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The activity for the induction of the sperm acrosome reaction localises in the outer layers and exists in the high-molecular-weight components of the egg-jelly of the newt, *Cynops pyrrhogaster*

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Summary

Localisation of the acrosome reaction inducing activity in egg-jelly was examined in the newt, *Cynops pyrrhogaster*. The jelly has six layers: the J0, J1, J2, J3, J4 and st layers. Jelly was mechanically dissected and placed on a Millipore filter. When sperm were added from the outer surface side of the jelly, most of them exhibited the acrosome reaction after passing through the jelly. When egg-jelly was divided into four layers, strong activity for the induction of acrosome reaction was detected in the outer layers, J4+st. These findings suggest that the acrosome reaction is induced by a substance in the outer layers of the egg-jelly. Among jelly components separated by SDS-PAGE, a fraction of more than 500 kDa in molecular weight induced the acrosome reaction. Wheat germ agglutinin (WGA), *Griffonia simplicifoliar* agglutinin 1 (GS-1), *Maclura pomifera* agglutinin (MPA) and *Arachis hypogaea* agglutinin (PNA) inhibited the induction of the acrosome reaction by jelly extract, and WGA did so in a dose-dependent manner. Those lectins precipitated some molecules of over 500 kDa. These results suggest that the acrosome reaction is induced by the high molecular-weight components of egg-jelly in *C. pyrrhogaster*.

Keywords: Acrosome reaction, Egg-jelly, Internal fertilisation, Newt, WGA

Introduction

The acrosome reaction (AR) plays a significant part in fertilisation. It is induced by sperm–egg interaction in the egg coat, and the AR-inducing substances have been identified in some animals. In starfish, the AR inducing substance is a huge molecule that exists in the egg-jelly, and its active component is composed of repeated oligosaccharides (Hoshi *et al*., 1988, 1990). In sea urchin, fucose sulphate rich glycoprotein in egg-jelly induces the AR (SeGall & Lennarz, 1979), and N-linked carbohydrates are necessary for the induction (Keller & Vacquier, 1994*a*; *b*). In mouse, ZP3, one of the molecules comprising the zona pellucida, activates the induction of the AR (Bleil & Wassarman, 1980), and the protein portion of ZP3 may be significant in this process (Endo *et al*., 1987). These reports suggest that the induction of the AR is regulated in a species-specific manner.

Anuran amphibians generally undergo external fertilisation, which begins after the eggs have been spawned into water. In contrast, most newts undergo internal fertilisation (Elinson, 1986; Wake & Dickie, 1998). In the Japanese newt, *Cynops pyrrhogaster*, an egg is inseminated with sperm in the cloaca of the female, and fertilisation is achieved without the eggs ever contacting the water (Tsutsui, 1931; Street, 1940). The fertilisation processes of these species are supposed to have adapted to internal fertilisation (Elinson, 1986; Ukita *et al*., 1999; Onitake *et al*., 2000). The AR of anuran amphibians, such as the toad, is induced at or near the vitelline envelope (Yoshizaki & Katagiri, 1982). The acrosome is maintained by the glycoprotein in egg-jelly (Arranz & Cabada, 2000). Sperm without an acrosome are observed in the egg-jelly of the urodele, *Pleurodeles waltlii* (Picheral, 1977), and in C. pyrrhogaster, sperm cannot bind to the vitelline envelope before the induction of the AR (Nakai *et al*., 1999). These reports indicate that there are differences in the regulation of induction of the AR between anurans and urodeles. Recently, we found that the AR could be induced by the egg-jelly extract of *C. pyrrhogaster* (Takai & Onitake, 1990; Nakai *et al*., 1999; Onitake *et al*., 2000), indicating that the substances for the induction of the AR are included in egg-jelly of *C. pyrrhogaster*.

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Amphibian egg-jelly is composed of several layers (Good & Daniel, 1943; Katagiri, 1965; Omata, 1993; Humphries, 1966; MacLaughlin & Humphries, 1978; Freeman, 1968; Shivers & James, 1970; Gusseck & Hedrick, 1971; Yurewicz *et al*., 1975; Carroll *et al*., 1992). Though these layers have qualitative differences (Yurewicz *et al*., 1975; Bonnell *et al*., 1996; Bonnell & Chandler, 1996), the role of each is unknown. In this study, we investigated the localisation of the AR-inducing activity in newt egg-jelly. Moreover, the molecular features of the active substance were estimated.

Materials and methods

Gametes

Sexually mature newts of *Cynops pyrrhogaster* were collected in Yamagata prefecture, Japan. Ovulation was induced by two injections of gonadotropin (hCG, Teikoku Zoki, Japan) at a dose of 50 IU every 24 h. Mature eggs were obtained from the uterus by surgical operation. To obtain dry sperm, the vasa deferentia were removed and the contents pushed out from the duct by fine forceps. Eggs and dry sperm were stored in a moist chamber at room temperature until use.

Histology

The mature eggs were fixed in methanol fixative. Paraffin sections of 20 µm were prepared and stained in Delafield's haematoxylin and eosin.

Insemination of the separated egg-jelly (Fig. 1)

Egg-jellies were removed from mature eggs by surgical operation. They were cut in half with fine scissors and mechanically divided into four layers; that is, the J4+sticky (st) layer, J3, J2 and J1+J0. The inner surface side of the egg-jelly was placed on a Millipore filter (0.45 µm pore size; Advatec, Japan), which had in it a hole of about 1 mm in diameter. One microlitre of dry sperm was added to the outer surface side of the egg-jelly, and allowed to sit for 15 min. An aliquot of modified Steinberg's salt solution (58.2 mM NaCl, 0.67 mM KCl, 0.83 mM $MgSO_4$, 12 mM Ca $(NO_3)_2$, 3 mM Hepes-NaOH; pH 7.8) was dropped on the inner surface of the egg-jelly through the hole in the Millipore filter. Sperm passing through the egg-jelly were collected from the drop.

Acrosome reaction

Sperm were fixed in 2.5% glutaraldehyde in Steinberg's salt solution (pH 7.8) for 30 min. An aliquot of the sperm suspension was placed on the glass slide.

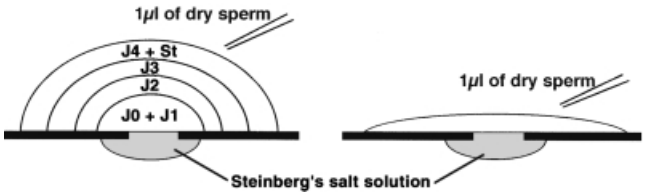

Figure 1 Preparation of jelly layers to estimate the acrosome reaction inducing activity. Egg-jellies were mechanically dissected (left figure). When the induction of the acrosome reaction was examined in each layer, whole egg-jelly was mechanically divided into four pieces (right figure); the st +J4, J3, J2 and J1+J0 layers. The inner surface of the sample was put on a Millipore filter with a hole 1 mm in diameter. One microlitre of dry sperm was added directly to the outer surface and allowed to sit for 15 min. A drop of Steinberg's salt solution was added through the hole on the other side, and sperm passing through the egg-jelly were collected from the drop.

Acrosome-reacted sperm could easily be observed by microscopy (BH2, Olympus, Japan) (Nakai *et al*., 1999) because the acrosome of *C. pyrrhogaster* is large in size. The percentage of acrosome-reacted sperm was calculated. In some cases, sperm were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for more than 4 h at 4 °C, rinsed with cold buffer, and post-fixed in similarly buffered 1% OsO_4 for 2 h. Samples were dehydrated in an ethanol series. After immersion in isoamyl acetate, they were dried in a critical point apparatus (HCP-1, Hitachi, Japan). The samples were then coated with platinum using a magnetron sputterer (JUC-5000, JEOL, Tokyo) and observed with a scanning electron microscope (JSM-5400, JEOL, Tokyo).

Solubilisation of egg-jelly

Egg-jellies were quickly freeze-dried in liquid nitrogen and dissolved at 10 µg/µl in 8 M guanidine hydrochloride. The solution was dialysed in modified Steinberg's salt solution and filtered with a Millipore filter (0.45 µm pore size; Advantec, Japan). Dry sperm were treated with the solubilised egg-jelly for 5 min at room temperature, then observed with a phase-contrast microscope.

SDS-PAGE

Sample buffer (2% SDS, 62.5 mM Tris-HCl; pH 6.8) was added in equal volume to the solubilised egg-jelly. Twenty microlitres of the sample was electrophoresed by SDS-PAGE with 2–15% or 3–3.5% polyacrylamide gel according to the method of Laemmli (1970). The gel was cut into six pieces along each lane with a fine knife. Each gel fragment was placed in 2 ml of glycine buffer

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(192 mM glycine, 0.1% SDS, 25 mM Tris-HCl; pH 8.3) and dialysed in the glycine buffer at 4 °C for 3 days. Each solution was treated with 1% trichloroacetate and centrifuged at 15 000 *g* at 4 °C for 30 min. The pellet was rinsed with methanol and dissolved in modified Steinberg's salt solution. Activity for the induction of the AR was examined.

Inhibition assay with lectins

For extracting jelly substances, 30 µl of modified Steinberg's salt solution was added to each mature egg. Eggs were shaken at 4 °C for an hour and centrifuged at 16 000 *g* at 4 °C for 30 min. The supernatant was collected and stored at 4 °C until used for the following experiments.

Nine types of lectins were tested: *Bauhinia purpurea* agglutinin (BPA), *Canavalia ensiformis* agglutinin (ConA), *Griffonia simplicifoliar* agglutinin 1 (GS-1) and 2 (GS-2), *Maclura pomifera* agglutinin (MPA), *Arachis hypogaea* agglutinin (PNA), *Glycine max* agglutinin (SBA), *Ulex europaeus* agglutinin-1 (UEA-1) and *Triticum vulgaris* agglutinin (WGA) (EY Lab., UK).

To confirm the effect of these lectins against substances for the induction of the AR, three types of experiments were independently pursued: (a) To examine the capability of lectins inducing the AR, 1 µl of dry sperm was treated with a solution containing 50 µg/ml of each lectin for 5 min at room temperature. (b) To examine the inhibitory activity on the induction of the AR by lectins binding to sperm, 1 µl of dry sperm was treated with 50 µg/ml of each lectin. Samples were placed for 5 min at room temperature and washed three times with modified Steinberg's salt solution. They were treated with jelly extract. (c) To examine the inhibition of lectins by binding to the substances for the induction of AR in the jelly extract, lectins were added to jelly extract at the respective concentrations of 100, 50 and 10 µg/ml. Samples were placed for 120 min at room temperature and centrifuged at 15 000 *g* for 30 min at 4 °C. One microlitre of dry sperm was added to the supernatant. Acrosome-reacted sperm were observed by phase contrast microscopy (MIT-2, Olympus, Japan).

Analysis of jelly extract with lectins

Jelly extract treated with each lectin was centrifuged 15 000 *g* at 4 °C for 30 min. The pellet was washed and treated with solution containing 50 mg/ml carbohydrate specifically bound to each lectin; that is, N-acetylgalactosamine to BPA, MPA and SBA; D-(+)-mannose to ConA; galactose to GS-1 and PNA; N-acetylglucosamine to GS-2 and WGA; and L-(–) fucose to UEA-1. One microlitre of dry sperm was added to the solution, and the induction of the AR was observed. Along with these procedures, an aliquot of the solution was electrophoresed by SDS-PAGE.

Characterisation of the AR-inducing activity in jelly extract

To examine the heat stability of the AR-inducing activity, jelly extract was incubated at 100 °C for 5 min. One microlitre of dry sperm was added to the heat-treated jelly extract for 5 min and fixed in 2.5% glutaraldehyde, and the induction of the AR was estimated.

When the involvement of a protein in the induction of the AR was examined, one-hundredth volume of 2% trypsin in modified Steinberg's salt solution (ST) was added to JE and the solution was incubated at 37 °C for 30 min. Then, one-hundredth volume of 1% soybean trypsin inhibitor (SBTI) in ST was added to the solution and the activity for the induction of the AR was estimated. In the control, one-hundredth volumes of 2% trypsin and/or 1% SBTI were added to jelly extract or ST. The solutions were incubated at 37 °C for 30 min.

To examine the involvement of carbohydrate moieties in the induction of the AR, one-tenth volume of 10% sodium periodate was added to jelly extract. The solution was incubated at 15 °C for 30 min.

Results

The morphology of egg-jelly in *C. pyrrhogaster*

Based on the histological and morphological features, jellies of *Cynops pyrrhogaster* consisted of six layers: J0, J1, J2, J3, J4 and st, from the innermost to the outermost layer (Fig. 2). J0 and J2 layers were stained with

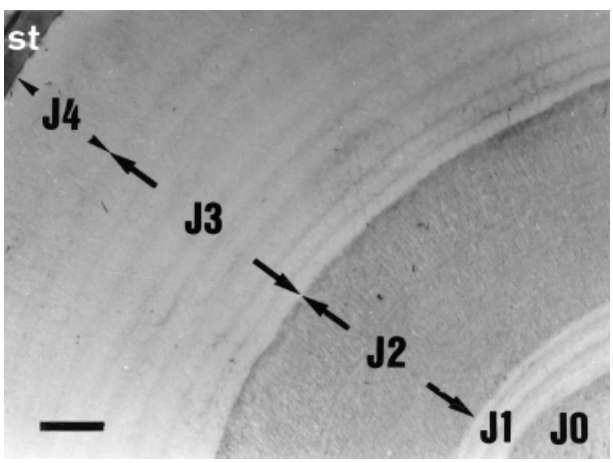

Figure 2 A section of egg-jelly of *C. pyrrhogaster*. The mature egg was fixed in methanol fixative. A section of 6 µm was prepared and stained with a hematoxylin-eosin. Six layers, the J0 to J4 and st layers, respectively, were visible in the egg-jelly. Scale bar represents 100 µm.

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haematoxylin, and the sticky layer was strongly stained with eosin. J1 and J3 layers had a layered composition, while J4 layer was uniform in appearance.

Induction of AR of sperm after passing through egg-jelly

Egg-jelly was obtained from the mature egg and put on a Millipore filter with a hole (Fig. 1). One microlitre of dry sperm was added to the outer surface side of the jelly, and sperm passing through it was collected from the inner surface side through the hole 15 min after the addition. Twenty-seven trials were independently performed, and the mean number of collected sperm was 18.0 ± 4.5. When the egg-jelly dissected into four layers was examined, the number of sperm collected was 25.7 ± 6.5 for st+J4 layers, 135.5 ± 15.2 for J3 layer, 41.7 ± 20.0 for J2 layer and 20.0 ± 11.4 for J1 + J0 layers. The large number of sperm in the J3 layer suggested that this layer is relatively easy for sperm to pass through. However, no significant difference was seen among the other layers and the intact egg-jelly.

As shown in Fig. 3, only 5.3% of dry sperm were acrosome-reacted. The percentage of acrosome-reacted sperm increased to 95.4% after passing through the whole egg-jelly. This result is in accord with those in previous studies (Nakai *et al*., 1999; Onitake *et al*., 2000); the activity for the induction of the AR existed in egg-jelly extract of *C. pyrrhogaster*.

When the localisation of the activity in egg-jelly was examined, 87.1% of sperm were acrosome-reacted in the J4+ outermost st layers. The activity was equal to that in the intact egg-jelly. The percentage was less than 50% in the J3, J2 and J1+J0 layers, respectively – significantly lower than that in the intact egg-jelly. Because every sperm was in contact with each egg-jelly layer for at least 15 min, this result suggests that the activity for the induction of the AR is localised in the outer layers of egg-jelly.

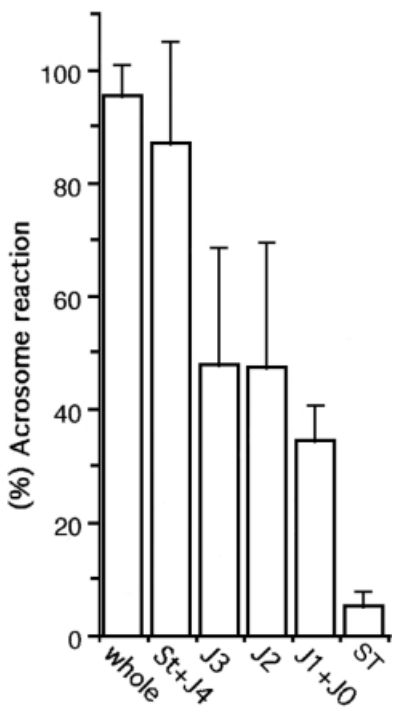

Figure 3 Induction of the acrosome reaction in sperm after its passage through various layers of the egg-jelly. Sperm passing through the jelly layers were fixed in 2.5% glutaraldehyde, and the induction of the acrosome reaction was observed by phase contrast microscopy. ST indicates the induction of the acrosome reaction in sperm suspended in modified Steinberg's salt solution as the negative control.

Identification of the AR-inducing substances in egg-jelly

For the analysis of the AR-inducing substances in egg-jelly, we solubilised egg-jelly components and used them for the examinations. When dry sperm was added to the solubilised egg-jelly, 80.4% of sperm were acrosome-reacted. The activity was also high, about 10 times as strong as that in the jelly extract, when the original jelly was diluted to 64 times. 19.4% of sperm were acrosome-reacted in the modified Steinberg's salt solution.

The jelly layer is composed of large glycoconjugates forming stable networks and small diffusible molecules binding to these networks (Bonnell *et al*., 1993, 1994, 1996). In order to identify the AR-inducing substances, the solublised egg-jelly was electrophoresed by SDS-PAGE with a 2–15% acrylamide gel. The gel was cut into sixths across the lanes, and the molecules were extracted from each gel fragment. Activity for the induction of the AR was detected only in the fraction containing molecules more than 500 kDa in molecular weight (Fig. 4), suggesting that these large components of egg-jelly are involved in the induction of the AR of *C. pyrrhogaster*. Smear bands under about 60 kDa in molecular weight were observed in lanes 1–4 (Fig. 4*b*). These were supposed to be the disintegrated large glycoconjugates of egg-jelly.

Inhibition assay by lectins

A carbohydrate component is involved in the induction of the AR in some animals (SeGall & Lennarz, 1979; Hoshi *et al*., 1988, 1990; Keller & Vacquier, 1994*a*, *b*). In this study, we examined whether carbohydrates were involved in the induction of the AR of *C. pyrrhogaster* using lectins. First, to examine the activity for the induction of AR in lectins, dry sperm were treated with the lectins. The percentage of acrosome-reacted sperm after the treatment with each lectin was as low as that of the negative control using Steinberg's

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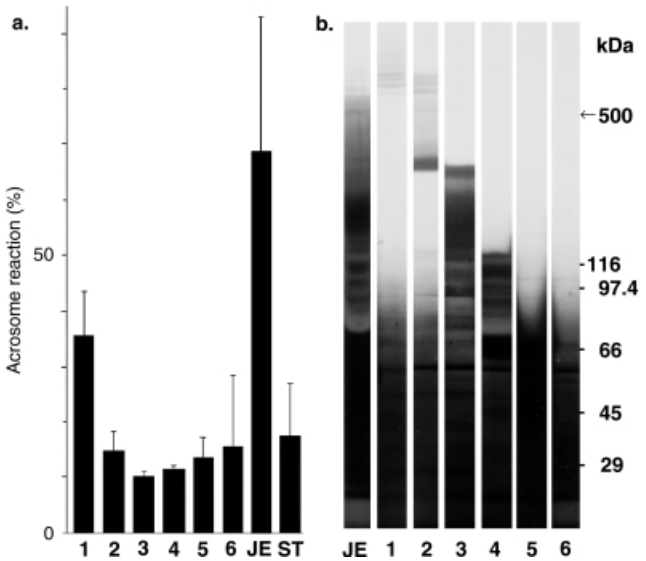

Figure 4 The activity for the induction of the acrosome reaction in the extract of dissolved egg-jellies fractionated by SDS-PAGE. The dissolved egg-jellies were fractionated by SDS-PAGE with 2–15% acrylamide gel. The gel was cut into six equal pieces across the lane. Egg-jelly components in each fragment were extracted in glycine buffer. (*a*) The induction of the acrosome reaction in the fractions. Dry sperm were added to each fraction. They were fixed for 5 minutes, and the acrosome reaction was observed by phase contrast microscopy. (*b*) SDS-PAGE of egg-jelly components with 2–15% acrylamide gel. JE indicates the whole jelly extract; ST indicates the modified Steinberg's salt solution. The numbers show egg-jelly components extracted from the divided gels. The fraction of the larger number contains the smaller components.

salt solution (Fig. 5), indicating that none of the tested lectins had any activity for the induction of AR.

Next, when sperm were pre-treated with lectins including WGA, SBA, PNA, MPA, GS-1, ConA and BPA and subsequently treated with jelly extract, more than 50% of sperm were acrosome-reacted (Fig. 6). The induction of AR was inhibited by pre-treatment with UEA-1 or GS-2, suggesting that these two lectins bound to sperm and inhibited the induction of AR.

Finally, jelly extract was respectively pre-treated with WGA, SBA, PNA, MPA, GS-1, ConA and BPA and sperm were added (Fig. 7*a*). Induction of the AR was inhibited by the pre-treatment with WGA, MPA, GS-1 or PNA. In those lectins, WGA inhibited the induction of the AR in a dose-dependent manner (Fig. 7*b*). When egg-jelly components were precipitated by WGA, MPA, GS-1 or PNA, followed by addition of the solution of N-acetylglucosamine, N-acetylgalactosamine or galactose as the competitor to WGA, MPA, GS-1 or PNA, most sperm were acrosome-reacted (Fig. 7*c*), suggesting that these lectins may bind to the AR-inducing substances.

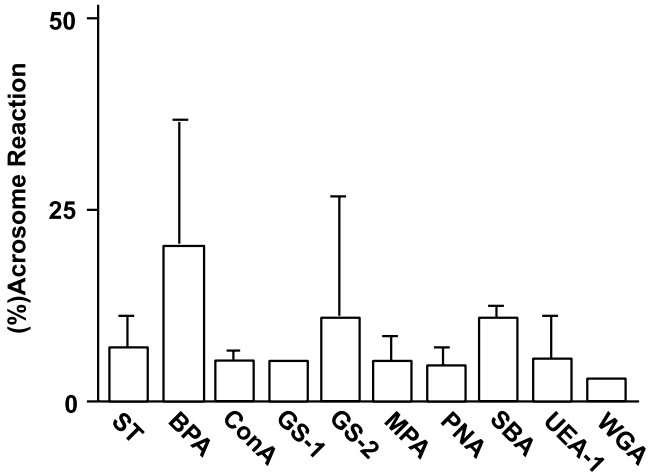

Figure 5 Induction of the acrosome reaction in lectin solutions. Dry sperm were added to 50 µg/ml of lectin solution. After 5 min, sperm were fixed and the induction of the acrosome reaction was observed by phase contrast microscopy. ST indicates modified Steinberg's salt solution.

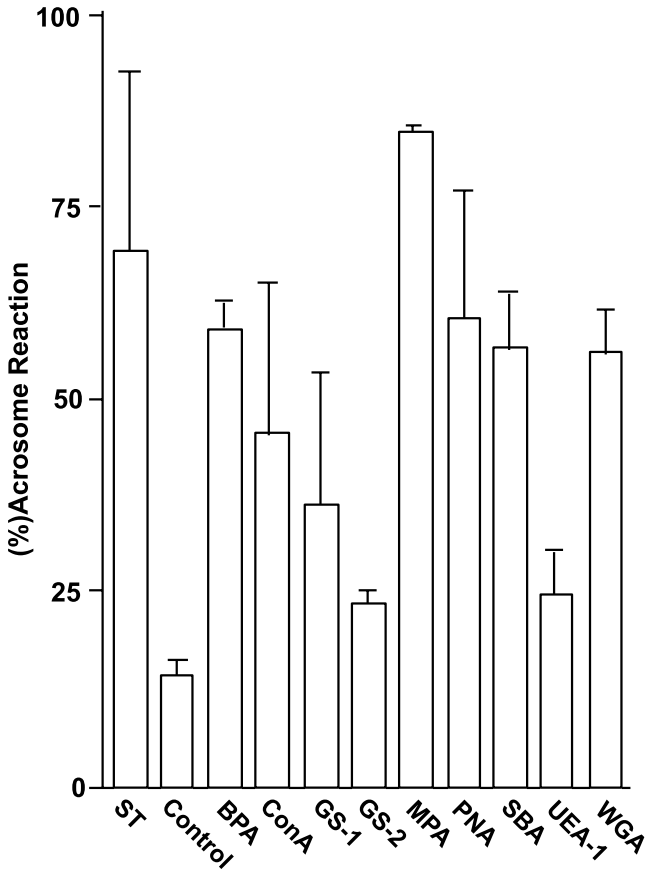

Figure 6 Induction of the acrosome reaction of lectin-treated sperm in jelly extract. Sperm were added to 50 µg/ml lectin solutions for 5 min, collected by the centrifugation, then treated with jelly extract. They were fixed in 2.5% glutaraldehyde for 5 min, and the induction of acrosome reaction observed by phase contrast microscopy. Control indicates the induction of acrosome reaction in sperm suspended in modified Steinberg's salt solution.

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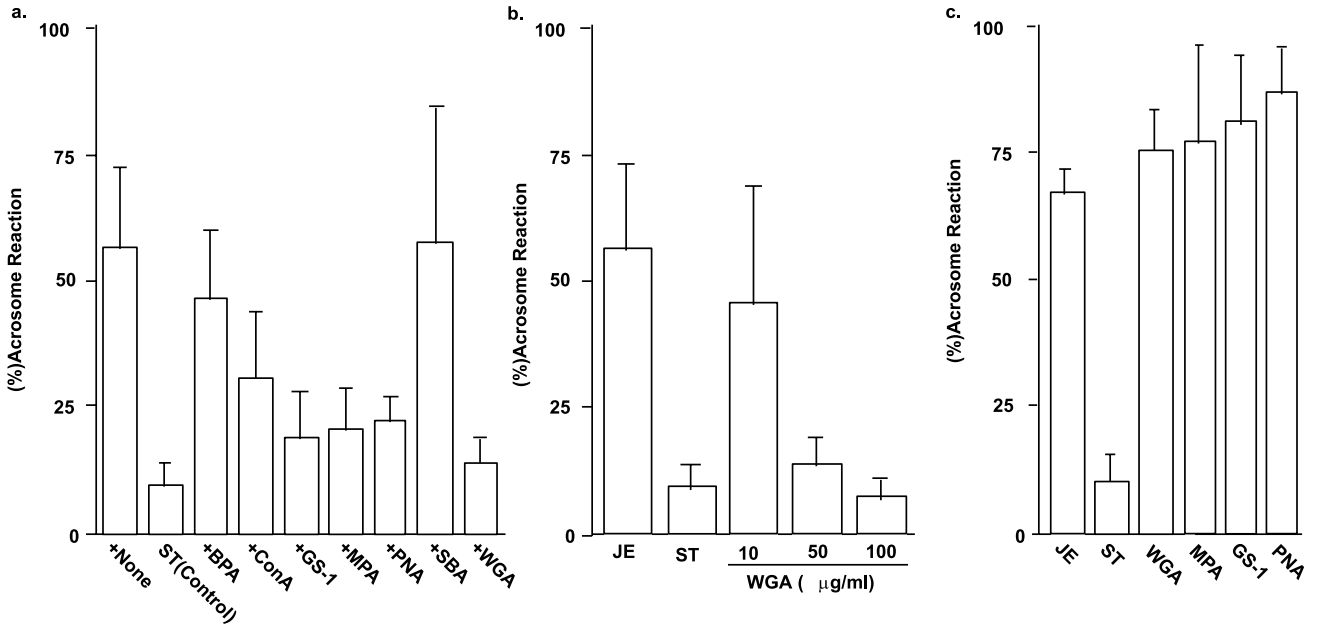

Figure 7 Induction of the acrosome reaction in jelly extract treated with lectins. Jelly extract (JE) was treated with 50 μg/ml lectin solutions, to which dry sperm were added. Sperm were fixed in 2.5% glutaraldehyde for 5 min. (*a*): Induction of the acrosome reaction was observed by phase contrast microscopy. ST indicates the induction of the acrosome reaction in sperm suspended in modified Steinberg's salt solution as the control. (*b*): JE was treated with WGA at 10, 50 and 100 μg/ml. The induction of the acrosome reaction was inhibited in a dose-dependent manner. (*c*): JE was treated with the lectins indicated and centrifuged at 15000 *g* at 4 °C for 30 min. The pellet was washed and treated with the solutions containing 50 mg/ml of N-acetylglucosamine in WGA, N-acetylgalactosamine in MPA, or galactose in GS-1 and PNA. Dry sperm were added to the solution and the induction of acrosome reaction was observed. JE and ST indicate jelly extract and the modified Steinberg's salt solution without treatment with lectins, respectively.

Jelly extract was treated with WGA, MPA, GS-1 or PNA, then centrifuged. Precipitates were electrophoresed by SDS-PAGE. The silver staining detected several bands of more than 500 kDa in molecular weight (Fig. 8). These bands are likely to represent the substances in the active fraction shown in Fig. 4*b*.

Characterisation of the AR-inducing activity in jelly extract

The effects of heat treatment, trypsin digestion and periodate treatment on the AR-inducing activity was examined. The activity was decreased by the heat treatment at 100 °C for 5 min (Fig. 9*a*). In the control, JE or modified Steinberg's salt solution (ST) was placed at room temperature for 5 min. The strong and faint activ-

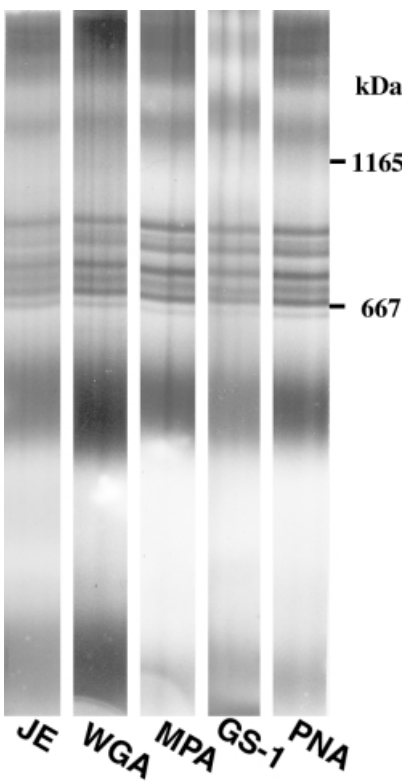

Figure 8 (*Right*) SDS-PAGE of the egg-jelly components precipitated with WGA, MPA, GS-1 or PNA. The respective lectins were added to the dissolved egg-jelly for 2 h, which was then centrifuged at 15000 *g*. The pellet was electrophoresed with 3–3.5% polyacrylamide gel, and processed by the silver staining. Seven major bands were detected from 500 to 900 kDa. The molecular weights of projectin (1165 kDa) and kettin (667 kDa) are indicated as molecular size markers. JE indicates jelly extract.

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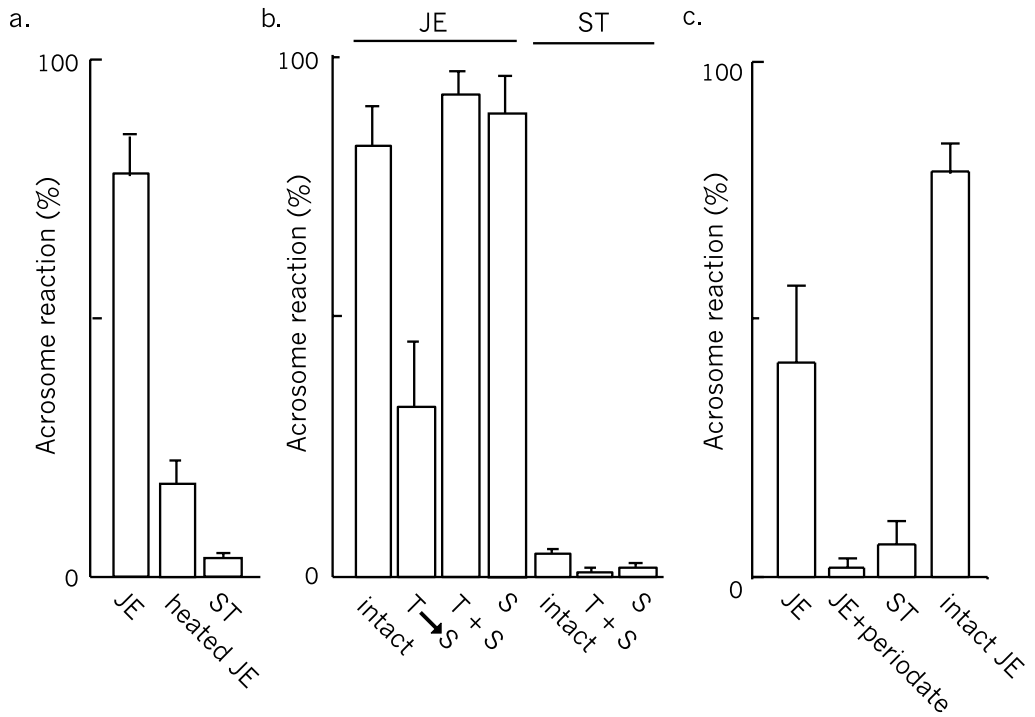

Figure 9 Characteristics of the acrosome reaction inducing activity in egg-jelly of *Cynops pyrrhogaster*. (*a*) The heat stability of the activity. Jelly extract (JE) was incubated at 100 °C for 5 min and sperm were treated with the heat-treated JE for 5 min. They were fixed in 2.5% glutaraldehyde for 30 min and the induction of the acrosome reaction was observed by microscopy. The activity was decreased by the heat treatment. (*b*) The effect of trypsin digestion on the activity. JE was incubated in 0.02% trypsin for 30 min. Then the JE was treated with 0.01% soybean trypsin inhibitor (SBTI) and sperm were treated with the trypsin-treated JE (T→S). In the control, JE or ST was incubated in 0.02% trypsin, 0.01% SBTI (T+S) or 0.01 % SBTI alone (S). The activity was decreased by trypsin digestion. (*c*) The effect of periodate treatment on the activity. JE was incubated in 1% sodium periodate at 15 °C for 30 min. They were dialysed in ST and sperm were treated with the periodate-treated JE. The activity remained high in the JE treated with ST (JE) and significantly decreased in JE treated with periodate (JE+periodate). In the control, strong and faint activities were observed in the JE without any treatment (intact JE) and ST.

ities were detected in JE and ST (Fig. 9*a*). This result indicates that the activity is not stable in heat treatment. When JE was treated with 0.02% trypsin at 37 °C for 30 min, the AR-inducing activity was decreased (Fig. 9*b*). The decrease was inhibited by the co-treatment of 0.01% soybean trypsin inhibitor (SBTI) with trypsin. This result indicates that a protein is responsible for the AR-inducing activity in jelly extract. The activity was also decreased by the periodate treatment at 15 °C for 30 min (Fig. 9*c*). This result suggests that carbohydrate moieties are involved in the AR-inducing activity.

Discussion

The AR is induced by the interaction between sperm and egg-coat in many animals, and regulated in a species-specific manner (SeGall & Lennarz, 1979; Hoshi *et al*., 1988, 1990; Keller & Vacquier, 1994*a*, *b*). In toad, AR is induced at or near the vitelline envelope (Yoshizaki & Katagiri, 1982), and the egg-jelly component has the ability to keep the acrosome intact in saline (Arrans & Cabada, 2000), suggesting that the jelly maintains the acrosome in the spawned sperm. In contrast, sperm may be induced to undergo the AR in the egg-jelly of *Pleurodeles waltlii* (Picheral, 1977). We have confirmed that the egg-jelly extract of *Cynops pyrrhogaster* is the source of activity for the induction of the AR (Nakai *et al*., 1999; Onitake *et al*., 2000). These results suggest that the mechanism of regulation of induction of the AR is different between anurans and urodeles.

We investigated here for the localisation of AR-inducing activity in the egg-jelly of *C. pyrrhogaster*, which was shown to consist of six different layers (Fig. 2). Our results showed that the activity for the induction of AR was localised in the outer layers of the egg-jelly (Fig. 3). The *Xenopus* egg has three layers of jelly. Each layer has its own morphological and qualitative features, and is composed of a network of fibril structures (Bonnell *et al*., 1996; Bonnell & Chandler, 1996). However, little is known about the role of each layer. The results of this study suggest that the amphibian

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egg-jelly layers are functionally differentiated as regards sperm–egg interaction.

Xenopus egg-jelly is composed of large glycoconjugates, which form network structures, and small diffusible molecules (Bonnell *et al*., 1996). When the jelly is solubilised and electrophoresed by SDS-PAGE, the high-molecular-weight glycoconjugates with fibril structures are detected as several bands of more than 450 kDa in molecular weight. In the newt, the activity for the induction of AR was detected only in the fraction including molecules of more than 500 kDa in molecular weight (Fig. 4*a*, *b*). The bands of more than 500 kDa were supposed to correspond to the components forming the network structure in the egg-jelly of *C. pyrrhogaster*, as they do in that of *Xenopus*, and those high-molecular-components are involved in the induction of the AR.

Carbohydrates are responsible for the induction of the AR in some species (Hoshi *et al*., 1988; Keller & Vacquier, 1994*a*). In this study, none of the lectins used induced the AR (Fig. 5). However, when sperm were pre-treated with UEA-1 or GS-2, the induction of AR by jelly extract was inhibited (Fig. 6). In sea urchin, WGA inhibits the induction of AR by binding to sperm, and blocks Ca^{2+} uptake and Na^{+}/K^{+} exchange (Podell & Vacquier, 1984). Therefore, it is possible that these two lectins may affect ion channels, the exchanges through which contribute to induction of the AR in amphibian sperm.

On the other hand, the induction of AR was inhibited by the pre-treatment of jelly extract with WGA, PNA, MPA or GS-1, and the inhibition by WGA occurred in a dose-dependent manner (Fig. 7*a*, *b*). Further, high activity for induction of the AR was detected in the precipitates of jelly extract with those lectins (Fig. 7*c*). These results suggest that these lectins, especially WGA, may bind to the AR-inducing substances in egg-jelly of *C. pyrrhogaster*. We observed the induction of the AR in sperm trapped on the sticky (st) layer (unpublished data), suggesting that the AR-inducing substance exists at least in the st layer. Recently, we found that WGA and MPA may bind to st layers (Okimura *et al*., in press). However, because there is a possibility of co-precipitation of the AR-inducing substances with egg-jelly components bound to each lectin, further study is needed to determine the significance of the carbohydrates bound to the lectin in the induction of the AR in sperm of *C. pyrrhogaster*.

These four lectins bind to molecules of more than 500 kDa (Fig. 8). This result fits with the result shown in Fig. 4, suggesting that the substances exist in the large components of the egg-jelly. This is the first report identifying candidates for the AR-inducing substance in amphibian egg-jelly. Egg-jelly of *Xenopus* is composed of a network of fibril components that sustain small diffusible molecules (Bonnell *et al*, 1996). It is supposed that the AR-inducing substances localise in the network structure of the egg-jelly in *C. pyrrhogaster*. However, in this study we did not determine a single molecule which would be the AR-inducing substance. The activity for the induction of the AR in the egg-jelly extract was heat-sensitive and lost by periodate treatment as well as protease treatment (Fig. 9), suggesting that both carbohydrate components and proteinaceous molecules are significant for the induction of AR in *C. pyrrhogaster*. These four lectins will become a useful probe with which to identify the AR-inducing substance.

Acrosome of the toad is maintained by the egg-jelly component (Arrans & Cabada, 2000). In contrast, the results of this study indicate that AR in the newt is induced as the sperm pass through the jelly layers, especially through the outermost layers. These differences in the effects of egg-jelly between the species may be caused by the adaptation of their fertilisation modes. Most anurans and ancient groups of urodeles undergo external fertilisation, whereas many other urodeles, including newts, undergo internal fertilisation (Elinson, 1986; Wake & Dickie, 1998). In some species, fertilisation occurs in the oviduct, and the eggs are developed in the uterus (Greven, 1998). In these species, the oviductal secretions are supposed to adapt to the viviparous mode. In amphibians, the fertilisation process has been investigated mainly in anurans, and remains largely unknown in species characterised by internal fertilisation. We found that the regulation mechanism in the initiation of sperm motility was adapted in the newt to the internal fertilisation mode (Mizuno *et al*., 1999; Onitake *et al*., 2000). Investigations of the whole process of fertilisation in the newt may lead to an understanding of adaptation to the fertilisation mode in amphibians.

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