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Purification, characterization and gene cloning of the killer toxin produced by the marine-derived yeast *Williopsis saturnus* WC91-2

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ABSTRACT

As the killer toxin produced by *Williopsis saturnus* WC91-2 could kill many sensitive yeast strains, including the pathogenic ones, the extracellular killer toxin in the supernatant of cell culture of the marine yeast strain was purified and characterized. The molecular mass of the purified killer toxin was estimated to be 11.0 kDa according to the data from SDS-PAGE. The purified killer toxin had killing activity, but could not hydrolyze laminarin. The optimal conditions for action of the purified killer toxin against the pathogenic yeast *Metschnikowia bicuspidate* WCY were the assay medium with 10% NaCl, pH 3–3.5 and temperature 16 °C. The gene encoding the killer toxin from the marine killer yeast WC91-2 was cloned and the ORF of the gene was 378 bp. The deduced protein from the cloned gene encoding the killer toxin had 125 amino acids with calculated molecular weight of 11.6 kDa. It was also found that the N-terminal amino acid sequence of the purified killer toxin had the same corresponding sequence deduced from the cloned killer toxin gene in this marine yeast, confirming that the purified killer toxin was indeed encoded by the cloned gene.

1. Introduction

In recent years, some yeast strains isolated from marine environments have been found to be able to cause diseases in marine animals, especially in immunocompromised animals or the animals in the stressed environments. For example, some *Candida* spp., *Metschnikowia bicuspidate*, *Cryptococcus* spp., *Sporobolomyces salmonicolor*, and *Trichosporon* sp. are the pathogens of amago (*Oncorhynchus rhodurus*), Chinook salmon (*Oncorhynchus tshawytscha*), the githead seabream (*Sparus aurata*), crab (*Portunus trituberculatus*), and teach (*Tinca tinca*), respectively (Gatesoupe 2007; Wang et al. 2007; Chi et al. 2010). Indeed, many marine yeast strains such as *Williopsis saturnus* WC91-2, *Pichia guilliermondii* GZ1, *Pichia anomala* YF07b, *Debaryomyces hansenii* hcx-1, *Aureobasidium pullulans* HN2.3, *Mrakia frigida* 2E00797, *Kluyveromyces siamensis* HN12-1 and *Pichia anomala* HN1-2 isolated from different marine environments also can produce killer toxin (low molecular mass proteins or glycoprotein toxins) against the eukaryotic pathogens (Wang et al. 2008; Hua et al. 2010; Buzdar et al. 2011; Sun et al. 2011).

Analyzing the killing mechanisms of killer toxins can provide important information for combating the yeast infections (Comitini et al. 2004; Schmitt and Breinig 2006). Although it is thought that the mechanisms of action of killer toxins produced by some yeasts 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. 2008), the detailed mechanisms of action of the killer toxins produced by some marine yeasts are still unknown (Hua et al. 2010; Buzdar et al. 2011). Therefore, it is very important to purify and characterize the killer toxins produced by the marine yeasts. In our previous study (Wang et al. 2008; Peng et al. 2010), it was found that the marine-derived yeast *W. saturnus* WC91-2 could produce high level of killer toxin against the pathogenic yeast *M. bicuspidata* WCY, the agent for the milky disease in crab (Wang et al. 2007). However, the killer toxin produced by *W. saturnus* WC91-2 was still not characterized. So, in the present study, the killer toxin produced by *W. saturnus* WC91-2 was purified and characterized.

2. Experimental

2.1. Yeast strains

The killer yeast employed in the present study was the marine-derived *W. saturnus* WC91-2 which can produce high level of killer toxin and the potential sensitive yeast strains used in this study included *M. bicuspidata* sensitive strain WCY which is the pathogenic yeast in crab (Wang et al. 2007, 2008), *Cryptococcus aureus*, *Lodderomyces elongisporus*, *Candida albicans*, *Yarrowia lipolytica* and *Rhodotorula mucilaginosa* isolated from different

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marine environments, and *Saccharomyces* sp. W0, which is a high ethanol-producing yeast (Chi and Arneborg 2000).

2.2. Media

The cell growth medium was 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 mmol/l Na_2HPO_4–citric acid buffer (Wang et al. 2007). The medium for 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 mmol/l Na_2HPO_4–citric acid buffer.

2.3. Production of killer toxin

The killer yeast was cultivated for 3 days at 22 °C in 250-ml Erlenmeyer flasks with 50 ml of the production medium. The cultures were aerobically incubated by shaking (180 rpm). After centrifugation (5000 × *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 15.0 ml by ultrafiltration with a 5-kDa cutoff™ membrane with a Labscale TFF System (Millipore). These partially concentrated supernatants were used as the killer toxin concentrates.

2.4. Measurement of killer toxin activity

The killing activity of the killer toxin was measured with a diffusion test, using 6-mm diameter sterile Oxford cups (6 mm × 10 mm) which were put on the assay medium seeded with the sensitive yeast strain WCY. Finally, 200 μl of the killer toxin concentrates or the purified killer toxin as described below was added to each cup and incubated at 16 °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 1.0 mm diameter (Santos et al. 2000). The purified killer toxin which was heated at 100 °C for 10 min was used as the control. Protein concentration was measured by the method of Bradford, and bovine serum albumin served as standard (Bradford 1976).

2.5. Purification of the killer toxin

The killer toxin concentrates were applied to DEAE Sepharose fast flow anion-exchange column (2.5 cm × 30 cm) to which the killer protein did not bind when equilibrated with 20.0 mmol/l Na_2HPO_4–citric acid buffer (pH 4.5). The eluate was collected and applied to Sephadex™ G-50 gel filtration chromatography column (2.5 cm × 100 cm). The column was eluted with 50.0 mmol/l Na_2HPO_4–citric acid buffer (pH 4.5). The active fractions were applied to CM Sepharose™ fast flow cation-exchange column equilibrated with 20.0 mmol/l Na_2HPO_4–citric acid buffer (pH 4.5). The killer toxin positive fractions were concentrated by filtration through an AmiconYM3 (MW cutoff 3000) membrane.

2.6. SDS-PAGE

The purity and molecular mass of the killer toxin in the concentrated fractions showing the killing activity were analyzed, respectively, in non-continuous 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 ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

2.7. Determination of N-terminal amino acid sequence of the purified killer toxin

To determine the N-terminal amino acid sequence, the band of the purified killer toxin on SDS-PAGE as described above was electroblotted to a polyvinlymethylformamide (PVDF) membrane (Bio-Rad) with a CAPS (3-cyclohexylamino]-1-propanesulfonic acid) buffer system. Electroblotting was conducted at 200 mA for 1 h. The membrane was stained with Ponceau S to visualize the protein. The band was excised and submitted for N-terminal amino acid sequencing with an ABI PROCISE™ 492cLC protein sequencer.

2.8. Assay of killing activity spectra of the purified killer toxin

The pathogenic yeast strain WCY and possible susceptible yeasts grown in the YPD medium were seeded on the assay medium plates. Two hundred microliters of the purified killer toxin was added to the sterile cups which had been put on the plates and incubated at 16 °C for 96 h and the diameter of the clear zone formed was measured. The purified killer toxin which was heated at 100 °C for 10 min was used as the control.

2.9. Effects of temperature, pH and NaCl on killer toxin activity

The optimal temperature for activity of the killer toxin was determined at temperatures of 16, 20, 25, 28 and 37 °C for 2–3 days on the assay medium plates as described above. Temperature stability of the purified killer toxin was tested by pre-incubating the killer toxin at different temperatures ranging from 20 to 70 °C in the water bath for 1 h and residual activity was measured as described above immediately. Here, the relative killer toxin activity of the pre-incubated sample at 4 °C was regarded as 100%.

The effect of pH on the killer toxin activity was determined by incubating the purified killer toxin on the assay medium plates (buffered with 20.0 mmol/l Na_2HPO_4–citric acid) with pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 using the methods described above. pH stability was tested via 24-h pre-incubation of the purified killer toxin in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3.0 to 7.0 and temperature of 4 °C. The remaining activities of the killer toxin were measured immediately after this treatment with the standard method as mentioned above.

The optimal NaCl concentrations for killing activity of the purified killer toxin were determined at NaCl concentrations from 0% to 10.0%.

2.10. Cloning of the gene encoding the killer toxin in W. saturnus WC91-2

Genomic DNA from *W. saturnus* WC91-2 was isolated according to the method described by Adams et al. (1998). To isolate the full length of the gene encoding the killer toxin from *W. saturnus* WC91-2, one set of the primers (the forward primer P1 was 5′-ATGAAATTTTCCTTCGTTTACGG-3′ and the reverse primer was P2: 5′-CTATTCCACACGTCTGTAAGAGCG-3′) designed based on the sequence information of the killer toxin gene (accession number: D13445.1) in *W. saturnus* var. *mrakii* using the software Primer5.0 were used for polymerase chain reaction (PCR) with the genomic DNA of *W. saturnus* WC91-2 as template. The reaction system (50.0 μl) was composed of 5.0 μl of 10× Taq buffer, 4.0 μl