Isolation and Characterization of the K5-Type Yeast Killer Protein and Its Homology with an Exo-β-1,3-glucanase

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K5-type yeast killer protein in the culture supernatant of Pichia anomala NCYC 434 cells was concentrated by ultrafiltration and purified to homogeneity by ion-exchange chromatography with a POROS HQ/M column followed by gel filtration with a TSK G2000SW column. The protein migrated as a single band on discontinuous gradient SDS-PAGE and had a molecular mass of 49,000 Da. The pI value of the K5-type killer protein was measured at pH 3.7 by high voltage vertical gel electrofocusing. The result of an enzyme immuno assay revealed that it was a glycosylated protein. Its internal amino acid sequencing yielded the sequences LNDFWQQGYHNL, IPIGYWAFQLLDNDPY, and YGGSDYGDVVIGIELL, which are 100% identical to exo-β-1,3-glucanase (accession no. AJ222862) of Pichia anomala (strain K). The purified protein was highly stable at pH values between 3 and 5.5 and temperatures up to 37°C.

Key words: killer yeast; Pichia anomala; K5 type killer protein; exo-β-1,3-glucanase

The killer phenotype, which was first discovered by Bevan and Makower\(^1\) in Saccharomyces cerevisiae, is widely distributed among yeast.\(^2\)–\(^4\) Yeast strains with killer phenotype (K\(^+\)) produce and excrete into the environment proteins or glycoproteins that are lethal to sensitive microbial cells.\(^5\),\(^6\) Thus these proteins are designated as killer proteins or killer toxins.\(^7\),\(^8\) Young & Yagiu\(^9\) have investigated the killer strains of the genera Saccharomyces, Candida, Hansenula, Kluyveromyces, and Pichia, and on the basis of killing and immunity cross-reactions among them classified the killers into ten typical (K1–K10) types to which another type (K11) was added later.\(^10\) In recent years several new killer yeast strains have been reported and their toxins characterized but they are not yet classified according to the above criteria.\(^11\)–\(^18\)

The genomes of the toxins have been mapped either on extra-chromosomal elements in the form of double-stranded RNA virus-like particles\(^8,19,20\) or double-stranded linear DNA plasmids\(^21,22\) or a chromosome.\(^15,23\) Killer toxins also have different killing mechanisms on sensitive cells. They disrupt cytoplasmic membrane function by ion channel formation\(^23\) or interfere with cell wall synthesis by inhibiting β-1,3-glucan synthase.\(^24\) In some cases toxin blocks both the DNA synthesis and the budding cycle\(^25\) or arrest cell division at G1 phase.\(^26\)

Killer toxin production confers an advantage on strains in competing with sensitive cells for nutrients available in their environment.\(^3\) Various potential applications have been studied and suggested for killer yeasts and their toxins. They can be used to eliminate undesirable contaminating yeasts during fermentations by conferring the killer character on starter strains\(^28\)–\(^30\) and can be considered as bio-control agents in the preservation of foods.\(^30\) In the medical field, the killer character can be a useful means of bio-typing the pathogenic yeasts\(^31\) and bacteria\(^32\) and the killer toxins have been suggested as potential antimicrobial agents in the treatment of infections.\(^33,34\) Although there is considerable amount of published information concerning the applications of yeast killer toxins, among the 11 typical types, only K1,\(^7\) K2\(^3\) and K6\(^37\) have been characterized. Herein we describe the purification and characterization of the K5 type yeast killer protein produced by the standard K5 killer type strain of Pichia anomala.\(^11,38\)

Materials and Methods

Yeast strains and culture media. Pichia anomala from the National Collection of Yeast Cultures, UK (NCYC 434, K5) was used as the source of K5 type killer protein and Saccharomyces cerevisiae NCYC 1006 was used as the killer-toxin-sensitive strain. Strains were routinely maintained on YEPD agar, comprising 1% (w/v) Bacto-yeast extract (Difco), 2% (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 grown either in YEPD or YNBG comprising 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and 2% (w/v) glucose (Merck).

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They were both supplemented with 5% (v/v) glycerol (Merck) 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.

Measurement of optimum culture conditions for killer toxin production. Optimum conditions for killer toxin production were found by the killer zone assay in a plate test as previously described[39] at various pHs covering the range 3.0–6.0, in intervals of 0.5 pH units and at 18–30°C in 2°C intervals. YEPD plates seeded with killer-toxin sensitive cells (1 x 10⁵ cells/ml) were inoculated with a 50 µl distilled water suspension of killer yeast cells. The toxin production was determined by examining the growth inhibition zones of the seeded strain surrounding the growth of the killer yeast strain after 48 h of incubation.

Protein determination. Protein concentration was estimated spectrophotometrically (model 1208, Shimadzu, Japan) with UV absorbance at 280 nm according to Warburg & Christian.[40]

Assessment of toxin activity. Agar diffusion assay. After each purification step, toxin activity was tested according to Brown et al.[41] Samples of 50 µl were spotted onto a YEPD plate (pH 4.5 at 22°C) seeded with a toxin-sensitive strain. The killer activity was measured by the occurrence of the clear zone of growth inhibition of the seeded killer-toxin sensitive strain. Killer toxin which gave a clear 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 according to Hodgson et al.[42] A hundred-microliter protein sample (100 µg/ml) or 100-µl control solution were added into the wells of a microtitre plate containing 100 µl of toxin-sensitive strain (1 x 10⁵ cells/ml in YEPD) and OD₆₀₀ was measured using an automatic plate reader (Spectramax 190, Molecular Devices, USA) before and after incubation of the plate at 22°C for 20 h. Toxin activity was expressed as the percentage reduction in growth of the sensitive strain with respect to a toxin-free control.

Crude toxin preparation. P. anomalaNCYC 434 (K5) cells were grown to stationary phase in 1 liter of YEPD medium at 20°C at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick Scientific, USA). The cells were removed by centrifugation (KR 22i, Jouan, France) at 5000 rpm for 10 min at 4°C and filtered through 0.45 µm and 0.2 µm cellulose acetate membranes respectively (Sartorius AG, Germany). The filtrate was further concentrated 50-fold by using a centrifugal 30 kDa-cutoff ultrafilter (Vivaspin, Sartorius AG) and stored in 20% v/v glycerol at −20°C.

Toxin purification. Toxin purification steps were done on a fully automated HPLC system BioCAD 700E (Perceptive 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.

Anion exchange chromatography. The crude protein obtained from the previous step was buffer exchanged to 30 mM N-methylpiperazine–HCl using 5 kDa-cutoff centrifugal ultrafilter (Vivaspin, Sartorius) and 750 µl of the sample (71 mg/ml) was applied to an anion exchange column (POROS HQ/M 4.6 mmD/100 mmL, Perseptive Biosystems). The column was washed to twenty column volumes (CV) with 30 mM N-methylpiperazine–HCl (pH 4.8) and elution was done with a linear gradient of 0 to 500 mM NaCl in the same buffer in 20 CV at a flow rate of 10 ml/min. The eluted fractions (1600 µl) were buffer-exchanged to 100 mM Na₂HPO₄–citric acid buffer, pH 4.5 using a 5 kDa-cutoff ultrafilter and assayed for killer activity. The active eluate was pooled, concentrated, and then subjected to gel permeation chromatography.

Gel permeation chromatography. Ninety microliters of the protein sample (4.6 mg/ml) was put on a TSK G2000SW column, 7.5 mmD/300 mmL (TosoHaas, Japan) equilibrated with 100 mM Na₂HPO₄–citric acid buffer, pH 4.5, containing 100 mM Na₂SO₄. Elution was done with the same buffer at a flow rate of 1 ml/min. The eluted fractions (1300 µl) were collected and assayed for killer activity. The active eluate was 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.

SDS and Native PAGE. Killer protein sample was heated at 100°C for 5 min in equal volume of sample buffer (125 mM Tris-Cl, 20% (v/v) glycerol, 4% (w/v) SDS, 0.02% (w/v) bromophenol blue and 10% (v/v) 2-mercaptoethanol, pH 6.8) and then electrophoresed on a 5–20% linear gradient SDS polyacrylamide gel in a discontinuous buffer system as described by Laemmli[43] using a dual vertical-slab gel electrophoresis unit (SE 600 Hoefer, USA). The electrophoresis was done at 15 mA/0.75 mm gel at 15°C. For non-denaturing conditions, SDS, reducing agent and the sample heating step were left out from the standard Laemmli protocol[44] and the samples were electrophoresed on a 15% gel. The gels were either visualized by Coomassie brilliant blue R-250[45] or with silver staining.[46] For the molecular weight determination the SDS gel was scanned using a GT9500 Colour Image Scanner (Epson, Japan) and the data were processed with GeoWorks 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 Biochemicals, USA; and all the other chemicals were from Merck).

Isoelectric focusing. The isoelectric point (pl) of the killer protein was measured by a high voltage vertical slab polyacrylamide gel electrophoresis system with a Hoefer SE 600 electrophoresis unit as previously described by Giulian et al. Focusing was done on a pH 3–10 gradient native 5.5% polyacrylamide gel (0.35 mm thick) containing 2.4% (w/v) ampholytes. The gel was prefocused at 10°C for 10 minutes at 100 V/h and then loaded with the protein sample mixed with an equal volume of sample loading solution (15% (v/v) glycerol and 2.4% (w/v) pH 3.0–10 ampholytes). The gel was focused at 10°C for 90 min at 2000 V/h and then stained with Coomassie brilliant blue R250. The pl value was measured using GelWorks 1D Intermediate Software (UVP Products). (Ampholytes and pl markers were from Pharmacia Biotech.)

Glyco-conjugate detection. Killer protein along with glycosylated (transferrin) and non-glycosylated (creatinase) control proteins were 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. Electrophoretic transfer was done at a constant current of 1.0 A for 1 h at a coolent temperature of 10°C. After blotting, the membrane was stained with Ponceau S (Roche Diagnostics) to confirm an efficient transfer. Glycoconjugate detection of the immobilized proteins on the membrane was done with a Dig-Glycan detection kit according to the instructions of the manufacturer (Roche Diagnostics).

Internal amino acid sequence analysis. Killer protein was put through SDS-PAGE on a 12.5% polyacrylamide gel under denaturing conditions. Following electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and the protein band was cut out from the gel. Internal amino acid sequence analysis of the protein was done by the custom service, Eurosequence b.v. (Groningen, The Netherlands). The Coomassie-blue stained protein band was digested in situ with trypsin and the extracted peptides were separated by reversed phase HPLC (Model 1100, Agilent, USA). The amino acid sequence of the selected peaks were analyzed by Edman degradation with an automated sequenator (Model 494 Procise, Applied Biosystem, USA). FASTA and FASTB programs were used to do a homology search of protein databases; NCBI/BLAST Swiss-Prot and NCBI/BLAST NR.

pH stability. Killer protein was precipitated in 2 volumes of acetone, pelleted by centrifugation, and suspended in 100 mM Na2HPO4-citric acid buffer at various pHs covering the range 2.5–7 in intervals of 0.5 pH units. 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.

Temperature stability. Killer protein samples in 100 mM Na2HPO4-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.

Results

Production of K5 type killer toxin

Toxin production by killer yeasts with a higher degree of activity depend on pH and temperature of the culture. To investigate the pH and temperature optima of cultivation for the production of K5 type toxin by P. anomala cells, a killer-zone assay was done at pHs 3.0–6.0 and at 18–30°C. Killer activity was manifested at every tested pH value and temperature with various degrees. The maximum activity was at pH 4.5 and at 20–22°C. For toxin production, P. anomala cells were grown in YEPD and YNBG under the same conditions with the addition of glycerol as a toxin stabilizer. Although YNBG medium which would facilitate the isolation of killer toxin by avoiding high molecular weight compounds supported the growth of yeast cells, toxin accumulation was only detected in the extracellular medium of YEPD.

Purification of the toxin

Cell-free culture liquid was concentrated by ultrafiltration and enriched for killer toxin by 7.2-fold. For ion exchange chromatography, pH-mapping experiments were initially done on anion and cation exchangers in the range 3.0–6.0 with increments of 0.2 pH units at which the toxin was produced in order to determine the optimal resolution and recovery. The toxin could not be resolved by cation exchanger at any pH. However resolution was achieved on a POROS HQ/M anion exchange column, pH 4.8 was determined to be optimal due to maximal resolution and killer toxin recovery. Among the tested fractions killer toxin was recovered in the fraction (indicated by arrow) corresponding to about 120 mM NaCl. No other killer activity was detected in the fractions eluted either before the gradient or after 400 mM NaCl (Fig. 1). The toxin was purified 280-fold in this step. The concentrated active fraction was then put through gel filtration chromatography using a TSK G 2000SW column. The fraction at 8.5 ml (indicated by arrow) showed killer-toxin activity (Fig. 2).

A final purification of 400-fold was achieved and the toxin had a specific activity of 200 U/mg and was homogenous on SDS-PAGE. The yield of K5 type killer toxin was 6 mg from 11 of culture filtrate. A summary of
Elution Profile of K5 Type Killer Toxin on a POROS HQ/M Column.

Concentrated culture supernatant of *P. anomala*NCYC 434 (K5) was applied to an anion exchange column. Column size: 4.6 mmD/100 mmL; sample: 750 μl (71 mg/ml); starting buffer: 30 mM N-methylpiperazine-HCl pH 4.5; gradient: 0–500 mM NaCl in the starting buffer in 20CV; flow rate: 10 ml/min; detection: UV (280 nm); fraction volume: 1600 μl. Fractions were tested for toxin activity against *S. cerevisiae*NCYC 1006 using an agar diffusion assay. Fraction indicated by arrow, corresponding to about 120 mM NaCl showed killer activity.

Elution Profile of K5 Type Toxin on a TSK G2000SW Column and Its Killer Activity against *S. cerevisiae*NCYC 1006.

(a) Pooled and concentrated active toxin fraction from anion exchange chromatography was applied to a gel-filtration column. Column size: 7.5 mmD/300 mmL; sample: 90 μl (4.6 mg/ml); elution buffer: 100 mM Na2HPO4-citric acid pH 4.5+ 100 mM Na2SO4; flow rate: 1 ml/min; detection: UV (280 nm); fraction volume: 1300 μl. Fraction indicated by arrow, eluted at 8.5 ml showed killer toxin activity. (b) Fifty microliter of the active fraction obtained from gel filtration chromatography gave a clear growth inhibition zone of ~7 mm in an agar diffusion assay which corresponds to 0.7 AU killer toxin (3.5 μg). Bar, 5 mm.
the purification steps is shown in Table 1. Subsequently native-PAGE profiles of extracellular proteins of YEPD and YNBG media were compared. Absence of a single band in the extract of YNBG, adjacent to the isolated protein, further confirmed the presence of a single type of killer protein in the culture medium and its purity (Fig. 3).

Characterization of the purified toxin

Molecular mass

On a discontinuous gradient SDS-PAGE with a wide range molecular mass markers the toxin moved in a single band in front of glutamate dehydrogenase (55,562 Da). The molecular mass of the toxin was estimated to be 49,000 Da (Fig. 4).

Isoelectric point

On a pH 3–10 gradient native polyacrylamide gel with broad range pH markers the toxin focused between soya bean trypsin inhibitor (pI 4.55) and amylglucosidase (pI 3.5). A pI value of 3.7 was decided for the toxin (Fig. 5).

Glycosylation

The result of the enzyme-immuno assay revealed that the toxin was a glycosylated protein since there was a positive signal on the membrane in the position corresponding to the toxin (Fig. 6).

Internal amino acid sequences

Tryptic peptides of the purified toxin were separated by reversed phase HPLC and the fractions 44 and 81 (Fig. 7) were collected and applied to an amino acid sequencer. Fraction 44 gave a sequence of LNDFWQQGYHNL and fraction 81 revealed two peptide sequences of IPITYWAYQQLDNDPY and YGGSDYGDVIGIELL. These internal amino acid sequences shared 100% homology with exo-β-1,3-
glucanase of *Pichia anomala* strain K (AJ222862; Grevesse et al.54) (Fig. 8).

**Stability**

The effects of pH and temperature on the toxin activity were found by using a microtitre assay. The toxin exhibited higher stability at lower pH values after incubation at 4°C for 18 h. There was no loss of toxin activity at pH 4.5; about 70% of the activity remained even at pH 2.5. However, toxin was readily inactivated at pH values above 6.5 (Fig. 9). The toxin at pH 4.5 was incubated at various temperatures between 4°C and 100°C for 1 h and no loss of activity was observed at temperatures up to 30°C. About 90% of the activity was...
retained at 37°C whereas at 100°C, half of the toxin activity was lost (Fig. 10).

**Discussion**

Characterization of the toxins will contribute to the understanding of differences in the killing activity of the strains. The purification of the K5 type yeast killer protein reported here under conditions that maintained the biological activity enabled its first characterization. This polypeptide toxin is larger (49 kDa) and more acidic (pI 3.7) than K1 (20.6 kDa, pI 4.34), K2; (21 kDa, pI 4.50) and K6 (42.3 kDa, pI 5.97) type toxins. Its internal amino acid sequences share complete homology with the exo-β-1,3-glucanase of *P. anomala* strain K which is a glycoprotein of 45.7 kDa with a pI of 4.7. This suggests that K5-type yeast killer toxin has an exo-β-1,3-glucanase activity. *P. anomala* NCYC 434 (K5) has a broad killing spectrum among fungi, with relatively higher growth inhibitory effect towards fungal cells with the cell walls predominantly composed of β-1,3-glucan (K5, K7) such as *Candida albicans*, *T. delbrueckii*, and *Kluyveromyces marxianus*. This also indicates the β-1,3-glucan residues of the cell wall as the target of the K5 type yeast killer toxin. Also killer toxin activity was only detectable when *P. anomala* NCYC 434 (K5) was grown in complex YEPD medium, not in YNB, which lacks yeast extract that contains a significant amount of cell wall β-D-glucans which — in turn — are known to induce exo-β-1,3-glucanases in yeasts. These findings reveal that the yeast killer toxin which was classified as the K5 type seems to be a rather potent exo-β-1,3-glucanase active against other fungi. The molecular mass and the pI value differences between the exo-β-1,3-glucanase of *P. anomala* strain K and the K5 type killer protein of *P. anomala* NCYC 434 may be due to the variations in the posttranslational modifications of these proteins as they are produced in different strains of the same species.

High stability of the K5 type toxin at pH values between 3–5.5 and temperatures up to 37°C and the broad killing-spectrum of *P. anomala* NCYC 434 (K5) highlight the potential use of this protein in environmental and industrial biotechnology as an antifungal agent.

This work and the studies on some other types of killer proteins which are underway in our laboratory will be of help in the classification of the killer yeasts according to the mode of action and biochemical properties of their toxins, and large scale purification of these toxins for industrial applications.

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