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Purification and Properties of Ovoperoxidase, the Enzyme Responsible for Hardening the Fertilization Membrane of the Sea Urchin Egg*

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The ovoperoxidase from the egg of the sea urchin, *Strongylocentrotus purpuratus*, has been purified to apparent homogeneity. Ovoperoxidase is secreted from the egg at fertilization and is responsible, *in vivo*, for hardening of the fertilization membrane by forming cross-links between protein tyrosyl residues. Purification was accomplished by activation of cortical granule exocytosis with acetic acid, followed by NH_4SO_4 precipitation, DEAE-Sephacel chromatography in the absence of divalent cations, and CM-Sephadex chromatography. The purified enzyme is a glycoprotein of M_r 70,000, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme exhibits a UV-visible spectrum typical of heme peroxidases (ϵ_{412} = 1.19 × 10^5 M^{-1} cm^{-1}).

Ovoperoxidase catalyzes the oxidation of tyrosine, guaiacol, iodide, and bromide, but not chloride, and can employ either H_2O_2 or, with 8% relative efficiency, ethyl peroxide as an oxidative substrate. Phenylhydrazine, 3-amino-1,2,4-triazole, azide, and sulfite all inhibit purified ovoperoxidase at concentrations similar to those that inhibit hardening *in vivo*. Inhibition by 3-amino-1,2,4-triazole is reversible, requires H_2O_2, and is slow relative to substrate turnover. The purified enzyme is sensitive to protease cleavage in the native state, yielding an active product of M_r ~ 50,000 which varies slightly depending upon the protease employed. Ovoperoxidase should provide a useful tool for the study of fertilization membrane formation as a paradigm of macromolecular assembly and modification.

The hallmark of sea urchin fertilization, recognized for over a century (Derbès, 1847), is the elevation of a fertilization membrane. This process depends upon the secretion and activation of a biochemical system that modifies the egg's surface coat in order to protect the embryo and prevent the entry of additional sperm (reviewed in Shapiro *et al.* (1981)). The resultant fertilization membrane is covalently cross-linked by dityrosine residues that harden it (Foerder and Shapiro, 1977; Hall, 1978) and render it stable to many disruptive agents. These dityrosine crosslinks are synthesized after fertilization in a reaction catalyzed by an ovoperoxidase that is released from secretory vesicles (the cortical granules) and inserted into the assembling fertilization membrane (Klebanoff *et al.*, 1979). The oxidizing substrate for the ovoperoxidase reaction appears to be hydrogen peroxide that is synthesized by the egg in a burst after fertilization (Foerder *et al.*, 1978). H_2O_2 synthesis accounts for at least two-thirds of the oxygen uptake first described by Warburg (1908) as the classic physiologic consequence of egg activation.

The coordinated activation of this complex peroxidative system provides a unique problem in biochemical regulation. Ovoperoxidase is stored within the egg, inside the cortical granules, prior to fertilization. The enzyme is released from cortical granules a few minutes after fertilization and assembled into the fertilization membrane (Foerder and Shapiro, 1977; Hall, 1978; Klebanoff *et al.*, 1979; Kay *et al.*, 1982). H_2O_2 is formed at an appropriate time to act as one substrate for dityrosine formation. Ovoperoxidase is one of several components that is juxtaposed in an appropriate spatial and temporal context outside of the egg in order to effect cross-linking of a newly assembled macromolecular matrix, the soft fertilization membrane (Kay and Shapiro, 1984). We have begun an exploration of the catalytic properties of the ovoperoxidase in order to understand its mechanism of association with other egg surface proteins in the formation of the fertilization membrane, its directed catalysis of appropriate dityrosine residues, and its potential role as a physiologic spermicidal agent. Peroxidases have spermicidal effects in model systems by virtue of their catalysis of halogen-mediated cytotoxicity (see Klebanoff *et al.* (1979) for references). Thus, the ovoperoxidase may play another role during fertilization membrane assembly, killing excess sperm in the vicinity of the egg. This paper describes an efficient means of purifying ovoperoxidase to homogeneity and an analysis of some kinetic and spectral properties of the purified enzyme.

EXPERIMENTAL PROCEDURES

Materials—*Strongylocentrotus purpuratus* were collected intertidally at the Strait of Juan de Fuca and stored prior to use in seawater aquaria at 10 °C. All subsequent steps were performed in a cold room at 10 °C. Egg shedding was induced by intracoelomic injection of 0.55 M KCl. Prior to further treatment, eggs were washed twice by suspension in seawater followed by settling and decantation.

Seawater was collected at Anacortes, Washington and filtered through a 0.45-μm Millipore filter before use. *Staphylococcus* V8 protease (Miles), thermolysin, tosylphenylalanyl chloromethyl ketone trypsin (Worthington), and bovine serum albumin (Sigma Fraction V) were used without further purification. Calcium ionophore A23187 was obtained from Calbiochem. All other reagents were of the highest purity available.

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard or by the method of Bradford (Bradford, 1976) using rabbit immunoglobulin G as a standard. Kinetic studies were performed on a Gilford model 240 spectrophotometer. UV-visible spectra were recorded on a Cary model 210 spectrophotometer. Both instruments were maintained at 25 °C during measurements.

Enzyme Assays—For routine assays, the guaiacol oxidation assay

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was employed. The reaction mixture contained 10 mM Tris chloride, pH 8.0, 18 mM guaiacol, and 0.3 mM H_2O_2 (Foerder and Shapiro, 1977). Fresh assay mixture was prepared daily. Assays were initiated by injection of enzyme solution, and the appearance of the reaction product was monitored at 436 nm, using an extinction coefficient for the product of 6.39×10^3 M^{-1} cm^{-1} (Bergmeyer *et al.*, 1974). Guaiacol units are defined as micromoles of product formed per min.

The kinetics of iodide oxidation were monitored at 353 nm, where the product I_3^- has an extinction coefficient of 2.66×10^4 M^{-1} cm^{-1} (Roman and Dunford, 1972). The reaction mixture contained 10 mM Hepes,[1] pH 7.5, and 10 mM KI. Enzyme stock was added to this mixture, allowed to equilibrate for 2 min, and the reaction initiated by addition of H_2O_2 to a concentration of 0.5 mM.

Tyrosine oxidation was measured at 315 nm, where the product dityrosine has an extinction coefficient of 6.3×10^3 M^{-1} cm^{-1} at pH 8.0 (Bayse *et al.*, 1972). The tyrosine concentration was 1.9 mM, and the optimal H_2O_2 concentration, 0.05 mM (see "Results"). Chloride and bromide oxidation were monitored using the method of Hager *et al.* (1966). All kinetic assays were performed at 25 °C.

Gel Electrophoresis—SDS-polyacrylamide slab gels (10% separating, 5% stacking) were prepared by the method of Laemmli (Laemmli, 1970; Studier, 1973). Gels were fixed and stained for protein with 0.1 g/100 ml Coomassie Blue R (Sigma) in 50% methanol, 10% acetic acid. Nondenaturing ("native") gels (5% separating, 4% stacking) were prepared as above except that SDS and 2-mercaptoethanol were omitted. To stain for peroxidase activity, these native gels were soaked in H_2O_2-free guaiacol assay mixture containing 0.1 g/100 ml sodium nitroferricyanide. The sodium nitroferricyanide stabilized the guaiacol product much as it does the peroxidase-catalyzed tetramethylbenzidine product (Mesulam, 1978). Upon the addition of 1 mM H_2O_2, an orange-brown color developed which was photographed within 15–20 min. Gels were photographed with Polaroid type 55 film using an orange filter for protein stains and a blue-green filter for ovoperoxidase activity stains.

Amino Acid and Sugar Analysis—Amino acid analyses were performed on a Durrum (model D 500) amino acid analyzer after hydrolysis for 24, 48, 72, and 96 h *in vacuo* at 100 °C in 6 N HCl. The hydrolysis time had little effect on the relative amino acid recovery. Cysteine was determined as cysteic acid by the method of Moore (1963). Tryptophan was estimated spectrophotometrically using an extinction coefficient at 288 nm of 4815 M^{-1} cm^{-1} (Edelhoch, 1967) after subtraction of a small contribution to absorbance at that wavelength by tyrosine, as determined by amino acid analysis. Norleucine was used as an internal standard throughout the analyses.

Neutral and amino sugars were determined by high-pressure liquid chromatography and mass spectroscopy, as described by Carter and Hakomori (1979). Sialic acid was determined by the thiobarbituric acid method (Spiro, 1966).

Preparation of Fertilization Product—The material released from the cortical granules, "fertilization product" (Carroll and Epel, 1975), was obtained by chemical induction of exocytosis after disruption of the vitelline layer. Eggs washed in seawater were allowed to settle, after which the supernatant was decanted. To each 100 ml of settled eggs, 100 ml of 20 mM dithiothreitol in seawater, adjusted to pH 9.4 with NaOH, was added and gently stirred for 5 to 10 min. The suspension was diluted into an additional 800 ml of seawater. The eggs were allowed to settle and then were washed three times with 4 liters of seawater. Eggs treated in this fashion have disrupted jelly coats and vitelline layers (Epel *et al.*, 1970).

For induction of cortical granule exocytosis, the treated eggs were brought to 5 times their original volume in seawater and gently stirred. This mixture was adjusted to pH 4.1–4.2 by adding 5.5 ml of 1 M acetic acid per liter of egg suspension. After 2 to 5 min of gentle stirring, 1.0 M MES, pH 8.0, was added to a final concentration of 20 mM, bringing the suspension to pH 6.2 and inducing exocytosis. A 200 mM solution of phenylmethylsulfonyl fluoride in 100% ethanol was added to a final concentration of 0.2 mM immediately thereafter. After settling the eggs, the supernatant was decanted. This supernatant, fertilization product, contains the exocytosed contents of the cortical granules.

Activation of egg exocytosis by the use of acetic acid in place of butyric acid (Loeb, 1913; Bryan, 1970) represents a significantly more convenient method, avoiding the unpleasant odor of butyric acid. Exocytosis, as measured by release of ovoperoxidase, was induced with equal efficiency by acetic acid, butyric acid, or the calcium ionophore A23187 (data not shown). The duration of exposure of the egg suspension to pH 4.1–4.2 is important for successful exocytosis. This parameter varies somewhat with the sample of sea urchins used and must be optimized for each batch of eggs. Incubation of eggs at low pH for times longer than the minimum required for complete activation leads to egg lysis.

RESULTS

Purification of Ovoperoxidase—Fertilization product prepared from several batches of eggs (as described under "Experimental Procedures") was brought to 50% saturation with solid NH_4SO_4 and centrifuged at $2,000 \times g$ for 60 min. The pellet was suspended in a minimum volume (5–10% of the original volume) of a buffer consisting of 20 mM imidazole, pH 7.5, containing 1 mM EGTA (Buffer A). The turbid solution was dialyzed twice against 20 volumes of buffer A containing 0.2 mM phenylmethylsulfonyl fluoride, resolubilizing all ovoperoxidase activity. A small amount of insoluble inactive material was removed by centrifugation at $20,000 \times g$ for 30 min.

The supernatant solution was mixed with sufficient DEAE-Sephacel (Sigma) in buffer A to bind all of the activity, as tested with small aliquots. Typically, 40 ml of DEAE were required to bind the ovoperoxidase present in 1 liter of fertilization product. The DEAE was then packed in a column and washed with buffer A until unbound protein was removed, as judged by A_{280} of the eluent. A linear gradient of 0–400 mM NaCl in buffer A was applied, eluting ovoperoxidase prior to the principal protein peak (Fig. 1). Those fractions showing enhanced specific activity were pooled, concentrated by ultrafiltration (Amicon PM-10), and dialyzed overnight against 40 volumes of 20 mM MES, pH 6.0, containing 1 mM EGTA (buffer B).

This solution was applied to a CM-Sephadex (Sigma) column (1×20 cm) equilibrated with buffer B, and the column was washed with buffer B to remove unbound material. Ovoperoxidase activity was eluted as a single peak by a linear gradient of NaCl (Fig. 2). Those fractions showing a ratio of A_{415}/A_{280} greater than 1.45 were pooled. Side fractions were pooled for repurification by repetition of the CM-Sephadex chromatography step. The isolated enzyme was stable for

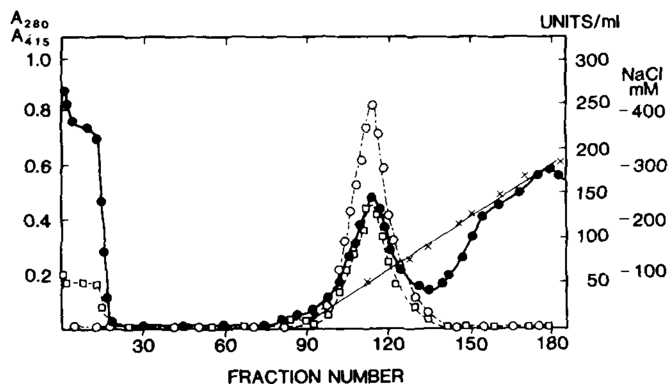


FIG. 1. **DEAE column chromatography of ovoperoxidase.** 300 ml of dialyzed NH_4SO_4 precipitate (final ionic strength, 30 meq of NaCl) were mixed with 200 ml of DEAE-Sephacel and packed in a 2.5×45 cm column (see "Results"). Protein was eluted with an 800-ml gradient of 0–400 mM NaCl in buffer A. The average fraction size was 5.7 ml. Fractions 100–132 were pooled for further purification (188 ml). ●, A_{280}; □, A_{415}; ○, ovoperoxidase activity (guaiacol units/ml); ×, NaCl (mM).

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ATA, 3-amino-1,2,4-triazole.

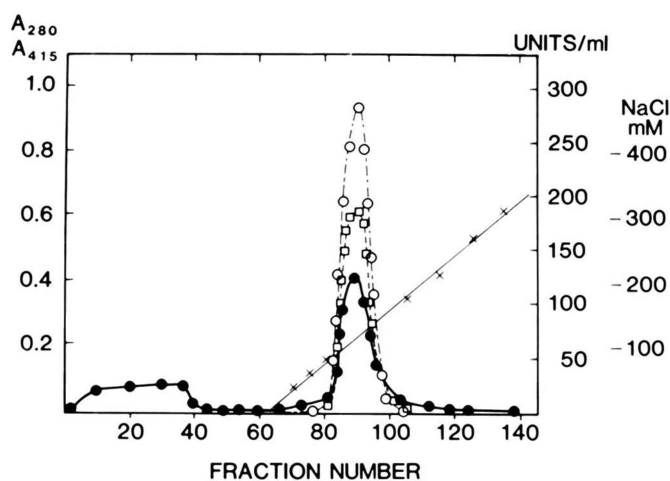


FIG. 2. **CM column chromatography of ovoperoxidase.** 172 ml of the pooled DEAE fractions were dialyzed against buffer B and were applied to a CM-Sephadex column (1.0 × 25 cm). Protein was eluted with a 500-ml gradient of 0-300 mM NaCl in buffer B. The average fraction size was 5.7 ml. Fractions 84-94 were pooled as peak fractions. The average value of R_z (A_{415}/A_{280}) across peak fractions was 1.46 ± 0.03. Symbols are as in Fig. 1.

TABLE I

Purification of ovoperoxidase

This table represents results of a typical purification. The range of final specific activity as seen in eight different preparations was 500-2000 guaiacol units/mg with an R_z of 1.4-1.5.

	Total protein	Total ovoperoxidase activity	Specific activity	R_z (A_{415}/A_{280})	Yield
	mg	*guaiacol units*	*guaiacol units/mg protein*		%
Fertilization product	21,000	3.5×10^4	16.8	NA[a]	100
NH_4SO_4 precipitate after dialysis	8,700	2.6×10^4	30.0	NA	75
DEAE peak fractions	57	2.4×10^4	418	0.81	69
CM peak fractions	29	1.4×10^4	502	1.46	52

[a] NA, not available.

weeks in solution at 4 °C as collected from the last chromatography step and could be stored in the same solution for months at −60 °C with negligible loss of activity.

This procedure resulted in a 30-fold purification of ovoperoxidase from fertilization product (Table I), which corresponds to a 600-fold purification from whole eggs. Ovoperoxidase was obtained by this method in 50-70% yield from fertilization product and was essentially homogeneous, *i.e.* greater than 95% pure by densitometric scans of Coomassie Blue-stained SDS-polyacrylamide gels.

The apparent M_r of ovoperoxidase was 70,000 + 1,000 (Fig. 3*A*). Minor variations in M_r, dependent on the percentage of acrylamide in the SDS gels, were observed, probably because ovoperoxidase is a glycoprotein (see below). Electrophoresis under nonreducing conditions in the presence of SDS showed only a slight change in apparent molecular weight, consistent with the presence of intrachain, but not interchain, disulfide linkages (Fig. 3*A*, *lane 6*). Electrophoresis in the absence of both SDS and 2-mercaptoethanol revealed two protein bands that coincided with the guaiacol stain for ovoperoxidase activity (Fig. 3*B*, *lanes 1* and *2*). The electrophoretic heterogeneity of ovoperoxidase may result from the presence of multiple isozymes or from heterogeneity of glycosylation of the enzyme. Ovoperoxidase is a glycoprotein, containing both

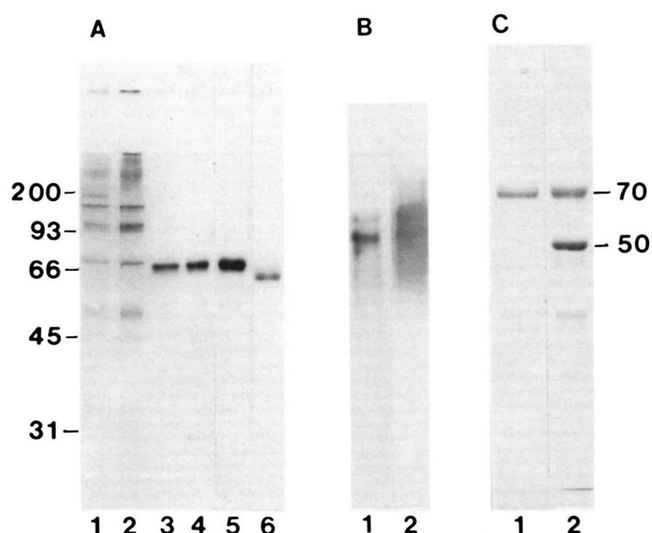


FIG. 3. **Purification of ovoperoxidase.** All size markers are indicated, for SDS gels, as kilodaltons. *A*, 10% SDS-polyacrylamide gel. *Lane 1*, fertilization product (20 μg); *Lane 2*, NH_4SO_4 precipitate after dialysis (20 μg); *Lane 3*, DEAE peak fractions (5 μg); *Lane 4*, CM-purified ovoperoxidase (5 μg); *Lane 5*, CM-purified ovoperoxidase (15 μg); *Lane 6*, CM-purified ovoperoxidase (5 μg) in the absence of 2-mercaptoethanol; *B*, 5% native polyacrylamide gel. *Lane 1*, ovoperoxidase (10 μg), Coomassie stained; *Lane 2*, ovoperoxidase (10 μg), peroxidase activity stained (see "Experimental Procedures"). *C*, 10% SDS-polyacrylamide gel. *Lane 1*, ovoperoxidase purified as described; *Lane 2*, ovoperoxidase as isolated by initial purification scheme (see "Results").

TABLE II

Composition of ovoperoxidase

The complete amino acid and carbohydrate composition of ovoperoxidase was determined as described under "Experimental Procedures." The data are expressed as moles per mol of ovoperoxidase, based on a protein M_r of 70,000. Fucose, glucose, galactose, *N*-acetylgalactosamine, and sialic acid were not detected.

Asx	68.3
Thr	21.8
Ser	44.6
Glx	53.6
Pro	42.0
Gly	43.1
Ala	40.2
Val	38.3
Met	7.6
Ile	31.0
Leu	49.9
Tyr	13.7
Phe	40.2
His	16.5
Lys	13.1
Arg	47.8
Cys	12.3
Trp	15.2
Mannose	8.7
N-Acetylglucosamine	3.9

mannose and *N*-acetylglucosamine moieties (Table II). It bound to concanavalin A and stained with periodic acid-Schiff reagent (Fairbanks *et al.*, 1971) in polyacrylamide gels (data not shown).

Prompt isolation of ovoperoxidase from fertilization product was important to the success of the preparation. Initial attempts to purify the enzyme yielded preparations with an apparent M_r of 50,000, rather than 70,000 as isolated by the procedure described above (Fig. 3*C*, *lane 2*). In the earlier procedure, fertilization product was obtained as previously described (Foerder and Shapiro, 1977) where trypsin was used,

instead of dithiothreitol, to remove the egg jelly and vitelline layer. Exocytosis was induced by fertilization instead of artificial activation. Most importantly, the fertilization product was concentrated by ultrafiltration, a procedure that took much longer than NH_4SO_4 precipitation. We suspected that proteolytic activity, known to be present in fertilization product (Shapiro, 1975), might have been responsible for the enzyme of lower apparent molecular weight obtained with such a protocol, even though protease inhibitors were used in both isolation procedures. To investigate the possibility that the M_r 70,000 enzyme could be converted to a M_r 50,000 product by limited proteolysis, purified ovoperoxidase was treated with several different proteases (Fig. 4). In each experiment, 7.5 μg of ovoperoxidase were incubated in seawater for 18 h at room temperature with 0.1–0.6 μg of protease. Thermolysin converted ovoperoxidase to a band of apparent molecular weight near 50,000, but not identical with the M_r 50,000 product previously isolated (Fig. 4, *lanes 1–3*). *Staphylococcus* V8 protease effected a smooth conversion of M_r 70,000 ovoperoxidase to a band co-migrating with the M_r 50,000 band that had been isolated in our initial experiments (Fig. 4, *lanes 4–6*). Trypsin led to a loss of the M_r 70,000 band with only traces of smaller macromolecular products being detected (data not shown). Neither chymotrypsin nor *Bacillus subtilis* neutral protease had any effect on the migration of ovoperoxidase in SDS-polyacrylamide gels.

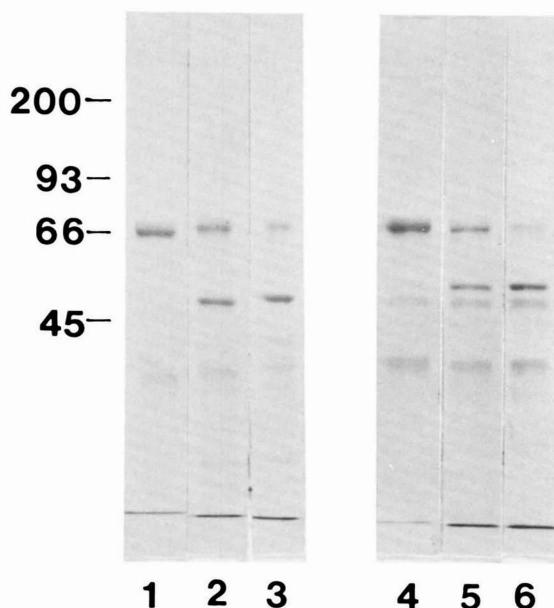


FIG. 4. **Proteolytic cleavage of ovoperoxidase.** All size markers are indicated as kilodaltons. Each lane contained 7.5 μg of ovoperoxidase that was incubated for 18 h at room temperature with the indicated amount of protease before SDS-polyacrylamide gel electrophoresis. *Lane 1*, no protease; *Lane 2*, Staphylococcus V8 protease (0.2 μg); *Lane 3*, *Staphylococcus* V8 protease (0.6 μg); *Lane 4*, no protease; *Lane 5*, thermolysin (0.1 μg); *Lane 6*, thermolysin (0.6 μg).

Spectral Properties—Purified ovoperoxidase exhibited a UV-visible spectrum typical of heme-containing peroxidases (Dunford and Stillman, 1976) with a protein absorbance at λ_{max} 278 nm and an intense Soret absorbance centered at 412 nm (Fig. 5, *left*). A quartet of less intense bands was also detected in the visible region (Fig. 5, *right*). Table III lists λ_{max} and extinction coefficient values determined for ovoperoxidase. Ovoperoxidase showed a slight pH dependence of its visible spectrum, with the Soret band becoming more intense and shifting from 410 to 416 nm as the pH was decreased from 9.0 to 4.5 (Table III). This shift was accompanied by the development of a band at 580 nm. In general, ovoperoxidase and lactoperoxidase (Morell, 1954) spectra

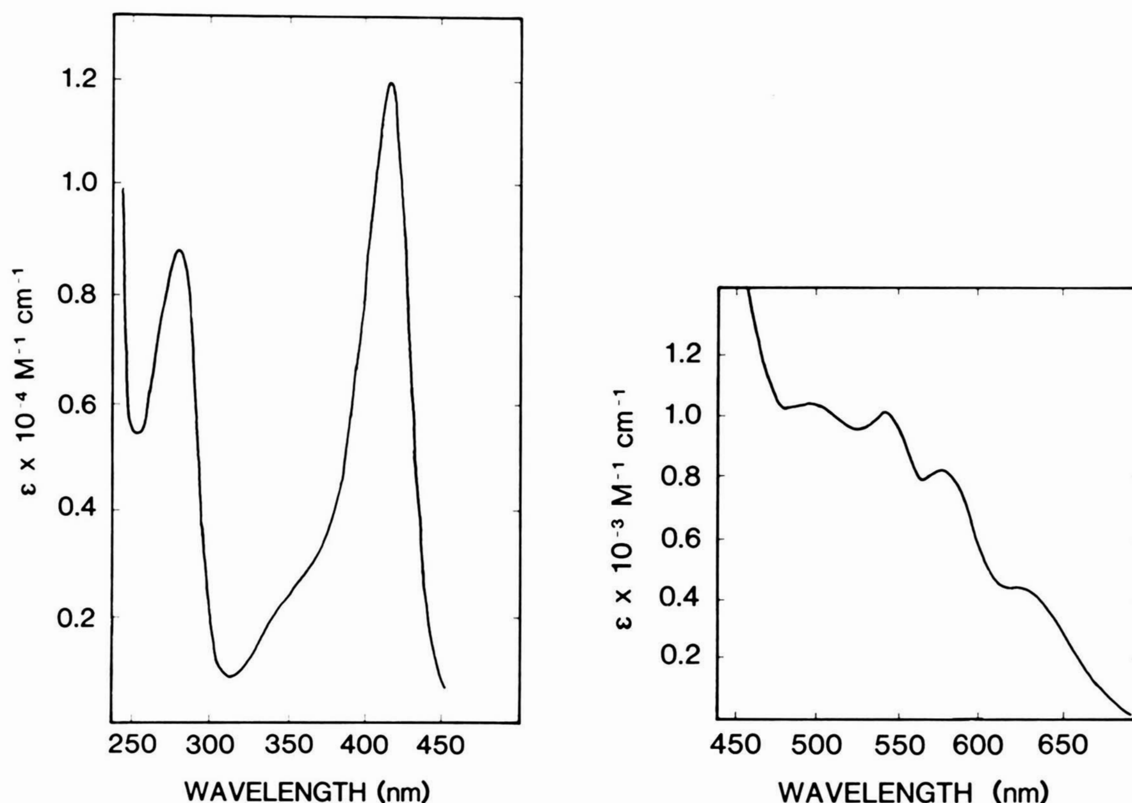


FIG. 5. **Spectra of ovoperoxidase.** Samples were dialyzed against 100 volumes of 20 mM acetate, pH 4.5, containing 80 mM CH_3SO_3Na. *Left*, UV-visible spectrum (250–450 nm); *right*, visible spectrum (400–800 nm).

TABLE III
Spectral properties of ovoperoxidase

The data are shown for ovoperoxidase at three pH values and, for comparison, lactoperoxidase at pH 5.6 as determined from Fig. 3 of Morell (1954). The λ_{max} are listed at the top of each column; when deviation occurs in peaks or inflection points, these wavelengths are listed in parenthesis next to the corresponding ϵ value.

Enzyme	pH	λ_{max}					
		278	414	496	540	578	630
		$\epsilon \times 10^{-4}\ M^{-1}\ cm^{-1}$					
Ovoperoxidase	9.0	6.6	10.4 (410)	1.20 (494)	1.04	0.67 (588)	0.61
	6.5	9.0	10.4	1.05	0.93 (543)	0.70	0.38 (618)
	4.5	8.9	11.9 (416)	1.04 (493)	1.02 (541)	0.83	0.40
Lactoperoxidase	5.6	NA[a]	10.8	0.87	0.85 (546)	0.63 (595)	0.58 (633)

[a] NA, not available.

TABLE IV
Catalytic activities of ovoperoxidase

Substrate	Turnover number	
	Ovoperoxidase[a]	Lactoperoxidase
	s^{-1}	
Guaiacol	20,000	71,000[b]
I^-	2,400	7,900[b]
Br^-	0.73	NR[c]
Tyrosine	64	75[d]

[a] Ovoperoxidase values were determined at the optimal pH and concentrations of H_2O_2 and substrate (see "Experimental Procedures").
[b] These values were calculated from Hosoya and Morrison (1962).
[c] Not reported.
[d] This value was calculated from Bayse *et al.* (1972).

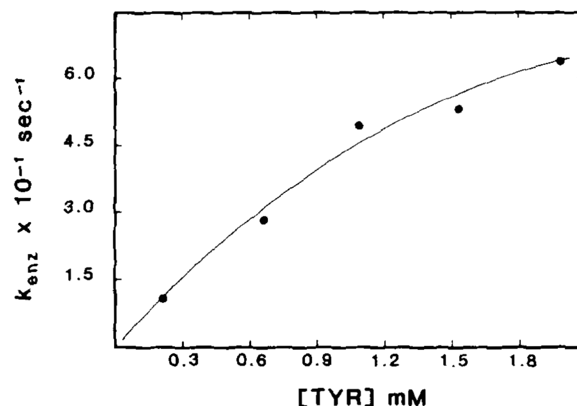
were similar in both the wavelength maxima and extinction coefficients of all peaks measured (Table III).

Kinetic Properties—Ovoperoxidase had activity toward a broad range of reducing substrates, as is characteristic of peroxidases. The turnover numbers of ovoperoxidase toward several substrates are summarized in Table IV. In each instance, the turnover number was calculated at the pH, reducing substrate, and H_2O_2 concentration giving maximal activity (see "Experimental Procedures") rather than at saturating substrate concentrations. For I^- and Br^-, concentrations in excess of 20 mM were inhibitory, and tyrosine was not saturating toward the enzyme at its maximum solubility in buffer (Fig. 6).

The optimum concentration of H_2O_2 varied significantly with the substrate examined. In the case of I^- and guaiacol, concentrations near 0.5 mM were optimal, with activity varying in a conventional hyperbolic saturation curve. However, with tyrosine, ovoperoxidase had a sharp optimum of activity at 0.05 mM H_2O_2 (Fig. 7) and was inhibited at higher levels. Ovoperoxidase could use ethyl hydrogen peroxide as an alternate substrate, although activity was reduced to 8% of that measured with H_2O_2 as substrate when guaiacol was the hydrogen donor. Cumene hydroperoxide and *t*-butyl hydroperoxide were not effective substrates.

Ovoperoxidase was more active toward all substrates at pH values above 7. The pH activity profile for ovoperoxidase-catalyzed I^- oxidation was typical of the substrates that were tested (Fig. 8). A plateau of activity was seen in the guaiacol and tyrosine assays at pH 8.0 (data not shown). The general shape of the curve is more consistent with multiple ionizations influencing the change in enzyme activity than with the simple ionization of a single functional group.

In order to obtain reproducible enzyme activities with ovoperoxidase, it is important that the enzyme be pre-equilibrated at the pH value of interest for several minutes prior to initiating the reaction. We have found ovoperoxidase to exhibit a slow pH-dependent change in catalytic activity when subjected to a rapid pH shift. The enzyme relaxes to its equilibrium activity, corresponding to Fig. 8, after periods ranging from a few seconds to minutes, depending on the initial and final pH values. This property of ovoperoxidase will be the subject of a subsequent report.[2]



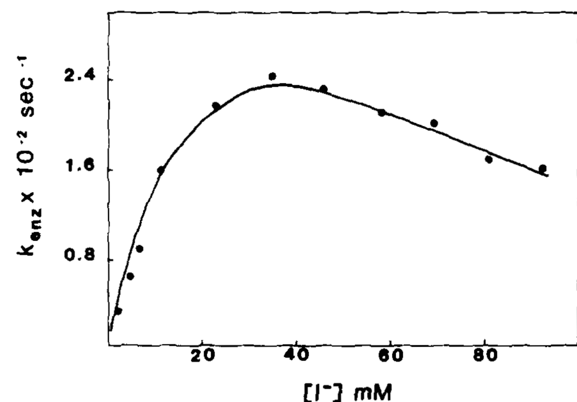
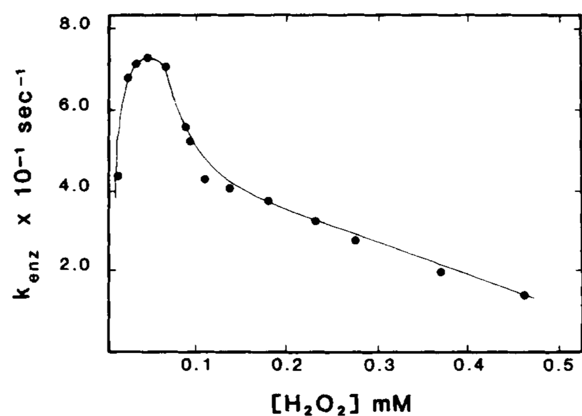


FIG. 6. **Substrate concentration dependence of ovoperoxidase activity.** *Top*, tyrosine assay described under "Experimental Procedures" using 20 mM Hepes, pH 8.0. *Bottom*, iodide assay as described under "Experimental Procedures" using 20 mM Hepes, pH 7.5. At each iodide concentration, the velocity of nonenzymatic oxidation of iodide was determined in the absence of ovoperoxidase, and this value was subtracted from the velocity observed in the presence of enzyme. The total ionic strength was maintained at 0.1 M with CH_3SO_3Na which was found to be noninhibitory in control experiments (data not shown).

[2] T. Deits and B. M. Shapiro, manuscript in preparation.



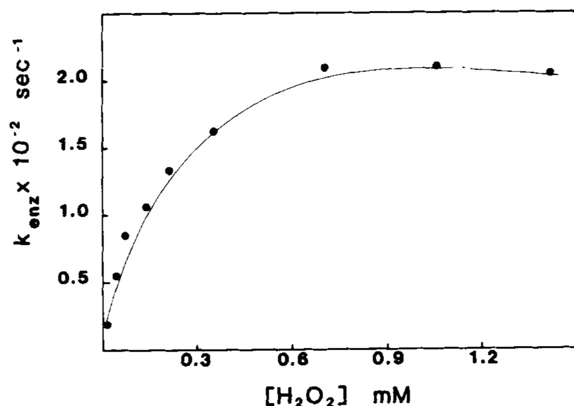


FIG. 7. **H_2O_2 concentration dependence of ovoperoxidase activity.** *Top*, tyrosine assay performed as described under "Experimental Procedures," using 20 mM Hepes, pH 8.0, 1.9 mM tyrosine. *Bottom*, iodide assay performed as described under "Experimental Procedures," using 20 mM Hepes, pH 7.5, 15 mM I^-. Total ionic strength maintained at 0.1 M with CH_3SO_3Na.

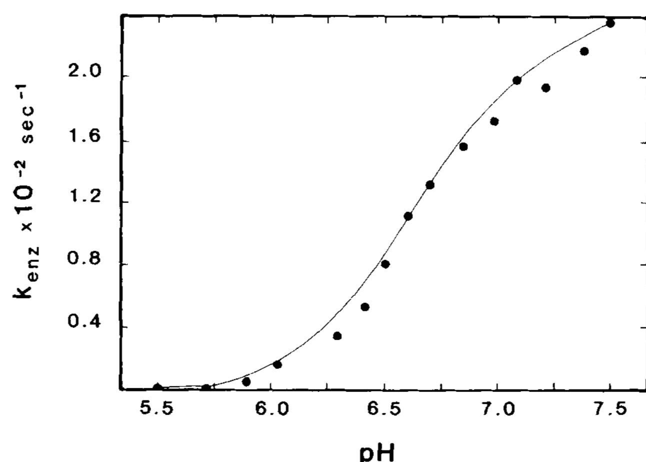


FIG. 8. **pH dependence of the iodide oxidation activity of ovoperoxidase.** Enzyme was pre-equilibrated at the indicated pH for 5 min prior to assaying, as described under "Experimental Procedures." A pH range of 5.5 to 7.5 was obtained with acetate, MES, and Hepes (20 mM), taking multiple measurements in the regions where the buffering capacities overlap. The product of the reaction, I_3^-, decomposes to iodate above pH 7.5 and becomes colorless.

Inhibition of Ovoperoxidase—Compounds previously reported as inhibiting the peroxidative activity of sea urchin eggs *in vivo* (Foerder and Shapiro, 1977) in most cases inhibited the purified ovoperoxidase at comparable concentrations (Table V). Phenylhydrazine, ATA, and azide inhibited the purified enzyme as was previously reported for the crude fertilization product. Glycine ethyl ester, at concentrations of 10–30 mM, inhibits a morphological transition in the developing fertilization membrane, termed the I to T transition, that occurs prior to hardening (Veron *et al.*, 1977). Glycine ethyl ester was reported to inhibit the activity of ovoperoxidase *in vivo* in this same concentration range (Foerder and Shapiro, 1977). However, fertilization product and purified ovoperoxidase obtained as described in the paper were inhibited by glycine ethyl ester only at concentrations more than 20-fold higher (Table V). The reasons for this discrepancy, as well as the relationship between the inhibition of ovoperoxidase and inhibition of the I to T transition, are currently under investigation.

Sulfite, reported as an inhibitor of hardening *in vivo* (Motomura, 1954, Veron *et al.*, 1977), is a substrate for peroxidases (Roman and Dunford, 1973) and behaves as a substrate toward ovoperoxidase (data not shown). The inhibition of hardening by sulfite and phenylhydrazine may be due to competition for oxidizing equivalents with the *in vitro* substrates of ovoperoxidase.

3-Amino-1,2,4-triazole Effects on Ovoperoxidase—Both catalase (Margoliash *et al.*, 1960) and lactoperoxidase (Castelfranco, 1960) are inhibited by ATA; in both instances, the inhibition is irreversible and leads to the alkylation of specific histidyl residues (Chang and Schroeder, 1973). The inhibition of ovoperoxidase by ATA had some interesting features. In the absence of H_2O_2, ATA inhibition of ovoperoxidase was partially reversible. Treatment of purified ovoperoxidase with ATA for 2 h, followed by dialysis to remove ATA, restored 80% of the activity when compared with control preparations treated similarly in the absence of ATA (Table VI). In the case of both lactoperoxidase and catalase, the presence of H_2O_2 is required to effect alkylation of the enzyme. Hydrogen peroxide alone irreversibly inactivated ovoperoxidase after prolonged exposure (Table VI); the addition of ATA facilitated this process.

The kinetically observed inhibition of ovoperoxidase by ATA required H_2O_2; this inhibition was slow relative to the rate of turnover of the enzyme in the I^- oxidation assay as

TABLE V

Inhibition of purified ovoperoxidase

The inhibition of crude ovoperoxidase (in fertilization product) was compared with the purified enzyme; the results were identical.

Inhibitor	$[I]_{0.5}$[a]
Phenylhydrazine	0.25 μM
ATA	30 μM
Azide	4.5 mM
Sodium sulfite	[b]
Glycine ethyl ester	700 mM

[a] Inhibitor concentrations were varied to determine the amount that inhibits 50% of the ovoperoxidase activity in the iodide assay. Enough ovoperoxidase to produce a maximal rate of 30 A_{353} per min per assay was used.

[b] Sulfite has been reported to inhibit *in vivo* hardening (Motomura, 1954; Veron *et al.*, 1977) and to inhibit the enzyme, ovoperoxidase, in the guaiacol assay $[I]_{0.5}$ = 28 μM (Foerder and Shapiro, 1977). In addition to acting as a substrate for peroxidase (see "Results"), sulfite interferes with the appearance of the colored product in the iodide assay, a fact which led us to re-examine the effects of this inhibitor on the guaiacol assay. Sulfite bleaches a portion of the colored reaction products generated in the guaiacol assay.

TABLE VI

Effect of H_2O_2 and ATA on the I^- oxidation activity of ovoperoxidase

Ovoperoxidase (1.8 μM) was treated with 2.0 mM ATA and the indicated concentration of H_2O_2 in 20 mM Hepes, pH 7.5, for 2 h and then dialyzed overnight against 20 mM Hepes, pH 7.5.

$[H_2O_2]$	Activity	
	−ATA	+ATA
μM	%	
0	100	77
0.1	87	68
1.0	82	72
10.0	71	44
50.0	36	8
100	29	5

shown by preincubation of the enzyme, substrates, and aminotriazole. Preincubation of ovoperoxidase, ATA, and H_2O_2 in the assay cuvette, followed by initiation of the reaction with I^-, gave no I_3^- in excess of the slow nonenzymatic background reaction (data not shown). However, when ovoperoxidase, ATA, and I^- were pre-equilibrated and the reaction was initiated with H_2O_2, a burst of I^- oxidation activity was observed which was complete within the 0.5-s mixing time of the assay (Fig. 9). This activity burst was observed at concentrations of ATA far in excess of those required to inhibit the steady-state activity of the enzyme. Fig. 9 presents data showing the magnitude of the burst of activity at ATA concentrations some 15- to 60-fold higher than the steady-state K_i values for ATA. Higher concentrations of ATA reduced the magnitude of the burst; increasing the substrate (I^-) concentration led to a larger burst effect. The magnitude of the burst was linearly related to enzyme concentration (Fig. 9, *top*, *inset*). Such plots yield from their slopes the average number of turnover events undergone by an enzyme molecule before inhibition is complete. At 2 mM ATA, the observed slope was 33 mol of I_3^-/mol of ovoperoxidase, and, at 0.5 mM, the slope was 101 mol of I_3^-/mol of ovoperoxidase. Thus, this burst of activity indicates that the inactivation of ovoperoxidase by ATA and H_2O_2 is dependent upon enzyme turnover.

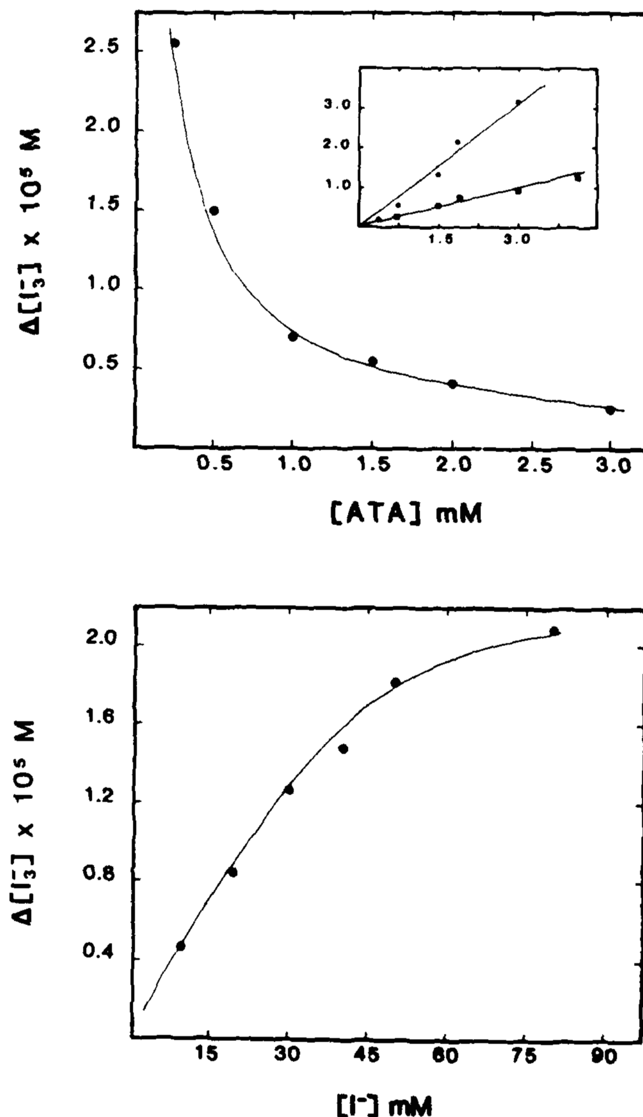


FIG. 9. **ATA and iodide concentration dependence of burst behavior of ovoperoxidase-catalyzed I^- oxidation.** The assay was performed in 10 mM Hepes, pH 7.5, 0.5 mM H_2O_2, and 0.1 M CH_3SO_3Na. *Top*, ATA concentration dependence. $[I^-]$ = 10 mM; [ovoperoxidase] = 0.14 μM. Enzyme was pre-equilibrated with ATA and I^- for 2 min prior to reaction initiation with H_2O_2. *Inset*, ovoperoxidase concentration dependence of burst effect; abscissa, [ovoperoxidase] × 10^6 M; ordinate, $[I_3^-]$ × 10^5 M. [ATA] = 2 mM (■), 0.5 mM (●). *Bottom*, iodide concentration dependence; [ATA] = 2.0 mM. Enzyme was pre-equilibrated with ATA and I^- for 2 min, and the reaction was initiated with H_2O_2. [Ovoperoxidase] = 0.14 μM.

DISCUSSION

This is the first description of the isolation and properties of a peroxidase that is involved in the physiology of fertilization (reviewed in Shapiro *et al.*, 1981). The enzyme has several important characteristics from a cellular and developmental point of view. During oogenesis, the enzyme is synthesized and directed to cortical granules where it resides until the exocytosis that follows sperm-egg fusion. After release, the enzyme interacts with other components of the egg coat, cortical granules, and seawater to form a noncovalently associated soft fertilization membrane (Kay *et al.*, 1982). Using H_2O_2 produced by the egg in a transient postfertilization burst (Foerder *et al.*, 1978), ovoperoxidase catalyzes the cross-linking of appropriately juxtaposed tyrosyl residues to form a hardened macromolecular complex, the fertilization membrane. This process constitutes a striking example of cellular coordination in which macromolecular assembly is followed by a redox reaction sequence, all of which proceed extracellularly. An examination of the properties of the individual components in this peroxidative system is central to understanding the regulatory mechanisms involved.

The purification protocol described above relies on the localization of ovoperoxidase in the cortical granule and its release after egg activation in order to arrive at a 600-fold purification in a few steps. Ovoperoxidase accounts for about 3% of the cortical granule protein or about 0.2% of total egg protein. Performing the DEAE chromatography step in the presence of divalent cations provided some hints about ovoperoxidase behavior *in vivo*. With DEAE chromatography in the absence of added divalent cations, ovoperoxidase eluted at 70 mM NaCl, prior to the major protein peak (Fig. 1). However, with DEAE chromatography in the presence of 10 mM Ca^{2+}, ovoperoxidase coeluted with the major protein peak at 250 mM NaCl. Once purified away from this major protein peak, the elution position of purified ovoperoxidase on DEAE-Sephacel was independent of divalent cation concentration (data not shown). Moreover, divalent cations had no effect on the catalytic activity of the purified enzyme.[3] These observations suggest that ovoperoxidase interacts with other solu-

[3] E. S. Kay, P. J. Weidman, and T. Deits, unpublished data.

ble components of the fertilization product in a divalent cation-mediated fashion, a phenomenon currently under study.[4]

A second observation emerging from our purification scheme that may bear on the interactions of ovoperoxidase *in vivo* is the sensitivity of the purified enzyme to proteolytic cleavage. Thermolysin, which cleaves at hydrophobic residues (Matsubara and Feder, 1971), and *Staphylococcus* V8 protease, which cleaves polypeptides at Asp and Glu residues (Drapeau *et al.*, 1972), efficiently cleave ovoperoxidase to fragments of slightly different size (Fig. 4). This fact suggests that a limited segment of the ovoperoxidase polypeptide chain is available for proteolytic attack in the native state. Thyroid peroxidase, a membrane-bound peroxidase, is similarly sensitive to protease (trypsin) treatment by which it can be solubilized to facilitate purification (Klebanoff *et al.*, 1962). The possibility that the portion of the ovoperoxidase molecule removed from the 70,000 molecular weight enzyme to yield the 50,000 form comprises a functional, regulatory, or binding domain remains to be explored.

Proteolysis plays some role in the elevation of the fertilization membrane *in vivo* (Carroll and Epel, 1975), and a limited proteolytic digestion of egg surface components occurs after fertilization (Shapiro, 1975). The sensitivity of purified ovoperoxidase to proteases might indicate that such processing of ovoperoxidase occurs during assembly of the fertilization membrane. Trypsin-like proteases with specificity for cleavage sites adjacent to basic and hydrophobic residues have been purified from sea urchin cortical granule exudate (Fodor *et al.*, 1975; Carroll and Epel, 1975). We do not know yet whether proteolysis plays a role in the modulation of ovoperoxidase activity *in vivo* or whether the protease-sensitive region of ovoperoxidase is protected from the cortical granule proteases present during assembly of the fertilization membrane.

The kinetic properties of the purified ovoperoxidase are consistent with its identification as a peroxidase, including its capacity to catalyze dityrosine formation (Table IV). Ovoperoxidase catalyzes guaiacol, I^-, and tyrosine oxidation with efficiencies comparable to those of lactoperoxidase (Table IV). Ovoperoxidase can also catalyze Br^- oxidation, albeit more slowly. Ovoperoxidase might act as an early block to polyspermy via a halogen-mediated oxidative killing mechanism similar to that of the polymorphonuclear leukocyte (Klebanoff *et al.*, 1979). The ability of purified ovoperoxidase to catalyze both I^- and Br^- oxidation is a necessary condition for such a mechanism. Although the catalysis of Br^- oxidation is significantly lower than that of I^- oxidation on the basis of turnover numbers, the concentration of both I^- and Br^- in seawater is significantly below the concentrations required for maximal activity (20 mM). Thus, the net rate of halide oxidation in seawater will depend on both the turnover number and the concentration of halide available, *i.e.* the rate is effectively second order in both enzyme and halide. The typical concentration of I^- in seawater is ~0.4 μM, and of Br^-, ~0.8 mM (Riley and Chester, 1971). The quantity of oxidized halide produced on a molar basis by ovoperoxidase will thus be comparable for these two substrates, despite the difference in turnover numbers. Both activities should, therefore, be taken into account in assessing the significance of ovoperoxidase-mediated cytotoxicity for sperm. The enzyme does not detectably oxidize Cl^-, the predominant anion in seawater.

Our working hypothesis for the observed burst of peroxidatic activity in the presence of ATA (Fig. 9) postulates that inhibition by ATA requires the initial formation of a complex of ovoperoxidase and H_2O_2 analogous to Compound I of other peroxidases (Margoliash *et al.*, 1960). ATA then competes with other substrates for reaction with this intermediate form, consistent with the reciprocal effects of increased ATA or substrate I^- concentrations on the magnitude of the burst (Fig. 9). The efficacy of ATA inhibition of ovoperoxidase *in vivo* then will depend strongly on the effective substrate concentration in the vicinity of the enzyme. Since binding and orientation of ovoperoxidase in the nascent fertilization membrane may influence this effective substrate concentration, the role of ATA *in vivo* remains to be clarified. Ovoperoxidase also exhibits a strong pH-dependent hysteretic effect.[2] This property necessitates equilibration of ovoperoxidase at the assay pH in order to accurately determine the enzyme activity. The pH-dependent hysteresis is currently under study; it may reflect one means of regulating the activity of ovoperoxidase after assembly into the fertilization membrane.

Ovoperoxidase provides a distinctive catalytic marker for events in fertilization. The purified enzyme should aid in obtaining a more detailed understanding of the process of macromolecular assembly and structural modification that occurs around the egg within minutes of fertilization, as well as indicating how a potent oxidizing agent like H_2O_2 is dealt with in close proximity to the early embryo.

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