PURIFICATION OF THE SPERM-BINDING FACTOR FROM THE EGG OF THE SEA URCHIN, HEMICENTROTUS PULCHERRIMUS[1]

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SUMMARY: The substance which seems to be responsible for the sperm-binding at fertilization was successfully purified from unfertilized eggs of the sea urchin, Hemicentrotus pulcherrimus. It completely cancelled the fertilizing capacity only of homologous sperm without reducing their motility. The antiserum against this substance made only homologous eggs incapable of binding sperm. The methods employed for purification were (1) extraction by urea, (2) fractionation by calcium acetate, (3) salting-out by ammonium sulfate, (4) gel filtration and (5) ion-exchange chromatography. This substance was electrophoresed on cellulose-acetate strip as a single band which was stained with Amido Black, and could not be split by 6 M guanidine hydrochloride.

Vitelline layer of unfertilized sea urchin egg plays important roles in fertilization. It is the site for the species-specific binding of sperm (1-6) and becomes barrier against supernumerary sperm after conversion into the fertilization membrane (7-10). We have obtained a factor responsible for the species-specific sperm-binding from several species of the sea urchins, and tentatively termed it sperm-binding protein (1-3, 11-13). In the present report, we describe the procedure for purification of this substance from the sea urchin, Hemicentrotus pulcherrimus.

MATERIALS AND METHODS

Materials: Eggs of the sea urchin, Hemicentrotus pulcherrimus were obtained by pouring isotonic (0.56 M) KCl into the opened body cavity. Spawned eggs were collected by gravity and washed carefully in sea water. Sperm were obtained by cutting ripe testis removed from dissected male. Jelly coat surrounding the egg was dissolved by titrating the egg suspension to pH 5.2 with dilute HCl. The jelly-less eggs, after washing in normal sea water, were sedimented by gravity to the bottom of the beaker, and the sea water was removed as thoroughly as possible. More than 20 volumes of 1 M urea was added to the sedimented eggs and the suspension was kept in room temperature for about 20 min with occasional stirrings. The supernatant was filtered through

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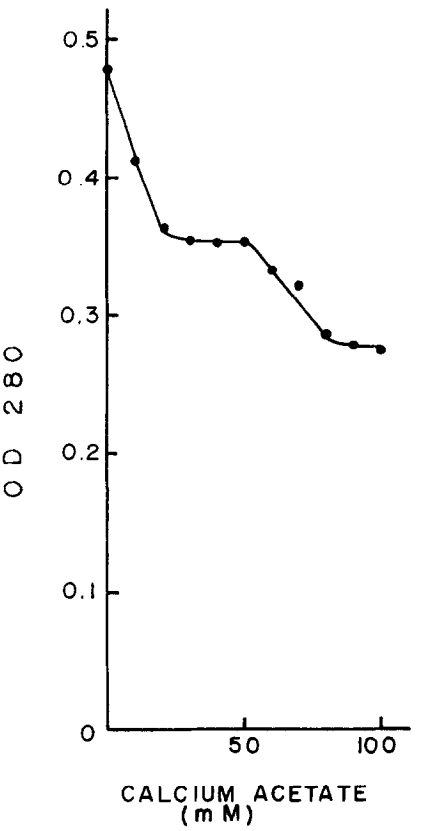

Fig. 1. Solubility of crude preparation of sperm-binding factor in varing concentrations of calcium acetate (cf. Materials and Method).

Electrophoresis on cellulose acetate strips: Electrophoresis was performed on Sepraphore III (German, Michigan, U.S.A.), 2.5 x 17 cm, at 0.8 mA/cm for 15 min. The buffer systems employed were as follows; (1) 18.55 g of H_3BO_4 and 120 ml of 0.5 N NaOH in 1,000 ml of distilled water, pH 8.05, and (2) 100 ml of pyridine and 4 ml of acetic acid in 1,000 ml of distilled water, pH 6.5. Amido Black (1 % in 7 % acetic acid) and Toluidine Blue (0.5 % in 3 % acetic acid) were used for staining protein and carbohydrate, respectively.

SDS-polyacrylamide gel electrophoresis: The procedure described by Weber and Osborn (15) was used. After electrophoresis, gels were stained with 0.25 % Coomassie blue.

RESULTS AND DISCUSSION

Fractionation with calcium acetate: According to the result presented in Fig. 1, the starting material was divided into two fractions, one of which was precipitated and the other remained soluble in 30 mM calcium acetate. The former was abbreviated as CaInS and the latter as CaS in the present

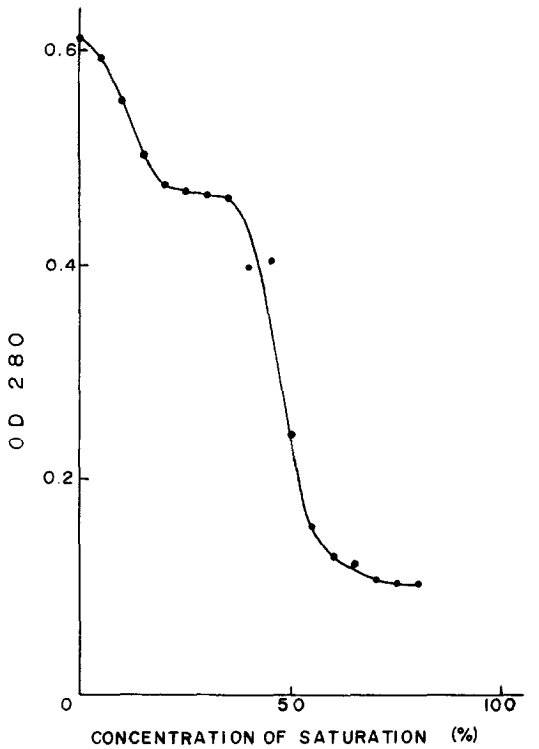

Fig. 2. Solubility of calcium-soluble fraction of sperm-binding factor (CaS) in varing concentrations of ammonium sulfate (cf. Materials and Method).

work. The antisera obtained against each fraction were examined for the inhibitory effect on the sperm-binding activity of the egg. Although both sera completely inhibited fertilization of Hemicentrotus eggs, only antiserum against CaS inhibited the sperm-binding (3, 14). When the antiserum against crude preparation, which contains both CaS and CaInS, was absorbed by each fraction and examined, the serum absorbed by CaS entirely lost its inhibitory effect on the sperm-binding (3, 14). It is certain, therefore, that the sperm-binding factor is contained in CaS.

Salting-out by ammonium sulfate: According to the precipitation curve by ammonium sulfate (Fig. 2), CaS was divided into the following two fractions; one precipitable by 20 % saturation (CaS-20) and the other by more than 55 % saturation (CaS-55).

Table 1. Effect of CaS-20 and of CaS-55 on the fertilizing capacity of the sperm. Percent fertilization of the egg is shown.*

Exp. No.	CaS-20	CaS-55	Control (Normal sea water)
1	3	90	94
2	5	99	94

* Sample (2 mg/ml) 0.2 ml, Sperm (5×10^3 dilution) 0.2 ml → 10 min → Jelly-less egg suspension 1 ml, Sperm suspension 0.1 ml

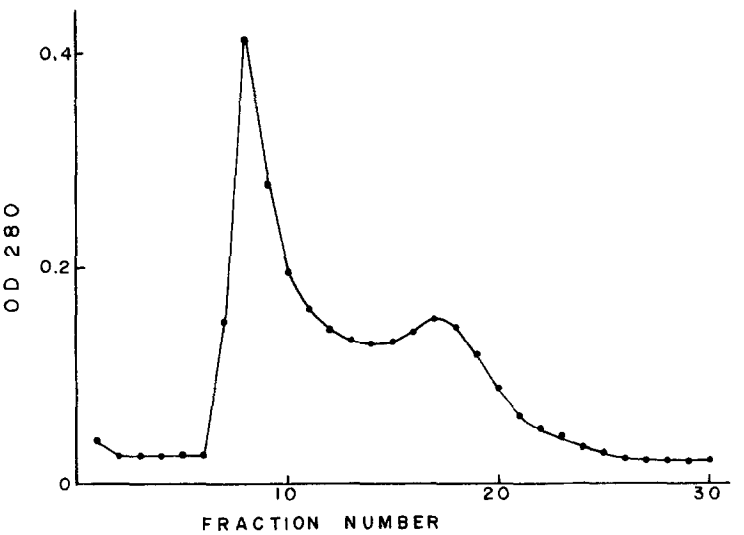

Fig. 3. Gel filtration of CaS-20 (CaS fraction precipitable by 20 % saturation of ammonium sulfate) through a Sepharose 4B column (1.2 x 33 cm) equilibrated with 0.01 M phosphate buffer, pH 7.2, containing 0.2 M NaCl.

The sperm-binding activity of each fraction was tested by the both two methods described in the Materials and Method. Anti-CaS antiserum lost its inhibitory effect on the sperm-binding after absorbed by CaS-20, whereas those absorbed by CaS-55 remained effective (16). The sperm pre-exposed to each fraction were bioassayed for its fertilizing capacity. As shown in Table 1, only CaS-20 rapidly diminished the fertilizing capacity of sperm.

Table 2. Effect of anti-CaS antiserum absorbed with CaS-20-A or -B on the fertilizability of the egg. Percent fertilization is shown.*

Exp. No.	Not absorbed	Absorbed by CaS-20-A	Absorbed by CaS-20-B
1	1	94	1
2	1	100	5
3	1	100	5

* Antiserum 0.5 ml, Jelly-less eggs 0.5 ml (approx. 300) → 30 min → Egg suspension 1 ml, Sperm suspension 0.2 ml (10^4 dilution)

Table 3. Effect of CaS-20-A and of CaS-20-B on the fertilizing capacity of the sperm. Percent fertilization of the egg is shown.*

Exp. No.	CaS-20-A	CaS-20-B	Control (Normal sea water)
1	10	100	88
2	28	100	92

* Sample (2 mg/ml) 0.2 ml, Sperm (5 x 10^3 dilution) 0.2 ml → 10 min → Jelly-less egg suspension 1 ml, Sperm suspension 0.2 ml

Though these sperm were swarming vigorously around the egg, they could not bind to it.

Gel filtration: Gel-filtration profile of CaS-20 through a Sepharose 4B column is shown in Fig. 3. Of the two peaks, one emerged just behind the void volume of the column and the other of lower molecular weight followed. The former was abbreviated as CaS-20-A, and the latter as CaS-20-B. When anti-CaS antisera were absorbed with each fraction, only those absorbed with CaS-20-A lost the fertilization-inhibiting effect (Table 2). It was also shown that only CaS-20-A could inhibit the fertilizing capacity of sperm without reducing the sperm motility (Table 3). It may be safely said that the sperm-binding factor is contained in CaS-20-A.

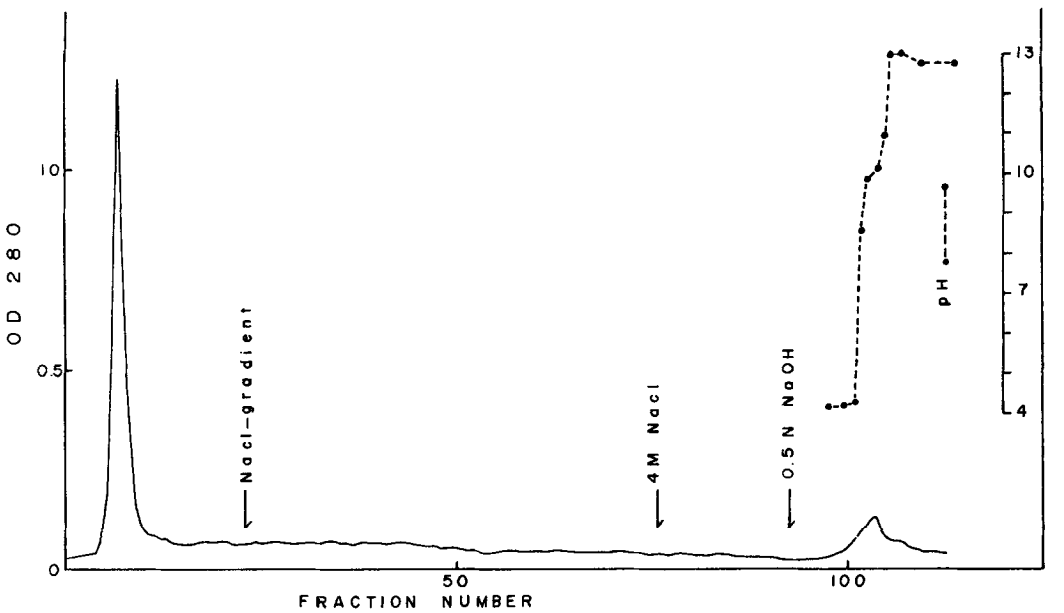

Fig. 4. CM cellulose column chromatography of CaS-20-A (CaS-20 fraction emerging in the void volume of the Sepharose 4B column). CM 23 was equilibrated with 0.1 M acetate buffer, pH 4.6, and a linear gradient, starting buffer to 4 M NaCl in the buffer, was employed.

Ion-exchange column chromatography: CaS-20-A was applied to a DEAE-Sephadex A-25 column equilibrated with 0.1 M acetate buffer, pH 4.6, and a linear gradient to 4 M NaCl in the buffer was employed. Any fraction did not emerge by the completion of the gradient. Only after the addition of 0.2 N NaOH, a fraction emerged, which exerted the fertilization-inhibiting effect on sperm. A similar profile was obtained with DE 23 column chromatography. When these anion-exchangers were replaced by a cation exchanger CM 23 equilibrated with the same starting solution, a reciprocal profile was obtained (Fig. 4). CaS-20-A emerged in the exclusion volume of the column. No other fraction derived from the sample was detected.

Electrophoresis on cellulose acetate strips: Electrophoresis of HpCaS-20 on cellulose acetate strips in borate buffer, pH 8.05, revealed four bands. Three of them were stained with Toluidine Blue, one of which showed metachroma-

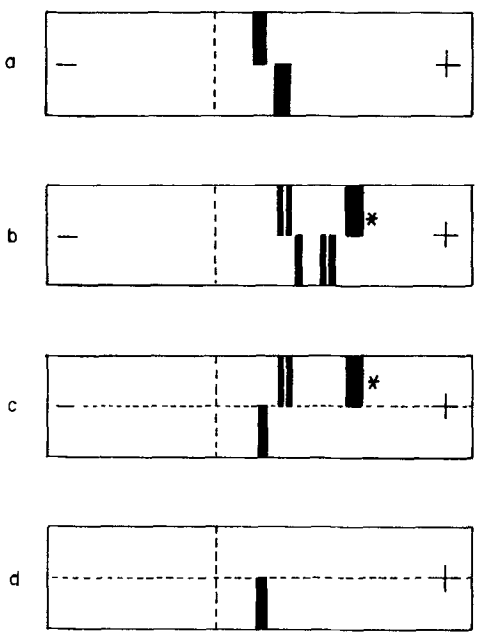

Fig. 5. Cellulose acetate electrophoresis of CaS-20, CaS-55 and CaS-20-A. (a) CaS-20 (upper) and CaS-55 (lower) stained with Amido Black. (b) CaS-20 (upper) and CaS-55 (lower) stained with Toluidine Blue. (c) CaS-20 stained with Toluidine Blue (upper) and with Amido Black (lower). (d) CaS-20-A stained with Toluidine Blue (upper) and with Amido Black (lower). Borate buffer, pH 8.05. 0.8 mA/cm width. x 15 min. * metachromasy

sy. The remainning one band was stained with Amido Black (Fig. 5 a, b and c), and was found to correspond to CaS-20-A (Fig. 5d). When pyridine-acetate buffer, pH 6.5, was used, CaS-20 was divided into two bands, one metachromasically stained with Toluidine Blue and the other stained with Amido-Black.

SDS-polyacrylamide gel electrophoresis of CaS-20-A: CaS-20-A was subjected to polyacrylamide gel electrophoresis in the presence of SDS for the examination of purity and for the determination of the molecular weight. The sample was dissolved in 1 M urea and dialyzed against 0.01 M phosphate buffer, pH 7.0, containing 0.1 % SDS and 0.1 % (V/V) mercaptoethanol. After incubation in a water bath at 50°C for 1 hr, it was applied to a gel column. But, the sample could not enter the gel. No band of smaller molecular size was detected.

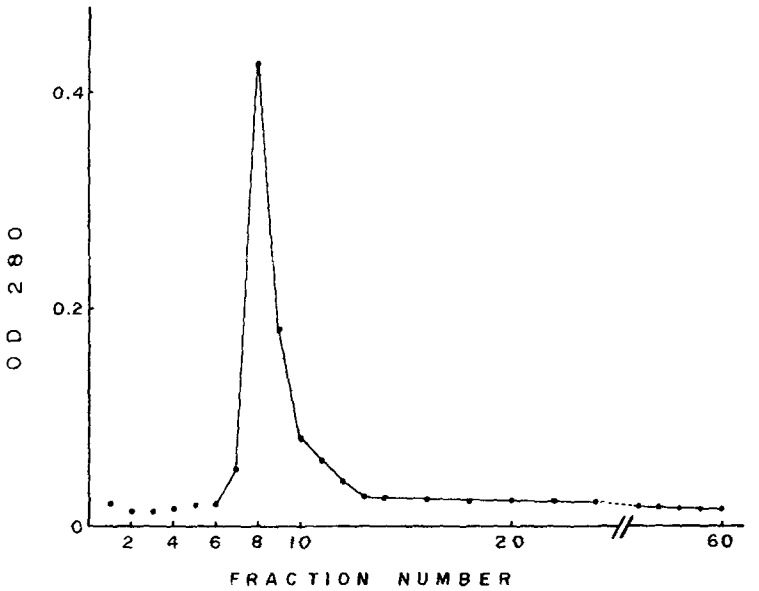

Fig. 6. Gel filtration of CaS-20-A through a Sepharose CL-4B column in the presence of 6 M guanidine hydrochloride. The gel volume was equal to that shown in Fig. 3.

Gel filtration of CaS-20-A in the presence of guanidine hydrochloride:

After dialysis against 6 M guanidine hydrochloride at 4°C for 24 hr, CaS-20-A was applied to a column of Sepharose CL-4B. The profile is shown in Fig. 6. CaS-20-A emerged still just behind the void volume of the column and any component of lower molecular size was not detected. Considered together with the result by SDS-gel electrophoresis, purified sperm-binding factor of Hemicentrotus pulcherrimus, HpCaS-20-A, seems to be a substance of an extraordinarily large molecular size and not composed of subunits. Some other physicochemical and biochemical features of this substance will be reported in the following paper.

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