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SOFTENING OF KIWIFRUIT DISCS: EFFECT OF INHIBITION OF GALACTOSE LOSS FROM CELL WALLS

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Key Word Index—*Actinidia deliciosa*; Actinidiaceae; kiwifruit; cell wall; fruit ripening; polysaccharides; galactose; pectin solubilization.

Abstract—Ripening-related cell wall changes that are characteristic of intact kiwifruit (*Actinidia deliciosa* var. *deliciosa*) were examined in excised outer pericarp discs, in the presence and absence of metabolic inhibitors. Cell wall materials (CWMs) were prepared from discs at time 0 and 24, 48 and 72 hr after excision. Control discs softened rapidly over 72 hr, showing a decrease in cell wall-associated galactose and an increase in pectin solubilization. When discs were treated with 50 mM galactose there was complete inhibition of galactose loss from the CWMs 24 hr after excision and a retardation of galactose loss after 72 hr, but no effect on either the rate of disc softening or pectin solubilization. Discs treated with aminooxyacetic acid, an inhibitor of ethylene biosynthesis, did not soften or show any sign of pectin solubilization after 72 hr but did show a loss of galactose from the CWM. The results suggest that the loss of cell wall-associated galactose and pectin solubilization in ripening kiwifruit are separate processes and that galactose loss, in part, may be independent of ethylene.

INTRODUCTION

Loss of galactosyl residues from cell walls during fruit ripening is well documented [1]. Galactosyl residues are normally lost from pectic polysaccharides where they occur as β-1-4 side-chains attached to the rhamnogalacturonan backbone [2]. Solubilization of cell wall pectin is widespread among ripening fruit but the *in vivo* mechanism of the process has not been well defined. In tomato, the action of polygalacturonase has been implicated in both pectin solubilization and depolymerization [3] but the apparent absence of this enzyme in some fruit [4] implies the existence of alternative mechanisms for pectin solubilization. The degradation of neutral pectin side-chains may have a marked influence on the rheological and physicochemical properties of pectin and of the cell wall. Recent work [5] has demonstrated that the removal by β-galactosidase of a few monomeric galactosyl residues from a chelator-soluble pectin caused a significant decrease in the apparent M_r of the pectin, and an increase in its solubility.

To date, few attempts have been made to establish a firm correlation between galactose loss, pectin solubilization and rate of fruit softening. Lidster *et al.* [6] tested the effects of vacuum infusion of a partially purified inhibitor of β-galactosidase on apple firmness and reported some retention of fruit firmness in the treated fruit. However, there was insufficient data to implicate a direct cause and effect relationship, since no attempt was made to show that the inhibitor prevented the loss of galactose from the cell wall. Apple is not the ideal fruit for such experiments, as apples do not soften markedly during ripening, galactose is not the predominant neutral sugar associated with the cell wall pectin and pectin solubilization is not pronounced [7]. Kiwifruit on the other hand softens considerably during ripening, shows a dramatic decrease in cell wall galactose and a marked solubilization of cell wall pectin [8]. In addition, β-galactosidase has been purified from kiwifruit which is capable of degrading β-1-4 galactosyl side-chains of a range of kiwifruit pectic polysaccharides [9]. The action of this β-galactosidase on a synthetic substrate can be inhibited *in vitro* by galactose and D-galactonic acid γ-lactone [9].

In the present study, discs were excised from the outer pericarp of unripe kiwifruit and allowed to soften over 72 hr. The effect of adding β-galactosidase inhibitors to the discs on disc softening, galactose loss and pectin solubilization was determined.

RESULTS AND DISCUSSION

Disc preparation

Kiwifruit discs showed no browning or other outward signs of deterioration during the 72 hr of the experiment. Mannitol (0.4 M) was provided in all washing solutions and agar plates to protect cells from rupturing [10]. There was an increase in fresh weight from 4.4 to 5.1 g (wt

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of 25 discs) over 72 hr as the discs swelled and absorbed moisture. This was expected, because cell wall swelling during ripening of intact kiwifruit is pronounced [8]. When ripening was inhibited with aminooxyacetic acid (AOA) (see later) the discs did not swell or increase in fresh weight during the 72 hr.

Ripening-related changes

The use of excised tissue discs for studying physiological processes has several advantages particularly where tissues need to be treated with metabolic precursors and inhibitors in order to test hypotheses. The extrapolation of measurements from a tissue slice to an intact organ such as a fruit must be done with caution unless evidence is obtained which can demonstrate that equivalent processes take place in both excised tissue and the intact organ. In tomato, a detailed comparison of ripening processes in intact fruit and excised pericarp discs [11] has demonstrated that excising the discs did not disrupt the normal physiological processes which accompanied ripening in intact fruit. The patterns of change in cell wall composition reported for ripening pericarp discs were duplicated in intact fruit. In the present study, we have shown that, as with tomato, several of the major features of ripening-related change which take place in intact kiwifruit occurred in tissue discs. The loss of firmness in the control discs was, as in intact fruit, accompanied by a depletion of starch (DMSO-soluble), an increase in pectin solubilization (phenol–acetic acid–water [PAW]-soluble) and a loss of galactose from the cell wall materials (CWM) (Table 1). Cell wall swelling, a characteristic feature of softening in the outer pericarp of kiwifruit [8], was also observed in the CWM prepared from discs (data not shown). Most of these changes were detected 24 hr after disc excision and became pronounced 48 and 72 hr after excision. Moreover, results of an experiment conducted during the 1991 season which compared ripening-related changes occurring in discs 48 hr after excision and discs freshly cut from intact kiwifruit 48 hr after a postharvest ethylene treatment, showed that pectin solubilization, starch degradation and galactose loss occurred to a similar degree in the two systems (Table 2).

Ethylene-induced ripening

The softening of kiwifruit discs and associated cell wall changes were far more rapid than similar changes in tomato discs. This was probably because of the wound-induced ethylene which is produced within hours of excising kiwifruit discs. Concentrations as low as 0.1 $\mu l\, l^{-1}$ in the storage environment have been shown to cause more rapid softening in kiwifruit than when ethylene is absent [12]. Wound-induced ethylene was measured in our experiments and the values showed that ethylene concentrations in each petri dish exceeded 1 $\mu l\, l^{-1}$ within 24 hr. When discs were treated with 1 mM AOA, an inhibitor of ethylene biosynthesis, they did not soften and the ripening-induced changes found in control discs were severely restricted (Fig. 1). A similar effect was produced when discs were treated with cycloheximide, a general inhibitor of translation (Fig. 1). These results, allied to those in Tables 1 and 2, suggested that disc softening and the accompanying cell wall changes were the result of

Table 1. Changes in tissue firmness, yield of PAW- and DMSO-soluble fractions and the galactose content of CWM of control discs at various times after excision from kiwifruit

Time (hr)	Disc firmness (N)	PAW-soluble (mg g^{-1} fr. wt)	DMSO-soluble (mg g^{-1} fr. wt)	Galactose ($\mu g\, mg^{-1}$ CWM)
0	47.6 ± 3.2	0.90 ± 0.06	38.6 ± 2.7	83.5 ± 1.6
24	17.1 ± 1.4	1.70 ± 0.08	33,6 ± 1.6	62.1 ± 1.7
48	2.5 ± 0.4	2.20 ± 0.16	20.5 ± 1.9	40.9 ± 1.6
72	1.50 ± 0.21	2.90 ± 0.24	12.8 ± 0.4	35.9 ± 2.4

Values are mean of three samples ± s.e.

Table 2. Comparison of ripening-related changes between intact fruit 48 hr after ethylene treatment and fruit discs 48 hr after excision

Treatment	Tissue firmness (N)	PAW-soluble (mg g^{-1} fr. wt)	DMSO-soluble (mg g^{-1} fr. wt)	Galactose ($\mu g\, mg^{-1}$ CWM)
Discs				
$t = 0$	18.7 ± 4.9	6.5 ± 0.3	103 ± 10.0	39.2 ± 1.9
48 hr	3.5 ± 1.3	13.8 ± 3.2	32 ± 5.0	21.8 ± 1.6
Intact fruit				
$t = 0$	19.6 ± 7.7	6.3 ± 0.24	116 ± 2.0	35.9 ± 2.8
48 hr	4.0 ± 1.0	12.9 ± 0.56	58 ± 1.4	23.8 ± 1.6

Values are mean of three samples ± s.e.

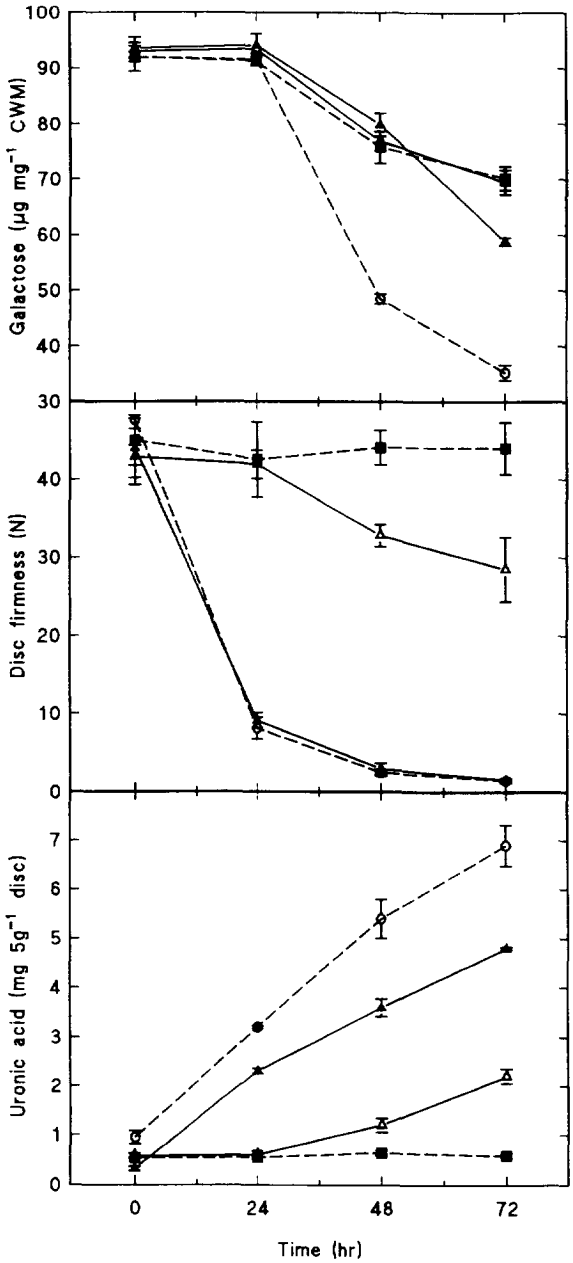

Fig. 1. Effect of 50 mM galactose, 1 mM aminooxyacetic acid and 50 μM cycloheximide on tissue firmness, galactose content and pectin solubilization in CWMs of kiwifruit discs 24, 48 and 72 hr after excision from intact fruit. --- ○--- control, ---■--- AOA, —▲—galactose, —△—cycloheximide.

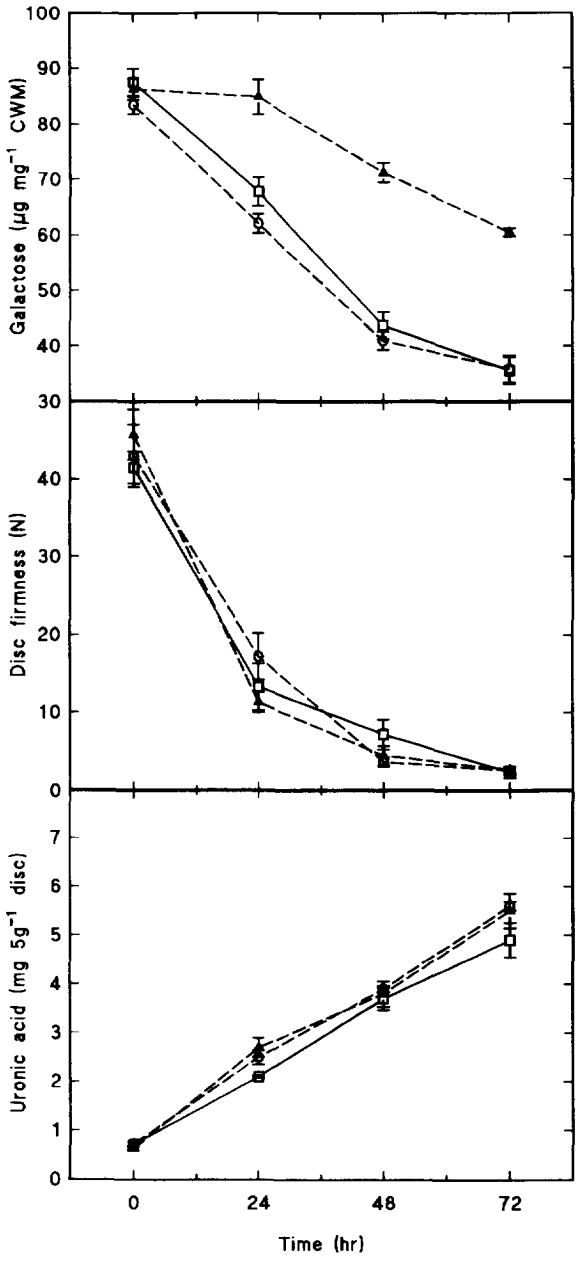

Fig. 2. Effect of galactose and glucose on tissue firmness, galactose content and pectin solubilization in kiwifruit discs 24, 48 and 72 hr after excision from intact fruit. --- ○--- control, ---▲---galactose, —□— glucose.

metabolically regulated processes, analogous to those occurring in intact fruit and were not induced as a consequence of procedures used to excise and treat the tissue.

Inhibition of galactose loss during softening

The timing of galactose depletion from the CWMs differed in each experiment (Figs 1 and 2). In experiment 1, there was no change in galactose content of control discs 24 hr after excision. However, 48 and 72 hr after excision, cell wall galactose levels decreased to 52.8 and 38.4% of that at time zero. In experiment 2, galactose content decreased within 24 hr, to 74.4% of that at time zero and then showed a similar rate of depletion as in experiment 1, 48 and 72 hr after disc excision. Fruit for experiment 2 were harvested at a maturity of 10.2% SSC compared to 6.3% SSC for the fruit of experiment 1. The difference in timing of galactose removal from cell walls of discs in the two experiments was likely to reflect the

greater maturity and *in vivo* ripening that had already taken place in the fruit of experiment 2 and suggested that the susceptibility of the wall to galactose loss is, in part, developmentally regulated.

The action of β-galactosidase on synthetic substrates *in vitro* can be inhibited by D-galactonic acid γ-lactone or galactose, with the former being the better inhibitor at low concentrations [9]. In the disc experiments, 50 mM galactose proved more effective (70% inhibition) than 1 mM D-galactonic acid γ-lactone (40% inhibition) as an inhibitor of galactose loss from the CWMs (data not shown). Galactose (50 mM) was, therefore, the inhibitor of choice for experiments 1 and 2 in the 1992 season.

Galactose was most effective in inhibiting galactose loss from CWM, 24 and 48 hr after excision of the discs (Figs 1 and 2). In experiment 2, there was no detectable change in the galactose content of the CWM 24 hr after excision, compared to a 36% decrease in the control discs. In experiments 1 and 2, 48 hr after excision, the galactose-treated discs lost 14.6 and 17.5%, respectively, of the galactose in CWM, compared to 70 and 62.9% in control discs of the equivalent experiments. Inhibition of galactose loss had no detectable effect on rate of softening at any time after excision. In experiment 2, even where there was 100% inhibition of galactose loss 24 hr after excision, discs softened as rapidly as control discs over the 24 hr period. To demonstrate that inhibition was specifically related to the addition of galactose and was not a general effect of adding 50 mM hexose to the discs, a separate treatment was done using 50 mM glucose. Results showed that 50 mM glucose had no effect on the rate of loss of galactose from the CWMs (Fig. 2).

Pectin solubilization vs galactose loss

In control discs, the uronic acid content of the PAW-soluble fraction increased eight-fold in 72 hr, indicating considerable solubilization of cell wall pectin (Figs 1 and 2). Inhibition of galactose loss with a 50 mM galactose treatment did not retard pectin solubilization at any sampling time (Fig. 2). Discs treated with AOA lost some galactose after excision but did not soften and demonstrated a negligible degree of pectin solubilization over 72 hr (Fig. 1). These results suggested that pectin solubilization and removal of galactose residues from the cell wall of the discs were separate processes and that at least some turnover of galactose polymers in maturing kiwifruit may be ethylene-independent. Additional evidence for this is provided by work with tomatoes, where it was shown that a significant decrease in cell wall galactose preceded the increase in ethylene production during the initial phases of ripening [13]. Our results also confirm previous studies that pectin solubilization and kiwifruit softening invariably occur in synchrony [8, 14], a fact reinformed by the results of treating discs with cycloheximide (Fig. 1). Cycloheximide did not completely inhibit softening but did retard the process considerably. Even so, the softening which did occur was accompanied by some pectin solubilization (Fig. 1).

DISCUSSION

Inhibition of the loss of cell wall-associated galactose from kiwifruit discs had no consistent effect on loss of tissue firmness or the degree of pectin solubilization. Previous studies with intact kiwifruit allowed to ripen on the vine [14] demonstrated that most cell wall galactose was lost before pectin solubilization. In fruit ripened by a post-harvest ethylene treatment, the beginning of pectin solubilization preceded galactose loss [8]. In the present work also, differences in the temporal relationship between loss of galactose and pectin solubilization suggests that the latter occurred only as a consequence of ripening and softening but that loss or turnover of galactose plays a role in several facets of cell wall metabolism throughout growth and development of the fruit.

The complexity of galactose metabolism in fruit is supported by studies on the β-galactosidase enzyme system. In apple, a β-galactosidase-related clone (pABG1) has been characterized and shown to be a member of a multi-gene family [15]. The mRNA homologous to this clone accumulated during ripening, whereas there was little change in enzyme activity during kiwifruit [16] and apple development and ripening [15]. The apparent inconsistency between enzyme activity and messenger mRNA expression has been ascribed to the presence of divergent enzyme isoforms, some of which may be expressed constitutively [15]. Such results imply that loss or turnover of galactose from the cell wall may occur throughout growth and development of the fruit. To date β-galactosidase is the only enzyme which has been shown to remove galactose from cell wall polysaccharides of apple and kiwifruit [9, 15]. The role of β-galactosidase in relation to cell wall metabolism awaits the analysis of transgenic plants with reduced activity of the enzyme and the possible selective manipulation of β-galactosidase isoforms.

In conclusion, while galactose loss from the cell wall may be an important event in the process that leads to fruit softening, the results presented in this paper indicate that the degree of galactose loss can be reduced with apparently no effect on tissue softening. Galactose loss may, therefore, be necessary but not sufficient for pectin solubilization and fruit softening.

EXPERIMENTAL

Plant material. Kiwifruit [*Actinidia deliciosa* (A. Chev.) C. F. Liang and A. R. Ferguson var. *deliciosa* Hayward] were harvested from the Kumeu Research Orchard, New Zealand. Experiments were done over two seasons on fruit harvested on 25 May and 5 June 1991, and on 6 and 22 May 1992. At each harvest, fruit of uniform size (120–140 g) were selected from a single vine and normal harvest parameters were measured as described in ref. [17].

Disc preparation and treatment. Eight fruit were surface sterilized in 2.5% NaOCl soln (10 min) and rinsed well in sterile dist. H_2O in a laminar flow cabinet where all subsequent operations were done. Ends of fruit were cut

off and discarded and 0.7 cm diameter longitudinal plugs of outer pericarp tissue removed with a corkborer from the body of the fruit. Approximately 250 discs, 4 mm in thickness, were cut with razor blades from plugs of tissue.

The following procedure is that used for the control treatment. All solns were sterilized by autoclaving before use. Freshly excised discs were suspended in 100 ml of 0.4 M mannitol with occasional stirring for 2 min. The soln was decanted and discs resuspended in a further 100 ml of 0.4 M mannitol for 3 min. This soln was also decanted and discs resuspended for a third time in 100 ml of 0.4 M mannitol for 4 min. Discs were gently blotted on a sheet of 3 MM paper to remove surface liquid and 25 discs transferred to the surface of a Petri dish of agar (0.7%) containing 0.4 M mannitol. The dishes were incubated at 20° for 72 hr.

The procedure for non-control treatments was identical, except that compounds required for each treatment were added to the mannitol washing soln and agar. The major results reported in this study are from two expts done during the 1992 season. Treatments for expt 1 were: control, 50 μM cycloheximide, 1 mM aminooxyacetic acid (AOA) (pH 5.8) and 50 mM galactose. Treatments for expt 2 were: control, 50 mM galactose, and 50 mM glucose. Four replicates, each of 25 discs, were taken for each treatment. One replicate was used to measure the firmness of the discs at time zero and 24, 48 and 72 hr after incubation using an Instron Model 4301 materials testing machine (Instron, USA). Disc firmness was measured by compression using a 13 mm diameter flat-tipped probe which compressed the discs at a rate of 20 mm min^{-1}. Peak-force was recorded for each disc. The remaining three replicates were used for cell wall analysis.

Preparation of cell wall fractions. Discs (*ca* 5 g per sample) were removed from the surface of the agar, transferred to plastic vials and frozen in liquid N_2. PhOH–HOAc–H_2O (2:1:1 w/v/v) (10 ml) was added and discs homogenized to a uniform slurry using a Polytron. The suspension was centrifuged (5000 g), the supernatant decanted and the residue resuspended in 10 ml H_2O, homogenized and centrifuged. Supernatants were combined, dialysed and freeze-dried to give the PAW-soluble fr. Cell wall pectin solubilized *in vivo* during ripening is recovered in the PAW-soluble fr. An increase in the uronic acid content of this fr., therefore, reflects a greater degree of *in vivo* pectin solubilization during ripening [8].

The PAW-insoluble residue was suspended with a Polytron in 9 ml DMSO and 10 ml 90% DMSO and left overnight. Following centrifugation, the supernatant was decanted and the residue washed with 10 ml 90% DMSO. The supernatant was recovered, combined with the first supernatant, dialysed and freeze-dried (DMSO-soluble fr.). The DMSO-insoluble residue was suspended in H_2O, poured into a glass fibre filter, washed exhaustively with H_2O and freeze-dried to give the cell wall material (CWM).

Chemical analyses. Monosaccharide composition of CWMs was determined by capillary GC of their alditol acetates following TFA hydrolysis [18]. Uronic acid content of PAW-soluble frs was determined by the method of ref. [19].

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