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Determination of the amino acid sequence of an intramolecular disulfide linkage-containing sperm-activating peptide by tandem mass spectrometry

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A sperm-activating peptide (SAP) was isolated from the egg jelly of the sea urchin *Stomopneustes variolaris*. The presence of an intramolecular disulfide linkage in the peptide was demonstrated by fast atom bombardment (FAB) mass spectrometry with the intact and reduced peptides. The amino acid sequence of the reduced peptide was determined to be Lys-Phe-Cys-Pro-Glu-Gly-Lys-Cys-Val by tandem mass spectrometry from the spectrum produced by a collision-induced decomposition method. Furthermore, it was also demonstrated that SAPs obtained from sea urchins *Arbacia punctulata* and *Glyptocidaris crenularis* are cyclic peptides containing one cystine residue by FAB mass spectrometry.

Sperm-activating peptide; Cystine-containing; Tandem mass spectrometry; Collision-induced decomposition

1. INTRODUCTION

Sperm-activating peptides (SAPs) isolated from the egg-conditioned media (egg jelly) of sea urchins have several effects on sea urchin spermatozoa. SAPs cause stimulation of sperm respiration and motility through intracellular alkalinization, transient elevations of cAMP, cGMP and Ca^{2+} levels in sperm cells, and transient activation and subsequent inactivation of the membrane form of guanylate cyclase [1,2]. A specific receptor for SAP-IIA (resact), Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH_2, is the membrane form of guanylate cyclase and the same as natriuretic peptide receptors and a heat-stable enterotoxin receptor [3-5]. It is reported that a receptor for SAP-I (speract), Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly, possesses several cysteine-rich domains which are analogous to that of the type I macrophage scavenger receptor [6].

Tandem mass spectrometry (MS/MS) has been successfully applied to sequence analysis of a linear peptide [7]. Despeyroux et al. have developed a method using MS/MS to determine the amino acid sequence of a cyclic peptide containing cystine [8]. In the present study, we isolated a new SAP which has two half-cystine residues from the egg jelly of the sea urchin *Stomopneustes variolaris*, and applied the method for determination of the amino acid sequence of the peptide. In addition, using a mass spectrometric method we found that SAP-IIA from *Arbacia punctulata* and SAP-IIB and its five derivatives from *Glyptocidaris crenularis* contain an intramolecular disulfide linkage.

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2. EXPERIMENTAL

2.1. *Isolation of* S. variolaris *SAP*

SAP was isolated from 66% ethanol extract of the egg jelly of the sea urchin *S. variolaris* as described in [9].

2.2. *High-performance liquid chromatography (HPLC)*

HPLC was carried out using C_8 columns as described in [9]. Programs I, II and III described in [9] were used for isolation of SAP.

2.3. *Fast atom bombardment (FAB) mass spectrometry*

FAB mass spectra were obtained with a JEOL JMS-HX100 double-focusing mass spectrometer or a JEOL JMS-HX110/110 four-sector tandem mass spectrometer equipped with an FAB ion source and a JEOL JMA-DA7000 data acquisition system, as described in [10]. In MS/MS mode, the precursor ion was selected by the first mass spectrometer and decomposed in a collision cell. The amount of the collision gas (He) introduced into the cell was adjusted to reduce the intensity of the precursor ion by half of its initial value. The daughter ion spectrum was obtained by collision-induced decomposition (CID) of precursor ion in a B/E-linked scan mode, where the ratio of the magnetic field and electric field of second mass spectrometer was kept constant.

2.4. *Peptide synthesis*

A peptide, Lys-Phe-Cys-Pro-Glu-Gly-Lys-Cys-Val, was synthesized using a solid-phase method with use of a MilliGen 9050 PepSynthesizer. Methylbenzhydrylamine polystyrene resin containing 9-fluorenylmethyloxycarbonyl (Fmoc)-L-Val (Fmoc-L-Val PAC Support) (0.3 meq/g) was used as starting material. The following amino acid active esters were used for the synthesis: Fmoc-L-Glu γ-*t*-butyl ester α-pentafluorophenyl ester (-OPfp), Fmoc-L-Pro-OPfp, Fmoc-Gly-OPfp, Fmoc-*S*-trityl-L-Cys-OPfp, Fmoc-L-Phe-OPfp, and N^{α}-Fmoc-

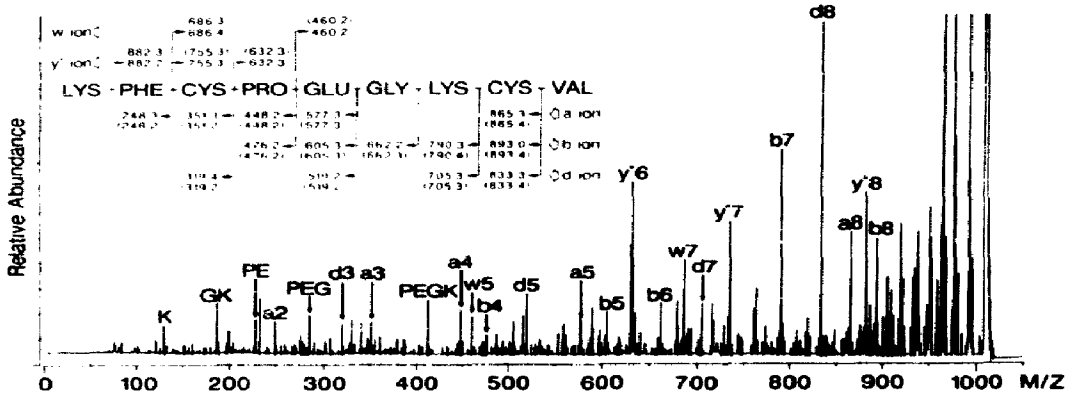

Fig. 1. FAB/MS/MS CID spectrum of the $[M+H]^+$ ion (m/z = 1010.4) observed in the spectrum of the reduced peptide SV-1. The insert shows an interpretation of the CID spectrum. The nomenclature used is based on that used in [7,12].

N$^{\alpha}$-*t*-butyloxycarbonyl-L-Lys-OPfp. The resin with protected peptide was treated with *m*-cresol, 1,2-ethanedithiol and thioanisole in trifluoroacetic acid (TFA) at 0°C for 2 h. After removal of TFA, the resulting peptide was extracted with 1 M acetic acid, washed with ethyl ether, and air-oxidized in dilute solution at pH 7.5 for 4 h.

Synthetic SAP-IIA was a generous gift from Dr. H. Shimomura. Synthetic SAP-IIB was previously synthesized in our laboratory by a liquid-phase method [11].

2.5. *Amino acid analysis*

Amino acid analysis was performed using a Hitachi L-8500 amino acid analyzer after hydrolysis for 20 h in constant-boiling HCl (5.7 N) at 110°C in vacuo.

2.6. *Determination of sperm respiration rates*

Sperm respiration rates were determined as described in [11].

3. RESULTS AND DISCUSSION

3.1. *Purification of an egg jelly peptide*

The ethanol extract of *S. variolaris* egg jelly obtained from 10 individuals was concentrated, delipidated, and then subjected to HPLC using Program I. An active fraction was purified further by HPLC using Program III and then Program II. An active peptide (SV-1) was purified in a final amount of 13.0 nmol. The amino acid composition was determined to be $Glu_{0.84}$ (1); $Pro_{1.27}$ (1); $Gly_{1.06}$ (1); 1/2$Cys_{1.65}$ (2); $Val_{1.00}$ (1); $Phe_{1.02}$ (1); $Lys_{2.01}$ (2) (mol/mol of Val).

3.2. *Primary structure of* S. variolaris *SAP*

FAB mass spectrum of the SAP gave a protonated molecular ion at m/z = 1008.5 which was identical with the theoretical value calculated from the amino acid composition with two half-cystines. In addition, intense sequence ions (m/z = 908.2, 880.4, 864.3, 733.3 and 717.2) were clearly observed, suggesting that the N-terminal sequence is Lys-Phe- and that the C-terminus is Val. To confirm the presence of an intramolecular disulfide linkage, the solution of the peptide (1 mM) in a reducing buffer (1% NH_4HCO_3, pH 6.8) was prepared, to which was added an equal volume of a reductive matrix (dithiothreitol/dithioerythritol, 5:1 w/w). In the FAB mass spectrum the reduced peptide gave a signal at m/z = 1010.4 which was nearly identical to the theoretical mass value (1010.5) of a peptide containing two cysteines instead of two half-cystines.

The amino acid sequence of the resulting linear peptide was then determined by MS/MS from the spectrum produced by the CID of the $[M+H]^+$ ion of the peptide [8]. As shown in Fig. 1, many sequence ions (types a_n, b_n, d_n, y''_n, and w_n) were observed giving sequence information to be established as Lys-Phe-Cys-Pro-Glu-Gly-Lys-Cys-Val. Furthermore, five internal sequence ions at m/z = 412.3 (PEGK), 284.2 (PEG), 227.3 (PE), 186.3 (GK) and 129.3 (K) were observed in the spectrum.

To confirm the proposed structure, a peptide was synthesized according to this sequence. The oxidized and reduced synthetic peptides were eluted separately from each other by HPLC, and the synthetic oxidized

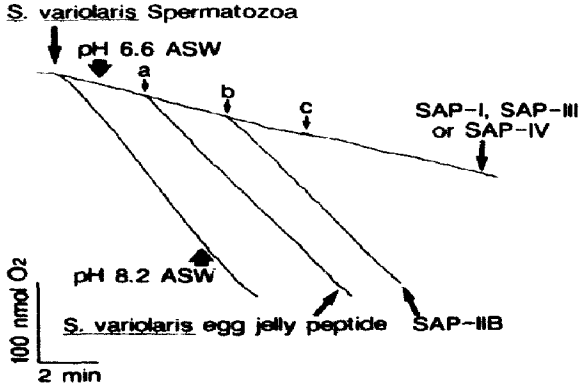

Fig. 2. Respiration-stimulating effect of peptide SV-1 on *S. variolaris* spermatozoa (10 mg wet weight/ml artificial sea water). Arrows (a, b and c) indicate the points at which *S. variolaris* egg jelly peptide (0.5 μM), SAP-IIB (1 μM) and other SAPs (SAP-I, SAP-III and SAP-IV) (1 μM) were added.

Table I

Observed mass values and amino acid sequences of sperm-activating peptides obtained from the egg jelly of sea urchins *A. punctulata* and *G. crenularis*

	AP-1	GC-1	GC-2	GC-3	GC-4	GC-5	GC-6
$[M+H]^+$ of intact peptide	1243.4 (1243.6)	1046.2 (1046.5)	888.2 (888.4)	760.1 (760.3)	1136.2 (1136.5)	902.2 (902.4)	774.0 (774.3)
$[M+H]^+$ of reduced peptide	1245.3 (1245.6)	1048.4 (1048.5)	890.1 (890.4)	762.1 (762.3)	1138.4 (1138.5)	904.1 (904.4)	776.2 (776.3)

AP-1: Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH_2 (SAP-IIA)
GC-1: Ser-Ala-Lys-Leu-Cys-Pro-Gly-Gly-Asn-Cys-Val (Ser-Ala-SAP-IIB)
GC-2: Lys-Leu-Cys-Pro-Gly-Gly-Asn-Cys-Val (SAP-IIB)
GC-3: Leu-Cys-Pro-Gly-Gly-Asn-Cys-Val (Des-Lys^1-SAP-IIB)
GC-4: Ser-Phe-Lys-Leu-Cys-Pro-Gly-Gly-Gln-Cys-Val (Ser-Phe-[Gln^7]SAP-IIB)
GC-5: Lys-Leu-Cys-Pro-Gly-Gly-Gln-Cys-Val ([Gln^7]SAP-IIB)
GC-6: Leu-Cys-Pro-Gly-Gly-Gln-Cys-Val (Des-Lys^1-[Gln^7]SAP-IIB)

The mass value in parentheses is shown as the theoretical mass value of each peptide. The amino acid sequences have been determined by automatic Edman degradation [11,13]. With regard to SAP-IIA, the experiment was performed using a synthetic peptide of which retention time on HPLC is identical with that of natural SAP-IIA [13].

peptide showed the same retention time as that of the natural peptide. (Amino acid composition of the synthetic peptide: $Glu_{1.10}$ (1); $Pro_{1.14}$ (1); $Gly_{1.13}$ (1); 1/2$Cys_{1.77}$ (2); $Val_{1.00}$ (1); $Phe_{0.99}$ (1); $Lys_{2.08}$ (2) (mol/mol of Val). Observed mass values ($[M+H]^+$): 1008.4 (oxidized); 1010.4 (reduced).)

In conclusion, the primary structure of peptide SV-1 was established to be Lys-Phe-Cys-Pro-Glu-Gly-Lys-Cys-Val by the mass spectrometric method without alkylation of peptide. This sequence is similar to that of SAP-IIB (alioresact) [11].

3.3. *Effects of the peptide on sperm respiration*

Peptide SV-1 was a quite potent stimulator for *S. variolaris* sperm respiration. The respiration was stimulated by the peptide as well as by SAP-IIB, but not by SAP-I, SAP-III and SAP-IV (Fig. 2). The peptide stimulated *G. crenularis* sperm respiration with 350 pM of ED_{50} value, which is comparable to those (500-1000 pM) with SAP-IIB and its five derivatives from *G. crenularis* [10]. This suggests that substitutions of Leu^2 by Phe, Gly^5 by Glu, and Asn^7 or Gln^7 by Lys do not alter the respiration-stimulating activity. These substitutions could occur by single-point mutation on a genetic code.

3.4. *Determination of a disulfide linkage in SAPs from* A. punctulata *and* G. crenularis

Although it has been demonstrated that SAP-IIA from *A. punctulata*, SAP-IIB and five SAP-IIB derivatives from *G. crenularis* contain two cysteine residues, the presence of a disulfide linkage in these peptides has not yet been confirmed. The observed mass values of intact peptides were nearly identical with the theoretical mass values calculated from the respective amino acid sequence with two half-cystines. Then, FAB mass spectrometry showed that reduction of these peptides by the reductive matrix increased the mass of these peptides by 2 atomic mass units (Table I).

The natural SAP-IIB was found to co-elute with the oxidized synthetic peptide on HPLC using C_8 column. However, the retention time of the natural peptide differed from that of the reduced synthetic peptide on HPLC. (Amino acid composition of the synthetic SAP-IIB: $Asp_{1.06}$ (1); $Pro_{1.09}$ (1); $Gly_{2.17}$ (2); 1/2$Cys_{1.75}$ (2); $Val_{1.00}$ (1); $Leu_{1.01}$ (1); $Lys_{0.94}$ (1) (mol/mol of Val). Observed mass value ($[M+H]^+$): 888.3 (oxidized); 890.1 (reduced).)

From these results, we concluded that SAP-IIA, SAP-IIB and five SAP-IIB derivatives are also cyclic peptides containing an intramolecular disulfide linkage.

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