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ScienceDirect
Postharvest Biology and Technology 43 (2007) 307–318
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

Patterns of enzymatic activity of cell wall-modifying enzymes during growth and ripening of apples

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Received 26 June 2006; accepted 14 October 2006

Abstract

Fruit softening is thought to result from extensive cell wall modifications that occur during ripening. These modifications are the result, at least in part, of the activity of members of cell wall-modifying enzymes from the same families involved in the cell wall loosening which promote tissue extension and growth. In this work, the activities of a set of pectolytic and non-pectolytic cell wall-modifying enzymes, namely polygalacturonase (PG; endo-and exo-acting), pectin methylesterase (PME), pectate lyase (PL), β-galactosidase (β-Gal), α-L-arabinofuranosidase (AFase), endo-1,4-β-glucanase (EGase), xyloglucan endotransglycosylase (XET) and expansin, were monitored during growth and ripening of 'Mondial Gala' apple (*Malus* × *domestica* Borkh.) fruit. After optimisation of protein extraction protocols and standard activity assays, activity could be detected in all the assays, except for endo-PG. The overall results suggest that fruit growth and ripening are possibly coordinated by members of the same families of cell wall-modifying enzymes, although different isoforms may be involved in distinct developmental processes. Based on the trend of total activity measured in vitro using equal amounts of protein per developmental stage, the role of EGase seems to be more prominent during growth than during ripening, and XET activity is most important only after the fruit stopped growing and is maintained throughout ripening. β-Gal and AFase activities increased after harvest as the fruit became over-ripe. On the other hand, exo-PG, PL and expansin activities increase from that in unripe fruit to fruit at harvest but are maintained at similar levels thereafter, throughout the over-ripe stages. The patterns of activity observed are discussed in relation to published information about ripening of apples and to results reported using other species.

Keywords: Cell wall; Enzymatic activity; Fruit development; Fruit ripening; Fruit softening; *Malus* × *domestica* Borkh

1. Introduction

Selective modification of the cell wall architecture is associated with almost every stage of development. It is an integral part of cellular growth, but it also occurs in several non-growing organs in events such as seed germination, anther dehiscence, penetration of pollen tubes in the pistils, abscission of leaves, flowers and fruit, development of intercellular spaces (e.g. aerenchyma), and fruit ripening. Fruit ripening involves changes in the composition and organization of pectin, hemicellulose and cellulose polysaccharides of the cell wall, which take place as a coordinated series of assembly and disassembly steps. Although growth has ceased, selective disassembly of the cell wall components and cell-to-cell separation is very pronounced during fruit ripening and is thought to be a key ripening-associated metabolic event that determines the timing and extent of loss of cell adhesion, which leads to fruit softening. The plant cell wall is a highly complex and dynamic structure composed of a network of hemicelluloses linked to cellulose microfibrils, embedded in a matrix of pectic polymers and other less abundant compounds, like phenols, structural proteins and enzymes (Brett and Waldron, 1996). Due to the nature of the polymers, a large number of linkages exist within the cell wall, maintaining and reinforcing its structure, thus various families of enzymes and their different isoforms are suggested to affect these processes. It has been proposed that

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doi:10.1016/j.postharvbio.2006.10.002

among the principal enzymes that act on the linkages between cell wall polymers are polygalacturonases (PG) (endo- and exo-), pectin methylesterases (PME), pectate lyases (PL), β-galactosidases (β-Gal), α-L-arabinofuranosidases (AFase), endo-1,4-β-glucanases (EGase), xyloglucan endotransglycosylase/hydrolases (XTH) and expansins. Although the same enzymes are believed to participate in different developmental processes, each particular event is probably regulated by the activity or by the selective regulation of different isoforms of the same enzyme, acting sequentially or overlapping. In addition, a single enzyme does not seem to be the uniquely responsible for the disassembly of the cell wall, which results in softening, so the action of these enzymes should be investigated collectively. With the advent of molecular biology, a number of genes have been recently identified, and a role for some enzyme families in fruit softening has been proposed, based on the correlation of mRNA accumulation and a given physiological stage or phenotype. This molecular information has been used to generate antisense or overexpressing transformants aimed to assess the physiological role of each enzyme. The results from genetic transformation supports the role of enzymes like expansins (Brummell et al., 1999), PLs (Jiménez-Bermúdez et al., 2002), and at least one β-Gal isoform (TBG4; Smith et al., 2002) in fruit softening, as antisense fruit proved to be firmer than controls and fruit over-expressing these transcripts showed a larger extent of softening. Nonetheless, a fruit that fails to soften has not been obtained by individual suppression of any transcript or activity. On the other hand, down-regulation of PG (Smith et al., 1988), PME (Tieman and Handa, 1994) or some β-Gal isoforms (reviewed in Smith et al., 2002) resulted in fruit with no significant differences in pulp firmness, despite some characteristics of the cell wall having been modified. The combined results illustrate that individual enzymes are not sufficient to produce an effect on fruit softening, so it has become evident that possible concomitant action of several isoforms and post-transcriptional regulatory events may be involved. In fact, although changes in levels of mRNA may predict changes in enzyme levels, the transcripts may not necessarily be translated and proteins detected in immunoassays may not necessarily be suitably modified by post-translational mechanisms to be fully active. Furthermore, the role of each enzyme cannot be explained by studying a single isoform since the presence of several isoforms, with distinct patterns of expression, may mask the total activity in a given developmental stage. Also, the presence of mRNA transcripts encoding for a specific isoform cannot be directly correlated to the resultant total enzymatic activity, due to different transcription rates. For example, from the seven expansin (FaEXP 1-7) mRNAs expressed during strawberry (*Fragaria ananassa* Duch.) fruit development, FaEXP3, which is expressed in small green fruit and in ripe fruit, is transcribed at much lower levels (1000-fold) than the other expansin mRNAs (Harrison et al., 2001). For these reasons, assays for monitoring the changes in the activity during the development of the fruit are needed to complement the studies on genetic expression.

In apples (*Malus* × *domestica* Borkh.), the activity of several cell wall-modifying enzymes has been reported and for some families, the activity fluctuations have been measured during fruit growth (Vincken et al., 1998) or ripening (reviewed by Johnston et al., 2002), although no work has investigated a set of enzymatic activities together using the same biological material. Furthermore, the ripening behaviour has been studied using fruit held under cold storage. The objective of this study was to determine the temporal patterns of activity of enzymes that have been implicated in cell wall modifications, to gain an insight into the changes in each activity during the complete development process; from fruit-set to over-ripe fruit.

2. Materials and methods

2.1. Plant materials

Apples (*Malus* × *domestica* Borkh cv. Mondial Gala) were obtained from trees grown at the experimental orchard of the Instituto Superior de Agronomia, Lisboa, Portugal, during two growing seasons. The fruit used were classified and assigned to classes according to their physiological stage, based on their time from anthesis or from harvest, size, skin colour, seed maturation and pulp firmness as: fruit set (stage 1), growing fruit (stage 2), unripe expanded fruit (stage 3), fruit at harvest (stage 4) and over-ripe fruit (stage 5). In all cases, samples were harvested, immediately frozen in liquid nitrogen and stored at −80 °C until extraction of proteins. Fruit at stages 1 and 2 were frozen and assayed with skin, whilst fruit from stages 3–5 were peeled and cut in small slices immediately before freezing. Fruit diameter was measured at the equatorial section using a vernier calliper. Seed maturation was assessed visually and colour of the fruit skin was accessed visually and measured using a colorimeter (Minolta Meter CR-300). Ground colour was expressed as the hue angle value in the Hunter scale (McGuire, 1992). Firmness of the pulp was determined using a Texture Analyser (TA-XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY) fitted with an 11 mm diameter flat probe. Peeled flat areas of the fruit were compressed 8 mm at a test speed of 1 mm s^{-1}.The compression force for each fruit was measured three times and the average of the maximum force necessary for the compression was used to define firmness. After the definition of developmental classes, based on the statistical analysis of 30 fruit, more than 60 fruit from each developmental stage and growing season were attributed to the classes defined. Sliced (stages 3, 4 and 5) or intact (stages 1 and 2) fruit were stored at −80 °C in bulks of mixed fruit from the same developmental stage. For extraction of proteins, 10 g samples of these fruit bulks were used per enzyme assay and per sample. For each enzyme activity, three independent assays were conducted per season (giving six replications). For expansin assays, four replications were made due to the high concentration of proteins required to detected activity