New Killer Toxins of Halophilic *Hansenula anomala*Shogo KAGIYAMA, Tomio AIBA, Kiyoshi KADOWAKI
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Two strains of killer yeasts, both identified as *Hansenula anomala*, were isolated from shoyu moromi. Both killer yeasts showed killer activity toward *Zygosaccharomyces rouxii* EA under high salt concentration conditions. The killer toxins produced by these strains were purified by ultrafiltration and ion-exchange chromatography followed by gel filtration. The molecular weights of the toxins were about 300 kd and both toxins were glycoproteins. The isoelectric point of the toxin, Kh-I, produced by one strain was pH 2.9 and that of the toxin, Kh-II, produced by the other strain was pH 3.6. The amino acid composition of Kh-I was different from that of Kh-II. Kh-II was more thermolabile than Kh-I. The killer activities of both toxins were not observed in the absence of NaCl in the medium but increased with increasing NaCl concentration. The killer spectra of Kh-I and Kh-II were different from those of the killer toxins known as K₁-K₁₁. The killer activities of these strains were not abolished by cycloheximide treatment and by cultivation at 37°C. No plasmid was detected in either killer yeast.

The killer character of *Saccharomyces cerevisiae* was first reported by Bevan and Makower.¹⁾ Thereafter a number of killer yeast strains were isolated from various natural habitats and classified according to their killing specificities.²⁾

The purification and properties of the killer toxins of *S. cerevisiae*,^{3~6)} *Candida glabrata*,⁷⁾ *Pichia kluyveri*,⁸⁾ *Kluyveromyces lactis*,⁹⁾ *Hansenula mrakii*¹⁰⁾ and *Hansenula saturnus*¹¹⁾ have been reported. But the existence of a killer character in halophilic yeasts has not previously reported. There are many kinds of killer strains in a wide variety of yeasts. This suggests that there should be killer strains among halophilic yeasts. If halophilic killer yeasts are isolated, they will be useful for microbial control in shoyu making. We tried to isolate killer yeasts from shoyu moromi and successfully isolated two different killer yeast strains, and found that they produced killer toxins with unique characteristics, whose activities appeared in medium containing a high concentration of NaCl, but not in its absence.

This paper describes the purification and

various physicochemical properties of the killer toxins produced by these yeasts.

MATERIALS AND METHODS

Yeast strains. *Zygosaccharomyces rouxii* EA from the yeast collection of Higeta Shoyu Co., Ltd. was used as the test strain for the detection of killer activity. *S. cerevisiae* NCYC 235, *S. cerevisiae* NCYC 738, *S. capensis* NCYC 761, *C. glabrata* NCYC 388, *H. anomala* NCYC 434, *K. fragilis* NCYC 587, *C. valida* NCYC 327, *H. anomala* NCYC 435, *H. mrakii* NCYC 500, *K. drosophilum* NCYC 575 and *C. glabrata* ATCC 15126 were used as standard killer strains with different killer sensitivities.^{2,12)}

Media. YEPD-1 medium (glucose 2%, polypeptone 2%, yeast extract 1%, 0.1 M citrate phosphate buffer, pH 4.8) and YEPD-2 medium (YEPD-1 medium containing NaCl 8%) were used for cultivation of the killer yeasts. YEPD-MB1 agar (YEPD-1 medium containing methylene blue 0.03% and agar 2%) and YEPD-MB2 agar (YEPD-MB1 agar supplemented with 8% NaCl) were used for the isolation of killer yeasts. For the curing test on the killer activity, YM agar (glucose 1%, polypeptone 0.5%, yeast extract 0.3%, malt extract 0.3%, agar 2%) was used.

Detection of killer yeasts. The detection of killer yeasts was performed by the cross-streak test,¹³⁾ as fol-

lows. *Z. rouxii* EA was inoculated on YEPD-MB1 and YEPD-MB2 agar plates and then the strain to be tested for killing activity was cross-streaked on them, followed by incubation at 23°C for 4 days. If the strain had killer activity, then the cells of *Z. rouxii* EA became blue due to methylene blue staining.

Taxonomic identification of yeasts. Taxonomic identification of the killer strains was carried out according to the methods of Kregar-van Rij.¹⁴⁾

Determination of killer activity. Killer activity was determined by the cup method. Killer yeast cultures were filtered through 0.2 µm nitrocellulose membrane filters. 0.1 ml portions of the filtrates were pipetted into cups (6 mm inside diameter) on YEPD-MB2 agar plates, each inoculated with 10⁵ cells of the test strain. The plates were incubated at 23°C for 4 days. The diameter of the inhibitory zone around each cup was measured and the area of the inhibitory zone was calculated. One unit (U) is the amount of killer toxin that inhibits one square cm of the test strain.

Biochemical methods. Carbohydrate was analyzed by gas chromatography (Yanako G-180) of trimethylsilyl derivatives,¹⁵⁾ after hydrolysis of the toxin sample in 2 N HCl at 100°C, on a column packed with Silicone GS-101 (Gasukurokogyo Inc.) (3 mm × 2 m glass column). The amino acid composition was determined with an amino acid analyzer (Shimadzu Liquid Chromatograph LC6A) after hydrolysis of the toxin sample in 6 N HCl at 110°C for 22 hr. The amounts of protein were determined by the method of Lowry *et al.*¹⁶⁾ with bovine serum albumin as a standard, and those of carbohydrate by the phenol-sulfuric acid method.¹⁷⁾ To examine the effect of proteolytic enzymes on the killer activity, pepsin, papain and chymotrypsin A₄ (Boehringer Mannheim GmbH, W. Germany), and the crude enzyme of *Aspergillus sojae* were used.

RESULTS

Isolation of killer yeasts

Six hundred and twenty-three osmophilic yeast strain isolated at random from shoyu moromi in our plants were tested as to killer activity using cells of *Z. rouxii* EA as the standard sensitive strain. Two strains showed activity on YEPD-MB2 agar but not on YEPD-MB1 agar.

Taxonomic characterization of killer yeasts

The 2 killer strains formed pseudomycelia and ascospores (hat-shaped). But true mycelia

and ballisporos were not formed. Both strains assimilated nitrate but did not produce acetic acid. So both strains were classified as *Hansenula*. Both strains fermented sucrose and were able to grow in vitamin-free medium. Both strains assimilated maltose, erythritol and soluble starch but not L-rhamnose. From the above results both killer strains were identified as *Hansenula anomala*.

Purification of killer toxins

The killer strains were grown at 23°C for 4 days in YEPD-2 medium. Thereafter, all the steps were performed at 5°C. The culture broths, about 10 l in volume, were filtrated with Hyflo Supercells. The filtrates were concentrated to 50 ml with hollow fiber H1P-10 (Amicon). For desalting, the concentrated solutions were applied to a column of Sephadex G-25 (5 × 90 cm) equilibrated with 50 mM citrate phosphate buffer (pH 4.8) and eluted with the same buffer. The active fractions, totaling 600 ml, were applied to a column of SP-Sephadex C-25 (5 × 50 cm) equilibrated with 10 mM citrate phosphate buffer (pH 4.8). After washing the column with buffer, elution was performed with a 1000 ml linear gradient of

FIG. 1. Gel Filtration of the Kh-I and Kh-II Toxins on Toyopearl HW60.

The Kh-I and Kh-II toxins were produced, respectively, by two independent isolates of *H. anomala*. ▲—▲, killer activity; -----, absorbance at 280 nm; —, carbohydrate concentration. Column size, 2.6 × 90 cm; flow rate, 60 ml/hr.

0~1M NaCl. The active fractions, 450 ml, were collected and concentrated by ultrafiltration (PM 10, Amicon) and then the concentrated sample (*ca.* 10 ml) was chromatographed on Toyopearl HW60 (2.6 × 90 cm) equilibrated with 100 mM citrate phosphate buffer (pH 4.8). Typical elution profiles for the killer toxin, Kh-I, produced by one of the isolates and that, Kh-II, produced by the other yeast strain isolated are shown in Fig. 1. It was evident from the elution profiles that the killer activities are identical with those of proteins and carbohydrates. These facts strongly suggest that the Kh-I and Kh-II toxins are glycoproteins. The active fractions were then collected, dialyzed and lyophilized.

Polyacrylamide gel electrophoresis of the active fractions was carried out with a 7.5% polyacrylamide slab gel according to the method of Davis.¹⁸⁾ After electrophoresis, the protein bands were visualized by staining with Coomassie Brilliant Blue G 250. It was confirmed that each lyophilized preparation, Kh-I and Kh-II, gave a single protein band on gel electrophoresis (data not shown).

Characteristics of the killer toxins

Molecular weight. The molecular weights of the Kh-I and Kh-II toxins were estimated from the elution profiles on Sepharose CL6B gel chromatography (Fig. 2). The molecular

FIG. 2. Determination of the Molecular Weights of the Kh-I and Kh-II Toxins by Gel Filtration on Sepharose CL6B.

a, Kh-II; b, thyroglobulin; c, ferritin; d, Kh-I; e, catalase; f, aldolase; a', Kh-II after hydro-carboxamide-methylation.

weight of Kh-I was estimated to be about 300 kd and that of Kh-II to be about 700 kd. These molecular weights are very large for secretion products of yeasts. Therefore, the toxins might have undergone subunit aggregation. To examine this, hydro-carboxamid-methylation¹⁹⁾ of the Kh-I and Kh-II preparations was performed. On hydrocarboxamid-methylation, the molecular weight of Kh-I did not change, but that of Kh-II decreased from 700 kd to 300 kd. This suggests that aggregation through disulfide bond formation of the Kh-II toxin occurred during the purification process and that the molecular weight of the intact Kh-II toxin is about 300 kd.

Isoelectric point. The isoelectric point was determined by isoelectric focusing.²⁰⁾ It was conducted at 5°C for 72 hr at 700 V in a glycerol gradient solution with carrier ampholytes (LKB, pH range, 2.5~4.0). The isoelectric point of the Kh-I toxin was estimated to be 2.9 and that of Kh-II to be 3.6.

Contents of protein and carbohydrate. The Kh-I toxin was composed of 53% protein and 47% carbohydrate, and Kh-II of 49% protein and 51% carbohydrate. These results indicate that both toxins are glycoproteins.

Carbohydrate composition. Figure 3 shows the results of GLC analysis of the products of TMS ethers obtained from acid hydrolysates of the Kh-I and Kh-II toxins. In Fig. 3, peaks 1a, 3a, 1b and 3b were identified as D-mannose, and peaks 2a, 5a, 2b and 5b as D-

FIG. 3. Gas Chromatography of TMS Sugars Obtained from Acid Hydrolysates of Kh-I and Kh-II Toxins.

1a, 3a, 1b, 3b, mannose; 2a, 5a, 2b, 5b, glucose; 4a, 4b, mannitol (internal standard).

TABLE I. AMINO ACID ANALYSIS OF THE Kh-I AND Kh-II TOXINS

Amino acid	No. of residues per molecule of	
	Kh-I ^a	Kh-II ^b
Aspartic acid	128	152
Threonine	92	91
Serine	90	128
Glutamic acid	271	106
Proline	108	53
Glycine	88	111
Alanine	58	82
Cystine	5	6
Valine	70	62
Methionine	18	5
Isoleucine	67	38
Leucine	54	130
Tyrosine	14	33
Phenylalanine	30	77
Histidine	46	23
Lysine	85	50
Arginine	14	24

^a Mass of killer protein per one molecule was estimated to be 159 kd.

^b Mass of killer protein per one molecule was estimated to be 149 kd.

glucose. These data indicate that the carbohydrate of the Kh-I toxin consists of 79% D-mannose and 21% D-glucose, the proportions being 86% D-mannose and 14% D-glucose for the Kh-II toxins.

Amino acid composition. The results of amino acid analysis of the Kh-I and Kh-II toxins are presented in Table I. They have different compositions. In particular, the contents of glutamic acid, proline, methionine, isoleucine and lysine of the Kh-I toxin were higher than those of Kh-II.

Effect of the NaCl concentration of the killer activity. Figure 4 shows the killer activities of the lyophilized preparation of the Kh-I and Kh-II toxins with the cup method when the NaCl concentration of YEPD-MB1 agar was changed in the range of 0~16%. In the absence of NaCl the killer activities of both toxins were not observed but increased in accordance with NaCl concentration.

pH stability of the killer activity. Solutions of the Kh-I and Kh-II toxins were adjusted to

FIG. 4. Effect of the NaCl Concentration on the Killer Activities of the Kh-I and Kh-II Toxins.

The killer activities of the lyophilized toxin preparation were determined by the cup method using *Z. rouxii* EA as the sensitive test strain. ○, the Kh-I toxin preparation; ●, the Kh-II toxin preparation.

FIG. 5. pH Stabilities of the Killer Activities of the Kh-I and Kh-II Toxins.

○, the Kh-I toxin preparation; ●, the Kh-II toxin preparation.

various pHs from 2.0 to 6.0 with 1 M citrate phosphate buffer and then kept at 5°C for 18 hr. After the incubation, each solution was assayed for killer activity. Figure 5 shows that both toxin preparations were stable from pH 2.5 to 5.0 under the above conditions.

Thermal stability of the killer activity. Solutions of the Kh-I and Kh-II toxins were incubated in the range of 15~45°C. After 15 min to 4.5 hr, the solutions were assayed for activity. The killer activities of the Kh-I and Kh-II preparations decreased with increasing of temperature (Fig. 6). After 4.5 hr incubation at 45°C, the Kh-I sample showed 40% of the initial activity, while incubation of the Kh-II

preparation at 35°C for 30 min led to complete loss of the activity. These results indicated that the Kh-I toxin is more stable at high temperature than Kh-II.

Effects of proteolytic enzymes. Solutions of 5 mg/ml pepsin, 2 mg/ml papain, 2 mg/ml chymotrypsin A₄ and 6.4 mg/ml crude enzyme of *A. sojae* were used. For the preparation of the crude enzyme of *A. sojae*, a bran medium (50 g of wheat bran mixed with 40 ml of tap water and then autoclaved for 30 min) was used. The bran medium inoculated

with *A. sojae* was incubated at 30°C for 48 hr and then the medium was mixed with 50 ml of 0.1 M citrate-phosphate buffer (pH 4.8), followed by filtration. The filtrate was lyophilized and used as the crude enzyme of *A. sojae*.

The lyophilized toxin preparations were mixed with 0.5 ml of enzyme solutions and then incubated at 15°C for 38 hr. After incubation, the killer activities were assayed. The effects of denatured enzymes, heated at 100°C for 15 min, were also examined.

The Kh-I toxin was inactivated by papain but not by pepsin or chymotrypsin A₄. The Kh-II toxin was not inactivated by pepsin, papain or chymotrypsin A₄. However, both toxin preparations lost about 60% of their killer activities on treatment with the crude enzyme of *A. sojae*.

Anti-yeast spectra of the Kh-I and Kh-II toxins. Table II shows anti-yeast spectra of the Kh-I and Kh-II toxins determined by the cross-streak method. The Kh-I toxin killed the K₆, K₁₀ and K₁₁ type killer yeasts, depending on the NaCl concentration in the medium, and the Kh-II toxin killed the K₂, K₃, K₇, K₁₀ and K₁₁ type killer yeasts. It is evident that the killer spectra of both the Kh-I

FIG. 6. Thermostabilities of the Killer Activities of the Kh-I and Kh-II Toxins.

A, the Kh-I toxin preparation; B, the Kh-II toxin preparation. ○, 15°C; ●, 25°C; △, 35°C; ▲, 45°C.

TABLE II. ANTI-YEAST SPECTRA OF THE Kh-I AND Kh-II TOXINS

Test strains	Killer type	Killer toxin			
		Kh-I		Kh-II	
		A ^a	B ^b	A ^a	B ^b
<i>S. cerevisiae</i> NCYC 235	K ₁	—	—	—	—
<i>S. cerevisiae</i> NCYC 738	K ₂	±	+	—	+
<i>S. capensis</i> NCYC 761	K ₃	—	—	—	+
<i>C. glabrata</i> NCYC 388	K ₄	±	+	±	+
<i>H. anomala</i> NCYC 434	K ₅	—	—	—	—
<i>K. fragilis</i> NCYC 587	K ₆	—	+	+	+
<i>C. valida</i> NCYC 327	K ₇	—	—	—	+
<i>H. anomala</i> NCYC 435	K ₈	—	—	—	—
<i>H. mrakii</i> NCYC 500	K ₉	—	—	—	—
<i>K. drosophilae</i> NCYC 575	K ₁₀	—	+	—	+
<i>C. glabrata</i> ATCC 15126	K ₁₁	—	+	—	+

^a Tested on YEPD-MB1 agar (NaCl 0%).

^b Tested on YEPD-MB3 agar (NaCl 4%).

+, killing; —, no killing; ±, weak killing.

and Kh-II toxins are different from those of the $K_1 \sim K_{11}$ types reported by Young and Yagiu.²⁾

Curing test

Cells of the killer yeasts were diluted with a sterile 0.9% NaCl solution and then 0.1 ml of each solution (containing about 100 cells) was spread on a YM agar plate. To examine the curing of the killer characteristics of the isolated *H. anomala* strains with cycloheximide, media containing 0.1 ppm of cycloheximide were prepared by the aseptic addition of the filter-sterilized antibiotic to autoclaved YM agar, and the plates were incubated at 30°C. To examine the cultivation temperature for the curing of the killer characteristics, plates without cycloheximide were incubated at 37°C. After 48 hr incubation, the plates were replicated onto YEPD-MB2 agar, which had been inoculated with the sensitive cells 2 hr before, followed by incubation at 23°C for 4 days.

The killer activities of the 2 killer yeast strains were not cured by cycloheximide treatment or incubation at 37°C.

Detection of plasmids

Detection of plasmids was attempted by the procedure of Gunge *et al.*²¹⁾ However, no plasmid was detected in either yeast.

DISCUSSION

It has been reported that the killer toxins in *S. cerevisiae*, *C. glabrata*, *P. kluyveri* and *K. lactis* are glycoproteins. But the molecular weights of the Kh-I and Kh-II toxins are three times greater than that of the toxin of *K. lactis* IFO 1267, which has the largest molecular weight so far reported. The proportions of carbohydrate in the Kh-I and Kh-II toxins are five as high as that in the *S. cerevisiae* toxin, besides which the isoelectric points of the Kh-I and Kh-II toxins were lower than those of most of the other known toxins. The pI of the Kh-I toxins was the lowest for the toxins so far isolated. Furthermore,

the killer spectra of both the Kh-I and Kh-II toxins are quite different from those of the $K_1 \sim K_{11}$ toxins. Therefore, the Kh-I and Kh-II toxins are new species of killer toxins. Moreover, judging from its thermal stability, amino acid composition and killer spectra, the Kh-I toxin is distinctly different from the Kh-II toxin. Invertase²²⁾ and acid phosphatase²³⁾ of *S. cerevisiae* are well known as secreted glycoproteins, whose molecular weights and sugar contents are similar to the Kh-I and Kh-II toxins. But these enzymes exist in the cell wall and are never secreted into the medium. There has been no report of such a large glycoprotein being secreted into the medium and it is very interesting that two killer strains secreting such large substances existed in shoyu moromi.

That the Kh-I and Kh-II toxins killed *Z. rouxii* EA depending on the NaCl concentration showed that the Kh-I and Kh-II toxins are halophilic factors. The effect of NaCl on the killer activity of the killer toxin of *S. cerevisiae* was also investigated by Palfree *et al.*³⁾ These authors reported, however, that the killer activity decreased with increasing NaCl concentration.

Killer plasmids were detected in the $K_1 \sim K_3$ types of killer yeasts. However, the killer activities of the present, two killer yeast strains, *H. anomala* strains Kh-I and Kh-II, were not cured by cycloheximide treatment or incubation at 37°C, and no plasmid was detected in either yeast. These results suggest that the genetic basis for the toxin production in these strains is a chromosomal gene and not a cytoplasmic factor.

In shoyu moromi, the concentration of NaCl is very high and activities of proteases derived from *Aspergillus sojae* are also high. In order to exhibit killer activity under such conditions, a killer toxin must be halophilic and tolerant to proteases. The isolated yeast strains, *H. anomala* strains Kh-I and Kh-II, are able to grow at such a high concentration of NaCl as that in shoyu moromi. In addition, the toxins of these killer yeasts are halophilic factors, and both toxin preparations exhibit

about 40% of the initial killer activity after treatment with the crude enzyme of *A. sojae*. These facts indicate that these killer yeasts can grow and express their activities in shoyu moromi. So, in shoyu brewing, we can use these killer yeasts to control lactic acid and alcoholic fermentation in moromi for good quality of the product.

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