Secretion, purification and activity of two recombinant pepper endo-β-1,4-glucanases expressed in the yeast *Pichia pastoris*

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Abstract Two pepper endo-β-1,4-glucanases, involved in fruit softening (cCel1) and leaf and flower abscission (cCel2), have been expressed in *Pichia pastoris*. Secretion was obtained by using either the mouse α-factor signal (cCel1) or the native signal sequence (cCel2). Times for optimal expression of the two proteins were different and cCel2 appeared very sensitive to proteolytic degradation. A one-step purification protocol yielded cCel2 in a pure form, while an additional chromatography step was necessary to purify cCel1. The two recombinant proteins are highly active and able to degrade carboxymethylcellulose in viscometric assays. Moreover, they have both a molecular mass (54 kDa) and an isoelectric point (7.2 for cCel2 and 8.5 for cCel1) equal to those of the native proteins, thus suggesting that post-translational modifications have properly occurred.

Key words: Endo-β-1,4-glucanase; *Pichia pastoris*; Heterologous expression; *Capsicum annuum*

1. Introduction

Though located outside the plasma membrane, the plant cell wall is now regarded as a cell compartment which is involved in a number of important functions, besides the long known provision of strength and shape to the cell [1]. For instance, precisely located weakenings of the cell wall result in a directional control of cell expansion, hence of cell shape.

Physiological processes such as abscission of leaves, flowers and fruits and softening of fleshy fruits are based on a more or less severe hydrolysis of the cell wall whose effects vary according to physiological process and plant type [2].

This variability is the consequence of different genetic programmes, but also reflects the biochemical complexity of the plant cell wall structure [3]. A very important consequence of this complexity is that plants require a large set of different enzymes to carry out the physiological processes where modifications of the cell wall properties are required [4–9].

In many of the studies dealing with the softening of fruits and abscission of organs, particular interest has been given to an enzyme which is able in vitro to hydrolyse the soluble cellulose derivative carboxymethylcellulose (CMC). For this reason the enzyme was thought to in vivo hydrolyse the crystalline cellulose and was therefore named cellulase. However, contrary to its bacterial and fungal counterparts, it does not contain the cellulose binding domain and it has never been shown to hydrolyse the crystalline cellulose [10].

Endo-β-1,4-glucanase (EGase; EC 3.2.1.4), which refers to the bond cleaved by the enzyme rather than to its substrate, is now the most commonly used name for this enzyme. This term seems to be more appropriate since, besides cellulose, the plant cell walls contain other polysaccharides with β-1,4-glucan links such as the xyloglucans. The uncertainty about these enzymes' substrate(s) is further complicated by the fact that a plant can have multiple forms of EGase [5,11,12].

In pepper we have recently demonstrated that, upon triggering the fruit ripening and the abscission of leaves and flowers, two EGase genes are expressed at very high levels. In particular, one of them (cCel1) is especially important for the softening of fruits, while the other (cCel2) appears relevant for the abscission of leaves and flowers [5,13].

In order to clarify possible differences between the biochemical characteristics of the two pepper EGases, we have endeavoured to in vitro express both cCel1 and cCel2 proteins by using the yeast *Pichia pastoris* system which is known to yield large amounts of active recombinant enzymes. In this paper the production of the two recombinant EGases in active form, their purification and a partial characterisation of their biochemical characteristics are described.

2. Materials and methods

2.1. Construction of the expression plasmids

Full-length cDNAs encoding the cCel1 and cCel2 EGases were isolated from two cDNA libraries representing pepper mRNAs of ripe fruits and activated leaf abscission zones, respectively ([13] and unpublished data). For the secretion of the recombinant enzymes, both the native EGase leading signal and the mouse α-factor secretion signal (supplied with the Pichia expression kit; Invitrogen, San Diego, CA) were used. In the case of the native EGase signals, *Eco*RV and *Not*I sites flanking the entire open reading frames (ORFs) of the two EGases were introduced by PCR using synthetic oligonucleotides: cCel1: (CXP1) 5′-GGCC**GATATC**CGCTTGTTCAACGAATA-3′ and (CXP2) 5′-TGTTGAGA**GCGGCCGC**TTACTGTGAAACATTCTTCGTGCTAA-3′; cCel2 (CXP5) 5′-TTGA**GATATC**CGCCCATGCTTTTAACAT-3′ and (CXP6) 5′-AGATGATT**GCGGCCGC**CTAGACTGCTGTAGATTCTGCAGATA-3′. Bold characters in the oligomers indicate the restriction sites. Denaturation, annealing and extension temperatures of 95°C (1 min), 55°C (1 min), 72°C (1.5 min), respectively, were used in the PCR experiments. After digestion, the two fragments were ligated at the *Sna*BI-*Not*I sites of the pPIC3 Pichia expression vector, yielding two constructs named Cel1pPIC3 and Cel2pPIC3, respectively.

In the case of the α-factor signal, the putative mature protein had to be cloned in frame with the α-factor sequence contained in the pPIC9 Pichia expression vector. For cCel1, the cDNA fragment corresponding to the mature protein was obtained using the already described CXP2 and CXP3 (5′-GGCC**GATATC**GCTCAAGATTATAAAGATG-3′) oligomers in a PCR experiment as above. This fragment was flanked by *Eco*RV-*Not*I sites for its cloning in frame with the α-factor leading signal. For cCel2, the PCR fragment to be cloned

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Abbreviations: EGase, endo-β-1,4-glucanase; BSA, bovine serum albumin; AOX1, alcohol oxidase 1; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CMC, carboxymethylcellulose; ORF, open reading frame

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into pPIC9 was flanked by *Sma*I-*Not*I sites, and was obtained using the following oligonucleotides: CXP7 5′-TCC**CCCGGG**TCTCAA-GATTACTCTAA-3′ and the above described CXP6. The two different constructs were named Cel1pPIC9 and Cel2pPIC9, respectively.

All the constructs, linearised with either *Sal*I or *Bgl*II endonucleases, were used for the transformation of *Pichia*. As negative controls, the pPIC3 and pPIC9 vectors linearised with the same enzymes were used for the transformation of the yeast.

2.2. Screening of recombinant colonies and expression

Recombinant clones, linearised with either *Sal*I or *Bgl*II, were picked and screened for their methanol utilisation phenotypes following the protocol supplied by the manufacturer.

In order to find the best EGase secreting clones, all the selected colonies were replica-plated onto minimal methanol (MM) plates and allowed to grow upside-down for 2 days at 30°C; 100 μl of methanol was supplied every 24 h inside the lid of the plate. On the third day, the plates were overlaid with a nitrocellulose disc previously wetted in 0.5% methanol, and left at 30°C for an additional 24 h. Then, the discs of nitrocellulose were lifted from the plates, rinsed with water, and probed with a polyclonal antibody raised against the cCel1 EGase which has been shown to cross-react with both the cCel1 and cCel2 proteins [5].

The most promising clones were used for a small-scale expression study by using the BMGY/BMMY (buffered complex glycerol/buffered complex methanol) media system in 50 ml plastic tubes containing 5 ml of medium. After shaking at 30°C and 250 rpm for 2 days, 1 ml of culture of each clone was centrifuged for 1 min at maximum speed in a microcentrifuge and the supernatant was precipitated with ammonium sulphate to 80% saturation. Proteins contained in the culture medium were assayed viscometrically for their EGase activity [14], which was converted to relative units of activity according to the method of Almin et al. [15]. In order to rule out a possible background carboxymethylcellulase activity of the *Pichia* cells, negative controls consisting of colonies transformed with plasmids carrying no EGase inserts were always included in the expression experiments. A colony expressing BSA (supplied with the Pichia Expression Kit) was also included as a positive control. Proteins from the culture medium were then separated by means of SDS-PAGE, using the buffer system of Laemmli [16], and either stained with Coomassie blue or electroblotted [17] and probed with the cCel1 antibody [5]. The best expressing clones were used for an intermediate-scale expression in baffled flasks using the same conditions described above and the BMG/BMM (buffered minimal glycerol/buffered minimal methanol) media system, supplemented with 3% casamino acids (Sigma, St. Louis, MO) in order to minimise the extracellular protease activity usually present in the *Pichia* cultures.

2.3. Protein purification and native isoelectric focusing (IEF)

The same purification protocol was used for both EGases. 100 ml of culture supernatant precipitated with ammonium sulphate was centrifuged at 15 000×*g* for 20 min, resuspended in 5 ml of 40 mM MES buffer pH 6.0 and loaded onto a 2×15 cm CMC (Sigma) column at room temperature. Proteins were eluted with a 0–0.5 M NaCl gradient in 50 mM MES buffer pH 6.0. Fractions containing EGase activity were pooled and precipitated with ammonium sulphate to 80% saturation. In the case of the only cCel1 enzyme, this procedure was followed by another chromatographic step consisting of a gel filtration column (1.6×50 cm) made with Sephacryl S200-HR (Pharmacia, Uppsala, Sweden).

All the purification steps were followed by means of SDS-PAGE stained with the silver method [18], and the identity of the two purified proteins was checked by means of Western analysis. Native IEF was carried out at 4°C in a 20×14 vertical gel system, as described in [19], using ampholine carriers of pH range 5.0–10.5 (Pharmacia). pH gradient and p*I* of the EGases were determined as described in [5].

3. Results and discussion

3.1. Construction of plasmids for the expression in Pichia pastoris of the pepper EGases cCel1 and cCel2

Secretion of the recombinant EGases by *Pichia pastoris* was chosen on the basis that in planta the two proteins to be studied must be secreted in order to perform their task at the level of the primary cell wall [13]. To this purpose, the secretion into the medium was planned using either the EGase native signal sequence or a mouse α-factor signal with both proteins.

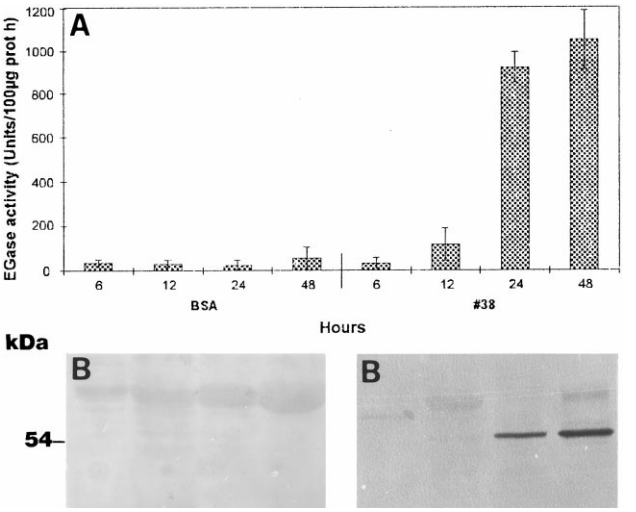

Fig. 1. Time course of cCel1 protein expression by clone 38. A steep increase of EGase activity (A) and secreted EGase protein (B) is observed between 12 and 24 h of induction. A parallel control was performed using a BSA expressing clone. EGase activity values are the average of four independent experiments ± standard error. Lanes in panel B correspond to the clones of the histogram above it.

In the case of the α-factor signal, the putative cleavage site of the native leading peptides had to be determined. A comparison among the sequences of the two pepper EGases (accession numbers: cCel1 X97188; cCel2 X97190) and those from avocado, bean [20], and pea [21], together with the hydrophobicity profiles of the sequences, allowed us to determine the location of the putative cleavage site of cCel1 between the 22nd and the 23rd amino acid, while for cCel2 it was determined to be between the 29th and the 30th amino acid (not shown).

The type of linearisation of the constructs to be used for the transformation of the *Pichia* cells can influence the recombination into the yeast genome. Briefly, the use of *Bgl*II endonuclease will yield two cuts at the level of the flanking regions of the alcohol oxidase gene 1 (AOX1), thus determining its replacement in the *Pichia* genome and the induction of the recombinant phenotype Mut^s (methanol utilisation slow). The use of *Sal*I for the linearisation of the constructs determines one single cut at the level of the HIS4 gene and the recombination will occur preferentially at the *his4* locus of the *Pichia* genome. In this case the most probable phenotype of the recombinant colonies will be Mut$^+$ (methanol utilisation fast). Due to the impossibility of predicting which linearisation method yields the highest level of recombinant protein expression, all the constructs were linearised in both manners. Therefore, transformation of *Pichia* GS115 cells was carried out using four different types of constructs for each EGase. Transformation of *Pichia* cells was also carried out with the pPIC3 and pPIC9 plasmids linearised in both manners and without inserts, to be used as negative controls.

3.2. Screening of the recombinant colonies

Since at least 100 recombinant colonies were screened for each type of construct, a minimum of 800 clones had to be

analysed in order to find those with the best expression performances. This time-consuming screening could be shortened by setting up a technique based on a Western analysis of the colonies grown on plates under inducing conditions (MM plates). Such a fast screening allowed us to restrict the expression studies in liquid medium to seven colonies for cCel1 and 15 colonies for cCel2 (not shown).

The next step of the screening consisted in a small-scale culture analysis. The parameters used to detect the best expressing clone were: the lowest background of secreted proteins; the highest amount of secreted EGase protein; the highest activity of the recombinant enzymes.

The selected cCel1 expressing clone (number 38) had the secretion of the recombinant protein driven by the mouse α-factor leading signal and was characterised by a Mut^+ phenotype while, contrary to this, the best cCel2 secreting clone (6B.31) had the secretion of the recombinant protein driven by the cCel2 leading signal, and showed a Mut^s phenotype (not shown).

3.3. Expression and purification of cCel1 and cCel2 proteins

The time course of expression showed different patterns for the two EGases. In the case of cCel1, a steep increase of both EGase activity (Fig. 1A) and protein level (Fig. 1B) was observed after 24 h of induction, and the rate of expression remained constant after 48 h of induction (not shown). Accordingly, the subsequent experiments of cCel1 expression were carried out with an induction time of 48 h.

The time course of cCel2 expression revealed that a high level of EGase activity and protein production was reached after 6 h of induction, then it increased slightly until 12 h, and decreased thereafter at a constant rate (Fig. 2A). As shown by Western analysis, this decrease was due to the degradation of the 54 kDa cCel2 protein to a peptide of 25 kDa (Fig. 2B). After 96 h of induction, nearly all the cCel2 protein was degraded, and the EGase activity dropped to the background level. On the basis of these results, an induction time of 12 h was adopted for the subsequent analyses of cCel2 expression.

The cationic exchange chromatography technique, based on the use of CMC, was used for the purification of the recombinant EGases (not shown). At room temperature, CMC showed a high binding capacity for these proteins, in spite of being characterised by a lower ion exchange capability compared to other media (e.g. SP-Sepharose). This may be due to the fact that the binding of the EGases to CMC can involve an affinity interaction, more specific than a simple

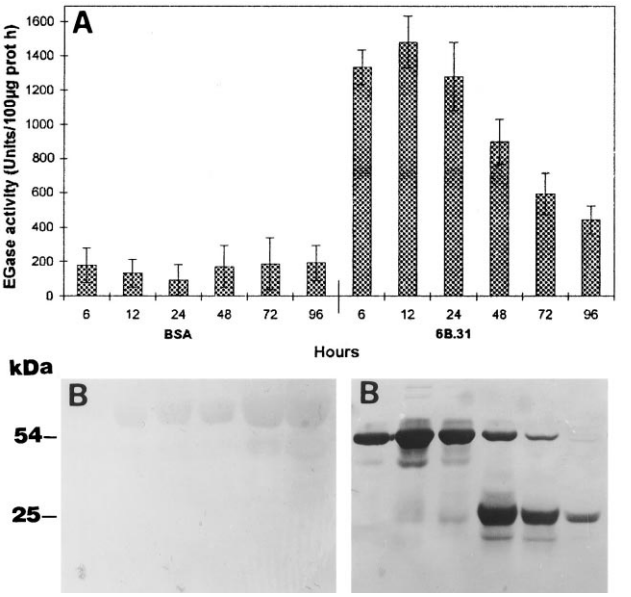

Fig. 2. Time course of cCel2 protein expression by clone 6A.33. A marked increase in EGase activity (A) and EGase protein production (B) is already observed after 6 h of induction. At 12 h, the expression reaches the maximum level and thereafter it decreases at a constant rate. Western analysis (B) shows that the loss of activity is due to degradation of the enzyme to a polypeptide of 25 kDa. At 96 h of expression, nearly all the active 54 kDa protein is degraded. A parallel control was performed using the BSA expressing clone. EGase activity values are the average of four independent experiments ± standard error. Lanes in panel B correspond to the clones of the histogram above it.

ionic link. This one-step purification protocol was enough to obtain a single protein band of 54 kDa in the case of the cCel2 enzyme (Fig. 3A, lane 2), while for the cCel1 protein another purification step was necessary consisting of a gel filtration chromatography in order to get rid of some minor contaminant proteins (not shown). After the gel filtration, a single 54 kDa protein band corresponding to the cCel1 enzyme was obtained (Fig. 3B, lane 3). The identity of the purified proteins was confirmed by Western analysis probed with the cCel1 antibody [5] (Fig. 3A, lane 3; Fig. 3B, lane 4). In both cases, the apparent molecular mass of the recombinant proteins (i.e. 54 kDa) was the same as that of the native proteins [5].

The purified recombinant proteins were subjected to native isoelectric focusing which evidenced that cCel1 has a p*I* of 8.5

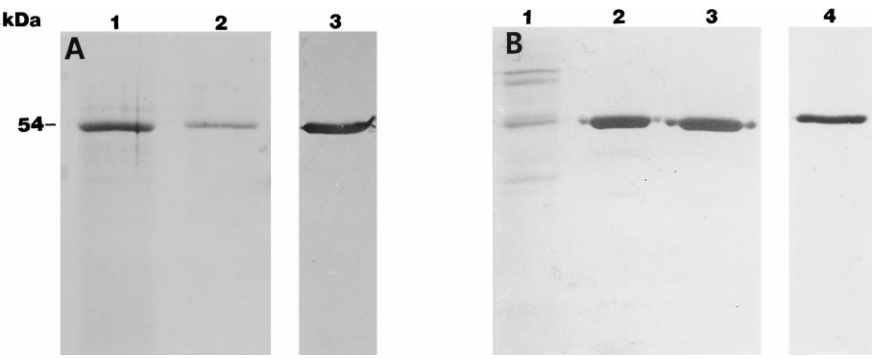

Fig. 3. Silver-stained SDS-PAGE of: (A) proteins secreted by the cCel2 expressing clone into the culture medium (lane 1) and proteins eluted from the CMC purification columns (lane 2); (B) proteins secreted by the cCel1 expressing clone (lane 1) and proteins eluted from the CMC (lane 2) and from the gel filtration columns (lane 3). The purified proteins were both recognised by the cCel1 antibody (A, lane 3; B, lane 4).

while cCel2 has a p*I* of 7.2 (not shown). Also in this case there is a complete identity with the p*I* values determined for the native proteins [5].

Since the recombinant EGases are highly active and have the same molecular mass and isoelectric point as their native counterparts, it is likely that the recombinant cCel1 and cCel2 enzymes are characterised by the same post-translational modifications of the native proteins.

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