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# Differential ethylene-inducible expression of cellulase in pepper plants

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## Abstract

Ethylene promotes the abscission of leaves and the ripening of fruits in pepper plants, and in both events an increase in cellulase activity is observed. However, two enzyme isoforms (pI 7.2 and 8.5, respectively) are differentially involved in the two physiological phenomena. The pI 8.5 form has been purified from ripe fruits. It is a glycoprotein with an apparent molecular mass of 54 kDa. Two short peptides were sequenced and a very high homology to a tomato cellulase was observed. Polyclonal antibodies, raised against the purified enzyme, have allowed us to demonstrate that the observed ethylene-induced increase in cellulase activity is paralleled by *de novo* synthesis of protein. Three cDNAs (CX1, CX2 and CX3), encoding different cellulases, were obtained and characterized and their expression investigated. Accumulation of all three mRNAs is induced by ethylene treatment, though to different levels. CX1 is mainly expressed in ripe fruits while CX2 is especially found in abscission zones. CX3 accumulates at very low levels in activated abscission zones. Comparisons with other known cellulases demonstrate clear heterogeneity within the higher plant cellulases. Differences in ethylene inducibility and molecular structure suggest different physiological roles for cellulase in pepper plants.

## Introduction

Ethylene is a plant hormone which is able to promote a number of physiological responses. Most striking among these are the promotive effects on the softening of climacteric fruits and on the abscission of leaves, flowers and fruits of many plants. The above events are part of a more general phenomenon, called cell separation, which occurs as a consequence of the cell wall weakening brought about by the activity of one or more hydrolases, for instance, polygalacturonases and cellulases [23]. Particularly important and widespread seems to be the role played by the latter enzymes. However, in contrast to bacterial cellulases, higher plants cellulases are not able to degrade crystalline cellulose [14], so they are also referred to as endo-β-(1,4)-glucanases (EC 3.2.1.4).

Data have been published that demonstrate the

This paper is dedicated to Prof. G. Dall'Olio on the occasion of his 70th birthday.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X83709 (CX1), X83710 (CX2) and X83711 (CX3).

ability of ethylene to induce transcription of mRNA for specific proteins, and notable among these are cellulases [5, 8, 17, 19, 31, 33]. Although very common, ethylene-inducible cellulase activity does not appear to be a general phenomenon, since the cellulase increase observed during softening of the non-climacteric strawberry fruits seems to be associated with the temporal fruit development rather than with ethylene induction [1]. Furthermore, in apple, a cellulase activity decrease was observed, after the production of climacteric ethylene, in fruits allowed to ripen on the trees [1].

Besides these differences in ethylene responsiveness, higher plant cellulases also show a number of biochemical and molecular diversities even within the same plant species. In peach, two isoforms can be found (pI 6.5 and 9.5) which are differently involved in cell separation phenomena [5]. Two isoforms (pI 4.5 and 9.5) have also been found in bean leaf abscission zones. No glycosylation was evidenced in the basic form which is highly inducible by ethylene, while the acidic form is induced by auxin [11]. In contrast, in ripening avocado fruits ethylene promotes a number of isoforms (pI 5.1–6.8) [16], and protein glycosylation has been demonstrated [3]. In tomato, two cDNAs have been found which encode cellulases that are structurally divergent and differentially expressed during fruit development and flower abscission [19].

Therefore, it is evident that, while apparently simple when measured as total activity, the cellulase involvement in cell separation events cannot be related to a single enzyme form with similar characteristics in higher plants.

We have endeavoured to study the cellulase involvement and expression in pepper plants during leaf abscission and fruit ripening. We report that ethylene accelerates both fruit ripening and leaf abscission. An increase in cellulase activity is observed during the cell separation events which occur in those processes. Two isoforms are differentially expressed in leaf abscission zones and in ripe fruits upon ethylene treatment. The form preferentially expression in the ripe fruits was purified and two short peptides have been sequenced. Polyclonal antibodies raised against this protein enabled us to demonstrate that the observed increases in cellulase activity are paralleled by *de novo* synthesis of protein. By means of RT-PCR we have been able to clone three cDNA fragments coding for three different cellulases, and their expression has been studied following ethylene induction of leaf abscission and fruit ripening.

## Materials and methods

### *Plant material*

Plants of *Capsicum annuum* var. *longum* (DC.) Sendt. used in the experiments were grown in pots at the botanic garden of Padova. Fruits at various stages of growth were collected and either used without further treatment or subjected to ethylene flushing. In the latter case, green ripe fruits washed with deionized water were placed in a chamber and flushed with ethylene (100 $\mu$l/l) in air at a flow rate of 6 l/h. All the fruits were deprived of their seeds, frozen in liquid nitrogen and stored at −80 °C. Whole plants were flushed with ethylene as described above. Abscission zones were excised from the leaf petioles either before or after the ethylene treatment, frozen in liquid nitrogen and stored at −80 °C.

Treatment with ethylene causes an acceleration and, especially, a synchronization of the onset of cell separation events in a population of variously aged organs.

### *Enzyme extraction and assays*

The plant material was ground in a mortar with liquid nitrogen. In the case of fruits, the powdered tissues were washed 2–3 times with cold acetone in order to minimize their lipid content. Both fruit acetonic powders and ground abscission zones were resuspended in 4 volumes (w/v) of extraction buffer (20 mM sodium phosphate, 1 M NaCl pH 6.1, and either 0.15% 2-mercaptoethanol (fruits) or 0.3% (v/v) 2-mercaptoethanol, 5% (w/v) polyvinylpolypyrrolidone (abscission

zones)). After stirring for 2 h at 4 °C, the homogenate was filtered through cheesecloth and, after centrifugation (14000 × *g*, 20 min), the supernatant was collected and proteins were precipitated by the addition of ammonium sulphate (80% saturation). The precipitated proteins were stored at 4 °C.

Qualitative analysis was performed using a modification of the cup-plate diffusion assay described by Carder [6]. A 1.5% (w/v) agar solution was made containing 0.1% (w/v) carboxymethylcellulose (CMC, medium viscosity, Sigma) in 20 mM sodium phosphate buffer pH 6. On cooling, 10 ml of liquid was poured into a 90 mm Petri dish to form a gel of 4 mm thickness. Before use, wells of 3 mm diameter were cut from the agar using a pipette tip and 5 μl of samples to be analysed were loaded onto each well. Up to 36 wells were obtained from one plate. The plates were then incubated at 37 °C for at least 5 h. After incubation, plates were flooded with 0.1% (w/v) congo red in aqueous solution for 30 min. The staining solution was then poured off and the plates were washed briefly in distilled water followed by flooding with 1 M NaCl for 10 min to enhance the zone of enzyme activity. The pH of the plate was lowered by the addition of 2% (v/v) acetic acid to alter the dye colour from red/orange to purple which gives a better contrast, thus facilitating detection of enzyme activity.

Quantitative enzyme determinations were performed viscometrically according to the method of Durbin and Lewis [11]. A known amount of protein in 0.2 ml was added to 1.8 ml of substrate consisting of 0.6% (w/v) CMC in 20 mM phosphate buffer (pH 6.0). The flow rate of this solution was measured before and after 3–5 h of incubation at 37 °C. Changes in viscosity of the solution were converted to relative units of enzyme activity according to the method of Almin *et al.* [2]. The pH optimum of the enzyme was determined using CMC solutions ranging from pH 4 to pH 8 in one unit pH steps.

Protein concentration was measured by the method of Lowry modified by Peterson [24] using bovine serum albumine (BSA, Fluka) as a standard.

*Cellulase purification*

Proteins extracted from ripe pepper fruits (see previous section) were centrifuged at 16000 × *g* for 20 min, the pellet was then dissolved in an appropriate volume of 40 mM sodium acetate (pH 5.5) and carefully applied to a first affinity column whose substrate consisted of fibrous cellulose (Sigma). The cellulose column was thoroughly washed with 40 mM sodium acetate (pH 5.5) until the protein content in the eluent was undetectable. Cellulase was released from the column by means of an elution buffer (40 mM sodium acetate, 0.3 M NaCl, 0.1 M cellobiose pH 5.5). Fractions were tested for cellulase activity using the cup-plate diffusion assay, the active fractions were pooled and the proteins were precipitated overnight at 4 °C by the addition of ammonium sulphate (80% saturation).

Proteins eluted from 4 cellulose affinity columns were pooled and applied to a second cellulose affinity column. Washing, elution and assays of cellulase activity were as described above. The active fractions were pooled and proteins precipitated by the addition of ammonium sulphate (80% saturation). After centrifugation at 25000 × *g* for 1 h the pellet was dissolved in 40 mM sodium acetate pH 5.0, applied to a gel filtration column made with Sephacryl S200 HR (Sigma) and the proteins were eluted with the same buffer. Fractions were tested for cellulase activity and the active fractions were pooled; the proteins were then precipitated with ammonium sulphate.

The last purification step was performed by preparative electrophoresis in a denaturing 10% polyacrylamide gel with the buffer system of Laemmli [18].

After electrophoresis the gel was stained with 0.25% Coomassie (w/v) in 50% methanol (v/v) and destained with a 50% methanol (v/v) solution. The band of 54 kDa corresponding to the cellulase was excised from the gel and electro-eluted.

*Antibody production*

Antiserum to the purified protein was raised in a young male rabbit. Fortnightly subcutaneous injections utilizing Freund's adjuvant (50% v/v) were performed at 2–3 injection sites bilaterally along the back of the animal [15]. The first injection contained approximately 100 $\mu$g of purified protein, while 50 $\mu$g were used for subsequent boosts.

Small volumes of serum (ca. 10 ml) were obtained from the animal after three days from each injections and assessment of antibody production (titre) was performed by western analysis.

*Gel electrophoresis, protein blotting and immunodetection*

Proteins were analysed by SDS-PAGE using 10% polyacrylamide gels with the buffer system of Laemmli [18]. Proteins (5–50 $\mu$g) were precipitated by the addition of an equal volume of 20% trichloroacetic acid (TCA, Sigma), allowed to stand at 4 °C for at least 30 min and centrifuged at 16000 × *g* for 20 min. To remove residual TCA the pellet was washed with cold acetone, then suspended in 20 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.1 M 2-mercaptoethanol, 0.025% (w/v) bromophenol-blue. After electrophoresis the gels were stained with silver using the method of Blum *et al.* [4].

For western analysis, proteins (10–50 $\mu$g) separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, 0.45 $\mu$m) as described by Towbin *et al.* [29]. Transfer was carried out overnight at 0.1 A and 4 °C using a Bio-Rad Trans Blot Cell and a buffer consisting of 25 mM Tris-HCl, 192 mM glycine pH 8.2, 10% (v/v) methanol. Proteins (100 $\mu$g) separated by native isoelectric focusing gels were electroblotted under acidic conditions using 0.7% (v/v) acetic acid as transfer buffer. In this case, the transfer was carried out overnight at 4 °C and 0.2 A using reversed polarities.

For the immunodetection, protein blots were first incubated at 4 °C for 2 h in 50 mM Tris-HCl, 85 mM NaCl, 2 mM $CaCl_2$ pH 8, 3% BSA (blocking solution). Cellulase protein was detected by incubation at 37 °C for 1.5 h in blocking solution with cellulase antiserum (1:500 dilution) followed by 4 washes with 20 mM $Na_2HPO_4$, 0.1 M NaCl, 0.05% (v/v) Tween 20, pH 7.4 (PBST). The blot was incubated for 1.5 h in blocking solution with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:8000 dilution) (Sigma) and, after 4 washes with PBST solution, developed with 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.6, added with 0.4 mM 4 nitroblue tetrazolium (Sigma), 0.38 mM 5 bromo-4 chloro-3-indolyl phosphate. The molecular mass of the proteins was estimated by comparison with known molecular weight markers (BioRad, Low Range Protein Molecular Weight Markers).

*Native isoelectric focusing (IEF)*

Native IEF was carried out at 4 °C in a vertical gel system as described previously [26], with the modification that a 20 cm × 14 cm cell was used.

Protein samples (100 $\mu$g) were mixed with 20% (v/v) glycerol and 2% (v/v) ampholine carriers (Pharmacia) of the same pH range used for the gel preparation, and the focusing was carried out by increasing the voltage up to 1000 V during the first 4 h and by leaving this voltage constant for the next 7 h.

The pH gradient and the pI of cellulase isoforms were both determined by cutting equal slices (20 × 5 mm) from each lane of the gel. The slices for the pH gradient were kept for 2 h in vials containing 1.3 ml of 10 mM KCl and the pH was then measured with a standard electrode. Those for cellulase assays were placed overnight at 4 °C in Eppendorf tubes containing 400 $\mu$l of 20 mM sodium phosphate, 1 M NaCl, pH 6.1, and this solution was then tested viscosimetrically for cellulase activity.

*Protein fragmentation and sequence of peptides*

200 $\mu$g of purified protein was dissolved in 1 ml of formic acid 70% (v/v). 400 $\mu$g of CNBr were

added and incubated for 24 h at room temperature. The reaction was stopped by the addition of an equal volume of water and the sample was dried using the Savant system.

The fragments obtained were resuspended in 50 mM Tris-HCl, 4% (w/v) SDS, 12% (w/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.01% (w/v) Serva Blue G and separated in a 16% Tricine-SDS-PAGE system, according to Schagger and von Jagow [28].

Peptides were electroblotted onto a polyvinylidene difluoride (PVDF) microporous membrane using a transfer buffer (pH 11) consisting of 10 mM 3-cyclohexylamino-1-propanesulphonic acid in 10% (v/v) methanol. After an overnight transfer at 0.1 A, the blotted membrane was stained with 0.1% (w/v) Serva Blue R, 1% (v/v) acetic acid, 40% (v/v) methanol, destained with 50% (v/v) methanol and dried.

Automated Edman degradation [12] of protein sample was performed using an Applied Biosystem sequencer (model 477A) equipped with online phenylthiohydantoin (PTH) amino acid analyser (model 120 A). Normal filter and PTH standard cycles for both reaction and conversion functions were as provided by manufacturer. The samples to be analysed were obtained by excision of the bands from the PVDF membrane.

*Glycated proteins detection*

10 $\mu$g of protein was run in a 10% (v/v) SDS polyacrylamide gel and electroblotted onto a nitrocellulose membrane according to standard procedures. The concanavalin A/peroxidase system suggested for glycoproteins by Weiss *et al.* [35] was followed, with the modifications that 3% (w/v) glycoprotein-free bovine serum albumin was used as the blocking agent and peroxidase activity was detected by staining with 3-amino-9-ethylcarbazole and $H_2O_2$.

*RNA extraction and northern blot analysis*

Total RNA was extracted from leaf abscission zones and fruits as described in Logemann *et al.* [21]. RNA quantification was checked by means of both ethidium bromide staining of agarose gels and hybridization with a 25S rRNA probe (not shown) from *Phaseolus coccineus* [22]. Total RNA samples were separated in 6% formaldehyde-1.2% agarose gels and blotted onto Hybond N membranes (Amersham) using 20 × SSC as blotting buffer. DNA probes were $^{32}$P-labelled using a random-primed DNA labelling kit (Promega). The membranes were prehybridized (2 h) and hybridised (16–20 h at 42 °C) in 50% formamide, 5% SDS, 5 × Denhardt's solution, 5 × SSC, sonicated herring sperm DNA (100 $\mu$g/ml). The membranes were subsequently washed with 0.1 × SSC, 1% SDS at 60 °C and exposed to X-ray films at −80 °C.

*RT-PCR and cloning of the amplified fragments*

Different oligonucleotides were chosen on the basis of known plant cellulase sequences [7, 30, 32]. The ones which gave successful results were: [1], 5′-GGC(A)GGATACTAT(C)GATGC-3′; [2], 5′-CCAGAAGACATGGATACACC-3′; [4], 5′–GAGCAGTAG(A)AATGGGCA-3′; [5], 5′-ACCATGTAT(C)GACATCTT-3′.

2 $\mu$g of total RNA was used as starting material for the RT-PCR experiments. The first-strand synthesis was carried out with the cDNA Cycle Kit (Invitrogen) using a specific internal primer (oligo 4 to obtain CX1 and CX2 and oligo 5 to obtain CX3). 5 $\mu$l of the first-strand reaction were used for the subsequent PCR amplification. PCR reactions with 200 pM of each primer and 20 mM $MgCl_2$ were performed in 50 $\mu$l volumes using a Perkin Elmer/Cetus apparatus (DNA Thermal Cycler). Denaturation, annealing and extension temperatures were 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, respectively. This cycle was repeated 30 times for the cloning experiments and 20 times to investigate the CX1, CX2 and CX3 expression levels. In the latter experiments 10 $\mu$l of each reaction mixture were analysed by Southern blotting and hybridized as described in the previous section with the omis-

sion of formamide. In order to clone the amplified cellulase fragments, the PCR products were separated by gel electrophoresis and the bands of interest cloned in the PCR Vector using the TA Cloning kit (Invitrogen).

*DNA sequencing and analysis*

DNA sequencing was performed using the T7 DNA sequencing kit (Pharmacia). Sequences were determined on both strands in all the cDNA clones. The amino acid sequences were deduced using the IG-Suite program (IntelliGenetics). The Clustal program was used to produce the alignments and the LFASTA program to determine the percentage of similarity among the cellulases.

## Results

Ethylene has a strong promotive effect on the abscission of leaves. Comparison of cellulase activity in abscission zones before and after 36 h of treatment with ethylene shows an increase in enzyme activity after induction of abscission (Fig. 1). Protein extracts from nonactivated and activated abscission zones have been submitted to isoelectric focusing (Fig. 2A). Prior to activation two isoforms are evident with isoelectric

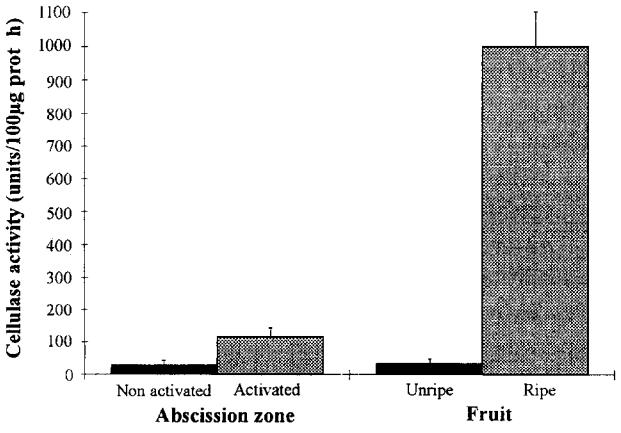


*Fig. 1.* Cellulase activity in abscission zones of leaves and in fruits.

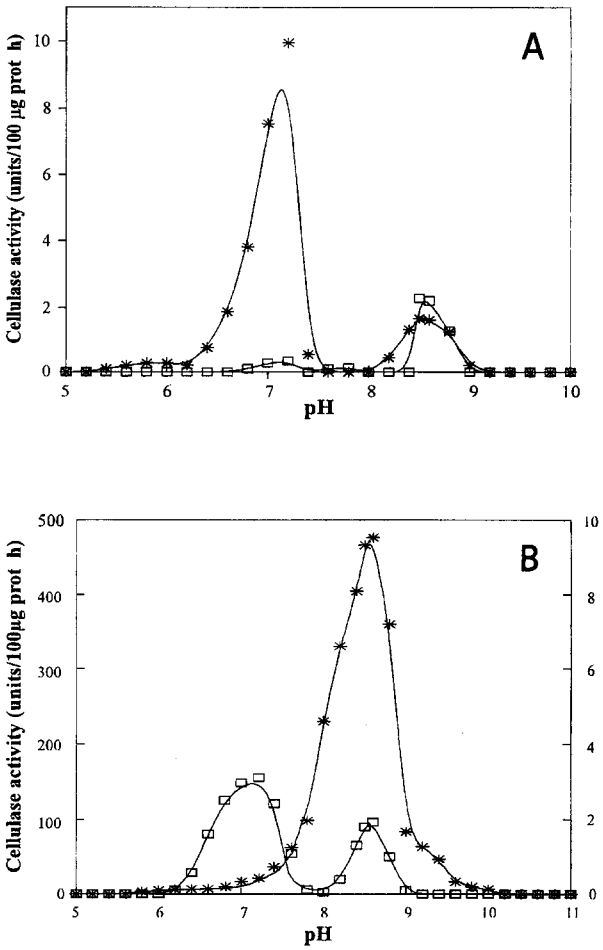


*Fig. 2.* Isoelectric focusing of protein extracts from abscission zones of leaves (A) and from fruits (B). A. The overall cellulase activity is supported by two isoforms both in control (□) and in ethylene activated (*) abscission zones. Flushing with ethylene induces a remarkable increase in the activity of the 7.2 isoform, while that of the 8.5 from remains low. B. Compared to the mature green fruits (□), where two isoforms (pI 7.2 and 8.5, respectively) account for the total enzyme activity, the 8.5 form seems to sustain the very high activity observed in fully ripe red fruits (*).

points of 7.2 and 8.5, respectively. The two isoforms are still present after activation of abscission, but while the 8.5 isoform maintains its previous low level, there is a large increase in activity of the 7.2 isoform.

Fruit ripening is also accompanied by an increase in cellulase activity (Fig. 1). In mature green fruits two isoforms with isoelectric points of 7.2 and 8.5, respectively, are normally found

(Fig. 2B). Interestingly, no peak for the 7.2 form can be detected in fully ripe fruits (Fig. 2B), so the observed high activity seems to be sustained by the basic isoform.

Fully ripe pepper fruits were used as the starting material for the purification of the 8.5 isoform. The main purification step was the use of a cellulose affinity column to detain the enzyme, followed by elution with a cellobiose containing solution. After two passages through the affinity column, the eluate was further purified by gel filtration (Sephacryl S 200 HR). After this step the enzyme appeared to have been purified 567-fold to a final specific activity of 57 224 units per 100 $\mu$g of protein (Table 1). Throughout the various steps the cellulase purification was monitored using activity assays, both a cup-plate diffusion assay and a viscosimetric method were used (not shown), and by means of silver-stained denaturing gels (Fig. 3). After gel filtration, the SDS gel showed one major protein band of about 54 kDa based on its position relative to known molecular mass standards (Fig. 3 lane 5). In a western analysis (Fig. 3, lane 6) this band gave a strong cross-reaction with anticellulase antibodies from avocado fruits [3]. A concanavalin A-based glycosylation assay yields a positive response (Fig. 3, lane 7), thus showing that the protein has a carbohydrate moiety.

In order to perform sequencing of the N-terminus, purification was completed by electrophoresis in a denaturing gel followed by electroelution of the protein banding at 54 kDa. Due to a block, the N-terminal amino acid sequence of this protein could not be determined. Therefore, the protein was fragmented by CNBr treatment and two peptides (named A and B) were analysed. These peptides (Fig. 7, single underline) exhibit an extremely high degree of homology (100% and 96%, respectively) to the deduced amino acid sequence of a cellulase from tomato fruit [19].

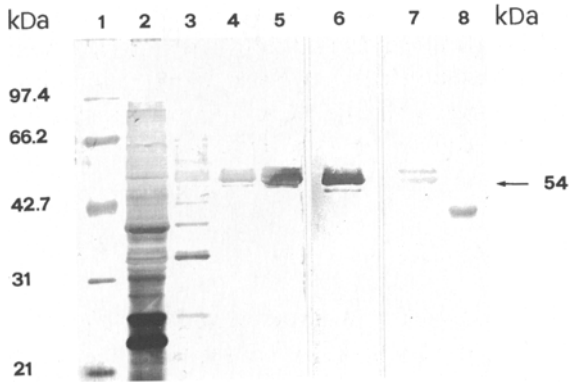


*Fig. 3.* Silver-stained SDS-PAGE of proteins from the different steps of cellulase purification (lanes 2–5). Lane 1, marker proteins; lane 2, proteins extracted from fruits and precipitated with ammonium sulphate at 80% saturation; lane 3, active fractions after the first affinity column; lane 4, active fractions after the second affinity column; lane 5, active fractions after the gel filtration. The cellulase band has an apparent molecular mass of 54 kDa. The same sample run in lane 5 has also been blotted onto a membrane and either probed with an anticellulase antibody from avocado fruits (lane 6) or submitted to a concanavalin A-based glycosylation assay (lane 7). Lane 8, marker proteins where the 42.7 kDa band (ovalbumin) was recognized as a glycated protein.

Polyclonal antibodies were raised against the purified cellulase. They were used to better define the relationships between ethylene treatments and either leaf abscission or fruit ripening in pepper plants. Since IEF analyses have shown that each cellulase isoform is preferentially involved in the two different phenomena, cross-reactivity of our antibody with the two isoforms was checked. Proteins, separated by IEF gels, were elec-

*Table 1.* Purification of cellulase from fully ripe red fruits of pepper. Yield values represent the average yield from 10 g starting material. Enzyme activity was determined by a viscosimetric assay.

| Steps | Cellulase act. (units) | Protein ($\mu$g) | Specific activity (U/100 $\mu$g prot) | Fold purification |
|---|---|---|---|---|
| 80% $[NH_4]_2SO_4$ precipitate | 4036.4 | 4000 | 100.9 | 0 |
| First affinity column | 16909 | 1545 | 1094.4 | 10.8 |
| Second affinity column | 11450 | 29.5 | 38813.6 | 384.7 |
| Gel filtration | 13791 | 24.1 | 57224.1 | 567.1 |

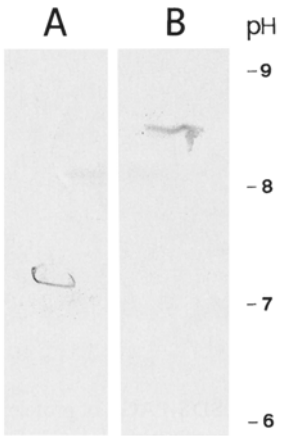


*Fig. 4.* Cross-reactivity of the pepper cellulase isoforms with the antibody raised against the cellulase protein purified from ripe pepper fruits. Proteins were extracted from either activated leaf abscission zones (A) or from ripe pepper fruits (B) where 7.2 and 8.5 are the main isoforms, respectively. After separation in native isoelectric focusing gels the proteins were electroblotted under acidic conditions onto a nitrocellulose filter which was subsequently probed with the pepper cellulase antibody. One band with an isoelectric point of ca. 7.2 is visible in leaf abscission zones (A), while one band is visible in ripe fruit at pH 8.5 (B). Numbers on the right indicate the pH gradient established upon the isoelectric focusing.

trotransferred to nitrocellulose and assayed in western analyses. Cross-reaction was observed with both cellulase isoforms (Fig. 4).

Exogenous ethylene has a strong promotive effect on the ripening of pepper fruits. A 96 h treatment is able to turn detached mature green berries into completely red ones (not shown). After an initial lag phase, the change in colour is accompanied by a steep increase in cellulase activity (Fig. 5A). A western analysis clearly demonstrates that such an increase is paralleled by *de novo* synthesis of protein (Fig. 5B). Leaf abscission is also promoted by exogenous ethylene, and the shedding of leaves is usually completed within 2 days of treatment. As with the ripening fruits, the observed increase in cellulase activity (Fig. 6A) is accompanied by *de novo* protein synthesis (Fig. 6B).

Total RNA, from both leaf abscission zones and fruits, has been used for RT-PCR experiments. Primers 2 and 4 give a similar amplification band with both types of RNA. Cloning of the

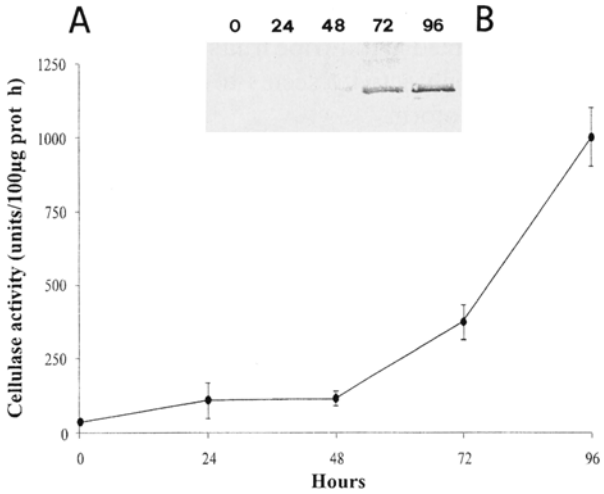


*Fig. 5.* Time course of cellulase activity (A) and cellulase protein induction (B) in pepper fruits flushed with ethylene. In the immunoblot (B) the probe was the antibody raised against the cellulase purified from ripe fruits. The remarkable increase in cellulase activity is paralleled by *de novo* synthesis of protein and corresponds to the changes in colour of fruits. Numbers indicate hours of ethylene treatment.

bands and sequence analysis of a number of clones have allowed us to discriminate two different cDNA fragments named CX1 (224 bp) and

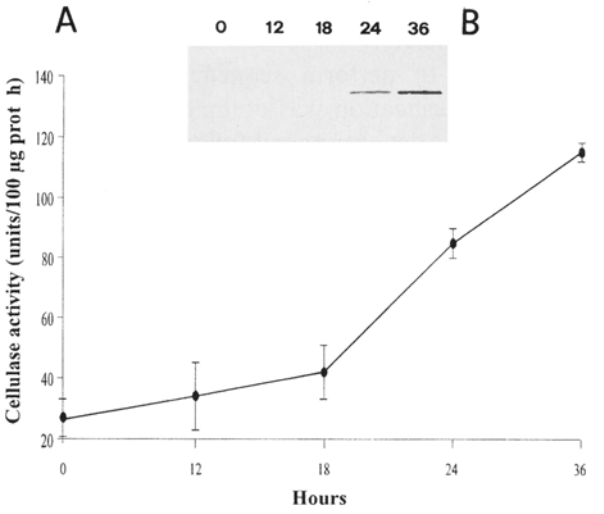


*Fig. 6.* Time course of cellulase activity (A) and cellulase protein induction (B) in leaf abscission zones following ethylene treatment. In the immunoblot (B) proteins were probed with the antibody anticellulase from pepper fruits. Numbers indicate hours of ethylene treatment.

CX2 (239 bp), respectively (not shown). Interestingly, the deduced amino acid sequence of fragment CX1 contains the sequence of peptide A, which belongs to the pI 8.5 cellulase protein purified from ripe fruits (Fig. 7, single underline). Primers 1 and 5 are able to generate a 974 bp PCR product (CX3) with RNA from leaf abscission zones (not shown).

Comparison of the three PCR products with sequences held in a database revealed a significant similarity to other known cellulase DNA sequences. In particular, CX1 has a higher sequence identity to bean abscission (76%) than to avocado fruit cellulase (64%). The opposite is true for CX2 which shows 77% identity to avocado, but only 67% to the bean cDNA. CX3 can be placed in a category by itself because of its low identity to both avocado cellulase (60%) and bean abscission cellulase (59.6%).

The relative expression of the three cellulase cDNAs in the two considered cell separation events has been analysed by northern analysis under highly stringent conditions. As expected, CX1-related mRNA (2.4 kb) is expressed in ripe

```
Bean cx      MGYHSVFIAVFLWSSMVCHNGLAMMDDGKLTSSSGPPN----YDYADALAKAILFFEGQRSGKLPSSQRVKWREDSALSDGKLQNVNLMGGYYDAGDNVKFGWPMAFSTS
tom cel1     ---------------MACSKNIWVIVIFFLCILAGPIIA---QDYNDSLGKAILFFEGQRSGKLPVSQRVKWRGDSALIDGIIEHVNLIGGYYDAGDNVKFGWPMAYSLT
cap cx1      ------------------------------------------------------------------------------------------------------------
cap cx2      ------------------------------------------------------------------------------------------------------------
cap cx3      ------------------------------------------------------------------------------------------ggyydagdnvkfvfpmaftAt
tom cel2     -----------------MAPKYTSIIFLFLLFNSFSCSFGGGHDYHDALRKSILFYEGQRSGKLPPDQRIKWRRDSALHDGASAGVDLTggyydagdnvkfvfpmaftTt
Avocado cx   ---------------MDCSSPLSLFHLLLVCTVMVKCCSASDLHYSDALEKSILFFEGQRSGKLPTNQRLTWRGDSGLSDGSSYHVDLVGGYYDAGDNLKFGLPMAFTTT
                                                    ^^^

Bean cx      LLSWAAVEYESEISSVNQLGYLQSAIRWGADFMLRAHTSPT-TLYTQVGDGNADHNCWERPEDMDTPRTVYKIDANSPGTEVAAEYAAALSAASIVFKKIDAKYSSTLLS
tom cel1     LLSWAAIEYQTQISSTNQLVHLQNAIRWGTNFLIRAHTSST-TLYTQVGDGNADHQCWERPEDMDTPRTLYKITSNSPGSEVAADVAAAFAAASIVFKNIDSNYSTKLLK
cap cx1      ------------------------------------------------------------PEDMDTPRTLYKITSNSPGSEVAAEVAAAFAAASIVFKNIDSNYSAKLLR
cap cx2      ------------------------------------------------------------PEDMDTPRNVYKVSPQNPGSDVAAETAAALAAASIVFKDSDPSYSSTLLH
cap cx3      llswsiidfkrnMg--SelgnavkavkwatdfllkataKEg-vVyvqvgdPfsdhscwerpedmdtlrtvykidQnHpgsdvageiaaalaaasivfrsldAsysnllld
tom cel2     llswsiidfkrnIg--NelgnavkavkwgtdfllkataRDg-vIyvqvgdAfsdhscwerpedmdtlrtvykidAnNpgsdvageiaaalaaasivfrsldSsysnllld
Avocado cx   MLAWGIIEFGCLMP--EQVENARAALRWSTDYLLKASTATSNSLYVQVGEPNADHRCWERPEDMDTPRNVYKVSTQNPGSDVAAETAAALAAASIVFGDSDSSYSTKLLH
                    ^^                    ↑                      ****** *..**.  . **..** . *** .******   *  **  **

Bean cx      HSKSLFDFADKNRGSYSGS-----CPFYCSYSGYQDELLWAAAWLYKASGESKYLSYIISN---QGWSQTVSEFSWDNKFVGAQTLLTEEFYGG-KKDLAKIKTDAESFI
tom cel1     RSRSLFAFADKYRGSYQAS-----CPFYCSYSGYKDELLWAAAWLYKAGGGNNYLNYASIN---QGWSQVASEFSWDDKFAGAQTLLAKEYLNG-KSNLEKFKKDADSFI
cap cx1      RSQSLFAFADKYRGSYQAS-----CPFYC-------------------------------------------------------------------------------
cap cx2      TAQKVFAFADKYRGSYSDSLSSVVCPFYC-------------------------------------------------------------------------------
cap cx3      ravkvfEfanrhrgaysssIhsavcpfycdfngyqdellwgaawlhkatrrrqyreyivknevilragdtinefgwdnkhaginvliskevlmgRapdlksfqvnadafi
tom cel2     ravkvfDfanrhrgaysssIhsavcpfycdfngyqdellwgaawlhkatrrrqyreyivknevilragdtinefgwdnkhaginvliskevlmgKapdlksfqvnadafi
Avocado cx   TAVKVFEFADQYRGSYSDSLGSVVCPFYCSYSGYNDELLWGASWLHRASQNASYMTYIQSNGHTLGADDDDYSFSWDDKRVGTKVLLSKGFLQDRIEELQLYKVHTDNYI
              .  .* ** . **.*  *⇑⇑⇑⇑⇑*****                                    ^^^                          ^

Bean cx      CAVMPGSNSRQIKTTPGGLLFTRDSSNLQYTTSSTMVLFIFSRILNRNHINGINCGSSHFTASQIRGFAKTQVEYILGKNPMKMSYMVGFGSKYPKQLHHRGSSIPSIKV
tom cel1     CGLMPESSSIQIKTTPGGLLYYRDSSNLQYVNGATMVLFMYTKVLEAAGIGGVTCGSVNFSTSKIKAFAKLQVDYILGNNPLKMSYMVGFGNKYPTKLHHRASSLPSIYN
cap cx1      ------------------------------------------------------------------------------------VGFGNKYPTQLHHRASS------
cap cx2      ------------------------------------------------------------------------------------------------------------
cap cx3      csilpgiAhpqvqyspgglivkpgvcnmqhvtslsflFlAysnylshanh-vvpcgsmSatpallkhiakrqvdyilgdnpqKmsym----------------------
tom cel2     csilpgiShpqvqyspgglivkpgvcnmqhvtslsflLlTysnylshanh-vvpcgsmTatpallkhiakrqvdyilgdnpqRmsymVGYGPHYPQRIHHRGSSVPSVAT
Avocado cx   CSLIPGTSSFQAQYTPGGLLYKGSASNLQYVTSTAFLLLTYANYLNSSGG-HASCGTTTVTAKNLISLAKKQVDYILGQNPAKMSYMVGFGERYPQHVHHRGSSLPSVQV
                                                      ^

Bean cx      HPAKVGCNAGLSDYYNSANPNPNTHVGAIVGGPDSNDRFNDARSDYSHAEPTTYINAAFVASISALLAKT
tom cel1     HPTRVGCNDGYSSWYNSNNPNPNTHVGAIVGGPNSGDQFIDSRSDYSHSEPTTYMNAAFIGSVAALIDQTKEGEHYGEINSQFNKTGFM
cap cx1      ------------------------------------ ------------------------------------------------------
cap cx2      ------------------------------------------------------------------------------------------
cap cx3      ------------------------------------------------------------------------------------------
tom cel2     HSARIGCKEGSRYFFSPN-PNPNRLIGAVVGGPNLTDSFPDARPYFQESEPTTYVNAPLVGLLAYFAAHSN
Avocado cx   HPNSIPCNAGFQYLYSSP-PNPNILVGAILGGPDNRDSFSDDRNNYQQSEPATYINAPLVGALAFFAANPVTE
                          ^
```

*Fig. 7.* Multiple alignment of the deduced amino acid sequences of a number of higher plant cellulases. Dashes within the sequences have been introduced to optimise alignment. In the area where all the considered sequences are represented, asterisks designate identical amino acids, while dots represent similar amino acids. The single solid underline identifies the fragments which correspond to peptide A (DTPRTLYKITSN) and B (VGFGNXYPTQLHHRASS) whose sequences have been determined by independent protein sequencing. A potential site for glycosilation in the sequence of the pepper CX1 is indicated by a double underline. Comparison between pepper CX1 and tomato Cel 1 is particularly evidenced by using boldface characters to show amino acid identity. The same comparison is shown between pepper CX3 and tomato Cel 2, but in this case amino acid identity is indicated by boldface small characters. Double arrows, arrow and arrowheads evidence significant gaps introduced to maintain an optimal amino acid alignment Bean cx [32]; tom cel 1 [19]; cap cx1, cap cx2, cap cx3: pepper cellulases; tom cel 2 [19]; avocado cx [30].

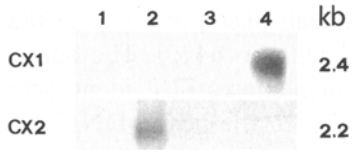


*Fig. 8.* Northern analysis of CX1, CX2 and CX3-related mRNA expression in leaf abscission zones and fruits. 30 $\mu$g of total RNA was separated on formaldehyde-agarose gels, blotted and hybridized with probes specific for each of the three cellulase mRNAs. Signals were observed only in the autoradiograms shown in the figure. 1, leaf abscission zones before activation; 2, activated leaf abscission zones; 3, mature green fruits; 4, fully ripe red fruits. The molecular weights of the mRNAs are indicated on the right.

fruits (Fig. 8). CX2-related mRNA (2.2 kb) seems to be specific for activated leaf abscission zones (Fig. 8), while no hybridization band can be detected, under the same experimental conditions, for the CX3-related mRNA (not shown). The more sensitive RT-PCR method has been used to better define expression of the three mRNAs. Aliquots withdrawn after 20 cycles of amplification were separated on an agarose gel, blotted and hybridised with the corresponding probe. Expression of CX1 in ripe fruits was confirmed; moreover, a lower level of expression is also found in abscission zones both before and, especially, after activation of abscission (Fig. 9). As regards CX2, besides the expected expression in the activated

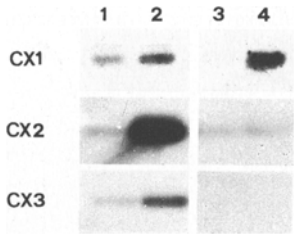


*Fig. 9.* RT-PCR analysis of the relative expression of the three cellulases in leaf abscission zones and fruits. Aliquots withdrawn after 20 cycles of amplification were separated on 1% agarose gels, blotted, and hybridized with the corresponding probe. A comparison of signal intensities is not possible because of different exposure requirements for the different Cxs signals. 1, leaf abscission zones before activation; 2, activated leaf abscission zones; 3, mature green fruits; 4, fully ripe red fruits.

leaf abscission zones, transcripts were detected to a much lesser extent before activation and in fruits as well (Fig. 9). Figure 9 shows that CX3 is only found in leaf abscission zones and that its expression is enhanced by ethylene activation of abscission.

## Discussion

In this study, evidence is presented that in pepper plants the enzyme cellulase is highly induced during the abscission of leaves and the ripening of fruits. The cell wall weakening which occurs in the two different organs involves the activity of two different isoforms (pI 7.2 and 8.5, respectively). Multiple cellulase forms have also been found in bean [9, 25] and in peach [5]. However, while in these plants cell separation appears to be always caused by the basic (pI 9.5) cellulase, in pepper both isoforms are important according to a different organ specificity, so that in fully ripe red fruits the 8.5 is the most important isoform, while 7.2 is the main isoform in activated leaf abscission zones.

The pI 8.5 cellulase was purified and partially characterized. It has an apparent molecular mass of ca. 54 kDa, like the cellulase purified from leaflet abscission zones of *Sambucus nigra* [34] and anthers of *Lathyrus odoratus* [27]. Although a number of plant cellulases have been purified, limited information is available about their glycosylation state. No carbohydrate residues have been detected in the bean abscission cellulase [11], while glycosylation has been demonstrated for the avocado fruits enzyme [3], and potential glycosylation sites have been evidenced in two cDNAs coding for tomato cellulase [19]. The pepper fruit enzyme gives a strong signal in a concanavalin A glycosylation assay, which shows the presence of some carbohydrate moieties on the protein.

Amino acid sequencing of the N-terminus could not be effected, so the protein was fragmented by CNBr treatment and the sequence of two short peptides was obtained. A comparison with the deduced amino acid sequences of other cellulases

confirms the identity of the purified protein and reveals that its similarity to a tomato fruit cellulase [19] is extremely high.

Cellulase has been shown to be encoded by a multigene family in a number of plants [7, 17, 19, 32]. This finding is in accordance with the wide range of physiological events which can require the intervention of cellulase activity as, for instance, cell and tissue expansion [13], adventitious root initiation (20), cell wall disruption in flowers reproductive organs [10], fruit ripening [14, 19], and the abscission of leaves, flowers and fruits [5, 17, 25, 34, 36, and others]. Since the above events can be regulated by different hormones, the presence of different genes coding for the same type of enzyme gives the plants a greater ability to pertinently respond to the various internal and external signals stimulating cell separation.

RT-PCR experiments have yielded three cDNA fragments whose sequences clearly demonstrate the presence in pepper of three different cellulase mRNAs. The deduced amino acid sequences of two fragments (i.e. CX1 and CX2) show a high similarity to the bean abscission and avocado fruit cellulases (74.3% and 88.6% respectively). Moreover, when compared to cellulases from tomato (Table 2), which belongs to the same plant family (Solanaceae), similarity between CX1 and Cel 1 is extremely high (94.6%), while it is much lower in all the other cases (Table 2). Surprisingly, the deduced amino acid sequence of the third fragment (CX3), which generally bears a poor similarity to other cellulases (Table 2), shows a striking 94.4% identity to tomato Cel 2, thus suggesting the presence of distinct groups within the general cellulase category.

*Table 2.* Percentage similarities among the deduced amino acid sequences of a number of higher plant cellulases. Comparisons were made considering sequences of 74, 79 and 324 amino acids in the case of CX1, CX2 and CX3, respectively. Light-shaded boxes indicate the high similarity to tomato cellulases; dark-shaded boxes indicate the high similarity to bean leaf abscission and avocado fruit ripening cellulases.

| | CX1 | CX2 | CX3 |
|---|---|---|---|
| CX1 | – | 64.6 | 58.2 |
| CX2 | 64.6 | – | 67.1 |
| CX3 | 58.2 | 67.1 | – |
| Avocado cx | 62 | 88.6 | 54.8 |
| Bean cx | 74.3 | 64.6 | 46.4 |
| tom cel 1 | 94.6 | 62 | 45.8 |
| tom cel 2 | 59.5 | 68.4 | 94.4 |

A multiple alignment of their deduced amino acid sequences with those of other higher-plant cellulases (Fig. 7) yields additional information. As with bean abscission cellulase and tomato Cel 1, pepper CX1 also lacks the five amino acids which are present in the other cellulases (Fig. 7, double arrows). Further differences in amino acid contents are found in other regions (Fig. 7, arrowheads), all of which mark a division within the higher plant cellulases, and strengthen the suggestion by Lashbrook *et al.* [19] that there are at least two different cellulase families in plants. It is interesting to note that different similarity patterns can be found if comparisons are made on the basis of either amino acid presence/absence or nucleotide identity. Unfortunately, due to the present fragmentary knowledge, the observed similarities cannot be related to organ specificity, isoelectric points or other characteristics.

Of the three pepper cDNAs, only CX1 encodes the amino acid sequence of a peptide obtained from the pI 8.5 cellulase purified from ripe fruits. A glycosylation assay gave a positive response for this protein, and the short CX1 fragment contains a potential glycosylation site (Fig. 7, double underline). Furthermore, a northern analysis showed that CX1-related mRNA is accumulated in ripe fruits. All these data demonstrate that the CX1 transcript encodes the pI 8.5 cellulase which is responsible for the dismantling of the cell wall which occurs in ripening pepper fruits.

The increase in CX2-related mRNA in leaf abscission zones suggests that this transcript is specifically involved in abscission. IEF results have shown that in abscission zones the pI 7.2 cellulase is especially increased by the activation of abscission. Accordingly, CX2 is likely to code for the 7.2 cellulase.

The level of the CX3 transcript was below the detection limit of northern analysis, so its expression was studied by the RT-PCR method which

allowed the isolation of this cDNA. CX3 is only found in abscission zones and as with the other two cellulase mRNAs, the transcript level is clearly increased by ethylene. Since the northern analysis suggests that the main mRNA involved in abscission of pepper leaves is the one encoded by CX2, the physiological role of the protein specified by CX3-related mRNA remains to be elucidated.

The presence of three cellulase transcripts is in contrast with the finding of only two isoforms. Unfortunately, the lack of full-length cDNAs makes it impossible to deduce the isoelectric points of the encoded proteins. It is possible that the third isoform (i.e. the CX3-related one) could not be detected due to its low abundance, even though the possibility remains that two cDNAs code for proteins with very similar isoelectric points.

Exogenous ethylene has a strong promotive effect on leaf abscission and fruit ripening which is paralleled by an increase in cellulase activity. The physiological responses are based on a differential expression of both mRNA and protein in the two different phenomena. This finding, and the observed molecular diversities, strongly suggest that the proteins specified by each mRNA have distinct biochemical and physiological functions.

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