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Source: Zoological Science, 12(5) : 559-564

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.559

ZOOLOGICAL SCIENCE 12: 559–564 (1995)

A Simple Method for Introducing Macromolecules into the Blastocoel of Living Starfish Embryos

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ABSTRACT—We have devised a simple method for introducing macromolecules into the blastocoel of living starfish embryos. In this method, embryos are treated for 15 min with Ca^{2+} free sea water (CFSW) containing 0.1–5.0 mg/ml of enzymes or immunoglobulins. These molecules are considered to flow through the septate junction, the septa of which are made diffuse by deprivation of Ca^{2+} ions from the environmental sea water.

Introduction of enzymes which affect the component molecules of the extracellular matrix (ECM) drastically deformed the larval morphology. Introduction of a monoclonal antibody recognizing an ECM component prevented the mesenchyme cells from stretching and migrating normally. These results show that our method can be used to study the function of various ECM components in embryonic development.

INTRODUCTION

In order to study the roles played by the extracellular matrix (ECM) in embryonic development, the blastocoel of echinoderm embryos has been injected with macromolecules such as enzymes and antibodies which react specifically with components of the ECM [2, 8, 15, 17]. However, microinjection methods can be used with only a limited number of embryos at one time [12, 13]. In order to overcome this limitation and open the way to biochemical, pharmacological and ultrastructural studies of the component molecules of the ECM, a method is needed to obtain a large number of homogeneously treated embryos.

Recently, Dan-Sohkawa *et al.* have reported that the barrier function of the wall epithelium of the starfish embryo is released in response to a hypertonic environment caused by small molecules, such as glycine, urea or NaCl [7]. When fluorescein isothiocianate-conjugated immunoglobulin G (FITC-IgG) is mixed with hypertonic sea water, it flows passively into the blastocoel through intercellular pathways [7]. This phenomenon can be used to introduce various macromolecules into the blastocoel simply by placing the embryos into hypertonic sea water containing the macromolecules. However, in order to avoid possible side effects of the small molecules, by which the sea water is made hypertonic, it is preferable if the macromolecules can be introduced without the small molecules.

We report, in this paper, a new condition allowing macromolecules to pass into the blastocoel of living starfish embryos. When the embryos are treated for 15 min with Ca^{2+} free artificial sea water (CFSW)(Jamarin Laboratory, Osaka) containing IgG or IgM, these immunoglobulins pass into the blastocoel without giving any apparent damage to the embryonic integrity. This passage coincides with opening of septate junctions of the wall epithelium. We also demonstrate the validity of our method by examining the effects of two types of enzymes, collagenase and a glycosidase mixture, and a monoclonal antibody (Mab) 4H11, which recognizes a fibrillar component of the blastocoelic ECM. A part of this work has been published in the Proceedings of the International Echinoderm Conference [10].

Accepted June 8, 1995
Received April 3, 1995

MATERIALS AND METHODS

Embryos

Mature eggs of the starfish *Asterina pectinifera* were obtained after Kanatani [9] by treating freshly isolated ovaries with 10^{-6} M 1-methyladenine. Spawned eggs were inseminated with diluted dry sperm and allowed to develop to various stages in artificial sea water (SW), Jamarin U (Jamarin Laboratory, Osaka), at 20°C [6].

Macromolecules

Enzymes used in this study were collagenase (Nitta Gelatin Co., Osaka) and a glycosidase mixture (Glycosidase "mixed", Seikagaku Kogyo Co., Tokyo). For immunoglobulins (Igs), both specific and non-specific ones were used. The non-specific Igs used were goat anti-rabbit FITC-IgG (Sigma) and FITC-monoclonal anti-mouse Thy 1.1 (FITC-IgM) (Mouse IgM isotype, Sigma). The specific IgGs used were FITC-labeled and non-labeled 4H11 Mab, the latter being prepared from hybridoma-injected mouse ascites by affinity chromatography on protein A-Sepharose (mouse monoclonal IgG purification kit: Pharmacia, Uppsala). A part of this Mab was conjugated with FITC after Clausen [4]. The specificity of 4H11 Mab to the fibrillar component of the ECM was checked beforehand on sections of embryos which had been fixed with paraformaldehyde and embedded and sectioned in polyester wax [14]. The concentrations of the 4H11 Mabs were determined in bovine serum albumin equivalent.

Introduction of macromolecules into the blastocoel

Packed embryos (0.1 ml) at various stages of development were washed once with 10 ml of CFSW. They were then incubated with 1 ml of CFSW containing 0.1–5.0 mg/ml of different macromolecules for 15 min. When enzymes were introduced, the embryos were incubated at 0°C to prevent the enzymes from functioning outside the blastocoel. For introduction of the Mabs and all other treatments, the temperature was 18–20°C. The incubation was stopped by dilution with 10 ml of SW. Subsequently, the embryos were washed twice with 10 ml of SW to remove the remaining macromolecules.

For the control experiment, embryos were washed once with 10 ml of SW and treated with SW containing the same concentration of the same molecules for the same length of time as the experimental group.

Fluorescence and light microscopy

The distribution of FITC-Igs in the blastocoel of the treated embryos was detected under a fluorescence microscope (Olympus BH-2). The effects of enzymes and non-labeled 4H11 Mab introduced into the blastocoel were examined with a light microscope (Olympus BHC). Micrographs were taken after fixing the embryos lightly with 4% paraformaldehyde in SW to stop them from swimming [11]. This fixation caused no significant morphological change in the embryos.

Transmission electron microscopy

Embryos treated either with CFSW or Jamarin U for 15 min were fixed immediately following treatment with 2% O_SO_4 in Jamarin U for 90 min on ice. After washing several times with Jamarin U, the embryos were dehydrated by passage through a graded ethanol series. The embryos were then embedded in Spurr's low viscosity resin [11], and ultrathin sections were cut. They were stained with 3% uranyl acetate in 30% methanol followed by treatment with Renold's lead citrate and observed with a H-300 transmission electron microscope (Hitachi).

RESULTS

CFSW permits passage of immunoglobulins into the blastocoel

Embryos at the mid gastrula stage retained their normal shape for at least 15 min in CFSW, while the integrity of the epithelia (the ectoderm and the endoderm) was slightly modified compared to the control embryos kept in Jamarin U (Fig. 1a, b). Under light microscope, the epithelia were rougher and more translucent. At the ultrastructural level, the septate junctions of the ectoderm of the treated embryos were diffuse (Fig. 1c), while those of the control embryos were unaffected (Fig. 1d). After being returned to Jamarin U, the treated embryos quickly recovered the smoothness of their epithelia. No difference in the reaction was observed from the blastula stage to the bipinnaria stage.

In order to examine whether the macromolecules flow into the blastocoel on treatment with CFSW, FITC-IgG was added as a visualization marker. When 0.5 mg/ml of non-specific FITC-IgG was applied to the mid gastrulae, the fluorescence was detected in the blastocoel within 15 min (Fig. 2a). It was distributed homogeneously throughout the blastocoel regardless of the IgG concentration at the time of treatment (data not shown). In control embryos, which

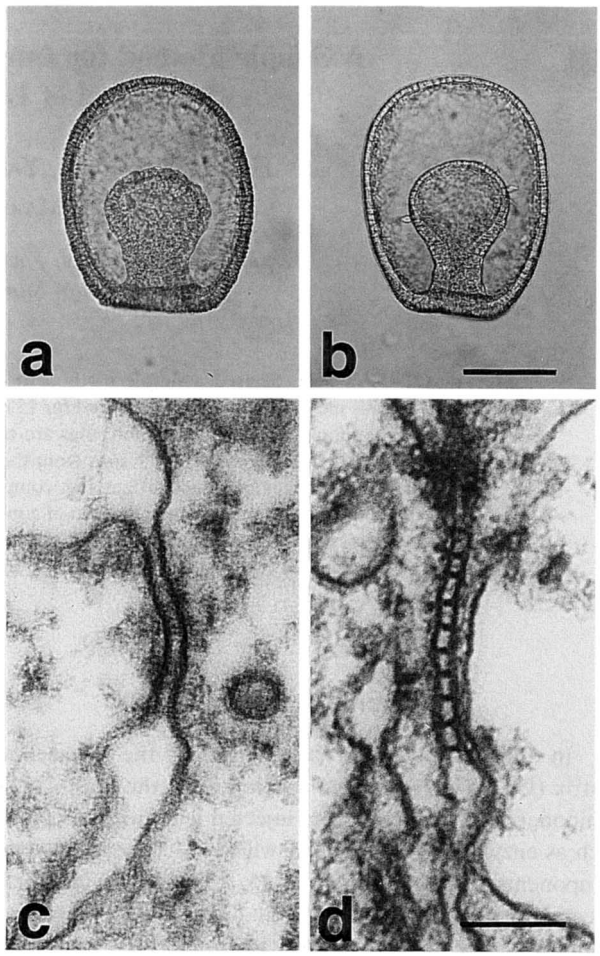

FIG. 1. Effect of CFSW on the embryonic epithelium at the mid gastrula stage. (a): Light micrograph of an embryo treated with CFSW for 15 min at 18–20°C. Note the rough and more translucent features of the epithelium as compared to that of the embryo in (b). (b): Light micrograph of an embyro treated with Jamarin U for 15 min at 18–20°C (control). (c): Electron micrograph of the septate junction binding the ectodermal cells of an embryo treated as in (a). The septa are more diffuse than those of the control embryo (d). Bar for (a) and (b), 100 μm; for (c) and (d), 0.1 μm.

received the FITC-IgG in Jamarin U at the same dose, no fluorescence was detected in the blastocoel (Fig. 2b). Application of non-specific FITC-IgM lead to the same results (photo not shown).

When FITC-4H11 Mab was given to the blastulae at concentrations ranging from 0.1 to 1.0 mg/ml in CFSW at 18–20°C, the intensity of the fluorescence increased in a dose-dependent manner. The fibrillar pattern of the staining was most obvious at 0.2 mg/ml (Fig. 2c).

Introduction of different kinds of macromolecules causes different morphological modifications

When collagenase was applied at concentrations between 0.1 and 2.0 mg/ml to embryos from the blastula to bipinnaria stages, the effect (Fig. 3b) was most prominent at 1 mg/ml in the mesenchyme migration stage (data not shown). Figure 3

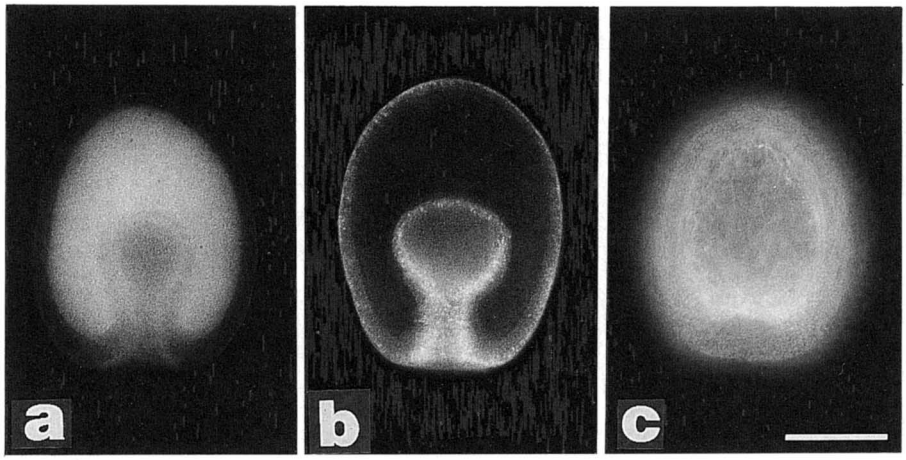

FIG. 2. Introduction of FITC-IgG into the blastocoel of living embryos. Fluorescence micrographs of (a); A mid gastrula embryo incubated for 15 min in CFSW containing 0.5 mg/ml of non-specific FITC-IgG. The fluorescence is distributed evenly throughout the blastocoel. (b): An embryo treated with Jamarin U containing the same concentration of the same FITC-IgG. Note the absence of fluorescence in the blastocoel. (c): An embryo 1 hr after treatment for 15 min with CFSW containing 0.2 mg/ml of specific FITC-IgG (4H11 Mab) at the blastula stage. The blastocoel is stained in a fibrillar pattern. The weak staining of the embryonic wall is considered to be non-specific, for it only appears occasionally under an unknown condition. Bar, 100 μm.

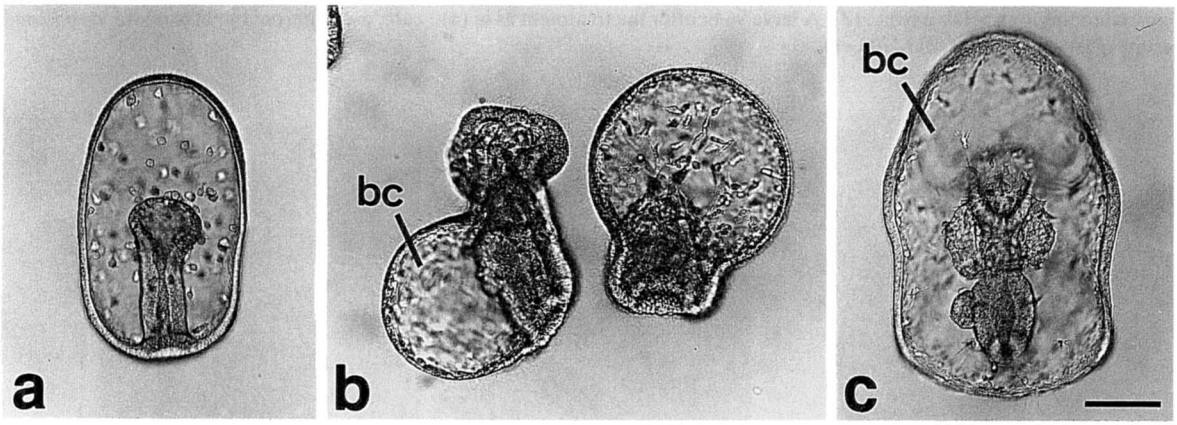

FIG. 3. Effect of collagenase introduced into the blastocoel of the late gastrula stage. (a): A late gastrula embryo to which the enzyme was introduced. (b): Embryos 24 hr after treatment with 1 mg/ml of collagenase at 0°C. The anterior (left embryo) or posterior (right embryo) portion of the blastocoel has almost completely collapsed. (c): A larva treated for 15 min at 0°C at the late gastrula stage with plain CFSW (blank experiment) and kept in Jamarin U for 24 hr. bc, blastocoel. Bar, 100 μm.

shows the effect of collagenase applied at 1 mg/ml in the late gastrula stage (Fig. 3a: a stage slightly later than the mesenchyme migration stage) at 0°C. The overall shape of the embryos was drastically deformed by 24 hr (Fig. 3b). Either the anterior or posterior portion of the blastocoel was markedly shrunken causing the archenteron to attach to the ectodermal wall. Mesenchyme cells were frequently absent from the remaining portions of the blastocoel (see the left embryo of Fig. 3b). Such deformations were not observed in the blank experiment, in which embryos were treated with CFSW not containing the collagenase (Fig. 3c).

When the glycosidase mixture was given to embryos at various stages between the blastula and bipinnaria at concentrations ranging from 0.5 to 5.0 mg/ml, the effect (Fig. 4b) was most prominent at 2.5 mg/ml on embryos at the mouth formation stage (Fig. 4a) (data not shown). The archenteron of the embryo treated with 2.5 mg/ml of the mixture at the mouth formation stage at 0°C lost its epithelial integrity within 2 hr as shown by the rounded feature of the constituent cells (Fig. 4b, arrows). Some of the cells had fallen into the archenteron lumen and were emitted from the blastopore. The mixture also caused a decrease in the volume of the blastocoel which, in turn, seemed to have generated the force to push the fallen cells out from the blastopore. The proportion of cells lost varied from embryo to embryo. The mixture seemed to affect the ectodermal cells much less severely as judged from the transparency and the smoothness of the embryonic wall. These embryos were capable of developing into bipinnariae lacking endodermal tissues to various extents depending on the proportion of cells lost. For instance, the larva shown in Figure 4c has no oesophagus and stomach. The size of the coelomic pouches was also

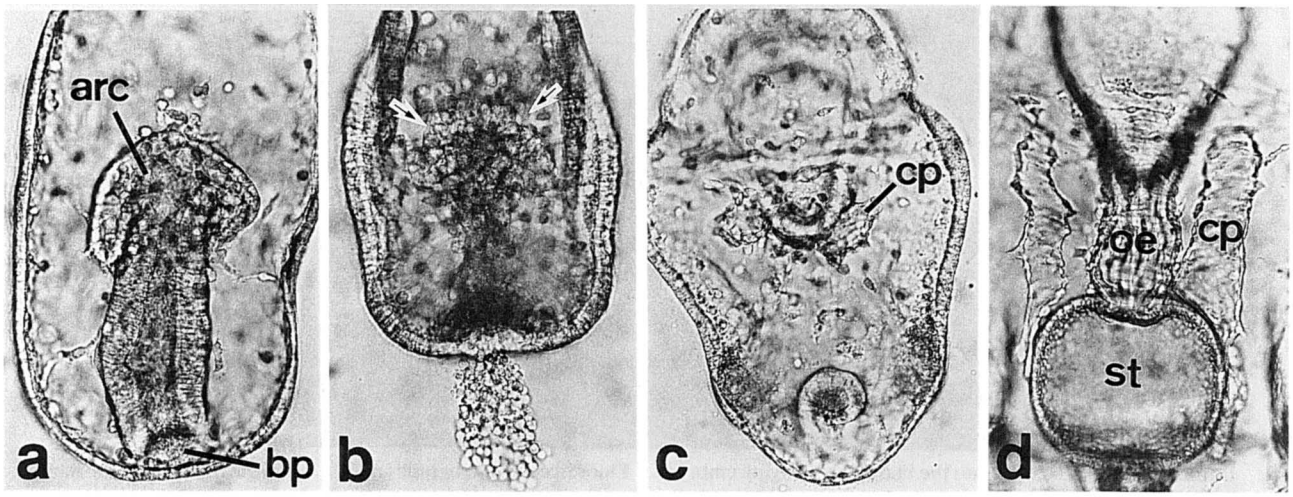

FIG. 4. Effect of the glycosidase mixture introduced into the blastocoel of embryos at the mouth formation stage. (a): An embryo looking quite normal 2 hr after treatment for 15 min at the mouth formation stage with Jamarin U containing 2.5 mg/ml of the glycosidase mixture at 18–20°C. (b): An embryo treated in the same manner as in (a) except that the mixture was dissolved in CFSW and the treatment was carried out at 0°C. The cells constituting the archenteron (arrows) are rounded while those of the ectoderm look quite normal. Some of the endodermal cells have been ejected from the blastopore. Also, the blastocoelic space is reduced. (c): A larva 96 hr after the treatment as in (b). The oesophagus and the stomach are missing altogether, and the volume of the coelimic pouches and the blastocoel are reduced as compared to the control larva (d). (d): A larva 96 hr after the treatment as in (a). arc, archenteron; bp, blastopore; cp coelomic pouch; oe, oesophagus; st, stomach. Bar, 100 μm.

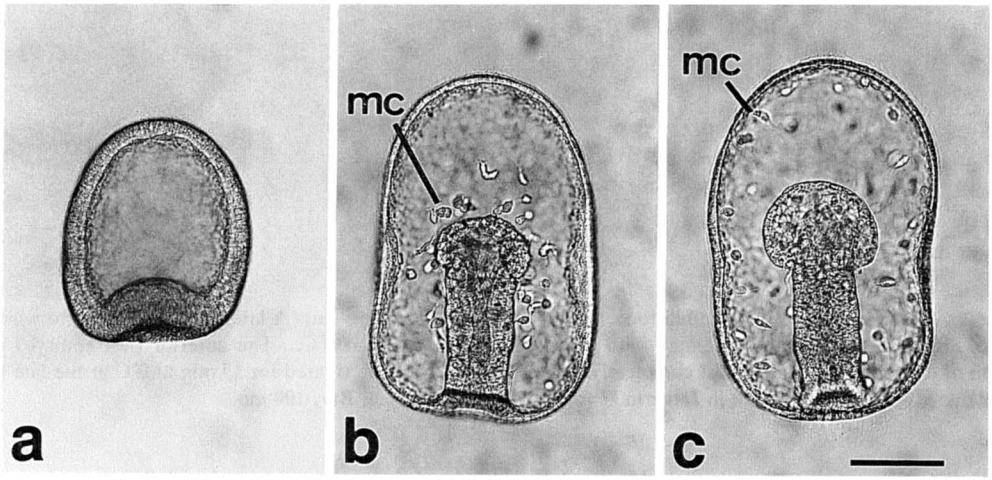

FIG. 5. Effect of 4H11 Mab introduced into the blastocoel of the very early gastrula stage. (a): An embryo 1 hr after treatment for 15 min at the very early gastrula stage with CFSW containing 1 mg/ml of 4H11 Mab. (b): An embryo 10 hr after the treatment as in (a). Note that the mesenchyme cells are confined to a space surrounding the archenteron. They are also prevented from stretching fully as compared to the control embryo shown in (c). (c); An embryo 10 hr after treatment with Jamarin U containing the same concentration of the same Mab as in (a). mc mesenchyme cell. Bar, 100 μm.

reduced compared to the control larva (Fig. 4d). No loss of endodermal tissues was observed in the control embryos, which were treated with Jamarin U containing the same dose of the glycosidase mixture (Fig. 4a, d).

In contrast to the low dose of the 4H11 Mab (0.2 mg/ml) given to the blastula for visualizing the fibrillar pattern in the blastocoel, as mentioned above, a higher dose was required to induce an apparent morphological effect on the embryonic development. Figure 5a shows an early gastrula 1 hr after introduction of 4H11 Mab (1 mg/ml in CFSW) into the blastocoel. While such embryos underwent gastrulation normally, the mesenchyme cells were prevented from migrating past a certain distance from the archenteron at the mesenchyme migration stage and later (Fig. 5b). These cells seemed unable to stretch fully when compared to those of the control embryos (Fig. 5c). No such suppression was observed in the control embryos, which were treated with Jamarin U containing the same dose of 4H11 Mab (Fig. 5c).

DISCUSSION

We have demonstrated here that macromolecules such as enzymes and immunoglobulins can be introduced into the blastocoel of living starfish embryos by incubating them with CFSW containing these macromolecules. This method not only enables us to treat a large number of embryos simply, but also under a homogeneous condition. Although the exact concentration of molecules introduced into the blastocoel is unknown, a large number of homogeneously treated embryos can be obtained by this method for studies to characterize the roles of different molecular components of the ECM *in vivo*.

As in the case of the hypertonic stimuli [7], the conditions used in the present method are considered to cause the macromolecules to flow passively into the blastocoel through paracellular pathways. This notion is based on the fact that the septate junctions are affected in a similar manner under the two conditions (Fig. 1c, d). It is not known at present the mechanism causing the same response of the septate junctions in response to the two different conditions.

We have demonstrated the validity of our method by showing specific modifications on the embryonic morphology induced by different macromolecules. The disappearance of the greater part of the blastocoelic cavity following introduction of collagenase indicates the possibility that collagen molecules function as the backbone of the molecular conformation of the ECM. This notion is supported by the fact that collagen molecules quickly accumulate in sea urchin embryos between the blastula and gastrula stages in parallel with the expansion of the blastocoelic cavity [1, 3, 5, 16, 18]. On the other hand, the loss of endodermal integrity caused by the introduced glycosidase mixture indicates that certain kind(s) of glycosaminoglycan molecules are functioning in cell-cell and/or cell-basal lamina adhesion. Furthermore, the partial shrinkage of the blastocoel caused by the same mixture indicates that all or a part of the substrate glycosaminoglycans of these enzymes are involved as component(s) of the hydrostatic skeleton of the blastocoel.

Another possible function of the ECM component was suggested by the 4H11 Mab experiment. The staining pattern of FITC-4H11 Mab introduced into the blastocoel indicates that this Mab recognizes a fibrillar component of the ECM. This component is considered to play a significant role in the migration of mesenchyme cells because its modification by Mab prevents mesenchyme cells from migrating outside a certain confined area around the archenteron (Fig. 5). This finding agrees with that of Burke and Tamboline [2] in which an injected Mab specific to a fibrillar component of the ECM of the sea urchin embryo inhibits migration of the secondary mesenchyme cells. It is not known, however, whether the fibrillar component of the ECM recognized by the two Mabs are identical or not.

As shown above, our method opens a way to analyzing the *in vivo* roles of known ECM component(s) in various aspects of embryonic development such as morphogenesis and cell migration. This method can also be extended to characterizing unknown ECM component(s) which is affected by the introduced macromolecules. Our method should promote further knowledge of the structure and function of the ECM components via studies encompassing wider ranges of both enzymes and antibodies of higher specificities.

ACKNOWLEDGMENTS

We are grateful to the members of Asamushi Marine Biological Station of Tohoku University and Professor Susumu Ikegami of Hiroshima University for supplying the starfish. Thanks are also due to Mr. Noriya Miyata for technical assistance with the photography. This investigation was partly supported by the Grant-In-Aid of the Ministry of Education, Science and Culture of Japan (No. 63540584).

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