



<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.

**Molecular Studies on Actinidin,
A Cysteine Protease from Kiwifruit**

Ellen Podivinsky

Department of Cellular and Molecular Biology

University of Auckland

Thesis submitted in fulfilment
of the requirements for
the degree of

Doctor of Philosophy

June, 1991

ABSTRACT

Research in this thesis describes the characterisation of mRNA sequences coding for actinidin, a cysteine protease found in abundance in the fruit of kiwifruit (*Actinidia deliciosa*).

The first step in the characterisation required the isolation of mRNA from ripe kiwifruit tissue. The suitability of a number of RNA extraction procedures was investigated. The method finally adopted differed from that used for unripe fruit tissue, and was chosen as a result of the nature of the polysaccharide that contaminated nucleic acids prepared from extracts of kiwifruit fruit tissue.

RNA extracted from ripe fruit was used to synthesise a partial cDNA library and clones for actinidin were isolated. A number of these cDNA clones were sequenced; three clones were almost full-length. The actinidin cDNA clones obtained fall into two broad sequence classes. The majority of them encode acidic proteins (pI~4.7), with 97% homology to the published amino acid sequence of actinidin. The second class encode basic proteins (pI~8.1), with 83% homology to the published amino acid sequence of actinidin. Both classes of actinidin cDNA sequence encode zymogens, which contain N- and C-terminal extensions not present in the mature form of the enzyme. The N-terminal extension of both sequence classes includes a putative signal peptide.

Northern hybridization analysis was used to investigate the tissue specificity of actinidin mRNA expression, and the expression of mRNA for the two actinidin sequence classes during fruit ripening. Both actinidin sequence classes were expressed differentially during the latter stages of kiwifruit fruit development and through post-harvest fruit ripening. The expression of both sequence classes increased from just prior to fruit maturity through ripening and reached a maximum as fruit attained the stage of 'eating' ripeness. The level of expression of the sequences encoding acidic actinidin reached a plateau at this point, while the expression of the sequence encoding basic actinidin appeared to decrease slightly as fruit continued to ripen. The sequences encoding acidic actinidin were expressed during ripening at a much higher level than those encoding basic actinidin. No actinidin mRNA was detected in other tissues except for very low levels of the acidic form in kiwifruit leaf, and low levels of the basic form in senescing petals.

A full-length, acidic, actinidin cDNA sequence was introduced into tobacco (*Nicotiana tabacum*) plants via *Agrobacterium tumefaciens*-mediated transformation. Using the binary vector pGA643, the sequence was introduced in both the sense and antisense orientation relative to the cauliflower mosaic virus 35S promoter and transgenic plants were obtained for both sequence orientations. The presence of the T-DNA cassette (containing the actinidin sequence) in the plant genomes was determined using PCR analysis, and confirmed by Southern hybridization. A number of the transgenic plants contained multiple insertions of the actinidin sequence, and most plants contained at least one intact copy of the T-DNA cassette. The transcription of the introduced actinidin sequence was investigated by Northern hybridization analysis. All of the plants containing actinidin in the sense orientation, and some of those incorporating the antisense construct, transcribed the actinidin sequence. Attempts to detect actinidin protein in the transgenic plants were unsuccessful.

Acidic actinidin was identified as one of the most abundant bands in the total protein profile from ripe kiwifruit fruit tissue. The identity of the protein was confirmed by N-terminal sequence analysis. The electrophoretic mobility of actinidin, both in the total cell homogenate and when partially purified, suggested that the first step in post-translational processing of the zymogen may be the removal of the N-terminal extension. Actinidin was also partially purified and used to raise antibodies. Poor specificity of the antibody for actinidin led to preliminary evidence for the glycosylation of actinidin.

ACKNOWLEDGMENTS

I thank Associate-Professor Richard Gardner for his supervision of the work described in this thesis and for assistance in the preparation of this manuscript, and Professor Dick Bellamy for his co-supervision.

There are many other people who assisted in the work described in this thesis, and who offered helpful advice and encouragement. My thanks go to the following:

Foremost, to all the members of the Plant Molecular Biology Group, and the Department of Cellular and Molecular Biology, for their help and advice. Especial thanks to Jeanette Keeling for her friendship and encouragement.

To Dr Michael Lay Yee, DSIR, Mt Albert, for assistance in the controlled ripening of kiwifruit, and for advice and encouragement.

To Dr Harry Smith and Dr Uta Praekelt, Leicester University, U.K., for the supply of the pAc1 clone, and for their advice on the extraction of RNA from unripe kiwifruit tissue.

To Dr Robert Redgwell, DSIR, Mt Albert, who performed the carbohydrate analysis on the kiwifruit RNA extract.

To Dr Richard Forster, DSIR, Mt Albert, who synthesised the cDNA that was used to construct the cDNA library, and gave assistance with the *in vitro* translation of kiwifruit RNA.

To Dr Lesley Beuning, DSIR, Mt. Albert who assisted in the sequencing of the partial actinidin cDNA clones and in the proof reading of this manuscript. Especial thanks for the advice, encouragement and friendship.

To Professor Ted Baker and his group at Massey University, for advice on the actinidin protein.

To Mr Keith Richards, this laboratory, for his help with the partial purification of actinidin.

To Dr David Christie, Biochemistry Department, University of Auckland, who performed the N-terminal sequencing of the actinidin protein.

To the staff of the MAF Animal Research Centre, Ruakura, who raised the actinidin antibodies.

To Nga Tama, this laboratory, for his help with the self-crossing and inheritance studies on the transgenic tobacco plants

To the staff of the Photographic Unit, MAF Horticultural Research Centre, Levin, for their help in the preparation of the photographic plates in this manuscript.

To Dr Simon Deroles, MAF, Levin, for his assistance with the proof reading of this manuscript and for encouragement and advice.

To Dr Graeme King, the members of the Plant Molecular Biology Group, MAF, Levin, and to Dr Rob Pringle, Centre Director, MAF, Levin, for the encouragement and assistance that enabled the completion of this thesis.

To my husband, Nick, for his constant support and patience, and to my family.

This work was supported in part by a grant from the Division of Fruit and Trees, DSIR, Mt. Albert.

TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	v
LIST OF FIGURES	xii
LIST OF TABLES	xiv
ABBREVIATIONS	xv
CHAPTER 1: General Introduction	
1.1 Introduction	1
1.2 Introduction to Kiwifruit	1
1.2.1 The History of Kiwifruit and Its Introduction into New Zealand	1
1.2.2 Kiwifruit Production in New Zealand	2
1.2.3 Taxonomic Classification of Kiwifruit	2
1.2.4 The Botany of Kiwifruit	3
1.2.5 Kiwifruit Development and Ripening	4
1.3 The Cysteine Proteases	6
1.3.1 Protease Nomenclature	6
1.3.2 The Evolution and Occurrence of Cysteine Proteases	6
1.3.3 The Catalytic Activity of Cysteine Proteases	7
1.3.4 Biological Roles of the Cysteine Proteases	8
1.3.5 Regulation of Cysteine Protease Activity	10

1.4 Actinidin	13
1.4.1 Analysis of the Actinidin Protein	13
1.4.2 The Structure of Actinidin	14
1.4.3 Characterisation of Actinidin Coding Sequences	15
1.4.4 Distribution and Expression of Actinidin in Kiwifruit	16
1.5 Aims of this thesis	17

CHAPTER 2: General Materials and Methods

2.1 Materials	19
2.1.1 Chemicals	19
2.1.2 Membranes	19
2.1.3 Radionucleotides	20
2.1.4 Oligonucleotides	20
2.1.5 Restriction Endonucleases and DNA Modifying Enzymes	20
2.1.6 Bacterial Strains	20
2.1.7 Bacterial Plasmids	21
2.1.8 Buffers	22
2.1.9 Media	23
2.1.10 Antibiotics	24
2.1.11 Autoradiography	24
2.2 Manipulation of bacteria	24
2.2.1 Preparation of Competent <i>E.coli</i> DH5 α Cells	24
2.2.2 Transformation of Competent <i>E.coli</i> DH5 α	25
2.2.3 Tri-parental Mating of <i>Agrobacterium tumefaciens</i>	25
2.2.4 <i>In situ</i> Hybridisation of <i>E.coli</i> Bacterial Colonies	26
2.3 Isolation of DNA	27
2.3.1 Large Scale Isolation of Plasmid DNA from <i>E.coli</i>	27
2.3.2 Small Scale Isolation of Plasmid DNA from <i>E.coli</i>	28
2.3.3 Rapid Screening of Plasmid DNA from <i>E.coli</i>	29

2.3.4	Small Scale Isolation of Plasmid DNA from <i>A. tumefaciens</i>	29
2.3.5	Isolation of Plant DNA	29
2.4	Manipulation of DNA	30
2.4.1	Phenol:Chloroform Extraction and Ethanol Precipitation of Nucleic Acids	30
2.4.2	Restriction Endonuclease Digestion of DNA	31
2.4.3	Dephosphorylation of Linear DNA	31
2.4.4	Agarose Gel Electrophoresis of DNA	32
2.4.5	Recovery of DNA from Agarose Gels	32
2.4.6	Radioactive Labelling of DNA with [α - 32 P]dCTP by Nick Translation	33
2.4.7	End-labelling of Linear Plasmid DNA with [α - 32 P]dNTP	33
2.4.8	Ligation of DNA	34
2.4.9	Plasmid DNA Sequencing	34
2.4.10	Amplification of DNA by the Polymerase Chain	36
2.4.11	Synthesis of RNA Transcripts from Plasmid DNA Template	37
2.4.12	Southern Hybridisation	37
2.5	Isolation of RNA from Plant Tissue	38
2.5.1	Method A: Guanidinium Isothiocyanate Extraction Followed by Caesium Chloride Ultracentrifugation	38
2.5.2	Method B: Guanidinium Isothiocyanate Extraction Followed by Lithium Chloride Precipitation	39
2.5.3	Method C: Extraction in Tris Buffer Followed by Treatment with Phenol and Sodium Acetate	40
2.6	Manipulation of RNA	41
2.6.1	Spectrophotometric Determination of RNA	41
2.6.2	Non-denaturing Gel Electrophoresis of RNA	41
2.6.3	Purification of Total RNA by Passage Through Cellulose	42
2.6.4	Selection of Poly(A) ⁺ RNA	42
2.6.5	Translation of RNA <i>in vitro</i>	43
2.6.6	Northern Hybridisation of RNA	43

2.7	Synthesis and Manipulation of Double-Stranded cDNA	44
2.7.1	Synthesis of Double-Stranded cDNA	44
2.7.2	Analysis of cDNA Products	46
2.7.3	'Blunt-Ending' of Double-Stranded cDNA	47
2.7.4	S1-Nuclease Digestion of Double-Stranded cDNA	47
2.8	Isolation of Proteins from Plants	47
2.8.1	Isolation of Total Proteins from Plants	47
2.8.2	Isolation and Purification of Actinidin Protein from Kiwifruit	48
2.9	Manipulation of Proteins	49
2.9.1	Assay of Protein Yields	49
2.9.2	SDS-PAGE of Proteins	49
2.9.3	Staining of Proteins in SDS-PAGE Gels	50
2.9.4	Staining for Glycoproteins	50
2.9.5	Acetylation of Proteins	51
2.9.6	ELISA of Antibodies	51
2.9.7	Immunoblotting of Proteins (Western Hybridisation)	52
2.10	Manipulation of Plants	53
2.10.1	Transformation of <i>Nicotiana tabacum</i> with <i>Agrobacterium tumefaciens</i>	53
2.10.2	Controlled Ripening of Kiwifruit Fruit	54

CHAPTER 3: Isolation of RNA from Kiwifruit and Construction of a cDNA Library from Ripe Fruit

3.1	Introduction	55
3.2	Controlled Ripening of Kiwifruit	56

3.3 Isolation of RNA from Kiwifruit	57
3.3.1 Considerations in the Choice of RNA Isolation Techniques	57
3.3.2 Comparison of Three Procedures for RNA Extraction	58
3.3.3 Isolation of poly(A) ⁺ RNA and <i>in vitro</i> Translation	62
3.4 Synthesis and Cloning of Double Stranded cDNA from Ripe Kiwifruit	65
3.4.1 Introduction to cDNA Synthesis and Cloning	65
3.4.2 Synthesis of Double Stranded cDNA from Ripe	66
3.4.3 Cloning of Kiwifruit Double Stranded cDNA	69
3.5 Concluding Discussion	70

CHAPTER 4: Isolation and Characterisation of Actinidin cDNA Clones

4.1 Isolation of Actinidin cDNA Clones	73
4.2 Sequencing of Actinidin cDNA Clones	74
4.3 Analysis of Actinidin cDNA Clone Sequences	75
4.3.1 pKIWI450 and 451	75
4.3.2 pKIWI452	76
4.3.3 Partial actinidin clones	78
4.3.4 Detailed Comparison of TypeI and TypeII	79
4.3.5 Comparison Between the Actinidin Isoforms and	81
4.4 Concluding Discussion	82

CHAPTER 5: Expression of Actinidin in Kiwifruit

5.1 Introduction	86
------------------	----

5.2 Northern Hybridisation Analysis of Actinidin Expression	87
5.2.1 Analysis of the Expression of Both Actinidin Classes	88
5.2.2 Expression of TypeII Actinidin	89
5.3 Concluding Discussion	90
5.3.1 Tissue Specificity of Actinidin Transcription	90
5.3.2 Developmental Transcription of Actinidin	91

CHAPTER 6: Introduction of Actinidin Coding Sequences into Tobacco Plants

6.1 Introduction	94
6.2 Transformation of Tobacco with Actinidin	95
6.3 Integration of Actinidin Coding Sequences into Tobacco	95
6.3.1 PCR Analysis	96
6.3.2 Southern Hybridisation	97
6.4 Transcription of Actinidin Coding Sequences in Tobacco	98
6.5 Translation of Actinidin Coding Sequences in Tobacco	98
6.5.1 Partial Purification of Actinidin Protein and Preparation of Actinidin Antibodies	99
6.5.2 ELISA Analysis	100
6.5.3 Western Hybridisation	100
6.7 Concluding Discussion	102

CHAPTER 7: Concluding Discussion

7.1 Extraction of RNA from Ripe Kiwifruit	106
---	-----

7.2 The Sequence of the cDNA Clones for Actinidin	108
7.3 Future Analysis of the Actinidin Protein	109
7.4 Possible Roles for Actinidin in Kiwifruit	110

APPENDIX A: RNA Isolation and Purification Techniques

A.1 RNA Isolation Procedures	113
A.1.1 CTAB Isolation Procedure	113
A.1.2 Isolation of RNA from Polyribosomes	114
A.1.3 Solvent Extraction	115
A.1.4 Combined Guanidinium/ CTAB Extraction	115
A.2 RNA Purification Procedures	116
A.2.1 Removal of Polysaccharides by Differential	116
A.2.2 Purification by Washing with NaOAc	116
A.2.3 Treatment with Methoxyethanol/ CTAB	117
A.2.4 Digestion of Samples with Driselase	117
A.2.5 Purification using Qiagen Columns	118

APPENDIX B: Analysis of the Actinidin Protein

B.1 Introduction	119
B.2 Identification of Actinidin from Total Kiwifruit Protein	120
B.3 Analysis of Actinidin for Glycosyl Groups	121
B.4 Concluding Discussion	121

APPENDIX C: Paper Submitted for Publication	124
--	-----

BIBLIOGRAPHY	125
---------------------	-----

LIST OF FIGURES

Chapter 1:

- 1.1 Mechanism of Protein Hydrolysis by Cysteine Proteases
- 1.2 Specificity Subsites within Papain
- 1.3 Changes in Actinidin Specific Activity During Kiwifruit Development

Chapter 3:

- 3.1 Controlled Ripening of Kiwifruit
- 3.2 Electrophoretic Analysis of RNA from Kiwifruit Fruit Tissue
- 3.3 Electrophoretic Analysis of Poly(A)⁺ RNA Isolated from Kiwifruit
- 3.4 *In Vitro* Translation of Kiwifruit RNA
- 3.5 The Effect of Template Concentration on the Yield of First Strand cDNA
- 3.6 Synthesis of Double Stranded cDNA from Kiwifruit RNA
- 3.7 Analysis of Second Strand cDNA Synthesised Using the Klenow Fragment of *E.coli* DNA Polymerase I
- 3.8 Size Fractionation of Kiwifruit Double-Stranded cDNA
- 3.9 Screening of Size-Fractionated Kiwifruit cDNA Clones
- 3.10 Determination of Insert Size in Kiwifruit cDNA Clones

Chapter 4:

- 4.1 Colony Hybridisation of Kiwifruit cDNA Clones with pAc1
- 4.2 Screening for Inserts in cDNA Clones that Hybridised to pAc1
- 4.3 Sequence and Position of the oAc Oligonucleotide Primers
- 4.4 Sequencing Strategy Adopted for pKIWI450 and pKIWI451
- 4.5 Sequencing Strategy Adopted for pKIWI452
- 4.6 Lineup of Actinidin cDNA Clone Sequences
- 4.7 A Comparison of Amino Acid Sequences for a Selection of Cysteine Protease
- 4.8 Hydrophilicity Plots for Proteins Encoded by TypeI and TypeII Actinidin Sequences
- 4.9 Sequence Mismatch Observed Between oAc Primers and pKIWI452
- 4.10 Matrix Comparison of TypeI and TypeII Actinidin Sequences
- 4.11 Relative Lengths of Actinidin cDNA Clones

- 4.12 A Comparison of the Full Length Nucleic Acid Sequences for TypeI and TypeII Actinidin
- 4.13 Comparison of the Inferred Amino Acid Sequences Encoded by TypeI and TypeII Actinidin
- 4.14 Charge Differences between the Inferred Amino Acid Sequences Encoded by TypeI and TypeII Actinidin

Chapter 5:

- 5.1 Cloning of pKIWI453 and pKIWI447
- 5.2 Synthesis of Actinidin Transcripts *in vitro*
- 5.3 Northern Hybridisation Analysis of TypeI and TypeII Actinidin mRNA Expression in Kiwifruit
- 5.4 Northern Hybridisation Analysis of TypeII Actinidin mRNA Expression in Kiwifruit

Chapter 6:

- 6.1 Construction of pKIWI448 and pKIWI449
- 6.2 Regeneration of Transgenic Tobacco Plants
- 6.3 PCR Analysis of Genomic DNA from Transgenic Tobacco Plants
- 6.4 Southern Hybridisation Analysis of Transgenic Tobacco Plants
- 6.5 Northern Hybridisation Analysis of Transgenic Tobacco Plants
- 6.6 Partial Purification of Actinidin
- 6.7 ELISA of Antibodies Raised Against Actinidin
- 6.8 Western Hybridisation Analysis on Transgenic Tobacco Plants

Appendix B:

- B.1 Total Protein Profile from Ripe Kiwifruit
- B.2 Staining of Actinidin for Glycosyl Groups

LIST OF TABLES

Chapter 3:

- 3.1 Removal of Polysaccharide Contamination from
Kiwifruit Total RNA

Chapter 4:

- 4.1 Size of Inserts in Actinidin Positive cDNA Clones

Chapter 6:

- 6.1 ELISA on Transgenic Tobacco Plants
- 6.2 Inheritance of the Kanamycin Resistance Phenotype

ABBREVIATIONS

APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
β -NAD	beta-nicotinamide adenine dinucleotide
BAP	6-benzylaminopurine
bp	base pair
BSA	bovine serum albumin
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
CTP	cytidine-5'-triphosphate
Dal	Daltons (1 dalton = 1 gram per mole)
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
ddNTP	dideoxyribonucleoside triphosphate
DEP	diethyl pyrocarbonate
dGTP	2'-deoxy-guanosine-5'-triphosphate
DNA	deoxyribose nucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
dTTP	2'-deoxy-thymidine-5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
EGTA	ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N' -tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
GTP	guanosine-5'-triphosphate
IPTG	isopropyl- β -D-thiogalactoside (0.45 dioxane)
kb	kilobase pair
mA	milliampere
MOPS	3-(N-morpholino)propane-sulphonic acid
mRNA	messenger ribose nucleic acid
MW	molecular weight
NAA	α -naphthalene acetic acid
OAc	ortho-acetate
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonyl fluoride
ppm	parts per million

PVP	polyvinylpyrrolidone
RNA	ribose nucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate (also called sodium lauryl sulfate)
TEMED	tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
UTP	uridine-5'-triphosphate
UV	ultraviolet
v/v	volume for volume
w/v	weight for volume
X-Gal	5-bromo-4-chloro-3-indolyl β -D-glucoside
X-Gluc	5-bromo-4-chloro-3-indolyl β -D-glucuronic acid
xg (max)	relative centrifugal field at maximum rotor radius