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## Synergistic antifungal activity of two chitin-binding proteins from spindle tree (*Euonymus europaeus* L.)

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**Abstract** Two structurally different chitin-binding proteins were isolated from bark and leaves of the spindle tree (*Euonymus europaeus* L.). Both the small hevein-like chitin-binding protein (Ee-CBP) and the classical class-I chitinase (Ee-chitinase) possess antifungal properties, Ee-CBP being far more potent than Ee-chitinase. In addition, Ee-CBP and Ee-chitinase display a pronounced synergistic effect when added together in the test medium. Determination of the biological activities indicates that the synergism between Ee-CBP and Ee-chitinase relies on a different mode of action. Cloning and sequencing of the corresponding genes further revealed that Ee-CBP and Ee-chitinase are simultaneously expressed in bark and leaf tissues, and hence can act

synergistically *in planta*. Moreover, analysis of the deduced sequences allowed the exact relationship between the structurally different Ee-CBP and Ee-chitinase to be corroborated. Both proteins are synthesized as similar chimeric precursors consisting of an N-terminal hevein domain linked to a C-terminal chitinase-like domain by a hinge region. However, whereas in the case of Ee-chitinase the C-terminal chitinase domain remains linked to the N-terminal hevein domain, the corresponding domain is cleaved from the Ee-CBP-precursor resulting in the formation of the hevein-type Ee-CBP. Since both precursors are—apart from the hinge region between the hevein and chitinase domains—very similar, the Ee-CBP/Ee-chitinase system offers a unique opportunity to study the importance of sequence and/or structural information comprised in the hinge region for the posttranslational processing of the respective precursor proteins.

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**Keywords** Antifungal activity · Chitin-binding protein · *Euonymus* · Hevein · Posttranslational processing

**Abbreviations** AMP: Antimicrobial protein  
CBP: Chitin-binding protein · GlcNAc: N-Acetylglucosamine · HCA: Hydrophobic cluster analysis · MeJA: Methyl jasmonate · PDB: Potato dextrose broth · RACE: Rapid amplification of cDNA ends · SPR: Surface plasmon resonance · UDA: Urtica dioica agglutinin · WGA: Wheat (*Triticum aestivum*) germ agglutinin

### Introduction

Plants express a wide variety of proteins that specifically bind to oligomers of N-acetylglucosamine (GlcNAc) and/or chitin. Though some of these chitin-binding proteins are members of the legume lectin and the Cucurbitaceae phloem lectin families (Van Damme et al.

1998) the great majority definitely belongs to the ubiquitous superfamily of chitin-binding proteins comprising hevein domains. These hevein domains are structural units equivalent to a 43-amino-acid residue chitin-binding protein that was originally identified in rubber tree (*Hevea brasiliensis* L.) latex and was therefore called the 'hevein' domain (Walujono et al. 1975). Since chitin is an important component of the cell wall of fungi and the exoskeleton of arthropods and nematodes, and, in addition, GlcNAc is a prevalent building block of bacterial peptidoglycan, it has been proposed on several occasions that chitin-binding proteins play a role in plant defense (Chrispeels and Raikhel 1991; Raikhel et al. 1993; Peumans and Van Damme 1995).

Chitin-binding proteins are either hololectins comprising exclusively hevein domain(s) or chimeric proteins in which one or more hevein domains are linked to an unrelated domain. All currently known hololectins are built up of protomers consisting of one, two, three, four or seven tandemly arrayed hevein repeats (Yamaguchi et al. 1997; Van Damme et al. 1998). Documented examples of chimerolectins are class-I and class-IV chitinases, which consist of a single N-terminal hevein domain fused to a C-terminal chitinase domain (Collinge et al. 1993; Beintema 1994; Ponstein et al. 1994), and the solanaceous lectins, which, besides multiple hevein domains, also comprise an extensin-like domain (Van Damme et al. 1998). Interestingly, some of the hololectins are the molecular skeletons of large chimeric precursors. For example, hevein and several other hevein-like proteins are formed by the proteolytic removal of a large C-terminal chitinase domain from a large precursor with an overall structure resembling that of class-I/IV chitinases (Van Damme et al. 1998). It should be mentioned, however, that some single-domain chitin-binding proteins are not derived from a large precursor but from a preproprotein with a normal-sized C-terminal propeptide. Irrespective of their origin, single-hevein-domain proteins have received a lot of attention because this group comprises several antimicrobial proteins (AMPs) with potent antifungal/antibacterial activities. Though all small AMPs consist of a single hevein domain there is a marked heterogeneity with respect to the number of cysteine residues and disulfide bonds. Most AMPs contain eight disulfide-linked cysteine residues and in this respect closely resemble the chitin-binding domain of the class-I/IV chitinases. There are also slightly truncated variants with only six disulfide-linked cysteine residues, like Ac-AMP from *Amaranthus caudatus* L. seeds (Broekaert et al. 1992) and Bv-IWF4 from the intercellular washing fluid of *Beta vulgaris* L. (Nielsen et al. 1997). Recently, two AMPs have been identified that contain ten cysteine residues forming five intra-chain disulfide bonds. These AMPs, which were isolated from bark tissue of *Eucommia ulmoides* Oliv (Huang et al. 2002) and *Euonymus europaeus* L. (spindle tree; Van den Bergh et al. 2002), respectively, not only exhibit a potent antimicrobial activity but also possess an unusual stability. Though

both the *Eucommia ulmoides* and *Euonymus europaeus* hevein-like peptides were completely sequenced, their corresponding genes have not been cloned yet. So, it is still unknown whether these AMPs with an extra disulfide bond are derived from a simple prepropeptide or a large chimeric class-I chitinase-like precursor.

This paper describes the molecular cloning and analysis of a cDNA encoding the *Euonymus europaeus* (spindle tree; family Celastraceae) chitin-binding protein (Ee-CBP). Evidence is presented that this AMP from bark tissue is derived from a large precursor with a C-terminal chitinase domain. Besides Ee-CBP, a class-I chitinase that shares a high sequence identity with the precursor of Ee-CBP but contains an N-terminal hevein-like domain with only eight disulfide-bound cysteine residues was isolated and cloned from leaf tissue of the spindle tree. Growth-inhibition assays indicated that the purified chitinase, called Ee-chitinase, and Ee-CBP act synergistically through a complementary mode of antifungal action. The functional implications and physiological relevance of differences in structure and post-translational processing between the two proteins are critically assessed.

## Materials and methods

### Biochemicals

Thyroglobulin, GlcNAc and the chitin oligomers *N,N'*-diacetylchitobiose (chitobiose), *N,N',N''*-triacetylchitotriose (chitotriose) and *N,N',N'',N'''*-tetracetylchitotetraose (chitotetraose) were from Sigma. Glycoproteins were desialylated in 0.1 M trifluoroacetic acid at 80°C for 1 h. After lyophilization, asialo-glycoproteins were diluted in methanol and lyophilized. This procedure was repeated three times to eliminate the remaining trifluoroacetic acid. Sensor chips (CM 5), Hepes-buffered saline (HBS: 10 mM Hepes, 150 mM NaCl, containing 0.05% surfactant P20 and 3.0 mM EDTA, pH 7.4) and all the chemicals required to activate the CM-dextran and immobilize the glycoproteins (100 mM *N*-hydroxysuccinimide, 400 mM *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride, and 1 M ethanolamine hydrochloride adjusted to pH 8.5 with NaOH) were from BIAcore AB (Uppsala, Sweden).

### Isolation and purification of two chitin-binding proteins

Bark and leaf material were collected in February and August from a single spindle tree (*Euonymus europaeus* L.) growing in a local forest (Egenhovenbos, Heverlee, Belgium). The antimicrobial protein Ee-CBP was purified from bark tissue essentially as described by Van den Bergh et al. (2002). Briefly, 100 g of powdered (lyophilized) bark was extracted in 1 l of 0.2 M NaCl. After centrifugation (8,000 g; 10 min) and filtration, the cleared supernatant was loaded onto a column (10 cm long, 5 cm i.d.; approximately 200 ml bed volume) of chitin (Sigma-Aldrich) equilibrated with 0.2 M NaCl. After loading the extract, the column was washed with 0.2 M NaCl until the  $A_{280}$  fell below 0.01. Bound proteins were eluted with 20 mM acetic acid and dialyzed for 48 h in the same buffer. During dialysis the small-sized Ee-CBP selectively permeated through the membrane into the medium (leaving behind other proteins that were retained on the chitin column). To recover Ee-CBP, the dialysate was applied onto a smaller column (5 cm long, 2.5 cm i.d.; 25 ml bed volume) of chitin and the bound Ee-CBP eluted with a small volume of 20 mM acetic acid. The total yield was approximately 10 mg of purified peptide.

For the purification of the *Euonymus europaeus* chitinase, leaves were chosen as the starting material because this tissue exhibited the highest chitinase activity. The whole extraction and purification procedure was performed at 4°C. Leaves (200 g) were homogenized with a Waring blender in 1 l of 0.2 M NaCl containing 0.1% (w/v) ascorbic acid as an antioxidant, centrifuged at 8,000 g for 10 min and filtered through filter paper. The cleared supernatant was adjusted to pH 7.0 with 1 N NaOH and applied onto a column (10 cm long, 5 cm i.d.; approximately 200 ml bed volume) of chitin. After loading the extract, the column was washed as described above, and chitin-binding proteins eluted from the column with 20 mM acetic acid. The most concentrated fractions were pooled, lyophilized and the resulting powder dissolved in 7 ml of 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. This concentrated protein solution was applied onto a column of Sephacryl 100 (60 cm long, 2.6 cm i.d.; approximately 300 ml bed volume) equilibrated with the same buffer to separate the different chitin-binding proteins by gel filtration. Fractions enriched in chitinase were pooled and dialyzed overnight against 25 mM sodium formate (pH 3.8) and applied onto a Mono-S cation-exchange column (type HR5/5 mounted on a Pharmacia FPLC system) equilibrated with 25 mM sodium formate buffer (pH 3.8). The column was washed with 4 ml of the same formate buffer and the proteins eluted with a linear gradient (60 ml) of increasing NaCl concentration (0–0.5 M). Under these conditions, the chitinase eluted in a single symmetrical peak. The fractions with the highest chitinase activity were collected and further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a C8 column (Aquapore RP-300; 50 mm long, 1 mm i.d.; Perkin Elmer). The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient (16 ml) of 0.1% TFA in 80% acetonitrile at a flow rate of 0.2 ml min<sup>-1</sup>. Peak fractions showing chitinase activity were collected, vacuum-dried and re-dissolved in Milli-Q water. Approximately 0.75 mg of pure enzyme (denoted Ee-chitinase) was obtained from 200 g leaf material.

#### Analytical methods

The purity of the Ee-CBP and Ee-chitinase was checked by SDS-PAGE on a 12.5–25% (w/v) acrylamide gradient gel using the discontinuous system described by Laemmli (1970). To determine the N-terminal amino acid sequence, the purified proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Immobilon-P transfer membrane; Millipore) using a semi-dry blotting system (Bio-Rad). The protein bands were excised from the blot and sequenced on a pulsed liquid-phase 491 Procise-cLC protein sequencer (Applied Biosystems, Foster City, CA, USA). The relative molecular masses of the two mature proteins were determined using an electrospray ion trap mass spectrometer (Esquire-LC; Bruker Daltonic, Bremen, Germany).

The chitinase and lysozyme activities of the two proteins were also measured. The chitinase assay was based on the release of acid-soluble fragments from the substrate carboxymethyl-chitin-Remazol-Brilliant-Violet 5R (Loewe Biochemica, Sauerlach, Germany; Wirth and Wolf 1990), whereas the lysozyme activity was tested using intact cells of *Micrococcus lysodeikticus* (Sigma-Aldrich) following the supplier's protocol.

#### Antifungal assays

The antifungal activity of Ee-CBP, Ee-chitinase and combinations of both proteins was tested using in vitro microtiter-plate assays according to Broekaert et al. (1990). Fungal strains tested were *Alternaria brassicicola* MUCL 20297, *Botrytis cinerea* MUCL 6492, *Fusarium culmorum* IMI 180420, *Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella eumusae* (two tropical fungi isolated from *Musa* sp.), *Fusarium oxysporum* f.sp. *matthioli* CBS 247.61, *Neurospora crassa* FGSC 2489, *Phoma exigua* CBS 431.74, *Phytophthora cryptogea* CBS 418.71, *Pythium ultimum* MUCL 30159, *Rhizoctonia solani* CBS 207.84 and *Trichoderma hamatum* ATCC 20765. Each

well was filled with 2-fold serial dilutions of 20 µl filter-sterilized chitin-binding protein (500 µg ml<sup>-1</sup> Ee-CBP or 1,000 µg ml<sup>-1</sup> Ee-chitinase) and mixed with 80 µl half-strength potato dextrose broth (PDB) containing 1.6×10<sup>4</sup> fungal spores. The inhibition of fungal growth was quantified spectrophotometrically at A<sub>595</sub>.

#### Surface plasmon resonance analysis

Specific interaction analysis of Ee-CBP with GlcNAc and oligomers thereof was performed by surface plasmon resonance (SPR) using a BIAcore 1000 biosensor (Pharmacia Biosensor AB). Ee-CBP was bound to the glycoprotein porcine thyroglobulin (Sigma-Aldrich) immobilized on the surface of the sensor chip (CM 5). Ee-CBP was used at a concentration of 1 mg ml<sup>-1</sup> in HBS (pH 7.4). GlcNAc and GlcNAc-oligomers at concentrations ranging between 1 and 25 mM in HBS (pH 7.4) were injected at the beginning of the dissociation phase for 5 min at a flow rate of 5 µl min<sup>-1</sup>. The resulting change in the SPR response was monitored at 25°C for 9.50 min. Results are expressed as the percentage of Ee-CBP remaining bound to the immobilized thyroglobulin after interaction with GlcNAc and GlcNAc-oligomers (as compared to measurements performed in the absence of GlcNAc and its oligomers). All results are the means of duplicate experiments.

#### Confocal fluorescence microscopy

Since Ee-CBP could not be labeled with an appropriate fluorescent dye (because of the absence of reactive groups) the binding of the protein to the fungal surface could not be visualized directly by fluorescence microscopy. Therefore, an indirect method was developed that was based on the visualization of the bound Ee-CBP by a labeled antibody. Since attempts to produce monospecific polyclonal antibodies against Ee-CBP were unsuccessful, advantage was taken of the availability of anti-wheat germ agglutinin (anti-WGA) antibodies that cross-react with Ee-CBP. Approximately 50 nmol anti-WGA was labeled with a 10-fold molar excess of the fluorescent probe 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester [5(6)-TAMRA, SE] dissolved in dimethyl sulfoxide (Molecular Probes, Leiden, The Netherlands). The reaction mixture was incubated for 1 h in the dark at room temperature under continuous stirring. After labeling, the conjugate was separated from the free dye on a Sephadex G-25 column (10 cm long, 1 cm i.d.; approximately 5 ml bed volume) in PBS. Small aliquots were collected and the concentration determined spectrophotometrically at A<sub>280</sub>. Fractions with the highest concentration of conjugate were selected for immunodetection of Ee-CBP by confocal fluorescence microscopy.

Pre-germinated spores (1.6×10<sup>4</sup> spores ml<sup>-1</sup>) of the fungi *Botrytis cinerea*, *Neurospora crassa* and *Pythium ultimum* were incubated overnight at 22°C with Ee-CBP (at a final concentration of 50 µg ml<sup>-1</sup>) in a multi-well cuvette (Greiner bio-one, Frickenhausen, Germany) coated with poly-L-lysine (Sigma-Aldrich). Positive and negative control samples contained, besides the fungal spores, WGA and sterile water instead of Ee-CBP, respectively. Approximately 1 µM of the fluorescent anti-WGA conjugate was applied to each well and incubated under gentle shaking for 2 h. The cuvette was washed three times with half-strength PDB and observed microscopically with fluorescent images of a Zeiss LSM 510 laser-scanning confocal microscope (ConfoCor 2; Carl Zeiss, Jena, Germany) equipped with a helium-neon laser with excitation at 543 nm. Since chitinases hydrolyze the fungal cell wall, the mode of action of Ee-chitinase was not investigated as such.

#### RNA isolation, cDNA synthesis and cloning

All RNA preparations were isolated from tissues of young (approx. 6 months old) spindle tree seedlings (kindly provided by the tree nursery Vereecke-De Cleene NV, Sleidinge, Belgium). Potted

seedlings were placed in a closed container (5 l) under continuous light and treated with methyl jasmonate (MeJA) by spotting 20  $\mu$ l of a 1% (v/v) solution on a strip of filter paper. After 24 h the leaves and herbaceous stems were collected separately, ground in liquid nitrogen and total RNA extracted as described by Eggermont et al. (1996). Full-length cDNA was synthesized from 5  $\mu$ g of total RNA from the respective tissues using the GeneRacer kit (Invitrogen, Carlsbad, CA, USA), following the instruction manual.

cDNA clones encoding Ee-CBP and Ee-chitinase were generated using a combination of 5' and 3' RACE-PCR. Degenerate primers for Ee-CBP were derived from the complete amino acid sequence of the mature protein whereas for Ee-chitinase highly conserved homologous regions of class-I chitinases were used. Two subsequent PCR rounds with a combination of the primers 5'-CARTGYGGMGNCARGCNGG-3' (derived from amino acid sequence QCGRQAG) and supplied GeneRacer 3' primer, and 5'-GCNGGNAAYMGNMGTGYGC-3' (derived from sequence AGNRRCA) and GeneRacer 3' nested oligonucleotides yielded a single 3' PCR-fragment for Ee-CBP using bark cDNA as a template. Similarly, the primer sets GeneRacer 5' primer and 5'-TTNGTNCKNCCRCARTANCC-3' (derived from sequence GYCGRTN), and GeneRacer 5' nested primer and 5'-CKNCCRCARTANCCRTAYTG-3' (derived from QY-GYCGR) amplified a single 5' PCR product partially encoding bark Ee-CBP. The same procedure was applied for the generation of a 3' RACE fragment partially encoding Ee-chitinase using a combination of the oligonucleotides 5'-WSNCAYGARACNACNGG-3' (derived from sequence SHETTG) and GeneRacer 3' primer, and 5'-GGNMGNNGGCCNHTNCA-3' (derived from sequence GRGPLQ) and GeneRacer 3' nested primer starting from leaf cDNA as a template. In addition, two subsequent PCR amplifications with the primer sets 5'-RCAYTCNADNSCNCRTTDDAT-3' (derived from sequence INGGIEC) and GeneRacer 5' primer, and 5'-GTCATCCARAACCANADNGC-3' (derived from sequence AIWFWMT) and GeneRacer 5' nested primer, generated a single 5' PCR-product. All PCR amplifications were run on an automatic thermal cycler (GeneAmp PCR System 2400; Perkin Elmer) using 1  $\mu$ l of template, appropriate primer sets (0.25  $\mu$ M each), 0.2 mM dNTPs and 2.5 units of *Ex Taq* polymerase (TaKaRa Biomedicals Otsu, Shiga, Japan) in a final volume of 50  $\mu$ l *Ex Taq* buffer. After analysis of the PCR products by agarose gel electrophoresis, fragments were cloned in pCR2.1-TOPO vector, transformed in competent *Escherichia coli* cells (TOPO TA Cloning; Invitrogen) and sequenced by the dideoxy method of Sanger et al. (1977).

#### Molecular modeling of Ee-CBP and Ee-chitinase

Hydrophobic cluster analysis (HCA; Gaboriaud et al. 1987; Lemese-Varloot et al. 1990) was used to delineate the conserved secondary structural features (strands of  $\beta$ -sheet and stretches of  $\alpha$ -helix) along the amino acid sequence of Ee-CBP/Ee-chitinase and the model protein hevein. HCA plots were generated using the program drawhca of L. Canard available at <http://www.lmcp.jussieu.fr/~soyer/www-hca/hca-form.html>.

Molecular modeling of Ee-CBP and Ee-chitinase was carried out on a Silicon Graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover (Accelrys, San Diego, CA, USA). The atomic coordinates of hevein (RCSB Protein Data Bank code 1HEV; Andersen et al. 1993) were used to build the three-dimensional models of Ee-CBP and Ee-chitinase. Steric conflicts were corrected during the model building procedure using the rotamer library (Ponder and Richards 1987) and the search algorithm implemented in the Homology program (Mas et al. 1992) to maintain proper side-chain orientation. An energy minimization of the final model was carried out by 50 cycles of steepest descent using the cvff forcefield of Discover3. To complete the modeling of the fifth disulfide bridge between Cys<sup>32</sup> and Cys<sup>44</sup> of Ee-CBP (Van den Bergh et al. 2002), an additional step of 50 cycles of conjugate gradient were performed keeping residues 1–32 of the polypeptide chain constrained. The program TurboFrodo (Bio-Graphics,

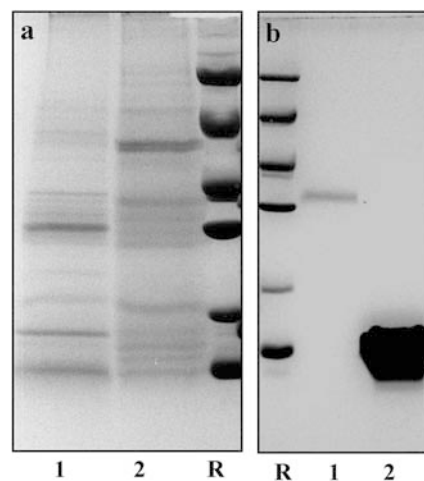
Marseille, France) was run on the O2 workstation to draw the Ramachandran plot and to perform the superposition of the models. PROCHECK (Laskowski et al. 1993) was used to assess the geometric quality of the three-dimensional models. Cartoons were rendered using Molscript (Kraulis 1991), Bobscript (Esnouf 1997) and Raster3d (Merritt and Bacon 1997).

## Results

### Characterization of Ee-CBP and Ee-chitinase

A small hevein-like protein (Ee-CBP) and a genuine chitinase (Ee-chitinase) were purified to homogeneity from spindle tree bark tissue and leaves, respectively, using a combination of affinity chromatography on chitin and conventional protein isolation techniques. The proteins migrated with an apparent molecular mass of approximately 14 kDa and 31 kDa, respectively, upon SDS-PAGE under reducing conditions (Fig. 1). More accurate estimations by mass spectrometry yielded  $M_r$  values of  $4,992.9 \pm 0.5$  Da and  $30,919 \pm 6$  Da for Ee-CBP and Ee-chitinase, respectively. Both proteins were assayed for exo- and endochitinase as well as lysozyme activity. Ee-CBP did not yield any positive reaction. In contrast, Ee-chitinase exhibited both exo- and endochitinase activity and was capable of lysing intact *Micrococcus lysodeikticus* cells.

N-terminal amino acid sequencing revealed that Ee-CBP and Ee-chitinase (Fig. 2) share a high sequence identity with hevein and the N-terminal chitin-binding domain of class-I chitinases. Though this sequence information allowed Ee-CBP and Ee-chitinase to be tentatively classified as a hevein-type AMP and a class-I



**Fig. 1a,b** SDS-PAGE analysis of crude extracts (**a**) and purified proteins (**b**) from spindle tree (*Euonymus europaeus*) in a 12.5–25% (w/v) gradient gel. **a** The gel was loaded with crude extracts from bark tissue (lane 1) and leaves (lane 2). **b** The gel was loaded with purified and reduced leaf Ee-chitinase (lane 1) and bark Ee-CBP (lane 2) proteins. Molecular-mass reference proteins in lane R were  $\alpha$ -lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and phosphorylase b (97 kDa)

		Signal peptide	Hevein-like domain	
Ee-CBPb	1	1	1	1
Ee-CBP1	1	1	1	1
Ee-chib	1	1	1	1
Ee-chil	1	1	1	1
		Hinge		
Ee-CBPb	61	61	61	61
Ee-CBP1	61	61	61	61
Ee-chib	57	57	57	57
Ee-chil	57	57	57	57
		C-terminal domain		
Ee-CBPb	121	121	121	121
Ee-CBP1	121	121	121	121
Ee-chib	110	110	110	110
Ee-chil	110	110	110	110
Ee-CBPb	181	181	181	181
Ee-CBP1	181	181	181	181
Ee-chib	170	170	170	170
Ee-chil	170	170	170	170
Ee-CBPb	241	241	241	241
Ee-CBP1	241	241	241	241
Ee-chib	230	230	230	230
Ee-chil	230	230	230	230
Ee-CBPb	301	301	301	301
Ee-CBP1	301	301	301	301
Ee-chib	290	290	290	290
Ee-chil	290	290	290	290

**Fig. 2** Comparison of the deduced amino acid sequences of cDNA clones from *E. europaeus* bark and leaves encoding Ee-CBP and Ee-chitinase. Clones Ee-CBPb and Ee-CBP1, encode Ee-CBP from bark and leaves, respectively. Clones Ee-chib and Ee-chil, encode Ee-chitinase from bark and leaves, respectively. *Hyphens* (-) indicate gaps introduced to obtain optimal alignments. *Circles* (°) indicate identical amino acids. *Bold asterisks* (\*) indicate invariant residues of the catalytic domain of class-I chitinases. *Boxes* denote distinct residues (according to Levorson and Chlan 1997). The mature Ee-CBP domain from bark and the N-terminal sequence determined for Ee-chitinase from leaves are indicated in *bold*

chitinase, respectively, the relationship between the two proteins remained unclear, especially concerning the possible origin of Ee-CBP from a class-I chitinase-like precursor. To answer these questions cDNA clones encoding Ee-CBP and Ee-chitinase were isolated and analyzed.

#### Isolation and characterization of cDNA clones encoding Ee-CBP and Ee-chitinase

##### *Ee-CBP-encoding cDNA clones*

A single, distinct 3' RACE-PCR product of 1,060 bp was amplified from mRNA prepared from bark of MeJA-treated seedlings after two successive PCR amplifications using primers derived from the complete amino acid sequence of the mature Ee-CBP peptide (Van den Bergh et al. 2002). In addition, a 5' RACE fragment of approximately 150 bp was retrieved after two nested PCR reactions. Subsequent cloning and sequencing of both PCR fragments revealed an 18-nucleotide overlap. PCR amplification and detailed sequence analysis showed that the complete cDNA comprises an open reading frame of 963 bp encoding a polypeptide of 320 amino acids.

An analysis of the deduced sequence revealed that the primary translation product starts with a 21-residue

signal peptide followed by a sequence that matches exactly the determined sequence of the 45-residue mature Ee-CBP polypeptide (Fig. 2) and a long (254-residue) C-terminal domain. According to these findings, Ee-CBP is—like several other single-hevein-domain proteins—synthesized as a class-I chitinase-like precursor of 320 residues. Co-translational processing of a signal peptide and post-translational cleavage of an extended (254-residue) C-terminal propeptide eventually yield the mature Ee-CBP polypeptide.

To check whether Ee-CBP is expressed in leaves, as well as in bark tissue, leaf mRNA was amplified by two subsequent PCR reactions using primer sets corresponding to the putative signal sequence and the C-terminal sequence of the primary translation product of the Ee-CBP clone from the bark. This approach yielded a single amplification product with a size virtually identical to that of the cDNA encoding the bark Ee-CBP. To check the identity of the presumed leaf paralog of Ee-CBP, the PCR amplification product was cloned and sequenced. A comparison of the deduced amino acid sequences of the bark and leaf clones encoding Ee-CBP (further referred to as Ee-CBPb and Ee-CBP1, respectively) revealed only minor differences. The two sequences share 94% and 95% sequence identity at the amino acid and nucleotide levels, respectively. The only obvious difference concerns the deletion in Ee-CBP1 of a 15-residue peptide at the C-terminus (Fig. 2). However, it is possible that this apparent deletion is just the result of a frame shift introduced in the PCR product corresponding to Ee-CBPb.

##### *cDNA clones encoding Ee-chitinase*

The 3' end of a PCR fragment encoding Ee-chitinase was generated by two successive PCR rounds with primer sets derived from well-conserved homologous regions of class-I chitinases, using mRNA from leaves induced with MeJA as a template. The resulting PCR product yielded a single band of approx. 560 bp upon agarose gel electrophoresis, and was subsequently cloned and sequenced. Similarly, a 5' RACE cDNA of approx. 780 bp was amplified by PCR and nested PCR. To obtain a PCR fragment encoding the complete Ee-chitinase sequence, additional PCR amplifications were performed with oligonucleotides corresponding to regions containing the initiation and termination codon, and their respective products cloned and sequenced. The same approach was followed to amplify cDNA clones encoding Ee-chitinase using mRNA from bark as a template. To avoid confusion, cDNA clones encoding Ee-chitinase from leaves and bark are further referred to as Ee-chil and Ee-chib, respectively.

Sequence analyses revealed that Ee-chil and Ee-chib are nearly identical and share 99% sequence identity, at both the amino acid and nucleotide level (Fig. 2). Both the leaf and bark clones contain an open reading

frame of 939 bp encoding a polypeptide of 312 amino acid residues. The primary translation product contains an N-terminal 19-amino-acid residue signal peptide preceding the N-terminus of mature Ee-chitinase. Co-translational removal of this signal peptide yields a protein with calculated molecular mass of 31,530 Da and 31,574 Da for the leaf and bark chitinase, respectively. Since this calculated value of the leaf protein is definitely higher than that determined by mass spectrometry (30,919 Da) it seems likely that the leaf Ee-chitinase is post-translationally processed by the cleavage of a short C-terminal propeptide. A similar removal of a short putative vacuolar-targeting C-terminal propeptide has been reported for several other class-I chitinases (Neuhaus et al. 1991).

#### Sequence similarity of Ee-CBP and Ee-chitinase

A BLASTp search in GenBank using the deduced amino acid sequences of Ee-CBPb and Ee-chil as a query revealed that the corresponding proteins share a high sequence identity with class-I chitinase precursors from a wide range of plants including, for example, *Leucaena leucocephala* (Lam.) De Wit, *Elaeagnus umbellata* Thunb, *Gossypium hirsutum* L. and *Hevea brasiliensis* L. There is no doubt, therefore, that both spindle tree proteins belong to the superfamily of plant chitinases.

Further analyses indicated that the two different types of spindle tree chitin-binding protein are more closely related to each other than to structurally related orthologues from other species (Fig. 2). It is striking, indeed, that the Ee-CBP clones share a higher sequence identity (approx. 70%) with the Ee-chitinase clones than with any other precursor of a hevein-type AMP or any other genuine class-I chitinase. The most obvious differences between the aligned sequences of Ee-CBPb/Ee-CBPi and Ee-chil/Ee-chib occur in the linker/spacer region between the hevein-like and the C-terminal domain (Fig. 2). The hinge region of the genuine chitinase is rich in glycine whereas the corresponding region of proEe-CBP contains no such typical glycine-rich motif.

#### Antifungal activity of Ee-CBP and Ee-chitinase

Several single hevein-domain proteins, as well as the two-hevein-domain lectin from *Urtica dioica*, exhibit an antifungal activity that relies (partly) on their respective chitin-binding domains. Since both Ee-CBP and Ee-chitinase also contain such chitin-binding domains, they have at least in principle the potency to interact with microorganisms containing chitin or GlcNAc-oligomers in their cell walls. To assess the possible antimicrobial activity of Ee-CBP and Ee-chitinase, their effect on fungi was investigated in some detail by in vitro microtiter-plate assays. In these assays both the effect of the individual proteins and a combination of Ee-CBP and Ee-chitinase on different fungi were corroborated.

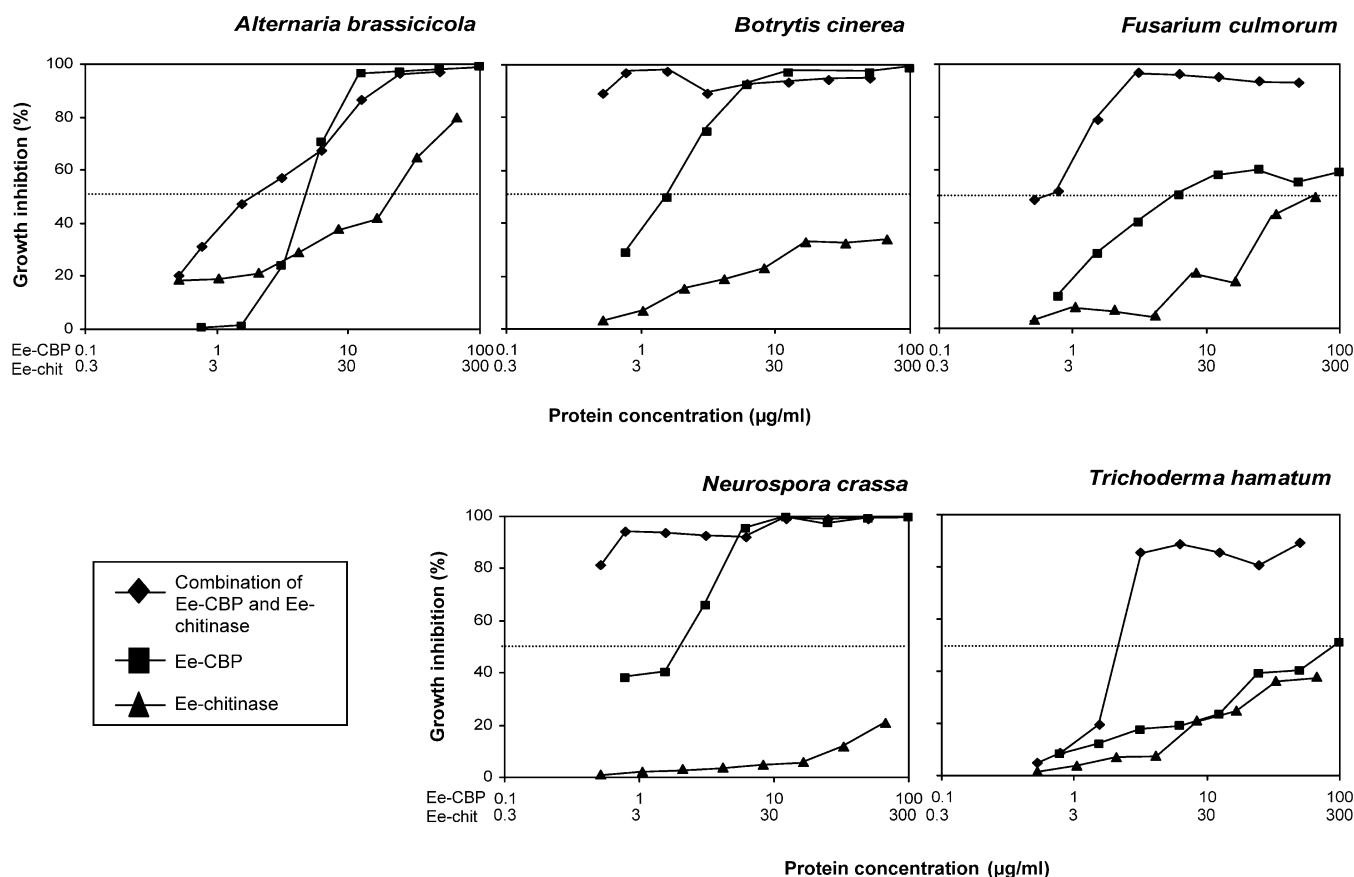
From the dose-response curves obtained in these microtiter-plate experiments, the inhibitory concentration (expressed in  $\mu\text{M}$  to correct for differences in  $M_r$  between the two proteins) and relative activity towards a series of test fungi were determined for Ee-CBP and Ee-chitinase. As shown in Table 1, Ee-CBP exhibited a stronger antifungal activity than Ee-chitinase towards most tested fungi. It should be mentioned, however, that the relative inhibitory activity of Ee-CBP and Ee-chitinase markedly differed as a function of the test fungus. For example, the required concentrations for the inhibition of *B. cinerea* were 0.2  $\mu\text{M}$  and  $>6.5 \mu\text{M}$  for Ee-CBP and Ee-chitinase, respectively, whereas for the inhibition of *T. hamatum* these values were 20  $\mu\text{M}$  and  $>6.5 \mu\text{M}$  for Ee-CBP and Ee-chitinase, respectively.

To check for possible synergistic activity, five fungal strains covering a broad range of relative sensitivity to Ee-CBP/Ee-chitinase (see Table 1) were selected for extensive inhibition assays with the individual proteins and a combination of Ee-CBP/Ee-chitinase. The fungi chosen were *A. brassicicola*, *B. cinerea*, *F. culmorum*, *N. crassa* and *T. hamatum*. Ee-CBP and Ee-chitinase were assayed on the fungi in half-strength PDB medium and applied (either individually or in combination) in a mass ratio of 1:3. As shown in Fig. 3, a combination of Ee-CBP and Ee-chitinase clearly inhibited fungal growth at a much lower concentration than the individual

**Table 1** Comparison of the antimicrobial activity of Ee-CBP from *Euonymus europaeus* bark tissue and leaf Ee-chitinase. The relative antimicrobial activity is expressed as  $(\text{IC}_{50} \text{ Ee-chitinase})/(\text{IC}_{50} \text{ Ee-CBP})$  in half-strength PDB. ND Not determined

Fungus	IC <sub>50</sub> ( $\mu\text{M}$ )		Relative activity
	Ee-CBP	Ee-chitinase	Ee-CBP/Ee-chitinase
<i>Alternaria brassicicola</i> <sup>a</sup>	0.6	2.3	3.8
<i>Botrytis cinerea</i> <sup>a</sup>	0.2	$>6.5$	32.5
<i>Fusarium culmorum</i> <sup>a</sup>	0.6	6.5	10.83
<i>Fusarium oxysporum</i> f.sp. <i>cubense</i> <sup>a</sup>	3	ND	ND
<i>Fusarium oxysporum</i> f.sp. <i>matthioli</i> <sup>a</sup>	1	$>6.5$	6.5
<i>Mycosphaerella eumusae</i> <sup>a</sup>	1.2	ND	ND
<i>Neurospora crassa</i> <sup>a</sup>	0.4	$>6.5$	16.25
<i>Phoma exigua</i> <sup>a</sup>	6.6	6.5	0.99
<i>Phytophthora cryptogea</i> <sup>b</sup>	5	ND	ND
<i>Pythium ultimum</i> <sup>a</sup>	6.6	$>6.5$	$>0.99$
<i>Rhizoctonia solani</i> <sup>c</sup>	5	$>6.5$	$>1.3$
<i>Trichoderma hamatum</i> <sup>a</sup>	20	$>6.5$	$>0.33$

<sup>a-c</sup>Concentration of protein required for 50% growth inhibition after incubation for 48 h<sup>(a)</sup>, for 1 week<sup>(b)</sup> and for 96 h<sup>(c)</sup> at 22°C



**Fig. 3** Inhibitory effect of Ee-CBP and Ee-chitinase in in vitro antifungal assays. Dose-response curves were derived from in vitro microtiter-plate assays with Ee-CBP, Ee-chitinase and a combination of Ee-CBP and Ee-chitinase (mass ratio 1:3). Results were evaluated after incubation for 48 h at 22°C. Fungi tested were *Alternaria brassicicola*, *Botrytis cinerea*, *Fusarium culmorum*, *Neurospora crassa* and *Trichoderma hamatum*

proteins. Since all tested fungi were more sensitive to the combined than to the individual proteins it is evident that Ee-CBP and Ee-chitinase act synergistically in the in vitro antifungal assay. Though the results of the antifungal assays do not allow conclusions to be drawn about the exact mode of action, the observed synergism may be indicative of a complementary mode of action of Ee-CBP and Ee-chitinase. One can reasonably expect that Ee-chitinase acts through a mechanism that relies—at least partly—on the enzymatic degradation of the fungal cell wall. Since Ee-CBP is completely devoid of exo- and endochitinase activity, this AMP must act through another yet unidentified mechanism.

#### Mode of antifungal action of Ee-CBP

At present, the mode of antimicrobial action of Ee-CBP and hevein-type AMPs in general is still unclear. Several hypotheses have been brought forward but eventually only two major concepts have been retained. According to the first concept, binding of AMPs to the glycoconjugates of the host cell wall, and in particular chitin, is believed to

be the direct cause of the antifungal effect. For example, due to their multivalency, hevein-type AMPs with multiple domains, e.g. *Urtica dioica* agglutinin (UDA), can cross-link the chitin microfibrils in a rather uncontrolled way. As a result, normal growth of the apical tip is disturbed because the nascent chitin chains can no longer cross-link with each other and with  $\beta$ -glucans to form the inner skeletal layer of the cell wall. Evidently, such a mechanism cannot apply to the monovalent single-hevein-domain AMPs. However, this does not preclude the possibility that the mode of action of the small AMPs is intimately linked to their carbohydrate-binding activity. One can imagine, for example, that binding of the monovalent AMPs to some compounds of the cell wall severely interferes with cell wall synthesis and eventually results in growth inhibition. According to the alternative concept, the small AMPs may penetrate through the pores of the microbial cell wall and reach the plasma membrane where they can interact with some compounds of the membrane. As a result of this interaction the plasma membrane is disrupted causing leakage and lysis of the cells. Both possible mechanisms were investigated in some detail (see below).

#### Interaction of Ee-CBP with GlcNAc and GlcNAc-oligomers

In a first approach to unravel the mode of action, the binding of Ee-CBP with chitoooligosaccharides was

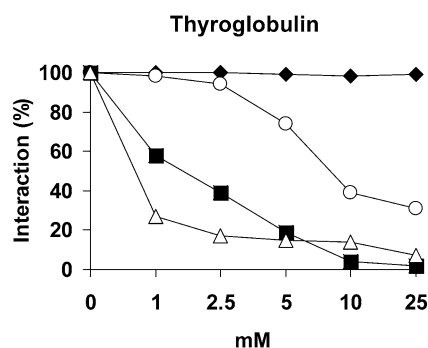
studied in detail by a direct method based on SPR measurements. Ee-CBP was immobilized on thyroglobulin coupled on the biosensor chip (CM 5) and its interaction with GlcNAc and oligomers thereof measured by SPR in the dissociation phase. Inhibition experiments showed that GlcNAc has no inhibitory effect on the interaction of Ee-CBP with the immobilized thyroglobulin, even at a concentration as high as 25 mM. Chitobiose definitely exhibited an inhibitory effect but its inhibitory potency was much lower than that of chitotriose and chitotetraose (Fig. 4). Since chitotetraose was not a better inhibitor than chitotriose one can reasonably conclude that the carbohydrate-binding site of Ee-CBP is most complementary to the trisaccharide. These observations leave no doubt that Ee-CBP specifically interacts with chito oligosaccharides and accordingly is capable of binding to native chitin (including the chitin present in the cell wall of most fungi). To corroborate whether an interaction with the chitin present in the fungal cell walls is essential for the observed antifungal activity of Ee-CBP, the binding of the protein to the surface of the fungal spores and hyphae was studied by confocal microscopy.

#### Immunodetection of Ee-CBP by confocal microscopy

Since Ee-CBP could not be properly labeled with a suitable fluorescent dye the binding of the protein to fungal cell walls was studied by confocal fluorescence microscopy using an indirect detection method. This indirect method was based on the detection of Ee-CBP bound to the fungal surface by anti-WGA antibodies labeled with the fluorescent probe 5(6)-TAMRA, SE. Fungi used in the binding studies were *B. cinerea*, *N. crassa* and *P. ultimum*. No aspecific interaction was observed after addition of the fluorescently labeled anti-WGA to the negative controls (in which Ee-CBP

was omitted), indicating that there was no cross-reactivity between the fungi and the antibodies (image not shown). After addition of WGA (positive control) the surface of the fungal cell wall exhibited a strong fluorescence whereby the contours of germinating spores and hyphae were clearly delineated (Fig. 5e), which demonstrated that the bound WGA was recognized by the fluorescent antibodies.

Spores and hyphae treated with Ee-CBP also produced a bright fluorescence after treatment with the labeled anti-WGA antibody. Spores were more prominently labeled than the hyphae due to the difference in cell wall thickness and rigidity (Fig. 5a–d). These observations clearly indicate that Ee-CBP binds to the surface of the fungal spores and hyphae. The binding studies also yielded some unexpected and intriguing observations. First, in the case of *B. cinerea*, which is most sensitive to Ee-CBP (see Table 1), not only the cell wall but also the intracellular space of the cells was stained (Fig. 5d), whereas within the positive control sample with WGA, only the contours of *B. cinerea* were illuminated (Fig. 5e). Second, neither WGA nor Ee-CBP was associated with the septa of the three tested moulds, although these septa contain (except in the oomycete, *P. ultimum*; see Fig. 5d) chitin. This finding apparently contrasts the previous observations of Koo et al. (1998) who noted a clear interaction of the fluorescently labeled hevein-type Pn-AMP1 with the septa and hyphal growth tips of the test fungi *B. cinerea* and *Phytophthora parasitica*. Third, Ee-CBP as well as WGA (image not shown) yielded similar fluorescence patterns with the fungus *P. ultimum*, the cell wall of which is devoid of chitin. This observation implies that the tested hevein-type proteins can also interact with glycoconjugates other than GlcNAc-oligomers and chitin, which in turn seriously complicates the elucidation of the mode of action of Ee-CBP.



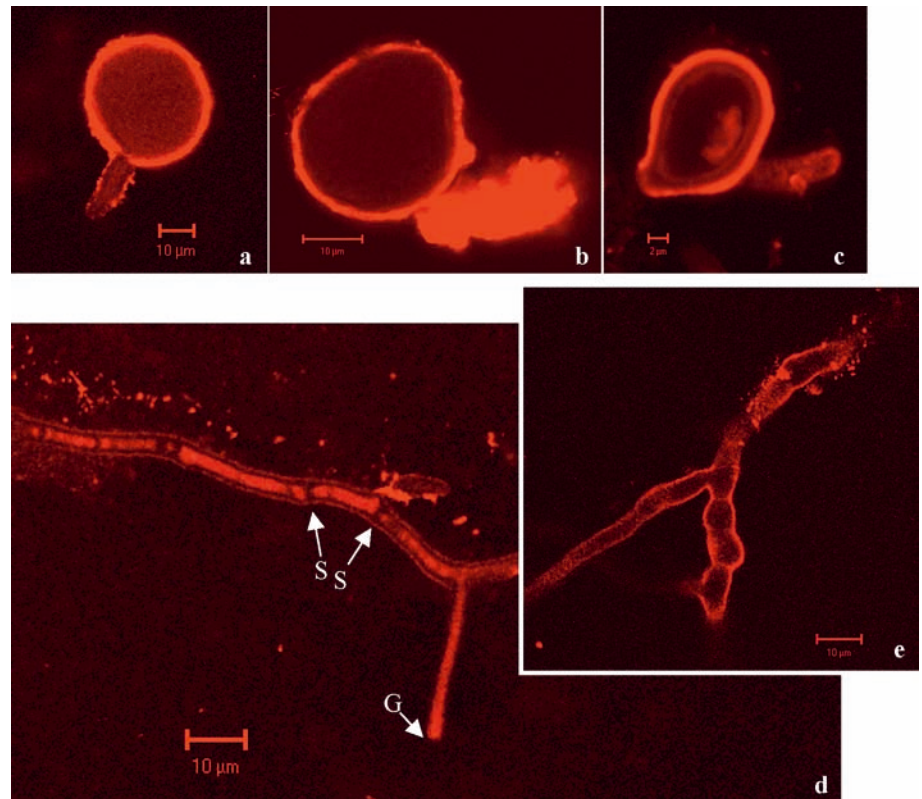
**Fig. 4** Surface plasmon resonance (SPR) analysis of the specificity of Ee-CBP towards GlcNAc and GlcNAc-oligomers. Graphs show the interaction of Ee-CBP with immobilized porcine thyroglobulin in the presence of 0–25 mM GlcNAc (♦), chitobiose [or (GlcNAc)<sub>2</sub>] (○), chitotriose [or (GlcNAc)<sub>3</sub>] (■) and chitotetraose [or (GlcNAc)<sub>4</sub>] (△) added during the dissociation phase at increasing concentrations ranging from 1 to 25 mM. Results are expressed as the percentage of Ee-CBP remaining bound to the immobilized glycoprotein. Values are the means of duplicate experiments

#### Molecular modeling of Ee-CBP and Ee-chitinase

Mature Ee-CBP consists of a single polypeptide chain of 45 amino acid residues with a high sequence identity (60.5%) and similarity (76.7%) to hevein from *Hevea brasiliensis* latex and the hevein domain(s) of other chitin-binding proteins. Hydrophobic cluster analysis (HCA) confirmed the structural similarity between Ee-CBP and hevein (Fig. 6). In addition, molecular modeling (using the <sup>1</sup>H-NMR coordinates of hevein as a model) indicated that Ee-CBP adopts the same overall fold and three-dimensional structure as hevein. As shown in Fig. 6, this structure is characterized by the presence of three antiparallel strands of  $\beta$ -sheet ( $\beta$ 1,  $\beta$ 2 and  $\beta$ 3) and two short stretches of  $\alpha$ -helix ( $\alpha$ 1 and  $\alpha$ 2) interconnected by loops. All four disulfide bonds present in hevein (Cys<sup>3</sup>–Cys<sup>18</sup>, Cys<sup>12</sup>–Cys<sup>24</sup>, Cys<sup>17</sup>–Cys<sup>31</sup> and Cys<sup>37</sup>–Cys<sup>41</sup>) occur at similar positions along the polypeptide chain of Ee-CBP. According to the model, the two extra Cys residues of Ee-CBP (Cys<sup>32</sup> and Cys<sup>44</sup>,



**Fig. 5a–e** Visualization by confocal fluorescence microscopy of the interaction of Ee-CBP with fungal spores and hyphae. Images shown are a germinating sporangium of *Pythium ultimum* (a), spore release (b) and a bulbous germinating spore (c) of *Neurospora crassa*, and hyphae of *Botrytis cinerea* treated with Ee-CBP (d) and WGA (e) as a positive control. The positions of septa (S arrows) and a growing tip (G arrow) of *B. cinerea* are indicated

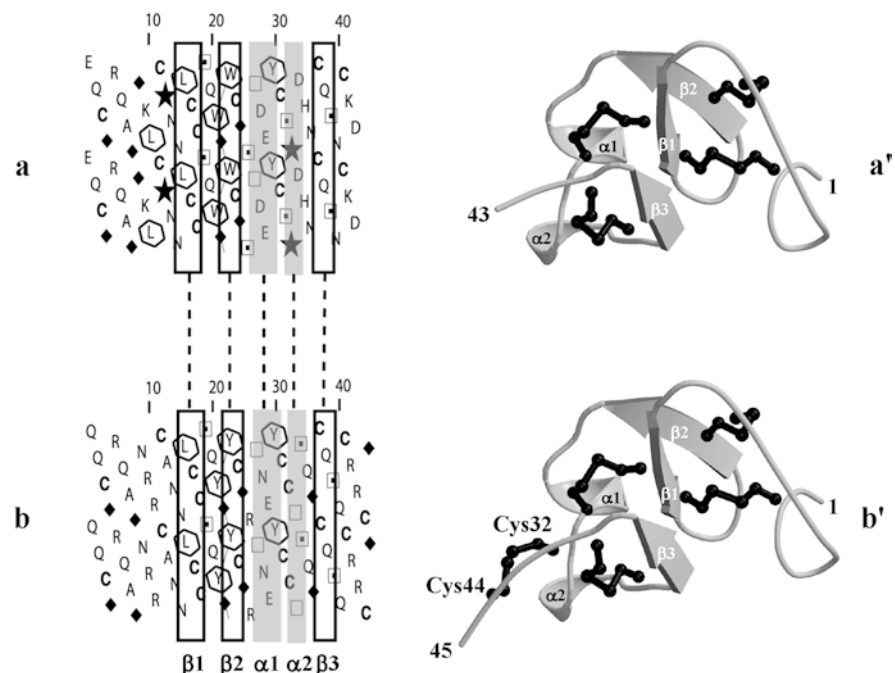


which are absent from hevein) form a fifth disulfide bridge located at the C-terminus of the polypeptide chain (Fig. 6), which is in perfect agreement with the reported absence of free thiol groups in the unreduced protein (Van den Bergh et al. 2002). HCA and molecular modeling confirmed that the N-terminal hevein domain of the Ee-chitinase also adopts the same overall fold and

three-dimensional structure as hevein (results not shown).

A closer examination of the sequence and modeled structure reveals that most of the amino acid residues, which are involved in the binding of either N-acetylneuraminic acid or GlcNAc to hevein (Saul et al. 2000) or UDA (Wright 1990), are conserved in Ee-CBP. These

**Fig. 6a,b** Comparison of the HCA plots of hevein (a) and mature Ee-CBP (b), and their corresponding ribbon diagrams (a' and b'). The strands of the  $\beta$ -sheet ( $\beta 1$ ,  $\beta 2$  and  $\beta 3$ ) and the  $\alpha$ -helices ( $\alpha 1$  and  $\alpha 2$ ) are numbered and delineated on both the HCA plots and ribbon three-dimensional structures. The half-cysteine residues involved in disulfide bonds are indicated in *black ball-and-sticks* (a' and b'). Cartoons were rendered with Molscript (Kraulis 1991), Bobscript (Esnouf 1997) and Raster3D (Merritt and Bacon 1997)



residues include Ser<sup>19</sup>, Tyr<sup>21</sup>, Tyr<sup>23</sup> and Tyr<sup>30</sup>, respectively. Furthermore, the aromatic rings of these residues are similarly oriented in both hevein and Ee-CBP and, accordingly, are fully capable of stacking against the pyranose ring of the GlcNAc residues of chitin just as reported for UDA-chitotriose (Montreuil 1984) and WGA-N-acetylneuraminyl-lactose (Wright 1990) complexes (results not shown).

## Discussion

Ee-CBP and Ee-chitinase, two chitin-binding proteins were isolated from, respectively, bark tissue and leaves from the same spindle tree. Antifungal assays indicated that Ee-CBP exhibits a potent *in vitro* antimicrobial activity, which even surpasses that of Ac-AMP, the strongest hevein-type AMP isolated hitherto (Broekaert et al. 1992). Ee-chitinase, a class-I chitinase, also possesses an *in vitro* antifungal activity but is far less potent than Ee-CBP. However, in spite of its relatively low antifungal activity, Ee-chitinase strongly enhances the antimicrobial effect of Ee-CBP through a marked synergism. The concept of synergism between different types of antifungal protein was originally introduced by Mauch et al. (1988) who discovered an enhanced antifungal activity with combinations of a chitinase and a  $\beta$ -1,3-glucanase from pea tissue. Most probably the contribution of each of the two spindle tree chitin-binding proteins to the synergism relies on a different but complementary mode of action. It is postulated that Ee-chitinase degrades the fungal cell wall by the catalytic degradation of nascent chitin microfibrils and by selective hydrolysis of the preformed chitin chains (Raikhel et al. 1993). As a direct result of the action of the chitinase, GlcNAc-oligomers are generated that act as readily accessible target glycans for the chitotriose/chitotetraose-binding Ee-CBP. Even though Ee-CBP is monovalent, its binding to accessible chito-oligosaccharides can prevent cross-linking of nascent chitin chains and of chitin and  $\beta$ -glucan microfibrils, resulting in a disturbance of cell wall morphogenesis and eventually of hyphal growth. The high affinity of Ee-CBP for chitin and particularly for trimers and tetramers of GlcNAc apparently relies on the presence of well-conserved aromatic amino acids and the N-terminal pyroglutamate residue (Harata and Muraki 2000; Muraki et al. 2000). It should be mentioned, however, that the chitin-binding activity of Ee-CBP solely cannot explain the inhibitory activity towards oomycetes, which do not contain chitin in their cell wall, and towards Gram-positive bacteria (Van den Bergh et al. 2002). This apparent discrepancy may be indicative of a second possible mode of action of Ee-CBP. Due to its small size and compact folding, Ee-CBP may be capable of migrating through the pores of the fungal cell wall and eventually reaching the plasma membrane. Upon arrival at the surface of the plasma membrane, Ee-CBP may interact with some surface glycoconjugates or alternatively penetrate the membrane

and impede fungal growth by altering membrane polarity (Huang et al. 2000; Van Parijs et al. 1991; Huang et al. 2002). The working mechanism of the antimicrobial effect based on binding to the plasma membrane, whether mediated by receptor-mediated ion fluxes or by insertion into the membrane with subsequent ion channel formation, has been studied in detail for cysteine-rich AMPs of the plant defensin and thionin groups. Following binding of these AMPs a Ca<sup>2+</sup> influx and K<sup>+</sup> efflux is triggered, which causes overall changes in membrane potential. Since a controlled Ca<sup>2+</sup> influx is believed to be essential for directing polar growth at the tip of the fungal hyphae via localized secretion of vesicles containing cell wall building blocks, interaction of the AMPs with this divalent cation leads to dissipation of the cytosolic [Ca<sup>2+</sup>] gradient and thus to hyphal growth inhibition (Thevissen et al. 1996; De Samblanx et al. 1997). Upon increasing the ionic strength of the fungal growth medium, the peptides come in competition with the added inorganic cations. Hence, the increased Ca<sup>2+</sup> influx, K<sup>+</sup> efflux and external pH changes are largely undone and the fungal growth inhibition effect diminished (Thevissen et al. 1996; De Samblanx et al. 1997). Since Ee-CBP is especially antagonized by divalent cations, this striking parallelism with thionins and plant defensins argues for an analogous mechanism of membrane polarization and concomitant permeabilization and leakage. This phenomenon was also observed by confocal microscopy of Ee-CBP exerting antifungal activity towards *B. cinerea*. The intracellular content of the fungal cells was stained by the fluorescent antibody, which implies that the probe had penetrated through the normally impermeable plasma membrane after an Ee-CBP-induced permeabilization. Possibly, the high isoelectric point of Ee-CBP (11.81) and most other single-domain hevein-type AMPs (Van den Bergh et al. 2002) also contributes to the membrane-associated antimicrobial activity because a high basicity may favour ionic interactions.

To corroborate whether the observed *in vitro* synergism between Ee-CBP isolated from bark tissue and Ee-chitinase from leaves can also occur *in planta*, a molecular analysis was done to check whether the corresponding mRNAs are simultaneously expressed in the same plant tissue to verify if a synergistic action is physically possible. cDNA cloning clearly demonstrated that the genes encoding both Ee-CBP and Ee-chitinase are expressed in bark as well as in leaf tissue after MeJA treatment. This obvious simultaneous expression of Ee-CBP and Ee-chitinase implies that a concerted, synergistic action between the two proteins is at least physically allowed. It should be mentioned here that MeJA-treated plants were chosen to check the possible presence of both Ee-CBP- and Ee-chitinase-encoding mRNAs because preliminary experiments indicated that this plant hormone increased the level of chitinase activity in the young tissues. The induction was not studied in detail but Northern blot experiments indicated that MeJA caused an increase in

the level of mRNAs encoding both Ee-CBP and Ee-chitinase in both leaves and bark (results not shown). Moreover, analysis of the proteins also confirmed that bark and full-grown leaves of trees growing in their natural habitat contain both Ee-CBP and Ee-chitinase. Besides the demonstration of the concomitant expression of Ee-CBP and Ee-chitinase, analysis of the corresponding cDNAs yielded interesting information about the structure, biosynthesis and topogenesis, and evolutionary relationship between the two spindle tree chitin-binding proteins. Both Ee-CBP and Ee-chitinase are synthesized as highly similar precursors consisting of a signal peptide and an N-terminal hevein-like domain connected by a spacer to a C-terminal class-I chitinase-like extension. Based on this overall structure, it can be predicted that both Ee-CBP and Ee-chitinase are synthesized in the ER and follow the secretory pathway to their final destination in the apoplastic space or vacuole (as indicated by the PSORT software, available at <http://psort.ims.u-tokyo.ac.jp/cgi-bin>). The most conspicuous difference between the primary translation products of Ee-CBP and Ee-chitinase is located in the hinge region between the N-terminal chitin-binding domain and the C-terminal chitinase domain. Since both the hevein domain and the C-terminal (catalytic) domain of Ee-CBP and Ee-chitinase are very similar it seems likely that the hinge region contains essential sequence or structural information for the distinct posttranslational processing of the respective precursor proteins. A comparison of the sequences indicates that only the precursor with a well-structured hinge region is cleaved whereas that with a short flexible hinge region is not.

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