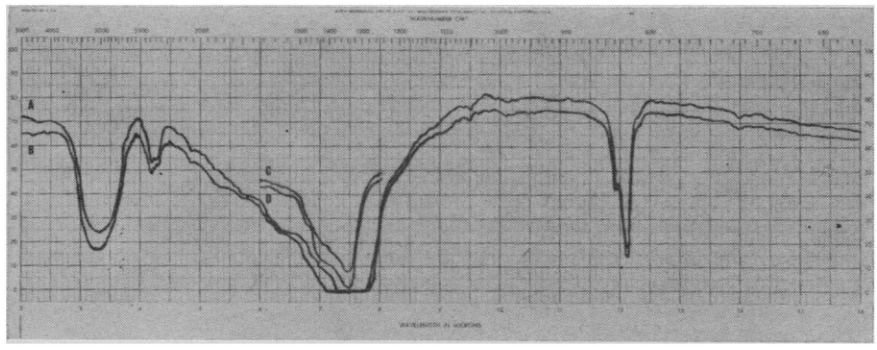

Fig. 1. Infrared spectra (determined with a Beckman model IR-5 spectrophotometer) of feeding deterrent B isolated from *M. infesta* and of ammonium nitrate in KBr pellet. (*A*) Deterrent B, 5 mg; (*B*) ammonium nitrate, 5 mg; (*C*) deterrent B, 2 mg; and (*D*) ammonium nitrate, 2 mg.

the methanolic solution, *n*-butyl alcohol (0.1 volume) was added, and the solution was allowed to stand at room temperature. Upon standing, methanol evaporated from the solution and colorless, needle-like crystals formed. The crystals were further purified by two more crystallizations from the methanol–butyl alcohol system, after which they were washed once with butyl alcohol and twice with ethyl ether. Washed crystals were dried in a vacuum (1 mm-Hg) at 40°C for 30 minutes to remove traces of ether. The yield of crystals from 100 ml of crude leaf extract was 48 mg.

Elemental analysis of the crystalline deterrent B disclosed the following percentages: H, 5.31; O, 59.31; N, 32.30; C, trace (< 1); and ash, trace. This composition corresponds reasonably well to that of ammonium nitrate (calculated percentages: H, 5.04; O, 59.96; and N, 35.00). The isolated material melted at 169° to 170°C (uncorrected, aluminum block) while known ammonium nitrate melted at 169.6°C. The ultraviolet absorption spectra of aqueous solutions of deterrent B and ammonium nitrate were identical, with molar extinction coefficients of approximately 15,400 and 7.0, respectively, at the 204 nm and 303 nm absorption maxima. The infrared spectrum of deterrent B also coincided with that of ammonium nitrate (Fig. 1). The R_F values for deterrent B and ammonium nitrate, respectively, in the three solvent systems used in the initial purification steps were as follows (15-cm solvent ascent): solvent I—0.56, 0.55; solvent II—0.55, 0.53; and solvent III—0.60, 0.55. Both deterrent B and ammonium nitrate were detected on the chromatograms as absorbing spots under short-wavelength ultraviolet light (peak near 254 nm). The feeding deterrent activity of the isolated compound was quantitatively similar to that of reagent grade ammonium nitrate at all levels of treatment (Table 1). Thus, the activity of isolated deterrent B appears to be due to ammonium nitrate rather than to impurities which might be present in trace amounts.

On the basis of preliminary tests, the feeding deterrent activities of potassium nitrate and sodium nitrate appear to be very similar to that of ammonium nitrate. In addition, nitrate assays of young *M. infesta* leaves indicate a level five to ten times as high as that in comparable *M. officinalis* leaves. Therefore, the hypothesis is advanced that in young, intact leaves of *M. infesta* and in crude aqueous extracts of such leaves, nitrate ion is the feeding deterrent. The ammonium salt was probably formed during the first chromatographic purification, inasmuch as this step employed an ammoniacal solvent.

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β-1,3-Glucanase of Sea Urchin Eggs: Release from Particles at Fertilization

Abstract. *β-1,3-Glucanase is a latent enzyme found in large and small particles in unfertilized eggs. At fertilization, the enzyme in the large particles is released into the surrounding perivitelline space. The enzyme may be involved in transformations of extracellular glycoproteins.*

In eggs of most species, contact with sperm results in the breakdown of a peripheral ring of cortical granules 1 to 2 μ in diameter (*1*). In the sea urchin egg, substances released from such granules are involved in the elevation and hardening of the fertilization membrane and in establishing the blockage of polyspermy (*1, 2*). Acidic glycoproteins contained within these cortical granules are also transformed into the 2 to 3 μ thick hyaline layer (*3*) necessary for cell adhesion and blastula formation (*4, 5*). Little is known about the chemical basis of these "cortical reactions." The major constituents of cortical granules have not been characterized, nor have enzymes capable of metabolizing such constituents been identified.

We have found a β-1,3-glucanohydrolase (β-1,3-glucanase) (E.C. 3.2.1.-39) in the eggs of the sea urchin, *Strongylocentrotus purpuratus* (*6*). This enzyme is particulate in the unfertilized egg and exhibits little activity unless released from the particle (that is, the enzyme is latent). After fertilization, a major portion of the activity is released rapidly from the egg into the perivitelline space and surrounding seawater. These properties and behavior suggest that the enzyme is associated with cortical granules and lead us to speculate that the β-1,3-glucanase may be involved in the "cortical reactions."

Enzyme activity is assayed as the release of free glucose from the algal β-1,3-glucan, laminarin (*7*), by means of the glucose oxidase (*8*) or Nelson-Somogyi (*9*) methods. Properties of this enzyme, determined with a 200-fold purified preparation, are described elsewhere (*10*). Briefly, the enzyme's properties are unlike those described for most β-1,3-glucanases (*11*), but are similar to a recently described fungal enzyme (*12*). The sea urchin enzyme is an exohydrolase specific for the β-1,3-glucosidic linkage. Its reac-

tion velocity (V_{max}) and substrate affinity (K_m) increase with increasing chain length. The *p*H optimum is 4.8 to 5.6, and at the *p*H of seawater (*p*H 7.8 to 8.2) activity is 50 percent of optimum. Enzyme activity is unaffected by sulfhydryl-reacting reagents, the detergent sodium lauryl sulfate (SLS), and freezing and thawing.

Up to 90 percent of the β-glucanase activity of unfertilized eggs can be retained within particles if the eggs are disrupted with a Dounce homogenizer in an appropriate homogenization medium [final concentration: 0.3*M* NaCl; 0.075 *M* ethylenediaminetetraacetate (EDTA); 0.05*M* maleate; *p*H 6.0]. Sucrose cannot be substituted for NaCl. Inclusion of this disaccharide results in release of about 50 percent of the enzyme from the particles.

Roughly one-half of the enzyme is contained in large particles sedimenting within 5 minutes at forces below 1200*g*. The remaining enzyme appears to be in a continuum of smaller-sized particles which can be completely sedimented by centrifugation at 28,000*g* for 20 minutes.

The particle-bound enzyme is in a latent condition, with little apparent activity unless the particle fraction is subjected to cycles of freezing and thawing or to hypotonic conditions, or treated with detergents such as SLS, Triton X-100, or saponin. The detergent SLS only "activates" the enzyme associated with the particle fraction and has little effect on the solubilized enzyme (Table 1). This behavior, also seen after freezing and thawing or osmotic shock, suggests that the increased activity results from the release of enzyme from the particles. This assumption is affirmed by sedimentation studies on enzyme activity after incubation in detergent (0.1 percent SLS). Thus, after this treatment, 98 percent of the total enzyme activity originally present in the particles was found in the 28,000*g* supernatant fraction.

Table 1. Latency and subcellular distribution of β-1,3-glucanase before and after fertilization. Eggs were disrupted by ten strokes of a Dounce homogenizer in the NaCl-EDTA-maleate homogenization medium. The enzyme assay, done at 30°C, was initiated by the addition of 0.25 ml of extract to a reaction mixture containing 0.2 percent laminarin (and 0.1 percent SLS where indicated) dissolved in the homogenization medium. The reaction was stopped 30 minutes later by the addition of $ZnCl_2$-$Ba(OH)_2$ (*9*), the supernatant was clarified by centrifugation and then analyzed by the glucose oxidase procedure (*8*). Activity is expressed as the number of micrograms of glucose per milligram of protein per 30 minutes. The fractions are: large particles, pellet after 3000*g* for 7 minutes; small particles, pellet after 28,000*g* for 15 minutes; supernatant, soluble fraction after 28,000*g* for 15 minutes.

Fraction	SLS	Activity	
		Unfertilized	Fertilized*
Total homogenate	−	7.9	19.8
	+	50.6	48.7
Large particles	−	5.3	9.4
	+	39.2	26.4
Small particles	−	3.0	2.9
	+	9.4	7.2
Supernatant	−	4.4	15.1
	+	5.6	17.3

* Two minutes after insemination.

A reasonable mechanism accounting for the observed enzyme release would be the breakdown of these enzyme-containing large particles. If so, the behavior of these particles would correspond most closely to that of the cortical granules which are the only particles known to lyse immediately after fertilization (*1–3*). At fertilization, the externally facing membrane of these cortical granules breaks down, resulting in discharge of their contents into the perivitelline space (*1–3*).

To determine whether the enzyme is also discharged into the perivitelline space after fertilization, β-1,3-glucanase activity was measured in normal eggs and in eggs from which the fertilization membranes had been removed before fertilization. Membranes were removed by incubation in 0.005 percent trypsin (crystallized twice, Worthington) for 15 minutes; the trypsin was removed by four to five washings (*13*). Fertilization of normal eggs resulted in no change in total enzyme activity. Treatment with trypsin had no effect on the enzyme content of the unfertilized egg. After fertilization of these eggs, however, 40 percent of the glucanase activity was "lost." This "lost" activity could be quantitatively recovered in the seawater supernatant surrounding the eggs.

This release of β-1,3-glucanase from the egg does not result from such artifacts as trypsin-induced cytolysis, since nonparticulate cytoplasmic enzymes, such as hexokinase and nicotinamide-adenine dinucleotide kinase are not excreted after fertilization. Furthermore, the fertilization membrane does not always retard passage of the enzyme, since in some batches of normal untreated eggs some glucanase activity is "lost" after fertilization and is recoverable in the surrounding seawater. These findings suggest that the β-1,3-glucanase which is released from particles after fertilization is excreted from the egg into the perivitelline space (in most batches) or into the surrounding seawater (in some batches of eggs and in eggs treated with trypsin before fertilization).

The timing of release of enzyme from particles and excretion into seawater is shown in Fig. 1. In this experiment, membraneless eggs were homogenized in the NaCl-EDTA-maleate media at the indicated times, and the soluble and particle fractions were separated by centrifugation. These fractions, as well as the supernatant

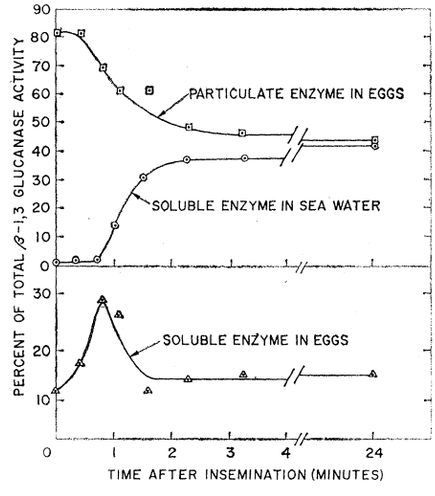

Fig. 1. Timing of release of β-1,3-glucanase from particles and its excretion into surrounding seawater after fertilization (14°C). Eggs in homogenizer tubes were packed by rapid sedimentation in a hand centrifuge (15 seconds), the supernatant seawater was collected by aspiration (5 seconds), homogenization medium was added to the cells (5 seconds), and cell disruption then begun (5 seconds). Indicated time corresponds to collection of seawater supernatant. "Particulate enzyme" represents all activity associated with particles sedimented by 28,000*g* for 15 minutes; "soluble enzyme in eggs" represents activity remaining in the 28,000*g* supernatant. Samples were assayed in the presence of 0.1 percent SLS as described in the legend to Table 1.

seawater, were then assayed for glucanase activity. Enzyme release from the particles is followed by its excretion into the surrounding seawater. The timing of the enzyme release parallels that of changes in light-scattering which accompany breakdown of cortical granules (*14*). This correspondence is consistent with the common identity of cortical granules with the large enzyme-containing particles.

The enzyme-containing particles are unstable in sucrose, and hence this compound cannot substitute for NaCl in the homogenization media. Since sucrose is also a known parthenogenetic agent causing lysis of the cortical granules (*4, 15, 16*), the instability of the particles in sucrose also lends support to the identification of the large glucanase-containing granules with the cortical granules. Additional evidence is that agents removing the cortical granules from intact eggs also result in removal of β-1,3-glucanase from the egg. Thus, incubation of unfertilized eggs in isotonic solutions of nonelectrolytes, such as 1*M* urea, discharges and dissolves the cortical granule contents (*4, 16*). If unfertilized eggs are incubated for 1 minute in 1*M* urea (*p*H 8.0), 45 percent of the glucanase is removed from the eggs and can be recovered in the urea supernatant.

Although final proof must await electron microscopic studies, the above evidence is consistent with the hypothesis that β-1,3-glucanase is associated with large particles which are the cortical granules. Undoubtedly, other enzymes, such as an acid phosphatase (*17*), are also present in the cortical granules. Utilization of membraneless eggs to collect and characterize enzymes and other constituents released at fertilization (*18*) may further clarify the chemical basis of the fertilization reactions.

The function of β-1,3-glucanase is the most intriguing aspect of this study. Behavior of the enzyme suggests that its activity is not solely concerned with fertilization, since 60 percent of the enzyme remains within cytoplasmic particles after insemination. Our studies show this remaining enzyme is gradually excreted from the egg during the cleavage and blastula stages and that the bulk of enzyme is gone by gastrulation.

Consideration of the enzymes' substrate specificity, its latent nature within particles, and its release from the egg suggest that it is involved with metabolism of extracellular or cell surface glycoproteins during both the fertilization reactions and the cleavage period. Since the solubilized glucanase is not retained within the egg or upon the egg surface, the critical action of this enzyme must occur coincidentally with its excretion. One model for this critical action is the hydrolytic unmasking of reactive groups, as in the fibrinogen reactions (*19*). Alternatively, if the enzyme acts as a transferase (rather than as a hydrolase) with its natural substrate (*20*), the critical action could be the rapid interchange of polysaccharide side chains and resultant modification of glycoproteins.

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Laser Diffraction Studies on Single Skeletal Muscle Fibers

Abstract. *Sarcomere movements during isometric tetanic contractions were resolved to 50 angstroms by diffraction techniques. After a latent period that followed the first stimulus, all sarcomeres shortened simultaneously and uniformly. Oscillations in length and tension in synchrony with the stimuli occurred during an incomplete tetanus. However, no oscillations in length were detected during the plateau of a fused tetanus.*

While it is widely accepted that contraction of the muscle cell involves a sliding movement of interdigitating filaments within the sarcomere, little is known about the kinetics of the mechanism underlying the propulsion of the filaments and the maintenance of steady-state tension. According to the sliding-filament model (*1*), contraction occurs through summated short-range forces acting between multiple sites along the A (myosin) and I (actin) filaments. Individual sites are assumed to go through repeated cycles of activity and each cycle contributes to the relative movement of the I filament or, during isometric conditions, to the maintenance of tension. Accordingly, the plateau tension of a tetanic contraction reflects an average number of force-producing sites brought into action from moment to moment at the sarcomere length considered.

Larson *et al.* (*2*), with laser diffraction techniques on whole muscle, have reported that small (1.0 to 1.7 percent of rest length) oscillatory changes in the length of the sarcomeres occur at a frequency unrelated to the rate of stimulation during the plateau of a smooth tetanus. This suggests, in terms of the sliding-filament model, that during an isometric contraction, the number of tension-producing links between the A and I filaments undergoes periodic fluctuations with instant-to-instant readjustment of the sarcomere length. Since no fluctuations in tension occur,