Biochemical characterization of MI-ENG1, a family 5 endoglucanase secreted by the root-knot nematode Meloidogyne incognita

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A β-1,4-endoglucanase named MI-ENG1, homologous to the family 5 glycoside hydrolases, was previously isolated from the plant parasitic root-knot nematode *Meloidogyne incognita*. We describe here the detection of the enzyme in the nematode homogenate and secretion and its complete biochemical characterization. This study is the first comparison of the enzymatic properties of an animal glycoside hydrolase with plant and microbial enzymes. MI-ENG1 shares many enzymatic properties with known endoglucanases from plants, free-living or rumen-associated microorganisms and phytopathogens. In spite of the presence of a cellulose-binding domain at the C-terminus, the ability of MI-ENG1 to bind cellulose could not be demonstrated, whatever the experimental conditions used. The biochemical characterization of the enzyme is a first step towards the understanding of the molecular events taking place during the plant–nematode interaction.

Keywords: cellulase; cellulose binding; endoglucanase; plant parasitism; root-knot nematode.

Natural plant cellulose is a polysaccharide composed of β-D-glucopyranosyl units joined by 1,4-glycosidic bonds. It is a carbon and energy source for a large number of cellulolytic free-living and rumen symbiotic microorganisms, which have developed a large panel of plant cell-wall degrading enzymes [1–3]. Moreover, the plant cell wall is a major mechanical barrier to the propagation and development of plant pathogens, many of which also produce cellulolytic and pectinolytic enzymes able to disrupt cell-wall polymers. The root-knot nematode, genus *Meloidogyne*, is an endoparasite that has evolved very tight interactions with its plant host. The success of parasitism depends for a large part on the capacity of the infective larva to penetrate the root tip and to migrate intercellularly towards the vascular cylinder.

Cellulolytic enzymes from microorganisms have been studied extensively in terms of structure and enzymatic properties. Cellulases catalyse the hydrolysis of β-1,4-glyco-sidic bonds. They are divided into endoglucanases that cleave the glucan chains at interior sites and cellobiohydrolases that release cellobiose from the chain ends. Microorganisms have developed two main strategies to degrade cellulose. Aerobic fungi and bacteria secrete batteries of individual but synergistically acting enzymes, while anaerobic microorganisms associated with the rumen of chewing animals utilize highly structured multienzyme complexes (cellulosomes) operating at the cell surface. Most cellulases secreted by aerobic organisms have a characteristic modular structure composed of a catalytic domain linked to a functionally independant cellulose-binding domain (CBD) by a flexible linker peptide [4]. In plants, most cellulases lack the ancillary CBD domain. Cellulase catalytic domains have been classified according to sequence-based homologies into 12 of the 77 glycoside hydrolase families [5] (http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf.html). Similarly, CBDs have been classified into 13 families according to similarities in primary structure [6].

Only a few cellulases have been purified from animals. Cellulase activities have been detected in molluscs [7,8] and snails [9], but whether cellulase is produced by the animal itself or an associated microorganism is difficult to establish until isolation of the corresponding gene. Endogenous production of cellulase by animals has only recently been demonstrated for plant parasitic nematodes [10,11] and termites [12]. In termites, two family 9 β-1,4-endoglucanase cDNAs have been cloned consisting of a single catalytic domain [13]. Concerning cyst nematodes, two categories of family 5 β-1,4-endoglucanase genes have been cloned in each of the two genera analysed, one with and one without CBD [10]. The first glycoside hydrolase gene, named *MI-ENG1*, isolated from the root-knot nematode *Meloidogyne* spp. encodes a β-1,4-endoglucanase composed of a catalytic domain and a CBD joined by a linker rich in proline and hydroxyamino-acid residues [11]. The MI-ENG1 catalytic domain belongs to the family 5 of glycoside hydrolases, which is the largest cellulase family. The MI-ENG1 CBD belongs to family II, which so far has been composed of bacterial hydrolase CBDs.

Here we present the biochemical characterization of MI-ENG1. The enzymatic properties of MI-ENG1 are described in terms of physico-chemical properties, mode of action, substrate specificity and cellulose binding ability. This work presents the first complete biochemical characterization of an animal family 5 glycoside hydrolase, and allows the comparison of its enzymatic properties with those of enzymes from microbe and plant origin. The role of this enzyme in nematode parasitism is discussed.

MATERIALS AND METHODS

Nematode cultures, bacterial strains, plasmids and growth conditions

Meloidogyne incognita nematodes were cultured on greenhouse-grown tomato plants and collected as described previously [11]. *Caenorhabditis elegans* nematodes were multiplied *in vitro* on *Escherichia coli* OP50 [14].

E. coli DH5α was used as the recipient strain for recombinant plasmids. *E. coli* cells were grown on Luria–Bertani medium [15]. Transformed bacteria were grown on Luria–Bertani medium supplemented with 100 μg·mL^{-1} ampicillin and 1 mM isopropyl thio-β-D-galactoside. The plasmid pENG1, containing the MI-ENG1 coding sequence, was isolated from a pcDNA II (Invitrogen) cDNA library from *M. incognita* J2 [11]. A plasmid containing a fusion of the CelA3 catalytic domain with the CipC CBD isolated from *Clostridium cellulolyticum* [16], called CelA-CipC, was kindly provided by Chantal Tardif (Bioénergétique et Ingénierie des Protéines, CNRS, IBSM-IFR1, Marseille, France). This vector was used as a positive control in substrate binding assays.

Preparation of protein extracts

Nematode homogenate was obtained by grinding frozen *M. incognita* preparasitic J2s or *C. elegans* in 50 mM sodium phosphate buffer (NaP$_i$). Juvenile stylet secretions were collected as described previously [11].

E. coli cells in the late logarithmic phase were harvested by centrifugation at 8000 *g* for 15 min. Cells expressing MI-ENG1 were resuspended in 50 mM NaP$_i$, pH 5.0. Cells expressing the CelA-CipC fusion protein were resuspended in 50 mM NaP$_i$, pH 7.0. The bacteria were disrupted by sonication at 4 °C (four treatments of 20 s each, separated by 30 s on ice). After centrifuging the total extracts at 10 000 *g* for 20 min, the protein soluble extracts were used for enzyme activity characterization. Protein concentrations were determined by the Bradford procedure [17].

Enzyme assays

Endoglucanase activity was assayed by measuring the decrease in viscosity of a carboxymethylcellulose (CM-cellulose) solution. Protein extracts were incubated in 50 mM NaP$_i$, 0.3% CM-cellulose at a given temperature. After incubation, the reaction was stopped by boiling for 20 min, then the sample was left to cool down to room temperature. The time required for the passage of the solution between two points marked on a 2-mL bulb pipette was measured. The reduction in viscosity was measured by comparing the time of passage of the incubated sample with that of a nonincubated one. To evaluate the effect of pH on activity, enzymatic assays were conducted in 50 mM NaP$_i$ ranging in pH from 4.5 to 8.0.

The substrates barley β-glucan, laminarin, lichenan, oat spelt xylan, medium viscosity CM-cellulose and Sigmacell 101 were obtained from Sigma and the microcrystalline cellulose Avicel PH-101 from Fluka. Tomato cell walls were extracted from tomato leaves following the procedure described by Keller *et al.* [18]. Each substrate (1%) was incubated for 2 h at 50 °C with the protein extracts in the presence of 2 mM phenylmethanesulfonyl fluoride in order to inhibit proteolysis. Released sugars were quantified by the ferricyanide method [19]. 4-Methylumbelliferyl β-D-glucoside (MUG) and 4-methylumbelliferyl

β-D-cellobioside (MUC) (1 mg·mL^{-1}) (Sigma) were used on agar plates to detect exoglucanase or cellobiase activity under UV light.

Thin-layer chromatography

Samples (10 μL) and standards (glucose and cellobiose 1 mM each) were spotted on thin-layer silica gel plates (CCM 60F254, Merck KGaA). Sugars were separated in one dimension as previously described [20] except that the separating mixture was butanol/acetic acid/water (8 : 10 : 1.5).

Binding assays

The recombinant MI-ENG1 (and positive control CelA-CipC) were tested for binding to cellulose by incubating soluble protein extracts in a 1% Sigmacell 101, 1% microcrystalline PH-101 cellulose (Fluka), 50 mM NaP$_i$ pH 5.0 solution (or pH 7.0 in the case of CelA-CipC), for 120 min at 50 °C and at room temperature. Samples were collected after 0, 10, 30, 60 and 120 min incubation and then centrifuged 5 min at 10 000 *g*. Supernatants were assayed for CM-cellulase activity. Negative controls without substrate were used to measure possible *E. coli* extract proteolysis during the incubation and to calculate the percentage of substrate-binding enzymes in the experimental samples.

Binding of MI-ENG1 to plant cells was assayed as described by Ding *et al.* [21], except that 2 mL of soluble protein extract (2 mg·mL^{-1}) were added to a 15-mL of fresh tobacco (*Nicotiana tabacum*) cell suspension culture. The culture was gently agitated for 36 h. Tobacco cells were fixed in 3% paraformaldehyde/NaCl/P$_i$ (32.9 mM Na$_2$HPO$_4$, 1.77 mM NaH$_2$PO$_4$, 0.14 M NaCl, pH 7.4) for 16 h at 4 °C, then 4 h at room temperature, washed twice in NaCl/P$_i$ and blocked in 3% BSA/NaCl/P$_i$ for 15 min. The cells were incubated with a 1 : 250 dilution of mouse polyclonal anti-(MI-ENG1–MBP fusion) Ig (where MBP is maltose-binding protein) in 1% BSA/NaCl/P$_i$, followed by a 1 : 100 dilution of anti-(mouse IgG) FITC-conjugated Ig in 1% BSA/NaCl/P$_i$. Each incubation step was followed by three 20-min washes in NaCl/P$_i$.

SDS/PAGE and zymogram

PAGE was performed as described by Laemmli [22] with a Mini Protean II Dual Slab cell electrophoresis apparatus (Bio-Rad Laboratories). Proteins in the gels were stained by Coomassie blue. For the CM-cellulase zymogram, PAGE was run under nondenaturing conditions except that SDS was added to the running buffer and 0.1% CM-cellulose was added into the polyacrylamide gel. CM-cellulase activity was visualized in gels with 0,1% Congo red, after three washes in 1 M NaCl to remove SDS and 15 h incubation at 37 °C in 50 mM NaP$_i$, pH 5.0.

Antibody production

A truncated inactive form of MI-ENG1 fused to the MBP was obtained by digestion of pENG1 with *Eco*RI and *Bam*HI then ligation of the digested fragment in frame with the MBP in the pMAL-p2 expression vector (New England Biolabs Inc.). The resulting recombinant protein was lacking the first 137 amino acids from MI-ENG1. The MI-ENG1–MBP protein was expressed in *E. coli* and purified on an amylose column (New England Biolabs Inc.) according to the supplier's instructions. Mouse polyclonal antibodies directed to MI-ENG1–MBP were

produced by Eurogentec. The activity and specificity of the serum obtained were checked by ELISA. ELISA tests showed that the anti-(MI-ENG1–MBP) serum cross-reacted with the CelA-CipC fusion protein with an affinity slightly lower than for MI-ENG1–MBP. This cross-reaction could be explained by the presence of common epitopes in the two family 5 endoglucanase catalytic domains.

Western blotting

Protein samples were loaded on a 13% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred to a nitrocellulose membrane (Hybond-C, Amersham) by electro-blotting. For immunodetection the membranes were incubated with 1 : 1000 mouse anti-(MI-ENG1–MBP) polyclonal Ig, followed by a 1 : 5000 diluted peroxidase-conjugated mono-clonal goat anti-(mouse IgG) Ig (Jackson ImmunoResearch Laboratories). The blots were stained in 0.224 mM 3,3′-diamino-benzidine, tetrahydrochloride in hydrogen peroxide (Pierce). The relative molecular masses of the proteins were estimated with prestained low range molecular mass markers (Bio-Rad).

RESULTS

Expression of MI-ENG1 in *E. coli* and *M. incognita*

A full-length cDNA clone previously isolated from *M. incognita* [11] and encoding a CM-cellulase of 506 amino acids named MI-ENG1 was studied for enzymatic properties.

Soluble extracts of *E. coli* DH5α cells containing either the empty vector pcDNAII or the pENG1 recombinant plasmid were analysed by zymogram run under nondenaturating conditions. The zymogram revealed after staining a unique band at about 54 kDa in *E. coli*/pENG1 cell extracts (Fig. 1A), which is consistent with the predicted 53.4-kDa molecular mass of MI-ENG1. The total CM-cellulase activity present in *M. incognita* J2 homogenate and J2 stylet protein secretions were also examined on this zymogram. Two major activity bands were detected in the homogenate at about 62 kDa and up to 120 kDa, whereas only the 62-kDa band was revealed in the stylet protein secretions. No clear band was detected in either sample around 54 kDa. As potential N-glycosylation sites are present on the MI-ENG1 deduced protein sequence [11], and as the linker region that separates the catalytic and the cellulose binding domains is rich in hydroxyaminoacids, we suggest that MI-ENG1 could be glycosylated in the nematode.

MI-ENG1 was detected in nematode homogenate and stylet secretions by Western-blot analysis using a polyclonal anti-serum raised against a truncated inactive form of MI-ENG1 linked to a MBP (Fig. 1B). The polyclonal anti-(MI-ENG1–MBP) serum reacted strongly with a protein whose molecular mass is 62 kDa in *M. incognita* homogenate (Fig. 1B) and with three other proteins of lower molecular masses (42, 40 and 34 kDa). The polyclonal serum did not react with *C. elegans* homogenate used as a negative control. Furthermore, no signal was observed on Western-blots treated with an anti-MBP monoclonal antibody, suggesting that the reaction obtained with anti-(MI-ENG1–MBP) Ig was not due to the presence of a MBP in the extracts analysed (not shown). The 62-kDa protein was the only protein recognized in stylet secretions by the anti-(MI-ENG1–MBP) serum (Fig. 1B), suggesting that the other proteins detected in the nematode homogenate could be degradation products of MI-ENG1 or nonsecreted cellulases sharing common epitopes with MI-ENG1. No protein was

detected at 54 kDa on Western blots, confirming the zymogram result and suggesting that the 62-kDa protein corresponds to the molecular mass of MI-ENG1 after post-translational modification such as glycosylation.

Physico-chemical properties of the endoglucanase

The endoglucanase activity of MI-ENG1 was tested by viscosimetry. The CM-cellulase activity expressed by *E. coli* cells producing MI-ENG1 was quantified by measuring the changes in viscosity of a CM-cellulose solution before and after incubation with the cell extracts. We ensured that the measured activity was proportional to the amount of enzyme and the incubation time.

Soluble cell extracts were incubated at 50 °C in 0.3% CM-cellulose solutions ranging from pH 3.0 to pH 8.5. For each pH condition the decrease in viscosity of the CM-cellulose solution was measured. The optimum activity of MI-ENG1 was observed at pH 5.0 (Fig. 2A) and the enzyme retained 80% of its activity between pH 4.5 and 6.5. There was a sharp decrease of activity below pH 4.0 and a slow gradual decrease at alkaline pH values.

The extracts were then incubated in 0.3% CM-cellulose, pH 5.0, at various temperatures. A maximum of activity was detected at 50 °C (Fig. 2B), and MI-ENG1 retained about 80% of its activity between 45 and 60 °C. The enzyme was inactivated above 65 °C, but retained 50% of activity when incubated between 30 and 50 °C. Therefore the enzyme is active in a large spectrum of temperature conditions, as was observed for pH conditions.

The effect of the chelating agent EDTA on MI-ENG1 endoglucanase activity was also tested. The enzyme was not inhibited when incubated in 5, 10, 15 or 20 mM EDTA (not shown), suggesting that MI-ENG1 does not require divalent cations to degrade CM-cellulose. Furthermore, addition of 5 mM calcium and magnesium in the incubation buffer did not produce any effect on MI-ENG1 activity. This is also the case for the majority of glycoside hydrolases from family 5, in contrast to family 9 glycoside hydrolases whose activity involves divalent cations [23–25]. Divalent metallic cations
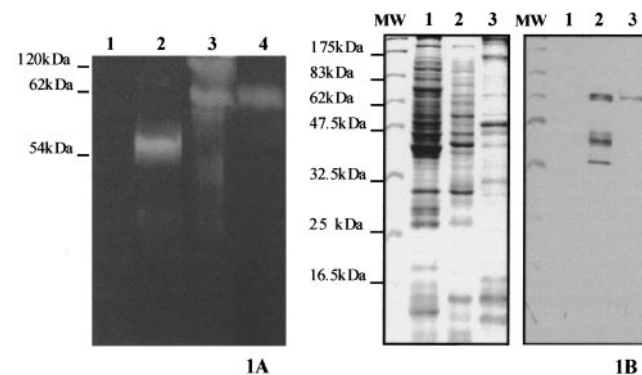

Fig. 1. CM-cellulase activity and MI-ENG1 detection in bacteria, nematode extracts and stylet secretions. (1A) Zymogram analysis of CM-cellulase activity in *E. coli* control extracts (lane 1), MI-ENG1 *E. coli* extracts (lane 2), *M. incognita* J2 total homogenate (lane 3), *M. incognita* J2 stylet secretions (lane 4). (1B) Detection of MI-ENG1 by Western-blotting in *C. elegans* total homogenate (lane 1), *M. incognita* J2 total homogenate (lane 2), *M. incognita* J2 stylet secretions (lane 3). Left panel, Coomassie blue stained PAGE gel. Right panel, Western-blot. MW, molecular mass markers.
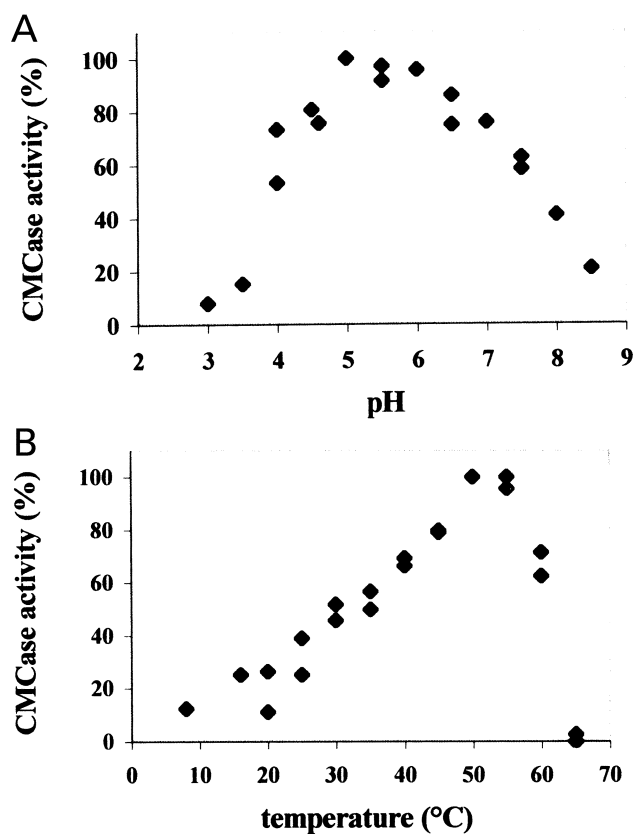
A

B

Fig. 2. Effect of pH and temperature on MI-ENG1 endoglucanase activity. (A) pH effect on CM-cellulase activity in NaP_i buffers ranging in pH from 3.0 to 8.5, incubation temperature 50 °C. (B) Temperature effect on CM-cellulase activity in NaP_i buffer pH 5.0. Relative activity is expressed as percentage of maximum activity. Values obtained in two repetitions of the experiment are shown.

(Fe^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+}), known to produce inhibitory effects on family 5 glycoside hydrolases, were tested for inhibition of MI-ENG1 activity. For each cation tested, cell extracts were incubated in 1 and 5 mM ion solutions. MI-ENG1 was inhibited by Zn^{2+} and Cu^{2+} cations; its activity decreased, respectively, by 30 and 50% in the presence of 5 mM $ZnCl_2$ or $CuSO_4$ (not shown). $SO4^{2-}$ and Cl^- ions did not affect enzyme activity.

As cellulases of most cellulolytic organisms are known to be sensitive to the presence of simple soluble sugars [4,26,27], we

evaluated the effect of several concentrations of glucose and cellobiose on the endoglucanase activity of MI-ENG1. Final concentrations of 25, 50, 75 or 100 mM glucose or cellobiose in the incubation buffer did not inhibit the enzyme activity (not shown), suggesting that MI-ENG1 is not subject to feed-back inhibition by these sugars.

Mode of action and substrate specificity

Soluble extracts of *E. coli* cells producing MI-ENG1 were incubated for 1 h in 0.3% CM-cellulose at 50 °C, and sampled periodically to measure both reduction of viscosity and release of reducing sugars. Figure 3 presents the correlation between the decrease in CM-cellulose viscosity and the reducing sugars produced at the same incubation time. A similar experiment was conducted on total *M. incognita* homogenate in order to compare the mode of action of MI-ENG1 to the total endoglucanase activity produced by the nematode (Fig. 3). For both *E. coli* and nematode extracts, quite few reducing sugars are liberated for high CM-cellulase activity, according to the endo-acting mode of cellulases. Moreover, the mode of action of MI-ENG1 is quite similar to that of the total endoglucanase activity present in *M. incognita* homogenate, as MI-ENG1 produced only 5 µg less reducing sugars than the total homogenate, when retaining 50% of CM-cellulase activity. No exoglucanase activity could be detected for MI-ENG1 using
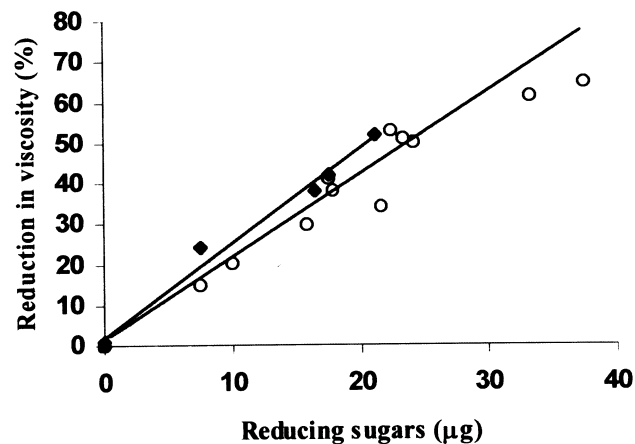

Fig. 3. Correlation between reduction in viscosity and released reducing sugars. (◆) MI-ENG1 *E. coli* cell extracts and (○) *M. incognita* total homogenate.

Table 1. Substrate specificity of MI-ENG1 endoglucanase. Results are mean values of three determinations. Reducing sugars determined after incubation of cell extracts for 2 h at 50 °C. One unit (U) of activity corresponds to µg of glucose equivalent for all glycan substrates or xylose equivalent for xylan substrates. Values given correspond to reducing sugars released by 100 µg of *E. coli*/pENG1 extracts. Each substrate was 1%. ND, not detectable. Cell-wall components extracted from fresh tomato cell suspension (see Materials and methods).

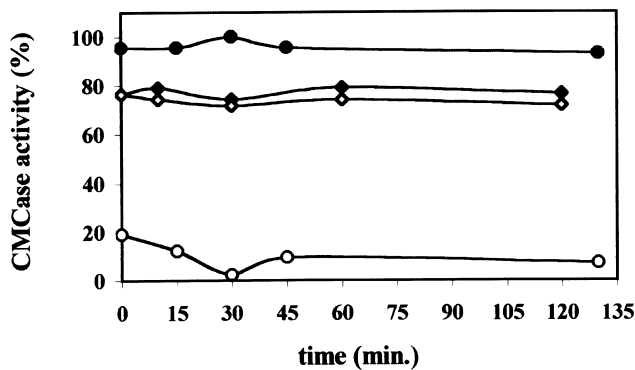| Substrate	Reducing sugars (U)	Relative activity (%)
Barley β-glucan	190 ± 14.1	152
CM-cellulose	129 ± 16.8	100
Laminarin	ND	0
Lichenan	27 ± 0.7	21.6
Crystalline cellulose (Sigmacell 101)	3.4 ± 0.6	2.7
Oat spelt xylan	2.2 ± 0.5	1.8
Tomato cellulose	1.9 ± 0.5	1.5
Crystalline cellulose (Avicel)	1.5 ± 1.2	1.2

Fig. 4. Cellulose binding assays. Relative CM-cellulase activity measured after incubation of MI-ENG1 (◇) and CelA-CipC (○) *E. coli* extracts with crystalline cellulose. For each extract, activity is expressed as percentage of maximum activity relative to the control without substrate (◆ and ●, respectively).

MUC as a substrate, nor could cellobiase activity be detected using MUG on agar plates, suggesting that the enzyme is only an endo-acting enzyme.

The products of CM-cellulose hydrolysis by MI-ENG1 were mainly cellobiose and cellotriose as deduced from thin-layer chromatography analysis (not shown).

MI-ENG1 was examined for its ability to hydrolyse carbohydrate polymers containing different types of linkages present in plant cell walls (Table 1). A high activity was detected on barley β-glucan and CM-cellulose, while no activity could be detected on laminarin (β-1,3 linkages), suggesting that MI-ENG1 is unable to cleave β-1,3 linkages and is exclusively a β-1,4-endoglucanase. A low activity was detected on lichenan. A difference in organization of β-1,3 and

β-1,4 linkages in lichenan as compared to β-glucan could explain this difference in enzyme activity.

Different types of crystalline celluloses as well as cell walls extracted from tomato leaves were tested for MI-ENG1 activity. Activity was barely detectable on Sigmacell or Avicel crystalline celluloses, as well as on tomato cell walls (Table 1), suggesting that the enzyme is not able to degrade plant cell walls by itself. Whereas several family 5 glycoside hydrolases show both cellulase and xylanase catalytic activities [27–30], MI-ENG1 showed no significant activity on xylan.

Cellulose, xylan, tobacco cells binding assays

The MI-ENG1 deduced protein sequence revealed a modular structure for the enzyme, with a family II CBD at the C-terminus [11]. We checked for the binding capacity of the endoglucanase on different polysaccharidic substrates. Binding to cellulose was first evaluated by incubating MI-ENG1 *E. coli* extracts in microcrystalline cellulose for 2 h at room temperature before monitoring CM-cellulase activity in the soluble fraction. As a control for proteolysis of MI-ENG1 during the incubation, cell extracts were incubated for 2 h in the absence of cellulose and CM-cellulase activity was measured. Figure 4 shows that CM-cellulase activity in the soluble fraction was constant during the incubation time for both the negative control (without cellulose) and the binding assay, suggesting that no proteolysis of the enzyme occurred during the 2 h of incubation but also that no decrease in activity occurred due to MI-ENG1 binding to the cellulose. The endoglucanase CELA from *Clostridium cellulolyticum* fused to an additional CipC CBD that binds to microcrystalline cellulose [16] was used as a positive control (Fig. 4). Relative CM-cellulase activity immediately decreased from 100% in the absence of cellulose to 20% when cellulose was present,

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                                                                        binding
                                                                     to cellulose
              1                                              60
MI-ENG1   ----------TASVQVP-DKWDNGARFQLVFKNNASTKKCAVKFSLTFASGQQITGIWNV        -
MI-CBP1   --------------VILRDKWVNGANCILAFKNNGNARACGVKFELTLGDNQRIQSIWNV        +
GR-ENG1   --------------VSTNTWNGGGQVNFEVKNTGSTTLCGVKFSVTLPAGTTVAGSWNM        +
HG-ENG1   --------------VPSNTWNGGGQVNFEIKNIGSVPLCGVVFSVSLPSGTTLGGSWNM        ?
TF-E5     --AAG----LTATVTKE-SSWDNGYSASVTVRNDTSSTVSQWEVVLTLPGGTTVAQVWNA        +
SH-Cel2   SPGAPGEPVTACEVAYATNVWSDGFTADVTVANTGSTPVDSWRLSFTLPSGQRVTNAWNA        +
AC-E1     -SGAR----CTASYQVN-SDWGNGFTVTVAVTNSGSVATKTWTVSWTFGGNQTITNSWNA        ?
                     *   *          *                         **
                                                                        binding
                                                                     to cellulose
              61                                            120
MI-ENG1   QNVTGNSFVLP---DYVTIEAGKQ-YTDAGMNINGPATPPQIKVLGDGK--CVF---        -
MI-CBP1   EKVGDKVYRIP---DYIQLGPGVE--NRDIGVVYNDVPELFHNQGLGQEEGCNIIE-        +
GR-ENG1   NAAGSNEYTLP---SYINIKAKEA--NKDAGMTLNG-SGKPTAKVLSTTA-CSG---        +
HG-ENG1   ESAGSGQYSLP---SWVRIEAGKS--SKDAGLTFNG-KDKPTAKIVTTKK-C-----        ?
TF-E5     QHTSSGNSHTFTGVSWNSTIPPGG--TASSGFIASG-SGEPTHCTINGAP-CDEGSG        +
SH-Cel2   TVSGPTGAVTATGLAHSAQVAAGASQTFGFQGTYSGTFSKPSGFSLNGAR-CA----        +
AC-E1     AVTQNGQSVTARNMSYNNVIQPGQNTTFGFQASYTG-SNAAPTVA------CAASF-        ?
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Fig. 5. Comparison of the deduced amino-acid sequence of the MI-ENG1 CBD with family II CBDs. Sequence alignement of MI-ENG1 CBD (GenBank accession number AF100549) with CBDs from MI-CBP1from *M. incognita* (AF049139), and endoglucanases GR-ENG1from *Globodera rostochiensis* (AF004523), HG-ENG1from *Heterodera glycines* (AF006052), CelE5 from *Thermomonospora fusca* (L01577), Cel2 from *Streptomyces halstedii* (U51222) and E1from *Acidothermus cellulolyticus* (P54583). Residues conserved in all sequences are marked with asterisks. Tryptophan residues usually conserved in family II CBDs are in bold.

showing an 80% loss of activity due to CelA-CipC binding to cellulose.

In order to evaluate a possible temperature effect on MI-ENG1 adhesion, cellulose-binding experiments were conducted at 10 °C, 35 °C and at the optimal activity temperature 50 °C. No binding of MI-ENG1 to cellulose could be observed whatever the temperature tested (not shown).

As it is known that some CBDs are able to bind to xylan or chitin [31–33], oat spelt xylan- and chitin-binding assays were carried out for MI-ENG1. No binding was observed for both polymers. Considering that commercial cellulose and xylan do not precisely mimic the plant cell-wall complexity, we checked the MI-ENG1 binding capacity on fresh tobacco cell suspensions. Fresh whole tobacco cells were incubated with MI-ENG1 *E. coli* extracts and adhesion was tested by immunofluorescence using the polyclonal antiserum directed against the fusion protein MI-ENG1–MBP. No binding of MI-ENG1 to tobacco cells could be observed (not shown). On the contrary, binding was clearly observed with CelA-CipC *E. coli* extracts used as a positive control (not shown). These results suggest that the binding of MI-ENG1 to plant material and purified cellulose or xylan is very weak or that the MI-ENG1 endoglucanase is not able to bind to these substrates under the experimental conditions tested.

Because all experimental procedures used to detect MI-ENG1 substrate binding capacity failed, we examined the protein sequence of the MI-ENG1 CBD in order to assess the possibility of an amino-acid exchange that could induce a loss of substrate-binding capacity. MI-ENG1 CBD was compared to protein sequences of several family II CBDs. From the sequence alignement, no amino-acid motif could clearly be identified as potentially responsible for the absence of binding (Fig. 5).

DISCUSSION

Cellulases have been extensively studied in the case of cellulolytic microorganisms from the soil and rumen of animals. However, the enzymatic properties of plant or phytopathogen cell-wall degrading enzymes have been poorly analysed. This study is the first extended biochemical characterization of a family 5 endoglucanase produced by a phytoparasitic animal. The biochemical characteristics of MI-ENG1 were analysed in order to have a better understanding of its role in parasitism. Moreover, this study allows the comparison of an animal cellulase with microbial cellulases from the same glycoside hydrolase family.

MI-ENG1 shares many enzymatic properties with known endoglucanases from plants, free-living microorganisms and phytopathogens. By analysing the products of enzymatic reaction and by measuring the liberated reducing sugars, we have demonstrated that MI-ENG1 is an endoglucanase devoid of any exoglucanase activity. Furthermore, substrate specificity analysis showed that MI-ENG1 cleaves β-1,4 linkages but is unable to cleave β-1,3 linkages.

The predicted molecular mass of MI-ENG1 is 53.4 kDa. However, zymogram and Western-blot analysis together suggested that MI-ENG1 is produced as an active endoglucanase with an approximate molecular mass of 62 kDa in total nematode homogenate and stylet secretions. This result suggests that MI-ENG1 undergoes post-translational modifications such as glycosylation when synthesized in the nematode. This hypothesis is supported by the presence of several potential N- and O-glycosylation sites in the deduced protein sequence of MI-ENG1. Glycosylation of family 5

glycoside hydrolases has been demonstrated in several cases [34,35]. In plant pathogens, it is assumed that such a glycosylation would protect hydrolases from proteolysis [36]. During infestation, plant pathogens secrete cellulases that are subjected to degradation by plant proteases. Protection of cellulases from proteolysis could then play a major role in the success of pathogen development *in planta*.

MI-ENG1 shares a lot of physico-chemical properties with family 5 endoglucanases characterized. Optimal MI-ENG1 activity was observed at pH 5.0, 50 °C. Several cellulases from plants [37], fungi [38,39] or bacteria [40,41] show optimal hydrolytic activities between pH 4.0 and pH 6.0. Similarly, extracellular endoglucanases secreted by the phytopathogenic enterobacteria *Erwinia* sp. have an optimal activity at pH 5.0 [42], or retain 80% of activity at pH 5.0 [43]. These pH conditions for enzymatic activity are in accordance with the pH 5.5 of the plant cell apoplasm. Furthermore, although no thermostabilizing domain could be shown in MI-ENG1, the enzyme is particularly active at high temperatures (up to 60 °C). This is also the case for several bacterial [40,44], fungal [39] or phytopathogen hydrolases such as the endoglucanase CELB from *E. carotovora* whose optimal temperature is 50 °C [45]. Optimal temperature and pH conditions revealed that MI-ENG1 is active over a large spectrum of conditions, as it retains 80% of its activity between pH 4.5 and 6.5 and 50% of its activity between 30 and 60 °C. This could be related to the fact that root-knot nematodes are wide-spread in tropical and temperate regions. Therefore, they are subjected to various edaphic and climatic conditions and are able to infect more than two hundred plant species. The ability of MI-ENG1 to be active at diverse pH and temperature conditions could reflect an ability of the enzyme to degrade host plant cellulosic materials in a large variety of environmental conditions.

The inhibitory effect of Zn^{2+} and Cu^{2+} metallic cations on MI-ENG1 activity is a common feature of cellulases and xylanases [46–48], including plant cellulases [24,49]. Furthermore, several family 5 glycoside hydrolases, especially from ruminal cellulolytic bacteria, are also inhibited by these cations [47,50]. Enzyme inhibition by metallic cations usually suggests the presence of at least one sulfhydryl group in the active site, usually a cysteine amino acid, whose oxidation by the cations destabilizes the conformational folding of the enzyme [51], or leads to the formation of disulfide bonds at an irregular position of the protein [24]. Five cysteine residues in the catalytic domain of MI-ENG1 could provide potential metallic cation reactive sites causing enzyme inhibition.

The endoglucanase activity of MI-ENG1 is not subjected to feed-back inhibition by soluble sugars such as glucose or cellobiose, suggesting that enzyme regulation could be provided by other polysaccharides or/and that regulation of the enzyme synthesis may exist. Such a regulation has been observed in most cellulolytic organisms [4] and in phytopathogenic organisms [27,52], where cellulase gene expression is regulated by soluble carbon sources which are easily metabolized.

The MI-ENG1 endoglucanase is not significantly active against crystalline cellulose, like all the family 5 glycoside hydrolases yet characterized. Up to now, cellulases able to hydrolyse microcrystalline cellulose have been found in families 9 and 48 of glycoside hydrolases from ruminal cellulolytic microorganisms [53–56] and termites [57]. No plant or biotrophic phytopathogen cellulase is yet known to degrade crystalline cellulose. A large number of cellulases are inactive or poorly active on this substrate when acting separately, but an efficient degradation can be observed when

glycoside hydrolases act synergistically or when they are associated in multienzymatic complexes [58]. Whereas cellulolytic ruminal microorganisms hydrolyse efficiently the plant biomass to produce energy and a carbon source, it is assumed that root-knot nematodes only need limited hydrolysis of the cell wall in order to separate adjacent cells when migrating intercellularly in the root tissue. Furthermore, one could hypothesize that plant parasitic nematodes do not produce a large hydrolytic multienzyme complex, but rather a more simple and qualitative panel of hydrolytic enzymes [10,59] (M. N. Rosso, L. Arthaud, T. N. Ledger and P. Abad, unpublished results).

One peculiarity of MI-ENG1 as compared to characterized microbial or plant cellulases is its inability to bind to cellulose under the experimental conditions tested, in spite of the presence of a CBD at the C-terminus. Whether the native MI-ENG1 binding features are different from the enzyme expressed in bacteria still has to be determined. Few data are available concerning the binding ability of CBDs isolated and binding in some cases is weak and depends on very specific physico-chemical conditions (J. T. Pembroke, personal communication). However, inability to bind to cellulose has been reported in the case of a family IIIc CBD [60]. The MI-ENG1 CBD belongs to family II CBDs. Comparing the MI-ENG1 CBD protein sequence to family II CBD sequences gave no explanation for the absence of binding ability on crystalline cellulose. Tryptophan residues have been shown to be involved in cellulose binding [58]. A peculiarity of CBDs isolated so far from nematodes is the presence of only two conserved tryptophan residues instead of three or four residues in subfamilies IIb and IIa, respectively [58]; the ability of such CBDs to bind to cellulose has been demonstrated in the case of the *M. incognita* cellulose binding protein (MI-CBP1) [21], and the cyst nematode endoglucanase GR-ENG1 (G. Smant, personal communication). CBDs of nematode origin carrying two tryptophan residues could then constitute a new subfamily inside the family II. Some CBDs have been shown to play an important role in cellulase activity and thermostability [60,61]. More studies such as CBD deletion or directed mutagenesis will be necessary in order to analyse whether the MI-ENG1 CBD has any effect on enzyme activity and whether residue substitutions restore cellulose binding ability.

To conclude, the enzymatic properties of *M. incognita* MI-ENG1 are in accordance with the environment the nematode faces during plant invasion. MI-ENG1 shares biochemical characteristics with the family 5 glycoside hydrolases characterized so far and with cellulases isolated from pathogens invading plant tissues intercellularly, such as bacteria or biotrophic fungi. The only peculiarity of the enzyme is its inability to bind efficiently to cellulose under the experimental conditions tested, which may reflect a weak binding or an absence of binding *in planta*. The lack of observed binding to cellulose together with the little effect of MI-ENG1 on crystalline cellulose or natural cell walls, suggest that MI-ENG1 plays a supporting role to other secreted cellulases. The ongoing isolation of new β-1,4-endoglucanase genes from *M. incognita* indeed suggests that the nematode secretes several cellulases during parasitism, some of them sharing identical molecular masses (M. N. Rosso, L. Arthaud, T. N. Ledger and P. Abad, unpublished results). Similarly, several cellulase genes have recently been cloned from plant-parasitic cyst nematodes [59]. The biochemical and molecular characterization of these new cell-wall degrading enzymes should lead to the understanding of their respective role in parasitism and the molecular events leading to nematode-specific plant tissue alterations.

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