accuracies in the microscope fine-focus adjustment result in an ~ 4 percent error in measurements of focal depth.
10. E. Frömter, *J. Membr. Biol.* **8**, 259 (1972); L. Reuss and A. Finn, *ibid.* **25**, 115 (1975); C. H. Van Os and J. E. G. Slegers, *ibid.* **24**, 341 (1975).
11. C. J. Bentzel, K. Loeschke, E. L. Benedetti, in *Proceedings of the International Union of Physiological Sciences, 27th International Congress* (French National Committee of Physiological Sciences, Paris, 1977), vol. 13, p. 68.
12. J. M. Diamond and W. H. Bossert, *J. Gen. Physiol.* **50**, 2061 (1967); L. A. Segel, *J. Theor. Biol.* **29**, 233 (1970); A. E. Hill, *Proc. R. Soc. London Ser. B* **190**, 115 (1975); J. J. Lim and J. Fischbarg, *Biochim. Biophys. Acta* **443**, 339 (1976); R. Huss and D. Marsh (*18*).
13. The perfusion fluid composition is given in table 1 in K. R. Spring and G. Kimura, *J. Membr. Biol.* **38**, 233 (1978).
14. Epithelial thickness is altered by the stretching of the gallbladder during mounting. In the experiments reported here, the tissue was tightly stretched to minimize movements during alterations in hydrostatic pressure. Minimal stretching results in a cell height of 32 to 36 μm; however, interspace shape and width are similar to those reported here.
15. K. R. Spring and A. Hope, in preparation.
16. C. L. Voute and H. H. Ussing, *J. Cell Biol.* **36**, 625 (1968); C. L. Voute, K. Mollgard, H. H. Ussing, *J. Membr. Biol.* **21**, 273 (1975); C. L. Voute and H. H. Ussing, *Exp. Cell Res.* **62**, 375 (1970); C. J. Bentzel, *Kidney Int.* **2**, 324 (1972).
17. The lateral space width was measured at one focal depth and the corresponding phototube current was recorded. The increase in phototube current over that measured at the level of the tight junction was divided by the measured space width (assuming zero width at the level of the junction). The slope of a plot of the change in current against the change in width was used to calculate the interspace width at the other focal depths. This calibration was repeated at each hydrostatic pressure difference. The width measurements are accurate within 0.15 μm.
18. R. Huss and D. Marsh, *J. Membr. Biol.* **23**, 305 (1975).
19. K. Loeschke, G. M. Eisenbach, C. J. Bentzel, *Excerpta Med. Int. Congr. Ser. No. 391* (1975), p. 406; J. M. Diamond, *J. Physiol. (London)* **161**, 503 (1962).
20. The basement membrane and connective tissue offer a finite hydraulic resistance, and the hydrostatic pressure within the lateral spaces will be slightly lower than that in the serosal medium. The amount of this pressure drop depends on the transepithelial volume flow induced by the hydrostatic pressure difference. Since the effective pressure gradient across the lateral membranes of the cells is actually less than that exerted transepithelially, the interspaces are probably even more compliant than our measurements indicate.
21. R. I. Kiemowitz and T. L. Stanton, *Clin. Res.* **20**, 598 (1972); J. Fischbarg, C. R. Warshavsky, J. J. Lim, *Nature (London)* **266**, 71 (1977); C. H. Van Os and G. Wiedner, in *Proceedings of the International Union of Physiological Sciences, 27th International Congress* (French National Committee of Physiological Sciences, Paris, 1977), vol. 13, p. 778.
22. We were greatly helped in the design and fabrication of the chamber by J. Sullivan. The television equipment was put into operation with the assistance of W. Whitehouse, and the optical system was selected by M. Nahmmacher.

3 October 1977; revised 13 December 1977

Separation of the Sperm Agglutinin and the Acrosome Reaction–Inducing Substance in Egg Jelly of Starfish

Abstract. *The egg jelly of the starfish* Asterias amurensis *was separated into the fractions J1, J2, and J3 on a Sephadex G-100 column. The J1 fraction induced the acrosome reaction and J2 induced sperm agglutination. Chemical analysis and chromatography revealed that sperm agglutinin is similar to asterosaponin A.*

In marine invertebrates, the egg jelly coat often shows physiological activity and plays an important role in fertilization. In sea urchins, egg jelly induces the acrosome reaction (*1*), sperm agglutination (*2*), and acceleration of sperm respiration (*3*). The first of these is an indispensable step for fertilization since the egg plasma membrane can fuse only with the membrane of the acrosomal process, which is newly formed during the acrosome reaction (*4*). However, the meaning, if any, of the phenomenon of sperm agglutination is unknown. The fertilizin-antifertilizin theory (*5*) describes the agglutination reaction as a binding, like that of an immune reaction, between specific substances on the gamete surfaces. We think that the attachment of the sperm to the jelly surface initiates the interaction between the two gametes, and that sperm agglutination may reflect only this initial interaction.

Egg jelly consists mainly of glycoprotein. That of sea urchins contains sialic acid (*6*) and fucose sulfate (*7*) as the main sugar components. Ishihara and Dan (*8*) have suggested the existence of fragments which induce the acrosome reaction but not sperm agglutination. A mucopolysaccharide-protein complex from starfish egg jelly has been obtained by phenol extraction (*9*). Which part of the egg jelly induces the acrosome reaction or sperm agglutination is still not known. Also it is not known whether sperm agglutinin and the acrosome reaction–inducing substance are identical to each other and what the chemical structure of the active substance is. We have succeeded in isolating the sperm agglutinin, free from the acrosome reaction–inducing substance, from the egg jelly of the starfish *Asterias amurensis*.

Suspensions of eggs, obtained by treating the ovaries with 1-methyladenine (*10*), were centrifuged at 6000*g* for 20 minutes, and the supernatant was used as the crude egg jelly. This was applied to a Sephadex G-100 column and separated into J1, J2, and J3 (Fig. 1a).

Spermatozoa, which had been washed with seawater containing 1 m*M* histidine, were diluted with 100 volumes of seawater. An equal volume of each fraction and a diluted sperm suspension were mixed and observed under microscopy. Only J1 induced the acrosome reaction and only J2 induced the sperm agglutination (Table 1 and Fig. 2). Fraction J3 also showed a weak agglutinating activity because of an overlap with J2, the sperm agglutinin; sperm agglutinin and the acrosome reaction–inducing substance are different from each other (Fig. 1). The sperm agglutination by jelly is irreversible in the starfish, whereas it is reversible in sea urchins. The agglutination caused by J2 was also irreversible.

The J1 fraction was separated on a Sepharose 4B column into subfractions consisting largely of (i) methylpentose-rich glycoproteins and (ii) hexose-rich glycoproteins.

The J2 fraction was undialyzable, but on Sephadex G-100 or G-50 it behaved as a small molecule; J2 has some affinity to Sephadex, a characteristic of many aromatic compounds. By ion-exchange column chromatography on DEAE-Sephadex A-25, J2 was separated into three fractions, one of which (2-2 in Fig. 1b) had agglutinating activity and contained a peptide moiety.

This sperm agglutinin was further purified by partition between *n*-butanol and 0.1*M* citrate buffer (*p*H 3.6). The agglutinating activity was recovered in the *n*-butanol layer. These two fractions were separated by thin-layer chromatography (TLC) on Kieselgel 60 (Merck) developed in a system consisting of chloro-

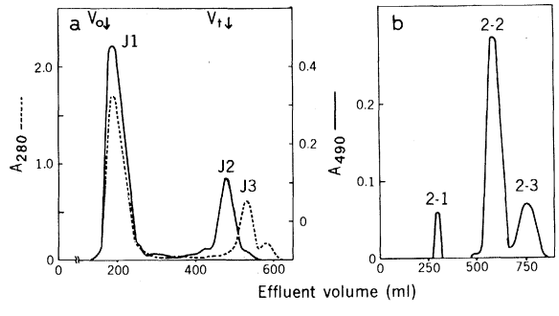

Fig. 1. (a) Elution profile of crude egg jelly from a Sephadex G-100 column. Abbreviations: V_0, void volume; V_t, total volume. (b) Elution profile of fraction J2 from a DEAE-Sephadex A-25 column eluted with a concave gradient from 0.5*M* pyridine, 0.25*M* acetic acid buffer (*p*H 5.2) to 1.0*M* pyridine, 0.5*M* acetic acid buffer (*p*H 5.2). (·····) Absorbance at 280 nm; (——) sugar content as determined by phenol-sulfuric acid methods.

0036-8075/78/0407-0058$00.50/0

form, methanol, acetic acid, and water (30:10:5:2, by volume). The *n*-butanol layer fraction gave two spots, T_1 and T_2. The aqueous layer fraction (T_3) remained at the origin with this solvent system. Each substance was isolated by preparative TLC. Only T_1 possessed agglutinating activity. Only T_3 contained any amino acids. These results show that the starfish sperm agglutinin is not a glycoprotein, contrary to what would be expected from the composition of sea urchin jelly (*11*).

The sperm agglutinin, T_1, was readily dissolved in water and organic solvents such as a mixture of chloroform and methanol (2:1, by volume), methanol, *n*-butanol, and ethanol. When crude egg jelly was precipitated by the addition of ethanol, as used for sea urchins, the agglutinating activity was recovered not in the precipitate but in the ethanol-soluble fraction. The agglutinin showed a strong ultraviolet absorption at 244 nm, which was indicative of heteroannular diene. *O*-Trimethylsilylated derivatives after methanolysis of the agglutinin were analyzed by gas-liquid chromatography (glass column, 2 m by 3 mm, packed with 1.5 percent OV-1 on Chromosorb W). The result showed that the sugar part consisted of 2 moles each of quinovose (6-deoxyglucose) and fucose, and also that a steroidal ring was present. Use of the sodium rhodizonate method (*12*) showed that 1 mole of sulfate was present per 4 moles of sugars. All the properties show that the sperm agglutinin is quite similar to asterosaponin A.

The effective concentrations of the sperm agglutinin ranged between 10^{-3} and $10^{-6}M$, if the molecular weight is assumed to be around 1200 as same as asterosaponin A. Although the critical micelle concentration of the agglutinin is unknown, it is safely estimated to be less than $10^{-5}M$ because the sperm agglutinin is undialyzable at this concentration. It is very likely that the agglutinin exists as multivalent micelles at the effective concentrations.

Several saponins have been obtained from starfishes; the two main ones were named asterosaponin A and asterosaponin B (*13*). The former consists of a sulfated steroidal ring and a sugar moiety containing 2 moles each of quinovose and fucose. The sperm agglutinin, T_1, and asterosaponin A showed the same mobility on TLC with two solvent systems: chloroform, methanol, acetic acid, and water (30:10:5:2, by volume) and *n*-butanol, acetic acid, and water (12:3:5, by volume). Although the steroidal ring of the sperm agglutinin is not completely identified yet, the gas chromatogram of trimethylsilylated derivatives after methanolysis of the sperm agglutinin was the same as that of asterosaponin A in the retention time of a steroidal ring and its relative response to sugars. It seems safe to conclude that the sperm agglutinin of this starfish is an asterosaponin A, although its exact chemical structure has not been ascertained.

A sample of asterosaponin A prepared from lyophylized whole ovaries of *A. amurensis* by Ikegami (*14*) failed to cause the sperm agglutination. Asterosaponin A is the general name for a number of saponins present in asteroids which contain 2 moles each of quinovose and fucose as the sugar moiety. Our preparation may perhaps differ in its sugar linkages or steroidal side chain from that of Ikegami (*14*).

The effect of the sperm agglutinin was examined on the spermatozoa of another starfish, *Distolasterias nipon*, belonging to the same family as *A. amurensis*. The highly purified sperm agglutinin, T_1, was not effective, but the partially purified agglutinin found in the butanol layer induced the sperm agglutination to some extent. It seems that the sperm agglutinin of *D. nipon* is not identical with but similar to the agglutinin of *A. amurensis*.

Recently Collins (*15*) has stated that there are two types of the sperm agglutination in sea urchins. One is a swarming of freely moving sperm, "isoagglutination," and the other is the agglutination with the acrosomal process. The former disappears spontaneously within 10 minutes, and instantaneously when fixatives or inhibitors of sperm motility are added. In the case of starfish, the sperm agglutination does not disappear either spontaneously or by the addition of such fixatives, and it is induced independently of the acrosome reaction.

Table 1. Effects of egg jelly fraction on the sperm. Equal volumes of 1 percent sperm suspension and an eluted fraction were mixed and observed with light microscopy (×100) for agglutination. Portions were fixed with formaldehyde and reacted spermatozoa were counted by electron microscopy. (+) Agglutinating activity, (−) no activity, and (±) weak activity.

Fraction tested	Acrosome reaction (%)	Sperm agglutination
Whole jelly	91.0	+
J1	85.4	−
J2	2.5	+
J3	4.3	±
Seawater	1.0	−

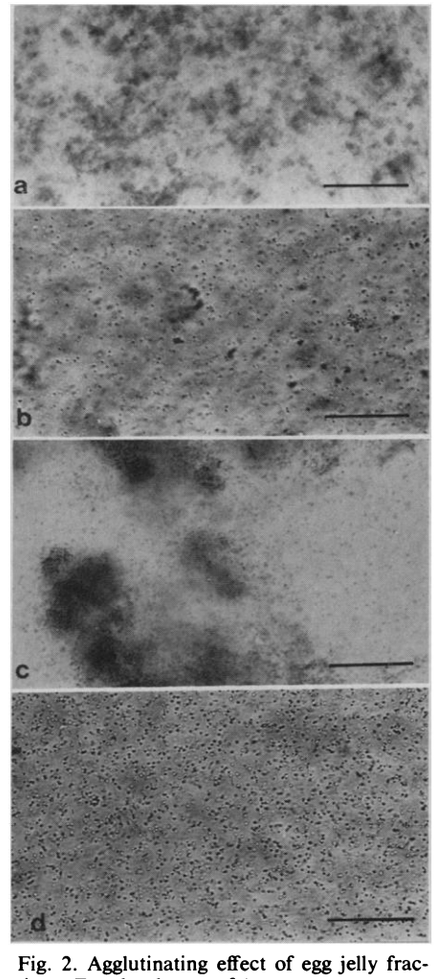

Fig. 2. Agglutinating effect of egg jelly fractions. Equal volumes of 1 percent sperm suspension and a fraction dissolved in seawater (1 mg/ml) were mixed and observed. (a) Whole jelly, (b) J1, (c) J2, and (d) seawater. Scale bar, 50 μm.

YOHKO UNO, MOTONORI HOSHI*

Biochemical Laboratory, Institute of Low Temperature Science, Hokkaido University, Sapporo 060, Japan

References and Notes

1. J. C. Dan, *Biol. Bull.* **103**, 54 (1952).
2. F. R. Lillie, *Science* **36**, 527 (1912).
3. H. Ohtake, *J. Exp. Zool.* **198**, 303 (1976).
4. L. H. Colwin and A. A. Colwin, *J. Biophys. Biochem. Cytol.* **10**, 231 (1961).
5. F. R. Lillie, *J. Exp. Zool.* **16**, 523 (1914); A. Tyler, *Physiol. Rev.* **28**, 180 (1948).
6. E. Vasseur, *Acta Chem. Scand.* **6**, 376 (1952).
7. S. Isaka, M. Akino, K. Hotta, M. Kurokawa, *Exp. Cell Res.* **54**, 247 (1969).
8. K. Ishihara and J. C. Dan, *Dev. Growth Differ.* **112**, 179 (1970).
9. T. Muramatsu, *J. Biochem.* **57**, 223 (1965).
10. H. Kanatani, *Exp. Cell Res.* **57**, 333 (1969).
11. A. Tyler, *ibid.* **10**, 377 (1956).
12. T. T. Terho and K. Hartiala, *Anal. Biochem.* **41**, 471 (1971).
13. Y. Hashimoto and T. Yasumoto, *Bull. Jpn. Soc. Sci. Fish.* **26**, 1132 (1960).
14. S. Ikegami, Y. Kamiya, S. Tamura, *Agr. Biol. Chem.* **36**, 1087 (1972).
15. F. Collins, *Dev. Biol.* **49**, 381 (1976).
16. We thank the Akkeshi Marine Biological Station of Hokkaido University and Tateyama Marine Laboratory of Ochanomizu University for the use of their facilities during parts of this study; Dr. Ikegami, at the University of Tokyo, for the asterosaponins, and Dr. J. C. Dan for critical reading of the manuscript. Supported in part by grants from the Ministry of Education of Japan, and the Itoh Science Foundation. This report is dedicated to Prof. Juro Ishida on the occasion of his 70th birthday.

* To whom correspondence should be addressed.

20 September 1977