



<http://researchspace.auckland.ac.nz>

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.

<http://researchspace.auckland.ac.nz/feedback>

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.

THE BIOSYNTHESIS AND CONTROL OF INDOLEACETIC ACID

by

Robert Malcolm Simpson

This thesis is presented in
fulfilment of the requirements
for the degree of Doctor of
Philosophy in Biochemistry at
the University of Auckland.

January, 1993.

ABSTRACT

Attempts were made to form indoleacetic acid in cellfree extracts of mung bean (*Vigna radiata*) shoots. The extracts were incubated with radiolabelled tryptophan and other substrates and cofactors thought to be involved in indoleacetic acid biosynthesis. After incubation indolepyruvate and indoleacetic acid were separated and quantified by HPLC. There was no significant difference in the conversion of tryptophan to indolepyruvate and indoleacetic acid between the incubations and control incubations using boiled extract.

The concentrations of indolepyruvate and indoleacetic acid in mung bean hypocotyl suspension cultures were measured using GC-MS SIM over the growth of the culture, a period of 29 days. Indoleacetic acid concentrations, although scattered, mostly remained at constant low levels in the range of 6 to 9ng/g fwt of culture. The indolepyruvate levels steadily increased to a maximum level after 14 days, then remained at this level, 10 to 12 ng/g fwt, for the remainder of the culture period. This plateau in indolepyruvate concentration matched the period that the suspension culture was in the logarithmic phase of growth.

An aromatic amino acid aminotransferase was purified over 33,000 fold from the shoots and primary leaves of mung beans, as determined using a tryptophan aminotransferase activity assay. The enzyme was a monomer, with a molecular weight of about 58kDa. The pH optimum was broad, with a maximum at about 8.6. The relative activities of the aromatic amino acids were: tryptophan 100, tyrosine 83 and phenylalanine 75, and the K_m s were 0.095, 0.08 and 0.07mM respectively. The enzyme was able to use 2-oxoglutarate, oxaloacetate

and pyruvate as the oxo acid substrate at relative activities 100, 128 and 116 and K_m s 0.65, 0.25 and 0.24mM respectively

In addition to the aromatic amino acids the enzyme was able to transaminate alanine, arginine, leucine and lysine to a lesser extent, and showed slight activity with asparagine, aspartate, histidine, valine and D-tryptophan and tyrosine. Inhibition studies showed that the alanine, aspartate and histidine activities were part of the aromatic amino acid aminotransferase activity.

The enzyme was not inhibited by indoleacetic acid, although naphthaleneacetic acid did inhibit slightly. There was evidence of substrate inhibition by hydroxyphenylpyruvate at high concentrations. Addition of the cofactor pyridoxal phosphate only slightly increased the activity of the enzyme.

The enzyme was blotted onto a PVDF membrane cleaved by *in situ* trypsin digest. Three of the tryptic fragments were sequenced. These fragments had approximately 60% sequence similarity with plant aspartate aminotransferases and tyrosine aminotransferases.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors: Dr Heather Nonhebel, who provided the light at either end of the tunnel, Dr David Christie, who assisted me in the darkness, and Dr Kevin Gould, who was of more help than he would believe.

Also of invaluable assistance were all those who aided me in removing cotyledons from more than twenty kilograms of mung beans. The most frequent helpers were Sushila Manilal, John Soo Ping Chow and Dave Hieber, but honorable mention has to go to Nick Rafaut, Charles Symes, Lucille Burton, Suzanne Borich, Mirella Daja, Jo Dodd, Hugh Senior, Cynthia Tse, Penny Sowerby, Catriona McKenzie and Simon Shaw (in absolutely random order), who all slaved away for hours at a time, no matter what conditions the mung beans were in.

Much gratitude has to go to Conrad Inskip and Rabendra Singh for keeping much of the aging and erratic equipment in the laboratory in working order, and to Sharon Fisher for reconciling the insatiable demand for chemicals to the insufficient funds available and for worrying about the lost samples.

I am greatly indebted to members of my lab, and others belonging to labs I considered my own, for their many ideas that kept the experiments rolling: Terry Cooney, Hilary Talbot, Jun-Sheng Zhu, John Soo Ping Chow, Sushila Manilal and Dave Hieber.

I am obligated to various members of the academic staff. This includes Professor Alistair Renwick and Dr Ken Scott, who both provided support as HOD, Dr Nigel Birch, who allowed me to use the FPLC and gave me much help in the abortive DNA work, Associate-Professor Brittain for his ideas, and Libby Hitchings who found me sufficient lab

demonstrating work to supplement my meagre income without overworking me.

Thanks must go to Bart Jansen, who wasted much time with me attempting to isolate RNA from mung beans, and to David Smith for help in drawing up some of the figures in this thesis.

Of course, I would have given up ages ago if it wasn't for the moral support of my friends. Those within the Department of Biochemistry have already been acknowledged as mung bean decotyledoners, but special mention must go to John Soo Ping Chow, Lucille Burton and Sushila Manilal. Then there are those from the Tramping Club: the ones who gladly (strangely) went tramping in Fiordland when no one else would: Dayne Laird, Neil Macdonald, Mike Clearwater and Chris North, those that just kept going after all the years: Peter Jenkins, Linda Kerr and Peter Maxwell; and the many, too many to name, who did the occasional tramp or social event or just cheered me up when I was down. And indubitably there are the discordians: David Smith, Peter Gleeson, Andrew Paxie (my long suffering flatmate), Mark Petrie, Clare West, Jonathon McSpadden and Nigel Bree.

I must thank the University Grants Committee and the Vice Chancellors Committee for the UGC Postgraduate Scholarship which kept me in bread and water for the first three years.

And last, but definitely not least, I express deep appreciation to my parents for the understanding, moral and financial support over the years and the real meat whenever I came to dinner.

TABLE OF CONTENTS

Abstract.....	ii
Acknowledgements.....	iv
List of Figures.....	xiii
List of Tables.....	xix
List of Abbreviations.....	xxii
1 INTRODUCTION.....	1
1.1 Auxins.....	1
1.1.1 Primary Mechanisms of Auxin Action.....	4
1.1.2 Auxin Binding Proteins.....	7
1.2 Biosynthesis.....	8
1.2.1 Tryptophan as a Precursor to IAA.....	8
1.2.2 Indolepyruvate Pathway.....	10
1.2.3 Tryptamine Pathway.....	12
1.2.4 Indoleacetaldoxamine Pathway.....	13
1.2.5 Indoleacetaldehyde Conversion.....	15
1.2.6 Which Pathway?.....	17
1.3 Regulation of IAA Levels.....	19
1.3.1 Is Control of IAA Levels Important?.....	19
1.3.2 Examples of Control of Plant Growth	
Regulators	21
Ethylene Biosynthesis.....	21
Gibberellin Biosynthesis.....	23
1.3.3 Mechanisms For Control of Indoleacetic Acid	
.....	25
1.3.4 General Metabolic Control as Related to	
Indoleacetic Acid.....	29

1.4	Aminotransferases.....	33
1.4.1	Functions.....	33
1.4.2	Properties.....	38
1.4.3	Tryptophan Aminotransferase.....	45
1.5	Aims of this Thesis.....	47
2.	INDOLEACETIC ACID PRODUCTION IN CELLFREE EXTRACTS	48
2.1.	Introduction.....	48
2.2.	Materials and Methods.....	52
2.2.1.	Materials.....	52
	Chemicals.....	52
	Plant Material.....	52
	Columns.....	52
2.2.2.	Resolution of DL-Tryptophan.....	53
2.2.3.	Separation of Indoleacetic Acid, Tryptophan and Indolepyruvate Mixtures.....	54
	Polymer Reverse Phase Column.....	54
	Sepralyte C18.....	55
2.2.4.	Determination of Indoleacetic Acid and Indolepyruvate Synthesis in Cellfree Extracts.....	56
	Plant Extraction.....	56
	Incubation.....	57
	Indoleacetic Acid and Indolepyruvate Measurements.....	58
	Analysis of Results.....	59
2.3.	Results and Discussion.....	61

3.	INDOLEACETIC ACID AND INDOLEPYRUVATE IN CELL SUSPENSION CULTURES	65
3.1.	Introduction	65
3.2.	Materials and Methods	68
3.2.1.	Materials	68
	Chemicals	68
	Plant Material	69
	Columns	69
	Silanisation of Glassware	69
3.2.2.	Cell Cultures	70
	Initiation	70
	Growth Curves	71
3.2.3.	Synthesis of Labelled Internal Standards	71
	Synthesis of [3H]-Indolepyruvate	71
	Synthesis of Deuterated Indolepyruvate	72
3.2.4.	Extraction and Isolation of Indoleacetic Acid and Indolepyruvate	73
	Harvesting Cells	73
	Extraction	74
	Sepralyte C18	74
	Pentafluoryl Benzyl Derivatization of Indolepyruvate	75
	Separation of PFB-IPyA and IAA	75
	Reverse Phase HPLC of PFB-IPyA	76
	Methylation of PFB-IPyA	76
	Normal Phase HPLC of PFB-MeIPyA	77
	Reverse Phase HPLC of IAA	77

Formation the Pentafluoryl Benzyl Derivative of IAA	7 8
Normal Phase HPLC of PFB-IAA	7 9
3.2.5. Quantification of Indoleacetic Acid and Indolepyruvate by GC-MS	7 9
3.3. Results and Discussion	8 2
4. PURIFICATION OF TRYPTOPHAN AMINOTRANSFERASE	8 9
4.1. Introduction	8 9
4.2. Materials and Methods	9 3
4.2.1. Materials	9 3
Chemicals	9 3
Plant Material	9 4
Columns	9 4
4.2.2. Assays	9 5
Tryptophan Aminotransferase Assays	9 5
Tyrosine Aminotransferase Assay	9 7
Aspartate Aminotransferase Assay	9 8
4.2.3. Protein Estimation	9 9
4.2.4. Gel Electrophoresis	1 0 0
4.2.5. Silver Staining	1 0 1
4.2.6. Purification	1 0 3
Preparation of Enzyme Extract	1 0 3
Ammonium Sulphate Fractionation	1 0 4
Gel Filtration	1 0 5
Anion Exchange Chromatography	1 0 5
Hydrophobic Interaction Chromatography	1 0 6
Storage of Final Enzyme Extract	1 0 7
4.3. Results and Discussion	1 0 8

5.	CHARACTERISATION OF TRYPTOPHAN AMINOTRANSFERASE	112
	112
5.1.	Introduction.....	112
5.2.	Materials and Methods.....	116
5.2.1.	Materials.....	116
	Chemicals.....	116
5.2.2.	Tryptophan Aminotransferase Preparation	
	116
5.2.3.	Assays.....	117
	Tryptophan, Aspartate and Tyrosine	
	Aminotransferase Assays.....	117
	Phenylalanine and Histidine Aminotransferase	
	Assays.....	117
	Alanine Aminotransferase Assay.....	118
	Serine Aminotransferase Assay.....	118
	Hydroxyphenylpyruvate Aminotransferase	
	Assay.....	119
	Indolepyruvate Aminotransferase.....	120
	Assaying by Glutamate Formation.....	121
	Inhibition Assays.....	123
5.2.4.	Indolepyruvate and Indoleacetic Acid	
	Formation in Cellfree Extracts Supplemented with	
	Tryptophan Aminotransferase.....	124
5.3.	Results and Discussion.....	125
5.3.1.	Specific Activities.....	125
5.3.2.	Michaelis Constants.....	129
5.3.3.	Inhibition Results.....	130
	Inhibition by Protein Amino Acids.....	130

Aminotransferase Inhibitors.....	132
Inhibition by IAA Analogues and Tryptophan Metabolites.....	134
Indolepyruvate and Indoleacetic Acid Formation in Cellfree Extracts Supplemented with Tryptophan Aminotransferase.....	135
5.3.4. Mechanism.....	136
5.3.5. Identity, Purity and Role of The Tryptophan Aminotransferase.....	137
6. SEQUENCING.....	138
6.1. Introduction.....	138
6.2. Materials and Methods.....	141
6.2.1. Materials.....	141
Chemicals.....	141
Plant material.....	142
Columns.....	142
6.2.2. Gels.....	142
SDS-Polyacrylamide Gel Electrophoresis.....	142
6.2.3. Protein Sequencing.....	143
Electroblotting onto PVDF Membranes.....	143
<i>In Situ</i> Trypsin Digestion.....	144
Microbore RP-HPLC.....	145
Sequencing.....	146
6.2.4. Computer Based Sequence Analysis.....	146
6.2.5. RNA Extraction.....	147
mRNA Extraction.....	147
RNA Extraction.....	148
6.3. Results and Discussion.....	151

6.3.1.	Protein Sequencing.....	151
6.3.2.	RNA Purification.....	153
7.	CONCLUSIONS	155
	Future Experiments.....	160
	Appendix A - Culture Medium.....	161
	References	164
	Publications.....	195

LIST OF FIGURES

	After Page
Fig. 1.1. Structures of Natural and Synthetic Auxins.	2
Fig. 1.2. Structure of Natural Auxin Conjugates.	2
Fig. 1.3. Possible Routes of Indoleacetic Acid Biosynthesis.	10
Fig. 1.4. Biosynthesis of Ethylene.	21
Fig. 1.5. Initial Gibberellin Biosynthesis: Formation of Gibberellin A ₁₂ -Aldehyde.	23
Fig. 1.6. Final Steps of Gibberellin Biosynthesis.	24
Fig. 1.7. Decarboxylative Pathway of Indoleacetic Acid Catabolism.	26
Fig. 1.8. Nondecarboxylative Pathway of Indoleacetic Acid Catabolism.	27
Fig. 1.9. Dicarboxylic Acid Transport System.	35
Fig. 1.10. Photorespiration.	35
Fig. 1.11. The Carbon Shuttle in C ₄ Plants.	36

Fig. 1.12. The Carbon Shuttle in Bundle Sheath Cells of the Three Types of C ₄ Plants.	36
Fig. 1.13. Aspartate Aminotransferase Mechanism.	44
Fig. 2.1. Resolution of D and L-Tryptophan by L-Proline Chiral Column.	53
Fig. 2.2. Separation of Tryptophan, Indoleacetic Acid and Indolepyruvate on Polymer Reverse Phase.	54
Fig. 2.3. Conversion of Tryptophan to Indoleacetic Acid and Indolepyruvate in Cellfree Extracts.	61
Fig. 3.1. The Quinolinium Ion.	80
Fig. 3.2. GC-MS SIM Trace for Indolepyruvate.	82
Fig. 3.3. GC-MS SIM Trace for Indoleacetic Acid.	82
Fig. 3.4. Calibration Curve For Dependence Between the Ratio of Unlabelled to Labelled Indoleacetic Acid and the Ratio of Peak Areas At m/z 130 and m/z 136.	82
Fig. 3.5. Indoleacetic Acid and Indolepyruvate Concentrations Throughout Development of Mung Bean Suspension Culture.	86

Fig. 3.6. Indolepyruvate Concentration and Growth Rate of Mung Bean Suspension Culture.	87
Fig. 4.1. Separation of Tryptophan and Aspartate Aminotransferase Activities on Sephacryl S-300HR.	109
Fig. 4.2. Separation of Tryptophan and Aspartate Aminotransferase Activities on Fastflow Q.	110
Fig. 4.3. Separation of Tryptophan and Aspartate Aminotransferase Activities on Mono Q.	110
Fig. 4.4. Polyacrylamide Gel Electrophoresis of Mono Q Tryptophan Aminotransferase Fraction.	110
Fig. 4.5. Separation of Tryptophan and Aspartate Aminotransferase Activities on Phenylsuperose.	110
Fig. 4.6. Polyacrylamide Gel Electrophoresis of Phenylsuperose Tryptophan Aminotransferase Fractions.	111
Fig. 4.7. Resolution of Tryptophan Aminotransferase Activities on Superose-12.	111
Fig. 5.1. Effects of pH on the Activity of Tryptophan Aminotransferase.	125

Fig. 5.2. Determination of Michaelis Constant of the Aminotransferase Towards Tryptophan.	129
Fig. 5.3. Determination of Michaelis Constant of the Aminotransferase Towards Phenylalanine.	129
Fig. 5.4. Determination of Michaelis Constant of the Aminotransferase Towards Tyrosine.	129
Fig. 5.5. Determination of Michaelis Constant of the Aminotransferase Towards 2-Oxoglutarate.	129
Fig. 5.6. Determination of Michaelis Constant of the Aminotransferase Towards Pyruvate.	129
Fig. 5.7. Determination of Michaelis Constant of the Aminotransferase Towards Oxaloacetate.	129
Fig. 5.8 Determination of Michaelis Constant of the Aminotransferase Towards Indolepyruvate.	129
Fig. 5.9. Determination of Michaelis Constant of the Aminotransferase Towards Hydroxyphenylpyruvate.	129
Fig. 5.10. Investigation of Inhibition by Histidine.	131

- Fig. 5.11. Formation of Indolepyruvate and Indoleacetic Acid by Cellfree Extracts Supplemented with Tryptophan Aminotransferase. 136
- Fig 5.12. The Effects of Changing 2-Oxoglutarate Concentration on the Tryptophan Michaelis Constant. 136
- Fig. 5.13 The Effects of Changing Tryptophan Concentration on 2-Oxoglutarate Michaelis Constant. 136
- Fig. 5.14. Dependence of Michaelis Constants on Substrate Concentrations. 136
- Fig. 5.15. Investigation of Inhibition by Alanine. 137
- Fig. 5.16. Investigation of Inhibition by Aspartate. 137
- Fig. 6.1. Alignment of Tyrosine and Plant Aspartate Aminotransferase Sequences. 139
- Fig. 6.2. Chromatograph of Peptides Released by *In Situ* Digestion of the Aromatic Amino Acid Aminotransferase. 151
- Fig. 6.3. Chromatograph of Peptides Extracted by Guanadine Hydrochloride from the *In Situ* Digestion of the Amino Acid Amino-transferase. 151

Fig. 6.4. Alignment of Sequenced Fragments of the Aromatic Amino Acid
Aminotransferase with Other Aminotransferases. 153

LIST OF TABLES

	Page
Table 1.1. Levels of Free and Conjugated Indoleacetic Acid <i>in Vivo</i> .	3
Table 1.2. Plant Aminotransferases.	40
Table 2.1. Final Substrate and Cofactor Concentrations Used in the Incubation of Cellfree Extract Mixtures.	57
Table 3.1. Yields and Amounts of Indolepyruvate and Indoleacetic Acid From Mung Bean Suspension Cultures.	83
Table 3.2. Sample Weights and Peak Areas for Indolepyruvate Analysis by GC-MS SIM.	85
Table 3.3. Sample Weights and Peak Areas for Indoleacetic Acid Analysis by GC-MS SIM.	86
Table 4.1. Relative Specificities of Plant Aromatic Aminotransferases.	91
Table 4.2. Recipe for 10% Polyacrylamide Gels.	101
Table 4.3. Silver Staining Protocol.	102

	Page
Table 4.4. Purification of Tryptophan Aminotransferase.	109
Table 5.1. Assay requirements of tryptophan aminotransferase.	125
Table 5.2. Relative Specificity of Protein Amino Acids of Tryptophan Aminotransferase.	126
Table 5.3. Relative Specificity of Oxo Acids of Tryptophan Aminotransferase.	127
Table 5.4. Relative Specific Activity of Reverse Reactions of Tryptophan Aminotransferase.	128
Table 5.5. Michaelis Constant of Tryptophan Aminotransferase Towards Various Substrates.	129
Table 5.6. Inhibition of Tryptophan Aminotransferase By Protein Amino Acids.	131
Table 5.7. Effects of Aminotransferase Inhibitors on Tryptophan Aminotransferase.	132
Table 5.8. Inhibition of Tryptophan Aminotransferase by Indoleacetic Acid Analogues and Tryptophan Metabolites.	133
Table A.1. Concentrations of Components of B5 Medium.	162

Table A.2. Constituents, Amounts and Strengths of Stock Solutions for Medium Preparation.	163
--	-----

LIST OF ABBREVIATIONS

2-OG	2-Oxoglutarate
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
μ -	Micro- (10^{-6})
cDNA	Complement DNA
conc.	Concentrated
fw	Fresh weight
g	Gram
h ν	Light radiation
i.d.	Internal diameter
k -	Kilo- (10^3)
m -	Milli- (10^{-3})
m/z	Mass to charge ratio
mRNA	Messenger RNA
n -	Nano- (10^{-9})
nd	Not determined
phn _j	Protein j of phn operon in <i>E. coli</i>
v	Observed enzyme rate
xg	Gravities (acceleration)
ACC	1-Aminocyclopropane-1-carboxylic acid
ADP	Adenosine dinucleotide phosphate
Ala	Alanine
Asn	Asparagine
Asp	Aspartate
BHT	2,6-Di- <i>tert</i> -butyl-4-methylphenol

Bq	Bequerel
BSA	Bovine serum albumin
CAPS	3-[Cyclohexylamino]-1-propane-sulphonic acid
C _n	n carbon compound
CPP	Copalyl pyrophosphate
D ₂ O	Deuterium oxide
Da	Dalton
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbamate
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenylhydrazine
DPM	Disintegrations per minute
E.C.	Enzyme convention numbering
EDTA	Ethylenediaminetetraacetic acid
EFE	Ethylene-forming enzyme
GA _n	Gibberellin A _n
GAP	Glyceraldehyde-3-phosphate
GC	Gas chromatography
GGPP	Geranylgeranyl pyrophosphate
Gln	Glutamate
Glu	Glutamine
Gly	Glycine
HPLC	High performance liquid chromatography
HR	High resolution
IAA	Indole-3-acetic acid
IAAld	Indoleacetaldehyde
IAN	Indoleacetonitrile

IAOx	Indoleacetaldoxime
Ile	Isoleucine
IPyA	Indolepyruvate
Kg	2-Oxoglutarate
K _m	Michaelis constant
K _m ^{amino acid}	Michaelis constant for the amino acid substrate
K _m ^{oxo acid}	Michaelis constant for the oxo acid substrate
L	Litre
Leu	Leucine
Lys	Lysine
M-	Mega- (10 ⁶)
Mal	Malate
MC	Mesophyll cells
Me	Methyl
MS	Mass spectroscopy
MW	Molecular weight
NAA	Naphthalene-2-acetic acid
NAD ⁺	β-Nicotinamide adenine dinucleotide
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADP ⁺	β-Nicotinamide adenine dinucleotide phosphate
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
OAA	Oxalacetate
PCR	Polymer chain reaction
PFB	Pentafluorobenzyl