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ORIGINAL ARTICLE

β-1,3-Glucanase Inhibits Activity of the Killer Toxin Produced by the Marine-Derived Yeast *Williopsis saturnus* WC91-2

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Abstract The marine-derived *Williopsis saturnus* WC91-2 was found to produce very high killer toxin activity against the pathogenic yeast *Metschnikowia bicuspidata* WCY isolated from the diseased crab. It is interesting to observe that the purified β-1,3-glucanase from *W. saturnus* WC91-2 had no killer toxin activity but could inhibit activity of the WC91-2 toxin produced by the same yeast. In contrast, the WC91-2 toxin produced had no β-1,3-glucanase activity. We found that the mechanisms of the inhibition may be that the β-1,3-glucanase competed for binding to β-1,3-glucan on the sensitive yeast cell wall with the WC91-2 toxin, causing decrease in the amount of the WC91-2 toxin bound to β-1,3-glucan on the sensitive yeast cell wall and the activity of the WC91-2 toxin against the sensitive yeast cells. In order to make *W. saturnus* WC91-2 produce high activity of the WC91-2 toxin against the yeast disease in crab, it is necessary to delete the gene encoding β-1,3-glucanase.

Keywords Killer toxin · β-1,3-Glucanase · Marine yeast · Pathogenic yeast in crab · Laminarin

Introduction

It has been well known that most of the diseases in marine animals are caused by marine bacteria and marine viruses (Zhu et al. 2006). However, in recent years, the studies have shown that some marine yeasts are also pathogenic to some marine animals (Gatesoupe 2007). For example, the pathogenic agent for the milky disease in crab is the yeast *Metschnikowia bicuspidata* WCY (Xu et al. 2003; Wang et al. 2007a), and the milky disease has caused large economic losses in maricultural industry in some regions of China. The yeast *Torulopsis mogii* is a pathogen to some shrimp (Sun and Sun 1998). *M. bicuspidata* var. *bicuspidata*, a pathogenic yeast of aquatic invertebrates, is capable of infecting aquaculture-reared and disease-free *Artemia* (Moore and Strom 2003). Much research has shown that killer yeasts can be applied to control the growth of pathogenic yeasts in humans, animals, and plants. Therefore, we are trying to find marine yeasts which can produce high level of killer toxin against the infection of the crab by the pathogenic yeast in the future in order to reduce the economic losses in maricultural industry in China. Killer toxins produced by some yeast strains are low molecular mass proteins or glycoprotein toxins which kill sensitive cells of the same or related yeast genera without direct cell–cell contact (Magliani et al. 1997). To date, the toxin-producing killer yeasts have been identified in the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Ustilago*, *Torulopsis*, *Williopsis*, and *Zygosaccharomyces*, indicating that the killer phenomenon is indeed widespread among yeasts (Magliani et al. 1997). Analyzing the mechanisms of killer toxins can also provide important information for combating yeast infections caused by certain human pathogenic strains of the yeasts *Candida albicans* and/or *Sporothrix schenkii* (Comitini et al. 2004). It is generally regarded that the mechanisms of action of killer toxins are binding of killer toxin to cell wall, the formation of transmembrane channels, ion leakage, arrest of cell division, interference with the synthesis of glucan in the cell wall, and cell death (Magliani et al.

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1997). However, it is very important to find the killer toxins that only interfere with the biosynthesis of glucan in the cell wall of the pathogenic yeasts because this kind of killer toxin is not toxic to animal and human cells which do not have cell wall. We screened multiple yeast strains from seawater, sediments, mud of salterns, guts of marine fish, and marine algae for killer activity against the yeast *M. bicuspidata* WCY (pathogenic to crab *Portunus trituberculatus*) and found that *Williopsis saturnus* WC91-2, *Pichia guilliermondii* GZ1, *Pichia anomala* YF07b, *Debaryomyces hansenii* hcx-1, and *Aureobasidium pullulans* HN2.3 could secrete toxin onto the medium and kill the pathogenic yeast (Wang et al. 2008). We found that *W. saturnus* WC91-2 has much higher killing activity and wider spectrum of killing activity than *P. anomala* YF07b and other yeasts obtained (Wang et al. 2008). In our previous studies (Wang et al. 2007a, b, c), it was found that the purified killer toxin from *P. anomala* YF07b has both β-1,3-glucanase activity and killer toxin activity. In this study, the purified β-1,3-glucanase from *W. saturnus* WC91-2 was found to have no killer toxin activity but could inhibit activity of the killer toxin produced by the same yeast. In contrast, the WC91-2 toxin produced by it had no β-1,3-glucanase activity. At the same time, the possible mechanisms of the inhibition are also investigated in the present study.

Materials and Methods

Yeast Strains The yeast strains employed in the present study were the marine-derived *W. saturnus* WC91-2 which can produce high level of killer toxin and *M. bicuspidata*-sensitive strain WCY which is the pathogenic yeast in crab (Wang et al. 2007a; Wang et al. 2008).

Media The cell growth medium was yeast extract–peptone–dextrose (YPD) medium containing 2.0% glucose, 2.0% peptone, and 1.0% yeast extract. The assay medium for killer toxin and its action was composed of 1.0% yeast extract, 2.0% peptone, 2.0% glucose, 1.5 mg methane blue per 100 ml, and 2.5–3.5% agar adjusted to pH 4.5 with 50.0 mM Na_2HPO_4–citric acid buffer (Wang et al. 2007a). The medium for β-1,3-glucanase and killer toxin production consisted of 1.0% yeast extract, 2.0% peptone, 2.0% glucose, 2.0% NaCl, and 15% glycerol adjusted to pH 4.5 with 50.0 mM Na_2HPO_4–citric acid buffer. The medium for killing the protoplasts of the pathogenic yeast consisted of 1.0% yeast extract, 2.0% peptone, 2.0% glucose, 1.0 M sorbitol, and 2.0% agar, adjusted to pH 4.5 with 50.0 mM Na_2HPO_4–citric acid buffer.

Production of β-1,3-Glucanase and Killer Toxin The killer yeast was cultivated for 3 days at 22°C in 500 ml Erlenmeyer flasks with 150 ml of the production medium. The cultures were incubated in a rotary bed shaker (130 rpm). After centrifugation (5,000×*g*, 10 min, 4°C), the supernatant of the yeast culture was thoroughly mixed with glycerol (the final glycerol concentration was 15 g/100 ml), and the mixture was concentrated to a volume of 15 ml by ultrafiltration with a 5-kDa cutoff™ membrane with a Labscale TFF System (Millipore). These partially purified concentrated supernatants were used as the WC91-2 toxin concentrates or the crude β-1,3-glucanase preparation (Wang et al. 2008).

Determination of β-1,3-Glucanase Activity The reaction mixture containing 250 μl of 0.25% (*w*/*v*) laminarin (purchased from Sigma) in 50.0 mM acetate buffer (pH 4.5) and 20 μl of the crude β-1,3-glucanase preparation or the purified β-1,3-glucanase described below was incubated at 30°C for 1 h. The reaction was stopped immediately by heating at 100°C for 10 min. The reducing sugar in the mixture was determined by the method of Nelson–Somogyi (Spiro 1966). One unit of β-1,3-glucanase activity was defined as the amount of enzyme causing release of reducing sugars equivalent to 1.0 mg glucose from laminarin in 30 min under the assay conditions. Protein concentration was measured by the method of Bradford, and bovine serum albumin served as standard (Bradford 1976).

Measurement of Killer Toxin Activity We assayed the WC91-2 toxin activity with a diffusion test, using 6-mm-diameter sterile Oxford cups (6×10 mm) which were put on the assay medium seeded with the sensitive yeast strain WCY. Finally, 200 μl of WC91-2 toxin concentrates or the purified killer toxin was added to each cup and incubated at 24°C for 72 h, and the diameter of the inhibition zone was used as a measure of the yeast killer activity, and killer toxin activity was expressed in arbitrary units (AU). One AU is defined as the amount of protein resulting in an inhibition zone with a 1 mm diameter (Santos et al. 2000). Protein concentration was measured as described above.

Purification of β-1,3-Glucanase and WC91-2 Toxin The crude β-1,3-glucanase preparation was applied to Sephadex™ G-75 column (Pharmacia 2.5×100 cm), and the column was eluted with 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5) by using ÄKTA™ prime with Hitrap™ (Amersham, Biosciences, Sweden). At a flow rate of 0.5 ml/min, 2.0-ml fractions were collected. The β-1,3-glucanase activity-positive fractions were combined and applied to diethylaminoethyl (DEAE) Sepharose fast flow anion-exchange column (2.5×30 cm) that had been equilibrated with 20.0 mM Na_2HPO_4–citric acid buffer (pH 4.5). The bound proteins were then eluted with a linear gradient of

NaCl solution in the range of 0–1.0 M in the equilibrating buffer. The active fractions were concentrated by filtration through an AmiconYM3 (M_W cutoff 10,000) membrane.

The WC91-2 toxins were applied to DEAE Sepharose fast flow anion-exchange column (2.5×30 cm) to which the killer protein did not bind when equilibrated with 20.0 mM Na_2HPO_4–citric acid buffer (pH 4.5). The eluate was collected and applied to Sephadex™ G-50 column (2.5×100 cm). The column was eluted with 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5). The active fractions were applied to CM Sepharose™ fast flow equilibrated with 20.0 mM Na_2HPO_4–citric acid buffer (pH 4.5). The killer toxin-positive fractions were concentrated by filtration through an AmiconYM3 (M_W cutoff 3,000) membrane.

SDS Polyacrylamide Gel Electrophoresis The purity and molecular mass of the β-1,3-glucanase and the WC91-2 toxin in the concentrated fractions showing the activities were analyzed, respectively, in noncontinuous denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) according to the instructions offered by the manufacturer with a two dimensional electrophoresis system (Amersham, Biosciences, Sweden), and the gels were stained by Coomassie brilliant blue R-250 (Varghese and Diwan 1983). The molecular mass standards for SDS-PAGE comprised β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa), restriction endonuclease Bsp981 (25 kDa), trypsin inhibitor (20.1 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

Laminarin Hydrolysis The reaction mixture containing 20 μl of 100 U/ml of the purified β-1,3-glucanase or 20 μl of 2,000 AU/ml of the purified WC91-2 toxin and 100 μl of 0.25% laminarin in 50.0 mM acetate buffer (pH 4.5) was incubated at 30°C for 1 h. After that, the mixture was inactivated by heating at 100°C for 10 min immediately. The end products of hydrolysis were determined by thin-layer chromatography (Gong et al. 2007). The reaction mixtures in which the purified β-1,3-glucanase or the purified WC91-2 toxin was inactivated prior to addition by heating at 100°C for 15 min were used as controls.

Effects of β-1,3-Glucanase on Activity of the Purified WC91-2 Toxin One point 5 μg of the purified WC91-2 toxin was mixed with various concentrations of the purified β-1,3-glucanase, and the final volume was adjusted to 200 μl with 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5). After incubation at room temperature for 30 min, the activity of the WC91-2 toxin in the mixture was measured using the diffusion test method. The mixture without addition of the purified β-1,3-glucanase was used as a control.

Binding of the Purified WC91-2 Toxin and β-1,3-Glucanase to the Sensitive Yeast Cells The cells of the yeast strain WCY grown to the logarithmic phase in YPD medium were collected and washed with sterile distilled water by centrifugation at 4,000×*g* for 5 min. About 10^7 of the washed cells was incubated with 1.5 μg of the purified WC91-2 toxin in 250.0 μl of 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5) at 20°C for 3 h with shaking. After that, the cells in the mixture were removed by centrifugation at 14,000×*g* for 30 s. The activity of the WC91-2 toxin remaining in the supernatant was measured using the diffusion test method. The mixture without addition of the sensitive yeast cells was used as a control.

About 10^7 of the washed cells of the yeast strain WCY was incubated with 6.0 μg of the purified β-1,3-glucanase in 200 μl of 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5) at 20°C for 3 h with shaking. After that, the cells in the mixture were removed by centrifugation at 14,000×*g* for 30 s. The supernatant obtained was mixed with 1.5 μg of the purified WC91-2 toxin, and the final volume was adjusted to 200.0 μl with 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5). The mixture was incubated at room temperature for 30 min. The activity of the WC91-2 toxin remaining in the supernatant was measured using the diffusion test method. The mixture without addition of the sensitive yeast cells was used as a control.

Binding of the Purified WC91-2 Toxin to Laminarin One point 5 μg of the purified WC91-2 toxin were mixed with 4.0 mg of laminarin, and the final volume was adjusted to 200.0 μl with 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5). After incubation at 20°C for 3 h, the activity of the WC91-2 toxin in the mixture was measured using the diffusion test method. The mixture without addition of laminarin was used as a control.

Preparation of the Yeast Protoplasts and Killing of the Protoplasts The protoplasts of the yeast strain WCY were prepared by treatment of the washed cells with Zymolyase 20T in SE buffer (50.0 mM Na_2HPO_4/NaH_2PO_4, 10.0 mM ethylenediaminetetraacetic acid, 10.0 mM 2-mercaptoethanol, pH 7.6) containing 1.0 M sorbitol for 30 min at 30°C. The complete formation of the protoplasts was observed and checked under the microscope (Olympus CX21FS1, Olympus Corporation, Tokyo, Japan). The protoplasts were collected and washed with 50.0 mM Na_2HPO_4–citric acid buffer (pH 4.5) containing 1.0 M sorbitol by centrifugation at 5,000×*g* and 4°C for 5 min. Then, the washed protoplasts were seeded on the medium for killing of protoplasts. The activity of the WC91-2 toxin

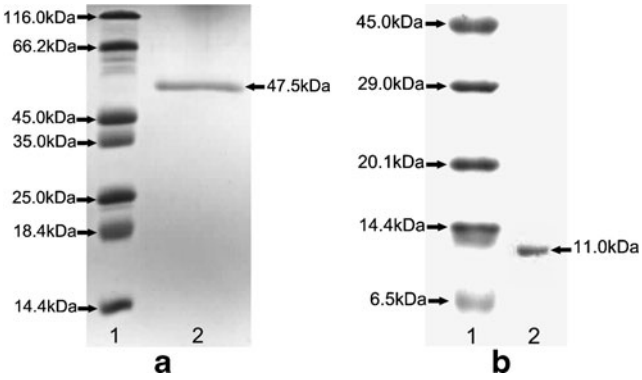

Fig. 1 SDS-PAGE analysis of the purified β-1,3-glucanase (**a**) and WC91-2 toxin (**b**). *Lane 1* in **a**: standard protein markers; *lane 2* in **a**: the purified β-1, 3-glucanase; *lane 1* in **b**: standard protein markers; *lane 2* in **b**: the purified WC91-2 toxin

was measured using the diffusion test method. The plate with the sterile Oxford cup of addition of 200 μl of sterile water was used as the control. Finally, the inhibition zones around the Oxford cups were observed and photographed.

Scanning Electron Microscopy The washed cells of the yeast strain WCY were seeded on the assay medium. After incubation at 24°C for 12 h, the sterile Oxford cup was put on the medium, and 200 μl of the purified WC91-2 toxin was added to the cup and incubated at 24°C for 5 h. The cells around the Oxford cup were collected and used as the sample for the scanning electron microscopy. The scanning electron microscope (JSM-840, JEOL, Japan) was used.

Results and Discussion

Purification of β-1,3-Glucanase and Killer Toxin After purification of β-1,3-glucanase from the concentrated supernatant of the yeast strain WC91-2, the purity and molecular mass of the β-1,3-glucanase in the concentrated fractions showing the activity were analyzed in noncontinuous denaturing SDS-PAGE (Laemmli 1970). The results in Fig. 1a indicated that the molecular mass of the purified β-1,3-glucanase was estimated to be 47.5 kDa. The optimal pH and temperature of the purified enzyme were 4.0 and 40°C, respectively (data not shown). However, in our previous study (Wang et al. 2007b), the molecular mass of the purified β-1,3-glucanase from the supernatant of a marine yeast *P. anomala* YF07b is 47.0 kDa, and the optimal pH and temperature of the enzyme are 4.5 and 40°C, respectively. This meant that size of the purified β-1,3-glucanase produced by the yeast strain WC91-2 was similar to that of the purified β-1,3-glucanase produced by *P. anomala* YF07b.

After purification of the WC91-2 toxin from the concentrated supernatant of the yeast strain WC91-2, the purity and molecular mass of the WC91-2 toxin in the concentrated fractions showing the activity were also analyzed in noncontinuous denaturing SDS-PAGE (Laemmli 1970). The results in Fig. 1b indicated that the molecular mass of the purified WC91-2 toxin was estimated to be 11.0 kDa. HM-1, a killer toxin produced by *Williopsis mrakii* is composed of 88 amino acids (10.7 kDa). In contrast, the active protein secreted by *W. saturnus* DBVPG 4561 migrates as a single band in SDS–PAGE and is characterized by a molecular weight of 62 kDa (Buzzini et al. 2004). A protein of 85 kDa with killer activity is secreted by *W. saturnus* var. *mrakii* MUCL 41968 (Guyard et al. 2002a). The mature killer toxin HM-1 deduced from the *HMK* gene in *W. mrakii* IFO 0895 and the mature killer toxin HYI deduced from the *HSK* gene in *W. saturnus* var. *saturnus* IFO 0117 are composed of 88 and 87 amino acid residues, respectively (Kimura et al. 1995). This suggested that the WC91-2 toxin produced by the yeast strain WC91-2 was similar to that produced by *W. mrakii* IFO 0895.

Killer Activity and Laminarin Hydrolysis of the Purified β-1,3-Glucanase and WC91-2 Toxin In our previous study (Wang et al. 2007b), it was found that β-1,3-glucanase produced by *P. anomala* YF07b has both β-1,3-glucanase activity and killing activity against the same sensitive yeast strain used in this study. However, it could be noted that the purified β-1,3-glucanase from the culture of the yeast strain WC91-2 had no killing activity against the pathogenic yeast WCY (Fig. 2a) although it could actively hydrolyze laminarin, producing only monosaccharides (Fig. 3). In contrast, the purified WC91-2 toxin from the culture of the yeast strain WC91-2 had high killing activity against the pathogenic yeast WCY (Fig. 2b) although it could not hydrolyze laminarin (Fig. 3). This meant that the β-1,3-glucanase produced by *P. anomala* YF07b was greatly

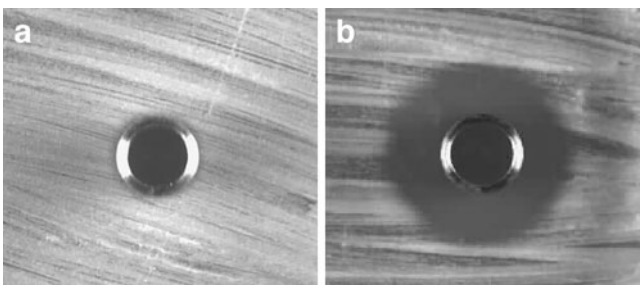

Fig. 2 Killer activity of the purified β-1,3-glucanase (**a**) and WC91-2 toxin (**b**). Two hundred microliters of the purified β-1,3-glucanase or the purified WC91-2 toxin was added to the Oxford cup, which was put on the assay medium seeded with the pathogenic yeast strain WCY and incubated at 15°C for 72 h, and the diameter of the inhibition zone was measured

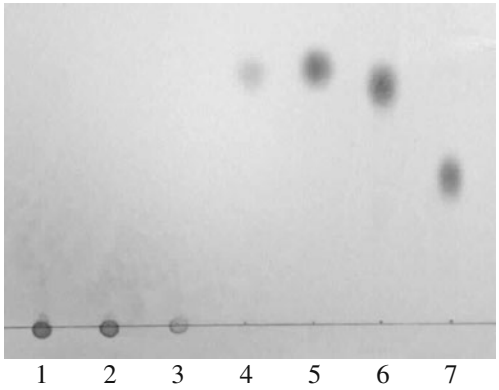

Fig. 3 Thin-layer chromatogram of the hydrolysis products of laminarin with the purified β-1,3-glucanase and WC91-2 toxin. *Lane 1*: control (laminarin + inactivated WC91-2 toxin by heating at 100°C for 10 min); *lane 2*: laminarin + WC91-2 toxin, incubated at 30°C for 1 h; *lane 3*: control (laminarin + inactivated β-1,3-glucanase by heating at 100°C for 10 min); *lane 4*: laminarin + β-1,3-glucanase, incubated at 30°C for 1 h; *lane 5*: glucose; *lane 6*: maltobiose; *lane 7*: D-raffinose

different from that produced by the yeast strain WC91-2 used in this study.

Inhibition of the WC91-2 Toxin by the β-1,3-Glucanase It has been confirmed that the crude WC91-2 toxin produced by the yeast strain WC91-2 has much higher killing activity against the pathogenic yeast strain WCY isolated from the diseased crab and wider spectrum of killing activity than that produced by *P. anomala* YF07b (Wang et al. 2008). So we thought that the purified β-1,3-glucanase and WC91-2 toxin may act synergistically in killing the sensitive yeast cells. However, unexpectedly, the results in Fig. 4 demonstrated that the size of inhibition zone of the WC91-2 toxin was continuously decreased from 15.0 to 0 mm as the amount of added β-1,3-glucanase was increased steadily from 0 to 6.0 μg in the mixture of the purified β-1,3-glucanase and WC91-2 toxin, indicating that the β-1,3-glucanase can inhibit the activity of the WC91-2 toxin. The possible reason for this phenomenon may be that the purified β-1,3-glucanase competed for binding to the same

Table 1 Binding of the purified WC91-2 toxin and β-1,3-glucanase to laminarin and the sensitive yeast cells

	Inhibition zone (mm)		
	Binding of the WC91-2 toxin to yeast cells of WCY	Binding of β-1,3-glucanase to yeast cells of WCY	Binding of the WC91-2 toxin to laminarin
Binding experiment	11±0.5	12±0.6	9±0.2
Control	13±0.8	10±0.4	11±0.3

Data are given as means ± SD, $n=3$

receptor on the sensitive yeast cells with the purified WC91-2 toxin. Because the β-1,3-glucanase had no killing activity against the sensitive yeast cells (Fig. 2a), less WC91-2 toxin in the mixture containing the β-1,3-glucanase could bind to the receptors which had been occupied by the β-1,3-glucanase so that activity of the WC91-2 toxin was decreased significantly. It has been reported that β-glucanase (Zymolyase) also exerts an antagonistic effect on WmKT activity produced by *W. saturnus* var. *mrakii* MUCL 41968 (Guyard et al. 2002b). Therefore, if both the purified WC91-2 toxin and β-1,3-glucanase can competitively bind to the receptor of the sensitive yeast cells, activity of the WC91-2 toxin must be reduced in the presence of β-1,3-glucanase.

Binding of the Purified WC91-2 Toxin to Laminarin and the Sensitive Yeast Cells It has been well documented that *Williopsis* killer systems interfere with the synthesis of β-1,3-D-glucan, the major cell wall polysaccharide polymer involved in determining cell morphology and in maintaining osmotic integrity. For example, HM-1 produced by *W. mrakii* kills susceptible strains by a unique mechanism, presumably involving interference with the synthesis of β-1,3-glucan, thus rendering the wall osmotically fragile or defective and ultimately resulting in lytic cell death (Magliani et al. 1997). It has been shown that laminarin (β-1,3-glucan) exists in the cell wall of most yeast cells

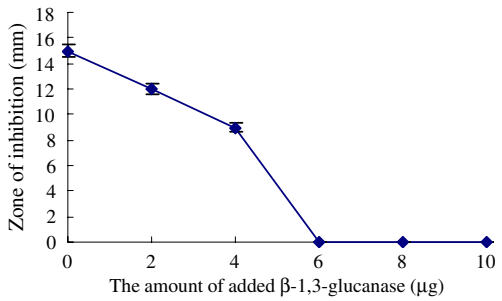

Fig. 4 Effects of the β-1,3-glucanase on activity of the WC91-2 toxin. Data are given as means ± SD, $n=3$

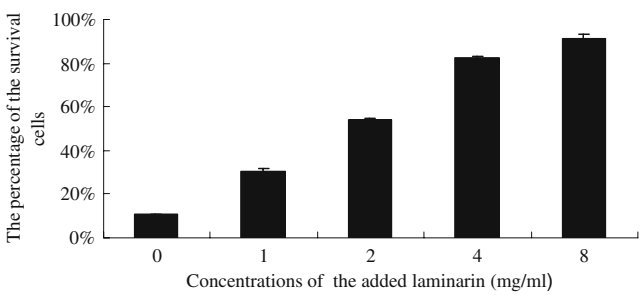

Fig. 5 Effects of laminarin on activity of the WC91-2 toxin against the sensitive yeast cells. Data are given as means ± SD, $n=3$

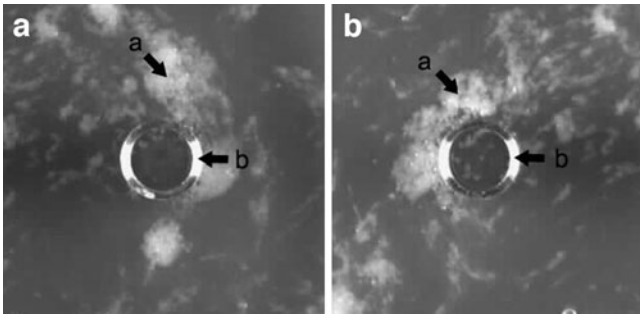

Fig. 6 Killing of the protoplasts of the sensitive yeast cells by the purified WC91-2 toxin. White a: The washed protoplasts of the yeast strain WCY were seeded on the medium for killing of protoplasts. The sterile Oxford cups were put on the medium, and 200 μl of the purified WC91-2 toxin was added to each cup and incubated at 24°C for 48 h. White b: The plate with the sterile Oxford cup of addition of 200 μl of sterile water was used as the control. Finally, the inhibition zones around the Oxford cups were observed and photographed. Black **a:** protoplasts; Black **b:** the Oxford cups

(Wang et al. 2007b; Santos et al. 2000; Izgu et al. 2006). In order to confirm that laminarin can be used as the receptor of the purified WC91-2 toxin, first, the purified WC91-2 toxin was incubated with laminarin. Then, activity of the WC91-2 toxin was measured. The results in Table 1 showed that the amount of the WC91-2 toxin remaining in the solution was decreased after the WC91-2 toxin was mixed with laminarin because the size of inhibition zone of the WC91-2 toxin in the solution was 9.0±0.2 mm while the size of inhibition zone of the WC91-2 toxin without the treatment using laminarin was 11.0±0.3 mm.

After the purified WC91-2 toxin was incubated with different concentrations of laminarin, the number of the viable cells was measured. We also found that the more the laminarin was added, the more the viable sensitive yeast cells were obtained (Fig. 5), indicating that the more the WC91-2 toxin was absorbed by laminarin, the less the sensitive yeast cells were killed. It has been regarded that the binding of killer toxin to the receptor (glucan) of the sensitive yeast cell wall is the first key step for action of the killer toxin (Magliani et al. 1997). For example, WmKT, the killer toxin produced by *W. saturnus* var. *mrakii* MUCL 41968 binds to the sensitive cells using surface-exposed β-glucans (Guyard et al. 2002b).

After the WC91-2 toxin was mixed with the sensitive yeast cells, the cells in the suspension were removed by centrifugation, and activity of the WC91-2 toxin in the supernatant obtained was determined. The results in Table 1 also revealed that the amount of the WC91-2 toxin remaining in the supernatant was decreased after the WC91-2 toxin was mixed with the sensitive yeast cells because the size of the inhibition zone of the WC91-2 toxin in the supernatant was 11±0.5 mm while the size of the inhibition zone of the WC91-2 toxin without the treatment using the sensitive yeast cells was 13±0.8 mm. Therefore, if the purified WC91-2 toxin binds to laminarin and the receptor (β-glucans) of the sensitive yeast cells, activity of the WC91-2 toxin must be reduced in the presence of laminarin and the sensitive yeast cells.

Binding of the Purified β-1,3-Glucanase to the Sensitive Yeast Cells After β-1,3-glucanase was absorbed by the sensitive yeast cells, the cells were removed by centrifugation. Then, the supernatant obtained was mixed with the purified WC91-2 toxin and activity of the WC91-2 toxin was measured. It could be seen from the results in Table 1 that the size of the inhibition zone of the WC91-2 toxin was 12±0.6 mm whereas the size of the inhibition zone of the WC91-2 toxin mixed with the supernatant in which β-1,3-glucanase was not absorbed by the sensitive yeast cells was 10±0.4 mm. This meant that β-1,3-glucanase indeed could be absorbed by the sensitive yeast cells, and this caused the reduced inhibition on activity of the WC91-2 toxin.

It has been reported that killer toxin produced by *Williopsis* spp. has many applications in pharmaceutical industries, fermentation industries, and biocontrol of post-harvest pathogens in agriculture (Wang et al. 2008; Magliani et al. 2002; Santos and Marquina 2004). In order to make *W. saturnus* WC91-2 produce higher activity of the killer toxin, it may be important to delete the gene encoding β-1,3-glucanase in *W. saturnus* WC91-2. This work is being undertaken in this laboratory.

The Activity of the WC91-2 Toxin Against the Protoplasts of the Sensitive Yeast Cells It has been reported that HM-1 produced by *W. mrakii* kills intact cells but not protoplasts (Magliani et al. 1997). The results in Fig. 6 demonstrated

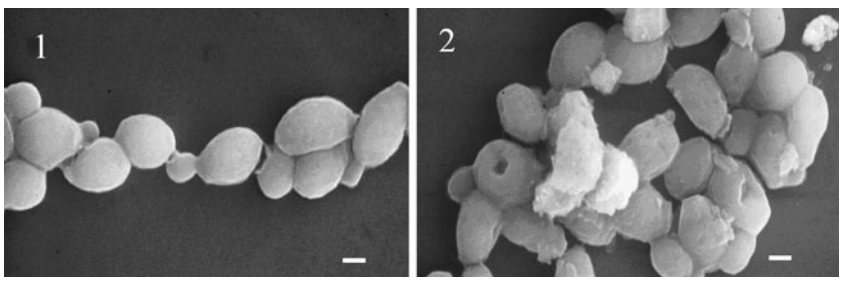

Fig. 7 Micrographs of the sensitive yeast cells *1* untreated with the purified WC91-2 toxin and those *2* treated with the purified WC91-2 toxin (*bar*=1.0 μm)

that the purified WC91-2 toxin from the yeast strain WC91-2 could not kill the protoplasts of the sensitive yeast cells either. This may imply that the binding receptor of the WC91-2 toxin indeed exists in the cell wall of the sensitive yeast. Guyard et al (2002b) also observed that the spheroplasts derived from the WmKT-sensitive yeast cells were resistant to WmKT and cell wall β-glucans are required for WmKT lethal effect.

The Scanning Electron Microscopy During the sensitive yeast cells were treated by the purified WC91-2 toxin, the cells were sampled and observed under the scanning electron microscope. It could be clearly seen from the results in Fig. 7 that after the sensitive yeast cells were exposed to the purified WC91-2 toxin, the invaginations of the cell wall occurred, the cell walls were destroyed, and integrity of the cell wall disappeared. This again confirmed that the killer toxin produced by *W. saturnus* WC91-2 acted on the cell wall of the sensitive yeast cells, causing defective integrity of the cell wall and cell death. Guyard et al. (2002b) also observed that WmKT treatment of *P. anomala* 41969 led to a rough cell surface, indicating that WmKT produced by *W. saturnus* var. *mrakii* MUCL 41968 has wall-degrading activity. They thought that the rough cell surface was the result of partial degradation of the cell wall β-glucans.

Therefore, the killer toxin produced by *W. saturnus* WC91-2 could be easily and safely applied to biocontrol growth of the pathogenic yeasts in animals and human and was not toxic to animals and human because animals and human cells did not contain cell wall.

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