Pectinesterase, Polygalacturonase, and β -galactosidase during Softening of Ethylene-treated Kiwifruit

Teresa F. Wegrzyn and Elspeth A. MacRae
*Department of Scientific and Industrial Research, Fruit and Trees,
Private Bag, Auckland 1, New Zealand*

Additional index words. ethylene, fruit softening, cell wall, *Actinidia deliciosa*

Abstract. The activities of several cell wall-associated enzymes of the outer pericarp were assayed during softening of kiwifruit [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* cv. Hayward] treated with ethylene. The activity of polygalacturonase (EC 3.2.1.15) increased slightly during fruit softening, while β -galactosidase (EC 3.2.1.23) activity remained constant. Salt-extracted pectinesterase (EC 3.1.1.11) activity increased during ethylene treatment, then dropped rapidly to low levels as fruit softened. Residual pectinesterase activity, extracted after digestion of the cell wall pellet with a fungal enzyme mix, decreased on softening. The rapid softening of kiwifruit in response to ethylene treatment may be initiated by an induction of pectinesterase activity, causing increased de-esterification of cell wall pectins, followed by degradation of solubilized pectin.

Mature kiwifruit show a rapid drop in firmness after harvest until the fruit reach a firmness of ≈ 25 N (2.5 kgf). The rate of softening then slows considerably. If the fruit are exposed to ethylene, the rate of softening of fruit increases, and there is less difference between individual fruit for firmness and soluble solids concentration (SSC) (Lallu et al., 1989). The outer pericarp of the fruit softens more rapidly than the core of the fruit (MacRae et al., 1989), and there is a difference in the timing of chemical changes in the cell walls of the different tissues (Redgwell et al., 1990). In the early stages of kiwifruit softening in response to ethylene, the most obvious changes in the cell wall components are swelling of the cell wall and loss of galactose from pectic polymers (Redgwell et al., 1990; 1991). De-esterification of pectins and some breakdown of middle lamella pectic polymers may also be occurring. By the time kiwifruit have reached a firmness of 6 N (0.6 kgf), pectic polymers have become smaller, and de-esterification has clearly taken place (Redgwell et al., 1991).

Previous studies on kiwifruit have focused on changes in pectin methylesterase (PE; EC 3.1.1.11) and polygalacturonase (PG) during fruit development (Matsui and Kitagawa, 1988), in whole ripe fruit (Fuke and Matsuoka, 1984; Giovane et al., 1990; Soda et al., 1986), or in kiwifruit after slicing (Varoquaux et al., 1990). Ogawa et al. (1990) measured β -galactosidase (β -gal) activity in kiwifruit during growth and maturation on

the vine. Given that there are differences in the rate of softening between the different tissues (MacRae et al., 1989), our aim was to follow enzymic changes in a specific tissue during softening in response to ethylene and to relate these to chemical changes already reported for the same tissue under a similar treatment (Redgwell et al., 1990; 1991). To this end, we measured PG, PE, and p-gal activity from the outer pericarp of the fruit.

In 1988, fruit were harvested at a maturity

of 8.2% SSC from the Dept. of Scientific and Industrial Research (DSIR) Research Orchard at Te Puke, New Zealand, then treated within 6 h with ethylene (16 h, 1000 $\mu\text{l-liter}^{-1}$, 20C) and ripened at 20C in trays provided with polyethylene liners. Samples were taken before ethylene treatment and at 1, 2, and 5 days after treatment. As the 1988 fruit were already quite soft at the time of the first sampling, ethylene dosage and sampling times were altered for the following season. In 1989, fruit from the DSIR Research Orchard at Kumeu, New Zealand, was harvested at 7.2% SSC, then treated and ripened as for 1988 fruit, except that ethylene treatment was for 18 h at 100 $\mu\text{l-liter}^{-1}$, and fruit samples were also taken immediately after ethylene treatment and at 10 days after harvest.

In both years, five replicates were made at each sample time, comprising the outer pericarp of two fruit (1988) or three fruit (1989). Before sampling, fruit firmness was measured for each fruit using a handheld penetrometer (Lallu et al., 1989). Samples were frozen immediately in liquid N and stored at -80°C . All extraction steps were carried out at 4°C , and all assays were carried out at 30°C .

Outer pericarp cell wall material was extracted by a modification of the method of Jen and Robinson (1984). About 15 g of frozen sample was blended to a powder with liquid N in a Waring blender or ground in a mortar. The powder was weighed without thawing and blended for 1 min with 50 ml of 5% polyethylene glycol 6000 (PEG, BDH) in distilled water containing 1 ng partially purified leupeptin/ml (Separation Science

Fig. 1. Changes in fruit firmness and activity of polygalacturonase and β -galactosidase in kiwifruit after harvest in 1989. All results are presented with SEM.

Received for publication 5 Aug. 1991. Accepted for publication 4 Mar. 1992. We thank J. Harman for advice on techniques, and D.R.K. Harding and J. Gibson (Separation Science Unit, Massey Univ., Palmerston North, New Zealand) for manufacture of leupeptin. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

Fig. 2. Salt-extracted (a and b) and digest-extracted (c and d) pectinesterase activity in kiwifruit after harvest in 1989 and 1991. All results are presented with SEM.

Table 1. Changes in fruit firmness in kiwifruit after harvest in 1991. Fruit treated with ethylene (+) were exposed to 100 $\mu\text{l}\cdot\text{liter}^{-1}$ for 15 h starting 7 h after harvest. Data are means of 20 fruit $\pm$ SEM.

Ethylene	Time after harvest (h)						
	0	10	13	22	48	120	456
	Fruit firmness (N)						
+	83	79	74	69	51	49	5
SEM	2	1	1	2	2	2	2
-	83	---	---	81	---	---	---
SEM	2	---	---	2	---	---	---

Unit, Massey Univ.), which was added to inhibit the protease actinidin (EC 3.4.22.14) (Soda et al., 1986). The homogenate was centrifuged for 30 min at $16,000\times g$. For PG extractions, the pellet was given a second wash with 1.5 volume of 5% PEG for 10 min and centrifuged again. The pellet was resuspended with stirring for 10 min in 25 ml of 1.25 M NaCl, 20 mM EDTA, 5 ng leupeptin/ml, then the pH was adjusted from pH ≈ 3.7 to pH 6.5 with NaOH and stirred for 45 min. The solution was centrifuged at $16,000\times g$ for 30 min. A further 5 ng leupeptin/ml was added to the salt-extracted supernatant before overnight dialysis against 1 liter of 100 mM NaCl, pH 6.5. After centrifugation of the dialysate at $16,000\times g$ for 20 min, the supernatant was assayed for PG, PE, and β -gal activity.

Residual pectinesterase was extracted by stirring the pellet remaining after salt extraction for 1 h at room temperature with 20 ml of 1% cellulase (EC 3.2.1.4) (Sigma), 1% pectinase (EC 3.2.1.15) (Sigma) in 1 M sodium citrate, and pH 5.5 (Wicker et al., 1988). After centrifugation at $12,000\times g$ for 20 min, the supernatant was dialyzed overnight against distilled water. The digestion mix was dialyzed for use as a control.

PE activity was determined by titrating a 1% citrus pectin solution containing 1:10 (v/v) of enzyme extract with 10 mM NaOH to

maintain the pH at 7.5 (Rouse and Atkins, 1955). For PG, 1 to 5 ml of enzyme extract was assayed in 0.25% polygalacturonic acid, 50 mM sodium acetate, 5% glycerol, and 5 ng leupeptin/ml. Aliquots of 0.4 ml were measured for reducing sugars by the cyanoacetamide method (Gross, 1982). For β -gal, 0.75 ml of enzyme extract was added to 2.6 ml of 75 mM phosphate/citrate buffer, pH 5.0, and 60 $\mu\text{g}\cdot\text{ml}^{-1}$ p -nitrophenol- β -D-galactopyranoside (Sigma). Aliquots of 0.1 ml were pipetted into 0.2 ml of 200 mM sodium carbonate and A_{405} recorded. No β -gal activity was recovered from the PEG supernatant. The activity was inhibited 90% by 1 mM D-galacturonic acid- γ -lactone (Pressey, 1983). All assays were tested with boiled enzyme as controls.

Protein levels were estimated using the modified Lowry method of Perbal (1988), after precipitation of the protein with 0.15% deoxycholate and 7.2% trichloroacetic acid. Data are presented on a fresh-weight basis, and all results were similar to those calculated on a protein basis.

As kiwifruit firmness decreased from 80 to 10N, PG activity increased steadily in 1988 (data not shown) and 1989 (Fig. 1). Co-extraction of 1989 samples taken at 24 and 126 h after harvest showed no inhibition of PG activity. Maximum PG activity in crude extract was similar to that obtained by Soda et al. (1986) for partially purified PG from whole kiwifruit but lower than that measured by Matsui and Kitagawa (1988) for kiwifruit during development. The increase we obtained was in the same range as that in PG reported for sweet pepper (*Capsicum annuum* L.) using a similar extraction protocol (Jen and Robinson, 1984) and avocado (*Persea americana* Mill.) (Awad and Young, 1979). However, it is still low by comparison with tomato (*Lycopersicon esculentum* Mill.), which shows a dramatic rise in PG activity during the climacteric (Grierson and Tucker, 1983). The increase noted in kiwifruit PG activity precedes both a decrease in

the size of pectic polymers that are its presumed substrate in vivo (Redgwell et al., 1991) and the loss of middle lamella material seen in ultrastructural studies (Hallett et al., 1992).

There was no significant change in B-gal activity as fruit softened in 1988 or in 1989 (Fig. 1). Extracted β -gal activity shows no relationship to the significant loss of galactose from the cell wall in vivo (Redgwell et al., 1990), although any increase in activity of active isozymes may be masked in the crude extract (Pressey, 1983) which, in our case, is likely to have included plastid β -gal activity. Ogawa et al. (1990) found a 4- to 5-fold increase in β -gal activity and a 10-fold increase in free galactose toward the end of kiwifruit maturation on the vine. However, it is difficult to make a comparison, as Ogawa et al. (1990) did not report any fruit characteristics that might allow a realistic comparison with our postharvest ripening results.

The 1988 fruit, which were 25 ± 0.6 N at their first sampling at 24 h after treatment with ethylene, showed a rapid decrease in PE activity (data not shown). Fruit in 1989 were at 67 ± 3 N when first sampled at the end of ethylene treatment and showed a transient increase in salt-extracted PE activity (Fig. 2a).

Significant PE activity was released when the outer pericarp cell wall pellet was digested with fungal enzyme digest mix (Wicker et al., 1988). The digestion of the cell wall was necessary to release residual PE, as washing the cell wall with citrate alone released no PE activity. The digest-released PE did not show the same increase in response to ethylene (Fig. 2c).

To confirm the trend for a transient increase in the salt-extracted PE activity in response to ethylene, we sampled kiwifruit in 1991 during ethylene treatment. Fruit from the DSIR Research Orchard at Kumeu were harvested at 5.7% SSC and treated 7 h after harvest with ethylene at 100 $\mu\text{l}\cdot\text{liter}^{-1}$ for 15 h. Fruit were sampled by combining plugs taken from the middle third of the outer pericarp of 20 fruit. Fruit firmness for each sample time was determined from a further set of 20 fruit (Table 1). Samples for PE extraction were taken before ethylene treatment, at 3 and 6 h after the start of treatment, at the end of treatment, and at 2, 5, and 19 days after treatment. A sample of fruit that had not been treated with ethylene was also taken 22 h after harvest (Table 1).

For fruit harvested in 1991, sodium tetrathionate replaced leupeptin as the actinidin inhibitor (Boland and Hardman, 1972) at concentrations of 10 mM in the initial homogenisation buffer, 5 mM in the salt and digestion buffers, and at 2 mM in the dialysis solution. Extractions were replicated four times. These results showed a similar trend in salt- and digest-extracted PE activity in response to ethylene (Fig. 2 b and d) as in 1989, despite being less mature at harvest (as determined by average SSC). The sample that had not been treated with ethylene had PE activities similar to those of fruit at har-

vest and at 3 h after ethylene treatment (data not shown). Co-extraction of samples with high and low PE activity resulted in rates similar to those of soft fruit for salt-extracted PE, but not for digest-extracted PE activity (data not shown).

The high levels of PE activity extracted from kiwifruit imply that the enzyme is under strict control in vivo. Balestrieri et al. (1990) were unable to measure PE activity in ripe kiwifruit unless they first extracted a potent water-soluble glycoprotein inhibitor of PE activity. Our initial homogenisation buffer included 5% PEG, which may have prevented removal of sufficient PE inhibitor.

A decrease in PE during softening has been reported for other fruit such as avocados (Awad and Young, 1979) and sweet pepper (Jen and Robinson, 1984). There is some suggestion that exposure to ethylene affects PE activity in bananas (*Musa xparadisica* L.). PE activity decreased in bananas kept in air, but not when kept in the presence of ethylene or under low O₂ conditions (Kanellis et al., 1989). Constant levels of PE activity during softening in bananas were also found by Brady (1976) when fruit were kept in the presence of ethylene.

Release of significant PE activity only after digestion of the cell wall with fungal enzymes suggests that there may be a differential association between some forms of PE and the cell wall. Giovane et al. (1990) found differences in glycosylation in salt-extracted PE from kiwifruit based on affinity of binding to heparin-sepharose. Glycosylation may be a factor in the partitioning of PE activity between salt- and digest-extracted fractions.

The activities measured in crude enzyme extracts of kiwifruit ripened in response to ethylene do not show the large scale induction of either PG or β -gal we might have expected from studies by others or from the chemical analysis of cell wall changes in kiwifruit. The results for PE activity suggest that the transient increase in activity seen in PE extracted under high salt conditions, in

combination with the de-esterification of pectin found after ethylene treatment (Redgwell et al., 1991), may be a specific response to ethylene exposure rather than part of the usual events of fruit softening.

Literature Cited

- Awad, M. and R.E. Young. 1979. Postharvest variation in cellulase, polygalacturonase, and pectinmethylesterase in avocado (*Persea americana* Mill, cv. Fuerte) fruits in relation to respiration and ethylene production. *Plant Physiol.* 64:306-308.
- Balestrieri, C., D. Castaldo, A. Giovane, L. Quagliuolo, and L. Servillo. 1990. A glycoprotein inhibitor of pectin methylesterase in kiwifruit (*Actinidia chinensis*). *European J. Biochem.* 193:183-187.
- Boland, M.J. and M.J. Hardman. 1972. Kinetic studies on the thiol protease from *Actinidia chinensis*. *Federation of European Biochem. Soc. Lett.* 27:282-284.
- Brady, C.J. 1976. The pectinesterase of the pulp of the banana fruit. *Austral. J. Plant Physiol.* 3:163-172.
- Fuke, Y. and H. Matsuoka. 1984. Changes in content of pectic substances, ascorbic acid and polyphenols, and activity of pectinesterase in kiwi fruit during growth and ripening after harvest. *Nippon Shokuhin Kogyo Gakkaishi* 31:31-37.
- Giovane, A., L. Quagliuolo, D. Castaldo, L. Servillo, and C. Balestrieri. 1990. Pectin methylesterase from *Actinidia chinensis* fruits. *Phytochemistry* 29:2821-2823.
- Grierson, D. and G.A. Tucker. 1983. Timing of ethylene and polygalacturonase synthesis in relation to the control of tomato fruit ripening. *Planta* 157:174-179.
- Gross, K.C. 1982. A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. *HortScience* 17:933-934.
- Hallett, I.C., E.A. MacRae, and T.F. Wegrzyn. 1992. Changes in kiwifruit cell wall ultrastructure and cell packing during postharvest ripening. *Intl. J. Plant Sci.* 153:349-60.
- Jen, J.J. and M.L. Robinson. 1984. Pectolytic enzymes in sweet bell peppers (*Capsicum annuum* L.). *J. Food Sci.* 49:1085-1087.
- Kanellis, A.K., T. Solomos, and A.K. Matoo. 1989. Changes in sugars, enzymic activities and acid phosphatase isoenzyme profiles of bananas ripened in air or stored at 2.5% O₂ with and without ethylene. *Plant Physiol.* 90:251-258.
- Lallu, N., A.N. Searle, and E.A. MacRae. 1989. Investigation into ripening and handling strategies for early season kiwifruit. *J. Sci. Food Agr.* 47:387-400.
- MacRae, E.A., N. Lallu, A.N. Searle, and J.H. Bowen. 1989. Changes in the composition of kiwifruit (*Actinidia deliciosa*) affected by maturity at harvest and postharvest treatments. *J. Sci. Food Agr.* 49:413-430.
- Matsui, T. and H. Kitagawa. 1988. Seasonal changes in pectinmethylesterase and polygalacturonase activity in kiwifruit. *Nippon Shokuhin Kogyo Gakkaishi* 35:851-855.
- Ogawa, H., H. Fukumoto, T. Yano, K. Yamamoto, and T. Tochikura. 1990. Purification and characterisation of β -galactosidase from kiwifruit. *Nippon Shokuhin Kogyo Gakkaishi* 37:298-305.
- Perbal, B. 1988. A practical guide to molecular cloning, p. 47-48. Wiley, New York.
- Pressey, R. 1983. β -galactosidases in ripening tomatoes. *Plant Physiol.* 71:132-135.
- Redgwell, R.J., L.D. Melton, and D.J. Brasch. 1990. Cell wall changes in kiwifruit following post-harvest ethylene treatment. *Phytochemistry* 29:399-407.
- Redgwell, R.J., L.D. Melton, and D.J. Brasch. 1991. Cell wall dissolution in ripening kiwifruit (*Actinidia deliciosa*): Solubilisation of the pectic polymers. *Plant Physiol.* 98:71-81.
- Rouse, A.H. and C.D. Atkins. 1955. Pectinesterase and pectin in commercial citrus juices, as determined by methods used at the citrus commercial experimental stations. *Bul. Fla. Agr. Expt. Sta.*, 570.
- Soda, I., T. Hasegawa, T. Suzuku, and N. Ogura. 1986. Detection of polygalacturonase in kiwifruit during ripening. *Agr. Biol. Chem.* 50:3191-3192.
- Varoquaux, P., I. Lecendre, F. Varoquaux, and M. Souty. 1990. Change in firmness in kiwifruit after slicing. *Sciences des Aliments* 1&127-139.
- Wicker, L., M.R. Vassallo, and E.J. Echeverria. 1988. Solubilization of cell wall bound, thermostable pectinesterase from Valencia orange. *J. Food Sci.* 53:1171-1174.