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β-GALACTOSIDASE ACTIVITY IN RIPENING APPLES

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Key Word Index—*Malus sylvestris*; Rosaceae; apple fruit; β-galactosidase; ripening; cell wall composition; polysaccharides.

Abstract—β-Galactosidase activity has been identified in soluble and cell wall preparations from apple cortex tissue. The enzyme degrades pectin galactan and has a pH optimum of 4·0 with *p*-nitrophenyl-β, D-galactopyranoside as substrate. Soluble polygalacturonide increased as the applies softened with ripening and these changes were preceded by loss of galactose residues from the cell wall and an increase in β-galactosidase activity.

INTRODUCTION

As APPLES soften during ripening, galactose and arabinose residues in the cortical cell walls decrease and the proportion of polygalacturonide which is freely soluble in aqueous extractants increases.[1] The enzyme activites which lead to these changes have not been identified. Pectinesterase activity in apple fruits has often been reported; attempts to find polygalacturonase have failed[2] and since polyuronide chains are not degraded during ripening[2] there is no reason to think that it is present. Autolytic activities which release galactose and arabinose from apple cell wall preparations, have been found[3] and it has been suggested that they have a role in wall breakdown and softening in whole fruits.[1]

This paper describes the identification of a β-galactosidase (E.C. 3.2.1.23) and the relationship between enzyme activity and changes in the polysaccharides of the cell walls of the cortex tissue of apples ripening in air at 12° and 3·5°.

RESULTS AND DISCUSSION

Identification of β-galactosidase

Autolytic glycosidase activity was maximal between pH 4 and 5[3] and a buffer of pH 4·5 was therefore chosen for assay of enzyme activity.

The abilities of soluble and cell wall preparations from apple cortex to degrade β-1, 4-galactan, *p*-nitrophenyl-α, D-galactopyranoside and *p*-nitrophenyl-β, D-galactopyranoside are shown in Table 1. *Escherichia coli* β-galactosidase will release terminal β-linked galactose residues from glycoproteins and glycopeptides[4] and it seemed possible that a similar enzyme was responsible for degradation of galactan and *p*-nitrophenyl-β, D-galactopyranoside by the apple preparations. Phenylthiogalactoside which inhibits the *E. coli*

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[4] SPIRO, R. G. (1966) *Methods Enzymol.* **8**, 26.

TABLE 1. GLYCOSIDASE ACTIVITIES IN PREPARATION FROM APPLE CORTEX TISSUE AND INHIBITION BY β-GALACTOSIDASE INHIBITOR

Substrate	Glycosidase activity Preparation fraction Soluble	Cell wall	Inhibition (%) of glycosidase activity by β-galactosidase inhibitor‡ Preparation fraction Soluble	Cell wall
Galactan	0·35*	0·66*	39	63
p-Nitrophenyl-β, D-galactopyranoside	0·90†	1·62†	95	95
p-Nitrophenyl-α, galactopyranoside	0·30†	0·16†	4	0

* Activity expressed as μmol glucose released (g fr. wt)/90 min.
† Activity expressed as μmol *p*-nitrophenol released (g fr. wt)/45 min.
‡ The inhibitor was freshly prepared from D-galactono-1.4-lactone[6] and used at a final concentration of 1 mM.

enzyme[5] had no effect on apple preparations at a final concentration of 1 mM. An inhibitor prepared from D-galactono-1,4-lactone[6] has been shown to act competitively on β-galactosidases from mammalian plant and molluscan sources.[7] The inhibition studies with this material (Table 1) indicate that there are separate α- and β-galactosidases in the preparations and that the β-galactosidase is able to degrade both the galactan and the β-galactoside. The difference in inhibition observed may be explained by differing affinities of the enzyme for the galactan and the β-galactoside substrates.

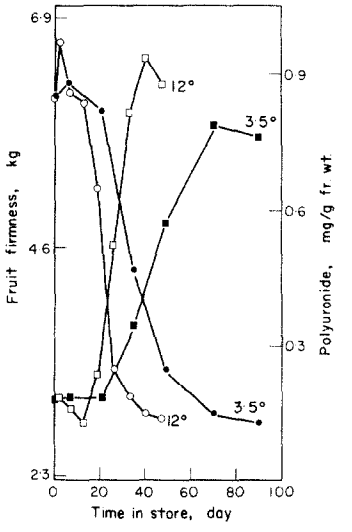

FIG. 1. CHANGES IN FRUIT FIRMNESS (○) AND RELEASE OF POLYURONIDE (□) BY THE APPLES DURING RIPENING AT 12° AND 3·5°.

The soluble and cell wall β-galactosidase activities have a pH optimum at 4·0 with the β-galactoside. The soluble enzyme activities have a second optimum at pH 8·0 (41% of the activity at pH 4·0).

[5] MONOD, J. and COHN, M. (1952) *Adv. Enzymol.* **13**, 67.
[6] LEVVY, G. A., MCALLAN, A. and HAY, A. J. (1962) *Biochem. J.* **82**, 225.
[7] CONCHIE, J., GELMAN, A. L. and LEVVY, G. A. (1967) *Biochem. J.* **103**, 609.

Changes in polysaccharide composition of the cell wall and β-galactosidase activity during ripening

The apples were stored at 12° and 3·5° (the commercial storage temperature for Cox's Orange Pippin in air) and the ripening of the fruit was followed by measurement of ethylene synthesis and flesh softening. The data confirm the well established observations that lowering the storage temperature slows the ripening of the fruit. The increase in soluble polyuronide was coincident with softening of the cortex tissue (Fig. 1) and was preceded by loss of galactose residues from the cell walls (Figs. 2 and 3). A small loss of arabinose from the cell walls was noted toward the end of the storage life of the fruit, in agreement with earlier findings[1] whilst the xylose content of the walls remained constant. The hydrolysis of the starch of the apple during ripening caused considerable changes in the glucose content of the preparations and changes in the glucose-containing components of the cell walls could not be determined.

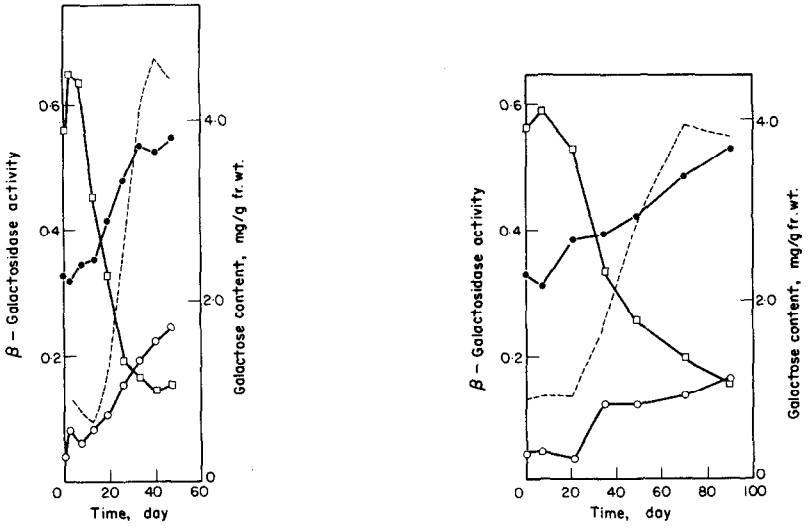

FIG. 2. CHANGES IN SOLUBLE POLYURONIDE (----), GALACTOSE CONTENT OF THE CELL WALLS (□) AND SOLUBLE AND CELL WALL β-GALACTOSIDASE ACTIVITIES (○, ●) DURING THE RIPENING OF THE APPLES AT 12°.
Enzyme activity expressed as μmol of *p*-nitrophenol released/g fr. wt tissue12 min.

FIG. 3. CHANGES IN SOLUBLE POLYURONIDE (----), GALACTOSE CONTENT OF THE CELL WALLS (□) AND SOLUBLE AND CELL WALL β-GALACTOSIDASE ACTIVITIES (○, ●) DURING THE RIPENING OF THE APPLES AT 3·5°.
Enzyme activity expressed as μmol of *p*-nitrophenol released/g fr. wt of tissue/12 min.

In cortical cell walls of the apple the polysaccharides are thought to be linked to one another and to protein components to form a polymeric network.[3] The data in Figs. 2 and 3 indicate that hydrolysis of the galactan of the wall is initiated before the release of high MW polyuronide is detectable. The soluble polyuronide contains negligible neutral carbohydrate residues[3] in contrast to the polymer in the wall which has neutral carbohydrates associated with it. This suggests that the galactan acts to stabilize the uronide in the cell wall and that hydrolysis of these galactan side chains leads to solubilization of the polymer.

An increase in the β-galactosidase activity associated with the wall occurred simultaneously with loss of galactose residues. Soluble enzyme activity also increased during storage and was coincident with the solubilization of the uronide. As stated by Frenkel *et al.*[8] specific protein synthesis is required for pome fruit ripening; the observed increase in activity coincident with the onset of the disappearance of galactose may reflect new protein synthesis accounting for the release of the polyuronide by hydrolysis of cross linking galactan side chains.

In a detailed study of cell wall structure,[9-11] it has been shown that a linear 1,4-linked galactan is linked to a rhamnogalacturonan (polyuronide) and suggested that the galactan probably serves as a cross link between the xyloglucan and rhamnogalacturonan components. Therefore, for the β-galactosidase to function in the metabolism of the wall an enzyme must hydrolyse the xyloglucan galactose linkages and release the non-reducing ends of the galactan chains. The activity of this enzyme may be envisaged as controlling the solubilization of polyuronide.

However, the mechanism of solubilization appears more complex for the loss of galactose residues from the wall preceded the release of polyuronide (Figs. 2 and 3) suggesting that hydrolysis of bonds which have to be characterized is required for the solubilization to occur.[3] One such linkage is that between the polyuronide and the protein components of the cell wall.[9-11]

EXPERIMENTAL

Source of fruit. (a) For the study of the β-galactosidase. The apples were Cox's Orange Pippin grown in New Zealand and stored in air at 3·5°. (b) For the storage experiment. A bulk sample of fruit was obtained on 20 Sept., 1972 from trees of Cox's Orange Pippin at East Malling Research Station and the fruit sorted into 19 groups of 16 apples. The fruit was placed in pairs in polythene tubes each of which was sealed and connected to lines delivering air at the rate of 5 l./hr in controlled temp. rooms at 12° and 3·5°. Duplicate samples of fruit were used for preparations.

Preparation of extracts. All manipulations were at 2°. Cortical slices excluding peel, were cut from opposite sides of fruit from stem to calyx in segments of each of 5 apples. In the storage experiment a single segment was cut from 10 apples. The cortical tissue was distintegrated in 9 vol. of Me_2CO 0·2 M Tris·HCl buffer (pH 8·9) 8:1 in a blender, filtered on Whatman 541 paper under suction and washed with a further 8 vol. of 80% Me_2CO. The Me_2CO extracted material was resuspended in 5 mM KH_2PO_4–NaOH buffer, pH 7 to 2× the weight of tissue and stored at −20°. The preparation was thawed slowly at room temp. and 30 ml filtered on Whatman 541 paper. The cell wall material was resuspended in buffer to 30 g and the soluble and cell wall preparations assayed.

Enzyme assays. (a) With galactan of potato pectin. The substrate, β-1,4-linked galactan,[12] was used at a concentration of 1 mg/ml of buffer. The incubation mixture contained 1 vol. of galactan, citrate phosphate buffer, pH 4·5 and 1 vol. of preparation. After 90 min incubation at 25°, 1 ml aliquots were used to determine the reducing sugar released in terms of a glucose standard.[13,14] Incubations with the cell wall preparations were terminated by filtration on Whatman 541 paper and filtrate assayed as above for reducing sugar. Substrate and preparation controls were included and incubations were in duplicate. (b) With *p*-nitrophenyl galactosides. The incubation mixture contained 4 vol. of citrate–phosphate buffer, pH 4·5 1 vol. of *p*-nitrophenyl galactoside soln (final concn 1 mM) and 5 vol. of preparation. After 45 min incubation at 25°, 0·5 ml aliquots of test soln were added to 1 ml 1 M Na_2CO_3 and 2 ml of H_2O. The mixture was centrifuged and the liberated *p*-nitrophenol determined by its A at 400 nm. Substrate and preparation controls were included. The incubations with cell wall preparations were terminated by filtration on Whatman 541 paper and the filtrate assayed as above for *p*-nitrophenol released. In the assay of activity in preparations from the fruit in the storage experiment, samples were taken at zero time and after 12 min incubation for the cell wall preparation and 30 min incubation for the soluble preparation. Hy-

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drolysis of substrate was linear during the period of the assay and directly proportional to the amount of enzyme added.

Buffers. Citric acid–Na_2HPO_4 (McIlvaine) buffer solutions[15] pH 3·0–8·95 were used in the determination of pH optimum.

Fruit firmness. Fruit firmness was measured using a standard penetrometer with an 8 mm plunger.

Determination of polyuronide. Soluble polyuronide was determined in the 5 mM phosphate buffer fraction by an automated procedure based on the carbazole H_2SO_4 method.[3]

Hydrolysis of cell walls and estimation of neutral monosaccharides. Acetone (4 vol.) was added to 1 vol. of thawed preparation. The suspension was filtered after 16 hr and washed with 80% Me_2CO, Me_2CO and Et_2O and allowed to dry. The cell wall material was hydrolysed and the monosaccharides analysed by GLC as their TMS–ether derivatives.[3]

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