Molecular Studies on Actinidin, A Cysteine Protease from Kiwifruit

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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 synthesis 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.

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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