Polyphenoloxidase Isozymes in Bitter Pit Affected Apple Tissues Either Natural or Locally Induced by Ammonium Oxalate Injections

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Abstract

Calcium-related disorders in fruits lead to the collapse of cells, resulting in tissue enzymatic browning, caused by polyphenol oxidase (PPO) and peroxidase enzymes. PPOs are responsible for the enzymatic browning reaction that occurs in damaged fruits or vegetables. It appears that oxidative browning reactions in plants generally decrease quality by changing nutritional and organoleptic properties. In this work, electrophoresis was used for detection and comparison of active PPO isoforms from apple cv. Golden fruit tissues. Three types of materials were studied: sound apple pulp, bitter pit affected pulp and corky lesions induced by ammonium oxalate subdermal injections. Three main isozyme bands revealed by catechol staining were detected in the pulp of sound apple. In the apple tissue affected by bitter pit, the intensity of the two bands of high molecular weight increased and two new isozymes appeared, one below the second of greater mass and another one in the zone of low molecular weight. However, in oxalate-induced corky tissue, the lowest molecular weight PPO isozyme found in the natural pits, did not appear while the remaining pattern was analogous. Both the natural process of bitter pit development and the chemically induced one, finally resulted in cork-like spots. These dehydrated brown tissues accumulate calcium and show greater PPO activity. However, these two processes must follow different metabolic routes or the speed at which the spots appear in the oxalate-induced process, does not allow for the adaptation of the isozymatic system as it occurs in the natural process.

INTRODUCTION

Polyphenol oxidases (PPO) and peroxidases are the main enzymes responsible for fruit quality loss due to phenolic degradation (Tomás-Barberan and Espin, 2007). Enzymatic fruit browning is mediated by polyphenol oxidase (EC 1.10.3.1). This enzyme is able to interact with a large number of phenolic compounds and thus has been referred to by several names including tyrosinase, catecholase, polyphenolase, and cresolase. The basic oxidation reaction normally results in the formation of an unstable quinone, which then through a number of steps, proceeds to form melanins. Melanins are brown to black in color and are responsible for undesirable spots on many fruits and vegetables such as bananas, apples, mushrooms, and potatoes (Yoruk and Marshall, 2003).

Low calcium levels are frequently correlated with internal browning in raw fruits and vegetables for reasons that are currently not fully understood. Symptoms are observed in young expanding leaves, such as "tipburn" of leafy vegetables, in enclosed tissues, "brown heart" of leafy vegetables or "black heart" of celery, and "bitter pit" in apples, where tissues are fed principally by the phloem rather than the xylem. Cytosolic calcium concentration is known to stabilize cell membranes, and is a key regulator of plant defences to such challenges as mechanical perturbation, cooling, heat shock, acute salt

stress, hyper-osmotic stress, anoxia, and exposure to oxidative stress (White and Broadley, 2003).

Corky spots that develop during bitter pit of apples have higher concentrations of calcium, magnesium and potassium, increased polyphenol oxidase activity and lower concentrations of all carbohydrates (Val, 2003). Bitter pit is characterized by brown corky flecks in the flesh of the fruit. Flecks are most frequently found just under the skin of the apple but may be scattered through the flesh extending as far as the core. Other biotic factors could induce browning of fruit tissues. Wound-inducible PPO activity was verified in a number of plants, including apple (Boss et al. 1995). The increase in PPO activity in infected or wounded tissues of many plants supports the assumption that PPO-mediated oxidative browning could be an important protection response of plants against infection or wounding.

Steenkamp et al. (1983), found high concentrations of oxalate and citrate in the bitter pit flecks, and by vacuum infiltration they even obtained, deteriorations of the lamina media of the fruit cells, similar to those observed in naturally occurring pits.

The protein pattern of oxalate-induced bitter pit-like lesions in apple, has close similarities with the naturally occurring flecks, particularly in the low molecular weight region of the polyacrylamide electropherograms. Oxalate wounds and bitter pit developed a new 18 kDa protein which may belong to a small heat stress (smHSP) family of proteins (Val et al., 2006).

There are few studies on the composition of apple tissues affected by corky physiopathologies and we have not found published data about the PPO isozyme pattern of such tissues. The authors aimed to ascertain PPO composition of apple pulp affected by bitter pit and other corky tissues, developed by oxalate injections, as compared to pulp from healthy fruits.

MATERIAL AND METHODS

Smothee Golden Delicious apple cultivar was chosen for this study because of its commercial importance and the great bitter pit incidence. The apples were collected at commercial maturity and stored for one month at 4°C before processing.

Ammonium oxalate Induction of bitter pit like flecks.

Corky spots similar to those of bitter pit, were induced by injecting 100 µL ammonium oxalate (0.4% w/v), 0.5 cm deep in the apple cortex. After 48-72 h at room temperature, large corky lesions were clearly visible inside the apple mesocarp.

Extraction of Corky Areas.

Either natural or oxalate-induced pitted tissue was surgically removed by careful separation of the spots from the surrounding sound tissue and epidermis. Each sample comprised a group of pits from different apples to obtain a representative fraction of the different tissues. Samples were then immediately placed in liquid nitrogen and stored at -80 °C. Sound tissue from non-affected fruits was also extracted and analysed separately.

Mineral analysis.

One g of fresh tissue from the apple mesocarp was wet digested and calcium, magnesium and potassium determined by atomic absorption spectroscopy.

Protein determination.

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin as the standard. Nondenaturing, native PAGE was performed without the use of SDS. Electrophoretic separation was performed on a 12% polyacrylamide gel for 4.0 h at a 100 V. Gels were stained for PPO activity with 27 mM catechol in 0.1 M sodium acetate buffer, pH 7.0.

Phenolics.

Total phenolic content in the extracts were determined according to the Folin-Ciocalteu procedure. Quantification was done on the basis of a standard curve with gallic acid. Results are expressed as milligrams of gallic acid equivalent (GAE) per 100 g of fresh weight.

Enzyme extracts were obtained by blending 1g of apple pulp with a 0.2 M (pH 7) phosphate buffer containing triton X-100 and PVP.

PPO activity was calculated as the initial linear change in absorbance at 420 nm of a solution containing 0.1 M catechol and an aliquot of the enzymatic extract. Results are expressed as a per cent of the slope of the sound apple. Linearity was generally restricted to the first 2 to 3 minutes of reaction.

RESULTS AND DISCUSSION

Phenolic compounds are naturally occurring secondary metabolites in plants, that give fruits and vegetables both desirable qualities like colour and antioxidant properties and undesirable ones like astringency and bitterness .

The increasing interest in dietary fruit phytochemicals (Kris-Etherton et al., 2002) has largely focused on phenolic compounds which, for their ubiquity and diversity, are considered the most important antioxidant components in fresh fruit (Vinson et al., 2001).

In Table 1, the composition of bitter pit tissues, brown flecks induced by ammonium oxalate injections and mesocarp of sound apples, are shown. Oxalate wounds and bitter pit, showed significantly higher Ca concentrations than sound tissues. Oxalate had the highest Ca content, while bitter pit areas accumulated more than twice Ca as compared with sound tissue. On the contrary, bitter pits accumulated the highest level of Mg, almost 10 times greater than the control, and Mg in oxalate corky zones was 4 times higher.

Table 1. Composition of the three apple tissues: bitter pit, oxalate induced flecks and sound pulp.

	units	Bitter Pit	Oxalate wound	Sound apple
Ca		6.74 a	12.86 b	2.51 c
Mg	(mg/100 g fresh pulp)	43.34 a	21.25 b	5.19 c
K		150.25 a	121.97 b	99.86 c
Phenolics	(GAE/100 g of fresh pulp)	60.02 a	73.27 b	77.79 b
PPO activity	(relative units)	361.1 c	274.5 b	100.0 a

Within each column, values followed by same letter do not differ significantly at $P \leq 0.05$.

Total phenolics content in the three tissues only showed significant differences between bitter pit and the other two tissues. It seems that the highest PPO activity

exhausted the available substrate in natural corky zones. However, oxalate-induced flecks had almost three times more PPO activity than the sound apple pulp.

Three main isozyme bands revealed by catechol staining were detected in the pulp of sound apple (Figure 1). In tissues of bitter pit-affected apples, the intensity of the two bands of high molecular weight increased and two new isozymes appeared, one below the second of greater mass and another one in the zone of low molecular weight. However, in oxalate-induced corky tissue, the lowest molecular weight PPO isozyme found in the natural pits, did not appear while the rest of the pattern was analogous.

Figure 1. Native PAGE and catechol stain of PPO isozymes from the bitter pits of Smoothie Golden delicious apples, wounds induced by oxalate injections and pulp from a sound fruit.

From these results, it could be scored as a hypothesis, that the spots of bitter pit would be probably originated from an exacerbated synthesis of oxalate in the zones of the apple with low calcium contents. This may be the result of a displacement of calcium from its functional sites by a local magnesium accumulation. Under these circumstances, calcium would precipitate as calcium oxalate. Under low calcium conditions, cellular defence regulation may be disrupted, and could lead to the accumulation of reactive oxygen species that would cause oxidation of phenolic compounds and the resulting discoloration (Adams and Brown, 2007). Furthermore, enzymatic browning could be enhanced by the high magnesium concentrations as shown in Table 1. In vitro experiments, carried out by Hopfinger et al (1984), demonstrated that enzymatic browning due to polyphenol oxidase (PPO) in apple fruits was stimulated by addition of 0.8-10 mM $MgCl_2$ to the reaction mixture. On the other hand, while the role of PPO in plants has yet to be defined, the wounded-inducible nature of apple PPO gene expression supports a role in plant defence (Boss et al., 1995).

CONCLUSION

Both the natural and the oxalate injection, chemically-induced processes of bitter pit development yielded cork-like spots that finally resulted in dehydrated brown tissues. These accumulated calcium and magnesium to a greater extent than sound apple pulp, showed a higher PPO activity and consumed the phenolics substrate. However, the two processes must follow different metabolic routes or alternatively, the rapidity at which the spots appear in the induced process, does not allow the adaptation of the isozymatic system as it happens in the natural process.

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