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PURIFICATION AND PROPERTIES OF AN EXO-β-D-1,3-GLUCANASE FROM SEA URCHIN EGGS

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SUMMARY

An exo-β-D-1,3-glucanase has been purified 200-fold from unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus* by DEAE-cellulose chromatography. Its pH optimum is 4.8–5.6. The enzyme appears to act specifically on β-1,3 glucosidic bonds with the release of glucose. With increasing length of laminaridextrin, both the substrate affinity and the initial rate constant v increase. Enzyme activity is unaffected by sulfhydryl reagents or by divalent metals. The molecular weight was estimated to be between 56 000 and 64 000 by sucrose density gradient centrifugation. It is speculated that the enzyme is involved in the early events of fertilization associated with breakdown of cortical granules.

INTRODUCTION

β-1,3-Glucanases (β-1,3-glucan glucanohydrolase, EC 3.2.1.39) are widely distributed in nature. They are either extracellular, as in bacteria[1] and fungi[2,3], or are localized to the digestive glands of higher organisms, as in the molluscs[4,5], annelids[6], and crustaceans[6]. They presumably function in the breakdown and utilization of polysaccharides containing β-1,3 glucosidic linkages. Recently we described the presence of a β-1,3-glucanase in sea urchin eggs[7]. This enzyme is particulate and latent in the unfertilized egg, and about 40% of the total activity is released from the egg at the time of fertilization. Its apparent localization in cortical granules and rapid release at fertilization have led to the suggestion that this portion of the enzyme activity may be involved in the conversion of polysaccharides of cortical granules into the polysaccharides of the fertilization and hyaline membranes at the time of fertilization[7].

In this paper we describe a purification and characterization of the β-1,3-glucanase isolated from unfertilized sea urchin eggs. It is an exo-β-1,3-glucanase. Its properties are generally similar to those of a glucanase isolated from the culture filtrate of a Basidiomycete species described by HUOTARI *et al.*[8].

MATERIALS AND METHODS

Adult sea urchins, *Strongylocentrotus purpuratus*, were collected on the California coast from October to January. The eggs were obtained at the beach by injection with 0.55 M KCl (see ref. 9). They were washed with sea water, and then frozen in 10–25-ml aliquots in glass vials by immersion in crushed, dry ice. No β-1,3-glucanase activity was lost on storage at −10° for up to 8 months.

Laminarin, a complex polysaccharide containing predominately β-1,3 glucosidic linkages[10,11] was used as substrate. It was the water-insoluble extract of *Laminaria cloustoni* fronds, and was obtained from Pierce Chemical Co., Rockford, Ill. Laminaribiose, laminaritriose, and laminaritetraose were kindly supplied by Dr. W. J. Whelan, Department of Biochemistry, Miami University School of Medicine.

Enzyme assays

β-1,3-Glucanase activity was measured as the rate of release of glucose; hence the activity measured was that of an exohydrolyase. Two methods were employed to detect glucose. The first involves coupling of glucose formation to the generation of NADPH in a system containing excess ATP, hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49) (ref. 12). This routine assay mixture contained in a volume of 1.5 ml: 1 mg laminarin, 0.76 μmole NADP (Calbiochem), 66 μmoles Tris–HCl, 4.5 μmoles ATP (Calbiochem), 0.9 international unit glucose-6-phosphate dehydrogenase (Calbiochem), 2.1 units yeast hexokinase (Sigma or Calbiochem), 15 μmoles $MgCl_2$, final pH 7.0, and a suitable amount of enzyme. The increment in absorbance at 340 mμ produced by the formation of NADPH was measured continuously at 37° in a Gilford Model 2000 multiple sample absorbance recorder. Appropriate deletion controls were always performed to insure that NADPH generation was dependent on the addition of both the β-1,3-glucanase and laminarin.

This coupled assay method was rapid and convenient, and was used in most of the studies described in this paper. However, the method could not be used when β-1,3-glucanase activity was measured at pH values below 6.5 due to instability of the coupling enzymes and reagents. In some cases, therefore, glucose was determined by the glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4) method. With this method β-1,3-glucanase activity was assayed in a total volume of 2.0 ml of a potassium phosphate–sodium citrate buffer[13] containing 3.5 mg laminarin and an appropriate amount of enzyme. The tubes were incubated at 37° for specified time intervals, and the reactions stopped by boiling the tubes for 5 min. Suitable aliquots were then assayed for glucose using the reagents of the "Glucostat" as outlined by Worthington Biochemical Corp. (Freehold, N.J.). Under the assay conditions used, glucose was not produced in the absence of either enzyme or laminarin.

Enzyme activity is expressed in terms of units, defined as the amount of enzyme that releases 1 μmole of glucose/min (free glucose assay) or produces 1 μmole of NADPH/min (coupled assay).

Characterization of reaction products

The reactions were carried out in phosphate–citrate buffer at pH 6.0 and were stopped by boiling for 5 min, following which the precipitated protein was removed

by centrifugation. Aliquots of such mixtures were analyzed for products by descending paper chromatography on Whatman No. 1 paper in 1-propanol–acetic acid–water (76:10:23, by vol.)[10]. Discrete reaction products as detected by spraying with *p*-anisidine[14] were compared with chromatographic properties of authentic glucose, laminaribiose, laminaritriose, and laminaritetraose.

Molecular weight

The molecular weight of the purified β-1,3-glucanase was estimated by the sucrose density gradient method of MARTIN AND AMES[15]. A linear gradient of 5 to 20% sucrose in 0.01 M potassium phosphate (pH 7.0) was used. The gradient was centrifuged for 12 h at 40 000 × *g* in a SW-50 head in a Spinco Model L ultracentrifuge. Ribonuclease (ribonucleate pyrimidine-nucleotide-2′-transferase (cyclizing), EC 2.7.7.16) and alcohol:NADPH oxidoreductase (EC 1.1.1.1) were used as marker enzymes of known molecular weight. They were obtained from Worthington Biochemical Corp. Ribonuclease and alcohol dehydrogenase were assayed by the method of KALNITSKY, HAMMEL AND DIERKS[16] and RACKER[17], respectively.

Protein

Protein was generally estimated by the method of LOWRY *et al.*[18], using bovine serum albumin as standard. Column fractions obtained during chromaotgraphic procedures were monitored for protein and nucleic acids by determining the absorbance at 260 and 280 mμ (see ref. 19).

RESULTS

Validity of assays

The accumulation of glucose, as measured with the glucose oxidase procedure, was linear from zero time when the enzyme source is either egg extract or the purified β-1,3-glucanase. A similar result was found at pH 7.0 with the coupled assay.

Fig. 1 shows the effect of increasing amounts of the purified enzyme on the rate of glucose release from laminarin at pH 7.0. The rate of the reaction was directly proportional to the amount of added enzyme only when relatively small amounts of enzyme were added. Addition of more laminarin and/or ancillary enzymes did not increase the reaction rates at the higher concentrations of enzyme. A similar nonlinearity of the reaction rate as a function of added enzyme was found with crude extracts. Therefore in all assays, conditions were used such that the rate of glucose release was proportional to added enzyme.

Purification

Extract. The following procedure, and all subsequent procedures, were performed at 2°. To extract the enzyme, approx. 50 ml of packed eggs were suspended in 70 ml of 0.05 M potassium phosphate (pH 7.4) and homogenized in a Sorvall "Omnimixer" for 1 min at top speed. The homogenate was then centrifuged at 30 000 × *g* for 10 min in a Sorvall RC2-B refrigerated centrifuge. The resulting supernatant fraction contained approx. 80–90% of the enzyme activity. This fraction was dialyzed against two changes of 4 l each of 0.05 M potassium phosphate (pH 7.4) for a total of 8 h.

DEAE-cellulose chromatography. DEAE-cellulose[20], obtained from Reeve-Angel

Co. (Clifton, N.J.) as a microgranular, preswollen preparation (DE-52), has given a high degree of reproducibility with excellent purification. The DEAE-cellulose was equilibrated with 0.05 M potassium phosphate (pH 7.4), and excess fluid was removed by filtering on a Buchner funnel. Small portions of this DEAE-cellulose (5 g wet weight) were added to the extract and allowed to equilibrate for 5–10 min. A sample of the extract was then centrifuged, and the supernatant was assayed for β-1,3-glucanase activity. This procedure was repeated until approx. 90% of the activity was adsorbed to the DEAE-cellulose (usually about 15 g of DEAE-cellulose). By this batch adsorption procedure approx. 15% of the extract protein was adsorbed. The DEAE-cellulose was then centrifuged for 5 min at 5000 $\times$ *g* and the supernatant solution discarded. The DEAE-cellulose now containing the adsorbed enzyme, was resuspended in 300 ml of 0.05 M potassium phosphate (pH 7.4) and recentrifuged. The washed DEAE-cellulose was then resuspended in 30 ml of 0.05 M potassium phosphate (pH 7.4), and the slurry was packed on top of a column (2 cm $\times$ 20 cm) of DEAE-cellulose that had been equilibrated with 0.05 M potassium phosphate (pH 7.4). The column was then developed with a 500-ml gradient of 0–1 M KCl in 0.05 M potassium phosphate (pH 7.4) at a flow rate of 15 ml/h.

A representative elution pattern of β-1,3-glucanase activity from DEAE-cellulose is shown in Fig. 2. The enzyme is strongly adsorbed to DEAE-cellulose and is eluted only when the KCl concentration reaches approx. 0.4 M.

TABLE I

SUMMARY OF PROCEDURE FOR PURIFICATION OF SEA URCHIN EGG β-1,3-GLUCANASE

In this purification 50 ml of packed eggs were used.

Step	*Volume (ml)*	*Protein (mg/ml)*	*Specific activity (units/mg protein)*	*Purification (fold)*	*Yield (%)*
Homogenate	145	43.5	0.0030	—	100
Extract	110	23.7	0.0059	1.9	81
DEAE-cellulose chromatography*	3.3	5.1	0.59	197	53

* Concentration by ultrafiltration.

The fractions containing the highest specific activity of β-1,3-glucanase activity were pooled and concentrated by ultrafiltration from a volume of 75 ml to a volume of approx. 4 ml. The enzyme solution was then dialyzed for 8 h against 4 l of 0.05 M potassium phosphate (pH 7.4). Table I summarizes the purification procedure employed. An overall purification of 200-fold was obtained with a yield of 53%. In a number of such purifications, the extent of purification varied between 87- and 225-fold, with the yields varying between 30 and 57%. This batch adsorption and gradient elution technique has proven exceptionally useful and time-saving. We have found that enzyme yields are higher and more reproducible than with the standard chromatographic procedure of placing the entire extract at the top of the column. This may be a result of the shorter elution times or because of the rapid removal of proteins during the batch procedure, such as proteases which might cause enzyme inactivation.

A number of other possible purification procedures have been attempted including ammonium sulfate fractionation, chromatography on BioGel P-150, selective heat inactivation, and precipitation of an enzyme–substrate complex with insoluble laminarin in a manner similar to that used for amylase[21]. Such procedures have either failed to achieve adequate or reproducible purification, or have resulted in enzyme inactivation.

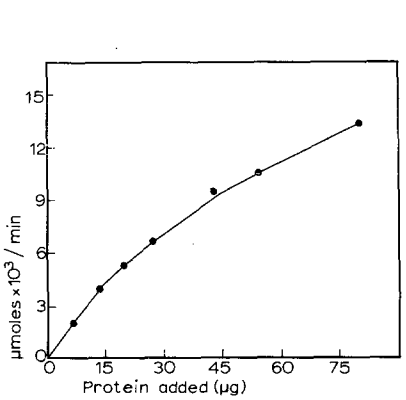

Fig. 1. The effect of the sea urchin egg β-1,3-glucanase concentration on the rate of glucose release from laminarin. Increasing amounts of purified enzyme, specific activity 0.26 unit/mg protein were assayed in the standard coupled assay as described in MATERIALS AND METHODS.

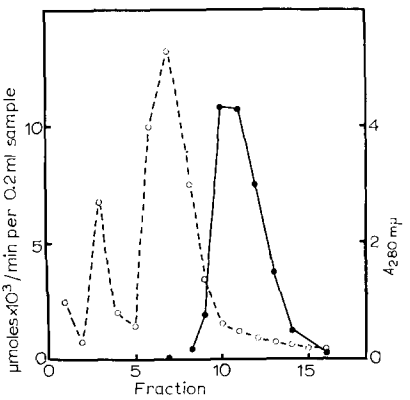

Fig. 2. Column chromatography of sea urchin egg β-1,3-glucanase on DEAE-cellulose. Details of the batch adsorption of laminarinase to DEAE-cellulose, and subsequent preparation of the column are described in the text. The column was developed with 500 ml of a 0–1 M KCl gradient in 0.05 M potassium phosphate (pH 7.4) at a flow rate of 15 ml/h. Fractions containing 15 ml were collected. ●—●, Enzyme activity; ○— — —○, absorbance at 280 mμ.

In several experiments, measurements were made to detect the presence of any endohydrolases. Protein not adsorbed to the DEAE-cellulose, as well as fractions eluted from the column, were assayed for enzyme-catalyzed increment in total reducing sugar by the method of NELSON[22]. Thus this method would detect the appearance of small oligosaccharides as well as glucose. Such assays were performed at pH 5.0 as described under MATERIALS AND METHODS, and incubations were for 60 min. Only that peak of enzyme activity detected by release of free glucose (Fig. 2) was detected when assays were also performed with the reducing sugar method. Thus, we could find no evidence for an endohydrolase that would hydrolyze laminarin with release of fragments other than glucose.

Properties

pH optimum. The β-1,3-glucanase is active over a broad pH range with maximum activity between pH values of 4.8 and 5.6 (Fig. 3). Activity at pH 7.0, at which pH the coupled assays were performed, is 40% of maximal.

Effect of temperature on reaction rate. Fig. 4 shows the effect of temperature on the rate of glucose release, using the coupled assay procedure. Although the enzyme may be presumed to function in the egg or sea water at approx. 10–15°, maximal activity is not observed at this temperature. Similar results were obtained when

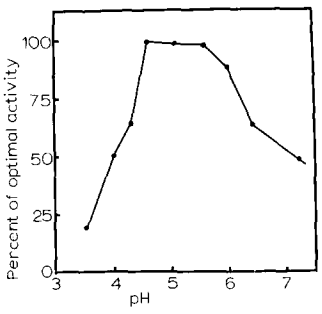

Fig. 3. pH optimum of purified β-1,3-glucanase. The activity of purified enzyme, specific activity 0.26 unit/mg protein, was determined in a phosphate–citrate buffer at various pH values as described in MATERIALS AND METHODS, using the glucose oxidase method. Activity is expressed as percent of the optimal activity observed.

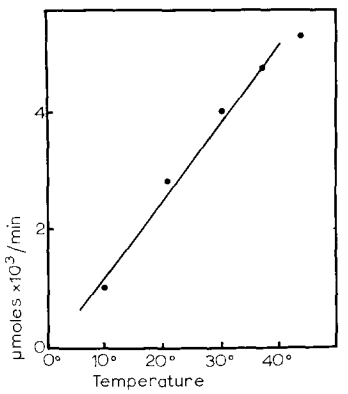

Fig. 4. Effect of temperature on β-1,3-glucanase activity. Enzyme activity was assayed by the coupled assay method as described in MATERIALS AND METHODS, using a water-jacketed Gilford absorbance recorder. At least 10 min was allowed for equilibration between changes of temperature. The assay mixture was equilibrated at the specified temperature prior to the addition of enzyme to initiate the reaction.

assays were performed at pH 5.0 and glucose release was detected using the glucose oxidase method as outlined in MATERIALS AND METHODS.

Substrate specificity and characterization of reaction products. Studies with different chain lengths of β-1,3-glucose polymers are shown in Table II. In the series of laminaridextrins studied, the initial reaction rate v increased with increasing chain length. The concentration of substrate required for half-maximal reaction rates are also given. If calculated on the basis of molarity, the long-chain laminarin would have a greater affinity for the enzyme than the short-chain laminaridextrins.

The relative inability of the β-1,3-glucanase to hydrolyze short-chain laminari-

TABLE II

EFFECT OF CHAIN LENGTH OF LAMINARIDEXTRINS ON K_m AND v OR SEA URCHIN EGG β-1,3-GLUCANASE

The enzyme used in these studies had a specific activity of 0.59 unit/mg. Assays were performed by the coupled assay as described in MATERIALS AND METHODS. Initial rates only were used. In all cases NADPH formation was not observed in the absence of added substrate or added enzyme.

Substrate	*Concentration for half-maximal activity (μg/ml)*	v*
Laminarin	5.0	100
Laminaritetraose	2.9	59
Laminaritriose	9.6	32
Laminaribiose	13 000	3

* Expressed as relative to laminarin.

dextrins, most notably the laminaribiose (Table II), is in keeping with results obtained from paper chromatography of hydrolysis products of laminarin using the purified enzyme (see MATERIALS AND METHODS for procedures employed). Thus, although the major reaction product that accumulated was glucose, small amounts of laminaribiose (R_{Glc} = 0.80) also accumulated. In addition, small amounts of a product (R_{Glc} = 0.56) were detected that migrated more slowly than laminaritriose (R_{Glc} = 0.67). The nature of this latter product is unknown, but it may represent a compound such as laminaribosylmannitol, or an oligosaccharide containing β-1,3 and β-1,6 glucosidic linkages. Such compounds have been isolated as products of laminarin hydrolysis by a bacterial β-1,3-glucanase[23].

Among compounds from which glucose was not released by purified enzyme were: sophorose, cellobiose, gentiobiose, maltose, lactose, milibiose, starch, and cellulose. In these studies on possible glucanase substrates, the reactions were carried out in the standard assay at pH 5.0 as described in MATERIALS AND METHODS.

Stability, activators and inhibitors. The purified enzyme preparations can be stored in 0.05 M potassium phosphate (pH 7.0) at 4° for up to 2 weeks without significant loss of activity. The enzyme can also be frozen and thawed without loss of activity. It is rapidly inactivated at 60° at either pH 6.0 or 7.0 along a first-order decay curve in which 50% of activity is lost within 5 min. The enzyme is very stable, however, to incubation at 25° with concentrations of up to 1 mg/ml of trypsin (EC 3.4.4.4). After 30 min of such incubation, there is no significant loss of activity.

The enzyme is not readily inactivated by sulfhydryl-reacting reagents. Thus incubation of the enzyme at pH 7.4 in 0.05 M potassium phosphate buffer in the presence of either 1 or 10 mM *N*-ethylmaleimide or 1 mM chloromercuribenzoate, for 15 min at 25° prior to assay did not affect enzyme activity.

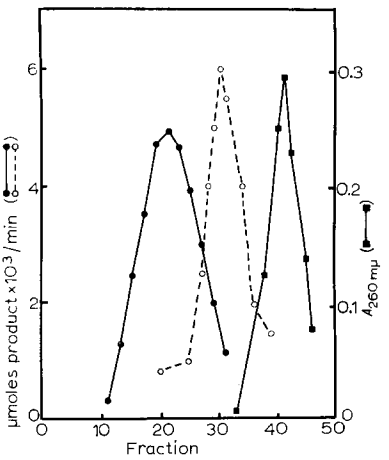

Fig. 5. Sucrose density gradient centrifugation of sea urchin egg β-1,3-glucanase. Ribonuclease (■—■), yeast alcohol dehydrogenase (●—●), and β-1,3-glucanase, specific activity 0.51 unit/mg protein (○— — —○), were layered on each of three separate 5–20% sucrose gradients as described in MATERIALS AND METHODS, and were centrifuged for 12 h at 40 000 rev./min in a SW-50 head. Fractions were collected and assayed for the 3 enzymes as described in MATERIALS AND METHODS. Activity of the glucanase and alcohol dehydrogenase is expressed as μmoles product formed per min in suitable aliquots, and ribonuclease activity is expressed as the amount of soluble $A_{260\ m\mu}$ released from yeast RNA under the specific assay conditions.

Various metal ions, including Mn^{2+}, Fe^{2+}, Ca^{2+}, and Co^{2+} and Ni^{2+} have been found to affect fungal β-1,3-glucanases[24,25]. Dialysis of the purified sea urchin enzyme against 40 000 vol. of 0.01 M Tris–HCl containing 0.01 M EDTA (pH 7.0) for 48 h resulted in no loss of enzyme activity. Preincubation of the enzyme at 37° for 15 min in 1 mM concentrations of the above ions, or addition of these ions during the assay had no effect on enzyme activity.

In a series of experiments in which the purified β-1,3-glucanase was added to various fractions obtained from the DEAE-cellulose chromatography, and assayed under optimal standard conditions of the coupled assay (see MATERIALS AND METHODS), no activation or inhibition of the enzyme could be demonstrated.

Molecular weight. Fig. 5 shows a representative result of a sucrose density centrifugation study used to estimate the molecular weight of the β-1,3-glucanase. Ribonuclease, with a molecular weight of 12 700 (see ref. 26) and alcohol dehydrogenase, molecular weight 150 000 (see ref. 27), were used as standards. A molecular weight of 64 000 was obtained using ribonuclease as standard, and 56 000 when the alcohol dehydrogenase was used as the standard.

DISCUSSION

The properties of the β-1,3-glucanase purified from unfertilized sea urchin eggs are generally similar to those described for β-1,3-glucanases of fungal and bacterial origins with respect to pH optimum and specificity for β-1,3 glucosidic bonds[1,2]. The sea urchin β-1,3-glucanase appears to differ from enzymes of microbial origin in that we can demonstrate no effects of divalent metals or sulfhydryl-reacting compounds on enzyme activity[24,25,28]. The sea urchin enzyme also exhibits a high affinity for long-chain laminaridextrins (half-maximal activity at a laminarin concentration of 5 μg/ml). Laminarin concentrations of 5 mg/ml have been reported to be necessary to saturate some β-1,3-glucanases of microbial origins[1,2]. On the other hand, HUOTARI *et al.*[8] have reported a similar high affinity for an exo-β-1,3-glucanase they have isolated in a homogeneous form from culture filtrates of a Basidiomycete species.

The sea urchin egg laminarinase is clearly an exohydrolase, since the major product formed is glucose. This is evidenced by the assays employed which depend on glucose formation. In addition, paper chromatography of products of laminarin hydrolysis by the enzyme indicated that glucose is the major product, along with smaller amounts of laminaribiose, and an unidentified sugar or oligosaccharide. In view of the presence of β-1,3-glucanases with both endo- and exohydrolytic actions in fungal species[5], the question arises as to whether, in addition to the exohydrolase described in these studies, there might also exist in extracts an enzyme activity capable of internal cleavage of laminarin. The finding that in various fractions obtained from DEAE-cellulose chromatography there was good correspondence between the presence of β-1,3-glucanase activity as measured by assays that detect glucose, and as measured by detection of reducing sugar, *i.e.*, oligosaccharides as well as glucose, indicates that no endohydrolase activity is present in sufficiently large amount to allow detection.

The function of this β-1,3-glucanase in sea urchin eggs is unknown at the present time. All previously reported β-1,3-glucanases would appear to play a role in digestion.

They are either found extracellularly, as in the case of bacteria[1] and fungi[2,3,8], or are localized in the digestive glands of higher animals as in the case of molluscs[4]. We have also found that the gut of the adult sea urchin contains a β-1,3-glucanase (exohydrolase) that is similar to that of the unfertilized egg with respect to purification properties on DEAE-cellulose chromatography, and inactivation by heat.

Although one can readily understand the role of such an enzyme in digestion of sea algae in the adult urchin, the role of a β-1,3-glucanase in the unfertilized egg is more difficult to understand. The physiologic events at the time of fertilization may give some clue to a possible role for this enzyme[7]. Thus at fertilization[29,30] a ring of peripheral (cortical) granules (1–2 μ in diameter) break down, and their contents, which include acid mucopolysaccharides, are converted into the transparent 2–3 μ-thick hyaline membrane of the early embryo. In addition, products of the cortical granules may be involved in the "hardening" of the fertilization membrane. We have presented evidence elsewhere[7] that a major portion of the β-1,3-glucanase of the unfertilized sea urchin egg is particulate and latent, and at the time of fertilization that portion associated with a large particle fraction is converted to a soluble form, and is released into the perivitelline space, *i.e.*, the space between the cell wall and the newly formed fertilization membrane. These results are consistent with the β-1,3-glucanase being associated with cortical granules. These findings have led us to propose that this enzyme may be involved in the metabolism of polysaccharide elements of the cortical granules during formation of either or both the hyaline and fertilization membranes.

In addition, the β-1,3-glucanase remaining in the egg after fertilization gradually disappears from the developing embryo such that by the time of gastrulation no enzyme is detectable. Thus, we have concluded that this enzyme plays a role during the early cleavage stages[7]. Indeed, a relationship between early events following fertilization and the presence of a β-1,3-glucanase may not be confined to sea urchin eggs, since a similar enzyme activity has been found in eggs of the limpet, *Acmaea scutum*[31].

The nature of potential substrates for a β-1,3-glucanase in cortical granules is unknown. MONROY AND VITTORELLI[32] have reported the presence of a glycoprotein in unfertilized sea urchin eggs, the polysaccharide portion of which is composed mainly of glucose. They report that this material disappears within 5 min after fertilization. Thus such a material may be a substrate for the glucanase. We are presently studying such a possibility.

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