Purification, characterization and synergism in autolysis of a group of 1,3-β-glucan hydrolases from the pilei of Coprinopsis cinerea fruiting bodies

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Using a combined chromatography method, we simultaneously purified three protein fractions (II-2, II-3 and II-4) with 1,3-β-glucanase activity from extraction of pilei of Coprinopsis cinerea fruiting bodies. MALDI-TOF/TOF amino acid sequencing showed that these three fractions matched a putative exo-1,3-β-glucanase, a putative glucan 1,3-β-glucosidase and a putative glycosyl hydrolase family 16 protein annotated in the C. cinerea genome, respectively; however, they were characterized as a 1,3-β-glucosidase, an exo-1,3-β-glucanase and an endo-1,3-β-glucanase, respectively, by analysis of their substrate specificities and modes of action. This study explored how these three 1,3-β-glucoside hydrolases synergistically acted on laminarin: the endo-1,3-β-glucanase hydrolysed internal glycosidic bonds of laminarin to generate 1,3-β-oligosaccharides of various lengths, the exo-1,3-β-glucanase cleaved the longer-chain laminarioligosaccharides into short-chain disaccharides, laminaribiose and gentiobiose, and the 1,3-β-glucosidase further hydrolysed laminaribiose to glucose. The remaining gentiobiose must be hydrolysed by other 1,6-β-glucosidases. Therefore, the endo-1,3-β-glucanase, exo-1,3-β-glucanase and 1,3-β-glucosidase may act synergistically to completely degrade the 1,3-β-glucan backbone of the C. cinerea cell wall during fruiting body autolysis. These three 1,3-β-glucoside hydrolases share a similar optimum pH and optimum temperature, supporting the speculation that these enzymes work together under the same conditions to degrade 1,3-β-glucan in the C. cinerea cell wall during fruiting body autolysis.

INTRODUCTION

Most basidiomycetes form a fruiting body for sporulation during their life cycle. The cell wall of the fruiting body is constructed mainly of chitin and 1,3-β-glucan with 1,6-β-linked branches (Kamada et al., 1991; Sakamoto et al., 2012). During the development of fruiting bodies, basidiomycetes produce some hydrolytic enzymes associated with these polysaccharides in cell walls. These fungal glycoside hydrolases act on cell wall components and are considered to be responsible for cell wall extension (Kamada et al., 1991; Lim & Choi, 2009; Muraguchi et al., 2008; Tao et al., 2013), cell wall remodelling.

Abbreviations: BGL1, 1,3-β-glucosidase 1 from C. cinerea (II-2 fraction); ENG, endo-1,3-β-glucanase from C. cinerea (II-4 fraction); EXG, exo-1,3-β-glucanase from C. cinerea (II-3 fraction).

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species (Bush, 1974; Kües, 2000). However, these glycoside hydrolases have not been purified to determine their properties or physiological functions in cell walls to date, although sequencing of theCoprinopsis cinerea genome was completed in 2010 (Stajich et al., 2010). Furthermore, stipe elongation and pileus expansion were also suggested to be caused by cell wall softening by some polysaccharide hydrolases (Kamada et al., 1991; Muraguchi et al., 2008; Tao et al., 2013). However, we recently purified an expansin-like protein from the juice of the snail stomach, which could reconstitute heat-inactivated stipe wall extension of Flammulina velutipes (Fang et al., 2014) and C. cinerea (Zhang et al., 2014) without hydrolytic activity. Our results do not support direct involvement of enzymic hydrolysis in stipe elongation. Therefore, it was necessary to isolate and purify a series of glycoside hydrolases from C. cinerea to elucidate their roles in stipe elongation, pileus expansion, cell wall remodelling and fruiting body autolysis. Here, we isolated and purified a group of 1,3-β-glucan hydrolases from pilei extractions of C. cinerea, and elucidated how they synergistically degraded 1,3-β-glucan for fruiting body autolysis.

METHODS

Strain and culture. Mycelium of C. cinerea ATCC 56838 grown on PDAY medium agar (300 g diced potatoes, 20 g glucose, 15 g agar, 5 g yeast extract and 1000 ml distilled water) was inoculated on sterilized grain medium (soaked, parboiled grains supplemented with 1 % calcium carbonate) in 250 ml wide-mouth bottles and incubated at 28 °C until the mycelium spread throughout the whole medium. The grain medium with mycelium was inoculated on sterilized rice straw medium (88 % dry rice straw, 5 % bran, 3 % corn meal, 2 % commodity fertilizer, 1 % lime, 1 % sugar and an appropriate amount of water) in polypropylene bags (17 cm × 25 cm) and incubated at 28 °C until the mycelium grew throughout the whole rice straw medium in the bags. The bags were then opened and transferred to a fruiting room to continue cultivation at 28 °C at 80 % humidity under a regime of 12 h light/12 h dark until the pilei of the fruiting bodies opened ∼75%.

Enzyme preparation. Approximately 200 g pilei from C. cinerea fruiting bodies, in which pilei opened ∼75%, was added to 200 ml prechilled grind buffer (25 mM HEPES, pH 7.0, 2 mM Na2S2O5 and 0.1 % Triton X-100) and homogenized in a Waring blender. The extraction was filtered through a nylon screen (70 μm mesh) and collected. After centrifugation, the fraction with 1,3-β-glucanase activity was precipitated between 40 and 60 % saturation of ammonium sulfate from the extraction. The precipitate was dissolved in 50 mM sodium acetate/acetate acid (pH 6.0), dialysed against 50 mM sodium acetate/acetate acid (pH 6.0) overnight and then fractionated on a Bio-Gel P-6 open column (50 × 2.5 cm; Bio-Rad) to remove non-specifically bound proteins.

The active fraction from the Bio-Gel P-6 open column was collected, applied to a DEAE-Sepharose fast flow open column (20 × 1 cm; GE) and eluted with a linear gradient of 0–0.3 M sodium chloride in 50 mM sodium acetate/acetate acid (pH 6.0). The active 1-1 fraction eluted from the DEAE-Sepharose open column was collected, concentrated on an Amicon Ultra-4 10K centrifuge filter (Millipore) and purified by TSKgel Phenyl-5PW hydrophobic interaction HPLC or TSKgel G2000SW molecular size exclusion HPLC. The active 1-2 fraction eluted from the DEAE-Sepharose open column was pooled, lyophilized, dissolved in 1 ml 1 M (NH4)2SO4 in 50 mM sodium acetate/acetate acid (pH 6.0), and applied to a TSKgel Phenyl-5PW HPLC column (10 μm, 10 mm × 250 mm; TOSOH) equilibrated with 1 M (NH4)2SO4 in 50 mM sodium acetate/acetate acid (pH 6.0) and eluted with a linear gradient of 1.0–0 M (NH4)2SO4 in 50 mM sodium acetate/acetate acid (pH 6.0).

The flow-through fraction containing 1,3-β-glucanase activity from the DEAE-Sepharose open column was pooled, adjusted to pH 4.5 with acetic acid, applied to a CM-Sepharose fast flow open column (20 × 1 cm; Amersham) equilibrated with 50 mM sodium acetate/acetate acid (pH 4.5) and eluted with a linear gradient of 0–0.5 M sodium chloride in 50 mM sodium acetate/acetate acid (pH 4.5). The active 1-3 fraction eluted from the CM-Sepharose open column was pooled, lyophilized, dissolved in ∼1 ml 0.1 M sodium chloride in 50 mM sodium acetate/acetate acid (pH 4.5), and eluted with 0.1 M sodium chloride in 50 mM sodium acetate/acetate acid (pH 4.5). The active 1-4 fraction eluted from the CM-Sepharose open column was pooled, concentrated and transferred to ∼1 ml 1 M (NH4)2SO4 in 50 mM sodium acetate/acetate acid (pH 4.5) on an Amicon Ultra-4 10K centrifugal filter (Millipore), and then applied to a TSKgel Phenyl-5PW HPLC column (10 μm, 10 mm × 250 mm; TOSOH) equilibrated with 1 M (NH4)2SO4 in 50 mM sodium acetate/acetate acid (pH 4.5) and eluted with a linear gradient of 1.0–0 M (NH4)2SO4 in 50 M sodium acetate/acetate acid (pH 4.5) (0–100 min) and 50 mM sodium acetate/acetate acid (pH 4.5) (100–200 min).

Open-column chromatography for the fractionation of 1,3-β-glucan hydrolases was carried out using a Biological low-pressure system (Bio-Rad) at 4 °C in a cooler. HPLC for the purification of 1,3-β-glucan hydrolases was conducted using an Agilent 1100 HPLC system at room temperature. The protein content of the elution was monitored by measuring the A280.

Enzyme protein assays. The purity and molecular masses of the proteins were determined by SDS-PAGE using 12 % polyacrylamide gels (Laemmli, 1970).

Protein concentrations in the enzyme preparations were determined using the Bradford method (Bradford, 1976) with BSA as the standard and measuring the A280 (Fukuda et al., 2008).

Amino acid sequencing of partial peptides of the purified proteins was determined by MALDI-TOF/TOF MS. The protein bands in the SDS-PAGE gel were excised and digested with trypsin (Promega). A 0.5 μl aliquot of the concentrated tryptic peptide mixture in 0.1 % trifluoroacetic acid was mixed with 0.5 μl CHCA matrix solution (5 mg Z-cycano-4-hydroxycinamic acid ml−1 in 50 % acetoni-trole/0.1 % trifluoroacetic acid) and spotted onto a freshly cleaned target plate. After air drying, the crystallized spots were analysed on an UltraflexExtreme MALDI-TOF/TOF mass spectrometer (BrukerDaltonics). Ionization was performed by MS and MS/MS by irradiation with a nitrogen laser (337 nm) operating at 1 kHz. Data were acquired at a maximum accelerating potential of 25 kV in the positive and reflection modes. MS and MS/MS data were analysed and peak lists were generated using FlexAnalysis3.1 (Bruker Daltonics). MS peaks were selected between 850 and 3700 Da and filtered with a signal-to-noise ratio >20. A peak intensity filter was used with ≤50 peaks per 200 Da. MS/MS peaks were selected based on a signal-to-noise ratio >10 over a mass range of 60–20 Da below the precursor mass. During MS/MS analysis, argon was used as the collision gas. MS/MS spectra were searched against the NCBI nr database by BioTools 3.2.
(BioTools Europe) with MASCOT 2.0 as the database search engine, and peptide and fragment ion mass tolerance of 50 p.p.m. High confidence identifications had statistically significant search scores (> 95% confidence, equivalent to MASCOT expect value \( P < 0.05 \)) and accounted for the majority of ions present in the mass spectra.

**Enzyme hydrolytic activity assays.** For measuring the amount of reducing sugar released from polysaccharides, a 200 \( \mu \)l reaction mixture containing the appropriate amount of enzyme and indicated polysaccharides or their derivatives in 50 mM sodium acetate/acetic acid (pH 6.0) was incubated at 37 \(^\circ\)C at 800 r.p.m. for 20 min. After incubation, 200 \( \mu \)l 3,5-dinitrosalicylic acid reagent was added to the reaction (Miller, 1959), which was then incubated at 100 \(^\circ\)C for 10 min and then placed on ice for 2 min. After centrifugation, the \( A_{520} \) was measured by a SpectraMax M2 spectrophotometer (Molecular Devices). One unit of enzyme activity was defined as the amount of enzyme that released 1.0 \( \mu \)mol reducing sugar (as the glucose standard) min\(^{-1}\) under the assay conditions. To evaluate the effects of pH, we varied the reaction pH using 50 mM sodium acetate (pH 4–6) and 50 mM Tris/HCl (pH 7–9) buffers. To determine the effects of temperature, we performed the reactions at different temperatures (20–70 \(^\circ\)C) in 50 mM sodium acetate buffer (pH 6.0). To determine the effects of substrate concentration and reaction kinetics, we varied the laminarin concentration (from 0.2 to 3.0 mM). Enzyme parameters used to calculate specific activity were determined by fitting data to the Michaelis–Menten equation.

For measuring the amount of glucose released from laminarioligosaccharides, a 10 \( \mu \)l reaction mixture containing an appropriate amount of laminarioligosaccharides and enzyme in 50 mM sodium acetate buffer (pH 6.0) was incubated at 37 \(^\circ\)C for 60 min. To determine the effects of substrate concentration and reaction kinetics, we varied the laminaritetrose concentration (from 2.0 to 25.0 mM). Enzyme parameters used to calculate specific activity were determined by fitting data to the Michaelis–Menten equation. Glucose content was measured by the glucose oxidase peroxidase method using a Glucose Assay kit (Robio) and detected by a SpectraMax M2 spectrophotometer (Molecular Devices) at \( A_{520} \) (Kumagai et al., 2014). One unit of laminariobiode-hydrolysing activity was defined as the amount of enzyme that liberated 2.0 \( \mu \)mol glucose min\(^{-1}\) because 2 mol glucose is produced from 1 mol laminaribiose. If laminaritriose or larger laminarioligosaccharides were used as the substrate, 1 U of enzyme was defined as the amount of enzyme that liberated 1.0 \( \mu \)mol glucose min\(^{-1}\).

For measuring the amount of \( p \)-nitrophenol released from \( p \)-nitrophenyl-\( \beta \)-D-glucopyranoside (PNPG) or other \( p \)-nitrophenyl glycoside derivatives, a 200 \( \mu \)l reaction mixture containing an appropriate amount of enzyme and PNPG or indicated \( p \)-nitrophenyl glycoside derivatives in 50 mM sodium acetate/acetic acid (pH 6.0) was incubated at 37 \(^\circ\)C for 20 min; the reaction was terminated by adding 200 \( \mu \)l ice-cold 0.5 M Na\(_2\)CO\(_3\) and the colour that developed as a result of \( p \)-nitrophenol liberation was measured by a SpectraMax M2 spectrophotometer (Molecular Devices) at \( A_{410} \) (Saha & Bothast, 1996). One unit of PNPG derivative-degrading activity was defined as the amount of enzyme that released 1.0 \( \mu \)mol \( p \)-nitrophenol min\(^{-1}\).

For TLC analysis of enzymic products from laminarioligosaccharides or laminarin, a 10 \( \mu \)l reaction mixture containing an appropriate amount of enzyme and laminarioligosaccharides or laminarin in 50 mM sodium acetate buffer (pH 6.0) was incubated at 37 \(^\circ\)C for 1 h and then added to 10 \( \mu \)l 10 % (v/v) trichloroacetic acid. An aliquot of 2 \( \mu \)l of the reaction was deposited on a TLC Silica Gel 60 plate (Merck), developed with a solvent comprising n-butyl alcohol/methanoic acid/water (2 : 3 : 1, v/v/v) and visualized by heating the plate to 110 \(^\circ\)C for 5 min after spraying with 20 % (v/v) sulfuric acid in ethanol (Kumagai et al., 2014).

**RESULTS**

**Purification studies**

The procedure that was applied for the fractionation of pilei extractions of *C. cinerea* fruiting bodies into a group of purified protein fractions is shown in Fig. 1(a). Two active fractions, I-1 and I-2, containing 1,3-\( \beta \)-glucanase activity were obtained by DEAE-Sepharose anion-exchange chromatography from pilei extracts of *C. cinerea* fruiting bodies (Fig. 2a). The active I-1 fraction failed to be purified by TSKgel Phenyl-5PW or TSKgel G2000SW HPLC to a homogeneous protein (data not shown). The active I-2 fraction was purified by TSKgel Phenyl-5PW HPLC to a
Identification of purified fractions

MALDI-TOF/TOF MS analysis showed that the amino acid sequences of two partial peptide segments produced by tryptic digestion from the II-2 protein completely matched a putative exo-1,3-β-glucanase consisting of 421 aa with a predicted molecular mass of 48 kDa as annotated in the C. cinerea genome (GenBank accession number EAU92553) (Table 1). Protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the putative exo-1,3-β-glucanase was previously categorized into the glyco_hydro_5 family, but is now in the glyco_hydro_2_c family. The SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) revealed a possible cleavage site located between aa 23 and 24 from the methionine translation start site in the mature II-2 protein. Thus, the mature II-2 protein consisted of 398 aa residues with a predicted molecular mass of 45 kDa, consistent with the result of the SDS-PAGE analysis.

MALDI-TOF/TOF analysis showed that the amino acid sequences of three partial peptide segments produced by tryptic digestion from the II-3 protein completely matched a putative glucan 1,3-β-glucosidase consisting of 786 aa with a predicted molecular mass of 85 kDa as annotated in the C. cinerea genome (GenBank accession number EAU86655) (Table 1). Protein BLAST showed that the putative glucan 1,3-β-glucosidase had a pectate lyase family 3 catalytic domain and was categorized into the glyco_hydro_55 family. The SignalP 4.1 server indicated that II-3 did not contain a signal peptide; thus, both the predicted and apparent molecular masses of the II-3 protein were similar to each other.

MALDI-TOF/TOF MS analysis showed that the amino acid sequences of two partial peptide segments produced by tryptic digestion from the II-4 protein completely matched a putative glycosyl hydrolase family 16 protein consisting of 385 aa residues with a predicted molecular size of 42 kDa as annotated in the C. cinerea genome (GenBank accession number EAU84955) (Table 1). The SignalP 4.1 server explored a possible cleavage site located between aa 25 and 26 from the methionine translation start site in the mature II-4 protein. Thus, the putative mature protein would consist of 360 aa with a predicted molecular mass of 39 kDa, approximately similar to its apparent molecular mass on SDS-PAGE.

Substrate specificity and mode of action

To determine the degradation mode of purified 1,3-β-glucanases, periodate-oxidized laminarin as a specific endo-1,3-β-glucanase substrate and pNPG as a specific exo-1,3-β-glucanase substrate (Goñi et al., 2011), and various β-glucoside polysaccharides and relative oligosaccharides, and some p-nitrophenyl β-glucosides were used for analysis of substrate specificity.

As shown in Table 2, the active II-2 fraction did not degrade periodate-oxidized laminarin (i.e. II-2 did not randomly hydrolyse the internal 1,3-β-linkages). In contrast,
the II-2 protein degraded laminarin, laminarioligosaccharides including laminaribiose and \( pNPG \), whereas the enzyme activity decreased as the glucose chain length increased (i.e. its ability to degrade laminarin was much lower than its ability to hydrolyse laminarioligosaccharides, especially laminaribiose, which was its most optimal substrate). As the II-2 protein did not degrade barley \( \beta \)-glucan or pustulan, or related cellobiose or gentiobiose, nor \( p \) -nitrophenyl-\( N \)-acetyl-\( \beta \)-D-glucosaminide, \( o \)-nitrophenyl-\( \beta \)-D-galactopyranoside or \( p \)-nitrophenyl-\( \beta \)-D-glucuronide, it must strictly be a \( 1,3-\beta \)-glucosidase (Bhatia et al. 2002), which was named \( 1,3-\beta \)-glucosidase 1 (BGL1). TLC analysis showed that BGL1 not only released glucose from laminaribiose but also produced larger laminaritriose, exhibiting transglycosylation activity (Fig. 4a). Some laminarioligosaccharides with a higher degree of polymerization than the substrates were observed with all of the substrates tested (Fig. 4a).

As shown in Table 2, the active II-3 fraction did not degrade periodate-oxidized laminarin; therefore, it was not an endo-1,3-\( \beta \)-glucanase. The II-3 fraction did not hydrolyse \( pNPG \) or the disaccharide laminaribiose either, indicating that it was not a glucan 1,3-\( \beta \)-glucosidase as annotated in the \( C. cinerea \) genome. The II-3 fraction hydrolysed 1,3-\( \beta \)-oligosaccharides with three or more glucosyl residues. Its hydrolytic activity increased with an increasing number of glucosyl residues, arranged from laminaritriose to laminaripentaose, and reached a maximum when laminaripentaose was used as the substrate. When laminarin was used as the substrate, the specific enzyme activity was much lower with only 3.5 % specific enzyme activity for laminaripentaose. TLC analysis of the enzymic products from laminarioligosaccharides produced by the II-3 fraction showed that the II-3 fraction did not cleave laminaritriose to produce glucose and laminaribiose (Fig. 4b). Consistent with these observations, II-3 had lower hydrolytic activity toward laminaritriose; enzymic products from laminarioligosaccharides with more than three glucosyl residues by the II-3 fraction mainly exhibited glucose, laminaritriose and relative larger laminarioligosaccharides with only trace or no laminaribiose (Fig. 4b). Thus, II-3 was apparently an exo-1,3-\( \beta \)-glucanase (EXG).
(Pitson et al., 1993; Sakamoto et al., 2012) rather than a 1,3-β-glucosidase. Furthermore, TLC analysis showed that EXG did not exhibit appearance of any laminarioligosaccharides with higher degrees of polymerization than the substrates; thus, II-3 must lack transglycosylation activity toward laminarioligosaccharides (Fig. 4b).

As shown in Table 2, the active II-4 fraction degraded periodate-oxidized laminarin in an internal cleaving manner; in contrast, the II-4 fraction did not degrade pNPG or laminaribiose, although it did degrade laminarioligosaccharides arranged from laminaritriose to laminarihexaose. In addition, the II-4 fraction degraded laminarin (1,3/1,6-β-glucan), barley β-glucan (1,3/1,4-β-glucan) and pachyman (1,3-β-glucan), but it did not degrade pustulan (1,6-β-glucan) or CM-cellulose (1,4-β-glucan). Thus, the II-4 fraction apparently acts at the 1,3-β-linkage rather than the 1,4-β- or 1,6-β-linkage. Notably, its hydrolytic activity toward laminarin (1,3/1,6-β-glucan) and barley β-glucan (1,3/1,4-β-glucan) was higher than that toward pachyman (1,3-β-glucan). TLC analysis showed that the II-4 fraction did not degrade laminaribiose to produce glucose, whereas it did cleave L3–L6 laminarioligosaccharides to produce a series of relative shorter laminarioligosaccharide substrates rather than glucose (Fig. 4c). Thus, the II-4 protein was characterized as an endo-1,3-β-glucanase (ENG) (Martin et al. 2007). TLC analysis also indicated that ENG lacked transglycosylation activity toward laminarioligosaccharides (Fig. 4c).

**Synergic action**

To explore how these three 1,3-β-glucoside hydrolases with different modes of action synergically acted on laminarin, enzymic products produced from laminarin by different combinations of *C. cinerea* BGL1, EXG and ENG were studied by TLC analysis.

As shown in Fig. 4(d), enzymic products from laminarin produced by BGL1 exhibited only a glucose spot and a stronger remaining laminarin sample spot in the TLC plates. Enzymic products from laminarin produced by EXG exhibited only a glucose spot, a weak gentiobiose spot and an apparent laminarin sample spot that remained in the TLC plates. Enzymic products from laminarin produced by ENG exhibited clear laminaribiose, laminaritriose and laminaritetraose spots, as well as a series of others at different lengths in a laminarioligosaccharide smear, leaving a weaker laminarin sample spot remaining, but no glucose spot on the TLC plates.

When both BGL1 and EXG co-existed in the reaction, BGL1 almost did not change the TLC pattern of the enzymic products from laminarin by EXG. When both BGL1 and ENG co-existed in the reaction, BGL1 promoted the degradation of laminaribiose, laminaritriose and laminaritetraose released from laminarin by ENG, making these product spots weaker and producing a related glucose spot in the TLC plates, but it did not eliminate the smears containing different lengths of laminarioligosaccharide released from laminarin by ENG on the TLC plates. When both EXG and
ENG co-existed in the reaction, EXG promoted the degradation of larger laminarioligosaccharides and eliminated the smear consisting of different lengths of laminarioligosaccharides released from laminarin by ENG on TLC plates to produce glucose; however, they left uncleaved laminaribiose and gentiobiose in the enzymic products. When BGL1, EXG and ENG simultaneously co-existed in the reaction, adding ENG co-existed in the reaction, EXG promoted the degradation of larger laminarioligosaccharides and eliminated the smear consisting of different lengths of laminarioligosaccharides, but also the laminaritriose released from laminarin by ENG such that the laminaritriose than other large laminarioligosaccharides, we chose laminarin and laminaritetraose as common substrates to determine the kinetic parameters of BGL1, EXG and ENG from *C. cinerea* (Fig. 6). The *Km* values for laminarin of BGL1, EXG and ENG were 4.96, 0.48 and 0.27, respectively, and the *Km* values for laminaritetraose of BGL1, EXG and ENG were 3.00, 3.27, and 15.75, respectively. Thus, amongst these three glycoside hydrolases, ENG had the highest affinity for laminarin whereas BGL1 had the highest affinity for laminaritetraose, which is consistent with their substrate specificities and action modes.

**Optimum pH and temperature, and kinetic parameters**

Experiments showed that these three glycoside hydrolases had a similar optimum pH and temperature. The hydrolytic activity towards laminarin of BGL1, EXG and ENG increased across an increasing temperature range from 30 to 60 °C, whereas the hydrolysing activity slowed down when the temperature reached 70 °C (Fig. 5a). The optimal pH for hydrolytic activity towards laminarin of BGL1, EXG and ENG was 5.0–6.0 (Fig. 5b).

As EXG and ENG did not act on the laminaribiose, and EXG even exhibited a lower hydrolysing activity towards laminaritriose than other larger laminarioligosaccharides, we chose laminarin and laminaritetraose as common substrates to determine the kinetic parameters of BGL1, EXG and ENG from *C. cinerea* (Fig. 6). The *Km* values for laminarin of BGL1, EXG and ENG were 4.96, 0.48 and 0.27, respectively, and the *Km* values for laminaritetraose of BGL1, EXG and ENG were 3.00, 3.27, and 15.75, respectively. Thus, amongst these three glycoside hydrolases, ENG had the highest affinity for laminarin whereas BGL1 had the highest affinity for laminaritetraose, which is consistent with their substrate specificities and action modes.
Members of the genus *Coprinopsis* possess various polysaccharide hydrolases that work in coordination with one another on cell wall components for the autolysis of mature fruiting bodies. The activities of these glycoside hydrolases have been detected in the extracts and autolysates of fruiting bodies of some species of *Coprinopsis* (Bush, 1974; Iten & Matile, 1970; Kamada et al., 1985; Lim & Choi, 2009). However, successful isolation, purification and characterization of these different hydrolases from *Coprinopsis* mycelium or fruiting bodies have not been reported to date. This study uses a combination of open chromatography and high-resolution HPLC methods to simultaneously obtain a group of 1,3-β-glucan hydrolases from *C. cinerea* pilei. The fact that these 1,3-β-glucan hydrolases could be purified from pileus extractions in high yields indicates that they are highly expressed in hydrolysing fruiting bodies and involved in the autolysis of pilei.

**DISCUSSION**

Fig. 4. TLC of the enzymic products from laminarioligosaccharides by (a) II-2, (b) II-3 and (c) II-4 from *C. cinerea*. A 10 µl reaction mixture contained 0.2 µg II-2, 0.04 µg II-3 or 0.01 µg II-4 and 150 µg laminarioligosaccharides in 50 mM sodium acetate buffer (pH 6.0). (d) TLC of the enzymic products from laminarin by individual or various combinations of II-2, II-3 and II-4 from *C. cinerea*. A 10 µl reaction mixture contained 0.4 µg II-2, 0.2 µg II-3 or 0.025 µg II-4 individually or different combinations of them at the same concentrations and 100 µg laminarin in 50 mM sodium acetate buffer (pH 6.0). ST, standard sugars; G1, glucose; G2, gentiobiose; L2–L6, laminaribiose to laminarihexaose.
The purified II-2 protein from *C. cinerea* pilei annotated previously as a putative exo-1,3-β-glucanase in the *C. cinerea* genome is now characterized as BGL1. The purified II-3 protein from *C. cinerea* pilei annotated previously as a putative glucan 1,3-β-glucosidase in the *C. cinerea* genome is now characterized as EXG. The purified II-4 protein from *C. cinerea* pilei annotated previously as the putative glycosyl hydrolase family 16 protein in the *C. cinerea* genome is now characterized as ENG. These differences between the predicted and detected features indicate that the 1,3-β-glucanases annotated in fungal genomes still need to be validated for their true catalytic features using purified proteins.

Laminarin, which is isolated from the brown alga *Laminaria digitata*, is a 1,3-β-linked D-glucan with a mean degree of polymerization of 25 glucosyl residues and 1,3,1,6-β-linked branches per molecule (Read et al., 1996). The endo-1,3,1,6-β-glucanase ENG from *C. cinerea* degrades laminarin by internal cleavage to generate varying lengths of laminarooligosaccharides. Although ENG specifically acted on the 1,3-β-linkage rather than the 1,4-β- or 1,6-β-linkage in β-glucans, ENG showed lower activity toward 1,3-β-glucan (pachyman) than 1,3,1,6-β-glucan (laminarin) and 1,3,1,4,6-β-glucan (barley 1,3,1,4,6-β-glucan). Therefore, an adjacent 1,4-β- or 1,6-β-glucosyl residue toward the 1,3-β-glucosyl linkage hydrolysis is favoured for cleaving the 1,3-β-linkage by ENG, which matches the characteristics of *C. cinerea* fruiting body wall glucans with 1,3-β- and 1,6-β-linkages (Kamada & Takemaru, 1983).

The exo-1,3-β-glucanase EXG from *C. cinerea* degrades laminarin, laminaritriose and larger laminarooligosaccharides, whereas it did not cleave laminaribiose. To date, known exo-1,3-β-glucanases catalyse the hydrolysis of the 1,3-β-glucosidase BGL1 from *C. cinerea* pilei could cleave laminarinibiose to release glucose, but did not cleave gentiobiose. BGL1 degraded laminarin to produce only glucose without gentiobiose; it also did not degrade the endo-1,3-β-glucosidase EXG belonging to *Phanerochaete chrysosporium* hydrolysed 6-O-glucosyl-laminaritriose to produce gentiobiose and L2. It was reported that glycohydro_55 family enzymes have an inverting hydrolytic mechanism (Ishida et al., 2009) and the 1,3-β-glucosidases with an inverting mechanism generally have a single-chain attack pattern of hydrolysis (Nelson, 1975). Thus, *C. cinerea* EXG belonging to glycohydro_55 family should cleave single glucose residues successively from the non-reducing terminus of laminarin until it reaches the last laminaribiose before it disassociated from the substrate. However, the enzymic products of laminarin produced by EXG only exhibited glucose and gentiobiose without laminaribiose. Therefore, it is necessary to further explore the mechanism of action of EXG by future experimentation.

The 1,3-β-glucosidase BGL1 from *C. cinerea* pilei could cleave laminarinibiose to release glucose, but did not cleave gentiobiose. BGL1 degraded laminarin to produce only glucose without gentiobiose; it also did not degrade the gentiobiose released from laminarin by *C. cinerea* EXG. These results imply that BGL1 was unable to bypass the glucose residues containing 1,6-β-branching sites, unlike...
EXG (Pitson et al., 1993); therefore, it cleaved only single glucose residues successively from the non-reducing terminus of laminarin until it reached a 1,6-β-branching site. BGL1 was previously categorized into the glyco_hydro_5 family, but is currently in the glyco_hydro_2_c family, both of which have a retaining hydrolytic mechanism (http://www.cazy.org/GH2.html; Moura-Tamames et al., 2009). It was suggested that exo-1,3-β-glucanases with a retaining mechanism frequently have a multi-chain attack pattern of hydrolysis (Nelson, 1975). The stronger sample

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**Fig. 6.** Substrate concentration dependence of the activity of (a) BGL1, (b) EXG and (c) ENG. The insets show Lineweaver–Burk plots of initial velocity versus various fixed substrate concentrations to estimate the $K_{m}$ constants. Reactions were performed in 10 μL (laminaritetrose, a2–c2) or 200 μL (laminarin, a1–c1) 50 mM sodium acetate buffer (pH 5.0) containing different concentrations of laminaritetrose or laminarin as the substrate and 0.2 μg II-2, 0.2 μg II-3 or 0.05 μg II-4. The data represent the mean ± SD of triplicate measurements.
spot of enzymic products on TLC plates may provide evidence for the multi-chain attack pattern (Nelson, 1975), i.e. BGL1 attacks a non-reducing terminal chain of the substrate producing a single cleavage; the next cleavage occurs with attachment to a different laminarin molecule or a different portion of the same molecule so it removes on average only a few glucose residues per encounter with a laminarin molecule after 1 h of hydrolysis, leaving larger partially digested laminarin fragments along with the undigested laminarin still remaining in the sample spot on TLC plates.

This study explored how three 1,3-β-glucoside hydrolases with different modes of action, i.e. an endoglucanase, an exoglucanase and a glucosidase, from C. cinerea synergically acted on laminarin. The endo-1,3-β-glucanase ENG hydrolyses internal glycosidic bonds at laminarin to generate 1,3-β-oligosaccharides of various lengths. However, the individual 1,3-β-glucosidase BGL1 did not degrade larger laminarioligosaccharides released from laminarin by the endo-1,3-β-glucanase ENG and the individual exo-1,3-β-glucanase EXG did not cleave laminaribiose released from laminarin by the endo-1,3-β-glucanase ENG even though it degraded larger laminarioligosaccharides to laminaribiose. When BGL1 and EXG co-existed in the reaction with ENG, EXG cleaved the longer-chain laminarioligosaccharides released from laminarin by ENG into short-chain disaccharides, laminaribiose and gentiobiose, and BGL1 further hydrolysed laminaribiose to glucose. The remaining gentiobiase must be hydrolysed by other 1,6-β-glucosidases. In fact, the partially purified II-1 fraction from pilei extracts exhibited extensive β-glucosidase activities, including 1,6-β-glucosidase activity, although it was not purified to homogeneity (data not shown). Therefore, this study implies that in this synergic way, endo-1,3-β-glucanase, exo-1,3-β-glucanase and 1,3-β-glucosidase may be involved in the complete degradation of the 1,3-β-glucan backbone in the C. cinerea cell wall during fruiting body autolysis. These three 1,3-β-glucoside hydrolases share a similar optimum pH and temperature, which also supports the speculation that these enzymes may work together under the same conditions for the degradation of 1,3-β-glucan in the C. cinerea cell wall during fruiting body autolysis.

Senescence of Lentinula edodes fruiting bodies after harvesting is the result of cell wall degradation. Two endo-1,3-β-glucanases, TLG1 and GLU1, and one exo-1,3-β-glucanase, EXG2, were suggested to be involved in lentilnian degradation after harvesting (Sakamoto et al., 2006, 2011, 2012). L. edodes GLU1 and TLG1 belong to a glyco_hydro_128 family and a thaumatin-like protein, respectively, rather than a glyco_hydro_16 family, so perhaps different basidiomycete species utilize different types of endo-1,3-β-glucanases to degrade 1,3-β-glucan in their cell walls. L. edodes EXG2 has 65% identity with C. cinerea EXG; it degraded 1,3,1,6-β-glucan (lentinan) to produce glucose, laminaribiose, gentiobiase and some other oligosaccharides (Sakamoto et al., 2005b, 2012), rather than only glucose and gentiobiase as the C. cinerea EXG did. It is notable that in those studies the combined action of GLU1 and EXG2 from L. edodes on 1,3-β-glucan produced glucose and disaccharides (laminaribiose) as end products; however, how the laminaribiose was further degraded to a single glucose was not addressed (Sakamoto et al., 2012). Protein BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) showed that C. cinerea BGL1 has 65% identity with an exo-1,3-β-glucanase EXG1 from L. edodes; however, L. edodes EXG1 has not been shown to possess 1,3-β-glucosidase activity toward laminarioligosaccharides or pNPG and was considered to play a role in changing stipe cell wall mechanical properties during stipe elongation rather than in lentilnian degradation during fruiting body senescence (Sakamoto et al., 2005a, 2012). To date, no other 1,3-β-glucosidases have been characterized in the senescence of fruiting bodies of L. edodes. As the senescence of L. edodes fruiting bodies after harvesting does not lead to complete collapse and liquefaction of fruiting bodies, as it does in C. cinerea, 1,3-β-glucosidase may play a significant role in the complete degradation of 1,3-β-glucan in cell walls during autolysis of mature fruiting bodies of C. cinerea.

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