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Cell Wall Metabolism in Ripening Fruit

II. CHANGES IN CARBOHYDRATE-DEGRADING ENZYMES IN RIPENING 'BARTLETT' PEARS[1]

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ABSTRACT

Mature 'Bartlett' pear (*Pyrus communis*) fruits were ripened at 20 C. Fruits at different stages of ripeness were homogenized, and extracts of the low speed pellet (crude cell wall) were prepared. These extracts contained polygalacturonase, pectin esterase, and activity against seven *p*-nitrophenyl glycoside substrates. Polygalacturonase, α-galactosidase, and α-mannosidase increased in activity as the fruit ripened. Cellulase and activities against pear wall xylan and arabinan were absent from the extracts.

The changes in cell wall composition which accompany the softening of ripening fruit apparently result from the action of enzymes produced by the fruit. Prominent among the enzymes implicated are PG[3] and PE because striking changes in wall pectin content are observed in ripening fruits (1, 13, 14, 21, 22), and activities of these two enzymes often increase as ripening continues (21). In addition, primarily because of their presence in extracts of ripening fruits, a variety of glycanases (10, 21, 26) and glycosidases (3, 20, 21) have been assigned an unspecified role in fruit cell wall metabolism.

This paper describes measurements of the activity of a variety of carbohydrate-degrading enzymes in extracts of ripening 'Bartlett' pears. The potential for depolymerization of pear cell wall polysaccharides was assessed using model substrates containing the same types of glycosidic linkages that have been described for pear cell walls (1). Glycosidase activities were followed using *p*-nitrophenyl substrates. The activities of several enzymes, notably PG, α-mannosidase, and α-galactosidase, increased substantially as the fruit approached the respiratory climacteric. The potential roles of the various enzymes in ripening-associated cell wall modification are discussed.

MATERIALS AND METHODS

Mature green 'Bartlett' pears (*Pryus communis*) were obtained from the University orchard at Davis. Fruits were selected for uniformity of size and freedom from blemishes. If not used immediately, fruits were stored at 0 C. No fruits were stored for longer than 1 month.

Batches of fruit were ripened as described previously (1). The progress of ripening was followed by monitoring respiratory rate (5) and production of ethylene (16).

Tissue Homogenization, Enzyme Extraction. Pear fruit at different stages of ripeness (based on flesh firmness, [1]) were peeled, cored, and diced. Tissue pieces were vacuum-infiltrated with cold (4 C) 100 mM Na-acetate (pH 6.0) containing 0.2% (w/v) $Na_2S_2O_4$ and 1% (w/v) soluble PVP (pharmaceutical grade, Mann Research Labs). Infiltrated tissues were homogenized in the same solution (2 ml/g fresh tissue) for 1 min in a Waring Blendor. The homogenate was centrifuged (10,000*g*, 30 min) in a Sorvall RC2-B refrigerated centrifuge. The supernatant solution was decanted and stored at −20 C.

The pelleted residue (crude cell wall) was suspended in cold 1 M Na-acetate (pH 6.0) containing 6% (w/v) NaCl. The suspension was stirred continuously while 2 N NaOH was added to adjust the pH to 8.2. The suspension was stirred overnight at 4 C and then centrifuged at 10,000*g*. The supernatant solution was filtered through GF/C glass fiber paper (Whatman). Aliquots of the filtrate were concentrated by dialysis against PEG (mol wt 20,000; Sigma) and then dialyzed (4 C) for 48 h against four changes of 4 liters distilled H_2O. The dialyzed material constituted the crude enzyme in various assays.

Enzyme Assays. PE activity was determined as described by Nagel and Patterson (17) with the aid of an automatic titrating pH meter (Radiometer Titrometer II).

Polysaccharide-degrading activities were followed by measuring the generation of reducing groups according to the method of Nelson (18), as modified by Somogyi (23). The standard reaction mixture contained enzyme and 0.1% (w/v) substrate in 1 ml of 100 mM Na-acetate (pH 5.0). Incubation was at 37 C. The substrates used were polygalacturonic acid (Sunkist Growers), carboxymethyl cellulose and larch xylan (Sigma), and araban (Koch-Light Laboratories, Ltd.).

Prior to use, the xylan was purified according to the technique of McNeil and Albersheim (as described by Taiz and Honigman [24]) to remove abundant glucose- and mannose-containing contaminants. The araban also had to be treated prior to use in order to remove uronic acid-containing materials. Five g araban were refluxed for 16 h in 250 ml 5 N NaOH containing 4 g $NaBH_4$. The preparation was cooled, neutralized with glacial acetic acid, and centrifuged for 5 min at 1,000*g*. Final clarification of the supernatant solution was accomplished by filtration through glass fiber (GF/C) paper. The filtrate was dialyzed against 10 mM K-phosphate (pH 7.0) and applied to a column (3 × 16 cm) containing DEAE-Sephadex that had been equilibrated in 10 mM phosphate. The araban was eluted in the column void volume. It was dialyzed against distilled H_2O and lyophilized.

Glycosidase activities were assayed by measuring the amount of *p*-nitrophenol released from various *p*-nitrophenyl glycosides following incubation at 37 C. The standard reaction mixture contained enzyme and 0.025% (w/v) substrate in 1 ml of 50 mM Na-acetate (pH 6.0). Incubation was terminated by addition of 2 ml of 1 N NH_4OH containing 2 mM EDTA. Free *p*-nitrophenol

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[3] Abbreviations: PG: polygalacturonase; PE: pectin esterase.

was measured by reading A at 400 nm. The p-nitrophenyl derivatives used were β-D-galactoside (Sigma), α-D-galactoside, α-D- and β-D-xylopyranosides, α-L- and β-L-fucopyranosides, and α-D-mannopyranoside (Koch-Light Laboratories), and α-L-arabinofuranoside (prepared according to the procedure of Fielding and Hough [8]).

Cellulase activity was also measured viscometrically. Reaction mixtures contained 2.5 ml crude enzyme, 5 ml 1% (w/v) carboxymethyl cellulose, and 2.5 ml 200 mM Na-acetate (pH 5.0) in a size 100 Ostwald viscometer. Incubation was at 30 C.

RESULTS AND DISCUSSION

Many of the activities described below for the NaCl extracts of pear cell wall material could also be identified in the pH 6.0 supernatant solution of tissue homogenates. However, a number of factors combined to restrict the data reported to the NaCl extracts. The initial supernatant solution contained substantial amounts of reducing sugars which contributed to high background readings in spite of extensive dialysis. More troublesome was the fact that the solution (especially from homogenates of ripe fruit) contained a good deal of polysaccharide material (chiefly pectin [1]). Because the solution also contained PG (see below), assays always indicated generation of reducing groups regardless of the enzyme activity being tested. Even when high reducing sugar levels did not interfere with a measurement, as in glycosidase assays, the NaCl preparations consistently gave higher activities than the initial supernatant solutions. Because the activities of enzymes in the initial supernatant preparations are not reported, the data presented (in terms of tissue fresh weight) are an underestimation of total activity in the pear fruit. Nevertheless, the data present an accurate picture of the variety of carbohydrate-degrading enzymes in 'Bartlett' pear tissue and of changes in the various enzyme activities as ripening occurs.

Pectic Enzymes. The PE and PG activities extractable from pear fruits at different stages of ripeness are illustrated in Figure 1. PE activity remains constant as the fruit experiences its most dramatic softening; only after the climacteric maximum does activity change, falling by 25%. In contrast, PG increases in parallel with respiration, ultimately reaching 10 to 12 times the initial level. As with PE, there is a decrease in PG activity in senescent tissue.

In many fruits (6, 9, 21) PE activity increases as ripening proceeds, thus giving rise to the idea that deesterification of pectin is a prerequisite for ripening-associated changes in wall pectin substances. However, there is little change in PE activity during the course of 'Bartlett' pear ripening. This is in agreement with other work on pears (17) but stands in contrast to a recent report concerning the Japanese pear (27). The rise in PG activity coincides with the conversion of cell wall pectic polysaccharides to water-soluble forms (1) thus suggesting a key role for PG in the wall changes which accompany pear ripening. This finding is in agreement with data for changes in PG activity in virtually every type of fruit studied (21).

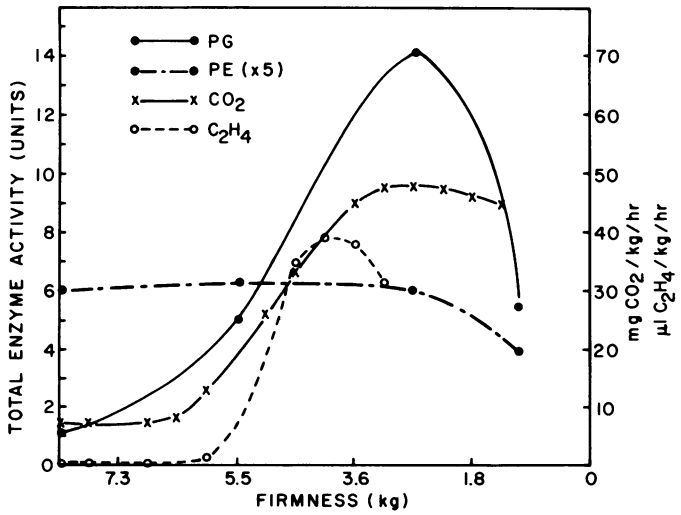

FIG. 1. Relationship of PG and PE activities to changes in fruit firmness, respiratory rate, and ethylene production in ripening 'Bartlett' pears. Enzymes were prepared from fruits having a pressure test of 8.2, 5.5, 2.7, and 1.1 kg.

Glycanases. Cellulase activity increases with the ripening of many fruits (10, 21), including the Japanese pear (27). Cellulolytic enzymes could degrade both cellulose and the β-1,4-glucan backbone of xyloglucan, a hemicellulosic polysaccharide prominent in walls of dicots (12), which has recently been identified in base-soluble preparations of pear cell walls (1). However, no cellulase was detected in extracts of the 'Bartlett' pear even when incubation was extended for 24 h. Both endo- and exocellulase could have been detected by the assays (viscometric and reductometric) used. This absence of cellulase is not surprising because cell wall cellulose and xyloglucan content do not change during 'Barlett' pear ripening (1).

The work of Chanda *et al.* (3) on hemicellulose from the Conference pear demonstrated the presence of a β-1,4-xylan. Recent work on the 'Bartlett' pear cell wall indicates the presence of a similar polymer (1). In spite of the fact that wall xylose content does not change during fruit ripening (1) it seemed important to determine if the pear fruit was capable of metabolizing its wall xylan. The larch xylan used for xylanase assay is largely β-1,4-linked and is therefore a good substrate for testing the pear's ability to degrade its wall xylan component. Even after prolonged incubation there was no evidence of xylanase activity in pear extracts.

The neutral sugar-containing wall component that undergoes the most extensive conversion to water-soluble form during pear fruit ripening is a branched arabinan (1) that is very similar in structure to the arabinan whose preparation is described under "Materials and Methods." However, as for cellulase and xylanase, incubation of pear extracts with the arabinan model substrate gave no indication of arabanase.

Glycosidases. Activities against seven of the 10 p-nitrophenyl glycosides tested were detected in pear extracts (Table I). The most active of these are α-galactosidase and α-mannosidase. α-

Table I. *Glycosidase Activities in Pear Fruit Extracts*

The data presented are from one experiment on a single batch of ripening pears. The experiment was performed three times with fruit that had been stored at 0 C for varying periods of time. Although total activities varied the patterns of change and relative activities of different glycosidases remained the same.

Enzyme	Fruit Firmness (in kg)[a]			
	8.2	5.5	2.7	1.1
α-Galactosidase	28.9[b]	35.0	57.5	57.5
β-Galactosidase	7.0	9.0	12.5	5.8
α-Glucosidase	0.5	0.6	0.8	0.3
β-Glucosidase	1.8	1.2	0.8	0.4
α-Mannosidase	6.4	12.5	33.6	3.0
α-Arabinosidase	0.5	0.5	0.7	0.2
β-Xylosidase	1.1	1.3	1.7	0.3

[a] Determined with a UC fruit firmness tester with a 0.79-cm tip. The respiratory peak was at the 2.7-kg point.

[b] Values represent µmol of p-nitrophenol released/min·kg fresh weight. No activity could be detected following incubation with p-nitrophenyl, α-xyloside, and α- and β-fucosides.

Galactosidase activity doubled during the course of ripening and remained high in postclimacteric tissue. α-Mannosidase increased 5-fold during ripening but fell to less than half the initial level in overripe fruit. The pattern of change for the less active glycosidases followed the general pattern of α-mannosidase with the exception of β-glucosidase which declined steadily as the fruit softened (Table I).

For the most part, the glycosidase activities described here are not unique to the 'Bartlett' pear. Changes in various glycosidase activities have been described for many fruits (2, 20, 21). The most complete records are those of Pharr *et al.* (20) and Wallner and Walker (26) who describe eight and seven glycosidases, respectively, in tomato. Both the pear and the tomato lack α- and β-fucosidase (26). In contrast to the pear, the tomato has α-xylosidase (20, 26). The only glycosidase in pear that has not been widely described for other fruits is α-arabinosidase and this is probably because the *p*-nitrophenyl substrate is not commercially available.

The cellular role of this array of glycosidases is open to speculation. Bartley (2) has proposed that a β-galactosidase present in homogenates of apple fruit is responsible for the large decrease in cell wall galactose which accompanies apple ripening (13). However, Wallner (25) has shown that β-galactosidase-containing preparations are not able to digest apple cell walls. It is not clear how glycosidases might act to disrupt cell walls. Even if a glycosidase acted as an exoglycanase, it is unlikely that it could cleave beyond cell wall constituent branch points and thus remove interpolysaccharide linkages. Furthermore, the data presented above indicate that 'Bartlett' pear α-arabinosidase and β-xylosidase do not act as exoglycanases because there was no generation of reducing groups when glycosidase-containing enzyme preparations were incubated with xylan and araban.

The most active glycosidases in the 'Bartlett' pear (Table I), as well as in the tomato (20), are α-galactosidase and α-mannosidase. It is difficult to assign a role in cell wall modification to these enzymes because analysis of pear fruit cell walls gives no indication of α-linked galactans or mannans. Furthermore, little change in wall mannose or galactose content occurs during Bartlett pear ripening (2). It may be that glycosidase activities determined by incubation with *p*-nitrophenyl substrates give an inaccurate picture of *in vivo* enzyme specificity. Pharr *et al.* (20) have shown that tomato fruit β-glucosidase is more active on the *p*-nitrophenyl substrate than on natural β-glucosidases. Furthermore, work with purified glycosidases has shown that single protein species can hydrolyze glycosides having different glycones (4, 7). At present no role in ripening-associated 'Bartlett' pear cell wall modification should be assigned to glycosidases.

Role of Carbohydrate-degrading Enzymes in Ripening-associated Cell Wall Changes. The relevance of the data presented above to understanding the control of cell wall changes during ripening of 'Bartlett' pears must be tempered by a number of considerations. During homogenization of the tissue, cellular compartmentation is disrupted. It is unclear how much of a given extracted activity was located in the tissue-free space (*i.e.* in a position to act on cell wall polymers) in the intact tissue. The designation of enzymes extracted from crude cell wall preparations by NaCl washing as "cell wall enzymes" (19, 20, 26) can be misleading because the possibility of cell wall trapping of cytoplasmic proteins, physically or by ion exchange, is substantial.

Some aspects of cellular control may have been eliminated during enzyme extraction and assay. Homogenization of fruit tissues was carried out in the presence of PVP to protect proteins from inactivation by soluble phenolic substances (15). Some of these substances may play a role as endogenous regulators. Finally, assays using a given substrate were carried out in solutions buffered to a pH value approximating the optimum pH for the type of enzyme under study. Changes in free space pH, such as occur during auxin stimulation of cell wall change and elongation in pea (11), could accompany ripening and might regulate *in situ* enzyme activity.

The data presented above must be assessed in conjunction with a description of the cell wall molecular changes which accompany pear ripening. As the pear ripens a substantial portion of its cell wall arabinose and galacturonic acid is converted to water-soluble forms (1). Arabanase was not detected in extracts of pear and thus is apparently not responsible for the loss of wall arabinose. The arabinose-containing material which becomes soluble during ripening is not degraded to low mol wt products (*i.e.* monosaccharides [1]). Treatment of cell walls prepared from mature, green (firm) 'Bartlett' pears with pure PG solubilizes substantial amounts of arabinose and galacturonic acid, thus changing the sugar composition of those walls to that of walls from ripe pears (1). This observation, coupled with the data on PG changes presented above, indicates a crucial role for PG in the cell wall changes which accompany 'Bartlett' pear ripening. Based on the available information, only PG of the enzyme activities described above, can presently be assigned a role in ripening-associated wall metabolism.

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