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Characterization of endoproteases from plant peroxisomes

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In this work, the characterization of endoprotease (EP) isoenzymes in peroxisomes is reported for the first time in cell organelles purified from pea leaves (*Pisum sativum* L.). A comparative analysis of the endo-proteolytic activity in peroxisomes purified from young (15-day-old) and senescent (50-day-old) leaves was carried out. Peroxisomes purified from senescent leaves showed a much higher endo-proteolytic activity than organelles from young plants. A 16 h incubation with exogenous substrates was the threshold time for the detection of a linear increase in the endo-proteolytic activity of peroxisomes from senescent leaves. Three EP isoenzymes (EP2, EP4 and EP5), having molecular masses of 88, 64 and 50 kDa respectively, were found in young plants by using SDS/polyacrylamide-gradient gels co-polymerized with gelatin. However, four additional isoenzymes (EP1, EP3, EP6 and EP7), with molecular masses of 220, 76, 46 and 34 kDa respectively, were detected in senescent plants. All the isoenzymes detected in peroxisomes from both young and senescent leaves were neutral proteases. By using different class-specific inhibitors, the electrophoretically separated EP isoenzymes were characterized as three serine-proteinases (EP1, EP3 and EP4), two cysteine-proteinases (EP2 and EP6) and a metallo-proteinase (EP7), and EP5 might be a metal-dependent serine-proteinase. Moreover, a peroxisomal polypeptide of 64 kDa was recognized by an antibody against a thiol-protease. The serine-proteinase isoenzymes (EP1, EP3 and EP4), which represent approx. 70% of the total EP activity of peroxisomes, showed a notable thermal stability, not being inhibited by incubation at 50 °C for 1 h.

INTRODUCTION

Plant senescence is a natural process that is genetically regulated, and in the course of which a wide variety of important changes in plant metabolism take place [1,2]. Senescence is mainly characterized by an intensive loss of proteins and chlorophyll, as well as severe increases in lipid peroxidation due to a notable enhancement in the metabolism of activated oxygen [3–6].

Proteolysis is a fundamental process during the whole life of the plant and its importance increases during senescence, when the nitrogen reserves of proteins are mobilized and translocated into the reproductive parts like seeds and fruits. There are many reports on the enhancement of proteolytic activity during plant senescence, measured either by using exogenous substrates or by following the degradation of endogenous Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) [7–10].

Originally, only vacuolar proteases were thought to be responsible for protein degradation in plant tissues [11,12]. However, the presence of proteolytic activity has also been demonstrated in other cell compartments, like chloroplasts [7,13,14], cell wall [15,16], microsomes [17], mitochondria [18], cytosol [14] and the Golgi apparatus [19].

In non-photosynthetic organisms, the occurrence of protease activity in peroxisomes has only been reported in organelles purified from rat liver [20,21]. Recently, the presence of proteolytic activity in plant peroxisomes has been demonstrated for the first time [18]. Exo- and endo-proteolytic activity was detected in peroxisomes from pea leaves and an exo-peptidase was characterized as a leucine aminopeptidase, belonging to the family of the serine-proteinases [18].

Peroxisomes are subcellular respiratory organelles, containing as basic enzymic constituents, catalase and H_2O_2-producing flavin oxidases [22,23]. These organelles have an essentially oxidative type of metabolism, and in recent years it has become increasingly clear that peroxisomes carry out essential functions in almost all eukaryotic cells [24–27]. Recently, an activated oxygen-mediated role for peroxisomes in the oxidative mechanism of natural and induced leaf senescence has been proposed [28,29].

In this paper, we report the characterization of the endoprotease (EP) activity of plant peroxisomes in organelles purified from *Pisum sativum* L. leaves, and a comparative analysis of the EP isoenzyme activity in peroxisomes from young and senescent leaves was carried out.

EXPERIMENTAL

Plant material

Pea seeds (*Pisum sativum* L., cv. Lincoln), purchased from Royal Sluis (Enkhuizen, Holland), were sown and grown in vermiculite for 14 days under greenhouse conditions (28–18 °C, day–night temperature; 80% relative humidity). The most healthy seedlings were selected and grown in aerated optimum-nutrient solutions [30] for 15 days (young plants) and 50 days (senescent plants) in the same greenhouse conditions indicated above.

Purification of peroxisomal fractions

All operations were carried out at 0–4 °C. Peroxisomes were isolated from pea leaves by two differential centrifugations and the final 12000 ***g*** particulate pellets, enriched in peroxisomes and

Abbreviations used: DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EP, endoprotease; *p*HMB, *p*-hydroxymercuribenzoate.

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mitochondria, were subjected to discontinuous sucrose density-gradient centrifugation (35–60 %, w/w), as described by López-Huertas et al. [31]. After centrifugation, gradients were eluted with a gradient fractionator (model 185; Isco, Lincoln, NE, U.S.A.) equipped with an optical unit and an absorbance detector. Catalase was used as the marker enzyme for peroxisomes [32]. The purified peroxisomes had intactness percentages of almost 90 % [31].

In order to assess possible contaminations of peroxisomes by other organelles, the gradient fractions were assayed for fumarase, cytochrome *c* reductase and acid phosphatase activities as marker enzymes for mitochondria, endoplasmic reticulum and vacuoles respectively [33–35], and an antibody against photosynthetic fructose-1,6-bisphosphatase [36] was used to localize chloroplastic fractions in the gradient.

To obtain peroxisomal soluble fractions (matrices), purified peroxisomes from several gradients were recovered by puncture with a syringe and were broken by hypotonic shock in 100 mM potassium phosphate buffer (pH 7.8)/1 mM EDTA. The suspensions were centrifuged at 120000 ***g*** for 30 min [31], and peroxisomal supernatants were then concentrated by ultrafiltration with a PM-10 membrane (Amicon).

Protein content was determined using a Bio-Rad protein assay reagent, with BSA as protein standard [37], and the density of the gradient fractions was calculated from the refractive index of the fractions, which was measured at room temperature using an Atago refractometer.

Determination of EP activity

EP activity was determined spectrophotometrically using azocasein as substrate [38]. Peroxisomal matrices from young and senescent plants were incubated at 37 °C with 0.15 % (w/v) azocasein in 0.1 M Tris/HCl, pH 8.5, for 2, 4, 8, 12, 14, 16, 20, 24 and 28 h. The excess of substrate was then precipitated on ice with 12 % (w/v) trichloroacetic acid and the absorbance at 340 nm of supernatants (10000 ***g*** for 10 min) was measured [39]. One unit of activity was defined as the amount of enzyme needed to cause a 0.01 increase in $A_{340\ nm}$ per 2 h [38].

Detection of EP isoenzymes

In order to detect EP activity in gels, electrophoretic separations of samples on SDS/polyacrylamide-gradient gels (6–12 %, w/v) containing 0.05 % (w/v) gelatin were carried out [40]. Before electrophoresis, peroxisomal matrices from young and senescent plants were incubated at 37 °C for 0, 3, 8 and 16 h. After electrophoresis, gels were transferred to a 2.5 % (v/v) Triton X-100 aqueous solution for 1 h at room temperature, to allow removal of SDS and renaturation of proteinases, and then were incubated with 250 mM Tris/HCl, pH 7.5, at 37 °C for 4 h. After incubation, gels were stained with 0.1 % (w/v) Coomassie Brilliant Blue R-250 in 50 % (v/v) methanol and 10 % (v/v) acetic acid for 1 h and finally destained with 40 % (v/v) methanol and 10 % (v/v) acetic acid. EP activity was detected on a dark-blue background as colourless bands, which were quantified by scanning gels on a Shimadzu CS-9000 densitometer. Other organellar fractions (mitochondria and chloroplasts plus vacuoles and endoplasmic reticulum), obtained from the density-gradients were also analysed by following the same procedure. Molecular masses of the EP isoenzymes were estimated by SDS/PAGE under the same conditions to detect the EP activity mentioned above [40] and using the Bio-Rad broad range of molecular mass markers.

Characterization of EP isoenzymes

In order to identify the different EP isoenzymes separated on SDS/gelatin gels, class-specific proteinase inhibitors were used [41,42]. Peroxisomal matrices were incubated, before electrophoresis, for 1 h on ice, in the presence of each of the following inhibitors: 2 mM PMSF, 20 mM EDTA, 2 mM *o*-phenanthroline, 30 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), 100 μM *p*-hydroxymercuribenzoate (*p*HMB), 100 μM leupeptin, 100 μM trypsin inhibitor, 50 μM aprotinin and 150 μM bestatin. After electrophoresis and Triton X-100 equilibration, gels were again incubated with each respective inhibitor in 250 mM Tris/HCl, pH 7.5, at 37 °C for 4 h. In the case of PMSF, which was prepared in a 50 % ethanol/50 % acetone solution, a separate control was used consisting of the incubation of the gel in the presence of the organic solvent but without the inhibitor.

To study the effect of reducing agents, as well as of some metal ions, on EP isoenzymes, gels were also incubated separately, after the electrophoresis and Triton X-100 equilibration, with 4 mM cysteine/5 mM 2-mercaptoethanol/8 mM dithiothreitol (DTT)/20 mM $CaCl_2$/20 mM $ZnCl_2$.

To detect the possible presence of aspartic proteinase activity, a slight modification of the gel staining was carried out, consisting of the incubation of the gel, after the electrophoresis, with 50 mM sodium acetate, pH 3.8, at 37 °C for 3 h, instead of 250 mM Tris/HCl, pH 7.5.

A preliminary study of the thermal stability of the EP isoenzymes was carried out. For this purpose, after the electrophoresis, gels were incubated at 50 °C for 1 h and then treated according to the procedure described above.

Western blotting

SDS/PAGE of samples was performed on either 10 % or 12 % acrylamide gels according to Laemmli [43], and the separated proteins were transferred to PVDF membranes (Immobilon P transfer Membranes), using a Bio-Rad Semi-Dry Transfer Cell. Blots were blocked with 1.5 % (w/v) fat-free milk powder in PBS, followed by incubation with antisera. Antibodies against a thiol-protease expressed in senescent ovaries of *P. sativum* (D. Orzaez, R. Blay and A. Granell, unpublished work), a cysteine-proteinase purified from germinating barley [45] and a thiol-endopeptidase from *Vigna mungo* cotyledons [46] were used. Goat anti-(rabbit IgG) with horseradish peroxidase (Bio-Rad) was used as the second antibody and membranes were stained with 4-chloro-1-naphthol.

RESULTS

The isolation of intact peroxisomes from both young and senescent leaves by sucrose density-gradient centrifugation is shown in Figure 1. In both kinds of plants a similar pattern for each marker enzyme was observed, although catalase and fumarase activities were lower in senescent plants compared with young plants. In both cases, as deduced from the catalase activity peak, peroxisomes banded in fractions 18–22 at an average equilibrium density of 1.24 $g \cdot cm^{-3}$, characteristic for these intact organelles in sucrose solutions [23,31]. No marker enzymes of other cell compartments were detected in the peroxisomal peaks from young and senescent leaves and this indicates the absence of cross-contamination by mitochondria, endoplasmic reticulum, vacuoles and chloroplasts.

The endo-proteolytic activity in peroxisomal matrices from young and senescent leaves, determined with azocasein at dif-

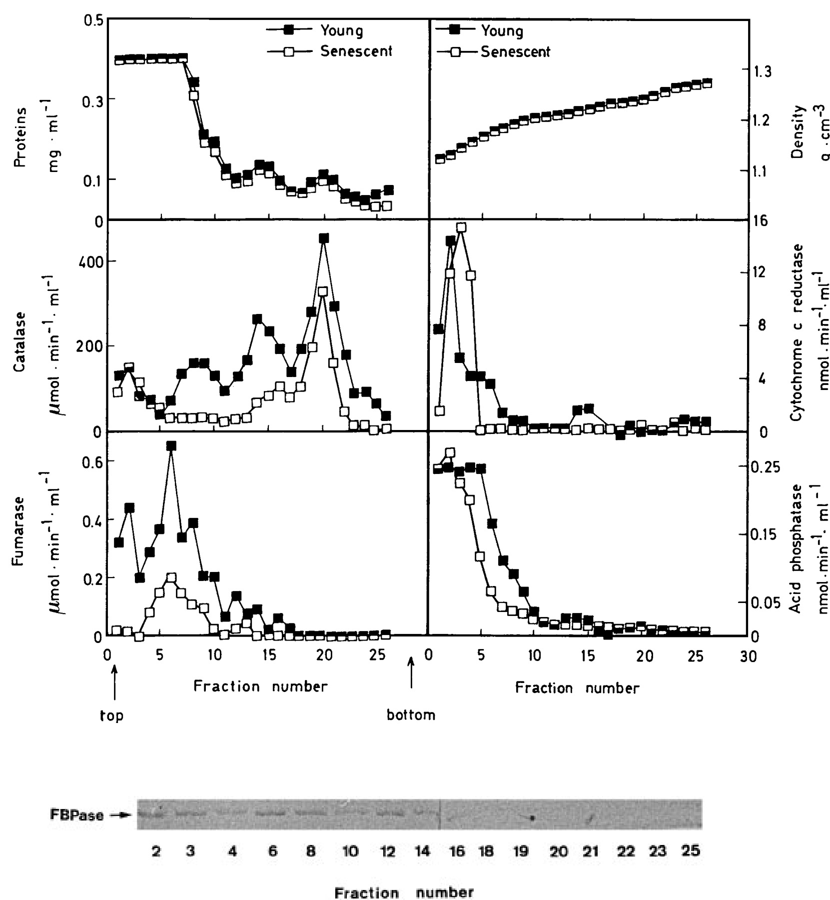

Figure 1 Purification of peroxisomes from young and senescent leaves of *P. sativum* L.

Organelles were purified by differential and sucrose density-gradient centrifugations, as described by López-Huertas et al. [31]. Gradient fractions (1.5 ml) were assayed for specific marker enzymes to localize cell organelles in the gradient: fumarase for mitochondria, cytochrome *c* reductase for endoplasmic reticulum, acid phosphatase for vacuoles and fructose-1,6-bisphosphatase (FBPase) for chloroplasts. The activity of all marker enzymes was determined spectrophotometrically, as indicated in the Experimental section, except for FBPase, the presence of which was detected by Western blotting (1/1000 dilution). A similar pattern of FBPase recognition was obtained in fractions eluted from young and senescent leaves.

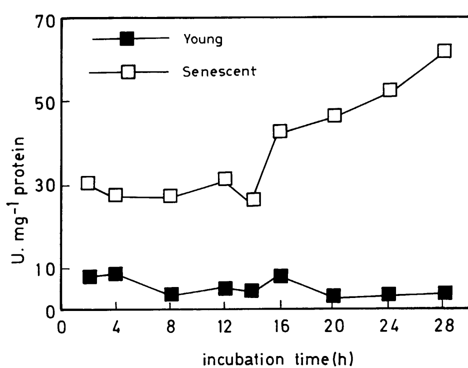

Figure 2 Endo-proteolytic activity (units · mg^{-1} of protein) of peroxisomal matrices purified from young and senescent leaves of *Pisum sativum* L.

The assay was carried out at different incubation times using azocasein as substrate.

ferent incubation times, is shown in Figure 2. Under our assay conditions, a low EP activity of approx. 10 units · mg^{-1} of protein was detected in peroxisomal matrices from young leaves at all the incubation times. By contrast, a 3-fold higher endo-proteolytic activity was observed in senescent plants, compared with young ones, at incubation times of less than 14 h. And for longer incubation times, a remarkable linear increase in the EP activity of senescent leaves was determined. This increase was maintained after 28 h of incubation, when a value of approx. 65 units · mg^{-1} of protein was obtained.

These data are consistent with those obtained when the endo-proteolytic activity of peroxisomal matrices from young and senescent plants was analysed by SDS/PAGE in the presence of gelatin. In young leaves, after 16 h incubation of peroxisomal matrices at 37 °C, only three EP isoenzymes were detected (Figure 3, lane A, marked with arrowheads). The three iso-enzymes were named EP2, EP4 and EP5 and had molecular masses of 88, 64 and 50 kDa respectively. On the other hand, in senescent leaves, besides the isoenzymes found in young leaves, four new EP isoenzymes were observed: EP1, EP3, EP6 and EP7, with molecular masses of 220, 76, 46 and 34 kDa respectively (Figure 3, lane B). When the peroxisomal matrices from young and senescent plants were incubated at 37 °C for 0, 3 and 8 h and then loaded on to SDS gels containing gelatin, a much lower endo-proteolytic activity was observed and only EP5 isoenzyme could be detected (results not shown).

A similar study was carried out with other cellular fractions obtained in the purification of peroxisomes. Samples of chloro-

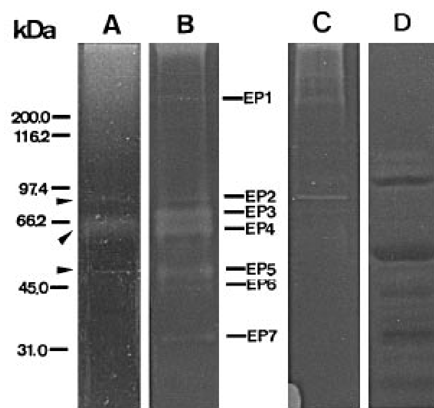

Figure 3 EP isoenzyme pattern in cell organelles from leaves of *P. sativum* L.

Endo-proteolytic activity was determined in gelatin-containing SDS/polyacrylamide-gradient gels (6–12%). Subcellular fractions were incubated at 37 °C for 16 h before electrophoresis. Each lane contained 15 μg of protein. Lane A, peroxisomal matrices from young leaves; lane B, peroxisomal matrices from senescent leaves; lane C, mitochondria from senescent leaves; lane D, chloroplast plus vacuoles from senescent leaves. Molecular mass markers are indicated on the left and positions of EPs on the right.

plasts plus vacuoles and mitochondria were obtained by pooling the gradient fractions with the highest activities of their respective marker enzymes. As shown in Figure 3, a different pattern of EP isoenzymes to that described for peroxisomes (lanes A and B) was found for mitochondria (lane C) and chloroplasts plus vacuoles (lane D).

The effect of different class-specific protease inhibitors on the electrophoretically separated EP isoenzymes was studied at pH 7.5 and the percentage of each isoenzyme was estimated by densitometry (Table 1). Results show that the most effective inhibitor was PMSF, a plant serine-proteinase inhibitor that completely abolished EP1, EP3 and EP4 activities. However, other serine-proteinase inhibitors, such as leupeptin and trypsin inhibitor, only depressed the activity of EP1. E-64 and *p*HMB, potent inhibitors of cysteine-proteinases, caused a high degree of inactivation of EP2 and EP6, the latter isoenzyme also being strongly inhibited by EDTA. Finally, EP7 was completely inactivated by EDTA and, apparently, except for PMSF and EDTA, none of the other inhibitors had any effect on the EP5 activity (Table 1).

The effect of reducing agents (DTT, mercaptoethanol and cysteine) and metal ions (Ca^{2+}, Zn^{2+}) on the EP isoenzymes was also studied, and results indicated that none of the isoenzyme activities was significantly affected by these compounds (Table 1). The absence of any endo-proteolytic band on gels incubated at pH 3.8 ruled out the occurrence of acid EPs in peroxisomes (Figure 4, lane A).

In order to test the thermal sensitivity of the peroxisomal EP isoenzymes, the SDS/polyacrylamide-gradient gels were incubated at 50 °C for 1 h. Under these conditions, a complete loss of EP2, EP5, EP6 and EP7 activities was obtained (Figure 4, lane B). By contrast, EP1, EP3 and EP4 were not modified by this treatment, suggesting a considerable stability of these three isoenzymes to high temperature (Figure 4, lane B).

Finally, to get deeper insights into the properties of the peroxisomal EP isoenzymes, Western blot assays were conducted with a specific antibody against a plant thiol-protease (D. Orzaez, R. Blay and A. Grannel, unpublished work). In peroxisomal

Table 1 Effect of class-specific protease inhibitors and activators on the electrophoretically separated EP isoenzymes from peroxisomes of senescent *P. sativum* L. leaves

Incubations with inhibitors and activators were performed as indicated in the text. The densitometric scanning of gel lanes was carried out and the percentage of remaining activity of each isoenzyme was calculated by comparison with the activity values obtained for the control lanes (100% activity). Values are means of at least three independent preparations of peroxisomes.

	Remaining activity (%)						
Isoenzymes ...	EP1	EP2	EP3	EP4	EP5	EP6	EP7
Inhibitors (mM)							
Control	100	100	100	100	100	100	100
PMSF (2)	0	216	0	0	31	85	100
EDTA (20)	13	103	169	38	60	29	0
o-Phenanthroline (2)	97	98	103	104	95	99	98
E-64 (30×10^{-3})	95	29	103	107	98	26	101
*p*HMB (0.1)	18	38	82	81	107	15	137
Leupeptin (0.1)	20	85	93	93	100	101	162
Trypsin inhibitor (0.1)	63	97	85	90	104	103	114
Aprotinin (5×10^{-3})	103	95	89	91	108	99	99
Bestatin (0.15)	98	104	100	98	98	102	100
Activators (mM)							
Cysteine (4)	97	100	95	104	94	99	98
Mercaptoethanol (5)	90	106	103	98	110	109	104
DTT (8)	91	107	105	107	100	100	91
Ca^{2+} (20)	98	87	94	97	101	101	93
Zn^{2+} (20)	103	94	100	108	99	102	106

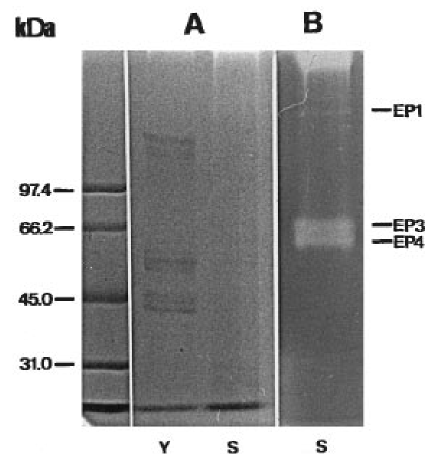

Figure 4 Effect of acidic pH and high temperature on the EP isoenzymes of peroxisomes from young and senescent pea leaves

Before electrophoresis, peroxisomal matrices (15 μg) were incubated at 37 °C for 16 h. Electrophoresis was carried out in SDS/polyacrylamide-gradient gels (6–12%) containing gelatin. After electrophoresis, gels were subjected to acid pH and high temperature and then were stained for EP activity as described in the Experimental section. Lane A, gel incubated in 50 mM sodium acetate, pH 3.8, at 37 °C for 3 h; lane B, gel incubated at 50 °C for 1 h. Molecular mass markers are indicated on the left and positions of EPs on the right. Y, peroxisomes from young plants; S, peroxisomes from senescent plants.

matrices from senescent plants, two polypeptide bands having molecular masses of 64 and 62.4 kDa were recognized by the antibody against the thiol-protease of *P. sativum* L. This antibody also cross-reacted with two mitochondrial polypeptides whose

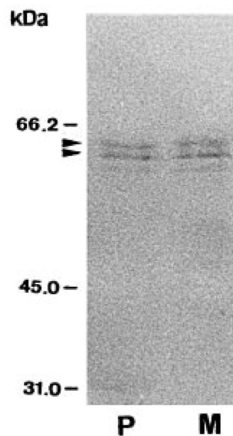

Figure 5 Western blotting of peroxisomes and mitochondria from senescent leaves of *P. sativum* L. with an antibody against a thiol-protease from pea senescent ovaries

Samples (15 μg) were run on large 10% acrylamide gels (20 cm × 20 cm) in a Protean II system (Bio-Rad Laboratories). A 1/200 dilution of the antibody was used. P, peroxisomes; M, mitochondria. Molecular mass markers are indicated on the left.

molecular masses were similar to those of the peroxisomal polypeptides (Figure 5).

DISCUSSION

In this work, the endo-proteolytic activity present in plant peroxisomes was characterized for the first time and the effect of senescence on the pattern of the peroxisomal EP isoenzymes was also reported. The presence of endo-proteolytic activity in peroxisomes was previously described in rat hepatocytes, although no EP isoenzymes were identified [20,21]. More recently, a peroxisomal protease from fibroblasts involved in the degradation of the cleaved leader peptide of thiolase has been reported [47].

Peroxisomes purified from pea leaves were free of contamination by mitochondria, endoplasmic reticulum, vacuoles and chloroplasts, as demonstrated by assays with different marker enzymes (Figure 1) and by the distinct pattern of EP isoenzymes in different organellar fractions (Figure 3). In peroxisomes purified from senescent pea leaves, decreases of approx. 45% and 65% for catalase and fumarase activities, respectively, two marker enzymes of peroxisomes and mitochondria, were obtained. These results were similar to those recently reported in senescent pea leaves by Pastori and del Río [29].

Results obtained from the incubation of peroxisomal matrices with azocasein for 2–14 h showed a much higher activity in senescent leaves than in young leaves, and this difference was exacerbated with increasing incubation times up to 28 h. This allowed us to estimate 14 h as the 'latency time' for peroxisomal EPs in senescent leaves (Figure 2), and 16 h was selected as the standard incubation time for the detection of endo-proteolytic activity in gelatin-containing SDS gels. The detection of proteolytic activity in gelatin-containing gels after SDS/PAGE has been broadly used in the study of plant EPs [42,48–50]. In this technique, samples are mildly treated, since they are not heated at 100 °C in the presence of either 2-mercaptoethanol or DTT, before electrophoresis. On the other hand, the incubation of gels, after electrophoresis, with the non-ionic detergent Triton X-100 removes SDS from gels and restores enzyme activity [40,42]. This

Table 2 Properties of EP isoenzymes from leaf peroxisomes of *P. sativum* L. plants

+, present; −, absent.

Isoenzyme	Age of plant		Molecular mass (kDa)	Type of EP isoenzyme
	Young	Senescent		
EP1	−	+	220	Serine-proteinase
EP2	+	+	88	Cysteine-proteinase
EP3	−	+	76	Serine-proteinase
EP4	+	+	64	Serine-proteinase
EP5	+	+	50	?
EP6	−	+	46	Cysteine-proteinase
EP7	−	+	34	Metallo-proteinase

method also allows the estimation of the size of EPs by comparison *in situ* with molecular mass standards, since both markers and samples are run and stained in the same gel.

To study and characterize the peroxisomal EP isoenzymes in SDS/gelatin gels, we followed a modification consisting of the incubation of samples at 37 °C before electrophoresis. In all previous reports on the detection of EP isoenzymes from different sources, the incubations to develop the endo-proteolytic activity were carried out in gels after electrophoresis [48,49]. However, our method rendered sharper proteolytic activity bands and provided clearer and more reliable results. Therefore since similar activity patterns were achieved, either by incubating samples before electrophoresis, or incubating gels after electrophoresis, the possibility that EP isoenzymes might be self-degraded in the incubated samples should be ruled out.

In peroxisomes from senescent leaves, seven EP isoenzymes were detected (EP1–EP7). In young plants only three isoenzymes were present (EP2, EP4 and EP5), which had identical molecular masses to EP2, EP4 and EP5 respectively from senescent tissues. Peroxisomal EP1 and EP2 also showed similar mobility to the EPs observed in the mitochondrial fraction from senescent leaves (Figure 3, lanes B and C respectively). However, considering the intensity of the detected bands in both organelles, and the profile of the marker enzymes for peroxisomes and mitochondria (Figure 1), these results can be explained by assuming that the two isoenzymes are located in both organelles, rather than to a cross-contamination. A similar pattern was also obtained when samples were directly loaded on to gels and, after electrophoresis, gels were incubated for 16 h at 37 °C (results not shown). All the isoenzymes detected in both young and senescent leaves were neutral, since they were visualized in gels incubated at pH 7.5 (Figure 3) and no isoenzymes could be observed by incubating gels at an acidic pH (Figure 4, lane A). These results indicated that no aspartic-proteinases were present in the peroxisomal matrices, and this agrees with previous data showing the occurrence of neutral endo-proteolytic activity in peroxisomes from rat liver [20,21].

Incubations of gels in the presence of class-specific protease inhibitors and several cofactors (reducing agents and metal ions) were performed in order to characterize the EP isoenzymes from pea-leaf peroxisomes and the conclusions obtained are presented in Table 2. EP1, EP3 and EP4 were considered as serine-proteinases [41,42,51] and EP1 and EP3 were only detected in senescent leaves. None of these isoenzymes belonged to the trypsin superfamily [49], as deduced from the data obtained in gels incubated with trypsin inhibitor, leupeptin and aprotinin (Table 1). However, EP1 and EP4 were inhibited by EDTA, and this

indicates that some metal ions might be also involved in the enzymic activity of these isoenzymes. Two peroxisomal polypeptide bands of 64 and 62.4 kDa were recognized by an antibody against a thiol-protease from senescent ovaries of *P. sativum*. The 64 kDa cross-reactive band had the same molecular mass as EP4, which indicates that this isoenzyme might be a thiol-containing serine-protease. In the mitochondrial fraction of senescent leaves two polypeptides were also recognized by the same antibody and they had similar molecular masses to those polypeptides observed in peroxisomes. However, data from densitometric analysis of Western blot membranes showed similar amounts of both polypeptides in each organelle (results not shown), and this, joined to the profile of catalase and fumarase activities in the density-gradients (Figure 1), supports the idea that both polypeptides are localized in peroxisomes and mitochondria.

EP2 and EP6, on the basis of their sensitivity to most of the class-specific protease inhibitors, were assigned to the cysteine-proteinase type [41,42,51], although they were not completely inhibited by E-64 and *p*HMB, two potent inhibitors of many cysteine-proteinases (Table 1). On the other hand, PMSF strongly enhanced the EP2 activity, whereas EP6 was inhibited by EDTA. This suggests that isoenzyme EP6, exclusively detected in senescent tissue, could perhaps need some metal ion as cofactor. When the activity stains were performed in gels previously incubated with reducing agents (cysteine, mercaptoethanol and DTT), which act by stimulating the activity of many cysteine-proteinases, no effect of these compounds on EP2 and EP6 was observed. Cross-reactivity assays of EP2 and EP6 with antibodies against cysteine-proteinases from other plant sources were carried out. An antibody against a 30 kDa cysteine-proteinase from germinating barley [45], and another one against a thiol endopeptidase from germinating *V. mungo* [46], were used. Nevertheless, these antibodies did not recognize the peroxisomal EPs.

The activity of EP7 was totally abolished by EDTA, which led us to think that this isoenzyme was a metallo-proteinase, although neither was affected by *o*-phenanthroline, another specific inhibitor of metallo-proteinases, nor was it increased by Ca^{2+} and Zn^{2+}, as would be expected for a metallo-proteinase [41]. Finally, EP5 did not show any typical behaviour against the inhibitors used. Only PMSF and EDTA partially inhibited its activity. This isoenzyme might be a metal-dependent serine-proteinase, although its final identity is still to be confirmed.

The effect of high temperature on the EP isoenzymes showed that EP2, EP5, EP6 and EP7 activities were abolished at 50 °C, whereas the remaining isoenzymes were resistant to this temperature. EP1, EP3 and EP4 are serine-proteinases and these results suggest that thermal stability could be a specific property of this type of peroxisomal proteases. Recently, a 33 kDa serine-proteinase from the filamentous fungus *Scedosporium apiospermum*, showing optimum activity at 37–50 °C, has been reported [52].

In plants, most endo-proteolytic isoenzymes reported so far are of the cysteine- or aspartic-proteinase type, and they have mainly been characterized in seeds and in germinating tissues, where they are involved in the mobilization of storage proteins [18,46,48–50,53,54]. Nevertheless, much less is known on plant serine-proteinases and their expression in specific developmental stages. Isoenzymes of serine-proteinases have been found in resting mature seeds [55], germinating and post-germinating tissues [52,56] and mature plants [57]. In peroxisomes from pea leaves, serine-proteinases, mainly EP3 and EP4, represent approx. 70 % of the total EP activity of the organelle, and they may have an important role during senescence, when both isoenzymes are present. In very recent experiments carried out in our laboratory with senescent leaves, an overall decrease in the number of polypeptides of peroxisomal matrices was observed when these samples were incubated at 37 °C for 16 h. This self-proteolytic effect was abolished when the incubation was performed in the presence of PMSF. These results suggest a role for these EPs in the protein turnover of peroxisomes of senescent tissues. The purification of serine-proteinases from pea leaf peroxisomes is under way in our laboratory in order to study their possible role in oxyradical-derived proteolysis of peroxisomal proteins.

Note added in proof (received 4 September 1997).

After the submission of this article, a cysteine endoprotease has also been demonstrated in castor bean glyoxysomes [58].

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