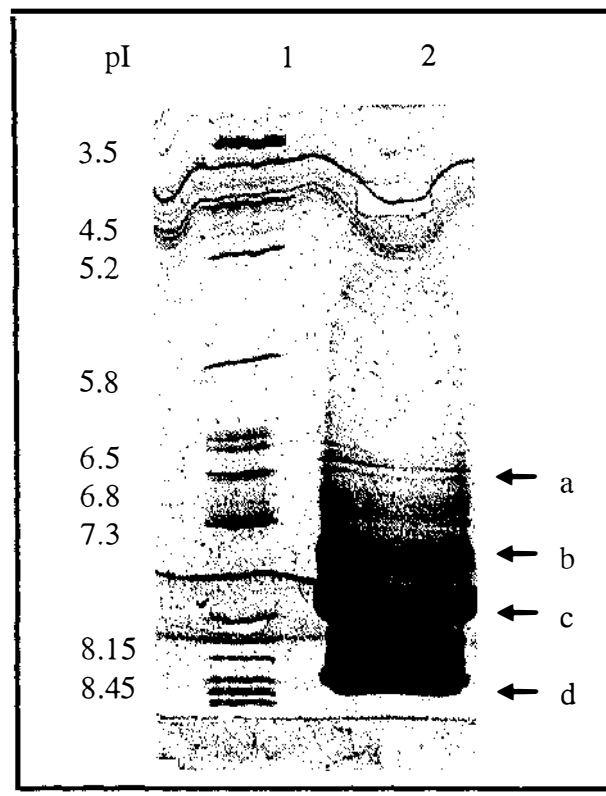

Figure 3.38

The separation of Apase Ib preparation by IEF gel.

The proteins were visualised with Coomassie blue stain. Lane 1 are standard proteins and Lane 2 is the Apase Ib preparation. The letters b, c, d indicate three major protein bands. Band a was identified by activity staining (not shown) as Apase Ib and sequenced.

Chapter Four: Discussion

4.1 Phosphorus (P) levels in leaves and cell wall acid phosphatase activity in roots of white clover

The first part of this study, on the effects of P-deprivation of the induction of Apases in the cell walls of roots of white clover, involved measuring changes in the P content of leaf tissue. This was performed as part of the 5-week time course to indicate sampling points for subsequent biochemical analysis. Significant decreases in P content in the foliage has been observed in many plant species as a pronounced consequence of the absence of, or inadequate exogenously supplied P (Besford 1979 b; Lee 1988; Garcia and Ascencio 1992; Fernandez and Ascencio 1994; Bosse and Köck 1998).

Besford (1979 b) examined changes in P content in leaves of seven plant species which were supplied with either low P (0.13 mM) or higher P (1.3 mM). The leaves of plants supplied with low phosphate contained considerably less P, with a 6-fold lower content measured in leaves of barley and cucumber, 3-fold lower in maize leaves, 2.7-fold lower in oat leaves, 2-fold lower in rice leaves, 5.7-fold lower in tomato leaves and 5-fold lower in wheat leaves when compared with the P content measured in leaves of these plants supplied with high P. Fernandez and Ascencio (1994) reported that P content was significantly lower in both roots and shoots of bean (*Phaseolus vulgaris)* and cowpea (*Vigna unquiculata*) after a 4-week growth period in low P nutrient solution (0.02 mM) when compared with control plants (supplied with 1.0 mM P). In these experiments, P content of the roots of beans reduced to 22%, and the roots of cowpea by 20% of the control while P content of shoots of beans and cowpeas were 40% and 14% of the control respectively. To estimate the influence of phosphate deficiency on the development of tomato seedlings, Bosse and Köck (1998) measured P content of seedlings grown in a nutrient medium containing either 4 mM P or no P. The phosphate deprivation revealed a clear decrease in P content in 14-day-old seedlings, the P content decreased to 14% of that measured in control plants.

In this thesis, the P levels measured in the youngest fully expanded leaves of white clover plants declined and reached a consistent minimum value (0.008% g^{-1} fresh weight) by the end of 4-week period maintained in P-deprived media. While this value is only 16% of the control (supplied with 5 mM P), the result is in agreement with the observation from other plant species that a decrease in, or deprivation of exogenous phosphate did result in a reduced P content in leaf tissue (Besford 1979 b; Garcia and Ascencio 1992; Fernandez and Ascencio 1994; Bosse and Köck 1998). Typical deficiency symptoms of white clover, such as the appearance of purplish or bronze spots in leaves (Wallace 1951), did not occur in these plants until at least 3 weeks in the P-deprived solution, but by 5 weeks plants were showing severe symptoms. In terms of the time course of decrease in P content in leaves, the maximum decrease was observed over the first 3 weeks, but with no further significant changes up to 5 weeks. Accordingly, root tissue was harvested at week 4 for subsequent Apase analysis, a time point by which the decrease in P content in leaf tissue had reached a maximum but before severe deficiency symptoms were evident.

Acid phosphatases (Apases) have been found in all plant species and tissues that have been studied (Duff et al 1994). The existence of acid phosphatases in cell walls of plants has been reported by many workers (Suzuki and Sato 1976; Hasegawa et al 1976; Crasnier et al 1980; Sugawara et al 1981; Dracup et al 1984; Crasnier and Giordani 1985; Tu et al 1988; Kaneko et al 1990; Duff et al 1991 a; Ferté et al 1993; Kaneko et al 1998) and salt elution is often used to extract acid phosphatases from plant cell walls. For example, about 65% of the Apase activity associated with sycamore cell wall fragments was solubilised by three washes of 1 M NaCl while the remaining enzyme could not be solubilised by further salt treatment, even with the salt concentration as high as 5 M (Crasnier and Giordani 1985). Acid phosphatases, which can be solubilised from cell walls with high concentration salt, are believed to be ionically bound (Brett and Waldron 1996) while those that remain, and can not be solubilised with high concentration salt, are probably covalently bound (Suzuki and Sato 1976; Dracup et al 1984; Crasnier and Giordani 1985). In this thesis, acid phosphatases which could be solubilised from the cell walls of white clover roots with 1 M NaCl treatment were the class of enzyme studied and are referred to as cell wall acid phosphatases.

The cell wall Apase activity of white clover roots increased over a 5-week period in P-deprived media while the cell wall Apase activity of control plants grown in P-supplied media maintained at consistent low level over the 5-week time course. This result is in agreement with results reported by many other workers: increased Apase activity in higher plants is observed in response to P-depletion in the external medium (Duff et al 1994). It has been found that the increased Apase activity in plant tissue occurs before deficiency symptoms become evident and it has been proposed that it is possible to use this enzyme as a rapid and simple indicator of phosphorus deficiency (Besford 1979 a). In this study, white clover plants did not show phosphorus deficiency symptoms until the plants had been in the P-deprived media for at least three weeks, but the cell wall Apase activity of roots had already increased to two-fold by the end of one week in the P-deprived media and was significantly higher than the control plants (P-supplied). These results indicate that increased cell wall Apase activity of roots could be used as a biochemical indicator of phosphorus deficiency for white clover although other workers have shown that there is no correlation between the timing or magnitude of Apase induction and the efficiency of P use by different white clover genotypes (Caradus and Snaydon 1987; Hunter and McManus 1999). However, of more significance to this thesis is the observation that the cell wall Apase activity was induced in roots in response to P-deprivation and it is this group of enzymes that was studied further.

4.2 Separation and partial purification of four isoforms of cell wall (1 M NaCl extractable) Apases from roots of white clover grown in P-deprived condition

Although there have been many reports which identify and characterise acid phosphatases from various plant species, organs, tissues and suspension cell cultures, studies on cell wall acid phosphatases are fewer when compared with those on the cytoplasmic acid phosphatases. Plant tissues from which cell wall acid phosphatases have been extracted with salt (NaCl, KCl) include potato tubers (Sugawara et al 1981 and Tu et al 1988), barley roots (Hall and Butt 1968; Panara 1990), wheat roots (Hasegawa et al 1976), corn roots (Tu et al 1988), subterranean clover roots (Dracup

et al 1984) and white clover leaves and roots (Hunter et al 1999). Cell wall acid phosphatases have also been identified using suspension cultured cells, such as tobacco cells (Suzuki and Sato 1976; Kaneko et al 1990; Kaneko et al 1998), sycamore cells (Crasnier et al 1980; Crasnier and Giordani 1985) and soya-bean cells (Ferté et al 1993). Many studies have shown that plants respond to environmental conditions of low phosphate availability by increasing the activities of phosphatases. However fewer studies have examined such changes in the cell-wall-bound Apases (Dracup et al 1984) or in cell wall Apases (Ueki and Sato 1977; Hunter and McManus 1999; Hunter et al 1999). Although Hunter et al (1999) examined the increase of cell wall Apases of whole plants in response to P-deprivation, their investigation of the cell wall acid phosphatases was confined to crude extracts. The aim of the present work was to purify and characterise this group of cell wall Apases that are induced in roots of whole plants in response to P-deprivation.

HPLC (High Pressure Liquid Chromatography) and FPLC (Fast Protein Liquid Chromatography) are commonly used for purification of acid phosphatases. In this study, FPLC with three purification steps (hydrophobic interaction, gel filtration and ion exchange chromatography) was used for the purification of cell wall Apases of roots of white clover grown in P-deprived conditions. Hydrophobic chromatography using Phenyl Superose separated the increased cell wall Apase activity of roots of P-deprived white clover into two components, Apase I and Apase II designated according to the sequence of their elution from the column. Since they were eluted off the Phenyl Superose column at a different percentage buffer B, they differ in hydrophobicity. This hydrophobic difference may result from a different content, or number of hydrophobic amino acid residues on the protein surface and suggests that Apase I and Apase II are distinct proteins. Further, there is a difference between Apase I and Apase II in terms of the timing of response to P-deprivation as shown by changes in the relative proportion of Apase I and Apase II over 4 weeks of P-deprivation. Apase II reached its maximal activity after a week of P-deprivation and remained at a similar level in following weeks. The activity of Apase I increased progressively during 4 weeks of P-deprivation and the highest activity was observed at the end of time course examined in this study. Apase II was the major component of the induced activity after 1 week in P-deprived condition, while by week 4 Apase I was the prevalent enzyme. This temporal difference

in induction between distinct Apases in roots has not been observed previously in other plant species, and its significance is assessed further when the substrate specificities of each are compared.

Multiple forms of Apases have been reported from studies characterising cell wall phosphatases from other species. Using roots from whole wheat plants, two cell wall forms of 60 kD and 32 kD were identified (Hasegawa et al 1976). However, these were separated using gel filtration chromatography and so the possibility of dimers can not be excluded. In tobacco cell cultures, Ueki and Sato (1977) used gel filtration and ion-exchange chromatography to separate 3 distinct Apases and Sugawara et al (1981) used ion-exchange to identify 6 distinct Apases in the cell walls of potato tubers.

Gel filtration chromatography, the second step in the purification process, revealed that Apase I and Apase II also differ in native molecular weight. The molecular weight of Apase I is 52 kD and Apase II, 92 kD. When white clover Apase I and Apase II were subjected to a Mono-S ion exchange chromatography, the final step in the purification process, each fractionated further into two isoforms, designated Apase Ia, Ib, Apase IIa and IIb.

The four Apase isoforms were purified 121-, 58-, 13- and 92-fold respectively after the final purification step of ion-exchange chromatography. The purification fold in this study appears to be low when compared with some Apases purified from other plant sources. For example, an acid phosphatase from wheat germ was purified 7000-fold (Waymack and Van Etten 1991) or an Apase from potato tubers has been purified 2289-fold (Gellatly et al 1994). However, a more direct comparison with other studies which have purified salt-extractable Apases from cell walls is more favourable. Ferté et al (1993) purified an Apase from cell suspension cultures of soybean to apparent homogeneity and reported a 34-fold purification, while Kaneko et al (1998) reported the purification of a single Apase from the walls of tobacco cells in suspension cultures as a 57-fold purification. It is likely that the elution of a specific group of proteins (those extracted with high salt from the cell wall) already represents a significant purification step (although a direct comparison of high-salt extractable protein fraction with total protein content has not been made). Further evidence for this enrichment comes from comparison of specific activity at the crude extract stage. In this thesis, a value of 5.80

μmoles/mg protein was determined, as compared with 0.27 μmole/mg protein in a crude extract from potato (Gellatly et al 1994) or 0.33 μmole/protein in a crude extract from onion bulbs (Guo and Pesacreta 1997). The potato tuber Apase was purified 2289-fold (and reported to be electrophoretically-pure), with a specific activity was 618 μmoles/mg protein (Gellatly et al 1994), and the onion bulb Apase was purified 609-fold (and reported to be electrophoretically pure) with a specific activity of 201 μmoles/mg protein (Guo and Pesacreta 1997). In this study, Apase Ia was purified to a specific activity of 705 μmoles/mg protein, IIb to 539 μmoles/mg protein and Ib to 339 μmoles/mg protein.

The major band in Apase Ia (the isoform with the highest specific activity) identified by gel electrophoresis using the mini-gel system, co-migrated with the acid phosphatase activity and so Ia was excised and submitted for amino acid sequencing (Figure 3.10). Two peptides were sequenced and database comparison revealed identity to the EDGP protein, a wound-induced extracellular glycoprotein from suspension cultured cells of carrot (Satoh et al 1992) (Figure 3.11). The second peptide had no apparent identity with any sequences in the database. This may be an acid phosphatase since Apase sequences identified from other plants have also been shown not to find matches (Tanaka et al 1990; Lebansky et al 1992; Kawarasaki et al 1996). The sequence obtained from Ia also did not show any homology with these sequences. A sequence from one Apase (from pea plumules, Guo et al 1998) did show some identity to the amino acid sequence of a kidney bean purple Apase, but again, the sequence identified in this thesis did not match these.

The discovery of two proteins in Ia fraction suggests that at least one other protein has co-purified with Apase Ia. Further sequencing of Ia or Ib, IIa or IIb, therefore, was not attempted until purity was assessed further.

4.3 Properties of four isoforms of cell wall Apases of white clover roots

In order to further investigate the relationship between Apase I and II, and the apparent isoforms of each (Ia, Ib, and IIa, IIb), a combination of native and denaturing SDS-PAGE was used initially to determine subunit isoformation for each enzyme.

Using non-denaturing gradient gel electrophoresis, two very closely located, strongly stained protein bands as well as a more faintly stained minor band were identified in the Apase Ia and Ib preparations. Activity staining revealed a single activity band of *ca.* 52 kD which co-migrated with the lower molecular weight protein of the two closely migrated bands identified by Coomassie blue staining. For the Apase IIa and IIb isoform preparations, nondenaturing mini-gel SDS-PAGE and Coomassie blue stain revealed a major staining band of *ca.* 112 kD which co-migrated with the major activity staining. Protein staining also detected minor protein contaminants which did not show any corresponding Apase activity.

Each isoform preparation was then reduced with 5% 2-mercaptoethanol and separated by SDS-PAGE and then activity stained. In these separations, the activity band of each isoform migrated to the identical place as the equivalent non-reduced isoforms (Figure 3.18). This result suggests that the four isoforms are active as monomers. After SDS-PAGE under reducing conditions, the relative mobility for each activity subunit for each of the four isoforms was calculated according to the migration from the origin. A least-squares program was used to fit these data to a protein standard curve and gave apparent molecular weights of 52 kD for Apase Ia and Ib, 112 kD for Apase IIa and IIb. The molecular weights determined by gel filtration chromatography were 52 kD for Apase I and 92 kD for Apase II. These molecular weights were determined by elution of enzyme activity and so confirm the monomeric identity of each isoform.

Plant acid phosphatases exhibited considerable heterogeneity with regards to their native molecular mass. However, the majority of these enzymes appear to exist as monomeric or dimeric forms having molecular masses ranging between 50,000 to 100,000 (Crasnier et al 1980; Jonsson 1981; Park and Van Etten 1986; Bhargava and Sachar 1987; Panara et al 1990; Biswas and Cundiff 1991; Ferté et al 1993; Staswick et al 1994; Guo and

Roux 1995; Ferreira et al 1998 b). The molecular weights of four Apase isoforms of white clover are within this range.

The use of SDS-PAGE, in combination with gel filtration chromatography, suggests that Apase I and II are monomers with different molecular weights further supporting the view that these proteins are distinct acid phosphatases. The identical molecular weights of Ia and Ib, and IIa and IIb suggest that these represent isoforms of Apase I and Apase II respectively. In terms of purity, non-denaturing SDS-PAGE of Apase Ia and Ib revealed the occurrence of two major staining proteins in these preparations which is consistent with the observation (from amino acid sequencing) that there are at least two proteins in the Ia preparation. Further, the acid phosphatase activity co-migrated with only one of these proteins. For IIa and IIb, protein staining after non-denaturing SDS-PAGE revealed a major staining protein of 112 kD with associated acid phosphatase activity. Nevertheless, the observation of more than one protein in the Ia preparation was sufficient cause to delay sequencing IIa and IIb until further purity was achieved. However, the combination of hydrophobic and ion-exchange column chromatography has separated Apase Ia and Apase Ib and Apase IIa and Apase IIb sufficiently to enable the analysis of the biochemical properties of each enzyme. Further, the use of IEF after these FPLC purification steps, in combination with activity staining, provides a means to identify each Apase isoform for accurate pI determination and for the identification of N-linked glycans using specific probes.

Biochemical characterisation of each isoform was therefore undertaken with a view

(I) to define differences between the isoforms of Apase I and II at the biochemical level, and

(ii) to accumulate biochemical data on Apase I and Apase II which may serve to indicate the likely functions of these enzymes in roots of white clover.

pH optima

Using ρNPP as substrate, Apase Ia, Ib, IIa and IIb, had optimal pH values of 5.8, 6.2, 5.8 and 6.8 respectively. These values are similar to pH optima reported for other plant

acid phosphatases, for example, pH 5.0-6.0 for cell wall isozymes of potato tubers (Sugawara et al 1981), pH 5.0 for cell wall Apase from wheat roots (Hasegawa et al 1976), pH 5.6 for an Apase secreted by tomato roots (Li and Tadano 1996), pH 5.8 for an Apase from potato tuber (Gellatly et al 1994), and pH 6.5 in pea plumules (Guo et al 1998) and confirm these enzymes are phosphatases with an acid pH optimum. The pH optima of Apase Ia and Apase IIa were examined using two buffer systems, a citrate buffer and a maleic acid-Tris buffer. Each of the isoforms exhibited the same pH optimum irrespective of which buffer system was used, which differs from Apases purified from soybean leaves (Staswick et al 1994) and soybean suspension cultures (Lebansky et al 1992) which displayed a shift in pH optima of 0.4 or 1 unit respectively in different buffer systems.

All four isoforms showed fairly broad pH activity profiles. Again this observation is consistent with those of Apases from other plant species. For example, more than 50% of the maximum activity was observed between pH 3 to 6 for an Apase secreted by lupin roots under phosphorus-deficiency conditions (Ozawa et al 1995), and a cell wall Apase from subterranean clover roots was active over the pH range of 4-7 (Dracup et al 1984). The Apases from wheat roots had pH optima of 4-5 (Hasegawa et al 1976) and a secretory Apase of tomato roots had a broad pH dependency with more than 50% activity at pH from 4-7 (Li and Tadano 1996). This broad pH optima of acid phosphatases may reflect the acidic cell wall environment which can vary over 1 pH unit. The broad pH optima may also be an advantage for these enzymes since they hydrolyse a variety of substrates which may require different pH to confer the optimal charge configuration for the amino acid(s) at the active site of the enzyme.

Isoelectric point

Isoelectric focusing on polyacrylamide gels in the range of pH 3-10 was used to determine the pI values of each of the 4 Apase isoforms in this study. The IEF gel was incubated in a β-naphthyl acid phosphate / Fast Garnet activity staining solution following isoelectric focusing which provides an accurate means of enzyme identification. The two isoforms of Apase I had close to or neutral pI values, while two

isoforms of Apase IIa and IIb had acidic pI values. Apase Ia gave rise to two bands with pI values of 7.0 and 7.3 and Apase Ib showed a major band with a pI of 6.7 and four more faintly stained bands on both the cathodic and anodic sides. Apase IIa exhibited a rather diffuse band with a pI value of 4.4. Apase IIb split into two closely located bands with pI values of 5.2 and 5.3.

Of the four isoforms, only Apase IIa showed one activity band on IEF gel but this was a rather diffuse band. Apase Ib displayed a major band at pI 6.7 with 4 minor bands which may arise through amidation of the protein during IEF (Olczak et al 1997). The other isoforms (Apase Ia, IIb) appear to be heterogeneous in terms of their behavior using isoelectric focusing since they comprise two equally staining pI bands. A similar phenomenon has been observed in other acid phosphatase studies. The molecular weight of the Apase from the endosperm of date palm (*Phoenix dactylifera*) seeds was 52 kD determined by gel filtration on a Sephacryl S-200 column. However when this enzyme was subjected to IEF gel, six forms with pI between 3.6 and 4.3 appeared (Sekhar and DeMason 1989). A 77 kD acid phosphatase of barley roots showed one major activity band and three minor bands after IEF (Panara et al 1990); isoelectrofocusing of a 93 kD Apase purified from yellow lupin seed revealed the presence of four molecular forms with pI values from 6.8 to 7.3 (Olczak et al 1997).

Many investigators have proposed reasons to explain the occurrence of multiple forms of acid phosphatase after IEF (as distinct from minor bands which probably arise form protein modification during IEF). It has been postulated that the range of pI values could indicate multiple phosphatases, but more likely it is the result of posttranslational modifications of one enzyme (Guo and Roux 1995). Since many acid phosphatases have been shown to be glycoproteins (Li and Tadano 1996, Nakazato et al 1997, Ferreira et al 1998 b and Olczak et al 1997), it is possible that the difference in pI between isoforms may be due to glycochain length or heterogeneity of sugar structures (Li and Tadano 1996). Such speculation is in good agreement with a theory that two evolutionary strategies may be involved in the differentiation of enzyme activity into isozymic forms (Biswas and Cundiff 1991). Firstly, isozymes are the products of different genes, which are possibly derived through evolution by gene duplication, mutation and chromosomal translocation. Alternatively, isozymes could be the products of the same structural gene

but are generated by alternative pathways of RNA splicing. In this thesis, the term isoform is used to denote the same gene but with gene products undergoing different posttranslational modifications, such as glycosylation. The very different molecular weights of monomeric Apase I and monomeric Apase II of white clover may imply their different gene origins, but two isoforms of each enzyme may result from modifications of same gene product. It will be interesting to determine the molecular origins for the isoforms of acid phosphatases from cell wall of roots of white clover once full amino acid sequence can be obtained. However through the use of specific sugar probes, the glycan status of each isoform was compared in this thesis.

N-linked glycosylation study

Although many plant acid phosphatases have been reported to be glycoproteins (Stahl et al 1994; Ferens and Morawiecka 1985; Park and Van Etten 1986; Bhargava and Sachar 1987; Duff et al 1991 b; LeBansky et al 1992; Miernyk 1992; Li and Tadano 1996; Olczak et al 1997; Pasqualini et al 1997), little information is available about the structural features of enzyme oligosaccharide chains. Olczak et al (1997) investigated the structural features of oligosaccharide chains of an Apase from yellow lupin seeds using different lectin sugar probes and observed the strong interaction between the enzyme and GNA or AAA, indicating the presence of terminal mannose and fucosylaiton of the N-glycosidic oligosaccharide chains in the structure. In present study, probes for two N-linked glycans, a digoxignin-labeled GNA lectin (which recognises terminal mannose or oligomannose N-linked glycan chains) and a monoclonal antibody, YZ1/2.23 (which recognises xylose/fucose -containing complex-type glycan structures) were used in the determination of the glycosylation status of the four Apase isoforms of white clover. Both Apase Ib and IIb were recognised by YZ1/2.23 but not by GNA lectin, indicating the existence of xylose/fucose-containing complex-type glycan structure in both enzymes. Apase Ia was recognised by both the GNA lectin and YZ1/2.23 antibody suggesting either (i) more than one class of structure at a single site, or (ii) more than one glycosylation site with different classes of structures at each site, or (iii) a hybrid glycan structure with epitopes recognised by both. Apase IIa seems not to be recognised by either sugar-specific probe. To determine whether this isoform is

glycoprotein or not, other sugar probes such as PNA (from *Arachis hypogaea*) which recognises O-glycosidically bound oligosaccharides, could be used. However, a more detailed examination of the purified protein is required including treatment with specific N-glycosidases to determine if any N-linked sugar structures are present.

With the exception of speculation that the different pI values of Apase isoforms may simply be due to heterogeneity in oligosaccharide chains attached to the enzyme, little is known about the roles these glycan side chains may play. Ferens and Morawiecka (1985) reported that purified Apase from rye germ could be activated by several plant lectins. Lectins are a class of proteins that bind carbohydrates. It was found that the Apase in crude rye germ extract was accompanied by agglutinating activity. The separation of the Apase from the agglutinating protein caused a significant loss of enzyme activity. The addition of a crude endogenous lectin preparation to rye germ Apase caused a gradual increase of enzyme activity. According to this result, as well as the fact that WGA (wheat germ agglutinin), SBA (soybean agglutinin), LL (lentil lectin), con A (concanavalin A) and *St*A (*Solanum tuberosum* agglutinin) can activate rye germ Apase activity, they proposed that these lectins may play a protective role by stabilisation of the most active enzyme conformation (Ferens and Morawiecka 1985). However, apart from this study there are no other which directly seek to ascertain function of oligosaccharide chains of the enzymes. The role of glycan chains on other plant enzymes, for example, peroxidase has been speculated upon (Van Huystee and McManus 1998). For example, the glycan moieties are proposed to be important in the regulation of enzyme conformation, protection from proteases and in subcellular targeting (for example to the wall). It is likely, therefore, that the glycan structures on Apases have a similar function.

Thermal stability

Thermal stability of acid phosphatases is often tested by heating enzyme preparations for a period of time at various temperatures and then measuring the remaining activity at a certain temperature, usually at 37 ^{0}C, and this approach was used in this study. The thermal stability difference lies between Apase I and Apase II, but not between two

isoforms of each group. Two isoforms of Apase II (IIa and IIb) are more thermal stable than two isoforms of Apase I (Ia and Ib). Apase IIa and IIb did not lose activity after incubation at 55 ^{0}C while Apase Ia and Ib lost about half of its original activity. This differential thermal stability of Apases have been observed with those of other plant Apases. Biswas and Cundiff (1991) investigated the heat stability of 4 Apase isozymes from germinating seeds of *Vigna sinensis* by preincubation for 60 min at 60 ^{0}C at pH 5. One isozyme retained 78% of its initial activity, while the other three retained about half of their initial activities. In another study, four isoforms of soybean seeds showed the same thermal stability, losing about 20% activity at 60 ^{0}C after 60 min incubation (Ferreira et al 1998 a). The different thermal stabilities of the isozymes suggest that these preparations comprise distinct Apases (for example the isozymes from germinating seeds of *V. sinensis*), while the similar thermal behaviour of isoforms suggests that they arise from the same protein (for example the isoforms from soybean seeds). The thermal stability study in this thesis supports the proposal that Apase I and Apase II are distinct proteins.

Inhibitors of white clover Apases

Inorganic phosphate (Pi) is an end product of hydrolysis of various phosphoesters by acid phosphatases. All the four Apase isoforms, in common with other phosphatases, are inhibited by Pi at the concentrations used of 1 to 20 mM. Again, there are differences between Apase I and Apase II rather than between isoforms with the two isoforms of Apase I being more sensitive to Pi than the two isoforms of Apase II. The activity of Apase I was completely abolished by 20 mM Pi, while Apase II still exhibited 20% activity. This inhibition is proposed to play a physiological role in which a constant level of phosphate in a cell is maintained through feedback regulation of Apases releasing P from storage forms. Such regulation of Apase is likely intracellular with Pi acting as a signal between the cytoplasm (where P is used in metabolism reactions) and the vacuole, the primary site of Apase activity. In the wall, such feedback regulation could be important in regulating the concentration of Pi at the site of the Pi transport for external

Pi for uptake, Pi may signal a decrease in the requirement for Pi and hence a decrease in Apase activity.

It has been known that many phosphatases are stimulated or inhibited by metal cations or other effectors (Ferens and Morawiecka 1985; Duff et al 1991 b; Biswas and Cundiff 1991; Gellatly et al 1994; Biswas et al 1996; Pasqualini et al 1997; Nakazato et al 1997). The effect of numerous divalent cations, the chelating agent (EDTA) and tartrate were tested in this study with the major purpose of comparing Apase I and II and to compare these enzymes with other cell wall enzymes in terms of sensitivity to metal cations. The four Apase isoforms of white clover did not seem to be metal-dependent proteins for the stimulation of activity, as the various metal ions tested were generally ineffective at significantly stimulating activity except, perhaps, Ba^{2+} which moderately activated the activity of Apase Ib (increased by 40%). Mg^{2+} has been known to be an activator for many acid phosphatases (Tu et al 1988, Duff et al 1989 and Gellatly et al 1994), but it was ineffective in this study.

The four Apase isoforms were strongly inhibited by Zn^{2+}, Cu^{2+} and molybdate and the presence of Fe^{3+} in the assay mixture reduced activity by *ca.* 50% for all four isoforms. Molybdate has been reported to be a potent inhibitor to a secretory acid phosphatase of lupin and tomato roots (Li and Tadano 1996), an acid phosphatase of black mustard cell-suspension cultures (Duff et al 1991 b) and of germinating seeds of *Vigna sinensis* (Biswas et al 1996). Zn^{2+}, Cu^{2+} and Fe^{3+} were also strong inhibitors to acid phosphatases of *Brassica nigra* (Duff et al 1989), *Vigna sinensis* (Biswas et al 1996), peanut seeds (Basha 1984) and red kidney bean (Cashikar et al 1997). It is interesting to note that the two isoforms of Apase I are more sensitive to Co^{2+} and Al^{3+} than the two isoforms of Apase II. In the presence of 5 mM Co^{2+} or Al^{3+}, the activities of Apase Ia, Ib, IIa and IIb were 61%, 35%, 89% and 104% or 65%, 42%, 79% and 95% respectively.

Little is known about mechanisms to account for inhibitory effects of metal ions on plant acid phosphatase activity. A few studies report such mechanisms of metal ion inhibition on animal acid phosphatases (Doi et al 1987; Doi et al 1988). Uteroferrine, an acid phosphatase isolated from the uterine secretion of pregnant sows, is inhibited by molybdate. A single monomeric species of molybdate, likely as a ligand of the binuclear iron cluster, binds to the uteroferrin to form an uteroferrin-molybdate complex in which

molybdate was incapable of undergoing redox chemistry by accepting an electron from the protein. Instead, it induces a conformation changes leading to inactivation of the enzyme. Some acid phosphatases of plant origin have been shown to contain metal ions (Uehara et al 1974; Beck et al 1986) and for this group of enzymes, inhibition of activity can be caused by interaction of the added metal cations with the ion cluster at active site of the enzymes. As for Apase isoforms of white clover, it has not been determined whether any metal ions are present at the active site. It is necessary to gather this information before further investigation of the mechanism of inhibitory effects of metal ions. However in terms of inhibition by Zn^{2+}, Cu^{2+} and molybdate the Apases from white clover are much similar to the cell wall Apases from wheat roots (Hasegawa et al 1976), and to some cell wall isoforms of potato tubers (Sugawara et al 1981).

Metal-binding agents such as EDTA are often used to identify the metalloprotein status of acid phosphatases. The effects of EDTA on Apases are commonly determined by the inclusion of EDTA in the enzyme reaction mixtures (Lin et al 1980; Lorenc-Kubis and Morawiecka 1980; Kruzel and Morawiecka 1982; Ching et al 1987; Duff et al 1989; De-Kundu and Banerjee 1990; Tanaka et al 1990; Miernyk 1992; Gellatly et al 1994; Guo and Roux 1995; Penheiter et al 1997).

In this study, the activity of the four isoforms was not effected by 5 mM EDTA added to the reaction mixture. Although EDTA did not effect the activity of the four isoforms in the condition of this study, it is perhaps premature to conclude that they are not metallloproteins since prolonged incubation with or prolonged dialysis against EDTA have been reported to cause partial losses of activity of acid phosphatases (Ferté et al 1993, Cashikar et al 1997). The soya-bean cell-wall phosphatase lost 20% activity after 20 min incubation in the presence of 1 mM EDTA and 40% after 48 hour of incubation (Ferté et al 1993). Red kidney bean purple acid phosphatase activity was reduced to 50% of its initial value after dialysis against EDTA (Cashikar et al 1997). Apart from the time factor, different metal chelators may have different effects on an acid phosphatase. When incubated with EDTA or L-tartrate, the acid phosphatase from rice grains was inactivated. However, *o*-phenanthroline and α,α'-dipyridyl did not inhibit the activity (Yamagata et al 1980). The violet-colored Apase of sweet potato was inactivated by α,α′-dipyridyl or *o*-phenanthroline, but not by EDTA or CyDTA (*trans*-1, 2-

cyclohexanediamine-N, N, N', N'-tetraacetic acid) (Uehara et al 1974). Therefore it is necessary to do prolonged incubations as well as examining different metal chelators (in addition to EDTA) before making any conclusions about the status of white clover Apases as metalloprotein.

L-(+)-tartrate, a strong competitive inhibitor of mammalian prostatic acid phosphatases, did not effect the activity of the four cell wall isoforms of white clover roots. This insensitivity towards tartrate has been observed with many plant Apases (Sugiura et al 1981; Guo and Roux 1995; Cashikar et al 1997; Olczak et al 1997; Guo et al 1998; Ferreira et al 1998 b); only a few of Apases from plant origin have been reported to be sensitive to tartrate (Yamagata et al 1980; Ferens and Morawiecka 1985; De-Kundu and Banerjee 1990). Although little is known about inhibitory action of tartrate on plant Apases, detailed study of inhibitory effect of tartrate on animal Apases is available. For example, Lindqvist et al (1993) reported that tartrate binds to rat prostatic acid phosphatase and interacts with the conserved residues Arg-11, His-12 and Arg-15 at active site leading to inactivation of the enzyme. Two active site carboxyl groups have been reported to be essential for catalytic activity of a human prostate acid phosphatase (Saini and Van Etten 1979). The treatment of homogenous human prostatic acid phosphatase with low concentration of Woodward's reagent K, a carboxyl residue modification reagent, leads to a rapid loss of enzymic activity. The enzyme is protected against inactivation by reagent K in the presence of various competitive inhibitors, such as L-(+)- tartrate. The activity of Apases from *Vigna radiata* seedlings (De-Kundu and Banerjee 1990), rye germ (Ferens et al 1985), rice grains (Yamagata et al 1980) is strongly inhibited by tartrate, and their active site may resemble those of animal acid phosphatases. The insensitivity of white clover Apase towards tartrate implies that its active site is distinct from those of Apases of *Vigna radiata*, rye germ and rice grains.

4.4 Substrate specificity and putative functions of each isoform

The four Apase isoforms of white clover were tested for their Michaelis constant (Km), maximal velocity (Vmax) and specificity constant (Vmax/Km) against a range of phosphoester substrates. The enzymes appear to have a broad substrate specificity, and

in terms of the specificity constant (Vmax/Km), with ATP the best substrate tested for all four Apase isoforms followed by pyrophosphate. The exception is Apase IIb, for which pyrophosphate is the third best within the substrates tested. Very similar substrate specificities were observed with a cell wall purple acid phosphatase from red kidney bean cotyledons (Cashikar et al 1997), red kidney bean seeds (Beck et al 1986) and a violet-colored acid phosphatase from sweet potato (Uehara et al 1974). The artificial substrate ρNPP has been reported to be the best substrate for many acid phosphatases (Pasqualini et al 1997, Giordani et al 1986, Hasegawa et al 1976, Basha 1984 and De-Kundu and Banerjee 1990). In marked contrast to this, ρNPP was at the third place on the substrate list for Apase Ia, fifth for Ib, fourth for Apase IIa and IIb.

All the four Apase isoforms could not hydrolyse the plant phosphate-storage compound phytate. This phenomenon was also observed with red kidney bean purple acid phosphatase (Cashikar et al 1997) and violet-colored acid phosphatase from sweet potato (Uehara et al 1974).

Duff et al (1994) has classified acid phosphatases into two categories: specialised acid phosphatases which have a clear but non-absolute substrate specificity and non-specific acid phosphatases which display absolutely no substrate preference. Phytase, PEP acid phosphatase and 3-PGA phosphatase have been reported to be specialised acid phosphatase (Randall and Tolbert 1971 a, b; Gibson and Ullah 1988; Duff et al 1989). However there is no clear standard to categorise acid phosphatases, it is somewhat arbitrary. Specificity constant, Km or percentage of the activity compared with ρNPP have been used in description of substrate specificity. An acid phosphatase from *Brassica nigra* suspension cells is defined as PEP acid phosphatase because it displayed a six-fold higher specificity constant value for PEP than those for any other nonsynthetic substrates (Duff et al 1989). An acid phosphatase from spinach leaves is named 3-PGA phosphatase since it showed 30% higher activity for 3-PGA than for next best substrate (Randall and Tolbert 1971 b). In this study, although all four isoforms showed a preference for ATP, the specificity constant for ATP is less than two-fold higher than that of the next best substrate. Therefore the four isoforms should belong to the non-specific acid phosphatase category.

The Km values of Apase Ia, Ib, IIa and IIb for ρNPP were 0.61, 0.45, 0.31 and 0.30 mM respectively. Similar Km values of other acid phosphatases for ρNPP are found to be in the range of 0.1-0.6 mM. For example, the Km value of an acid phosphatase from cotton seedlings was 0.5 mM (Bhargava and Sachar 1987), the Km value of an acid phosphatase isolated from cultured *B. nigra* cells was 0.57 mM (Duff et al 1989) and from lupin roots, 0.27 mM (Ozawa et al 1995).

Although much effort has been made to identify the substrate specificity of acid phosphatases *in vitro* in the hope that it may provide information about physiological roles *in vivo*, it is difficult, from the data available, to define the exact physiological roles of cell wall acid phosphatases in roots. However, Duff et al (1994) has proposed that the extracellular Apase of roots, which is localised mainly in apical meristems and outer surface cells, is undoubtedly involved in hydrolysing and mobilising Pi from organic phosphates in the soil for plant nutrition. The broad substrate specificities observed in this study may imply the involvement of these cell wall enzymes in providing inorganic phosphate from soil organic phosphates. There is a temporal separation in the induction of Apase I and Apase II in roots of white clover in response to P-deprivation, and the two proteins do differ in terms of molecular weight, pI and thermal stability. However, in terms of substrate specificity, the isoforms comprising each Apase metabolise a wide, broadly similar range and so offers no apparent clues to the significance of this temporal separation of response to P-deprivation. It may be that Apase I is concerned with mining of extracellular P, while Apase II has a larger role in scavenging leaked P compounds as membranes become more leaky during the prolonged P-deprived growth (Barrett-Lennard et al 1993). However more detailed sub-cellular localisation of each isoform identified in this study is needed to elucidate further the role of each Apase in the cell walls of roots of white clover.

4.5 Identification of acid phosphatases using amino acid sequencing

Amino acid sequence data describes the primary structure of a given protein polypeptide. Homology analysis of acid phosphatase sequences may help to identify the molecular origins of isoforms of the enzyme as well as providing information on any

proposed functions. Initial attempts to sequence Apase Ia identified the occurrence of at least one protein contaminant. Therefore, to further fractionate the isoforms, the final enzyme preparations of Apase Ia and Ib were subjected to isoelectric focusing after the three FPLC-based purification steps (Figures 3.37; 3.38). In this way, activity staining could be used to reveal the appropriate protein stained band for excision and sequencing. For both isoforms, the major protein bands did not correspond to the acid phosphatase bands, suggesting a very low abundance of the enzyme. Although purified to two major bands after non-denaturing SDS-PAGE, acid phosphatase is only a minor component of this preparation.

In order to generate enough pure protein for sequencing, more starting material can be used and the 3-step FPLC purification procedure repeated including the final IEF step. Alternatively, an additional chromatofocusing step can be added to the FPLC purification.

For Apase Ib, a short sequence containing 5 amino acids was obtained but when this amino acid sequence was compared with the protein sequence database (NCBI), no significant sequence similarity with other proteins was found. This suggests that either the purified enzyme is a novel protein or the amino acid sequence obtained in this study is too short for accurate sequence comparison. Comparison of this truncated sequence with a range of conserved sequence motifs among bacterial, eukaryotic and archaeal phosphatases (Thaller et al 1998) did not reveal any complete identity.

Chapter Five: Summary

Plants of white clover (cultivar Huia, genotype PgH_2) were grown for 5 weeks in either half-strength Hoaglands solution or subjected to phosphate starvation by omitting phosphate from the growth medium and phosphate levels were measured in the first fully expanded leaf. The deprivation of exogenous phosphate influenced P status of white clover markedly with a decrease in P content of leaves of -P plants when compared with control plants (+P). The first significant difference ($p < 0.001$) between the two populations occurred at week 2. In concert with decreased phosphate content in leaves, an increase in cell wall acid phosphatase activity was observed in roots of white clover plants maintained in -P media. Two acid phosphatases, designated Apase I and Apase II, were identified in the cell wall fraction using hydrophobic Phenyl Superose chromatography. A difference was observed between Apase I and Apase II in terms of the timing of response to P-deprivation during a 4-week time course. Apase II reached its maximal activity after one week of P-deprivation and remained at a similar level in the following weeks. The activity of Apase I increased progressively during 4 weeks of P-deprivation, with the highest activity observed at the end of the time course examined in this study.

To further purify and characterise Apase I and II, the Phenyl Superose fractions containing Apase I and Apase II were chromatographed separately through a Superose-12 gel filtration column. Apase I was eluted as a single peak of enzyme activity and comparison with molecular weight standards determined the molecular weight to be 52 kD. The single activity peak obtained after gel filtration was then subjected to ion-exchange chromatography and two peaks of activity, designated Apase Ia and Ib, were eluted within a NaCl gradient. Apase II was also eluted as a single peak after gel filtration, with a molecular weight of 92 kD, and eluted as two distinct isoforms (designated Apase IIa and IIb) after ion-exchange column chromatography. After the three FPLC chromatography steps, Apase Ia had been purified 121.3-fold to a specific activity of 705.2 $\mu mol.min^{-1}$, Ib by 58.3-fold to 338.9 $\mu mol.min^{-1}$, IIa by 13.4-fold to 78.1 $\mu mol.min^{-1}$ and IIb by 92.7-fold to 539.0 $\mu mol.min^{-1}$. Assessment of purity of

isoform Ia was undertaken using SDS-PAGE and Coomassie blue staining. Isoform Ia, after reduction with 2-mercaptoethanol, appeared as a single peptide of *ca.* 52 kD using SDS-PAGE, with the acid phosphatase activity associated with this band. While only a very few minor contaminants were observed, Apase Ia can not be considered electrophoretically pure. Although not strictly pure, the major band in Apase Ia, co-migrated with the acid phosphatase activity and so was excised and submitted for amino acid sequencing. Two peptides were sequenced and database comparisons revealed identity of one with EDGP, a stress-related protein identified in carrot root cells (Satoh et al 1992). The second peptide had no apparent identity with any sequences in the database. This result suggests that at least one another protein has co-purified with Apase Ia. Sequencing of Apase Ib, IIa and IIb at this stage of the purification was therefore not attempted.

Subunit analysis of each isoform was also carried out using SDS-PAGE. The enzyme preparations from ion exchange chromatography were either reduced with 2-mercaptoethanol or not, and then subjected to SDS-PAGE and Apases identified using activity staining. The activity band of each isoform after reduction appeared as the identical molecular weight as the equivalent non-reduced sample. These observations suggest that all four isoforms are active as monomeric structures. The two isoforms of Apase I showed the same molecular weight after gradient SDS-PAGE of 52 kD; the two isoforms of Apase II also showed the same molecular weight after SDS-PAGE of 112 kD. These results, together with elution after Apase I and Apase II from the gel filtration column suggest that Apase I and Apase II are two distinct proteins. The conclusion that Apase I and Apase II are two distinct proteins is supported by some of biochemical properties of the enzymes.

In terms of isoelectric point (pI), heat stability and sensitivity to inhibitors, differences between each distinct phosphatase rather than individual isoforms emerged. The two isoforms of Apase I showed neutral or close to neutral pI values (6.7-7.3), while the two isoforms of Apase II showed much lower pI values (4.4-5.3). The two isoforms of Apase I are more heat sensitive than the two isoforms of Apase II. After the incubation at 65 ^{0}C for 15 min, only 25% Apase I activity was remained, while 66% of Apase II activity remained. Molybdate, Zn^{2+}, Cu^{2+} are strong inhibitors of all the four isoforms.

Fe^{+3} is also an inhibitor although not as potent as other three. Different sensitivities were shown between Apase I and II when assayed in the presence of Al^{3+} and Co^{2+}. The activities of Apase Ia and Ib were reduced to nearly half (65%, 42% and 61%,35%) while Apase IIa and IIb remained 80-100% in the presence of Al^{3+} and Co^{2+}. The two isoforms of Apase I are more sensitive to inorganic phosphate, an end product of phosphatase hydrolysis, than the two isoforms of Apase II. The activity of Apase I was completely abolished by 20 mM Pi, while Apase II still exhibited 20% activity.

Using ρNPP as substrate, the pH optimum study showed that the four isoforms, Apase Ia, Ib, IIa and IIb, have the optimal pH values of 5.8, 6.2, 5.8 and 6.8 respectively although each showed to be active over a broad pH range (at least 1 pH unit).

The glycosylation status of each isoform was examined using two N-linked glycan probes, a GNA lectin (which recognises terminal mannose or oligomannose N-linked glycan chains) and a monoclonal antibody YZ1/2.23 (which recognises xylose/fucose-containing complex-type glycan chains). Apase Ia was recognised by both probes, Ib and IIb by antibody YZ1/2.23, while Apase IIa was not recognised by either the GNA lectin or the antibody YZ1/2.23. These results indicate Apase Ia, Ib and IIb are glycoproteins. As to the glycosylation status of Apase IIa , further study is needed, such as using other sugar probes, or using periodic acid-Schiff staining technique, or deglycosylation treatment with glycosidases before any conclusion can be made in terms of its glycosylation status.

Substrate specificity studies are consistent with the interpretation that each enzyme is a non-specific acid phosphatase with a large spectrum of activity. Each Apase isoform has shown to be able to hydrolyse seven out of eight substrates tested, with ATP and PPi the preferred substrates, and the range of Km values for the synthetic substrate ρNPP within the range reported for Apases characterised in other species. These results suggest that these isoforms may be involved in either accessing P from the external medium or salvaging leaked P from membranes. Further, the broad range of substrate specificities are shared by both Apase I and II even though the temporal induction of these enzymes differs, and so offers no clues as to the significance of this temporal induction.

Amino acid sequencing was carried out with Apase Ib after being further purified using IEF after 3 step FPLC separation. Activity staining was used to precisely locate the Apase band. A short sequence containing 5 amino acids was obtained but no significant sequence similarity with other proteins was found by database searches.

Future work:

The results reported in this study described the characteristics of the acid phosphatases from cell walls of white clover roots and also opened up some interesting aspects that can be pursued further.

The amino acid sequencing of each isoform should be pursued. Five amino acid sequence obtained from Apase Ib is perhaps too short for genetic analysis. A second sequence was obtained from Apase Ia after three FPLC-based purification steps which also has no identity with any sequence in the database - is this an Apase ? The amino acid sequence of the acid phosphatase isoforms would provide adequate information for us to confirm the exact relationship between Apase I and II and the relationships between the two isoforms of each. Because of the low abundance of the cell wall Apases of white clover, a large quantity of root material (several kilograms) must be used in order to get enough enzyme for amino acid sequencing.

The cell wall acid phosphatase of white clover roots should be localised. Although many researchers extract cell wall acid phosphatases by solubilising the enzymes using high salt solution and conclude they are ionically bound to cell walls, very little work has been undertaken to actually localise acid phosphatases in cell walls. Red kidney purple acid phosphatase (KBPAP) was localised in cell walls by activity-based histochemical method and KBPAP is a cationic protein that binds to the cell wall and require 0.5 M salt for elution (Cashikar et al 1997). Sycamore cell wall acid phosphatase activity which can be solubilised by 1 M NaCl was principally located on the external side of the wall using histochemical staining (Crasnier and Giodani 1985). However, in this thesis two distinct Apase activity have been identified in the wall and so *in situ* histochemical assays will not discriminate between the two forms. If Apase I and II can be purified, then antibodies can be raised to each and localisation can be performed using immuno-labelling methods. As well, the external media should be assayed for the occurrence of

either Apase I or Apase II or both. Localisation of each Apase will provide significant clues as to their respective functions.

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ERRATA

Page

11 line 3: phytases

14 line 6: "hydrolysed, when they were added to a nutrient solution, by the roots of intact seedling."

15 line 9: "is principally due to the extracellular"

29 line 2: Materials

31 line 1: minimise

32 line 4: H_2O

33 line 4: Salzburg

35 Figure 2.3 x axis: µg/ml

37 line 15: optimum

37 37^0C

39 line 19: (as the concentration where V = 50% of Vmax)

41 line 2,3 Waring, Dynamics

45 line 21: 100 µl

53 line 11: isoelectrically

67 table, row 4 and column 4: 55.4 instead of 82.3

68 line 5 and 6: Amino acid sequences were obtained from two distinct trypsin-generated peptides, obtained after separation using reverse-phase HPLC, and compared....

74 line 6: preparations

82 line 7: relatively

103 line 5, insert (after ion-exchange chromatography): In these separations, the maximum amount of protein was loaded onto the IEF gel to obtain enough material for amino acid sequencing.

107 line 6: does

115 line 20: from

118 line 3: their, line 4: has

120 line 18: affected, line 19: affect

121 line 5: affect

128 line 23: requires, line 27: activities

145 line 5: 447-453