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.

Table of Contents

List of Figures

List of Tables

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