A Cysteine Protease That Processes Insect Vitellin

PURIFICATION AND PARTIAL CHARACTERIZATION OF THE ENZYME AND THE PROENZYME

(Received for publication, April 12, 1996, and in revised form, October 14, 1996)

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A cysteine protease that initiates degradation of vitellin (Vt) in the orthopteran Blattella germanica, and its proprotease precursor, was purified from yolk and partially characterized. The protease, purified 300-fold, contains three peptides of M, 27,000, 29,000, and 31,000. A comparison of the purified enzyme’s action pattern on Vt in vivo and in vitro confirmed its role in Vt processing. Protease-deficient yolk (day 0 postovulation) contained peptides of M, 35,500, 37,000, 39,000, and 41,000, which were absent from yolk with protease activity. These were replaced by three peptides of approximately M, 29,000, at days 2–3, the same time in development that protease expression and acidification of yolk granules occur (Nordin, J. H., Beaudoin, E. L., and Liu, X. (1991) Arch. Insect Biochem. Physiol. 18, 177–192). Acidification of purified proprotease converted it to three peptides of approximately M, 29,000 with cysteine protease activity. This conversion also required participation of a cysteine protease. Activated proprotease had the same pH activity profile, susceptibility to inhibitors, and cathepsin classification (L) as the protease. These results indicate that the Vt-processing protease is derived from a proprotease, which is activated in vivo by a developmentally regulated decrease in intragranular pH.

Vitellins, Vt, are multisubunit phosphoglycolipoproteins, which serve as a primary nutritive source for development of embryos of most egg-laying animals (1). They are endocytosed by the oocyte and stored in membranous organelles, called yolk granules or yolk platelets, until utilized (2, 3). This delay in degradation, which occurs in germ cells, is clearly different from the temporal character of the event in somatic cells. In the latter, endocytosed ligands are degraded rather quickly in the lysosome (4).

Yolk granules contain proteases that degrade their constituent proteins (5–10). Cysteine (6, 7, 11, 12) aspartyl (10), and serine (13, 14) proteases have been identified. However, the mechanisms regulating yolk protease activation and how the timing of activation is coordinated with embryo development are important questions that remain to be answered.

Previous work demonstrated that Vt utilization in Blattella germanica eggs is initiated at days 3–4 postovulation by proteolytic processing to a distinct set of peptides, which are then accessed by the embryo during development (9, 15, 16). Similar processing events are characteristic of Vt utilization in other insect species also (see Ref. 9, and references therein).

Assays of B. germanica yolk with synthetic substrates revealed that protease activity, first detectable at days 2–3 postovulation, increased in specific activity through day 5, suggesting it could be important in Vt processing (9). Greater than 90% of this proteolytic activity was inhibitable by cysteine protease inhibitors (9), suggesting that this class of proteases play a major role in the processing event. These assays identified both cathepsin L- and B-like activities (17), the former constituting more than 85% of the total. The finding that the yolk of certain B. germanica translocation heterozygotes with defective embry development and Vt processing lack yolk protease activity also implicates them (9, 18).

Yolk granules prepared from eggs 4–6 days postovulation accumulate high concentrations of the dye acridine orange, a probe for vesicle acidity, while those from eggs at days 0–3 do not (19, 20). The acidification was determined to be the result of proton translocation, suggesting that a vacuolar ATPase was involved (20). The temporal association of Vt processing with granule acidification suggested that acidification was part of the mechanism controlling protease activation. Other experiments showed that when the yolk of freshly ovulated eggs (which contains no detectable protease activity) was acidified in vitro, Vt processing occurred (20). These facts led us to speculate that the B. germanica Vt-processing protease is stored either as an acid-activated mature enzyme or, more likely, as a proprotein that is converted to a mature, catalytically active form; in either case, the population of catalytically active molecules would be controlled in vivo by granule acidification.

As part of the overall project aimed at understanding how the Vt-processing event is controlled, the major B. germanica yolk (Vt processing) protease was purified and partially characterized. The storage (precursor) form of this enzyme was also identified, purified, and its activated form compared with the processing protease. Proprotease activation was also examined in the context of possible regulation in vivo, by acidification. A preliminary report of some of this work has been published (17).
**EXPERIMENTAL PROCEDURES**

**Materials**—Special chemicals and reagents and their suppliers were as follows. Z-Phe-Arg-NMec, goat anti-rabbit antibody-HRP conjugates, rabbit anti-benzyloxycarbonyl-phenylalanyl-phenylhydrazine, ovalbumin, Founce 5, streptavidin-HRP, the M200 Q HR/5.5 FPLC column, and activated Sephacryl 4B (6-aminohexanoic acid N-hydroxysuccinimide ester-Sepharose conjugate) were from Sigma. The Pro-Blue staining kit was from Integrated Separations (Natick, MA). Extract-Gel D detergent removing gel was from Pierce, Z-Arc-Arg-NMec and Z-Gly-Phe glacial semi-carbazone were from Bachem Bioscience Inc. (King of Prussia, PA). Biotinyl-Phe-Ala-CHN₂, Z-Phe-Ala-CHN₂, and Z-Phe-Tyr(CH₃)₂ were from Biosyn Ltd. (Belfast, Northern Ireland). Endo H, Pefabloc SC, and leupeptin (N-acetyl-Leu-Leu-Arg-al) were from Boehringer Mannheim. The GlycoTrack™ detection kit for glycoproteins was from Oxford GlycoSystems Inc. (Rosedale, NY). Renaissance chemiluminescence detection kit was from DuPont NEN. Microcon and Centriprep tubes and YM-10 membranes were from Amicon Inc. (Beverly, MA). Precoated TLC sheets (silica gel 60 F254, thickness 0.2 mm) were from EM Sciences (Gibbstown, NJ). Palladium black was from Aldrich.

**Insects and Tissue Preparations**—B. germanica, from Carolina Biological Supply Co. (Burlington, NC), were reared in the laboratory at 30 °C (21). Oothecae (egg cases) were collected daily. Day zero postovulation is the first 24 h following oothecal extrusion. Manduca sexta larval muscle extract was a kind gift from Dr. J. Lawrence (Department of Biology, University of Massachusetts, Amherst). Protein concentration measurements were conducted (9) using two broad-spectrum substrates: Z-LNE and azoalbumin. Z-Phe-Arg-NMec (hydrolyzed preferentially by cathepsins L, but slowly by cathepsin B) and Z-Arc-Arg-NMec (hydrolyzed by cathepsin B only, Ref. 23) were used. With Z-LNE, 1 unit of enzymatic activity caused an increase in absorbance of 1.0/min at 326 nm; and with azoalbumin, an increase in absorbance of 1.0/h at 366 nm. With AMC, 1 pmol of AMC/s from Z-Phe-Arg-NMec or Z-Arc-Arg-NMec (determined by a standard curve). AMC formation was measured at 20±s intervals by fluorescence spectrophotometry at 370 nm (excitation) and 460 nm (emission).

**Synthesis of Sepharose Gly-Phe-Glycinal Semicarbazone**—A modification of the procedure of Rich et al. (24) was employed to produce venom temperature. Z-Gly-Phe-glycinial semicarbazone (48 mg) was dissolved in 1 mM HCl at 4°C and then washed in a column (0.8 cm diameter; bed volume 3 ml) with 60 ml of 0.1 M NaHCO₃, pH 8.0. Gly-Phe-glucinal semicarbazone (56 mg), in 5 ml of methanol, was diluted with 3 ml of 0.1 M NaHCO₃, pH 8.0. The mixture was combined with the activated Sepharose in a small tube and incubated overnight on a nutator at 20°C, transferred back to the column, and washed with 100 ml of 50% aqueous methanol and 100 ml of water. It was agitated again for 4 h in 10 ml of 0.13 M ethanolamine buffer, pH 9.0 at 20°C and then washed with 100 ml of water containing 0.1% azide, prior to storage at 4°C.

**Preparation of Yolk Extracts**—Seventy oothecae (approximately 2.1 g; day 6 for protease, day 0 for proteogelase) stored up to 3 weeks at −20 °C, were thawed in a 12-ml centrifuge tube, suspended in 6.2 ml of ice-cold CBS (10 mM citrate buffer, pH 5.5, containing 0.15 M NaCl and 1 mM EDTA; protease) or HBS (10 mM Hepes, pH 7.5, containing 0.15 M NaCl and 1 mM EDTA; proteogelase). Buffers at neutral pH were required for proteogelase purification to preclude protease activation. Oothecae were squashed against the tube’s wall with a spatula and then incubated on a rotator at 20°C, transferred back to the column, and washed with 100 ml of 50% aqueous methanol and 100 ml of water. It was agitated again for 4 h in 10 ml of 0.13 M ethanolamine buffer, pH 9.0 at 20°C and then washed with 100 ml of water containing 0.1% azide, prior to storage at 4°C.

**Protease Assays**—Protease assays were conducted as described (9) using two broad-spectrum substrates: Z-LNE and azoalbumin. Z-Phe-Arg-NMec (hydrolyzed preferentially by cathepsins L, but slowly by cathepsin B) and Z-Arc-Arg-NMec (hydrolyzed by cathepsin B only, Ref. 23) were used. With Z-LNE, 1 unit of enzymatic activity caused an increase in absorbance of 1.0/min at 326 nm; and with azoalbumin, an increase in absorbance of 1.0/h at 366 nm. With AMC, 1 pmol of AMC/s from Z-Phe-Arg-NMec or Z-Arc-Arg-NMec (determined by a standard curve). AMC formation was measured at 20±s intervals by fluorescence spectrophotometry at 370 nm (excitation) and 460 nm (emission).

**Stability of protease in 1% SDS at pH 7.5** permitted preparative SDS-PAGE (Bio-Rad Prep Cell, model 491) to be used as an alternative last step. The separation gel (1×2.8 cm diameter) was composed of 5% acrylamide in 10 mM sodium phosphate buffer pH 7.5, containing 2.65% cross-linker. The sample buffer was 6.7 mM phosphate, pH 7.5, containing 0.05% Brij 35 and 5 μM E-64, and 0.02 μg of protein was assayed in 1 ml of each buffer containing 0.05% Brij 35 and 5 μM Z-Phe-Arg-NMec. Activated protease (0.25 μg of protein) was assayed in the same manner.

**Protease Assay—**To determine the enzyme’s pH optimum, 0.25 μl of each fraction were acidified as described above for 30 min to activate the putative protease. Control aliquots were incubated in HBS containing β-MSH. Fractions containing acid-activatable protease were identified from the previous step. A pool of purified protease (0.25 g of protein) was chromatographed on a Mono Q HR/5.5 column, equilibrated with HBS, using a Pharmacia model LCC-500 FPLC system at room temperature. A segmented elution gradient (maximum NaCl concentration 1 M) was programmed at a flow rate of 1.0 ml/min. Fractions of 1.9 ml were assayed with Z-Phe-Arg-NMec before and after acid activation, and for protein content. The Mono Q pool (13 ml, 0.5 g of protein) which was approximately 0.25 M in NaCl, was chromatographed on a phenyl Sepharose CL-4B column (0.8 cm diameter, 2 ml bed volume) in HBS containing 0.25 M NaCl. The column was given washes (2 ml each) of HBS containing 0.1 M NaCl and 0.05 M NaCl, respectively. Protoprotease was eluted using a step gradient with 1 ml each of 10, 8, 6, 4, and 2 M Hepes, pH 7.5. The column was then washed with 5 ml of water. Fractions (0.8 ml) were assayed for acid-activatable protease, conductivity, and protein content.

**Preparation of Yolk Extracts**—Seventy oothecae (approximately 2.1 g; day 6 for protease, day 0 for proteogelase) stored up to 3 weeks at −20 °C, were thawed in a 12-ml centrifuge tube, suspended in 6.2 ml of ice-cold CBS (10 mM citrate buffer, pH 5.5, containing 0.15 M NaCl and 1 mM EDTA; protease) or HBS (10 mM Hepes, pH 7.5, containing 0.15 M NaCl and 1 mM EDTA; proteogelase). Buffers at neutral pH were required for proteogelase purification to preclude protease activation. Oothecae were squashed against the tube’s wall with a spatula and then incubated on a rotator at 20°C, transferred back to the column, and washed with 100 ml of 50% aqueous methanol and 100 ml of water. It was agitated again for 4 h in 10 ml of 0.13 M ethanolamine buffer, pH 9.0 at 20°C and then washed with 100 ml of water containing 0.1% azide, prior to storage at 4°C.

**Protein concentration measurements** (22) used an ovalbumin standard.
Comparative effects of selected inhibitors on the enzymatic activities of yolk protease and activated proprotease

| Inhibitor   | Protease class | Concentration | Activity remaining (%) |
|-------------|----------------|---------------|------------------------|
|             |                |               | Crude Extract | Purified protease | Activated proprotease |
| None        |                |               | 100<sup>a</sup>,<sup>b</sup> | 100 | 100 |
| Aprotinin    | Serine         | 10 µM         | 100<sup>a</sup> | 105 | 98 |
| EDTA        | Metallo        | 1 mM          | 100<sup>a</sup> | 100 | 90 |
| Pepstatin   | Aspartic       | 3.5 µg/ml     | 95<sup>a</sup> | 82 | 83 |
| Phenylmethylsulfonyl fluoride | Serine/papain | 1 µM         | 94<sup>a</sup> | 82 | 68 |
| Pefabloc SC | Serine         | 0.5 mg/ml     | 81<sup>a</sup> | 78 | 71 |
| Soybean trypsin inhibitor | Serine | 100 µg/ml | 85<sup>a</sup> | 86 | 87 |
| TPCK        | Chymotrypsin   | 100 µµl       | 25<sup>a</sup>,<sup>b</sup> | 11 | 0.66 |
| TLCK        | Ser or Cys     | 100 µµl       | 0.45<sup>b</sup> | 7.8 | 0.98 |
| E-64        | Cysteine       | 5 µµl         | 14<sup>a</sup> | 0 | 0.5 |
| Leupeptin   | Ser or Cys     | 25 µg/ml      | 4<sup>a</sup> | 0 | 0.5 |
| Antipain    | Ser or Cys     | 100 µµl       | 2.5<sup>a</sup> | 0 | 3.9 |
| HgCl<sub>2</sub> | Cysteine | 200 µµl   | 0.98<sup>a</sup> | NA | NA |
| Z-Phe-Ala-CHN<sub>2</sub> | Cathepsin B and L | 10 µµl        | 11<sup>a</sup> | 0.95 | 30.5 |
| Z-Phe-Tyr(O-but)-CHN<sub>2</sub> | Cathepsin L | 10 µµl        | 22<sup>a</sup> | 8.5 | 0.37 |

<sup>a</sup> Crude extract was assayed at pH 3.7 with azoalbumin.
<sup>b</sup> Crude extract was assayed at pH 4.2 with CBZ-Lys-p-nitrophenyl ester.

* Purified protease and activated protease were assayed at pH 5.5 with CBZ-Phe-Arg-NMec.

Proteolytic Processing of Vt in Vitro—Ten µl of the Hg<sup>2+</sup> form of the protease was reactivated (specific activity 212 units of Z-LNE/mg) and mixed with 10 µl of CBS, pH 5.3, and incubated under toluene vapors with 300 µg of protease-free Vt at 30 °C. Five-µl aliquots were withdrawn periodically and analyzed by SDS-PAGE. The Hg<sup>2+</sup> protease, incubated with β-MSH-free CBS, pH 5.3, prior to addition of protease-free Vt, served as a control.

Electrophoresis—SDS-PAGE was conducted by a modification (25) of the Laemmli method (26) at 75 mA/gel as described previously (16). Nondenaturing PAGE was run at 200 V by a modification (Sigma) of the Laemmli method (26) at 75 mA/gel as described previously (16).

Purification. Processed Vt peptides (1.1 µg of protein) and purified protease (1.7 ng of protein) were brought to 100 µl with 0.25 mM citrate buffer, pH 7.5. Aliquots of 10 µl were withdrawn every 20 min and assayed with Z-Phe-Arg-NMec. Controls contained water substituted for protease or protease.

Action of Purified Day 6 Protease on Day 0 Proprotease—Aliquots of purified protease (1.1 µg of protein) and purified protease (1.7 ng of protein) were brought to 100 µl with 0.25 mM citrate buffer, pH 7.5. Aliquots of 10 µl were withdrawn every 20 min and assayed with Z-Phe-Arg-NMec. Controls contained water substituted for protease or protease.

Effect of Proprotease Concentration on Its Activation—Purified protease (1.1 µg of protein) was diluted 10-, 10<sup>2</sup>-, or 10<sup>3</sup>-fold in activation buffer to give total volumes of 0.01, 0.1, and 10 ml, respectively. Aliquots of 0.5, 5, and 500 µl (55 ng of proprotease each) were then withdrawn at intervals of 10, 30, 60, 120, and 240 min and adjusted to 500 µl with 0.25 mM citrate buffer, pH 5.5, containing 40 µM β-MSH. (The uniform final volume equalized the possible effect of activation buffer on the protease assay.) This solution was assayed for protease activity with Z-Phe-Arg-NMec.

RESULTS

Protease Purification—Tests of the crude extract with inhibitors at concentrations effective with the various protease classes (31) showed that the predominant activity is a cathepsin L-like cysteine protease (Table I). Sephacryl S-300 chromatography of the extract afforded a good separation of protease from processed Vt polypeptides (Fig. 1). Three non-protein peaks (fractions 59–65, 75–81, and 84–96) were not investigated further.

Properties of the Protease—The course of purification. Processed Vt peptides (M<sub>r</sub> 53,000, 45,000, and 42,000) are major contaminants in crude preparations (lane 1), but the putative protease is evident at M<sub>r</sub> 29,000 in the Sephacryl S-300 pool (lane 2) and the purified enzyme (lane 3) contained three peptides of approximately M<sub>r</sub> 29,000 (± 2 kDa).
$M_r$ 29,000 (Fig. 3) with no other probe-sensitive component evident. However, preincubation of the enzyme with E-64 prevented reactivity with the probe (lane 4), demonstrating clearly that the three peptides are cysteine proteases. Nondenaturing PAGE (27) also yielded three bands of $M_r$ 28,600, 29,400, and 37,600 (data not shown), values in close agreement with SDS-PAGE results. A 20-fold increase in specific activity accompanies a decrease in pH from 7.0 to 5.0 (Fig. 4). Previous work demonstrated that the yolk granules become acidified at days 3–4 postovulation (20), their pH decreasing from neutral to about 5.5. Thus, the profile is consistent with protease activation in vivo being regulated by granule acidification. Purified protease had the same relative susceptibility to inhibitors as the crude extract (Table I). TLCK and TPCK, inhibitors of trypsin and chymotrypsin-like serine proteases respectively (31), also inhibit some cysteine proteases (32).

Proteolytic Processing of Vt—The time course of Vt processing by the protease in vitro was compared with the in vivo pattern. Table II summarizes the results of these studies. In both situations the $M_r$ 102,000, 95,000, and 50,000 subunits of Vt were degraded completely and "limit" peptides of $M_r$ 53,000, 22,000, 21,000, and 20,000 accumulated. In addition, peptides of $M_r$ 88,000, 68,000–77,000, 40,000–43,000, and 30,000 were produced. Unique limit peptides in vitro were $M_r$ 88,000, 70,000, and 50,000–53,000. Processing of the $M_r$ 50,000 subunit in vitro was obscured by other products of that approximate size, but this subunit is processed beginning at day 5 (16, 18). Addition of fresh enzyme to the in vitro incubation did not alter the product distribution. Thus, despite the enzyme's activity with synthetic substrates, it catalyzes only limited proteolysis of Vt in vitro, which is what occurs in vivo.

Evidence for a Proenzyme Precursor—Previous work (20) demonstrated that Vt in day 0 yolk was processed at acidic pH. To check for the presence of a proenzyme, reactivities of day 0 and day 6 yolk with biotinyl-Phe-Ala-CHN$_2$ (29) were compared with biotinyl-Phe-Ala-CHN$_2$ (data not shown).

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FIG. 4. pH activity profiles of protease and activated proprotease. Enzymatic activities were determined with Z-Phe-Arg-NMec. Buffers employed at the various pH values and assay conditions are described under “Experimental Procedures.” Each datum point is the average of three assays. Data are normalized to 1.0 at pH 5.5.

**TABLE II**

Comparison of vitellin processing in vivo and by purified protease in vitro

For details, see “Experimental Procedures.”

| Peptide  | In vivo | In vitro |
|----------|---------|----------|
| Intermediate Peptides | 88, 68, 77, 30 | 43 |
| Limit Peptides | 53, 44, 42, 23, 22, 21 | 88, 70, 53, 50, 30, 23, 22, 21 |

(Fig. 5). Day 6 yolk (*lane 1*) contains a broad reactive band at approximately M<sub>r</sub> 29,000 and a trace component at approximately M<sub>r</sub> 50,000. The M<sub>r</sub> 29,000 band’s mobility matched that of the purified protease (*lane 2*). Day 0 yolk (*lane 3*) was labeled more extensively by the probe, especially the three Vt subunits at M<sub>r</sub> 102,000, 95,000, and 50,000, which have been degraded by day 6 in vivo (16). However, *lane 3* also contains three probe-reactive peptides at approximately M<sub>r</sub> 40,000 (*arrowheads*), absent when day 0 yolk was preincubated with E-64 (*lane 4*). Although peptides of M<sub>r</sub> 50,000 and larger were labeled nonspecifically, only the M<sub>r</sub> 40,000 components were E-64-sensitive, suggesting that they contain active site cysteine residues. Because the protease is also resolvable into multiple bands on SDS-PAGE (*lane 2* and Figs. 2 and 3), the M<sub>r</sub> 40,000 triplet was considered as the candidate proprotease.

**Purification of the Proprotease**—The putative proprotease was stable in day 0 yolk extracts at pH 7.5 at 4°C for at least 1 week. Values lower than 7.0 resulted in protease activation, and those higher than 8.0 resulted in irreversible inactivation. Sephacryl S-300 chromatography (Fig. 6) gave one major protein peak (fractions 21–30) and a peak of activable protease activity (fractions 31–40), which was absent before acidification. FPLC separated the putative proprotease, which eluted between 0.2 and 0.3 M NaCl, from additional proteins and proved to be a critical step in the purification (data not shown). Optimal resolution was achieved when the protein load per run was kept below 10 mg. In the final step, phenyl-Sepharose CL-4B chromatography resolved the putative proprotease, which eluted at approximately 2 mM Hepes, from trace contaminants (data not shown). The candidate proprotease consists of four peptides of M<sub>r</sub> 35,500, 37,000, 39,000, and 41,000 (Fig. 7, *lane 4*), their mobilities essentially the same as those in day 0 yolk probed with biotinyl-Phe-Ala-CHN<sub>2</sub> (Fig. 5). The proprotease migrated as a single band on nondenaturing PAGE at each acrylamide concentration employed (data not shown), and the calculated molecular weight (27) was 45,600. After acidification, the M<sub>r</sub> 45,600 peptide disappeared but new ones were evident at M<sub>r</sub> 36,800 and 34,800. The shift in molecular weights is close to the difference between the proprotease (40,000) and the protease (29,000), obtained by SDS-PAGE.

Protease and acid-activated proprotease displayed similar pH activity profiles (Fig. 4), relative susceptibilities to numerous inhibitors (Table I), and heat inactivation characteristics (data not shown). Reactivity of the proprotease with biotinyl-Phe-Ala-CHN<sub>2</sub> demonstrates that the active site is accessible to the inhibitor in the proenzyme and is probably in a conformation similar to the protease (34). They retained 63% and 67% of their enzymatic activities, respectively, after exposure to 3 M urea. While cathepsins B are inactivated under these condi-

![Fig. 4](image-url)  
**FIG. 4.** pH activity profiles of protease and activated proprotease. Enzymatic activities were determined with Z-Phe-Arg-NMec. Buffers employed at the various pH values and assay conditions are described under “Experimental Procedures.” Each datum point is the average of three assays. Data are normalized to 1.0 at pH 5.5.

![Fig. 5](image-url)  
**FIG. 5.** Electroblotting of biotinylated yolk proteins from eggs at day 0 and day 6 postovulation. Treatment of proteins with E-64, biotinylation, SDS-PAGE, electrotransfer, and peptide detection with streptavidin-HRP and Luminol are described under “Experimental Procedures.” *Lane 1*, 30 μg of day 6 yolk extract; *lane 2*, 5 μg of Vt-processing protease purified from day 6 yolk; *lanes 3* and 4, 30 μg of day 0 yolk extract incubated without or with 100 μM E-64 (final concentration), respectively, prior to biotinylation.

![Fig. 6](image-url)  
**FIG. 6.** Sephacryl S-300-HR column chromatography of day 0 yolk extract. Clarified extract (6.5 ml) was chromatographed at 4°C on a Sephacryl S-300-HR column (2.6 × 91 cm) and eluted with HBS. Fractions of 8.8 ml were collected. To locate proprotease (fractions 28–38), aliquots of each fraction were acidified and then assayed with Z-LNE as described under “Experimental Procedures.” Contents of fractions 20–25 were also pooled and used as a source of protease-free Vt.
tns, cathepsins L are far less sensitive to this reagent (23). In addition, protease and activated proprotease had only 2.5% and 5.5% of the enzymatic activity, respectively, with Z-Arg-Arg-NMec, a cathepsin B substrate, compared to that with the cathepsin L substrate Z-Phe-Arg-NMec (23).

Conversn of Proprotease to Protease—In vivo, the relationship between the proprotease and the protease is seen in an experiment using yolk extracts (day 0 to day 6 postovulation) and the active site probe biotinyl-Phe-Ala-CHN2 (Fig. 8, panels A and B). Although numerous yolk peptides were derivatized nonspecifically, a reactive band at approximately M, 40,000, present at oothecal extrusion (panel A, lanes 0 and 1, closed arrowhead), decreased in intensity during development and was absent after day 3. However, during days 4–6, the intensity of a doublet at M, 29,000 increased (panel A, open arrowhead). Since the staining intensity of several bands changed during the 7 days of embryo development, the specificity of derivatization was evaluated by preincubation of the yolk with E-64. With inhibitor present (panel B), no bands were detected at M, 40,000 or 29,000 kDa. Thus, these peptides contained E-64-reactive cysteine residues. Appearance of the M, 29,000 components also correlated temporally with Vt processing and protease activation in vivo (9).

In vitro, the mobility of purified proprotease on SDS-PAGE (Fig. 9, lane pH 5.3, 0 h) shifted to that of the protease following incubation at pH 5.3 (compare pH 5.3, 12 h lane and Prostate lane) but not at neutral pH (pH 7.5, 12 h). The conversion was blocked by E-64 but not by Pefabloc SC, pepstatin, or EDTA (Fig. 9), demonstrating that activation requires both acidification and participation of a cysteine protease. These results were confirmed by the experiment illustrated in Fig. 10. When proprotease and protease were incubated separately for 60 min, the increment in enzymatic activity was negligible when compared to the activity obtained upon mixing them at these individual concentrations. Taken together, the results of these several experiments indicate that the protease is derived from the putative proprotease in day 0 yolk.

Another experiment demonstrated that the activation rate is intermolecular. Protease was diluted and assayed as described under “Experimental Procedures.” At dilutions of 104-, 102-, and 10-fold, the relative rates of activation were 1, 5.3, and 48 (data not shown). The dependence of the activation rate on enzyme concentration rules out a single intramolecular (zero order) process.

The Proprotease Is a Glycoprotein—Purified proprotease reacted with biotin hydrazide, showing that the proenzyme is a glycoprotein (Fig. 11, lanes C, minus endo H). Removal of carbohydrate by endo H, indicated by a slight increase in mobility (lanes C, plus and minus endo H) suggests that most, if not all, occurs as high Man-type oligosaccharides (30), the class of oligosaccharides found in B. germanica Vt (16, 35) and other insect glycoproteins (36, 37). The probe also labeled the ando H-treated protein, but with less intensity than with the control, a result reflecting either incomplete removal of oligosaccharides or to the remaining single GlcNAc residues (30). Mature protease was not reactive with the probe (lanes B, minus and plus endo H), showing that the oligosaccharides are located exclusively on the pro-region of the proprotease. As expected, the Vt control was deglycosylated by endo H (lanes A, minus and plus endo H). A mobility shift in the M, 50,000 subunit was not observed.

Vt-processing Peptides Are Not Ubiquitinated—A 26 s “proteolytic complex” that degrades ubiquitinated proteins in vitro has been isolated from Drosophila melanogaster embryos, and it was suggested (38) that ubiquitination might be important in yolk polypeptide degradation. To check for this possibility in B. germanica, extracts of yolk prepared daily from eggs at days
Like the propapain is also converted to papain, molecules have an essential thiol at the active site (34) and with the mosquito triple peptides of similar molecular weights are found in the bands. Other examples of mature proteases consisting of multiple peptides were detected in the extracts tested (data not shown).

**DISCUSSION**

Cathepsin-like cysteine proteases are derived from proprotein precursors (40). An excellent example is propapain (M, 39,000), which is processed in vivo to the cathepsin L papain (M, 24,000) (41). In addition to similarity in molecular weights with the B. germanica yolk proenzyme and protease, all four molecules have an essential thiol at the active site (34) and propapain is also converted to papain, in vitro, at acidic pH (34). Like the B. germanica proprotease, propapain is glycosylated exclusively on its propeptide region (42). Whether this modification is critical to production of a functional enzyme, as it is with propapain (42), remains to be established. Although tests with various inhibitors demonstrated that the proprotease is more closely related to the cathepsins L than cathepsins B (20, 43, 44), the distinction is not absolute. Because its molecular weight heterogeneity was retained throughout purification, degradation during the procedure is not causing the multiple bands. Other examples of mature proteases consisting of multiple peptides of similar molecular weights are found in the mosquito Culex nigripalpus (45), the bacterium Erwinia chrysanthemi (46), eggs of Ornithodorus moubata (7, 8), the silkworm Bombyx mori (14), and human neutrophils (47).

Numerous similarities between the activated proprotease and the protease lead to the conclusion that the proprotease in day 0 yolk is the enzyme’s precursor. Most importantly, degradation of Vt in vivo and by the protease in vitro is limited, and both events afford several common intermediates and products (Table II). The differences that were noted could reflect unique sets of scissile bonds being exposed to proteolysis in each case, possibly due to differences in the structures/solution conformations of Vt in vivo and in vitro, or to the fact that Vt’s oligosaccharides are trimmed coincident with proteolysis in vivo (16). Digestion of B. mori Vt with a purified yolk cysteine protease has been shown to give a peptide distribution similar, but not identical, to that seen in vivo (48). Electrophoretic gels of yolk during embryonic development (Fig. 8) demonstrated that the conversion of the putative proenzyme to the processing protease, begins at day 2–3 postovulation, which correlates with the time in embryo development when both granule acidification and protease activation occur (9, 19, 20).

It is now clear that yolk granule acidification is of general physiological importance and that Vt proteolysis is initiated by a developmentally regulated decrease in intragranular pH. Vt-degrading yolk proteases from O. moubata (7, 8), B. mori
(48), *Aedes aegyptii* (49), and *Musca domestica* (50) are all converted from proenzymes to active, mature enzymes in vitro at acid pH, and yolk granules of *O. moubata* (51) and the blowfly *Phormia regina* (20) also acidiﬁc in concert with Vt utilization. Other well documented examples of acidification-dependent Vt degradation include the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* (52) and frog *Xenopus laevis* (53). The pH range of acidiﬁed granules measured for the latter two examples, pH 5.6–6.2 (52, 54), is probably adequate for proteolytic processing of proenzymes and is in the range for catalytic activity of both the activated B. *germanica* proprotease and protease (Fig. 4). Bafilomycin sensitivity of granule acidiﬁcation in *X. laevis* (54) is consistent with our ﬁnding (20) that granule acidiﬁcation occurs by proton translocation and it provides additional evidence that a vacuolar ATPase is responsible for pumping protons into yolk granules. Although the source of the proton pump and the mechanism initiating intragranular proton accumulation remain to be determined, the present work links the Vi-processing protease of *B. germanica* to the acidiﬁcation-dependent, proteolysis of a proprotease precursor.

The dynamics of vitellogenesis and Vt processing in *B. germanica*, as they are currently understood, can be summarized as follows. Vitellogenin is synthesized in, and secreted from, the fat body of vitellogenic females (35) and endocytosed by the oocytes (55). Following vitellogenin’s uptake by the oocyte, fusion of small Vt-containing vesicles leads to formation of large, mature yolk granules (56), which are stored for approximately 72 h prior to acidiﬁcation and initiation of Vt processing. Recently acquired immunoelectron microscopy data (57) demonstrate that protease is located over these mature granules from days 0–2 postovulation.

Ultrastructural studies have also demonstrated the presence of vitellinophages initially at about day 2 postovulation, primarily on the ventral periphery of the yolk mass (56). These cells then intercalate the tightly packed granules, extending folio- and lamellipodia over granule surfaces. Portions are engulfed and sequestered as large vacuoles (56) in a sequence of events strikingly similar to that observed in embryogenesis of the stick insect *Carausius morosus* Br. (57). The *B. germanica* vacuoles then become vesiculated and partitioned into smaller vesicles (56) with the same size distribution as granules isolated from yolk (20). A role for vitellinophages in the acidiﬁcation and protease activation events remains to be established, but providing acidiﬁcation machinery (e.g. a vacuolar ATPase) to the granules is one possibility.

**Acknowledgments**—We thank Professor Franco Giorgi, University of Pisa, Italy for critical readings of the manuscript and Dr. Alan J. Barrett, Cambridge University, United Kingdom, for helpful discussions.

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