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Isolation and identification of a specific and reversible inhibitor of starfish development

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An inhibitor of development of the starfish *Asterina pectinifera* was purified to homogeneity from a culture of the bacterium *Bacillus subtilis*, and was identified as adenosine. Adenosine at 6 μg/ml was shown to halt embryonic development specifically at the 256-cell stage when all the embryonic cells differentiate into epithelial cells. By returning treated embryos to normal seawater, they developed normally to the bipinnaria stage.

Blastula; Adenosine; Starfish embryo, *Bacillus subtilis*)

1. INTRODUCTION

Early embryonic development proceeds through a course of successive events that are well ordered in both space and time. In fertilized eggs of the starfish *Asterina pectinifera*, cell division proceeds rapidly and almost synchronously without growth for a total of eight or nine cleavages [1]. Completion of this rapid cleavage period is followed by the immediate activation of a new developmental program, blastulation [2].

Studies on the molecular mechanisms of these developmental changes would be facilitated by the availability of chemicals having various influences on development. We have been searching for a substance(s) capable of arresting the embryonic development of the starfish at the 256–512-cell stage when all the embryonic cells differentiate into epithelial cells to form a blastula. After screening approx. 10000 filtrates of fermentation broth, we have found that a strain of the bacterium *Bacillus subtilis* produces a substance which arrests development at this specific stage. Here we describe the isolation and identification of the inhibitory substance.

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2. MATERIALS AND METHODS

2.1. *Materials*

Adenosine and silica gel $60PF_{254}$ were purchased from Merck, adenine and inosine from Nakarai Chemicals, uridine, ATP, *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine and adenosine 3′:5′-monophosphate (cAMP) from Boehringer-Mannheim, cytidine, thymidine, guanosine, AMP and ADP from Kohjin, 1-methyladenine from Sigma, and artificial seawater (ASW) from Jamarin Laboratories. The broth of *B. subtilis* was prepared as described [3]. Starfish were obtained on the coast of the Inland Sea of Japan during their breeding season and kept in running ASW.

2.2. *Bioassay*

The inhibitory activity of the broth filtrate and

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various fractions purified from it was determined by adding a small number of fertilized eggs of the starfish *A. pectinifera* to serially diluted sample solutions and observing the cytological changes in the embryos cultured at 20 ± 2°C as described in [4,5]. One unit of activity was defined as the maximum volume (in ml) of a solution which showed sufficient effectiveness in arresting development at the 256-cell stage.

2.3. *Physical and chemical analyses*

High-performance liquid chromatography (HPLC) was carried out using a Water Radialpak C_{18} column (8 × 100 mm). NMR spectra were measured on a JEOL FX200 instrument, mass spectra on a JEOL HX-100 instrument, and UV spectra with a Shimazu UV-3000 spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. *Purification and identification of an inhibitor of bacterial origin*

We had previously isolated iturin A-2, a cyclic peptide [6], from a culture of *B. subtilis* and showed it to be an inhibitor of cytokinesis of fertilized starfish eggs [3,7]. During the course of purification, we found that a fraction separated chromatographically from iturin prevented the development of *A. pectinifera* embryos just at the beginning of blastulation. We isolated this inhibitory substance as outlined in fig.1. From 1.0 l of the bacterial broth, 6.2 mg colorless needles were obtained as an active substance, which inhibited embryonic development specifically at the 256-cell stage at a concentration of 6 μg/ml or greater (fig.2). The purity of the substance was confirmed by HPLC (fig.3).

The substance was soluble in dimethyl sulfoxide, sparingly soluble in water, methanol and *n*-butyl alcohol, and insoluble in chloroform and ethyl acetate. The UV spectrum (H_2O, maximum at 258 nm), the mass spectrum obtained with an FAB ionization system (molecular ion, 267), and ^1H- and ^{13}C-NMR spectra (fig.4) showed clearly that the substance was pure adenosine. HPLC of the substance showed that it co-migrated with authentic adenosine. Furthermore, the inhibitory activity of authentic adenosine on the development of *A. pectinifera* embryos was the same as that of the isolated material.

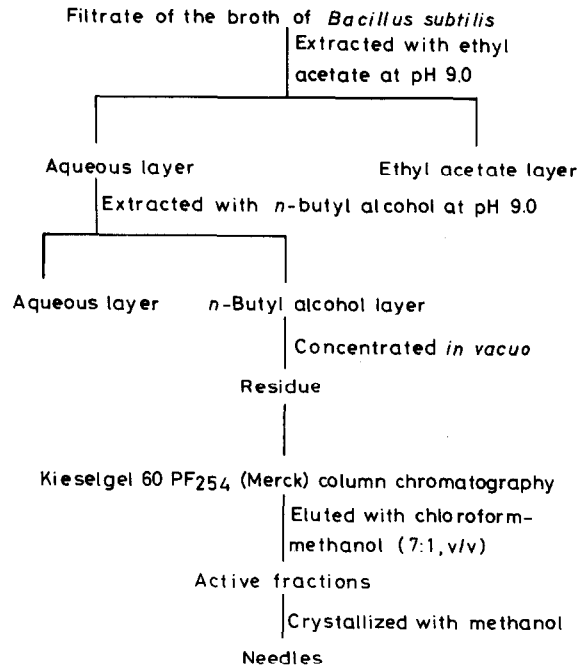

Fig.1. Procedure used for purification of the inhibitor of development of *Asterina pectinifera* embryos.

3.2. *Effects of adenosine and related substances on starfish development*

When fertilized *A. pectinifera* eggs were cultured continuously from fertilization in ASW containing adenosine at a concentration of 6 μg/ml or greater, the eighth cell division occurred at 7.5 h after fertilization with a 2 h delay as compared with normal embryos, and further development was arrested. Up to the 128-cell stage, however, development was not retarded by the presence of adenosine even at a very high concentration (500 μg/ml). When embryos that had remained at the 256-cell stage by being kept in adenosine solution for 10 h from fertilization were washed with ASW and then cultured in fresh ASW, they became normal gastrulae within 24 h after adenosine removal.

When fertilized *A. pectinifera* eggs were exposed to a 100 μg/ml concentration of inosine, guanosine, uridine, cytidine, thymidine, adenine, 1-methyladenine, *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine, AMP, cAMP, ADP or

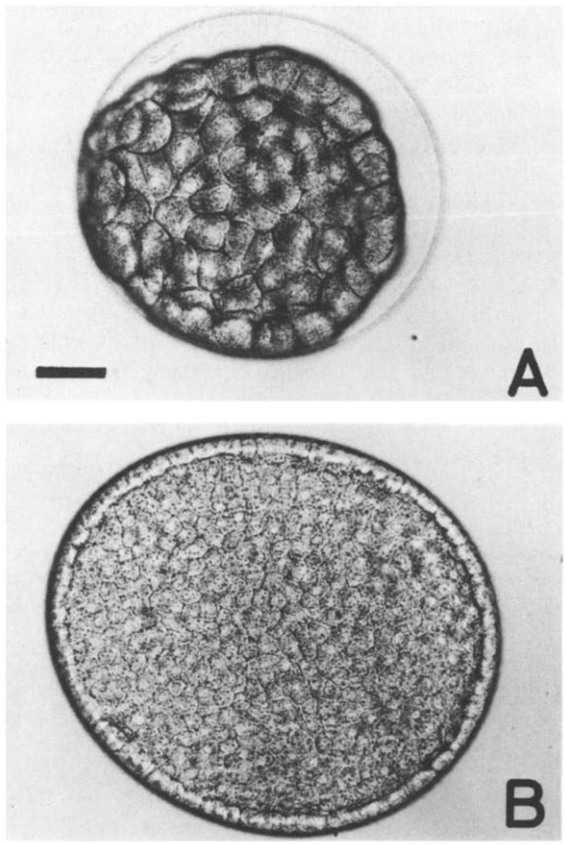

Fig.2. Influence of the purified inhibitor (50 μg/ml) on development of fertilized *A. pectinifera* eggs. An embryo in the inhibitor (A) and its control (B) were allowed to develop for an identical period, 10 h from fertilization. Bar, 30 μm.

ATP, they developed to the gastrula stage, suggesting that the action of adenosine is rather specific.

When uridine (25 μg/ml), cytidine (25 μg/ml), inosine (50 μg/ml) or guanosine (200 μg/ml) was introduced into a culture of fertilized *A. pectinifera* eggs which were placed in ASW containing 25 μg/ml of adenosine, they were able to develop to the gastrula stage, suggesting that uptake of sufficient amounts of adenosine was prevented by these nucleosides through a single broad-specificity transport system for purine and pyrimidine nucleosides [8] in the embryonic cell membranes.

In embryos that had remained at the 256-cell stage by being kept in adenosine solution, the rates

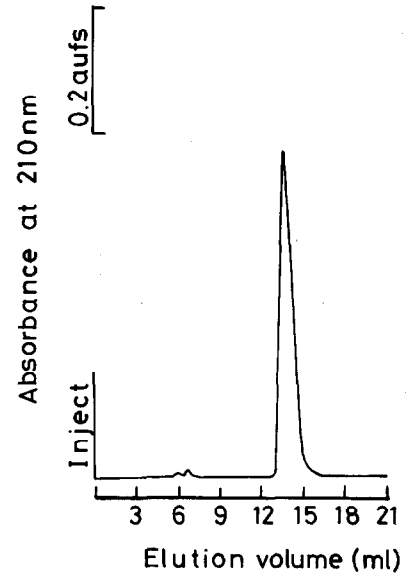

Fig.3. HPLC using a Waters Radialpak C_{18} column (8 × 100 mm) with 20% aqueous methanol. 20 μg purified inhibitor was applied to the column at a flow rate of 3 ml/min. aufs denotes absorbance units full scale.

of protein, DNA and RNA synthesis were suppressed by more than 95% (in preparation). By returning the treated embryos to ASW, protein synthesis was reinitiated and returned to the control level within 10 h after removal of adenosine. These results suggest that the quiescence induced in starfish embryos by external application of

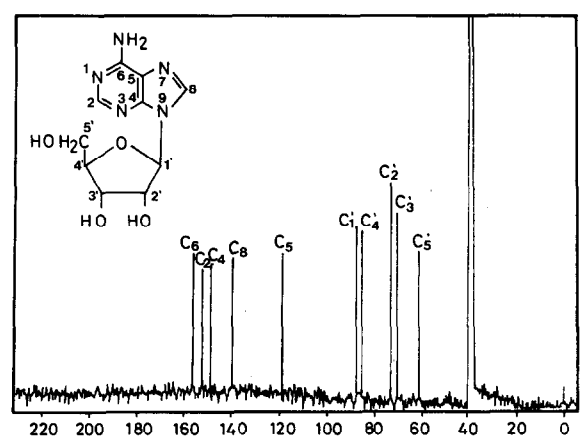

Fig.4. ^{13}C-NMR spectrum (fully decoupled) of the purified inhibitor in dimethyl sulfoxide-d_6.

adenosine is a useful system for gaining new insights into translational regulation, which is of critical importance during the early period of development.

ACKNOWLEDGEMENT

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