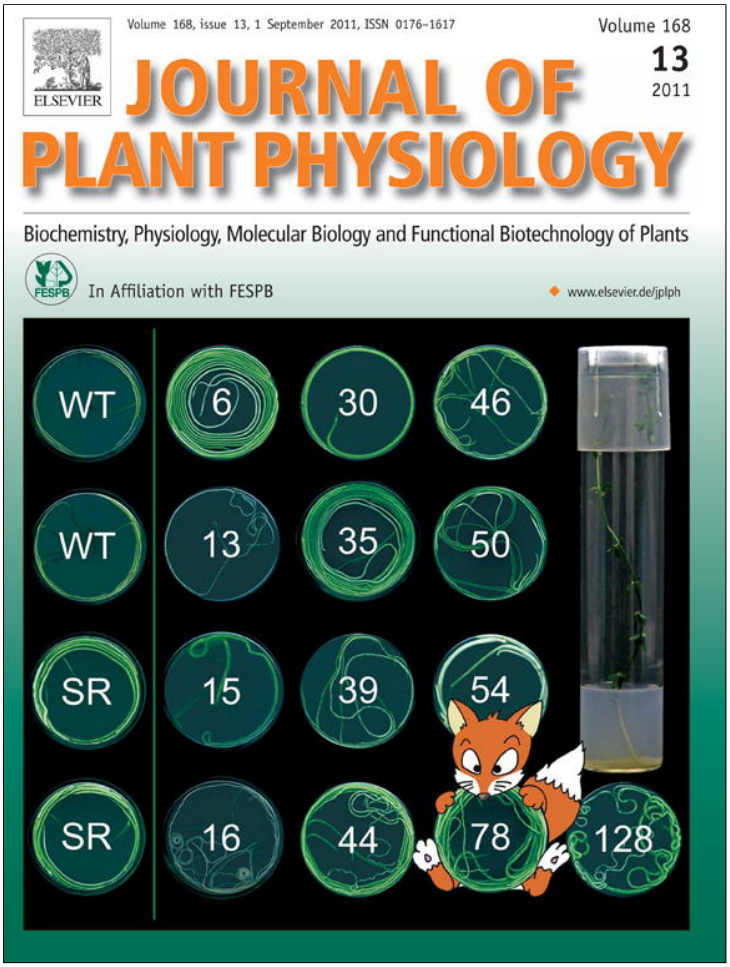

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2-D zymographic analysis of Broccoli (*Brassica oleracea* L. var. Italica) florets proteases: Follow up of cysteine protease isotypes in the course of post-harvest senescence

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ABSTRACT

Zymographic analysis of Broccoli florets (*Brassica oleracea* L. var. Italica) revealed the presence of acidic metallo-proteases, serine proteases and cysteine proteases. Under conditions which were denaturing for the other proteases, the study was restricted to cysteine proteases. 2-D zymography, a technique that combines IEF and zymography was used to show the presence of 11 different cysteine protease spots with molecular mass of 44 and 47–48 kDa and pIs ranging between 4.1 and 4.7. pI differences could be ascribed to different degrees of phosphorylation that partly disappeared in the presence of alkaline phosphatase. Post-harvest senescence of Broccoli florets was characterized by decrease in protein and chlorophyll contents and increase of protease activity. In particular, as determined by 2-D zymography, the presence of cysteine protease clearly increased during senescence, a finding that may represent a useful tool for the control of the aging process.

Introduction

Broccoli (*Brassica oleracea* L. var. Italica) is an important vegetable of high nutritional value and a common component of human diet. In recent years, due to the health benefits, broccoli consumption is strongly increased. Broccoli is rich in dietary fibers, minerals, anti-oxidants, phyto-chemicals and vitamins, – in particular flavonoids, glucosinolates and vitamin C – playing important roles in disease prevention and health maintenance (Ames et al., 1993; Rice-Evans et al., 1994; Stoner and Morse, 1997; Kushad et al., 1999).

Broccoli presents green floral heads composed of hundreds of florets organized in whorls on a fleshy stem. After harvesting, broccoli quality is greatly reduced due to chlorophyll degradation and the rapid yellowing of floral heads (Matile et al., 1999; Costa et al., 2005). Plant senescence is a natural complex process involving the degradation of both chlorophyll and macromolecules – such as proteins, lipids, nucleic acids – and the dismantling of the cellular architecture. The degradation of macromolecules during senescence contributes to the mobilization and the recycling of nutrients to still active growing parts of the plants (Smart, 1994; Buchanan-Wollaston, 1997). In harvested broccoli, senescence is induced artificially by several stress responses due to the removal of nutrient supplies.

In the course of plant senescence, proteolysis of chloroplast proteins and degradation of chlorophylls are the major biochemical changes (King and Morris, 1994; Valpuesta et al., 1995; Wagstaff et al., 2002), and in Broccoli florets chlorophyll and protein contents decline while proteolytic activity increases (Page et al., 2001; Coupe et al., 2003).

Proteases play an important role in the mobilization of amino acids out of dying cells into active regions of the plants during senescence. Similar to the role of caspases in animal cell death, a specific protease may act either as a mediator of signal transduction or as an effector of cell death during plant senescence (Beers et al., 2000). In the course of senescence, proteases are up regulated and several proteases, in particular cysteine proteases, have been shown to be involved in the degradation of proteins (Stephenson and Rubinstein, 1998; Granell et al., 1998).

Nine cDNAs of cysteine proteases were identified in senescent Broccoli floret tissues: (SAG12, LSC7, LSC790 and LSC833) (Page et al., 2001) (BoCP1, BoCP2, BoCP3, BoCP4) (Coupe et al., 2003) and BoCP5 (Eason et al., 2005). The first four BoCPs described by Coupe et al. (2003) are the dehydration responsive cysteine proteases, while BoCP5 is very similar to the aleurain-like protease. Four additional senescence-associated cysteine endoproteases (EP2, EP4, EP5 and EP7) have been described by Wang et al. (2004).

The aim of this study was the analysis of Broccoli's proteases and in particular of cysteine proteases since they play an important role

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during senescence and could represent a useful biochemical marker of senescence.

To get more insight into the characteristics of Broccoli floret proteases during post-harvest senescence without the need to purify them, we applied the crude extracts of Broccoli florets to 2-D zymography (2-DZ).

In 2-DZ, proteases are separated in the first dimension according to their pI and in the second dimension according to their apparent molecular mass (Mr) in a SDS-containing polyacrylamide gel copolymerized with an appropriate protein substrate (here gelatin). Proteolytic enzymes, their isoforms and post-translational isomers present in the extract are detected as clear, unstained, spots on a dark blue background. On these grounds, 2-DZ may represent a useful technique to detect the whole pattern of proteolytic enzymes present in the sample under study. Some limitations of 2-DZ may derive from: (1) underestimation of number of proteinases due to poor degradation of substrate or to failure to reactivate the enzymes after SDS removal; (2) the irreversible enzyme inactivation by urea.

At the present, the application of 2-DZ is not very frequent. 2-DZ has been applied to investigate the proteolytic enzymes present in human pure pancreatic juice (Kaino et al., 1998), colon carcinoma (Pucci-Minafra et al., 2001), as well as in plant (Delannoy et al., 2008), bacteria (Choi et al., 2004) or marine organisms (Saitoh et al., 2007). More recently, we applied 2-DZ for the detection and direct characterization with mass spectrometry of cysteine proteases present in pineapple and kiwi fruits (Larocca et al., 2010a,b).

In the present study, 2-DZ was applied for the first time for the analysis of the cysteine proteases of Broccoli florets during post-harvest senescence. 2-DZ showed the presence of several cysteine protease isotypes with different degrees of phosphorylation. The follow up of the activities suggested that they were involved in the aging process of Broccoli florets.

Methods

Chemicals

All reagents used were of the highest grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA), Carlo Erba (Milan, Italy), Bio-Rad Laboratories (Segrate, Italy) and GE Healthcare (Uppsala, Sweden).

Broccoli: source and storage

Mature Broccoli (*Brassica oleracea* L. var. Italica) heads were harvested from a local field grown (Abriola, near the town of Potenza, Italy) and stored (within 1 h of harvest) in plastic bags at room temperature (20 °C) in the dark. At intervals after harvest (0, 1, 2, 3 and 4 days), the floret tissue was shaved from the branchlets, pooled and frozen in liquid nitrogen and stored at −70 °C. Analysis of chlorophyll and protein content, as well as protease activity, were run in triplicate (three independent experiments). Values are reported as mean (±S.D.).

Chlorophyll determination

Chlorophyll contents were assessed using a spectrophotometric method according to Porra et al. (1989). Broccoli florets (0.3 g of frozen tissue) were extracted in 10 mL of 80% (v/v) acetone. Samples were incubated for 1 h at room temperature (vortexing 30 s every 10 min) and centrifuged at 10,000 × *g*, at 4 °C for 5 min. After centrifugation, absorbance was read at 646, 664 and 750 nm with a Ultrospec 2000 spectrophotometer (Pharmacia Biotech).

Protein assay

Protein concentration was determined according to the method of Bradford (1976) and the Bio-Rad reagent using bovine albumin as standard protein. Soluble proteins were extracted as follow: 2 mL of 1 mM DTT, 1 mM EDTA in 50 mM Tris–HCl (pH 6.8) were added to 0.2 g of frozen Broccoli tissue. After incubation on ice for 30 min (vortexing 30 s every 5 min) samples were centrifuged at 10,000 × *g*, at 4 °C for 15 min and the supernatants were retained.

Assessment of total proteolytic activity in solution

Total proteolytic activity was assessed using the spectrophotometric method based on azocasein. Briefly, 50 μL of the Broccoli floret extracts (obtained as described above) were added to 0.5 mL of 0.15% (w/v) azocasein (Sigma, St. Louis, MO, USA), 2 mM DTT, 30 mM cysteine, in 40 mM phosphate buffer (pH 7.0) at 40 °C. The reaction was stopped after 4 h by the addition of 0.55 mL 6% (w/v) trichloracetic acid (TCA). After centrifugation, the supernatants were separated from the undigested substrate and the absorbance at 440 nm of the released dye was recorded. One unit of total proteolytic activity (*U*) was defined as the amount of enzyme yielding 0.01 unit of absorbance per min at 440 nm under the assay conditions. The assay included an appropriate blank, in which TCA was added before the substrate.

To determine the effects of specific protease inhibitors (Sigma) on total protease activity, the extracts were incubated with iodoacetamide (IAA) for cysteine proteases; pepstatin A for aspartic proteases; phenylmethyl sulphonyl fluoride (PMSF) for serine proteases and 1,10 phenanthroline (1,10 PA) for metallo-proteases. Stock solutions were 200 mM in water, 200 μM in methanol, 20 mM in ethanol, 200 mM in ethanol, respectively. Aliquots of 5 μL of each inhibitor stock solution were mixed separately with 45 μL of Broccoli extract containing the enzymes, incubated for 1 h at 20 °C and then added to azocasein. Final concentrations of the inhibitors were 20 mM IAA, 20 μM pepstatin, 2 mM PMSF and 20 mM 1,10 PA, respectively. Control assays contained only the solvent used to dissolve the inhibitor. Residual activity was calculated in relation to uninhibited activity.

One-way ANOVA was carried out to test for significant differences and results were considered to be statistically significant at $P < 0.05$.

1-D gel gelatin zymography (1-DZ)

Proteolytic activities in floret extracts were detected by gelatin gel zymography. Aliquots of extracts containing 10 μg of proteins were supplemented with 30 μL of electrophoresis non-reducing loading buffer: 4% (w/v) SDS, 12% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 50 mM Tris–HCl (pH 6.8). Samples were then separated under non-reducing conditions in a 12% (w/v) polyacrylamide gel copolymerized with 0.1% (w/v) gelatine. Stacking gels contained 4% (w/v) polyacrylamide. Electrophoresis was carried out at 4 °C at 250 V constant using a Bio-Rad Protean II apparatus (Bio-Rad Laboratories), Low range pre-stained standard proteins (Bio-Rad) were used as molecular weight markers.

For the inhibition studies, specific inhibitors were added to the extract and incubated for 1 h at 20 °C before the electrophoresis. Final concentrations of the inhibitors were 20 mM IAA, 2 mM PMSF and 20 mM 1,10 PA, respectively. After electrophoresis, the gels were washed (2× 40 min) in 2.5% (w/v) Triton X-100, 40 mM Tris–HCl buffer (pH 6.8) (washing buffer) in order to remove SDS, then incubated for 16 h at 30 °C in developing buffer: 1% (w/v) Triton X-100, 2 mM DTT, 10 mM cysteine, 5 mM $CaCl_2$, 40 mM Tris–HCl buffer (pH 6.8), in the absence and in the presence of specific inhibitors. For the development of enzyme activity, the gels were

stained with Coomassie Brilliant Blue R-250, then destained in methanol/acetic acid/H_2O and scanned using an ImageMaster DTS (Pharmacia Biotech, Uppsala, Sweden) scanner. Proteolytic activities were detected as clear, unstained bands on a blue background and were quantified by computerized image analysis through two dimensional scanning densitometry using the Image Master 1D program.

2-D gelatin zymography (2-DZ)

Protease activities present in Broccoli floret extracts were detected by gelatin 2-D zymography. Samples were applied under non-reducing conditions. Aliquots of extracts (35 μg of proteins) were mixed with the rehydration solution containing 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.5% (v/v) immobilized pH gradient (IPG) buffer, plus a trace of bromophenol blue, to a final volume of 250 μL. IEF was performed on IPG Dry-Strips of 13 cm in linear pH gradient of 4–7 (GE-Heathcare). IPG Dry-Strips were rehydrated with a sample-containing rehydration solution for 12 h at 20 °C. IEF was run using an IPGphor unit (Amersham Biosciences) at 20 °C for a total of 26,450 Vh. After IEF, IPG-strips were equilibrated for 20 min by gentle shaking in equilibration buffer: 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 50 mM Tris–HCl (pH 8.8). In the second dimension, proteins were separated in a 12% (w/v) polyacrylamide gel copolymerized with 0.1% (w/v) gelatin. Run was carried out on the Hoefer SE 600 vertical electrophoresis unit at 4 °C first for 30 min at 200 V and then for 4 h at 280 V. Pre-stained low range standard protein (Bio-Rad) were used as molecular weight markers. After electrophoresis, gels were washed two times in washing buffer and then incubated for 14 h at 30 °C in developing buffer in the absence and in the presence of specific inhibitors. For the development of enzymatic activities, gels were stained with Coomassie Brilliant Blue R-250 (for 30 min at room temperature) and destained in methanol/acetic acid/H_2O. Proteolytic activity was detected as a clear, unstained, spot on a blue background.

Gels were scanned using a ScanMaker 9800 XL-Microtek (Hsinchu, Taiwan) in a grey scale. The images were digitally converted from positive to negative image.

Image analysis were carried out by the ImageMaster 2D Elite V. 2002.01 software (Amersham Biosciences). To reduce the chance of errors during the analysis, spot detection was performed automatically. Correlation between spot volume (corresponding to the sum of pixel intensity within the digested spot) and loaded samples was achieved using dilutions (in water) of broccoli floret extracts obtained at the 4th day after harvest. 20 μL of each diluted sample (corresponding to 5, 20, 35, 60 and 100 μg of protein, respectively) was mixed with 230 μL of rehydration solution and analyzed in triplicate by 2-DZ.

Proteolytic activity of the digested spots detected at different days after harvest was expressed in terms of arbitrary scanning units (spot volume). Values are reported as the mean of three independent experiments.

Results

Biochemical analysis of post-harvest broccoli florets

Green Broccoli heads were harvested from a local field grown (Abriola, near the town of Potenza, Italy) and immediately stored at 20 °C in the dark. During storage, the Broccoli florets remained green for 2 days after harvest, then started to become yellow. The Broccoli heads turned completely yellow within 4 days after harvest.

Accordingly, the chlorophyll content declined markedly showing a decrease of more than 75% after 4 days (Fig. 1A). Protein content decreased rapidly as well, showing a profile similar to that observed for chlorophyll (Fig. 1B). Conversely, protease activity increased in the course of the senescence process of Broccoli florets (Fig. 1C). In particular, the strongest increase, approximately 4 times the initial value, was observed after 2 days of harvesting.

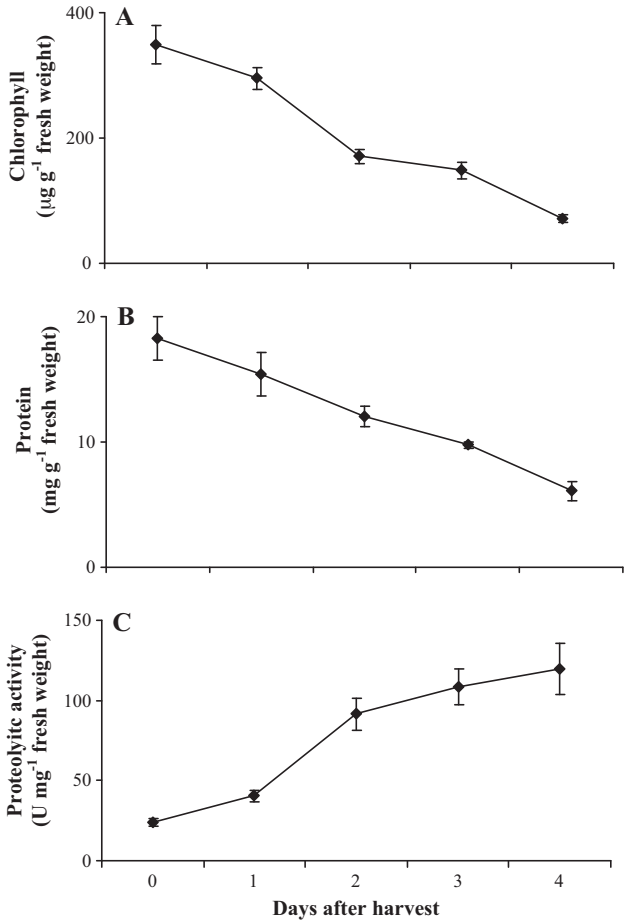

Fig. 1. Chlorophyll content, protein content and protease activity in post-harvest Broccoli florets. (A) Chlorophyll level was measured spectrophotometrically using the extinction coefficients; (B) protein content was determined using a Bio-Rad dye; and (C) protease activity was measured using azocasein as substrate, one unit of total proteolytic activity (*U*) was defined as the amount of enzyme yielding 0.01 unit of absorbance per min at 440 nm under the assay conditions (pH 7.0, 40 °C). Data are the mean (±S.D.) of three independent experiments.

Characterization of protease activity in post-harvest Broccoli florets in solution

Protease activity was determined by measuring the hydrolysis of azocasein in solution. Results obtained in the absence or in the presence of specific inhibitors are shown in Table 1. About 50% of proteolytic activity was inhibited by IAA, a specific inhibitor of cysteine proteases. Other proteases present were inhibited by inhibitors of metallo-proteases and serine proteases, while aspartic proteases were not revealed.

Detection of proteolytic activities by 1-D gel gelatin zymography

Analysis by monodimensional gelatin zymography (1-DZ) was done to determine composition and apparent Mr of senescence-associated proteases present in the Broccoli floret extracts after harvest. 1-D zymographic analysis revealed the presence in the extract of five bands corresponding to proteolytic activities with

Table 1
Effect of class-specific inhibitors on protease activity of Broccoli floret extracts.

Inhibitor[a]	Protease class	Residual activity[b] (%) Days after harvest				
		0	1	2	3	4
Control		100c	100c	100c	100c	100c
PMSF (2 mM)	Serine proteases	103.1c ± 6.1	105.9c ± 5.2	87.4d ± 7.1	84.4d ± 6.1	86.8d ± 3.7
1,10 PA (20 mM)	Metallo-proteases	104.9c ± 4.2	110c ± 5.7	92.4cd ± 8.3	91.9cd ± 11.3	85.2d ± 7.3
Pepstatin (20 μM)	Aspartic proteases	107.3c ± 10.4	109c ± 6.6	103.8c ± 5.4	102.5c ± 5.1	104.5c ± 1.5
Iodoacetamide (20 mM)	Cysteine proteases	71.7d ± 5.6	58.1d ± 7.8	51.2e ± 8.1	54.3e ± 3.6	49.6e ± 4.2

[a] Aliquots of extract were incubated for 1 h at 20 °C with specific inhibitors.
[b] Protease activity was assessed on azocasein in solution (pH 7.0, 40 °C). 100% activity (control) was the activity determined in the absence of inhibitors (mean ± SD, $n = 3$). The mean values with different letters (in the same column) are significantly different ($P < 0.05$) as analyzed by one way analysis of variance (ANOVA).

Mr of 65, 60, 56, 47 and 43 kDa, respectively (Fig. 2A). The active digestion bands of 56 and 47 kDa were the first to be detected and the only ones at days 0/1 after harvest. The intensity of the proteolytic bands of 60, 56, 47 and 43 kDa increased after 2 days after harvest (lane 3), whereas the upper band of 65 kDa was detected only after 4 days after harvest (line 5).

In the presence of IAA, a strong reduction of intensity was observed in the proteolytic bands with Mr 43 and 47 kDa (Fig. 2B).

In Fig. 2C, corresponding to the samples incubated with 1,10 phenanthroline (1,10 PA), a specific inhibitor of metallo-proteases, the intensity of the proteolytic bands of 65 and 60 kDa decreased to about 45% and 60%, respectively.

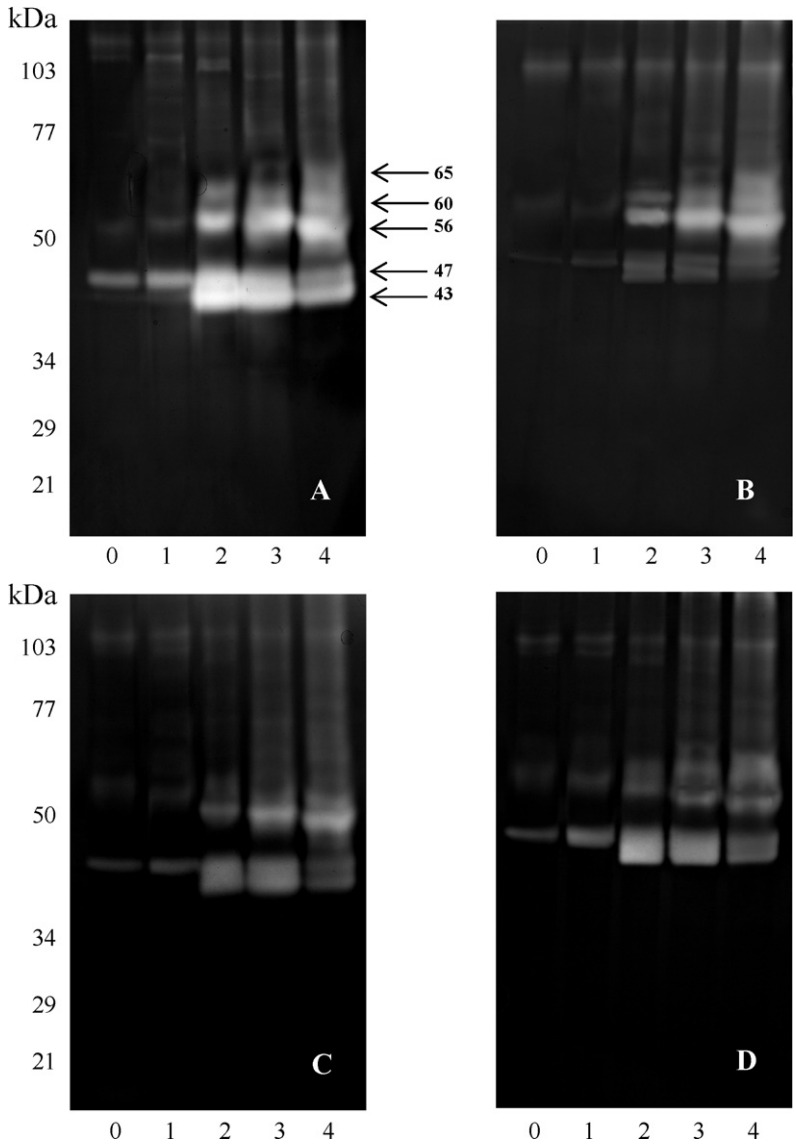

Fig. 2. 1-D zymography of Broccoli floret extracts. 1-D zymography (12% polyacrylamide/0.1% gelatin) of the extract obtained from the post-harvest Broccoli florets, in the absence (gel A, control) and in the presence of specific inhibitors: gels: (B) 20 mM iodoacetamide; (C) 20 mM 1,10 phenanthroline; (D) 2 mM phenylmethylsulphonyl fluoride. Molecular weights corresponding to the activity bands (right) were determined using the low range prestained standard proteins of Bio-Rad Laboratories. Lanes: 0–4 (days post-harvest). 10 μg of proteins were applied to each lane.

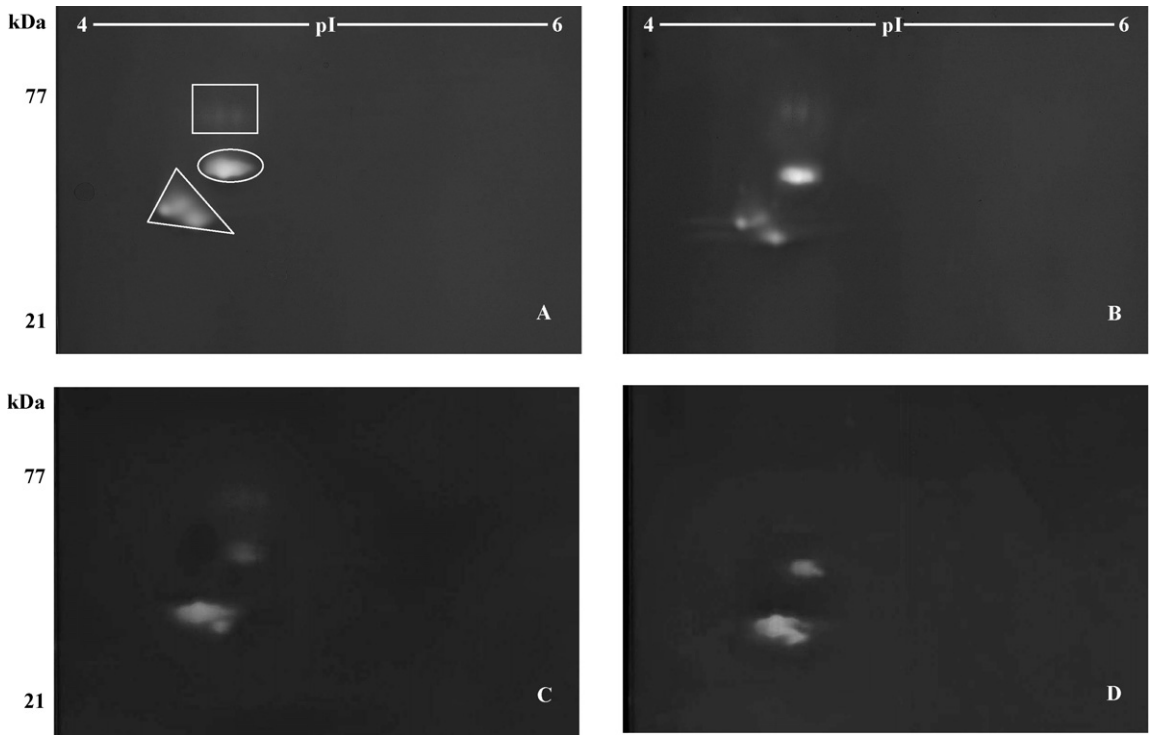

Fig. 3. 2-D zymography of proteases present in the Broccoli floret extracts at the 4th day post-harvest: effect of inhibitors. Detection of proteases (35 μg of proteins) in the absence (gel A) and in the presence of specific inhibitors: gels: (B) 20 mM iodoacetamide; (C) 2 mM phenylmethylsulphonyl fluoride; (D) 20 mM 1,10 phenanthroline. IPG-strips before IEF were rehydrated in the presence of 4 M urea and 2 M thiourea. 1st dimension: IEF on IPG dry-strips of 13 cm in linear pH gradient of 4–7; 2nd dimension: zymography (12% polyacrylamide/0.1% gelatin). The figures show only the regions of the gels with isoelectric point between pH 4 and 6 and molecular mass between 21 and 77 kDa.

The gelatinolytic activity corresponding to the band of 56 kDa was inhibited by PMSF, an inhibitor of a serine proteases (Fig. 2D). In this study, pepstatin A, the specific inhibitor for aspartic protease, was not used because this protease class cannot be detected by gelatin zymography (Wang et al., 2004).

Detection of proteolytic activities by 2-D gelatin zymography (2-DZ)

To observe the complete enzymatic pattern, including enzyme isoforms and isomers, present in the extracts of Broccoli florets, non-reducing 2D gelatine zymography was performed. The crude extracts were analyzed in the first dimension according to their pI, using IPG strips (IPG-strips, GE-Healthcare) of 13 cm in linear pH gradient of 4–7, and in the second dimension by their Mr in a 12% SDS-polyacrylamide gel copolymerized with 0.1% gelatin.

In preliminary runs the IEF was performed using a pI range of 3–11, but since the spots corresponding to the digestion of the substrate were present only in the acidic region of the gel, the runs were then performed in the pI range of 4–7.

The 2-DZ of the Broccoli floret extract corresponding to the 4th day after harvest is shown in Fig. 3A. IEF was performed in the presence of 4 M urea and 2 M thiourea in the rehydration solution containing 2% CHAPS (w/v), 0.5% (v/v) IPG buffer. Zymograms showed the presence of nine spots (clear unstained zones indicating the presence of proteolytic enzymes), subdivided in three groups (triangled, circled and boxed spots, respectively) in the range of 43–69 kDa and 4.2–4.7 pI. On the basis of the results obtained incubating the gels in the presence of specific inhibitors, the three triangled spots (with Mr between 44 and 48 kDa and pI between 4.2 and 4.4), which were inhibited in the presence of 20 mM IAA, could correspond to the cysteine protease bands of 43 and 47 kDa detected in 1-D gel zymography (Fig. 3B).

The circled spots (Mr 58 kDa and pI between 4.5 and 4.7), which were partially inhibited by 2 mM PMSF (Fig. 3C), could correspond to the serine protease band of 56 kDa observed in 1-D zymography. Finally, the boxed upper spots of 69 kDa could correspond to metallo-proteases since they were inhibited in the presence of 20 mM 1,10 PA (Fig. 3D).

Zymographic analysis of cysteine protease isoforms and isomers in Broccoli florets

In the present work, we have focused our attention on the activity of cysteine proteases. To this end 2-DZ was performed using 7 M urea and 2 M thiourea, instead of 4 M urea and 2 M thiourea, in the rehydration solution.

As shown in Fig. 4B, the increase of urea concentration improved number and resolution of the spots corresponding to cysteine proteases, and conversely induced the irreversible denaturation of serine proteases and metallo-proteases. In particular, 2-DZ revealed the presence of 11 spots corresponding to the cysteine proteases (Fig. 4B). They could be subdivided in two groups: the first group (spots 1–6) with Mr 48 kDa and pI 4.17–4.44, and the second group (spots 7–11) with Mr 44 kDa and pI 4.39–4.71. The highest activity was detected in spot 9 with Mr 44 kDa and pI 4.57 (Table 2). As it is clearly evident in Fig. 4B, the digested spots corresponding to metallo-proteases and serine proteases (boxed and circled spots in Fig. 4A, respectively) disappeared after treatment with 7 M urea.

All the digested spots detected in Fig. 4B were inhibited in the presence of the specific inhibitor IAA in developing buffer (Fig. 4C), confirming the presence of cysteine proteases.

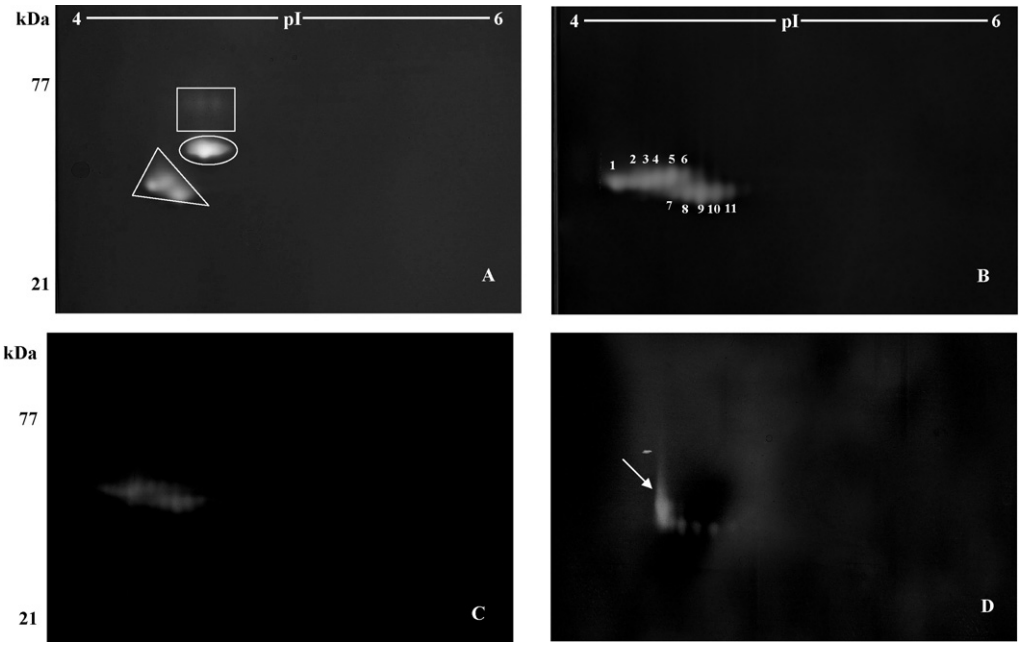

Fig. 4. Phosphorylated isomers of cysteine proteases in the Broccoli floret extract at the 4th day post-harvest as detected by 2-D zymography in the presence of 7 M urea and 2 M thiourea. IPG-strips before IEF were rehydrated in the presence of 4 M urea and 2 M thiourea (gel A) or in the presence of 7 M urea and 2 M thiourea (gels B, C and gel D). In (C) gel was incubated with the specific inhibitor iodoacetamide. In (D) sample before IEF was incubated with 50 U of alkaline phosphatase (Sigma, P8361) for 6 h at 37 °C, pH 8.0. Arrow indicate pI shift of spots after dephosphorylation.

The isomers of Broccoli cysteine proteases are in part phosphorylated

The presence of multiple spots with approximately the same Mr but different pI values could be ascribed to post-translational modifications such as phosphorylation and the relative increase of negative charges.

In Fig. 4D is shown the 2-DZ corresponding to the Broccoli floret extract at the 4th day post-harvest after incubation with bovine alkaline phosphatase (AP). This zymogram showed less spots than the control (Fig. 4B). AP treatment caused the pI shift of the spots 1–4 towards a more basic pI. This clearly indicates that the pI shift is due to the loss of negatively charged group due to the effect of AP, and suggests the presence of several isomers with different degree of phosphorylation. In particular, the removal of phosphate from the various isomers leads to a single spot with pI of 4.38 (spot with arrow) corresponding to the unphosphorylated form (spot 5) detected in Fig. 4B.

Follow up of cysteine protease activity during Broccoli post-harvest senescence

In order to study the changes of cysteine protease patterns during senescence, the floret extracts obtained at 0, 1, 2, 3 and 4 days after harvest were analyzed by 2-DZ (Fig. 5).

Zymograms corresponding to 0 and 1 day after harvest (Fig. 5A and B) showed 6 spots of 48 kDa corresponding to the active band of 47 kDa detected by 1-D gel zymography at all times during senescence (Fig. 2A). After 2 days post-harvest, other 5 spots with Mr of 44 kDa (Fig. 5C), corresponding to the 1-DZ band of 43 kDa (Fig. 2A), were detected. As clearly shown in Fig. 5D and E, protease activity notably increased at days 3 and 4 postharvest.

Quantitative analysis of each digested spot detected in the gel was performed by evaluating the linear relation between the spot pixel intensity (spot volume) and the enzyme amount loaded in each gel. Fig. 6 shows that the spot volume calculated for each spot is directly proportional to the enzyme amount

Table 2
Image analysis of the digested spots detected by 2-D zymography in Broccoli floret extracts in the course of post-harvest senescence.

Spot number	Proteolytic activity (arbitrary scanning units × 10^3)[a]			Days after harvest		pI_{Exp}/MW_{Exp}[b]
	0	1	2	3	4	
1	44.25 ± 3.21c	45.56 ± 3.74c	65.06 ± 3.29d	119.57 ± 9.17e	315.75 ± 16.24f	4.170/47.113
2	8.25 ± 0.49c	11.26 ± 1.11d	36.67 ± 2.08e	51.57 ± 2.47f	219.48 ± 10.25g	4.209/47.351
3	4.73 ± 0.76c	9.35 ± 1.56d	44.40 ± 2.94e	57.11 ± 3.13f	176.47 ± 6.16g	4.264/47.526
4	5.38 ± 0.81c	6.57 ± 0.62c	38.67 ± 2.02d	48.58 ± 3.13e	192.21 ± 18.44f	4.332/47.794
5	10.90 ± 1.14c	11.83 ± 1.37c	41.06 ± 3.21d	71.50 ± 6.49e	351.80 ± 33.99f	4.382/47.913
6	3.34 ± 0.21c	3.78 ± 0.31c	16.71 ± 1.14d	32.95 ± 1.56e	131.78 ± 9.86f	4.443/47.102
7			14.23 ± 0.99c	28.90 ± 1.13d	148.69 ± 10.39e	4.398/44.220
8			32.67 ± 2.19c	39.14 ± 1.15d	181.18 ± 14.41e	4.495/44.203
9			44.60 ± 3.05c	101.82 ± 7.16d	538.65 ± 61.29e	4.573/44.079
10			22.67 ± 1.03c	37.22 ± 2.12d	190.36 ± 11.37e	4.643/44.215
11			19.19 ± 0.77c	25.39 ± 1.70d	145.80 ± 13.28e	4.711/44.307

[a] Proteolytic activity of the digested spots was expressed in terms of arbitrary scanning units (spot volume) corresponding to the sum of intensity values of every pixel of the digested spot detected in the gel. Values are reported as mean of three independent experiments.

[b] pI and MW, determined using the ImageMaster 2D Elite V. 2002.01 software (Amersham Biosciences), correspond to the broccoli floret extracts at the 4th day after harvest.

The mean values with different letters (in the same row) are significantly different ($P < 0.05$) as analyzed by one way analysis of variance (ANOVA).

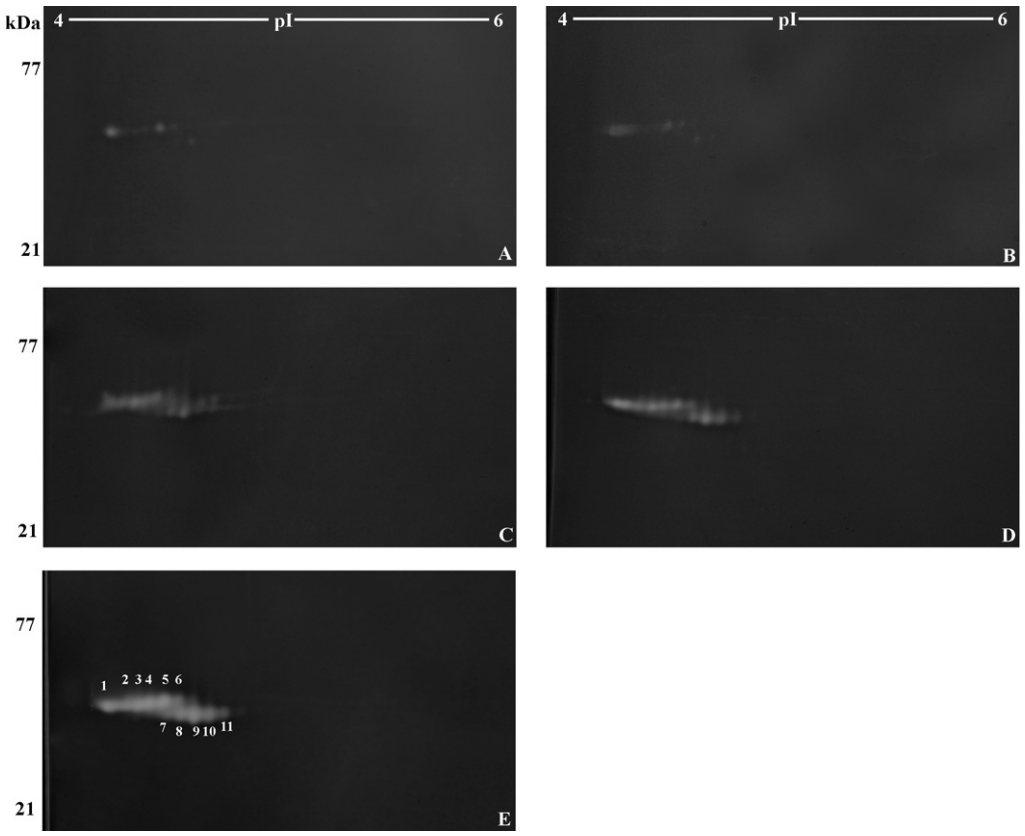

Fig. 5. Time course of cysteine protease activity in the Broccoli florets during senescence as detected by 2-D zymography: soon after post-harvest (A), and after 1 day (B), 2 days (C); 3 days (D) and 4 days (E) post-harvest. Samples (35 μg of proteins) were analyzed using the following conditions: 1st dimension: IEF on IPG dry-strips of 13 cm in linear pH gradient of 4–7; 2nd dimension: gelatin zymography (12% polyacrylamide/0.1% gelatin).

loaded on the gels, with a linear regression R^2 value mean >0.96.

As shown in Fig. 7A and B, protease activity (expressed as arbitrary scanning units, i.e. spot volumes) notably increased at days 2, 3 and 4 postharvest. In particular at the 4th day after harvest, spots 9 and 7 increased 12 and 10.5 times when compared to the activities measured at the 2nd day after harvest, respectively. Conversely, spots 6, 7, 10 and 11 increased about 8 times, and the remaining spots 8, 1, 2 and 3 increased 5 times. The lowest increase was observed in the case of spot 3 (4 times). Data corresponding to all zymographic spots are shown in Table 2.

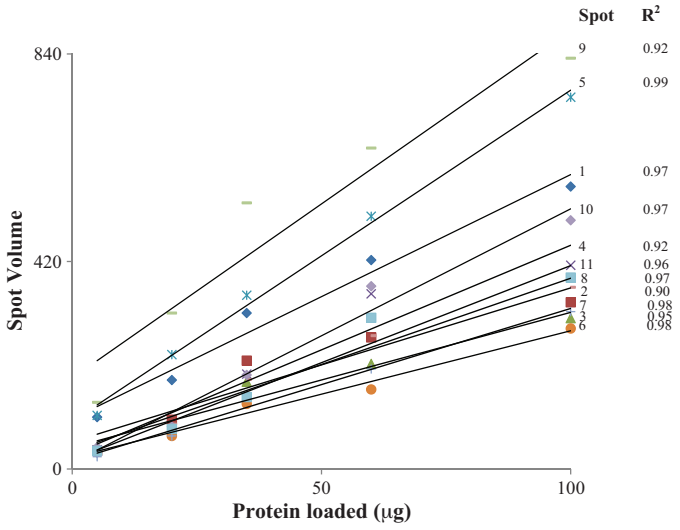

Fig. 6. Correlation between spot volume and enzyme activity in loaded samples. Correlation between spot volume (corresponding to the sum of pixel intensity within the digested spot) and loaded samples was performed using dilutions (in water) of broccoli floret extracts obtained at the 4th day after harvest. 20 μL of each dilution (corresponding to 5, 20, 35, 60 and 100 μg of protein, respectively) was mixed with 230 μL of rehydration solution. The reproducibility of spot volume quantification was determined by the analysis of three 2-DZ gels.

Discussion

In the present study, we report for the first time on the application of 2-D zymography for the characterization of cysteine proteases in Broccoli (*Brassica oleracea* L., var. Italica) florets during senescence.

The main event during senescence in plants is the degradation of macromolecules that contribute to the mobilization of nutrients from dying cells and their recycling in growing parts of the plants (Smart, 1994; Buchanan-Wollaston, 1997). In this context, the activity of proteolytic enzymes represent an essential element of senescence. During senescence, protease-encoding genes are up regulated and most of the newly expressed enzymes are cysteine proteases (Page et al., 2001; Coupe et al., 2003; Eason et al., 2005; Wang et al., 2004). As shown in our work, the decrease in protein and chlorophyll contents observed in Broccoli florets during senescence corresponds to the increase of protease activity with a typical pattern already shown by other authors (Coupe et al., 2003; Wang et al., 2004). As determined using azocasein in solution, after 2 days post-harvest the protease activity in Broccoli florets increased markedly. This finding was consistent with the results obtained by 1-DZ and 2-DZ, where the increased proteolytic activity was found to correlate with both the higher intensity of proteolyic bands or spots and the appearance of new active bands or spots.

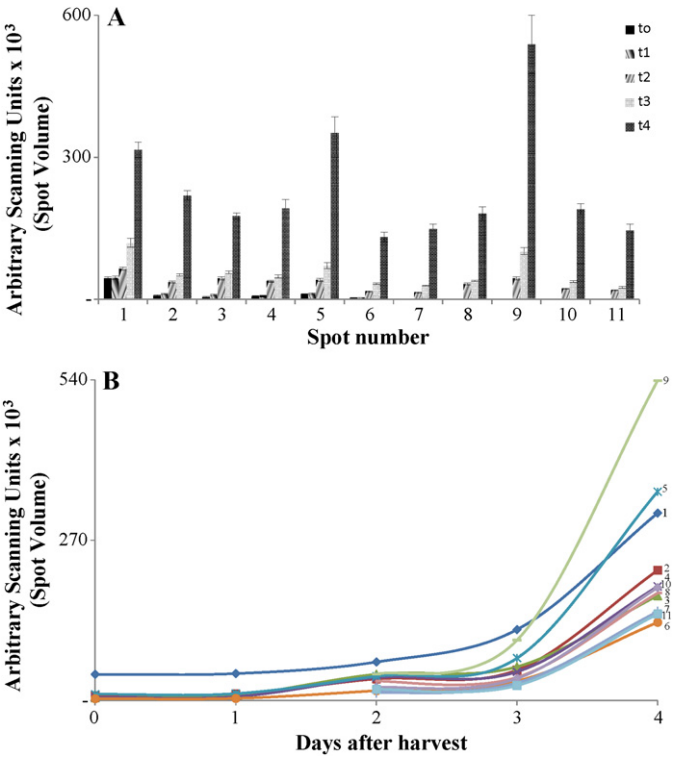

Fig. 7. Quantitative analysis of enzymatic patterns in the course of post-harvest senescence. Proteolytic activity of the digested spots was expressed in terms of arbitrary scanning units (spot volume). Values are reported as mean of three independent experiments. In (A) the values are presented as histograms with the corresponding error bars, while in (B) proteolytic activity profile is presented in the form of lines. Data are shown in Table 2.

2-DZ run, performed at lower urea concentrations in order to retain all proteolytic activities, showed the presence of multiple spots corresponding to different protease classes such as metalloprotease, serine proteases and cysteine protease. All detected proteases were acidic.

The increase of urea concentration in the rehydration buffer improved sample solubilization and spots resolution of cysteine proteases, and induced the denaturation of other protease classes. As already reported, cysteine proteases are resistant to denaturing agents such as urea and SDS (Bhattacharya and Bhattacharya, 2009).

In agreement with the statements made by other authors (Coupe et al., 2003; Wang et al., 2004), our study shows that cysteine proteases are present in all stages of Broccoli senescence and suggests their involvement in the control of senescence.

Moreover, the use of 2-DZ in the present study clearly indicated that cysteine proteases occur in the form of several phosphorylated isomers. Their specific role in senescence remains to be determined.

Our results indicate the presence of seven different cysteine proteases. Spots 5 and 6 (Fig. 4B or 5E) with Mr of 48 kDa could correspond, both for their Mr and for their presence during all stages of senescence, to endoprotease EP4, indicated by Wang et al. (2004) or to the mature forms of cysteine proteases BoCp2 and BoCp3 (accession numbers: Q8W181, Q8W180, respectively), identified by Coupe et al. (2003).

However, in the case of the cysteine protease focused as spot 5, we have found the presence of 4 isoforms with different phosphorylation level (spots 1–4), suggesting that specific isotypes may have different functional role during senescence. The proteolytic spots 7–11 of 44 kDa could correspond to endoproteases EP5 (Wang et al., 2004) and to cysteine proteases BoCp1, BoCp4, BoCp5 (accession numbers: Q8W182, Q8W179, Q8W178, respectively) (Coupe et al., 2003).

In conclusion, 2-DZ can offer a map of the enzymes present in a biological sample or in a food matrix, including all their isoforms and isomers. Data shows that quantitative analysis of the enzymatic spots performed using the ImageMaster 2D Elite software can provide a linear measure of enzyme abundance.

An advantage of 2-DZ is that direct identification of proteases is possible without complex purification. When coupled to proteomic analysis of the unstained spots, a technique that we called zymoproteomics (Larocca et al., 2010a,b), direct identification of the enzymes can be achieved. In our previous work, cysteine proteases such as bromelain and actinidin were indeed directly identified in Ananas and Kiwi fruits after increasing the concentration of urea and by coupling 2-DZ and mass spectrometry (Larocca et al., 2010a,b).

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