# Application of monoclonal antibodies to the isolation and characterization of a killer toxin secreted by *Hansenula mrakii*

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A strain of yeast, *Hansenula mrakii*, secretes a toxin that kills sensitive yeasts, such as *Saccharomyces cerevisiae*. Monoclonal antibodies raised against the toxin had both binding and neutralizing activities. The toxin in culture media was isolated by an affinity column of monoclonal antibody. The toxin is a basic polypeptide with an isoelectric point at pH 9.1, and devoid of mannosides. It is composed of 88 amino acid residues with a molecular size of 10721 Da. The monoclonal antibodies could be applicable to the analysis of biologically active sites on the toxin, in an attempt to synthesize chemically a small peptide with killer activity and little immunogenicity.

*Killer toxin* (Hansenula mrakii) *Monoclonal antibody* *Affinity chromatography* *Amino acid sequence*

## 1. INTRODUCTION

Some yeast strains secrete into culture media the factors, designated killer toxins, which kill sensitive strains of yeast [1]. Recently, Ashida et al. [2] described another killer toxin that was produced by *Hansenula mrakii*. The toxin of *H. mrakii* is stable against heat and unaffected over a wide range of pH changes, contrasting with most of the killer toxins [3–5].

We raised monoclonal antibodies against the toxin secreted by *H. mrakii*, and applied them to its isolation and characterization.

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of killer toxin*

The killer strain of *H. mrakii* (IFO 0895 [LKB169]), obtained from Institute for Fermentation, Osaka, Japan, was cultured at 30°C for 1 day in minimal medium. The toxin in culture medium, detectable by bioassay with the sensitive strain (*Saccharomyces cerevisiae*, diploid, no. 5059 from our stock culture) [6], was isolated by gel filtration in Sephadex G-50 (fig.1).

### 2.2. *Monoclonal antibodies*

A female BALB/c mouse received intracutaneously 125 $\mu$g killer toxin emulsified in Freund's complete adjuvant. After 1 month, the mouse was injected with 50 $\mu$g toxin intravenously. Hybridization, subsequent culture and cloning were carried out by a modification [7] of the method of Köhler and Milstein [8]. Antibodies were screened by enzyme immunoassay that sandwiched them between the toxin fixed on a solid-phase support and rabbit anti-mouse $\gamma$-globulin antibody labeled with horseradish peroxidase (details in [9]). The neutralizing activity was tested by the method given in the legend to fig.2.

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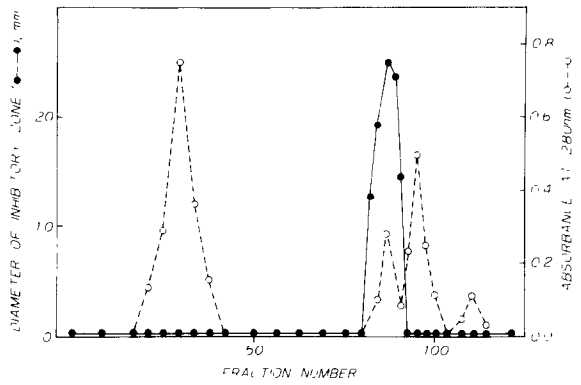


Fig.1. Isolation of the killer toxin in culture media of *H. mrakii* by gel filtration in Sephadex G-50. Fractions were monitored for the activity to kill *S. cerevisiae* (●) and the absorbance at 280 nm (○).

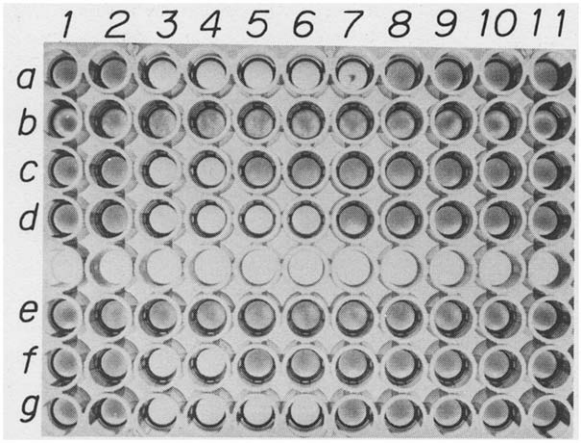


Fig.2. Neutralization of killer toxin with monoclonal antibody. A varying amount of toxin in 25 μl YPAD medium was mixed with an equal volume of medium containing monoclonal antibody and incubated at 37°C for 1 h in wells of a polyvinyl microtiter plate. The mixture was then tested for the activity to kill *S. cerevisiae* (2000 cells in 50 μl medium). Lanes: 1, 2, 11, toxin-free medium; 3–10, 10, 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.05 μg/ml of killer toxin, respectively. Row a received 250 μg/ml monoclonal antibody to hepatitis B surface antigen (negative antibody control). Rows b, c and d received monoclonal antibody against killer toxin (no. 6207) at 250, 25 and 2.5 μg/ml, respectively. Rows e, f and g received the other monoclonal antibody against killer toxin (no. 6217) at 250, 25 and 2.5 μg/ml, respectively.

### 2.3. *Affinity chromatography*

Monoclonal antibody against killer toxin (monoclonal no. 6207, see section 3) was coupled to Sepharose 4B by means of CNBr [10].

### 2.4. *Characterization of killer toxin*

Electrophoresis in a polyacrylamide gel slab in the presence of SDS was performed as in [11]. Polypeptides in the gel slab were transferred onto a nitrocellulose sheet (Millipore, Bedford, MA), and the toxin detected by incubation with monoclonal antibody and then with goat anti-mouse γ-globulin antibody labeled with horseradish peroxidase using the immunoblot system (Bio-Rad, Richmond, CA). Isoelectrofocusing was carried out in 5% polyacrylamide gel containing 0.5% Ampholine [12].

### 2.5. *Biochemical analyses*

A preparation of killer toxin (20 μg), purified by affinity chromatography, was hydrolysed and applied to a Hitachi high-speed amino acid analyser

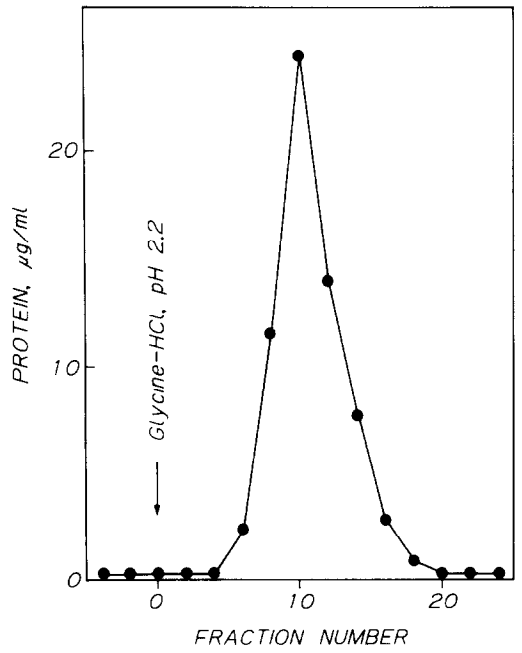


Fig.3. Purification of killer toxin (●) by affinity column of monoclonal antibody. After application of cell-free medium from the culture of *H. mrakii*, the column was eluted with glycine-HCl buffer (0.2 M, pH 2.2).

(model 835, Hitachi, Tokyo). The content of tryptophan was determined spectrophotometrically [13]. Protein was determined by the method of Lowry et al. [14], employing bovine serum albumin as the standard. Mannosides were determined by the capacity to bind with jackbean concanavalin A (Miles, Elkhart, IN) according to Wood and Sarinana [15].

### 2.6. *Amino acid sequence*

Purified killer toxin (500 $\mu$g) was dissolved in 500 $\mu$l Tris-HCl buffer (0.13 M, pH 7.6) containing 6 M guanidine hydrochloride and 10 mM dithiothreitol and incubated at 37°C overnight. The reduced toxin was digested with trypsin (Worthington, Freehold, NJ), chymotrypsin (Worthington), *Staphylococcus aureus* V8 protease (Sigma, St. Louis, MO) or lysyl endopeptidase (Wako, Osaka), added at a weight ratio of 1:100. Amino acid sequences of the obtained peptides were determined by an automatic Edman 470A protein sequencer (Applied Biosystems, Foster, CA).

## 3. RESULTS

### 3.1. *Monoclonal antibodies*

Antibody to killer toxin was detected by enzyme immunoassay in 2 out of 400 wells containing hybridoma cells. They were designated as monoclonal nos 6207 and 6217. Hybridoma cells were propagated in the peritoneal cavity of mice made ascitic by the injection with 2,6,10,14-tetramethylpentadecane. Antibodies in ascites fluid were precipitated with 2 M $(NH_4)_2SO_4$, and purified by gel filtration on Sephadex G-200 (Pharmacia, Uppsala). Both monoclonal antibodies neutralized the killing activity of toxin (fig.2).

### 3.2. *Purification of killer toxin by affinity chromatography*

The killer toxin in cell-free concentrate of media from the culture of *H. mrakii* was purified by the affinity column of monoclonal antibody to killer toxin (fig.3).

### 3.3. *Physicochemical characters of killer toxin*

When a preparation of killer toxin, purified by affinity chromatography, was subjected to electrophoresis, it was detected as a single band (fig.4).

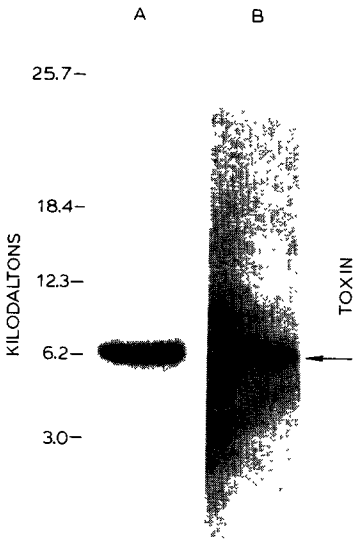


Fig.4. Electrophoresis of purified killer toxin. A strip of gel was stained for protein with Coomassie brilliant blue (lane A). Another strip was transferred onto a nitrocellulose sheet and immunoblotted for the toxin (lane B).

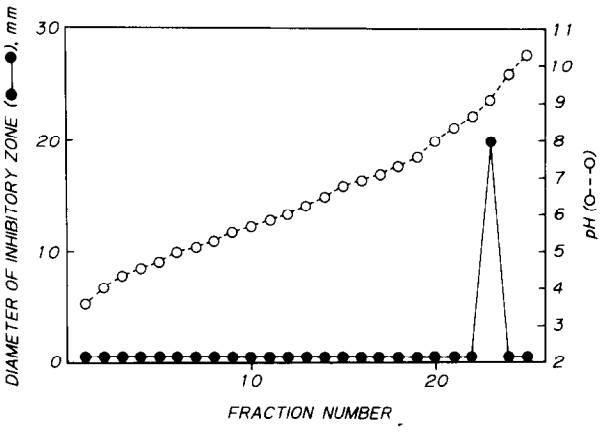


Fig.5. Isoelectrofocusing of purified killer toxin. After elektrofocusing, gel slices were eluted with distilled water and tested for the activity to kill *S. cerevisiae* (●) and pH (○).

The toxin had an isoelectric point at pH 9.1, thereby indicating its basic nature (fig.5). It did not bind with concanavalin A, indicating the lack of mannosides.

### 3.4. *Amino acid sequence of killer toxin*

The complete amino acid sequence of killer toxin is given in fig.6. It is composed of 88 amino acid residues with a calculated molecular size of 10721 Da. The amino acid composition of the toxin estimated by amino acid analysis was very close to that determined from the sequence (table 1).

## 4. DISCUSSION

By means of a monoclonal antibody, the killer toxin secreted by *H. mrakii* was purified and characterized. The toxin is a basic protein with an isoelectric point at pH 9.1, and is composed of 88 amino acid residues with a molecular size of 10721 Da. It is devoid of mannosides.

The toxin secreted by *H. mrakii* is smaller than $K_1$ killer toxin (18.5 kDa) [16] or the toxin from *Pichia kluyveri* (19 kDa) [5] and larger than that from *Hansenula saturnus* (8.5 kDa) [17]; it compares in size with the toxin from *Kluyveromyces lactis* (10 kDa) [4]. It is basic in nature unlike most of the reported toxins that are acidic proteins [5,17,18].

Yeasts harboring the plasmid containing recombinant complementally DNA have been applied to the synthesis of a number of biologically active

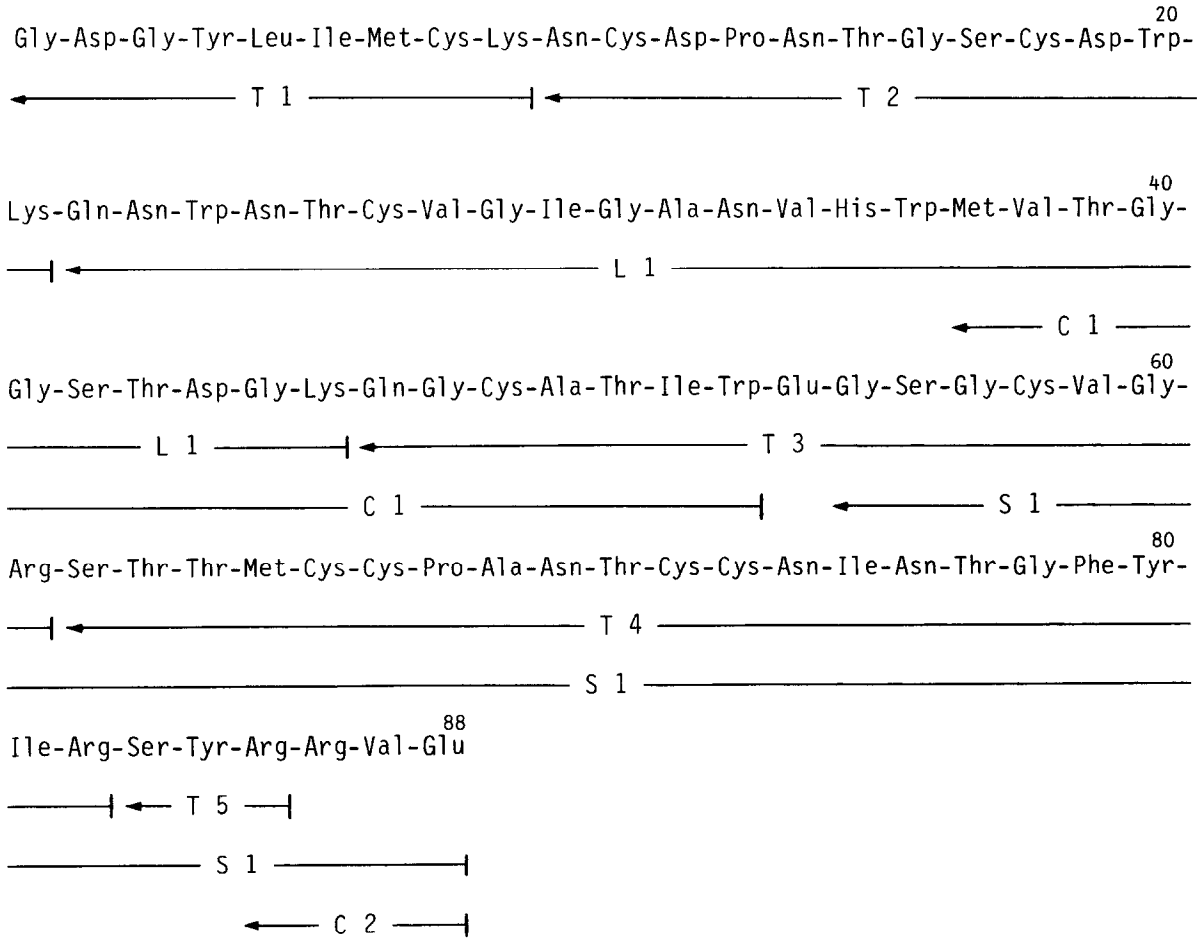


Fig.6. Amino acid sequence of the killer toxin from *Hansenura mrakii*. Tryptic peptides (T), chymotryptic peptides (C), peptides obtained by digestion with *Staphylococcus aureus* V8 protease (S) and peptides obtained by digestion with lysyl endopeptidase (L), subjected to Edman degradation, are shown, The sequence from the 1st to 30th position was confirmed by Edman degradation of undigested toxin.

Table 1

Amino acid composition of the killer toxin of *H. mrakii*

| Amino acid | Residues per polypeptide | |
|---|---|---|
| | Analysis[a] | Sequence[b] |
| Aspartic acid | 11 | 12 |
| Threonine | 8 | 9 |
| Serine | 5 | 5 |
| Glutamic acid | 4 | 4 |
| Proline | 2 | 2 |
| Glycine | 13 | 13 |
| Alanine | 4 | 3 |
| Valine | 5 | 5 |
| Methionine | 3 | 3 |
| Isoleucine | 5 | 5 |
| Leucine | 1 | 1 |
| Tyrosine | 3 | 3 |
| Phenylalanine | 1 | 1 |
| Lysine | 3 | 3 |
| Histidine | 1 | 1 |
| Arginine | 4 | 4 |
| Tryptophan | 3 | 4 |
| Cysteine | 9 | 10 |
| Total residues | 85 | 88 |

[a] The number of glycine was estimated at 13 to make a polypeptide with a molecular size estimated from the sequence analysis. The other amino acids were calculated pro rata and the nearest moles were adopted.

[b] See fig.6

substances [1]. Killer toxins would be applicable to this field, in order to protect the desired strain from contaminating strains that are sensitive to toxins.

There are possibilities that killer toxins will be used as fungistatic agents, should they prove to be effective on pathogenic organisms. In order that they may be given to patients during a long period without untoward effects, they have to be small peptides with little immunogenicity. Monoclonal antibodies with neutralizing activities, like we have raised against the toxin of *H. mrakii*, would be particularly useful in identifying the amino acid sequence responsible for toxic activities. Now that its complete amino acid sequence has been determined, attempts along this line could be accelerated by identifying the region(s) of high local hydrophilicity along the sequence [19].

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REFERENCES

[1] Tipper, D.J. and Bostian, K.A. (1984) Microbiol. Rev. 48, 125–156.
[2] Ashida, S., Shimazaki, T., Kitano, K. and Hara, S. (1983) Agric. Biol. Chem. 47, 2953–2955.
[3] Pfeiffer, P. and Radler, F. (1984) Arch. Microbiol. 137, 357–361.
[4] Sugisaki, Y., Gunge, N., Sakaguchi, K., Yamasaki, M. and Tamura, G. (1983) Nature 304, 464–466.
[5] Middelbeek, E.J., Hermans, J.M.H. and Stumm, C. (1979) Antonie van Leeuwenhoek 45, 437–450.
[6] Woods, D.R. and Bevan, E.A. (1968) J. Gen. Microbiol. 51, 115–126.
[7] Oi, V.T. and Herzenberg, L.A. (1980) in: Immunoglobulin-Producing Hybrid Cell Lines (Mishell, B.B. and Shiigi, S.M. eds) pp. 351–572, Selected Methods in Cellular Immunology, Freeman, San Francicso.
[8] Köhler, G. and Milstein, C. (1975) Nature 256, 495–497.
[9] Tachibana, K., Tanaka, E., Usuda, S., Okamoto, H., Imai, M., Nakamura, T., Miyakawa, M. and Mayumi, M. (1984) J. Immunol. Methods 75, 43–51.
[10] Porath, J., Axén, R. and Eruback, S. (1967) Nature 215, 1491–1492.
[11] Mishiro, S., Imai, M., Takahashi, K., Machida, A., Gotanda, T. Miyakawa, Y. and Mayumi, M. (1980) J. Immunol. 124, 1589–1593.
[12] Takahashi, K., Imai, M., Miyakawa, Y., Iwakiri, S. and Mayumi, M. (1978) Proc. Natl. Acad. Sci. USA 75, 1952–1956.
[13] Palfree, R.G.E. and Bussey, H. (1979) Eur. J. Biochem. 93, 487–493.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Wood, J.G. and Sarinana, F.O. (1975) Anal. Biochem. 69, 320–322.
[16] Bostian, K.A., Elliott, Q., Bussey, H., Burn, V., Smith, A. and Tipper, D.J. (1984) Cell 36, 741–751.
[17] Ohta, Y., Tsukada, Y. and Sugimori, T. (1984) Agric. Biol. Chem. 48, 903–908.
[18] Bussey, H. (1981) Adv. Microbiol. Physiol. 22, 93–122.
[19] Hopp, T.P. and Woods, K.R. (1981) Proc. Natl. Acad. Sci. USA 78, 3824–3828.