

Cloning, Heterologous Expression, and Functional Characterization of a Chitinase Gene, *Lbchi32*, from *Limonium bicolor*

Zhi Hua Liu · Chuan Ping Yang · Xiao Tian Qi ·
Li Li Xiu · Yu Cheng Wang

Received: 5 November 2009 / Accepted: 12 February 2010 / Published online: 30 May 2010
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Abstract In the present study, an endochitinase gene, *Lbchi32*, was cloned from *Limonium bicolor*. The cDNA sequence of *Lbchi32* was 1,443 bp in length and encoded 319 amino acid residues. The DNA sequence of *Lbchi32* was 2,512 bp in length and contained three exons and two introns. The *Lbchi32* gene was inserted into a pPIC9 vector and transferred into *Pichia pastoris* strains GS115 and KM71 for heterologous expression. SDS-PAGE analyses indicated that LbCHI32 was expressed in both GS115 and KM71 and that it was secreted extracellularly. The optimal reaction conditions for LbCHI32 activity are 45°C, pH 5.0, and 5 mM Ba²⁺. The LbCHI32 enzyme can efficiently degrade chitin, chitin derivatives, and the cell walls of different pathogenic fungi, including phytopathogenic *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Valsa sordida*, *Septoria tritici*, and *Phytophthora sojae*. These findings suggest that *Lbchi32* has potential use in the degradation of chitin and chitin derivatives.

Keywords Chitinase · Gene cloning · Heterologous expression · *Limonium bicolor*

Introduction

Chitin, which is a polymer of *N*-acetylglucosamine, is the second most abundant natural polymer on earth after cellulose. It is currently attracting considerable

Z. H. Liu · C. P. Yang · X. T. Qi · L. L. Xiu · Y. C. Wang (✉)
Key Laboratory of Forest Tree Genetics Breeding and Biotechnology, Northeast Forestry University, Ministry of Education, 26 Hexing Road, Harbin 150040, China
e-mail: wangyucheng1029@yahoo.com.cn

Z. H. Liu
Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, China

attention as an unutilized biomass resource. Chitinase can hydrolyze chitin to chitin oligosaccharides, including oligomers, chitobiose, and *N*-acetylglucosamine, which can be utilized as carbon or nitrogen sources (Schrempf 2001). This suggests that chitinase can play an important role in the utilization of chitin. In addition, chitin is also the main component of the cell walls of phytopathogenic fungi. Chitinase can catalyze the hydrolysis of fungal cell walls and thereby exhibits antifungal activities. Accordingly, this enzyme could be used directly as an antifungal agent against fungal phytopathogens (Robert et al. 2002; Ohnuma et al. 2004). Therefore, there is the potential for chitinases to play important roles in both industrial production and the biocontrol of plant diseases.

Chitinases are widely distributed in various organisms; among these, plant chitinases comprise a large family. Previous studies have shown that chitinases are involved in plant defense mechanisms against fungal pathogens. For instance, the chitinase (*Chi1*) from *Triticum aestivum* “Ning7840” has been found to be involved in a generalized resistance response to *Fusarium graminearum*, suggesting an antifungal role (Kong et al. 2005). A chitinase gene from mung bean (*Phaseolus mungo*) can exert antifungal activity against various fungi, including *Fusarium solani*, *F. oxysporum*, *Mycosphaerella arachidicola*, *Pythium aphanidermatum*, and *Sclerotium rolfsii* (Ye and Ng 2005). Overexpression of *Mcchit1*, a chitinase gene isolated from *Momordica charantia*, in *Nicotiana benthamiana* was found to improve resistance significantly to the plant pathogenic fungus *Phytophthora nicotianae*. Furthermore, *Mcchit1*-transformed cotton exhibits elevated resistance to *Verticillium wilt*, indicating that the *Mcchit1* gene is a useful gene in the engineering of plant resistance to fungal diseases (Xiao et al. 2007). Also, a chitinase gene (*Vvchit1a*) from *Vitis vinifera* was induced in *V. vinifera* leaves infected with *Bacillus cinerea* or *Pseudomonas syringae* pv. *pisi*, a bacterium that induces an incompatible interaction (Robert et al. 2002).

Determination of the chitin and chitin derivative degradation activity is very important in characterizing chitinases. Previous studies of plant chitinases, however, have focused mainly on their roles as antifungal agents, and little research had been performed on plant chitinase activities with respect to the degradation of chitin and chitin derivatives.

In the present study, we cloned a chitinase gene, *Lbchi32*, from *Limonium bicolor*, a biennial flowering plant belonging to the Plumbaginaceae. To investigate the role of *Lbchi32* in decomposing chitin, chitin derivatives, and the fungal cell walls, we expressed the gene in the yeast *Pichia pastoris*. We found that the recombinant LbCHI32 protein produced by transgenic yeast can exhibit high levels of activity in the degradation of chitin resources and fungal/oomycete cell walls. Our results suggest that plant chitinases, similarly to fungal chitinase, also exhibit high decomposing activity against chitin and chitin derivatives and thus have potential value in chitin exploitation. Further, the ability of LbCHI32 to decompose the cell walls of different fungi suggests that the *Lbchi32* gene is also involved in antifungal responses in *L. bicolor*.

Materials and Methods

Plant Material, Strains and Culture Conditions

Limonium bicolor seedlings were grown in pots containing a mixture of turf peat and sand (2:1 v/v). Plants were grown in a greenhouse under controlled conditions of 65–75% relative humidity, 14 h light, and an average temperature of 24°C. The leaves of *L. bicolor* were harvested for DNA and RNA extractions.

Two *P. pastoris* strains, GS115 (Mut⁺, His⁻) and KM71 (Mut^S, His⁻) (Invitrogen, Carlsbad, USA), were used as the hosts for expression of the *Lbchi32* chitinase gene. Five phytopathogenic fungal strains, including *F. oxysporum* (soybean root rot), *Septoria tritici* (wheat leaf wither), *Valsa sordida* (poplar bark rot), *Rhizoctonia solani* (rice sheath blight), *Sclerotinia sclerotiorum* (sclerotium disease on soybean), and one strain of oomycete plant pathogen, *Phytophthora sojae* (soybean root rot), were obtained from the Microbial Genetic Engineering Laboratory, Harbin Institute of Technology, Harbin, China. The mycelia of the five phytopathogenic fungal strains were inoculated into potato dextrose medium and incubated at 25°C for 48 h with shaking at 180 rpm. Mycelium of the oomycete strain was inoculated into carrot medium and incubated at 25°C for 48 h with shaking at 180 rpm. After cultivation, the mycelia of these strains were harvested. Cell wall fragments were prepared from the mycelia of each strain, according to the method of Morissette et al. (2006), and were used as substrates for fungal cell wall degradation analyses.

Cloning of the Chitinase *Lbchi32* Gene

A cDNA library was constructed from the leaves of *L. bicolor*, and 2358 ESTs were obtained by random sequencing of the library clones (Wang et al. 2008). The EST (GenBank no. EH793640) containing a full-length open reading frame (ORF) of endochitinase *Lbchi32* was identified from the cDNA library by EST analysis. Primers for amplification of the DNA sequence of *Lbchi32* were designed as follows: chi32-1, 5'-ATGGGGAGGCATTGGAGACTGGT-3'; chi32-2, 5'-TTATGACGATGCAGAGCCGGATG-3'. Total DNA was isolated from *L. bicolor* leaves according to the procedure described by Sambrook et al. (2002), and was used as a PCR template. The PCR product was cloned into a pMD18-T vector (Takara, Dalian, China) for sequencing.

Bioinformatics Analysis of the *Lbchi32* Gene

The catalytic domain of the LbCHI32 protein was identified by the BlastP program of NCBI, and the theoretical molecular mass and isoelectric point of the LbCHI32 protein were calculated using the ProtParam tool (<http://us.expasy.org/tools/protparam.html>). A signal peptide was predicted using SignalP3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) and glycosylation sites were predicted using the ELM Server (<http://elm.eu.org/>). Amino acid sequences of chitinases from various plant species (including *L. bicolor*) were aligned by the ClustalX program.

Construction of Recombinant pPIC9-chi32 and Yeast Transformation

The primers for amplification of the ORF of the *Lbchi32* gene were designed as follows: chi32-L, 5'-ATCGCTCGAGAAAAGAGAGGCTGAAGCTTACGCGCTGGACCTGACGGACCAGCTCG-3' (*Xho*I site underlined); chi32-R, 5'-CGATGCGGCCGCTGACGATGCAGAGCCGGATGGGTTG-3' (*Not*I site underlined). The ORF of the *Lbchi32* gene was cloned into a pPIC9 expression vector (Invitrogen, Carlsbad, USA), and the recombinant vector (pPIC9-chi32) was transformed into GS115 or KM71 cells by pulsed electroporation using a Bio-Rad GenePulser (1150 V, 25IF, 200 \times).

SDS-PAGE Analysis of Recombinant Chitinase LbCHI32

Three types of yeast transformants, the GS115 strain harboring pPIC9-chi32 (GS115-chi32), the KM71 strain harboring pPIC9-chi32 (KM71-chi32), and yeast transformants not harboring pPIC9 (GS115-pPIC9, used as a control), were cultured following the procedures described in the *Pichia* expression kit manual (K1710-01; Invitrogen). Methanol was added to the culture medium at a final concentration of 1% (v/v) every 24 h to maintain the induction. The supernatants were harvested after culturing for 1, 2, 3, 4, 5, and 6 days at 30°C. After the addition of 1 \times loading buffer, the supernatants were boiled for 5 min, centrifuged for 10 min at 8,000 rpm, and loaded onto a 12% SDS-PAGE gel.

Assay of LbCHI32 Activity

Chitinase activity was measured according to Liu et al. (2008). The strain transformed with empty pPIC9 was induced by 1% (v/v) methanol at 30°C for 1–7 days at 1-day intervals, and the fermentation medium was harvested for measurement of chitinase activity.

The yeast transformant GS115-chi32 was induced by 1% (v/v) methanol at 30°C for 6 days. The yeast cells were centrifuged, and the supernatant was used in the following experiments. For determination of the optimum temperature for LbCHI32 activity, the reaction mixtures (pH 4.5) were incubated at 35–80°C for 30 min at 5°C intervals, boiled for 5 min, supplemented with 0.05% (w/v) potassium ferrocyanide, and boiled for 10 min. The supernatant (enzyme solution) was incubated at 35–80°C for 30 min at 5°C intervals (pH 4.5) and chilled on ice; the remaining activity was then measured. Determination of the optimum pH for LbCHI32 enzyme activity was performed at 50°C over a pH range of 3–12, at intervals of 1 pH unit. The pH of the reaction mixture was adjusted using a series of buffers: 0.01 M citric acid–0.02 M Na₂HPO₄ (pH 3–5), 0.02 M Na₂HPO₄–NaH₂PO₄ (pH 6–8), 0.02 M glycine–NaOH (pH 9–10), and 0.05 M Na₂HPO₄–0.01 M NaOH (pH 11–12).

The Effect of Ions on LbCHI32 Activity

LbCHI32 activity was measured in a reaction buffer that was supplemented with 5 mM of a metal ion in the form of a chloride or sulfate salt. Different buffer

solutions were prepared, each spiked with a different metal salt (KCl, BaCl₂, CaCl₂, CoCl₂, NaCl, LiCl, MgSO₄, MnSO₄, FeSO₄, CuSO₄, or ZnSO₄). The relative activity of LbCHI32 was calculated as the activity in the ion-enhanced buffer divided by the activity of LbCHI32 under normal conditions (the control).

Chitin Derivative and Fungal Cell Wall Degradation Analyses

The substrates used for LbCHI32 degradation analysis, including 1% cell wall fragments from each fungus and 1% of chitin derivatives (chitosan, N,O-Carb-Chitosan, colloidal chitin, or N,O-Carb-chitin), were dissolved in 0.02 M HAc-NaAc buffer (pH 4.5). Cell wall fragments from the six fungal/oomycete plant pathogens (*R. solani*, *F. oxysporum*, *V. sordida*, *Sclerotinia sclerotiorum*, *Septoria tritici*, and *Phytophthora sojae*) were prepared as described by Morissette et al. (2006). The chitinase activity of LbCHI32 toward the substrates was measured using the Schales method (Imoto and Yagishita 1971).

All of the above experiments were completed, at a minimum, in triplicate, and average values were calculated based on results from three independent experiments. Data were analyzed using SPSS version 11.0 statistical software (SPSS Inc., Chicago, IL). Sample variability is given as the standard deviation.

Results

Cloning and Sequence Analysis of Chitinase *Lbchi32*

The cDNA sequence of *Lbchi32* is 1,443 bp in length, and the ORF length is 960 bp. It encodes 319 amino acids with a calculated molecular mass of 32.4 kDa and a predicted isoelectric point of 5.38. The DNA sequence of the *Lbchi32* chitinase gene was 2,512 bp in length and contained three exons and two introns. The lengths of the three exons were 391, 842, and 379 bp, and the two introns were 1,203 and 349 bp. The splice sites between exons and introns followed the GT–AG splicing rule. The cDNA and DNA sequences of the *Lbchi32* gene have been deposited in the GenBank database under accession numbers DQ431249 and EU744179, respectively.

SignalP prediction showed a signal peptide (25 amino acids) in the N-terminal sequence of LbCHI32. The LbCHI32 protein (ABD92820) is predicted to be cleaved by a signal peptidase between positions A25 and L26 (SAAl|LDL). There are three potential N-linked glycosylation sites, at C57-P62, Q208-A213, and K244-S249 of the LbCHI32 amino acid sequence. Pfam analysis revealed that the LbCHI32 protein had a catalytic domain common to members of the family 19 chitinases (InterPro acc. no.: IPR000726), indicating that it is a member of the family 19 glycosyl hydrolases (endochitinase).

Analysis of Sequence Identity

BlastP prediction showed that the catalytic domain of LbCHI32 consists of approximately 220–230 amino acid residues and three catalytic residues (E128,

BAH30273	QK	MAAFLGHVASKTSCGYGVATGGPLAWGLCYNR	E	MSPMQSYCDESWKFYPCSPGAEEYGRGALP	Y	WN	184
NP_172076	QK	MAAFLGHVASKTSCGYGVATGGPLAWGLCYNR	E	MSPMQSYCDESWKFYPCSPGAEEYGRGALP	Y	WN	184
ABV89660	QK	MAAFLGHVASKTSCGYGVATGGPLAWGLCYNR	E	MSPSQSYCDETWKFYPCSPGAEEYGRGALP	Y	WN	184
AAP80800	MK	VAAFLAHVGAKTSCGVDGIDGGPLAWGLCFKR	E	MSPSQDYCDYYKYMPGAPGAQYGRGALP	Y	WN	187
EEF43061	QK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPSDIYCDYYKYTYPTPGVSYHGRGALP	Y	WN	188
XP_002316022	QK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPSKTYCDDYYKYTYPTPGVSYHGRGALP	Y	WN	184
AAQ56598	MK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPSKLYCDDYYKYTYPTPGVSYHGRGALP	Y	WN	179
ABL86685	MK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPSKLYCDDYYKYTYPTPGVSYHGRGALP	Y	WN	179
ABL86686	MK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPSKLYCDDYYKYTYPTPGVSYHGRGALP	Y	WN	180
AAQ56599	MK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPSKIYCDYYKYTYPTPGVSYHGRGALP	Y	WN	180
ACM45714	MK	VT AFLGHVGSKTSCGYGVATGGPTAWGLCYNR	E	MSPS QLYCDDYYKYTYPCSPGASYHGRGALP	Y	WN	185
NP_188317	MK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPDLQYCDYYKLYTYPTPGVSYHGRGALP	Y	WN	187
AAL06524	MK	VAAFLGHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPDQLYCDYYKLYTYPTPGVSYHGRGALP	Y	WN	192
XP_002276563	MK	VAAFLGHVGSKTTCGYGVATGGPLAWGLCYNR	E	MSPSKSYCDDYYKYTYPTPGVEYFGRGALP	Y	WN	183
ABK95866	MK	LA A FLAHVGCKTSCGYGVATGGPLAWGLCYNR	E	MSPSQT YCD D FYKY TYPT PGAEY YGRGALP	Y	WN	182
XP_002269474	MK	L A A FLAHVGCKTSCGYGVATGGPLAWGLCYNR	E	MSPSKSYCDDFYKYTYPTPGAEY YGRGALP	Y	WN	182
EEF46716	MK	L A A FF SHVGSKTSCGYGVATGGPLAYGLCYNR	E	MSPSQSYCDDFYKYTYPTPGAEY YGRGALP	Y	WN	184
ABN08775	MM	L A A FLAHVGSKTSCGYGVATGGPLAWGLCYNR	E	MSPA QTYCD D Y Y KLY TYPT PGAEY YGRGALP	Y	WN	184
ABD92820	QL	L A A FLAHVGSQTSCGYGVATGGPTAWGLCYNR	E	LSPSQKYCDDSYKYTYPTPGADY YGRGALP	Y	WN	185
ACN39823	QK	L A A FP GHVAAETS CGYSVA VGGPYAWGLCYNE	E	MSPDQLYCDQN—YLFPCSPGASYHGRGALP	Y	WN	166
EAZ07679	MK	VAAFLGHVGA K T S C G Y S V A T G G P L A W G L C Y N R	E	LSPSQSYCDNS—NELYPVEGVEY YGRGALP	Y	WN	183
		:*:.*,*:*:* * *: :*:*: :. *: * , **:		*: * , **:		:* * , * , ***:*: *	

Fig. 1 Catalytic residues of chitinase Ib from 21 plant species. BAH30273, NP_172076, AAL06524: chitinases from *Arabidopsis thaliana*; ABV89660: chitinase from *Brassica rapa*; AAP80800, AAQ56598, AAQ56599: chitinases from *Gossypium hirsutum*; EEF43061, EEF46716: chitinases from *Ricinus communis*; XP_002316022, ABK95866: chitinases from *Populus trichocarpa*; ABL86685, ABL86686: chitinases from *Gossypium barbadense*; ACM45714, NP_188317: chitinases from *Pyrus pyrifolia*; XP_002276563, XP_002269474: chitinases from *Vitis vinifera*; ABN08775: chitinase from *Medicago truncatula*; ABD92820: chitinase from *Limonium bicolor*; ACN39823: chitinase from *Picea sitchensis*; EAZ07679: chitinase from *Oryza sativa*. Three highly conserved catalytic residues (E, E, and Y) are boxed. Asterisk (*), identity. Colon (:), high similarity. Period (.), low similarity

E150, Y183) (Fig. 1). To determine the conservation of the three catalytic residues, multiple sequence alignment of the chitinases from 21 plant species was conducted using the ClustalX program. This revealed that the amino acid sequences of these chitinases were conserved, with identities ranging from 71 to 80%. Furthermore, the chitin-catalyzing domains of the different chitinases were highly conserved, with the three catalytic residues (E, E, and Y) being particularly highly conserved (Fig. 1).

Heterologous Expression of *Lbchi32* in *Pichia pastoris*

Pichia pastoris GS115 and KM71 transformants harboring pPIC9-chi32 were generated, and the expression of *Lbchi32* was induced by methanol. After induction culture, SDS-PAGE analysis was conducted to determine whether the *LbCHI32* protein was synthesized in *P. pastoris*. Compared with the control *P. pastoris* transformed with an empty pPIC9, both the GS115 and KM71 transformants showed a clearly visible protein band with a molecular mass of approximately 32 kDa on an SDS-PAGE gel (Fig. 2). This result indicates that the *LbCHI32* protein was successfully synthesized in the GS115 and KM71 yeast cells, and that it was secreted extracellularly.

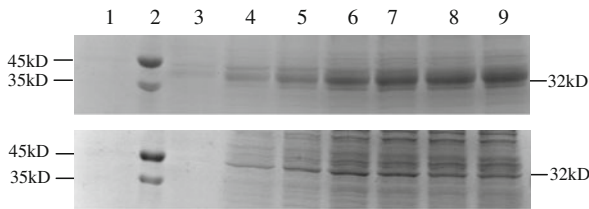


Fig. 2 SDS-PAGE analysis of the heterologous expression of Lbchi32 in the GS115 and KM71 strains of *Pichia pastoris*. *Top panel*, *Pichia pastoris* GS115 transformed with *Lbchi32* (GS115-chi32). *Bottom panel*, *P. pastoris* KM71 transformed with *Lbchi32* (KM71-chi32). *Lane 1*, supernatant of *P. pastoris* transformed with empty pPIC9 induced for 1 day. *Lane 2*, protein marker. *Lane 3*, supernatant of *P. pastoris* transformed with empty pPIC9 induced for 4 days. *Lanes 4–9*, supernatants of the recombinant yeasts (GS115-chi32 or KM71-chi32) after induction for 1, 2, 3, 4, 5, and 6 days, respectively

Properties of Recombinant LbCHI32

Chitinase activity was not detected in the culture medium of *P. pastoris* GS115 transformed with an empty pPIC9 vector after methanol induction, indicating that the chitinase detected in transgenic yeast cells is due to the expression of the exogenous gene *Lbchi32*.

Both the GS115 and KM71 transformants harboring *Lbchi32* showed chitinase activity; however, the GS115 yeast exhibited a higher chitinase activity than the KM71 yeast (Fig. 3). The chitinase activity of the LbCHI32 transgenic yeast strain GS115-chi32 showed a peak value at 6 days following methanol induction, with a chitinase activity of 0.71 U/ml (Fig. 3). The transformant that expressed the chitinase at the highest level (named GS115-chi32) was selected for further experimentation. LbCHI32 activity reached a peak at 45°C and was dramatically reduced at temperatures over 55°C. When the temperature reached 60°C, all enzyme activity was lost. Therefore, the optimal reaction temperature for LbCHI32 was determined to be 45°C (Fig. 3). The enzyme activity of recombinant LbCHI32 was stable when incubated between 35 and 65°C for 30 min, and decreased rapidly when temperatures exceeded 60°C. The chitinase was inactivated when incubated at 65°C for 30 min (Fig. 3). At 45°C, the enzyme activity of recombinant LbCHI32 was stable at pHs between 3 and 10, with a peak of activity at pH 5. Chitinase activity was reduced rapidly when the pH exceeded 10 and was inactivated when the pH reached 11 (Fig. 3).

The activity of recombinant LbCHI32 was strongly inhibited by Mn^{2+} and K^{+} but was stimulated by Ba^{2+} and Cu^{2+} ; the activity of LbCHI32 reached 0.86 U/ml when activated by Ba^{2+} and 0.77 U/ml by Cu^{2+} . Co^{2+} , Na^{+} , Li^{+} , Mg^{2+} , and Zn^{2+} did not induce any substantive activation of LbCHI32 (Fig. 4).

The activity of recombinant LbCHI32 toward different substrates was measured. LbCHI32 showed hydrolytic activity against all the test substrates, indicating that LbCHI32 can decompose the tested substrates. The activity of LbCHI32 toward different substrates ranged from 0.73 to 0.47 U/ml (Fig. 5).

The optimal reaction system for LbCHI32 appears to comprise a temperature of 45°C (Fig. 3), a pH of 5.0 (Fig. 3), and a Ba^{2+} concentration of 5 mM (Fig. 4).

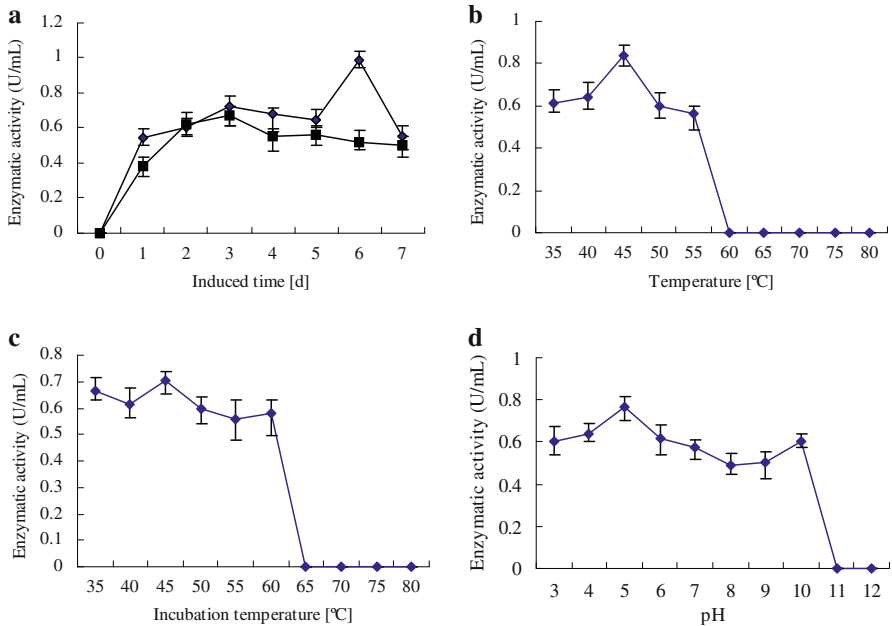


Fig. 3 Activities and properties of recombinant chitinase LbCHI32. **a** Chitinase activity of *Pichia pastoris* GS115-chi32 (diamond) and KM71-chi32 (square) at different induction times. **b** Effect of temperature on the chitinase activity of LbCHI32. **c** Effect of temperature on the chitinase stability of LbCHI32. **d** Effect of pH on the chitinase activity of LbCHI32. Error bars represent the standard deviation for three independent experiments

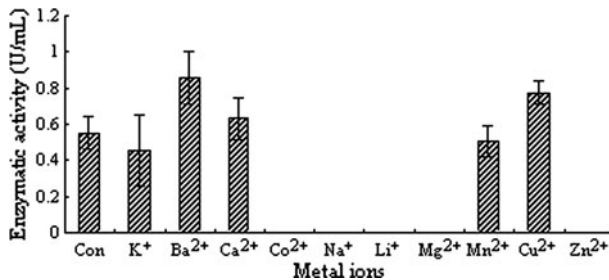


Fig. 4 Effect of metal ions on the chitinase activity of LbCHI32. Error bars represent the standard deviation for three independent experiments

Using this reaction system, the highest activity achieved by the recombinant chitinase LbCHI32 was 1.09 U/ml when the cell wall chitin of the fungal pathogen *F. oxysporum* was used as a substrate.

Discussion

Pfam prediction showed that LbCHI32 belongs to glycoside hydrolase family 19 (Fig. 1). Chitinases of family 19 (also known as classes IA/I and IB/II) are enzymes

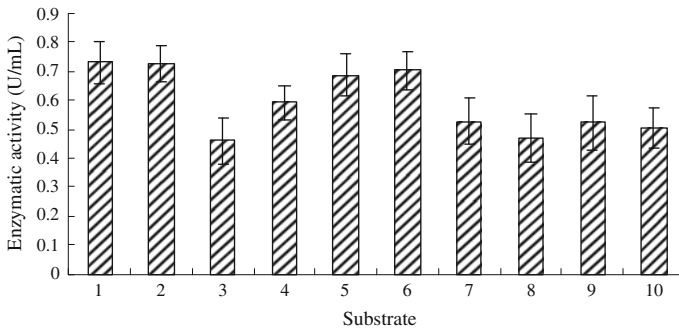


Fig. 5 Activity of chitinase LbCHI32 on substrates: 1, N,O-Carb-chitin; 2, Colloidal chitin; 3, Chitosan; 4, N,O-Carb-Chitosan; 5–10, cell wall chitin of *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Phytophthora sojae*, *Septoria tritici*, and *Valsa sordida*. Error bars represent the standard deviation for three independent experiments

from plants that function in the defense against fungal and insect pathogens by destroying their chitin-containing cell wall. Class IA/I and IB/II enzymes differ with respect to the presence (IA/I) or absence (IB/II) of an N-terminal chitin-binding domain (Hahn et al. 2000; Fukamizo 2000; Robertus and Monzingo 1999; Robertus et al. 1998; Monzingo et al. 1996). LbCHI32 lacks an N-terminal chitin-binding domain and therefore belongs to class IB.

In eukaryotic cells, N-glycosylation is the most common modification of secretory and membrane-bound proteins. N-linked glycosylation is a cotranslational process involving the transfer of an oligosaccharide chain to an asparagine residue in the protein (Apweiler et al. 1999; Mellquist et al. 1998; Nilsson and von Heijne 2000; Shakin-Eshleman et al. 1996). Kasturi et al. (1997) showed that amino residues Trp, Asp, and Glu are uncommon before the Ser/Thr position. Efficient glycosylation usually occurs when ~60 residues or more separate the glycosylation acceptor site from the C terminus. According to an ELM program prediction, three N-glycosylation sites (C57-P62, Q208-A213, and K244-S249) occur in the LbCHI32 protein, suggesting that glycosylation may occur in these regions. Furthermore, after glycosylation modification, the molecular mass of the LbCHI32 protein is larger than that predicted. Similarly, recombinant BjCHI1 and BjCHI2 in *P. pastoris* showed obviously larger molecular masses than those calculated from SDS-PAGE, and the larger masses were due to glycosylation of the enzyme (Tang et al. 2004). In the present study, the recombinant LbCHI32 exhibited a molecular mass larger than the 32 kDa observed on SDS-PAGE (Fig. 2). Therefore, this result indicates that LbCHI32 might be modified by glycosylation or some other post-translational modification.

In the present study, to assay the enzyme activity of the recombinant LbCHI32, four types of chitin derivative and six types of phytopathogen cell wall were used as substrates. The results showed that the decomposing ability of the LbCHI32 enzyme toward different chitin derivatives was similar to that toward different phytopathogen cell walls (Fig. 5). Therefore, these data indicate that *Lbchi32* not only

probably plays a role in fungal plant pathogen defense but also has the potential to decompose organic chitin resources.

The effect of metal ions on chitinase activities has been studied previously. For instance, the activity of a chitinase from *Bacillus* sp. DAU101 was strongly inhibited by Zn^{2+} , Cu^{2+} , and Hg^{2+} but stimulated by Co^{2+} (Lee et al. 2007). Similarly, the activity of two chitinases from *Paecilomyces variotii* DG-3, *chi32* and *chi46*, can be enhanced by Co^{2+} (Nguyen et al. 2009). In contrast, the activity of chitinase from *Chaetomium cupreum* was inhibited by Co^{2+} and Zn^{2+} and enhanced by Mg^{2+} , K^{2+} , and Ba^{2+} (Wang and Yang 2009). The activity of an *Enterobacter aerogenes* chitinase was activated by Ba^{2+} , Ca^{2+} , Mn^{2+} , and Zn^{2+} but was highly inhibited by Co^{2+} and Mg^{2+} (Tang et al. 2001). In the present study, the activity of LbCHI32 was strongly inhibited by Mn^{2+} and K^{+} , stimulated by Ba^{2+} and Cu^{2+} , and not affected by Co^{2+} , Na^{+} , Li^{+} , Mg^{2+} , and Zn^{2+} . These results indicate that the activity of chitinase can be strongly influenced (activated or inhibited) by metal ions; however, the same ion may have different effects on the activity of different chitinases. Therefore, it is important to determine the effects of metal ions on chitinase activity for the characterization of a particular chitinase.

In conclusion, a chitinase gene was cloned from *L. bicolor* and its chitinase activity was characterized. Expression of the *Lbchi32* gene in *P. pastoris* can provide valuable information on the development of a new, cost-effective technique for large-scale production of LbCHI32. In view of the need for safer and environmentally friendly biocontrol tools, the recombinant chitinase LbCHI32 produced in *P. pastoris* has the potential to be used agriculturally in the biocontrol of fungal plant pathogens.

Acknowledgments This work has been supported by Heilongjiang Province International Scientific and Technological Cooperation Project (WB07N02) and Natural Science Foundation of Heilongjiang Province (QC07C56).

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