Cloning of a Cysteine Protease Required for the Molting of *Onchocerca volvulus* Third Stage Larvae*

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We have investigated the involvement of a cysteine protease in the development of *Onchocerca volvulus* fourth stage larvae (L4) by testing the effect of cysteine protease inhibitors on the survival of third stage larvae (L3), and the molting of L3 to L4 *in vitro*. When larvae were cultured in the presence of specific inhibitors, the peptidyl monofluoromethylketones, viability of either L3 or L4 was not affected. However, the inhibitors reduced the number of L3 that molted to L4 *in vitro* in a time- and dose-dependent manner. Molting was completely inhibited in the presence of 50–250 μM inhibitor. Ultrastructural examination of L3 that did not molt in the presence of inhibitors indicated that new L4 cuticle was synthesized, but there was no separation between the L3 and the L4 cuticles. The endogenous cysteine protease was detected in molting larvae after binding to labeled inhibitors, and by antibodies directed against a recombinant *O. volvulus* L3 cysteine protease that was cloned and expressed. The enzyme was detected in cuticle regions where the separation between the cuticles occurs in molting larvae. These studies suggest that molting and successful development of L4 depends on the expression and release of a cysteine protease.

Nematodes characteristically grow and develop through four molts; in parasitic nematodes, the last two are always in the final host. The process of molting involves the separation of the cuticle from the underlying epidermis (apolysis), the formation of a new cuticle arising from the outermost surface of the epidermis, and the shedding of the old cuticle (ecdysis) (1). The actual mechanics of molting can be very different among the various nematode genera. Some nematodes reabsorb the old cuticle during molting (2), some shed the old cuticle intact (3), and some do not undergo ecdysis until entering the mammalian host (4). Proteases are thought to play an essential role in molting by digesting the old cuticle, degrading cuticular anchoring proteins, or activating peptide molting hormones or other molting enzymes by processing of proenzymes (5–7). A 44-kDa zinc metalloprotease in *Hemionchus contortus* was shown to be responsible for the digestion of the ring region of the second stage larval cuticle before molting (8, 9). Proteases active during molting have been also observed in other species, including *Phlocenema decipiens* (10), *Ancylostoma* (11), and *Filariae* (5, 12). In *Dirofilaria immitis* and *Brugia pahangi*, metalloproteases were shown to be intimately associated with molting as well as activities that might facilitate larval migration (5, 12).

*Onchocerciasis*, or river blindness, is one of the leading causes of infectious blindness and severe chronic dermatitis, affecting about 18 million people in West Africa and Latin America (13). The *Onchocerca volvulus* parasite is transmitted by bites of *Simulium* black flies. Our ability to study the molting process of *O. volvulus* third stage larvae (L3) has been enhanced after the conditions for *in vitro* cultivation of larval stages were developed (14). Ultrastructural examination of larval stages during molting have shown that the formation of the new cuticle of fourth stage larvae (L4) is already under way on days 1 and 2 of culture. The new epicuticle was evident in some areas beneath the basal layer of the old cuticle and irregularly shaped “lakes” (separated areas between the cuticles) appeared below the old basal layer. On day 2 and 3 in culture, these lakes fused into a continuous layer of separation, and by day 4 or 5, most of the larvae have completed the molt (14). The appearance of the lakes and their fusion into a continuous separation before ecdysis suggested the possible activation of enzymatic processes that promote the separation of the old L3 cuticle from the new one (15).

In our previous studies we had identified and cloned a functional *O. volvulus* cysteine protease inhibitor, onchocystatin (16). Interestingly, onchocystatin was localized by immunogold electron microscopy in the hypodermis and the cuticles of L3 and L4 during the molting of L3 to L4, especially around the region of cuticle separation. As the postulated role for cystatin has been the regulation of cysteine protease activities (17), these studies indirectly suggested that a cysteine protease may be involved in the molting process of *O. volvulus* larvae. We now show that a cysteine protease is indeed one of the enzymes involved in the molting of *O. volvulus* larvae and is an essential enzyme for the successful development of L4.

**MATERIALS AND METHODS**

In Vitro Culturing of L3 in the Presence of Cysteine Protease Inhibitors—One approach to study the role of cysteine proteases in the molt-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[274]/EBI Data Bank with accession number(s) U71150.

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† The abbreviations used are: L4, fourth stage larvae; L3, third stage larvae; GST, glutathione S-transferase of *Schistosoma japonicum*; PAGE, polyacrylamide gel electrophoresis; Z, benzoyloxycarbonyl; FMK, fluoromethylketone; Mu, morphourea; CHN2, diazomethane; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OVCP, *O. volvulus* cysteine protease; LOVCP, larval OVCP.

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ing process was to culture the larvae in the presence of specific irreversible inhibitors of cysteine proteases and examine their effect during the molting of L3 to L4. We have used the peptide monofluoromethylketone (FMK) compounds containing a peptide sequence targeting the active sites of the cysteine proteases, Cathepsin B and L (18).

*Simulium yahense* black flies were infected with *O. volvulus* microfilariae, and after 7–8 days L3 were harvested as described previously (14, 19). L3 were cultured in groups of 10 larvae in 96-well plates containing 100 μl of culture medium (1:1 NCTC 135 and Iscove’s modified Dulbecco’s medium plus 20% heat-inactivated fetal calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 5 μg of amphotericin B (Fungizone)/ml). Using these conditions, we routinely achieved 40–60% successful L3 to L4 molting by day 5 in culture. L3 were cultured in vitro for 6 days at 37°C in 5% CO2 incubator in the presence of increasing concentrations of cysteine protease inhibitors: benzylxoy carbonyl-Phe- Ala-fluoromethylketone (Z-Phe-Ala-FMK), Z-Phe-Ala-FMK, Z-Phe-Arg-FMK, and Z-Phe-Phe-FMK, and the number of molting larvae was determined on day 6. Molting was manifested by shedding of the thick L3 cuticle and a marked increase in the motility of the larvae. Larval viability was also assessed visually after the uptake of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide and its reduction into the blue formazan derivative as described previously (20, 21). Larvae were scored live when they stained blue uniformly along their entire length and dead when the larvae remained unainted or partially blue. The results shown for each inhibitor concentration were the average number of larvae that molted or the average number of larvae that did not molt. Larvae scored alive, and dead were counted in groups of 50–100 L3. Each experiment was repeated at least twice. The standard error between experiments never exceeded 10% and did not vary with the concentration of the inhibitors.

**Ultrastructure of Larvae Cultured in the Presence of Cysteine Protease Inhibitors**—Larvae that did not molt in the presence of cysteine protease inhibitors were collected and fixed overnight at 4°C with 3% glutaraldehyde in 0.1 x phosphate buffer (pH 7.3), washed in the same buffer, and processed for electron microscopy examination as described previously (14). For examination of the ultrastructure of larvae undergoing normal molting, L3 were cultured in vitro for 5 days and larvae from day 1 to 5 in culture were collected. The larvae were fixed as described above.

**Molecular Biology Methods**—General procedures for preparation of plasmid DNA, subcloning, Southern blotting, hybridization, and preparation of probes were followed as described by Sambrook et al. (22). For subcloning PCR products, the DNA fragments were made blunt-ended with Klenow DNA polymerase for 5 min at 37°C, or cut with the appropriate restriction enzyme. The products were fractionated on 1.2% agarose gels and transferred by electrophoresis on Hybide. The products were ligated into pBluescript SK+ (Stratagene, La Jolla, CA) previously digested with SmaI for blunt end ligation, or other restriction enzymes for ligation of cohesive ends.

Automated DNA sequencing was done by the Microchemistry Facility at the New York Blood Center using the fluorescent dye termination method and Taq DNA polymerase on an ABI model 373A sequencer (Applied Biosystems Inc., Foster City, CA). In order to confirm this clone was expressed in larvae and to obtain full-length cDNA of the *O. volvulus* larval cysteine protease, we first cloned the 3' region of the cDNA by a modification of the 3' rapid amplification of cDNA ends (RACE) protocol (25). A first-strand cDNA using oligo(dT) adaptor primer (SWE17) and L3 *O. volvulus* poly(A)™-competent cells (Life Technologies, Inc.). The ligation junction was amplified by PCR using a specific primer corresponding to the sequence coding for the onchocerca cysteine proteases and which contained the conserved amino acid of the histidine active site of cysteine proteases in addition to the asparagine sequence which was derived from the reverse primer (data not shown). A *BLAST* homology search with the nucleotide sequence of the PCR product showed that this sequence was a partial DNA fragment from the 3' end of a genomic clone. The DNA fragment was subcloned into pBluescript SK+ –, and the plasmid DNA of the transformant was purified and sequenced from both directions using the T3 and T7 primers and specifically designed internal primers. Sequence analysis of the 5' region (2230 bp) of the genomic clone indicated that this region contained a specific but partial genomic sequence coding for the onchocerca cysteine protease, OVCp. The remaining of the 5' coding region of the OVCp gene was purified and sequenced after a specific fragment was generated by PCR from *O. volvulus* genomic DNA using a specific reverse primer to the genomic sequence (OVCp10) and a forward primer specific to the 5' coding region of the cDNA sequence (see below) including the first methionine (OVCp-Met).

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**Sequence Analysis**—A*O. volvulus* poly(A)™-competent cells (Life Technologies, Inc.). The ligation junction was amplified by PCR and subcloned into the pGEM-4T-3 expression vector (Pharmacia Biotech, Inc.) which was a gift from Dr. Perler, New England Biolabs. 9 × 10⁵ plaque-forming units were screened by DNA hybridization at 37°C following the protocols of Sambrook et al. (22). Four genomic clones containing DNA inserts of 5–6.5 kb were isolated and purified. The largest clone (clone 9) containing the open reading frame was chosen and its DNA was digested with the appropriate restriction enzymes for ligation of cohesive ends. The genomic DNA was digested with the same enzyme. The genomic DNA containing the probe peptide and the mature enzyme of onchocerca A Molting Protease of *O. volvulus*

*O. volvulus* can only be obtained in limited quantity from clinical samples, a recombinant approach was taken to clone the larval cysteine protease, and later we confirmed its identity by immunolocalization and Western blot analysis with antibodies against the recombinant polypeptide. A specific DNA fragment was initially isolated from genomic DNA prepared from *O. cervicalis* adult worms by PCR using degenerate primers designed for the cysteine and the asparaginase active site motifs of cysteine proteases as described by Sakaran et al. (23) and Eakin et al. (24). The PCR amplification was performed in a volume of 100 μl for 40 cycles, with the following profile: 1 min at 94°C, 2 min at 25°C, and 3 min at 72°C, followed by a final 7 min extension at 72°C. A DNA fragment of about 700 bp was purified and named OCl700. This fragment would provide cDNA for assaying onchocerca at conserved active site residues. OCl700 contained two exons and one intron. In its 5' region, an exon of 139 bp containing 45 amino acids was identified, while the 3' end of the sequence CGSCWC originating from the forward primer. In its 3' region an exon of 153 bp was identified with an open reading frame encoding 51 amino acids, which had a significant homology to other cysteine proteases and which contained the conserved amino acid of the histidine active site of cysteine proteases in addition to the asparagine active site which was derived from the reverse primer (data not shown).

**Expression of Recombinant OVCp and Antibody Production**—The region containing the polyhedrin and the major enzyme of onchocerca (Fig. 4, underlined arrows) was amplified by PCR and subcloned into the pGEX-4T-3 expression vector (Pharmacia Biotech, Inc.) which was successfully used for the expression of an active onchoceytoitin (16). The ligation mixture was used to transform *E. coli* subcloning efficiency DH1α™-competent cells (Life Technologies, Inc.). The ligation junction of the pGEXLOVCP plasmid was sequenced to verify the correct read-
ing frame. Transformants expressing the GST-LOVCP fusion peptidase were identified after SDS-gel electrophoresis (SDS-PAGE) by staining with Coomassie Blue and by Western blot analysis using rabbit anti-GST antibodies, followed by goat anti-rabbit antibodies conjugated to horseradish peroxidase and detection by 3, 3', 5, 5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Large scale purification of GST-LOVCP fusion polypeptides was carried out according to the procedure described before (16). 250 ml of culture yielded about 0.5 mg of pure GST-LOVCP fusion polypeptide. The GST-LOVCP fusion polypeptide was not active as a cysteine protease when tested in a continuous fluorometric assay with Z-Phe-Arg-AMC (Z-Phe-Arg-4-amino-7-methylcoumarin) or Z-Arg-Arg-AMC (data not shown). Antiserum to the GST-LOVCP fusion polypeptide was prepared by subcutaneous injection of a rabbit with 100 μg of protein mixed in complete Freund’s adjuvant (Difco), followed by repeated injection 3 and 4 weeks later in incomplete Freund’s adjuvant (Difco). The rabbit was bled 2 weeks after the third injection.

Identification of a Cysteine Protease in O. volvulus Molting Larvae—Initially, we used an indirect approach in order to identify the endog-

cene cysteine protease in molting larvae. We took advantage of the irreversibly binding of labeled cysteine protease inhibitors to the en-

zyme during the molting process (28, 29). Larvae were cultured in the presence of 50 μM iodinated Mu-Tyr-O-methyl-homoPhe-FMK (28), and then we collected the worms after 2 days, extracted the proteins in SDS-PAGE sample buffer (final concentration of 2% SDS, 5% 2-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8) and separated them on a 12% SDS-PAGE gel. The iodinated proteins were then identified by autoradiography. In addition, stage-specific soluble extracts were prepared from about 100 L3 or 100 larvae collected on day 1, 2, or 3 in culture by homogenization on ice in a prechilled 100 mM sodium acetate buffer, pH 5.0. These extracts and culture supernatant collected from wells containing larvae cultured for 2 days were then incubated with 1 μM biotinylated inhibitor, biotin-Phe-Ala-diazomethane (biotin-Phe-Ala-CHN2, after adding the inhibitor on day 0, 1, 2, or 3. After 24 h the larvae were collected and fixed as described above. The biotin-Phe-Ala-CHN2 was detected in thin sections of the embedded worms by 15 nm gold particles coated with streptavidin (Amersham Life Sciences). The cysteine protease in these worms was detected with antibodies to the GST-LOVCP fusion polypeptide.

RESULTS

The Effect of Specific Cysteine Protease Inhibitors on L3 Molting—The presence and location of the cysteine protease inhibitor, onchocystatin, suggested the possible involvement of a cysteine protease in the development of O. volvulus L4. To test this possibility, we studied the effect of four specific irreversible inhibitors (peptidyl monofluoromethylketone compounds) of the cysteine proteases, cathepsin B and cathepsin L, on the viability of molting L3 and the ability of L3 to molt to L4 in vitro. Parasite viability was assessed visually after the uptake of MTT by the larvae. L3 were cultured in the presence of increasing concentrations of the inhibitors, and the number of molting larvae was determined on day 6. All inhibitors reduced, in a dose-dependent manner, the molting ability of L3 in vitro (Fig. 1). In comparison with 50% molting under normal culture conditions, 50–80% of molting was inhibited by 100 μM Z-Phe-Ala-FMK, Z-D-Phe-Ala-FMK, Z-Phe-Arg-FMK, and Z-Phe-Phe-FMK at 250 μM completely inhibited molting. Z-Phe-Phe-FMK at 250 μM inhibited only 70% of the molting. The inhibition was not due to a lethal effect of the inhibitors on L3. In parallel experiments larvae were treated with MTT on different days during culture in the presence of various concentrations of the inhibitors, and their viability were assessed after 24 h. The viability curves in Fig. 1 represent the data from larvae cultured for 3 days in the presence of the compounds. L3 that were cultured in the highest concentration (250 μM) of Z-Phe-Ala-FMK, Z-Phe-Arg-FMK, and Z-Phe-Phe-FMK were still viable. With Z-D-Phe-Ala-FMK, only concentrations greater than 100 μM had any significant effect on L3 viability. For the viability curves in Fig. 1, we used the data from day 3 in culture because this has been shown to be the optimal time to determine if larvae are both viable and ready for molting. Larvae usually complete their L3 to L4 molt by day 5 (14). Similar viability results were also obtained on successive days; the larvae were motile and viable during all days in culture (data not shown). Since the cysteine protease inhibitors were not lethal to L3 or L4 larvae at all concentrations, we could assay their effects on the molting process.

To identify the point during the molting process that is most sensitive to protease inhibitors, culture medium containing 100 μM Z-Phe-Ala-FMK or Z-Phe-Arg-FMK was added to larvae on day 0, or on days 1, 2, and 3 by replacing the normal culture.
medium. The larvae were then allowed to remain in culture until day 6, when the number of molting larvae was determined. When added on day 0 or day 1 both inhibitors were able to inhibit completely the molting (Fig. 2). However, when Z-Phe-Arg-FMK was added on day 2 or day 3, only a partial inhibition was observed; 16 and 14% of the larvae molted, respectively, in comparison with 50% that molted under normal culture conditions. Interestingly, when Z-Phe-Ala-FMK was added on day 2, the inhibition was complete, but became partial when added on day 3 (72% inhibition). Similar results were obtained with the other inhibitors (data not shown).

We then tested a second generation of inhibitors (kindly provided by Drs. Robert Smith and Mary Zimmerman of Prototek, Inc.) in which the amino acid at position P1 is not natural. This modification enhances half-life of the compounds both in vitro and in vivo. Indeed, these compounds were effective at even lower concentrations. The molting of *O. volvulus* L3 to L4 was inhibited by 48–88% and 81–100% at 10 and 50 μM, respectively (Table II).

![Ultrastructure of Larvae That Did Not Molt in the Presence of Cysteine Protease Inhibitors](image)

**Table II**

| Inhibitors                  | Concentration | Molting Inhibition |
|-----------------------------|---------------|-------------------|
| Mu-Phe-nitroargin-FMK       | 10 μM         | 5%                | 88%              |
| Mu-Phe-homoPhe-FMK          | 50 μM         | 0%                | 100%             |
| Mu-Leu-homoPhe-FMK          | 10 μM         | 14%               | 88%              |
| Mu-Leu-homoPhe-FMK          | 50 μM         | 7%                | 84%              |
| Mu-But-homoPhe-FMK          | 10 μM         | 8%                | 81%              |
| Mu-But-homoPhe-FMK          | 50 μM         | 7%                | 84%              |
| Mu-Tyr-O-methylhomoPhe-FMK  | 10 μM         | 19%               | 81%              |
| Mu-Tyr-O-methylhomoPhe-FMK  | 50 μM         | 8%                | 84%              |
| Medium control              | 0 μM          | 42%               |                  |
| Medium + 0.5% Me₂SO control | 0 μM          | 44%               |                  |

*Fig. 1. Molting and viability of *O. volvulus* L3 in the presence of cysteine protease inhibitors.* 50–100 L3s were cultured in the presence of increasing concentrations of 4 different FMK inhibitors and the molting rate was determined on day 6. 50% L3 larvae molt by day 5 under normal control culture conditions. Larval viability after 3 days in culture in the presence of the inhibitors was assessed visually after the uptake of MTT by the larvae, and its reduction into the blue formazan derivative. Each experiment was repeated at least twice; the standard error between experiments never exceeded 10%.

*Fig. 2. Molting of *O. volvulus* L3 in the presence of Z-Phe-Ala-FMK and Z-Phe-Arg-FMK inhibitors that were added at different days during molting.* The inhibitors were added during the molting of 50 L3's on day 0, 1, 2, or 3 and were kept in culture until day 6, when the number of molting larvae was determined. 50% of larvae molted in normal culture conditions, without inhibitors.

*Ultrastructure of Larvae That Did Not Molt in the Presence of Cysteine Protease Inhibitors.* — *O. volvulus* larvae that did not molt in cultures containing the cysteine protease inhibitors continuously were collected and processed for electron microscopy. As shown in Fig. 3, in larvae that did not molt in the presence of Z-Phe-Ala-FMK (d), Z-Phe-Arg-FMK (e), or Mu-Tyr-O-methylhomoPhe-FMK (f) as well as the other inhibitors (data not shown), the development was arrested prior to the molting stage.

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*R. Smith, personal communication.*
CDNA (OVCP-Met) using genomic DNA of (OVCP10) and a primer specific to the first 6 amino acids of the volvulus gene was obtained by PCR using an internal primer as the template. The 3' PCR products using oligo(dT) adaptor-primed first strand L3 cysteine protease, LOVCP, was obtained from two separate cysteine protease gene (gOVCP) contains eight exons and seven introns. The composite sequence of the L4 epicuticle and L3 cuticle was partial or complete as seen under normal culture conditions on days 1–4 (Fig. 3, a–c). In addition we found that many larvae had unusual swollen L3 cuticles (Fig. 3, e and f).

Cloning of the O. volvulus Cysteine Protease, OVCP—A specific 642-bp fragment, designated OC700, was obtained by PCR amplification of O. cervicalis genomic DNA using degenerate primers based on the cysteine and the asparagine active site motifs that are well conserved among the papain-family cysteine proteases (32). Translation of all the possible open reading frames indicated that OC700 encoded portions of a cysteine protease including 51 amino acids with a high degree of similarity to the papain family, containing the conserved histidine active site motif of cysteine proteases plus the cysteine and asparagine active site motifs encoded by the primers. The fragment also contained a 359-bp intron. A Taq/HindIII 135-bp fragment from the 3' exon was used as a hybridization probe to screen an O. volvulus genomic DNA library (a gift from Dr. F. Perler, New England Biolabs). Out of four genomic clones, the largest clone (clone 10 = 6.5 kb) was chosen to be analyzed further. A 2230-bp region of this genomic clone contained a partial gene encoding a cysteine protease with six exons and five introns encoding all three active site motifs: cysteine, histidine, and asparagine. In a 642-bp overlap, the O. volvulus genomic clone was 90.4% identical to the OC700 genomic fragment from O. cervicalis (93.6% identity in the amino acids encoded by the two exons, 6/51 differences in the 3' exon; 0/43 in the 5' exon). Intron similarity was only 87.7%, with 315 out of 359 bp identical (data not shown). The 5' end of the O. volvulus gene was obtained by PCR using an internal primer (OVCP10) and a primer specific to the first 6 amino acids of the cDNA (OVCP-Met) using genomic DNA of O. volvulus as the template. The cDNA was obtained by PCR as described below. This additional fragment contained the remaining two exons and two introns. The composite sequence of the O. volvulus cysteine protease gene (gOVCP) contains eight exons and seven introns (see Fig. 4, arrowheads).

The composite full-length cDNA sequence of the O. volvulus L3 cysteine protease, LOVCP, was obtained from two separate PCR products using oligo(dT) adaptor-primed first strand cDNA of L3 as the template. The 3' region was obtained using a specific primer to the histidine active site motif (CPF6) and the 3' adaptor primer, and the 5' region was obtained using the C. elegans SL1 sequence and a specific primer to the 3' non-coding region of the first fragment (CPR3). The nucleotide sequence of LOVCP and the predicted amino acid sequence are shown in Fig. 4. The 1113-bp cDNA sequence contains a single open reading frame extending from the postulated initiator methionine at positions 24–26 to the stop codon, TAA, at positions 942–944, and two putative polyadenylation signals, AAT-TAA, at positions 993–998 and 1024–1029. The open reading frame encodes 306 amino acids. The entire coding region predicts a proform of the enzyme with a molecular mass of 34.5 kDa. The 23 nucleotides of the 5'-untranslated region includes the 22 bp of the SL1 sequence, which is separated from the postulated initiating codon ATG (bp 24–26) by 1 nucleotide. A purine (adenine) in the −3 position to the first ATG is flanked by sequences in agreement with the Kozak initiation consensus (33). Sequence analysis from the postulated initiator methionine indicates a putative signal peptide of 20 amino acid residues, which is common in many cysteine proteases. Using the −1, −3 prediction method of von Heijne (34), a potential signal peptide cleavage site is indicated at residue 21, lysine, which would result in a proform of the enzyme with 286 amino acids and a predicted mass of 32 kDa. Comparison with the other members of the papain superfamily (35) suggested that the LOVCP proenzyme is processed into the mature protease by cleavage at the lysine residue (amino acid 66, Fig. 4), perhaps by autohydrolysis, as has been demonstrated for cathepsin L (36, 37). Cleavage of the propeptide region (45 amino acids) would result in a putative mature enzyme with 241 amino acids and a predicted mass of 27 kDa. The conserved cysteine, histidine, and asparagine active site residues are at position 31, 178, and 200, respectively, in the mature protein (Fig. 4, C, H, and N in bold at positions 96, 244, and 265 in the cDNA sequence, respectively). The mature enzyme contains two putative N-glycosylation sites (residues 187–189 and 286–288). An additional putative N-glycosylation site is present in the signal peptide (residues 16–18).

A homology search of LOVCP amino acid sequence against the PIR Protein Data Base showed a significant sequence similarity with other members of the cysteine protease family of the papain superfamily including papain, actinidain, cathepsin C, mammalian cathepsin L, and cathepsin B, and many of the proteozoa, plant, and Schistosoma mansoni cathepsin-like enzymes, ranging from 22% to 35% identity in the amino acid sequences (data not shown).

An interesting feature of the onchocerca cysteine protease is...
acids containing putative nucleotide SL1 sequence of sequence into GEX vector are marked with from this motif. The primers used for the subcloning of the OVCP enzyme. A termination codon (TGA) at residues 942–944 is marked by the putative cleavage site of the propeptide to release the mature Top arrow indicates the splicing sites in the genomic clone gOVCP.

The Protease Gene Is Single-copy—Identification of the Endogenous Larval Cysteine Protease—

The endogenous cysteine protease in molting larvae was identified initially with labeled inhibitors. One of the peptidyl monofluoromethylketone that has a tyrosyl residue, Mu-Tyr-O-methyl-homoPhe-FMK, was labeled with $^{125}$I and used in vitro to label the target cysteine protease during molting. This inhibitor reacts covalently and specifically with the cysteine residue in the active site and does not react with inactive or denatured enzymes or with other proteases or proteins (28).

Larvae were cultured for 2 days in the presence of the radiolabeled inhibitor, collected, and washed four times in PBS. Lysates were prepared and subjected to electrophoresis in a 12% acrylamide gel. An autoradiogram of the gel clearly showed one radio labeled band with a molecular mass of 72 kDa (Fig. 6c).

A major band of 72 kDa was also identified in the soluble and nondenatured extracts of larvae collected on day 2 and 3 in the culture supernatant collected from larvae that were cultured for 2 days in vitro. To detect the endogenous active enzyme, the samples were reacted with biotin-Phe-Ala-CHN$_2$ (29) before running on a SDS-PAGE gel and electrophoresis onto nitrocellulose filter. The inhibitor did not detect an active enzyme in extracts from L3 or L3 collected after only 1 day in culture (Fig. 6b). The similarity between the radiolabeled band and biotinylated bands confirmed that the endogenous active cysteine protease in molting larvae binds to the inhibitors is a 72-kDa protein species in SDS-PAGE gels.

As the cloned cysteine protease cDNA encoded a preproform of the enzyme with a molecular mass of 34.5 kDa, it was of interest to find out the relationship between the 72-kDa protein identified by the inhibitors and the native protein corresponding to the recombinant enzyme. Soluble extracts, prepared from L3 cultured for 1 day in normal medium before being metabolically labeled for 24 h with $^{[35]$S]methionine, were incubated with protein A-Sepharose and evaluated by 4–20% SDS-PAGE electrophoresis and fluorography. Three proteins of 72, 48, and 37 kDa were recognized (Fig. 6c). Crude extracts prepared from L3 collected on day 2 in culture were also analyzed by immunoblot analysis with the same antibodies. Three proteins of 72, 37, and 23 kDa were recognized (Fig. 6d).

The similarity between the 72-kDa protein band recognized
by the antibodies and the 72-kDa band recognized by the inhibitors confirmed our assumption that we have cloned the larval cysteine protease targeted by the cysteine protease inhibitors in vitro.

Localization of the Onchocerca Cysteine Protease in Larval Stages of the Parasite—We previously proposed that a cysteine protease has a critical role in the molting process of the Onchocerca volvulus infective larvae, L3 (15, 16). The results from this study clearly support this hypothesis. We have shown the inhibitory effects of a panel of FMK compounds on the molting of L3 to L4. The FMK are known to be highly specific inhibitors of cysteine proteases of various organisms.

Fig. 6. Identification of the endogenous cysteine protease in O. volvulus larval extracts. A, extracts of 100 larvae cultured in the presence of 50 μM iodinated Mu-Tyr-O-methyl-homoPhe-FMK for 2 days were separated by SDS-PAGE electrophoresis. The iodinated proteins were identified by autoradiography. Molecular size markers (kDa) are indicated on the left. B, extracts equivalent to 40 L3 (lane 5) or 40 L3 day 1 (lane 4), day 2 (lane 3), or day 3 (lane 2) in culture and supernatant from 50 larvae cultured for 2 days (lane 1) were reacted with biotin-Phe-Ala-CHN2, as described under "Materials and Methods." The reaction mixtures were separated by SDS-PAGE electrophoresis, the proteins were electroblotted onto nitrocellulose paper, and the biotin was detected by streptavidin-alkaline phosphatase and nitro blue tetrazolium. Molecular size markers (kDa) are indicated on the left. The 72-kDa protein (arrow) was recognized by the inhibitors and antibodies raised against the recombinant LOVCP protein. Molecular mass markers (kDa) are indicated on the left. The 37-kDa protein (arrowhead) was recognized by immuno precipitation and by immunoblot analysis with antibodies raised against the recombinant LOVCP cysteine protease.

Fig. 7. Ultrastructural localization by immunoelectron microscopy of the larval endogenous cysteine protease. Thin sections of O. volvulus: L3 (a), successive developmental stages during molting of L3 in vitro; day 1 (b), day 2 (c), day 3 (d) in culture; and L4 (e) were incubated first with rabbit anti-GST-LOVCP antibodies and then with protein A coupled to 15 nm gold particles for indirect antigen localization (bar, 0.25 μm). Note the labeling in the areas of L4 epicuticle/cuticle (small arrows, b, c, and d) and the basal layer of the L3 cuticle (arrowheads, b, c, and d), where the separation between cuticles takes place, and in the cuticle of L4 (e).

DISCUSSION

We previously proposed that a cysteine protease has a critical role in the molting process of the O. volvulus infective larva, L3 (15, 16). The results from this study clearly support this hypothesis. We have shown the inhibitory effects of a panel of FMK compounds on the molting of L3 to L4. The FMK are known to be highly specific inhibitors of cysteine proteases of...
During the molting process on days 1–3 than in earlier events. At this time the separation between the old and new cuticles is in progress to completion (Fig. 3, a and b). This conclusion is supported by the ultrastructural studies of the larvae that did not molt (Fig. 3, d–f). The FMK inhibitors induced morphological abnormalities in the cuticles of these larvae. It appeared that the separation between the old and new cuticles was inhibited, inducing in many larvae a very unusual swelling of the old cuticles, yet the synthesis of the cuticle of the L4 was not affected. In addition, in immunogold labeling experiments we were able to localize the cysteine protease with an antisera raised against the recombinant cysteine protease that was cloned from L3 of O. volvulus, LOVCP. Notably, these antibodies reacted in molting larvae with a protein present in the same areas where onchocystatin, the endogenous cysteine protease inhibitor of O. volvulus, was localized (16). Both proteins were localized in the areas around the region where the separation between the cuticles takes place, in some areas of the cuticle of the newly formed L4, and in the lower part of the old cuticle, where the separation had occurred (Fig. 7).

We propose that the biological effects of the FMK inhibitors were specifically due to the inhibition of a cysteine protease needed for the degradation of cuticular proteins in the region where the L3/L4 cuticle separation occurs. The inhibition of L4 development correlates with the timing of the appearance of the lakes in the cuticles, a process that precedes ecdysis, or larval exsheathment, the emergence of the L4 from the old cuticle of L3. A cathepsin L-like cysteine protease was recently shown to be also important for the molting of B. pahangi L3 (3).

An endogenous cysteine protease that has a probable function in the excystment of Paragonimus westermani metacecariae was recently described by Chung et al. (38). In this parasite, the authors have suggested that the dormant metacecariae begins to secrete the cysteine proteases on its arrival in duodenum, probably in response to the host signal of pH change. Although the mRNA encoding the onchocerca larval cysteine protease was isolated from vector-derived L3, enzyme activity was detected predominantly after the L3 were cultured at 37 °C for 24 h. This was confirmed by immunogold labeling of larvae at different stages in the molting process (Fig. 7), as well as by detection of an active enzyme with a biotinylated inhibitor (Fig. 6b). One possible explanation is that the transcription of the protease mRNA, and the translation of enzymatically inactive protease proform, may occur in the L3 and during the 1st day in culture. However, conversion of the protease to an active form occurs only after the parasite has started the molting process and the protease has been sequestered in the cuticle for 24 h. Another possibility is that the enzyme is complexed with the endogenous inhibitor, onchocystatin, and is dissociated from the inhibitor after 24 h when the separation between cuticles starts. The timing of the enzyme activation in both cases would be consistent with the detection of the active enzyme by the labeled inhibitor in larval extracts of larvae cultured for more than 24 h. Cuticle degrading proteases in an inactive complex were also found in the molting fluid of silkmoth larvae. Activation of the molting enzymes was thought to occur by dissociation of an inhibitor, the activation of proenzymes, or release of enzymes from a compartmentalized state (reviewed in Ref. 39). In O. volvulus larvae, cultured in the presence of a biotinylated derivative of a fluoromethylketone inhibitor, the enzyme-inhibitor complexes were co-localized in granules in the glandular part of the esophagus (Fig. 8, d and e), indicating that the enzyme is probably compartmentalized.

The fact that increased concentrations of the Z-Phe-Phe-FMK were required to demonstrate biological effects is consistent with limitations in the transport of this inhibitor across the cuticle of the larvae. Since these inhibitors were not lethal to the larvae, their effects appeared to be specific to the molting process and, therefore, indirectly indicated that a specific cysteine protease is present and active in the larvae during molting. A putative target enzyme was identified in extracts of larvae collected on day 2 and 3 (Fig. 6b). The inhibitors, Z-Phe-Ala-FMK and Z-Phe-Arg-FMK had to be present during the first 24–48 h, the beginning of the molting process, to exert a complete inhibitory effect on molting (Fig. 2). After the 3rd day in culture, only a partial inhibitory effect was observed. The partial effects on days 2 and 3 could be due to a subpopulation of larvae that were faster in their molting process and therefore were not affected anymore when the inhibitors were added. Many times during in vitro culturing, we have seen larvae that have already molted by day 3 (14). These findings implied that the effect of the inhibitors was more specific to the changes in the cuticle that occur during the molting process on days 1–3 than in earlier events.

**Fig. 8. Ultrastructural localization by immunoelectron microscopy of the larval endogenous cysteine protease.** Thin sections of O. volvulus molting larvae were incubated first with rabbit anti-GST-LOVCP antibodies and then with protein A coupled to 15 nm gold particles for indirect antigen localization (bar, 0.25 μm). Note the labeling in the secretory vesicles in the hypodermis of larvae day 3 in culture (big arrow, a), in the basal lamina lining the pseudocoelom in larvae day 2 in culture (b), and in cells of epidermal cord, C (c). In thin sections of larvae from day 2 in culture that were cultured in the presence of biotin-Phe-Ala-CHN2, for 24 h, the biotin was detected with streptavidin-gold (d). Note the presence of the label in the granules, g, which surround the glandular esophagus, Eo. Thin sections of these larvae were also probed with rabbit anti-GST-LOVCP antibodies and then with protein A coupled to 15 nm gold particles for indirect enzyme localization (e, bar 0.25 μm). The endogenous cysteine protease was co-localized in the granules, g, which surround the glandular esophagus. In addition, the label was detected in the basal lamina lining the pseudocoelom (arrows).
talized in these organelles before being transported via the pseudocoelom fluid and the secretory vesicles to the cuticle (Fig. 8, a and b). In previous immunogold labeling studies, we found that onchocystatin was also localized to the same internal organelles as the enzyme (data not shown).

The PCR approach used to clone the cysteine protease of *O. volvulus* L3 (LOVCP) allowed us to circumvent the considerable difficulty in obtaining adequate quantities of the onchocerca enzyme for partial amino acid sequence determination. We believe that LOVCP encodes the cysteine protease involved in molting of onchocerca larvae for several reasons. First, we found only one copy of the gene (Fig. 5) by Southern blot analysis at low stringency; it is expressed in L3 and molting larvae, antibodies raised against the recombinant enzyme localized the protein in the cuticles of molting larvae (Figs. 7 and 8), and antibodies raised against the recombinant enzyme recognized the same 72-kDa protein band that was detected by the cysteine protease inhibitors (Fig. 6). The sequence of the onchocerca cysteine protease clearly places the enzyme as a member of the papain family of cysteine proteases. The amino acid sequence of the *O. volvulus* cysteine protease deduced from the cDNA sequence has significant identity to many members of the papain superfamily of cysteine proteases, sharing 22–35% identical amino acids (data not shown). However, the onchocerca enzyme is unique in having a 5-amino acid repeat, CGSCW, which is part of the cysteine active site motif, CGSC-WAF, and two additional repeats of GSC. The significance or function of the 5-amino acid repeat is unknown. Expression of an active enzyme and comparative studies using deleted and site-directed mutants of the cDNA coding for LOVCP in *E. coli* or *Pichia pastoris* may provide clues to the possible function of these repeated amino acids.

By homology the predicted amino acid sequence of LOVCP is mostly similar to cathepsin C or cathepsin L (data not shown). The predicted molecular mass of the mature *O. volvulus* enzyme encoded by LOVCP is 27 kDa, whereas the molecular mass of the active enzyme detected by the cysteine protease inhibitors, and of one of the protein bands recognized by antibodies directed against the recombinant enzyme, is 72 kDa. The 37-kDa protein band recognized by the antibodies may correspond to the preproform of the enzyme (predicted molecular mass of 34.5 kDa), and the 23-kDa protein band may correspond to the mature protease (Fig. 6). The reasons for the size differences between the endogenous and recombinant proteases are not clear. It may be due to post-translational modification such as N-linked glycosylation, the endogenous enzyme forming dimmers resistant to the reducing agents in the SDS-PAGE buffers, or if the enzyme is bound to another protein to help export it to the hypodermis and the cuticle. Size differences between the endogenous and recombinant cysteine proteases were also observed in *Trypanosoma cruzi* (37).

The cloning of a cysteine protease required for the molting of *O. volvulus* L3 provides us with the opportunity to study the structure-function relations between the *O. volvulus* enzyme and its endogenous inhibitor, onchocystatin, and will also be useful for studying the regulation of cysteine protease activity during molting. Acknowledgments—We thank Dong-Hun Lee for DNA sequencing and preparation of synthetic oligonucleotides and Massah Abdullah and Johnette Brown for technical assistance. We are grateful to Dr. Robert Smith and Prototek Inc. for the FMK compounds. We are also grateful to Dr. Francine Perler and Dr. Colvin Redman for comments on the manuscript and to Dr. Xiquiang Hong for useful discussions.

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