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Apple β-Galactosidase[1]

Activity against Cell Wall Polysaccharides and Characterization of a Related cDNA Clone

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A β-galactosidase was purified from cortical tissue of ripe apples (*Malus domestica* Borkh. cv Granny Smith) using a procedure involving affinity chromatography on lactosyl-Sepharose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that two polypeptides of 44 and 32 kD were present in the fraction that showed activity against the synthetic substrate *p*-nitrophenol-β-D-galactopyranoside. The enzyme preparation was incubated with polysaccharide extracts from apple cell walls containing β-(1→4)-linked galactans, and products of digestion were analyzed by gas chromatography. Small amounts of monomeric galactose were released during incubation, showing that the enzyme was active against native substrates. Amino acid sequence information was obtained from the purified protein, and this showed high homology with the anticipated polypeptide coded by the ethylene-regulated *SR12* gene in carnation (K.G. Raghothama, K.A. Lawton, P.B. Goldborough, W.R. Woodson [1991] Plant Mol Biol 17: 61–71) and a harvest-related pTIP31 cDNA from asparagus (G. King, personal communication). Using the asparagus cDNA clone as a probe, an apple homolog (pABG1) was isolated. This clone contains a 2637-bp insert, including an open reading frame that codes for a polypeptide of 731 amino acids. Cleavage of an N-terminal signal sequence would leave a predicted polypeptide of 78.5 kD. Genomic DNA analysis and the isolation of other homologous apple clones suggest that pABG1 represents one member of an apple β-galactosidase gene family. Northern analysis during fruit development and ripening showed accumulation of pABG1-homologous RNA during fruit ripening. Enzyme activity as measured in crude extracts increased during fruit development to a level that was maintained during ripening.

Ripening of fruit is usually accompanied by loss of fruit firmness, in a process that may be mediated by the action of hydrolytic enzymes on the polysaccharides of the cell wall. Although a number of specific cell wall hydrolases have been isolated and studied, the relative importance of individual enzymes in the softening process has yet to be determined. For example, the enzyme polygalacturonase has been studied in detail, but its role has not been well defined, and molecular evidence has suggested that this enzyme alone does not cause softening in tomato (Giovannoni et al., 1989). These and other results have stimulated further research into other glycosidases such as the enzyme β-galactosidase (EC 3.2.1.23).

In apples (*Malus domestica*), the loss of fruit firmness during ripening has been associated with increased activity of β-galactosidase and a decrease in the Gal content of the cell wall (Bartley, 1974; Wallner, 1978). However, differences in enzyme activity are quite small, and in at least one case, invariant activity during maturation and ripening was reported (Dick et al., 1990). Treatments with polyphenolics, which have been shown to inhibit β-galactosidase activity (Dick and Bearne, 1988), can delay softening and increase shelf life (Lidster et al., 1985). A number of apple glycosidases, including several β-galactosidases, have recently been purified and partially characterized (Dick et al., 1990).

Most studies of fruit cell wall glycosidases have included assay of activity with synthetic *p*-nitrophenyl-pyranoside substrates (e.g. Pressey, 1983; Dick et al., 1990; Ogawa et al., 1990). However, there have also been attempts to detect activity against likely native substrates extracted from the same tissue, because data from such experiments can provide more realistic indications of the in vivo role of the enzyme. β-Galactosidase activity against extracted cell walls has been detected with the tomato (Pressey, 1983), the muskmelon (Ranwala et al., 1992), and kiwifruit enzyme (Ross et al., 1993) but not with β-galactosidases from apple (Wallner, 1978) or citrus fruit (Burns, 1990).

In this paper we report the isolation of a β-galactosidase from ripening apple flesh. This enzyme preparation shows activity against a natural apple cell wall polysaccharide in vitro, suggesting that the enzyme may have a role in the degradation of fruit cell walls. We present amino acid sequence information from the β-galactosidase protein and report the isolation and characterization of a cDNA clone that is believed to encode an apple β-galactosidase. The clone shows homology to a number of β-galactosidase precursors (e.g. human, Morreau et al., 1989) and several previously unidentified cDNA clones that have been isolated from other plant systems (Raghothama et al., 1991; G. King, personal communication).

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Abbreviation: NaOAc, sodium acetate.

MATERIALS AND METHODS

Plant Tissue

Apples (*Malus domestica* [Borkh] cv Granny Smith) were harvested at 4-week intervals during fruit development, beginning 4 weeks after full bloom and continuing until commercial harvest (mature, unripe fruit). Two samples were also taken after commercial harvest from fruit producing either between 1 and 10 or more than 100 ppm internal ethylene (Saltveit, 1982). For each sample cortical tissue was cut into pieces of approximately 2 cm^3, frozen in liquid nitrogen, and stored at −80°C, pending enzyme or RNA extraction. Enzyme was purified from fruit at the time of commercial harvest; RNA was purified from tissue throughout fruit development.

Enzyme Purification and Measurement of Activity

All operations were conducted at 4°C unless otherwise stated. At each stage of purification, β-galactosidase activity and protein concentration were determined. Frozen, mature, unripe apple tissue (500 g) was mixed with 2% (w/w) polyvinylpolypyrrolidone and homogenized in approximately 1 L of 25 mM NaOAc, 0.1% (v/v) β-mercaptoethanol, pH 4.5, in a Waring blender. The homogenate was centrifuged at 16,300*g* for 20 min. The β-galactosidase activity and protein content of the supernatant were measured, and the supernatant was discarded. The pellet was washed twice with H_2O, and resuspended in 300 mL of 25 mM NaOAc, 100 mM NaCl, 0.1% (v/v) β-mercaptoethanol, pH 4.5. This mixture was stirred for 1 h at 4°C and centrifuged at 16,300*g* for 20 min. The supernatant was retained, the pellet was resuspended in an additional 200 mL of the same buffer and recentrifuged, and the two supernatants were combined (hereafter called the "salt extract").

The salt extract was loaded on an affinity column of lactosyl-Sepharose 4B (bed volume approximately 200 mL; Simos et al., 1989), which had been pre-equilibrated in 25 mM NaOAc, 0.1% (v/v) β-mercaptoethanol, pH 4.5. After the column was washed with 25 mM NaOAc, 100 mM NaCl, 0.1% (v/v) β-mercaptoethanol, pH 4.5, the enzyme was eluted with 50 mM NaOAc, 50 mM NaCl, 50 mM lactose, 0.1% (v/v) β-mercaptoethanol, pH 4.5. The active fractions were combined and concentrated by dialysis overnight against CM-cellulose. The size of the native, active protein was determined by gel permeation on a Superose 12 column (Pharmacia), with a column buffer of 50 mM NaOAc, 50 mM NaCl, 0.1% (v/v) β-mercaptoethanol, pH 4.5.

For determination of enzyme activity during fruit development and ripening, salt extracts were prepared from samples (5 g) of cortical tissue and assayed for enzyme activity. All measurements of enzyme activity against *p*-nitrophenyl-β-D-galactopyranoside were made using incubations in microtiter plate wells as described previously (Ross et al., 1993). Protein concentrations were determined using BSA as a standard (Bradford, 1976). Purification was monitored by SDS-PAGE, followed by silver staining (Blum et al., 1987).

Enzyme Characteristics

The pH optimum of the "purified" enzyme against *p*-nitrophenyl-β-D-galactopyranoside was determined in phosphate/citrate buffers covering a range of pH. Inhibition of enzyme activity with D-galactono-(1→4)-lactone, Gal, lactose, Glc, and Ara was also investigated. Enzyme was preincubated for 15 min at 30°C with each compound at concentrations of either 1, 10, or 100 mM and then assayed as usual.

Preparation of Natural Substrates

Methods previously developed for kiwifruit (Redgwell et al., 1992a, 1992b) were used, and two substrates were prepared from the cortical tissue of apple: (a) the major Gal-containing polysaccharide ("galactan") and (b) a Na_2CO_3-soluble pectic polysaccharide fraction.

Enzyme Action against Native Substrates

Reaction mixtures consisting of 2 mg of substrate, 1.0 mL of 50 mM NaOAc, 50 mM NaCl, pH 5.0, containing purified apple β-galactosidase (sufficient enzyme to hydrolyze 15 nmol of *p*-nitrophenyl-β-D-galactopyranoside/min at 30°C) and 20 μL of toluene, were incubated at 37°C for 72 h. Separate blanks were used for each substrate and the enzyme preparation. For comparison, digestions with a kiwifruit β-galactosidase (Ross et al., 1993) and a galactan fraction extracted from kiwifruit cell walls were also included. After incubation, cold acetone (2 mL) was added to each reaction mixture to precipitate undegraded polysaccharide and protein. The supernatant was recovered after centrifugation and concentrated to approximately 0.5 mL in a stream of air. The solution was passed through columns (1.0-mL bed volumes) of QAE-Sephadex (formate form) and SP-Sephadex (H^+ form), and the effluent was dried and dissolved in 100 μL of 10% (v/v) isopropanol. This solution was used for the identification and quantitation of substrate products by capillary GLC.

GLC

Prior to analysis by GLC, polysaccharides were hydrolyzed with 2 M TFA for 1 h at 121°C, and sugar mixtures were converted into alditol acetates (Albersheim et al., 1967). Analysis was performed on a SP-2330 fused silica capillary column (30 × 0.32 mm) held at 120°C for 2 min, and then the temperature was raised to 220°C at 10°C min^{-1}.

β-Galactosidase Peptide Sequencing

Amino-terminal sequence analysis of the purified 44-kD β-galactosidase subunit was performed with an ABI 473A protein sequencer at the School of Biological Sciences (The University of Auckland). Internal proteolytic fragments were generated by digestion with trypsin and separated by HPLC on a 220- × 2.1-mm Aquapore RP-300 column, using a solvent gradient (*A* = 0.1% [v/v] TFA in water, *B* = 0.1% [v/v] TFA in aqueous 80% [v/v] CH_3CN; 10–70% *B* over 45 min), at a flow rate of 0.2 mL min^{-1}. Individual peaks were collected for sequence analysis.

Nucleic Acid Extraction

RNA was extracted from the cortical tissue of Granny Smith apple using a modification of the method of Boedtker

et al. (1976). Samples (20 g) were ground to a powder in liquid N_2 and transferred to a flask containing 20 mL of 25 mM NaOAc, 250 mM NaCl, 5 mM EDTA, 5% (w/v) SDS, pH 6.0. The aqueous phase was extracted twice with phenol:chloroform at 65°C, and nucleic acids were precipitated with ethanol. Following centrifugation at 12,000*g* for 30 min, the pellet was resuspended in 2 mL of 50 mM Tris-HCl, 10 mM EDTA, 1% (w/v) sodium lauryl sarcosine, pH 7.0. CsCl (0.5 g) was dissolved in the samples, which were then pipetted onto 2-mL cushions of 5.7 M CsCl in Beckman SW-41 ultracentrifuge tubes. The tubes were filled with sterile paraffin oil and centrifuged for 17 h at 15,000 rpm at 6°C in a SW-41 rotor. The pellet was resuspended in water and precipitated with ethanol prior to cDNA library construction or studies of expression.

Genomic DNA was extracted from young, expanding apple leaves using the method of Doyle and Doyle (1990).

cDNA Library Construction, Clone Isolation, and Sequencing

Total RNA was isolated from mature, unripe apple fruit, and poly(A^+) RNA was selected using a commercial mRNA purification kit (Pharmacia P-L Biochemicals Inc., Milwaukee, WI). A cDNA library was constructed in the Uni-ZAP XR vector following the manufacturer's instructions (Stratagene, La Jolla, CA). For library screening, duplicate Hybond N^+ (Amersham International Little Chalfont, UK) filter-lifts from six plates, each containing approximately 5000 plaques, were probed with ^{32}P-labeled insert from the pTIP31 plasmid, a clone isolated from an asparagus cDNA library (G. King, personal communication). Hybridization conditions were as described by the membrane manufacturers, with the highest wash stringency being in 1× SSC, 0.1% (w/v) SDS, 65°C. Six positive plaques were isolated and pBluescript excised using the ExAssist/SOLR system (Stratagene). One of these clones (pABG1) was fully sequenced in both directions by the dideoxynucleotide method with dye terminators on an ABI 373 DNA sequencer. Sequence analysis was performed using software from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

Gel Blot Analyses of RNA and Genomic DNA

Total RNA was extracted from apple fruit throughout development and ripening. The RNA samples (20 μg) were denatured and fractionated on a 1.2% agarose gel containing 0.66 M formaldehyde (Fourney et al., 1988) and transferred to Hybond N^+ membrane by capillary transfer in 50 mM NaOH. Prehybridization and hybridization conditions were as recommended by the membrane manufacturer. The membrane was probed with ^{32}P-labeled pABG1 insert produced by random priming (Boehringer Mannheim). Following hybridization before autoradiography, blots were washed twice in 2× SSC, 0.1% (w/v) SDS at room temperature and twice in 1× SSC, 0.1% (w/v) SDS at 65°C.

For Southern analysis, genomic DNA (10 μg) was digested to completion with *Eco*RI, *Hin*dIII, and *Pst*I (Boehringer Mannheim) and fractionated on a 0.6% (w/v) agarose gel. The DNA was transferred to Hybond N^+ by capillary transfer in 0.4 M NaOH. Hybridization and washing conditions were as described for RNA analysis. The 1× SDS, 0.1% (w/v) SDS (65°C) washing treatment represents a hybridization stringency that allows approximately 25% mismatch between pairing nucleic acids. After autoradiography, the membrane was washed at a higher stringency (0.1× SSC, 0.1% [w/v] SDS, 65°C; allowing 5% mismatch) and exposed to x-ray film again.

RESULTS

Enzyme Purification

Enzyme from the cortex of apples was purified up to 180-fold after affinity chromatography, with recovery of 20.8% of the extractable activity (Table I). Silver-stained SDS-PAGE showed that the active fractions following affinity chromatography had predominant polypeptides of 44 and 32 kD (Fig. 1). Occasionally a minor band of 77.5 kD was also visible on SDS-PAGE (data not shown). Gel permeation chromatography indicated that enzyme activity was associated with one protein peak with an estimated size of 59 kD (SE ±0.2, $n = 3$; data not shown). More than 30% of the total extractable enzyme activity was present in the supernatant of the initial purification step (Table I), which was usually discarded during purification. However, when this fraction was also taken through the purification protocol, the purified protein was found to have the same molecular mass as that purified from the salt-extracted fraction.

The enzyme was active against *p*-nitrophenyl-β-D-galactopyranoside but showed no activity against the following other *p*-nitrophenol substrates (0.33 μg μL^{-1}, pH 4.5): α-D-galactopyranoside, α-L-arabinofuranoside, β-D-xylopyranoside, α-L-rhamnopyranoside, α-L-fucopyranoside, β-L-fucopyranoside, α-D-mannopyranoside, β-D-mannopyranoside, *o*-β-D-glucopyranoside, *p*-β-D-glucopyranoside, acetyl-β-D-glucosamide. The pH optimum for enzymic action against *p*-nitrophenyl-β-D-galactopyranoside substrate was approximately 4.0 (data not shown). D-Galactono-(1→4)-lactone was a powerful inhibitor of the enzyme, even at 1 mM (88% inhibition), and Gal also showed some inhibition of activity (44% inhibition at 10 mM). The concentrations of D-galactono-(1→4)-lactone and Gal required for 50% inhibition of activity

Table I. *Purification and recovery of β-galactosidase from apple cortical tissue*

1 unit = 1 μmol *p*-nitrophenol released/min.

	Total Activity	Total Protein	Specific Activity	Purification Factor
	unit	*mg*	*unit mg^{-1} protein*	
Total tissue	13.4 (100%)	103.7	0.13	1
Salt extract	8.96 (66.8%)	24.2	0.37	2.9
Affinity chromatography	2.36 (17.6%)	0.11	21.5	165
Concentration by dialysis	2.79 (20.8%)	0.12	23.2	180

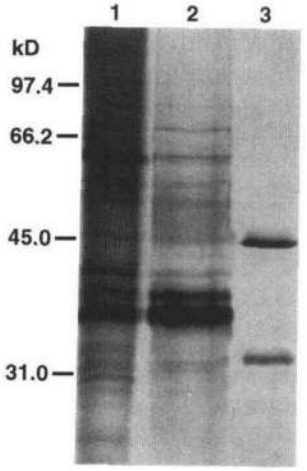

Figure 1. Silver-stained SDS-PAGE of aliquots taken at specific points during the purification protocol: Soluble extract, lane 1; salt extract, lane 2; eluent from lactosyl-Sepharose, lane 3.

were 0.12 and 13 mM, respectively. No inhibition of activity was observed with lactose, Glc, and Ara.

Activity against Native Substrates

Products of enzyme digestion of cell wall substrates were examined by TLC (data not shown) and quantified by capillary GLC (Table II). Only Gal was detected after incubation of the apple β-galactosidase with the galactan fraction prepared from apple cell walls. A trace of Gal could also be detected after incubation with apple Na_2CO_3-soluble pectin, and the apple β-galactosidase was also active against a galactan fraction from kiwifruit. Although the incubations included equal enzyme activity as measured against *p*-nitrophenyl-β-D-galactopyranoside, kiwifruit β-galactosidase was 20 times more active against kiwifruit galactan and 10 times more active against apple galactan than was apple β-galactosidase against the same substrates. There was no detectable Gal in any of the substrate or enzyme blanks.

β-Galactosidase Protein Sequence

The sequence of 15 amino acids at the N terminus of the 44-kD polypeptide was obtained (Table III). In addition, sequence information was obtained from three peptides isolated following tryptic digestion. Although a search of the EMBL protein data bases with the N-terminal sequence did not reveal any highly homologous sequences, peptides TPEP1 and TPEP3 showed homology with amino acid sequences coded by two unidentified, ethylene- and senescence-related clones from carnation and asparagus (pSR12, Raghothama et al., 1991; pTIP31, G. King, personal communication). With this sequence available, regions in the carnation and asparagus polypeptides that show some homology to the other apple peptide sequences could be located by eye (Fig. 2). The asparagus pTIP31 clone (provided by G. King) was, therefore, used as a nucleic acid probe in screening an apple fruit cDNA library to isolate pABG1, an apple homolog.

Table II. *Release of Gal by apple and kiwifruit β-galactosidase acting on apple and kiwifruit polysaccharides*

Two milligrams of substrate were incubated at 37°C with 1.0 mL of purified enzyme, capable of releasing 15 nmol *p*-nitrophenol min^{-1} at 30°C. tr, Trace.

	Gal Content of Polysaccharide	Gal Released
	mol %	*μg/2 mg substrate*
Apple β-galactosidase		
Apple galactan	20.8	2.0
Kiwifruit galactan	75.0	2.4
Apple Na_2CO_3-soluble pectin	7.6	tr
Kiwifruit β-galactosidase		
Apple galactan	As above	18.4
Kiwifruit galactan	As above	40.0
Kiwifruit Na_2CO_3-soluble pectin	12.0	15.0

pABG1, an Apple β-Galactosidase cDNA Clone

Escherichia coli DH5α colonies harboring the pABG1 plasmid appear blue when grown in the presence of isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indoyl-β-D-galactoside, a chromogenic substrate for β-galactosidase. The nucleic acid and deduced amino acid sequence of the pABG1 cDNA is shown (Fig. 3). The clone contains a 2637-bp insert and an open reading frame that extends from nucleotide 175 to nucleotide 2370. The positions of two in-frame Met residues near the 5′ terminus of the clone are indicated. If translation is initiated at the 5′ Met (which is flanked by a consensus initiation sequence AAAATGG; Kozak, 1983), then the open reading frame encodes a 81-kD polypeptide of 731 amino acids. This polypeptide shares 70 and 67% amino acid identity with pTIP31 and SR12, respectively.

The sequence contains a hydrophobic N-terminal leader sequence. According to an algorithm proposed for determining signal-sequence cleavage sites (Von Heijne, 1983), this leader sequence is likely to be cleaved at or very close to the Ala residue, which is aligned to the first residue of the N-terminal protein sequence. Cleavage of the signal sequence would leave a predicted mature polypeptide of 78.5 kD, with an approximate isoelectric point of 5.8. The 3′ untranslated region contains a putative polyadenylation sequence located approximately 30 bases upstream of the poly(A^+) tail. Also present in the 3′ untranslated region are several motifs that

Table III. *Protein sequence information obtained from the purified 41-kD apple β-galactosidase*

TPEP peptides were obtained after tryptic digestion of this subunit. Parentheses indicate where it was not possible to determine the residue at this position.

N terminus	A-K-V-T-Y-D-H-R-A-L-V-I-D-G-K
TPEP 1	S-T-P-E-M
TPEP 2	()-T-G-G-P-F-I-A-T-S-Y-()-Y
TPEP 3	V-L-V-S-G-S-I-H-Y-P

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CARNATION  29   GNVWYDYRAIKINDQRRILLSGSIHYPRSTPEM   61
ASPARAGUS  25   ASVTYDRKSVIINGQRRILISGSIHYPRSTPEM   57
APPLE           AKVTYDRALVEDGK VLVEGSIHYP STPEM

CARNATION 307   TA.GRFVSTSYDY   318
ASPARAGUS 302   TAGGPFISTSYDY   314
APPLE           T.GGPFIATSY Y
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Figure 2. Lineup of apple β-galactosidase peptide sequences with those coded by the *SR12* gene from carnation (Raghothama et al., 1991) and the pTIP31 mRNA from asparagus (G. King, personal communication). Numbers indicated are with respect to the start Met residue in these sequences. Shaded blocks indicate sequences common to more than one species.

show high homology with downstream sequence elements, which have been suggested to contribute to mRNA instability (Newman et al., 1993). Regions where the peptide coded by pABG1 shows homology to the amino acid sequences obtained from the β-galactosidase protein are also indicated (Fig. 3). There are some sequence differences (10 over the total 41 residues), particularly at the N terminus.

DNA Gel Blot Analysis

DNA gel blot analysis of apple genomic DNA digested with several restriction enzymes and probed with the ^{32}P-labeled pABG1 insert is shown in Figure 4. Restriction enzyme sites in the cDNA sequence suggests at least four *Eco*RI bands, three *Hin*dIII bands, and four *Pst*I bands should be observed. However, when the membrane was washed at moderate stringency (1× SSC, 0.1% [w/v] SDS, 65°C), all three enzyme digest lanes showed more hybridizing bands than predicted, and these were of various intensities. After the membrane was washed at high stringency (0.1× SSC, 1% [w/v] SDS, 65°C) a number of these bands disappeared, whereas others were maintained (Fig. 4). These observations indicate the presence in the apple genome of other sequences that are related to ABG1.

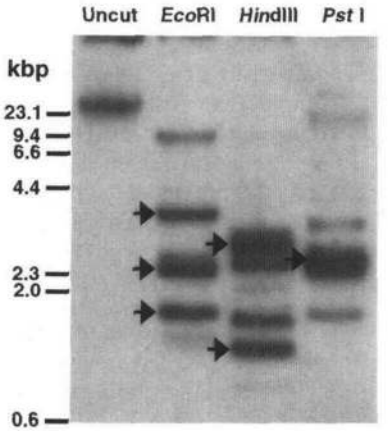

Figure 4. Genomic DNA gel blot analysis of the pABG1 clone. Genomic DNA (10 μg) digested with the indicated restriction enzyme was loaded on each lane. The blot was probed with the radiolabeled cDNA insert from the pABG1 clone and washed at a stringency that allowed for 25% mismatch between pairing nucleic acids. When washed at higher stringency (5% mismatch), some bands were lost, whereas those indicated with arrows were maintained.

Expression and Activity during Apple Fruit Development and Ripening

During fruit development and in mature, unripe fruit, there was no detectable expression of pABG1-homologous RNA

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CTCAACTCTGCCACTCTCTCTCTGTCTCTTTCCAAAATATCAAAAGCACCAAACAAAAGAAACCAAATTCAAATCCCAAAACACAATATATATTTTAAGT   100
TTTTGGTACAAACAAAGCAAGTATATTTATATATAAAGGCCATTGCTTTTGAGCGTTTCAGAAGCAAGGGAAAAAATGGGTGTTGGAATTCAAACAATGTG   200
                                                                  M  G  V  G  I  Q  T  M  W
GAGCATTCTGCTATTGTTTTCCTGCATTTTTTCTGCAGCTTCAGCTTCTGTGAGTTATGACCACAAGGCTATAATAATTAATGGGCAGAAAAGGATTTTA   300
 S  I  L  L  L  F  S  C  I  F  S  A  A  S  A  S  V  S  Y  D  H  K  A  I  I  N  G  Q  K  R  I  L
                                          *  K  *  T  *  *  *  R  *  L  V  *  D  *  *     V  *
ATTTCTGGCTCCATTCACTATCCCAGAAGCACTCCCGAGATGTGGCCGGATTTAATTCAGAAGGCCAAAGATGGAGGCTTGGATGTTATACAGACCTATG   400
 I  S  G  S  I  H  Y  P  R  S  T  P  E  M  W  P  D  L  I  Q  K  A  K  D  G  G  L  D  V  I  Q  T  Y
 V  *  *  *  *  *  *     *  *  *  *  *
TGTTTTGGAATGGCCATGAACCTTCTCCGGGAAATTATTATTTCGAGGAAAGATATGATTTGGTCAAGTTTATCAAGCTGGTGCAACAAGAAGGCCTATT   500
V  F  W  N  G  H  E  P  S  P  G  N  Y  Y  F  E  E  R  Y  D  L  V  K  F  I  K  L  V  Q  Q  E  G  L  F
TGTTAATCTCCGGATTGGCCCTTATGTTTGCGCTGAATGGAACTTCGGGGGATTCCCAGTTTGGCTGAAATATGTCCCTGGAATCGCTTTTCGAACGGAC   600
 V  N  L  R  I  G  P  Y  V  C  A  E  W  N  F  G  G  F  P  V  W  L  K  Y  V  P  G  I  A  F  R  T  D
AATGAGCCTTTCAAGGCGGCAATGCAAAAATTTACAGAGAAGATTGTCAGCATGATGAAGGCAGAGAAGCTGTTTCAAACTCAAGGAGGTCCTATAATTC   700
 N  E  P  F  K  A  A  M  Q  K  F  T  E  K  I  V  S  M  M  K  A  E  K  L  F  Q  T  Q  G  G  P  I  I
TCTCTCAGATAGAAATGAATTTGGACCTGTGGAATGGAATTGGTGCACCTGCAAAAGCTTACACCAATGGGCAGCTCAGATGGCTGTAGGCCTAGA   800
 L  S  Q  I  E  N  E  F  G  P  V  E  W  E  I  G  A  P  G  K  A  Y  T  K  W  A  A  Q  M  A  V  G  L  D
CACTGGAGTTCCATGGATTATGTGCAAGCAAGAGGATGCCCCCGATCCCGTTATTGACACTTGCAATGGTTTCTACTGTGAGAATTTCAAGCCAAATAAG   900
  T  G  V  P  W  I  M  C  K  Q  E  D  A  P  D  P  V  I  D  T  C  N  G  P  Y  C  E  N  F  K  P  N  K
GACTATAAGCCCAAAATGTGGACAGAAGTCTGGACTGGTTGGTATACAGAATTCGGTGGGGCAGTTCCCACTAGACCTGCAGAAGATGTGGCATTTTCAG   1000
 D  Y  K  P  K  M  W  T  E  V  W  T  G  W  Y  T  E  F  G  G  A  V  P  T  R  P  A  E  D  V  A  F  S
TTGCTAGGTTCATACAAAGCGGCGGTTCGTTTTTGAACTATTACATGTACCACGGAGGAACGAATTTTGGCCGAACAGCCGGAGGTCCCTTCATGGCCAC   1100
V  A  R  F  I  Q  S  G  G  S  F  L  N  Y  Y  M  Y  H  G  G  T  N  F  G  R  T  A  G  Q  P  F  M  A  T
                                                                  *  -  *  *  *  *  *  I  *  *
TAGCTATGACTACGACGCCCCCTTAGACGAATATGGACTACCCCGGGAACCAAAGTGGGGACATTTGAGAGATCTGCACAAAGCCATTAAATCATGTGAG   1200
  S  Y  D  Y  D  A  P  L  D  E  Y  G  L  P  R  E  P  K  W  G  H  L  R  D  L  H  K  A  I  K  S  C  E
  *  *  *  *  *
TCTGCTTTAGTGTCCGTTGATCCTTCAGTGACTAAACTCGGAAGTAATCAAGAGGCTCATGTATTCAAATCAGAGTCGGATTGCGCTGCATTCCTCGCAA   1300
 S  A  L  V  S  V  D  P  S  V  T  K  L  G  S  N  Q  E  A  H  V  F  K  S  E  S  D  C  A  A  F  L  A
ATTATGACGCAAAATACTCTGTTAAAGTGAGCTTTGGAGGCGGGCAGTATGACCTGCCGCCATGGTCCATCAGCATTCTTCCGGACTGCAAAACCGAAGT   1400
N  Y  D  A  K  Y  S  V  K  V  S  F  G  G  G  Q  Y  D  L  P  P  W  S  I  S  I  L  P  D  C  K  T  E  V
TTACAACACTGCAAAGGTTGGTTCGCAAAGCTCGCAAGTTCAGATGACACCAGTACATAGTGGATTTCCTTGGCAGTCATTCATCGAAGAAACCACGTCT   1500
 Y  N  T  A  K  V  G  S  Q  S  S  Q  V  Q  M  T  P  V  H  S  G  F  P  W  Q  S  F  I  E  E  T  T  S
TCTGATGAGACCGATACAACTACATTGGACGGGTTGTATGAGCAAATAAATATCACTAGGGATACTACAGACTACTTGTGGTACATGACAGATATCACAA   1600
 S  D  E  T  D  T  T  T  L  D  G  L  Y  E  Q  I  N  I  T  R  D  T  T  D  Y  L  W  Y  M  T  D  I  T
TAGGTTCTGATGAAGCGTTTCTAAAGAACGGAAAGTCCCCACTCTTACGATCTTTCAGCAGGTCATGCCTTGAATGTTTCATCAATGGTCAGCTATC   1700
 I  G  S  D  E  A  F  L  K  N  G  K  S  P  L  L  T  I  F  S  A  G  H  A  L  N  V  F  I  N  G  Q  L  S
AGGAACCGTGTATGGGTCATTGGAGAATCCTAAATTATCATTCAGTCAAAACGTGAACCTGAGATCTGGCATCAACAAACTTGCATTGCTTAGCATTTCC   1800
 G  T  V  Y  G  S  L  E  N  P  K  L  S  F  S  Q  N  V  N  L  R  S  G  I  N  K  L  A  L  L  S  I  S
GTGGGTCTGCCGAATGTTGGTACTCACTTTGAGACATGGAACGCGGGAGTTCTAGGCCCGATCACGTTGAAGGGTCTGAATTCAGGAACATGGGACATGT   1900
 V  G  L  P  N  V  G  T  H  F  E  T  W  N  A  G  V  L  G  P  I  T  L  K  G  L  N  S  G  T  W  D  M
CAGGGTGGAAATGGACGTACAAGACTGGTCTGAAAGGTGAAGCTTTAGGCCTCCATACTGTTACTGGGAGTTCTTCTGTTGAATGGGTAGAAGGACCATC   2000
 S  G  W  K  W  T  Y  K  T  G  L  K  G  E  A  L  G  L  H  T  V  T  G  S  S  S  V  E  W  V  E  G  P  S
GATGGCTGAAAAACAACCCCTTACATGGTACAAGCTACTTTTAATGCACCACCAGGTGATGCTCCATTAGCTTTAGATATGGGAAGCATGGGAAAAGGT   2100
  M  A  E  K  Q  P  L  T  W  Y  K  A  T  F  N  A  P  P  G  D  A  P  L  A  L  D  M  G  S  M  G  K  G
CAGATATGGATAAATGGACAGAGCGTGGGACGCCACTGGCCCGGGTACATTGCACGCGGCAGCTGTGGCGATGTTCTTATGCCGGAACTTATGATGATA   2200
 Q  I  W  I  N  G  Q  S  V  G  R  H  W  P  G  Y  I  A  R  G  S  C  G  D  C  S  Y  A  G  T  Y  D  D
AGAAATGCAGAACTCATTGTGGCGAGCCCTCTCAGAGATGGTACCACATTCCTCGATCATGGTTGACCCCGACTGGGAATCTTTTGGTGGTGTTCGAAGA   2300
K  C  R  T  H  C  G  E  P  S  Q  R  W  Y  H  I  P  R  S  W  L  T  P  T  G  N  L  L  V  F  E  E
ATGGGGTGGTGATCCGTCAAGGATTTCGTTGGTTGAAAGAGGTACAGCCCTGACGCGAAGAAGCTCTAGGTTGAGGCTGTCTGCAGATCCAGCAGATAC   2400
 W  G  G  D  P  S  R  I  S  L  V  E  R  G  T  A  L  D  A  K  K  L  *
GTAGATTACTAAATACGTGAAGTGGTTGTGTACATAGACAATCTATTAATTGTTGGAAAAAAATAGCTCCACATGATATACGAAGGATTACATACAAAGT   2500
TTGTAGTCAGTAGATTTGCGGAAGCATTTTCCATTGTAAGTTTGTAACAACTTATGGAAAAGATTTCCTTTTCCCTTACAAGAATAAATGGAAAACTAAT   2600
AGAGACTACTTTTATCAAAAAAAAAAAAAAAAAAAAA  2637
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Figure 3. Nucleic acid sequence of the pABG1 cDNA insert, with the amino acid sequence of the coding region. Two possible "start" Met codons are indicated in bold type, as is a putative polyadenylation sequence and a number of motifs in the 3′ untranslated region that show homology to downstream sequence elements (Newman et al., 1993). Also indicated are the regions of homology between the apple peptide sequences and the polypeptide encoded by pABG1 (* indicates an identical residue; otherwise the different residue is given).

(Fig. 5). However, after harvest, pABG1-homologous RNA accumulated to a level at which hybridization with an RNA species of 2.6 kb was easily detectable in both the 1- to 10-ppm and >100-ppm ethylene samples. Enzyme-specific activity, as measured in the crude salt extracts, was low in the earliest (4 week) sample but increased during the following 8 weeks to a level that was maintained during the later stages of fruit development and after harvest (Fig. 5).

DISCUSSION

The loss of Gal from the cell walls of ripening fruit has been well documented (Bartley, 1976; Seymour et al., 1990; Redgwell et al., 1992a). There is currently widespread interest in β-galactosidase as the enzyme most likely to be responsible for this change. The enzyme has now been purified from a number of different fruit, including tomato (Pressey, 1983), orange (Burns, 1990) kiwifruit (Ogawa et al., 1990; Ross et al., 1993), and apple (Dick et al., 1990; this report), and activity against cell wall substrates has been shown in tomato (Pressey, 1983), muskmelon (Ranwala et al., 1992), kiwifruit (Ross et al., 1993), and now apple.

β-Galactosidases have been purified from a wide range of plant tissues using affinity chromatography on lactosyl-Sepharose (Simos et al., 1989; Ross et al., 1993). Consistently, two subunits are obtained, which range in molecular mass from 42 to 46 and 31 to 35 kD. From Granny Smith apples we have obtained 180-fold purification of β-galactosidase to provide a preparation that shows bands of 44 and 32 kD. The lack of other bands after SDS-PAGE suggests that the level of purification achieved was close to homogeneity. Previously, a β-galactosidase of 79 kD was purified from Spartan apples (Dick et al., 1990), with other proteins of 146 and 161 kD also detected that had lesser activity. It is possible that the ephemeral SDS-PAGE band that we observed at 77.5 kD is an aggregation of the two smaller polypeptides. The discrepancy between the molecular mass estimates obtained by SDS-PAGE and gel permeation appears to be a common feature of β-galactosidase preparations (Sekimata et al., 1989; Giannakouros et al., 1991; Ross et al., 1993).

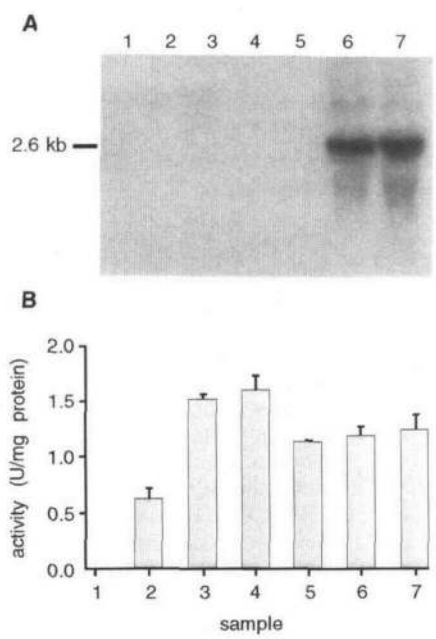

Figure 5. RNA gel blot analysis of pABG1 gene expression (A) and β-galactosidase activity during apple fruit development and ripening (B). Each lane in A contained 20 μg of total RNA extracted from cortical tissue of developing fruit. For B, activity was measured in salt extracts from the cortical tissue of developing fruit, and data presented are the means of four replicates ± SE (1 unit = 1 μmol *p*-nitrophenol released per min). Lanes: 1, 4 weeks after full bloom; 2, 8 weeks; 3, 12 weeks; 4, 16 weeks; 5, harvest maturity; 6, postharvest, producing 1 to 10 ppm ethylene; 7, postharvest, >100 ppm ethylene.

The purified enzyme released Gal from a galactan prepared from apple cell walls and a trace of Gal from a Na_2CO_3-soluble pectic fraction. However, β-galactosidase from kiwifruit was much more effective in releasing Gal from both polysaccharides. Although the significance of this difference awaits further experimentation, it is noteworthy that activity was detected at all, since in a previous study in which an apple β-galactosidase was incubated with an extract from apple cell walls, no activity was detected (Wallner, 1978). The apple galactan and Na_2CO_3-soluble pectic fractions are likely to contain β-(1→4) linkages (Aspinall and Fanous, 1984). Furthermore, the galactan fraction accounted for most of the Gal in apple cell wall material (R.J. Redgwell, unpublished data) and is, therefore, likely to be the major source of the Gal lost during ripening. Because monomeric Gal was the only product that we detected from enzymic digestion, we conclude that this enzyme acts in an exo-fashion, removing single galactosyl residues from the nonreducing end of polysaccharides.

As with a kiwifruit β-galactosidase (Ross et al., 1993), the amount of Gal released by the apple enzyme in vitro is unlikely to be sufficient to account for the observed in vivo loss of Gal from apple cell walls during ripening (Bartley, 1976). Previously, we speculated concerning possible causes for the low in vitro enzyme activity (Ross et al., 1993). Recent evidence has suggested that even only limited in vivo β-galactosidase activity on the pectins of the cell wall could have a significant effect on pectin solubility, through decreasing the ability of pectin molecules to aggregate (De Veau et al., 1993). Thus, limited β-galactosidase action on the side chains of the pectic backbone could have major implications for the matrix of the cell wall in rendering substrates susceptible for hydrolysis by other cell wall-degrading enzymes.

Work on plant β-galactosidases is entering the molecular arena. The apple protein sequence that we obtained suggests a carnation clone (SR12; Raghothama et al., 1991) and an asparagus clone (pTIP31; G. King, personal communication) code for β-galactosidase in these species. These clones were isolated following differential screening of cDNA libraries and were induced following ethylene treatment (carnation) or harvest (asparagus). The challenge with this approach is the identification of the differential clones that are isolated. In this respect, the data we provide from the apple β-galactosidase protein are especially useful in efforts to assign functionality to two previously unidentified clones. There is supporting evidence for the presence of β-galactosidase activ-

ity in senescing carnations and asparagus spears. The cell walls of carnation petals lose considerable quantities of Gal during senescence (de Vetten and Huber, 1990). Similarly, cell wall analysis in asparagus stem tissue after excision has indicated a storage-related decrease in (1→4) linked Gal in all fractions rich in pectic polysaccharides (Waldron and Selvendran, 1992).

We were able to use the pTIP31 cDNA directly as a probe to isolate an apple homolog. The mismatches between peptide sequences suggest that the pABG1 clone may not in fact encode the exact protein that was purified from apple flesh. However, we believe that β-galactosidase in apples is encoded by a multigene family, and pABG1 is one of the members. Several lines of evidence support this conclusion. First, following a high-stringency wash of the genomic DNA gel blot, several previously detectable bands were lost, whereas others were maintained. This indicates the presence of other related sequences in the apple genome with lower homology to pABG1. Second, preliminary analysis of DNA sequences and restriction enzyme sites in the other pTIP31 homologs isolated in our laboratory has indicated a number of different family members. Although a range of clones are available, we have yet to identify a member that encodes the apple peptides without discrepancy. Finally, the observation that colonies harboring the pABG1 plasmid are blue on 5-bromo-4-chloro-3-indoyl-β-D-galactoside suggests that pABG1 encodes β-galactosidase enzyme, although this evidence is not conclusive identification, since other clones containing a cDNA insert that interrupts the *lac*Z gene also occasionally appear blue. Confirmation that pABG1 encodes an apple β-galactosidase awaits expression of the functional protein in a system that does not involve *lac* genes.

The SR12 (carnation), pTIP31 (asparagus), and pABG1 (apple) transcripts are of sufficient lengths to code for proteins of 82.8 kD (Raghothama et al., 1991), 92.2 kD (G. King, personal communication), and 81 kD, respectively. Whereas the putative leader sequence in the apple polypeptide could be cleaved to leave a mature protein of 78.5 kD, the apple protein sequence was obtained from a 44-kD polypeptide. Since the sequence homologies among the apple, carnation, and asparagus clones occur in a region close to the 5′ end of the respective coding regions, we suggest that during processing of the mRNA, or during either enzyme extraction or SDS-PAGE, a large polypeptide is cleaved from the C terminus of the apple protein. This other subunit may be the 32-kD band visible during SDS-PAGE. We have sought amino acid sequence from this subunit to confirm this, but its N terminus appears to be blocked.

Northern analysis of pABG1 expression during apple fruit development and ripening indicates that pABG1 is a ripening-related gene, with an accumulation of transcripts coincident with autocatalytic ethylene production by the fruit. This pattern of ethylene- or senescence-related expression is consistent with that observed with the carnation and asparagus homologs (Raghothama et al., 1991; King and Davies, 1992; G. King, personal communication). Our measurements of enzyme activity in apple fruits indicate relatively consistent levels of activity during the later stages of fruit development and ripening. This result is consistent with previous studies (Dick et al., 1990). The apparent inconsistency between mRNA expression and enzyme activity could be explained by the presence of divergent enzyme isoforms that may be expressed at more constitutive levels.

In conclusion, the clone reported here appears to represent one member of an apple β-galactosidase gene family. There is good evidence that β-galactosidase enzymes are capable of acting on cell wall polysaccharides, but the significance of their action in the overall softening process remains to be shown.

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LITERATURE CITED

Albersheim P, Nevins DJ, English PD, Karr A (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohydr Res **5:** 340–345

Aspinall GO, Fanous HK (1984) Structural investigations on the non-starchy polysaccharides of apples. Carbohydr Polym **4:** 193–214

Bartley IM (1974) β-Galactosidase activity in ripening apples. Phytochemistry **13:** 2107–2111

Bartley IM (1976) Changes in the glucans of ripening apples. Phytochemistry **15:** 625–626

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99

Boedtker H, Frischauf AM, Lehrach H (1976) Isolation and translation of *Calvaria* procollagen messenger ribonucleic acids. Biochemistry **15:** 4765–4770

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Burns JK (1990) α- and β-Galactosidase activities in juice vesicles of stored Valencia oranges. Phytochemistry **29:** 2425–2429

De Veau I, Gross KC, Huber DJ, Watada AE (1993) Degradation and solubilisation of pectin by β-galactosidases purified from avocado mesocarp. Physiol Plant **87:** 279–285

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:** 387–395

De Vetten NC, Huber DJ (1990) Cell wall changes during the expansion and senescence of carnation (*Dianthus caryophyllus*) petals. Physiol Plant **78:** 447–454

Dick AJ, Bearne SL (1988) Inhibition of β-galactosidase of apple by flavonoids and other polyphenols. J Food Biochem **12:** 97–108

Dick AJ, Opuku-Gyamfua A, DeMarco AC (1990) Glycosidases of apple fruit: a multi-functional β-galactosidase. Physiol Plant **80:** 250–256

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus **12:** 13–15

Fourney RM, Miyakoshi J, Day RS III, Paterson MC (1988) Northern blotting: efficient RNA staining and transfer. Focus **10:** 5–7

Giannakouros T, Karagiorgos A, Simos G (1991) Expression of β-galactosidase multiple forms during barley (*Hordeum vulgare*) seed germination. Separation and characterization of enzyme isoforms. Physiol Plant **82:** 413–418

Giovannoni JJ, DellaPenna D, Bennett AB, Fischer RL (1989) Expression of a chimeric polygalacturonase gene in transgenic *rin* (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. Plant Cell **1:** 53–63

King GA, Davies KM (1992) Identification, cDNA cloning and analysis of mRNAs having altered expression in tips of harvested asparagus spears. Plant Physiol **100:** 1661–1669

Kozak M (1983) Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol Rev **47:** 1–45

Lidster PD, McRae KB, Dick AJ (1985) Effects of a vacuum infusion of a partially purified β-galactosidase inhibitor on apple quality. Hortscience **20:** 80–82

Morreau H, Galjart NJ, Gillemans N, Willemsen R, van der Horst GTJ, d'Azzo A (1989) Alternative splicing of a β-galactosidase mRNA generates the classic lysosomal enzyme and a β-galactosidase-related protein. J Biol Chem **264:** 20655–20663

Newman TC, Ohme-Takagi M, Taylor CB, Green PJ (1993) DST sequences, highly conserved among plant SAUR genes, target reporter transcripts for rapid decay in tobacco. Plant Cell **5:** 701–714

Ogawa H, Fukumoto H, Yano T, Yanamoto K, Tochikura T (1990) Purification and characterisation of β-galactosidase from kiwifruit. J Jpn Soc Food Sci Technol **37:** 298–305

Pressey R (1983) β-Galactosidases in ripening tomatoes. Plant Physiol **71:** 132–135

Raghothama KG, Lawton KA, Goldborough PB, Woodson WR (1991) Characterization of an ethylene regulated flower senescence-related gene from carnation. Plant Mol Biol **17:** 61–71

Ranwala AP, Suematsy C, Masuda H (1992) The role of β-galactosidase in the modification of cell wall components during muskmelon fruit ripening. Plant Physiol **100:** 1318–1325

Redgwell RJ, Melton LD, Brasch DJ (1992a) Cell wall dissolution in ripening kiwifruit (*Actinidia deliciosa*). Solubilization of the pectic polymers. Plant Physiol **98:** 71–81

Redgwell RJ, Melton LD, Brasch DJ, Coddington JM (1992b) Structures of the pectic polysaccharides from the cell walls of kiwifruit. Carbohydr Res **226:** 287–302

Ross GS, Redgwell RJ, MacRae EA (1993) Kiwifruit β-galactosidase: isolation and activity gainst kiwifruit cell wall polysaccharides. Planta **189:** 499–506

Saltveit ME Jr (1982) Procedures for extracting and analysing internal gas samples from plant tissues by gas chromatography. Hortscience **17:** 878–881

Sekimata M, Ogura K, Tsumuraya Y, Hashimoto Y, Yamamoto S (1989) A β-galactosidase from radish (*Raphanus sativus* L.) seeds. Plant Physiol **90:** 567–574

Seymour GB, Colquhoun IJ, DuPont S, Parsley KR, Selvendran RR (1990) Composition and structural features of cell wall polysaccharides from tomato fruits. Phytochemistry **29:** 725–731

Simos G, Giannakouros T, Georgatsos JG (1989) Plant β-galactosidases: purification by affinity chromatography and properties. Phytochemistry **28:** 2587–2592

Von Heijne G (1983) Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem **133:** 17–21

Waldron KW, Selvendran RR (1992) Cell wall changes in immature asparagus stem tissue after excision. Phytochemistry **31:** 1931–1940

Wallner SJ (1978) Apple fruit β-galactosidase and softening in storage. J Am Soc Hortic Sci **103:** 364