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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning and functional characterization of a complex endo-β-1,3-glucanase from *Paenibacillus* sp.

Yueh-Mei Cheng · Tang-Yao Hong · Chia-Chi Liu · Menghsiao Meng

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Abstract A β-1,3-glucanase gene, encoding a protein of 1,793 amino acids, was cloned from a strain of *Paenibacillus* sp. in this study. This large protein, designated as LamA, consists of many putative functional units, which include, from N to C terminus, a leader peptide, three repeats of the S-layer homologous module, a catalytic module of glycoside hydrolase family 16, four repeats of the carbohydrate-binding module of family CBM_4_9, and an analogue of coagulation factor Fa5/8C. Several truncated proteins, composed of the catalytic module with various organizations of the appended modules, were successfully expressed and characterized in this study. Data indicated that the catalytic module specifically hydrolyze β-1,3- and β-1,3–1,4-glucans. Also, laminaritriose was the major product upon endolytic hydrolysis of laminarin. The CBM repeats and Fa5/8C analogue substantially enhanced the hydrolyzing activity of the catalytic module, particularly toward insoluble complex substrates, suggesting their modulating functions in the enzymatic activity of LamA. Carbohydrate-binding assay confirmed the binding capabilities of the CBM repeats and Fa5/8C analogue to β-1,3-, β-1,3–1,4-, and even β-1,4-glucans. These appended modules also enhanced the inhibition effect of the catalytic module on the growth of *Candida albicans* and *Rhizoctonia solani*.

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Introduction

Fungal cell walls, composed of complex polymers of β-1,3- and β-1,6-glucans, mannoproteins, and small amounts of chitin, are essential for cell integrity by affording mechanical protection against environmental stresses (Klis 1994; Smits et al. 2001). The structural complexity implies that breakdown of the cell wall may require the synergistic action of various enzymes (Gacto et al. 2000). One of theses enzymes is β-1,3-glucanase that is widely distributed among higher plants and bacteria. Divergence of the amino acid sequences has classified the plant β-1,3-glucanases into glycoside hydrolase family 17 (GH17), but the bacterial enzymes into GH16, although the two enzyme classes have analogous catalytic activity (Henrissat 1991; Henrissat and Bairoch 1993). Because β-1,3-glucan is the most abundant component of the fungal cell wall, extensive hydrolysis of the polymer by β-1,3-glucanase may weaken the mechanical strength of the cell walls, leading to fungal cell lysis. In plants, β-1,3-glucanases are components of the defense system against the invasion of pathogenic fungi (Castresana et al. 1990; Grenier et al. 1993; Yi and Hwang 1997). To bacteria, secretion of β-1,3-glucanase has been thought to assimilate fungal cell wall as a nutrition source (Watanabe et al. 1992).

Given the potential for hydrolyzing fungal cell walls, β-1,3-glucanase is useful in many application aspects. Regarding basic research, β-1,3-glucanase has been used as a reagent for obtaining yeast protoplasts (Evans and Conrad 1987; Ferrer 2006; Gacto et al. 2000), and for

defining the architectural traits of fungal-cell-wall structure (Kollár et al. 1997; Kapteyn et al. 2000). As for industrial application, β-1,3-glucanase could be utilized in the processes of producing yeast extract (Ryan and Ward 1985.), soluble β-1,3-glucan, which is a potential immunoactivator (Ríos-Hernández et al. 1994; Mohagheghpour et al. 1995), and in wine extract clarification (Villettaz et al. 1984; Pretorius and van der Westhuisen 1991). In relation to agricultural application, it could serve as a biocontrol agent in protecting plants from fungal invasion as illustrated in numerous publications. For instance, β-1,3-glucanases isolated from *Trichoderma harzianum* were inhibiting growth of phytopathogenic *Sclerotium rolfsii* (El-Katatny et al. 2001) and *Pythium* spp (Thrane et al. 1997), the enzymes from *Pseudomonas* sp. inhibited *Rhizoctonia solani* (Nagarajkumar et al. 2004) and *Fusarium solani* (Lim et al. 1991), and that from *Bacillus amyloliquefaciens* was active against *Colletotrichum lagenarium* (Kim and Chung 2004). Potential for controlling the growth of *Candida* sp., opportunistic pathogens present on human skin and mucosa, by β-1,3-glucanases has also been demonstrated recently (Izgü et al. 2007).

A β-1,3-glucanase, with apparent molecular mass of 44 kDa, was purified previously from cultural medium of a strain of *Paenibacillus* sp. This enzyme could disrupt fungal-cell-wall structures and, as a result, inhibited the mycelial growth of *Pythium aphanidermatum* and *R. solani* (Hong and Meng 2003). To further explore the application potential of this bacterial strain, we set out to clone its β-1,3-glucanase genes. A putative open reading frame, which encodes a β-1,3-glucanase with 1,793 amino acid residues, was investigated in this study. Analysis of the deduced amino acid sequence revealed that this complex protein contains a putative glycoside hydrolase plus several auxiliary modules including repeats of S-layer homolog (SLH), repeats of carbohydrate-binding module (CBM), and an analogue of coagulation factor. The enzymatic properties of the hydrolase module and the associated functions of the CBM repeats and coagulation factor analogue were characterized in this study. Inhibition effects of the protein on the growth of *Candida albicans* and *R. solani* were investigated in this study as well.

Materials and methods

Materials Various β-1,3-glucans, including laminarin (from *Laminaria digitata*), curdlan (from *Alcaligenes faecalis*), and zymosan A (from *Saccharomyces cerevisiae*), were purchased from Sigma, while pachyman was prepared from commercial fruiting bodies of the basidiomycete *Poria cocos* (Sun Ten Pharmaceutical). Barley β-glucan, lichenan, xylan, and carboxymethylcellulose (CMC), were also purchased from Sigma, while crystalline cellulose Avicel PH101 was a product of Fluka.

Bacterial strains The *Paenibacillus* strain, deposited in the Culture Collection and Research Center of Taiwan with stock number **CCRC 17245**, was originally isolated from garden soil (Hong and Meng 2003). *E. coli* BL21(DE3) (Novagen) and Top10F' (Invitrogen) cells were used for protein expression and DNA manipulation, respectively.

Isolation of β-1,3-glucanase gene Extraction and manipulation of DNA were performed according to standard protocols (Sambrook et al. 1989). The chromosomal DNA library of the *Paenibacillus* strain was constructed by inserting *Kp*nI-digested DNA fragments into *pUC*18. The DNA library was then introduced into *E. coli* Top10F' cells, followed by selective growth on LB agar plate, which also contained ampicillin (100 μg/ml), IPTG (0.25 mM), and X-Gal (0.004%, *w*/*v*). White colonies were picked up and grown on another LB agar plate containing pachyman (0.8%, *w*/*v*) and aniline blue (0.006%, *w*/*v*) on which the β-1,3-glucanase-producing colony would form a surrounding halo (Mahasneh and Stewart 1980).

Nucleotide sequence Both strands of the cloned DNA (12.4 kb) were sequenced using the BigDye terminator cycle sequencing ready reaction kit with an ABI Prism 3773 auto sequencer (Applied Biosystems). Nucleotide sequence of the *Paenibacillus* β-1,3-glucanase gene and its deduced amino acid sequence are available in GenBank with the accession number DQ987544.

Protein expression vectors Four truncated proteins (designated as CBF, CB_3, SC, and C, respectively) were first produced in this study. The corresponding coding regions were amplified from the 12.4-kb cloned DNA using respective pairs of primer (Table 1) by PCR. PCR reactions were carried out 35 cycles (95°C, 30 s; 60–67°C, 30 s; 72°C, 1–3 min) followed by a 10-min extension at 72°C in 50-μl reaction buffer that contained 0.32 μM primer (each), 0.2 mM dNTP (each), 10 ng template DNA and 2.5 U of *Pfu* Turbo polymerase (Stratagene). The PCR-amplified fragments were then digested with *Eco*RI and *Kpn*I and inserted into plasmid pETDuet (Novagen) to generate pET-CBF, pET-CB_3, pET-SC, and pET-C, respectively.

The expression vector for the mutated protein CF, consisting of the catalytic module and the C-terminal coagulation factor analogue, was derived from pET-CBF template by a PCR-based deletion mutagenesis. Briefly, a set of 5′-phosphorylated divergent primers (Table 1) was used in PCR with 35 cycles of amplification (95°C, 30 s; 64°C, 30 s; 72°C, 7min), followed by a 10-min extension at

Table 1 Nucleotide sequences of primers used in PCR for amplification of desired DNA fragments

Amplified fragments	Primers[a]	DNA sizes (bp)
CBF	5′-ACGCAGAATTCGGGCAAATGGGAGCTGGTC	3,879
	5′-GGACGTGGTACCGACCCGCGAAGAATGGTACGT	
CB_3	5′-CGGAATTCGATGGGCAAATGGGAGCTGGTC	2,481
	5′-GGGGTACCCCGGTTATCGGTCGCATCGGC	
C	5′-CGGAATTCGATGGGCAAATGGGAGCTGGTC	1,065
	5′-GGGGTACCATCCGGCGTATGCAGGAAATTC	
SC	5′-CGGAATTCGGACCGTACCGCCATCGGC	2,103
	5′-GGGGTACCATCCGGCGTATGCAGGAAATTC	
pET-CF	5′-p-TATGCAGGGAATACGGTCTCC	1,419[b]
	5′-p-GGCTTCGGCCGGGATGGG	

[a] Underlined sequences within the primers are the engineered cutting sites of *Eco*RI and *Kpn*I, respectively.
[b] The number represents the length of the coding region of CF protein.

72°C. The amplified linear fragment was purified and self-ligated to become expression vector pET-CF.

Protein expression and purification To express truncated protein CBF, CF, C, or SC, the *E. coli* BL21(DE3) cells, harboring desired expression vector, were cultivated in 2×TY medium (tryptone 16 g/L, yeast extract 10 g/L, NaCl 5 g/L), which also contained 100 μg/ml ampicillin, at 30°C to an $OD_{600}\approx1.0$. IPTG was then added to final 1 mM into the medium and the cultivation was continued for 4–6 h. For the expression of CB_3, a different approach was taken due to the aggregation tendency of the protein. The *E. coli* cells carrying pET-CB_3, were grown in ampicillin-containing TB medium (tryptone 12 g/L, yeast extract 24 g/L, glycerol 0.4%, *v*/*v*) at 26°C. Sorbitol, NaCl, and betaine were added to final 500, 690, and 10 mM, respectively, into medium at a culture optical density at 600 nm of approximate 2.0. IPTG (final 2 mM) was added 1 h later, and the cultivation was continued for another 15 h.

At the end of protein induction, cells were harvested by centrifugation and suspended in lysis buffer (20 mM Tris–HCl, [pH 8.0], 500 mM NaCl, 10% glycerol, 5 mM phenylmethylsulfonyl fluoride, and 5 mM β-mercapthoethanol). The cell suspension was disrupted by ultrasonic treatment, and the extract was clarified by centrifugation at 13,000×*g* for 20 min. To isolate the recombinant protein, the supernatant was mixed with Ni-NTA affinity resin (Promega) in the presence of 10 mM imidazole, and the mixture was incubated at 4°C with gentle agitation for 1 h. The mixed resin was then washed repeatedly with wash buffer (10 mM Na_2HPO_4–KH_2PO_4, [pH 7.0], 500 mM NaCl, 10% glycerol, and 40 mM imidazole), and eluted with 500 mM imidazole-containing wash buffer. The protein recovered from the immobilized nickel affinity resin was further purified by gel filtration chromatography using a Sephacryl S-300 column (60 cm×2.6 cm), equilibrated with 10 mM sodium acetate [pH5.8] and 10% glycerol.

Protein concentration was determined by the Coomassie blue method using bovine serum albumin (BSA) as the standard (Bradford 1976).

Enzymatic activity assay Unless otherwise stated, the standard activity assay for β-1,3-glucanase was carried out at 30°C for 15 min, using laminarin (5 mg/ml) as the substrate in 10 mM sodium acetate buffer [pH 5.8]. The glucose equivalents releasing from enzyme reactions were determined colorimetrically by the dinitrosalicylic acid method (Wood and Bhat 1988). One unit of activity is defined as the amount of enzyme required for releasing 1 μmol of glucose equivalents per min under the indicated reaction conditions. To determine the kinetic parameters, the initial velocities under varying laminarin concentrations (0.5–10 mg/ml) were measured, and the values of K_M and V_{max} were obtained from Lineweaver–Burk plots.

The nearly insoluble substrates such as CMC, xylan, barley β-glucan, and lichenan were preheated at 90°C for 10 min, while Avicel PH101 was pretreated with phosphoric acid (Hong et al. 2000) prior to being used in enzymatic assays to render them more accessible by the enzymes.

Detection of hydrolytic products The hydrolytic products of laminarin after β-1.3-glucanase treatment were analyzed by thin-layer chromatography (TLC). The purified catalytic module (0.6 μg) and laminarin (0.1%, *w*/*v*) were incubated in 1 ml 50 mM sodium acetate buffer [pH 5.5] at 30°C for various time intervals. The reaction was stopped by repeated extractions with phenol/chloroform (1:1). Each 10 μl of the reaction products in the water phase was then spotted on a silica plate (silica gel 60, Merck), developed with ethyl acetate/glacial acetic acid/water (2:1:1; Sakellaris et al. 1990), and visualized by *p*-anisaldehyde (Fried and Sherma 1982).

Binding activity assays Binding strength of the truncated β-1,3-glucanases to insoluble polysaccharides was estimat-

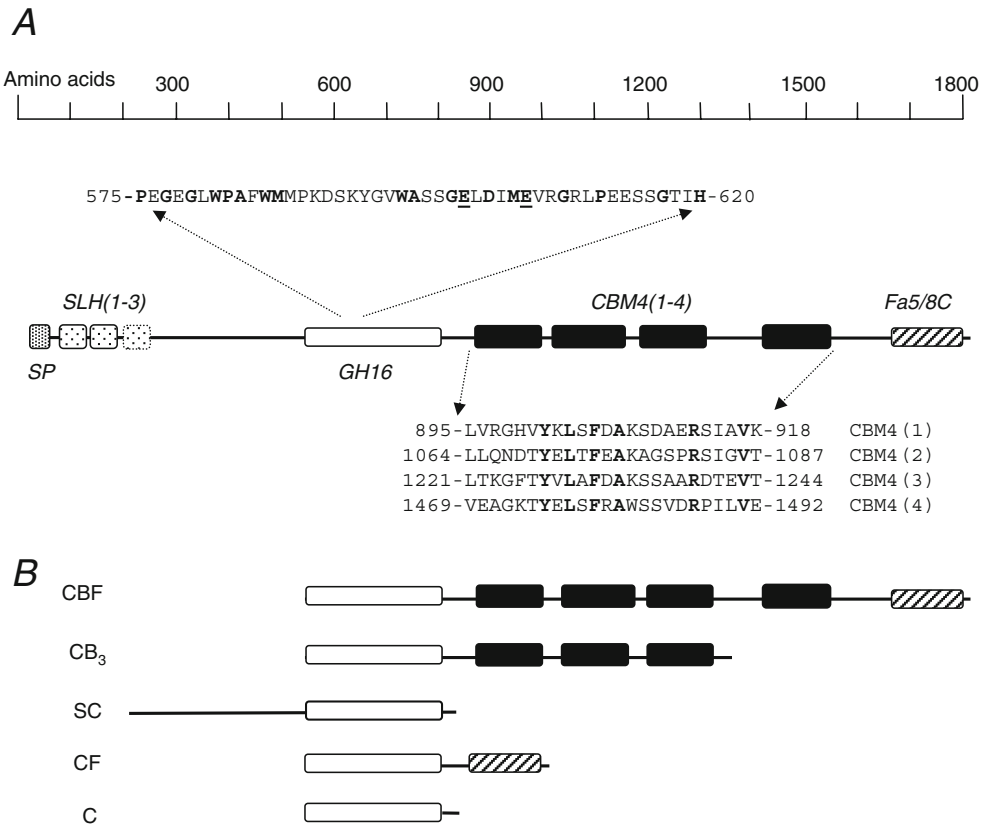

Fig. 1 **a** Organization of functional units of LamA. *SP* signal peptide, *SLH* S-layer homologous modules, *GH16* catalytic module belonging to glycoside hydrolase family 16, *CBM4* carbohydrate-binding modules family_4_9, *Fa5/8C* analogue of the C-terminal domain of coagulation factor 5/8. The *ruler on the top* represents numbers of amino acid residues. Signature sequences for GH16 and CBM_4_9 are shown *above* and *below* the protein illustration, respectively. Residues in *bold* are evolutionally conserved, while the putative catalytic glutamate residues are *underlined*. **b** Schematic representation of the module composition of truncated proteins expressed in this study. Constructions of the protein expression vectors are described in "Materials and Methods". Every truncated protein has a histidine tag fused to the N terminus

ed by mixing 0.6 μg of the purified enzymes with various washed substrates (2 mg) in 1.0 ml 10 mM sodium acetate buffer [pH 5.8] at 4°C for 1 h. After centrifugation (1,000×*g*, 5 min), the residual activity for laminarin hydrolysis in the supernatant was determined. Decreasing degrees of the enzymatic activity in supernatant reflect fractions of the enzyme bound to the insoluble polysaccharides (Yamamoto et al. 1998). Binding potency of the protein samples to laminarin was also qualitatively evaluated by affinity electrophoresis method, in which 0.3% (*w*/*v*) laminarin was included in the native polyacrylamide gel (Tomme et al. 1996). Retardation of protein migration on the gel would depend on the binding strength of the protein to laminarin. Electrophoresis was carried out under 80 V in a cold room.

Antifungal activity assay Overnight culture of *C. albicans*, grown in YPD medium (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L) at 30°C, was suspended in 5 mM sodium acetate buffer [pH 5.8] to an OD_{600}=0.5. An aliquot of the cell suspension (10 μl) was then mixed with 100 μl purified enzyme solution (in 10 mM sodium acetate buffer [pH 5.8], and 10% glycerol), and incubated at 30°C with intermittent shaking for 3 h. Surviving cells were counted according to colony forming numbers on YPD agar plates. The morphologies of enzyme-treated cells were examined using field emission scanning electron microscope (JSM-6330F, Jeol).

Inhibitory effect on growth of *R. solani* was observed by placing potato dextrose agar discs (~5 mm diameter) of fully grown mycelia in wells of six-well plate that contained indicated proteins in 10 mM potassium phosphate buffer [pH 6.0] and 10% glycerol at room temperature. The mycelium growth was recorded 2 days later.

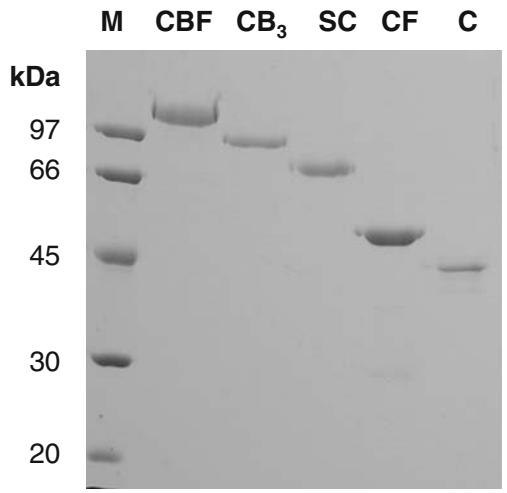

Fig. 2 Purity of the proteins shown on the gel of 10% SDS-PAGE. The proteins were purified by an immobilized Ni^{2+} affinity chromatography followed by a Sephacryl S-300 gel filtration as described in "Materials and Methods"

Table 2 Substrate specificity (U/nmol of protein) of the various truncated proteins of LamA

Substrates	CBF	CB_3	SC	CF	C
Laminarin	3.7±0.4[a]	2.6±0.2	4.6±0.3	1.7±0.1	1.4±0.2
Pachyman	1.3±0.1	0.8±0.1	0.6±0.1	0.4±0.1	0.3±0.1
Curdlan	2.0±0.3	1.0±0.1	0.7±0.1	0.6±0.1	0.3±0.1
Zymosan A	1.6±0.1	0.8±0.1	0.7±0.1	0.5±0.1	0.4±0.1
Barley β-glucan[b]	1.5±0.1	0.9±0.1	0.7±0.1	0.5±0.1	0.4±0.1
Lichenan[b]	1.3±0.1	0.9±0.1	0.7±0.1	0.4±0.1	0.3±0.1
CMC[b]	0	0	0	0	0
Xylan[b]	0	0	0	0	0

[a] Activity assay was performed by incubating 1 μg specified enzyme with 0.5% (*w*/*v*) various substrates in a final 1 ml 10 mM sodium acetate buffer [pH 5.8] at 30°C. Data are means of three independent experiments.
[b] Barley β-glucan, lichenan, xylan, and CMC were preheated at 90°C for 10 min to promote their solubility before activity assay.

Results

Cloning and sequence analysis of the β-1,3-glucanase gene To clone β-1,3-glucanase genes from the *Paenibacillus* strain, a shotgun genomic library, made by inserting *Kpn*I-digested chromosomal fragments into *pUC*18, was introduced into *E. coli* Top10F' cells. Among thousands of *E. coli* transformants grown on LB agar plate containing pachyman and aniline blue, a colony was selected based on its ability to form a surrounding halo. A ~12.4-kb recombinant DNA fragment was retrieved from this positive colony and its nucleotide sequence was determined. This DNA fragment contains seven putative open reading frames (ORFs). Possible genes for a β-1,3-glucanase, a chemotaxis sensory transducers, and an incomplete sugar ABC transporter permease are on one strand, while those for a 5-keto-4-deoxyuronate isomerase, a transcriptional regulator, a 2-deoxy-D-gluconate 3-dehydrogenase, and an aryl-alcohol dehydrogenase, are on the complementary strand (data not shown). This specific β-1,3-glucanase, designated as LamA, contains 1,793 amino acid residues, consisting of several putative functional modules including a signal peptide, three consecutive SLH, a β-1,3-glucanase catalytic module, four repeats of CBM_4_9, and an analogue of blood coagulation factor 5/8 type of *Clostridium beijerincki* (Fa5/8C, Genbank number: EAP61142) according to BLAST analyses (http://tw.expasy.org/tools/blast/; Fig. 1a). Still there is a region immediately preceding the catalytic module without predictable function. The catalytic module shares similarity in amino acid sequence to members of bacterial GH16, while the following four CBMs are similar to each other and belong to CBM_4_9 family. The domain organization of LamA is similar to Lic16A, a β-1,3-glucanase recently isolated from *Clostridium thermocellum*, in which a leader peptide, a threefold repeat of SLH, an unknown module, a catalytic module of GH16, and a fourfold repeat of CBM_4_9 are connected sequentially from N to C terminus in total of 1,324 amino acid residues (Fuchs et al. 2003). To our knowledge, LamA and Lic16A are the two most complex β-1,3-glucanases documented today. Sequence comparison revealed that the regions encompassing SLHs, GH16, and CBMs are similar respectively among the two enzymes with local identities around 20–35%. On the other hand, the unknown modules share a similarity of less than 20%. Therefore, the presence of

Table 3 Kinetic constants of truncated proteins of LamA toward laminarin

Enzyme	K_M (mg/ml)	V_{max} (U/nmol of protein)
CBF	2.9±0.3	5.2±0.4
CB_3	2.4±0.4	3.2±0.3
SC	2.4±0.4	6.6±0.5
CF	1.8±0.1	1.9±0.2
C	1.1±0.1	1.7±0.1

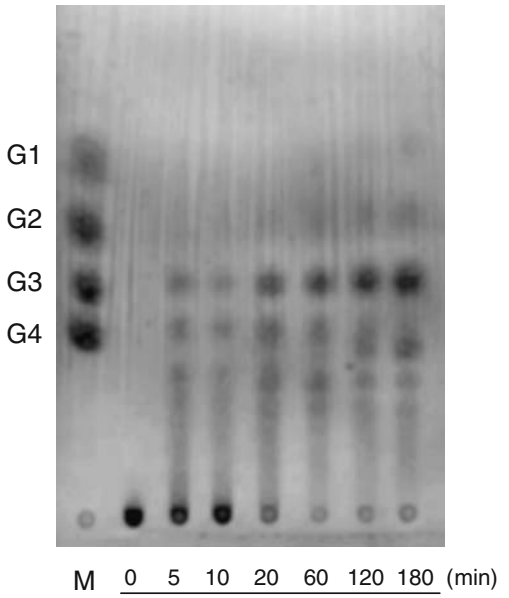

Fig. 3 TLC analysis of the digested products of laminarin by the catalytic module (truncated protein C) of LamA. Glucose (*G1*), laminaribiose (*G2*), laminaritriose (*G3*), and laminaritetraose (*G4*) were used as standards

Fa5/8C in LamA marks the major structural difference of the two complex β-1,3-glucanases.

Expression and purification of proteins derived from LamA Five truncated proteins with a variety of module organizations were successfully expressed in *E. coli* after failing to express LamA in full length, in order to characterize the enzymatic activity of the catalytic module and the relevant functions of CBMs, Fa5/8C analogue, and the region without predictable function (Fig. 1b). All the five truncated proteins have a histidine tag fused to the N terminus. The calculated molecular masses of expressed CBF, CB_3, CF, SC, and C are approximately 144.6, 92, 51.6, 77, and 38.2 kDa, respectively. Purification of the truncated proteins was achieved by immobilized metal affinity chromatography and Sephacryl S-300 gel filtration chromatography as described in "Materials and Methods" (Fig. 2).

Activity characterization of the β-1,3-glucanase Optimal working pH toward laminarin hydrolysis was found to be around pH 5.5–6.0 for truncated proteins CBF and C, while their optimal temperature was 50°C and 40°C, respectively, for 10-min reactions carried out at pH 5.5 (data not shown). The glucose equivalents releasing upon hydrolysis of laminarin at 30°C in pH 5.8 buffer increased steadily with incubation time up to 120 min for all the five truncated proteins (data not shown).

Substrate specificity of LamA was examined by including polysaccharides with various linkages and building blocks in reactions carried out in pH 5.8 at 30°C (Table 2). Laminarin, a soluble β-1,3-glucan, was the most favorable substrate for all the truncated proteins. Despite at slower rates, insoluble β-1,3-glucans, e.g. pachyman, curdlan, and zymosan A, and β-1,3–1,4-glucans, e.g. barley β-glucan and lichenan were also degradable by the enzymes. Nonetheless, CMC, xylan, or amylose could not be hydrolyzed to any appreciable extent. This results confirmed the identify of LamA as a β-1,3-glucanase (laminarinase). The amino acid sequences flanking the catalytic module could enhance the hydrolyzing activity toward all kinds of usable substrates. It is worth noting that the enhancement was more obvious for insoluble substrates than for laminarin, except for SC which had the most significant influence on laminarin hydrolysis. Comparing the enzymatic activities of CBF, CB_3, CF, and C also suggests that the rate enhancements exerted by CBM repeats and Fa5/8C module were cumulative.

Kinetic parameters of the truncated proteins for laminarin hydrolysis were determined from substrate concentration dependence of velocity. Based on double reciprocal plots, K_M values of CBF, CB_3, SC, CF, and C were calculated to be 2.9, 2.4, 2.4, 1.8, and 1.1 mg/ml, respectively (Table 3), suggesting that the appended modules have negative influences on the catalytically relevant binding of laminarin. On the other hand, the value of V_{max} increased from 1.7 of the catalytic module to 1.9, 6.6, 3.2, and 5.2 U/nmol of CF, SC, CB_3, and CBF, respectively. Similar effects on K_M and V_{max} were observed in the case of the CBM, a member of CBM_6 family, of an endo-β-1,3-glucanase isolated from *Streptomyces sioyaensis* (Hong et al. 2002). TLC analysis of the products of laminarin digested by the catalytic domain showed that laminaritriose was the major end product (Fig. 3). The gradual decrease of chain length of the resulting oligosaccharide products with incubation time suggested that LamA is an endo-β-1,3-glucanase.

Polysaccharide binding capability Greater catalytic activities, particularly to insoluble substrates, of CBF and CB_3 than C (Table 2) implied that the appended modules preceded by the catalytic module may increase the accessibility of the insoluble substrates by the enzyme. Binding strength of the five truncated proteins to insoluble polysaccharides was examined in this study by determining the remaining enzymatic activity in the supernatant after incubating the purified enzymes with various tested substrates at 4°C for 1 h. The rationale of the assay is that the stronger the binding of the protein to insoluble polysaccharides, the less the enzymatic activity remains in

Table 4 Binding affinity of truncated proteins of LamA to various polysaccharides

Substrate	CBF	CB_3	SC	CF	C
Pachyman	69±20[a]	67±19	41±22	77±8	69±16
Curdlan	59±4	40±10	5±2	24±7	15±12
Zymosan A	53±4	16±6	7±3	25±11	15±6
Barley β glucan	51±13	37±13	21±13	21±6	22±2
Lichenan	53±10	47±29	21±12	28±8	28±6
Avicel	50±15	42±24	24±14	26±3	12±5
Xylan	0	0	0	0	0

[a] Binding affinity is determined according to the ratio (%) of the enzymatic activity remained in the supernatant after incubation of proteins (each 0.6 μg/ml) with specified polysaccharides (2 mg/ml) at 4°C for 1 h, and presented as the reverse number of the ratio. Enzymatic activity was determined by using laminarin as the substrate. The enzymatic activity before incubation was taken as 100%.

the supernatant. It should be noted that no glucose equivalent was released during the 1-h incubation at 4°C. The enzymes, in all versions, had the ability to bind β-1,3-, β-1,3–1,4-glucans, and cellulose to various extents, with pachyman the most favorably binding substrate (Table 4). By contrast, the proteins showed no affinity to xylan. In general, the binding strength was in the order of CBF> CB_3>CF>C, suggesting that the binding forces provided by the CBM repeats and Fa5/8C module were additive. SC was distinct in that it had generally weaker affinities than C to the tested polysaccharides, suggesting that the sequence preceding the catalytic module may have a restriction on the access of the latter to insoluble substrates.

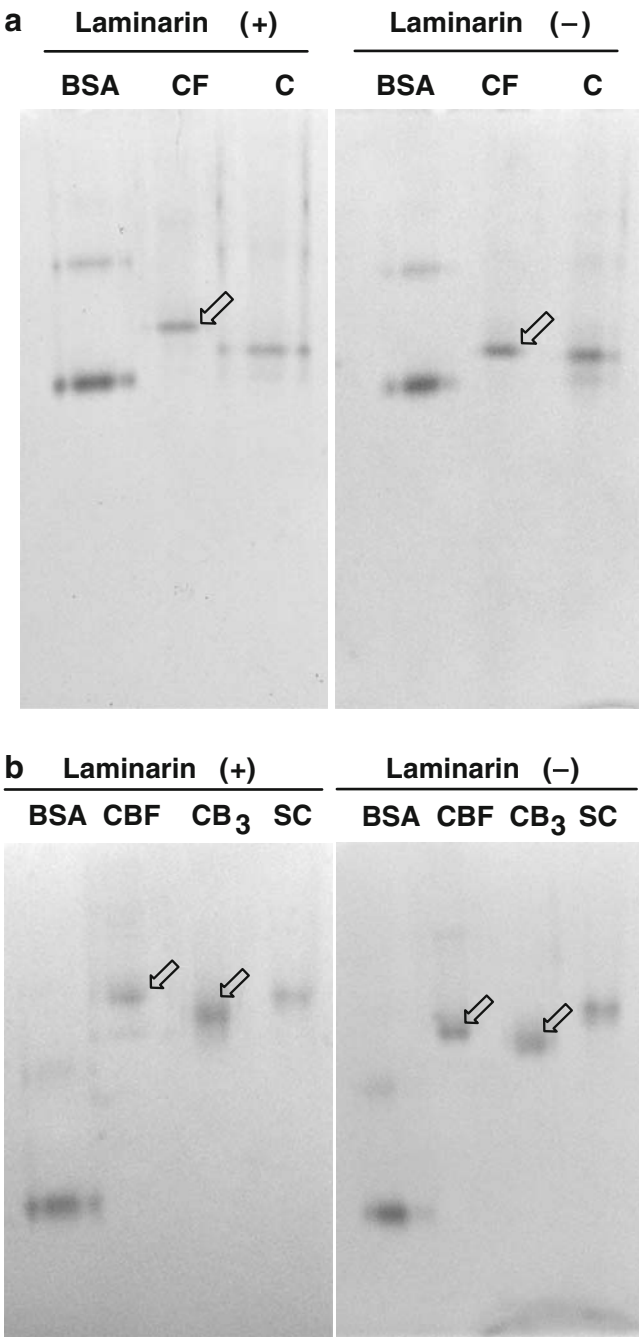

Fig. 4 Retardation of protein mobility on laminarin-embedded polyacrylamide gels. Specified proteins (each ca. 0.2 μg) were applied to 10% (panel **a**) or 8% (panel **b**) polyacrylamide gel electrophoresis under 80 V at 4°C. Laminarin (3 mg/ml) was present or absent in the separation gels as indicated. Trace SDS (0.02%) was also added in the gels to reduce the smearing of the proteins

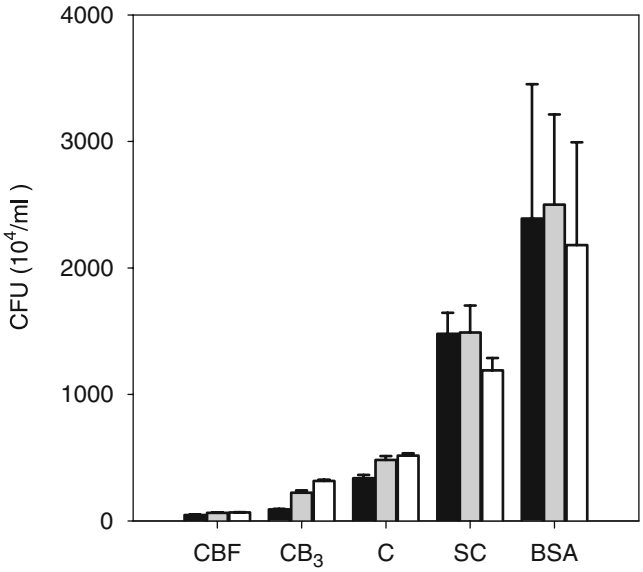

Fig. 5 Inhibition of *C. albicans* by truncated proteins of LamA. The colony forming units (CFU) of the yeast on YPD agar plate were determined after treatment by the indicated proteins under conditions as described in "Materials and Methods". The *black*, *grey*, and *blank bars* indicate the experiments with 1, 0.5, and 0.25 μM protein solutions, respectively

To further determine the contribution of the appended modules to the binding of soluble β-1,3-glucan, an affinity gel electrophoresis was performed in this study. A significant binding between the protein and laminarin, which was present in the polyacrylamide gel, would retard the protein's mobility during electrophoresis under a rather native condition. Apparently, the migrations of CBF, CB_3, and CF were slowed down by laminarin in comparison with the behaviors of the catalytic module and BSA (Fig. 4), suggesting that both the CBM repeats and Fa5/8C module could bind laminarin. It should be noted that SC also migrated at a slower rate in the laminarin-embedded gel, although the difference was barely noticeable.

Antifungal activity To test the application potential of LamA on growth control of pathogenic fungi, the inhibition effects of the truncated proteins on *C. albicans* and *R. solani* were examined. The cells of *C. albicans* were treated by tested proteins, including BSA as a control, at concentrations of 0.25, 0.5, or 1 μM for 3 h. Protein inhibition effects were then assessed by counting the surviving cell numbers (Fig. 5). Truncated protein CBF, CB_3, and C had

significant effects on decreasing the cell viability. Among them, CBF was most potent with the surviving rate of approximately 2%. Analogy to the binding effect on insoluble polysaccharides, the sequence preceding the catalytic module exerted a negative influence on killing *C. albicans*. The morphologies of the yeast cells after treatment with CBF or BSA were observed under SEM (Fig. 6). The cells treated by BSA had normal appearances with intact cell wall structure; nonetheless, those by CBF showed aberrant cell structures disposed with cell debris.

As for *R. solani*, an agar plug full of mycelia was placed in the center of a well containing tested proteins in 10 mM phosphate buffer [pH 6.0] and 10% glycerol. The growth of mycelia after 2 days was directly observed (Fig. 7). In general, all the truncated proteins of LamA delayed the growth of *R. solani* in comparison with BSA.

Discussion

The gene encoding a β-1,3-glucanase of 1,793 amino acids was isolated from a strain of *Paenibacillus* sp. in this study. Besides a catalytic module, this complex β-1,3-glucanase (LamA) also contains several modules such as SLH, CBM, Fa5/8C, and regions without predictable function (Fig. 1). Successful expression of five truncated proteins in *E. coli* allowed biochemical characterization of this complex protein in the first step.

Modules of glycoside hydrolase family 16 are present in bacterial β-1,3- and β-1,3–1,4-glucanases. The β-1,3-glucanases specifically hydrolyze β-1,3-glucosidic linkages, while β-1,3–1,4-glucanases catalyze the specific hydrolysis of β-1,4-glucosidic bonds adjacent to β-1,3 linkages in β-D-glucans or lichenan. Crystal structures of the family members, such as an endo-β-1,3-glucanase from *Nocardiopsis* sp. (Fibriansah et al. 2007) and β-1,3–1,4-glucanases from *Fibrobacter succinogenes* (Tsai et al. 2005) and *Bacillus macerans* (Hahn et al. 1995; Gaiser et al. 2006), showed classical folds of sandwich-like β-jelly roll. The catalytic module of LamA catalyzed the hydrolysis of β-1,3-glucosidic linkages, at an endolytic mode, with greatest preference for laminarin. The apparent K_M and V_{max} of the catalytic module toward laminarin were determined to be 1.1 mg/ml and 1.7 U/nmol of protein, respectively. In general, presence of the appended modules increased the magnitudes of both K_M and V_{max} (Table 3). With the substrate-binding ability, such appended modules might presumably increase the encountering frequency between the catalytic module and the available β-13-linkages, leading to an increase in V_{max}. However, their presence might also modify the entrance of binding pocket in the catalytic module, and somehow increased the magnitudes of apparent K_M. As regards insoluble substrates, the enzyme exhibited similar efficiencies for the hydrolysis of β-1,3-glucans and β-1,3–1,4-glucans. Similar substrate preferences have also been demonstrated on GlcA of *B. circulans* WL-12 (Aida et al. 1995), Curd1 of *S. sioyaensis* (Hong et al. 2002),

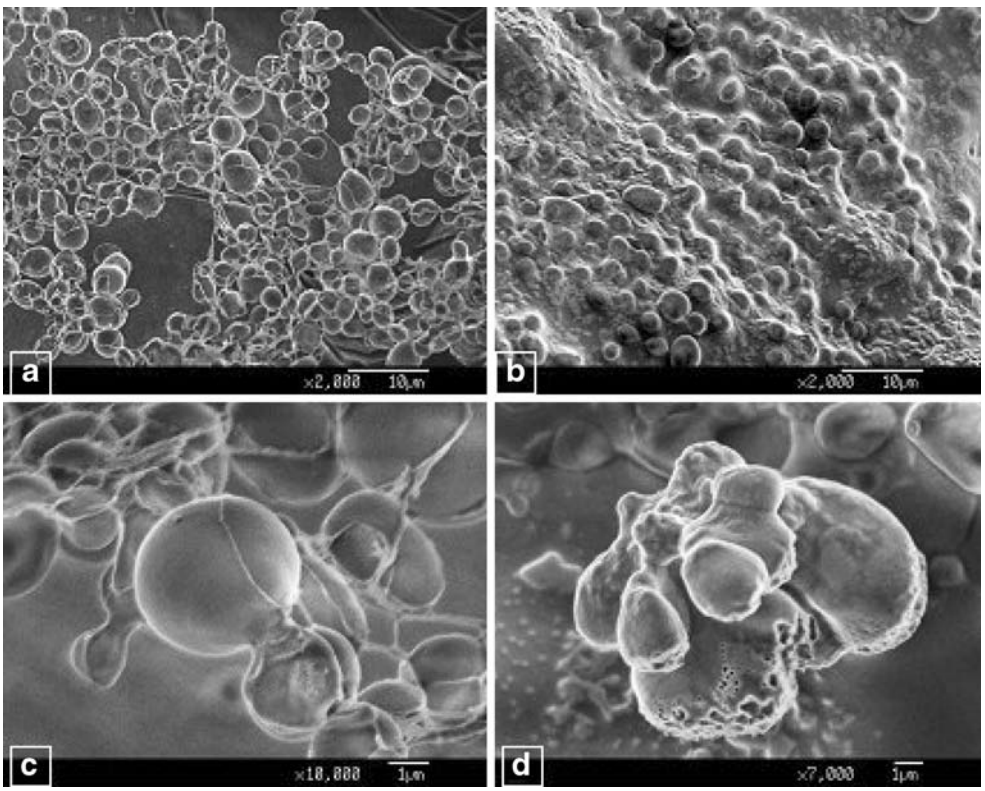

Fig. 6 Morphology of *C. albicans* after treatment with CBF or BSA. Cells were incubated with 0.14 mg/ml BSA (panel **a** and **c**) or CBF (panel **b** and **d**) for 3 h as described in "Materials and Methods". The morphology of cells was observed under scanning electron microscope

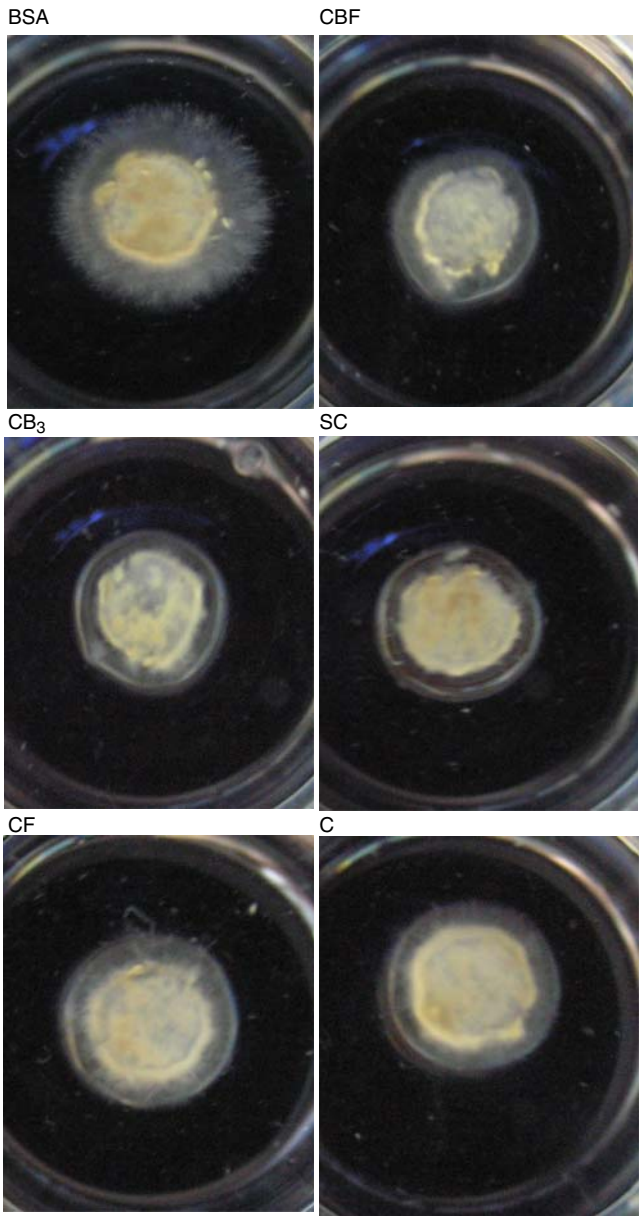

Fig. 7 Growth inhibition of *R. solani* by truncated proteins of LamA. Agar disks (ca. 5 mm diameter) of fully grown hypha were placed in 6-well plate that contained 10 mM potassium phosphate buffer [pH 6.0], 10% glycerol, and 1.8 μM indicated proteins. The radial growth of mycelia at room temperature was photo-recorded after 2 days

LamA of *Thermotoga neapolitana* (Zverlov et al. 1997), and the β-1,3-glucanases from *B. circulans* IAM1165 (Aono et al. 1995), *Cellvibrio mixtus* (Sakellaris et al. 1990), and *Pyrococcus furiosus* (Gueguen et al. 1997). In terms of the organization of functional modules, LamA is very similar to Lic16A; nonetheless, their substrate preferences are different. The most favorable substrate for LamA was laminarin, whereas Lic16A preferred barley β-glucan and lichenan. Zymosan A could be hydrolyzed as efficiently as other composite polysaccharides by LamA, but it was not degradable by Lic16A (Fuchs et al. 2003).

Modules of CBM_4 are approximately 150 amino acid residues found in bacterial secreted glycoside hydrolases such as cellulase, xylanase, β-1,3-glucanase, and β-1,3–1,4-glucanase. They can be either at N or C terminus of the catalytic module with single or multiple copies. As elucidated on the CBMs of *Cellulomonas fimi* 1,4-β-glucanase CenC (Johnson et al. 1996) and *Thermotoga maritima* laminarinase Lam16A (Boraston et al. 2002), members of the family are thought to have structures of sandwich jelly-roll fold. These structural data also suggested the importance of the conserved polar and aromatic amino acid residues in binding the oligosaccharides. With the capability to bind to a variety of polysaccharides, modules of CBM_4 may promote the association of the enzyme with composite substrates, and consequently enhance the efficiency of substrate degradation. The four CBM_4_9 repeats of LamA have sequence identities within 12–27% among each other, and share several aromatic and aliphatic residues in the putative substrate-binding pocket (Fig. 1a). Binding assay in this study confirmed the binding capability of the CBM_4_9 repeats to β-1,3-, β-1,3–1,4-, and β-1,4-glucanases but xylan (Table 4), and this capability presumably conferred greater specific activities to the catalytic module toward degradable substrates (Table 2). However, the binding specificity of each of the four CBM_4_9 repeats remains to be addressed.

The family of discoidin module, which includes repeats of Fa5/8C of blood coagulation factors V and VIII, has been implicated in cell-adhesion, developmental processes, and binding to polysaccharides present on cell surface. The C-terminal Fa5/8C analogue of LamA shares approximately 40% similarity in amino acid sequences to a tandem discoidin repeat found on a chitosanase isolated from *Paenibacillus fukuinensis* D2 (Kimoto et al. 2002). The role of the D2 discoidin repeat was unclear since it was dispensable for the *Paenibacillus* chitosanase activity. It was therefore interesting to know whether the analogue of Fa5/8C can assist LamA in assimilating hydrolysable polysaccharides. Binding assays indeed suggested that the Fa5/8C analogue could enhance the binding of the catalytic module to laminarin and insoluble β-1,3-, and β-1,3–1,4-glucans, and this capability should account for the enhancement of the catalytic activities to those degradable substrates. The region preceding the catalytic module does not have a predictable function. Its presence could significantly enhance the hydrolyzing activity of the catalytic module to laminarin; nonetheless, it also seemed to restrict the access of the catalytic module to some insoluble substrates. The base for the apparently controversial phenomenon remains to be investigated.

The 44-kDa β-1,3-glucanase, purified previously from culture medium of the *Paenibacillus* sp., has amino acid sequence AGNWNLVWSDEFDG at its N terminus (Hong

and Meng 2003). No exact match of the sequence was found on LamA, suggesting that this 44-kDa protein was not a degraded form of the cloned β-1,3-glucanase. The major product after laminarin hydrolysis was also different between the two *Paenibacillus* enzymes. LamA produced primarily laminaritriose, but the 44-kDa enzyme produced glucose. This strain of *Paenibacillus* sp. may therefore produce at least two β-1,3-glucanases with different characteristics to digest β-1,3-glucans synergistically. Similar situations as multiple β-1,3-glucanase being produced by a single bacterial strains have been demonstrated in *Cellulosimicrobium cellulans* (Ferrer 2006) and *Bacillus circulans* (Aida et al. 1995).

Invasive *Candida* infections are serious threats to immunocompromised patients with as high as 75–100% mortality (Venkatesan et al. 2005). Although a number of drugs are clinically available, most can trigger side effects such as hepatoxicity. In addition, the emergence of drug-resistant strains also limits their efficacy in treating the patients. For these reasons, there is a continued need for new antifungal agents with potent activity and improved safety. Enzymes able to degrade the yeast cell walls may have potential for being supplementary agents to the current therapeutic drugs. Panomycocin, an exo-β-1,3-glucanase isolated from *Pichia anomala*, was recently demonstrated to have potent activity against a variety of *Candida* species (Izgü et al. 2007). In this study, we found that the truncated proteins of LamA, particularly CBF, could kill *C. albicans* efficiently. The CBM_4_9 repeats and Fa5/8C analogue might anchor to the yeast cell walls, and consequently increase the accessibility of susceptible β-1,3-glucan structures to the catalytic module. The various truncated proteins of LamA also showed inhibition on the mycelium growth of *R. solani*, a pathogen infecting a broad range of many crops, suggesting the application potential of LamA as an antifungal agent in many aspects.

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References

Aida K, Okada T, Kasahara N, Nikaidou N, Tanaka H, Watanabe T (1995) Comparative studies of β-1,3-glucanase A1 and B of *Bacillus circulans* WL-12: purifications and enzymatic properties. J Ferment Bioeng 80:283–286

Aono R, Hammura M, Yamamoto M, Asano T (1995) Isolation of extracellular 28- and 42-kilodalton β-1,3-glucanase and comparison of three β-1,3-glucanase produced by *Bacillus circulans* IAM1165. Appl Environ Microbiol 61:122–129

Boraston AB, Nurizzo D, Notenboom V, Ducros V, Rose DR, Kilburn DG, Davies GJ (2002) Differential oligosaccharide recognition by evolutionarily-related β-1,4 and β-1,3 glucan-binding modules. J Mol Biol 319:1143–1156

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Castresana C, de Carvalho F, Gheysen G, Habet, s M, Inze D, van Montagu M (1990) Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* β-1,3-glucanase gene. Plant Cell 2:1131–1143

El-Katatny MH, Gudelj M, Robra KH, Elnaghy MA, Gübitz GM (2001) Characterization of a chitinase and an endo-β-1,3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. Appl Microbiol Biotechnol 56:137–143

Evans CT, Conrad D (1987) An improved method for protoplast formation and its application in the fusion of *Rhodotorula rubra* with *Saccharomyces cerevisiae*. Arch Microbiol 148:77–82

Ferrer P (2006) Revisiting the *Cellulosimicrobium cellulans* yeast-lytic β-1,3-glucanases toolbox: a review. Microb Cell Fact 17:5–10

Fibriansah G, Masuda S, Koizumi N, Nakamura S, Kumasaka T (2007) The 1.3 Å crystal structure of a novel endo-β-1,3-glucanase of glycoside hydrolase family 16 from alkaliphilic *Nocardiopsis* sp. strain F96. Proteins 69:683–690

Fried B, Sherma J (1982) Thin-layer chromatography: Techniques and applications. Marcel Dekker, New York

Fuchs KP, Zverlov VV, Velikodvorskaya GA, Lottspeich F, Schwarz WH (2003) Lic16A of *Clostridium thermocellum*, a non-cellulosomal, highly complex endo-β-1,3-glucanase bound to the outer cell surface. Microbiology 149:1021–1031

Gacto M, Vicente-Soler J, Cansado J, Villa TG (2000) Characterization of an extracellular enzyme system produced by *Micromonospora chalcea* with lytic activity on yeast cells. J Appl Microbiol 88:961–967

Gaiser OJ, Piotukh K, Ponnuswamy MN, Planas A, Borriss R, Heinemann U (2006) Structural basis for the substrate specificity of a *Bacillus* 1,3–1,4-b-glucanase. J Mol Biol 357:1211–1225

Grenier J, Potvin C, Asselin A (1993) Barley pathogenesis-related proteins with fungal cell wall lytic activity inhibit the growth of yeasts. Plant Physiol 103:1277–1283

Gueguen Y, Voorhorst WGB, van der Oost J, de Vos WM (1997) Molecular and biochemical characterization of an endo-β-1,3-glucanase of the hyperthermophilic archaeon *Pyrococcus furiosus*. J Biol Chem 272:31258–31264

Hahn M, Olsen O, Politz O, Borriss R, Heinemann U (1995) Crystal structure and site-directed mutagenesis of *Bacillus macerans* endo-1,3–1,4-b-glucanase. J Biol Chem 270:3081–3088

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309–316

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781–788

Hong TY, Meng M (2003) Biochemical characterization and antifungal activity of an endo-1,3-β-glucanase of *Paenibacillus* sp. isolated from garden soil. Appl Microbiol Biotechnol 61:472–478

Hong Y, Proctor A, Schultz J (2000) Acid-treated soy hull carbon structure and adsorption performance. JAOCS 77:785–790

Hong TY, Cheng CW, Huang JW, Meng M (2002) Isolation and biochemical characterization of an endo-1,3-β-glucanase from *Streptomyces sioyaensis* containing a C-terminal family 6 carbohydrate-binding module that binds to 1,3-β-glucan. Microbiology 148:1151–1159

Izgü F, Altinbay D, Türeli AE (2007) In vitro susceptibilities of *Candida* spp. to panomycocin, a novel exo-β-1,3-glucanase

isolated from *Pichia anomala* NCYC 434. Microbiol Immunol 51:797–803

Johnson PE, Joshi MD, Tomme P, Kilburn DG, McIntosh LP (1996) Structure of the N-terminal cellulose-binding domain of *Cellulomonas fimi* CenC determined by nuclear magnetic resonance spectroscopy. Biochemistry 35:14381–14394

Kapteyn JC, Hoyer LL, Hecht JE, Müller WH, Andel A, Verkleij AJ, Makarow M, Van den Ende H, Klis FM (2000) The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. Mol Micriobiol 35:601–611

Kim PI, Chung KC (2004) Production of an antifungal protein for control of *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens* MET0908. FEMS Microbiol Lett 234:177–183

Kimoto H, Kusaoke H, Yamamoto I, Fujii Y, Onodera T, Taketo A (2002) Biochemical and genetic properties of *Paenibacillus* glycosyl hydrolase having chitosanase activity and discoidin domain. J Biol Chem 277:14695–14702

Klis FM (1994) Review: cell wall assembly in yeast. Yeast 10:851–869

Kollár R, Reinhold BB, Petráková E, Yeh HJ, Ashwell G, Drgonov á J, Kapteyn JC, Klis FM, Cabib E (1997) Architecture of the yeast cell wall: β(1,6)-glucan interconnects mannoprotein, β(1,3)-glucan, and chitin. J Biol Chem 272:17762–17775

Lim HS, Kim YS, Kim SD (1991) *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. Appl Environ Microbiol 57:510–516

Mahasneh AM, Stewart DJ (1980) A medium for detecting β-(1,3)-glucanase activity in bacteria. J Appl Bacteriol 48:457–458

Mohagheghpour N, Dawson M, Hobbs P, Judd A, Winant R, Dousman L, Waldeck N, Hokama L, Tusé D, Kos F (1995) Glucans as immunological adjuvants. Adv Exp Med Biol 383:13–22

Nagarajkumar M, Bhaskaran R, Velazhahan R (2004) Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiol Res 159:73–81

Pretorius IS, van der Westhuisen TJ (1991) The impact of yeast genetics and recombinant DNA technology on the wine industry: a review. Am J Enol Vitic 12:3–29

Ríos-Hernández M, Dos-Santos N, Silvia-Cardoso J, Bello-Gárciga JL, Pedroso M (1994) Immunopharmacological studies of β-1,3-glucan. Arch Med Res 25:179–180

Ryan EM, Ward OP (1985) Study of the effect of β-1,3-glucanase from basidiomycete QM 806 on yeast extract production. Biotechnol Lett 7:409–412

Sakellaris H, Pemberton JM, Manners JM (1990) Gene from *Cellvibrio mixtus* encoding an β-1,3-endoglucanase. Appl Environ Microbiol 56:3204–3208

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Smits GJ, van den Ende H, Klis FM (2001) Differential regulation of cell wall biogenesis during growth and development in yeast. Microbiology 147:781–794

Thrane C, Tronsmo A, Jensen DF (1997) Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, inducing of and biological activity against plant pathogenic *Pythium* spp. Eur J Plant Pathol 103:331–344

Tomme P, Creagh AL, Kilburn DG, Haynes CA (1996) Interaction of polysaccharides with the N-terminal cellulosebinding domain of *Cellulomonas fimi* CenC. 1. Binding specificity and calorimetric analysis. Biochemistry 35:13885–13894

Tsai LC, Shyur LF, Cheng YS, Lee SH (2005) Crystal structure of truncated *Fibrobacter succinogenes* 1,3–1,4-β-D-glucanase in complex with β-1,3–1,4-cellotriose. J Mol Biol 354:642–651

Venkatesan P, Perfect JR, Myers SA (2005) Evaluation and management of fungal infections in immunocompromised patients. Dermatol Ther 18:44–57

Villettaz JC, Steiner D, Trogus H (1984) The use of a β-glucanase as an enzyme in wine clarification and filtration. Am J Enol Vitic 35:253–256

Watanabe T, Kasahara N, Aida K, Tanaka H (1992) Three N-terminal domains of β-1,3-glucanase A1 are involved in binding to insoluble β-1,3-glucan. J Bacteriol 174:186–190

Wood TM, Bhat KM (1988) Methods for measuring cellulase activities. Methods Enzymol 160:87–112

Yamamoto M, Ezure T, Watanabe T, Tanaka H, Aono R (1998) C-terminal domain of β-1,3-glucanase H in *Bacillus circulans* IAM1165 has a role in binding to insoluble β-1,3-glucan. FEBS Lett 433:41–43

Yi SY, Hwang BK (1997) Purification and antifungal activity of a basic 34 kDa β-1,3-glucanase from soybean hypocotyls inoculated with *Phytophthora sojae* f. sp. glycines. Mol Cells 7:408–413

Zverlov VV, Volkov IY, Velikodvorskaya TV, Schwarz WH (1997) Highly thermostable endo-1,3-β-glucanase (laminarinase) LamA from *Thermotoga neapolitana*: nucleotide sequence of the gene and characterization of the recombinant gene product. Microbiology 143:1701–1708