# Cerato-platanin shows expansin-like activity on cellulosic materials.

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# Cerato-platanin shows expansin-like activity on cellulosic materials

**Ivan Baccelli · Simone Luti · Rodolfo Bernardi · Aniello Scala · Luigia Pazzagli**

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**Abstract** Cerato-platanin (CP) is a non-catalytic protein with a double ψβ-barrel fold located in the cell wall of the phytopathogenic fungus *Ceratocystis platani*. CP is released during growth and induces defence-related responses in plants. CP is also the first member of the "cerato-platanin family" (CPF) (Pfam PF07249). In the CPF, the molecular mechanism of action on plants and above all the biological role in fungal life are little-known aspects. However, an expansin-like function has recently been suggested concerning CP. Expansin-like proteins have the ability to act non-hydrolytically on cellulose. In the present work, the expansin-like activity of CP and Pop1, a CP family member, was investigated. Like expansins, CP and Pop1 were able to weaken filter paper in a concentration-dependent manner and without the production of reducing sugars. A metal-dependent polysaccharide monooxygenase-like activity was excluded. The optimum of activity was pH5.0, 38 °C. CP was also able to cause fragmentation of the crystalline cellulose Avicel and the breakage and defibration of cotton fibres. However, the interaction did not involve a stable bond with the substrates and CP did not significantly enhance the hydrolytic activity of cellulase. On the other hand, CP and Pop1 bound quickly to chitin. We consider CP as a novel one-domain expansin-like protein. We propose a structural role for CP in the fungal cell wall due to the ability to bind chitin, and hypothesize a functional role in the interaction of the fungus with the plant for the weakening activity shown on cellulose.

**Keywords** Non-hydrolytic activity · Loosenin · Swollenin · Disruption activity · Amorphogenesis · PAMP

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I. Baccelli (✉) · A. Scala
Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Università degli Studi di Firenze,
Via della Lastruccia 10,
50019 Sesto Fiorentino, Italy
e-mail: ivan.baccelli@unifi.it

S. Luti · L. Pazzagli
Dipartimento di Scienze Biomediche Sperimentali e Cliniche, Università degli Studi di Firenze, Viale Morgagni 50,
50134 Firenze, Italy

R. Bernardi
Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, Università di Pisa, Via del Borghetto 80,
56124 Pisa, Italy

## Introduction

Cerato-platanin (CP) is a small protein (12.4 kDa) produced by the phytopathogenic fungus *Ceratocystis platani*, the causal agent of canker in plane trees (Pazzagli et al. 1999). CP is a component of the cell wall of *C. platani*, but is also released during growth (Bernardi et al. 2011; Boddi et al. 2004; Scala et al. 2004). The three-dimensional structure of this protein has been defined: CP has a double ψβ-barrel fold similar to that occurring in endoglucanases, in the plant defence protein barwin and in domain I of expansins (de Oliveira et al. 2011). However, CP does not show hydrolytic activity and is known to the scientific community for its eliciting activity on plants (Fontana et al. 2008; Martellini et al. 2012; de Oliveira et al. 2011; Pazzagli et al. 1999; Scala et al. 2004).

Currently, a substantial number of proteins homologous to CP exists. These proteins are grouped into a protein family of which CP is the first member (the "cerato-platanin family", Pfam PF07249), and like CP some of them are known for the resistance-inducing activity in plants (Djonović et al. 2006; Frías et al. 2011; Seidl et al. 2006;

Yang et al. 2009; Zaparoli et al. 2009). In the "cerato-platanin family", the molecular mechanism of action on plants and above all the biological role in fungal life are little-known aspects. Nevertheless an expansin-like function has recently been suggested concerning CP, on the basis of the structural similarity and of the relation between gene expression and hyphal growth/chlamydospores formation found in *C. platani* (Baccelli et al. 2012).

Expansins are proteins mainly found in plants, where they have cell wall-loosening activity and various roles in growth and developmental processes (Sampedro and Cosgrove 2005). However, expansin-like proteins have also been found in fungi and bacteria (often called swollenins or loosenins) (Bouzarelou et al. 2008; Brotman et al. 2008; Chen et al. 2010; Dermatsev et al. 2010; Kerff et al. 2008; Lee et al. 2010; Quiroz-Castañeda et al. 2011; Saloheimo et al. 2002; Yao et al. 2008).

Expansins are believed to disrupt non-covalent bonds of wall polysaccharides without any hydrolytic activity (Cosgrove 2005; Yennawar et al. 2006). Their action was shown for the first time as a weakening activity on filter paper, a hydrogen-bonded network of cellulose fibrils (McQueen-Mason and Cosgrove 1994). Consequently, almost all the expansin-like proteins from fungi and bacteria are nowadays tested in vitro on cellulosic materials, for obvious biotechnologically relevant purposes, i.e. to increase the efficiency of bioconversion of lignocellulose to ethanol (Arantes and Saddler 2010; Jäger et al. 2011). These objectives are supported by a number of experimental data concerning the synergism with cellulases (Chen et al. 2010; Kim et al. 2009; Lee et al. 2010; Quiroz-Castañeda et al. 2011; Wang et al. 2010, 2011; Zhou et al. 2011). However, the biological role of expansin-type protein domains from microbes seems that of facilitating the interaction with plants, as colonization or symbiosis establishment (Brotman et al. 2008; Dermatsev et al. 2010; Kerff et al. 2008).

In the present work, the expansin-like activity on pure cellulose and the carbohydrate-binding properties of CP were investigated. During this study, another protein of the CP family, cerato-populin (Pop1) (Comparini et al. 2009), was also assayed.

## Materials and methods

### Production and purification of CP and Pop1

The proteins CP and Pop1 used in this study were produced by heterologous expression in the yeast *Pichia pastoris* strain GS115 (*his4*) (*Pichia* expression kit, Catalog no. K1710-01, Invitrogen—Life Technologies Ltd., Paisley, UK) as described by Carresi et al. (2006) and Martellini et al. (2012), and purified as described by the same authors.

### Effect on cellulosic materials

Whatman no. 1 filter paper (Whatman-GE Healthcare, Milan, Italy) was used as first substrate to assay the effect of CP and Pop1. Filter paper was cut into 1-cm-diameter discs (6 mg each), and a single disc was incubated in 2 ml of 50 mM sodium acetate buffer (pH5.0) containing CP or Pop1 at three different concentrations ($3\times10^{-7}$, $3\times10^{-6}$ and $3\times10^{-5}$M). Buffer only or buffer containing the protein bovine serum albumin (BSA) at the highest concentration ($3\times10^{-5}$M) were used as negative controls. The experiments were performed in Pyrex 20-ml glass culture tubes (Sigma-Aldrich, St. Louis, MO, USA) sealed with parafilm and incubated oblique at 38 °C, for 48 h, onto an orbital shaker (IKA, Staufen, Germany) at 320 rpm. At the end of the incubation period, pictures were taken, discs were removed and the absorbance at 500 nm on 1 ml of suspension was measured in order to quantify the paper fragments produced in each trial. Each measurement was taken immediately after shaking the suspension in the measuring cuvette, and three different measurements were taken for every suspension. The remaining 1 ml was used to verify the production of reducing sugars.

In order to test whether the action on cellulose was the result of a Cu-dependent polysaccharide monooxygenase-like activity, as recently found for the family-61 glycoside hydrolase (GH61) (Žifčáková and Baldrian 2012), the experiment with filter paper was repeated by assaying the incubation conditions reported for these enzymes. The paper disc was firstly incubated for 1 h in 50 mM sodium acetate buffer (pH5.0) only, or in the presence of 15 mM $Na_2EDTA$, used as a metal chelator in order to prevent the action of CP (incubations at 38 °C, 320 rpm); CP was then added to a final concentration of $3\times10^{-5}$M to buffer/EDTA and buffer only, and the latter was also amended by adding $CuSO_4$ to a final concentration of 30 μM and ascorbic acid (chemical reductant) to 1 mM in order to increase the action of CP (Harris et al. 2010; Quinlan et al. 2011). Incubations were performed at 38 °C, 320 rpm, for 48 h.

The ability of CP to act on cellulose was also assayed with different substrates such as microcrystalline cellulose (Avicel PH-101, catalogue no. 11365 Fluka, Sigma-Aldrich) and cotton.

CP $3\times10^{-5}$M was incubated with 1 mg of Avicel in 0.5 ml of 50 mM acetate buffer (pH5.0). Buffer or buffer containing BSA $3\times10^{-5}$M were used as a control. The experiments were performed in Pyrex 18-ml glass tubes sealed with parafilm and incubated at 38 °C, for 24 h, with shaking at 320 rpm. The physical structure of Avicel after treatment was observed using a light microscope (Optika Srl, Bergamo, Italy).

Cotton fibres from pharmaceutical-grade cotton wool were firstly mercerized according to Saloheimo et al.

(2002). In summary, 1 mg of cotton fibres was treated with 25 % NaOH for 15 min at 4 °C, washed several times with distilled water and subsequently incubated in Pyrex 18-ml glass tubes with 0.5 ml of 50 mM acetate buffer (pH5.0) containing CP $3\times10^{-5}$M, buffer only, or buffer containing BSA $3\times10^{-5}$M. After 24 h at 38 °C, with shaking at 320 rpm, the fibres were removed, suspended in distilled water with glass beads, and sonicated for 1 min at 25 μm, using a Soniprep 150 sonicator (MSE Ltd, London, UK). The physical structure of the fibres after sonication was observed by light microscopy.

### Effect on chitin and colloidal chitin

CP and Pop1 $3\times10^{-5}$M were incubated with 1 mg of chitin from crab shells (catalogue no. C9752, Sigma-Aldrich) or 1 mg of lyophilized colloidal chitin prepared as described by Roberts and Selitrennikoff (1988) from shrimp shells chitin (catalogue no. C9213, Sigma-Aldrich). Incubations were performed in 0.5 ml of 50 mM acetate buffer pH5.0, at 38 °C, 320 rpm, for 72 h. At the end of the incubation period, chitin and colloidal chitin were analyzed by light microscope to observe any effect caused by CP and Pop1. Buffer and buffer containing BSA $3\times10^{-5}$M were used as controls.

### Hydrolytic activity assay

The presence of reducing sugars after activity on filter paper was verified by dinitrosalicylic acid (DNS) method (Miller 1959) at the end of the 48-h incubation period with CP and Pop1 $3\times10^{-5}$M. One millilitre of suspension was firstly centrifuged and 300 μl of supernatant were added to 300 μl of 1 % DNS solution. The samples were then incubated at 90 °C for 15 min, added with 100 μl of 40 % potassium sodium tartrate, cooled at room temperature, and the absorbance at 575 nm was then measured. The absorbance values were compared to a glucose calibration curve made in 50 mM acetate buffer (pH5.0) to determine the concentration of reducing sugars.

### Analysis of optimal pH and temperature

Optimum pH and temperature for the activity of CP on cellulose were determined by measuring the activity on filter paper. Filter paper discs were prepared as previously described; CP was used at the concentration of $3\times10^{-5}$M. Optimal pH was established at 38 °C incubating CP in 2 ml of 50 mM citrate–phosphate buffer pH3.0, 5.0 or 7.0, and Tris–HCl pH9.0, with shaking at 320 rpm. The temperature optimum was determined both in 50 mM citrate–phosphate buffer pH5.0 and distilled water (pH5.7), performing the incubation at 25, 38 and 50 °C with shaking at 320 rpm. For each experiment, a corresponding control with buffer only was incubated.

The absorbance at 500 nm of the paper fragments produced in CP-treated samples and control samples was measured on 1 ml after 48 h of incubation in the various conditions. Each control value was subtracted from the corresponding CP-treated sample value and used for the graphs.

### Polysaccharides binding assay

The binding ability of CP and Pop1 was tested on cellulose (filter paper and Avicel), chitin, colloidal chitin and β-1,3-glucan. CP and Pop1 $3\times10^{-5}$M were incubated with a disc (1-cm diameter) of Whatman no. 1 filter paper (6 mg each), 40 mg of Avicel PH-101 or 40 mg of chitin from crab shells in 50 mM acetate buffer pH5.0, 38 °C, with shaking at 320 rpm. The experiments were performed in 2 ml of buffer in Pyrex 20-ml tubes for the incubations with paper (48 h), and with 1 ml of buffer in Pyrex18-ml tubes for the incubations with Avicel and chitin (72 h). At the end of the incubation period, the tubes were centrifuged and 10 μl of supernatant (unbound fraction) were vacuum dried, resuspended in 10 μl of sample buffer and loaded into a 15 % SDS–PAGE gel according to Laemmli (1970). As a control, 10 μl from a tube containing the same protein concentration used for the experiments were processed and loaded. The gels were stained with colloidal Coomassie blue.

In order to further evaluate the binding ability of CP and Pop1to polysaccharides, experiments with filter paper, chitin and β-1,3-glucan (catalogue nos. C9752 and G6513, Sigma-Aldrich) were performed monitoring the binding after 1, 5, 15, 30 min, 1, 3, 6 and 24 h of incubation.

The binding of CP to filter paper was also analyzed in distilled water (38 °C) and with different temperatures (25 and 50 °C). A test was also performed with a concentration tenfold lower. The binding of CP to Avicel was also analyzed in distilled water (38 °C, 24 h).

Binding capacity ($B_{max}$) and dissociation constant ($K_d$) were determined for CP with colloidal chitin, which was prepared as described in the previous paragraph. Binding was analyzed by depletion isotherm as described by Georgelis et al. (2011). In summary, variable amounts of CP were added to 50 mM acetate buffer pH5.0 containing a fixed amount of binding substrate. The mixture was incubated at 38 °C, for 1 h, with shaking. The samples were then centrifuged at 14,000×*g* for 10 min to collect the binding substrate and the protein in the supernatant was quantified by bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Illkirch Cedex, France), using BSA for calibration. Soluble protein was subtracted from the protein initially added to obtain the protein bound to chitin.

Dissociation constant and binding capacity were calculated by fitting the data to Langmuir isotherm.

### Treatment of filter paper with CP and cellulase

In order to test whether the action of CP on cellulose could enhance the action of hydrolytic enzymes, discs of Whatman no. 1 filter paper (6 mg) prepared as described previously were incubated in Pyrex 18-ml tubes containing CP $3\times10^{-6}$ or $3\times10^{-5}$M in 1 ml of 50 mM acetate buffer (pH5.0). Buffer only or buffer containing BSA $3\times10^{-5}$M were used as negative controls. After 48 h of incubation at 38 °C, 320 rpm, 1 U of cellulase from *Trichoderma viride* (Cellulase E.C 3.2.1.4, catalogue no. C1794, Sigma-Aldrich) was added to each tube and the tubes were further incubated for 3 h at 38 °C, 320 rpm. The reducing sugars released were then measured by DNS method (Miller 1959) as previously described.

The experiments were also repeated decreasing the amount of cellulase from 1 to 0.2 U/ml, or diminishing the incubation time with CP (24 h or no incubation).

## Results

### Weakening activity on filter paper by CP and Pop1

CP and Pop1 were able to weaken filter paper in a concentration-dependent manner (Fig. 1). The action on filter paper discs was clearly visible as production of paper fragments in suspension. To clearly observe the production of paper fragments, an incubation was needed with vigorous shaking in at least 2 ml of buffer. The fragments were more abundant as the concentration increased, and CP produced a higher amount of fragments than Pop1. Unlike the incubations with CP and Pop1, when paper discs were incubated with buffer or BSA, a very poor amount of fragments was detectable. The spectrophotometric analysis of the suspensions allowed quantification of the paper fragments produced, showing that the minimum active concentration was $3\times10^{-7}$M for CP and $3\times10^{-6}$M for Pop1 (Fig. 2).

As expected on the basis of previously obtained results with CP on carboxymethylcellulose (de Oliveira et al. 2011), during the action of CP and Pop1 on filter paper, no detectable amount of reducing sugars was produced (data not shown). It has recently been shown that glycosyl hydrolases GH61 can internally cleave cellulose molecules in a direct oxidative way that does not lead to the generation of new reducing ends (Westereng et al. 2011). In order to exclude this mechanism, we tested whether CP could take advantage, on filter paper, of the incubation conditions suitable to these enzymes. GH61 are metal-dependent enzymes and require an external electron donor. Any trace of copper from the solution or substrate can be enough to activate these enzymes, but GH61s are generally blocked by the presence of a metal chelator, such as EDTA (Harris et al. 2010; Quinlan et al. 2011). However, in its weakening activity, CP was neither blocked in the presence of EDTA nor amplified in the presence of copper ($CuSO_4$) and ascorbic acid (electrons donor) (Electronic supplementary material (ESM), Fig. S1).

### Effect on Avicel, cotton fibres and chitin

The effect of CP was analyzed on other cellulosic materials: Avicel (crystalline cellulose) and cotton fibres. Cotton fibres were firstly mercerized and the effect of CP was observed after sonication, in accordance to other similar works carried out with expansin-like proteins (Quiroz-Castañeda et al. 2011; Saloheimo et al. 2002).

CP was able to act both on Avicel and cotton. When Avicel was incubated with CP, it showed fragmentation into

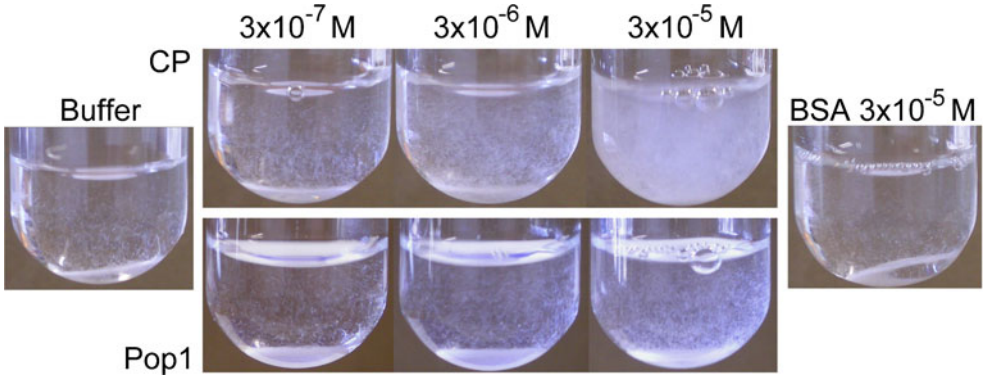


**Fig. 1** Weakening activity of CP and Pop1 on filter paper. Each tube contained a filter paper disc (6 mg) in 2 ml of 50 mM sodium acetate buffer (pH5.0), and CP or Pop1 at three different concentrations ($3\times10^{-7}$, $3\times10^{-6}$ and $3\times10^{-5}$M). Buffer only or buffer containing the protein bovine serum albumin (BSA) at the highest concentration ($3\times10^{-5}$M) were used as negative controls. The action was visible as paper fragments released in suspension from the paper disc (discs visible on the bottom of the tubes) after 48 h, at 38 °C, with shaking at 320 rpm

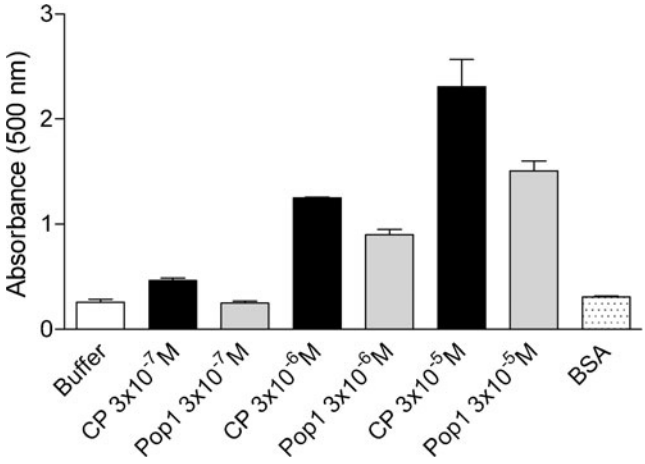


**Fig. 2** Quantification by spectrophotometer analysis of the paper fragments produced. Absorbance at 500 nm was measured on 1 ml of suspension at the end of the incubation period with paper discs (48 h, 38 °C, 320 rpm). *Error bars* indicate the standard deviation of three measurements

smaller particles that were observable on most of the sample (Fig. 3a). Some particles remained apparently intact. However when Avicel was incubated with buffer or BSA it showed principally intact particles and only some small particles. The effect was quantified in about 15±6 intact particles (length≥50 μm) per field analysed at ×100 after incubation with CP, against about 33±8 intact particles after incubation with buffer or BSA ($n$=3).

Cotton incubated with CP showed breakage of the fibres and defibration (Fig. 3b). Conversely, fibres remained essentially undamaged by sonication when incubated with buffer only or BSA. The effect was quantified in about 95 broken/defibrated fibres per 100 fibres analysed after incubation with CP, and about 1 or 10 damaged fibres per 100 fibres analysed after incubation with buffer or BSA, respectively. No swollen areas or enlarged "bubbles", as described by Saloheimo et al. (2002) and Quiroz-Castañeda et al. (2011), were observed on CP-treated fibres.

On chitin and colloidal chitin CP and Pop1 did not cause any apparent activity (expansion or weakening) as observed by light microscope (ESM, Fig. S2).

## Optimum pH and temperature

Since the spectrophotometric analysis turned out to be a reliable method of quantifying the activity of CP and Pop1 on filter paper, the same system was employed to determine the optimal values of pH and temperature for the weakening activity shown by CP. The best conditions turned out to be pH5.0 and 38 °C (Fig. 4). However, CP worked with all the pH values tested (Fig. 4a) and with all the temperatures (Fig. 4b). In addition, the incubation in distilled water increased the production of paper fragments by CP as compared to the incubation in buffer pH5.0 (Fig. 4b).

## Binding to polysaccharides

The binding ability of CP and Pop1 was tested on cellulose (filter paper and Avicel), chitin, colloidal chitin and β-glucan. The ability of CP and Pop1 to bind the substrates was detected by SDS-PAGE, analyzing aliquots of the supernatants in order to visualize the unbound fraction.

Results showed that neither CP nor Pop1 were able to bind cellulose (Fig. 5a). Further experiments performed with CP showed that even changing the incubation conditions (temperature and buffer) or the time of sampling (few minutes), or diminishing the concentration, the binding between

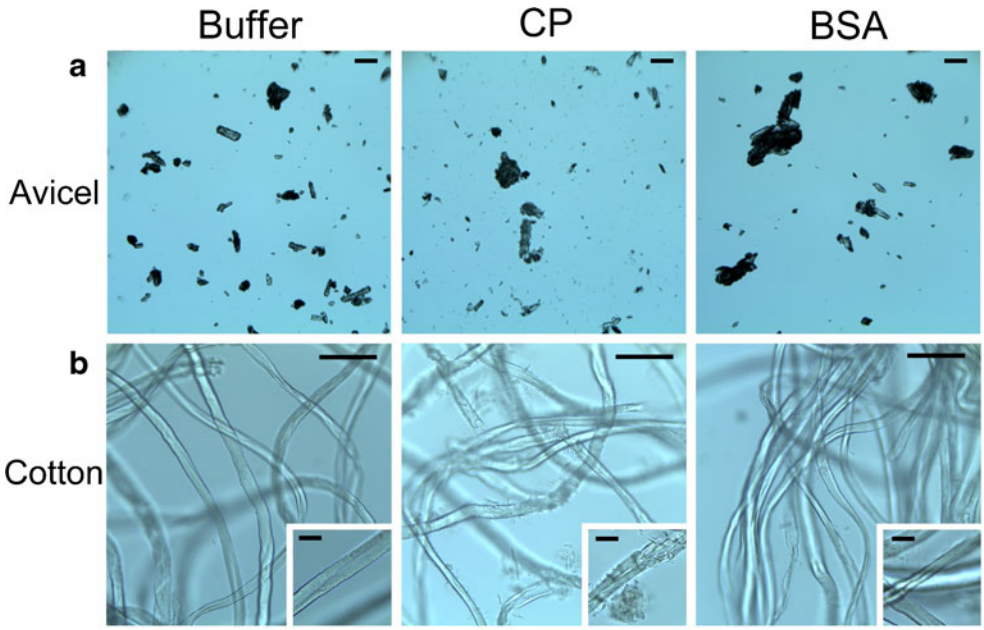


**Fig. 3** Light microscopy of Avicel and cotton fibres treated with CP $3\times10^{-5}$M. **a** Avicel (1 mg) incubated with CP in 0.5 ml of 50 mM acetate buffer (pH5.0) for 24 h, at 38 °C, shaking at 320 rpm. Bars= 100 μm. **b** Mercerized cotton fibres (1 mg) incubated for 24 h (38 °C, 320 rpm) with CP in 0.5 ml of 50 mM acetate buffer (pH5.0), and then sonicated for 1 min at 25 μm. Bars=100 and 25 μm (images for details). Buffer only or buffer containing BSA $3\times10^{-5}$M were used as controls

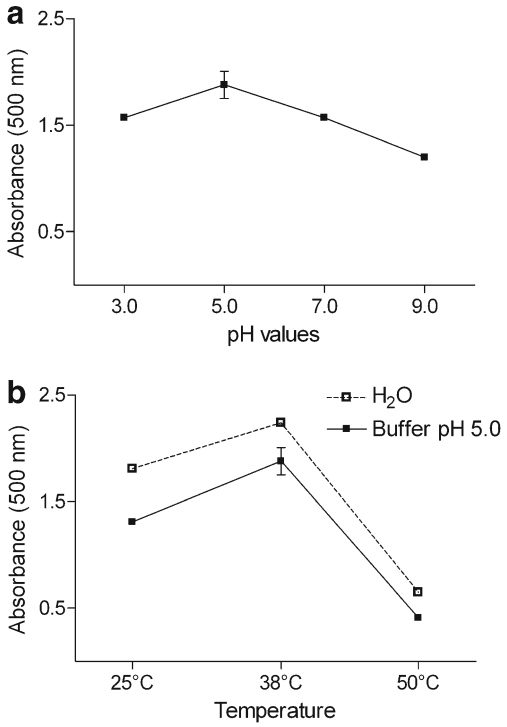


**Fig. 4** Optimum pH and temperature for the activity of CP on cellulose. The values were determined by measuring the absorbance at 500 nm of the paper fragments produced after 48 h of incubation with CP $3\times10^{-5}$M. **a** The optimum pH was established at 38 °C incubating CP in 2 ml of 50 mM citrate–phosphate buffer (pH3.0, 5.0 or 7.0) or Tris–HCl buffer (pH9.0), with shaking at 320 rpm. **b** The optimum temperature was determined both in 50 mM citrate–phosphate buffer (pH5.0) and distilled water, at 25, 38 and 50 °C, with shaking at 320 rpm. Controls (buffer only) were prepared for each experiment, and their absorbance value was subtracted from the value obtained in the CP-treated samples. *Error bars* indicate the standard deviation of three measurements

CP and cellulose did not occur (Table 1). CP and Pop1 were also unable to bind β-1,3-glucan (Table 1).

On the contrary, both CP and Pop1 were able to bind chitin and colloidal chitin (Table 1). CP bound to chitin fragments very quickly (1 min) and compared to Pop1 the binding occurred earlier and to a greater extent (Fig. 5b). The binding capacity of CP and Pop1 was the same both in the presence of chitin fragments and colloidal chitin (ESM, Fig. S3a). Therefore, we used colloidal chitin to determine the kinetic curve: the binding parameters for CP turned out to be $B_{max}$=2.07±0.19 μmol/g of chitin, and $K_d$=40.56± 9.50 μM (ESM, Fig. S3b). The $K_d$ value of Pop1 was of at least tenfold higher (>400 μM), due to the low binding affinity, in accordance with the SDS-PAGE result (Fig. 5b).

## Combined action on filter paper by CP and cellulase

As expansin-like proteins can enhance the yield of reducing sugars produced by hydrolytic enzymes when applied together on cellulosic materials (Chen et al. 2010; Kim et al. 2009; Lee et al. 2010; Quiroz-Castañeda et al. 2011; Wang et al. 2010, 2011; Zhou et al. 2011), accordingly the synergistic effect of CP and cellulases was tested in the present study.

CP was incubated with filter paper for 48 h at 38 °C using two different concentrations ($3\times10^{-6}$ and $3\times10^{-5}$M) and applying the conditions used for the previous experiments. Subsequently, 1 U of cellulase from *T. viride* was added to the tubes, and further incubation (3 h, 38 °C) was performed.

Results showed that the incubation with CP increased the yield of reducing sugars produced by cellulase by about 50 % more than the incubation with buffer only (Fig. 6). However, the same enhancing effect was also found with BSA. We were also unable to find a significant yield increase compared to BSA even when reducing the amount of cellulase to 0.2 U/ml (the minimum active concentration), or

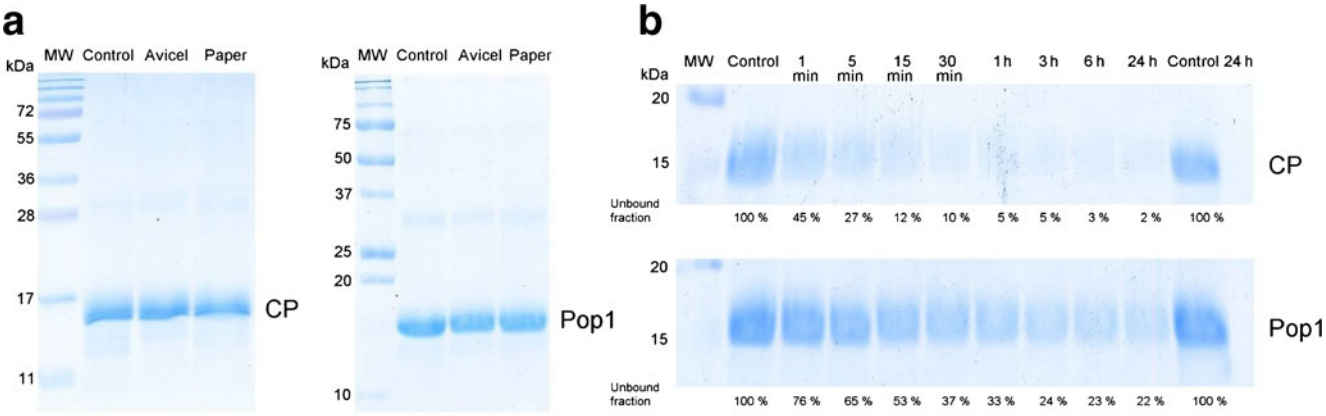


**Fig. 5** Binding assay with cellulose and chitin. **a** CP and Pop1 $3\times10^{-5}$ M incubated with filter paper (6-mg disc) for 48 h, or 40 mg of Avicel PH-101 for 72 h. **b** Binding over time (from 1 min to 24 h) of CP and Pop1 $3\times10^{-5}$M to chitin from crab shells. All the incubations were performed in 50 mM acetate buffer (pH5.0), at 38 °C, with shaking at 320 rpm. Aliquots of the supernatants (unbound fraction) were centrifuged and analyzed by 15 % SDS–PAGE and Coomassie blue staining. Percentage values were calculated with Kodak MI software (Carestream Health, Inc.). *Control*, protein not incubated. *Control 24 h*, protein incubated for 24 h without any substrate

**Table 1** Binding assays performed with CP

| Substrate | Incubation conditions | Binding result |
|---|---|---|
| Filter paper | Acetate buffer (pH5.0), 48 h, 25 °C | Negative |
| | Acetate buffer (pH5.0), 48 h, 38 °C | Negative (see Fig. 5a) |
| | Acetate buffer (pH5.0), 48 h, 50 °C | Negative |
| | Acetate buffer (pH5.0), 48 h, 38 °C, CP $3\times10^{-6}$M | Negative |
| | Acetate buffer (pH5.0), 1 min, 38 °C | Negative |
| | Acetate buffer (pH5.0), 5 min, 38 °C | Negative |
| | Acetate buffer (pH5.0), 15 min, 38 °C | Negative |
| | Acetate buffer (pH5.0), 30 min, 38 °C | Negative |
| | Acetate buffer (pH5.0), 1 h, 38 °C | Negative |
| | Acetate buffer (pH5.0), 3 h, 38 °C | Negative |
| | Acetate buffer (pH5.0), 6 h, 38 °C | Negative |
| | Acetate buffer (pH5.0), 24 h, 38 °C | Negative |
| | Distilled water, 48 h, 25 °C | Negative |
| | Distilled water, 48 h, 38 °C | Negative |
| | Distilled water, 48 h, 50 °C | Negative |
| Avicel | Acetate buffer (pH5.0), 72 h, 38 °C | Negative (see Fig. 5a) |
| | Distilled water, 24 h, 38 °C | Negative |
| Chitin | Acetate buffer (pH5.0), 72 h, 38 °C | Positive |
| | Acetate buffer (pH5.0), 1, 5, 15, 30 min; 1, 3, 6, 24 h, 38 °C | Positive (see Fig. 5b) |
| Colloidal chitin | Acetate buffer (pH5.0), 72 h, 38 °C | Positive (see Fig. S3) |
| β-1,3-glucan | Acetate buffer (pH5.0), 1, 5, 15, 30 min; 1, 3, 6, 24 h, 38 °C | Negative |

All the assays were performed with the concentration $3\times10^{-5}$M (except where specified). Acetate buffer (pH5.0) was used at the concentration 50 mM. Incubations were carried out with shaking at 320 rpm. Aliquots of the supernatants (unbound fraction) were centrifuged and analyzed by 15 % SDS–PAGE and Coomassie blue staining comparing to a control (CP not incubated)

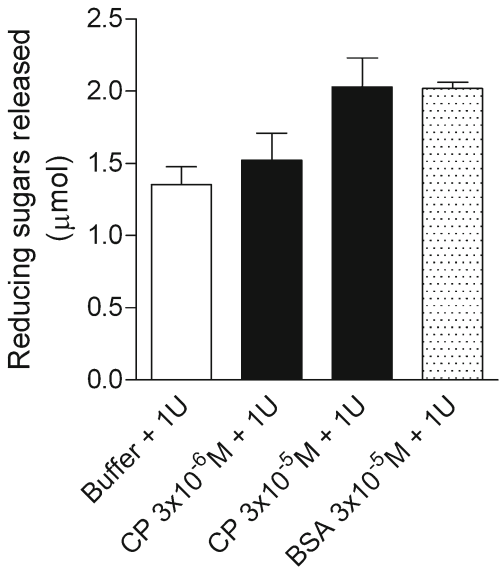


**Fig. 6** Synergistic effect of CP and cellulase on filter paper. Filter paper discs were incubated with CP $3\times10^{-6}$ or $3\times10^{-5}$M in 1 ml of 50 mM acetate buffer (pH5.0) for 48 h, at 38 °C, with shaking at 320 rpm. Subsequently, 1 U of cellulase from *Trichoderma viride* was added to each tube and further incubation was performed (3 h at 38 °C, 320 rpm) before measuring reducing sugars released by DNS method. Buffer only or buffer containing BSA $3\times10^{-5}$M were used as negative controls. Concentration of reducing sugars was determined comparing to a glucose calibration curve. *Error bars* indicate the standard deviation of three measurements

diminishing the incubation time with CP (24 h or no incubation) before adding 1 U of cellulase (data not shown).

## Discussion

In the present work, we have shown a novel activity concerning CP never previously reported for proteins of the cerato-platanin family. CP, like the orthologous protein Pop1, weakened filter paper in a concentration-dependent manner. No detectable amount of reducing sugars was released by CP and Pop1 during the action on filter paper, thus confirming a lack of β–1,4–glucanase activity that had already been reported for CP on carboxymethylcellulose (de Oliveira et al. 2011). At the same time, we also excluded the possibility that the weakening activity was the result of an oxidative mechanism like that recently found for the glycosyl hydrolases GH61 that does not lead to the generation of new reducing ends (Westereng et al. 2011; Žifčáková and Baldrian 2012). In fact, CP was neither blocked in the presence of a metal chelator (EDTA), which normally prevents GH61s, nor enhanced by the addition of metal (copper) and of a redox-active cofactor (ascorbate), in contrast to that observed with GH61s (Harris et al. 2010; Quinlan et al. 2011).

In order to further understand the nature of the weakening activity shown by CP and Pop1, other substrates such as Avicel, cotton, chitin, colloidal chitin and β-1,3-glucan were used. Avicel and cotton turned out to be susceptible to the

action of CP, suggesting an interaction with these cellulosic materials. The interaction, however, did not involve a stable bond with the substrates, since both CP and Pop1 were recovered from the supernatants of the incubation mixtures, as revealed by SDS-PAGE. The proteins did not bind even β-1,3-glucan. However, both CP and Pop1 bound quickly to chitin and colloidal chitin. CP bound to chitin with the following binding parameters: $B_{max}$=2.07±0.19 μmol/g of chitin, $K_d$=40.56±9.50 μM, whereas Pop1 had an affinity of about tenfold lower.

A weakening activity on cellulose without enzymatic activity is a common feature of expansins and expansin-like proteins (Kerff et al. 2008; McQueen-Mason and Cosgrove 1994; Saloheimo et al. 2002; Yennawar et al. 2006). In particular, the effect caused on filter paper by CP and Pop1 was very similar to that reported by Chen et al. (2010) for the expansin-like protein Swo1 from *Aspergillus fumigatus*, while on Avicel the effect was very similar to that reported both by Chen et al. (2010) and Wang et al. (2011). Conversely, on cotton CP did not cause the formation of swollen areas or enlarged "bubbles" as reported for two expansin-like proteins (Quiroz-Castañeda et al. 2011; Saloheimo et al. 2002), but breakage and defibration.

Pop1 acted to a lesser extent than CP and the minimum active concentration was $3\times10^{-7}$M for CP and $3\times10^{-6}$M for Pop1. The concentrations range used in this study (from $3\times10^{-7}$ to $3\times10^{-5}$M) was substantially in accordance with those used to assay other expansin-like proteins from fungi and bacteria (Chen et al. 2010; Georgelis et al. 2011; Kerff et al. 2008; Kim et al. 2009; Saloheimo et al. 2002). However, it is worth emphasizing that CP, when used as a resistance inducer, is reported active at a minimum concentration of about $2\times10^{-5}$M (Fontana et al. 2008), i.e. almost 100-fold higher than the minimum required to show the weakening activity on filter paper.

Optimum pH and temperature for the activity of CP were also determined by spectrophotometric analysis that turned out to be a reliable method of quantifying the weakening activity on filter paper. The optimum values were pH5.0 and 38 °C, substantially in accordance with expansins and expansin-like proteins (Chen et al. 2010; Kim et al. 2009; Quiroz-Castañeda et al. 2011; Sampedro and Cosgrove 2005; Wang et al. 2011).

Canonical expansins are small proteins of about 26 kDa consisting of two compact domains: the N-terminal domain (D1) constitutes the double ψβ-barrel fold; the C-terminal domain (D2) forms an Ig-like β-sandwich fold (Kerff et al. 2008; Yennawar et al. 2006). Georgelis et al. (2011) showed that the essential role of D2 consists in the binding of the bacterial expansin EXLX1 to cellulose and to matrix polysaccharides, and proposed D2 as the founding member of a new family of carbohydrate binding modules. Since CP lacks D2, this could account for its inability to bind cellulose.

However when D1 from EXLX1 was separated from D2 it did not weaken paper (Georgelis et al. 2011), but it should be noted that LOOS1, the only expansin-like protein found in nature without D2, showed instead both binding ability and disrupting activity on cellulose (Quiroz-Castañeda et al. 2011). Thus, the behaviour of CP differed from that of both EXLX1 and LOOS1.

Georgelis et al. (2011) report that several polar residues in D1 could contribute to the weakening activity of EXLX1. Most notably Asp-82, an amino acid considered essential for this activity and also well conserved among expansins. Cerato-platanins have the residue Asp-77 well conserved among members of the family (de Oliveira et al. 2011), and given the results presented in this paper, its role will be the object of further investigation.

In literature, experimental evidences concerning the synergism between expansin-like proteins and cellulases exist (Chen et al. 2010; Kim et al. 2009; Lee et al. 2010; Quiroz-Castañeda et al. 2011; Wang et al. 2010, 2011; Zhou et al. 2011). In all these studies, BSA was used as a nonspecific control protein. In the present work, CP did not enhance the cellulose hydrolysis in the presence of cellulase beyond that observed with BSA, which leads us to think of a general protein effect caused by CP (e.g. increased enzyme stability). A similar result had previously been reported only for EXLX1, even if diminishing the cellulase concentration made the synergistic effect evident (Kerff et al. 2008; Kim et al. 2009). Here, even diminishing the cellulase concentration the result did not change. Since CP is the only protein with an expansin-like domain that does not show a synergistic effect with cellulases and the only protein active on cellulose without binding to it, the lack of synergistic effect could somehow be related to its inability to bind. The other assumptions are that the paper fragments produced by CP were too coarse to produce any significantly increase in the activity of the commercial preparation of cellulase used, corresponding principally to endoglucanase (Quiroz-Castañeda et al. 2011), ore that pure cellulose is not the best substrate to see a stimulatory effect, as observed for example by Harris et al. (2010) with GH61A.

Interestingly, coarse fragments of chitin or colloidal chitin were bound both by CP and Pop1, but they did not appear damaged and/or altered by the incubations, as observed by optical microscope. The binding result was partially expected for two reasons. Firstly, de Oliveira et al. (2011) showed that the residues involved in binding to β-(1,4)-linked *N*-acetyl-D-glucosamine oligomers are the most conserved in the CP family; here, we unequivocally demonstrated the ability of both CP and Pop1 to bind rapidly long chitin polymers. Secondly, the ability to bind chitin had been already reported for expansin-like proteins from fungi, although it is not clear whether these proteins caused changes in the chitin structure (Chen et al. 2010; Quiroz-Castañeda et al. 2011).

In conclusion, we think that CP can be considered a novel one-domain expansin-like protein that lacks the

ability to bind cellulose, but that retains the ability to weaken cellulose by means of a non-enzymatic mechanism that needs to be clarified. This could be the trademark of cerato-platanins, as the results obtained with Pop1 seem to confirm. Pop1 has about 38 % of its amino acids different from those of CP (Comparini et al. 2009), and this could justify the difference found in the weakening activity and in the chitin binding. However both CP and Pop1 are enough representative of the CP family, as shown by the phylogenetic tree (ESM, Fig. S4), and thus there are substantial reasons to believe that the activity on cellulose without binding to it, although with some differences, is a common feature of cerato-platanins.

In partial support of this hypothesis, Epl1, another member of the CP family, has recently been reported unable to bind cellulose (while it was able to bind chitin), but no test aiming to evaluate an activity on cellulose was made (Frischmann et al. 2013). In view of the results shown for CP and Pop1, we think that hereafter it is worth performing for cerato-platanins a test with filter paper following the methods described in the present paper.

Finally, we can speculate that CP has a double biological role in fungal life. The localization in the cell wall and the ability to bind chitin demonstrate a clear structural role in the fungal cell wall; however, an involvement of CP in the remodelling and enlargement of the cell wall during the hyphal growth and the formation of chlamydospores cannot be excluded (Baccelli et al. 2012). An additional role could be hypothesized in the fungus-plant interaction: the weakening activity on the plant cellulose could facilitate the host colonization.

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