Isolation and structure of the *Streptococcus faecalis* sex pheromone, cAM373

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The *Streptococcus faecalis* sex pheromone, cAM373, which induces a mating response of donor cells harboring plasmid pAM373 and is also produced by *Staphylococcus aureus*, was isolated and its structure determined. Supernatant from an overnight culture of a recipient strain was subjected to successive purification procedures, and 4.4 μg cAM373 was obtained. The isolated pheromone showed activity at a concentration as low as 5×10^{-11} M. Sequence analysis indicated that cAM373 was a heptapeptide, H-Ala-Ile-Phe-Ile-Leu-Ala-Ser-OH, and that its M_r was 733. A synthetic replicate of the peptide showed the same biological activity and chromatographic behavior as the native cAM373.

(Streptococcus faecalis, Staphylococcus aureus) *Sex pheromone* *Conjugative plasmid transfer* *Amino acid sequence*

1. INTRODUCTION

Recipient strains of *Streptococcus faecalis* excrete small peptidal sex pheromones which induce mating responses in donor strains harboring certain conjugative plasmids; donors harboring different conjugative plasmids respond to different sex pheromones. Donor cells induced by sex pheromone synthesize a proteinaceous substance on the cell surface, which facilitates the formation of mating aggregates. Since donor cells mixed with a cell-free filtrate of recipients undergo self-aggregation or clumping, sex pheromone is also called clumping-inducing agent (CIA) (review [1]). Recently, two of the pheromones, cPD1 and cAD1, which specifically induce the mating response of donor strains harboring plasmids pPD1 (determines bacteriocin) or pAD1 (determines hemolysin) respectively, have been isolated and characterized. Their sequences were determined [2,3] and found to be:

cPD1: H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH

cAD1: H-Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly-OH

Another sex pheromone, cAM373, also excreted by recipient strains of *S. faecalis* and involved in the conjugative transfer of the plasmid pAM373, is characterized by the fact that its activity is also detected in cultre filtrates of certain strains of *S. sanguis* and *S. faecium* and all of the *Staphylococcus aureus* strains examined [4]. However, since pAM373 does not transfer in culture broth from *S. faecalis* to these organisms, it is conceivable that cAM373 activity does not play a pheromonal role

Abbreviations: CIA, clumping-inducing agent; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; FAB, fast atom bombardment; Boc, *t*-butoxycarbonyl; Bzl, benzyl

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in these species. It is noteworthy that almost all coagulase-negative staphylococci (e.g. *St. epidermidis*) do not excrete cAM373 activity [4]. This raises the possibility that in the genus *Staphylococcus* cAM373 activity might contribute to virulence. Here we report the isolation, structure elucidation and total synthesis of the sex pheromone cAM373.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and media*

The responder strain for assaying cAM373 was *S. faecalis* FA373 which harbors the conjugative plasmid pAM373 [4]. Strain *S. faecalis* JH2-2 (pAM351) [5], which harbors a derivative of plasmid pPD1, pAM351, but can be a recipient for plasmid pAM373, was used as a source of cAM373.

Todd-Hewitt Broth (Oxoid) was used in the CIA assays. For cAM373 production, THG medium (18.2 g Oxoid Todd-Hewitt broth, 20 g glucose, 1 l distilled water) was employed.

2.2. *Bioassay*

CIA activity was assayed using the microtiter dilution method described in [6]. One unit of activity was defined as the lowest amount that could induce clumping of responder cells in 100 μl of assay medium in a microtiter dilution well.

2.3. *Isolation of cAM373*

The pheromone-producing strain JH2-2 (pAM351) was grown (0.25% inoculum) in THG medium (4 l per batch) with gentle stirring under anaerobic conditions at 37°C for 20 h (stationary phase). The grown cells were pelleted by centrifugation and the supernatants corresponding to 2 batches (8 l) were passed through a charcoal column (Wakojunyaku, 3.6 × 35 cm). The active material was adsorbed and recovered with 50% pyridine. The eluate was 5-fold diluted with water and applied to a DEAE-Sephadex A-25 column (Pharmacia, 3.4 × 11 cm, acetate form); the column was eluted with a gradient of 0.05 (150 ml) to 0.2 (150 ml) M ammonium acetate in 20% ethanol. The active material obtained in this step corresponding to 4 batches (16 l culture broth) was combined and subjected to reverse-phase HPLC on an LRP-2 column (Whatman, 2.0 × 30 cm). The column was eluted with a gradient of 15–45% acetonitrile in 10 mM ammonium acetate for 60 min at 10 ml/min, and the active fractions were 2-fold diluted with 0.1% heptafluorobutyric acid and charged on the LRP-2 column again at 10 ml/min. The column was eluted with a gradient of 20–50% acetonitrile in 0.1% heptafluorobutyric acid for 60 min at 10 ml/min, and the active fractions were combined.

The active material thus obtained was divided into two portions and each portion (equivalent to 8 l culture broth) was further purified using two cycles through reverse-phase HPLCs on an SSC-ODS-742 column (Senshukagaku, 1.0 × 25 cm) at 4 ml/min with gradients of (i) 15–24% (5 min) and 24–29% (25 min) acetonitrile in 0.1% TFA, and then (ii) 10–21% (5 min) and 21–26% (25 min) acetonitrile in 10 mM triethylammonium phosphate buffer (pH 5). The fractions containing partially purified cAM373 were further divided into four aliquots and each aliquot (equivalent to 4 l culture broth) was then subjected to HPLC on a Senshupak CN-4251-N column (Senshukagaku, 1.0 × 25 cm) and eluted with a gradient of 5–25% (40 min) acetonitrile in 0.1% TFA at 4 ml/min. The active fractions thus obtained corresponding to 4 batches (16 l culture broth) were combined again and applied to final purification with reverse-phase HPLC on an SSC-ODS-262 column (Senshukagaku, 0.6 × 10 cm), and a gradient of 10–22% (10 min) and 22–28% (30 min) acetonitrile in 0.1% TFA at 1 ml/min afforded cAM373 as a single peak.

2.4. *Amino acid sequence analysis*

1 nmol cAM373 was subjected to a gas-phase protein sequencer (model 470A, Applied Biosystems) [7], and the phenylthiohydantoin amino acid derivatives were identified with a PTH analyzer (model 120A, Applied Biosystems).

2.5. *FAB mass spectrum*

2 μl of 50% acetonitrile solution containing cAM373 (2 μg) were added to a matrix of glycerol (about 2 μl) containing 1 μl of 1 N HCl on a stainless-steel probe tip and the tip was introduced into the ion source of a mass spectrometer. Analysis was performed with a JMS DX-303 mass spectrometer (Jeol) using xenon as the fast atom.

2.6. *Synthesis of cAM373 replicate*

The protected heptapeptide, Boc-Ala-Ile-Phe-Ile-Leu-Ala-Ser-OBzl, was synthesized in solution by the fragment condensation between N-terminal tripeptide and C-terminal tetrapeptide. N-terminal tripeptide was synthesized by the step-wise chain elongation method. To synthesize C-terminal tetrapeptide, Boc-Leu-Ala-OH and H-Ser-OBzl were coupled and the resulting tripeptide was treated with TFA following acylation with Boc-Ile-OH. All the coupling reactions were carried out using HONB (*N*-hydroxy-5-norbornene-2,3-dicarboximide)-DCCD (*N,N'*-dicyclohexylcarbodiimide) or the HONB-activated ester method [8].

After deblocking all the protecting groups from the protected heptapeptide by TFA treatment and hydrolysis, the desired material was collected from water. The purity of the product was checked by thin-layer chromatography with silica gel 60 F_{254} (Merck) and amino acid analysis. [The R_f values of synthesized cAM373 were 0.71 (solvent system, ethyl acetate-*n*-butanol-acetic acid-water, 1:1:1:1) and 0.76 (solvent system, *n*-butanol-pyridine-acetic acid-water, 4:1:1:2). Amino acid ratio of hydrolysate of cAM373 with 6 N HCl for 100 h: Ser, 1.0; Ala, 2.07; Ile, 1.98; Leu, 1.0; Phe, 0.95.]

3. RESULTS AND DISCUSSION

The *S. faecalis* sex pheromone cAM373 from 16 l of culture supernatant was obtained through a purification procedure consisting of 8 steps, by which we generated 35000000-fold purification and obtained 4.4 μg pure peptide. The total weight, total activity and specific activity of the active material in each step are summarized in table 1. During the purification, the 7th step using a Senshupak CN-4251-N column was most effective, and enabled 150-fold purification. In each step of the purification, the biological activity was monitored by the CIA assay [6]. The specific activity of the purified cAM373 was 3.4 pg/unit (about 5×10^{-11} M), a value similar to those found for cPD1 [2] and cAD1 [3].

Since inactivation experiments of cAM373 with proteolytic enzymes showed that the active substance should be a peptide [4], the isolated substance was subjected to sequence analysis with a gas-phase protein sequencer equipped with a PTH analyzer. As a result of analysis, the amino acid sequence, H-Ala-Ile-Phe-Ile-Leu-Ala-Ser-, was identified, and no amino acids were detected after the 8th step. The FAB mass spectrum of cAM373 showed a quasi-molecular ion peak at *m/z* 734 $(M+H)^+$; consequently, the M_r of cAM373 was deduced to be 733, which explains the above-mentioned sequence with a free C-terminus.

Table 1

Isolation of cAM373

Purification step	Total weight (mg)	Total activity (units)	Specific activity (ng/unit)
Culture broth (16 l)	610000[a]	5120000	119000
Charcoal, activated	8300[b]	2560000	3200
DEAE-Sephadex	1100[b]	2560000	430
LRP-2 (i)	180[b]	2560000	70
LRP-2 (ii)	85[b]	1800000	47
SSC-ODS-742 (i)	22[b]	1280000	17
SSC-ODS-742 (ii)	1.9[c]	1280000	1.5
CN-4251-N	0.013[c]	1280000	0.01
SSC-ODS-262	0.0044[c]	1280000	0.0034

[a] Dry weight
[b] Calculated from absorbance at 280 nm
[c] Calculated from absorbance at 220 nm

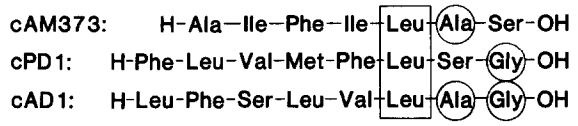

Fig.1. Amino acid sequence of *Streptococcus faecalis* sex pheromones. Coincident residues are encircled (coincident in two of three peptides) or boxed (consensus in all three peptides).

Accordingly, the structure of cAM373 was determined as H-Ala-Ile-Phe-Ile-Leu-Ala-Ser-OH. A heptapeptide possessing this structure was chemically synthesized and showed the same biological activity and chromatographic behavior as the naturally obtained cAM373.

Although the pheromone cAM373 differs from the other two pheromones in the number of amino acid residues, it shares some structural characteristics with the pheromones cPD1 and cAD1; i.e. Leu as the 3rd residue from the C-terminus, absence of acidic and basic residues, unusual hydrophobicity of the molecule, and Ser as the only hydrophilic residue (fig.1). In spite of these resemblances, cross-activity was never observed between these pheromones (not shown). It was revealed that the N-terminal part of the peptides was responsible for the specificity of pheromones to plasmids through synthetic studies on the structure-activity relationships of cPD1 and cAD1 [9]. This may hold true in the case of cAM373, since the three peptides appear to differ in the N-terminal region whereas they conserve relatively high homology in the C-terminal part (fig.1).

Whether the substance showing cAM373 activity in cultures of bacterial species other than *S. faecalis* is identical with *S. faecalis* cAM373 and what role the activity plays in such microorganisms are very interesting questions. In this connection, the purification and characterization of the cAM373 activity present in cultures of a *St. aureus* strain are now in progress.

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REFERENCES

[1] Clewell, D.B., White, B.A., Ike, Y. and An, F.Y. (1984) in: Microbial Development (Losik, R. and Shapiro, L. eds) pp.133–149, Cold Spring Harbor Press, Cold Spring Harbor, NY.

[2] Suzuki, A., Mori, M., Sakagami, Y., Isogai, A., Fujino, M., Kitada, C., Craig, R.A. and Clewell, D.B. (1984) Science 226, 849–850.

[3] Mori, M., Sakagami, Y., Narita, M., Isogai, A., Fujino, M., Kitada, C., Craig, R.A., Clewell, D.B. and Suzuki, A. (1984) FEBS Lett. 178, 97–100.

[4] Clewell, D.B., An, F.Y., White, B.A. and Gawron-Burke, C. (1985) J. Bacteriol. 162, 1212–1220.

[5] Ike, Y., Craig, R.A., White, B.A., Yagi, Y. and Clewell, D.B. (1983) Proc. Natl. Acad. Sci. USA 80, 5369–5373.

[6] Dunny, G.M., Craig, R.A., Carron, R.L. and Clewell, D.B. (1979) Plasmid 2, 454–465.

[7] Hewick, R.M., Hunkapiller, M.W., Hood, L.E. and Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990–7997.

[8] Fujino, M., Kobayashi, S., Obayashi, M., Fukuda, T., Shinagawa, S. and Nishimura, O. (1974) Chem. Pharm. Bull. (Tokyo) 22, 1857–1863.

[9] Kitada, C., Fujino, M., Mori, M., Sakagami, Y., Isogai, A., Suzuki, A., Clewell, D.B. and Craig, R.A. (1985) in: Peptide Chemistry-1984 (Izumiya, N. ed.) pp.43–48, Protein Research Foundation, Osaka.