Fruit Development in *Capsicum annuum*: Changes in Capsaicin, Lignin, Free Phenolics, and Peroxidase Patterns

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Pepper fruits, of *Capsicum annuum* cv. Padrón, undergo changes in content of capsaicinoids, lignin, and free phenolics during the maturation process. Although capsaicinoids increase with development, the maximal levels of free phenolics and lignin are observed during the early stages of development. A decrease of peroxidase activity was observed during maturation, and this was related with a decrease in other physiological parameters studied, namely chlorophylls and pH. Subcellular fractionation studies reveal that most peroxidase activity is localized in the soluble fraction throughout development. The changes in the peroxidase activity were accompanied by changes in the different isoenzymes. Acidic isoenzymes increased whereas the basic isoenzymes decreased over the same period, and the changes in these isoenzymes were related with capsaicin metabolism.

Keywords: *Pepper; peroxidase; capsaicinoids; lignin; free phenolics*

INTRODUCTION

Peroxidase (EC.1.11.1.7.) is the enzyme which catalyzes the oxidation of a large number of aromatic structures at the expense of H_2O_2. Widely distributed in plants, peroxidases have been implicated in numerous physiological processes, namely the control of plant cell growth and differentiation due to their participation in the catabolism of auxins, the polymerization of cinnamoyl alcohols to lignin, and the H_2O_2-detoxifying reactions (Gaspar et al., 1985).

Peroxidases are glycoproteins that may be classified into two main groups according to their pI: acidic and basic (Ros Barceló, 1998). A given fruit can show several isoenzymes with very different isoelectric points (Silva et al., 1990). An analysis carried out on some fruits throughout the course of ripening detected different behavior. Thus, in strawberry (Civello et al., 1995) and banana fruits (Haard and Tobin, 1971) the same isoenzymes are present in several ripening stages. However, in the tomato, the pattern of peroxidase isoenzymes changes during ripening (Thomas et al., 1981).

It has recently been suggested that peroxidases might participate in the capsaicinoid metabolism of the pepper fruit. Capsaicinoid accumulation is especially associated with a particular developmental stage of a single organ of the plant: the fruit. Like other plant alkaloids, they accumulate and later undergo a rapid turnover and degradation during fruit development (Iwai et al., 1979). Basic peroxidases with a high pI may be directly related to capsaicinoid metabolism because both capsaicin and dihydrocapsaicin, and their phenolic precursors, are easily oxidized by this enzyme (Bernal et al., 1993a, 1993b, 1995). The ability of the acidic peroxidase to oxidize capsaicin has also been demonstrated recently by Pomar et al. (1997).

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Padrón is a cultivar of pepper commercially available in the NW region of Spain, Galicia, and it is highly appreciated for fresh market consumption. The fruits are harvested and commercially available when immature and their flavor is not extremely hot, as they contain only slight levels of capsaicin which is the main compound causing the pungency in peppers (Estrada et al., 1997). This low capsaicinoid content of the fruit is the most important trait of its quality.

Considering that peroxidase activity may be correlated with the process of fruit ripening, and that peroxidases are involved in capsaicin metabolism, in this paper we study the changes of peroxidase activity, the modifications in the pattern of peroxidases throughout the course of pepper fruit ripening, and the possible implication of different isoenzymes in the capsaicin catabolism.

MATERIALS AND METHODS

Plant Material. *Capsicum annuum* L. cv. Padrón, was grown in a greenhouse on the campus of the University of La Coruña from April to September. Anthesis began about 3 months after germination; individual flowers were numbered and their date of flowering was recorded. Pepper fruits were harvested every 7 days from 14 (stage 1) to 42 (stage 5) days after flowering and individual weights of the whole peppers were measured. The fruits were subsequently stored at −30 °C until processed.

Physicochemical Analyses and Color. The soluble solids of the pepper fruit were determined with an ATAGO refractometer, and the resulting data are reported as °Brix at 20 °C. The pH was determined by homogenizing 40 g of the fruit in 100 mL of distilled H_2O, in a Waring blender, and then filtering. The color of the pepper fruit homogenate was measured spectrophotometrically at 470 and 652 nm for carotenoids and chlorophylls, respectively (Lichthentaler, 1987).

Extraction and Quantification of Capsaicinoids by HPLC. Capsaicinoids were extracted from Padrón pepper fruits using the technique described by Collins et al., (1995), with modifications (Estrada et al., 1997)

10.1021/jf000190x CCC: $19.00
Published on Web 11/14/2000

Table 1. Compositional and Physical Changes during Development of Pepper Fruit[a]

	days after flowering				
	14	21	28	35	42
soluble solids (°Brix at 20°)	3.10 ± 0.20	3.60 ± 0.40	3.80 ± 0.40	4.02 ± 0.10	8.40 ± 0.40
pH	6.13 ± 0.01	6.20 ± 0.01	6.27 ± 0.02	6.37 ± 0.02	5.01 ± 0.03
chlorophyll mg/g FW	0.35 ± 0.01	0.43 ± 0.07	0.90 ± 0.18	0.64 ± 0.21	0.25 ± 0.03
carotenoid mg/g FW	0.19 ± 0.06	0.21 ± 0.08	0.26 ± 0.12	0.29 ± 0.11	4.76 ± 0.90
ratio FW/DW	16.19	15.35	13.48	12.12	9.57

[a] Values are mean of 5 fruits ± SD.

Pepper fruits were oven-dried at 60 °C for 2−5 days; they were then ground using a laboratory mill and stored in sealed plastic tubes at room temperature prior to extraction. The capsaicinoids were extracted from 1.0 g of the ground pepper in 10 mL of acetonitrile by heating at 80 °C for 4 h. The suspended material was allowed to settle, and the supernatant was extracted and centrifuged at 100*g* for 10 min and then filtered (0.45 μm Whatman on a 10-mL disposable syringe) into a 2-mL glass sample vial which was capped and stored at 5 °C until analyzed. A 10-μL aliquot was used for each HPLC injection.

The samples were analyzed using a Waters LC616 system equipped with a Waters 717 plus autosampler, a Waters temperature control module, a Waters 996 photodiode array detector, and Millennium software for data processing. Reverse-phase HPLC was carried out on a Spherisorb ODS2 C_{18} column (5-μm particle size, 150 mm × 46 mm). A precolumn guard cartridge Spherisorb ODS2 C_{18} column was also used. The capsaicinoids were determined under controlled HPLC operating conditions: temperature of 25 °C, a flow rate of 1 mL min^{-1}, and a 14 min run. The mobile phase was isocratic, with 50% solvent A (100% acetonitrile-HPLC grade) and 50% B (10% acetonitrile-HPLC grade).

Standards of capsaicin (approximately 98%) and dihydrocapsaicin (approximately 90%) were obtained from Sigma Chemical Co. (St. Louis, MO) and were used for retention-time verification and instrument calibration. The mean retention times of capsaicin and dihydrocapsaicin under these conditions were 7.36 and 10.38 min, respectively.

Extraction and Determination of Free Phenolics. A procedure similar to that used in capsaicinoid extraction was carried out to separate the free phenolics from Padrón pepper fruits, but with 80% MeOH instead of acetonitrile, according to the technique described by Díaz et al. (1997). Phenolics in HPLC chromatograms were identified and quantified by co-injection and by comparing retention time and peak areas with those of an external standard.

Extraction of Cell Walls and Lignin Determination. Cell walls were extracted from the powdered samples according to Díaz and Merino (1998), with modifications. A 100-mg portion of powder was incubated in 50 mL of MeOH at 70 °C for 2 h. After powder decantation, the supernatant containing pigments and phenolics was removed and discarded. Fresh MeOH was added to the pellet and a second 2-h incubation was performed. Next, the supernatant was removed and a minimal amount of fresh MeOH was added to resuspend the pellet. The suspension was centrifuged at 1500*g* for 15 min, and the pellet was resuspended in 6 mL of distilled water. The suspension was stirred for 1 h and centrifuged at 1500*g* for 10 min, followed by the resuspension of the pellet (performed 2×) in 6 mL of distilled water. The last pellet was resuspended once in 6 mL of 0.5 M phosphate buffer (pH 7.0) containing 5% EtOH and 0.02% protease (Pronase E, Sigma Chemicals P-5147). This suspension was incubated for 18 h at 37 °C and centrifuged at 1500*g* for 10 min, after which the resuspension of the pellet in 6 mL of solvent and centrifugation under the same conditions was performed consecutively: 3× with distilled water as the solvent, 3× with 95% EtOH, and 2× with absolute EtOH. The final pellet (cell wall material) was dried at room temperature for 1−2 days and weighed. Its lignin content was determined using the acetyl bromide/acetic acid method (Johnson et al., 1961).

Enzyme Procedures. All steps in the enzyme extraction were performed at 4 °C. Each sample of pepper fruit (5 g) was homogenized in a Waring blender in the presence of 10 mL of buffer I: 5 mM Tris, 0.25M sucrose, 1mM magnesium acetate, 1M KCl, pH 7.2, supplemented with 0.25 g of PVPP. The homogenate was maintained 90 min at 4 °C. After filtration through 4 layers of nylon gauze, the homogenate was centrifuged at 12 100*g* for 30 min. The resulting supernatant was dialyzed for 24 h in buffer (50 mM Tris HCl, pH 7.5) and was considered to be a soluble crude and ionically bound peroxidase fraction.

The location of most of the peroxidase activity was determined by subcellular fractionation using fruits in different stages of development according to the method of Bernal et al. (1993c). Four different fractions were obtained using this technique: soluble, ionically bound cell wall and membrane, and covalently bound cell wall peroxidases.

The determination of peroxidase activity with 4-methoxy-α-naphthol (4MN) was carried out as described by Ferrer et al. (1990).

Blotting. The histochemical localization of peroxidase in pepper fruits was studied by tissue printing on nitrocellulose membranes (Bernal et al., 1994). The transversal slice of tissue was obtained by cutting the extremity at the pedicle of frozen peppers with a scalpel. Thawed slices were placed on the nitrocellulose membrane and then slightly pressed for 30 s. The slices were removed and the dry membrane was revealed according to Bernal et al. (1994).

Isoelectric Focusing (IEF) and Staining for Peroxidase Activity. IEF was performed on a Pharmacia Multiphor II using Ampholine PAGplate (pH = 3.5−9.5) polyacrylamide gels. A kit of isoelectric point markers (IEF Mix 3.6−9.3, Sigma) was used to calculate the pI of the peroxidase. Peroxidase activity was detected in gels by incubating them for 15 min at 25 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM 4MN followed by the addition of H_2O_2 to a final concentration of 0.33 mM (Ferrer et al., 1990).

Densitometry Quantification of Peroxidase Isoenzymes. IEF gels were scanned using a Personal Densitometer SI (Molecular Dynamics), and these scans were analyzed using the software Image QuaNT v. 4.2. Densitometric profiles of the zymograms were obtained and the area in relative units (pixels) was quantified for each isozyme using the Peak Finder function.

RESULTS AND DISCUSSION

Composition and Physical Changes. Table 1 summarizes some physical and compositional characteristics which define fruit maturity as they change throughout the growth of pepper fruits. Soluble solids exhibited slight increases during maturation until the fourth stage. In the last stage, however, they underwent a sharp increase. The pH also climbed slightly from the first to the fourth stage, but declined rapidly in the last stage. These pH values were higher than those found in the New Mexican type pepper whose pH extract was more acidic (from 5.4 to 4.6) when it turned from green to red (Biles et al., 1993). The changes observed in chlorophylls and carotenoids throughout fruit development were largely attributable to the chloroplast-to-chromoplast transition in the fruit cells (Mínguez-Mosquera and Hornero-Méndez, 1994). The ratio between dry weight and fresh weight revealed that fruit matura-

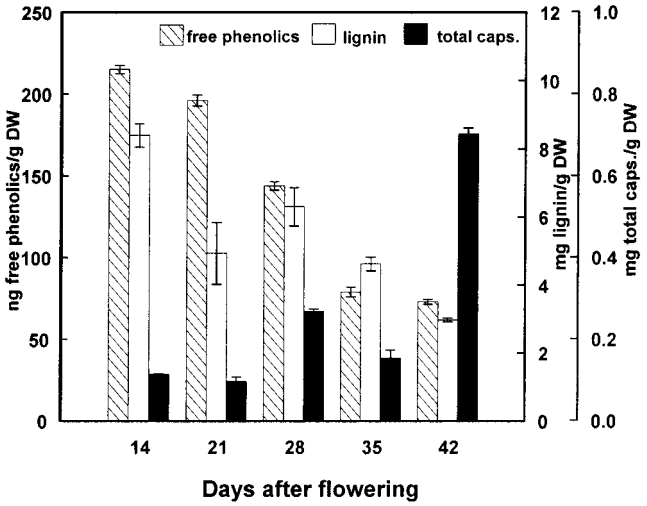

Figure 1. Changes in capsacinoid, lignin, and free phenolics contents during ripening of pepper fruits. Bars show SD.

Table 2. Evolution of Free Phenolics during Ripening of Pepper Fruits

	free phenolics[b]			
day[a]	protocatechuic acid	chlorogenic acid	coumaric acid	ferulic acid
14	130 ± 4	66 ± 2	19 ± 2	nd
21	134 ± 5	48 ± 3	14 ± 2	nd
28	113 ± 3	31 ± 2	nd	nd
35	79 ± 3	nd	nd	nd
42	63 ± 2	nd	nd	10 ± 1

[a] Number of days after flowering. [b] Levels of free phenolics reported as ng/g DW ± SD. nd, not detected.

tion processes are accompanied by a loss of water. Indeed, it has been reported that the dry matter increases with maturity (Govindarajan, 1985).

Capsaicinoids, Free Phenolics, and Lignin. Figure 1 we shows the changes in capsaicinoids, free phenolics, and lignin in pepper fruits during ripening. Capsaicinoids were detected 14 days after flowering in the first stage. Their levels remained low for 21 days, but 28 days after flowering capsaicinoids increased moderately, and finally, at the end of development, the cv. Padrón pepper exhibited a dramatic increase in capsaicinoid levels with the highest values found in the fifth stage, 42 days after flowering. The level of capsaicinoids in the cultivar Padrón was lower than that in other fruit varieties (Iwai et al., 1979; Salgado-Garciglia & Ochoa-Alejo, 1990) but accumulation patterns were similar.

We have found differences in the levels of the predominant compounds of free phenolics. These differences were associated with successive stages of fruit development. The pattern of free phenolic accumulation was totally different from that of capsaicinoids, as the levels were much greater in the early stages than in the final stages (Figure 1).

Over the course of the development of the maturing fruit we identified four free phenolics: protocatechuic acid, chlorogenic acid, coumaric acid, and ferulic acid (Table 2). The total concentrations of these four phenolics during the fruit maturation process are presented in Figure 1. They all exhibited a decrease in content with development except for ferulic acid, the precursor to capsaicinoids which only appeared in the last stage with maturation. Considerable variations were generally observed in the levels of phenolic compounds in fruits at different stages during growth and maturation, and from one variety to another (Romeyer et al., 1983). In the cv. Padrón fruit the decrease in the levels of free phenolics may be linked to capsaicinoid synthesis. In contrast, Sukrasno and Yeoman (1993), who, in a study of *Capsicum frutescens*, observed the presence of glycosilated phenolics in substantial amounts only before the onset of capsaicinoid accumulation due to the subsequent decrease in phenolics, suggested that the glycosilated phenolics may act as a source of intermediates for capsaicinoid synthesis. However, Sakamoto et al. (1994), detecting free phenolics in two cultivars of *C. annuum* fruits, reported their increase throughout development, but this was not correlated with the production of capsaicinoids in either cultivar.

Lignin accumulation presented the same pattern as free phenolics and thus differed from capsaicinoids. Maximal levels were observed in the first stage, then decreased until maturation when the level was half the amount detected at the beginning of development (Figure 1). Such a decrease in lignin content in the Padrón pepper coincided with the softening process that pepper fruits undergo (data not shown). Softening is a process that entails a major chemical restructuring of the walls of the cells that comprise the body of the fruit (Gross et al., 1986). It was only at the time of maturation that we detected the presence of ferulic acid (Table 2). Ferulic acid can also be found in small quantities in the various glucidic fractions on the cell wall, and could be related to the major biochemical modifications in the cell wall in which pectin−ferulic acid bonds may play a regulatory role (Ozawa et al., 1987). On the other hand, it has been proposed that, as capsaicin synthesis in vivo coincides with fruit softening, such softening may provide a huge supply of substrates for capsaicin synthesis. Holden et al. (1987) reported that the sink of bound phenolics in the cell wall may turn over and could thus provide substrates for the capsaicin biosynthetic pathway. Also, Hall et al. (1987) and Sukrasno and Yeoman (1993) suggest that capsaicin biosynthesis in pepper fruits competes with an active accumulation of lignin-like substances in cell walls, which are probably derived from phenylpropanoid precursors and from capsaicin itself, a process which takes place at the onset of capsaicin accumulation.

Peroxidase Activity During Ripening. At the beginning of fruit development, 14 days after flowering, soluble and ionically bound peroxidase activity showed the highest levels, decreasing in the second stage and remaining constant during the third stage (Figure 2). It decreased in the fourth stage and continued to decline until maturation.

The decrease in peroxidase activity correlates with the decrease in the other physiological parameters cited above, namely chlorophylls and pH, which define fruit maturity. Similar changes in peroxidase activities have been observed in other ripening fruits. Thus, in Habanero chile peppers (*Capsicum chinense* Jacq.) peroxidase activity decreased during development after 50 days from the setting of the fruit (Contreras-Padilla and Yahia, 1998). In tomato fruit, the maximum value of soluble peroxidase was reached at the mature green stage and declined with ripening (Thomas et al., 1981). Also, in the strawberry fruit peroxidase activity markedly decreased as the fruit ripened (Civello et al., 1995). However, in other cases, such as in the pulp of papaya

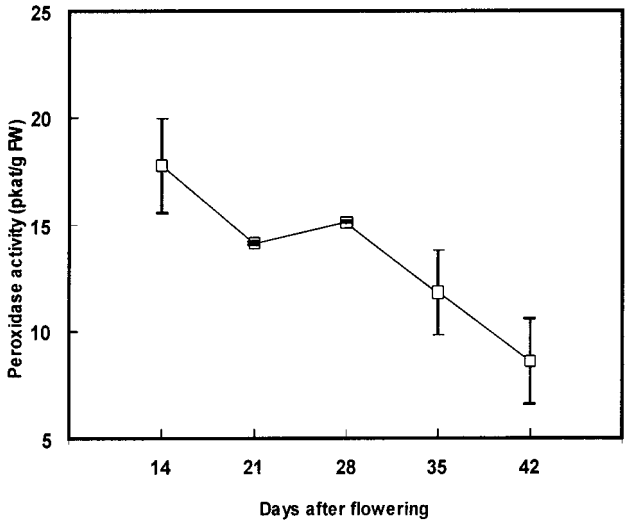

Figure 2. Peroxidase activity in pepper fruit at different stages of development. Bars show SD.

Table 3. Distribution of Peroxidase Activity by Subcellular Fractionation in Pepper Fruits

day[a]	soluble[b]	ionically bound membrane[b]	ionically bound cell wall[b]	covalently bound cell wall[b]
14	77.14	18.84	1.88	2.12
21	80.52	16.91	2.17	0.38
28	90.27	8.26	0.84	0.61
35	92.13	6.36	0.81	0.68
42	95.63	2.80	0.70	0.84

[a] Days after flowering. [b] The enzymatic activities are expressed in percentage.

fruit, the soluble and bound peroxidase increased during ripening 2.5 and 4.22-fold, respectively (Silva et al., 1990)

Blotting of frozen and thawed slices of pepper fruits on nitrocellulose membranes and subsequent staining with 4-methoxy-α-naphthol/H_2O_2 revealed that most of the peroxidase activity was present in both the placental and outermost epidermal cells. Up until the third stage in fruits the enzymatic activity was concentrated principally around the seeds, and in the final stages the majority of activity was found in the placental and epidermal cells.

The subcellular fractionation of pepper fruits in different stages of development revealed that most of the peroxidase activity, using 4MN as a substrate, was located in the soluble fraction. The remaining activity was associated ionically with both the membrane and the cell wall fraction. Covalently bound cell-wall peroxidase made only a small contribution (Table 3). This activity varied in all fractions over the course of fruit development, increasing in the soluble fraction and decreasing in the other fractions. The most important fraction in the fruits is usually the soluble one, as in tomato fruits (Matoo and Vickery, 1977), but in some cases, as in the strawberry, the membrane-bound fraction is the most abundant (Civello et al., 1995).

These changes in the peroxidase activity were also accompanied by the variations that the different isoenzymes undergo throughout the fruit maturation process. Thus, the acidic peroxidases increased during this process whereas the basic isoenzymes, whose highest levels were attained in the first stage, continued to decrease until they almost disappeared in the fifth

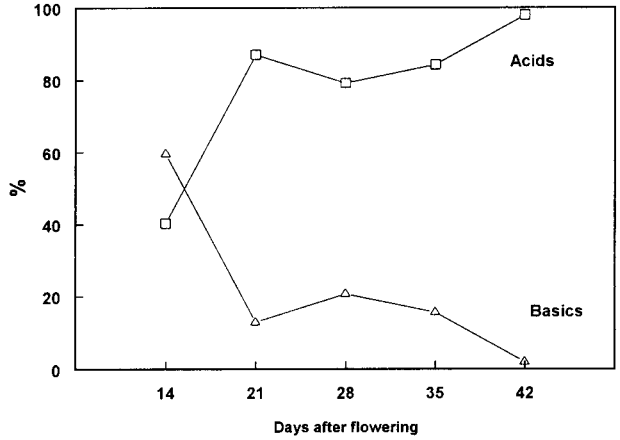

Figure 3. Densitometric quantification of basic and acidic peroxidase isoenzymes of pepper fruits cv. Padrón at different growth stages (expressed in percentage).

stage. Densitometric analysis made it possible to quantify the variation in the different isoenzymes: acidics and basics. This variation is presented in percentage in Figure 3. These results suggest that during the fruit ripening process a differential expression of peroxidase isoenzyme groups took place. A comparison of the results obtained by Biles et al. (1997) in the chile pepper fruit, showed that there was an increase in acidic peroxidases which is similar to what we have observed here, but they did not detect the basic peroxidases. This last difference may be due to the fact that they studied a different cultivar.

It is important to gather information on the temporal and quantitative changes in the activities of peroxidase isoenzymes during fruit maturation, because the two main forms present in plants, the basic and the acidic peroxidases, exhibit different substrate specificity and also play different physiological roles. Thus, basic peroxidase was found to be most effective in IAA catabolism (Zheng & Van Huystee, 1992), and the acidic isoforms are believed to be associated with lignification (Ros Barceló, 1997; Ros Barceló et al., 1998). However, peroxidases have recently been considered to play a role in the metabolism of alkaloids. Basic peroxidases of a high pI may be directly related to capsaicinoid metabolism because both capsaicin and dihydrocapsaicin, and their phenolic precursors, are easily oxidized by this enzyme (Bernal et al., 1993a and b; Bernal et al., 1995). This basic peroxidase (Bernal et al., 1993b), like capsaicin (Suzuki et al., 1980; Fujiwake et al., 1980), is located in the vacuole of the placental tissue of pepper fruits. On the other hand, the decrease observed in the basic peroxidases could be related to the increase in the capsaicinoid level throughout ripening. The capsaicinoids are phenolic compounds, and some authors point to an inverse relationship between the level of phenolic compounds and the activity of basic peroxidases (Gaspar et al., 1985). In the pepper fruit cv. Padrón, we observed the highest level of capsaicinoids at the end of the maturation process. This was produced by an increase in their synthesis rate and also by a decrease in the degradation levels, because there is a drop in the activity of the isoenzymes involved in oxidation which are the basic ones.

The ability of the acidic peroxidase to oxidize the capsaicin has recently been demonstrated in vitro by Pomar et al. (1997). The results obtained with this purified peroxidase suggest that capsaicin is a good

substrate for this isoenzyme. Because the lignin level does not increase in pepper fruit throughout maturation this would suggest that, although the percentage of acidic peroxidases increases over the course of growth, the increment of these isoenzymes would not be related to lignification processes as has been suggested by other researchers (Abeles and Biles, 1991). Moreover, although it might be considered that the primary role of acidic peroxidase is to participate in capsaicin metabolism, because of the increase in the expression of the acidic isoenzymes over development, these isoenzymes in the former stages of maturation are not able to oxidize the capsaicin, as they are both found in different subcellular compartments. Only in the last stage, when the loss of cellular compartmentalization occurs, could the acidic isoenzymes participate in the capsaicin catabolism. In conclusion, we report the correlation between the pattern of accumulation of capsaicin and the activity of the different peroxidase isoenzymes. Thus, even though the acidic peroxidase could be involved in the metabolism of the capsaicin in the last stage, it is the basic isoenzymes which are indeed involved in that process, because when they disappear the capsaicin content undergoes its highest accumulation.

ACKNOWLEDGMENT

The authors are grateful to Mrs. M. Lema Grillé (S.X.A.I.N., University of A Coruña) for technical assistance.

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Received for review February 11, 2000. Revised manuscript received September 5, 2000. Accepted September 5, 2000. This work was supported by XUGA 10301/A/95.

JF000190X