A low molecular weight peptide (speract) associated with sea urchin eggs has been purified to apparent homogeneity by charcoal adsorption, DEAE-Sephadex chromatography, Bio-Gel P-2 filtration, and Dowex AG 50W-X4 chromatography. Gametes from 5000 female sea urchins were required for the isolation of approximately 9 mg of the peptide. The isolated peptide is homogeneous based on \[^3H\]acetic anhydride labeling, gel filtration, and reverse phase high pressure liquid chromatography. Speract is composed entirely of neutral and acidic amino acids with glycine as the major component, and it appears to have a blocked NH$_2$ terminus based on its insensitivity to leucine aminopeptidase, its failure to react with dansyl chloride, and its chromatographic behavior on strong cation exchange resins. Speract is a potent stimulator of sea urchin sperm oxygen consumption, causing significant increases of sperm respiration rates at concentrations as low as 10$^{-12}$ M and producing 20-fold increases of oxygen consumption at maximal concentrations of 10$^{-6}$ M. Sperm cyclic GMP and cyclic AMP concentrations are also increased by speract, but concentrations of at least 10$^{-10}$ M and 10$^{-5}$ M are required for half-maximal elevations, respectively. The peptide, purified from Strongylocentrotus purpuratus eggs, also cross-reacts with spermatozoa from Lytechinis pictus sea urchins, suggesting that speract does not show species specificity. These results represent the first report of the purification of a peptide associated with eggs that may affect spermatozoa under natural conditions.

Since 1928 when Gray first demonstrated a stimulation of sea urchin sperm respiration rates by factors associated with the sea urchin egg (1), various studies have described the activating properties of "egg water" (2-13). The component or components responsible for these effects have not been successfully purified or characterized, although a number of studies have characterized the sperm respiratory stimulating component of egg water as being dialyzable, alcohol-soluble, and heat-stable (6-9). At one time the stimulating factor was reported to be echinochrome A, the pigment from ripe eggs, but a number of subsequent studies have disputed these reports (10-12).

Here, we report the purification to apparent homogeneity of the sperm respiratory stimulating component associated with the eggs of the sea urchin Strongylocentrotus purpuratus. The component is a low molecular weight peptide which we have named speract, because of its general sperm-activating properties. It is composed of acidic and neutral amino acids and appears to contain a blocked or modified NH$_2$-terminal amino acid. Speract is a potent stimulator of sea urchin sperm respiration with half-maximal stimulations at 10$^{-11}$ M concentrations; it also produces rapid elevations of sperm cyclic AMP and cyclic GMP concentrations.

**EXPERIMENTAL PROCEDURES**

**Materials**

S. purpuratus sea urchins were obtained from Pacific Bio-Marine, Venice, CA. Radioactive cyclic nucleotides ([H]-labeled), \[^3H\]acetic anhydride, and \[^3H\]H$_2$O were obtained from Amersham. Activated coconut charcoal (50 to 200 mesh) was from Fisher, and DEAE-Sephadex from Pharmacia. Dowex AG 50W-X8, Dowex AG 50W-X4, and Bio-Gel P-2 and P-4 were from Bio-Rad Laboratories. Dansyl C1 and dansyl amino acid standards were purchased from Pierce Chemical Co. and polyamide thin layer plates were obtained from Brinkman. All enzymes were purchased from Sigma or Calbiochem-Behring. All other compounds were acquired from either Sigma or Fisher.

**Methods**

**Gamete Collection and Incubation**—Spermatozoa or eggs were obtained after the injection of sea urchins with 0.5 M KCl, and were washed as previously described (13). Spermatozoa were stored at 0-2°C as a suspension (100 to 150 mg weight/ml). Eggs were diluted approximately 1 volume of eggs to 4 volumes of buffer in acidified sea water (pH 5.0) containing 10 mM 2-(N-morpholino)ethanesulfonic acid to a final concentration of 20 to 60 mg wet weight eggs/ml. These conditions facilitated rapid removal of the egg jelly coat and resulted in a high yield of the low molecular weight factor capable of stimulating sperm respiration rates (12). After 20 min, the eggs were removed by gentle centrifugation (2000 × g for 20 min) and the supernatant fluid was stored for purification of speract. In all experiments, the cells were incubated in artificial sea water buffered to pH 6.5 with either 20 mM N-(2-acetamido)-2-aminoethanesulfonic acid or 20 mM N-(2-acetamidomido)-iminodiacetic acid. All incubations were carried out at 15-16°C in the presence of artificial sea water composed of 454 mM NaCl, 9.7 mM KCl, 24.9 mM MgCl$_2$, 9.6 mM CaCl$_2$, 27.1 mM MgSO$_4$, 4.4 mM NaHCO$_3$, 10 mM Tris, and either 20 mM N-(2-acetamido)-2-aminoethanesulfonic acid or 20 mM N-(2-acetamidomido)iminodiacetic acid.

**Determination of Respiration Rates**—Respiration rates were determined using a Gilson K-IC Table Top Oxygraph equipped with a 1.25-mL capacity temperature control chamber fitted with a Clark-type electrode. One hundred microliters of the sperm suspensions were added to 1.15 ml of sea water at 15°C, and after basal respiration has remained constant for 3 min, various agents were added and the new respiration rates were determined over the next 3 min.

**Determination of Cyclic Nucleotide Concentrations**—The incubation mixture for the determination of cyclic nucleotide concentrations consisted of 4 to 6 mg (wet weight) sperm, 20 mM N-(2-acetamido)-2-aminoethanesulfonic acid or 20 mM N-(2-acetamidomido)iminodiacetic acid.
mido)iminodiacetic acid or 20 mm N-(2-acetamido)-2-aminooacetatesulfonic acid in artificial sea water, 100 μM 1-methyl-3-isooctylxanthine and the indicated concentration of speract in a final incubation volume of 0.5 ml. Incubations were started by the addition of sperm and were terminated by the addition of 1 ml of 0.5 N HClO containing tracer amounts of cyclic [3-3H]AMP or cyclic [3-3H]GMP for estimation of cyclic nucleotide recoveries. Zero time cyclic nucleotide concentrations were estimated by adding sperm directly to the incubation mixture containing 0.5 N HClO. The samples were then frozen and thawed five times and the cyclic nucleotides were subsequently purified on Dowex AG 50X-8 (H+ form) columns (0.7 × 25 cm) using the procedure of Schulz et al. (14).

The column fractions containing cyclic AMP or cyclic GMP were lyophilized, and then dissolved in 1.0 ml of deionized water. Cyclic AMP and cyclic GMP were determined by radioimmunoassay (15) with modifications by Harper and Brooker (16).

Enzymatic Sensitivity of Speract—In order to determine the sensitivity of speract to various proteolytic enzymes, aliquots of solutions containing speract were tested for ability to stimulate sperm respiration after incubation with various enzymes. Incubations were carried out for 90 min at 37°C in sealed tubes with 100 μl of speract in a final incubation volume of 150 to 170 μl. The incubation mixture contained 50 μg of the respective enzymes, and the final concentrations of the following reagents: 80 mM Tris (pH 7.9), 8 mM CaCl₂ for trypsin, carboxypeptidase A, pronase, and subtilisin; 80 mM Tris (pH 7.9) for thermolysin; and 75 mM sodium acetate (pH 4.75), 3 mM dithiothreitol, and 1 mM EDTA for papain. Incubations with leucine aminopeptidase were carried out in 80 mM Tris (pH 7.9), 5 mM MgCl₂. The reactions were generally stopped by placing the tubes in a boiling water bath for either 15 or 30 min. EDTA (11 mM) and N-ethylmaleimide (7.6 mM) were added to stop the thermolysin and papain reactions, respectively.

Dansyl Chloride Analysis—Derivatization of samples of isolated speract was carried out by the procedure of Gray (17). Samples were hydrolyzed for 12 to 18 h at 110°C in 6 N HCl under vacuum either before or after derivatization. The dansyl derivatives were separated by two-dimensional thin layer chromatography on polyamide plates (18) and identification was made by comparison with standards.

Amino Acid Analysis—Amino acid analysis was carried out using a Beckman 121 amino acid analyzer equipped with a Durrum DC-6A ion exchange column (6 × 320 mm). Lyophilized samples were reconstituted in 1.0 ml of 6 N HCl and hydrolyzed at 110°C for 24 h under a vacuum prior to analysis.

P-4 Gel Filtration—Samples of purified speract were applied to a Bio-gel P-4 column (1 × 15 cm) equilibrated in 150 mM sodium phosphate at pH 6.7. The sample was eluted with the equilibration buffer and 0.3-ml fractions were collected. Each fraction was tested for respiratory stimulating activity and for absorbance at 206 nm. Peaks of absorbing material were subjected to acid hydrolysis and subsequent amino acid analysis.

Acetic Anhydride Labeling—Acetic anhydride labeling of speract was accomplished with the following procedure: An aliquot of purified speract (74 nmol of glycine) was lyophilized to dryness in a 1.0-ml glass vial. Fifty μl of acetic anhydride (500 μCi/mmol) was added, and the vial was sealed and blended on a Vortex mixer. After 14 h at room temperature, 500 μl of deionized water was added and the sample was incubated 6 h more. The sample was then frozen at −70°C and lyophilized using a liquid nitrogen cold trap to collect the volatile radioactivity. Portions of the lyophilized sample were chromatographed on a Bio-Gel P-2 column (1.5 × 86 cm).

High Pressure Liquid Chromatography Analysis—High pressure liquid chromatography analysis of purified speract was carried out using either a Waters Associates system consisting of two 600A solvent delivery units, an M-660 solvent programmer, and a U6K injector or a Beckman model 322 chromatograph system. All separations were performed on a C₃ Ultrasil column (4.6 mm × 25 cm) from Beckman and the column effluent was monitored for absorbance at 206 nm using a Beckman model 155 variable wavelength detector with a 20-μl flow cell. In general, separations were carried out using the following program: with a constant flow rate of 1.0 ml/min, the column was first eluted with a mixture of 10% Solvent B (90% Solvent A) for 2 min, then a linear gradient from 10 to 60% Solvent B was developed over the next 25 min to 1 h. Solvent A consisted of 10 mM KH₂PO₄ in water and Solvent B was acetonitrile.

RESULTS

Purification of Speract

By using the ability of speract to stimulate sea urchin sperm respiration as an assay, the purification of speract was accomplished as outlined below.

Charcoal Column Chromatography—The supernatant fluid obtained from the egg treatment was applied to a column of activated charcoal (2.6 × 32 cm) which had been pre-equilibrated with water. The respiratory stimulating activity completely adsorbed to the charcoal, and after extensive washing with H₂O and 50% ethanol, speract was eluted with 70% ethanol containing 0.2% NH₄OH (Fig. 1). The fractions containing speract were pooled, flash-evaporated at 30°C, and the remaining aqueous solution was frozen and lyophilized.
DEAE-Sephacel Chromatography—The dried residue from the pooled charcoal column fractions was reconstituted in 10 mM triethanolamine buffer at pH 7.6 to a dilution of less than 1 mg of residue/ml, and the pH of the solution was readjusted to 7.6 with 0.5 N NaOH. This solution was then applied to a DEAE-Sephacel column (1.6 × 29.0 cm) which had been equilibrated with 10 mM triethanolamine buffer at pH 7.6. The column was washed with 200 ml of the equilibration buffer and the respiratory stimulating activity was eluted from the column using a linear gradient from 0 to 300 mM NaCl in 10 mM triethanolamine at pH 7.6. Speract eluted from the column at approximately 100 mM NaCl (Fig. 2).

P-2 Gel Filtration—The fractions from the DEAE-Sephacel chromatography which contained speract were desalted on a Bio-Gel P-2 column (5.0 × 23 cm) equilibrated with 0.2 M pyridine acetate at pH 5.0 and the fractions containing speract were then pooled and lyophilized.

Dowex 50 Chromatography—The lyophilized fractions containing speract were dissolved in 4.0 ml of 100 mM HCl. The acidified sample was then applied to a column (0.7 × 15 cm) of Dowex AG 50W-X4 (H⁺) which had equilibrated with 100 mM HCl. Speract did not bind to the resin and was eluted with 100 mM HCl (Fig. 3). The fractions containing speract were pooled, lyophilized, reconstituted in H₂O, and stored at −35°C.

**Table I**

| Purification step   | Total biological activity | Recovery per purification step | Total recovery |
|---------------------|---------------------------|--------------------------------|---------------|
| Crude egg water     | 84 × 10^6                 | 100                            | 100           |
| Charcoal column chromatography | 84 × 10^6 | 100                            | 100           |
| DEAE-Sephacel chromatography | 77.3 × 10^6 | 92                            | 92            |
| P-2 chromatography  | 33.2 × 10^6               | 43                             | 40            |
| Dowex 50 chromatography | 11.7 × 10^6 | 35                            | 14            |

* Biological activity is expressed in units, defined as the amount of speract which produced a half-maximal increase in S. purpuratus sperm respiration under standard conditions of 15°C, 1.25 ml final volume, pH 6.6 sea water, and a final sperm concentration of 8 to 16 mg wet weight/ml. Approximately 3.6 × 10^6 units of activity represent 1 nmol of speract.

**Table II**

| Enzyme treatment       | Percentage of respiratory stimulating activity remaining after 90 min |
|------------------------|-----------------------------------------------------------------------|
| Thermolysin            | 17 (n = 7)                                                            |
| Subtilisin              | 7.4 (n = 4)                                                            |
| Papain                 | 0 (n = 4)                                                              |
| Pronase                | 2.6 (n = 4)                                                            |
| Carboxypeptidase A     | 57 (n = 3)                                                             |
| Trypsin                | >95 (n = 4)                                                            |
| Leucine aminopeptidase | >97 (n = 3)                                                            |

**Fig. 3. Dowex 50 chromatography of speract.** The speract sample of approximately 5.0 × 10^10 units of biological activity was applied to the AG 50W-X4 column (0.7 × 15 cm) and eluted as described under “Experimental Procedures.” Fractions (3 ml) were collected and 10 µl of a 100-fold dilution were used to assay for respiratory stimulating activity.

**Fig. 4. Thin layer chromatography of 0.2 nmol of speract which had been derivatized with dansyl chloride prior to (top chromatogram) or after (lower chromatogram) 12-h acid hydrolysis in 6 N HCl (110°C).** Samples were derivatized with 2 mg/ml of dansyl Cl in acetone as described under "Experimental Procedures." After derivatization, the samples were evaporated under a nitrogen stream, dissolved in 20 µl of acetone/glacial acetic acid (3:2), and applied to polyamide plates (20 × 20 cm) for two-dimensional thin layer chromatography. The dansyl derivatives were visualized by inspection with a long wavelength UV lamp. The numbered spots were identified as follows: 1, Dns-NH₂; 2, Dns-Val; 3, Dns-Leu; 4, Dns-Ala; 5, Dns-Phe; 6, Dns-Thr; 7, Dns-Gly; 8, Dns-Ser; 9, Dns-Glu; 10, Dns-Asp; 11, Dns-OH (dansyl sulfonic acid); 12, origin.

were pooled, lyophilized, reconstituted in H₂O, and stored at −35°C.

**Recovery**

Based on half-maximal stimulation of sperm respiration,
the recovery of speract through this purification scheme was approximately 14% (Table I).

**Characterization of Speract**

In order initially to establish the nature of the isolated respiratory stimulating material, the sensitivity of speract (based on ability to stimulate respiration) to various enzymatic treatments was examined (Table II). After a 90-min incubation with subtilisin, thermolysin, pronase, and papain, the majority of the respiratory stimulating activity was destroyed while trypsin and leucine aminopeptidase failed to inactivate the factor. Based on these data it appeared that the respiratory stimulating component might be a peptide where Lys or Arg residues either were not present or did not donate a carbonyl function to a peptidyl linkage. Insensitivity to leucine aminopeptidase suggested the presence of a blocked primary amino groups in the presumed peptide (19). Furthermore, NH₂-terminal analysis by the dansyl chloride method failed to identify any dansyl amino acids unless the isolated material was hydrolyzed prior to dansyl chloride derivatization (Fig. 4).

![Figure 5. Gel filtration of [³H]acetic anhydride-labeled speract on a Bio-Gel P-2 column (1.5 x 86 cm). Purified speract was treated with [³H]acetic anhydride as described under “Experimental Procedures.” A portion (350,000 cpm) of the radioactive material was applied in 100 μl of H₂O to the P-2 column, then eluted with 150 mM sodium phosphate at pH 6.0 with a flow rate of 10 ml/h, and 1.4-ml fractions were collected. Respiratory stimulating activity of the fractions was also tested using 20 μl of a 50-fold dilution as indicated by O- - O. Under identical conditions, samples of unlabeled speract eluted at the same position as the radioactive peak.](image-url)

![Figure 6. Reverse phase high pressure liquid chromatography of speract on an Ultrasil C₈ column (4.6 mm x 25 cm). Speract was eluted using a linear gradient from 10% acetonitrile to 60% acetonitrile over 25 min starting at 0.00 min. All other details of the chromatography run were as given under “Experimental Procedures.” The absorbance peak at 16.5 min corresponded exactly with the peak of respiratory stimulating activity (not shown).](image-url)

**TABLE III**

| Amino acid composition of speract | Amino acid | Mole % | Residues/mol² |
|----------------------------------|------------|-------|---------------|
| Asp                              | 12.5 ± 0.8 | 2.1 ± 0.1 |
| Thr                              | 4.7 ± 0.5  | 0.8 ± 0.1 |
| Ser                              | 4.8 ± 0.7  | 0.8 ± 0.1 |
| Gln                              | 7.1 ± 1.0  | 1.2 ± 0.2 |
| Gly                              | 34.5 ± 2.2 | 5.8 ± 0.3 |
| Ala                               | 6.0 ± 0.3  | 1.0 ± 0.1 |
| Val                               | 7.6 ± 0.6  | 1.3 ± 0.1 |
| Leu                               | 9.9 ± 0.5  | 1.7 ± 0.1 |
| Phe                               | 9.7 ± 1.5  | 1.7 ± 0.4 |

² Values represent mean ± S.E. for four analyses from two separate preparations.

**Assessment of Homogeneity**

The isolated respiratory stimulating material appears homogeneous based on several criteria.

**P-4 Gel Filtration**

The isolated material migrated as a single peak of protein on Bio-Gel P-4 columns. Monitoring absorbance of the column effluent at 206 nm indicated one major peak of UV-absorbing material which co-migrated with the respiratory stimulating activity. The amino acid composition of this peak also appeared identical with the original material. A minor peak of 206 nm absorbing material appeared slightly beyond the total volume of the column. This peak varied in size in various runs and no detectable amino acids were observed before or after acid hydrolysis of this peak.

**[³H]Acetic Anhydride Labeling**

Incubation of the isolated speract with [³H]acetic anhydride yielded a single nonvolatile radioactive peak on Bio-Gel P-2 gel filtration (1.5 x 86 cm) which eluted at the same position as unlabeled speract (Fig. 5).

**High Pressure Liquid Chromatography Analysis**

Further analysis of the isolated speract yielded one major peak of 206 nm absorbing material which co-chromatographed with the respiratory stimulating activity and corresponded to the only detectable peak of amino acid-containing material detected after acid hydrolysis of the various fractions (Fig. 6). This step again failed to alter the amino acid composition from that of the original material.

**Amino Acid Composition of Speract**

The amino acid composition of purified speract is consistent with the previously observed properties of the molecule (Table III). The lack of Lys or Arg residues explains the resistance of speract to trypsin inactivation and the presence of Leu and Val explains its sensitivity to thermolysin treatment. The amino acid composition from three different preparations was identical and each preparation had the same relative biological specific activity of 6 x 10⁶ units/mmol of glycine. One unit of activity is defined as the amount of speract which produces a half-maximal increase in sperm respiration under standard conditions (1.25 ml final volume, pH 6.6 sea water, 15°C, and a final sperm concentration of 8 to 16 mg wet weight/ml).

**Potency of Purified Speract**

In addition to the effects of speract on sperm respiration, it is also capable of producing rapid elevations of sperm cyclic AMP and cyclic GMP concentrations (Fig. 7). Speract is more potent toward the stimulation of sperm respiration with half-
maximal effects occurring at \(3 \times 10^{-11} \text{ M}\) (based on a molecular weight of 1885) than toward elevating cyclic nucleotide concentrations. Values represent the mean ± S.E. \((n = 6)\) of each parameter as measured in the presence of the indicated concentration of speract (based on a molecular weight of 1885). In cases where vertical bars are not shown, the standard error lies within the area occupied by the symbol.

Fig. 7. Concentration-dependent effects of speract to stimulate respiration and to elevate cyclic AMP and cyclic GMP concentrations in \(S.\) purpuratus spermatozoa. Measurements of oxygen consumption and assay of cyclic nucleotides were carried out as described under “Experimental Procedures.” Time points of 15 min were used in the estimates of cyclic nucleotide concentrations. Values were used in the estimates of cyclic nucleotide concentrations. Half-maximal elevations of cyclic GMP are at 2 \(\times 10^{-9} \text{ M}\) while half-maximal elevations of cyclic AMP concentrations required at least \(2 \times 10^{-8} \text{ M}\) speract. Speract is also capable of stimulating sperm fatty acid oxidation (20) with half-maximal effects at concentrations of \(10^{-11} \text{ M}\) (data not shown).

**DISCUSSION**

A peptide associated with eggs has been purified that stimulates sperm respiration rates and elevates sperm cyclic AMP and cyclic GMP concentrations. This peptide, which we have named speract because it appears to act as a general sperm activator, is probably similar or identical with the sperm respiratory stimulating component previously studied in crude extracts of sea urchin eggs (1–12). The isolated speract appears homogeneous based on several criteria. Gel filtration profiles on Bio-Gel P-2 column indicate one peak of protein. [\(^3\)H]-Acetic anhydride labeling of the isolated material using a procedure designed to acetylate the hydroxyl groups of threonine and serine (21) produced one radioactive band with the same chromatographic behavior as unlabeled speract. The isolated material retained a constant amino acid composition through further chromatographic steps, including reverse phase high pressure liquid chromatography (Fig. 6), a technique which is considered a high resolution analytical technique for peptide analysis (22, 23). The lack of prior reports related to the stimulation of steroidogenesis. If cyclic GMP is actually involved as a second messenger for speract, closer analysis of the compartmentalization of cyclic GMP may reveal a similar situation.

The occurrence of factors associated with either the female reproductive tract or the egg itself which can stimulate the metabolism, motility, or cyclic nucleotide metabolism of spermatozoa appears to be widespread in nature (27, 28). It is now clear that in at least one animal, the sea urchin, the factor is a peptide. It is also clear that the peptide has extremely high biological potency. Since the jelly coat of eggs is actually a female secretion, it remains to be determined whether or not the peptide is synthesized by follicular cells of the ovary and deposited as a part of the jelly coat, or whether the peptide is synthesized and secreted by the egg itself.

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