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BGN16.3, a novel acidic β-1,6-glucanase from mycoparasitic fungus *Trichoderma harzianum* CECT 2413

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A new component of the β-1,6-glucanase (EC 3.2.1.75) multienzymatic complex secreted by *Trichoderma harzianum* has been identified and fully characterized. The protein, namely BGN16.3, is the third isozyme displaying endo-β-1,6-glucanase activity described up to now in *T. harzianum* CECT 2413. BGN16.3 is an acidic β-1,6-glucanase that is specifically induced by the presence of fungal cell walls in *T. harzianum* growth media. The protein was purified to electrophoretical homogenity using its affinity to β-1,6-glucan as first purification step, followed by chomatofocusing and gel filtration. BGN16.3 has a molecular mass of 46 kDa in SDS/PAGE and a pI of 4.5. The enzyme only showed activity against substrates with β-1,6-glycosidic linkages, and it has an endohydrolytic mode of action as shown by HPLC analysis of the products of pustulan hydrolysis. The expression profile analysis of BGN16.3 showed a carbon source control of the accumulation of the enzyme, which is fast and strongly induced by fungal cell walls, a condition often regarded as mycoparasitic simulation. The likely involvement β-1,6-glucanases in this process is discussed.

Trichoderma harzianum is a filamentous fungus that has been proposed as a potential biocontrol agent against phytopathogenic fungi [1] and more recently as opportunistic, avirulent plant symbiont [2]. The antagonism by *T. harzianum* has been explained by different mechanisms [3]. One of them, mycoparasitism, involves the production of several hydrolytic enzymes for the local degradation of the host fungal cell wall and further penetration inside its hyphae as main steps [1].

Several mycoparasitic strains included in different taxonomic groups in the *Trichoderma* genus [4,5] secrete complex sets of enzymes [6]. Within these enzymes we can find hydrolytic activities able to degrade most components of fungal cell walls (chitinases, glucanases, proteases, lipases, etc.). These are usually present as isozyme groups composed by proteins with the same activity but different catalytic and molecular properties [7–12].

Chitinases and β-1,3-glucanases are considered the main enzymes responsible for the degradation of the host cell walls, as chitin and β-1,3-glucan are their two major components. However, other enzymes hydrolyzing less abundant, but structurally important components (as β-1,6-glucan), can contribute to the efficient disorganization and further degradation of the cell wall by *Trichoderma*. β-1,6-glucan has been described in budding yeasts as the link between cell wall proteins and the main β-1,3-glucan/chitin polysaccharide [13] supporting an important role for this polymer in the structure of the fungal cell wall.

Although β-1,6-glucanases are widely distributed among filamentous fungi, few of them have been purified and characterized [10,14–17] and few gene sequences have been published [18–22].

We have previously described two β-1,6-glucanases in *T. harzianum* CECT 2413: BGN16.1 and BGN16.2

Abbreviations
CECT, Spanish type culture collection; CWDE, cell wall degrading enzyme.

[10,16]. Both enzymes are secreted under conditions where chitin is present as the only carbon source. In this paper we report on the purification and characterization of a third isozyme: an acidic β-1,6-glucanase [EC 3.2.1.75], namely BGN16.3, which is specifically secreted in the presence of fungal cell walls, completing the characterization of the β-1,6-glucanase isozyme system of *T. harzianum* CECT 2413. The expression profile of BGN16.3 is also analyzed.

Results

Enzyme production and purification

The purification and characterization of two β-1,6-glucanases from *T. harzianum* have been previously reported. Both proteins were produced in the presence of chitin as carbon source [10,16]. The β-1,6-glucanase described in this work (BGN16.3) was purified from culture filtrates of *T. harzianum* CECT 2413 grown in minimal medium supplemented with 0.5% cell walls of *Botrytis cinerea* as the only carbon source. Under these conditions, two β-1,6-glucanases were detected by chromatofocusing and activity staining (Fig. 1), one of them corresponding to BGN16.2 (pI 5.8), which could also be detected under chitin inductions, meanwhile the other was a novel acidic isozyme which was named BGN16.3 and showed a pI value around 4.5.

To purify BGN16.3 the filtrate of fungal cell walls-supplemented cultures (1000 mL) was concentrated by ammonium sulfate precipitation. The concentrate was subjected to pustulan adsorption and further digestion. Enzymes released after digestion of the polymer were subjected to chromatofocusing and an acidic peak (pH 4.1) with β-1,6-glucanase activity was obtained. Fractions within this peak were pooled, concentrated and subjected to FPLC gel filtration producing the final purified protein with a yield of 31%. The purified β-1,6-glucanase was analyzed by SDS/PAGE (Fig. 2A) and a single protein band was observed using Coomassie blue staining, suggesting a highly homogeneous preparation. BGN16.3 was followed along all the purification steps using gel β-1,6-glucanase activity assay after SDS/PAGE (Fig. 2B). Purification factors and yields at each step are summarized in Table 1.

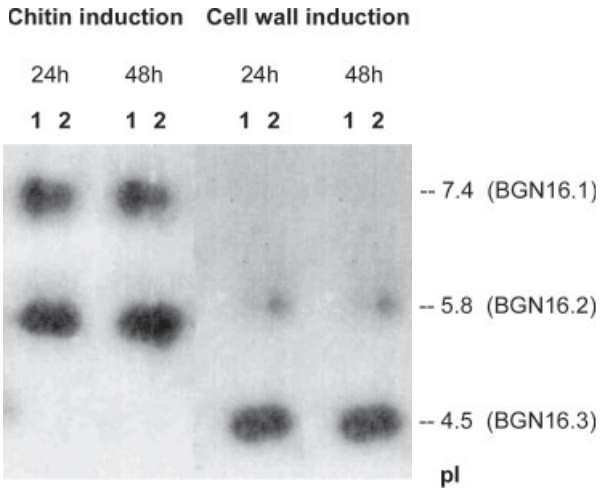

Fig. 1. Isoelectrofocusing and further β-1,6-glucanase specific staining of extracellular proteins produced by *T. harzianum* CECT 2424 (1) and *T. harzianum* CECT 2413 (2) after 24 or 48 h growing on chitin or *B. cinerea* cell walls as sole carbon source.

Physicochemical parameters

The molecular mass of the purified BGN16.3 was approximately 46 kDa by SDS/PAGE, however, when it was determined by S-200-HR gel filtration a value in the range of 25–30 kDa was obtained.

The isoelectric point of the purified protein determined by isoelectrofocusing and acidic chromatofocusing were 4.5 and 4.1, respectively.

No evidence was found of the presence of carbohydrates (glycosylation) in the purified protein as staining with periodic acid/Schiff's reagent [23] was negative and no mobility shift was detected on SDS/PAGE after treatment with endoglycosidase-F (Sousa, unpublished results).

Kinetic parameters

The enzyme activity was measured at different pustulan concentrations and Lineweaver–Burk representation was used to calculate Michaelis constants. A K_m of 1.1 mg pustulan·ml^{-1} and a V_{max} of 390 μmol of product per min^{-1}·(mg protein)$^{-1}$ were estimated.

The optimal temperature for the BGN16.3 activity was 50 °C and the inactivation temperature (50% of the activity lost after 30 min incubation in the absence of substrate) was calculated also 50 °C. This suggests substrate protection against temperature inactivation as previously described for other β-1,6-glucanases [10,16]. Optimal pH was determined to be 5.0 and at least 20% of maximum enzymatic activity was maintained between pH 4.0 and 7.0.

Substrate specificity and reaction products

The purified BGN16.3 was tested for activity towards several glucan substrates (Table 2) by measuring the release of reducing sugars. The highest activity was

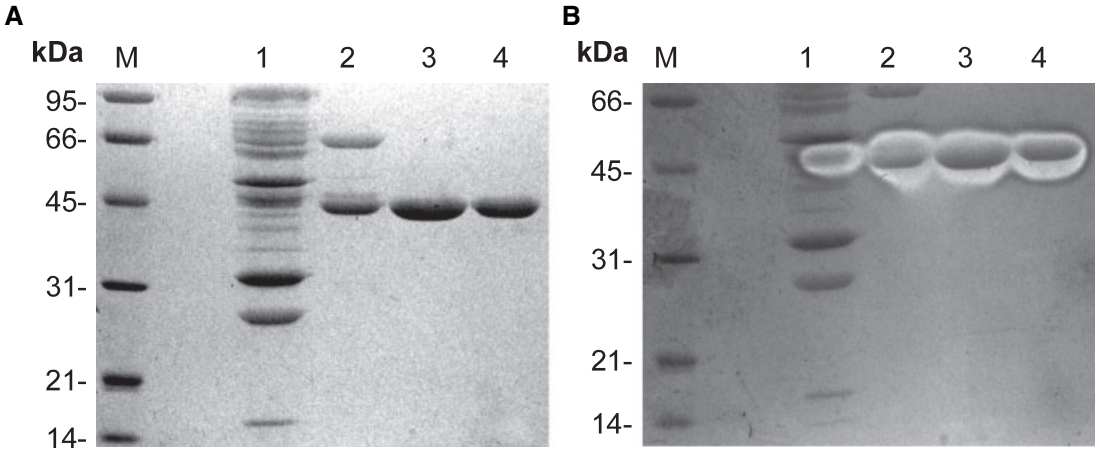

Fig. 2. Purification of BGN16.3. SDS/PAGE analysis (A) and activity staining by pustulan-agarose overlay (B) of the different purification steps of BGN16.3. Proteins were stained with Coomassie blue. Lane 1, crude extract; lane 2, pustulan digestion; lane 3, chromatofocusing eluate peak IP 4.1; lane 4, gel filtration eluate. The numbers of the left indicate the molecular masses of protein standards (lane M).

Table 1. Purification of a β-1,6-glucanase (BGN16.3).

Step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity ($U \cdot mg^{-1}$)	Yield (%)	Purification (fold)
Crude enzyme	10	35.65	257	7.2	100	1
Pustulan digestion	1.8	2.02	215	106	75	15
Chromatofocusing eluate	0.425	0.550	100.4	182	39	25
Gel filtration eluate	0.400	0.425	80	188	31	26

detected for pustulan (linear β-1,6-glucan) and a lower activity was measured towards yeast glucan (18% of the maximum activity) and laminarin (8% of maximum) which are β-1,3-glucans with β-1,6-glycosidic linkages at branches at the ratios of 4 : 1 and 7 : 1, respectively [24]. No activity was found towards colloidal chitin, pachyman, starch, cellulose, nigeran or dextran, concluding that BGN16.3 is a specific β-1,6-glucanase.

Table 2. Substrate specificity of the purified BGN16.3. 100% activity corresponds to 185 U (mg protein)$^{-1}$.

Substrate	Linkage type	β-1,6-Glucanase relative activity (%)
Pustulan	β-1,6 (Glc)	100
Glucan (*S. cerevisae*)	β-1,3: β-1,6 (Glc)	18
Laminarin	β-1,3: β-1,6 (Glc)	8
Pachyman	β-1,3 (Glc)	0
Carboxymethylcellulose	β-1,4 (Glc)	0
Colloidal chitin	β-1,4 (GlcNAc)	0
Nigeran	α-1,3: α-1,4 (Glc)	0
Soluble starch	α-1,4: α-1,6 (Glc)	0

The most abundant oligomers detected by HPLC after pustulan hydrolysis were di-, tri- and tetra-β-1,6-glucosides as shown in Fig. 3. Low levels of glucose could only be detected after longer incubations, supporting an endolytic mode of action for BGN16.3. This was confirmed later finding the lack of enzymatic activity of BGN16.3 on gentiobiose (β-1,6-disaccharide, not shown).

Protein sequences

The N-terminal and an internal peptide of the purified protein were sequenced. Two 14 and 13 amino acid sequences were obtained, respectively. These were:

N-terminal: Ala-Ala-Gly-Ala-Gln-Ala-Tyr-Ala-Ser-Asn-Gln-Ala-Gly-Asn

Internal peptide: Gly-Leu-Asn-Ser-Asn-Leu-Gln-Ile-Phe-Gly-Ser-Pro-Trp

Both sequences were compared to the existing sequences in GenBank using BLASTP program. In the case of the N-terminal no highly similar glucanase sequences could be found, furthermore there was not high similarity to the amino terminal ends of any of

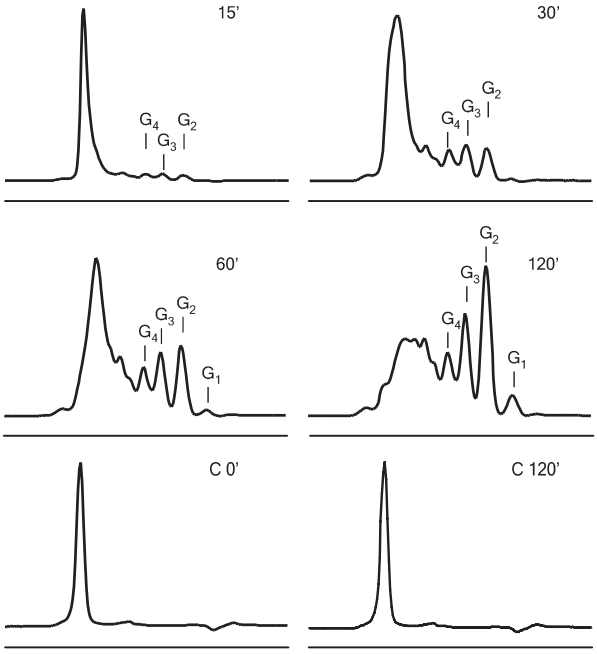

Fig. 3. HPLC analysis of the mechanism of substrate degradation by BGN16.3 on pustulan. The enzyme was incubated with pustulan for 120 min, and aliquots of the reaction were taken at different times. G_n refers to glucose oligomers (n = degree of polymerization). Lower panels are substrate controls (C) where the enzyme was not present. The incubation time is indicated in minutes in the upper right corner of each graph.

the cloned β-1,6-glucanases confirming BGN16.3 as a novel enzyme.

BGN16.3 internal peptide showed seven of 13 amino acids identity with a fragment of a *Neurospora crassa* β-1,6-glucanase named Neg1 [19]. No significant similarity was found to BGN16.2 sequence previously cloned from *T. harzianum* [18].

Regulation of the BGN16.3 production

To study the regulation of the expression of BGN16.3 under several different physiological conditions, we used different induction media (replacement media) after growth for 48 h in modified Czapek minimal medium supplemented with glucose. Western blotting with polyclonal antibodies raised against BGN16.3 was used in order to detect the presence of the enzyme in seven different conditions after 48 h in the replacement media. When glucose, glycerol, sorbitol or chitin was used as a carbon source in the replacement media, the presence of the protein could not be detected. However it was clearly detected if 0.5% pustulan or 0.5% *B. cinerea* cell walls were used as the sole carbon sources. A fainter band could be seen if no carbon source was added to the minimal medium (Fig. 4A). Similar results were obtained by β-1,6-glucanase activity staining after SDS/PAGE (not shown) on the same samples. Further analyses were carried out on those conditions where BGN16.3 could be detected studying the expression of the enzyme at shorter time points: 12 and 24 h. Twelve hours after induction with fungal cell walls BGN16.3 could already be clearly detected, it was also detected in the absence of carbon source, but not in the presence of pustulan. In this latter condition 24 h induction was required to detect the protein in the supernatants (Fig. 4B).

Induction of BGN16.3 at a different pH or by nitrogen starvation was also tested, with negative results (not shown).

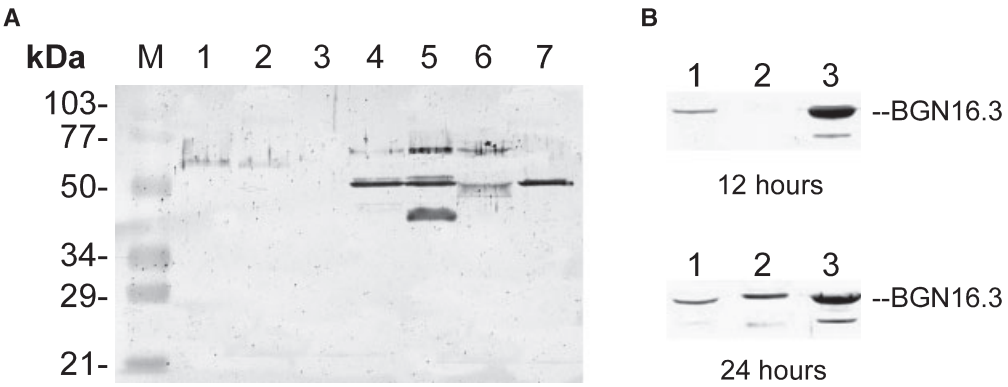

Fig. 4. Expression profile of BGN16.3 under different induction conditions. (A) Western blot analysis on total extracellular protein from cultures of *T. harzianum* CECT 2413 grown for 48 h on 2% glucose (1), 2% glycerol (2), 0.5% chitin (3), 0.5% pustulan (4), 0.5% *B. cinerea* cell walls (5) or no carbon source (6). The purified BGN16.3 was used as positive control (7). (B) Accumulation of BGN16.3 was analyzed at shorter times in the absence of carbon source (1), or in pustulan (2) or *B. cinerea* cell walls (3) inductions.

Discussion

The implication of cell wall degrading enzymes (CWDEs) in mycoparasitic processes carried out by *Trichoderma* is widely accepted. Several dozen enzymes putatively involved in the process have been identified, many of them purified and their genes cloned [25].

Two extracellular β-1,6-glucanases had been previously purified from *T. harzianum* CECT 2413 [10,16]. In this paper we report the purification of a third β-1,6-glucanase (BGN16.3), advancing the knowledge on this diverse isozyme system. Interestingly the BGN16.3 was identified using fungal cell walls in the induction media, a condition often regarded as a simulation of mycoparasitism, whereas it could not be detected in chitin inductions, the condition most frequently used to isolate enzymes from *T. hazianum* [7,10,16].

The presence of different proteins displaying identical hydrolytic activity but with high sequence dissimilarities is a common fact in the CWDE complex secreted by *Trichoderma* strains during mycoparatisic interactions. In some strains, more than 10 different chitinolytic enzymes and a similar number of β-1,3-glucanase isozymes have been described [9,25]. Differences in their substrate specificity and/or regulatory properties [7,26,27] support the idea of a synergic and/or complementary functional role for the different isozymes during antagonistic processes to overcome the problem of the complex nature of the fungal cell wall. It is also interesting to consider the simultaneous production of proteins with diverse structure but identical substrate as a mechanism to avoid specific inhibitors produced by the fungal host during the antagonistic interaction. This phenomenon has been described in plant–pathogen interactions [28]. Similar situations are likely to occur in the fungus-to-fungus mycoparasitic process.

The molecular mass of BGN16.3 is 46 kDa as determined by SDS/PAGE. Furthermore, the activity detected for BGN16.3 after SDS/PAGE and renaturation suggests the monomeric nature of this protein. The divergence with the molecular mass calculated from gel filtration is probably due to an affinity of the protein towards Sephacryl as previously described for other extracellular proteins produced by *T. harzianum* [7].

Biochemical values obtained for this novel enzyme are similar to the ones already described in the other two endo-β-1,6-glucanases from *T. harzianum* [10,16], although some differences can be found in isoelectric point, K_m value and substrate specificity, as summarized in Table 3. BGN16.3 can degrade mixed β-1,3-/β-1,6-glucans (i.e. laminarin, a β-1,3-glucan polymer with β-1,6- branches), BGN16.1 can do this as well, but not BGN16.2. However, unlike BGN16.1, BGN16.3 cannot degrade isolated fungal cell walls of *S. cerevisiae*. The fact that BGN16.3 cannot release reducing sugars from the whole cell wall of *S. cerevisiae*, but releases reducing sugars from β-glucan obtained from this cell wall (by alkali lysis), suggests that the enzyme is unable to reach its substrate in the whole cell wall, probably due to the complex structure of the fungal cell wall. This inability of BGN16.3 (and probably other purified cell wall degrading enzymes) to reach its substrate would not affect its participation in the mycoparasitic process, as *Trichoderma* coordinately produces a complex set of different enzymes with synergistic action, able to complete the degradation of the host cell wall [1,11].

Table 3. Biochemical properties of the three β-1,6-glucanases purified from *T. harzianum* CECT 2413.

	BGN16.1	BGN16.2	BGN16.3
Molecular mass (kDa)	51	43	46
pI	7.4–7.7	5.8	4.1–4.5
Optimum temperature (°C)	50	50	50
Glycosylation	ND	ND	ND
K_m (mg pustulan·mL^{-1})	0.8	2.4	1.1
Degrades laminarin	+++	–	+
Degrades *S. cerevisiae* cell wall	+	–	–
Degrades *B. cinerea* cell wall	–	–	–

BGN16.3 accumulation is mainly controlled by the carbon source in the induction media, as could be expected for a glucanolytic extracellular enzyme. When glucose is present in the induction media, no enzyme is produced due to catabolite repression. Pustulan and cell walls can induce the accumulation of BGN16.3 as well as carbon source starvation. Western blots showed a faster and higher accumulation of BGN16.3 when *T. harzianum* was grown on fungal cell walls rather than in pustulan or in the carbon source depletion condition.

This regulation pattern is different from that previously described for BGN16.1, which accumulates abundantly under chitin induction, as do most of the extracellular enzymes described from *T. harzianum*. The fact that BGN16.3 accumulates strongly and specifically in fungal cell wall inductions suggests this enzyme may play a role in mycoparasitism.

A thorough comparative study of the biochemical properties of these three β-1,6-glucanases and the conditions for the induction of each of them (including the motifs present in their regulatory 5′ region) could give light to the detailed biological function of the different components of the β-1,6-glucanolytic system of *T. harzianum*.

Interestingly, there has recently been evidence for the implication of a β-1,6-glucanase, Glu1, in the mycoparasitic interaction of *V. fungicola* with *Agaricus bisporus* [22]. In this process, the penetration into the host occurs by a local degradation of its fungal cell wall [29,30], as also occurs in *Trichoderma* mycoparasitic interactions. These results support an important role for *endo*-β-1,6-glucanases in the degradation of the fungal cell wall complex structure during mycoparasitic interactions. Further experiments will be carried out to assess this possible role for BGN16.3.

The induction of the expression of BGN16.3 using fungal cell walls has proven to be a valid approach to identify novel enzymes produced by *T. harzianum*. The use of fungal cell walls instead of chitin for inductions would be closer (though maybe still not identical) to a mycoparasitism situation, and has allowed us to identify of novel enzyme as shown here.

Experimental procedures

Strains and culture conditions

T. harzianum CECT 2413 [31] and *T. harzianum* CECT 2424 [4] were obtained from the Spanish Type Culture Collection (Burjasot, Valencia, Spain). *Botrytis cinerea* was isolated in our laboratory from infected strawberries. Both strains were maintained in PDA [Potato/Dextrose/Agar (Difco, Detroit, MI, USA)] plates. For protein production a two step growing method was used: *Trichoderma* strains were grown (approximately 10^6 conidia per 400 mL media) in modified Czapek minimal medium (0.5 g·L^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g·L^{-1} $FeSO_4 \cdot 7H_2O$, 0.425 g·L^{-1} KCl, 0.115 g·L^{-1} $MgCl_2 \cdot 6H_2O$, 2.1 g·L^{-1} NH_4Cl, 0.92 g·L^{-1} $NaHPO_4$) supplemented with 2% glucose, in a rotatory shaker at 180 r.p.m. After 48 h the mycelium was filtered, thoroughly washed with 2% magnesium chloride and water, and transferred to a new flask containing Czapek minimal medium supplemented with different carbon sources (replacement medium) and incubated for 48 h at 25 °C in a rotatory shaker at 180 r.p.m. In case of mycoparasitic simulation, 0.5% *B. cinerea* cell walls, prepared as previously described [10], were used as carbon source. For carbon source starvation, modified Czapek minimal medium without any supplement was used as replacement medium.

Enzyme assays

β-1,6-Glucanase activity was determined by measuring the amount of reducing sugars released from pustulan by the Somogyi and Nelson procedure [32,33] using glucose as standard. One unit of β-1,6-glucanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalents, expressed as glucose, per min under standard assay conditions.

Thermal stability of the enzyme was determined incubating the purified protein at temperatures from 30 to 70 °C in 50 mM sodium acetate buffer (pH 5.5) for 30 min and then measuring the remaining enzymatic activity adding pustulan as substrate and incubating as described. Inactivation temperature was defined as the temperature with a reduction of 50% of the specific activity.

Optimum pH determination was performed using citrate–acetic acid buffer for pH values between 3 and 5, phosphate buffer for pH values between 6 and 8 and Tris/HCl buffer was used for pH 9. In all cases the concentration was 50 mM.

Protein purification

(a) All purification steps, unless indicated, were performed at 4 °C. *T. harzianum* CECT 2413 cultures grown at 28 °C for 48 h on *B. cinerea* cell wall as the only carbon source were filtered through filter paper and centrifuged for 10 min at 12 000 ***g***. The supernatant was precipitated with ammonium sulfate (90% saturation) and the precipitate recovered by centrifugation at 25 000 ***g*** for 15 min, resuspended in a small volume of distilled water and dialyzed against 50 mM sodium acetate buffer, pH 5.5.

(b) Dialyzed samples were adsorbed to alcohol precipitated pustulan with magnetic stirring. Pustulan was then precipitated by centrifugation at 12 000 ***g*** for 10 min. The adsorption was repeated twice with the nonadsorbed supernatant. Pustulan pellets were washed three times with 50 mM sodium acetate buffer (pH 5.5), containing 1 M NaCl and resuspended in the same buffer. These samples were incubated overnight at 37 °C in the presence of 1 mM phenylmethanesulfonyl fluoride and 1 mM sodium azide for pustulan digestion. Clarified solutions were centrifuged at 12 000 ***g*** for 10 min and the supernatants recovered and dialyzed against 25 mM imidazole/HCl buffer (pH 6.5).

(c) A 0.5 mL sample of the dialyzed solution was applied to a Polybuffer Exchanger PBE 94 column (Amersham Biosciences, Barcelona, Spain) equilibrated with 25 mM imidazole/HCl buffer pH 6.5. Proteins were eluted at a flow rate of 10 mL·h^{-1} with polybuffer 74 (1 : 10 pH 4.0) and collected fractions (1.6 mL each) were assayed for β-1,6-glucanase activity as described above. Active fractions were pooled and concentrated with a Centricon 10 (Amicon, Beverley, MA, USA) device.

(d) The concentrated pool was subjected to FPLC gel filtration with a Protein Pack 125 column (Waters, Milford, MA, USA) using 50 mM sodium acetate buffer 0.1 M KCl as eluent. The flow rate was 0.1 mL·min^{-1} and fractions were collected every minute. Fractions giving absorbance at 280 nm were assayed for β-1,6-glucanase activity as described above. Active fractions were pooled and concentrated using Centricon 10 devices.

Gel electrophoresis and β-1,6-glucanase activity staining

SDS/PAGE was performed by the method of Laemmli [34] with 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Detection of β-1,6-glucanase specific activity in agar replicas of the SDS/PAGE gels was carried out as described previously [35].

Isoelectrofocusing was performed using Pharmalyte gels (Amersham Biosciences) following manufacturer's directions. β-1,6 activity staining after electrofocusing was performed as described earlier [35]. Standard marker proteins with pI values within the range 3.5–9.3 (Amersham Biosciences) were used to determine the apparent pI for BGN16.3.

Substrate specificity

The purified BGN16.3 activity was tested against several polymers with glycosidic linkages using 0.5 $mg \cdot mL^{-1}$ of each substrate. Activity on these substrates was measured by reducing sugar quantification using the Somogyi–Nelson method, except for chitinase activity that was determined as described previously [7].

Hydrolysis products determination

The resulting products from pustulan hydrolysis by the purified BGN16.3 were applied to a HPLC Aminex HPX-42 A column (Bio-Rad, Barcelona, Spain) maintained at 45 °C. Water was used as eluent at a flow rate of 0.4 $mL \cdot min^{-1}$; diffraction index of the eluate was used for the detection of the products. Glucose and cellulose oligosacharides (2–4 polymerization degree) were used as standards. Substrate controls were carried out in each determination.

Preparation of antisera

Polyclonal antibodies were raised by subcutaneous injection of 250 μg of purified BGN16.3 into rabbits (New Zealand) in complete Freund's adjuvant. At 2-week intervals, rabbits received additional injections with 125 μg of protein in incomplete Freund's adjuvant. Blood samples were taken three times after the second injection with 2-week intervals. Samples were centrifuged 5 min at 3000 *g* and the supernatant was stored at −20 °C and used for western blotting.

Protein partial sequences

N-Terminal and internal peptide sequencing from the purified BGN16.3 was carried out by Eurosequence b. vs. (Groningen, the Netherlands) following Edman degradation method in an Applied Biosystem 494 Sequencer.

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