PLANT SCREENING FOR TYROSINE/PHENYLALANINE AMMONIA LYASE AND BIOCHEMICAL CHARACTERISATION, PURIFICATION AND CLONING OF THE TYROSINE/PHENYLALANINE AMMONIA LYASE ENZYME FROM *TRICHOSPORON CUTANEUM*

by

ANDREA MICHELLE GOLDSON

B.Sc.,The University of The West Indies, Jamaica, 1996
M.Phil.,The University of The West Indies, Jamaica, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Food Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

June 2012

ABSTRACT

Phenylalanine ammonia lyase (PAL, EC 4.3.1.24) catalyzes the conversion of phenylalanine (Phe) to *trans* cinnamic acid and, in some instances tyrosine (Tyr) to *para* hydroxycinnamic acid (pHCA). The specificity of PAL for Phe relative to Tyr varies by over 10^6 between biological sources. An understanding of the basis for this astounding range of substrate preference is required to rationally engineer a highly efficient Tyr-specific enzyme for use in synthesizing pHCA for health, pharmaceutical and flavour applications.

Of the plant seedlings screened for PAL activity, *Triticum aestivum* displayed the highest level of activity with both substrates. A unique 103 kDa PAL polypeptide was detected by Western blot analysis, along with two others at 74 and 83 kDa. Dual substrate activity was identified for the first time in the dicot *Lens culinaris*. Microbial *Trichosporon cutaneum* PAL (TcPAL) possessed the highest level of activity with Tyr of the sources investigated. Induction with Tyr (2 mM) produced the highest ratio of Phe:Tyr activity (1.6 ± 0.3: 0.4 ± 0.1 µmol/h g wet weight). TcPAL displayed Michaelis Menten kinetics with Phe (K_m 5.0 ± 0.7 mM) but allosteric kinetics with Tyr (K' 1.7 ± 0.8 mM, Hill coefficient 1.8 ± 0.2). A greater specificity for Phe was demonstrated by a Phe [V_{max}/K_m] / Tyr [V_{max}/K_m] ratio of 2. The enzyme had a pH optimum of 8 - 8.5, temperature optimum of 32 °C, showed no metal dependence, and had a monomer molecular mass of 79 kDa.

Of the three methods investigated for purifying TcPAL, anion exchange

chromatography using a Hi-Trap Q-Sepharose column produced the highest yield (20%) and purification fold (50).

The PAL gene from *Trichosporon cutaneum* was cloned into the pET30a vector and sequenced. Five amino acid residues different from a previous report were identified, namely Arg74Glu, Val274Ala, Ala298Val, Ser322Pro and Arg486Lys. The gene had an intron of 1062 base pairs, starting at position 121. A His-Gln motif, which appears to be characteristic of yeast PALs with dual substrate activity, was identified in the substrate selectivity region. Residues that could potentially enhance the tyrosine activity of the enzyme were identified for future mutagenesis studies.

PREFACE

A version of Chapter 2 has been published in the Journal of the Science of Food and Agriculture, 2008. Figures 2.1, 2.2 and 2.4 Journal of the Science of Food and Agriculture, 2008, by permission.

Goldson*, A., Lam*, M., Scaman, C.H., Clemens, S. and Kermode, A. 2008. Screening of phenylalanine ammonia lyase in plant tissues, and retention of activity during dehydration. J. Sci. Food Agric. 88: 619-625.

* Authors contributed equally:

Specific contributions to manuscript

A. Goldson
Screening of plant seedlings for phenylalanine ammonia lyase (PAL)
PAL activity during post-germination growth of red spring wheat seedlings
Western blot analysis of red wheat seedling during post-germination growth

M. Lam
Water activity and moisture determination
Dehydration experiments

C.H. Scaman
Thesis supervisor for A. Goldson and M. Lam

S. Clemens
Technical assistance with Western blots

A. Kermode
Project collaborator

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

A	Ampere
At	*Arabidopsis thaliana*
AU	Absorbance unit
Bo	*Bambusa oldhamii*
Bp	Base pair
BSA	Bovine serum albumin
°C	Degree celsius
cDNA	Complementary deoxyribonucleic acid
d	Day
Da	Dalton
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
FPLC	Fast protein liquid chromatography
g	Gravitational acceleration constant
H	Hill coefficient
HAL	Histidine ammonia lyase
hr	Hour(s)
IMAC	Immobilized metal affinity chromatography
IU	International units
kb	Kilobases (1000 bp)
kcat	Catalytic rate constant
kcat/Km	Specificity constant
kJ	Kilojoule
Km	Michaelis Menten constant
L	Litre
LB	Luria-Bertani medium
m	Meter
mA	Milliampere
mg	Milligram

mm	Millimeter
mM	Millimolar
min	Minutes
MIO	4-methylidene-imidazol-5-one
mL	Milliliters
mRNA	Messenger ribonucleic acid
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PAGE	Polyacrylamide amide gel electrophoresis
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
pmol	Picomole
PMSF	Phenylmethysulfonyl fluoride
Pf	Purification fold
RNA	Ribonucleic acid
rms	Root mean square
rpm	Revolutions per minute
s	Seconds
SDS	Sodium dodecyl sulphate
TAL	Tyrosine ammonia lyase
Tc	*Trichosporon cutaneum*
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
µg	Microgram
µm	Micrometre
µmol	Micromole
µL	Microliter
µM	Micromolar
U	Units of activity
UV	Ultraviolet
V	Volts
W	Watt

w/v	Weight per volume
x g	Relative centrifugal force

Common amino acid abbreviations

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Nucleotide base abbreviations

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

ACKNOWLEDGEMENTS

A special thank you to my supervisor Dr Christine Scaman for her, patience, guidance and supervision during my course of study here at the University of British Columbia. Her dedication and passion to research are exceptional. I have learned much and grown in so many different ways. Dr Li-Chan and Dr Kermode, thank you for serving as members of my supervisory committee.

To the Canadian Bureau of International Education and the Government of Canada, thank you for the financial assistance provided to me through the Canadian Commonwealth Scholarship.

To the members of my group, especially Reena Mistry and Venkatrao Konasani, other members of the lab, Yazheng Liu, Ingrid Elisia and Xiumin Chen, thank you for your words of encouragement, support and friendship.

To all my friends who have helped me at some point of the journey I say thank you. Special thanks go to Beatrice and Stanford Raymond, UBC International Bible Study Fellowship, Graduate Christian Union, Trinity Baptist Church and Portmore Gospel Assembly.

Thanks to Dr Pedro Aloise and Valerie Skura for your technical assistance.

Words cannot express my gratitude to my mom, Ingrid Goldson, who has always been there for me, encouraging me and cheering me on.

To my husband, Nicholas Barnaby, thank you for all your love, patience and encouragement. My meeting you is another of God's Divine appointments in my life.

To my Lord and Saviour, Jesus Christ, thank You for your guidance, wisdom, provision and protection. Without you I am nothing but with You I can do all things through Christ which strengthens me.

This dissertation is dedicated to my parents,

Ingrid Loraine Goldson

Lloyd George Goldson (Aug. 14, 1941 - Jan. 23, 2006)

As well as my husband

Nicholas Dave Anthony Barnaby

Thank you for your love and support

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Enzymes play an essential role in regulating and modulating reactions and have found various industrial applications. They are classified on the basis of type of reaction catalyzed. The aromatic amino acid ammonia lyases, the focus of the current research, are one such classification consisting of the enzymes histidine ammonia lyase (HAL) and phenylalanine ammonia lyase (PAL). HAL (EC 4.3.1.3), a degradative enzyme commonly found in bacteria and animals, catalyzes the deamination of histidine, leading to the formation of urocanic acid (Poppe & Retey 2005). PAL (EC 4.3.1.24), which has mainly been identified in plant sources as well as in a few microbes, catalyzes the deamination of phenylalanine producing *trans* cinnamic acid (Koukol & Conn 1961; Hanson & Havir 1970). In some instances the enzyme is also able to deaminate tyrosine producing *para* hydroxycinnamic acid (Neish 1961, Figure 1.1) in which case it may be classified either as tyrosine ammonia lyase (TAL, EC 4.3.1.23) or phenylalanine /tyrosine ammonia lyase (EC 4.3.1.25). The enzyme typically exhibits higher catalytic efficiencies for phenylalanine. There is no known source of the enzyme that acts only on tyrosine. Sources of PAL that are able to metabolize tyrosine are mainly monocotyledonous plants, for example rice, maize (Rösler et al. 1997) and bamboo (Hsieh et al. 2010). Bacterial sources of PAL that are able to metabolize tyrosine include *Rhodobacter capsulatus* (Kyndt et al. 2002), *Rhodobacter sphaeroides* (Watts et al. 2004) and *Sacchrothrix espanaensis* (Berner et al. 2006) which exhibit a greater catalytic efficiency for tyrosine than the plant sources.

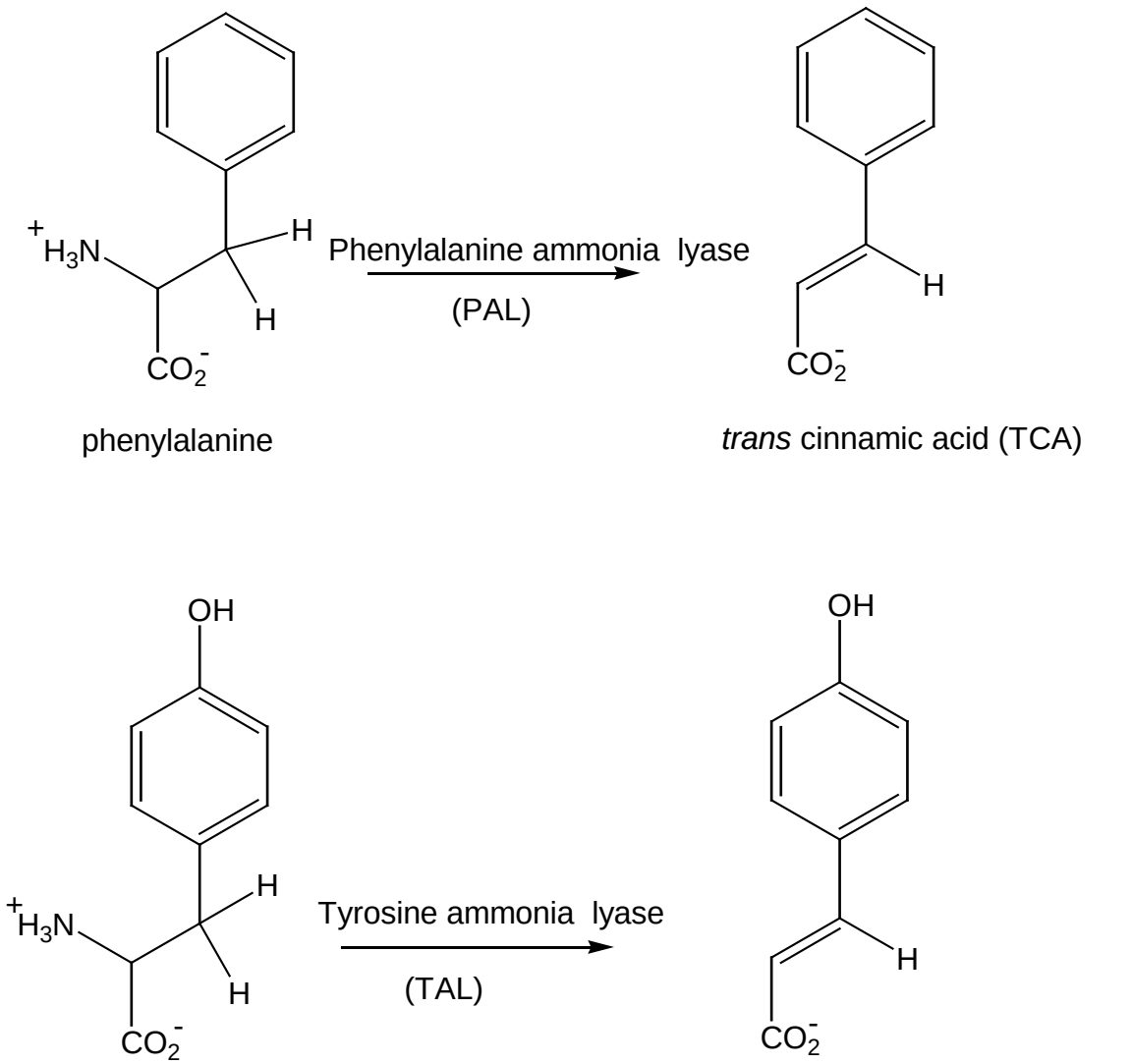


Figure 1.1 Non-oxidative deamination of the aromatic amino acids, phenylalanine and tyrosine to produce *trans* cinnamic acid and *para* hydroxycinnamic acid respectively.

The yeasts *Sporobolomyces roseus* (Camm & Towers 1969), *Sporobolomyces pararoseus* (Parkhurst & Hodgins 1971), *Trichosporon cutaneum* (Vanelli et al. 2007a) and *Rhodotorula glutinis* (Havir & Hanson 1975) also exhibit activity with tyrosine. More recently a thermostable PAL enzyme was isolated from the wood rotting fungus *Phanerochaete chrysosporium* (Xue et al. 2007a). *Rhodotorula* sp. has been the predominant source of the enzyme industrially due to its high levels of PAL and non requirement for specialized media for growth (Evans et al.1987).

Para hydroxycinnamic acid, the product of TAL activity, is an important monomeric unit, which has found applications in the health, pharmaceutical and food industry (Qi et al. 2007). It is used in the synthesis of *para* hydroxystyrene (Verhoef et al. 2009), liquid crystal polymers (Verhoef et al. 2007) and the flavour component vanillin (Priefert et al. 2001). Liquid crystal polymers are a class of polymers with desirable mechanical properties, flame retardancy and chemical inertness (Wang et al. 2003). The ability of PAL to convert tyrosine directly to *para* hydroxycinnamic acid is of significant interest, as it reduces the number of steps required for the production of *para* hydroxycinnamic acid. In the absence of activity with tyrosine, the production of *para* hydroxycinnamic acid from phenylalanine is a two-step process involving deamination of phenylalanine to *trans* cinnamic acid followed by hydroxylation via hydroxylase to produce *para* hydroxycinnamic acid.

Current interest in this family of enzymes stems from their unusual mechanistic features as well as commercial applications. Interest in PAL is two fold. First there is

interest in catalytic mechanism and substrate selectivity. The enzyme is able to catalyse the removal of a non-acidic proton from the substrate. How is the enzyme able to distinguish between phenylalanine or tyrosine? Secondly, a more selective and efficient TAL is of interest for industrial applications. The use of biotechnology in the production of aromatic chemicals continues to increase as it allows for the use of greener technologies and renewable energy sources (Schmid et al. 2001, Tang 2003, Breinig et al. 2005, Trotman et al. 2007; Qi et al. 2007).

In the present study, phenylalanine/tyrosine ammonia lyase activity of several sources of the enzyme was investigated. PAL enzyme from *Trichosporon cutaneum* exhibited the highest levels of activity with tyrosine and phenylalanine, was selected as a model to further investigate the enzyme's ability to utilize both substrates. Currently the information on phenylalanine/tyrosine ammonia lyase activity of this enzyme is limited to a publication by Vanelli et al. (2007a) and a patent by Breinig et al. (2005). This thesis describes in detail; characterization of the enzyme, and its purification and cloning with the intent of producing mutant clones with higher catalytic efficiencies for tyrosine. Potential mutation sites, which could enhance the enzyme's ability to utilize tyrosine, were identified by sequence comparison of PAL and TAL enzymes in the National Center for Biotechnology Information (NCBI) database. The identification of key amino acid residues responsible for substrate selectivity will assist in further elucidation of the mechanism by which different PAL/TAL enzymes are able to use different substrates at various levels of efficiency. Further insight on how to improve the enzyme's catalytic efficiency for tyrosine. An enzyme with a higher catalytic efficiency

for tyrosine is desirable for utilization as a biocatalyst in the synthesis of polymers, which possess the *para* hydroxycinnamic acid monomeric unit or derivatives thereof.

1.2 PHENYLALANINE AMMONIA LYASE SUBSTRATE SELECTIVITY

1.2.1 Phenylalanine ammonia lyase (EC 4.3.1.24)

PAL is of great significance in plant metabolism as it serves as the key enzyme of the phenylpropanoid pathway diverting the flow of carbons from protein synthesis to the synthesis of phenolic compounds. PAL is considered to be the enzyme that provides a link between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism (Dixon & Paiva 1995). The first reaction of the phenylpropanoid pathway is catalyzed by PAL, in which phenylalanine is deaminated resulting in the formation of *trans* cinnamic acid. *Para* hydroxylation of *trans* cinnamic acid via the P-450 enzyme system leads to the formation of *para* hydroxycinnamic acid. *Para* hydroxycinnamic acid (via its coenzyme A ester) is the starting material for lignin. *Ortho*-hydroxylation results in the production of coumarins. Other derivatives of *para* hydroxycinnamic acid include ferulic, caffeic and sinapic acids and phytoalexins, which play a critical role in plant pathogen interactions (Morrison & Buxton 1993). Novel flavour components, for example vanillin, fragrances, pharmaceuticals and other chemicals of commercial interest are derived from *para* hydroxycinnamic acid.

PAL has also been identified in various microbial sources. Fungi (Kim et al. 1996, Hattori et al. 1999, Xue et al. 2007a, Vanelli et al. 2007a), cyanobacteria (Moffitt et al. 2007) and other bacteria, for example, *Streptomyces maritimus* (Xiang & Moore 2005),

Photorhabdus luminescens (Williams et al. 2005), *Sorangium cellulosum* (Hill et al. 2003) and *Streptomyces verticillatus* (Emes & Vining 2007) are known to have PAL activity. In cyanobacteria, the first reported incidence of PAL was in *Nostoc punctiforme* (Moffitt et al. 2007). Subsequently PAL was also identified in the cyanobacterium *Anabaena variabilis* (Moffitt et al. 2007). Cyanobacterial PALs are structurally similar to PALs of plant and yeast origin as well as histidine ammonia lyase (Moffitt et al. 2007).

Microbial PAL is essential in the synthesis of antimicrobial agents, antibiotics and antifungal components. In *Streptomyces maritimus*, *trans* cinnamic acid is a key intermediate in the biosynthesis of the antibiotic enterocin (Xiang & Moore 2005), and 3,5-dihydroxy-4-isopropyl-stilbene by *Photorhabdus luminescens* (Williams et al. 2005). In the red yeast, *Rhodotorula glutinis*, *trans* cinnamic acid is converted to benzoate and other cellular materials (Kane & Fiske 1985). Microbes have the ability to oxidize a variety of aromatic compounds through the dihydroxylated intermediates catechol and protocatechuate, as well as the beta-ketoadipate pathway (Durham et al. 1984). Protocatechuate is produced by the hydroxylation of benzoate. The fate of *trans* cinnamic acid in the cyanobacteria *Anabaena variabilis* and *Nostoc punctiforme* is not presently known.

1.2.2 Phenylalanine ammonia lyase/tyrosine ammonia lyase (EC 4.3.1.25)

In some instances, PAL is also able to utilize tyrosine as a substrate (EC 4.3.1.25; P/TAL). This dual substrate activity has been identified in plants, bacteria and fungi

(Camm & Towers 1973). The level of activity with tyrosine is typically much lower than that observed with phenylalanine but the magnitude of difference varies significantly. There are no known genes that code specifically for activity with tyrosine. An enzyme that is able to act solely on tyrosine has not been identified. Activity with tyrosine is always found in conjunction with phenylalanine. In the enzyme from maize both activities were found to reside on the same polypeptide (Rösler et al. 1997). This was proven unequivocally when the full-length cDNA encoding the PAL enzyme from *Zea mays* was isolated and the coding region expressed in *Escherichia coli*. The purified enzyme exhibited activity with phenylalanine and tyrosine.

Yeast strains of *Sporobolomyces roseus* (Camm & Towers 1969), *Sporobolomyces pararoseus* (Parkhurst & Hodgins 1971) and *Rhodotorula glutinis* (Havir & Hanson 1975) were among the first microbes identified as exhibiting dual substrate activity. Activity with both substrates was stimulated in the presence of phenylalanine and tyrosine. In *Sporobolomyces roseus* enzyme inhibition of both activities occurred in the presence of *trans* cinnamic acid or *para* hydroxycinnamic acid (Camm & Towers 1969). Purification of extracts from both *Sporobolomyces pararoseus* (Parkhurst & Hodgins 1971 and *Rhodototorula glutinis* (Havir and Hanson 1975) yielded a single major protein band, suggesting that dual substrate activity was due to the presence of a single enzyme (Parkhurst & Hodgins 1971).

More recently, dual substrate activity was detected in *Trichosporon cutaneum* (Vanelli et al. 2007a). Sparnins et al. (1979) were the first to report the deamination of tyrosine

to *para* hydroxycinnamic acid by *Trichosporon cutaneum* when tyrosine served as the sole source of carbon for the organism. Based on this evidence it was predicted by Vanelli et al. (2007a) that *Trichosporon cutaneum* would possess significant levels of activity with tyrosine. Of the microbes investigated (*Rhodotorula rubra*, *Saccharomycopsis fibuligera*, *Rhodotorula glutinis*, *Sporidiobolus pararoseus*, *Sporidiobolus ruineniae*, *Rhodotorula minuta*, *Rhodotorula graminis*, *Trichosporon cutaneum*) the PAL enzyme from *Trichosporon cutaneum* had the highest catalytic efficiency for tyrosine (Vanelli et al. 2007a). While the enzyme has a lower overall activity compared to the *Rhodotorula glutinis* enzyme, it has a higher PAL/TAL ratio at lower substrate concentrations (0.1 - 1 mM) (Vanelli et al. 2007a). Sequence analysis has revealed that the enzyme has 56 - 62% similarity to various other fungal PAL/TAL enzymes (Vanelli et al. 2007a).

1.2.3 Tyrosine ammonia lyase (EC 4.3.1.23)

Enzymes with a greater catalytic efficiency for tyrosine than phenylalanine are referred to as tyrosine ammonia lyase (TAL). In 2002, the first bacterial source of PAL that has activity with tyrosine was detected in *Rhodobacter capsulatus* (Kyndt et al. 2002). This had a 150-fold greater catalytic efficiency (k_{cat}/K_m) for tyrosine than phenylalanine (Kyndt et al. 2002). These findings are controversial and have proven difficult to replicate (Xue et al. 2007b). Subsequently other bacterial sources exhibiting tyrosine activity have been identified, including several species of the purple phototropic bacteria, namely, *Rhodobacter sphaeroides* (Louie et al. 2006) and *Halorhodospira halophila* (Kyndt et al. 2003). The enzyme from *Rhodobacter sphaeroides* also shows a

catalytic efficiency for tyrosine, which was 50-fold greater than for phenylalanine (Louie et al. 2006). This observation of a higher catalytic efficiency for tyrosine in *Rhodobacter* bacteria may indicate that the enzyme may have a specialized role in these bacteria and may provide some insight as to how different sources of the enzyme are able to preferentially utilize one substrate over the other. This may prove to be a useful tool for understanding the substrate recognition mechanisms of the aromatic ammonia lyase family of enzymes (Xue et al. 2007b).

In *Rhodobacter*, *para* hydroxycinnamic acid serves as a protein cofactor and precursor to the chromophore of the photoactive yellow protein (PYP) (Cusanovich & Meyer 2003). The photoactive yellow protein is a small (14 kDa) water-soluble cytoplasmic protein that was originally isolated from *Halorhodospira halophilia* (Meyer et al. 1987). It is believed to serve as a blue light photoreceptor associated with genes for gas vesicle formation. Gas vesicles act as flotation devices bringing cells to the surface of the water where there is an abundance of light and oxygen. *Para* hydroxycinnamic acid is synthesized from tyrosine and is next activated by a specific ligase for binding to the PYP apo-protein (Cusanovich & Meyer 2003). The TAL gene is located upstream of the PYP gene in *Rhodobacter capsulatus* (Kyndt et al. 2004).

PAL enzyme from the actinomycete *Saccharothrix espanaensis* exhibits significant activity with tyrosine. The catalytic efficiency for tyrosine was 750 times greater than that for phenylalanine, and is the largest catalytic efficiency ratio that has been reported for tyrosine. The *Actinomycetales* are well known, as most antimicrobial agents that are

currently being utilized originated from this order. *Saccharothix espanaensis* produces two heptadecaglycoside antibiotics, saccharomicins A and B (Kong et al. 1998). These antibiotics represent a new class of antibiotics due to their potency (Singh et al. 2000). They are active both *in vitro* and *in vivo* against multiple resistant strains of *Staphylococcus aureus* as well as vancomycin-resistant enterococci (Singh et al. 2000). Structurally, the saccharomicins are oligosaccharide antibiotics consisting of 17 monosaccharide units and a unique aglycon N-(m,p-dihydroxy-cinnamoyl)taurine in which caffeic acid is linked to the amino sulfonic acid taurine via an amide bond (Berner et al. 2006).

Table 1.1 gives a comparative overview at the kinetic data of PAL enzymes that have been identified as having activity with both tyrosine and phenylalanine. The Michaelis Menten constant (K_m), the turnover number (k_{cat}), and the catalytic efficiency (k_{cat}/K_m) are used to characterize the enzyme. K_m is defined as the substrate concentration at which the reaction velocity is half of the maximum velocity (V_{max}), an indicator of the enzyme's ability to bind to the substrate. The turnover number relates the maximum velocity to the total active site concentration and gives a direct measure of the catalytic production of product under optimum conditions. Catalytic efficiency is a measure of the substrate specificity of the enzyme, with a high value indicating that the enzyme is better able to act on a particular substrate.

Table 1.1 Kinetic parameters for PAL enzymes exhibiting activity with both tyrosine and phenylalanine.

PAL and TAL enzymes	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	(k_{cat}/K_m) Tyr / (k_{cat}/K_m) Phe	
Saccharothrix espanaensis[a]	L-Tyr	15.5	0.015	9.68×10^{-4}	745	TAL
	L-Phe	2,860	0.0038	1.30×10^{-6}		
Rhodobacter capsulatus[b]	L-Tyr	15.6	27.7	1.77	150	TAL
	L-Phe	1,277	15.1	0.0118		
Trichosporon cutaneum[c]	L-Tyr[i]	600	1.92×10^{-4}	3.20×10^{-7}	55	TAL
	L-Phe	4900	2.83×10^{-5}	5.78×10^{-9}		
Rhodobacter sphaeroides[d]	L-Tyr	74.2	4.32	0.058	53	TAL
	L-Phe	11,400	13.10	0.0011		
Zea mays[e]	L-Tyr	19	0.92	0.0473	1.3	P/TAL
	L-Phe	270	10.6	0.037		
Bambusa oldhamii[f]	L-Tyr	97	0.54	5.56×10^{-3}	0.71	PAL
	L-Phe	2072	16.32	7.88×10^{-3}		
Rhodotorula glutinis[g]	L-Tyr	110	0.46	4.14×10^{-3}	0.50	PAL
	L-Phe	250	2.09	8.36×10^{-3}		
Petroselium crispum[h]	L-Tyr	2,500	0.3	1.20×10^{-4}	9.4×10^{-5}	PAL
	L-Phe	17.2	22	1.28		

[a-h] References: Berner et al. 2006; Kyndt et al. 2002; Vanelli et al. 2007a; Louie et al. 2006; Rosler et al. 1997; Hsieh et al. 2010; Gatenby et al. 2002; Appert et al. 1994.
[i] The reported value for tyrosine is K'

1.3 STRUCTURAL FEATURES OF PAL

1.3.1 Gene and protein structure

PAL exists universally as a family of genes in higher plants. In all plants analyzed, PAL occurs as a tetramer and multiple forms have often been isolated (Bolwell et al. 1985 and Sarma et al. 1998). Isoforms of the enzyme are a common occurrence with typically three or four isoforms being present in a particular species (Appert et al. 1994; Fukasawa-Akada et al. 1996; Butland et al.1998; Kumar & Ellis 2001; Cochrane et al. 2004). PAL multigene families occur for example in parsley (*Petroselinum crispum*; Appert et al. 1994 and Logemann et al. 1995). PAL is encoded by at least five genes in tomato leaves (Lee et al. 1992). Four PAL isoforms have been identified in parsley (Appert et al. 1994), *Arabidopsis thaliana* (Cochrane et al. 2004) and green bamboo (Hsieh et al. 2010). In raspberry (*Rubus idaeus*), PAL is encoded by a family of two genes (Kumar and Ellis 2001). The presence of isoforms as well as post translational modifications (Cheng et al. 2001), offers some explanation as to the presence of different forms of PAL. This may also offer some explanation as to the reported presence of two separate PAL enzymes in tobacco (*Nicotiana tabacum*) callus (Beaudoin-Eagan & Thorpe 1985) and castor bean (*Ricinus communis*) endosperm (Gregor 1976). Many studies have also indicated that genes are differentially expressed in response to environmental stress (Pellegrini et al. 1994 and Liang et al. 1989). In contrast to PAL found in plants, only one gene is apparent in microbial sources of the enzyme. A single gene was identified in the yeast *Ustilago maydis* (Kim et al. 2001) and *Rhodosporidium toruloides* (Anson et al. 2007). A gene encoding TAL from the bacteria *Rhodobacter sphaeroides* has also been isolated (Nishiyama et al.

2010).

Microbial and plant PAL enzymes are typically made up of four identical subunits. The molecular weight of the enzyme is approximately 330 kDa, with subunits ranging from 77 - 83 kDa (Gilbert & Tully 1982). The homotetrameric nature of the enzyme was confirmed from three-dimensional structures of the enzyme from the yeast *Rhodosporidium toruloides* (Calabrese et al. 2004 and Wang et al. 2005). Each subunit possesses 716 residues, and has a molecular mass of 77 kDa each (Calabrese et al. 2004). In the enzyme from *Rhodobacter sphaeroides,* each homotetramer contains four active sites. The formation of the active site cavity for each monomer involves the three other monomeric units, which adopt a predominantly α-helical fold. The domain that carries the 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) prosthetic group is formed from the N-terminal region of the polypeptide chain, whereas the C-terminal forms a peripheral α-helical layer, which participates in additional intersubunit contacts, providing stabilization of the homotetramer (Louie et al. 2006). Homotetramers have also been identified in PAL found in parsley (Ritter & Schulz 2004), potato, maize (Havir & Hanson 1973) and green bamboo (Hsieh et al. 2010).

There are a few exceptions in which the enzyme has been reported as not being a homotetramer. These include the enzyme from wheat (Nari et al. 1974), the fungus, *Rhizocotania solani* (Kalghatgi & Subba Rao 1975) and French bean (Bolwell & Rodgers 1991). The wheat enzyme has two pairs of non-identical subunits of 75 and 85 kDa each (Nari et al. 1974) while the fungal PAL has two pairs of non-identical subunits

with molecular weights of 70 and 90 kDa (Kalghatgi & Subba Rao 1975). PAL purified from suspension cultured cells of the French bean (*Phaseolus vulgaris* L) possessed both a high molecular weight PAL (83 kDa) and a more typical subunit of 77 kDa (Bolwell & Rodgers 1991). The induction properties of both subunits were different from each other. It is known that PAL is subject to considerable post-translational processing, for example N-glycosylation (Bolwell & Rodgers 1991 & Shaw et al. 1990) and phosphorylation (Allwood et al. 1999 & Bolwell 1992). In partially purified preparations, which were prepared under conditions minimizing degradation, the 77 kDa subunit was accompanied by additional bands which were shown to be forms of PAL (Bolwell et al. 1986). The high molecular weight of 83 kDa subunit could be attributed to glycosylation as well as a direct transcription product which had not been mapped to a particular gene (Bolwell & Rodgers 1991). Table 1.2 gives a summary of the molecular subunits from various PAL sources.

Table 1.2 Molecular weights of PAL from different sources.

Organism	Molecular subunit kDa	References
Rhodosporidium toruloides	80	Jia et al. 2008
Trichosporon cutaneum	78	Vanelli et al. 2007a
Petroselinum crispum	78	Appert et al. 1994
Rubus idaeus	78	Kumar & Ellis 2001
Arabidopsis thaliana	76	Cochrane et al. 2004
Bambusa oldhamii	76	Hsieh et al. 2010
Zea mays	75	Rosler et al. 1997
Anabaena variabilis	64	Moffitt et al. 2007
Nostoc punctiforme	64	Moffitt et al. 2007
Streptomyces maritimus	60	Xiang & Moore 2005
Rhodobacter capsulatus	55	Kyndt et al. 2002
Rhodobacter sphaeroides	55	Watts et al. 2006
Lycopersicon esculentum	80	Sarma et al. 1998
Rhizocotania solani	70; 90	Kalghatgi & Subba Rao 1975
Triticum aestivum L	75; 85	Nari et al. 1974
Phaseolus vulgaris L	77; 83	Bolwell & Rodgers 1991

1.3.2 Sequence homology

The aromatic ammonia lyases possess several homologous regions within the active site of the enzyme. A HAL/PAL motif consisting of approximately 460 amino acids residues is common in bacterial HAL (~500 amino acids), and plant and fungal PAL (~710 amino acids). Among the different species an amino acid sequence identity of ≥ 30% is common within this region (Pilbák et al. 2006). In eukaryotic PAL, an additional domain, the C-terminal multi-helix domain is approximately 100 residues in length, has been identified (Pilbák et al. 2006). The domain forms a peripheral α-helical layer and participates in additional intersubunit contact, providing stabilization of the enzyme. It is also involved in the formation of an outer lid loop (Louie et al. 2006). This C-terminal multi-helix domain domain is absent from the prokaryotic PALs *Streptomyces maritimus* and *Photorhabdus luminescens* (Pilbák et al. 2006).

The HAL/PAL core domain is believed to be critical for catalysis, while the C-terminal multi helix region has a regulatory role (Pilbák et al. 2006). The greatest divergence between HAL and PAL enzymes is observed within the N-terminal region of the enzyme, which contributes to the domain carrying the 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) prosthetic group (Pilbák et al. 2006). This is evident from variations within, the sequences as well as the number of amino acids present (Lee et al. 2003).

1.3.3 The active site loops

The 3,5-dihydro-5-methylidene-4H-imidazol-4-one prosthetic group and the Tyr110 residue (relative to parsley and its equivalents) are key features of the aromatic ammonia lyases. Crystal structures of HAL and PAL have revealed that there are two loops located in close proximity to the active site of the enzyme. The two loops are referred to as the inner lid loop and the outer lid loop (Louie et al. 2006).

The inner lid loop is formed from the 3,5-dihydro-5-methylidene-4H-imidazol-4-one motif and contains several conserved amino acid residues, including Tyr110 (Louie et al. 2006). This loop is composed of amino acid residues spanning the region 102 through 124 of the protein sequence (Wang et al. 2005). In *Petroselinum crispum* PAL, SMNKGTDS**Y**GVTTGFGATSHRRT were identified as the amino acids in this region. Several hypotheses have been made regarding the function of the loop in substrate binding and catalysis. One group has proposed that upon substrate binding, the flexible inner lid loop becomes more rigid, thereby producing an induced fit (Ritter & Schulz 2004). In support of this, a Tyr110Phe mutation resulted in significant decline of activity in both PAL (Röther et al. 2002) and HAL enzymes (Röther et al. 2001). Also introduction of Phe into the mobile loop may result in this residue binding to the active site of the enzyme, thereby inhibiting any incoming substrate from reacting with the active site resulting in enzyme inhibition (Ritter & Schulz 2004). Tyr110 and the amino acid residues within the active site of the enzyme are the most conserved residues in this family of enzymes (Figure 1.2).

It has been proposed that the outer loop which projects from the C-terminal domain (Louie et al. 2006) acts as an opening and closing mechanism for the active site on another monomeric unit (Wang et al. 2005). Substrate or substrate analogs may provide anchorage for the loop upon binding, leading to changes in enzyme conformation required for catalysis (Wang et al. 2005). One thing that is conclusive is that the loop plays a critical role in substrate binding and catalysis (Pilbák et al. 2006).

These loops are well defined in the *Rhodobacter sphaeroides* TAL, forming a compact arrangement within the active site of the enzyme (Louie et al. 2006, Figure 1.3). In addition to being present in the aromatic ammonia lyase family, these key structural residues are found in the aminomutase family (Pilbák et al. 2006). The aminomutases possess some mechanistic similarity with the aromatic ammonia lyases, catalyzing the synthesis of aromatic β-amino acids directly from α-amino acids via a 3,5-dihydro-5-methylidene-4H-imidazol-4-one prosthetic group (Cooke et al. 2009).

```
Petroselinum crispum       EARAGVKASSDWVMDSMNKGTDSYGVTTGFGATSHRRTK--QGGALQKEL 133
Nicotiana tabacum          EARAGVKASSDWVMDSMNKGTDSYGVTTGFGATSHRRTK--NGGALQKEL 131
Arabidopsis thaliana       TARAGVNASSDWVMESMNKGTDSYGVTTGFGATSHRRTK--NGVALQKEL 141
Triticum aestivum          SARGRVKESSDWVMNSMMNGTDSYGVTTGFGATSHRRTK--EGGALQREL 117
Zea mays                   SARGRVKASSDWVMDSMMNGTDSYGVTTGFGATSHRRTK--EGGALQREL 132
Bambusa oldhamii           SARGRVKESSDWVMNSMMNGTDSYGVTTGFGATSHRRTK--EGGALQREL 130
Gingko biloba              AAKSRVEESSNWVLHQMTKGTDTYGVTTGFGATSHRRTS--QGVELQKEL 144
Trichosporon cutaneum      SVAGPVRASVDFKES--KKHTSIYGVTTGFGGSADTRTS--DTEALQISL 113
Oryza sativa               EARPRVKASSEWILDCIAHGGDIYGVTTGFGGTSHRRTK--DGPALQVEL 120
Rhodobacter sphaeroides    PARDRCRASEARLGAVIREARHVYGLTTGFGPLANRLISGENVRTLQANL 86
Saccharothrix espanaensis  EEIVRMGASARTIEEYLKSDKPIYGLTQGFGPLVLFDAD--SELEQGGSL 84

Petroselinum crispum       IRFLNAGIFGSGAEAG---NNTLPHSATRAAMLVRINTLLQGYSGIRFEI 180
Nicotiana tabacum          IRFLNAGVFGNGTET----SHTLPHSATRAAMLVRINTLLQGYSGIRFEI 177
Arabidopsis thaliana       IRFLNAGIFGSTKET----SHTLPHSATRAAMLVRINTLLQGFSGIRFEI 187
Triticum aestivum          IRFLNAGAFGTGTDG-----HVLPAAATRAAMLVRVNTLLQGYSGIRFEI 162
Zea mays                   IRFLNAGAFGTGDDG-----HVLPAAATRAAMLVRINTLLQGYSGIRFEI 177
Bambusa oldhamii           IRFLNAGAFGTGSDG-----HVLAAEATRAAMLVRINTLLQGYSGIRFEI 175
Gingko biloba              IRFLNAGVFGSCEGN------VLPEATTRAAMLVRTNTLPQGYSGIRWAL 188
Trichosporon cutaneum      LEHQLCGFLPTDATYEGMLLAAMPIPIVRGAMAVRVNSCVRGHSGVRLEV 163
Oryza sativa               LRHLNAGIFGTGSDG-----HTLPSEVTRAAMLVRINTLLQGYSGIRFEI 165
Rhodobacter sphaeroides    VHHLASGVG----------PVLDWTTARAMVLARLVSIAQGASGASEGT 125
Saccharothrix espanaensis  ISHLGTGQG----------APLAPEVSRLILWLRIQNMRKGYSAVSPVF 123

Petroselinum crispum       LEAITKFLNHNITPCLPLRGTITASGDLVPLSYIAGLLTGRPNSKAVGPT 230
Nicotiana tabacum          LEAITKLINSNITPCLPLRGTITASGDLVPLSYIAGLLTGRPNSKAVGPN 227
Arabidopsis thaliana       LEAITSFLNNNITPSLPLRGTITASGDLVPLSYIAGLLTGRPNSKATGPN 237
Triticum aestivum          LETIATLLNANVTPCLPLRGTITASGDLVPLSYIAGLVTGRPNSMATAPD 212
Zea mays                   LEAIAVLLNANVTPCLPLRGTITASGDLVPLSYIAGLVTGRPNSTAVAPD 227
Bambusa oldhamii           LEAIAKLLNANVTPCLPLRGTITASGDLVPLSYIAGLVTGRENSVAVAPD 225
Gingko biloba              LETIEKLLNAGITPKLPLRGTITASGDLVPLSYIAGLLTGRPNSKVRTRD 238
Trichosporon cutaneum      LQSFADFINRGLVPCVPLRGTISASGDLSPLSYIAGAICGHPDVKVFDTA 213
Rhodobacter sphaeroides    IARLIDLLNSELAPAVPSRGTVGASGDLTPLAHMVLCLQGRGD--FLDRD 171
Saccharothrix espanaensis  WQKLADLWNKGFTPAIPRHGTVSASGDLQPLAHAALAFTGVGEAWTRDAD 173
```

Figure 1.2 Conserved amino acid residues in PAL and TAL enzymes. Residues in bold font include, Tyr 110 and its equivalents, the substrate selectivity (FL, HQ, HL) region as well as the active site residues ASG which are involved in the formation of the MIO prosthetic group. Sequence alignments were performed using ClustalW.

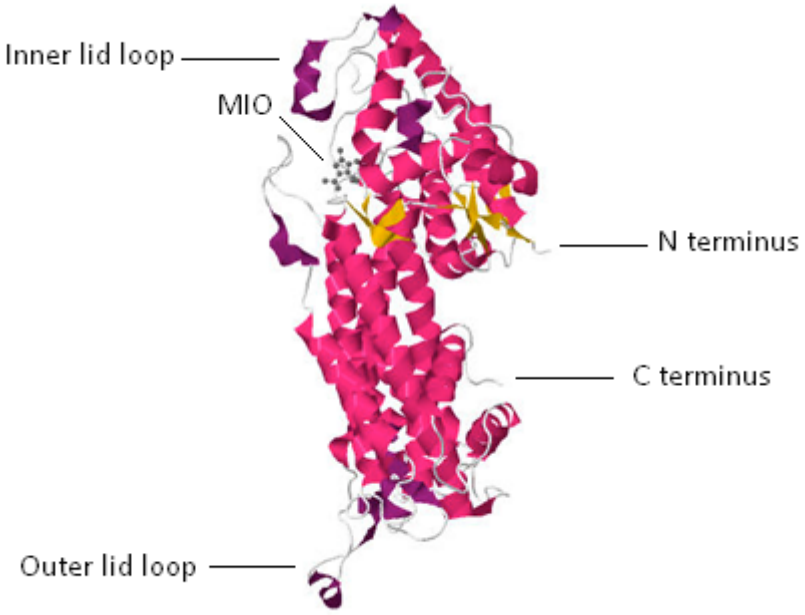


Figure 1.3 Ribbon representation of the *Rhodobacter sphaeroides* TAL monomer highlighting the tyrosine loops and the active site of the enzyme (PDB ID: 2O6Y Louie et al. 2006).

Based on the functional similarity of HAL and PAL, the presence of the same electrophilic prosthetic group and sequence conservation, it has been proposed that the genes encoding HAL and PAL diverged from a common ancestral gene, which had HAL functionality (Taylor et al. 1990). There are 12 amino acid residues that are conserved in the active site of HAL enzymes. In PAL enzymes, two amino acid substitutions occur, His83Leu and Glu414Gln (Kyndt et al. 2002). It has also been proposed that bacterial PAL may have diverged from eukaryotic PAL (Ritter & Schulz 2004, Figure 1.4). The bacterial PAL enzyme from *Rhodobacter capsulatus* has a lower similarity to known yeast PAL enzymes (*Rhodotorula glutinis*, 20%) but a higher identity (32%) with the plant PAL *Pinus taeda* (Kyndt et al. 2002).

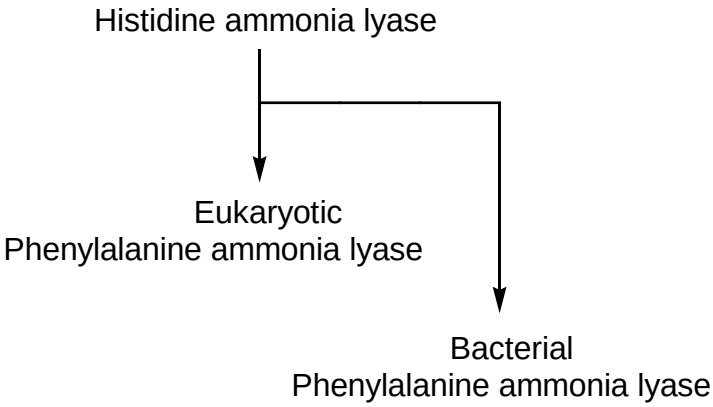


Figure 1.4 Schematic representation of the ancestry of HAL and PAL enzymes (Ritter & Schulz 2004).

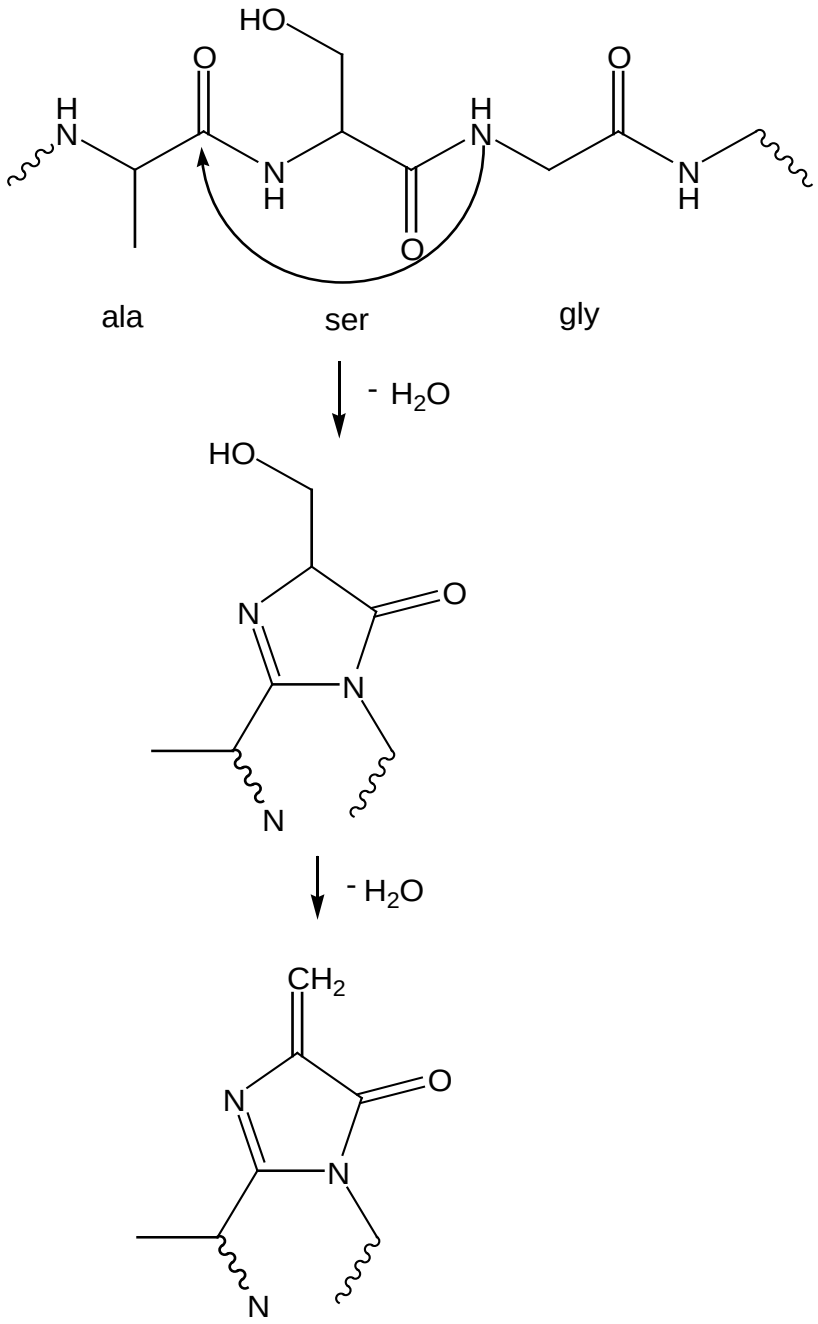
1.4 3,5-DIHYDRO-5-METHYLIDENE-4H-IMIDAZOL-4-ONE PROSTHETIC GROUP

Two post translational modifications of a protein segment involving serine are known. One involves the formation of a N-terminal pyruvyl group by removal of a N-terminal peptide portion and dehydration (Gallagher et al. 1993; Xiong & Pegg 1999) while the other is a modification involving enzymatic electrophile 3,5-dihydro-5-methylidene-4H-imidazol-4-one (Schwede et al. 1999). The aromatic ammonia lyases have a unique mode of catalysis, which involves 3,5-dihydro-5-methylidene-4H-imidazol-4-one. For approximately four decades it was believed that dehydroalanine served as the electrophilic prosthetic group at the active site of both PAL and HAL (Wickner 1969; Givot et al. 1969; Hanson & Havir 1970). Most of the earlier mechanistic features of the enzyme were based on this proposal. In the mechanism proposed by Hanson and Havir (1970), dehydroalanine served as an electrophilic catalyst increasing the leaving capacity of the amino acid moiety within the substrate by addition of the amino group to the electrophile. The mechanism, while accounting for the order in which the products are released from the reaction, did not explain the removal of a non-acidic β-proton from the substrate.

From the elucidation of the crystal structure of HAL from *Pseudomonas putida* (Schwede et al. 1999), several key features of the enzyme were discovered, including the finding that the electrophilic prosthetic group, 3,5-dihydro-5-methylidene-4H-imidazol-4-one is the true catalytically essential moiety required for substrate activation, and not dehydroalanine, as was initially proposed (Schwede et al. 1999; Calabrese et al. 2004; Ritter & Schulz 2004). Further evidence supporting the presence of a 3,5-

dihydro-5-methylidene-4H-imidazol-4-one group at the active site of PAL was obtained from spectroscopic data (Röther et al. 2000).

The 3,5-dihydro-5-methylidene-4H-imidazol-4-one prosthetic group is a common feature within all the aromatic ammonia lyases and is formed post-translationally by autocatalytic cyclization and dehydration of an internal Ala-Ser-Gly motif present within the active site of the enzyme (Poppe 2001; Baedeker & Schulz 2002). Two water molecules are eliminated, resulting in the formation of 4-methylidene-imidazole-5-one (MIO) (Figure 1.5, Poppe & Rétey 2003).



4-methylidene-imidazole-5-one (MIO) prosthetic group

Figure 1.5 Formation of the 4-methylidene-imidazol-5-one (MIO) prosthetic group via cyclization and dehydration of a conserved alanine, serine, glycine triad found in all aromatic amino acid lyases. Adapted from Schwede et al. (1999).

1.5 ENZYME MECHANISM

The reaction catalyzed by PAL poses a mechanistic challenge with regards to how ammonia is eliminated from the aromatic amino acids. This is due to a non-acidic and inactive β proton being removed from the methylene group, which is adjacent to an aromatic ring (Walsh 1979). Removal of this inert proton is not kinetically feasible without some mechanistic aid (Poppe & Retey 2003). In order to surmount the energy barriers associated with these reactions, electrophilic catalysis has evolved. Examples of enzymatic electrophiles include organic cofactors, metal ions, protons of acidic side-chains and post-translational modification of a protein segment (Poppe & Rétey 2003).

The basis of how this enzymatic electrophile is able to facilitate the removal of a non-acidic proton is not totally clear. The mechanism requires the presence of a base for catalysis. The tyrosine amino acid residue (Tyr300), present in the active site of the *Rhodobacter sphaeroides* enzyme, is believed to facilitate this role (Poppe & Retey 2003). Mutation of Tyr300Phe resulted in deactivation of the enzyme confirming that this conserved amino acid residue is essential for enzyme catalysis (Schroeder et al. 2008). Mutation of the residue corresponding to Tyr300Phe in HAL and PAL also resulted in a 55- and 235-fold decline in enzyme activity, respectively (Röther et al. 2001, 2002). The study by Schroeder et al. (2008) utilized additional methods of evaluating the effect of Tyr mutations, including kinetic analysis, fluorescence spectroscopy and mass spectrometric analysis, which confirmed that Tyr300 is an essential residue in the enzyme.

1.5.1 The E1cb mechanism

Two mechanisms have been proposed for the deamination of phenylalanine, an E1cb-like elimination and a Friedel-Crafts acylation. Both mechanisms have been intensely debated and must also account for the activity of recently characterized enzymes tyrosine and phenylalanine aminomutases (Christenson et al. 2003; Walker et al. 2004; Steele et al. 2005). The E1cb mechanism was postulated over 40 years ago by Hanson and Havir (1970). It involves nucleophilic attack on the substrate α-amino group by 3,5-dihydro-5-methylidene-4H-imidazol-4-one (Figure 1.6). 3,5-Dihydro-5-methylidene-4H-imidazol-4-one may act as a catalytic electrophile, eliminating ammonia and the non-acidic β-proton from the substrate (Schroeder et al. 2008). The methylidene carbon of MIO could potentially react with the α-amino group of the substrate by nucleophilic addition, increasing its ability to leave as well as accounting for the order of release of product, cinnamate being released prior to ammonia (Hermes et al. 1985). This mechanism is supported by studies on the crystal structure of *Pseudomonas putida* HAL (Schwede et al. 1999), *Rhodosporidium toluroides* PAL (Calabrese et al. 2004) and the parsley PAL enzymes (Ritter & Schulz 2004). Studies performed by Peterkofsky (1962) also supported the existence of an amino-enzyme complex with the order of release of products with ammonia being released last. The studies were however not able to confirm that ammonia covalently bonds to the prosthetic electrophile, which is a requirement of this mechanism.

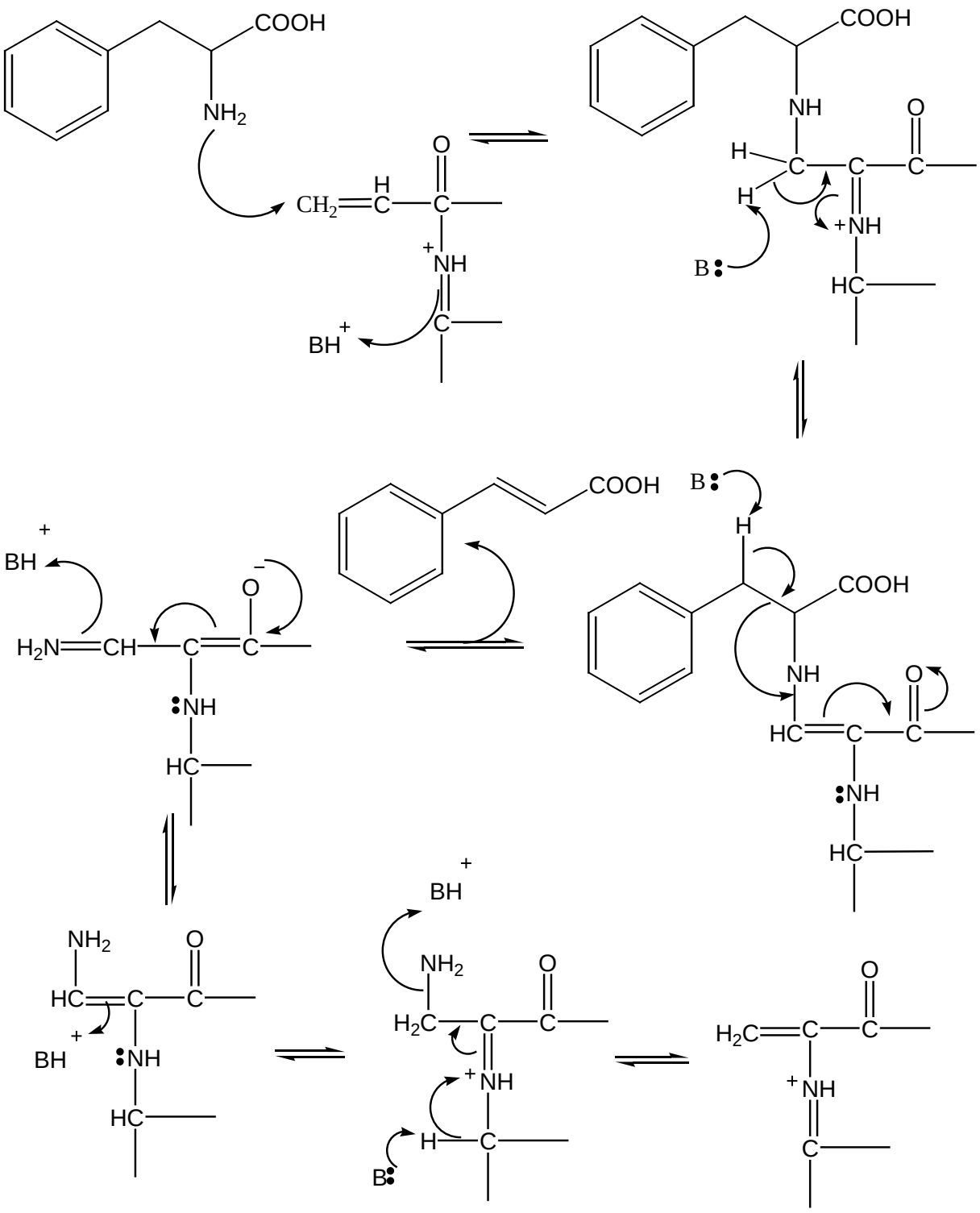


Figure 1.6. E_1cB mechanism involving nucleophilic attack on the substrate amino group. Adapted from Hanson and Havir, 1970.

1.5.2 Friedel Crafts acylation

The alternative mechanism that has been proposed is a Friedel Crafts acylation (Figure 1.7), which would involve reaction of the electron deficient methylidene carbon of the 3,5-dihydro-5-methylidene-4H-imidazol-4-one group (an electrophile) with the electron rich aromatic ring of the substrate (Poppe & Rétey 2005). This would result in loss of aromaticity of the substrate ring, producing a delocalized carbocation within the benzyl ring system of the enzyme bound substrate (Watts et al. 2006). The cationic intermediate that is formed is positively charged, making the nearby β protons more acidic. Removal of the proton would be facilitated by an enzymic base, with subsequent elimination of ammonia. Energy is required for the formation of the cationic intermediate due to loss of aromaticity. This may be facilitated by aromatization of the 3,5-dihydro-5-methylidene-4H-imidazol-4-one ring, or interaction of the carbonyl group of 3,5-dihydro-5-methylidene-4H-imidazol-4-one with a positively charged ion. Both mechanisms are plausible, but the Friedel Crafts acylation is supported by its ease of reactivity with alternative substrates, for example 3-hydroxyphenylalanine (*m*–tyrosine), a facilitator of electrophilic attack (Röther et al. 2002). Disruption of the 3,5-dihydro-5-methylidene-4H-imidazol-4-one prosthetic group by mutation of Ser to Ala allowed it to react readily with 4-nitrophenylalanine in which the β-proton is more acidic (Schuster & Retey 1995). Modeling studies and experimental data also support this mechanism, and it is believed that this is the more plausible mechanism.

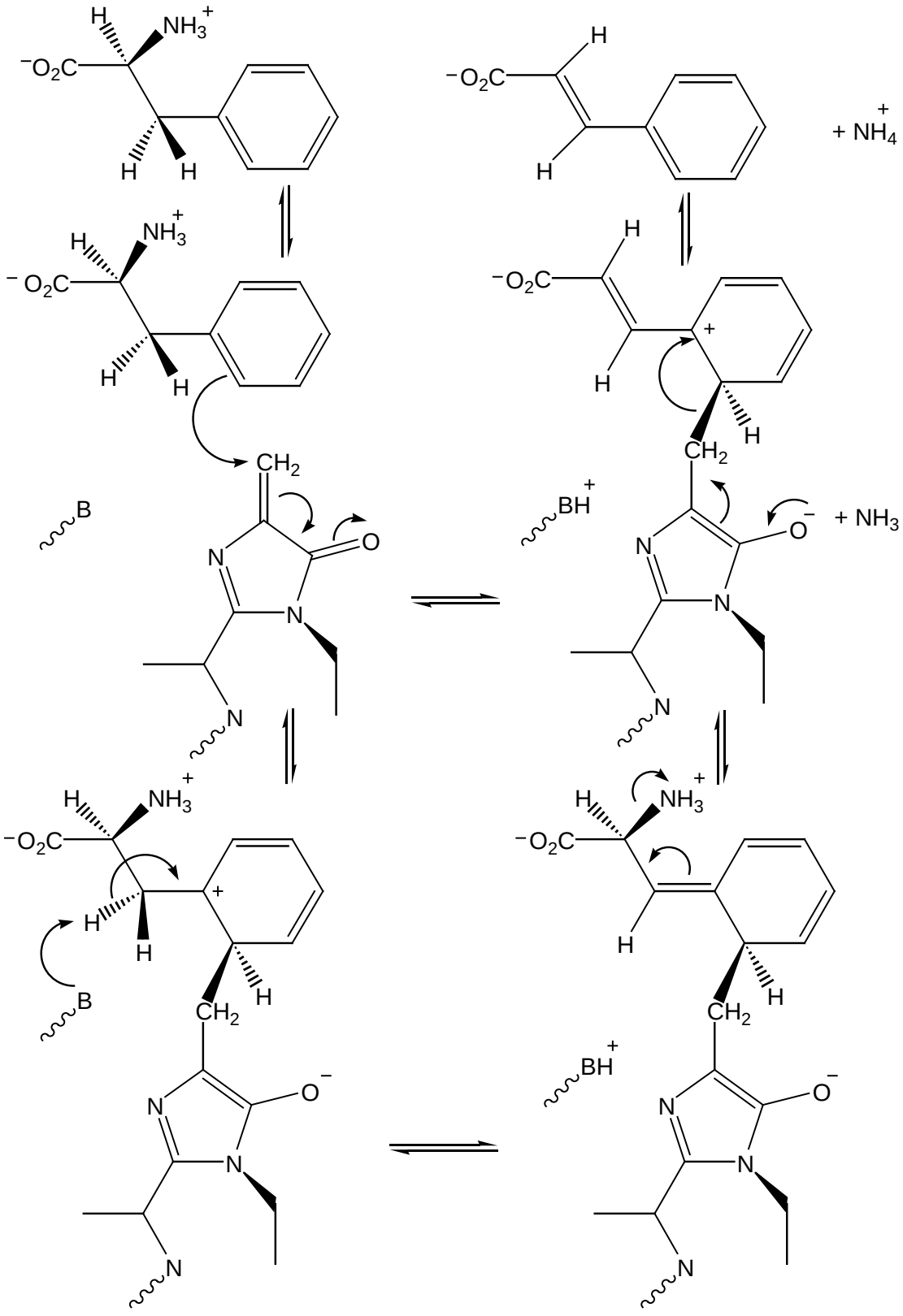


Figure 1.7. Friedel-Crafts acylation with electrophilic attack of MIO on the substrate aromatic ring. Adapted from Rother et al. 2002.

1.6 SUBSTRATE SELECTIVITY SWITCH REGION

Mutagenesis studies of the aromatic amino acid ammonia lyases have demonstrated that relatively minor changes in their primary structure can result in significant changes in the substrate specificity of the enzymes. The polarity and the size of the amino acid residues plays an important role in determining the enzyme's ability to react with either phenylalanine or tyrosine. Residues with a polar side chain favour tyrosine ammonia lyase activity due to the presence of the hydroxyl group in tyrosine (Watts et al. 2006).

Amino acids surrounding the active site of PAL have been targeted to determine critical/essential amino acid residues within the enzyme. Site directed mutagenesis studies of the TAL enzyme from *Rhodobacter sphaeroides* have led to the discovery of an important residue that determines substrate selectivity of the enzyme. A histidine in the active site predetermines that the enzyme will have a substrate preference for tyrosine over phenylalanine (Watts et al. 2006 and Louie et al. 2006). Substitution of His89 with phenylalanine, a characteristic residue of PALs, in the highly selective TAL enzyme from *Rhodobacter sphaeroides*, resulted in a switch in kinetic preference from tyrosine to phenylalanine (Louie et al. 2006). Further confirmation of the switch in kinetic preference from tyrosine to phenylalanine was obtained by purification and kinetic analyses of the mutant clone (Watts et al. 2006).

Arabidopsis has a very low catalytic efficiency for tyrosine (k_{cat}/K_m, 75 M^{-1} s^{-1}) but a high catalytic efficiency for phenylalanine (k_{cat}/K_m, 2.55 x 10^4 M^{-1} s^{-1}) (Cochrane et al. 2004). A corresponding mutation made in the PAL enzyme from *Arabidopsis thaliana* resulted

in the enzyme losing PAL activity and gaining TAL activity (Watts et al. 2006). A mutant was generated in which Phe144 was replaced with histidine. The mutant enzyme displayed a marked 80-fold reduction in catalytic efficiency for phenylalanine from 2.7 x 10^{6} M^{-1} s^{-1} to 3.4 x 10^{4} M^{-1} s^{-1}, when compared to that of the wild-type enzyme, coupled with an 18-fold increase in catalytic efficiency for tyrosine, thus confirming the presence of the substrate selectivity switch region (Watts et al. 2006).

This is an example of a single point mutation being able to essentially modify the substrate kinetic preference of the enzyme. This amino acid residue may be used as a predictive tool in determining the presence or absence of PAL/TAL activity. An unknown enzyme from *Nostoc punctiforme* PCC 73102 was determined as a PAL based on information from the substrate selectivity switch motif. This prediction was subsequently confirmed experimentally (Watts et al. 2006).

Prior mutagenesis studies have pointed out the importance of this region in determining the substrate specificity of the aromatic ammonia lyase group of enzymes. A His83Leu mutation in the HAL from *Pseudomonas putida* resulted in enzyme inactivation (Rother et al. 2001). An attempt to convert the *Petroselium crispum* PAL enzyme to a HAL by a Leu138His mutation resulted in PAL with a 100-fold increase in K_m for phenylalanine and no difference in K_m for histidine (Rotter et al. 2002). Leu138 is the corresponding amino acid moiety to histidine 83 in *Pseudomonas putida* HAL. Additional mutations have confirmed the influence of of His89 on PAL/TAL activity. Mutants His89Ala and His89Trp are devoid of TAL activity. This is most likely due to the loss of the tyrosine-

hydroxyl/histidine interaction, due to steric hinderance (Watts et al. 2006). It is believed that His89 in *Rhodobacter sphaeroides* TAL and its equivalents are involved in binding and catalysis and substrate orientation (Rother et al. 2001). PAL/TAL enzymes have either histidine or phenylalanine at the switch position followed by leucine (Watts et al. 2006). Some exceptions do exist in which there is a glutamine residue at the leucine position (Watts et al. 2006). This is seen, for example, in the enzyme from *Rhodosporidium toruloides*.

1.7 OTHER CRITICAL AMINO ACID RESIDUES

Val409 has been identified as another key amino acid residue in bacterial PALs, which affects substrate specificity. Sequence comparison of the TAL enzyme from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* has revealed that Val409 is conserved between the two Rhodobacter species. A Val409Met or Val409Ile mutation, in the *Rhodobacter sphaeroides* TAL enzyme, demonstrated a greater preference for phenylalanine when compared to the wild-type enzyme (Xue et al. 2007b). Enzyme activity was determined by an end point assay using high performance liquid chromatography to determine the formation of production of *para* hydroxycinnamic acid and *trans* cinnamic acid. In the wild-type enzyme, the ratio of *para* hydroxycinnamic acid to *trans* cinnamic acid was 29, compared to a ratio of 1.7 in the mutant gene (Xue et al. 2007b). Crystal structures of the *Rhodobacter sphaeroides* TAL enzyme have revealed that the Val409 residue is located in the active site of the enzyme in close proximity to the aromatic ring of the substrate. Val409 is positioned at the opposite side of the aromatic ring in comparison to His89, which was also proposed by Louie et al.

(2006) and Watts et al. (2006), as playing an important role in the substrate specificity of the enzyme (Xue et al. 2007b). In addition to His89, Val409 in the *Rhodobacter sphaeroides*, the TAL enzyme appears to play a key role in the preferential binding of tyrosine over phenylalanine. Methionine and isoleucine both have longer hydrophobic side chains than valine. It is believed that the mutants His89Phe, Val409Ile and Val409Met create a more favourable environment for the interaction of the aromatic side chain of phenylalanine with the active site of the enzyme. Sequence alignments of TALs with other PAL and HAL enzymes revealed that the residue corresponding to Val409, for example Ile472 (*Rhodosporidium toluroides* PAL) is conserved amongst all PAL enzymes (Xue et al. 2007b).

1.8 ENZYME ASSAYS

1.8.1 Spectrophotometric assay

Spectrophotometric assays are commonly used for the determination of PAL activity. The reaction velocity is determined by an end point or continuous assay. Of the two, the continuous assay is the more accurate, as the initial velocity of the reaction may be determined and linearity monitored. It may not always be convenient to use the continuous assay, especially when separation techniques, for example high performance liquid chromatography or electrophoresis, are employed. In these instances, the end point assay also known as a discontinuous assay, is used. The velocity of the reaction is determined at a specific time point. One assumption of this method is that linearity is maintained within the selected time interval, and this may be a source of error. The velocity of the reaction may be altered due to changes in

temperature, pH, substrate concentration and product inhibition. The end point assay should therefore be used with caution (Copeland 2000).

In the method developed by Zucker (1965), the formation of *trans* cinnamate/cinnamic acid is followed at a wavelength of 290 nm. In another method developed by Koukol and Conn (1961), cinnamic acid is extracted from the reaction mixture with diethyl ether under acidic conditions. The residue resulting after removal of diethyl ether is dissolved in sodium hydroxide (0.05 M) and spectrophotometric determinations made at 268 nm. This method is laborious, with challenges arising in producing reproducible results. To overcome these challenges, a direct assay method is utilized in which the absorbance of *trans* cinnamic acid or *para* hydroxycinnamic acid is measured under acidic conditions. A similar method was established by O'Neal and Keller (1970), who estimated the formation of cinnamic acid after the reaction was stopped with trichloroacetic acid. One possible error with the spectrophotometric assay is the formation of a complex that also absorbs at 290 nm. This occurs when borate buffer is used in the preparation of crude extracts. In the presence of α-keto acids, phenylpyruvate is produced with its enol tautomer-borate complex. This complex absorbs strongly at 290 nm (Erez 1973). The degree of interference in the PAL assay is dependent on the concentration of α-keto acids in the extracts (Erez 1973).

1.8.2 Radioactive assay

In a radioactive assay, C^{14}-L-phenylalanine replaces phenylalanine and is used to determine the quantity of product formed. An end point assay is used and the reaction

is terminated by the addition of trichloroacetic acid (50%) followed by extraction with benzene. Protein in the benzene phase is removed by centrifugation and the benzene is subsequently removed in a vacuum oven (Innerarity et al. 1972).

1.8.3 High performance liquid chromatography

The concentration of *trans* cinnamic acid and *para* hydroxycinnamic acid may be determined by high performance liquid chromatography (HPLC) utilizing Zorbax columns (Watts et al. 2006; Xue et al. 2007b) which are silica based. HPLC is a more sensitive method of assaying for enzyme activity due to its lower detection limit. In the PAL from *Phaseolus vulgaris*, low activities with phenylalanine and tyrosine were detected for periods of up to four days. Accurate quantification of TAL activity was based on detection of *para* hydroxycinnamic acid, which had a detection limit of 3-5 *p*mol (Scott et al. 1992).

1.9 PROTEIN PURIFICATION

Phenylalanine ammonia lyase has been purified using various methodologies. Typically the enzyme is purified by protein precipitation, followed by ion exchange chromatography. The PAL enzyme from *Rhizoctonia solani* was purified by protamine sulphate precipitation followed by DEAE-cellulose chromatography (Kalghatgi & Subba Rao, 1975). A similar methodology was employed for the PAL enzyme from *Rhodotorula glutinis.* The enzyme was fractionated with ammonium sulphate and sodium citrate, followed by chromatography on DEAE-cellulose and Sephadex G-200 (Fritz et al. 1976). A procedure using sonication, salt precipitation, gel filtration and

hydrophobic interaction chromatography has also been utilized successfully in the purification of *Rhodotorula glutinis* PAL (D'Cunha et al. 1996). Similar methods are used for the purification of PAL from plant sources. PAL from cell cultures of *Phaseolus vulgaris* L. was purified by ammonium sulphate precipitation, gel filtration and DEAE-cellulose chromatography. A purification fold of 197 and a 12% yield was reported (Bolwell et al. 1985).

Ways of improving and reducing the number of steps required for the purification of PAL are continually being investigated. One method that was recently used successfully is purification of the PAL enzyme from *Rhodotorula glutinis* using an aqueous two phase partitioning system. A polyethylene glycol 1000/Na_2SO_4 system gave the highest yields (80.6%) with a 9.3-fold purification (Yue et al. 2007). This is a mild and simple method of purifying the enzyme.

1.10 APPLICATIONS OF PAL

Due to its central role in the phenylpropanoid pathway, PAL is one of the most extensively studied plant enzymes. *Trans* cinnamic acid and *para* hydroxycinnamic acid are precursors to a wide array of compounds, which have applications in the synthesis of polymers, novel flavours, fragrances, pharmaceuticals, biocosmetics and health and nutrition products (Qi et al. 2007). TAL activity presents a biological route for production, directly catalyzing the conversion of tyrosine to *para* hydroxycinnamic acid. This alleviates the need to express both PAL and 4-coumaric acid hydroxylase, a membrane bound cytochrome P450 enzyme that converts phenylalanine to *para*

hydroxyl-cinnamic acid (Schoeder et al. 2008). A PAL enzyme that demonstrates high substrate efficiency for tyrosine may prove useful as a biocatalyst in synthesizing *para* hydroxycinnamic acid. TAL has been successfully used in metabolic engineering of flavonoids and resveratrol biosynthesis pathways, which require *para* hydroxycinnamic acid as a precursor molecule (Watts et al. 2004; Jiang et al. 2005; Zhang et al. 2006; Qi et al. 2007; Trotman et al. 2007).

PAL has also been used in the preparation of low phenylalanine diets (Yamada et al. 1981), quantitative analysis of serum phenylalanine (Koyama 1984) and the synthesis of amino acids. PAL not only catalyzes the degradation of phenylalanine but is also able to synthesize phenylalanine from *trans* cinnamic acid in the presence of high concentrations of ammonia (Yamada et al. 1981). This reverse reaction has also been used in the synthesis of L-phenylalanine methyl ester, one of the precursors of the sweetener aspartame. The PAL enzyme from *Rhodotorula glutinis* was used to catalyze the formation of L-phenylalanine methyl ester from *trans*-cinnamyl methyl ester (D Cuhna et al. 1994).

1.10.1 Vinyl phenol derivatives

Polyhydroxystyrene is an important monomeric unit in the production of commercial polymers. Interest in its production stems from its diverse commercial applications, which includes the manufacture of photoresists in photolithography and semiconductors (Flanagin et al. 1999). It is typically produced from petroleum-based feedstocks. Currently biological routes for the production of polyhydroxysytrene using a

renewable energy source are being investigated. Biological processes using alternative substrates, for example glucose, are the most economically feasible. Glucose is converted via the shikimate pathway to L-tyrosine, which is then deaminated with a PAL enzyme possessing tyrosine activity to yield *para* hydroxycinnamic acid (Qi et al. 2007). Subsequent decarboxylation of *para* hydroxycinnamic acid leads to the formation of poly-hydroxystyrene, also known as 4-vinyl phenol (Figure 1.8). Some vinyl phenol derivatives have found applications in the formation of volatile aromatic derivatives, which contribute to the aroma of various fermented foods and beverages. These include for example, 4-vinylguaiacol and 4-vinylphenol (Vanbeneden et al. 2007). Decarboxylation of *para* hydroxycinnamic acid is facilitated by a *para* hydroxycinnamic decarboxylase (PDC) (Trotman et al. 2007).

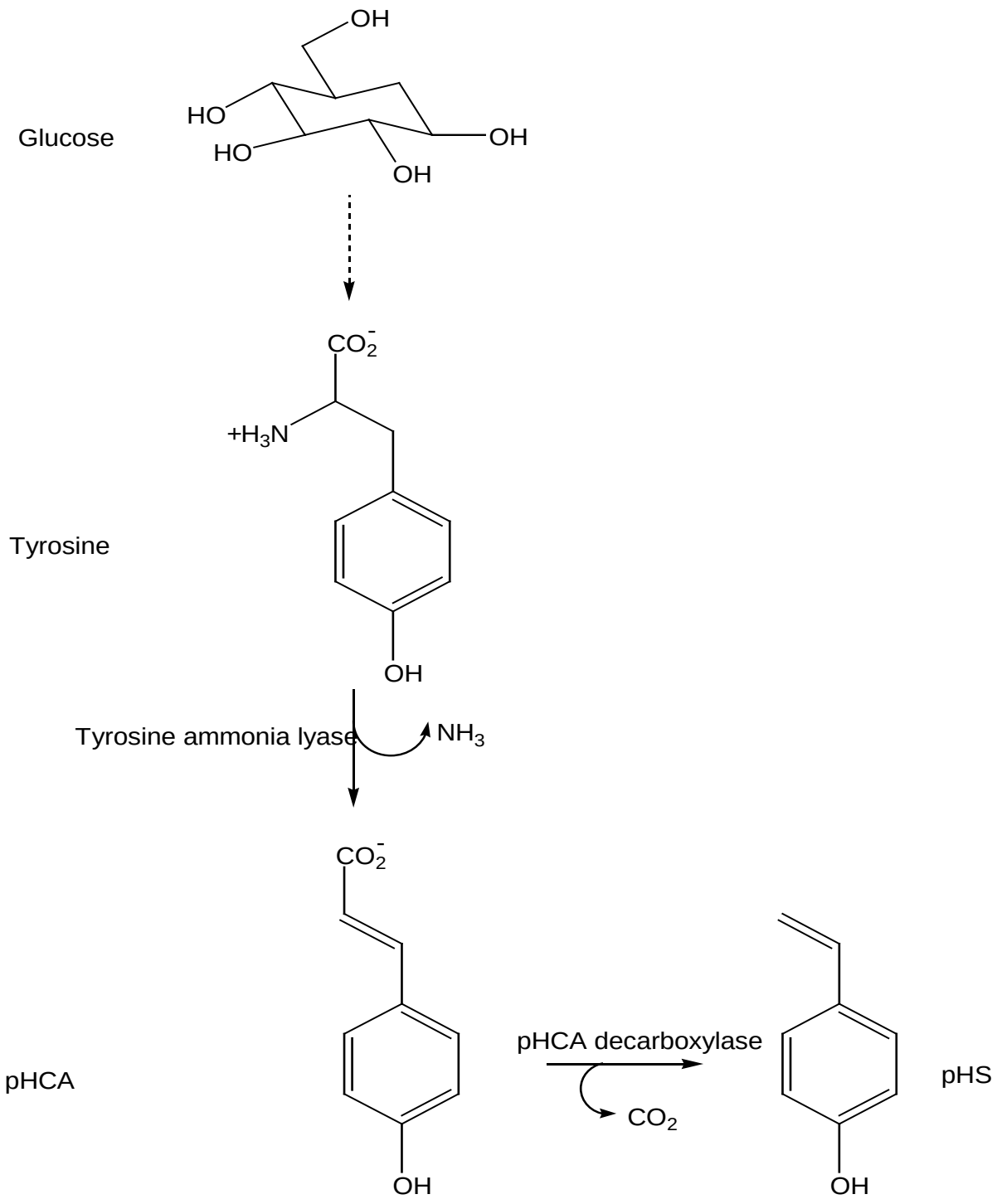
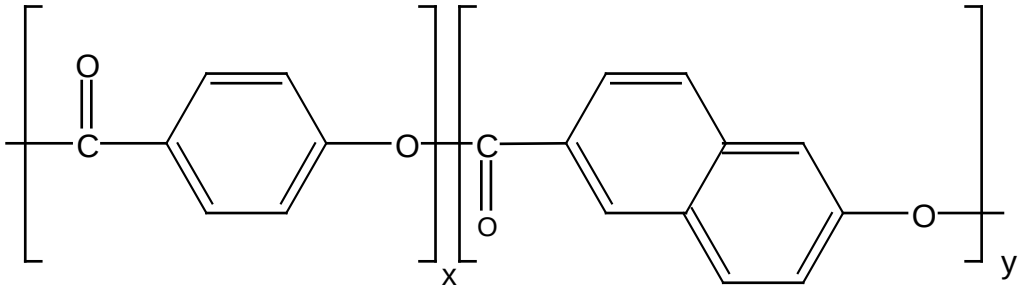


Figure 1.8 Pathway for the conversion of glucose to polyhydroxystyrene by a recombinant *Escherichia coli* strain expressing phenylalanine ammonia lyase (active with tyrosine) and *para* hydroxycinnamic decarboxylase, adapted from Qi et al. (2007).

Both PAL and PDC genes have been successfully cloned and expressed in *Escherichia coli* strains (Qi et al. 2007). The PDC gene has been cloned and co-transformed with a plasmid encoding a bifunctional PAL enzyme from the yeast *Rhodotorula glutinis*. Successful co-expression of the fungal PAL and the bacterial PDC gene in a phenylalanine over-producing *Escherichia coli* strain was accomplished. Best yields of polyhydroxystyrene were reported with the PDC gene from *Lactobacillus plantarum*. This is an initial step in producing a microbial strain able to convert glucose directly to polyhydroxystyrene (Qi et al. 2007).

1.10.2 Liquid crystal polymers

PAL enzymes have been investigated for use in the synthesis of liquid crystal polymers (Verhoef et al. 2007). Liquid crystal polymers are a relatively unique class of partially crystalline aromatic polyesters based on *para* hydroxybenzoic acid and related monomers such as *para* hydroxycinnamic acid (Figure 1.9). They are high performance plastics capable of forming regions of highly ordered structure while in the liquid state (Jin et al. 1995). Their favourable mechanical properties at high temperatures, excellent chemical resistance and strong thermal resistance has led to their use in a number of industrial applications, such as medical devices, electronic connectors, telecommunication (Wang et al. 2003) and aerospace applications in addition to chemical and food packaging applications (Guerriero et al. 2011).



HBA
p-Hydroxybenzoic acid

HNA
6-Hydroxy-2-naphthoic acid

Figure 1.9 Monomeric units of liquid crystal polymers

A solvent tolerant bacterium *Pseudomonas putida* S12 has been developed as a host for the biological production of aromatic compounds from renewable sources (Verhoef et al 2007; Nijkamp et al 2005; Verhoef et al 2009; Wierckx et al 2005). This bacterium is tolerant of organic solvents, for example toluene and l-octanol, making it an excellent platform host for the production of toxic hydrophobic compounds (Verhoef et al. 2010). For the production of *para* hydroxybenzoate, the PAL gene from *Rhodosporidium toluroides* was introduced into the *Pseudomonas putida* S12 host strain (Verhoef et al. 2007).

1.10.3 Phenylalanine ammonia lyase and phenylketonuria

Phenylketonuria is one of the most common inborn errors of metabolism. It was first described by Asborn Folling in 1934 (Centerwall & Centerwall 2000). People suffering from phenylketonuria are unable to metabolize the essential amino acid phenylalanine. This leads to elevated levels of phenylalanine and phenylalanine derived metabolites in the blood in excess of 1200 µM (Scriver et al. 1995). Normal blood levels of phenylalanine fall typically in the range of 80 to 120 µM (Hendriksz & Walter 2004). The disease is due to mutations in the phenylalanine hydroxylase gene (Scriver et al. 2000). Untreated, phenylketonuria leads to impaired neurological function as well as a reduction in cognitive development (Menkes 1967).

Currently patients are treated mainly by dietary regulation, which is very restrictive. Disadvantages of dietary regulation include biological side effects, restricted food intake, low palatability of synthetic dietary supplements and lifelong treatment (Walter et

al. 2002). Alternative treatment options are being investigated and include the use of enzyme therapy. Oral enzyme therapy with PAL in combination with a low protein diet has been suggested as a possible alternative to current treatment options that are available to people suffering from phenylketonuria (Scriver et al. 1989). Enzyme therapy is expected to increase the protein tolerance of patients.

Some success has been reported from preliminary trials in which phenylketonurics were given an oral dose of PAL encapsulated in enteric-coated gelatin capsules (Hoskins et al. 1980). Hyperphenylalaninemia in rats was also attenuated by oral administration of microencapsulated PAL (Bourget & Chang 1984). A recombinant PAL from the yeast gene encased in its original *Escherichia coli* expression cells when fed to a mouse model reduced blood phenylalanine levels by 30 - 40% (Sarkissian & Gamez 2005). A 50% reduction was attained when the naked recombinant PAL and a protease inhibitor were fed together (Sarkissian et al. 1999). Covalent polyethylene glycol derivatization of PAL has also resulted in enhanced stability of the enzyme (Gamez et al. 2005).

1.11 RESEARCH OUTLINE

The enzyme phenylalanine ammonia lyase (PAL) in some instances displays dual substrate activity, using both phenylalanine and tyrosine as substrates. The level of catalytic efficiency varies and is typically greater for phenylalanine. *Trichosporon cutaneum* has been identified as one of the few microbial sources of the enzyme displaying dual substrate activity with tyrosine and phenylalanine. The level of activity with tyrosine was significant when compared to other sources of the enzyme. The enzyme will be used as a model to further investigate the tyrosine ammonia lyase activity of this class of enzymes as well as factors influencing substrate specificity. An effective and efficient means of purifying the enzyme will be established.

1.11.1 Objectives

The main objectives of this research project are the biochemical characterisation and purification of the PAL/TAL enzyme from *Trichosporon cutaneum.*

1.11.2 Hypotheses

Hypothesis statement 1

PAL/TAL enzyme from *Trichosporon cutaneum* which has dual substrate activity with phenylalanine and tyrosine, possesses similar biochemical characteristics to other PAL enzymes devoid of activity with tyrosine, and can be used as a model to further investigate the TAL activity of this family of enzymes and to confirm the findings of a prior study (Vanelli et al. 2007a).

Hypothesis statement 2

Trichosporon cutaneum PAL/TAL will display typical Michaelis Menten kinetics in the presence of phenylalanine and atypical Michaelis Menten kinetics in the presence of tyrosine.

Hypothesis statement 3

Trichosporon cutaneum PAL/TAL can be purified utilizing methods that have been published for other yeast PAL enzymes.

Hypothesis statement 4

Amino acid residues that potentially affect TAL activity in *Trichosporon cutaneum* can be identified by sequence alignment of PAL/TAL enzymes in the National Center for Biotechnology Information database.

CHAPTER 2: INVESTIGATION OF THE DUAL SUBSTRATE SPECIFICITY OF PHENYLALANINE AMMONIA LYASE FROM VARIOUS PLANT SOURCES

2.1 INTRODUCTION

PAL catalyzes the conversion of phenylalanine to *trans* cinnamic acid (Koukol & Conn 1961) which is modified through phenylpropanoid metabolism to secondary metabolites, for example, lignin, flavonoid pigments and phytoalexins (Morrison & Buxton 1993). PAL enzymes that are active with tyrosine have been detected in monocotyledonous plants as well as dicotyledonous plants, for example, parsley (Appert et al. 1994), beans (Scott et al. 1992) and *Arabidopsis* (Cochrane et al. 2004). Levels of activity with tyrosine vary but tend to be lower when compared to phenylalanine; for example in extracts of *Phaseolus vulgaris* activity with tyrosine was found to be 0.6 - 1.3% of PAL activity (Scott et al. 1992).

The catalytic efficiency measures the ratio of k_{cat}/K_m and indicates the enzyme's preference for a particular substrate. In parsley, a 10,000 fold higher catalytic efficiency is seen with the substrate phenylalanine versus tyrosine (Appert et al. 1994). Similar results have been observed with Arabidopsis PAL isoforms (Cochrane et al. 2004). Another class of bifunctional PALs have the ability to use both substrates with almost equal efficiency (Watts et al. 2006). This phenomenon has been most clearly observed in the enzyme characterized from maize (Rösler et al. 1997) and bamboo (Hsieh et al. 2010). Equal efficiency is common among monocotyledonous plants such as corn, wheat and rice (Neish 1961). In maize, the K_m value for phenylalanine was fifteen times higher than that observed with tyrosine (Havir et al. 1971). The turnover numbers for phenylalanine is approximately 10 times higher than that for tyrosine resulting in

comparable catalytic efficiencies (k_{cat}/K_m) for both substrates (Rösler et al. 1997). Table 2.1 summarizes the levels of PAL activity reported by Young et al. (1966) in monocotyledonous and dicotyledenous plants. Highest levels of PAL activity with the substrates phenylalanine and tyrosine was reported in *Hordeum vulgare L.* (barley) followed by *Triticum aestivum* L (wheat). The ratio of activity between the two substrates (PAL/TAL) in barley was ~3 while in wheat the ratio was ~4.

PAL activity has been found to be dependent on the germination stage of the plant as well as the segment of the plant investigated. In wheat, dual enzyme activity was detected in extracts from the leaf, inflorescence and stems of the plant. Activities with both substrates increased with the age of the plants, with maximum enzyme activities being observed between days 40 and 45 post-germination (Guerra et al. 1985). Greatest PAL activity was observed in the older primary-stem, primary-inflorescence, and flag-leaf extracts whereas activity with tyrosine remained relatively constant in preparations from the inflorescence and stem but increased in the flag leaf (Guerra et al. 1985).

Table 2.1 Plant PAL enzymes exhibiting activity with both tyrosine and phenylalanine. The reported values are the percent conversion/0.2 g acetone powder.

Monocotyledons	**Section**	**L-phenylalanine (0.15%)[a]**	**L-tyrosine (0.10%)[a]**	**Ratio Phe/Tyr**
Hordeum vulgare L.	Shoots	12.0	3.9	3.1
Hordeum vulgare L.	Roots	12.4	4.0	3.1
Triticum aestivum L.	Shoots	9.0	2.1	4.3
Triticum aestivum L.	Roots	11.2	2.9	3.9
Eriophorum spissum Fern.	Leaves	0.6	0.1	6
Carex paupercula Michx.	Leaves	1.1	0.4	2.8
Juncus baltica Willd.	Leaves	0.5	0.1	5
Dicotyledons	**Section**	**L-phenylalanine (0.15%)[a]**	**L-tyrosine (0.10%)[a]**	**Ratio Phe/Tyr**
Spinacea oleracea	Leaves	1.4	0.8	1.8
Hydrangea sp.	Leaves	0.4	0.1	4.0
Sorbus americana Marsh.	Leaves	0.3	0.1	3.0
Brassica napobrassica	Root	0.3	0.1	3.0
Daucus carota L.	Root	0.3	0.1	3.0
Acer saccharum Marsh.	Leaves	0.2	0.1	2.0
Quercus borealis	Leaves	0.4	0.1	4.0
Medicago sativa L.	Stem & Leaves	4.0	0.1	40
Pisum sativum L.	Stem & Leaves	1.4	0.2	7.0

Reference: Adapted from Young et al. 1966

[a] For the enzyme assay acetone powder (2 g) was suspended in sodium borate buffer (0.05 M, 3 mL, pH 8.8) with either L- phenylalanine (0.15%) or L-tyrosine (0.10%).

In durum wheat, significant level of enzyme activity was also found during grain development with one or two maxima during the hydrical step. The hydrical step is associated with protein storage and starch accumulation in the endosperm and is defined as phase II of grain development, which generally takes place 12-35 days after anthesis (Régnier & Macheix 1996). After the hydrical stage there was a decrease in enzyme activity. Enzyme activity was not detected in mature durum seeds. Maximal enzyme activity with tyrosine peaked a few days prior to that with phenylalanine. This shift in the maxima of enzyme activity may be indicative of the enzyme acting on different substrates in the phenolic biosynthetic pathway (Régnier & Macheix 1996).

PAL plays an important role in plant defence mechanisms and is induced by various biotic (infection by viruses, bacteria, fungi, etc) or abiotic (low and high temperature, UV-B light, wounding, etc) stresses and the application of exogenous hormones including jasmonic acid, abscissic acid, salicylic acid and ethylene (Campos-Vargas & Saltveit 2002), resulting in the accumulation of phenolic compounds, for example, phenolic acids and flavonoids (Solecka & Kacperska 2003; Sgarbi et al. 2003; Wen et al. 2005).

Chitosan and chitin oligomers, which are fungal elicitors, enhanced PAL/TAL activities in soybean leaves. Peak activity was observed 36 hours after treatment of the leaves (Khan et al. 2003). Wheat grown using solar lamps exhibited an increase of 1 - 3 fold over the PAL/TAL activities of plants irradiated with metal halide or high pressure sodium lamps (Guerra et al. 1985). Low temperature exposure produced enhanced

PAL/TAL activity in eggplant fruits (*Solanum melongena* L.). Fruits stored at 1 °C over different time intervals resulted in a peak in PAL activity after 2 days of storage at 1 °C. TAL activity increased after transfer from 1 °C to 20 °C (Kozukue et al. 1979).

Strawberry plants which were treated with the plant hormone gibberellic acid, produced an enhancement in PAL/TAL activity as well as improvement in the weight, size and colour of the fruit (Montero et al. 1998). Application of salicylic acid to grape berry tissues at 1 and 3 hours after treatment also resulted in an increase in PAL activity (Wen et al. 2005). These results are similar to that reported in lettuce leaves (Campos-Vargas & Saltveit 2002). Salicylic acid is a signalling molecule, which is required for plant systemic resistance (Nugroho et al. 2002 and Chaman et al. 2003). The objective of this study was to identify plant sources of PAL exhibiting activity with phenylalanine and tyrosine as well as investigating ways of enhancing PAL activity.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Twenty seedlings from various grains and seeds were screened for phenylalanine ammonia lyase activity. Seeds and grains (buckwheat, fall rye, winter wheat, red spring wheat, mustard, lentils, alfaalfa, mung beans, winter field peas, marrowfat, azuki, black turtle, kidney, great northern, pinto, romano) were purchased from local suppliers and surface sterilized with chlorine bleach (500 mL L^{-1}) prior to germinating at room temperature in natural light on Metro-Mix 220 soil (Grace Horticultural Products) for an average of seven days. Larger seeds, which took a longer time to germinate, were

assayed after 9 to 10 days of germination. Seedlings were excised and evaluated for enzyme activity with the substrates tyrosine and phenylalanine. The enzyme activity of different regions of red spring wheat seedlings was also investigated. Experiments were performed at least in duplicate.

2.2.2 Plant extraction

Plant samples were homogenized in extraction buffer (50 mM Tris-HCl, pH 8.8, 1 mM EDTA, 10 mM 2-mercaptoethanol) and 2.5% (w/v) polyvinylpolypyrrolidone [PVPP] in a 1:2 ratio (fresh weight : buffer). After homogenization, samples were kept at 4 ºC for 1 hour, and centrifuged at 23,500 *x g* for 15 minutes at 4 ºC. Aliquots of supernatant were desalted using a PD-10 column (GE Healthcare) which was eluted with extraction buffer. Resulting extracts were used for enzyme assays. Protein was determined by use of the Bradford assay using bovine serum albumin as the standard protein (Bradford 1976).

2.2.3 Enzyme assays

The PAL or TAL activity of enzyme extracts was measured using a Unicam UV/Vis spectrometer (Abell & Shen 1987). Enzymatic assays for PAL was initiated by addition of the enzyme extract (200 µL) to a solution (600 µL) containing L-phenylalanine at a final concentration of 30 mM and 10 mM Tris-HCl (pH 8.5) at a temperature of 37 °C for 15 minutes. The reaction was terminated by the addition of 25% trichloroacetic acid (200 µL). Samples were centrifuged at 13,000 *x g* for 15 minutes and the absorbance of *trans* cinnamic acid measured at 290 nm. TAL activity was measured similarly but

using tyrosine as substrate at a final concentration of 2 mM. The absorbance of the *para* hydroxycinnamic acid produced was measured at 315 nm. *Trans* cinnamic acid (0 - 100 µM) and *para* hydroxycinnamic (0 - 50 µM) standard curves were constructed using appropriate dilutions and buffers.

2.2.4 Gel electrophoresis and Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions according to Laemmli (1970), using 10% acrylamide resolving gels and PageRulerTM prestained protein ladders (#SM0671, Fermentas). Samples were diluted with loading buffer, placed in capped microcentrifuge tubes, and heat-denatured at 100 °C for 10 min. Lanes were loaded with 50 or 100 µg protein. Gels were run at a constant voltage (120 V) for 90 minutes. Western blots were carried out using a nitrocellulose membrane, pore size 0.45 µm (Amersham Life Science), using a semi-dry electroblotter. The blot was blocked with 5% non-fat milk powder (10 mL) in phosphate buffered saline Tween-20 (PBST) for 1 hour on a gyratory shaker. It was then probed with rabbit anti-poplar PAL polyclonal antibodies (1:1000) in 3% non-fat milk powder (1.5 mL) in PBST buffer for 3 hours. The blot was washed using PBST buffer washes (3 x 10 mL, 20 minutes each), and then incubated for 1 hour (1:2000 dilution in PBST) with goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, A 3687). Excess secondary antibody was removed with 2 x 10 mL of PBST for 20 minutes followed by a 10 minute wash with phosphate buffered saline (PBS) buffer. Alkaline phosphatase activity was detected on the blot using nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-

toluidine salt (BCIP) in alkaline phosphate buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

2.2.5 Induction of PAL activity

Ultraviolet irradiation

Seven day old wheat seedlings were exposed to an artificial light source of UV-B (280 – 315 nm) provided by ten UV-B 313 40 W fluorescent tubes (Q-Panel Lab Products, Cleveland, OH, USA) installed 1.10 m above the greenhouse bench, in a 1.20 m long x 1.20 m wide x 1.25 m high frame enclosed with Mylar film (Type D, 0.127 mm thick) (GE Polymershapes Coquitlam, BC, Canada). Within this frame, smaller frames (32.5 cm x 32.5 cm x 60.0 cm high) were covered with one layer of cellulose acetate film (diacetate type, 0.127 mm) (McMaster-Carr, New Brunswick, NJ, USA). Total UV-B exposure was equivalent to 11 kJ m^{-2} d^{-1} biologically effective UV-B. UV-C treatment was delivered by two GE G25T8, 25 W bulbs, having a nominal lamp voltage and current of 46 V rms and 0.580 A rms respectively at a temperature of 25 °C. Seedlings were irradiated for 4 hours.

Light vs dark germination

Wheat seedlings were allowed to germinate under dark or light conditions at room temperature for seven days and then assayed for PAL activity.

Wounding of wheat sprouts

Seven day old wheat seedlings were excised and the base of stems suspended in test tubes containing extraction buffer (Tris HCl buffer, 50 mM, pH 8.8). PAL activity was monitored over a 24 hour time period.

Osmotic stress

Wheat seedlings were subjected to osmotic stress by application of sugar solutions of chitosan, glucose and trehalose (10 mg/mL).

2.2.6 Statistical analysis

One-way analysis of variance was used to compare means. Significance of difference was defined at $P < 0.05$.

2.3 RESULTS AND DISCUSSION

PAL activity was reported on both a fresh weight basis and per mg protein basis (Figure 2.1). Fresh weight basis refers to the level of enzyme activity in relationship to the weight of the hypocotyl. On a protein basis, the enzyme activity is reported in relationship to the total protein content of the hypocotyl. Due to the low levels of PAL activity in buckwheat and azuki beans, only activity on a fresh weight basis was determined for these species. Red spring wheat seedlings had significantly higher PAL activity than all other plant sources examined both on a fresh weight and protein basis. The roots/endosperm from the seedling had the highest level of enzyme activity (Table 2.2).

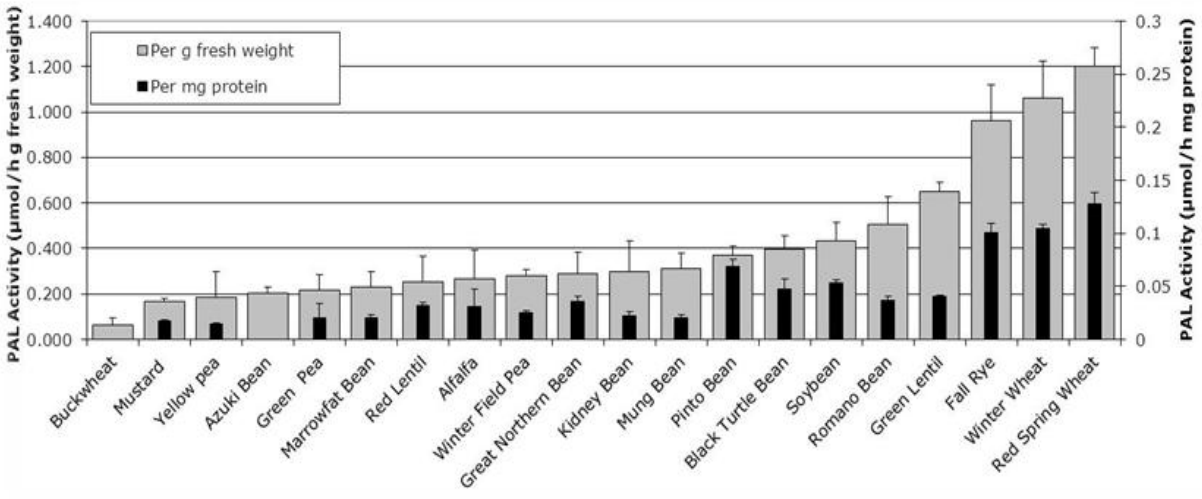


Figure 2.1 Screening of various seeds and grains for phenylalanine ammonia lyase (PAL) activity. Grains and seeds (buckwheat, fall rye, winter wheat, red spring wheat, mustard, lentils, alfaalfa, mung beans, winter field peas, marrowfat, azuki, black turtle, kidney, great northern, pinto, romano) were assayed on day 7 of germination. Soybean, green and yellow peas were assayed on day 9 to 10 of germination. Maximum PAL activity was observed in red spring wheat seedlings. Values are the mean ± standard deviation of three or four replications.

Table 2.2 PAL activity in different regions of red spring wheat seedlings

Region of seedling	**PAL activity [a](µmol/hr g fw)**
Entire seedling	1.64 ± 0.06
Shoots	1.60 ± 0.22
Seeds	2.29 ± 0.07

[a]Units: micromole per hour gram fresh weight
Experiments were performed twice with duplicate samples
The reported values are the mean ± standard deviation of four samples

The plant material was also evaluated for activity with tyrosine. Dual substrate activity was readily detected in red spring wheat, winter wheat, fall rye, mung beans, red lentil and green lentil seedlings (Table 2.3). Activity with tyrosine was not detected in the other seedlings investigated. Maximum enzyme activity occurred in red spring wheat (*Triticum aestivum* L). Activity with phenylalanine was always significantly higher than activity with tyrosine. This is the first reported incidence of PAL activity with tyrosine in lentils (*Lens culinaris*). While PAL activity in mung beans (*Vigna radiate*) has been reported (Koizuka et al. 1995 & Hoagland 1985), its ability to also act on tyrosine has only been reported by Hassan et al. (2008). In their study, the effect of sodium chloride on PAL activity was investigated. A general increase in enzyme activity was observed (Hassan et al. 2008). In research conducted by Koizuka et al. (1995) an increase in the level of expression of mRNA for the PAL gene was observed in wounded mung bean cotyledons. Hoagland (1985) investigated the inhibitory effect of *o*-benzylhydroxylamine on the enzyme. Ammonium sulfate fractionation (50 - 75%) of a mung bean extract resulted in an active fraction with a PAL activity of 65 x 10^3 I U /g fw (Ahmed & Swain 1970).

Monocotyledenous plants typically have higher levels of PAL activity, with tyrosine due to the enzyme's importance in lignification. Cereals are known for their high efficiency of converting tyrosine to lignin (Young et al. 1966). Higher levels of PAL activity were observed in the grains fall rye (*Secale cereal*), winter wheat and red spring wheat (*Triticum aestivum* L).

Table 2.3 A comparative look at the dual substrate activities observed in seedlings.

	Enzyme activity (µmol/hr g fw)[a]		
Sample	**With phenylalanine**	**With tyrosine**	**PAL/TAL activity ratio**
Green Lentils	0.65 ± 0.05	0.007 ± 0.002	93
Red Lentils	0.26 ± 0.11	0.010 ± 0.003	25
Mung Bean	0.31 ± 0.07	0.020 ± 0.003	16
Fall Rye	0.96 ± 0.16	0.023 ± 0.005	41
Winter Wheat	1.06 ± 0.16	0.028 ± 0.004	38
Red Spring Wheat	1.20 ± 0.08	0.033 ± 0.006	37

[a] Units: micromole per hour gram fresh weight
Experiments were performed twice with duplicate samples
The reported values are the mean ± standard deviation of four samples

2.3.1 PAL activity of red spring wheat as a function of germination day

PAL activity observed in red spring wheat was further investigated as a function of germination day (Figure 2.2). PAL activity plateaued between days 3 and 9 following transfer of seeds to germination conditions, with the lowest standard deviation between days 6 and 8.

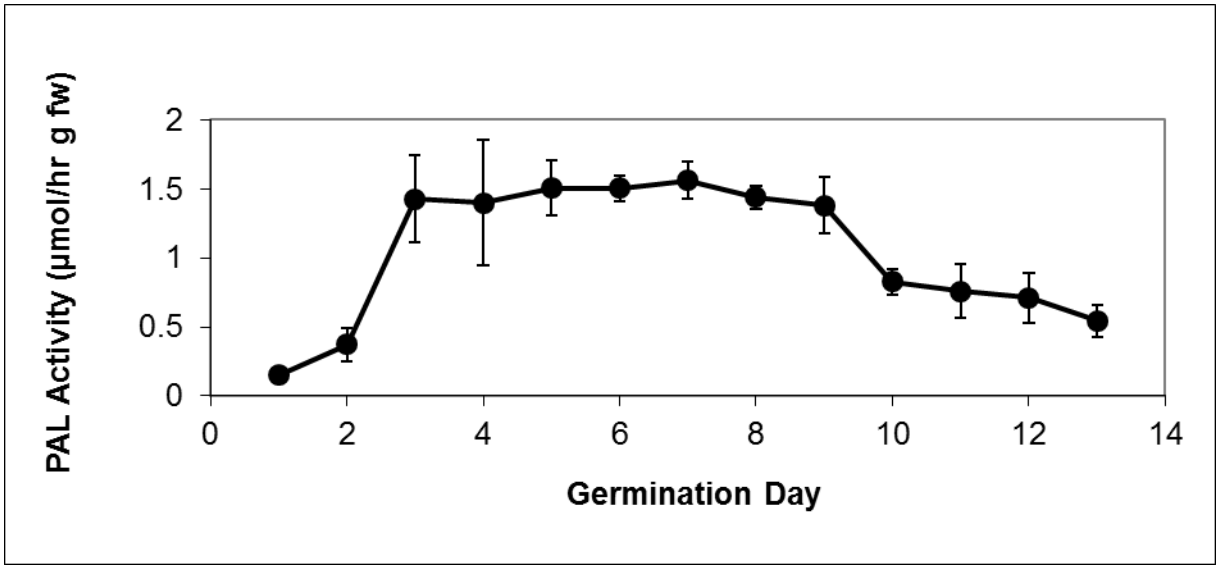


Figure 2.2 Variation of PAL activity during post-germinative growth of red spring wheat seedlings. Maximum activities are observed between day 3 and 10 of post germination. Values for day 5 and days 10-13 are mean ± range of two samples, while values for the remaining days are mean ± standard deviation of three or four samples.

2.3.2 PAL-related polypeptides in wheat seedlings

A comparison was made of the PAL-related polypeptides of red spring wheat seedlings with that of other grains and three leguminous seedlings (Romano, Black Turtle, Great Northern) by Western blot analysis (Figure 2.3). Grains or seeds were selected for Western blot analysis due to their high levels of PAL activity. For a comparative look at the PAL-related polypeptides in monocotyledons versus that of dicotyledons, Romano, Black Turtle, and Great Northern were also selected for Western blot analysis. Their levels of activity were representative of other dicotyledonous seedlings assayed for PAL activity. Of the three, Romano bean seedlings had the highest PAL activity and Great Northern the lowest. Grains were analysed on day 7 of germination while seeds were analysed on day 9 to 10 of germination. Three PAL-related polypeptides (74, 83 & 103 kDa) were detected in wheat seedlings; the relative abundance of these polypeptides appeared to change as seedlings developed, with the 103 kDa polypeptide becoming detectable only at day 7 (Figure 2.4). The 103 kDa polypeptide was exclusive to red spring wheat. Previously, polypeptides of 75 and 85 kDa were separated by electrophoresis of highly purified wheat PAL (Nari et al. 1972), which may correspond to the two smaller bands reported in this work. Only one polypeptide was observed in the other seedlings investigated. The 103 kDa polypeptide may account for the high level of PAL activity on day 7 of germination observed in wheat seedlings. This variant may be formed due to post-translational modification, for example glycosylation (Shaw et al. 1990) or phosphorylation (Allwood et al. 1999). Two potential N-glycosylation sites, Asn88 and Asn244, were identified in the protein sequence of wheat PAL (obtained from the NCBI database) utilizing the program NetNGlyc (Figure 2.5). These

predictions are based on the presence of the amino acid sequon Asn-Xaa-Ser/Thr. Xaa may be any amino acid (Gupta et al. 2004).

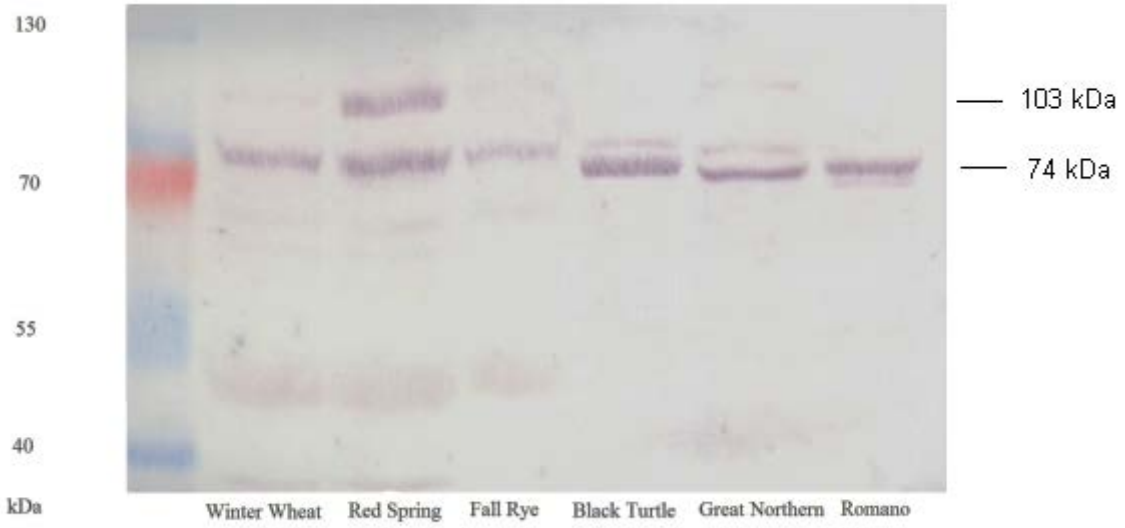


Figure 2.3 Western blot of a SDS-PAGE gel showing different isoforms of PAL in grain (day 7 post germination) and legume (day 9 post germination) samples. Winter wheat, red spring wheat and fall rye are monocotyledonous while black turtle, great northern and romano seedlings are dicotyledonous. Lanes were loaded with 100 µg of protein.

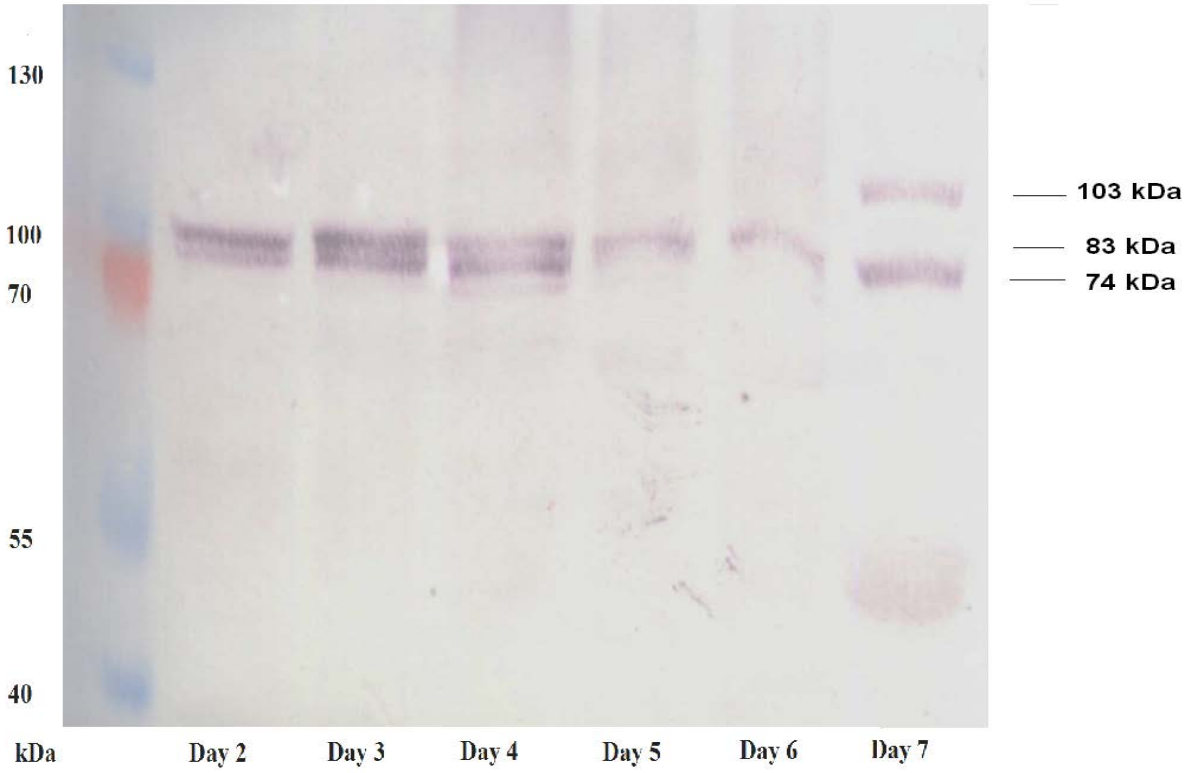


Figure 2.4 Western blot to detect PAL-related polypeptides in red spring wheat seedling during different days in germination conditions. Lanes were loaded with 100 µg of protein.

MACAWRSRSRADPLNWGKAAEELSGSHLEAVKRMVEEYRKPVVTMEGATTIAMVAAVAAGSDTRVELDESARGRVKESSD 80
WVMNSMM**N**GTDSYGVTTGFGATSHRRTKEGGALQRELIRFLNAGAFGTGTDGHVLPAAATRAAMLVRVNTLLQGYSGIRF 160
EILETIATLLNANVTPCLPLRGTITASGDLVPLSYIAGLVTGRPNSMATAPDGSKVNAAEAFKIAGIQHGFFELQPKEGL 240
AMV**N**GTAVGSGLASMVLFEANVLSLLAEVLSGVFCEVMNGKPEFTDHLTHKLKHHPGQIEAAAIMEHILEGSSYMMLAKK 320
LGELDPLMKPKQDRYALRTSPQWLGPQIEVIRAATKSIEREINSVNDNPLIDVSRGKAIHGGNFQGTPIGVSMDNTRLAI 400
AAIGKLMFAQFSELVNDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCSELQFLGNPVTNHVQSAEQHNQDVNSLGLI 480
SSRKTAEAIDILKLMSSTFLVALCQAIDLRHLEENVKNAVKSCVKTVARKTLSTDNNGHLHNARFCEKDLLLTIDREAVF 560
AYADDPCSANYPLMQKMRAVLVEHALANGEAEAHVETSVFAKLAMFEQELRAVLPKEVEAARSAVENGTAAQQNRIAECR 640
SYPLYRFVRKELGTEYLTGEKTRSPGEEVDKVFVAMNQGKHIDALLECLKEWNGEPLPLC

Figure 2.5 Potential N-glycosylation sites identified in the protein sequence of wheat PAL utilizing the program NetNGlyc are highlighted and in bold font.

2.3.3 Induction of PAL activity

PAL activity is responsive to a wide range of physiological factors and has been induced by various environmental factors, such as low nutrient levels, wounding, light irradiation and fungal infection. The effect of light irradiation, wounding and osmotic stress on PAL activity of wheat seedlings was investigated here. Light germinated seedlings had significantly higher ($P < 0.05$) enzyme activity compared to those allowed to germinate in the dark (Table 2.4). Plants allowed to develop under enhanced UV-B regimes or allowed to acclimate to doses of supplemental UV-B radiation generally have been reported as showing a simultaneous increase in soluble phenolics and photosynthetic tolerance to UV (Bieza & Lois 2001). Irradiation of wheat seedlings resulted in enhanced PAL activity (Table 2.5). The effect was more pronounced in etiolated, irradiated seedlings. There was a greater increase in the PAL activity of etiolated seedlings irradiated with UV-B (52%) compared to UV-C (35%). In light germinated seedlings the increase in PAL activity from irradiation of seedlings with UV-B and UV-C was 8% and 5% respectively. Time course analysis of excised sprouts revealed enhanced activity up to 24 hours after initial excision (Table 2.6). When exposed to osmotic stress, trehalose produced the highest induction of PAL activity (Table 2.7). Treatments with trehalose and glucose were significantly different ($P < 0.05$). No siginificant difference was observed with chitosan ($P < 0.05$).

Table 2.4 PAL activity in dark and light germinated seedlings.

Induction	**PAL activity [a](µmol/hr g fw)**
Dark germination	1.49 ± 0.05
Light germination	2.44 ± 0.002

[a] Units: micromole per hour gram fresh weight
Experiments were performed twice with duplicate samples
The reported values are the mean ± standard deviation of four samples
Samples were significantly different at $P < 0.05$

Table 2.5 Induction of PAL activity by UV irradiation of red spring wheat seedlings.

	Enzyme activity [a](µmol/hr g fw)		
Light germinated seedlings	**Time zero**	**4 Hr Exposure**	**% Increase**
Sunlight	1.64 ± 0.01	1.67 ± 0.15	1.8
UV B	1.84 ± 0.11	1.99 ± 0.21	8.2
UV C	2.65 ± 0.00	2.79 ± 0.09	5.3
Etiolated seedlings	**Time zero**	**4 Hr Exposure**	**% Increase**
UV B	1.33 ± 0.00	2.02 ± 0.33	51.9
UV C	1.67 ± 0.16	2.26 ± 0.08	35.3

[a] Units: micromole per hour gram fresh weight
Experiments were performed twice with duplicate samples
Different batches of seedling were used for each trial
The reported values are the mean ± the standard deviation of four samples
Enzyme activity of etiolated seedlings were significantly different after 4 hr irradiation ($P < 0.05$)

Table 2.6 Induction of PAL activity by wounding of red spring wheat seedlings.

Wounding	PAL activity [a](μmol/hr g fw)	% Increase
Control	1.43 ± 0.02	-
12 hr	1.15 ± 0.13	-
16 hr	1.35 ± 0.03	-
20 hr	1.92 ± 0.05	34.3
24 hr	2.90 ± 0.08	50.7

[a] Units: micromole per hour gram fresh weight
Trials were performed with light germinated seedlings
Experiments were performed twice with duplicate samples
The reported values are the mean ± standard deviation of four samples
Enzyme activity of wounded sprouts were significantly different at 20 hr and 24 hr ($P < 0.05$)

Table 2.7 Induction of PAL activity in red spring wheat seedlings by osmotic stress.

Osmotic Stress	PAL activity [a](µmol/hr g fw)	% Increase
Control	1.99 ± 0.26	-
Trehalose (10 mg/mL)	2.84 ± 0.10	42.7
Glucose (10 mg/mL)	2.70 ± 0.18	35.7
Chitosan (10 mg/mL)	2.15 ± 0.30	8.0

[a] Units: micromole per hour gram fresh weight
Experiments were performed twice with duplicate samples
Trials were performed with light germinated seedlings
Treatment with chitosan was not significantly different at $P < 0.05$
Treatment with trehalose and glucose were significantly different at $P < 0.05$
The reported values are the mean ± the standard deviation of four samples

2.4 CONCLUSION

The screening of several grains and seeds has revealed the presence of a number of PAL enzymes that are also active with tyrosine. Enzymes active with tyrosine were identified in both monocotyledonous and dicotelydonous plants. The presence of dual substrate activity was however at higher levels within monocotyledonous species. This is the first reported incidence of PAL activity with tyrosine in lentils. Levels of activity with tyrosine are however much lower than that with phenylalanine for both monocotyledonous and dicotyledenous plants. Of the two, monocotyledenous plants had a higher level of enzyme activity with tyrosine. This is due to the importance of the PAL enzyme in lignification in these plants. A comparative look at dual substrate activities in a number of cereal crops, namely, winter wheat, fall rye and red spring wheat is reported. An additional PAL-related polypeptide (103 kDa) in red spring wheat, which has not been previously reported, was also identified. This polypeptide may be a contributing factor to the higher levels of PAL activity in wheat seedlings. It is uncertain as to whether this polypeptide is due to expression of different PAL genes (isoforms) in wheat or if it is due to post-translation modification for example, N-linked glycosylation, proteolysis and/or phosphorylation. PAL activity was induced by light irradiation, osmotic stress and wounding. Highest levels of induction were observed by irradiation of seedlings with UV-B, wounding after 24 hours and treatment with trehalose. There appeared to be a limit to the maximal PAL activity that can be induced in the wheat seedlings. Future studies could involve a combination of these optimal conditions to try and further enhance the induction of PAL activity.

CHAPTER 3: BIOCHEMICAL CHARACTERIZATION AND PURIFICATION OF THE NATIVE PAL ENZYME FROM *TRICHOSPORON CUTANEUM*

3.1 INTRODUCTION

Phenylalanine ammonia lyase (PAL) enzymes have been purified and characterized from a variety of plant tissues and microbial cell cultures. The PAL/TAL enzyme from *Trichosporon cutanuem* was investigated. This enzyme has a catalytic efficiency ratio of 0.8 The substrate specificity (V_{max}/K_m) of both substrates was determined and used to calculate the PAL/TAL ratio of the enzyme. Enzymes with a greater catalytic efficiency for tyrosine may serve as useful biocatalysts in the synthesis of *para* hydroxycinnamic derivatives, for example, liquid crystal polymers and polyhydroxystyrene.

PAL from different sources have been purified to homogeneity (Bowell et al. 1985 and Kim et al. 1996). The enzyme has been purified from plant tissues (Havir & Hanson 1968, Marsh et al. 1968, Nari et al. 1972, Sarma et al. 1998), cell suspension cultures (Havir 1981, Jorrin & Dixon 1990) and fungi (Dahiya 1993, Hodgins 1971, Parkhurst & Hodgins 1971, Vanelli et al. 2007a). Purification usually requires extensive fractionation of cell extracts to obtain a homogenous preparation. Several purification protocols have been established. They may typically involve cell disruption, nucleic acid precipitation with protamine sulphate (for microbial PAL), protein fractionation with ammonium sulphate, followed by two or three steps of chromatography, for example, ion exchange, gel filtration or hydrophobic chromatography (Abell & Shen 1987; Emes & Vining 1970, Fritz et al. 1976; Hodgins 1971, Kalghatgi & Subba Rao 1975, Parkhurst & Hodgins 1972, Tables 3.1 & 3.2). An example of a purification procedure applied to the yeast PAL from *Rhodotorulu glutinis* involved the use of ammonium sulphate

precipitation, gel filtration and hydrophobic interaction chromatography, which resulted in 33% yield (D'Cunha et al. 1996).

Table 3.1 Purification of PAL from different microbial sources.

Enzyme	Purification	Pf[g]	Yield (%)
Rhizoctonia solani[a]	Protamine sulphate precipitation	13.2	6
	Ammonium sulphate fractionation	17.1	6
	Acetone precipitation	31.6	6
	DEAE cellulose chromatography[g]	227	6.5
Rhodotorula glutinis[b]	Aqueous two phase system	9.3	81
Rhodotorula glutinis[c]	Sonication	1	100
	Ammonium sulphate precipitation	2.5	80
	Gel filtration (Sephacryl-S-400)	73	61
	Hydrophobic interaction chromatography	195	33
Ustilago maydis[d]	Protamine sulphate precipitation	1.3	80
	Ammonium sulphate precipitation	3.4	31
	DEAE cellulose chromatography	20.6	29
	Gel filtration chromatography	318	19
	Preparative PAGE[h]	1178	3
Trichosporon cutaneum[e]	Anion exchange chromatography	4.2	25
	Hydrophobic interaction chromatography	14	18
	Gel filtration chromatography	14	6
Sporidiobolus pararoseus[f]	Acid precipitation	5.6	100
	Ultrafiltration	5.8	100
	DEAE-Sephacel chromatography	32.6	80.3

[a-f] References: Kalghatgi & Subba Rao 1975; Yue et al. 2007; D'Cunha et al. 1996; Kim et al. 1996; Vanelli et al. 2007a; Monge et al. 1995
[g] DEAE: Diethylaminoethyl
[h] PAGE: Polyacrylamide gel electrophoresis
[i] Pf: Purification fold

Table 3.2 Purification of PAL from different plant sources.

Enzyme	Purification	[k]Pf	Yield (%)
Oryza sativa[a]	Protamine sulphate precipitation	1	100
	Phenyl sepharose chromatography	9.7	13.1
	DEAE[h] sepharose chromatography	14.3	14.3
	Chromatofocusing	40.9	6.0
Petroselinum hortense[b]	Ammonium sulphate precipitation	8	71
	DEAE sepharose chromatography	41	28
	Sephadex G-200 chromatography	180	17
	Hydroxyapatite chromatography	440	16
Glycine max[c]	Ammonium sulphate precipitation	2.2	93
	Agarose chromatography	20	55
	Hydroxylapatite chromatography	37.5	45
	Preparative chromatography	53.1	10
Phaseolus vulgaris[d]	Ammonium sulphate precipitation	2.5	75
	Sephacryl S-200 chromatography	2.4	70
	Affinity chromatography with L-phenylalanine	23.8	68
Ocimum basilicum[e]	Ammonium sulphate precipitation	1.6	94
	DEAE-Sephacel (1st separation)	32.5	35
	DEAE-Sephacel (2nd separation)	57	15
	Preparative PAGE[i]	180	7
Bambusa oldhamii[f]	Protamine sulphate precipitation	1.3	91
	Ammonium sulphate precipitation	2.6	46
	Sephacryl S-200 chromatography	9.7	32
	Phenyl Sepharose chromatography	23	22
	Mono Q (FPLC[j]) Anion exchange	135	10
Helianthus annus L[g]	Streptomycin sulphate	1.1	100
	Ammonium sulphate precipitation	2.4	86
	Sephacryl S-300 chromatography	5.6	72
	DEAE-trisacryl chromatography	9.5	65
	Octyl-Sepharose chromatography	84.6	61
	Chromatofocusing PBE 94	175	24

[a-g]References: Sarma & Sharma 1999; Zimmermann & Hahlbrock 1975; Havir 1981; da Cunha 1998; Hao et al. 1996; Hsieh et al. 2010; Jorrín et al. 1988
[h] DEAE: Diethylaminoethyl
[i] PAGE: Polyacrylamide gel electrophoresis
[j] FPLC: Fast protein liquid chromatography
[k] Pf: Purification fold

Problems that have been encountered during purification include enzyme instability. The enzyme is sometimes prone to degradation, which can lead to loss of enzyme activity, low yields and discrepancies with regards to the actual native and subunit structure of the enzyme (Kim et al. 1996). In some instances PAL is associated with carbohydrates via glycosylation. This is seen for example in maize, which has an appreciable quantity of carbohydrates bound to the enzyme. These carbohydrates include arabinose, ribose, fucose, galactose, mannose, and glucose. Carbohydrates have a stabilizing effect on the enzyme by protecting it from proteolytic degradation. They are also involved in enzyme localization (Havir 1979).

Purified rice PAL was reported to be inherently unstable. Consequently, lower molecular weight partial degradation products (50 kDa) appeared during SDS-PAGE. Fragmentation intensified with excess incubation at high temperatures, storage or repeated freeze-thaw cycles. The actual molecular weight of the subunit is 84 kDa (Sarma & Sharma 1999). Partial degradation products were also seen in the purification of *Phaseolus vulgaris* PAL (Bolwell et al. 1986). The purified enzyme from *Cucurbita pepo* was also reported to be extremely unstable unless it was stored in 30% glycerol (El Shora 2002). PAL from illuminated buckwheat hypocotyls showed major losses in both activity and protein content during the individual steps of purification, especially after freezing and thawing (Belunis & Hrazdina 1988). However, PAL from plants, *Glycine max* (Havir 1981), *Bambusa oldhamii* (Hsieh et al. 2010), *Zea mays* (Reid et al. 1972) and *Gossypium hirsutum* (Dubery & Smit 1994) were reported to be stable throughout enzyme purification.

Purification of PAL from some yeast, for example *Sporidiobolus pararoseus*, the presence of carotenoids in cell extracts can interfere with protein purification. Carotenoids are not completely soluble, are difficult to remove by centrifugation and may limit the resolution capabilities of resins used for chromatographic separation resulting in reduced purification efficiency. To avoid this problem, acid precipitation followed by anion exchange chromatography has been utilized with success (Monge et al. 1995). Acid precipitation had a positive stabilizing effect on the enzyme by removal of contaminating proteins and nucleic acids from the crude extract (Monge et al. 1995). Alginate, glutaraldehyde, and polyhydric alcohols such as, glycerol, sorbitol and polyethyleneglycol have been used to increase the stability of PAL from *Rhodotorula rubru* (Evans et al. 1987). By using glycerol (20%) in the elution buffer, the stability of the *Sporidiobolus pararoseus* PAL enzyme was also improved (Monge et al. 1995).

Methods of improving the efficiency of PAL purification are continually being investigated. The enzyme from *Rhodotorula glutinis* is of interest commercially. This enzyme has been purified utilizing a simple and rapid method, which involves gel-filtration of the salt precipitated crude extract followed by hydrophobic interaction chromatography. The addition of glycerol throughout the purification process enhanced enzyme stability. The final preparation had a high specific activity, with yield of 33% and a purification fold of 195 (D'Cunha et al. 1996). Manganese (0.01%) has been reported to improve the stability of *Rhodotorula glutinis* PAL. When added to the storage buffer of the enzyme, over 80% of its original activity was retained over a 3 month period. Control samples had less than 3% of the original activity after 1 month

of storage. The optimal temperature for activity retention was 0 - 2 °C (Wall et al. 2008).

The aqueous two-phase system (Yue et al. 2007) which is a mild method of protein purification has also been investigated. It is employed in the extraction and purification of protein/enzymes from crude cell extracts and may be used on an industrial or laboratory scale. Interfacial tension between the two layers is reduced and is less than that which would be encountered in water/organic solvent systems. This leads to less denaturation of the extracted protein (Diamond & Hsu 1992). A characteristic feature of the aqueous two phase system is that both phases are aqueous but still immiscible. The formation of both phases is determined by the pH, temperature and ionic strength of the two components. Separation of the phases occurs when the polymer present exceeds a certain limiting concentration. The polymer layer stabilizes the extracted protein and also concentrates the desired protein. Specialized systems have been developed to favour the enrichment of the desired protein in one layer. This is achieved by varying factors such as temperature, salt concentrations and the degree of polymerization (Walter et al. 1985).

Here I report the biochemical characterization of PAL from the yeast, *Trichosporon cutaneum*, including substrate specificity, pH dependence, metal dependence and enzyme kinetics studies. I investigated the effects of acid precipitation, anion exchange chromatography and aqueous two-phase partitioning on enzyme purification. I provide confirmatory data to that reported by Vanelli et al. (2007a) with regards to the molecular

weight, kinetic data and the gene sequence of the enzyme. In order to gain more insight as to the mechanism by which PAL is sometimes also able to utilize tyrosine as a substrate, two microbial sources of the enzyme, *Rhodobacter capsulatus* and *Trichosporon cutaneum,* were selected for further investigation. These sources of the enzyme were selected due to the limited information available in the literature regarding their activity with tyrosine. The enzyme from *Trichosporon cutaneum* has a relatively high activity with tyrosine in comparison to other sources of the enzyme investigated, possibly providing an enzyme source with potential for future studies involving enhancement of activity with tyrosine through mutagenesis studies.

3.2 MATERIALS AND METHODS

3.2.1 Yeast culture

The yeast strain *Trichosporon cutaneum* (ATCC 58094) was purchased from the American Type Culture Collection (Manassass, VA). The yeast strain was maintained in glycerol stocks (10%) which were stored at -80 °C. These glycerol stocks were utilized to prepare cultures of *Trichosporon cutaneum*. Shake cultures of *Trichosporon cutaneum* were grown aerobically at 30 ºC at 100 rpm, in a medium containing (per litre): Na_2HPO_4 (1.97 g), KH_2PO_4 (4.92 g), NH_4Cl, (2 g), casamino acids, (0.5 g), yeast extract, (0.5 g) and $MgSO_4$, (0.1 g) at a pH of 6.4. For induction studies tyrosine, phenylalanine or glucose were added at a final concentration of 2 mM. Control media were devoid of tyrosine, phenylalanine or glucose. The medium was distributed in culture tubes at 5 mL portions and 100 mL portions in 500 mL flasks. Cells were initially pre-incubated in 5 mL media portions and then transferred to 100 mL portions,

which were incubated for 24 hours at 30 ºC on a shaker. Growth was followed turbidimetrically at 650 nm and by wet weight determinations after centrifugation.

3.2.2 Cell extraction

Cells were harvested by centrifugation at 23,500 *x g* for 15 minutes at 4 ºC. The cell pellet was then resuspended in extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, protease inhibitor cocktail tablets EDTA free, Sigma Aldrich, 1 tablet per 100 mL) in a 1:4 ratio (wet weight : buffer) and the cells were disrupted by vortexing (maximum speed) with glass beads for 6 minutes, with intermittent cooling on ice. Cell rupture was determined by staining of lysed cells with trypan blue and by observation under a microscope. Viable cells do not retain the blue colour of the stain whereas cells with disrupted cell walls appear blue in colour. The cell suspension was then centrifuged for 20 minutes at 14,200 *x g* to remove unbroken cell mass. Aliquots of supernatant (2.5 mL) were desalted using a PD-10 column (GE Healthcare) and then eluted with extraction buffer (3.5 mL). The eluant from the column was used for protein concentration assay and PAL/TAL activity determination. Protein was determined by use of the Bradford assay using bovine serum albumin as the standard protein (Bradford 1976).

3.2.3 Enzyme assays

The PAL/TAL activity of enzyme extracts was measured using a Unicam UV/Vis spectrometer (Abell & Shen 1987). For preliminary induction studies, the enzyme was assayed using the end point assay described in Section 2.2.3. Substrate

concentrations of phenylalanine (40 mM) or tyrosine (2 mM) in 10 mM Tris-HCl (pH 8, Trizma hydrochloride, reagent grade, Sigma-Aldrich) were used for PAL and TAL assays, respectively. All other experiments were conducted using a continuous spectrophotometric assay which was conducted at a temperature of 30 °C for 2 minutes. Enzymatic assays were initiated by addition of the enzyme extract (200 µL) to substrate solution (800 µL). For the kinetic analyses solutions containing L-phenylalanine (0.5 mM to 100 mM) or L-tyrosine (0.5 mM to 10 mM) in 10 mM Tris-HCl (pH 8, Trizma hydrochloride, reagent grade, Sigma-Aldrich) were used. Stock solutions were made by a series of dilutions of phenylalanine (100 mM) or tyrosine (10 mM). The absorbance of *trans* cinnamic acid was measured at 290 nm whereas *para* hydroxycinnamic acid was measured at 315 nm. Kinetic constants were determined using the GraphPad Statistical Software, which combines biostatistics, curve fitting (nonlinear regression) and scientific graphing into a comprehensive program.

3.2.4 Temperature optimum, pH optimum and metal dependence studies

The temperature and pH optimum of the enzyme were determined by performing the enzyme assay over a two minute interval at different temperatures (28 °C, 32 °C, 36 °C and 40 °C) and pH values (7.3, 7.6, 8.2, 8.6 and 8.9) in Tris-HCl buffer (50 mM). Metal dependence studies were performed using the chloride salts (0.1 M) of: sodium, potassium, magnesium and iron.

3.2.5 Gel electrophoresis and Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis was performed as outlined in Section 2.2.4.

3.2.6 Acid precipitation

Acid precipitation was carried out by addition of an acetic acid solution (5% v/v) to crude cell free extracts (3.5 mL) of *Trichosporon cutanuem.* The pH of the extract was reduced with the acetic acid solution (500 µL) from 8.0 to 6.5, just above the isoelectric point of PAL. The enzyme has a theoretical isoelectric point of 6.2, which was determined from the protein sequence of the enzyme using the Expasy proteomics server. After 10 minutes of gentle agitation at pH 6.5, the solution was centrifuged at 13000 rpm for 5 minutes. The pH was then adjusted back to 8.0 (Monge et al. 1995).

3.2.7 Anion exchange chromatography

All purification steps were carried out at 4 °C. Crude cell free extract of *Trichosporon cutanuem* was applied to Fast Protein Liquid Chromatography anion exchange HiTrapQ Sepharose column (2.5 x 1.0 cm) which was pre-equilibrated with Tris HCl buffer (0.1 M, pH 8). The adsorbed enzyme was eluted with a linear gradient of KCl from 0 to 0.3 M in the same buffer (Monge et al. 1995). Fractions with PAL activity were pooled and exchanged with extraction buffer using Amicon Ultra centrifugal filter units from Millipore with a molecular cut off weight of 30 kDa.

3.2.8 Aqueous two-phase system

Aqueous two phase systems were prepared in centrifuge tubes (15 mL) by adding the appropriate quantity of polyethylene glycol 1000 (PEG1000), salts, distilled water and crude cell free extracts of *Trichosporon cutaneum*. Systems were selected based on the optimum yields obtained by Yue et al. (2007) and consisted of:

PEG1000 (1.85 g, 18.5%)/ammonium sulphate (1.60 g, 16.0%);

PEG1000 (1.1 g, 11%)/sodium sulphate (1.4 g, 14%);

PEG1000 (1.2 g, 12%)/sodium carbonate (1.1 g, 11%);

PEG1000 (1.1 g, 11%)/sodium sulphate (1.4 g, 14%)/sodium carbonate (5.3 g, 5.3%).

Water was added to the mixture of salt and polyethylene glycol to give a total weight of 8 g and the resulting mixture vortexed. After complete dissolution of each phase, crude cell free extract (2 mL) was added and the mixture further vortexed. The aqueous two phase system was centrifuged for 5 minutes at 2000 × *g* and the phases carefully separated. The interface, which consisted of a thin film, was discarded. Volumes of the separated phases were measured. The purification fold (P_F) was defined as the ratio of PAL specific activity (S_A) of each phase to the initial PAL S_A of crude extract. Activity yield (Y) was defined as the total activity in each phase to the total activity in crude extract. All experiments were run in duplicate at room temperature (25 °C) and pH 8.0.

3.3 RESULTS AND DISCUSSION

3.3.1 Enzyme activity induction

Phenylalanine ammonia lyase that has activity with both phenylalanine and tyrosine was detected in the yeast *Trichosporon cutaneum. Trichosporon cutaneum* was found to have significant activity with tyrosine (Table 3.3). The enzyme activity ratio for phenylalanine versus tyrosine was four, in comparison to higher ratios ranging from sixteen to ninety three, in the plant seedlings investigated (Chapter 2, Table 2.3).

In yeasts, PAL is not a constitutive enzyme but is inducible by the addition of substrates, for example L-phenylalanine, to the culture medium (Nakamichi et al. 1983, Marusich et al. 1981, Ogata et al. 1967). In the presence of tyrosine there was 84% induction of PAL activity. Less induction was seen in the presence of phenylalanine. A PAL/TAL activity ratio of ~4 was apparent in control and tyrosine induced sample. There was however an anomaly in the data obtained for phenylalanine-induced cultures in that a PAL/TAL ratio of ~1 was obtained. This anomaly may be due to inherent errors associated with the end point assay not being able to accurately determine the initial rate of the reaction. The fact that the enzyme was induced by tyrosine and not phenylalanine may also suggest that the enzyme is inherently a TAL (EC 4.3.1.23) rather than a PAL enzyme. In the presence of glucose, PAL enzyme activity appeared to be severely inhibited, as seen from the very low values obtained for PAL activity and TAL activity (Table 3.3).

The regulation of PAL in *Rhodosporidium toruloides* has been investigated previously (Gilbert & Tully 1982). PAL catalytic activity was produced after the addition of phenylalanine (Gilbert & Tully 1982) or tyrosine (Gilbert et al. 1983) to the yeast cultures with enzyme activity rapidly decreasing during the stationary phase. Decline in enzyme activity is believed to be due to induction of proteinase activity or leakage of lysosomes. There was a higher uptake of [^{3}H]leucine in the enzyme than that of total protein. The result of their study is consistent with de novo synthesis of the enzyme upon induction with phenylalanine, as opposed to activation of a proenzyme. Enzyme activity was inhibited in the presence of glucose, as previously reported by Gilbert & Tully (1982). Phenylalanine, glucose and other rapidly metabolizable substrates play an important role in regulating catabolic enzymes by a variety of mechanisms, which includes catabolite repression, inducer exclusion from cells and enzyme inactivation (Gilbert & Tully 1982).

The regulation of functional mRNA coding for PAL from *Rhodosporidium toruloides* has also been investigated (Gilbert et al. 1983). The levels of functional mRNA coding for PAL was six times higher in yeast grown on phenylalanine compared to glucose-phenylalanine minimal medium. Functional PAL mRNA was absent from yeast grown on a glucose-ammonia minimal medium in the absence or presence of phenylalanine (Gilbert et al. 1983). These results indicate that phenylalanine, ammonia and glucose play an important role in the regulation of PAL synthesis, by adjusting the levels of functional PAL mRNA (Gilbert et al. 1983). Based on the findings from these studies,

tyrosine, phenylalanine and glucose also plays a significant role in the regulation of PAL activity in *Trichosporon cutaneum*.

Table 3.3 A comparative look at the induction of phenylalanine ammonia lyase from *Trichosporon cutaneum* with phenylalanine, tyrosine and glucose.

Inducer	Enzyme activity with phenylalanine (µmol/hr g fw)[a]	Enzyme activity with tyrosine (µmol/hr g fw)[a]	Enzyme activity ratio
Control	0.89 ± 0.03	0.24 ± 0.05	4
Tyr [2 mM]	1.64 ± 0.25	0.43 ± 0.06	4
Phe [2 mM]	0.38 ± 0.15	0.27 ± 0.07	1
Glucose [2 mM]	-0.04 ± 0.15	0.01 ± 0.04	-

[a]Units: micromole per hour gram fresh weight of cell mass
The reported values are the average results from three independent cultures ± std dev.

3.3.2 The PAL protein from *Trichosporon cutaneum*

The PAL enzyme is typically tetrameric with four identical subunits. The molecular weight of the enzyme is approximately 330 kDa, with subunits ranging from 77 - 83 kDa (Gilbert & Tully 1982). Examination of the crude cell-free extracts of *Trichosporon cutaneum* by SDS gel electrophoresis and Western blot analysis showed the presence of a 79 kDa band. A molecular subunit of 77.5 kDa was reported by Vanelli et al. (2007a). The protein band was detected in both induced and uninduced cultures of the yeast. This is evident from the SDS page and Western blot analysis of crude yeast extracts (Figure 3.1). The presence of trace quantities of the enzyme in control samples suggests that there may be basal levels of enzyme before induction, or that the enzyme was slightly induced by casamino acids, a mixture of amino acids, used to supplement the growth media. Greater protein induction was observed in tyrosine-induced cultures as opposed to phenylalanine induced cultures (Table 3.3, Figure 3.1). This is contrary to that reported by Vanelli et al. (2007a) who did not detect the PAL protein band in phenylalanine- or succinate- grown cells. The susceptibility of the enzyme to degradation is evident from the Western blot analysis. Minor bands were observed at 68, 63, 56, 46 kDa (Figure 3.1). Figure 3.2 is the Western blot of a freshly prepared extract. Degradation bands are not apparent in this Western blot and may also be due a lower protein load (50 µg).

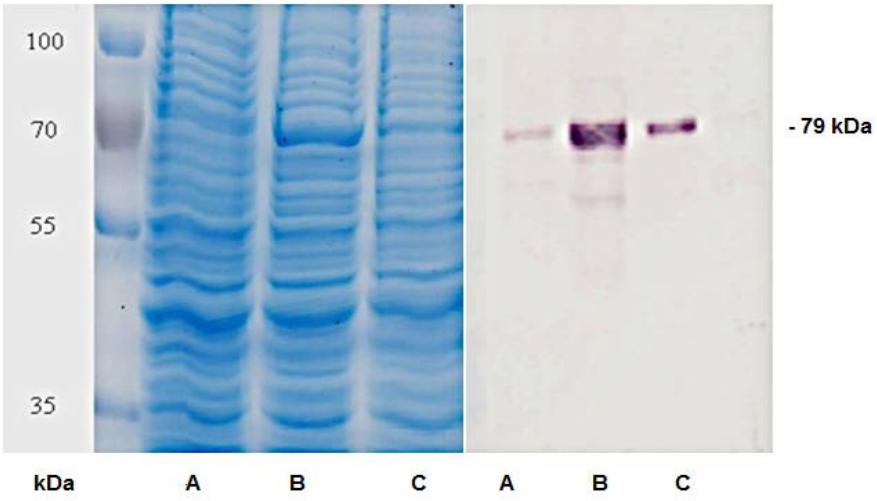


Figure 3.1 SDS-PAGE and Western blot analysis of crude extracts from *Trichosporon cutaneum* that were induced with tyrosine (lane B) and phenylalanine (lane C). Lane A is the control sample. Lanes were loaded with 100 µg protein. The primary band observed was 79 kDa.

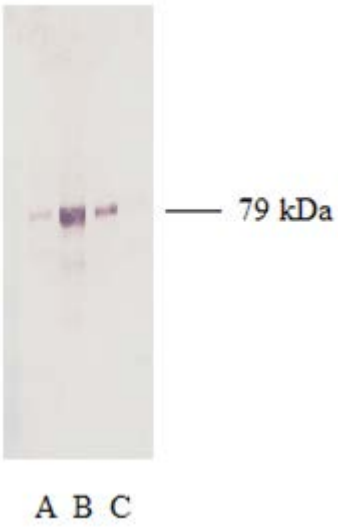


Figure 3.2 Western blot analysis of freshly extracted cultures of *Trichosporon cutaneum* that was induced with tyrosine (Lane B), phenylalanine (Lane C). Lane A is a control. Each lane was loaded with 50 µg of protein. The desired band was observed at 79 kDa

3.3.3 pH and temperature optimum

The pH optimum of *Trichosporon cutaneum* PAL for activity on phenylalanine and tyrosine as the substrates was found to occur in the range of 8 to 8.5 (Figure 3.3). The similarity of their pH optima suggests that both PAL and TAL activities reside on the same polypeptide and that there is a common protonation state for catalysis. The pH optimum of other PAL enzymes fall within the range of that found for the *Trichosporon cutaneum* PAL enzyme (Table 3.4). The *Rhodobacter* TAL enzymes appear to have a slightly higher pH optimum when compared to that of the *Trichosporon cutaneum* PAL enzyme. In the enzyme from *Rhodobacter capsulatus*, the pH optima of both activities were found to be slightly different, with an optimum of 8.5 for TAL and 9.4 for PAL (Kyndt et al. 2002). The enzyme from *Rhodobacter sphaeroides*, displayed a pH optimum in the range of 8.5 and 9 (Watts et al. 2006).

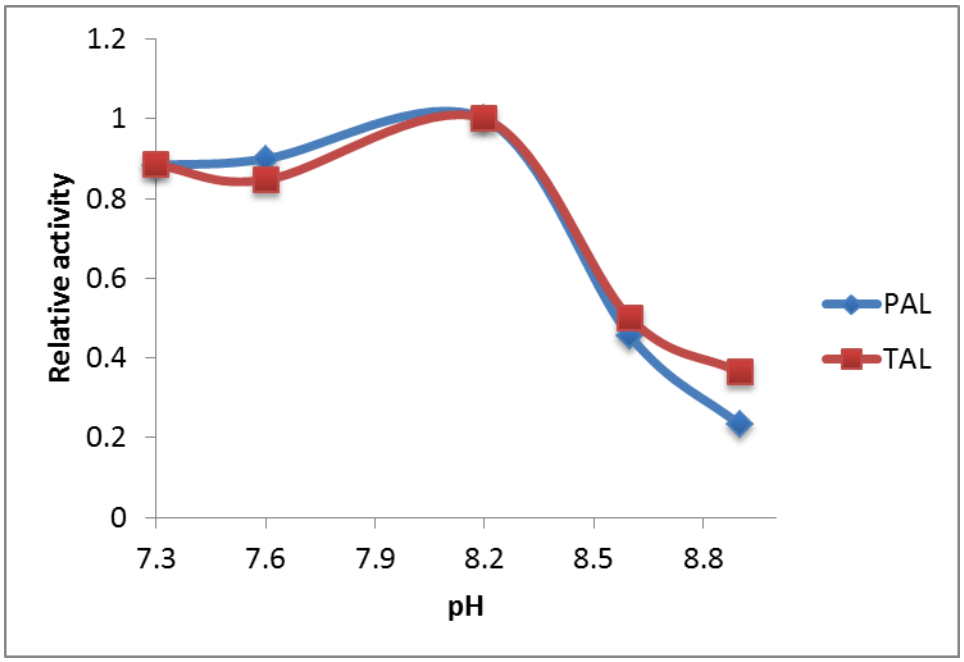


Figure 3.3 pH dependence studies on the PAL and TAL activities in the yeast *Trichosporon cutaneum*. Experiments were conducted at a temperature of 32 °C with the buffer Tris HCl (10 mM). The reported values are the average of three independent trials.

Table 3.4 The pH optima for PAL enzymes lies within the range of 8 to 9. The TAL enzymes *Rhodobacter capsulatus*, *Rhoodobacter sphaeroides* and *Bambusa oldhami* have a higher pH optimum when compared to other PAL enzymes.

Organism	**PAL**	**TAL**	**Reference**
Trichosporon cutaneum	**8.2**	**8.2**	**Current work**
Rhodobacter capsulatus	9.4	8.5	Kyndt et al. 2002
Rhodobacter sphaeroides	9	8.5 - 9	Watts et al. 2006
Zea mays	8 - 8.5	8 - 8.5	Rosler et al. 1997
Bambusa oldhami	9	-	Hsieh et al. 2010
Nostoc punctiforme	8.5	-	Moffitt et al. 2007
Anabaena variabilis	8.5	-	Moffitt et al. 2007
Streptomyces maritimus	8	-	Xiang & Moore 2005
Arabidopsis thaliana	8.4 - 8.8	-	Cochrane et al. 2004

A temperature optimum of 32 °C was observed for the *Trichosporon cutaneum* PAL enzyme with both substrates (Figure 3.4). This is low in comparison to other reported enzymes with dual substrate activity. PAL from plants, *Zea mays* (Rösler et al. 1997) and *Bambusa oldhamii* (Hsieh et al. 2010) have temperature optima in the range of 55 °C to 60 °C, and 50 °C respectively. The microbial TAL, *Rhodobacter sphaeroides* did not show any improvement in enzyme activity when assay temperatures were varied between 25 °C and 55 °C (Xue et al. 2007b). A novel thermostable TAL enzyme, able to retain full activity for up to 3 hours at 60 °C, was identified in the wood rotting fungus, *Phanerochaete chrysoporium*. This enzyme shows maximal activity at 55 °C to 60 °C (Xue et al. 2007a).

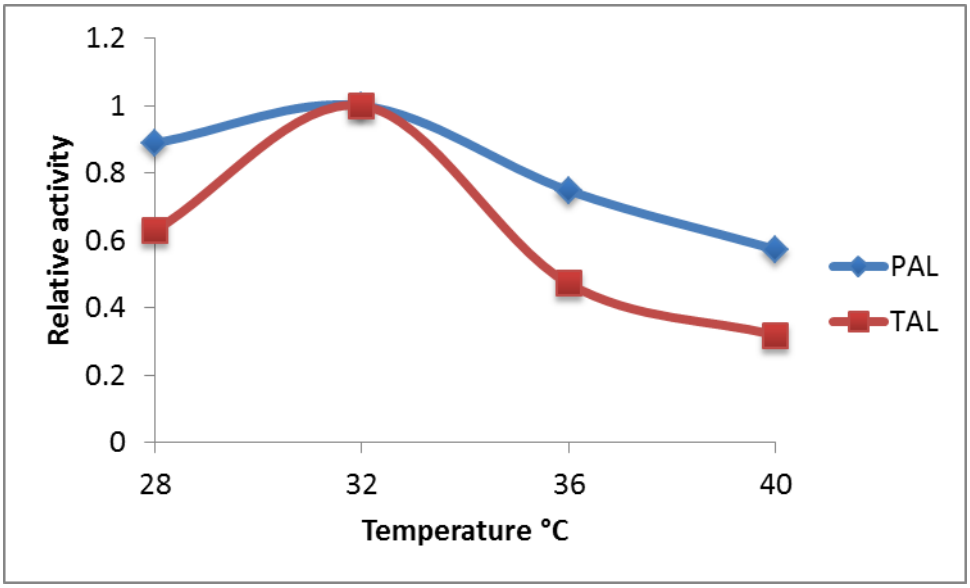


Figure 3.4 Temperature activity studies were performed at a pH of 8.2 with the buffer Tris HCl (10 mM) on the PAL and TAL activities in the yeast *Trichosporon cutaneum.* The reported values are from one set of trials.

3.3.4 Metal dependence studies

While PAL enzyme from some sources display metal dependence, others do not. The effect of metal cations on the PAL activity of *Trichosporon cutaneum* was therefore investigated. Ethylenediaminetetraacetic acid (EDTA), a chelating agent, was used in the extraction buffer to limit the possibility of rate enhancement of enzyme activity by metal cations that may inherently be present in buffer solutions. Relative to controls, no rate enhancement was observed in the presence of divalent metals, suggesting that the enzyme does not require any metal ion cofactors. There was a decline in enzyme activity in the presence of potassium chloride (35%) and iron chloride (39%) for the substrate phenylalanine. Less of an effect was seen with tyrosine, with enzyme activity remaining high (Table 3.5). The cause of this discrepancy in the data is uncertain. It would be expected that there would be the same level of decline in enzyme activity with both substrates.

Similarly, there are no metal cofactor requirements for the rust *Ustilago maydis* (Kim et al. 1996) or the bacterium *Rhodobacter sphaeroides* (Watts et al. 2006). In *Ustilago maydis*, PAL activity was inhibited by the heavy metal ions, silver, copper and mercury (Kim et al. 1996). Treatment with the chelating agent, ethylenediaminetetraacetic acid was not inhibitory. A copper chelator, 8-hydroxyquinoline, however, produced modest reduction in enzyme activity. Some enzyme inhibition was reversible. Mercury inhibition was partially reversed by treatment of the enzyme with β mercaptoethanol. Treatment of the enzyme with 8-hydroxyquinoline was however unable to reverse the inhibitory effects of copper cations (Kim et al. 1996). Enzyme activation in the presence

of metal cations has been reported in *Cucurbita* (El-Shora 2002). The enzyme was activated by magnesium and manganese cations, with magnesium producing the better results. A decline in enzyme activity was seen in the presence of calcium (El-Shora 2002).

Table 3.5 Study on the effect of cations on PAL activity in *Trichosporon cutaneum.*

Metal salt (0.1 M)	**Enzyme activity with phenylalanine (µmol/hr g fw)[a]**	**%[b]**	**Enzyme activity with tyrosine (µmol/hr g fw)[a]**	**%[b]**
Control	1.64 ± 0.25	-	0.64 ± 0.24	-
NaCl	1.24 ± 0.26	76	0.54 ± 0.17	84
KCl	0.58 ± 0.24	35	0.47 ± 0.11	73
$MgCl_2$	1.16 ± 0.43	71	0.54 ± 0.28	84
$FeCl_2$	0.64 ± 0.15	39	0.68 ± 0.10	106

[a]Units: micromole per hour gram fresh weight of cell mass
[b] Percentage of control
The reported values are the average of three independent experiments ± std dev
It is uncertain why there is a relatively high standard deviation in some of the data reported.

3.3.5 Enzyme kinetics

Enzyme catalysed reactions become saturated: Their rate of catalysis does not show a linear response to increasing substrate concentrations. As substrate concentrations increase, the enzyme becomes saturated, with the substrate and the rate of the reaction achieving a maximum velocity (V_{max}). V_{max} depends on the concentration of enzyme present within the reaction mixture (Briggs & Haldane 1925). The substrate concentration that produces an initial velocity that is one half of V_{max} is referred to as the Michaelis Menten constant (K_m), which is named after the scientists that developed the study of enzyme kinetics (Michaelis & Menten 1913). K_m provides a measure of the substrate's affinity or strength of binding of the enzyme with the substrate. A lower K_m is indicative of a higher substrate affinity (Nelson & Cox 2005).

One of the assumptions of Michaelis-Menten kinetics is an absence of cooperativity. In an enzyme that is multimeric, the assumption is made that the binding of a substrate to one binding site, does not have an effect on the activity of neighbouring sites. This may not necessarily be true. If binding of the substrate molecule to the first site increases the binding of the substrate to successive sites, this is referred to as positive cooperativity. If binding to the first site decreases binding of the substrate to successive sites, this is referred to as negative cooperativity. A Hill coefficient greater than one indicates positive cooperativity, less than one, negative cooperativity and equal to one, no cooperativity. The Hill coefficient is determined from the Hill equation:

$$\theta = \frac{[L]^n}{K_d + [L]^n} = \frac{[L]^n}{(K_a)^n + [L]^n}$$

Where:

θ is the fraction of occupied sites where the ligand can bind to the active site

[*L*] is unbound ligand concentration

K_d is the apparent dissociation constant

K_A is the ligand concentration occupying half of the binding sites

n is the Hill coefficient, describing cooperativity

An alternative formulation of the Hill equation is:

$$\log\left(\frac{\theta}{1-\theta}\right) = n \log [L] - \log K_d$$

PAL preparations from different sources exhibit considerable variations in their kinetic behaviour. Purified preparations from potato tubers (Havir & Hanson 1968), maize shoots (Marsh et al. 1968), wheat seedlings (Nari et al. 1974) and *Rhizoctonia solani* (Kalghatgi & Subba Rao 1975), show significant deviations from Michaelis-Menten kinetics, whereas those obtained from sweet potato roots (Minamikawa & Uritani 1965), green bamboo (Hsieh et al. 2010), rice (Sarma & Sharma 1999), *Ustilago hordei* (Subba Rao et al. 1967), *Rhodotorula glutinis* (Hodgins 1971), *Sporobolomyces pararoseus* (Parkhurst & Hodgins 1972) and *Streptomyces verticillatus* (Emes & Vining 1970) obey the classical Michaelis-Menten kinetics.

PAL from *Trichosporon cutaneum* is active with L-tyrosine and L-phenylalanine. Crude extracts of the enzyme displayed typical Michaelis-Menten kinetics with L-phenyl-alanine ($K_{m,}$ 7.9 ± 1.3 mM, Figure 3.5) but atypical Michaelis Menten kinetics with L-

tyrosine (K', 10.6 ± 1.7 mM, Hill coefficient, 2.6 ± 0.2, Figure 3.6). When compared to that of the crude extracts, the purified enzyme (Section 3.3.7) showed similar kinetic properties but had a significantly lower value for K' (K_m, 5.0 ± 0.7 mM for phenylalanine and K', 1.7 ± 0.8 mM, Hill coefficient, 1.8 ± 0.2, for tyrosine). The enzyme had a higher affinity for tyrosine compared to phenylalanine after purification. It is plausible that the hydroxyl group of tyrosine is able to bind more strongly to the active site of the enzyme due to its ability to hydrogen bond. In the chorismate mutase enzyme of *Saccharo-myces cerevisiae*, the allostery effect of tyrosine due to the presence of a hydroxyl group facilitating strong binding is well documented (Helmstaedt et al. 2001). Tyrosine enhanced cooperativity there (Helmstaedt et al. 2001). Attempts to visualize the binding of tyrosine to the active site of the TAL enzyme in *Rhodobacter sphaeroides* via crystallography studies were unsuccessful due to high concentrations of ammonium ion in the crystallization medium (Louie et al. 2006). Vanelli et al. (2007a) also reported typical Michaelis Menten kinetics (K_m, 4.9 ± 0.9 mM) with phenylalanine, and atypical Michaelis Menten kinetics (K', 0.6 mM, Hill coefficient, 2.6 ± 0.4) with tyrosine, for the purified enzyme. As can be seen from the data, the kinetic parameters from the present study for the purified enzyme (Phe, K_m 5.0 ± 0.7 mM; Tyr, K' 1.7 ± 0.8 mM, Hill coefficient, 1.8 ± 0.2) are comparable to that reported by Vanelli et al. (2007a). The kinetics for PAL from crude extracts (Phe, K_m 2.3 mM; Tyr, K' 0.43 mM) reported by Vanelli et al. (2007a) were however lower than the results from the present study.

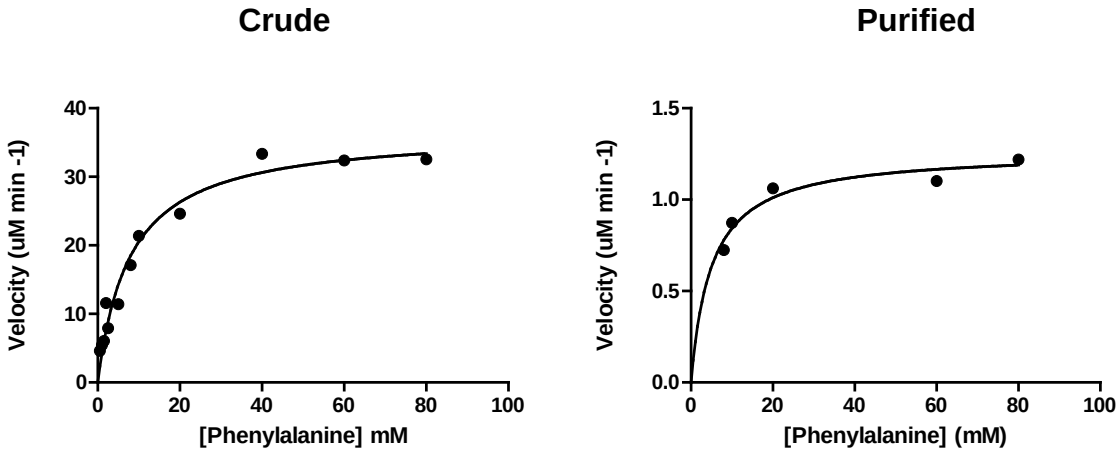


Figure 3.5 Enzyme kinetics with phenylalanine as substrate.
The enzyme displayed typical Michaelis Menten kinetics.
Crude enzyme: K_m 7.9 mM ± 1.3; V_{max} 36.7 mM min $^{-1}$ ± 1.9.
Purified enzyme: K_m 5.0 mM ± 1.1; V_{max} 1.3 mM min $^{-1}$ ± 0.06.

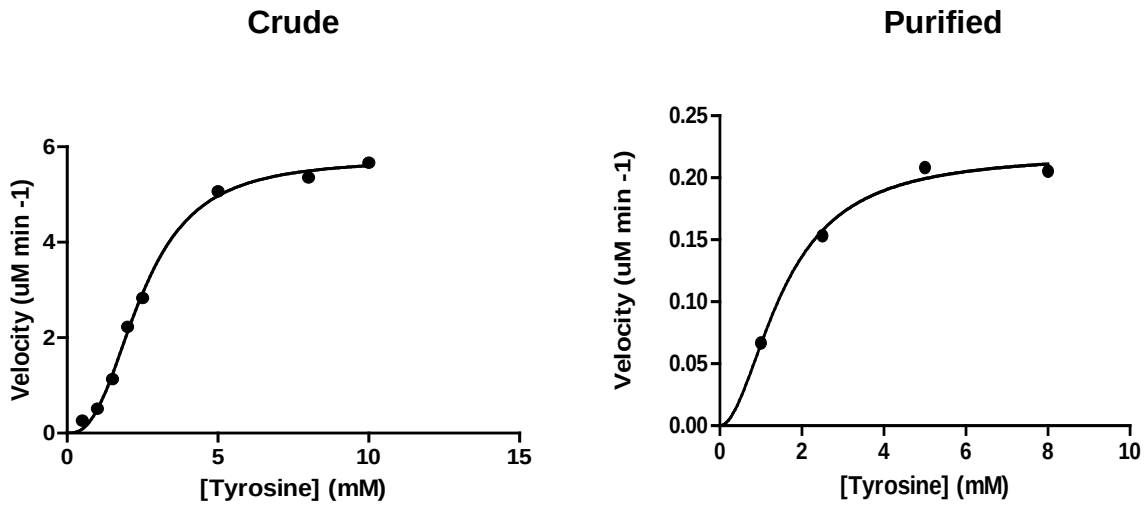


Figure 3.6 Enzyme kinetic data with tyrosine as substrate. The crude and purified enzyme displayed allosteric interaction as indicated by the sigmoidal curve. Crude enzyme: K', 10.6 mM ± 1.7, V_{max}, 5.8 mM min^{-1} ± 0.1, H, 2.6 ± 0.2. Purified enzyme: K', 2.4 mM ± 0.6, V_{max}, 0.2 mM min^{-1} ± 0.0, H, 1.9 ± 0.5.

Variations in kinetic constants before and after purification have in some cases been attributed to the enzyme existing as a protein complex formed by different PALs. This was observed in the enzyme from *Bambusa oldhami* (Hsieh et al. 2010). Fluctuations observed in the kinetic constants of PAL enzymes have been explained based on association/dissociation of PAL subunits (Jorrin et al. 1990), the occurrence of PAL isoforms with differing substrate affinity or the presence of two substrate binding sites differing in their substrate affinity and interaction with each other (Iredale & Smith 1973). It has also been suggested that during purification or storage, modifications of structure, which could be caused by proteolytic enzymes as well as inactivation or modification of one or more active sites, could lead to variations in kinetic behaviour (Marsh et al. 1968).

Only one species of PAL was apparent in *Trichosporon cutaneum*, which suggests that the observed variations may possibly be due to subunit interactions or the presence of different substrate binding sites. Ideally the pure enzyme is anticipated to have a higher affinity for phenylalanine and tyrosine with the removal of other components, which could impede the ability of the substrates to effectively bind to the active site. A decrease in the K_m has also been reported for other enzymes after purification. This was seen for example in extracellular lipase from *Pseudomonas aeruginosa* (Borkar et al. 2009) and alcohol oxidase in *Candida boidinii* (Anděrová et al. 1993). The decrease in K_m for alcohol oxidase was attributed to dissociation of compounds, for example methanol-oxidation products, from the enzyme molecule (Anděrová et al. 1993).

The enzyme specificity was determined from the ratio V_{max}/K_m (Table 3.6). For crude extracts, the enzyme specificity for phenylalanine and tyrosine were 3.3 ± 1.7 (mM min^{-1} and 0.4 ± 0.1 mM min^{-1}, respectively. In the purified enzyme values of 0.9 ± 0.7 mM min^{-1} and 0.5 ± 0.4 mM min^{-1}, were obtained for phenylalanine and tyrosine, respectively. When compared to the crude enzyme (7.8 ± 2.4), there was a decrease in the PAL/TAL ratio for the purified enzyme (2 ± 0.6) demonstrating a decline in the enzyme specificity for phenylalanine. It is possible that after purification there is a modification in enzyme structure, which affects the enzyme's ability to bind with phenylalanine. Vanelli et al. (2007a) reported a PAL/TAL ratio of 0.8, which would suggest that the enzyme has a slightly higher preference for tyrosine versus phenylalanine. From the present study a higher enzyme specificity was observed for phenylalanine in both the crude and purified enzyme, which implies that the enzyme is a PAL/TAL and not a TAL enzyme. V_{max} was also higher for phenylalanine compared to tyrosine in both crude (34.5 ± 6 μM min^{-1}; 4.1 ± 1.6 μM min^{-1}, respectively) and purified extracts (4.4 ± 3 μM min^{-1}; 0.7 ± 0.4 μM min^{-1}, respectively) of the enzyme. The V_{max} ratio of PAL and TAL activity in crude and purified extracts of the enzyme were 9.1 ± 3.4 and 6.7 ± 2.2 respectively.

Table 3.6 Kinetic parameters for crude and purified extracts of the PAL enzyme from *Trichosporon cutaneum.* The enzyme efficiency was calculated as the ratio V_{max}/K_m.

Phenylalanine V_{max} (μM min^{-1})	Phenylalanine K_m (mM)	Phenylalanine V_{max}/K_m	Tyrosine V_{max} (μM min^{-1})	Tyrosine K' (mM)	Tyrosine V_{max}/K'	PAL/TAL Ratio
Crude Enzyme						
40.8 ± 3.8	7.8 ± 2.4	5.2	5.8 ± 0.1	10.6 ± 1.7	0.5	9.7
28.8 ± 1.4	12.6 ± 1.8	2.3	3.9 ± 0.1	8.6 ± 1.6	0.5	5.1
33.8 ± 2.1	14.6 ± 2.6	2.3	2.6 ± 0.1	9.8 ± 1.7	0.3	8.6
Purified Enzyme						
1.3 ± 0.1	5.0 ± 1.1	0.3	0.2 ± 0.02	2.4 ± 0.6	0.1	2.7
4.7 ± 0.6	5.7 ± 3.0	0.8	1.0 ± 0.1	2.0 ± 0.4	0.5	1.5
7.2 ± 0.8	4.2 ± 2.0	1.7	0.8 ± 0.1	0.9 ± 0.4	0.9	1.9

For each set of reported values, both substrate enzyme activity assays were performed on the same extract. The total activity (U) of the crude extract was 0.05 ± 0.01 while that of the pure enzyme was 0.01 ± 0.00. Yield of the enzyme after purification was 20%.

Allosteric interactions are important in the regulation of enzyme activity (Hammes & Wu 1974). Control of protein function is achieved by protein conformational change, which is induced by effector molecules (Helmstaedt et al. 2001). Effector molecules bind to regulatory sites, which are distinct from the active site. Allosteric behaviour is most often observed in the regulation and control of enzymes of metabolic pathways forming the basis of feedback inhibition and activation (Helmstaedt et al. 2001). The exact mechanism by which allosteric control of proteins is achieved varies. Downstream products or upstream substrates participate in regulating enzyme catalysis. Aromatic amino acids appear to play an essential role in the regulatory mechanism of microbes. In the enzyme chorismate mutase, tyrosine and phenylalanine act as negative effectors and tryptophan as a positive heterotropic ligand (Kradolfer et al. 1977). In plants, different isoenzymes are typically present, which differ in their regulatory behaviour (Helmstaedt et al. 2001).

Allosteric interaction is a physical mechanism, which may result in a cooperative effect. Cooperativity is defined as an observed deviation from Michaelis-Menten or classical binding curves. The observed positive cooperativity with tyrosine may be due to interactions of the *para*-hydroxyphenyl group with the four active sites present in the enzyme. Positive cooperativity has also been observed in the PAL enzyme from *Glycine max* (Havir 1981). Crude extracts of the enzyme displayed negative cooperativity (H, 0.61), which changed to positive cooperativity (H, 1.2) after purification (Havir 1981).

Positive allostery allows enzymes more sensitivity to changes in the substrate concentration of their environment. Large changes in enzyme activity can occur in the presence of a narrow change in substrate concentration. While positive cooperativity is a rare occurrence in PAL, the enzymes from several plant and fungal sources have shown negative cooperativity with regards to substrate binding (Table 3.7). The PAL enzyme from potatoes provided one of the first examples of subunit cooperativity in which the binding of a first substrate molecule reduced the affinity for a second molecule (Havir & Hanson 1968). This negative cooperativity (Koshland et al. 1966), minimizes the influence of fluctuations in the phenylalanine pool and may serve to maintain relatively high reaction rates even at low substrate concentrations (Hanson & Havir 1981). Negative cooperativity appears to be common in PALs with kinetic constants falling within the micromolar range. In some cases the variability of kinetic properties has been attributed to differences in the stability of different isoforms of the enzyme that may be present (Havir & Hanson 1968), or the presence of a mixture of isoforms that differ in their affinity for the substrate. Negative cooperativity is sometimes lost during purification or after enzyme storage (Havir 1981). Cooperative interaction shown by PAL is thought to play an important role in the short-term regulation of phenylpropanoid metabolism.

Table 3.7 PAL enzymes displaying negative cooperativity. The reported apparent kinetics constant are at low and high substrate concentrations respectively with Hill coefficients less than 1.

Enzyme Source	K□ (μM)	H[a]	References
Rhizoctonia solani	180 and 5000	0.50	Kalghatgi & Subba Rao 1975
Citrus sinensis	13 and 52	0.75	Dubery & Schabort 1988
Gossypium hirsutum	10 and 75	0.87	Dubery & Smith 1994
Glycine max	12 and 61	0.61	Havir 1981
Raphanus sativus	15 and 95	0.48	Fourcroy 1980
Cucumis sativus	43 and 290	0.65	Iredale & Smith 1973
Petroselinum hortense	32 and 240	0.60	Zimmermann & Hahlbrock 1974
Fagopyrum esculentum	42 and 220	NR[b]	Belunis & Hrazdina 1988
Medicago sativa	30 and 85	NR[b]	Jorrin and Dixon 1990
Phaseolus vulgaris	44 and 241	NR[b]	Bowell et al. 1985
Ipomeoa batatas	20 and 290	NR[b]	Haard & Wasserman 1976
Solanum tuberosum	40 and 260	NR[b]	Hanson & Havir 1972
Lycopersicon esculentum	60 and 1120	NR[b]	Sarma et al. 1998

[a]Hill Coefficient
[b]Not reported

3.3.6 Purification of *Trichosporon cutaneum* PAL by acid precipitation

Purification of PAL by acid precipitation was also investigated. Acetic acid (5%) was added to crude extracts of the enzyme to precipitate contaminating carotenoids (as evident from the presence of a yellow suspension) and nucleic acids. A purification fold of 1.3 was obtained with 94% yield. In the yeast *Sporidiobolus pararoseus*, acid precipitation produced higher purification folds of 5.6 (Monge et al. 1995). Differences in the level of purification obtained between the two sources of PAL are uncertain. The *Trichosporon cutaneum* PAL enzyme was able to withstand changes in pH from 8 to 6.5, as seen in the retention and recovery of enzyme activity (Table 3.8).

3.3.7 Purification by anion exchange chromatography

Anion exchange chromatography has been used extensively for the purification of PAL. It is based on the separation of ions and polar molecules based on their charge. PAL is negatively charged at a pH of 8 and binds to the column. Undesired proteins are eluted first. Bound PAL is then eluted by increasing the ion concentration of the elution buffer. *Trichosporon cutaneum* PAL was purified using a HiTrap Q Sepharose column, which has long chains of dextran coupled to a 6% highly cross-linked agarose matrix. The ion exchange group present is a quaternary amine group ($CH_2N(CH_3)_3$) which is a strong ion exchanger. Dextran chains allow for increased exposure of the charged group, resulting in increased loading capacity of the column. The particle size of the beads (34 µm) allows for fast adsorption and desorption. A wide range of purity and yields has been obtained from anion exchange chromatography, as seen in Tables 3.1 and 3.2.

The stabilization of enzymes with glycerol is well documented. Different mechanisms have been proposed with regards to the stabilizing effect of glycerol. These include a glycerol induced conformational change leading to increased stability of the enzyme (Bradbury & Jakoby 1972), formation of a water glycerol structure around the protein (Ruwart & Suelter 1971) and a decrease in the hydrogen bond rupturing capacity of the medium (Gerlsma & Sturr 1972). This suggests that a dominant factor in protein stabilization may be due to enhancement of the structure of the medium or of the solvation layer of the protein (Gekko & Timasheff 1981). Denaturation increases the surface contact between the protein and solvent and also exposes hydrophobic residues to solvent contact. Glycerol however favors the more native form of the protein (Gekko & Timasheff 1981). The use of glycerol for PAL stabilization during purification was therefore investigated. Glycerol (20%) was introduced into the elution buffers. There was however no apparent purification of the enzyme as determined from comparing the specific activity of the crude extract with that of the fractions obtained.

In the absence of glycerol, PAL eluted as a broad peak with 20% yield and 50-fold purification (Figure 3.7). The SDS PAGE of the purified protein revealed the presence of the desired band at 80 kDa (Figure 3.8). Two additional major bands were observed, one at 94 kDa and the other at 37 kDa, which may be impurities co-eluting with the desired protein. Several other minor bands were also apparent. A more concentrated sample of the purified fraction (Figure 3.9) that was stored at –80 °C for 5 months showed evidence of PAL degradation. The original band observed at 80 kDa appeared to have degraded to produce two bands occurring at 74 kDa and 64 kDa. Vanelli et al.

(2007a) reported degradation bands at 62 kDa, 56 kDa, 24 kDa and 14 kDa for the PAL enzyme from *Trichosporon cutaneum*. This is a two-step procedure in comparison to the four-step procedure reported by Vanelli et al. (2007a) for the same enzyme. A crude extract of the enzyme is first prepared and then purified by anion exchange chromatography. In the methodology used by Vanelli et al. (2007a) anion exchange chromatography, hydrophobic interaction chromatography and gel filtration chromatography were utilized. An overall yield of 6% was obtained from the purification. Anion chromatography produced a purification fold of ~4. Further purification with hydrophobic interaction chromatography and gel filtration produced purification folds of ~14. In the protocol used by Vanelli at al. (2007a) a HQ (quaternized polyethyleneimine) column was utilized for anion chromatography. Better yields and purification folds were obtained by the use of a Sepharose Q anion exchange column in the present study. Sepharose columns have been reported as having a very high binding capacity with the dextran coating increasing binding strength (Staby & Jensen 2001).

A reduction in the number of steps for purification is advantageous, as in a number of species, the native PAL subunit is inherently unstable *in vitro*, and may degrade during purification (Sarma et al. 1998). Vanelli et al. (2007a) reported degradation of the enzyme and loss of activity during purification. A purification fold of 14 was reported with the S_A of the crude extract being 0.02 (U/mg) compared to a S_A of 0.33 U/mg in the purified fraction. The total units of activity recovered in the purified fraction was 0.3 compared to 5.1 in the crude extract, a 94% loss. A few minor bands were observed at

62, 56, 23.5 and 14 kDa after purification which were identified as degradation products from NH_2-terminal sequencing (Vanelli et al. 2007a). The purification protocol in the current study presents a simple and efficient method to purify PAL through the use of anion exchange chromatography.

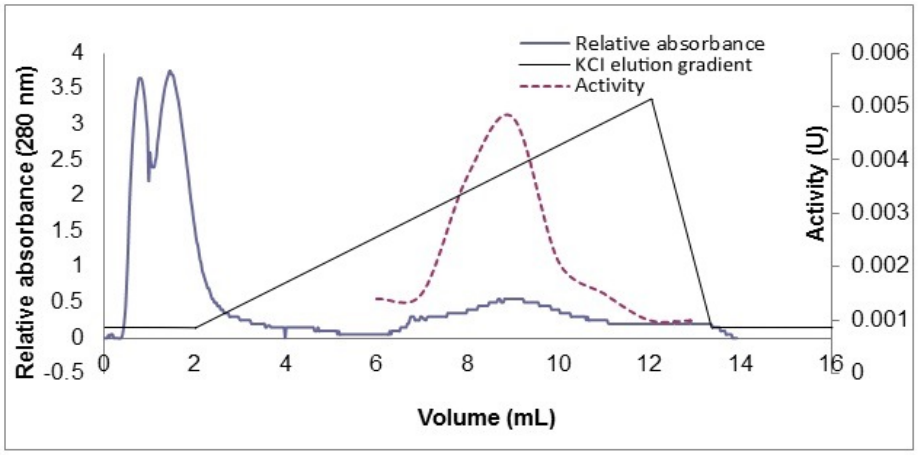


Figure 3.7 Chromatographic profile illustrating the purification of the PAL enzyme from *Trichosporon cutaneum* by anion exchange chromatography.

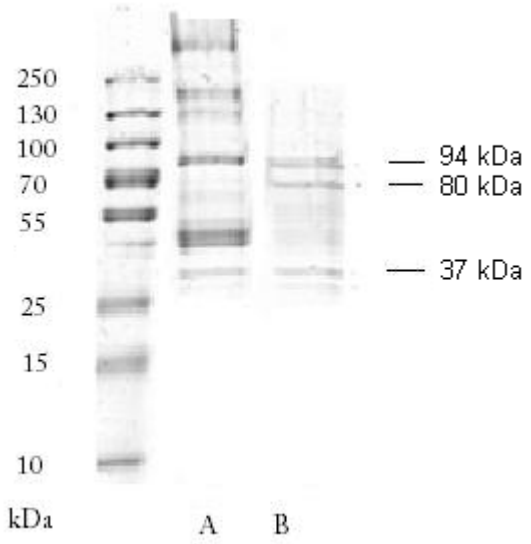


Figure 3.8 SDS PAGE illustrating the purification of the PAL enzyme from *Trichosporon cutaneum* by anion exchange chromatography. Lane A is the crude extract. Lane B is the purified protein. *Trichosporon cutaneum* PAL was observed at 79 kDa. Lanes were loaded with 25 µg protein. The protein gel was stained with Coomassie blue.

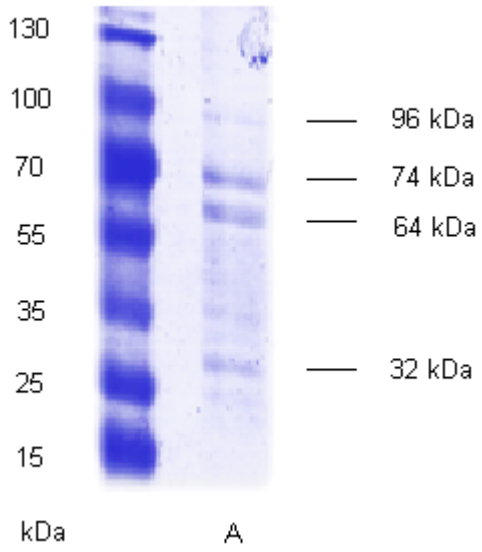


Figure 3.9 SDS PAGE of purified PAL from *Trichosporon cutaneum* after storage at -80 °C for 5 months. The PAL band observed at 80 kDa in Figure 3.8 appears to have degraded to produce the 74 kDa and 64 kDa band. Lane A was loaded with 35 µg protein. The protein gel was stained with Coomassie blue.

3.3.8 Purification by an aqueous two-phase system

Minimum purification was achieved with the aqueous two-phase system. Of the four systems investigated, highest purification fold (1.5) was obtained with the polyethylene glycol 1000/sodium sulphate system. PAL preferentially migrated to the top phase. There was no enzyme activity in the bottom layer. Carotenoids migrated to the interphase of the system. Significant reduction in yield of the enzyme was observed when a second partitioning was performed with sodium carbonate. This decline in enzyme activity was also apparent in the polyethylene glycol 1000/sodium carbonate system, suggesting that sodium carbonate has an adverse effect on enzyme activity. The decline in enzyme activity may be due to denaturation of PAL. Ammonium sulphate has been reported to cause deactivation of PAL resulting in reduced yields (Yue et al. 2007). Aqueous two-phase partitioning has been used successfully in the purification of PAL from *Rhodotorula glutinis*. A 9-fold purification was reported for the PEG1000 (1.1 g, 11%)/sodium sulphate (1.4 g, 14%)/sodium carbonate (5.3 g, 5.3%) system (Yue et al. 2007) while in this case a 0.8 purification fold was obtained for *Trichosporon cutaneum* PAL. Table 3.8 summarizes the results from the three different methods of purification investigated for the PAL protein from *Trichosporon cutaneum*.

Table 3.8 [a]Purification of the PAL enzyme from *Trichosporon cutaneum* by acid precipitation, aqueous two phase partitioning and anion exchange chromatography.

Method		Total Activity [b](U)	Total Protein (mg)	Specific activity (U/mg)	Purification fold (P_f)	Actual Yield (%)
Acid precipitation						
	Crude	0.16 ± 0.03	1.04 ± 0.74	0.20 ± 0.10	-	100
	Post	0.15 ± 0.04	0.61 ± 0.28	0.26 ± 0.06	1.3	94
Anion exchange						
	Crude	0.05 ± 0.01	0.38 ± 0.09	0.15 ± 0.00	-	100
	Post	0.01 ± 0.00	0.0013 ± 0.00	7.39 ± 0.73	49.6	20
Aqueous two phase						
	Crude	0.07 ± 0.03	0.39 ± 0.14	0.21 ± 0.05	-	100
PEG/sodium sulphate		0.02 ± 0.00	0.08 ± 0.00	0.33 ± 0.03	1.5	28
PEG/ammonium sulphate		0.02 ± 0.00	0.10 ± 0.08	0.10 ± 0.04	0.48	28
PEG/sodium carbonate		0.01 ± 0.00	0.31 ± 0.04	0.02 ± 0.01	0.10	14

[a] Results are the average ± range of two independent trials.
[b] 1 U of activity = 1 µmol of released *trans* cinnamic acid per minute.

3.4 CONCLUSION

The PAL enzyme from *Trichosporon cutaneum* displays several characteristic features typical of other microbial enzymes, exhibiting a pH optimum in the range of 8.0 to 8.5 and a molecular subunit of 79 kDa. The enzyme requires no metal cofactors. While displaying typical Michaelis-Menten kinetics with phenylalanine, positive allosteric sigmoidal kinetics was observed with tyrosine. The latter is the main distinguishing feature of the enzyme in comparison to other microbial sources of PAL.

L-Phenylalanine appears to be the natural substrate of the enzyme under physiological conditions as determined from the V_{max}/K_m ratio, which measures enzyme efficiency and can be used to compare enzymes. It should be noted that the enzyme efficiency for phenylalanine decreased after enzyme purification but was still greater than the enzyme efficiency for tyrosine. The V_{max} for L-phenylalanine ranged from seven to thirteen times higher than that for L-tyrosine.

Purification of the PAL enzyme from *Trichosporon cutaneum* was accomplished by use of anion exchange chromatography. A 20% yield and 50-fold purification was achieved from the protocol. Acid precipitation and the aqueous two phase system were also investigated and may be utilized for preliminary purification of the enzyme. The PEG1000/sodium sulphate system gave the best results for the aqueous two-phase system but was not further investigated due to a low purification fold of 1.5.

The microbial production of aromatic chemicals from renewable sources has gained significant interest in recent years (Frost & Lievense 1994). The first example of the bacterial production of *para* hydroxycinnamic acid from glucose was seen in the expression of the PAL gene from the yeast *Rhodosporidium toruloides* in an *E. coli* strain overproducing phenylalanine (Watts et al. 2006). This strain produced both *trans* cinnamic acid and *para* hydroxycinnamic acid, ~380 µM and 150 µM, respectively (Watts et al. 2006). The PAL enzyme from *Trichosporon cutaneum* may also be considered for use as a biocatalyst in the production of *para* hydroxycinnamic acid. Stabilization of the enzyme by encapsulation may be necessary to improve the economic feasibility of the process (Trotman et al. 2007). Mutagenesis studies may also be utilized to increase the catalytic efficiency of PAL for tyrosine. *Para* - hydroxycinnamic acid and its derivatives are of interest commercially due to their use in the production of flavour components and food additives (Gosset 2009).

CHAPTER 4: SEQUENCING OF THE PAL GENE FROM *TRICHOSPORON CUTANEUM* AND IDENTIFICATION OF POTENTIAL MUTAGENESIS SITES FOR ENHANCING ENZYME ACTIVITY WITH TYROSINE

4.1 INTRODUCTION

Interest in *para* hydroxycinnamic acid and its derivatives stems from their applicability in a wide array of health, pharmaceutical, flavour and industrial products. Whereas *para* hydroxycinnamic acid may be produced via the conventional "PAL route", synthesis via tyrosine offers a shorter, economical and more feasible pathway in microbial hosts. In the "PAL route", phenylalanine is first deaminated producing *trans* cinnamic acid, which is then hydroxylated producing *para* hydroxycinnamic acid. Synthesis of *para* hydroxycinnamic acid from tyrosine eliminates the first step of the "PAL route". TALs with higher catalytic activity are more desirable for biotechnology applications. The PAL/TAL enzyme from *Rhodotorula glutinis* has been investigated for its commercial use in the biosynthesis of *para* hydroxycinnamic from tyrosine (Gatenby et al. 2002; Qi et al. 2007; Vanelli et al. 2007b; Trotman et al. 2007).

Other sources of the enzyme, for example, *Phanerochaete chrysosporium* have been investigated (Xue et al. 2007a). Desired features of this enzyme include enhanced stability and a higher catalytic efficiency for tyrosine compared to phenylalanine. The k_{cat} and K_m values for tyrosine were 1.3 s^{-1} and 44 μM respectively, with values of 3.3 s^{-1} and 161 μM for phenylalanine (Xue et al. 2007a). The catalytic efficiency (k_{cat}/K_m) for tyrosine (30 mM^{-1} s^{-1}) was slightly higher than that of phenylalanine (21 mM^{-1} s^{-1}), which indicated that the enzyme had a slight preference for tyrosine as observed from the PAL/TAL ratio of 0.7 (Xue et al. 2007a).

Genes that encode the PAL gene have been cloned and sequenced from both plant (Schulz et al.1989) and microbial sources (Oerum & Rasmussen 1992) in different bacterial vectors. Plant PALs that have been successfully cloned and expressed in *Escherichia coli* include the PAL enzyme from parsley (Appert et al. 2004) and four putative *Arabidopsis* PAL genes (Cochrane et al. 2004). The four putative PAL genes in *Arabidopsis* encode proteins that share 74% identity and 78% amino acid similarity (Cochrane et al. 2004). The recombinant proteins were expressed, purified to homogeneity, and characterized with regard to their kinetic and physical parameters. *Arabidopsis thaliana* PAL1, 2 and 4 had similar K_m values ranging from 64 - 71 μM while PAL2 had a lower K_m and higher k_{cat} for phenylalanine. Activity with tyrosine was low in PAL1, 2 and 4 but absent in PAL3 (Cochrane et al. 2004). Phylogenetic analysis of the four PAL genomic sequences revealed that there are two distinct groups, one for PAL1 and 2 and the other for PAL3 and 4. PAL1 and 2 share 90.4% amino acid identity whereas PAL3 and 4 have 83.5% identity. The intron number varies between the two groups with PAL1 and 2 possessing a single intron and PAL3 and 4 possessing two. This may indicate that there are differences in the post-transcriptional modification of isoforms (Cochrane et al. 2004).

Currently, not much literature is available on the cloning and expression of the TAL gene. Bacterial TALs, *Rhodobacter capsulatus* (Kyndt et al. 2002) and *Rhodobacter sphaeroides* (Xue et al. 2007b) have been successfully cloned and functionally expressed in *Escherichia coli*. Target proteins have been purified utilizing affinity chromatography with Ni^{2+}-NTA agarose. The TAL gene from *Rhodobacter sphaeroides*

has also been cloned in *Arabidopsis thaliana* with the intent of altering the *para* hydroxycinnamic pathway of the plant. By introduction of the TAL gene in *Arabidopsis*, a direct mechanism for the production of *para* hydroxycinnamic acid was produced, eliminating the need for hydroxylation of *trans* cinnamic acid via cinnamate 4-hydroxylase. Expression of *Rhodobacter sphaeroides* TAL in *Arabidopsis thaliana* resulted in enhanced accumulation of anthocyanins, quercetin glycosides, sinapoyl and *p*-coumaroyl derivatives, indicating that there was enhancement in phenylpropanoid production (Nishiyama et al. 2010).

Fungal TALs that have been successfully cloned include *Rhodotorula glutinis* and the thermostable TAL enzyme from the white rot fungus *Phanerochaete chrysosporium* (Xue et al. 2007a). An arabinose-inducible promoter was utilized for tightly controlled expression of the enzymes in *Escherichia coli*. Approximately 50% soluble protein expression was obtained for both enzymes (Xue et al. 2007a).

PAL enzyme isolated from plant sources, *Zea mays* (Rosler et al. 1997) and more recently *Bambusa oldhamii* (Hsieh et al. 2010), which are active with tyrosine, were successfully cloned. *Bambusa oldhamii* is a monocotyledonous plant belonging to the Poaceae family, which is common in East Asia. Four genes designated as BoPAL1 to BoPAL4, were cloned in a bacterial and yeast expression system (Hsieh et al. 2010). BoPAL3 and BoPAL4 had open reading frames of 2142 bp (77 kDa) and 2106 bp (76 kDa) respectively. One intron and two exons were found in BoPAL2 - 4 but no intron was found in BoPAL1. The lack of an intron in BoPAL1 was unexpected given the fact

that an intron region is typical of most of the PAL genes that have been characterized. *Arabidopsis thaliana* (Wanner et al. 1995), *Salvia miltiorrhiza* (Song & Wang 2009), *Oryza sativa* (Minami et al. 1989), *Coffea canephora* (Mahesh et al. 2006), all possess an intron. The absence of an intron has also been reported in the PAL gene from *Ginkgo biloba* (Xu et al. 2008).

The cloning of the PAL enzyme from *Trichosporon cutaneum* has been reported by Vanelli et al. (2007a). They reported high levels of protein expression with approximately 30% total soluble protein (Vanelli et al. 2007a). The original intent of the present experiments was to mutate the native gene at specific sites with the objective of producing a mutant gene with high levels of tyrosine ammonia lyase activity and to investigate the mechanism by which some PAL enzymes are able to utilize tyrosine as substrate. The gene was cloned, however analysis of the gene sequence revealed the presence of an intron. Protein over-expression experiments could therefore not be conducted. Five different amino acid residues not previously reported, namely Glu74, Ala274, Val298, Pro322 and Lys486, were identified in the sequence of the gene.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

DNA restriction enzymes and T4 DNA ligase were purchased from Fermentas. Phusion Polymerase was purchased from New England Bio Labs (NEB, Beverly, MA). DNA and PCR purification kits were purchased from Qiagen. The plasmid extraction kit and DNA agarose gel cleanup kits were purchased from Invitrogen and Qiagen. The primers

were chemically synthesized by Operon Biotechnologies, Inc. Millipore water was used in all experiments. Reagents were prepared according to the manual “Molecular cloning: A laboratory manual” (Sambrook et al. 1989).

4.2.2 Bacterial strains and plasmids

Trichosporon cutaneum (ATCC 58094) was purchased from American Tissue Culture Collection (Manassas, VA). *Trichosporon cutaneum* (ATCC 46446) also has PAL activity but was not investigated as it was reported as having a lower PAL/TAL ratio than strain ATCC 58094 (Vanelli et al. 2007a). The *Escherichia coli* DH5α and BL21(DE3) strains were used for all cloning and DNA manipulations. The pET-30a (+) vector was obtained from Novagen (Madison, WI). Recombinant plasmids were amplified in *Escherichia coli* DH5α cells. All other reagents were purchased from Sigma-Aldrich.

4.2.3 Cell growth, induction and preparation of cell free extracts of the yeast

Yeast cultures, cell extraction and enzyme assays were prepared as outlined in Chapter 3 sections 3.3.1, 3.3.2 and 3.3.3.

4.2.4 Bacterial media and growth condition

Escherichia coli bacterial strains were routinely cultured in Luvia Bertani (LB, Mediatech, Herndon, VA), (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g sodium chloride per litre) liquid medium. For solid media, 2% agar was added to LB liquid medium before autoclaving. Kanamycin (50 µg/mL) was added to LB media and plates

when growing bacteria containing plasmids. Transformants were cultured in LB medium (50 mL) supplemented with kanamycin (50 µg/mL) at 37 °C. Cells were harvested and washed with cold Tris-HCl (50 mM, pH 8.0).

4.2.5 Preparation of competent *Escherichia coli*

Colonies of *Escherichia coli* DH5α were inoculated into Super Optimal Broth (SOB), (20 g casein enzymic hydrolysate, 2.4 g magnesium sulphate, 5 g yeast extract, 0.186 g potassium chloride, 0.5 g sodium chloride per litre, pH 7), (250 mL) in a 1L flask. Cells were cultured at 19 °C with vigorous shaking (100 rpm) to an OD 600 = 0.5 (24-36 hours). The flask was then placed in ice for 10 minutes and the cells pelleted by spinning at 2700 *x g* for 10 minutes at 4 °C. Cells were gently resuspended in ice cold Terrific Broth (TB), (12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g monopotassium phosphate, 12.54 g dipotassium phosphate) and stored on ice for 10 minutes. Thereafter the cells were again pelleted by spinning at 2700 *x g* for 10 minutes at 4 °C. The resulting pellet was resuspended in ice cold Terrific Broth (20 mL) and ice cold dimethyl sulphoxide (1.4 mL). Aliquots of competent cells (200 µL each) were distributed in sterile microcentrifuge tubes for transformation, with the remainder stored at -80 °C (Inoue et al. 1990).

4.2.6 Preparation of genomic DNA from *Trichosporon cutaneum*

Trichosporon cutaneum cells were grown in the minimal medium (250 mL) containing tyrosine (2.0 mM) at 30 °C to an OD600 of 3. Cells were pelleted by centrifugation (9000 *x g*, 15 minutes, 4 °C). Genomic DNA was extracted utilizing a Qiagen DNA

purification kit and utilized as template for PCR amplification. The concentration and purity of the extracted DNA was determined from the A260/A280 ratio using a nanodrop spectrophotometer (1 OD 260 nm = 50 µg/mL double stranded DNA).

4.2.7 Polymerase chain reaction

Primer sequences were designed based on the DNA sequence of the *Trichosporon cutaneum* PAL (TcPAL) gene being cloned. This information was obtained from the protein sequence of TcPAL reported by Breinig et al. (2005). EcoRI and HindIII restriction sites were introduced into the PAL gene, allowing it to be cloned in frame with the pET30a vector. The PAL gene was cloned under the control of a T7 lac promoter in the prokaryotic pET30a vector in the *Escherichia coli* BL21(DE3) strain. The PAL gene was PCR amplified using Phusion DNA polymerase (New England Biolabs) from *Trichosporon cutaneum* genomic DNA with the 5′ primer TcPAL- F: 5'-CGCGAATTCATGTTTATTGAGACC-3' as the forward primer (containing a EcoRI site underlined) and 3′ primer TcPAL- R: 5'-GAAGCTTTTAGAACATCTTGCCAAC-3' as the reverse primer (containing a HindIII site underlined). The reaction mixture contained the two primers at a final concentration of 0.5 µM each, 100 ng of template DNA, High Fidelity reaction buffer (10 µL), 200 µM dNTPs and 1.0 unit of Phusion polymerase (50 µL). The PCR program was set as follows: 98 °C, 2.0 min; followed by 35 cycles of 98 °C, 30 s; 58 °C, 30 s; and 72 °C 1.0 min, followed by a final extension of 6.0 min at 72 °C in sterile microcentrifuge tubes (0.5 mL). Control reactions were performed in the absence of DNA template. An aliquot (5 µL) of the reaction mixture was taken from the

PCR reaction mixture and loaded onto a 1% agarose gel to verify the PCR reaction product.

4.2.8 Ligation reaction

The amplified PCR fragment (2114 bp) was purified using a Qiagen PCR purification kit, digested with HindIII and EcoRI (Fermentas), and inserted between the HindIII and EcORI sites of the pET30a vector which was predigested with HindIII and EcoRI (Fermentas) to give plasmid pET-30a (+) TcPAL. The ligation mixture was used to transform *Escherichia coli* DH5α competent cells. A control reaction was also performed in the absence of the amplified target DNA.

4.2.9 Introduction of DNA into bacterial cells by heat shock

Freshly prepared *Escherichia coli* DH5α competent cells were transformed with the plasmid construct pET-30a (+) TcPAL by use of heat shock treatment. The ligation mixture (2 µL) was added to the cells (50 µL) and incubated on ice for 30 minutes. Cells were then heat shocked for 20 s at 37 ºC and chilled on ice again. LB broth (0.95 µL) was added to the cells and incubated for 1 hour at 37 ºC on a shaker at 100 rpm. Cells were centrifuged and resuspended in LB broth and streaked on LB plates containing kanamycin (50 µg/mL) and incubated overnight at 37 ºC. Positive clones were identified by restriction analysis following plasmid isolation. Agarose gel electrophoresis was performed to confirm the presence of the constructed target gene. Electrophoresis was performed using either 1 or 1.5% (w/v) agarose gels in Tris-borate EDTA (TBE) buffer along with a standard DNA marker. All bands were visualized under

UV light after staining with ethidium bromide and were visualized on a UV transilluminator (254 nm). Photographic records were taken. Clones were submitted to the UBC Nucleic Acid Protein Service (NAPS) Unit, Michael Smith Laboratories, for DNA sequencing.

4.2.10 DNA sequencing and computer-assisted sequence analysis

Database comparison was performed with the BLAST search tools on the server of the National Center for Biotechnology Information, National Library of Medicine, NIH (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were performed using ClustalW. Intron analysis was performed using GENSCAN.

4.3 RESULTS AND DISCUSSION

The PAL/TAL enzyme from *Trichosporon cutanuem* was identified as a source of TAL activity and was selected for cloning and potential mutagenesis studies. The yeast possesses significant TAL activity in comparison to other sources of the enzyme investigated. Currently there is only one publication by Vanelli et al. (2007a) and a patent (Breinig et al. 2005) regarding the TAL activity of this enzyme. Significant work has already been conducted on the enzyme from *Rhodotorula glutinis* (Abell & Shen, 1987; Gatenby et al. 2002; Xue et al. 2007a; Trotman et al. 2007) and *Rhodobacter sphaeroides* (Xue et al. 2007b). *Rhodobacter capsulatus* TAL was also considered but when investigated, no TAL activity was detected (Kyndt et al. 2002). *Trichosporon cutaneum* PAL was therefore selected for further investigation of the TAL activity of this family of enzymes.

4.3.1 Sequencing of the PAL gene

An open reading frame of the PAL gene from *Trichosporon cutaneum* was amplified from genomic DNA using PCR. A 2114 bp fragment size was generated (Figure 4.1) and cloned into the pET30a vector. Five positive clones were identified using restriction analysis (Figure 4.2). Clones were submitted for DNA sequence analysis.

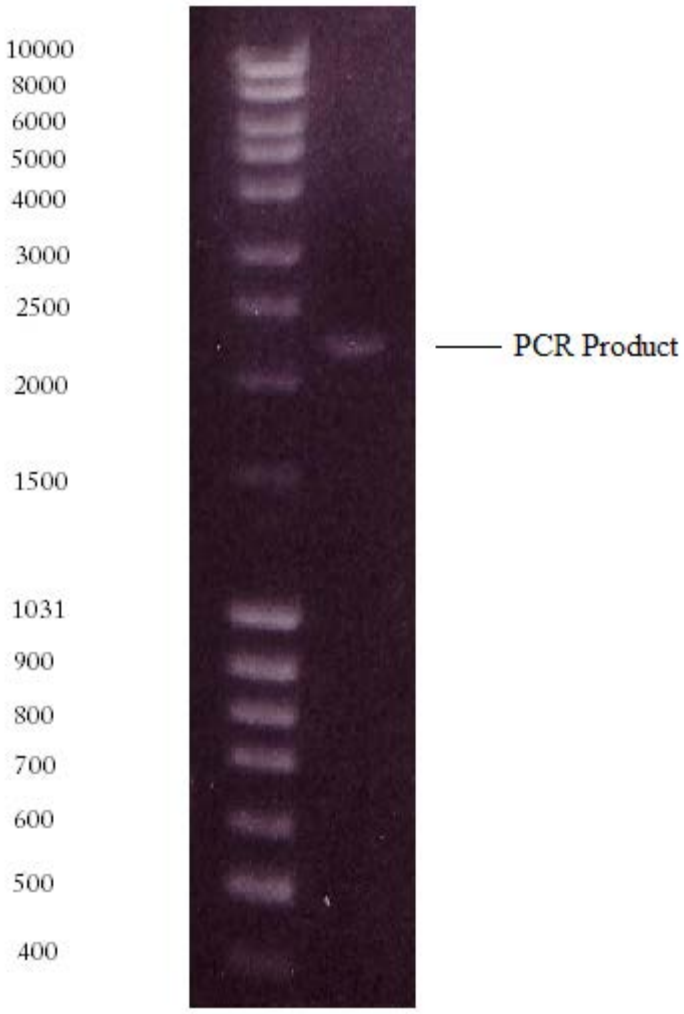


Figure 4.1 Amplification of the PAL gene from *Trichosporon cutaneum* by PCR. The amplified product was observed at ~2200 base pairs.

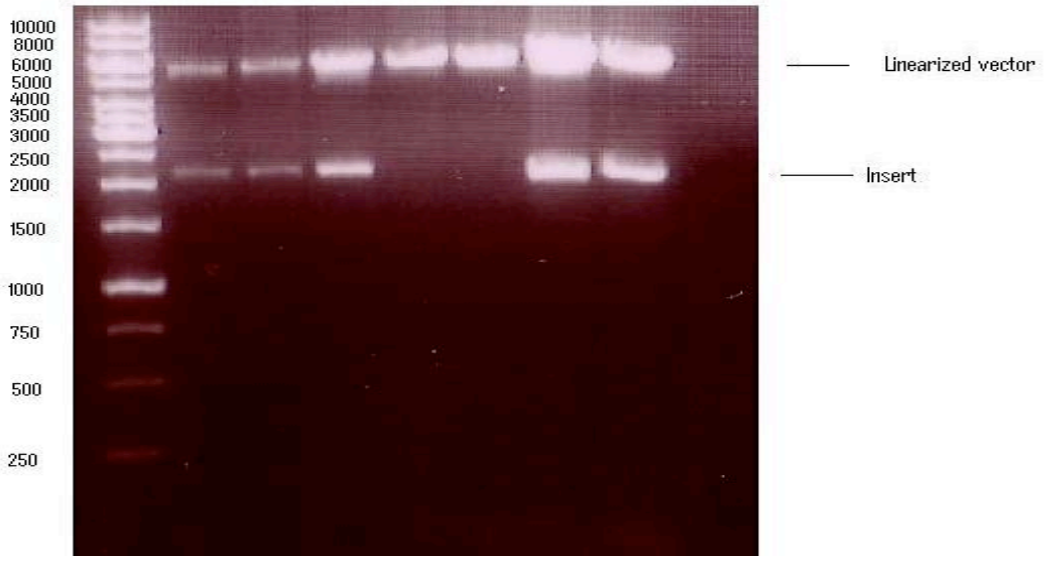


Figure 4.2 Five positive clones were identified with an insert size of ~2200 base pairs.

The gene shows high similarity with other PAL enzymes in the NCBI database and shares identity with the yeast PALs, *Sporidiobolus salmonicolo*r (89%) and *Rhodosporidium toruloides* (68%), as well as the monocotyledenous plant PALs *Zea mays* (74%) and *Oryza sativa* (72%). PAL/TAL activity has been reported in the yeasts *Rhodosporidium toruloides* (Jiang et al. 2005), *Sporidiobolus salmonicolor* (Bandoni et al. 1967) and the monocotyledonous plants, *Zea mays* and *Oryza sativa* (Rosler et al. 1997, Chapters 1.2.2 and 2.1*).*

Analysis of the gene sequence by GENESCAN utilizing the parameter matrices for Arabidopsis and maize predicted the presence of an intron within the sequence at position 121 through 1182 (1062 bp). The translated protein sequence also confirmed the presence of an intron within the sequence. Studies on yeast have revealed that intron containing genes are able to produce more RNA and protein improving transcriptional and translational yields (Juneau et al. 2006). They are required for competitive growth of yeast (Juneau et al. 2006).

Sequencing of the PAL gene from two independent clones revealed five amino acid residues that differ from that previously reported by Breinig et al. (2005) for this species. These amino acid residues include Arg 74 Glu, Val 274 Ala, Ala 298 Val, Ser 322 Pro and Arg 486 Lys (Table 4.1, Figure 4.3). Of the five amino acids identified, Glu, Ala and Lys are straight chain amino acids, while Pro has a cyclic ring and Val an isopropyl moiety. The substitutions involving the straight chain amino acids Glu, Ala and Lys are quite significant in terms of side chain differences. The presence of a

distinctive cyclic side chain in Pro confers some rigidity to the structure in comparison to other amino acids. The isopropyl moiety in Val confers steric hindrance. In Arg 74 Glu, Arg is more basic than Glu. Both Arg and Lys are basic in the Arg 486 Lys substitution. While none of these residues have been identified as a critical residue in the enzyme, any change in the amino acid sequence could have an impact on protein structure affecting enzyme functionality.

The five different amino acid residues identified in the sequence of the cloned gene may be due to mutations or may be the actual residues present in the native enzyme. Future work could involve cloning the gene by designing primers that would eliminate the intron region of the gene, utilizing cDNA for cloning experiments, as well as investigating the use of several vectors, for example, one that is devoid of a histidine tag or a yeast vector to obtain an active form of the protein.

The presence of a histidine tag has been reported in some cases to affect enzyme activity (Mohanty & Wiener 2004). The N-terminal His-tagged *Arabidopsis thaliana* PAL (AtPAL2 & AtPAL4) had very low protein yields as estimated by SDS-PAGE. Better protein yields were obtained for AtPAL1, 2 & 4 with the use of a pET Blue-1 expression vector system. None of the vector systems investigated were successful for the overexpression of AtPAL3 (Cochrane et al. 2004).

Table 4.1 A comparative look at the different amino acid residues identified in the cloned gene versus that reported by Breinig et al. (2005).

Breinig et al. (2005)	Current work	Residue position number
Arg	Glu	74
Val	Ala	274
Ala	Val	298
Ser	Pro	322
Arg	Lys	486

```
PATENT          MFIETNVAKPASTKAMNAGSAKAAPVEPFATYAHSQATKTVSIDGHTMKVGDVVAVARHG 60
CLONE           MFIETNVAKPASTKAMNAGSAKAAPVEPFATYAHSQATKTVSIDGHTMKVGDVVAVARHG 60
                ************************************************************

PATENT          AKVELAASVAGPVRASVDFKESKKHTSIYGVTTGFGGSADTRTSDTEALQISLLEHQLCG 120
CLONE           AKVELAASVAGPVQASVDFKESKKHTSIYGVTTGFGGSADTRTSDTEALQISLLEHQLCG 120
                ************:***********************************************

PATENT          FLPTDATYEGMLLAAMPIPIVRGAMAVRVNSCVRGHSGVRLEVLQSFADFINRGLVPCVP 180
CLONE           FLPTDATYEGMLLAAMPIPIVRGAMAVRVNSCVRGHSGVRLEVLQSFADFINRGLVPCVP 180
                ************************************************************

PATENT          LRGTISASGDLSPLSYIAGAICGHPDVKVFDTAASPPTVLTSPEAIAKYGLKTVKLASKE 240
CLONE           LRGTISASGDLSPLSYIAGAICGHPDVKVFDTAASPPTVLTSPEAIAKYGLKTVKLASKE 240
                ************************************************************

PATENT          GLGLVNGTAVSAAAGALALYDAECLAIMSQTNTVLTVEALDGHVGSFAPFIQEIRPHAGQ 300
CLONE           GLGLVNGTAVSAAAGALALYDAECLAIMSQTNTALTVEALDGHVGSFAPFIQEIRPHVGQ 300
                **********************************.*********************.**

PATENT          IEAARNIRHMLGGSKLAVHEESELLADQDAGILRQDRYALRTSAQWIGPQLEALGLARQQ 360
CLONE           IEAARNIRHMLGGSKLAVHEEPELLADQDAGILRQDRYALRTSAQWIGPQLEALGLARQQ 360
                ********************.***************************************

PATENT          IETELNSTTDNPLIDVEGGMFHHGGNFQAMAVTSAMDSARIVLQNLGKLSFAQVTELINC 420
CLONE           IETELNSTTDNPLIDVEGGMFHHGGNFQAMAVTSAMDSARIVLQNLGKLSFAQVTELINC 420
                ************************************************************

PATENT          EMNHGLPSNLAGSEPSTNYHCKGLDIHCGAYCAELGFLANPMSNHVQSTEMHNQSVNSMA 480
CLONE           EMNHGLPSNLAGSEPSTNYHCKGLDIHCGAYCAELGFLANPMSNHVQSTEMHNQSVNSMA 480
                ************************************************************

PATENT          FASARRTMEANEVLSLLLGSQMYCATQALDLRVMEVKFKMAIVKLLNETLTKHFAAFLTP 540
CLONE           FASARKTMEANEVLSLLLGSQMYCATQALDLRVMEVKFKMAIVKLLNETLTKHFAAFLTP 540
                *****:******************************************************

PATENT          EQLAKLNTHAAITLYKRLNQTPSWDSAPRFEDAAKHLVGVIMDALMVNDDITDLTNLPKW 600
CLONE           EQLAKLNTHAAITLYKRLNQTPSWDSAPRFEDAAKHLVGVIMDALMVNDDITDLTNLPKW 600
                ************************************************************

PATENT          KKEFAKEAGNLYRSILVATTADGRNDLEPAEYLGQTRAVYEAVRSELGVKVRRGDVAEGK 660
CLONE           KKEFAKEAGNLYRSILVATTADGRNDLEPAEYLGQTRAVYEAVRSELGVKVRRGDVAEGK 660
                ************************************************************

PATENT          SGKSIGSSVAKIVEAMRDGRLMGAVGKMF 689
CLONE           SGKSIGSSVAKIVEAMRDGRLMGAVGKMF 689
                *****************************
```

Figure 4.3 CLUSTAL 2.1 multiple sequence alignment comparing the protein sequence obtained for the clone compared to that in the patent (Breinig et al. 2005). The highlighted residues are the five different amino acid residues identified in the cloned gene.

While *Escherichia coli* remains a common host for high-level expression of heterologous genes there are a number of factors that affect gene expression. These include the source of the target genes, codon usage and mRNA secondary structures. Genes that possess a high proportion of common codons, are highly expressed while those with rare codons are poorly expressed (Andersson & Sharp 1996). Rare codons can result in slow translation, pre-mature translation termination and translation errors, which in turn lead to the inhibition of protein synthesis or cell growth (Kurland & Gallant 1996). The codon useage in yeast and bacteria are different and it is possible that codon optimization could be achieved if a yeast vector is used in place of a bacterial vector. *Pichia pastoris*, an eukaryotic expression host, was developed as an alternate to the *Escherichia coli* expression system. It has the advantage of being capable to produce complex molecules that have proven difficult to express and/or properly fold in bacteria (Yadava & Ockenhouse 2003). *Aspergillus* has also been utilized as an eukaryotic expression host (Devchand & Gwynne 1991). Protein over-expression and the production of an active enzyme in any one of these scenarios would provide further insight as to the actual sequence of the gene.

4.3.2 Sequence analysis

Amino acid sequences of 13 aromatic ammonia lyases were extracted from the National Center for Biotechnology Information (NCBI) database and their amino acid sequences compared utilizing the sequence alignment programs Clustal W and T-Coffee. These enzymes were selected based on their established ability to use either phenylalanine exclusively or both phenylalanine and tyrosine as substrates.

PAL from plant sources, *Arabidopsis thaliana*, *Petroselinum crispum*, *Nicotiana tabacum* and *Ginkgo bilboa* are predisposed to having activity only with phenylalanine. In the monocotyledenous plants, *Triticum aestivum*, *Bambusa oldhamii*, *Oryza sativa* and *Zea mays* the PAL enzyme has activity with both phenylalanine and tyrosine. The microbial PALs, *Rhodotorula glutinis*, *Sporidibolus salmonicolor* and *Trichosporon cutaneum* are also active with both substrates. The enzyme from *Rhodobacter sphaeroides* and *Saccharothrix espanaensis* are TALs.

Based on sequence comparisons, several substitutional differences were proposed to enhance the tyrosine substrate selectivity of the PAL enzyme from *Trichosporon cutaneum*. The proposed residues included valine 91, glutamine 117, arginine 160, isoleucine 185, serine 195, tyrosine 196 and glycine 199 (Figure 4.4). Of the selected mutations only valine 91 is present in the tyrosine loop of *Trichosporon cutaneum* PAL. It is possible that any mutation within this region may have an adverse effect on enzyme activity. Selections were based primarily on comparing the amino acid residues present in the TAL enzymes *Saccharothrix espanaensis* and *Rhodobacter sphaeroides* with other PAL enzymes in the database. *Saccharothrix espanaensis* and *Rhodobacter sphaeroides* have been established as having a higher catalytic efficiency for tyrosine. I hypothesize that mutation of valine 91 and glutamine 117 to leucine, arginine 160 to serine, isoleucine 185 to valine, serine 195 to alanine, tyrosine 196 to histidine and glycine 199 to leucine, which are the corresponding residues in *Saccharothrix espanaensis* and *Rhodobacter sphaeroides*, will result in enhanced tyrosine selectivity of the enzyme (Table 4.2). TAL enzymes are predisposed to having a leucine moiety

adjacent to the histidine moiety of the substrate selectivity switch region. In PAL *Trichosporon cutaneum* a glutamine residue is apparent. By converting glutamine to leucine I hypothesize that the enzyme will have a greater selectivity for tyrosine. One mutation was also selected to enhance the activity of the enzyme with phenylalanine. This mutation is also within the substrate selectivity switch region of the enzyme. By converting histidine 116 to phenylalanine it is anticipated that the enzyme will have even greater activity with phenylalanine. This is an already established mutation site by Watt et al. (2006) and would serve as a positive control.

```
Arabidopsis thaliana      TDSYGVTTGFGA-TSHRRTKN-GVALQKELIRFLNAGIFGSTKETS-------HTLPHSA 164
Petroselinum crispum      TDSYGVTTGFGA-TSHRRTKQ-GGALQKELIRFLNAGIFGNGSDN---------TLPHSA 155
Nicotiana tabacum         TDSYGVTTGFGA-TSHRRTKN-GGALQKELIRFLNAGVFGNGTETS-------HTLPHSA 154
Gingko bilboa             TDTYGVTTGFGA-TSHRRTSQ-GVELQKELIRFLNAGVFGSCEGN---------VLPEAT 165
Triticum aestivum         TDSYGVTTGFGA-TSHRRTKE-GGALQRELIRFLNAGAFGTGTDG--------HVLPAAA 139
Bambusa oldhamii          TDSYGVTTGFGA-TSHRRTKE-GGALQRELIRFLNAGALDTGDDG--------HVLPAAA 152
Oryza sativa              TDSYGVTTGFGA-TSHRRTKE-GGALQRELIRFLNAGAFGTGTDG--------HVLPAEA 151
Rhodoturula glutinis      VRLHIALSPFSSRRASTDSRG-PFAFENSLLEHQLCGVLPTSMDGFALGSGLENSLPLEV 84
Sporidiobolus salmonicolor -----VTTGFGG-SADTRTDD-PLALQKSLLEHQLCGVLPTSLSGFSLGRGLENALPIEV 60
Trichosporon cutaneum     TSIYGVTTGFGG-SADTRTSD-TEALQISLLEHQLCGFLPTDAT---YEGMLLAAMPIPI 140
Zea mays                  GDIYGVTTGFGG-TSHRRTKD-GPALQVELLRHLNAGIFGTGSDG--------HTLPSEV 142
Rhodobacter sphaeroides   RHVYGLTTGFGPLANRLISGENVRTLQANLVHHLASGVG-------------PVLDWTT 102
Sacchrothrix espanaensis  KPIYGLTQGFGPLV--LFDADSELEQGGSLISHLGTGQG-------------APLAPEV 100

Arabidopsis thaliana      TRAAMLVRINTLLQGFSGIRFEILEAITSFLNNNITPSLPLRGTITASGDLVPLSYIAGL 224
Petroselinum crispum      TRAAMLVRINTLLQGYSGIRFEILEAITKFLNQNITPCLPLRGTITASGDLVPLSYIAGL 213
Nicotiana tabacum         TRAAMLVRINTLLQGYSGIRFEILEAITKLINSNITPCLPLRGTITASGDLVPLSYIAGL 214
Gingko bilboa             TRAAMLVRTNTLPQGYSGIRWALLETIEKLLNAGITPKLPLRGTITASGDLVPLSYIAGL 225
Triticum aestivum         TRAAMLVRVNTLLQGYSGIRFEILETIATLLNANVTPCLPLRGTITASGDLVPLSYIAGL 199
Bambusa oldhamii          TRAAMLVRINTLLQGYSGIRFEILETIAALLNANVTPCLPLRGTITASGDLVPLSYIAGL 212
Oryza sativa              TRAAMLVRINTLLQGYSGIRFEILEAIAKLLNANVTPCLPLRGTITASGDLVPLSYIAGL 211
Rhodoturula glutinis      VRGAMTLRVNSLTRGHSAVRIVVLEALTNFLNHGITPIVPLRGTISASGDLSPLSYIAAS 144
Sporidiobolus salmonicolor VRGAMVIRCNSLLRGHSAIRLSVLETLVKLINLNITPVVPLRGSISASGDLSPLSYIAGA 120
Trichosporon cutaneum     VRGAMAVRVNSCVRGHSGVRLEVLQSFADFINRGLVPCVPLRGTISASGDLSPLSYIAGA 200
Zea mays                  TRAAMLVRINTLLQGYSGIRFEILEAITKLLNTGVSPCLPLRGTITASGDLVPLSYIAGL 202
Rhodobacter sphaeroides   ARAMVLARLVSIAQGASGASEGTIARLIDLLNSELAPAVPSRGTVGASGDLTPLAHMVLC 160
Sacchrothrix espanaensis  SRLILWLRIQNMRKGYSAVSPVFWQKLADLWNKGFTPAIPRHGTVSASGDLQPLAHAALA 160
```

Figure 4.4 Residues in bold were selected for future mutagenesis studies to enhance the tyrosine ammonia lyase activity of *Trichosporon cutaneum*. Residues were selected based on sequence of alignment of various PAL and TAL enzymes in the database.

Table 4.2 Proposed targeted amino acid residues for site directed mutagenesis. Mutation of histidine to phenylalanine is expected to enhance the PAL activity of *Trichosporon cutaneum.*

Amino acid	**Mutation**	**Predicted results**
Valine 91	Leucine 91	Enhanced tyrosine activity
Glutamine 117	Leucine 117	Enhanced tyrosine activity
Arginine 160	Serine 160	Enhanced tyrosine activity
Isoleucine 185	Valine 185	Enhanced tyrosine activity
Serine 195	Alanine 195	Enhanced tyrosine activity
Tyrosine 196	Histidine 196	Enhanced tyrosine activity
Glycine 199	Leucine 199	Enhanced tyrosine activity
Histidine 116	Phenylalanine 116	Enhanced phenylalanine activity

4.3.3 Substrate selectivity switch residues

A single active site residue that is important for substrate selection of PAL/TAL enzymes was identified in *Rhodobacter sphaeroides* (Watts et al. 2006). A histidine residue (His 89) was found to be conserved in TALs but replaced in PALs. In sequences of PAL, His 89 varied between a phenylalanine or histidine residue. PALs with a strong specificity for phenylalanine possess a Phe amino acid residue in place of histidine. The presence of Phe in PALs is predicted to be favourable as it facilitates a non-polar interaction with the phenyl group of phenylalanine. Hydrogen bonding with the hydroxyl group of tyrosine is facilitated in the presence of the His 89 amino acid residue. Adjacent to His 89 a Leu amino acid residue is apparent. His-Leu are characteristic of TALs whereas Phe-Leu are characteristic of PALs. In *Trichosporon cutaneum* His-Leu was replaced by His-Gln. This motif was also observed in the PAL enzymes of *Rhodotorula glutinis* and *Sporidiobolus salmonicolor* (Figure 4.4). PAL enzymes with the His-Gln motif possess dual substrate activity with phenylalanine and tyrosine, with greater enzyme specificity for phenylalanine. The similarity observed in the substrate selectivity switch region of *Sporidiobolus salmonicolor* led to my speculating that this enzyme is potentially active with phenylalanine and tyrosine. A short communication by Bandoni et al. (1967) confirmed the presence of both PAL (0.45 µM product /mg protein) and TAL (0.05 µM product /mg protein) activity in *Sporidiobolus salmonicolor.*

4.3.4 Essential amino acid residues

A number of essential amino acid residues have been identified in the TAL gene from *Rhodobacter sphaeroides,* which includes His 89, Val 409, Ala 149, Ser 150, Gly 151, Tyr 60 and Tyr 300 (Schroeder et al. 2008, Watts et al. 2006; Louie et al. 2006). These residues were identified by crystallography and sequence comparison with other HAL and PAL enzymes in the database. By using a combination of site directed mutagenesis, kinetic analysis, mass spectrometry, and fluorescence spectroscopy, the role of conserved active sites were examined (Schroeder et al. 2008). His 89 forms the basis of the substrate selectivity switch region of the enzyme (Watts et al. 2006; Louie et al. 2006). Sequence comparisons of the TAL enzyme from *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* revealed that Val 409 is conserved between the two Rhodobacter species. Mutations of Val 409 to methionine or isoleucine resulted in the enzyme having a greater preference for phenylalanine instead of tyrosine (Xue et al. 2007b). Sequence alignment of TALs with other PALs and HALs, shows that the residue which corresponds to Val 409 is conserved amongst some PAL enzymes. In *Rhodotorula glutinis* PAL, this residue corresponds to Ile 472 (Xue et al. 2007b) while in *Trichosporon cutaneum* PAL, Ile 446 was apparent. Ala 149, Ser 150, and Gly 151 are the amino acid triad, which is involved in the formation of the MIO prosthetic group. Mutation of Ser 150 to alanine disrupts the ability of the enzyme to form the MIO prosthetic group thereby inactivating the enzyme (Schroeder et al. 2008). In *Trichosporon cutaneum* PAL, the active site is composed of Ala 187, Ser 188 and Gly 189.

The Tyr 110 residue in parsley and its equivalents is a key feature of the aromatic ammonia lyases and is one of the most conserved residues in this family (Rother et al. 2001 and Rother et al. 2002). It is also a key feature of the aminomutase family. The surrounding region of Tyr 110 is referred to as the tyrosine loop which is believed to play a critical role in substrate binding and catalysis. This residue, while not apparent in all PALs, was detected in *Trichosporon cutaneum* PAL at the position Tyr 89. A Tyr110Phe mutation in parsley PAL resulted in a 75,000 fold decrease in k_{cat} of the enzyme (Rother et al. 2002) reiterating the importance of this residue in PAL functionality. Its counterpart in HAL, Tyr 53 when mutated, Tyr53Phe resulted in a 2650 fold decrease in the k_{cat} of the enzyme (Rother et al. 2001). Critical amino acid residues identified in PAL *Trichosporon cutaneum* by sequence comparison with the PAL enzyme from *Rhodobacter sphaeroides* are summarized in Table 4.3.

Other mutagenesis studies have been performed on the PAL enzyme from the yeast *Rhodotorula glutinis* (Tang 2003). These mutations were reported to result in a change in substrate preference from phenylalanine to tyrosine (Table 4.4). One of the mutations (Gln138Leu) involves the substrate selectivity switch region (HQ) of the enzyme and resulted in an increase of the TAL/PAL to 7.2. These proposed mutations (Figure 4.5) may also be investigated in *Trichosporon cutaneum* PAL to increase the level of tyrosine ammonia lyase activity of the enzyme.

Table 4.3 Essential amino acid residues identified in *Rhodobacter sphaeroides* TAL and the corresponding residues in *Trichosporon cutaneum* PAL.

RsTAL	**TcPAL**	**Notes**
Tyr60[a]	Tyr89	Critical residue
His89Leu90[b]	His116Q117	Substrate selectivity
Ala149Ser150Gly151[a]	Ala187Ser188Gly189	Active site
Val409[c]	Ile446	Critical residue
Tyr300[a]	Tyr338	Critical residue

[a-c] References: Schroeder et al. 2008; Watts et al. 2006; Xue et al. 2007b

Table 4.4 Critical amino acid residues identified in *Rhodotorula glutinis* by mutagenesis (Tang 2003).

Strain	Mutations	TAL/PAL ratio	Corresponding residue *Trichosporon cutaneum*
Wild Type PAL	None	0.5	
EP18Km-6	Ile540Thr	1.7	Met514
RM120-1	Asp126Gly Gln138Leu Ile540Thr	7.2	Asp105 Gln118 Met514
RM120-2	Gly198Asp Ile540Thr	2.1	Gly174 Met514
RM120-4	Ser181Pro Val235Ala Ile540Thr	2.0	Ser157 Asp211 Met514
RM120-7	Ser149Pro Ile202Val Ile540Thr	0.8	Ala127 Cys178 Met514
RM492-1	Val502Gly Ile540Thr	2.0	Val476 Met514

Clustal sequence alignment

```
Rhodotorula       MAPSLDSISHSFANGVASAKQAVNGASTNLAVAGSHLPTTQVTQVDIVEKMLAAPTDSTL 60
Trichosporon      ---------------MFIETNVAKPASTKAMNAG----SAKAAPVEPFATYAHSQATKTV 41
                                 :   .:..: ***:   **    :::.: *: . .   : : .*:

Rhodotorula       ELDGYSLNLGDVVSAARKGRPVRVKDSDEIRSKIDKSVEFLRSQLSMSVYGVTTGFGGSA 120
Trichosporon      SIDGHTMKVGDVVAVARHGAKVELAAS--VAGPVRASVDFKESKKHTSIYGVTTGFGGSA 99
                  .:**::::****:.**:*  *.:  *  : . :  **:* .*:   *:**********

Rhodotorula       DTRTEDAISLQKALLEHQLCGVLPSSFDSFRLGRGLENSLPLEVVRGAMTIRVNSLTRGH 180
Trichosporon      DTRTSDTEALQISLLEHQLCGFLPT--DATYEG-MLLAAMPIPIVRGAMAVRVNSCVRGH 156
                  ****.*: :** :********.**:  *:   *  *  ::*: :*****::**** .***

Rhodotorula       SAVRLVVLEALTNFLNHGITPIVPLRGTISASGDLSPLSYIAAAISGHPDSKVHVVHEGK 240
Trichosporon      SGVRLEVLQSFADFINRGLVPCVPLRGTISASGDLSPLSYIAGAICGHPDVKVFDTAASP 216
                  *.*** **:::::*:*:*:.* ******************.**.**** **. .  .

Rhodotorula       EKILYAREAMALFNLEPVVLGPKEGLGLVNGTAVSASMATLALHDAHMLSLLSQSLTAMT 300
Trichosporon      PTVLTSPEAIAKYGLKTVKLASKEGLGLVNGTAVSAAAGALALYDAECLAIMSQTNTVLT 276
                   .:* : **:* :.*:.* *..**************: .:***:**. *:::**: *.:*

Rhodotorula       VEAMVGHAGSFHPFLHDVTRPHPTQIEVAGNIRKLLEGSRFAVHHEEEVKVKDDEGILRQ 360
Trichosporon      VEALDGHVGSFAPFIQEIR-PHAGQIEAARNIRHMLGGSKLAVHEESELLADQDAGILRQ 335
                  ***: **.*** **::::  **. ***.* ***::* **::***.*.*: ..:* *****

Rhodotorula       DRYPLRTSPQWLGPLVSDLIHAHAVLTIEAGQSTTDNPLIDVENKTSHHGGNFQAAAVAN 420
Trichosporon      DRYALRTSAQWIGPQLEALGLARQQIETELN-STTDNPLIDVEGGMFHHGGNFQAMAVTS 394
                  ***.****.**:** :. *  *:  :  * . **********.   ******** **:.

Rhodotorula       TMEKTRLGLAQIGKLNFTQLTEMLNAGMNRGLPSCLAAEDPSLSYHCKGLDIAAAAYTSE 480
Trichosporon      AMDSARIVLQNLGKLSFAQVTELINCEMNHGLPSNLAGSEPSTNYHCKGLDIHCGAYCAE 454
                  :*:.:*: * ::***.*:*:**::*. **:**** **..:** .******** ..** :*

Rhodotorula       LGHLANPVTTHVQPAEMANQAVNSLALISARRTTESNDVLSLLLATHLYCVLQAIDLRAI 540
Trichosporon      LGFLANPMSNHVQSTEMHNQSVNSMAFASARRTMEANEVLSLLLGSQMYCATQALDLRVM 514
                  **.****::.***.:** **:***:*: ***** *:*:******.:::**. **:***.:

Rhodotorula       EFEFKKQFGPAIVSLIDQHFGSAMTGSNLRDELVEKVNKTLAKRLEQTNSYDLVPRWHDA 600
Trichosporon      EVKFKMAIVKLLNETLTKHFAAFLTPEQL-AKLNTHAAITLYKRLNQTPSWDSAPRFEDA 573
                  *.:**  :   : . : :**.: :* .:*  :*  :.  ** ***:** *:* .**:.**

Rhodotorula       FSFAAGTVVEVLSSTS--LSLAAVNAWKVAAAESAISLTRQVRETFWSAASTSSPALSYL 658
Trichosporon      AKHLVGVIMDALMVNDDITDLTNLPKWKKEFAKEAGNLYRSILVATTADGRNDLEPAEYL 633
                   .. .*.:::.*  ..   .*: :  **   *:.* .* *.:  :  : . ..  . .**

Rhodotorula       SPRTQILYAFVREELGVKARRGDVFLGKQEVTIGSNVSKIYEAIKSGRINNVLLKMLA 716
Trichosporon      G-QTRAVYEAVRSELGVKVRRGDVAEGKSGKSIGSSVAKIVEAMRDGRLMGAVGKMF- 689
                  . :*: :*  **.*****.*****  **.  :***.*:** **::.**: ..: **:
```

Figure 4.5 Sequence alignment of PAL *Rhodotorula glutinis* and PAL*Trichosporon cutaneum* with proposed mutation sites highlighted.

4.4 CONCLUSION

The PAL gene was cloned into the pET30a vector and sequenced. Five amino acid residues not previously reported were identified in the sequence of the gene, namely, Glu74, Ala 274, Val 298, Pro322 and Lys486. The His Gln motif identified in the substrate selectivity switch region of *Trichosporon cutaneum* PAL may potentially be utilized to assist in the identification of other uncharacterized PAL/TAL enzymes in the microbial genomic database. This motif was also identified in the protein sequence of *Sporidiobolus salmonicolor,* which also possesses dual substrate activity (Bandoni et al. 1967). Several mutation sites that could potentially enhance the TAL activity of *Trichosporon cutaneum* were proposed for future mutagenesis studies.

CHAPTER 5: OVERALL CONCLUSION AND FUTURE PERSPECTIVES

5.1 OVERALL CONCLUSION

PAL catalyzes the first step of the phenylpropanoid pathway, producing secondary metabolites, which play important functional roles in plants. Flavonoids are derived from the phenylpropanoid pathway and are used by plants for UV protection (Bieza & Lois 2001), defence against plant pathogens (Dixon & Paiva 1995) and colouration (Springob et al. 2003). They are also associated with various health benefits such as antioxidant activity, enzyme inhibition, anti-inflammatory and estrogenic activities to cytotoxic antitumor activities (Harborne & Williams 2000). Research attempting to elucidate the complexity of the metabolic network associated with flavonoid biosynthesis to enhance and alter flavonoid composition in dietary plants continues (Tanaka et al. 1998; Dixon et al. 1998). Phenylpropionic acids that are abundantly available from agricultural waste products are also being exploited in processes such as microbial vanillin production (Marasco & Schmidt-Dannert 2003). Their use as precursors in the synthesis of more highly valued flavonoid compounds is of interest commercially.

Ways of enhancing phenylpropanoid production in plants continue to be investigated. Cloning of the TAL gene from *Rhodobacter sphaeroide*s into *Arabidopsis* resulted in enhanced metabolic influx into the phenylpropanoid pathway and increased accumulation of flavonoids and phenylpropanoids (Nishiyama et al. 2010). By the cloning of a TAL gene into *Arabidopsis*, the rate-limiting steps of phenylpropanoid metabolism catalyzed by PAL and cinnamate 4 hydroxylase was reduced to one by the

direct conversion of tyrosine to *para* hydroxycinnamic acid. This approach can be used to facilitate the metabolic engineering of the phenylpropanoid pathway in different types of plants (Nishiyama et al. 2010).

In plants dual substrate activity has mainly been detected in monocotyledonous plants. Preliminary screening of plant seedlings for PAL/TAL activity revealed the highest levels of PAL and TAL activities in wheat seedling. A unique PAL polypeptide was identified at 103 kDa in *Triticum aestivum*. In the dicotyledenous plants, this is the first reported incidence of dual substrate activity in *Lens culinaris*.

Trichosporon cutaneum PAL is one of the few microbial sources that has been identified as having both PAL and TAL activity. To gain further insight about *Trichosporon cutaneum* PAL, as well as the possibility of enhancing its activity with tyrosine, the enzyme was characterized, purified and cloned here. The enzyme is inducible with highest levels of induction being achieved with tyrosine. Glucose however has an inhibitory effect on the enzyme. Kinetic analyses revealed that the enzyme possessed typical Michaelis Menten kinetics with the substrate phenylalanine but allosteric interaction with tyrosine. Positive allostery is uncommon in this family of enzymes and is the main characteristic feature of this enzyme. The kinetic constants for the purified enzyme were K_m, 5.0 ± 0.7 mM for phenylalanine and K', 1.7 ± 0.8 mM, Hill coefficient, 1.8 ± 0.2, for tyrosine.

This is the first report on the pH and temperature optimum of the enzyme, which was found to be in the range of 8 - 8.5 and 32 °C respectively. The pH optimum is typical of that observed for other PAL enzymes. The temperature optimum was however lower than that reported for other PAL/TAL enzymes. The enzyme does not show metal dependence with the chloride salts of sodium, potassium, magnesium and iron.
The enzyme's propensity to degrade was seen in the fluctuation of PAL/TAL activities during storage and purification as well as from SDS PAGE and Western blot analysis.
The native enzyme was purified utilizing an aqueous two phase system, acid precipitation as well as anion exchange chromatography. This is the first report on the purification of *Trichosporon cutaneum* PAL by aqueous two phase partitioning and acid precipitation. In the aqueous two phase partitioning system relatively modest yields and increased purification fold was observed with the PEG1000/Na_2SO_4 system. The enzyme remained in the top phase. Limited success was achieved with acid precipitation, although the enzyme remained active after acid treatment. The yields and purification fold obtained from these two methods may potentially be improved and can be investigated further.

While the purification of *Trichosporon cutaneum* PAL by anion exchange chromatography has been previously reported by Vanelli et al. (2007a), the results from the present study produced better yield and purification fold by the use of a Sepharose Q anion exchange column. A purification fold of 50 was achieved with over 20% yields. Anion exchange chromatography provided best yields for enzyme purification and presents a facile and rapid method for enzyme purification.

The gene was cloned into the pET30a vector and sequenced. Five different amino acid residues not previously reported for this species were identified in the sequence of the gene, namely, Glu 74, Ala 274, Val 298, Pro 322 and Lys486. It is not certain as to whether these residues are present in the native enzyme or if this is a mutant gene.

Comparison of the *Trichosporon cutaneum* PAL gene using the BLAST search tools of the National Center for Biotechnology Information database, revealed that *Sporidiobolus salmonicolor* potentially has PAL/TAL activity. This was later confirmed from a literature search. Very limited information is available on this enzyme and the opportunity is presented for its characterization with regards to TAL activity.

Potential mutation sites with regards to enhancing TAL activity in *Trichosporon cutaneum* PAL were identified by comparing the gene sequence of *Trichosporon cutaneum* PAL with other PAL/TAL enzymes in the National Center for Biotechnology database. Future work involving the PAL gene from *Trichosporon cutaneum* includes cloning of the gene by utilizing cDNA or a truncated form of the DNA eliminating the intron region of the gene as well as the use of the yeast vector *Pichia pastoris* and mutageneis studies to enhance the tyrosine activity of the enzyme. Cloning of the *Trichosporon cutaneum* PAL gene into plants to further enhance the production of phenylpropanoids can also be investigated.

The PAL enzyme from *Trichosporon cutaneum* could potentially be used in the treatment of PKU by oral enzyme therapy. High amounts of enzyme activity can be

obtained by tyrosine induction. *Trichosporon cutaneum* PAL exhibits good stability at body temperature (37 °C) with 75% retention of PAL activity compared to the optimum of 32 °C. The enzyme would require protection from the acidic conditions of the stomach due to its pH optimum of ~8.2. Enzyme encapsulation is one means of stabilizing the enzyme (Trotman et al. 2007). Mutagenesis studies are an approach to increase the enzyme's affinity for phenylalanine for the treatment of PKU.

PAL with a high catalytic activity for tyrosine would be desirable for use of the enzyme in the biosynthesis of *para* hydroxycinnamic acid. *Para* hydroxycinnamic acid is derivative of a number of industrially important chemicals in the food and pharmaceutical industries (Gosset 2009). The catalytic efficiency of the enzyme for tyrosine may be improved by site directed mutagenesis.

The importance of PAL is seen in nature as well as its commercial applicability. The results from this study have provided further knowledge and insight as to the dual substrate activity of the PAL family of enzymes. Dual substrate activities were observed in several plant species as well as the *Trichosporon cutaneum* PAL enzyme. Amino acid residues which may be important in the enzyme's ability to utilize tyrosine have been identified. The HQ motif observed in the substrate selectivity switch region of *Trichosporon cutaneum* PAL may be utilized to identify other uncharacterized PAL/TAL enzymes.

REFERENCES

Abell, C.W. and Shen, R.S. 1987. Phenylalanine ammonia-lyase from yeast *Rhodotorula glutinis*. Methods Enzymol. 142: 242-248.

Ahmed, S.I. and Swain, T. 1970. Effect of light on the activity of enzymes of the aromatic pathway in peas and mung beans. Phytochemistry 9: 2287-2290.

Allwood, E.G., Davies, D.R., Gerrish, C., Ellis, B.E. and Bolwell, G.P. 1999. Phosphorylation of phenylalanine ammonia-lyase: evidence for a novel protein kinase and identification of the phosphorylated residue. FEBS Lett. 457: 47-52.

Anděrová, M., Anděra, L., Volfová, O., Panoš, J. and Löbl, V. 1993. Influence of increasing methanol concentration on alcohol oxidase activity in *Candida boidinii.* Folia Microbiol. 38: 55-58.

Andersson, G.E. and Sharp, P.M. 1996. Codon usage in the *Mycobacterium tuberculosis* complex. Microbiology 142: 915-925.

Anson, J.G., Gilvert, H.J., Oram, J. D., Minton, N.P. 1987. Complete nucleotide sequence of the *Rhodosporidium toruloides* gene coding for phenylalanine ammonia-lyase. Gene 58:189-199.

Appert, C., Logemann, E., Hahlbrock, K., Schmid, J. and Amrhein, N. 1994. Structural and catalytic properties of the four phenylalanine ammonia-lyase isoenzymes from parsley (*Petroselinum crispum* Nym.). Eur. J. Biochem. 225: 491-499.

Bandoni, R.J., Moore, K., Subba Rao, P.V. and Towers, G.H.N. 1967. Phenylalanine and tyrosine ammonia-lyase activity in some basidiomycetes. Phytochemistry 7: 205-207.

Belunis, C.J. and Hrazdina, G. 1988. Phenylalanine ammonia-lyase from illuminated buckwheat hypocotyls and production of monoclonal antibodies. Phytochemistry 27: 2485-2489.

Berner, M., Krug, D., Bihlmaier, C., Vente, A., Muller, R., and Bechthold, A. 2006. Genes and enzymes involved in caffeic acid biosynthesis in the actinomycete *Saccharothrix espanaensis*. J. Bacteriol. 188: 2666-2673.

Bieza, K. and Lois, R. 2001. An *Arabidopsis* mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. Plant Physiol. 126: 1105-1115.

Bolwell, G.P. and Rodgers, M.W. 1991. L-Phenylalanine ammonia- lyase from French bean (*Phaseolus vulgaris* L.). Characterisation and differential expression of antigenic multiple Mr forms. Biochem. J. 279: 231-236.

Bolwell, G.P., Bell, J.N., Cramer, C.L., Schuch, W., Lamb, C.J. and Dixon, R.A. 1985. L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*. Characterization and differential induction of multiple forms from elictor-treated suspension cultures. Eur. J. Biochem. 149: 411-419.

Bolwell, G.P., Sap, J., Cramer, C.L., Lamb, C.J., Schuch, W. and Dixon, R.A. 1986. L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*: Partial degradation of enzyme subunits *in vitro* and *in vivo*. Biochim. Biophys. Acta 881: 210-221.

Borkar, P.S., Bodade, R.G., Rao, S.R. and Khobragade, C.N. 2009. Purification and characterization of extracellular lipase from a new strain: *Pseudomonas aeruginosa* SRT 9. Braz. J. Microbiol. 40: 358-366.

Bourget, L. and Chang, T.M. 1984. Artificial cell-microencapsulated phenylalanine ammonia-lyase. Appl. Biochem. Biotechnol. 10: 57-59.

Bradbury, S.L. and Jakoby, W.B. 1972. Glycerol as an enzyme-stabilizing agent: Effects on aldehyde dehydrogenase (thiol groups/polyhydric alcohols/arsenite). Proc. Nat. Acad. Sci.USA 69: 2373-2376.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Breinig, S., Qi, W.W., Sariaslani, F., Vanelli, T. and Xue, Z. 2005. DNA and amino acid sequences of a tyrosine-inducible tyrosine ammonia lyase enzyme from the yeast *Trichosporon cutaneum*. United States Patent US6951751.

Briggs, G.E. and Haldane, J.B. 1925. A note on the kinetics of enzyme action. Biochem. J. 19: 338-339.

Butland, S.L., Chow, M.L. and Ellis, B.E. 1998. A diverse family of phenylalanine ammonia-lyase genes expressed in pine trees and cell cultures. Plant Mol. Biol. 37: 15-24.

Calabrese, J.C., Jordan, D.B., Boodhoo, A., Sariaslani, S. and Vannelli, T. 2004. Crystal structure of phenylalanine ammonia lyase: multiple helix dipoles implicated in catalysis. Biochemistry 43: 11403-11416.

Camm, E. and Towers, G. 1973. Phenylalanine ammonia lyase. Phytochemistry 12: 961-973.

Camm, E. and Towers, G. 1969. Phenylalanine and tyrosine ammonia lyase activity in *Sporobolomyces roseus*. Phytochemistry 8: 1407-1413.

Campos-Vargas, R. and Saltvei, M.E. 2002. Involvement of putative chemical wound signals in the induction of phenolic metabolism in wounded lettuce. Physiol. Plant. 114: 73-84.

Centerwall, S.A. and Centerwall, W.R. 2000. The discovery of phenylketonuria: the story of a young couple, two retarded children, and a scientist. Pediatrics 105: 89-103.

Chaman, M.E., Copaja, S.V. and Argandona, V.H. 2003. Relationships between salicylic acid content, phenylalanine ammonia-lyase (PAL) activity, and resistance of barley to aphid infestation. J. Agric. Food Chem. 51: 2227-2231.

Cheng, S.H., Sheen, J., Gerrish, C. and Bowlell, G.P. 2001. Molecular identification of phenylalanine ammonia-lyase as a substrate of a specific constitutively active Arabidopsis CDPK expressed in maize protoplasts. FEBS Lett. 503: 185-188.

Cochrane, F., Davin, L. and Lewis, N. 2004. The *Arabidopsis* phenylalanine ammonia lyase gene family: kinetic characterization of the four PAL isoforms. Phytochemistry 65: 1557-1564.

Cooke, H.A., Christianson, C.V. and Bruner, S.D. 2009. Structure and chemistry of 4-methylideneimidazole-5-one containing enzymes. Curr. Opin. Chem. Biol 13: 460-468.

Copeland, R. A. 2000. Experimental measures of enzyme activity In *Enzymes:* A practical introduction to structure, mechanism and data analysis, 2nd ed, 199-200, John Wiley Press, New York.

Cusanovich, M.A. and Meyer, T.E. 2003. Photoactive yellow protein: a prototypic PAS domain sensory protein and development of a common signaling mechanism. Biochemistry 42: 4759-4770.

da Cunha, A. 1998. Purification, characterization and induction of L-phenylalanine ammonia lyase in *Phaseolus vulgaris*. Eur. J. Biochem. 178: 243-248.

Dahiya, J.S. 1993. Isolation and characterization of phenylalanine ammonia-lyase enzyme from the fungus *Leptosphaeria maculans*. Indian J. Exp. Biol. 31: 874-877.

D'Cunha, G.B., Satyanarayan, V. and Nair, P.M. 1996. Purification of phenylalanine ammonia lyase from *Rhodotorula glutinis*. Phytochemistry 42: 17-20.

D'Cunha, G.B., Satyanarayan, V. and Nair, P.M. 1994. Novel direct synthesis of L-phenylalanine methyl ester by using *Rhodotorula glutinis* phenylalanine ammonia lyase in an organic-aqueous biphasic system. Enzyme Microb. Technol. 16: 318-322.

Devchand, M. and Gwynne, D.I. 1991. Expression of heterologous proteins in Aspergillus. J. Biotech. 17: 3-9.

Dixon, R.A., Howles, P.A., Lamb, C., He, X.Z. and Reddy, J.T. 1998. Prospects for the metabolic engineering of bioactive flavonoids and related phenylpropanoid compounds. Adv. Exp. Med. Biol. 439: 55-66.

Dixon, R. and Paiva, N. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085-1097.

Dubery, I.A. and Smit, F. 1994. Phenylalanine ammonia-lyase from cotton (*Gossypium hirsutum*) hypocotyls: properties of the enzyme induced by a *Verticillium dahliae* phytotoxin. Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 1207: 24-30.

Dubery, I.A. and Schabort, J.C. 1988. Kinetic and regulatory properties of phenylalanine ammonia-lyase from *Citrus sinensis*. Int. J. Biochem. 20: 217-222.

Durham, D.R., McNamee, C.G. and Stewart, D.B. 1984. Dissimilation of aromatic compounds in *Rhodotorula graminis*: biochemical characterization of pleiotropically negative mutants. J. Bacteriol. 160: 771-777.

El-Shora, H.M. 2002. Properties of phenylalanine ammonia lyase from marrow cotyledons. Plant Sci. 162: 1-7.

Emes, A.V. and Vining, L.C. 1970. Partial purification and properties of L-phenylalanine ammonia lyase from *Streptomyces verticillatus*. Can. J. Biochem. 48: 613-622.

Erez, A. 1973. Possible errors in quantitative determination of phenylalanine ammonia lyase activity by spectrophotometric methods. Plant Physiol. 51: 409-411.

Evans, C.T., Conrad, D., Hanna, K., Peterson, W., Choma, C., Misawa, M. 1987. Novel stabilization of phenylalanine ammonia lyase catalyst during bioconversion of *trans*-cinnamic acid to L-phenylalanine. Appl. Microbiol. Biotechnol. 25: 399-405.

Flanagin, L.W., Singh, V. and Grant, W. 1999. Molecular model of phenolic polymer dissolution in photolithography. J. Poly. Sci. Part B Poly. Phys. 37: 2103-2113.

Fourcroy P. 1980. Properties of L-phenylalanine ammonia-lyase and turnover rate in etiolated and far-red illuminated seedlings of radish. Biochim Biophys Acta. 613: 488-498.

Fritz, R.R., Hodgins, D.S. and Abell, C.W. 1976. Phenylalanine ammonia-lyase. Induction and purification from yeast and clearance in mammals. J. Biol. Chem. 251: 4646-4650.

Frost, J.W. and Lievense, J. 1994. Prospects for biocatalytic synthesis of aromatics in the 21st century. New J. Chem: 18:341-348.

Fukasawa-Akada, T. Kung, S.D. and Watson, J.C. 1996. Phenylalanine ammonia-lyase gene structure, expression, and evolution in *Nicotiana*. Plant Mol. Biol. 30: 711-722.

Gallagher, T., Rozwarski, D.A., Ernst, S.R. and Hackert, M.L. 1993. Refined structure of the pyruvoyl-dependent histidine decarboxylase from *Lactobacillus* 30a. J. Mol. Biol. 230: 516-528.

Gamez, A., Sarkissian, C.N., Wang, L., Kim, W., Straub, M., Patch, M.G., Chen, L., Striepeke, S., Fitpatrick, F., Lemontt, F., O'Neill, C., Scriver, C.R. and Stevens, R. 2005. Development of pegylated forms of recombinant *Rhodosporidium toruloides* phenylalanine ammonia-lyase for the treatment of classical phenylketonuria. Mol. Ther. 11: 986-989.

Gatenby, A.A., Sariaslani, F.S., Tang, X., Qi, W.W. and Vanelli, T. 2002. Bioproduction of hydroxycinnamic acid. US Patent 6368837.

Gekko, K., and Timasheff, S.N. 1981. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. Biochemistry 20: 4667-4676.

Gerlsma, S.Y. and Stuur, E.R. 1974. The effect of combining two different alcohols on the heat-induced reversible denaturation of ribonuclease. Int. J. Pept. Protein Res. 6: 65-74.

Gilbert, H.J. and Tully, M. 1982. Synthesis and degradation of phenylalanine ammonia-lyase of *Rhodosporidium toruloides*. J. Bacteriol. 150: 498-505.

Gilbert, H.J., Stephenson, J.R. and Tully, M. 1983. Control of synthesis of functional mRNA coding for phenylalanine ammonia-lyase from *Rhodosporidium toruloides*. J. Bacteriol. 153: 1147-1154.

Givot, I.L., Smith, T.A., and Abeles, R.H. 1969. Studies on the mechanism of action and the structure of the electrophilic center of histidine ammonia lyase. J. Biol. Chem. 244: 6341-6353.

Gosset, G. 2009. Production of aromatic compounds in bacteria. Curr. Opin. Biotechnol. 20: 661-668.

Guerra, D., Anderson, A. and Salisbury, F. 1985. Reduced phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities and lignin synthesis in wheat grown under low pressure sodium lamps. Plant Physiol. 78**:** 126-130.

Guerriero, G., Alderliesten, R., Dingemans, T. and Benedictus, R. 2011. Thermotropic liquid crystalline polymers as protective coatings for aerospace. Prog. Org. Coat. 70: 245-251.

Gupta, R., Jung, E., and Brunak, S. 2004. Prediction of N-glycosylation sites in human proteins. In preparation.

Haard, N.F. and Wasserman, B. 1976. Induction of phenylalanine ammonia lyase in sweet potato (*Ipomoea batatas*) root by light irradiation and black rot infection. Physiol. Plant Pathol 8: 207-213.

Hammes, G.G. and Wu, C.W. 1974. Kinetics of allosteric enzymes. Annu. Rev. Biophys. Bioeng. 3: 1-33.

Hanson K. R. and Havir E. A. 1981. Phenylalanine ammonia-lyase. In *The Biochemistry of Plants* (Edited by Stumpf P. K. and Conn E. E.). 7: 577-625. Academic Press, New York.

Hanson, K.R. and Havir, E.A. 1972. The enzymatic elimination of ammonia. In: *The Enzymes*, Ed. by P. Boyer, Vol 7, 3rd ed, pp. 75-166. Academic Press, New York.

Hanson, K.R. and Havir, E.A. 1970. L-phenylalanine ammonia-lyase. IV. Evidence that the prosthetic group contains a dehydroalanyl residue and mechanism of action. Arch. Biochem. Biophys. 141: 1-17.

Hao, Z., Charles, D.J., Yu, L. and Simon, J.E. 1996. Purification and characterization of a phenylalanine ammonia-lyase from *Ocimum basilicum.* Phytochemistry 43: 735-739.

Harborne, J.B. and Williams, C. A., 2000: Advances in flavonoid research since 1992. Phytochemistry 55: 481-504.

Hassan, N.M., Serag, M.S., El-Feky, F.M. and Nemat Alla, M.M. 2008. *In vitro* selection of mung bean and tomato for improving tolerance to NaCl. Ann. Appl. Biol. 152: 319-330.

Hattori, T., Nishiyawa, A. and Shimada, M. 1999. Induction of L-phenylalanine ammonia-lyase and suppression of veratryl alcohol biosynthesis by exogenously added l-phenylalanine in white-rot fungus *Phanerochaete chrysosporium*. FEMS Lett. 179: 305-309.

Havir, E.A. 1981. Phenylalanine ammonia-lyase: Purification and characterization from soybean cell suspension cultures. Arch. Biochem. Biophys. 211: 556-563.

Havir, E.A. 1979. L-phenylalanine ammonia-lyase. Binding of polysaccharide by the enzyme from maize. Plant Sci. Lett. 16: 297-304.

Havir, E.A. and Hanson, K.R. 1975. L-Phenylalanine ammonia-lyase (maize, potato, and *Rhodotorula glutinis*). Studies of the prosthetic group with nitromethane. Biochemistry 14: 1620-1626.

Havir, E.A. and Hanson, K.R. 1973. L-Phenylalanine ammonia-lyase (maize and potato). Evidence that the enzyme is composed of four subunits. Biochemistry 12: 1583-1591.

Havir, E.A. and Hanson, K.R. 1968. L-Phenylalanine ammonia-lyase. I. Purification and molecular size of the enzyme from potato tubers. Biochemistry 5: 1896-1903.

Havir, E.A. and Hanson, K.R. 1968. L-phenylalanine ammonia-lyase. II. Mechanism and kinetic properties of the enzyme from potato tubers. Biochemistry 7: 1904-1914.

Havir, E.A., Reid, P.D. and Marsh, H.V. Jr 1971. L-Phenylalanine ammonia-lyase (maize). Evidence for a common catalytic site for L- phenylalanine and L- tyrosine. Plant Physiol. 48**:** 130-136.

Helmstaedt, K., Krappmann, S. and Braus, G.H. 2001. Allosteric regulation of catalytic activity: *Escherichia coli* aspartate transcarbamoylase versus yeast chorismate mutase. Microbiol. Mol. Biol. Rev. 65: 404-421.

Hendriksz, C.J. and Walter, J.H. 2004. Update on phenylketonuria. Current Paediatrics 14: 400-406.

Hermes, J.D., Weiss, P.M. and Cleland, W.W. 1985. Use of nitrogen-15 and deuterium isotope effects to determine the chemical mechanism of phenylalanine ammonia-lyase. Biochemistry 24: 2959-2967.

Hill, A.M., Thompson, B.L., Harris, J.P. and Segret, R. 2003. Investigation of the early stages in soraphen A biosynthesis. ChemBioChem 4: 1358-1359.

Hoagland, R.E. 1985. O-Benzylhydroxylamine: an inhibitor of phenylpropanoid metabolism in plants. Plant Cell Physiol. 26: 1353-1359.

Hodgins, D.S. 1971. Yeast phenylalanine ammonia-lyase. Purification, properties, and the identification of catalytically essential dehydroalanine. J. Biol. Chem. 246: 2977-2985.

Hoskins, J.A., Ambrus, J.L., Jack, G., Peiris, R.J.D., Starr, D.J.T., Wade, H.E., Wright, E.C., and Stern, J. 1980. Enzymatic control of phenylalanine intake in PKU. Lancet 1: 392-394.

Hsieh, L.S., Ma, G.J., Yang, C.C. and Lee, P.D. 2010. Cloning, expression, site-directed mutagenesis and immunolocalization of phenylalanine ammonia-lyase in *Bambusa oldhamii*. Phytochemistry 71: 1999-2009.

Innerarity, L.T., Smith, E.C. and Wender, S.H. 1972. Indoleacetic acid inhibition of a phenylalalnine ammonia lyase preparation from suspension cultures of WR-132 tobacco. Phytochemistry 11: 83-88.

Inoue, H., Nojima, H. and Okayama, H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene 96: 23-28.

Iredale, S.E. and Smith, H. 1974. Properties of phenylalanine ammonia lyase extracted from *Cucumis sativus* hypocotyls. Phytochemistry 13: 575-583.

Jia, S.R., Cui, J.D and Li, Y. 2008. Production of l-phenylalanine from *trans*-cinnamic acids by high-level expression of phenylalanine ammonia lyase gene from *Rhodosporidium toruloides* in *Escherichia coli.* Biochem. Eng. J. 42: 193-197.

Jiang, H., Wood, K.V. and Morgan, J.A. 2005. Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 71: 2962-2969.

Jin, X.M., Carfagna, C., Nicolais, L. and Lanzettag, R. 1995. Synthesis, characterization, and in vitro degradation of a novel thermotropic ternary copolyester based on *p*-hydroxybenzoic acid, glycolic acid, and *p*-hydroxycinnamic acid. Macromolecules 28: 4785-4794.

Jorrin, J. and Dixon, R. A. 1990. Stress responses in alfalfa (*Medicago sativa* L.) II. Purification, characterization and induction of phenylalanine ammonia lyase isoforms from elicitor-treated cell suspension cultures. Plant Physiol. 92: 447-455.

Jorrín, J., López-Valbuena, R. and Tena, M. 1988. Purification and properties of phenylalanine ammonia lyase from sunflower (*Helianthus annus* L.) hypocotyls. Biochim. Biophys. Acta, Gen. Subj. 964: 73-82.

Juneau, K., Miranda, M., Hillenmeyer, M., Nislow, C. and Davis, R. 2006. Introns regulate RNA and protein abundance in yeast. Genetics 174: 511-518.

Kalghatgi, K.K. and Subba Rao, P.V. 1975. Microbial L-phenylalanine ammonia lyase. Purification, subunit structure and kinetic properties of the enzyme from *Rhizoctonia solani*. Biochem. J. 149: 65-72.

Kane, J.F. and Fiske, M.J. 1985. Regulation of phenylalanine ammonia lyase in *Rhodotorula glutinis*. J. Bacteriol. 161: 963-966.

Khan, W., Balakrishnan, P. and Smith, D. 2003. Chitosan and chitin oligomers increase phenylalanine ammonia lyase and tyrosine ammonia-lyase activities in soybean leaves. J. Plant Physiol. 160: 859-863.

Kim, S.H., Virmani, D., Wake, K., McDonald, K., Kronstad, J.W. and Ellis, B.E. 2001. Cloning and disruption of a phenylalanine ammonia-lyase from *Ustilago maydis*. Curr. Genet. 40: 40-48.

Kim, S.H., Kronstad, J.W. and Ellis, B.E. 1996. Purification and characterization of phenylalanine ammonia-lyase from *Ustilago maydis*. Phytochemistry 43: 351-357.

Koizuka, N., Tanaka, Y. and Morohashi, Y. 1995. Expression of α-amylase in response to wounding in mung bean. Planta 195: 530-534.

Kong, F.M., Zhao, N., Siegel, M.M., Janota, K., Ashcroft, J.S., Koehn, F.E., Borders, D.B. and Carter, G.T. 1998. Saccharomicins, novel heptadecaglycoside antibiotics effective against multidrug-resistant bacteria. J. Am. Chem. Soc. 120: 13301-13311.

Koshland, D.E., Jr., Nemethy, G. and Filmer, D. 1966. Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry 5: 365-385.

Koukol, J. and Conn, E. 1961. The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase in *Hordeum vulgare*. J. Biol. Chem. 236: 2692-2695.

Koyama, H. 1984. A simple and rapid enzymatic determination of L-phenylalanine with a novel L-phenylalanine oxidase (deaminating and decarboxylating) from Pseudomonas sp. P-501. Clin. Chim. Acta. 136: 131-136.

Kozukue, N., Kozukue, E. and Kishiguchi, M. 1979. Changes in the contents of phenolic substances, phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) accompanying chilling-injury of eggplant fruit, and development of browning is closely related to chlorogenic acid, PAL and TAL. Sci. Hort. 11: 51-59.

Kradolfer, P., Zeyer, J., Miozzari, G. and Hutter, R. 1977. Dominant regulatory mutants in chorismate mutase of *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 2: 211-216.

Kumar, A. and Ellis, B.E. 2001. The phenylalanine ammonia-lyase gene family in raspberry: structure, expression, and evolution. Plant Physiol. 127: 230-239.

Kurland, C. and Gallant, J. 1996. Errors of heterologous protein expression. Curr. Opin. Biotechnol. 7: 489-493.

Kyndt, J., Meyer, T., Cusanovich, M. and Van Beeumen, J. 2002. Characterization of a bacterial tyrosine ammonia lyase, a biosynthetic enzyme for the photoactive yellow protein. FEBS Lett. 512: 240-244.

Kyndt, J.A., Vanrobaeys, F., Fitch, J.C., Devreese, B.V., Meyer, T.E., Cusanovich, M.A. and Van Beeumen, J.J. 2003. Heterologous production of *Halorhodospira halophila* holo-photoactive yellow protein through tandem expression of the postulated biosynthetic genes. Biochemistry 42: 965-970.

Kyndt, J.A., Hurley, J.K., Devreese, B., Meyer, T.E., Cusanovich, M.A., Tollin, G. and Van Beeumen, J.J. 2004. *Rhodobacter capsulatus* Photoactive yellow protein: Genetic context, spectral and kinetics characterization and mutagenesis. Biochemistry 43: 1809-1820.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lee, B.K., Park, M.R., Srinivas, B., Chun, J.C., Kwon, I.S., Chung, I.M. Yoo, N.H., Choi, K.G. and Yun, S.J. 2003. Induction of phenylalanine ammonia-lyase gene expression by paraquat and stress-related hormones in *Rehmannia glutinosa*. Mol. Cells 16: 34-39.

Lee, S.W., Robb, J. and Nazar, R.N. 1992. Truncated phenylalanine ammonia-lyase expression in tomato (*Lycopersicon esculentum*). J. Biol. Chem. 267: 11824-11830.

Liang, X., Dron, M., Cramer, C.L., Dixon, R.A. and Lamb, C.J. 1989. Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. J. Biol. Chem. 264: 14486-14492.

Logemann, E., Parniske, M. and Hahlbrock, K. 1995. Mode of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley, Proc. Natl. Acad. Sci. USA 92: 5905-5909.

Louie, G.V., Bowman, M.E., Moffitt, M.C., Baiga, T.J., Moore, B.S., and Noel, J.P. 2006. Structural determinants and modulation of substrate specificity in phenylalanine-tyrosine ammonia-lyases. Chem. Biol. 12: 1327-1338.

Mahesh, V., Rakotomalala, J.J., Le Gal, L., Vigne, H., de Kochko, A., Hamon, S., Noirot, M. and Campa, C. 2006. Isolation and genetic mapping of a *Coffea canephora* phenylalanine ammonia-lyase gene (CcPAL1) and its involvement in the accumulation of caffeoyl quinic acids. Plant Cell Rep. 25: 986-992.

Marasco, E. and Schmidt-Dannert, C. 2003. Towards the biotechnological production of aroma and flavour compounds in engineered microorganisms. Appl. Biotech. Food Sci. Policy 1: 145-147.

Marsh, H.V. Jr, Havir, E.A. and Hanson, K.R. 1968. L-Phenylalanine ammonia-lyase. 3. Properties of the enzyme from maize seedlings. Biochemistry 5: 1915-1918.

Marusich, W.C., Jensen, R.A. and Zamir, L.O. 1981. Induction of L-phenylalanine ammonia-lyase during utilization of phenylalanine as a carbon or nitrogen source in *Rhodotorula glutinis*. J. Bacteriol. 146: 1013-1019.

Menkes, J.H. 1967. The pathogenesis of mental retardation in phenylketonuria and other inborn errors of amino acid metabolism. Pediatrics 39: 297-308.

Meyer, T.E. Yakali, E., Cusanovich, M.A. and Tollin, G. 1987. Properties of a water-soluble, yellow protein isolated from a halophilic phototrophic bacterium that has photochemical activity analogous to sensory rhodopsin. Biochemistry 26: 418-423.

Michaelis, L. and Menten, M.L. 1913. Die kinetik der invertinwirkung. Biochem. Z. 49: 333-369.

Minami, E., Ozeki, Y., Matsuoka, M., Koizuka, N. and Tanaka, Y. 1989. Structure and some characterization of the gene for phenylalanine ammonia-lyase from rice plants, Eur. J. Biochem. 185: 19-25.

Minamikawa, T. and Uritani, I. 1965. Phenylalanine ammonia-lyase in sliced sweet potato roots. J. Biochem. 57: 678-688.

Moffitt, M.C., Louie, G.V., Bowman, M.E., Pence, J., Noel, J.P. and Moore, B.S. 2007. Discovery of two cyanobacterial phenylalanine ammonia lyases: Kinetic and structural characterization. Biochemistry 46: 1004-1012.

Mohanty, A.K. and Wiener, M.C. 2004. Membrane protein expression and production: effects of polyhistidine tag length and position. Protein Expr. Purif. 33: 311-325.

Monge, R.I., Lara, M. and Lopez-Munguia, A. 1995. Purification and stabilization of phenylalanine ammonia lyase from *Sporidiobolus pararoseus*. Biotechnol. Tech. 9: 423-428.

Montero, T., Mollá, E., Martín-Cabrejas, M. and López-Andreu, F. 1998. Effects of gibberellic acid (ga_3) on strawberry PAL (phenylalanine ammonia-lyase) and TAL (tyrosine ammonia-lyase) enzyme activities. J. Sci. Food Agric. 77**:** 230-234.

Morrison, T.A. and Buxton, D.R. 1993. Activity of phenylalanine ammonia lyase, tyrosine ammonia-lyase, and cinnamyl alcohol dehydrogenase in the maize stalks. Crop Sci. 33: 1264-1268.

Nakamichi, K., Yamada, N.S. and Chibata, I. 1983. Induction and stabilization of L-phenylalanine ammonia lyase activity in *Rhodotorula glutinis.* Eur. J. Appl. Microbiol. Biotechnol. 18: 158-162.

Nari, J., Mouttet, C., Pinna, M.H. and Ricard, J. 1972. Some physico-chemical properties of L-phenylalanine ammonia-lyase of wheat seedlings. FEBS Lett. 23: 220-224.

Nari, J., Mouttet, C., Francis, F. and Ricard, J. 1974. Subunit interactions in enzyme catalysis: kinetic analysis of subunit interactions in the enzyme L-phenylalanine ammonia lyase. Eur. J. Biochem. 41: 499-515.

Neish, A.C. 1961. Formation of M- and P-coumaric acids by enzymatic deamination of the corresponding Isomers of tyrosine. Phytochemistry 1: 1-24.

Nelson, D.L. and Cox, M.M. 2005. Lehninger - Principles of biochemistry. 4th ed. New York: W.H. Freeman and Company.

Nishiyama, Y., Yun, C.S., Matsuda, F., Sasaki, T., Saito, K. and Tozawa, Y. 2010. Expression of bacterial tyrosine ammonia-lyase creates a novel *p*-coumaric acid pathway in the biosynthesis of phenylpropanoids in Arabidopsis. Planta 232: 209-218.

Nugroho, L.H., Verberne, M.C. and Verpoorte, R. 2002. Activities of enzymes involved in the phenylpropanoid pathway in constitutively salicylic acid-producing tobacco plants. Plant Physiol. Biochem. 40: 755-760.

Oerum, H. and Rasmussen, O.F. 1992. Expression in *E. coli* of the gene encoding phenylalanine ammonia lyase from *Rhodosporidium toruloides*. Appl. Microbiol. Biotechnol. 36: 745-748.

Ogata, K., Uchlyama, K. and Yamada, H. 1967. Metabolism of aromatic amino acid in microorganisms. I. Formation of cinnamic acid from phenylalanine. Agric. Biol. Chem. 31: 200-206.

O'Neal, D. and Keller, C.J. 1970. Partial purification and some properties of phenylalanine ammonia-lyase of tobacco (*Nicotiana tabacum*). Phytochemistry 9: 1373-1383.

Parkhurst, J. and Hodgins, D. 1971. Phenylalanine and tyrosine ammonia-lyase activity in *Sporobolomyces pararoseus*. Phytochemistry 10: 2997-3000.

Parkhurst, J.R. and Hodgins, D.S. 1972. Yeast phenylalanine ammonia-lyase. Properties of the enzyme from *Sporobolomyces pararoseus* and its catalytic site. Arch. Biochem. Biophys. 152: 597-605.

Pellegrini, L., Rohfritsch, O., Fritig, B. and Legrand, M. 1994. Phenylalanine ammonia-lyase in tobacco. Plant Physiol. 106: 877-886.

Peterkofsky, A. 1962.The mechanism of action of histidase: amino-enzyme formation and partial reactions. J. Biol. Chem. 237: 787-795.

Pilbák, S., Tomin, A., Rétey, J. and Poppe, L. 2006. The essential tyrosine-containing loop conformation and the role of the C-terminal multi-helix region in eukaryotic phenylalanine ammonia-lyases. FEBS Journal 273: 1004-1019.

Poppe, L. 2001. Methylidene-imidazolone: a novel electrophile for substrate activation. Curr. Opin. Chem. Biol. 5: 512-524.

Poppe, L. and Rétey, J. 2003. Properties and synthetic applications of ammonia-lyases. Curr. Org. Chem. 7: 1297-1315.

Poppe, L. and Retey, J. 2005. Friedel-Crafts-type mechanism for the enzymatic elimination of ammonia from histidine and phenylalanine. Angew. Chem. Int. Ed. Engl. 44: 3668-3688.

Priefert, H., Rabenhorst, J. and Steinbüchel, A. 2001. Biotechnological production of vanillin. Appl. Microbiol. Biotechnol. 569: 296-314.

Qi, W.W., Vannelli, T., Breinig, S., Ben-Bassat, A., Gatenby, A.A., Haynie, S.L. and Sariaslani, F.S. 2007. Functional expression of prokaryotic and eukaryotic genes in *Escherichia coli* for conversion of glucose to *p*-hydroxystyrene. Metab. Eng. 9: 268-276.

Régnier, T. and Macheix, J. 1996. Changes in wall-bound phenolic acids, phenylalanine and tyrosine ammonia-lyases, and peroxidases in developing durum wheat grains (*Triticum turgidum* l. var. durum). J. Agric. Food Chem. 44: 1727-1730.

Reid, P.D., Havir, E.A. and Marsh, H.V. 1972. L-Phenylalanine ammonia-lyase (Maize): Partial purification and response to gibberellic acid and cycloheximide of L-phenylalanine and L-tyrosine ammonia-lyase activities. Plant physiol. 50: 480-484.

Ritter, H. and Schulz, G.E. 2004. Structural basis for the entrance into the phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase. Plant Cell 16: 3426-3436.

Rösler, J., Krekel, F., Amerhein, N. and Schmid, J. 1997. Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. Plant Physiol. 113: 175-179.

Röther, D., Merkel, D. and Rétey, J. 2000. Spectroscopic evidence for a 4-methylidene imidazol-5-one in histidine and phenylalanine ammonia-lyases. Angew. Chem. Int. Eds. 39: 2462-2464.

Röther, D., Poppe, L., Viergutz, S., Langer, B. and Rétey, J. 2001. Characterization of the active site of histidine ammonia-lyase from *Pseudomonas putida*. Eur. J. Biochem. 268: 6011-6019.

Röther, D., Poppe, L., Morlock, G., Viergutz, S. and Rétey, J. 2002. An active site homology model of phenylalanine ammonia-lyase from *Petroselinum crispum*. Eur. J. Biochem. 269: 3065-3075.

Ruwart, M.J. and Suelter, C.H. 1971. Activation of yeast pyruvate kinase by natural and artificial cryoprotectants. J. Biol. Chem. 246: 5990-5993.

Sambrook, J., Fritschi, E.F. and Maniatis, T. 1989. Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, New York.

Sarkissian, C.N. and Gamez, A. 2005. "Phenylalanine ammonia lyase, enzyme substitution therapy for phenylketonuria, where are we now?" Mol. Genet. Metab. 86: S22-6. (suppl 1).

Sarkissian, C.N., Shao, Z., Blain, F., Peevers, R., Su, H., Heft, R., Chang, T.M.S. and Scriver, C.R. 1999. A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase. Proc. Natl. Acad. Sci. USA 96: 2339-2344.

Sarma, A.D., Sreelakshmi, Y. and Sharma, R.1998. Differential expression and properties of phenylalanine ammonia-lyase isoforms in tomato leaves. Phytochemistry 49: 2233-2243.

Sarma, A.D. and Sharma, R. 1999. Purification and characterization of UV-B induced phenylalanine ammonia-lyase from rice seeding. Phytochemistry 50: 729-737.

Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. 2001. Industrial biocatalysis today and tomorrow. Nature 409: 258-268.

Schroeder, A.C., Kumaran, S., Hicks, L.M., Cahoon, R.E., Halls, C., Yu, O. and Jez, J.M. 2008. Contributions of conserved serine and tyrosine residues to catalysis, ligand binding, and cofactor processing in the active site of tyrosine ammonia lyase. Phytochemistry 69: 1496-1506.

Schulz, W., Eiben, H.G. and Hahlbrock, K. 1989. Expression in *Escherichia coli* of catalytically active phenylalanine ammonia lyase from parsley. FEBS Lett. 258: 335-338.

Schuster, B. and Rétey, J. 1995. The mechanism of action of phenylalanine ammonia-lyase: the role of prosthetic dehydroalanine. Proc. Natl. Acad. Sci. USA 92: 8433-8437.

Schwede, T.F., Rétey, J. and Schulz, G.E. 1999. Crystal structure of histidine ammonia-lyase revealing a novel polypeptide modification as the catalytic electrophile. Biochemistry 38: 5355-5361.

Scott, D., Hammond, P., Brearley, G. and Price, C. 1992. Identification by high-performance liquid chromatography of tyrosine ammonia-lyase activity in purified fractions of *Phaseolus vulgaris* phenylalanine ammonia-lyase. J. Chromatogr. 573**:** 309-312.

Scriver, C.R., Waters, P.J., Sarkissian, C., Ryan, S., Prevost, L., Cote, D., Novak, J., Teebi, S. and Nowacki, P.M. 2000. PAHdb: a locus-specific knowledgebase. Hum. Mutat. 15: 99-104.

Scriver, C., Kaufman, S., Eisensmith, R.C. and Woo, S.L. 1995. The hyperphenyl-alaninemias. In: Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. Editors, The

Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 1015-1075.

Scriver, C.R., Kaufman, S. and Woo, S.L.C. 1989. in The Metabolic Basis of Inherited Disease, ed. by Scriver, C.R., Beaudt, A.L., Sly, W.S., and Valle, D. McGraw-Hill, New York, 495-546.

Sgarbi, E., Fornasiero, R.B., Lins, A.P. and Bonatti, P.M. 2003. Phenol metabolism is differentially affected by ozone in two cell lines from grape (*Vitis vinifera* L.) leaf. Plant Sci. 165: 951-957.

Shaw, N.M., Bolwell, G.P. and Smith, C. 1990. Wound-induced phenylalanine ammonia-lyase by L-phenylalanine in potato (*Solanum tuberosum*) tuber discs. Significance of glycosylation and immunolocalization of enzyme subunits. Biochem. J. 267:163-170.

Shen, R.S. and Abell, C.W. 1977. Phenylketonuria: A new method for the simultaneous determination of plasma phenylalanine and tyrosine. Science 197: 665-667.

Singh, M.P. Petersen, P.J., Weiss, W.J., Kong, F. and Greenstein, M. 2000. Saccharomicins, novel heptadecaglycoside antibiotics produced by *Saccharothrix espanaensis*: Antibacterial and mechanistic activities. Antimicrob. Agents Chemother. 44: 2154-2159.

Solecka, D. and Kacperska, A. 2003. Phenylpropanoid deficiency affects the course of plant acclimation to cold. Physiol. Plant. 119: 253-262.

Song, J. and Wang, Z. 2009. Molecular cloning, expression and characterization of a phenylalanine ammonia-lyase gene (SmPAL1) from *Salvia miltiorrhiza*. Mol. Biol. Rep. 36: 939-952.

Sparnins, V.L., Burbee D.G., and Dagley, S. 1979. Catabolism of L-tyrosine in *Trichosporon cutaneum*. J. Bacteriol. 138: 425-430.

Springob, K., Nakajima, J., Yamazaki, M. and Saito, K. 2003. Recent advances in the biosynthesis and accumulation of anthocyanins. Nat. Prod. Rep. 20: 288-303.

Staby, A. and Jensen, I.H. 2001. Comparison of chromatographic ion-exchange resins: II. More strong anion-exchange resins. J. Chromatogr. A. 908: 149-161.

Subba Rao, P.V., Moore, K. and Towers, G.H. 1967. Degradation of aromatic amino acids by fungi. II. Purification and properties of phenylalanine ammonia-lyase from *Ustilago hordei*. Can. J. Biochem. 45: 1863-1872.

Tanaka, Y., Tsuda, S. and Kusumi, T. 1998. Metabolic engineering to modify flower color. Plant Cell Physiol. 39: 1119-1126.

Tang, X. 2003. Polynucleotide encoding a mutant *Rhodotorula glutinis* tyrosine ammonia lyase polypeptide. E. I. du Pont de Nemours and Company United States Patent and Trademark Office Granted Patent US6521748.

Taylor, R.G., Lambert, M.A., Sexsmith, E., Sadler, S.J., Ray, P.N., Mahuran, D.J. and McInnes, R.R. 1990. Cloning and expression of rat histidase: homology to two bacterial histidases and four phenylalanine ammonia-lyases. J. Biol. Chem. 265: 18192-18199.

Trotman, R.J. Camp, C.E., Ben-Bassat, A., Dicosimo, R., Huang, L., Crum, G.A., Sariaslani, F.S. and Haynie, S.L. 2007. Calcium alginate bead immobilization of cells containing tyrosine ammonia lyase activity for use in the production of *p*-hydroxy-cinnamic acid. Biotechnol. Prog. 23: 638-644.

Vanbeneden, N., Gils, F. Delvaux, F. and Delvaux, F. R. 2007. Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: Occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. Food Chem. 107: 221-230.

Vannelli., T., Xue, Z., Breinig, S., Qi, W. and Sariaslani, F. S. 2007a. Functional expression in *Escherichia coli* of the tyrosine-inducible tyrosine ammonia-lyase enzyme from yeast *Trichosporon cutaneum* for production of *p*-hydroxycinnamic acid. Enzym. Microb. Tech. 41: 413-422.

Vannelli, T., Qi, W.W., Sweigard, J., Gatenby, A.A. and Sariaslani, F.S. 2007b. Production of *p*-hydroxycinnamic acid from glucose in *Saccharomyces cerevisiae* and *Escherichia coli* by expression of heterologous genes from plants and fungi. Metabol. Eng. 9: 142-151.

Verhoef, S., Ruijssenaars, H.J., de Bont, J.A., Wery, J. 2007. Bioproduction of *p*-hydroxybenzoate from renewable feedstock by solvent- tolerant *Pseudomonas putida* S12. J. Biotechnol. 132: 49-56.

Verhoef, S., Wierckx, N., Westerhof, R.G., de Winde, J.H. and Ruijssenaars, H. J. 2009. Bioproduction of *p*-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. Appl. Environ. Microbiol. 75: 931-936.

Verhoef, S., Ballerstedt, H., Volkers, R.J.M., de Winde, J.H. and Ruijssenaars, H.J. 2010. Comparative transcriptomics and proteomics of *p*-hydroxybenzoate producing *Pseudomonas putida* S12: novel responses and implications for strain improvement. Appl. Microbiol. Biotechnol. 87: 679-690.

Wall, M.J., Quinn, A.J. and D'Cunha, G.B. 2008. Manganese ($Mn^{2+)}$ dependent storage stabilization of *Rhodotorula glutinis* phenylalanine ammonia-lyase activity. J. Agric. Food Chem. 56: 894-902.

Walsh, C. 1979. Enzymatic reaction mechanisms, W.H. Freeman and Company, San Francisco.

Walter, J.H., White, F.J., Hall, S.K., MacDonald, A., Rylance, G., Boneh, A., Francis, D. E., Shortland, G.J. Schmidt, M. and Vail, A. 2002. How practical are recommendations for dietary control in phenylketonuria? Lancet 360: 55-57.

Walter, H., Brooks, D.E. and Fisher, D. 1985. Partitioning in aqueous two-phase systems, Academic Press, New York.

Wang, L., Gamez, A., Sarkissian, C.N., Straub, M., Patch, M.G., Won Han, G., Striepeke, S., Fitzpatrick, P., Scriver, C.R., and Stevens, R.C. 2005. Structure-based chemical modification strategy for enzyme replacement treatment of phenylketonuria. Mol. Genet. Metab. 86: 134-140.

Wang, X., Engel, J. and Liu, C. 2003. Liquid crystal polymer (LCP) for MEMS: processes and applications. J. Micromech. Microeng.13: 628-633.

Wanner, L.A., Li, G., Ware, D., Somssich, I.E. and Davis, K.R. 1995. The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant. Mol. Biol. 27: 327-338.

Watts, K., Lee, P. and Schmidt-Dannert, C. 2004. Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*. Chembiochem. 5: 500-507.

Watts, K.T., Mijts, B.N., Lee, P.C., Manning, A.J. and Schmidt-Dannert, C. 2006. Discovery of a substrate selectivity switch in tyrosine ammonia-lyase, a member of the aromatic amino acid lyase family. Chem. Biol. 13: 1317-1326.

Wen, P.F., Chen, J.Y. Kong, W.F. Pan, Q.H., Wan, S.B. and Huang, W.D. 2005. Salicylic acid induced the expression of phenylalanine ammonia-lyase gene in grape berry. Plant Sci. 169: 928-938.

Wickner, R.B. 1969. Dehydroalanine in histidine ammonia lyase. J. Biol. Chem. 244: 6550-6552.

Williams, J.S., Thomas, M. and Clarke, D.J. 2005. The gene stlA encodes a phenylalanine ammonia-lyase that is involved in the production of a stilbene antibiotic in *Photorhabdus luminescens* TT01. Microbiol. 151: 2543-2550.

Xiang, L. and Moore, B.S. 2005. Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. J. Bacteriol. 187: 4286-4289.

Xiong, H. and Pegg, A.E. 1999. Mechanistic studies of the processing of human S-adenosylmethionine decarboxylase proenzyme. Isolation of an ester intermediate. J. Biol. Chem. 274: 35059-35066.

Xu, F., Cai, R., Cheng, S., Du, H., Wang, Y. and Cheng, S. 2008. Molecular cloning, characterization and expression of phenylalanine ammonia-lyase gene from *Ginkgo biloba*. Afr. J. Biotechnol. 7: 721-729.

Xue, Z., McCluskey, M., Cantera, K., Ben-Bassat, A., Sariaslani, F. S. and Huang, L. 2007a. Improved production of *p*-hydroxycinnamic acid from tyrosine using a novel thermostable phenylalanine/tyrosine ammonia lyase enzyme. Enzyme Microb. Technol. 42: 58-64.

Xue, Z., McCluskey, M., Cantera, K., Sariaslani, F.S. and Huang, L. 2007b. Identification, characterization and functional expression of a tyrosine ammonia-lyase and its mutants from the photosynthetic bacterium *Rhodobacter sphaeroides*. J. Ind. Microbiol. Biotechnol. 34: 599-604.

Yadava, A. and Ockenhouse, C.F. 2003. Effect of codon optimization on expression levels of a functionally folded malaria vaccine candidate in prokaryotic and eukaryotic expression systems. Infect. Immun. 71: 4961-4969.

Yamada, S., Nabe, K., Izuo, N., Nakamichi, K. and Chibata, I. 1981. Production of L-phenylalanine from *trans*-cinnamic acid with *Rhodotorula glutinis* containing L-phenylalanine ammonia-lyase activity. Appl. Environ. Microbiol. 42: 773-778.

Young, M.R., Towers, G.H.N. and Neish, A.C. 1966. Taxonomic distribution of ammonia-lyases for L-phenylalanine and L-tyrosine in relation to lignification. Can. J. Bot. 44: 314-349.

Yue, H., Yuan, Q. and Wang, W. 2007. Purification of phenylalanine ammonia-lyase in PEG1000/Na_2SO_4 aqueous two-phase system by a two-step extraction. Biochem. Eng. J. 37: 231-237.

Zhang, Y., Li, S.Z., Pan, X., Cahoon, R.E., Jaworski, J.G., Wang, X. Jez, J.M., Chen, F. and Yu, O. 2006. Using unnatural protein fusions to engineer resveratrol biosynthesis in yeast and mammalian cells. J. Am. Chem. Soc. 128: 13030-13031.

Zimmermann, A. and Hahlbrock, K. 1975. Light-induced changes of enzyme activities in parsley cell suspension cultures: Purification and some properties of phenylalanine ammonia-lyase (E.C. 4.3.1.5). Arch. Biochem. Biophys. 166: 54-62.

Zucker, M. 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiol. 40: 779-784.

APPENDIX: CLUSTAL 2.0.12 multiple sequence alignment of two independent clones vs patent DNA

```
R1              ATGTTTATTGAGACCAATGTCGCCAAGCCCGCTTCCACCAAGGCGATGAACGCCGGTTCG 60
R2              ATGTTTATTGAGACCAATGTCGCCAAGCCCGCTTCCACCAAGGCGATGAACGCCGGTTCG 60
Patent          ATGTTTATTGAGACCAATGTCGCCAAGCCCGCTTCCACCAAGGCGATGAACGCCGGTTCG 60
                ************************************************************

R1              GCCAAGGCCGCTCCTGTGTGAGTACCCACCACTAACTGGGGAGTCACCGCTGACATGCAG 120
R2              GCCAAGGCCGCTCCTGTGTGAGTACCCACCACTAACTGGGGAGTCACCGCTGACATGCAG 120
Patent          GCCAAGGCCGCTCCTGTGTGAGTACCCACCACTAACTGGGGAGTCACCGCTGACATGCAG 120
                ************************************************************

R1              TGAGCCGTTCGCTACCTATGCCCACTCCCAGGCTACCAAGACCGTCAGCATCGACGGCCA 180
R2              TGAGCCGTTCGCTACCTATGCCCACTCCCAGGCTACCAAGACCGTCAGCATCGACGGCCA 180
Patent          TGAGCCGTTCGCTACCTATGCCCACTCCCAGGCTACCAAGACCGTCAGCATCGACGGCCA 180
                ************************************************************

R1              CACCATGAAGGTCGGTGACGTCGTCGCCGTCGCCCGCCACGGCGCCAAGGTCGAGCTCGC 240
R2              CACCATGAAGGTCGGTGACGTCGTCGCCGTCGCCCGCCACGGCGCCAAGGTCGAGCTCGC 240
Patent          CACCATGAAGGTCGGTGACGTCGTCGCCGTCGCCCGCCACGGCGCCAAGGTCGAGCTCGC 240
                ************************************************************

R1              GGCCTCGGTCGCCGGCCCCGTCCAGGCCTCGGTCGACTTCAAGGAGTCCAAGAAGCACAC 300
R2              GGCCTCGGTCGCCGGCCCCGTCCAGGCCTCGGTCGACTTCAAGGAGTCCAAGAAGCACAC 300
Patent          GGCCTCGGTCGCCGGCCCCGTCCGGGCCTCGGTCGACTTCAAGGAGTCCAAGAAGCACAC 300
                ********************** ***********************************

R1              GTCGATCTACGGCGTCACCACCGGCTTTGGCGGCTCGGCCGACACGCGCACCAGCGACAC 360
R2              GTCGATCTACGGCGTCACCACCGGCTTTGGCGGCTCGGCCGACACGCGCACCAGCGACAC 360
Patent          GTCGATCTACGGCGTCACCACCGGCTTTGGCGGCTCGGCCGACACGCGCACCAGCGACAC 360
                ************************************************************

R1              CGAGGCGCTCCAGATCTCGCTCCTCGAGCACCAGCTCTGCGGCTTCCTCCCCACCGACGC 420
R2              CGAGGCGCTCCAGATCTCGCTCCTCGAGCACCAGCTCTGCGGCTTCCTCCCCACCGACGC 420
Patent          CGAGGCGCTCCAGATCTCGCTCCTCGAGCACCAGCTCTGCGGCTTCCTCCCCACCGACGC 420
                ************************************************************

R1              CACCTACGAGGGCATGCTCCTCGCCGCGATGCCGATCCCCATCGTCCGCGGCGCCATGGC 480
R2              CACCTACGAGGGCATGCTCCTCGCCGCGATGCCGATCCCCATCGTCCGCGGCGCCATGGC 480
Patent          CACCTACGAGGGCATGCTCCTCGCCGCGATGCCGATCCCCATCGTCCGCGGCGCCATGGC 480
                ************************************************************

R1              CGTCCGCGTCAACAGCTGCGTCCGCGGCCACTCGGGCGTCCGCCTCGAGGTCCTCCAGTC 540
R2              CGTCCGCGTCAACAGCTGCGTCCGCGGCCACTCGGGCGTCCGCCTCGAGGTCCTCCAGTC 540
Patent          CGTCCGCGTCAACAGCTGCGTCCGCGGCCACTCGGGCGTCCGCCTCGAGGTCCTCCAGTC 540
                ************************************************************

R1              GTTTGCCGACTTTATCAACAGAGGCCTCGTCCCCTGCGTGCCCCTCCGCGGCACCATCTC 600
R2              GTTTGCCGACTTTATCAACAGAGGCCTCGTCCCCTGCGTGCCCCTCCGCGGCACCATCTC 600
Patent          GTTTGCCGACTTTATCAACAGAGGCCTCGTCCCCTGCGTGCCCCTCCGCGGCACCATCTC 600
                ************************************************************

R1              GGCCTCGGGCGACCTCTCGCCCCTCTCGTACATTGCCGGTGCGATCTGCGGCCACCCCGA 660
R2              GGCCTCGGGCGACCTCTCGCCCCTCTCGTACATTGCCGGTGCGATCTGCGGCCACCCCGA 660
Patent          GGCCTCGGGCGACCTCTCGCCCCTCTCGTACATTGCCGGTGCGATCTGCGGCCACCCCGA 660
                ************************************************************

R1              CGTCAAGGTGTTCGACACCGCGGCGTCGCCCCCCACGGTTCTCACCTCCCCCGAGGCGAT 720
R2              CGTCAAGGTGTTCGACACCGCGGCGTCGCCCCCCACGGTTCTCACCTCCCCCGAGGCGAT 720
Patent          CGTCAAGGTGTTCGACACCGCGGCGTCGCCCCCCACGGTTCTCACCTCCCCCGAGGCGAT 720
                ************************************************************

R1              CGCCAAGTACGGCCTCAAGACCGTCAAGCTCGCCTCCAAGGAGGGCCTCGGCCTCGTCAA 780
R2              CGCCAAGTACGGCCTCAAGACCGTCAAGCTCGCCTCCAAGGAGGGCCTCGGCCTCGTCAA 780
Patent          CGCCAAGTACGGCCTCAAGACCGTCAAGCTCGCCTCCAAGGAGGGCCTCGGCCTCGTCAA 780
                ************************************************************

R1              CGGCACGGCCGTCTCGGCGGCCGCGGGCGCGCTCGCGCTCTACGACGCCGAGTGCCTCGC 840
```

```
R2              CGGCACGGCCGTCTCGGCGGCCGCGGGCGCGCTCGCGCTCTACGACGCCGAGTGCCTCGC 840
Patent          CGGCACGGCCGTCTCGGCGGCCGCGGGCGCGCTCGCGCTCTACGACGCCGAGTGCCTCGC 840
                ************************************************************

R1              CATCATGAGCCAGACCAACACTG**C**GCTCACGGTCGAGGCGCTCGACGGCCACGTCGGCTC 900
R2              CATCATGAGCCAGACCAACACTG**C**GCTCACGGTCGAGGCGCTCGACGGCCACGTCGGCTC 900
Patent          CATCATGAGCCAGACCAACACTG**T**GCTCACGGTCGAGGCGCTCGACGGCCACGTCGGCTC 900
                ********************** ***********************************

R1              GTTTGCCCCCTTCATCCAGGAGATCCGCCCTCACG**T**CGGCCAGATCGAGGCCGCTAGAAA 960
R2              GTTTGCCCCCTTCATCCAGGAGATCCGCCCTCACG**T**CGGCCAGATCGAGGCCGCTAGAAA 960
Patent          GTTTGCCCCCTTCATCCAGGAGATCCGCCCTCACG**C**CGGCCAGATCGAGGCCGCTAGAAA 960
                ********************************** ************************

R1              CATTAGACACATGCTCGGTGGCTCCAAGCTCGCCGTGCACGAGGAG**C**CCGAGCTCCTCGC 1020
R2              CATTAGACACATGCTCGGTGGCTCCAAGCTCGCCGTGCACGAGGAG**C**CCGAGCTCCTCGC 1020
Patent          CATTAGACACATGCTCGGTGGCTCCAAGCTCGCCGTGCACGAGGAG**T**CCGAGCTCCTCGC 1020
                ********************************************** *************

R1              CGACCAGGACGCCGGCATCCTCCGCCAGGACCGCTACGCGCTCCGCACCTCGGCGCAGTG 1080
R2              CGACCAGGACGCCGGCATCCTCCGCCAGGACCGCTACGCGCTCCGCACCTCGGCGCAGTG 1080
Patent          CGACCAGGACGCCGGCATCCTCCGCCAGGACCGCTACGCGCTCCGCACCTCGGCGCAGTG 1080
                ************************************************************

R1              GATCGGCCCGCAGCTCGAGGCGCTCGGCCTCGCCCGCCAGCAGATCGAGACCGAGCTCAA 1140
R2              GATCGGCCCGCAGCTCGAGGCGCTCGGCCTCGCCCGCCAGCAGATCGAGACCGAGCTCAA 1140
Patent          GATCGGCCCGCAGCTCGAGGCGCTCGGCCTCGCCCGCCAGCAGATCGAGACCGAGCTCAA 1140
                ************************************************************

R1              CTCGACCACCGACAACCCGCTCATCGATGTCGAGGGCGGCATGTTCCACCACGGCGGCAA 1200
R2              CTCGACCACCGACAACCCGCTCATCGATGTCGAGGGCGGCATGTTCCACCACGGCGGCAA 1200
Patent          CTCGACCACCGACAACCCGCTCATCGATGTCGAGGGCGGCATGTTCCACCACGGCGGCAA 1200
                ************************************************************

R1              CTTCCAGGCCATGGCCGTCACCTCGGCCATGGACTCGGCCCGCATCGTCCTCCAGAACC 1259
R2              CTTCCAGGCCATGGCCGTCACCTCGGCCATGGACTCGGCCCGCATCGTCCTCCAGAACC 1260
Patent          CTTCCAGGCCATGGCCGTCACCTCGGCCATGGACTCGGCCCGCATCGTCCTCCAGAACC 1259
                ************************************************************

R1              TCGGCAAGCTCAGCTTTGCCCAGGTCACCGAGCTCATCAACTGCGAGATGAACCACGG**C**C 1319
R2              TCGGCAAGCTCAGCTTTGCCCAGGTCACCGAGCTCATCAACTGCGAGATGAACCACGG**C**C 1319
Patent          TCGGCAAGCTCAGCTTTGCCCAGGTCACCGAGCTCATCAACTGCGAGATGAACCACGG**N**C 1319
                ************************************************************ *

R1              TCCCCTCCAACCTCGCCGGCTCCGAGCCTAGCACCAACTACCACTGCAAGGGTCTCGACA 1379
R2              TCCCCTCCAACCTCGCCGGCTCCGAGCCTAGCACCAACTACCACTGCAAGGGTCTCGACA 1379
Patent          TCCCCTCCAACCTCGCCGGCTCCGAGCCTAGCACCAACTACCACTGCAAGGGTCTCGACA 1379
                ************************************************************

R1              TCCACTGCGGCGCCTACTGCGCCGAGCTCGGCTTCCTCGCCAACCCCATGAGCAACCACG 1439
R2              TCCACTGCGGCGCCTACTGCGCCGAGCTCGGCTTCCTCGCCAACCCCATGAGCAACCACG 1439
Patent          TCCACTGCGGCGCCTACTGCGCCGAGCTCGGCTTCCTCGCCAACCCCATGAGCAACCACG 1439
                ************************************************************

R1              TCCAGAGCACCGAGATGCACAACCAGAGCGTGAACTCGATGGCGTTCGCGTCCGCCCGCA 1499
R2              TCCAGAGCACCGAGATGCACAACCAGAGCGTGAACTCGATGGCGTTCGCGTCCGCCCGCA 1499
Patent          TCCAGAGCACCGAGATGCACAACCAGAGCGTGAACTCGATGGCGTTCGCGTCCGCCCGCA 1499
                ************************************************************

R1              **A**GACGATGGAGGCCAACGAGGTCCTCTCGCTCCTCCTCGGCTCGCAGATGTACTGCGCGA 1559
R2              **A**GACGATGGAGGCCAACGAGGTCCTCTCGCTCCTCCTCGGCTCGCAGATGTACTGCGCGA 1559
Patent          **G**GACGATGGAGGCCAACGAGGTCCTCTCGCTCCTCCTCGGCTCGCAGATGTACTGCGCGA 1559
                 ***********************************************************

R1              CCCAGGCCCTCGACCTCCGCGTCATGGAGGTCAAGTTCAAGATGGCCATCGTCAAGCTCC 1619
R2              CCCAGGCCCTCGACCTCCGCGTCATGGAGGTCAAGTTCAAGATGGCCATCGTCAAGCTCC 1619
Patent          CCCAGGCCCTCGACCTCCGCGTCATGGAGGTCAAGTTCAAGATGGCCATCGTCAAGCTCC 1619
                ************************************************************

R1              TCAACGAGACCCTCACCAAGCACTTTGCGGCCTTCCTCACGCCCGAGCAGCTCGCCAAGC 1679
R2              TCAACGAGACCCTCACCAAGCACTTTGCGGCCTTCCTCACGCCCGAGCAGCTCGCCAAGC 1679
```

```
Patent          TCAACGAGACCCTCACCAAGCACTTTGCGGCCTTCCTCACGCCCGAGCAGCTCGCCAAGC 1679
                ************************************************************

R1              TCAACACCCACGCCGCCATCACGCTGTACAAGCGCCTCAACCAGACGCCCAGCTGGGACT 1739
R2              TCAACACCCACGCCGCCATCACGCTGTACAAGCGCCTCAACCAGACGCCCAGCTGGGACT 1739
Patent          TCAACACCCACGCCGCCATCACGCTGTACAAGCGCCTCAACCAGACGCCCAGCTGGGACT 1739
                ************************************************************

R1              CGGCCCCGCGCTTCGAGGACGCCGCCAAGCACCTCGTCGGCGTCATCATGGACGCCCTCA 1799
R2              CGGCCCCGCGCTTCGAGGACGCCGCCAAGCACCTCGTCGGCGTCATCATGGACGCCCTCA 1799
Patent          CGGCCCCGCGCTTCGAGGACGCCGCCAAGCACCTCGTCGGCGTCATCATGGACGCCCTCA 1799
                ************************************************************

R1              TGGTCAACGACGACATCACCGACCTCACCAACCTCCCCAAGTGGAAGAAGGAGTTCGCCA 1859
R2              TGGTCAACGACGACATCACCGACCTCACCAACCTCCCCAAGTGGAAGAAGGAGTTCGCCA 1859
Patent          TGGTCAACGACGACATCACCGACCTCACCAACCTCCCCAAGTGGAAGAAGGAGTTCGCCA 1859
                ************************************************************

R1              AGGAGGCCGGCAACCTCTACCGCTCGATCCTCGTCGCGACCACCGCCGACGGCCGCAACG 1919
R2              AGGAGGCCGGCAACCTCTACCGCTCGATCCTCGTCGCGACCACCGCCGACGGCCGCAACG 1919
Patent          AGGAGGCCGGCAACCTCTACCGCTCGATCCTCGTCGCGACCACCGCCGACGGCCGCAACG 1919
                ************************************************************

R1              ACCTCGAGCCCGCCGAGTACCTCGGCCAGACGCGCGCCGTCTACGAGGCCGTCCGCTCCG 1979
R2              ACCTCGAGCCCGCCGAGTACCTCGGCCAGACGCGCGCCGTCTACGAGGCCGTCCGCTCCG 1979
Patent          ACCTCGAGCCCGCCGAGTACCTCGGCCAGACGCGCGCCGTCTACGAGGCCGTCCGCTCCG 1979
                ************************************************************

R1              AGCTCGGCGTCAAGGTCCGCCGCGGCGACGTCGCCGAGGGCAAGAGCGGCAAGAGCATCG 2039
R2              AGCTCGGCGTCAAGGTCCGCCGCGGCGACGTCGCCGAGGGCAAGAGCGGCAAGAGCATCG 2039
Patent          AGCTCGGCGTCAAGGTCCGCCGCGGCGACGTCGCCGAGGGCAAGAGCGGCAAGAGCATCG 2039
                ************************************************************

R1              GCTCGAGCGTCGCCAAGATCGTCGAGGCGATGCGCGACGGCCGCCTCATGGGCGCTGTTG 2099
R2              GCTCGAGCGTCGCCAAGATCGTCGAGGCGATGCGCGACGGCCGCCTCATGGGCGCTGTTG 2099
Patent          GCTCGAGCGTCGCCAAGATCGTCGAGGCGATGCGCGACGGCCGCCTCATGGGCGCTGTTG 2099
                ************************************************************

R1              GCAAGATGTTCTAA 2113
R2              GCAAGATGTTCTAA 2113
Patent          GCAAGATGTTC--- 2110
                ***********
```