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SCIENCE DIRECT
Enzyme and Microbial Technology 39 (2006) 669–676
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Killer toxin of *Pichia anomala* NCYC 432; purification, characterization and its *exo*-β-1,3-glucanase activity

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Received 24 September 2005; received in revised form 20 November 2005; accepted 29 November 2005

Abstract

Pichia anomala NCYC 432 secretes a killer toxin which is inhibitory to a variety of yeasts including pathogenic *Candida* spp. The killer toxin in the culture supernatant was concentrated by ultrafiltration and purified to homogenity by two successive gel filtration chromatographies with a TSK G2000SW column. Biochemical characterization of the toxin showed that it is a glycosylated protein with a molecular mass of 47 kDa and p*I* values of 3.4 and 3.7. The toxin showed high stability at pH values between 3 and 5.5 and up to 37 °C. Its N-terminal amino acid sequencing yielded the sequence GDYWDYQNDKIR which is 100% identical with that of mature *exo*-β-1,3-glucanase (accession no. AJ222862) of *P. anomala* strain K. The toxin displayed high activity against laminarin thus showing a β-glucanase activity. The Michaelis constants K_m and V_{max} for laminarin hydrolysis were 0.3 mg ml^{-1} and 350 μmol min^{-1} mg^{-1}.

Keywords: Killer yeast; *Pichia anomala*; Killer toxin; *exo*-β-1,3-Glucanase

1. Introduction

Yeast strains with killer phenotype (K+) produce extra cellular protein toxins called killer proteins or killer toxins which are lethal to sensitive microbial cells [1,2]. Killer phenomenon is widespread among many yeast genera such as *Saccharomyces, Candida*, *Hansenula*, *Pichia*, *Debaryomyces*, *Ustilago*, *Cryptococcus*, *Metschnikowia*, *Williopsis*, *Kluyveromyces* and *Zygosaccharomyces* [3–6]. Toxin production confers considerable advantage to the yeast strains in competing with sensitive strains for nutrients available in their environment [7].

The genes responsible for killer phenotype are either carried on extra chromosomal elements in the form of dsRNA [2] or ds linear DNA [8,9] or a chromosome [10,11].

Killer yeast strains are immune to their own toxins due to a mechanism called self-immunity but can be sensitive to the toxins of others. In the past years killer yeasts were classified into 11 distinct (K1–K11) groups based on the killing and immunity reactions among them [3,12].

Killer toxins kill the sensitive cells by different mechanisms. They hydrolyze [13] or inhibit the synthesis [14] of the major cell wall component β-1,3-glucans or cause ion leakage by ion channel formation on cytoplasmic membrane [15]. In some cases, toxin blocks both the DNA synthesis and budding cycle [16] or arrest the cells in G1 phase of the cell cycle [17].

Several potential applications for the killer yeasts and their toxins have been studied and suggested. In fermentation industries starter strains with killer character can be used to combat undesirable contaminating yeasts [18,19]. Killer strains are considered useful in biological control of spoilage yeasts in the preservation of food [20] and in the biotyping of medically important pathogenic yeasts [21] and bacteria [22]. Also killer toxins have been proposed as novel antimicrobial agents in the treatment of human and animal infections [23–26].

There are several reports in the literature of wide range intergeneric killing spectrum of *pichia* toxins and their relative high stability in comparison to toxins of other killer yeasts [27–29]. Among the species with killer character, *Pichia anomala*, NCYC 432 was extensively studied for various potential applications mentioned above [30–37].

Here in, we describe the characterization of *P. anomala* NCYC 432 killer protein.

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doi:10.1016/j.enzmictec.2005.11.024

2. Materials and methods

2.1. Yeast strains and cultivation conditions

The killer strain *P. anomala* NCYC 432 and killer toxin sensitive *S. cerevisiae* NCYC 1006 were provided from National Collection of Yeast Cultures (NCYC) Norwich, UK. Pathogenic yeasts tested for sensitivity to purified toxin are listed in Table 2. All yeast strains were routinely maintained at 4 °C on YEPD agar, comprising, 1% (w/v) Bacto-yeast extract (Difco), 1% (w/v) Bacto-peptone (Difco), 2% (w/v) dextrose (Merck), and 2% (w/v) Bacto-agar (Difco) at pH 5.5. For killer-activity determination it was buffered to pH 3.0–6.0 at intervals of 0.5 pH units with 100 mM citrate–phosphate buffer. *P. anomola* cells in liquid culture were cultivated in YEPD broth supplemented with 5% (v/v) glycerol and buffered to pH 4.5 with 100 mM citrate–phosphate buffer. For pH and temperature stability tests of the toxin, *S. cerevisiae* cells were grown in unbuffered YEPD.

2.2. Toxin activity assays

Agar diffusion assay: Toxin activity at various stages of the study was tested according to Brown et al. [38]. Samples of 20 μl were spotted onto YEPD plate (pH 4.5 at 22 °C) seeded with toxin sensitive or a pathogenic test strain. Killer activity was determined by measuring the growth inhibition zones of the seeded strain. Killer toxin which gave a clear inhibition zone of 10 mm in diameter was defined as 1 arbitrary unit (AU).

Microtitre plate assay: pH and temperature stability of the purified toxin were tested as previously described [39]. One hundred micro liter protein sample (0.05 mg ml^{-1}) or 100 μl control solution were added into the wells of a microtitre plate containing 100 μl of toxin sensitive strain (1×10^5 cells ml^{-1}) in YEPD. Optical density at 600 nm was measured before and after incubation of the plates at 22 °C for 24 h using an automatic plate reader (Spectramax 190, Molecular Devices, USA). Toxin activity was expressed as the percentage reduction in growth of the sensitive strain with respect to a toxin-free control.

2.3. Determination of protein content

Protein concentration was estimated according to the method of Bradford [40] using bovine serum albumin as standard.

2.4. Preparation and concentration of crude killer protein

P. anomala NCYC 432 was cultivated in 1 l YEPD broth at 20 °C with shaking at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick Scientific, USA). When the cells reached to stationary phase they were removed by centrifugation at 5000 rpm for 10 min at 4 °C and the culture supernatant was filtered through 0.45 and 0.2 μm cellulose acetate membranes respectively (Sartorius AG, Germany). The filtrate was concentrated 40-fold by using a centrifugal ultrafiltration device with a molecular weight cutoff 30 kDa (Vivaspin, Sartorius AG) and stored in 20% (v/v) glycerol at −20 °C until required.

2.5. Purification of killer protein

Toxin purification was done on a fully automated HPLC system BioCAD 700E (Perseptive Biosystems, USA) which included an Advantec model SF2120 fraction collector (Advantec MFS, Japan). Detections were done with UV absorbance at 280 nm at an ambient temperature of 20 °C. The crude protein obtained from the previous step was buffer exchanged to 100 mM Na_2HPO_4–citric acid buffer, pH 4.5 containing 100 mM Na_2SO_4 using 5 kDa cutoff centrifugal ultrafilter and 90 μl of the protein sample (2.6 mg ml^{-1}) was loaded onto a TSK G2000SW column, 7.5 mm *D* × 300 mm *L* (TosoHaas, Japan) which was pre-equilibrated with the sample buffer. Elution was done with the same buffer at a flow rate of 1 ml min^{-1}. Fractions of 1 ml volume were collected and assayed for killer activity. The active fraction from different runs pooled and re-chromotographed under the same conditions then concentrated and buffer exchanged to the same elution buffer containing 20% (v/v) glycerol but the salt and stored at −20 °C for further analysis. All the chemicals were HPLC grade and obtained either from Fluka or Merck, Germany.

2.6. SDS polyacrylamide gel electrophoresis

Purified toxin was subjected to acetone precipitation and resuspended in 125 mM Tris-Cl, pH 6.8. Protein sample was heated at 100 °C for 5 min in equal volume of sample buffer (20% (v/v) glycerol, 4% (v/w) SDS, 0.02% (v/w) bromophenol blue and 10% (v/v) 2-β-mercaptoethanol pH 6.8) and then electrophoresed on a 5–20% linear gradient, 7.5 mm thick SDS polyacrylamide gel in a discontinues buffer system as described by Laemmli [41] using a slab gel electrophoresis unit SE 600 (Hoefer, USA). For non-denaturing conditions, reducing agent was left out from the standard Laemmli protocol [42] and the samples were electrophoresed on a linear 15% polyacrylamide gel. The gels were visualized by silver staining [43] and for molecular weight determination it was scanned by GT 9500 color image scanner (Epson, Japan) then the data were processed with gel work 1D intermediate software (UVP Products, UK). (Molecular weight markers were obtained from Roche Diagnostics, Germany; Acrylamide, bisacrylamide, sodium dodecyl sulphate TEMED and ammonium persulphate were obtained from Pharmacia Biotech., Sweden; β-mercaptoethanol was from Sigma, USA; Coomassie blue R250 was from ICN Biochemical's, USA; and all the other chemicals were from Merck).

2.7. NH_2-terminal amino acid sequencing

The protein band recognized by Coomassie-blue R-250 staining was cut out from SDS gel. Amino acid sequence analysis of the protein was done by the custom service Eurosequence b.v. (Groningen, The Netherlands). After isolation from the gel followed by sample cleanup the protein was submitted to Edman degradation [44] using an automated sequenator (Model 494 Procise, Applied Biosystems, USA). FASTA [45] program was used to do a homology search of protein database NCBI/BLASS Swiss-prot.

2.8. Glycoconjugate detection

The killer protein was run on a 15% SDS polyacrylamide gel and then transferred onto a nitrocellulose membrane (0.2 μm) using a tank buffer system (TE 62 X Transphor II electrophoresis unit; Hoefer) as previously described by Towbin et al. [46] Glycodetection of the immobilized protein on the membrane was done with a Dig-Glycan detection kit according to the instructions of the manufacturer (Roche Diagnostics).

2.9. Isoelectric focusing

Acetone precipitated killer protein was resuspended in dd H_2O and mixed with an equal volume of sample loading solution (15% (v/v) glycerol and 2.4% (w/v) pH 3.0–10 ampholytes). Electrofocusing was done on a prefocused pH 3–10 gradient native 5.5% polyacrylamide gel (0.35 mm thick) containing 2.4% (w/v) ampholytes by a high voltage vertical slab polyacrylamide gel system with a Hoefer SE 600 electrophoresis unit as previously described by Giulian et al. [47]. The gel was stained with Coomassie brilliant blue R250 [47] and the p*I* value was measured using GelWorks 1D Intermediate Software (ampholytes and p*I* markers were from Pharmacia Biotech.).

2.10. pH and temperature stability

Killer protein was precipitated with acetone and resuspended in 100 mM Na_2HPO_4–citric acid buffer at various pHs (2.5–7.0, in 0.5 pH unit intervals). After incubation of the samples at 4 °C for 18 h, toxin activity was measured against a toxin-sensitive strain using a microtitre plate assay. The percentage reduction in growth in each well was determined with respect to toxin-free buffer control of equivalent pHs. For temperature stability, killer protein samples in 100 mM Na_2HPO_4–citric acid buffer, pH 4.5 were exposed to 4, 18, 25, 30, 37, 50, 70 and 100 °C for 1 h and toxin activity was measured against a toxin-sensitive strain using a microtitre plate assay.

2.11. exo-β-1,3-Glucanase assay

Glucanase activity was determined as described by Bara et al. [48]. Purified toxin (20 μl) was mixed with 250 μl of 50 mM sodium acetate buffer (pH 4.5) containing 0.25% laminarin (from *Laminaria digitata*, Sigma: l-9634). After 1 h incubation at 30 °C, the reaction was stopped by boiling for 5 min. Glucose formation was measured with Glucose HK kit (Sigma) according to the instructions of the manufacturer. One unit (U) of β-1,3-glucanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min. Michaelis–Menten constants (K_m and V_{max}) were determined from Lineweaver–Burk representation of data obtained by measuring the initial rate of laminarin hydrolysis under the assay conditions described above and using a range of 25–175 μg ml^{-1}.

3. Results

3.1. Production and purification of the killer toxin

P. anomala cells were grown in YEPD broth (pH 4.5 and 20–22 °C) supplemented with glycerol as a toxin stabilizer [49] and then the cell-free culture liquid was concentrated by ultrafiltration and enriched for killer toxin by 4.3-fold. Crude toxin samples were put through gel filtration chromatography using a TSK G2000SW column, the fraction at 9 ml indicated by arrow showed killer toxin activity (Fig. 1A). The active fraction from different runs pooled and reinjected onto the same column in order to purify the fraction further and the killing activity was tested by an agar diffusion assay (Fig. 1B). The purity of the toxin was confirmed by non-denaturing SDS-PAGE [50] (Fig. 2). A final purification of 120-fold was achieved and the toxin had a specific activity of 1800 AU mg^{-1}. The yield of killer toxin was 0.5 mg from 1 l of culture filtrate. A summary of the purification steps is shown in Table 1. SDS-PAGE of the sample collected after these purification procedures showed a single protein band with an apparent molecular mass of 47,000 Da (Fig. 3)

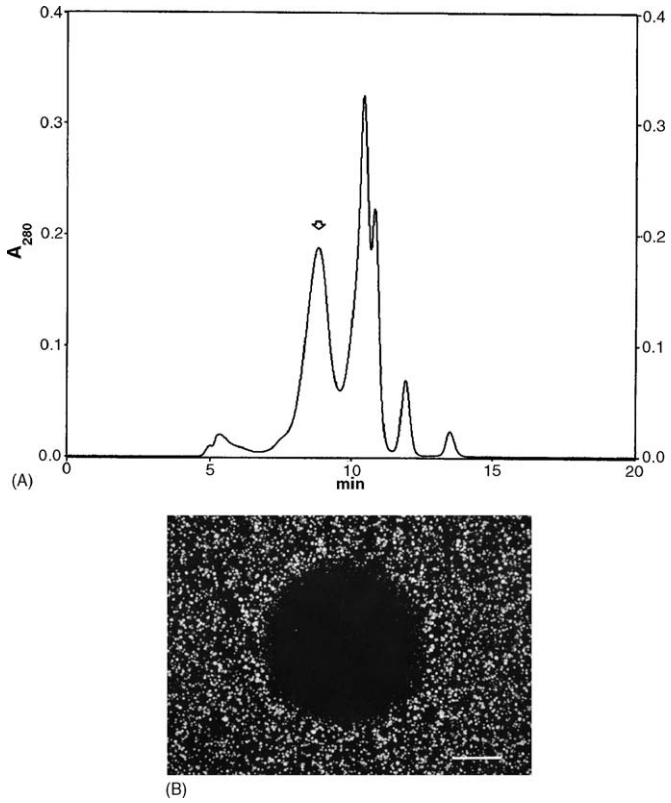

Fig. 1. (A) Elution profile of *P. anomala* NCYC 432 killer toxin on a TSK G2000SW column. Concentrated culture supernatant of *P. anomala* NCYC 432 was applied to a gel-filtration column. Column size: 7.5 mm $D \times 300$ mm L; sample: 90 μl (2.6 mg ml^{-1}); elution buffer: 100 mM Na_2HPO_4–citric acid pH 4.5 + 100 mM Na_2SO_4; flow rate: 1 ml min^{-1}; detection: UV (280 nm); fraction volume: 1000 μl. Fraction indicated by arrow, eluted at 9 ml showed killer toxin activity. (B) Killer activity of the purified toxin. Twenty micro liter of the toxin (50 μg ml^{-1}) obtained after second gel filtration chromatography was spotted onto a YEPD plate seeded with toxin sensitive *S. cerevisiae* cells. Killer toxin which gave a clear inhibition zone of 10 mm in diameter was defined as 1 arbitrary unit (AU). Bar: 5 mm.

3.2. N-terminal amino acid sequence and properties of the toxin

The sequence of the N-terminal 12 amino acid residues of the toxin was determined by Edman degradation and it gave a sequence of GDYWDYQNDKIR. This sequence exactly matched to the deduced amino acid sequence which starts just after the predicted cleavage site of signal peptide of the *exo*-β-1,3-glucanase precursor of *P. anomala* strain K (AJ222862) [51]. The result of the enzyme-immuno assay revealed that the toxin carries a polysaccharide moiety since there was a positive signal on the membrane in the position corresponding to the protein (Fig. 4). On a pH 3–10 gradient native polyacrylamide gel the toxin showed two spaced bands with apparent p*I*'s of 3.4 and 3.7, respectively (Fig. 5).

3.3. pH and thermo stability

The effects of pH and temperature on the toxin activity were found by using a microtitre assay. Stability of the killer toxin was higher at acidic pHs, with the optimal pH at 4.5. Even at pH 2.5 it retained 75% of its activity. Whereas when maintained

Table 1
Purification of the killer toxin

Step	Volume (ml)	Protein		Activity of killer toxin		Total purification (fold)	Total recovery (%)
		(mg ml^{-1})	(mg)	(AU)	(AU mg^{-1})		
Cell-free culture liquid	1000	0.4	400	6000	15	1	100
Concentrated culture liquid	25	2.6	65	4200	64.6	4.3	70
First TSK G2000SW	12	0.12	1.44	2100	1458	97.2	35
Second TSK G2000SW	10	0.05	0.5	900	1800	120	15

1 AU is defined as the toxin concentration that gives a clear inhibition zone of 10 mm in diameter.

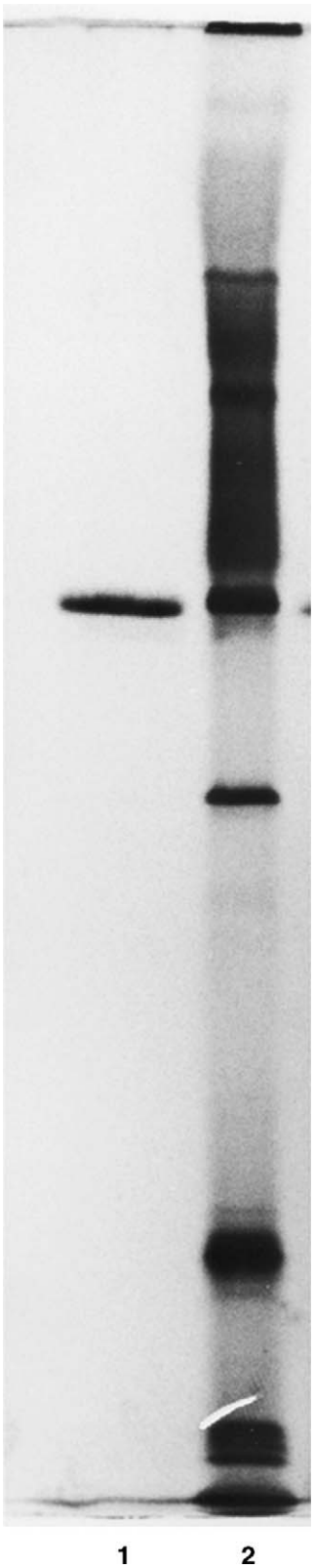

Fig. 2. Non-reducing SDS-PAGE of the purified toxin. Lane 1: purified toxin (2 μg), Lane 2: concentrated culture liquid (15 μg).

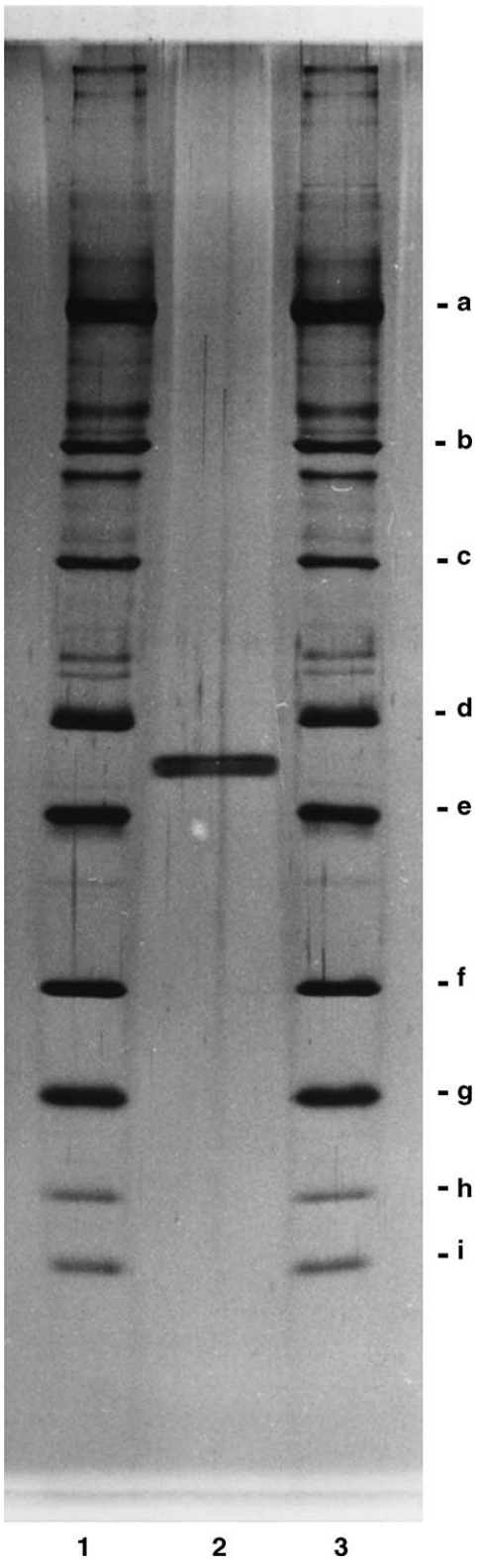

Fig. 3. SDS-PAGE (5–20% gradient) of the purified toxin. Lanes 1 and 3 are molecular mass markers (Da): (a) α_2-macroglobulin 170,000; (b) β-galactosidase 116,353; (c) fructose-6-phosphate kinase 85,204; (d) glutamate dehydrogenase 55,562; (e) aldolase 39,212; (f) triose phosphate isomerase 26,626; (g) trypsin-inhibitor 20,100; (h) lysozyme 14,307; (i) aprotinin 6,500. Lane 2 is purified toxin (2 μg).

Fig. 4. Glycoconjugate detection of the purified toxin blotted onto nitrocellulose membrane. Lane 1 is the positive control transferrin, Lane 2 is the negative control creatinase and Lane 3, purified toxin (2 μg).

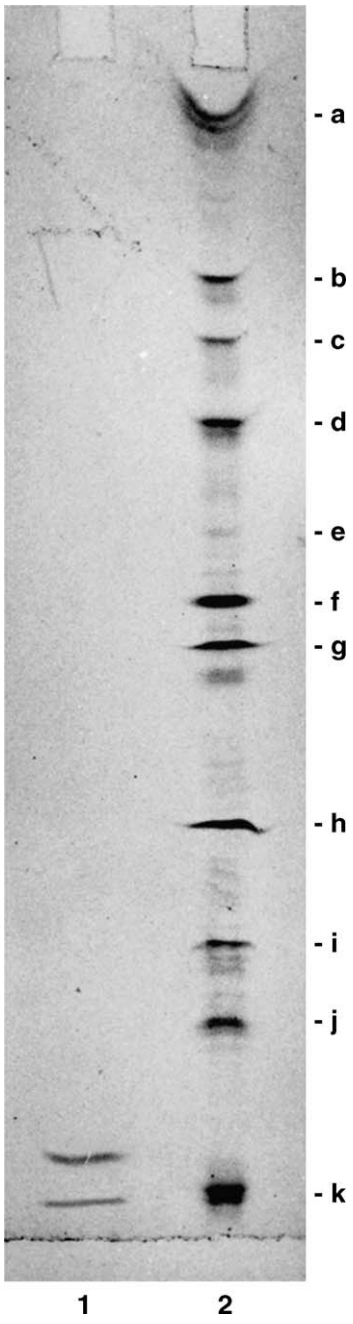

Fig. 5. Polyacrylamide gel electrofocusing of the toxin in native state. Lane 1 is killer toxin (2 μg) and Lane 2 is p*I* markers: (a) trypsinogen 9.3; (b) lentil lectin-basic band 8.65; (c) lentil lectin-middle band 8.45; (d) lentil lectin-acidic band 8.15; (e) myoglobin-basic band 7.35; (f) myoglobin-acidic band 6.85; (g) human carbonic anhydrase 6.55; (h) bovine carbonic anhydrase 5.85; (i) β-lactoglobulin A 5.2; (j) soya bean trypsin inhibitor 4.55; (k) amyloglucosidase 3.50.

at pH 6.0, the toxin lost 60% of its activity and it was almost completely inactive at pH 7.0 (Fig. 6A). The toxin at pH 4.5 was incubated at various temperatures between 4 and 100 °C for 1 h. There was no significant loss of activity up to 37 °C and only 50% activity loss was observed at 100 °C (Fig. 6B).

3.4. β-1,3-Glucanase assay

Purified toxin showed *exo*-glucanase activity on laminarin and had a specific activity of 50 U mg^{-1} K_m and V_{max} values of the purified toxin were determined as 0, 3 mg ml^{-1} and 350 μmol min^{-1} mg^{-1}.

3.5. Anti-Candidal activity of the toxin

Clinically significant *Candida* spp. were tested for their susceptibility to the purified toxin by a killer zone assay in a plate test. All the strains (13 in toto) were susceptible to the 20 μl toxin solution (0.05 mg ml^{-1}) at various degrees as summarized in Table 2.

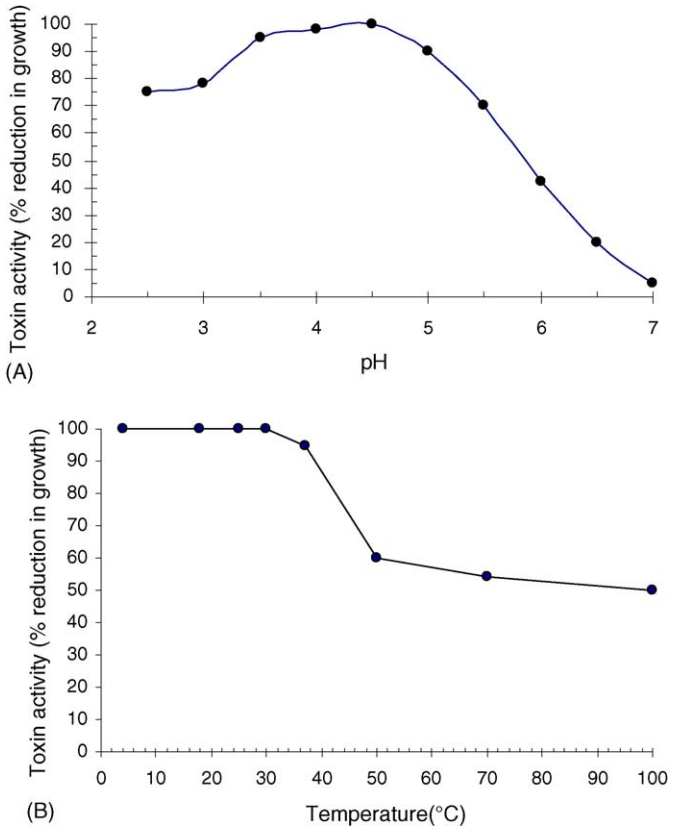

Fig. 6. Effect of pH (A) and temperature (B) on activity and stability of the toxin. At indicated pHs or temperatures toxin activity was expressed as the percent reduction in growth of the toxin sensitive *S. cerevisiae* cells with respect to a toxin free control. Values represent the mean of three separate determinations. Standard deviations were less than 2% for both experiments.

Table 2
Human pathogenic *Candida* spp. tested for sensitivity to the purified killer toxin

Species	Strain number/ source[a]	Isolation	Toxin sensitivity[b] (mm; growth inhibition)
C. albicans	ATCC 10231	Bronchi	19
	ATCC 14053	Blood	16
	ATCC 22972	Sputum	14
	ATCC 24433	Nail	14
	ATCC 36802	Blood	16
	ATCC 90028	Blood	20
	Clinical isolate (F+)	Bronchi	15
	Clinical isolate (F+)	Bronchi	14
C. glabrata	ATCC 90030	Blood	20
C. guilliermondii	Clinical isolate	Sputum	10
C. parapsilosis	ATCC 90018	Blood	11
C. pseudotropicalis	Clinical isolate	Sputum	14
C. tropicalis	Clinical isolate	Abscess	18

[a] ATCC, American Type Culture Collection, Rockville, MD, USA; Clinical isolates were obtained from GATA (Gülhane Military Medical Academy), Ankara, Turkey; F+, flucanazole resistance.
[b] Values represent the mean of three seperate determinations.

4. Discussion

Previously, *P. anomala* NCYC 432 killer toxin has been found inhibitory to a wide-range of pathogenic fungi. In those studies the growth inhibitory effect of the killer toxin was tested either by spotting the NCYC 432 cell suspensions or the crude toxin preparations onto the plates seeded with the test strains [30–37]. Although suggested as a potential antimicrobial agent in biomedicine and in the preservation of foods, no biochemical characterization of *P. anomala* NCYC 432 killer toxin had been achieved so far and its mode of action remained to be explored. In this study, for the first time we have purified and studied the characteristics of this toxin.

The purified toxin was homogenous on SDS-PAGE under both non-reduced and reduced conditions but split into two bands on a native IEF gel. The doublet formation could be explained by a charge difference in the protein induced by post-translational modifications.

The N-terminal sequence of this polypeptide toxin shared complete homology with the deduced amino acid sequence which starts just after the predicted KEX2-type endoproteinase processing site of the *exo*-β-1,3-glucanase precursor of *P. anoma*la strain K [51]. This also revealed that the mature *exo*-β-1,3-glucanase of *P. anomala* strain K which is a glycoprotein with a predicted mass of 45.7 kDa and a p*I* value of 4.7, has the same N-terminal amino acid sequence with *P. anomala* NCYC 432 killer toxin. This homology and in vitro glucanase assay of *P. anomala* NCYC 432 killer toxin that resulted in the hydrolysis of laminarin in an *exo*-like fashion strongly suggested that, *exo*-β-1,3-glucanase activity is involved in the killing action of this toxin. When *P. anomala* NCYC 432 cells were cultivated in YNBG growth medium (yeast nitrogen base without amino acids) which is free of glucans both the glucanase and killer activity were not detected in the spent medium (data not shown). It is known that the production of glucanases by the cells is dependent on the presence of glucans in the culture medium. The lack of killer activity in the absence of glucanase activity also showed the correlation between the glucanase and the killer activity of this toxin. The cytocidal activity of the purified toxin on several human pathogenic *Candida* spp. further supported such an activity since the cell walls of the tested strains are predominantly composed of β-1,3-glucans [32,52]. It was shown that *P. anomala* strains produce antifungal metabolites like ethyl acetate and ethanol [53]. Sensitivity of the *Candida* spp. to the purified killer toxin also proved that the growth inhibitory activity of *P. anomala* NCYC 432 cells on pathogenic strains was due to the toxin action but not to its metabolites.

β-1,3-Glucanase activity has been also reported for the killer toxins of *Willopsis saturnus* var. *mrakii* MUCL 41968 (85 kDa, glycosylated) [54], *Kluyveromyces phaffii* DBVPG 6076 (33 kDa, glycosylated) [55] and *P. anomala* NCYC 434 (49 kDa, p*I* 3.7, glycosylated) [13].

To date several *exo*-β-1,3-glucanases have been purified and suggested as potential antifungals. When compared with some well characterized ones, the apparent K_m (0.3 mg ml^{-1}) of the *P. anomala* NCYC 432 toxin was lower than that of *C. albicans* (3.9 mg ml^{-1}) [56], *Trichoderma harzianum* (2.1 mg ml^{-1}) [57] and *T. harzianum* TC (1.72 mg ml^{-1}) [58] but was slightly higher than those reported for *T. viride* (0.12 mg ml^{-1}) [59], *T. asperellum* (0.087 mg ml^{-1}) [48] and *T. harzianum* TY (0.1 mg ml^{-1}) [60].

The high *exo*-β-1,3-glucanase activity and reasonable pH and thermo stability of the *P. anomala* NCYC 432 killer toxin highlight its potential use against human and animal fungal infections and as a biocontrol agent in food industry. The results reported here concerning the characteristics of *P. anomala* NCYC 432 killer toxin also will be of help in evaluating the killing mechanisms of the yeasts.

Acknowledgements

We thank Drs. H. Bak and J. Wichert from Eurosequence b.v. (Groningen, the Netherlands) for performing amino acid sequence analysis. This work was supported by a grant (AFP 2002-07-02-00-01) from the institute of Natural and Applied Sciences, Middle East Technical University, Ankara, Turkey.

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