Oviductin. Purification and Properties of the Oviductal Protease That Processes the Molecular Weight 43 000 Glycoprotein of the *Xenopus laevis* Egg Envelope†

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ABSTRACT: The *Xenopus laevis* egg envelope is modified during egg transit through the pars recta oviduct. The physicochemical properties and ultrastructure of the envelope change, and the M_r 43 000 envelope glycoprotein (gp43) is processed to M_r 41 000. We purified a gp43 processing protease from oviductal secretory granules and studied its effects on the egg envelope. The M_r 66 000 protease, designated oviductin, hydrolyzed the arginyl–X bond of N^{α}-*tert*-butoxycarbonylphenylalanylserylarginyl-7-methylcoumaryl-4-amide (K_m = 58 μM, k_{cat} = 3.80 s^{-1}). Diisopropyl fluorophosphate, EDTA, and EGTA inhibited oviductin irreversibly; soybean trypsin inhibitor, aprotinin, guanidine hydrochloride (K_i = 7.5 mM), and *p*-aminobenzamidine (K_i = 4.1 μM) also inhibited, but iodoacetamide, E-64, pepstatin, or 1,10-phenanthroline did not. The N-terminal amino acid sequence of oviductin was up to 64% identical to those of several serine proteases. Oviductin accounted for all of the gp43 processing activity we detected in secretory granules, and oviductin-catalyzed processing of gp43 rendered coelomic egg envelopes physically (as determined by thermal solubility) similar to those of oviposited eggs. We conclude (1) a unique serine protease secreted by the oviduct processes gp43 of the *Xenopus laevis* egg envelope, and (2) this processing causes physical changes in the egg envelope which occur during egg transit through the oviduct.

During animal fertilization, spermatozoa penetrate the glycoproteinaceous investment overlying the egg plasma membrane. In *Xenopus laevis* (South African clawed frog), four functionally distinct forms of this investment, or envelope, have been defined (Wolf et al., 1976; Grey et al., 1977; Gerton & Hedrick, 1986; Hedrick & Nishihara, 1991; Hedrick & Hardy, 1991). These envelope forms are (1) oocyte envelope (OE),[1] which surrounds oocytes in the ovary, (2) the coelomic egg envelope (CE), which surrounds ovulated eggs that have not entered the oviduct, (3) the vitelline envelope (VE), which surrounds oviposited eggs, and (4) the envelope surrounding fertilized eggs.

Eggs with CE are not fertilizable (Brun, 1974; Katagiri, 1987). During egg transit through the pars recta oviduct (first 1–2 cm in *Xenopus laevis*), the egg envelope acquires physicochemical and ultrastructural characteristics of the VE, and the eggs are rendered fertilizable (Grey et al., 1977; Hedrick & Nishihara, 1991; Katagiri, 1987). These changes in egg envelope properties correlate temporally with processing of the M_r 43 000 glycoprotein of the CE (gp43) to M_r 41 000 (gp41) (Gerton & Hedrick, 1986).

Purification of the gp43 processing enzyme is necessary to understand the relationship between gp43 processing, changes in egg envelope physical properties, and the acquisition of egg fertilizability. The amino acid compositions and C-terminal amino acids of gp43 and gp41 differ (Bakos et al., 1990a), suggesting that gp43 processing is proteolytic. Fluid recovered from surgically ligated pars recta oviduct contains several amidase activities and gp43 processing activity (Bakos et al., 1990b). In this paper, we describe purification of the processing enzyme from pars recta secretory granules, and characterization of its enzymatic properties. We find that the enzyme is a unique serine protease. By using the purified enzyme to process gp43 to gp41 in isolated CE and OE, we also find that this processing event changes the physical properties of CE and OE such that they solubilize with a temperature dependence similar to that of VE isolated from oviposited eggs.

Experimental Procedures

Materials. Female *Xenopus laevis* were purchased from Lombardo's Animal Imports (Glen Ridge, NJ) or from a colony maintained at the University of California, Berkeley. Gonadotropins were obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, or purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin (TPCK-treated), kallikrein, and clostripain were also purchased from Sigma Chemical Co. Peptidyl-7-methylcoumaryl-4-amides (peptidyl-NH-CouMe) were purchased from The Peptide Institute (Osaka, Japan), Peptides International (Louisville, KY), Peninsula (San Francisco, CA), and Chemalog (South Plainfield, NJ). Electrophoresis chemicals (ultra-pure or electrophoresis grade) were from Bio-Rad Laboratories (Richmond, CA) and Amresco (Solon, OH). All other chemicals were reagent grade.

Egg/Oocyte Envelope Isolation. Ovulation was induced by sequential injection with pregnant mare serum gonadotropin and human chorionic gonadotropin (Gerton & Hedrick, 1986; Hedrick & Nishihara, 1991; Hedrick & Hardy, 1991). Coelomic eggs were recovered, 10 h after induction of ovu-

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[1] Abbreviations: OE, oocyte envelope(s); CE, coelomic egg envelope(s); VE, vitelline envelope(s); gp43, M_r 43 000 envelope glycoprotein; gp41, M_r 41 000 envelope glycoprotein; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; TLCK, 1-chloro-3-(tosylamino)-7-aminoheptan-2-one; DFP, diisopropyl fluorophosphate; E-64, [[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NH-CouMe, 7-methylcoumaryl-4-amide; Boc, N^{α}-*tert*-butoxycarbonyl; Bz, benzoyl; Pyr, L-pyroglutamyl; Suc, succinyl.

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lation, from the peritoneal cavities of frogs with ligated oviducts (Grey et al., 1977; Gerton & Hedrick, 1986; Hedrick & Hardy, 1991). VE were isolated from oviposited eggs from which jelly layers had been removed with 2-mercaptoethanol (Gusseck & Hedrick, 1971; Hedrick & Hardy, 1991). Late-stage oocytes were obtained by chopping ovaries with a meat grinder and panning the chopped tissue to select for the larger cells (Hedrick & Hardy, 1991). Envelopes were isolated by sieving oocyte or egg lysates through nylon screens (Wolf et al., 1976; Hedrick & Hardy, 1991) and then washed with H_2O and stored frozen at −20 °C.

Enzyme Assays. Amidolysis of peptidyl-NH-CouMe was determined spectrofluorometrically at 25 °C (Zimmerman et al., 1977). To 380 μL of assay buffer (routinely, 25 mM Tris-HCl, pH 8.0, containing 200 mM NaCl) were added 10 μL of an 8 mM solution of substrate in dimethyl sulfoxide and 10 μL of enzyme solution. Excitation and emission wavelengths were 380 and 460 nm, respectively. Generally, oviductin samples (e.g., secretory granule contents and column fractions) were prepared for assay by diluting 10-fold at 0 °C in the equilibration buffer used for affinity chromatography.

Amidolysis by purified oviductin of peptidyl-NH-CouMe at various pHs was determined using the following buffers: pH 5.0, 50 mM sodium acetate; pH 5.5–6.5, 50 mM sodium 2-(*N*-morpholino)ethanesulfonate; pH 7.0–7.5, 50 mM sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; pH 8.0–8.5, 50 mM Tris-HCl; pH 9.0, 50 mM ethanolamine hydrochloride.

To assess gp43 processing, the course of the processing reaction was evaluated qualitatively by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE). Samples of CE and VE were run as standards to define the beginning and end of the processing reaction. Isolated CE in H_2O were centrifuged (1500*g*, 5 min, 25 °C), and the envelope pellet was suspended in 2 volumes of H_2O. The suspension was homogenized using a Potter–Elvehjem homogenizer until a pipettable slurry was obtained, and stored at 4 °C. For each time point, equal volumes (10–30 μL for one-dimensional gels, 50–100 μL for two-dimensional gels) of envelope slurry and enzyme solution (usually 5.0 mIU/mL in affinity column equilibration buffer) were mixed and incubated at 25 °C. At the desired times, the suspensions were centrifuged (10000*g*, 4 °C, 30 s), and the envelope pellet was dissolved at 100 °C in the appropriate sample buffer for analysis by one-dimensional or two-dimensional electrophoresis.

Electrophoresis. SDS–PAGE was as described (Laemmli, 1970). To remove salts which interfered with electrophoresis, oviductin samples were precipitated with trichloroacetic acid. Briefly, sufficient Triton X-100 [10% (v/v) in H_2O] was added to produce a 0.5% solution of the detergent, which serves as a carrier during precipitation. Trichloroacetic acid [100% (w/v), in H_2O] was then added to a final concentration of 10% (w/v), and after 20 min at 0 °C, the precipitate was sedimented (10000*g*, 5 min, 4 °C) by centrifugation. The pellets were washed twice by centrifugation (10000*g*, 5 min, 4 °C) with 90% (v/v) acetone and dissolved in sample buffer. Proteins dissolved in sample buffer were reduced when desired by addition of 2-mercaptoethanol to 5% (v/v). After electrophoresis, gels were stained with Coomassie brilliant blue R-250; when increased sensitivity was required, gels were subsequently silver-stained (Morrisey, 1980), with modifications (Hardy et al., 1987).

Two-dimensional gel electrophoresis of egg envelopes, consisting of isoelectric focusing in the first dimension and SDS–PAGE in the second dimension, was done as described previously (Gerton & Hedrick, 1986).

Protein Assay. Protein concentrations were measured with bicinchoninic acid (Smith et al., 1985), using bovine serum albumin as a standard.

Preparation of Secretory Granule Lysate. Secretory granules were isolated from excised pars recta by differential centrifugation (Takamune et al., 1986), except ultracentrifugation on Percoll was omitted as it did not substantially enrich the secretory granule preparation (determined by comparing electrophoresis patterns). The subcellular fraction sedimenting at 12100*g*, was suspended in 25 mM Tris-HCl, pH 8.0, containing 25 mM $CaCl_2$ (1 mL/10 frogs) and frozen and stored at −20 °C for up to 6 months. After being thawed, the lysed secretory granules were centrifuged at 150000*g* at 4 °C for 30 min, and the supernatant solution, designated secretory granule lysate, was either used immediately for enzyme purification or frozen at −20 °C until required.

Oviductin Purification. Secretory granule lysate from up to 140 frogs was applied at 0.05–0.15 mL/min to a 0.4 × 3 cm plastic column containing *p*-aminobenzamidine–Sepharose (Sigma Chemical Co.) equilibrated in 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 0.5 mg/mL poly(vinyl alcohol) (average molecular weight 10000; Sigma Chemical Co.) at 4 °C. The applied sample was washed through with equilibration buffer until the A_{280nm} of the effluent returned to base line. Bound proteins were eluted with 500 mM guanidine hydrochloride in 25 mM Tris-HCl, pH 8.0, and 0.5 mg/mL poly(vinyl alcohol); the flow rate was reduced to 0.05 mL/min, and 0.2-mL fractions were collected in plastic tubes. Fractions containing greater than 50% of the activity of the peak tube were pooled and kept at 0 °C for up to 3 h.

The activity peak pool from affinity chromatography (up to 1.0 mL) was applied to a 0.5 × 20 cm glass fast protein liquid chromatography column (HR 5/20; Pharmacia, Inc., Piscataway, NJ) packed with hydroxylapatite (high resolution, Calbiochem, La Jolla, CA). Flow rate (0.5 mL/min) and elution gradients were controlled with a Dionex metal-free high-performance liquid chromatograph, at 24 °C. The column was equilibrated in 10 mM sodium phosphate, pH 7.4, 100 mM guanidine hydrochloride, and 0.5 mg/mL poly(vinyl alcohol). The second solvent used for constructing the elution gradient was 500 mM sodium phosphate, pH 7.4, and 100 mM guanidine hydrochloride; sample elution was monitored continuously at 280 nm. Three minutes after the start of sample injection, the injected materials were eluted with a linear 10–108 mM gradient of sodium phosphate, pH 7.4, over 20 min, followed by a linear 108–206 mM gradient over the next 10 min. Fractions were collected in plastic tubes which were placed on ice upon completion of the elution gradient. Fractions containing greater than 50% of the activity of the peak tube were pooled and stored at −20 °C either frozen (for amino acid sequencing) or in liquid phase in 50% glycerol (for enzymology).

Kinetics. The K_m and k_{cat} for N^{α}-*tert*-butoxycarbonyl-phenylalanylserylarginyl-7-methylcoumaryl-4-amide (Boc-Phe-Ser-Arg-NH-CouMe) amidolysis were determined from Eadie–Hofstee plots and the K_i for *p*-aminobenzamidine and guanidine hydrochloride from Dixon plots (Segel, 1975) of the kinetic data.

Amino Acid Sequencing. The N-terminal amino acid sequence of oviductin was determined by Edman degradation in the gas phase using an Applied Biosystems 470A protein sequencer with an on-line Model 120 PTH analyzer.

Envelope Solubilization Experiments. Thermal solubilization of isolated egg envelopes was assessed by measuring

Table I: Effect of Injection with Pregnant Mare Serum Gonadotropin on the Yield of Boc-Phe-Ser-Arg-NH-CouMe Amidolytic Activity in Secretory Granule Lysate

no. of animals	gonadotropin (units/animal)	total protein (mg)	total act. (mIU)[a]	sp act. (mIU/mg)	normalized act. (mIU/animal)
60	35	10.5	803	76.5	13.4
106	0	15.9	488	30.7	4.6
140	35	12.4	1660	135	11.9

[a] 1 IU = 1 μmol of product formed per minute.

Table II: Relative Rates of Peptidyl-NH-CouMe Hydrolysis by Secretory Granule Lysate and by Purified Oviductin

substrate	relative rate (%) lysate	oviductin
Boc-Phe-Ser-Arg-NH-CouMe	100	100
Boc-Leu-Ser-Thr-Arg-NH-CouMe	14	14
Boc-Leu-Thr-Arg-NH-CouMe	10	12
Pro-Phe-Arg-NH-CouMe	8	9
Pyr-Gly-Arg-NH-CouMe	7	9
Boc-Ile-Glu-Gly-Arg-NH-CouMe	3	2
Boc-Val-Leu-Lys-NH-CouMe	3	2
Boc-Val-Pro-Arg-NH-CouMe	2	3
Boc-Leu-Gly-Arg-NH-CouMe	1	2
Suc-Ala-Ala-Pro-Phe-NH-CouMe	<1	<1
Suc-Ala-Pro-Ala-NH-CouMe	<1	<1
Bz-Arg-NH-CouMe	<1	<1
Leu-NH-CouMe	<1	<1

the intrinsic fluorescence of solubilized envelope glycoproteins (Bakos et al., 1990a). Particulate egg envelopes were suspended at 100 μg of envelope protein/800 μL of 20 mM sodium borate, pH 9.0. The suspension was heated for 5 min and centrifuged at 10000*g* and 4 °C for 1 min, and the fluorescence of the supernatant solution (290-nm excitation, 340-cm emission) was measured. The supernatant solution was recombined with the pellet was mixed, and the suspension was reheated (2–5 °C increment). The fluorescence of the supernatant solution was measured after each sequential heating.

Results

Initial Characterization of Secretory Granule Lysate. We first isolated secretory granules from oviducts of frogs not previously injected with gonadotropin, lysed the granules, and then used a panel of peptidyl-NH-CouMe substrates to survey amidase activities in the lysate. The substrate hydrolyzed at the greatest rate was Boc-Phe-Ser-Arg-NH-CouMe. The amount of Boc-Phe-Ser-Arg-NH-CouMe amidase in secretory granule lysate was increased nearly 3-fold when animals were injected with pregnant mare serum gonadotropin 4 days prior to secretory granule isolation (Table I). In all subsequent experiments, we used lysates of secretory granules isolated from gonadotropin-injected frogs.

Secretory granule lysate exhibited several amidase activities (Table II). The Boc-Phe-Ser-Arg-NH-CouMe amidolytic activity was more than 7-fold greater than those detected with 12 other peptidyl-NH-CouMe substrates. Amidolysis of Boc-Phe-Ser-Arg-NH-CouMe by secretory granule lysate was maximal at pH 8.0, and this activity decayed exponentially at 24 °C and pH 8.0 in the absence of added Ca^{2+} ($t_{1/2}$ = 23 min). The amidase activity was also relatively stable to rapid freezing in the presence of 20% glycerol (90% of the activity remained after each freeze–thaw cycle). A component(s) of the lysate readily processed gp43 of the CE to gp41, and the processing activity consistently coeluted with Boc-Phe-Ser-Arg-NH-CouMe amidolytic activity in gel filtration high-performance liquid chromatography on TSK2000, TSK3000, and Superose 12 columns (not shown).

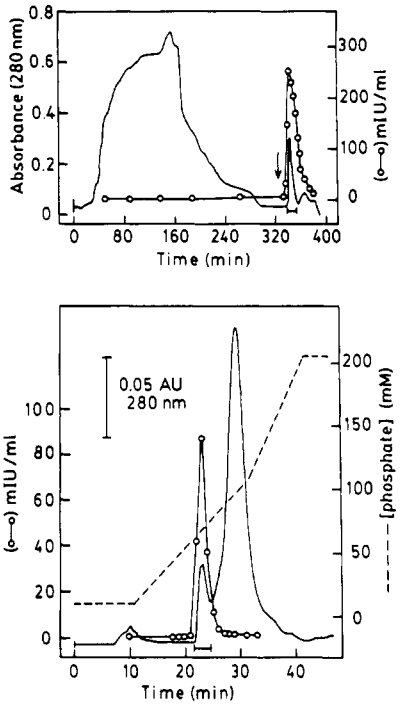

FIGURE 1: Purification of the gp43 processing enzyme as described under Experimental Procedures. Upper panel: affinity chromatography of secretory granule lysate on *p*-aminobenzamidine–Sepharose. The downward arrow marks the start of elution with 500 mM guanidine hydrochloride. Peak activity fractions (horizontal bar) were pooled for subsequent hydroxylapatite chromatography. Lower panel: hydroxylapatite high-performance liquid chromatography in 100 mM guanidine hydrochloride. Peak activity fractions (horizontal bar) were pooled for subsequent characterization of the purified enzyme.

Purification of the Processing Enzyme. On the basis of the observations described above, especially the strict correlation between gp43 processing the Boc-Phe-Ser-Arg-NH-CouMe amidolysis, we purified the Boc-Phe-Ser-Arg-NH-CouMe amidase from secretory granule lysate. The purification is illustrated in Figure 1. The enzyme bound to a *p*-aminobenzamidine–Sepharose affinity column and was specifically eluted with 500 mM guanidine hydrochloride (500 mM NaCl was ineffective). Further purification by high-performance liquid chromatography on hydroxylapatite produced a fraction highly enriched in amidase. The purification is summarized in Table III.

The purified enzyme migrated with M_r 66000 in SDS–PAGE under both nonreducing conditions (Figure 2) and reducing conditions (not shown). A minor M_r 100000 contaminant was present in the purified enzyme preparation; the amount of this contaminant varied with the preparation and did not correlate with the amount of Boc-Phe-Ser-Arg-NH-CouMe amidolytic activity present.

Table III: Summary of Purification

purification step	volume (mL)	total protein (μg)	total act. (mIU)	recovery (%)	sp act. (mIU/mg)	purification (*x*-fold)
secretory granule lysate	5.5	10500	803	100	76.5	1.0
p-aminobenzamidine–Sepharose	0.87	560	187	23	334	4.4
hydroxylapatite	1.5	70	177	22	2530	33

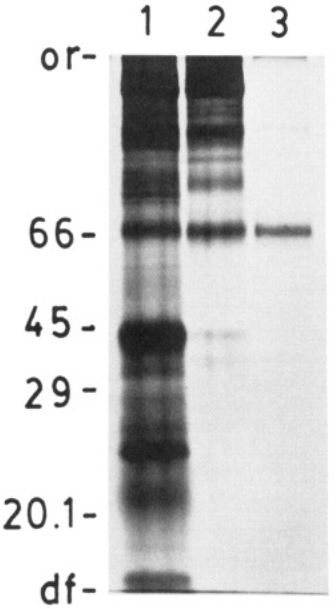

FIGURE 2: SDS–PAGE (10% gel, silver-stained, samples not reduced) of activity peak pools from enzyme purification. 760 μIU of amidase activity was applied to each gel lane. Lane 1, 9.5 μg of secretory granule lysate; lane 2, 2.3 μg of amidase pool after affinity chromatography; lane 3, 0.31 μg of amidase pool from hydroxylapatite chromatography.

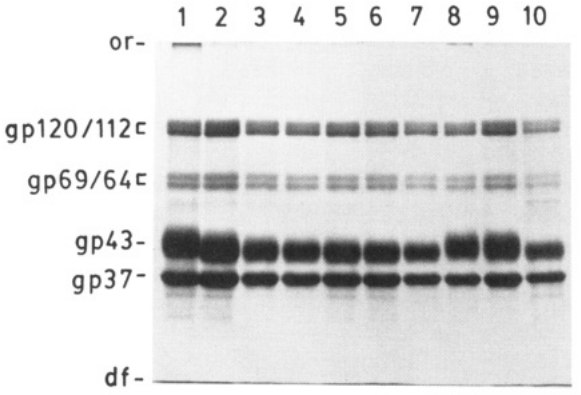

FIGURE 3: Processing of gp43 to gp41 by purified amidase. On analysis by one-dimensional electrophoresis, the processing is characterized by an increase in the mobility and a decrease in the apparent size heterogeneity of the gp43 band. Lanes 1–7, 30 μg of particulate CE incubated in 2.8 mIU/mL enzyme (37 μIU, 15 ng total) for 0, 10, 20, 30, 40, 50, and 60 min, respectively. Lane 8, 30 μg of CE incubated 60 min in buffer only (no enzyme). Lane 9, same as lane 7 with 1 mM *p*-aminobenzamidine added during enzyme treatment. Lane 10, 20 μg of VE. Envelopes were treated with enzyme, prepared for electrophoresis, and separated by electrophoresis on an 8.75% polyacrylamide gel as described under Experimental Procedures.

The purified amidase, designated oviductin, readily processed gp43 of CE to gp41 (Figure 3). Throughout the purification, only fractions containing oviductin exhibited gp43 processing activity. To test at higher resolution whether gp43 processing catalyzed in vitro by oviductin mimicked the in vivo processing reaction, we compared egg envelope preparations by two-dimensional gel electrophoresis. When analyzed by this method, the egg envelope glycoproteins exhibit isoelectric heterogeneity (Gerton & Hedrick, 1986). Clear differences in the isoelectric points of gp43 and gp41 spots provide a convenient means of assessing gp43 processing: the gp43 spots are converted into relatively more acidic and slightly more mobile gp41 spots (Gerton & Hedrick, 1986). The two-dimensional gel electrophoretic pattern of gp41 produced by incubating CE with purified oviductin was identical to that

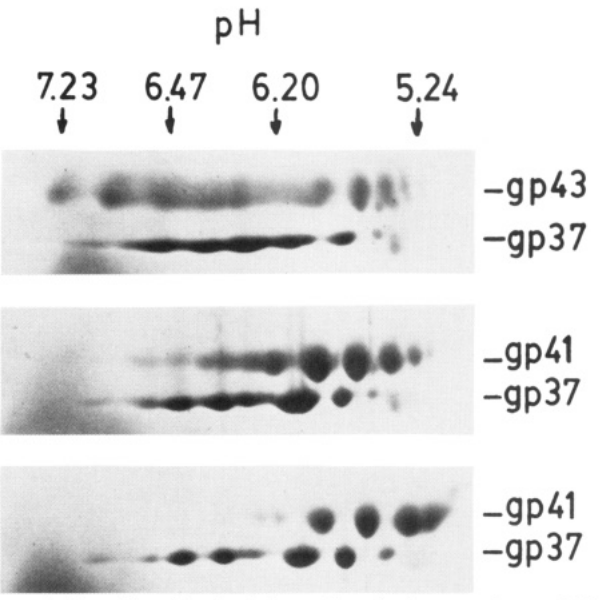

FIGURE 4: Two-dimensional gel electrophoresis of egg envelopes. Only the gp37/gp43 regions of the gels are shown. Upper gel: 200 μg of CE incubated in buffer only. Middle gel: 200 μg of CE incubated with 3.3 mIU/mL (0.5 mIU total) oviductin. Lower gel: 200 μg of VE incubated in buffer only. Incubations were for 2 h at 25 °C as described under Experimental Procedures. Note that three gp43 spots with p*I* between 6.47 and 7.23 are absent in the middle (CE + oviductin) and lower (VE) gels. In addition, the relative intensities of spots with p*I* between 5.24 and 6.20 are increased in these gels in comparison with those in the upper (untreated CE) gel.

of gp41 in isolated VE (Figure 4). The two-dimensional gel electrophoretic patterns of the other CE glycoproteins were unaffected by the oviductin treatment (not shown).

Characterization of Purified Oviductin. (*A*) *Catalytic Properties.* The substrate specificity of the purified enzyme is summarized in Table II. The amidase preferentially hydrolyzed Boc-Phe-Ser-Arg-NH-CouMe. The relative rates at which the enzyme hydrolyzed other peptidyl-NH-CouMe substrates were similar to those exhibited by crude secretory granule lysate. Thus, oviductin appeared to account for all of the amidase activities detected in secretory granule lysate under the conditions of our assay.

Oviductin hydrolyzed Boc-Phe-Ser-Arg-NH-CouMe maximally at pH 8.0. The shape of the titration curve (activity vs pH) was characteristic of a serine protease; activity was greater than 90% of maximal from pH 7.5 to 8.5, with a sharp decline in activity (to less than 30% of maximal) below pH 6.5. Similarly shaped titrations were observed whether or not the assay buffer contained added NaCl (200 mM), but the maximal activity was higher at the higher ionic strength. Maximal activities at pH 8.0 in 200–400 mM solutions of monovalent salts were 200 ± 10% of that in the absence of added salt, and the concentration dependence of the stimulation was similar for four of five salts tested (Figure 5). The enzyme was also mildly stimulated by Ca^{2+} (38% at 10 mM, 11% at 100 mM) under conditions wherein the stimulation by ionic strength was already maximal (200 mM NaCl). The K_m of the enzyme for Boc-Phe-Ser-Arg-NH-CouMe amidolysis was 58 μM with a k_{cat} of 3.80 s^{-1}.

(*B*) *Inhibition.* Inhibition of oviductin is summarized in Table IV. Aprotinin, soybean trypsin inhibitor, EDTA, and EGTA were the most effective inhibitors tested. Inhibition by EGTA was irreversible, since enzyme activity was measured in the presence of 25 mM Ca^{2+}. Guanidine hydrochloride and *p*-aminobenzamidine competitively inhibited with *Ki* of 7.5 mM and 4.1 μM, respectively. The kinetics of the enzyme's

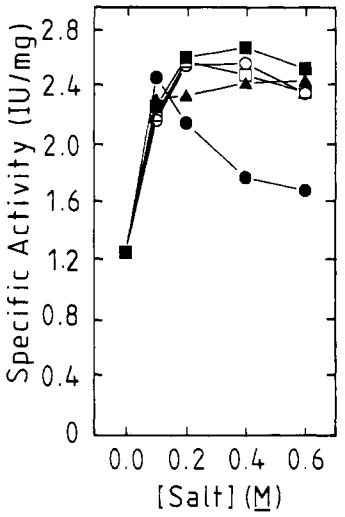

FIGURE 5: Effects of monovalent salt concentration on amidolytic activity of oviductin at pH 8.0. (○) Na Cl; (■) NaF; (▲) LiCl; (□) KCl; (●) $NaNO_3$.

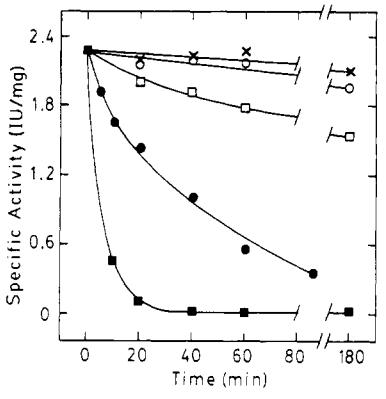

FIGURE 6: Inhibition of oviductin by DFP and TLCK. The enzyme was incubated at 24 °C with 50 μM DFP (●), 500 μM DFP (■), 500 μM TLCK (□), or 500 μM TPCK (×) or with no added inhibitor (○) for the indicated times and assayed.

Table IV: Inhibition of Oviductin

inhibitor	% inhibn[a]	inhibitor	% inhibn[a]
TLCK	6	leupeptin	55
TPCK	4	antipain	35
DFP	37	pepstatin	0
iodoacetamide	0	E-64	0
EDTA	90	soybean trypsin inhibitor	84
EGTA	94	aprotinin	97
1,10-phenanthroline	7		

[a] Oviductin was incubated for 20 min at 24 °C with inhibitors and then assayed (Boc-Phe-Ser-Arg-NH-CouMe amidolysis) in 25 mM Tris-HCl, pH 8.0, containing 200 mM NaCl and 25 mM $CaCl_2$. The concentration of soybean trypsin inhibitor was 2.5 μM; all other inhibitor concentrations were 50 μM.

inhibition by tosyllysine chloromethyl ketone (TLCK) and diisopropyl fluorophosphate (DFP) are shown in Figure 6. Even at 500 μM, TLCK did not inhibit significantly, while DFP rapidly and irreversibly inhibited the enzyme.

(C) N-Terminal Amino Acid Sequence. Inhibition by DFP suggested that oviductin is a serine protease but this observation appeared to be contradicted by the lack of inhibition by TLCK. To reconcile these apparently conflicting data, the N-terminal amino acid sequence of the enzyme was determined. Two different oviductin preparations were sequenced, with identical results. The yield in the first sequencing cycle was greater than 90% (molar basis) of the amount of enzyme sequenced. The sequence of the enzyme's N-terminal 28 amino acids is shown (Figure 7) aligned with the N-terminal

	5 10 15 20 25 30	Positional Identity
Oviductin (*X. laevis*)	IVGGR ESKKG QHPWT VSLKR -NG-- KHFCG G	
Trypsin (spiny dogfish)	IVGGY ECPKH AAPWT VSLN- -VG-- YHFCG G	64%
Plasmin (bovine)	IVGGC VSKPH SWPWQ VSLRR -SS-- RHFCG G	61%
Chymotrypsin A (bovine)	IVNGE EAVPG SWPWQ VSLQD KTG-- FHFCG G	55%
Trypsin (bovine)	IVGGY TCGAN TVPYQ VSLN- -SG-- YHFCG G	50%
Factor IX (human)	VVGGE DAKPG QFPWQ VVLNG -KV-- DAFCG G	50%
Kallikrein (human)	IVGGT NSSWG EWPWQ VSLQV KLTAQ RHLCG G	48%
Invariable	G P C G	100%

FIGURE 7: N-Terminal amino acid sequence of oviductin aligned with those of several serine proteases. Positional identities were calculated from optimal pairwise alignments with the oviductin sequence. For ease in comparing multiple sequences in this figure, additional gaps were introduced. References: spiny dogfish trypsin (Titani et al., 1975), plasmin (Schaller et al., 1985), chymotrypsin A (Braun & Hartley, 1966), bovine trypsin (Mikes et al., 1966), factor IX (Yoshitake et al., 1985), plasma kallikrein (Chung et al., 1986), invariant residues (Young et al., 1978).

sequences of several serine proteases. The sequences are clearly similar, with positional identities ranging to greater than 60% for the comparisons with spiny dogfish trypsin and bovine plasmin. In addition, all of the positions invariably conserved in serine proteases are conserved in oviductin.

Proteolysis of gp43 by Other Proteases. Since oviductin preferentially hydrolyzed Boc-Phe-Ser-Arg-NH-CouMe, we tested whether the ability of other proteases to hydrolyze this substrate correlated with the ability to proteolyze gp43. Five proteases (including oviductin) were tested for both Boc-Phe-Ser-Arg-NH-CouMe amidolytic and gp43 processing activity. The specific activities (Boc-Phe-Ser-Arg-NH-CouMe amidolysis) of trypsin, acrosin, and clostripain ranged from 10-fold to 67-fold higher than that of oviductin. Conversely, the specific activity of kallikrein with this substrate was 177-fold lower than that of oviductin. At 2.5 mIU/mL (Boc-Phe-Ser-Arg-NH-CouMe amidolysis), oviductin readily processed gp43 to gp41, as expected; the reaction appeared to be complete by 30 min. At 2.5 mIU/mL (Boc-Phe-Ser-Arg-NH-CouMe amidolysis), trypsin and clostripain also proteolyzed gp43, and the processing catalyzed by these proteases was qualitatively indistinguishable from that catalyzed by oviductin. Acrosin possessed no detectable gp43 processing activity, even after incubation for 100 min. At 2.5 mIU/mL (Pro-Phe-Arg-NH-CouMe amidolysis, the preferred substrate for this enzyme), kallikrein also failed to proteolyze gp43. None of the proteases altered the electrophoretic properties of the other CE glycoproteins under the conditions we used.

Effects on Egg Envelope Solubility. The effect of gp43 processing on the thermal solubilization of CE and OE is shown in Figure 8. Isolated OE and CE were incubated with oviductin under conditions which supported complete conversion of gp43 to gp41. These envelopes dissolved with the same temperature dependence as did the high-temperature component of a biphasic VE solubilization profile (see Figure 8 legend).

Discussion

We purified the oviductal enzyme which processes gp43 of the *Xenopus laevis* coelomic envelope. Starting material for the purification was a lysate of secretory granules isolated from the pars recta oviduct of gonadotropin-injected frogs. The enzyme, designated oviductin, accounted for all of the measured peptidyl-NH-CouMe amidolytic activity in the lysate. We conclude that the gp43 processing enzyme is a unique M_r 66 000 serine protease, based both on the catalytic properties and on the N-terminal amino acid sequence of the purified enzyme.

Oviductin was inactivated irreversibly upon removal of Ca^{2+} and required the presence of 200 mM salt for maximal am-

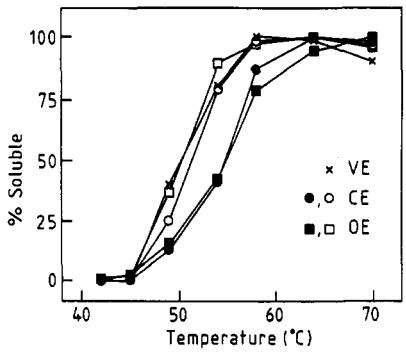

FIGURE 8: Thermal solubilization of egg envelopes. Solubilization of envelopes was determined fluorometrically as described under Experimental Procedures, and is expressed as a percentage of the total amount of envelope glycoprotein solubilized. Solubilization of the VE was biphasic, with 75% of the envelope solubilized below 42 °C. The second component (the fraction which solubilized above 42 °C) of this biphasic solubilization curve was normalized over the range of zero to 100% to simplify comparison with the results obtained for CE and OE. (×) High-temperature (greater than 42 °C) component of VE solubilization; (●) CE; (○) CE incubated 100 min with 2.5 mIU/mL oviductin; (■) OE; (□) OE incubated 100 min with 2.5 mIU/mL oviductin.

idolysis of Boc-Phe-Ser-Arg-NH-CouMe. The latter was an ionic strength requirement and not a specific ion requirement, since different salts produced similar results. These enzymatic properties may be functionally relevant, since the egg jelly layers, which are applied over the egg envelope after the egg has passed through the pars recta oviduct (Katagiri, 1987), chelate divalent metal ions (Ishihara et al., 1984), and anuran eggs are usually spawned into a hypotonic environment.

Oviductin hydrolyzed arginyl–X and lysyl–X bonds of peptidyl-NH-CouMe substrates. Six of nine substrates with arginyl–X bonds were hydrolyzed at a greater rate than was the lysyl–X bond of Boc-Val-Leu-Lys-NH-CouMe, suggesting that the enzyme may discriminate between arginyl–X and lysyl–X bonds. In addition, since oviductin hydrolyzed the arginyl–X bond of Boc-Phe-Ser-Arg-NH-CouMe more than 7-fold faster than the arginyl–X bonds of eight other substrates, the enzyme exhibits significant specificity for amino acids two or more residues to the amino side of the scissile bond. These observations may be a reflection of oviductin's function as a specific processing enzyme. Oviductin's substrate specificity could explain its insignificant inactivation by TLCK, an irreversible inhibitor of trypsin-like serine proteases (Shaw, 1967).

Although oviductin preferentially hydrolyzed Boc-Phe-Ser-Arg-NH-CouMe, its specific activity against this substrate was lower than those of trypsin, clostripain, and acrosin. Despite acrosin's superior amidolysis of Boc-Phe-Ser-Arg-NH-CouMe, it did not hydrolyze gp43. Thus, it seems that Boc-Phe-Ser-Arg-NH-CouMe amidolytic activity may be necessary, but is not sufficient, for a protease to exhibit gp43 processing activity. These results indicate that the fluorogenic substrate specificity of oviductin cannot be used to infer specific properties of the oviductin processing site in gp43, other than to suggest that an arginyl–X is the scissile bond. Rather, it seems probable that oviductin is specific for a unique processing site within gp43 and that Boc-Phe-Ser-Arg-NH-CouMe is simply the best among several fluorogenic substrates which poorly mimic this site. We do not know whether trypsin and clostripain hydrolyzed gp43 precisely at the oviductin processing site. It will be necessary to determine the primary structure of gp43 before this question can be answered.

The Boc-Phe-Ser-Arg-NH-CouMe amidolytic activity of secretory granule lysate was increased nearly 3-fold when pregnant mare serum gonadotropin was injected prior to secretory granule isolation. Since oviductin appears to be the only amidase detected in the lysate under our assay conditions, this increase in amidolytic activity represents either an increase in the amount of oviductin in secretory granules or an enhancement of the activity of oviductin already in the granules. We cannot yet distinguish between these possibilities, although the latter seems unlikely. In *Bufo arenarum*, pars recta extract prepared from animals previously injected with pituitary gland renders coelomic eggs fertilizable, but extract from uninjected animals does not (Miceli et al., 1978). Similar injection increases the number of secretory granules in *Bufo japonicus* pars recta (Katagiri et al., 1982). Our results are consistent with these observations and suggest that oviductin synthesis is controlled by pituitary hormone. This hypothesis requires further study.

The thermal solubilities of the CE and VE differ (Bakos et al., 1990a). Since prior incubation with oviductin caused CE and OE to solubilize with the same temperature dependence as a subset of VE, gp43 processing by oviductin is part of the mechanism subserving a change in envelope thermal solubility which occurs during egg transit through the oviduct. One can envision a two-step process wherein gp43 processing produces a modest change in envelope thermal solubility, followed by a second event which further alters the envelope. This second event, though unknown, could be as simple as loss of bound metal ions, since ionic conditions can profoundly alter the thermal solubility of VE (Bakos et al., 1990a).

In electron microscopic views, CE appear to be comprised of discrete bundles of fibrils, or fascicles (Grey et al., 1977; Hedrick & Nishihara, 1991). In the VE, the fibrils are partially dispersed; consequently, the VE appears more granular (Grey et al., 1977; Hedrick & Nishihara, 1991). In preliminary experiments (D. M. Hardy, C. A. Larabell, and J. L. Hedrick, unpublished results), we incubated coelomic eggs with purified oviductin under conditions which supported processing of gp43 to gp41. The fibrils comprising the envelopes of these eggs were clearly dispersed in comparison to those of coelomic eggs incubated with both oviductin and 1 mM *p*-aminobenzamidine. The observed changes were not dependent on factors released from the egg, since we obtained similar results using isolated envelopes. We are presently unable to determine whether processing of gp43 by oviductin accounts for *all* of the ultrastructural changes which occur during egg transit through the pars recta oviduct. This question will be the subject of future study.

Oviductal processing of the egg envelope occurs in other anurans (Katagiri, 1987); processing proteases have been partially purified from *Bufo japonicus* (Takamune & Katagiri, 1987) and *Bufo arenarum* oviduct (Miceli et al., 1978). A single M_r 66 000 component of a preparation of the *Bufo japonicus* processing enzyme was covalently labeled with [^{3}H]DFP (Takamune & Katagiri, 1987), and a M_r 68 000 component of fluid secreted by the *Xenopus laevis* pars recta can be similarly labeled (Bakos et al., 1990b). It seems likely that the enzyme labeled in these studies was oviductin.

These initial studies with purified oviductin have demonstrated that gp43 hydrolysis by this enzyme produces changes in the physical properties of the egg envelope which are similar to those which occur during transit of the egg through the oviduct. The results are consistent with the hypothesis that intermolecular associations between gp43 and the other envelope glycoproteins become altered when oviductin processes gp43 to gp41. These altered molecular associations may then be manifest in the different envelope thermal solubilities and

ultrastructures. With the availability of purified oviductin, it will be possible to study (1) the relationship between oviductin-induced changes in envelope properties and the acquisition of egg fertilizability during oviductal transit and (2) the molecular basis of changes in envelope structure and function effected by gp43 processing.

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