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Localization and General Properties of Developing Peach Seed Coat and Endosperm Peroxidase Isoenzymes

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Abstract. Changes of soluble and ionically bound peroxidase and indoleacetic acid (IAA) oxidase activities were followed during peach seed development. Soluble peroxidase activity was located mainly in the embryo plus endosperm tissue, whereas wall ionically bound activities were found predominantly in the integument tissue. The different peroxidase isoenzymes present in the extracts were characterized by polyacrylamide gel electrophoresis and isoelectric focusing; the main soluble isoenzyme of embryo plus endosperm tissue was an anionic isoperoxidase of R_F 0.07. Basic ionically bound isoenzymes were located only in the integument tissue, but two soluble anionic isoenzymes of R_F 0.23 and 0.51 were also present in this tissue. In parallel, peroxidase protein content was estimated specifically using polyclonal antibodies. The kinetic data and the changes of seed IAA oxidase activity during fruit development suggested that basic peroxidase isoenzymes from ionically bound extracts of integument might be involved in IAA degradation.

The peach fruit is a useful model for studying growth-related changes in peroxidase isoenzyme patterns and their relationships with some physiological processes because its growth occurs in three well defined development stages (Valpuesta et al. 1991). The seed apparently plays a critical role in growth and viability of the fruit during stage I (Tukey 1936). Changes in indoleacetic acid (IAA) levels and peroxidase activities have been determined previously along with the fruit growth (Valpuesta et al. 1989).

The main soluble anionic peroxidase isoenzyme from peach seeds was purified, and rabbit polyclonal antibodies were obtained against this isoenzyme (Quesada et al. 1990). Changes in total IAA oxidase activity of peach complete seed peroxidases and levels of specific peroxidase proteins, detected by polyclonal antibodies, were followed during fruit growth (Alba et al. 1993).

Here we report the first differential distribution of peroxidase isoenzymes and changes in the levels of specific protein in each seed tissue, integument (the outer coat of seed) and embryo plus endosperm, during fruit development. The kinetic behavior of IAA oxidase activity of the isoenzymes with the highest activity with this substrate was analyzed in order to study the possible role of peroxidases during peach seed development.

Materials and Methods

Plants

Fruits were obtained from a commercial cultivar (cv. Redhaven) of peach (*Prunus persica* L. Batsch) during fruit growth. Several samples were collected during the three stages of fruit development defined previously for the cultivar Redhaven (Alba et al. 1995). Stage I extends from 0 to 55 days, stage II from 55 to 79 days, and stage III from 79 to 98 days after anthesis. Seeds were removed from frozen fruit and dissected mechanically into integument and embryo plus endosperm as a unique section.

Crude Extract Isolation Procedure

Soluble protein extracts and those ionically bound to cell walls were obtained by grinding the seeds and their different sections with 50 mM sodium acetate buffer, pH 4, or the same buffer containing 1 M NaCl (Alba et al. 1993). Both types of extracts were dialyzed exhaustively against 25 mM sodium acetate buffer, pH 4, and were then concentrated

Abbreviations: IAA, indoleacetic acid; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; ELISA, enzyme-linked immunosorbent assay.

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with CX-10 immersible filters or by filtration through YM-10 Amicon membranes.

Isolation of Peroxidase Isoenzymes

The main anionic isoenzyme of soluble extracts was purified as described previously (Quesada et al. 1990). DEAE-Sephacel chromatography in a batch system was used to isolate a group of ionically bound cationic isoenzymes (Alba et al. 1993).

Enzyme Activities and Protein Measurement

Peroxidase activity was measured as the increase in absorbance at 460 nm upon incubation with 0.6 mM *o*-dianisidine and 8.8 mM hydrogen peroxide in 50 mM sodium phosphate buffer, pH 6.0, at 25°C (Quesada et al. 1990). IAA oxidation was monitored by following the absorbance changes at 254 nm. (Rubery 1972). The initial rate, after the end of a lag period of 2–10 min, was usually determined in a mixture containing 0.66 mM IAA, 1 mM $MnCl_2$, 0.1 mM *p*-coumaric acid, and 50 mM sodium phosphate buffer, pH 5.7 (De Forchetti and Tigier 1983). The enzyme unit was defined arbitrarily as the amount of enzyme which produced an 0.01 absorbance change at 460 nm/min for peroxidase and at 254 nm/min for IAA oxidase.

Protein content was determined by the folin phenol reagent method (Lowry et al. 1951) or by absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis (PAGE) and Isoelectric Focusing (IEF)

Peroxidase isoenzymes were separated by anionic (Davis 1964) and cationic (Reisfeld et al. 1962) PAGE. Electrophoresis was performed on 7.5% (w/v) polyacrylamide gels.

IEF was performed in a Mini IEF Cell from Bio-Rad, working with polyacrylamide slabs and ampholines (pH 3–10).

Peroxidase isoenzymes were stained in PAGE and IEF gels with a mixture of 45 mM H_2O_2, 3.8 mM benzidine, 500 mM sodium acetate, pH 4.6, at room temperature for 5 min (De Forchetti and Tigier 1983).

Immunological Techniques

Rabbit polyclonal antibodies were obtained against the anionic peach seed peroxidase isoenzyme of R_F 0.23 (Quesada et al. 1990). When tested, these antibodies also displayed an excellent cross-reaction with the cationic isoenzymes present in the seeds, and they have been used to estimate quantitatively both groups of peroxidase isoenzymes (Alba et al. 1993). Quantitative ELISA was performed with selected samples representative of every developmental stage. The specific protein calibration curve was obtained according to Tigier et al. (1991b) and Alba et al. (1993).

Results and Discussion

Changes of total peroxidase activity from integument and embryo plus endosperm were followed during the time of fruit growth. The results for soluble and ionically bound fractions are shown in Fig. 1. Peroxidase activity from the embryo plus endosperm tissue was mainly the result of soluble isoenzymes extracted easily at low ionic strength. The highest activity was displayed at stage I, and the minimum activity was found at the transition from stage II to stage III (Fig. 1*A*). On the contrary, ionically bound peroxidase activity was detected mainly in the integument tissue, and the highest activities were found at mid stage I and during stage II (Fig. 1*B*). IAA oxidase activities showed a similar distribution (Fig. 1, *C* and *D*).

As described by Stahly and Thompson (1959), IAA tends to be localized in the endosperm of peach seeds, its maximum level was detected in the complete seed, at the transition from stage II to stage III (Miller et al. 1987 and Valpuesta et al. 1989); the minimum soluble peroxidase and IAA oxidase activities were found in the embryo plus endosperm tissue.

At the three stages considered in the present study, the zymograms obtained were similar. Fig. 2 shows the result obtained 43 days after anthesis when both soluble and bound extracts display high activity. Qualitative and quantitative differences were found in peroxidase patterns of soluble and ionically bound fractions from complete seed, integument, and embryo plus endosperm when an identical amount of protein was loaded per lane.

Soluble extracts contain only anionic isoenzymes with R_F values of 0.07, 0.23, and 0.51 (Fig. 2*A, lane a*); no cationic isoenzymes were present in this extract. The more abundant isoenzyme of R_F 0.23 was detected in the seed coat extracts (*lane b*) and at a low level in the embryo plus endosperm tissues (*lane c*), probably resulting from the difficulty of isolating the different tissues. The more anionic isoenzyme (R_F 0.51) was found only in the seed coat extracts (Fig. 2*A, lane b*), whereas the less anionic isoenzyme (R_F 0.07) was detected only in the embryo plus endosperm extracts (*lane c*).

The ionically bound extracts anionic isoenzyme patterns showed only two bands (R_F 0.04 and 0.25) in the complete seed (Fig. 2*B, lane a*); these bands were located in the integument tissue (*lane b*), but no band was detected in embryo plus endosperm (*lane c*). Ionically bound complete seeds and the integument tissue extracts also contained cationic isoenzyme with a main isoenzyme of R_F 0.22 and a poor stained band of 0.41 (Fig. 2*C*).

The isoelectric points of the main peroxidase isoenzymes were detected by extracting the bands of cationic and anionic PAGE slabs and developing IEF (Fig. 3). The correspondences between R_F and pI were: anionic soluble R_F 0.23 (Fig. 2*A*) with pI 3.5 (Fig. 3); anionic ionically bound R_F 0.25 (Fig. 2*B*) with pI 3.5 (Fig. 3); cationic ionically bound R_F 0.22 and R_F 0.41 (Fig. *C*) with pI 7.5 and pI 7.8, respectively. Anionic soluble R_F 0.51 is probably not detected with this ampholine. Anionic R_F 0.04 (Fig. 2*B*) with little mobility is not detected in IEF, and perhaps it is a cationic isoenzyme.

As was shown, peroxidase isoenzymes were not distributed uniformly in peach seeds. The more anionic

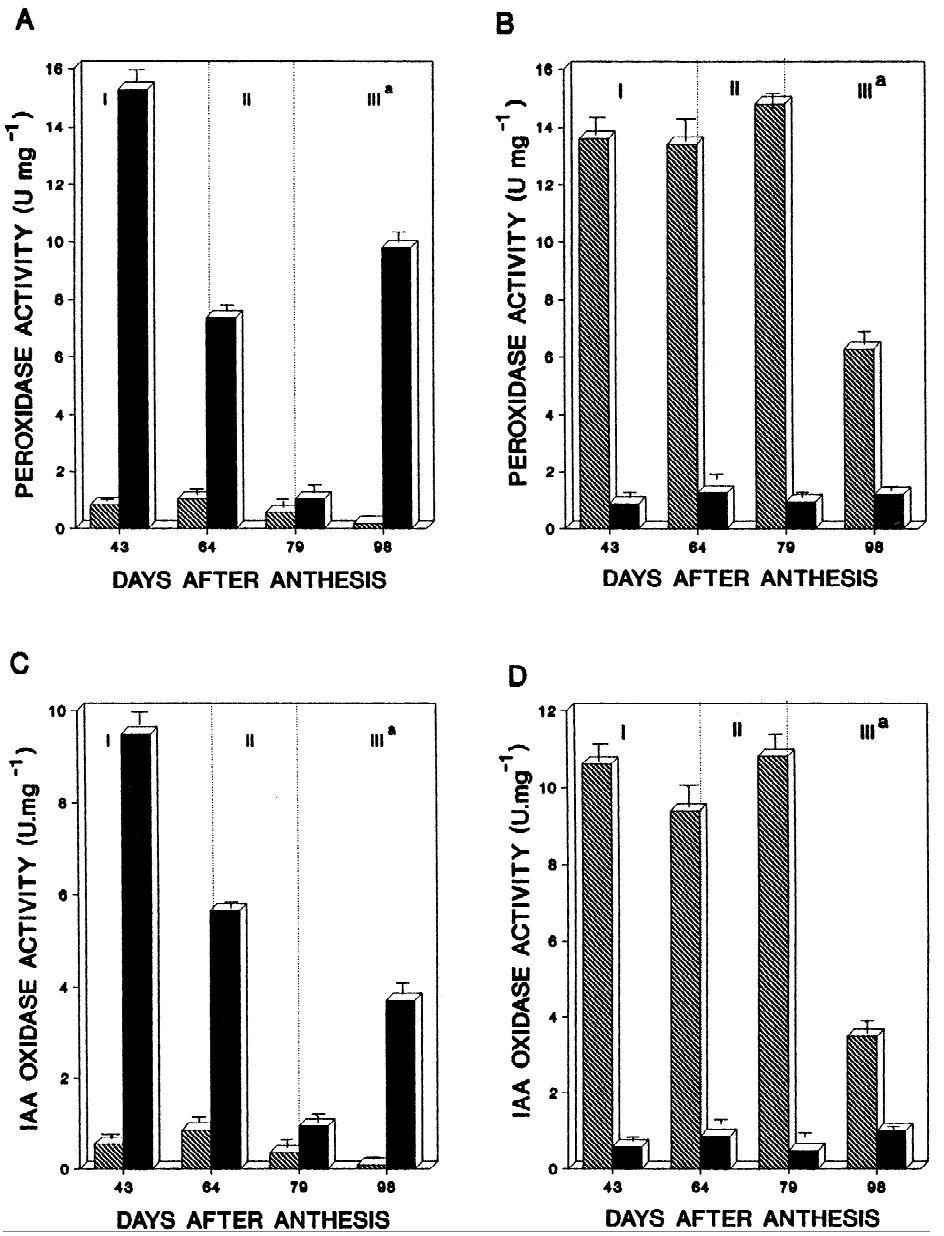

Fig. 1. Changes in peroxidase (*panel A*, soluble extract; *panel B*, ionically bound extract) and IAA oxidase (*panel C*, soluble extract; *panel D*, ionically bound extract) activities from (▨) the integument and (■) embryo plus endosperm extracts during fruit growth. *a*, growth stages.

soluble isoenzymes (R_F 0.23 and 0.51) were localized mainly in the integument, whereas the less anionic isoenzyme (R_F 0.07) was detected only in embryo plus endosperm tissues and it was probably responsible for the high total peroxidase activity shown in Fig. 1*A*. The ionically bound isoenzymes were detected only in the integument, contrary to the results with soybean seeds. In these seeds the majority of peroxidase activity is localized in the seed coat. Most of this activity is caused by an anionic soluble isoenzyme of pI 4.1, which is related to the oxidation of phenolic compounds and extensin modification (Gillikin and Graham 1991).

Changes in peroxidase-specific protein levels from soluble and ionically bound extracts of the integument and embryo plus endosperm, detected with polyclonal antibodies, were followed along with fruit growth (Table 1). The specific protein determined by ELISA, in soluble and ionically bound extracts of the integument was significantly higher than that detected in embryo plus endosperm, the highest levels being those of the ionically bound extracts. A good correlation exists between activities and specific protein content in the case of these ionically bound extracts. Thus, the differences in activity observed are not the result of activators or inhibitors present, at least in the ionically bound extracts. This correlation is not evident in the case of the soluble ex-

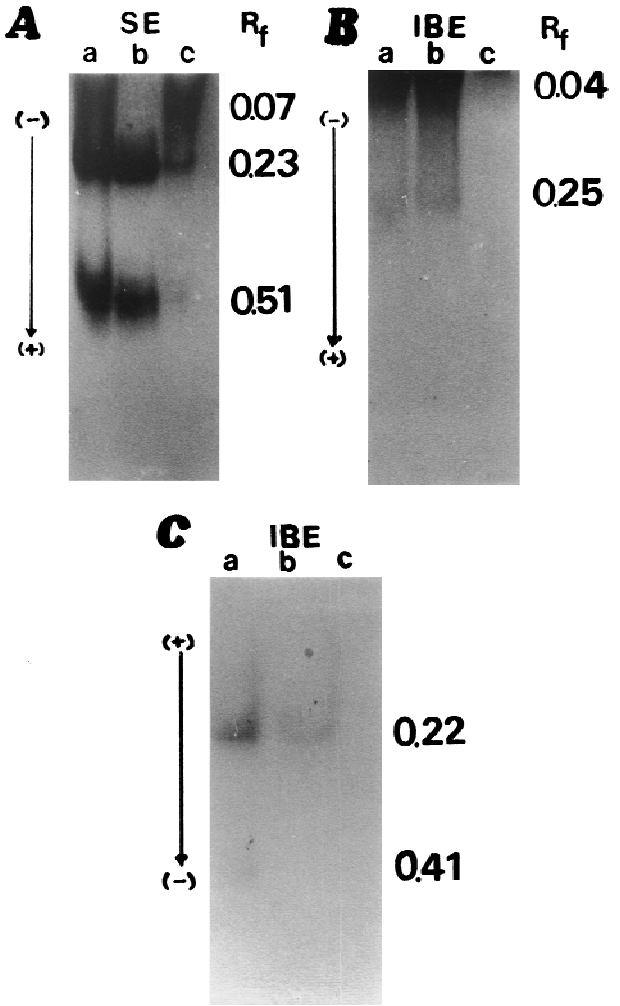

Fig. 2. Peroxidase isoenzymes zimograms from the complete seed, the integument, and embryo plus endosperm extracts. *Panel A,* anionic PAGE of soluble protein extracts (SE). *Panel B,* anionic PAGE of ionically bound protein extracts (IBE). *Panel C,* cationic PAGE of ionically bound protein extracts. *Lanes a,* complete seed. *Lanes b,* integument. *Lanes c,* embryo plus endosperm.

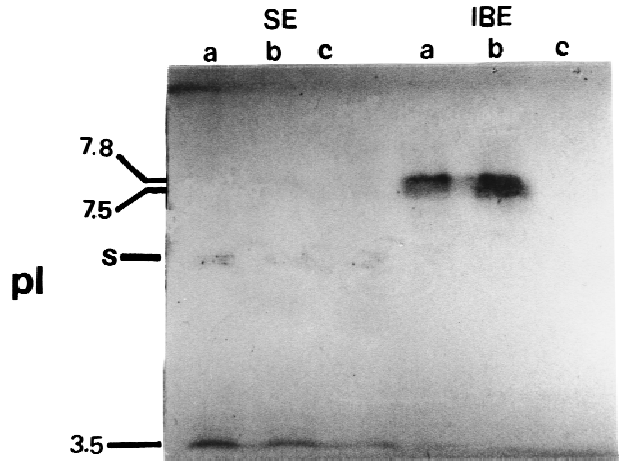

Fig. 3. Peroxidase isoenzymes IEF patterns. SE, soluble protein extracts. IBE, ionically bound protein extracts. *Lanes a,* from complete seed; *b,* integument; *c,* embryo plus endosperm. Anionic SE R_F 0.23 (Fig. 2*A*) with pI 3.5 (Fig. 3); anionic IBE R_F 0.25 (Fig. 2*B*) with pI 3.5 (Fig. 3); cationic IBE R_F 0.22 and R_F 0.41 (Fig. 2*C*) with pI 7.5 and pI 7.8, respectively. Anionic SE R_F 0.51 is probably not detected with this ampholine. Anionic R_F 0.04 (Fig. 2*B*) with mobility not detected in IEF; perhaps it is a cationic isoenzyme.

Table 1. Changes in soluble and ionically bound specific peroxidase proteins, from integument and embryo plus endosperm extracts, detected by polyclonal antibodies at 43, 79, and 98 days after anthesis.

	Soluble extracts		Ionically bound extracts	
Days after anthesis	Integument	Embryo plus endosperm	Integument	Embryo plus endosperm
43	12.9 ± 1.0	5.7 ± 0.2	17.2 ± 2.0	0.1 ± 0.08
79	4.3 ± 0.8	1.1 ± 0.1	16.9 ± 2.0	0.6 ± 0.09
98	4.3 ± 0.2	3.8 ± 0.1	5.7 ± 0.8	0.3 ± 0.04

Note. The values represent the specific protein (in μg/mL) detected by ELISA methods. Each value represents the mean (± S.E.) of three extracts, from different growth stages, assayed three times each.

tracts because the high specific protein levels were found for soluble extracts of integument in spite of the low activity they displayed. This may be because of the presence of a high content of anionic isoenzyme of R_F 0.23 (used as an antigen for antibody production) and/or the poor cross-reaction with the band of R_F 0.07 of the embryo plus endosperm soluble extract. However, in general immunological homology among peroxidases of different plant origin is high when these proteins are compared using polyclonal antibodies (Clark and Conroy 1984, Conroy et al. 1982, Quesada et al. 1990). This fact, jointly with the excellent staining obtained in the PAGE of the isoenzyme present in the integument (Fig. 2*A, lane b*) in comparison with the staining of band of R_F 0.07 (*lane c*), may indicate the presence of activity inhibitors in the integument soluble extract.

Previous studies dealing with the role of peroxidase activity during peach seed development were focused mainly on soluble isoenzymes, the isoenzyme of R_F 0.23 being purified and studied (Quesada et al. 1990, Tigier et al. 1991b). The present study shows that enzymes ionically bound to integument are also important and merit further effort. As a first approach they were partially purified from the ionically bound extract of complete seeds by ion exchange chromatography with DEAE-Sephacel in a batch system, and they were used to study their ability to degrade IAA. At their optimum pH (5.5) these isoenzymes showed a sigmoidal kinetic behavior

with an $s_{0.5}$ of 0.27 mM and n 2, for IAA as substrate. The anionic isoenzymes also present in this extract showed an optimum pH of 5.3, an $s_{0.5}$, and n of 0.35 and 2, respectively, for the same substrate.

Integument acidic peroxidase isoenzymes showed lower IAA oxidase activities than cationic isoenzymes of the same tissue. Based on the results of the kinetic studies, we suggested that the basic isoenzymes from peach seeds are most effective in IAA degradation as we had reported previously (Alba et al. 1993). Similar results were described for other cationic peroxidase isoenzymes of different tissues (De Forchetti and Tigier 1990, Gaspar et al. 1991, Tigier et al. 1991a).

IAA oxidase, an activity displayed by most plant peroxidases, at least in vitro, was challenged as a mechanism of IAA degradation in vivo (Normanly et al. 1995) using as argument results obtained with peroxidase overproducer transgenic tobacco (Lagrimini 1991, Lagrimini et al. 1990).

More recent studies on the IAA degradation mechanism of this peroxidase overexpressed in transgenic plants suggest that IAA oxidation may be an important physiological role for this peroxidase. In fact, IAA appears to be a unique substrate for plant peroxidases when, in their reaction with IAA, it behaves like oxygenase with high substrate specificity (Gazaryan et al. 1996).

However, whether IAA tends to be located in the endosperm of peach seeds as it was in the integument tissue, also found in previous studies with cherry seeds (Valpuesta and Bukovac 1983), remains unclear.

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