Isolation and amino acid sequence of a mating pheromone produced by mating type α cells of *Saccharomyces kluyveri*

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Received 18 November 1983

A peptide, termed α^{sk2} pheromone, was isolated from culture filtrates of mating type α cells of *Saccharomyces kluyveri* as a mating pheromone, having both shmoo inducing (in *a* cells of *S. kluyveri* and *S. cerevisiae*) and agglutinability inducing (in *a* cells of *S. cerevisiae*) actions. The amino acid sequence of α^{sk2} pheromone was determined as H–Trp–His–Trp–Leu–Ser–Phe–Ser–Lys–Gly–Glu–Pro–Met–Tyr-OH by mass spectrometry, sequence analysis and enzymatic digestions.

Saccharomyces kluyveri *Mating pheromone* α^{sk} *pheromone* *Amino acid sequence*

1. INTRODUCTION

In the sexual process of heterothallic strains of *Saccharomyces* yeasts, mating reaction occurs between the two opposite mating *a* and α type cells. In *S. cerevisiae*, mating pheromones of α substance–I_A [1] or α factors [2], called α^{sc} pheromones here, secreted from α cells have been isolated and chemically characterized as regulating substances, which induce sexual agglutinability and formation of large pear-shaped cell form called shmoos in the opposite mating type *a* cells.

Authors in [3] reported that α cells of *S. kluyveri* also secrete a pheromone(s) which shows shmoo inducing action on *a* cells of both *S. cerevisiae* and *S. kluyveri*. Recently, we isolated from the culture filtrate of α cells of *S. kluyveri* a peptide, α_k substance, termed α^{sk1} pheromone here, which induces sexual agglutinability in *a* cells of *S. cerevisiae* [4], and proposed the amino acid sequence of the peptide [5] shown in fig.1 (I).

Abbreviations: HPLC, high-performance liquid chromatography; SIMS, secondary ion mass spectrometry

X-His-Trp-Leu-Ser-Phe-Ser-Lys-Gly-Glu-Pro-Met(O)-Tyr-OH

I . α^{sk1} Pheromone (α_k Substance)

H-Trp-His-Trp-Leu-Ser-Phe-Ser-Lys-Gly-Glu-Pro-Met-Tyr-OH

II . α^{sk2} Pheromone

Fig.1. Amino acid sequence of α^{sk} pheromones.

The α^{sk1} pheromone possesses an as yet unknown residue X at its N-terminus, and its methionine residue is oxidized to the sulfoxide. Examination of the biological activity of this peptide and its synthetic analogues revealed that the peptide with a methionine residue showed stronger activity than that with a methionine sulfoxide residue [6]. This evidence indicated that α^{sk1} pheromone might be an artifact caused by oxidation during the purification procedure. To clarify this, we improved the purification procedure to be more effective and used mild conditions by application of HPLC. This re-investigation resulted in the isolation of a new peptide, α^{sk2} pheromone (II), with higher activity than that of I, as the main active principle.

We describe here the isolation, biological activi-

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

ty and amino acid sequence of α^{sk2} pheromone produced by the mating type α cells of *S. kluyveri*.

2. MATERIALS AND METHODS

α Cells of *S. kluyveri* IFO 1894 were cultured in a jar fermentor as in [5] and the active principles of the culture filtrate (75 l) were purified by adsorption on an Amberlite CG-50 column eluted with ethanol–0.07 M hydrochloric acid (8:2, v/v), Sephadex LH-20 chromatography with methanol–acetic acid (98:2, v/v), adsorption on a column of Biorex 70 eluted with 1-propanol–0.05 M pyridinium formate buffer (pH 2.4) (3:7, v/v) and then Sephadex G-25 chromatography with 10% acetic acid, to give a crude active material (100 mg).

Biological activity of the pheromones in inducing sexual agglutinability of inducible *a* cells of *S. cerevisiae* was investigated using strains of *S. cerevisiae* T55s-41 (inducible *a* cells) and T56 (constitutive α cells) as in [5,6]. For a reference pheromone, α-mating factor (Protein Research Foundation) was used as α^{sc} pheromone.

HPLC was performed on a Spectro Physics SP 8100 Liquid Chromatograph with an SP 8440 UV/VIS detector monitored at 220 or 280 nm on a packed column of Hypersil ODS or Nucleosil $5C_{18}$ in solvent systems described below.

Amino acid compositions were determined by acid hydrolysis of each peptide (20–10 nM) with 5.7 N HCl containing 3% thioglycollic acid in an evacuated tube at 110°C for 20 h, followed by amino acid analysis on a JEOL JLH-6AH amino acid analyzer.

Mass spectrometric analyses by SIMS were performed on a Hitachi M80 spectrometer equipped with an SIMS ion source. Xenon gas was used as a source of primary ions and the accelerating voltage of secondary ions was set at 1.5 kV. Samples were applied as a glycerol matrix on an Ag sample holder.

Sequence analysis was performed on an Applied Biosystems 470A Protein Sequenator using 24 nM peptide. For digestions with enzyme, the peptide was incubated with TPCK-trypsin (Worthington Biochemicals) in 0.05 M ammonium acetate buffer (pH 6.5) at 27°C for 18 h or with thermolysin (Daiwakasei) in 0.05 M ammonium acetate buffer (pH 6.5) containing 10 mM $CaCl_2$ at 40°C for 12 h at an enzyme:substrate ratio of 1:20 (w/w).

3. RESULTS AND DISCUSSION

3.1. *Isolation*

The crude active material obtained from the culture filtrate of α cells of *S. kluyveri* was fractionated by HPLC on a column of Hypersil ODS (20 × 300 mm) using a solvent system of 1-propanol–acetonitrile–0.1% trifluoroacetic acid (10:40:50) with a flow rate of 10 ml/min. Fractions were collected according to retention times of 2–5 min each of the eluate. Strong activity was detected in fraction V of the collected eluate at 28–32 min, and weak activity was observed in the fractions VI (32–36 min), VII (36–38 min) and VIII (38–53 min).

On analytical HPLC on Nucleosil $5C_{18}$ (6 × 150 mm) with a solvent system of acetonitrile–0.1% trifluoroacetic acid (29:71) with a flow rate of 1 ml/min, fraction V showed several peaks detected by UV absorption. Among them, a peak at a retention time of 16.1 min was found to constitute the main active principle which was different from α^{sk1} pheromone in retention time. By the same HPLC analysis, fraction VIII was found to contain a peptide identical with α^{sk1} pheromone which showed a peak at a retention time of 15.0 min.

The main active component showing the peak at 16.1 min was obtained in a pure state (14.1 mg) through preparative HPLC on a column of Nucleosil $5C_{18}$ (8 × 200 mm) with the same solvent system as above, and was named α^{sk2} pheromone.

3.2. *Biological activity*

The agglutinability-inducing action of the purified α^{sk2} pheromone on the inducible *a* cell of *S. cerevisiae*, together with that of α^{sc} pheromone, is shown in fig.2. The α^{sk2} pheromone showed detectable activity even at 1 ng/ml, indicating that its activity is 10–100-times higher than that of α^{sk1} pheromone [6]. The α^{sc} pheromone was more active at lower concentrations but less active at higher concentrations than α^{sk2} pheromone. Further, we also observed a strong shmoo-inducing action of α^{sk2} pheromone on *a* cells of both *S. cerevisiae* and *S. kluyveri* at 1 μg/ml (not shown).

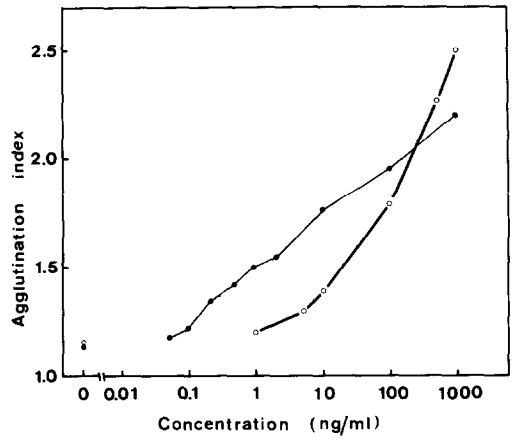

Fig.2. Agglutination-inducing action of α^{sk2} and α^{sc} pheromones on inducible *a* cells of *S. cerevisiae*. T55s-41 (inducible *a*) cells were treated with α^{sk2} or α^{sc} pheromone, and sexual agglutinability of the treated cells was expressed by agglutination indexes measured after mixing with α tester cells (T56) having high sexual agglutinability [6]. (○—○) α^{sk2}, (●—●) α^{sc}.

3.3. *Amino acid sequence*

On amino acid analysis of the acid hydrolysate, the following amino acids (molar ratio to Leu = 1.0) were detected in α^{sk2} pheromone: Ser (1.8), Glu (1.1), Pro (1.0), Gly (1.1), Met (1.0), Leu (1.0), Tyr (1.0), Phe (1.0), Lys (1.1), His (0.9) and Trp (1.5). Mass spectrometric analysis by SIMS gave a quasimolecular ion $(M + H)^+$ at m/z 1666. This value coincides with the molecular mass of a peptide composed of 2 Ser, 1 Glu, 1 Gly, 1 Pro, 1 Leu, 1 Met, 1 Tyr, 1 Phe, 1 Lys, 1 His and 2 Trp with free N- and C-termini. Thus, α^{sk2} pheromone was confirmed as being composed of the above 13 amino acids and as possessing a methionine residue.

Table 2

Amino acid composition of the peptide fragments obtained by enzymatic digestions of α^{sk2} pheromone

Amino acids	Tryptic fragments		Thermolytic fragments			
	Tr-1	Tr-2	Th-1	Th-2	Th-3	Th-4
Ser	2			1	1	
Glu		1			1	
Pro		1			1	
Gly		1			1	
Met		1				1
Leu	1			1		
Tyr		1				1
Phe	1				1	
Lys	1				1	
His	1		1			
Trp	2		2			

Sequence analysis was carried out with a protein sequenator and the resulted PTH amino acids were unambiguously identified as shown in table 1. Digestion of the peptide with trypsin gave two peptide fragments and that with thermolysin 4 peptide fragments. Each peptide fragment was purified by HPLC on a column of Nucleosil $5C_{18}$ (8 × 200 mm) with solvent systems of acetonitrile–0.1% trifluoroacetic acid (5:95–25:75, stepwise). The amino acid composition of each peptide fragment was determined and the results are shown in table 2. These results showed a good agreement with the sequence deduced from the analysis with a sequenator.

Table 1

Amino acid sequence analysis of α^{sk2} pheromone

Degradation steps	1	2	3	4	5[a]	6	7[a]	8	9	10[b]	11	12	13
PTH-amino acids identified	Trp	His	Trp	Leu	Ser	Phe	Ser	Lys	Gly	Glu	Pro	Met	Tyr

[a] In the 5th and 7th steps, a degradation product of PTH-serine was detected
[b] In the 10th step, only PTH-glutamic acid was detected

PTH amino acids were identified by HPLC on a Zorbax ODS column (4.6 × 300 mm) with a solvent system of acetonitrile–0.04 M sodium acetate buffer (pH 4.8)–water (programmed from 36.5:10.0:53.5 to 90.0:2.0:8.0, in 19 min)

The amino acid sequence of α^{sk2} pheromone was thus confirmed as II (fig.1). The α^{sk2} pheromone has a free N-terminus of tryptophan and non-oxidized methionine residue and showed higher activity than α^{sk1} pheromone. From this evidence, α^{sk2} pheromone should be regarded as a genuine mating pheromone secreted by α cells of *S. kluyveri*.

ACKNOWLEDGEMENTS

We would like to thank Mr S. Fukushima of Nikkaki Co. for amino acid sequence analysis with the protein Sequenator, and Mr M. Chijimatsu of this institute for amino acid analyses.

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