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STRUCTURE AND ACTION OF SPERM ACTIVATING PEPTIDES FROM THE EGG JELLY OF A SEA URCHIN, ANTHOCIDARIS CRASSISPINA

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SUMMARY: Three Sperm Activating Peptides (SAPs) have been isolated to homogeneity from the jelly coat of the eggs of a Japanese sea urchin, Anthocidaris crassispina, and two of them have been sequenced. At pH 6.8 they can stimulate the sperm respiration 20-30 fold, to the level in normal seawater (pH 8.2), and the half-maximal activation was achieved by SAPs as low as around 100 pM. The stimulative activity was both pH- and Na^+-dependent. The chymotryptic fragments (res. 3-10) were 10^4-10^5 times less active, and the thermolytic fragment (res. 4-10) was 10^6 times less active than the parent SAP. CD spectra of SAPs indicate that they have unordered structure in aqueous solution.

The jelly coat of sea urchin egg has a few important functions in addition to the protection of the egg itself. A fucose sulfate polymer, one of the components of the jelly, triggers the acrosome reaction of sperm (1), and a sialoglycoprotein is responsible for the agglutination of sperm (2). Recently two peptides which stimulate the sperm respiration and motility have been isolated from the egg jelly of Hemicentrotus pulcherrimus and their primary structures have been determined (3). This is an advance from the previous work on Sperm Activating Substance (4). Later the same peptide was found in Strongylocentrotus purpuratus (5). These peptides seem to activate the sperm respiration by accelerating the Na^+-H^+ exchange and eventual

ABBREVIATIONS: SAP, Sperm Activating Peptide; ASW, artificial seawater; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; MES, 2-[N-morpholino]ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; E/S, enzyme-to-substrate ratio; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate.

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increase of intracellular pH (6,7). The peptides also increase the sperm cAMP and cGMP concentration (8). This paper describes the purification, primary and secondary structure, and some characteristic actions of new SAPs from another Japanese sea urchin, A. crassispina.

EXPERIMENTAL PROCEDURE

Materials: Sea urchins, A. crassispina and H. pulcherrimus were collected at Japan Sea coast (Tsukumo Bay) near Noto Marine Laboratory. Artificial seawater, Jamarin was purchased from Jamarin Laboratory (Osaka). Thermolysin and chymotrypsin were purchased respectively from Seikagaku Kogyo (Tokyo) and Miles-Seravac (England). CH_3CN of HPLC grade was from Wako Chemical Comp. (Osaka). Other reagents were of analytical grade.

Methods: The gametes were obtained by intracoelomic injection of 0.5 M KCl. The purification procedures for SAPs from egg jelly was basically the same as previouly reported (3), except the final HPLC step added in this experiment. Amino acid analysis was carried out with an automatic amino acid analyzer, Hitachi 835-50 after hydrolysis with constant-boiling HCl. The amino acid sequences of A-1 and A-2 were determined by automated Edman degradation with a Beckman Sequencer 890C using the Beckman Program 102974 (3). The COOH-terminal residues were confirmed by hydrazinolysis (9). The activity of the peptides to stimualte the respiration of sperm was measured with a Yanaco PO-100A Oxgen Consumption Recorder with a rotating platinum electrode, in Jamarin buffered to pH 6.8 with 25 mM HEPES (3,10). The sperm density in the incubation mixture (3.0 ml) was 1.2×10^9/ml. CD spectra of the peptides (0.1-0.2 mg/ml) were measured in a quarz cell of 5 mm path length with a spectropolarimeter JASCO J-500A.

RESULTS

Purification and sequence of SAPs: The peptides purified by several steps of column chromatographies and TLC were designated as A-1 (R_f=0.78) and A-2 (0.72). Both preparations were slightly cross-contaminated, and so further purified by HPLC, which enabled the complete separation of A-1 and A-2 as well as the isolation of the third peptide A-20 (Fig. 1). Each peak was rechromatographed with 0.1% TFA and CH_3CN-gradient and the main peak was lyophilized. From 2006 female sea urchins, 24.8 mg of partially purified SAP was obtained prior to the step of TLC, and from 4 mg of that, highly purified A-1 (0.15 mg), A-2 (0.40 mg) and A-20 (0.08 mg) were recovered. By automated Edman degradation following PTH-amino acids were consecutively released: from A-1 (40 nmol), (1) Gly (11.5 nmol), (2) Phe (7.9 nmol), (3) Asp (not determined, ND), (4) Leu (4.5 nmol), (5) Thr (ND), (6) Gly (1.5 nmol), (7) Gly (1.0 nmol), (8) Gly (ND), (9) Val (1.4 nmol), (10) Gly (0.8 nmol); and from A-2 (60 nmol), (1) Gly (30.0 nmol), (2) Phe (ND), (3) Asp (ND), (4) Leu

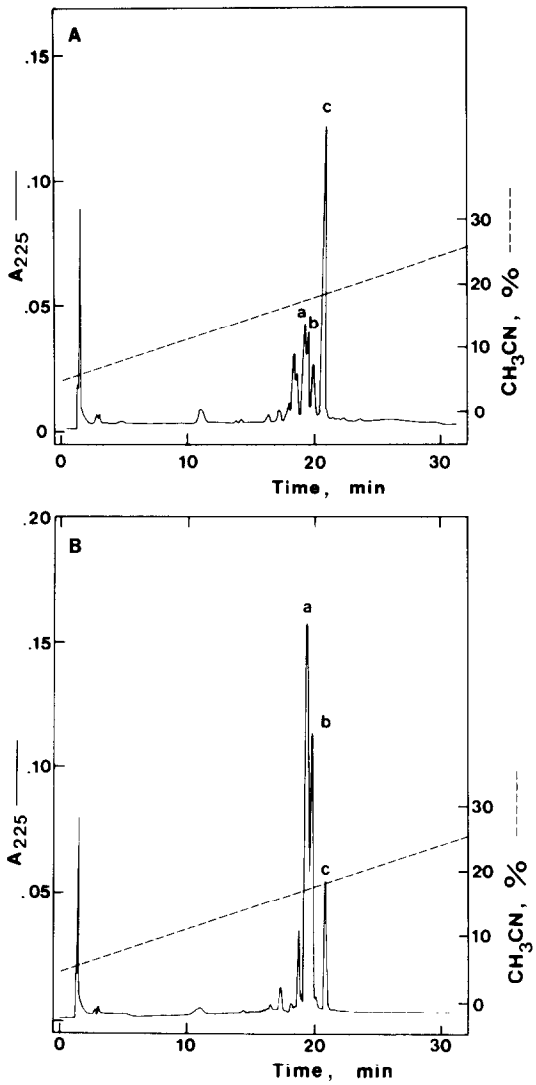

Fig.1. HPLC of partially purified A-1 (A) and A-2 (B). An apparatus of Shimadzu LC-4A with a data processor, chromatopack C-R1A was used. A reversed phase column, Zorbax C8 (4.6 x 250 mm) kept at 40°C was equilibrated with 5 mM Na-phosphate buffer (pH 5.7) containing 5% CH_3CN. Elution was carried out by the linear gradient of CH_3CN from 5% to 30% in 40 min with the flow rate of 1.5 ml/min. The eluate was monitored continuously by the absorbance at 225 nm. The peaks are designated as: a) A-2, b) A-20, and c) A-1.

(28.3 nmol), (5) Ser (ND), (6) Gly (8.6 nmol), (7) Gly (9.2 nmol), (8) Gly (ND), (9) Val (5.7 nmol), and (10) Gly (1.1 nmol). The repetitive yields through residue 7 were 65% for A-1 and 82% for A-2. Their amino acid sequences are shown in Table 1 with the amino acid composition and NH_2-terminal residue of A-20. The sequence of A-2 was confirmed by the analyses on the two thermolytic fragments: amino acid analysis, dansylation,

Table 1
Amino acid sequences of Sperm Activating Peptides from the egg jelly coat of two sea urchins

Symbol of SAP	Amino acid sequence	Calculated Mr
A-1	Gly-Phe-Asp-Leu-Thr-Gly-Gly-Gly-Val-Gly	879
A-2	Gly-Phe-Asp-Leu-Ser-Gly-Gly-Gly-Val-Gly	865
A-20	Gly-(Phe,Asp,Leu,Ser,Gly,Ser,Ser,Val,Gly)	925
H-1	Gly-Phe-Asp-Leu-Thr-Gly-Gly-Gly-Val-Gly	879
H-2	Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly	892

The sequences of A-1 and A-2 are determined in this experiment, and that of H-1 is revised from the previously reported (3). A-1, A-2 and A-20 are from Anthocidaris crassispina, and H-1 and H-2 are from Hemicentrotus pulcherrimus.

hydrazinolysis, digestion with aminopeptidase M, carboxypeptidases A and P (data not shown). SAPs H-1 and H-2 from H. pulcherrimus (3) were also further purified by HPLC, and the amino acid composition and the COOH-terminal residues were reinvestigated. The results are also shown in Table 1. H-1 turned out to be identical to A-1, with additional tenth Gly residue as COOH-terminus, comparing with the 9-residue sequence previously reported (3).

Proteolysis of SAPs: The digest of A-2 by thermolysin (E/S=1/200, w/w, 1 h) and those of A-1 and A-2 by chymotrypsin (E/S=1/50, w/w, 48 h) were applied to HPLC to separate the fragments. Analyses on the fragments revealed that thermolysin selectively cleaved the Asp^3-Leu^4 bond, while chymotrypsin cleaved only the Phe^2-Asp^3 bond.

Respiratory stimulation of sperm by SAPs and proteolytic fragments: The suppressed respiration of Hemicentrotus sperm at pH 6.8 (10-15 nmol O_2/min) was remarkably increased by SAPs of 10 nM order to the level observed in normal seawater (pH 8.2) or a little higher than that (about 270 nmol O_2 /min). From the sigmoidal curves by semilogarithmic plots of activity and concentration (Fig. 2), the half-maximal concentration (AC_{50}) of A-1, A-2 and A-20 were given as 90 pM, 73 pM and 110 pM, respectively. By a sharp contrast, the chymotryptic fragments, A-1(Cht) and A-2(Cht) with residues 3-10, were 10^4-10^5 times less active than the parent SAPs. Furthermore, one

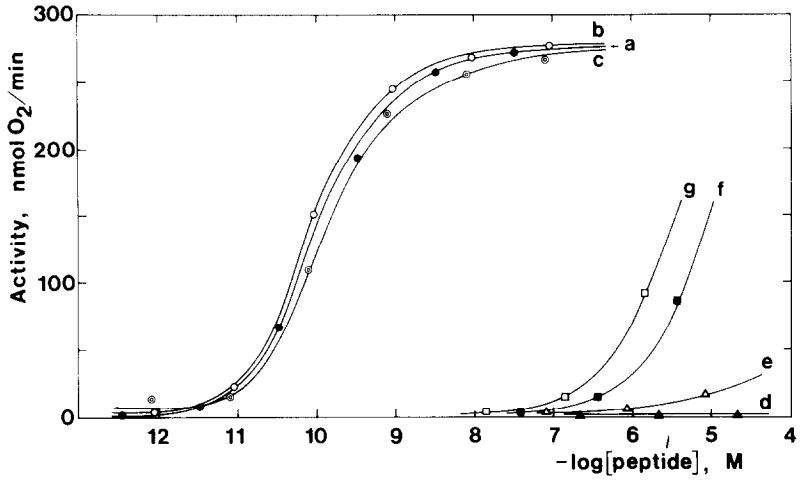

Fig. 2. Activity-concentration profile of the peptides. The respiration-enhancing activities of SAPs A-1 (a), A-2 (b), A-20 (c), thermolytic fragments A-2N (d) and A-2C (e), and chymotryptic fragments (octapeptides) A-1(Cht) (f) and A-2(Cht) (g) are plotted against the logarithm of their concentrations. The activity is expressed by the net increase in the initial rate of O_2 consumption in the incubatoin mixture of 3 ml.

of the thermolytic fragments, A-2N (Gly-Phe-Asp) was inactive even at 20 μM, and the counterpart, A-2C (res. 4-10), was only poorly active (20 nmol O_2 /min) at 8μM, or about 10^6 times less active than A-2. The activity of SAPs was also measured on the homologous Anthocidaris sperm. The AC_{50} values were about 10 times higher than the values on Hemicentrotus sperm.

pH- and Na^+-dependence of SAP activity: Jamarin was buffered with 25 mM MES (pH 6.0, 6.5) and HEPES (pH 7.0, 7.5, 8.0 and 8.4). The stimulative activity of A-1 and A-2 was observed between pH 6.0 and 8.0 with the optimum value at pH 7.0. To obtain various concentration of NaCl (15-450 mM) in ASW, choline chloride was substituted for NaCl. The basal respiration was slightly Na^+-dependent, but the SAP-stimulated one was much more dependent on Na^+. The respiration rate increased with the rising Na^+ concentration and saturatued above 150 mM, with half-maximal stimulation at about 70 mM.

CD spectra of SAPs: CD spectra of A-1 and A-2 in H_2O and in 0.5 M NaCl have a maximum at 219 nm and a minimum at 229 nm (Fig. 3). These spectra seem to have no suggestion of ordered structure such as α-helix and β-sheet but rather unordered structure. However, the COOH-terminal sequence rich in Gly residues may produce β-turn like structure in the molecule under proper

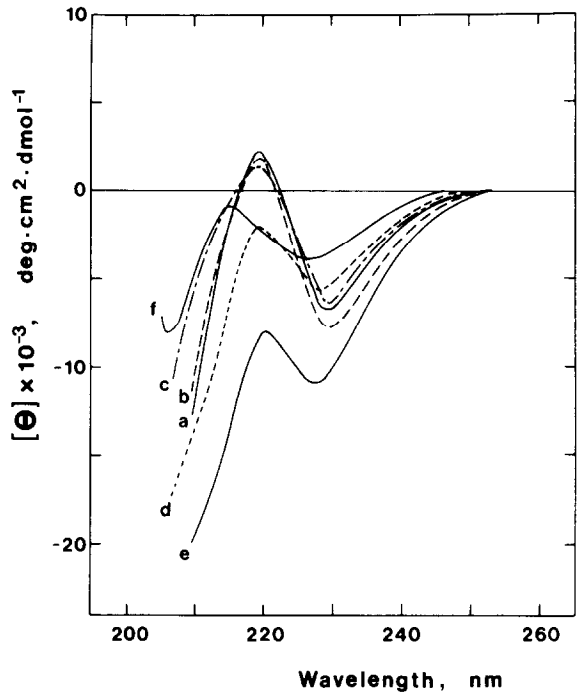

Fig. 3. CD spectra of A-1, A-2 and A-1(Cht). Each solution (0.1 or 0.2 mg/ml) in a quarz cell of 5 mm path length was measured with a spectropolarimeter JASCO J-500A with a data processor. a) A-1 in H_2O, b) A-2 in H_2O, c) A-1 in 0.5 M NaCl, d) A-1 in 25 mM Tris-HCl (pH 8.4) containing 0.5 M NaCl, e) A-1 in 1% SDS, f) A-1(Cht) in H_2O.

conditions. At slightly alkaline pH value or in the presence of 1% SDS the spectra showed bathochromic shift, suggesting more rigid conformation.

DISCUSSION

New SAPs have been isolated and sequenced from another species of sea urchin, A. crassispina. The primary structures of SAPs are very close to each other with the substitution only at the fifth residue: Thr for A-1 and H-1, Ser for A-2 and Asn for H-2. The sequence of A-20 has not been determined yet, but presumably is Gly-Phe-Asp-Leu-Ser-Gly-Ser-Ser-Val-Gly, deduced from the chemical synthesis of this sequence with comparable activity as natural A-20 (manuscript in preparation). The substitutions found in natural SAPs do not alter their activity.

Two Japanese sea urchins have two or three molecular species of SAP, while an American sea urchin, S. purpuratus, reportedly, has only one SAP (Speract) which is identical to H-2 (5). Another Japanese sea urchin, S. intermedius

also has a few SAPs, one of which seems to be identical to H-2, based on its amino acid composition (11).

The mechanism of action of SAP is still not unveiled, but most probably, it combines to the surface of the sperm and accelerates the influx of Na^+ and efflux of H^+. This causes the increase of intracellular pH and eventually the respiration rate (6,7,12,13). SAP itself might be an ionophore like monensin, or it might bind to a receptor on the sperm plasma membrane and activate the ion-transfer system in or near the receptor. The CD spectrum of A-1 in H_2O was not altered by 0.5 M NaCl, indicating no conformational change in A-1. This supports the latter mechanism claiming a messenger-like or hormone-like nature of SAP. Dephosphorylation of a major sperm membrane protein (160 kDa) does not seem to be induced by SAP (14).

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