

The role of the histidine-35 residue in the cytotoxic action of HM-1 killer toxin

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Diethylpyrocarbonate modification and site-directed mutagenesis studies of histidine-35 in HM-1 killer toxin (HM-1) have shown that a specific feature, the imidazole side chain of histidine-35, is essential for the expression of the killing activity. In subcellular localization experiments, wild-type HM-1 was in the membrane fraction of *Saccharomyces cerevisiae* BJ1824, but not the HM-1 analogue in which histidine-35 was replaced by alanine (H35A HM-1). Neither wild-type nor H35A HM-1 was detected in cellular fractions of HM-1-resistant yeast *S. cerevisiae* BJ1824 *rhk1Δ::URA3* and HM-1-insensitive yeast *Candida albicans* even after 1 h incubation. H35A HM-1 inhibited the activity of partially purified 1,3- β -glucan synthase from *S. cerevisiae* A451, and its extent was almost the same as wild-type HM-1. Co-immunoprecipitation experiments showed that wild-type and H35A HM-1 directly interact with the 1,3- β -glucan synthase complex. These results strongly suggest that histidine-35 has an important role in the cytotoxic action of HM-1 that participates in the binding process to the HM-1 receptor protein on the cell membrane, but it is not essential for the interaction with, and inhibition of, 1,3- β -glucan synthase.

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INTRODUCTION

HM-1 killer toxin (HM-1) is a strong anti-yeast protein produced by the yeast *Williopsis saturnus* var. *mrakii* IFO 0895 and belongs to the K9-type killer toxin group (Young & Yagi, 1978; Kimura *et al.*, 1995). HM-1 kills susceptible yeasts effectively, consists of 88 amino acids, including five disulfide cross-links, and is very stable against heat treatment and at pH 2–11 (Yamamoto *et al.*, 1986; Komiyama *et al.*, 1996).

Some killer toxins bind to the yeast cell wall as the first receptor (Bussey *et al.*, 1979; Hutchens & Bussey, 1983; Tipper & Schmitt, 1991; Marquina *et al.*, 2002). HM-1 is believed to bind in this way, then bind to a putative receptor on the cell membrane and finally inhibit 1,3- β -glucan synthase (Kasahara *et al.*, 1994; Takasuka *et al.*, 1995; Kimura *et al.*, 1998).

Previously, by using an alanine-scanning method, we showed that several amino acid residues in HM-1 are required for the expression of killing activity (Miyamoto *et al.*, 2005). In particular, replacement of histidine-35, which is the only histidine residue in the HM-1 molecule,

caused a loss of HM-1 killing activity, but it did not affect strongly its secretion efficiency. In this study, to examine the importance of specific features of histidine-35 in the killing activity, we replaced histidine-35 with other amino acids by using site-directed mutagenesis, and measured the amounts of HM-1 secreted and killing activities. To clarify the importance of histidine-35 in the binding process to yeast cells, we examined subcellular localization of wild-type HM-1 and H35A HM-1 in yeast whole cells and spheroplasts. We also determined the mode of action of the 1,3- β -glucan synthase inhibition and the interaction with the 1,3- β -glucan synthase complex by wild-type HM-1 and H35A HM-1. The results showed that histidine-35 is required for the binding of HM-1 to the sensitive yeast receptor in the membrane fraction and this also involves the Rhk1 protein.

METHODS

Materials. HM-1, the HM-1 gene, plasmid YEp51, the HM-1-resistant strain of *Saccharomyces cerevisiae* BJ1824 *rhk1Δ::URA3* and other strains, including *Candida albicans*, were obtained as reported previously (Kimura *et al.*, 1993; Komiyama *et al.*, 1996). Anti-1,3- β -glucan synthase catalytic subunit (Fks1p) mouse monoclonal antibody 1F4 was a kind gift from Dr. Watanabe of Japan Roche Research Institute (Kamakura, Japan). Anti-aldehyde dehydrogenase (Ald4p) rabbit polyclonal antibody was prepared by Nordic Immunological Laboratories. The YPD medium consisted of 1% yeast extract, 2% peptone and 2% glucose. Rabbit polyclonal and

Abbreviations: CD, circular dichroism; DEPC, diethylpyrocarbonate; HM-1, HM-1 killer toxin.

mouse monoclonal antibodies against HM-1 were prepared by Nippon Bio-Test Laboratories. An LA PCR *in vitro* mutagenesis kit was obtained from Takara Shuzo (Shirai *et al.*, 2000), and the oligonucleotide primers used were: 5'-TAACCATCCAAGCGACATTAGCT-3' for H35A HM-1 toxin and 5'-TAACCATCCANNNACATTAGCT-3' for H35X HM-1 toxin, where N is one of the 4 nucleotides and X is one of the 20 amino acids.

General methods. Recombinant DNA methods were carried out according to Sambrook *et al.* (1989). The DNA was sequenced by using the dideoxy method with fluorochrome-labelled dNTPs and a Long Read tower DNA sequencer (Amersham Pharmacia Biotech). 3D structures of HM-1 were analysed from the NMR data (Antuch *et al.*, 1996). HM-1 and its analogues were quantified by using ELISA with the purified HM-1 and rabbit polyclonal or mouse monoclonal antibodies (Komiya *et al.*, 2004).

Chemical modification of histidine residue. HM-1 was reacted with 5 mM diethylpyrocarbonate (DEPC) in PBS at pH 7.4 [PBS (-)] at 20 °C for 30 min, and then excess DEPC was quenched with 20 mM histidine (Miles, 1977). The amount of modified residue was calculated as the increase in the absorbance at 240 nm ($\epsilon = 3200 \text{ M}^{-1} \text{ cm}^{-1}$).

Mutant HM-1 gene construction and expression. Construction of mutant HM-1 genes and the expression of mutant HM-1 analogues were carried out as reported previously (Shirai *et al.*, 2000). The mutant gene-containing plasmids were used to transform HM-1-resistant *S. cerevisiae* BJ1824 *rhk1Δ::URA3*. Amino acid replacement at histidine-35 was confirmed by nucleotide sequencing after transformation. Yeasts bearing the genes for HM-1 and its analogues were grown for 48 h at 30 °C in liquid medium consisting of 1% yeast extract, 2% peptone, 2% galactose, 0.5% sucrose (YPGal+suc). After cultivation, the supernatant was collected by centrifugation, passed through a cellulose acetate filter (pore size 0.45 μm), and then dialysed against deionized distilled water for 16 h at 4 °C. The dialysates were concentrated using an evaporator and then were kept at 4 °C.

SDS-PAGE and Western blotting. SDS-PAGE and immunoblotting were carried out as reported previously (Tsang *et al.*, 1983; Schäger & von Jagow, 1987). The HM-1 and its analogues were detected using anti-HM-1 rabbit polyclonal antibody as the primary antibody and horseradish peroxidase- or alkaline phosphatase-conjugated anti-rabbit IgG as the secondary antibody. Fks1p was detected using anti-Fks1p mouse monoclonal antibody 1F4 as the primary antibody and alkaline phosphatase-conjugated anti-mouse IgG as the secondary antibody. As detection dyes, 3-amino-9-ethyl-carbazole for horseradish peroxidase and Western Blue stabilized substrate for alkaline phosphatase (Promega) were used.

Circular dichroism (CD) spectrum. The far-UV CD spectra of HM-1 were measured between 190 and 260 nm by using a JASCO J-820 spectropolarimeter at 25 °C. Sample solutions consisted of 10 μM wild-type or H35A HM-1 and PBS (-).

Measurement of killing activity of HM-1. The killer eclipse assay was used as described by Kishida *et al.* (1996), using *Hansenula anomala* IFO 0569 as test strain. This yeast (3 μl) at 1×10^6 cells ml^{-1} was spotted onto agar plates consisting of YPGal+suc, mutant HM-1 gene-bearing yeasts were planted on the edges of the test strain spots, and then the spots were incubated for 24 h at 30 °C. To estimate killing activities and IC_{50} values (the concentration required for 50% inhibition of the growth) test yeast *S. cerevisiae* A451 cells at exponential phase were incubated for 12 h in YPD medium with various concentrations of HM-1 or its analogues at 30 °C with reciprocal shaking at 120 r.p.m. The optical densities of the culture

broths were then measured at 600 nm using a spectrophotometer, and the IC_{50} values were read from the semi-logarithmic graphs (Komiya *et al.*, 1996).

Subcellular localization of HM-1. HM-1 localization in yeast cells was determined as described by Suzuki *et al.* (2001). Exponentially growing *S. cerevisiae* and *C. albicans* yeast cells (3×10^7 cells ml^{-1}) were collected by centrifugation at 6000 r.p.m. for 3 min at 25 °C and washed with 0.5 ml water, then suspended in 250 μl 50 mM phosphate buffer pH 6.0 containing 1.2 M sorbitol with or without wild-type HM-1 or H35A HM-1 (10 μg ml^{-1}). The mixtures were incubated at 30 °C for 1 h and were centrifuged at 3000 r.p.m. for 3 min at 4 °C. The supernatant became the 'unbound' fraction. The collected cells were washed five times with 0.5 ml 50 mM phosphate buffer pH 6.0 containing 1.2 M sorbitol. The washed cells were suspended in 0.1 M sodium carbonate pH 11 containing 1.2 M sorbitol and kept on ice for 30 min to extract cellular components that weakly bind on the surface of plasma membrane. The cells were collected by centrifugation at 3000 r.p.m. for 3 min at 4 °C and the supernatant became the 'periplasmic fraction'. The collected cells were suspended in 50 mM Tris/HCl pH 7.5 containing 1 mM EDTA, 1 μM PMSF, 1 μg leupeptin ml^{-1} and 1 μg pepstatin A ml^{-1} , and vortexed with glass beads. The mixture was centrifuged at 1000 g for 5 min at 4 °C and the pellet was collected as the 'cell wall fraction'. The supernatant was centrifuged at 100 000 g for 30 min at 4 °C, and the pellet was collected as the 'membrane fraction' and the supernatant as the 'cytosolic fraction'. Each fraction was subjected to SDS-PAGE and Western blotting. Completion of each cellular fractionation was determined by Western blotting using anti-Fks1p mouse monoclonal antibody 1F4 for the membrane fraction and anti-aldehyde dehydrogenase Ald4p rabbit polyclonal antibody for the cytosolic fraction. Spheroplasts were prepared using Zymolyase 100T, as described previously (Komiya *et al.*, 2002).

Partial purification of 1,3-β-glucan synthase. Partial purification of 1,3-β-glucan synthase used the method described previously (Takasuka *et al.*, 1995). Briefly, exponentially growing *S. cerevisiae* A451 cells (3×10^7 cells ml^{-1}) were collected by centrifugation and were disrupted with glass beads in 50 mM Tris/HCl pH 7.5 containing 10 mM EDTA, 0.5 M NaCl, 1 μM PMSF, 1 μg leupeptin ml^{-1} and 1 μg pepstatin A ml^{-1} , and centrifuged at 1000 g for 5 min at 4 °C. The supernatant was centrifuged at 100 000 g for 30 min at 4 °C. The precipitate was suspended in buffer A (50 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 33% glycerol) containing 0.16 M NaCl, 20 μM guanosine 5'-[γ-thio]triphosphate (GTP_γS), 5 mM DTT, 0.5% CHAPS and 0.1% cholesteryl hemisuccinate (CHS), and was incubated for 30 min at 4 °C. The suspension was centrifuged at 100 000 g for 30 min at 4 °C, and then 20 mM KF and 5 mM uridine diphosphate glucose (UDP-Glc) were added to the supernatant, and the mixture was incubated for 30 min at 30 °C. 1,3-β-Glucan polymer was formed and was collected by centrifugation at 1500 g for 5 min at 4 °C. The pellet was washed twice with buffer B (buffer A, 4 μM GTP_γS, 1 mM DTT, 0.4% CHAPS and 0.08% CHS) and 10 mM UDP-Glc. 1,3-β-Glucan synthase was extracted from the pellet by incubation with buffer B for 10 min at 30 °C.

Measurement of 1,3-β-glucan synthase activity. 1,3-β-Glucan synthase activities were examined as described previously (Takasuka *et al.*, 1995). Briefly, 2 μl partially purified 1,3-β-glucan synthase was incubated in 40 μl reaction mixture consisting of 20 mM Tris/HCl pH 7.5, 0.4 mM EDTA, 20 mM KF and 5 mM UDP-Glc containing 0.02 kBq UDP-[U-¹⁴C]glucose for 60 min at 30 °C. After incubation, 250 μl cold 10% trichloroacetic acid was added. The solution was kept at 4 °C for 10 min and then was filtered through glass. The filters were washed four times with cold 10% trichloroacetic acid and then twice with 95% ethanol. The radioactivities retained on

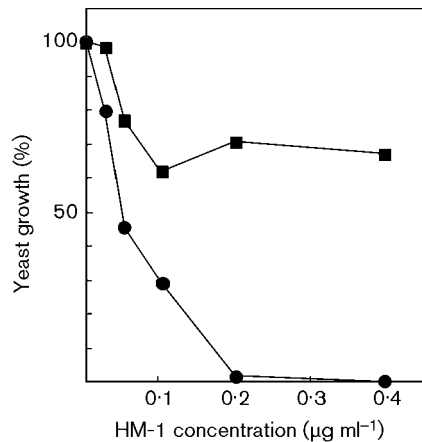


Fig. 1. Yeast killing activity of HM-1 and DEPC-modified HM-1 against *S. cerevisiae* A451. Procedures for the modification of HM-1 with DEPC and killing activity measurement are described in Methods. ●, wild-type HM-1; ■, DEPC-modified HM-1.

the filter were counted using a liquid scintillation counter. To measure the inhibition of 1,3- β -glucan synthase, wild-type or H35A HM-1 was added to the reaction mixture at various concentrations. The reactions were done in triplicate.

Co-immunoprecipitation of HM-1 and 1,3- β -glucan synthase.

Co-immunoprecipitation was carried out as described by Harlow & Lane (1999). After mixing 0.6 μ g partially purified 1,3- β -glucan synthase with 20 ng wild-type HM-1 or H35A HM-1, 1 μ l anti-HM-1 rabbit serum or 0.5 μ l anti-Fks1p mouse monoclonal antibody 1F4 was added. BSA (0.6 μ g) was used in place of HM-1 and 1,3- β -glucan synthase as the negative control. Then the solution was combined with 10 μ l protein G-Sepharose 4 Fast Flow (Amersham Pharmacia) and kept on ice for 1 h with occasional mixing. The mixture was then centrifuged and the precipitate washed four times with PBS (-) containing 0.1% CHAPS and 0.02% CHS. The precipitate was incubated with SDS-PAGE sample buffer at 96 °C for 5 min and the supernatant subjected to SDS-PAGE and Western blotting.

RESULTS

Modification of HM-1 with DEPC

In our previous study (Miyamoto *et al.*, 2005), the importance of histidine-35 in HM-1 for the expression of yeast killing activity was shown by using site-directed mutagenesis. Therefore, modification of histidine-35 in HM-1 with DEPC was expected to cause loss of the yeast killing

activity. After incubation of HM-1 with 5 mM DEPC for 30 min, 1.13 moles of histidine modified for each mole of HM-1 was calculated as an increase in absorbance at 240 nm (Schäger & von Jagow, 1987). Fig. 1 shows the yeast killing activity of wild-type and DEPC-modified HM-1. The strong killing activity of wild-type HM-1 was greatly decreased by DEPC modification, although about 30% of the killing activity remained. These results indicate that one mole of histidine is essential for the killing activity of HM-1.

SDS-PAGE of HM-1 analogues secreted by mutant gene-bearing yeasts

To verify the importance of the specific characteristic of the imidazole group of histidine-35 in the yeast killing activity by HM-1, we replaced histidine-35 with 19 other amino acids (indicated by their one-letter symbols) using site-directed mutagenesis. Supernatants of the concentrated culture of the HM-1 gene- or histidine-35-substituted HM-1 gene-bearing yeasts were subjected to SDS-PAGE and Western blotting (Fig. 2). All HM-1 analogues, except H35P and H35W HM-1, had 9.5 kDa bands corresponding to the molecular size of wild-type HM-1, but the band intensities varied among the supernatants. These results indicate that HM-1 analogue expressions were successful and the amount of secretion varied among the mutants. H35S and H35T HM-1 showed an extra band that migrated to a higher molecular mass position.

Quantification of secreted HM-1 analogues

Using concentrated culture supernatants, the amounts of HM-1 analogues secreted by mutant gene-bearing yeasts were measured by ELISA (Table 1). H35A, H35K and H35R HM-1 are secreted into the culture broth at about half the amount of wild-type HM-1. The amounts of H35M, H35N, H35Q, H35S and H35T HM-1 were about eightfold lower than that of wild-type HM-1, and H35C, H35D, H35E, H35F, H35G, H35I, H35L, H35P, H35V, H35W and H35Y HM-1 were secreted at even smaller amounts.

Estimation of killing activity by killer eclipse assay

To estimate the killing activity of histidine-35-substituted HM-1 analogues against sensitive yeasts, we used a killer eclipse assay (Fig. 3). Test yeast strain, *H. anomala* IFO 0569, had high sensitivity to wild-type HM-1. The wild-type HM-1 gene-bearing yeasts showed strong killing activity

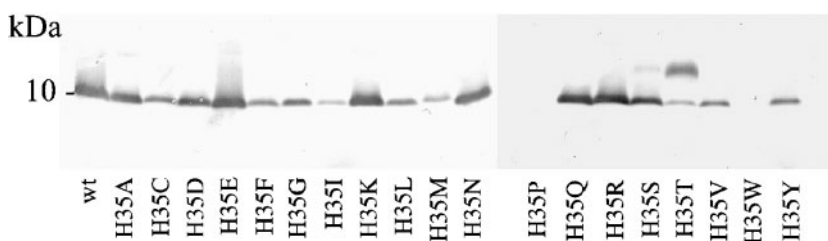


Fig. 2. Western blotting of HM-1 analogues. Aliquots (5 μ l) of 20 times concentrated supernatants of cultured broth underwent 15% SDS-PAGE and then immunoblotting as described in Methods. Amino acids are indicated by one-letter symbols. wt, wild-type.

Table 1. Amounts and IC₅₀ values of HM-1 analogues secreted by HM-1 analogue gene-bearing yeasts

Amino acids are indicated by one-letter symbols. ND, Not detected.

Analogue	Concentration (ng ml ⁻¹)	IC ₅₀ (ng ml ⁻¹)
Wild-type	2160	85
H35A	870.0	2575
H35C	7.95	ND
H35D	58.7	ND
H35E	120	ND
H35F	38.9	ND
H35G	67.0	ND
H35I	40.0	ND
H35K	1190	ND
H35L	99.0	ND
H35M	253	ND
H35N	279	ND
H35P	7.10	ND
H35Q	185	ND
H35R	1230	ND
H35S	230	ND
H35T	200	ND
H35V	79.0	ND
H35W	0.45	ND
H35Y	53.1	ND

against *H. anomala*, but all histidine-35-substituted HM-1 gene-bearing yeasts showed no killing activity.

Killing activity estimation by IC₅₀ values

Table 1 shows the killing activities of HM-1 analogues measured by IC₅₀ values. The IC₅₀ value of H35A HM-1 was 30 times larger than for wild-type HM-1. The other histidine-35-substituted HM-1 gene-bearing yeasts showed no killing activity even at the highest concentrations under our experimental conditions. Therefore, we could not estimate the exact IC₅₀ values of these HM-1 analogues.

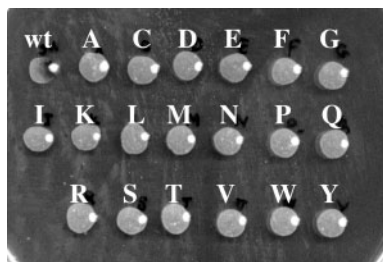


Fig. 3. Killer eclipse assay of HM-1 analogues. *H. anomala* IFO 0569 was used as a test strain. The assay method is described in Methods. wt, wild-type. Substituted amino acids at position 35 in HM-1 are indicated by their one-letter symbol.

CD spectrum of wild-type and H35A HM-1

To examine the possibility that histidine replacement with alanine perturbed the conformation of the secondary structure of HM-1, we carried out a CD spectroscopic analysis of wild-type HM-1 and H35A HM-1. H35A HM-1 had a CD spectrum similar to wild-type HM-1 (Fig. 4), even though H35A HM-1 lost its yeast killing activity.

Subcellular localization of wild-type HM-1 and H35A HM-1

To verify how histidine-35 of HM-1 is related to the location of the protein in yeast cells, we examined subcellular localization of wild-type HM-1 and H35A HM-1, after incubation with yeast cells, using Western blotting. The purity of each subcellular fraction was determined using anti-Fks1p antibody 1F4 for the membrane fraction and anti-aldehyde dehydrogenase Ald4p antibody for the cytosolic fraction. Fig. 5(c) shows that the transmembrane enzyme Fks1p (200 kDa) was detected only in the membrane fraction and cytosolic enzyme Ald4p (56 kDa) was detected in the cytosolic fraction, but some nonspecific bands were also seen in each fraction. Wild-type HM-1 bands were detected in the cell wall fraction and cytosolic fraction of HM-1 sensitive *S. cerevisiae* BJ1824 (lane 1) and *C. albicans* ATCC 10231 (lane 5), and in the membrane fraction of *S. cerevisiae* BJ1824 (lane 1) (Fig. 5a). But H35A HM-1 bands were not detected in any cellular fractions of *S. cerevisiae* and *C. albicans* after 1 h incubation with whole yeast cells (lanes 2, 6). Wild-type and H35A HM-1 bands were not detected in the cellular fractions of *S. cerevisiae* BJ1824 *rhk1Δ::URA3* (lanes 3, 4) that is an HM-1-resistant yeast strain (Kimura *et al.*, 1997). In periplasmic fractions, we could not find any band of HM-1 (data not shown). Fig. 5(b) shows the subcellular localizations of wild-type and H35A HM-1 using the spheroplasts. A wild-type HM-1 band was detected in the membrane fraction and weakly in

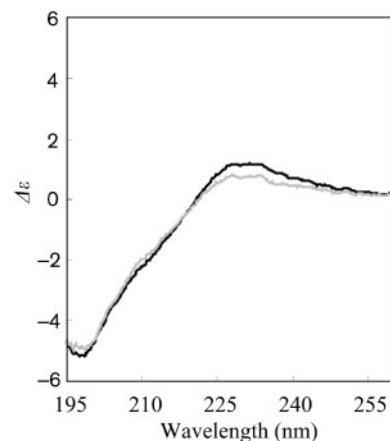


Fig. 4. CD spectra of wild-type and H35A HM-1. Spectra were measured as described in Methods. Black line, wild-type HM-1; grey line, H35A HM-1.

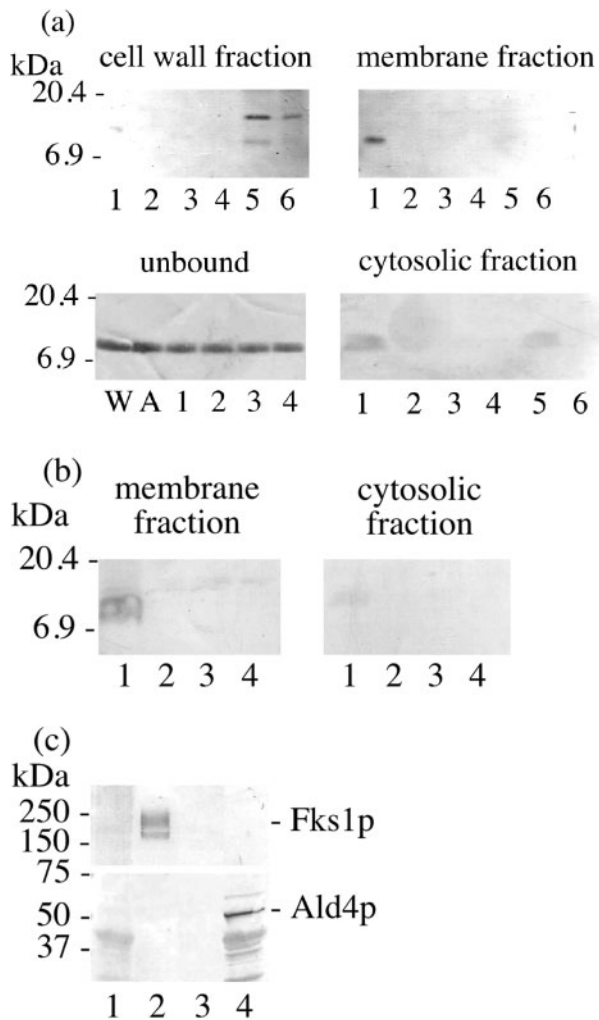


Fig. 5. Subcellular localizations of wild-type HM-1 and H35A HM-1 in *S. cerevisiae* BJ1824 and BJ1824*rhk1Δ::URA3*. (a) Yeast whole cells, *S. cerevisiae* BJ1824 (lanes 1, 2), *S. cerevisiae* BJ1824*rhk1Δ::URA3* (lanes 3, 4) or *C. albicans* (lanes 5, 6), were incubated with wild-type HM-1 (lanes 1, 3, 5) or H35A HM-1 (lanes 2, 4, 6) and each cell was fractionated as described in Methods. Each fraction was applied to SDS-PAGE and HM-1 bands were detected by Western blotting using anti-HM-1 rabbit serum as the primary antibody. Ten nanograms each of wild-type (W) and H35A (A) HM-1 were electrophoresed as a control. (b) Yeast spheroplasts were used with the same methods as for (a). (c) Completion of each cellular fractionation of *S. cerevisiae* BJ1824 was determined by Western blotting using anti-Fks1p mouse monoclonal antibody 1F4 for the membrane fraction and anti-Ald4p rabbit polyclonal antibody for the cytosolic fraction. Cell wall, membrane, periplasmic and cytosolic fractions of *S. cerevisiae* BJ1824 are shown in lanes 1, 2, 3 and 4, respectively.

the cytosolic fraction of *S. cerevisiae* BJ1824 (lane 1), but the H35A HM-1 band was not detected in the cellular fractions (lane 2). Wild-type and H35A HM-1 bands were not detected in any fractions of *S. cerevisiae* BJ1824

rhk1Δ::URA3 (lane 3, 4). These results show that wild-type HM-1 localizes in the cell membrane whether a cell wall is present or not, but H35A HM-1 does not localize in the cell membrane.

1,3- β -Glucan synthase inhibition by wild-type HM-1 and H35A HM-1

Wild-type HM-1 strongly inhibits 1,3- β -glucan synthase activity of susceptible yeasts (Takasuka *et al.*, 1995). In this study we examined the effect of H35A HM-1 on 1,3- β -glucan synthase activity (Fig. 6). H35A HM-1 inhibited 1,3- β -glucan synthase activity in a concentration dependent manner and at almost the same effectiveness as wild-type HM-1. This result indicates that replacement of histidine-35 with alanine has no effect on the inhibition of 1,3- β -glucan synthase activity by HM-1.

Co-immunoprecipitation of HM-1 and 1,3- β -glucan synthase

We believe that whether or not HM-1 interacts with 1,3- β -glucan synthase directly has not been reported before, and so we carried out a co-immunoprecipitation analysis to verify the direct interaction between HM-1 and 1,3- β -glucan synthase *in vitro* (Fig. 7). Incubations of HM-1 with partially purified 1,3- β -glucan synthase and co-immunoprecipitation with anti-Fks1p antibody were detected using anti-HM-1 rabbit serum as the primary antibody (Fig. 7a). Lane 5 shows that wild-type HM-1 co-immunoprecipitated with 1,3- β -glucan synthase subunit Fks1p, but without Fks1p wild-type HM-1 was not detected (lane 4). By similar incubations H35A HM-1 also co-immunoprecipitated with 1,3- β -glucan synthase, but the intensity of the band was weaker than for wild-type HM-1 (lane 7). These results indicate that wild-type and H35A HM-1 interact with 1,3- β -

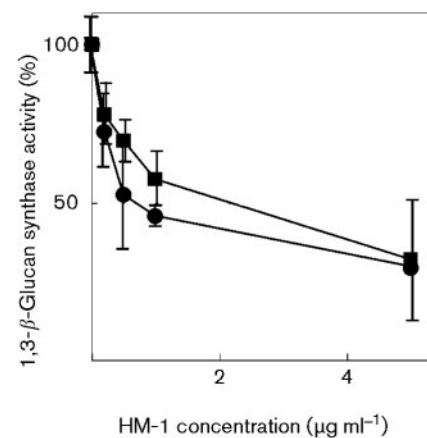


Fig. 6. Inhibition of partially purified 1,3- β -glucan synthase activity by wild-type and H35A HM-1. 1,3- β -Glucan synthase activity was measured in the presence of wild-type HM-1 or H35A HM-1 as described in Methods. ●, wild-type HM-1; ■, H35A HM-1.

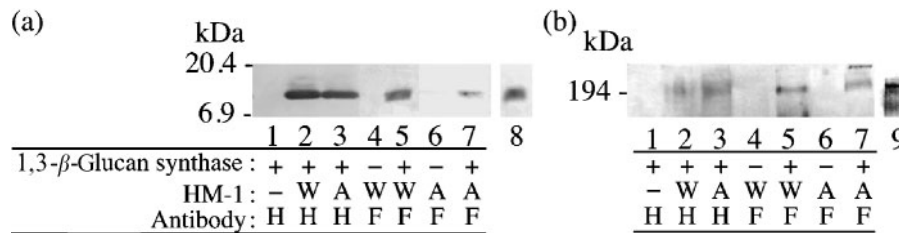


Fig. 7. Co-immunoprecipitation analysis of the interaction between HM-1 and 1,3- β -glucan synthase. After mixing partially purified 1,3- β -glucan synthase (lanes 1, 2, 3, 5, 7) or BSA (lanes 4, 6), with wild-type HM-1 (lanes 2, 4, 5) or H35A HM-1 (lanes 3, 6, 7) or BSA (lane 1), anti-HM-1 rabbit serum (lanes 1–3) or anti-Fks1p mouse monoclonal antibody (lanes 4–7) was added. Protein G-Sepharose 4 Fast Flow was added and centrifuged to precipitate protein molecular complexes. The extracted complexes were subjected to SDS-PAGE and Western blotting using anti-HM-1 rabbit serum (a) or anti-Fks1p mouse monoclonal antibody (b) as the primary antibody for the detection. Wild-type HM-1 (lane 8) and partially purified 1,3- β -glucan synthase (lane 9) were electrophoresed as a control. W, wild-type HM-1; A, H35A HM-1; H, anti-HM-1 rabbit serum; F, anti-Fks1p mouse monoclonal antibody.

glucan synthase directly *in vitro*. In the case of co-immunoprecipitations with anti-HM-1 antibody and detections by anti-Fks1p antibody, the high molecular mass band indicative of Fks1p (molecular mass 200 kDa) precipitated with wild-type and H35A HM-1 (Fig. 7(b), lane 2, 3), but without HM-1, Fks1p was not detected (lane 1). This result also supports our finding that wild-type and H35A HM-1 interact with 1,3- β -glucan synthase *in vitro*.

DISCUSSION

Previously, by using an alanine-scanning method, we showed that histidine-35 is essential for the yeast killing activity of HM-1 (Miyamoto *et al.*, 2005). This conclusion is supported by the DEPC modification experiment of this study (Fig. 1), which modified the sole histidine residue in HM-1 and resulted in the loss of 70% of the yeast killing activity.

To examine the importance of this histidine residue in HM-1 killing activity, we further substituted histidine-35 by 19 other amino acids, and estimated the amounts of secreted histidine-substituted analogues and their killing activities. All such analogues were successfully expressed and showed bands of the same molecular mass as wild-type HM-1, but in different quantities (Fig. 2). In the case of substitution by hydrophobic amino acid residues, the amounts of HM-1 analogues secreted tended to decrease. In particular, bands of H35P and H35W HM-1 were not found by Western blotting. Probably, in the synthesis process, these amino acid residues destroyed the core structure of HM-1 due to their hydrophobicity or by the steric hindrance of their side chain, and the analogues folded into an unstable structure that was degraded by proteolysis (Kowalski *et al.*, 1998; Ellgaard & Helenius, 2003). H35S and H35T HM-1 had an extra high molecular mass band. Histidine-35 substitution with serine or threonine creates a consensus sequence for the N-linked glycosylation site -N-X-S/T- in HM-1, so it is possible that H35S and H35T HM-1 have a glycosylated side chain attachment at the position of asparagine-33. Substitutions by

hydrophilic amino acids at the histidine-35 residue yielded varying amounts of HM-1 analogues (Table 1). The amounts of H35A, H35K and H35R HM-1 production were half of wild-type HM-1, but the amounts of other analogues produced were much lower than for wild-type HM-1. These results indicate that position 35 requires a relatively small and/or positively charged amino acid side chain for efficient synthesis and secretion of HM-1.

We estimated the strength of killing activities of HM-1 analogues by killer eclipse assay and IC_{50} measurement. Wild-type HM-1 gene-bearing yeasts had strong killing activity against *H. anomala*, but all histidine-35-substituted HM-1 gene-bearing yeasts showed no killing activity (Fig. 3, Table 1). It is notable that positively charged amino acids lysine and arginine, and aromatic amino acids tyrosine and phenylalanine, did not recover the killing activity. Replacement of histidine-35 with alanine did not affect the secondary structure of HM-1 (Fig. 4). These results indicate that the yeast killing activity of HM-1 requires the specific features of the imidazole group of the histidine side chain at position 35.

Some killer toxins bind to yeast cell wall molecules as a first receptor (Hutchens & Bussey, 1983; Tipper & Schmitt, 1991; Kimura *et al.*, 1998; Marquina *et al.*, 2002). The killing action of *S. cerevisiae* killer toxin K1 is believed to be a multi-step process (Bussey, 1981; Martinac *et al.*, 1990; Kurzweilova & Sigler, 1994; Schmitt & Compain, 1995). The first step is the binding of toxin to the cell wall 1,6- β -glucan, and the next step is interaction with the plasma membrane receptors. The tertiary structure of *Pichia farinosa* killer toxin SMKT is similar to that of HM-1 (Kashiwagi *et al.*, 1997; Kunishima *et al.*, 1997). SMKT localizes on the surface of the plasma membrane and is removed from the membrane by sodium carbonate treatment (Suzuki *et al.*, 2001). HM-1 interacts weakly with the yeast cell wall (Kasahara *et al.*, 1994), but what component interacts with it after this is not clear. Therefore, we examined the subcellular localization of HM-1 in yeasts using whole cells and spheroplasts. Fig. 5(a, b)

(lane 1) shows that wild-type HM-1 localizes in the membrane fraction of HM-1 sensitive yeast cells and spheroplasts. In contrast to SMKT, HM-1 bound strongly to the cell membrane and was not removed by sodium carbonate treatment. However, H35A HM-1 did not localize to the membrane and other fractions in HM-1 sensitive yeast (Fig. 5a, b; lane 2). These results indicate that histidine-35 in HM-1 has an important role in the interaction with molecules of the cell membrane, the putative HM-1 receptors.

C. albicans ATCC 10231 bound to wild-type HM-1 in cell wall and cytosolic fractions, but not in the membrane fraction, and its interactions were weaker than for *S. cerevisiae* BJ1824 (Fig. 5a, lane 5). These may cause the difference in sensitivity of HM-1 against *S. cerevisiae* (MIC = 0.4–1.6 $\mu\text{g ml}^{-1}$) and *C. albicans* (MIC > 300 $\mu\text{g ml}^{-1}$) (Yamamoto *et al.*, 1988).

HM-1-resistant yeast *S. cerevisiae* BJ1824 *rhk1* Δ ::*URA3* did not bind to wild-type HM-1 and H35A HM-1 (Fig. 5a, b; lanes 3, 4). The α -1,3-mannosyltransferase Rhk1p participates in the HM-1 sensitivity of yeasts and contributes to the maintenance of the polysaccharide composition of the cell wall and its structure (Kimura *et al.*, 1997, 1999). The HM-1 resistance of *S. cerevisiae* BJ1824 *rhk1* Δ ::*URA3* strain is explained by the deficiency of the Rhk1p causing perturbation of the cell wall structure so that HM-1 cannot bind there. In this study, we showed that wild-type HM-1 interacts with spheroplast membranes, but cannot interact with *RHK1* deleted spheroplast membranes (Fig. 5b). These results indicate that wild-type HM-1 can interact with the putative HM-1 receptor on the cell membrane regardless of whether the cell wall is present or not, and the receptor is likely to need Rhk1p-associated modification in its molecule for interaction with HM-1.

Since a possibility that histidine-35 has an important role in the interaction with 1,3- β -glucan synthase remained, we checked the interaction of HM-1 with 1,3- β -glucan synthase. H35A HM-1 was able to inhibit 1,3- β -glucan synthase to the same extent as wild-type HM-1 (Fig. 6), and H35A HM-1 interacted directly with 1,3- β -glucan synthase *in vitro* (Fig. 7). These results indicate that histidine-35 in HM-1 is not essential for the interaction and inhibition of 1,3- β -glucan synthase. After solubilization of HM-1-treated yeast membrane fraction with various detergents, most HM-1 was not extracted in the same fraction as 1,3- β -glucan synthase (data not shown). These results support the idea that the presence of a putative HM-1 receptor other than 1,3- β -glucan synthase is required on the cell membrane for the interaction with HM-1.

In conclusion, the importance of histidine-35 for the expression of the killing activity of HM-1 was shown by using chemical modification and site-directed mutagenesis. Subcellular localization and co-immunoprecipitation experiments indicated that the role of histidine-35 in HM-1 is related to the binding process of HM-1 to its

putative receptor protein, not to the inhibition of 1,3- β -glucan synthase activity.

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