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Sea urchin sperm peroxidase is competitively inhibited by benzohydroxamic acid and phenylhydrazine

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Sea urchin sperm contain a phenylhydrazine-sensitive peroxidase that is believed to use hydrogen peroxide produced by the fertilized egg to reduce sperm fertility and thereby assist in the prevention of polyspermy. *Strongylocentrotus purpuratus* sperm were treated initially with hypotonic phosphate buffer (pH 7.0) to remove catalase and then extracted with 0.5% Triton X-100 in 0.5 *M* acetate buffer (pH 5.0). Peroxidase activity in this detergent extract was assayed using 3,3′,5,5′-tetramethyl benzidine (TMB) as oxidizable substrate. Kinetic studies showed that the K_m for TMB is 250 μM. Benzohydroxamic acid and phenylhydrazine are known to be competitive inhibitors of a variety of plant and animal peroxidases. These substances were found to competitively inhibit the sea urchin sperm peroxidase: for benzohydroxamic acid, K_i = 51.2 μM, mean inhibitory dose (ID_{50}) = 146.7 μM; for phenylhydrazine, K_i = 201 nM, ID_{50} = 303 nM. These findings (*i*) indicate that the biochemical properties of the sea urchin sperm peroxidase resembles those of peroxidases found in somatic tissues where oxygen radicals are produced by phagocytes to kill bacteria and (*ii*) support our hypothesis that the sperm peroxidase has a functional role in the prevention of polyspermy during fertilization.

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Le sperme d'oursin de mer contient une peroxydase sensible à la phénylhydrazine qui utiliserait le peroxyde d'hydrogène produit par l'œuf fécondé pour réduire la fécondité du sperme et participer ainsi à la prévention de la polyspermie. Nous avons d'abord traité le sperme de *Strongylocentrotus purpuratus* avec un tampon phosphate hypotonique à pH 7,0 pour enlever la catalase et nous l'avons ensuite extrait avec le Triton X-100 à 0,5% dans un tampon acétate 0,5 *M* à pH 5,0. Nous avons mesuré l'activité peroxydasique dans cet extrait par détergent, utilisant la 3,3′,5,5′-tétraméthyl benzidine (TMB) comme substrat oxydable. L'étude cinétique montre que le K_m pour la TMB est de 250 μM. L'acide benzohydroxamique et la phénylhydrazine sont des inhibiteurs compétitifs reconnus de diverses peroxydases végétales et animales. Ces réactifs inhibent de façon compétitive la peroxydase du sperme d'oursin de mer: l'acide benzohydroxamique, K_i = 51,2 μM et dose moyenne inhibitrice (DI_{50}) = 146,7 μM; la phénylhydrazine, K_i = 201 nM et DI_{50} = 303 nM. Ces données (*i*) montrent que les propriétés biochimiques de la peroxydase du sperme d'oursin de mer ressemblent à celles des peroxydases présentes dans les tissus somatiques où les radicaux oxygène sont produits par les phagocytes pour tuer les bactéries et (*ii*) confirment notre hypothèse que la peroxydase du sperme exerce un rôle fonctionnel dans la prévention de la polyspermie durant la fécondation.

[Traduit par la revue]

Introduction

The establishment of the block to polyspermy during fertilization is a critical event in the process of reproduction. Normally, only one spermatocyte enters the egg's cytoplasm and fuses with the female pronucleus. Penetration of the egg by more than one spermatocyte, polyspermy, is a pathological event that results in abnormal development and death of the embryo (1).

In sea urchins, the fertilizing sperm stimulates several responses by the egg, which act together at the egg's surface to prevent supernumerary sperm from entering (2). The first of these to be recognized was the elevation of the fertilization envelope to form an absolute mechanical barrier to sperm penetration (1). The fertilization envelope is derived in part from the vitelline layer that is attached to the plasma membrane of the unfertilized egg plus secretory products that are released by exocytosis of the egg's cortical granules (cortical reaction) at fertilization (reviewed in Refs. 2–5). This process begins at the site where the fertilizing sperm fuses with the egg's plasma membrane and propagates around the entire surface of the egg. However, the fertilization envelope does not elevate rapidly enough from the entire surface of the sea urchin egg (1–2 min for completion) to be exclusively responsible for the prevention of polyspermy in the presence of excess sperm (1, 2). During this period, other faster egg responses operate to restrict sperm penetration until the cortical reaction is completed: rapid sodium-dependent electrical depolarization of the egg's plasma membrane (6, 7), possible production of putative arachidonic acid derived oxidation products (8, 9), and the release of H_2O_2 into the ambient sea water (10, 11).

The fertility of sea urchin sperm is rapidly reduced upon exposure to H_2O_2 at concentrations comparable to

ABBREVIATIONS: TMB, 3,3′,5,5′-tetramethyl benzidine; ID_{50}, mean inhibitory dose; DMSO, dimethyl sulfoxide.

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those released by sea urchin eggs during the cortical reaction at fertilization (10). Under these physiological conditions inactivation of sperm fertility by H_2O_2 appeared to be promoted by a phenylhydrazine-sensitive peroxidase endogenous to the sperm. Sperm also appeared to contain a catalase (aminotriazole sensitive) that tends to protect them from H_2O_2. Subsequent biochemical studies demonstrated that sea urchin sperm contain a distinct phenylhydrazine-sensitive peroxidase and a aminotriazole-sensitive catalase that could be separated by differential extraction (12). Phenylhydrazine is known to be a potent competitive inhibitor of peroxidases obtained from somatic tissues of animals and plants (13–16). Benzohydroxamic acid is another competitive inhibitor of various peroxidases (17–19). In the present study we show that phenylhydrazine and benzohydroxamic acid are competitive inhibitors of the peroxidase activity extracted from sea urchin sperm. A preliminary account of this study has been presented previously (20).

Materials and methods

Strongylocentrotus purpuratus were obtained from Pacific Bio-Marine Supply Co. (Venice, CA) and maintained, in Buffalo, NY, at 10–15°C in a marine aquarium from Aquarium Systems, Inc. (Wickliffe, OH) until used. The animals were induced to spawn by an intracoelomic injection of 0.5 *M* KCl. Dry sperm (semen) were collected, by inverting spawning animals over small glass dishes, and stored on ice until used.

The semen was centrifuged at 1470 × *g* for 15 min to sediment the sperm (21). The supernatant (seminal plasma) was removed by aspiration. The sperm pellet was resuspended in 20 volumes of hypotonic (0.1 *M*) sodium phosphate buffer (pH 7.0), extracted initially for 2 h to remove the catalase, and then reextracted for 1 h with 0.5% Triton X-100 in 0.5 *M* sodium acetate (pH 5.0) to solubilize the peroxidase as previously described (12).

Peroxidase activity in the detergent extract was assayed spectrophotometrically at room temperature (24–26°C) using TMB as the oxidizable substrate (12, 22–24). For the assay, aliquots (0.4 mL) of sperm extract were added to 0.1 mL of TMB (1–6 mg·mL^{-1}) dissolved in DMSO and 0.5 mL of inhibitor (phenylhydrazine or benzohydroxamic acid) dissolved in 0.5 *M* sodium acetate (pH 5.0). The initial burst of peroxidase activity was monitored at 60 s by absorbance at 650 nm in a Beckman DB spectrophotometer. The reference side contained all reagents except for H_2O_2. The extinction coefficient of 5400 for oxidized TMB (25) was used to calculate the amount of substrate oxidized by the peroxidatic reaction. Each experiment was repeated three to four times with different preparations of sperm peroxidase. Each data point presented represents the mean from these trials (*n*).

The following biochemical reagents were used in this study: TMB, DMSO, and Triton X-100 purchased from Sigma Chemical Co. (St. Louis, MO); H_2O_2 and phenylhydrazine from Fisher Scientific Co. (Pittsburg, PA); and benzohydroxamic acid from Aldrich Chemical Co. (Milwaukee, WI).

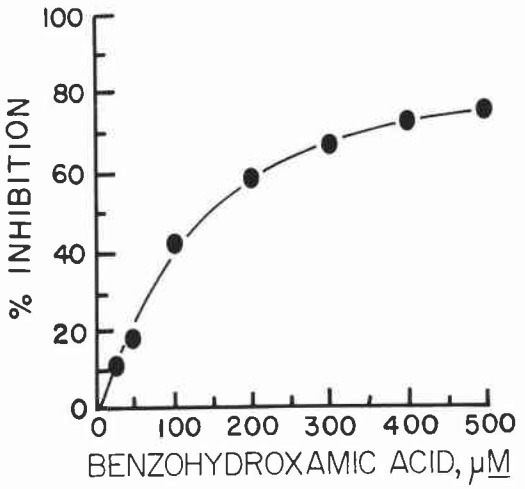

FIG. 1. Inhibition of TMB peroxidase activity in detergent extract of *Strongylocentrotus purpuratus* sperm by benzohydroxamic acid. $n = 3$.

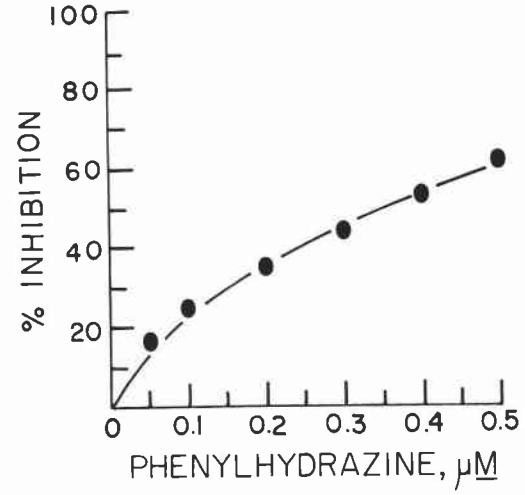

FIG. 2. Inhibition of TMB peroxidase activity in detergent extract of sperm by phenylhydrazine. $n = 4$.

Results

The TMB–peroxidase activity in the detergent extract of sperm is strongly inhibited by benzohydroxamic acid (Fig. 1) and phenylhydrazine (Fig. 2). The latter results confirm previous observations that the sperm peroxidase is sensitive to inhibition by phenylhydrazine (12).

The mode of inhibition by these substances was investigated by kinetic parameters (26). Analysis of the experimental data by Lineweaver–Burk plots shows that inhibition of the sperm peroxidase is competitive with both benzohydroxamic acid (Fig. 3) and phenylhydrazine (Fig. 4). Dixon plots of the kinetic data also show that these substances are competitive inhibitors of the sperm peroxidase (data not shown). The K_m for TMB is

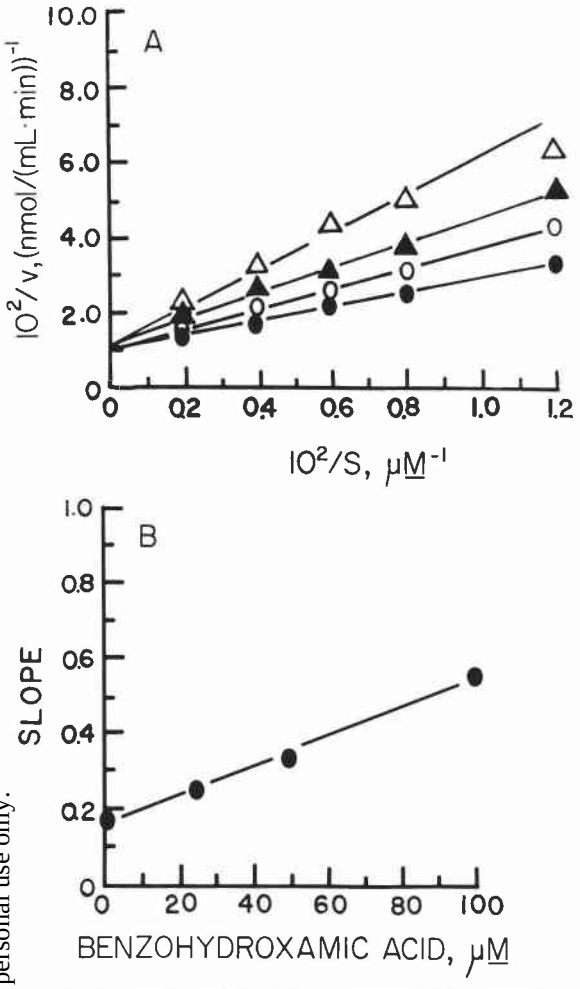

FIG. 3. Competitive inhibition of sperm TMB peroxidase activity by benzohydroxamic acid. $n = 3$. (A) Lineweaver–Burk plots of substrate (TMB) concentration against the rate of TMB oxidation, with and without benzohydroxamic acid (●, 0 μ*M*; ○, 25 μ*M*; ▲, 50 μ*M*; △, 100 μ*M*). (B) Slopes of curves obtained in Lineweaver–Burk plot versus inhibitor concentration for K_i determination. K_i ($-x$ axis intercept) is equivalent to the concentration of inhibitor that doubles the slope of the $1/v$ versus $1/S$ plot (26).

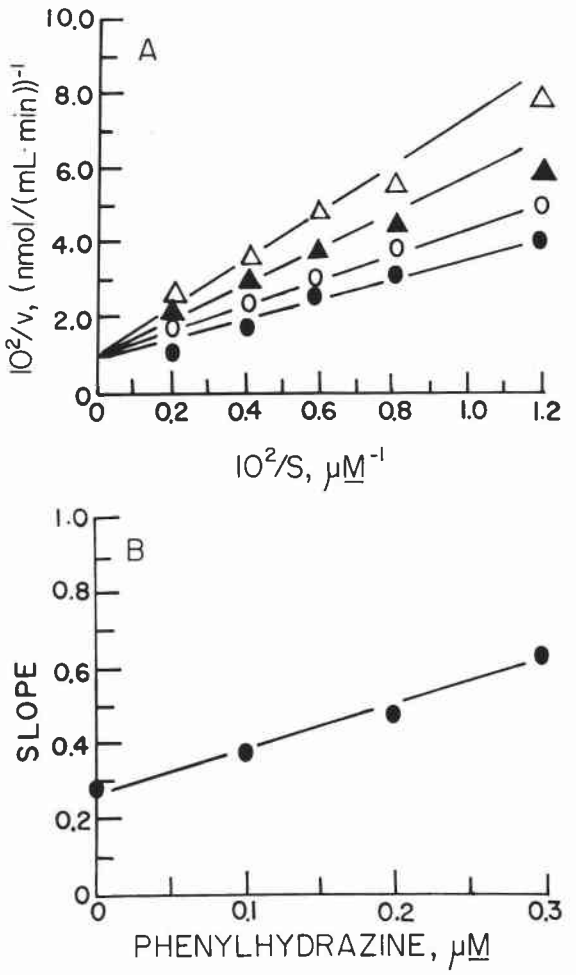

FIG. 4. Competitive inhibition of sperm TMB peroxidase activity by phenylhydrazine. $n = 4$. (A) Lineweaver–Burk plots of substrate concentration against the rate of TMB oxidation, with and without phenylhydrazine (●, 0 μ*M*; ○, 0.1 μ*M*; ▲, 0.2 μ*M*; △, 0.3 μ*M*). (B) Slopes of curves obtained in Lineweaver–Burk plot versus inhibitor concentration for K_i determination.

250 ± 51.1 μ*M* ($n = 8$). It was determined in these experiments by extrapolation of the kinetic data obtained in the absence of inhibitors (Figs. 3A and 4A) to the $-x$ axis intercept. Comparison of the K_i and ID_{50} values from these studies shows that phenylhydrazine is a more potent inhibitor of the sperm peroxidase than benzohydroxamic acid (Table 1). Furthermore, there is good agreement between the observed ID_{50} values for phenylhydrazine and benzohydroxamic acid and those calculated from the experimentally determined K_m for TMB and the K_i values for each inhibitor.

Discussion

The results described above show that benzohydroxamic acid and phenylhydrazine are competitive inhibitors of the sea urchin sperm peroxidase. Since these substances are known to be competitive inhibitors of peroxidases obtained from the somatic tissues of plants and animals (13–19), our findings suggest that the biochemical properties of the sperm peroxidase resembles those of peroxidases found in other cellular systems. A more precise analysis of the molecular mechanisms responsible for the observed inhibition of the sperm peroxidase by phenylhydrazine and benzohydroxamic acid will be possible after the enzyme has been purified. Benzohydroxamic acid is a useful affinity

TABLE 1. Summary of inhibitor kinetics for sea urchin (*S. purpuratus*) sperm peroxidase

Inhibitor	n	Inhibition mode	K_i, μM	ID_{50} μM^a	ID_{50} μM^b
Phenylhydrazine	3	Competitive	0.201±0.067	0.303±0.087	0.581±0.218
Benzohydroxamic acid	3	Competitive	51.2±1.3	146.7±16.1	186.9±28.6

[a]Inhibitor concentration that produced 50% inhibition of peroxidase activity in each experiment.
[b]Calculated from the equation (26) $ID_{50} = (1 + S/K_m)K_i$, where K_m and K_i have been determined experimentally in each trial. K_m for TMB in phenylhydrazine series is 272.9 ± 32.7 μM and benzohydroxamic acid series 193.7 ± 39.8 μM.

ligand to purify peroxidases by means of affinity chromatography (27). Our findings suggest that this methodological approach could be used to help purify the peroxidase from detergent extracts of sea urchin sperm.

The sperm peroxidase is believed to have a functional role in the prevention of polyspermy, by using H_2O_2 released by fertilized eggs during the cortical reaction to reduce the fertility of sperm in the vicinity (10, 12). The addition of phenylhydrazine (10) and benzohydroxamic acid (28) to fertilization cultures promotes polyspermy. These observations, together with the results of the present study, are consistent with our hypothesis. The mechanism by which the sperm peroxidase uses H_2O_2 to reduce sperm fertility is unknown at present. This process might involve the production of strong oxidants such as hypochlorous acid, formation of cytotoxic oxygen radicals, synthesis of toxic lipid peroxides, and (or) the formation of bioregulatory products (prostaglandins and leukotrienes) derived from the enzymatic oxidation of arachidonic acid (2, 8, 9, 29–32). Additional work is required to resolve this question.

All aerobic cells produce H_2O_2 and oxygen radicals as by-products of aerobic respiration. These metabolites are potentially highly toxic substances, and aerobic cells have evolved elaborate defense mechanisms to protect themselves from the damaging effects of oxygen radicals (reviewed in Refs. 29 and 30). However, there are situations where cells deliberately produce H_2O_2 for specific physiological purposes. For example, phagocytic cells such as leukocytes and macrophages in somatic tissues produce large amounts of H_2O_2 when they are stimulated by contact with invading microorganisms or tumor cells (30). The released H_2O_2 is used by myeloperoxidase that also is secreted by the activated phagocyte to kill the invading target cells (30). In this context, peroxidases, H_2O_2, and oxygen radicals can be viewed as biological defense weapons. The fertilized sea urchin egg also produces H_2O_2 to perform important physiological roles, this time in early development. One role is to promote hardening of the elevated fertilization envelope (5). This process depends, in part, upon the formation of dityrosine cross-links within the fertilization envelope, a reaction catalyzed by ovoperoxidase that is secreted by the egg's cortical granules (4, 5, 33–35). However, hardening of the fertilization envelope is not required for this investment to act as a mechanical barrier to polyspermy (2), but may protect the early embryo from predation and other environmental hazards (4). It was suggested that H_2O_2 and ovoperoxidase released by sea urchin eggs during fertilization might contribute to the prevention of polyspermy by killing sperm in a manner analogous to that used by phagocytes (secretion of myeloperoxidase and release of H_2O_2) to kill bacteria (30, 34). A peroxide-mediated block to polyspermy has been demonstrated in sea urchins (10, 11), but currently available evidence indicates that the peroxidase responsible for reducing sperm fertility is endogenous to the sperm (2, 10, 12, 28). It is possible that a similar polyspermy preventing mechanism might operate in mammals. Hydrogen peroxide and oxygen radicals are known to be extremely toxic to mammalian sperm (31, 36–38). Fertilized mammalian eggs utilize secreted ovoperoxidase to mediate cross-linking reactions to harden the zona pellucida (39), an extracellular coat comparable to the sea urchin egg's fertilization envelope (4, 5). In view of these factors, it is tempting to speculate that the fertilized mammalian egg might release H_2O_2 to reduce sperm fertility and also to harden the zona pellucida. Such functional cooperation by the interacting gametes increases the probability of normal monospermic fertilization, a situation with obvious selective advantage within the context of evolution.

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