cell cultures produce TA, GEA, GE, and GA at the same levels as those found in *G. jasminoides* cell cultures. This unique capability to adapt to both tropical and temperate circumstances suggests that these plants could survive under the reciprocal changes of a climate.

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Enzyme Activities Responsible for the Defense Mechanism of the Fertilization Envelope

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The fertilization envelope (FE) is formed by modification of the preexisting vitelline envelope (VE) with the addition and actions of cortical granule exudates through the cortical reaction process [1, 2]. The cortical reaction is a series of physiological and morphological phenomena involving exocytosis of the cortical granules or alveoli, elevation and toughening of the FE, and the establishment of polyspermy blocking by degradation of the sperm receptors of the VE surface [2, 3]. In the process of transformation of the VE into the FE, the outermost layer of the VE of fish eggs is replaced by cortical alveolus materials, so that the FE surface is different in ultrastructure, cytochemistry, and immunohistochemistry from the VE surface [4, 5]. It is very important to clarify the function(s) of the properties that the FE acquires.

The authors first examined the kinds of enzymes present in the FE extract from artificially activated eggs from the fish *Salvelinus leucomaenis,* using an agar plate assay for enzyme activities. The extract consists of the main components of the FE outermost layer, as confirmed by electron microscopy and agglutination test for fish sperm or human B-type erythrocytes (data not shown; [4 – 6]). The enzyme assay revealed that the FE extract may contain enzyme activities responsible for digesting each of ten kinds of substrate tested, forming a halo around each well (Fig. 1). The presence of these enzyme activities implies that the FE extract may have the ability to actively protect the embryo by attacking bacteria and fungi. In fact, suspension of the FE extract and various bacteria (*Vibrio anguillarum, Aeromonas hydrophila,* or *Escherichia coli*) demonstrated that the FE extract had bactericidal action on *V. anguillarum* (Fig. 2). The VE extract had no bactericidal action and revealed little or no formation of haloes in the agar plates (data not shown). In addition, the present results indicate that the FE extract may contain cellulase (or CM-cellulase), chitinase (or CM-chitinase), β-1,3-glucanase (or laminaranase), β-1,3(4)-glucanase (or lichenase), xylanase, mannase, dextranase, protease, and lysozyme activities, as deduced from the formation of haloes revealing degradation of the substrates used, although the authors cannot be certain all of these enzymes are contained in the FE extract, since it is known that plant chitinases act as a lysozyme when incubated with *Micrococcus lysodeikticus* [7]. However, it seems very unlikely that the present chitinase and lysozyme activities were exerted by the same enzyme protein, since chitinase activity is hardly inactivated at all by boiling, whereas lysozyme activity is abolished.

It is not clear how the present FE extract exerted its bactericidal action on *V. anguillarum* alone among the three species of gram-negative bacteria. The

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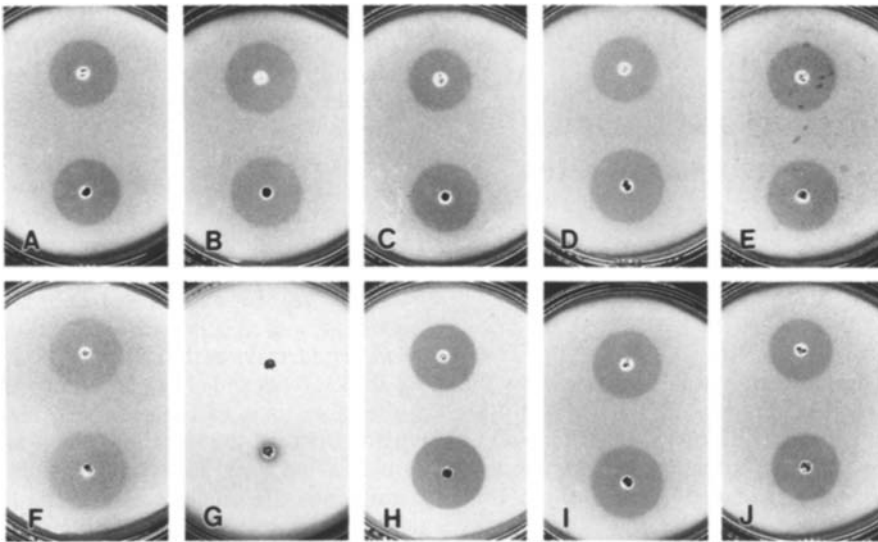

Fig. 1. Agar plate assay for enzyme activities in Petri dishes. Each of the plates contains one of the following substrates: A) barley β-glucan; B) lichenan; C) laminaran; D) carboxymethylcellulose (CM-cellulose); E) carboxymethylchitin (CM-chitin); F) xylan; G) *M. lysodeikticus* dried cells; H) casein; I) dextran; J) mannan. Boiling (upper wells) of the FE extract for 10 min did not markedly inactivate the enzyme activities, except lysozyme activity. Each of the plates contains 10 mg of the substrate in 2 % agar dissolved in 50 m*M* phosphate buffer (pH 7) containing 0.85 % NaCl (PBS). Each well contains 2 mg of the lyophilized FE extract (lower wells) or boiled FE extract (upper wells) which was extracted as described previously [5, 6]

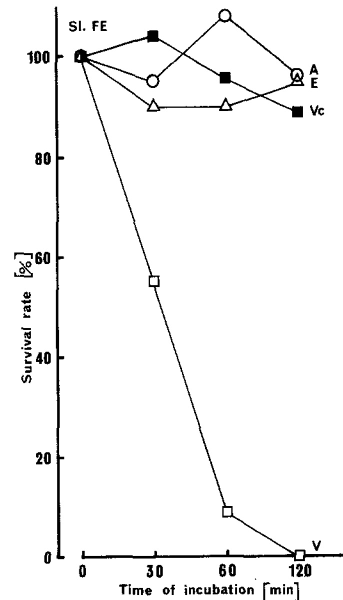

Fig. 2. The relationship between incubation time and survival rate of bacteria in the presence of FE extract. *A Aeromonas hydrophila, E Escherichia coli, V Vibrio anguillarum, Vc Vibrio* control. The bacterial suspension (5.2 to 6.1 $\times$ 10^4 bacterial cells ml^{-1}) were mixed with FE extract (5 mg dry weight ml^{-1} PBS) for testing, and then incubated at 30 °C prior to plating. Colonies were counted after 48 h. The zero time point was taken to mean plating of a 0.1-ml aliquot of the mixture immediately after mixing the FE extract and bacterial suspension. Controls were bacteria in sterile PBS incubated under the same conditions

relationship between the present enzyme activities and degradation of bacterial cells walls remains unsolved. However, since the bactericidal action was confirmed by electron microscopy to be due to bacteriolysis (data not shown), there is a high likelihood that the bactericidal effect may be caused by enzymatic actions contained in the FE extract. In addition, since bacterial polysaccharides (both specific and non specific) make up a group of polymers in which structural variation is almost unlimited [8], the resistance of the bacteria to the actions of the FE extract would be related to structural variations in the cell-wall polysaccharides rather than in peptidoglycan, which is the only cell-wall polymer common to both gram-negative and gram-positive bacteria.

In conclusion, it is considered that the mechanism by which the FE is able to protect the developing embryo from invaders or pathogens present in the water surrounding the FE is probably enzymatic action, the properties of which are acquired through the cortical reaction process and originate in cortical alveolus material, in addition to protecting the embryo as a physical barrier. This information may lead to new insights into the functions of the FE and the cortical alveoli. Data on antifungal or fungicidal action of the FE extract will be reported in detail in another paper.

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