Cuticle of *Caenorhabditis elegans*: Its Isolation and Partial Characterization

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ABSTRACT The adult cuticle of the soil nematode, *Caenorhabditis elegans*, is a proteinaceous extracellular structure elaborated by the underlying layer of hypodermal cells during the final molt in the animal's life cycle. The cuticle is composed of an outer cortical layer connected by regularly arranged struts to an inner basal layer. The cuticle can be isolated largely intact and free of all cellular material by sonication and treatment with 1% sodium dodecyl sulfate (SDS). Purified cuticles exhibit a negative birefringence due to ordered material in the basal cuticle layer. The cuticle layers differ in their solubility in sulfhydryl reducing agents, susceptibility to various proteolytic enzymes and amino acid composition. The struts, basal layer, and internal cortical layer are composed of collagen proteins that are extensively cross-linked by disulfide bonds. The external cortical layer appears to contain primarily noncollagen proteins that are extensively cross-linked by nonreducible covalent bonds. The collagen proteins extracted from the cuticle with a reducing agent can be separated by SDS-polyacrylamide gel electrophoresis into eight major species differing in apparent molecular weight.

The cuticle of the small, free-living soil nematode, *Caenorhabditis elegans*, is a multilayered extracellular structure that completely surrounds the animal except for small openings into the pharynx, anus, excretory pore, and vulva. The entire cuticle is sloughed off and replaced at each of four postembryonic molts under the direction of an underlying layer of hypodermal tissue, which, for the most part, consists of a single large syncytium that extends throughout the length of the animal (1). Both the synthesis and secretion of cuticle components by the hypodermis are tightly coupled to the molting cycles (2, 3). The molts are not equivalent, however, for at least four of the developmental stages of *C. elegans* possess cuticles that differ from one another in morphology (4, our unpublished results). *C. elegans* is an organism well-suited to genetic studies (5), and many mutants have been isolated that exhibit gross morphological changes in cuticle structure (6; unpublished results). These facts lead us to believe that the formation of the *C. elegans* cuticle is a promising system in which to study the genetic specification of a complex extracellular structure.

However, little work has been reported on the ultrastructure or biochemical composition of the *C. elegans* cuticle (7, 8). Most biochemical studies of nematode cuticles have focused on the large parasitic nematode, *Ascaris lumbricoides*, where surgical isolation of single cuticles is convenient. The *Ascaris* cuticle has been shown to be composed principally of collagen proteins cross-linked by disulfide bonds (9). The cuticle of the small free-living nematode, *Panagrellus silusiae*, has also recently been shown to consist primarily of collagen proteins cross-linked by disulfide bonds (10).

We describe here methods for obtaining pure cuticles from synchronous populations of *C. elegans* adults. We also characterize the morphological features of the adult cuticle and present information on its biochemical composition.

MATERIALS AND METHODS

Nematode Strains and Cultivation

The wild type Bristol strain of *Caenorhabditis elegans*, designated N2, was originally obtained from Sydney Brenner (Cambridge, England). *fer-1(hc1ts)I* and *him-8(el489)IV* have been described previously (11, 12).

The dauer larva is a developmental alternative to the L3 juvenile, which is formed in response to adverse environmental conditions (4). Large numbers of dauer larvae were obtained using egg-white plates (a modification of a method communicated to us by David Baillie, Simon Fraser University), which are prepared by stirring the white of one chicken egg with 50 ml of boiling distilled water for several seconds, homogenizing the mixture for 1 min in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.), and layering 3–4 ml of the resulting liquid slurry onto a standard 100-mm NGM plate (5). When dry, these plates are seeded with ~1,500 dauer larvae and incubated at 20°C. Approximately 1×10^5 dauer larvae are usually recovered per plate after 5–7 days' incubation time.

For purification of dauer larvae, animals from individual egg-white plates

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were incubated with 5–10 ml of 1% sodium dodecyl sulfate (SDS) for 30–60 min (4), collected by low speed centrifugation, resuspended in 0.5–1 ml of M9 buffer (5), and spun through a 2-ml cushion of ice-cold 15% ficoll for 10 min at 300 *g*. Intact dauer larvae pellet through the ficoll while egg matter and worm carcasses remain at the interface. This isolation procedure was usually repeated. Stock solutions of dauer larvae were stored at 16°C in M9 buffer until use.

Large synchronous adult populations were grown from dauer larvae on 100-mm petri dishes containing 30 ml of 8P media (13), using *E. coli* strain OP50 as a food source. Adult animals were harvested after 28 h of growth from dauer at 25°C (or 40 h at 20°C), which is several hours after the completion of the final molt (2).

A population enriched for males was obtained using *him-8*, which produces 36% male progeny (12). These populations were further enriched for males by layering synchronous adult animals onto a 30-μm mesh nylon filter suspended over a dish of gently stirred M9 buffer (Michael Klass, University of Houston, personal communication). Because of their smaller size, males are able to crawl through the mesh openings while hermaphrodites are retained. 98% of the animals collected after 4-h were males.

Isolation of Cuticles

The methods outlined below are for a typical preparation of 1×10^5 adults. All extraction steps except those involving SDS were performed on ice. Nematodes ($2–3 \times 10^4$/tube) were washed free of bacteria with M9 buffer, suspended in 5 ml of sonication buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [PMSF]), and given ten 20-sec bursts of a Biosonik III sonifier (Bronwill Scientific, Inc., Rochester, N. Y.). Cuticle pieces were collected by centrifugation for 4 min at 300 *g* and washed several times with 10 ml of sonication buffer. Cuticles were then transferred to a 1.5-ml microfuge tube, suspended in 1 ml of ST buffer (1% SDS, 0.125 M Tris-HCl, pH 6.8) and heated for 2 min at 100°C. After several hours of incubation at room temperature, cuticles were spun down in a Brinkmann microcentrifuge (60 sec; Brinkmann Instruments, Inc., Westbury, N. Y.) and extracted again with ST buffer, as described above. As will be demonstrated in Results, cuticles treated this way appear free of cellular and membranous material. However, cuticle preparations from wild-type (N2) animals are contaminated by empty eggshells derived from fertilized eggs contained within the hermaphrodites and released during sonication. For many experiments, this contaminant was eliminated by using a temperature-sensitive fertilization-defective mutant of *C. elegans, fer-1*, and growing the animals under restrictive conditions (i.e., at 25°C). The disulfide cross-linked proteins of the cuticle were solubilized by heating purified cuticles for 2 min at 100°C in 0.5 ml of ST buffer, 5% β-mercaptoethanol (BME) and agitating gently for several hours at room temperature. The insoluble cuticle material was extracted again with ST buffer, 5% BME, as described above, washed several times with distilled water, and lyophilized. All protein samples were stored frozen at −20°C.

Gel Electrophoresis

Discontinuous SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out in a Model 220 electrophoresis apparatus (Bio-Rad Laboratories, Richmond, Calif.) using, with only minor modifications, the buffer systems described by Laemmli and Favre (14). Proteins were visualized by staining for 1 h with 0.1 or 0.25% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid, and destained by diffusion in 5% methanol and 10% acetic acid. Apparent molecular weights were determined using the following proteins as molecular weight markers: myosin (212,000), β-galactosidase (116,000), phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), and myoglobin (17,200). Individual lanes of slab gels were scanned at 595 nm in a Beckman Model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a linear transport gel scanner, and the relative proportions of the different protein bands estimated by weighing cut outs of the individual peaks.

Electron Microscopy

Nematodes and cuticle preparations were fixed overnight in 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4, postfixed for 1 h in 1% OsO_4 in 0.1 M sodium phosphate, pH 7.4, and dehydrated through a graded ethanol series, all at 0°C, as described by Mackenzie et al. (15). Nematodes were cut in half with a scalpel after 1 h in the first fixative. Samples for scanning electron microscopy were critical point–dried with CO_2, coated with alternating layers of carbon and gold, and observed in a JSM-2 scanning electron microscope. Samples for transmission electron microscopy were agar-embedded before dehydration and final embedding in Araldite 502 resin (Ladd Research Industries, Inc., Burlington, Vt.). Thin sections were stained with uranyl acetate, followed by lead citrate, and observed in a Jeol 100-B transmission electron microscope.

Light Microscopy

A Zeiss WL microscope equipped with phase-contrast optics and crossed polarizers was used for these studies. Photomicrographs were made with an Olympus camera (Olympus Corporation of America, New Hyde Park, N. Y.) attached to the microscope.

Enzyme Digestion Studies

For light microscopy studies, SDS-purified cuticles were washed once with an appropriate buffer (see below) and incubated with the following enzymes at a density of 2×10^4 cuticles/ml: *Clostridial* collagenase (Type III; Sigma Chemical Co., St. Louis, Mo.), 2 mg/ml in 100 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$; pronase (Worthington Biochemical Corp., Freehold, N. J.), 400 μg/ml in 100 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$; elastase (Type I; Sigma), 5 mg/ml in 200 mM Tris-HCl, pH 8.8; chitinase (Sigma), 12 mg/ml in 50 mM potassium phosphate, pH 6.2; and lipase (Type VII; Sigma), 500 μg/ml in 50 mM Tris-HCl, pH 7.5. Incubations were carried out at 37°C in microcentrifuge tubes or at room temperature (~22°C) on glass microscope slides ringed with petroleum jelly.

For electrophoresis studies to determine effects of collagenase treatment, BME-soluble cuticle proteins in ST buffer, 5% BME were precipitated with 90% acetone (16), air-dried, suspended in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$, 10 mM N-ethylmaleimide, and incubated with and without (controls) *Clostridial* collagenase (Type VI, Sigma) for 2–12 h at 37°C, at enzyme to substrate ratios of 1:3 or 1:10. Noncollagen proteins normally used as molecular weight markers were treated with collagenase in parallel incubations. Reactions were terminated by dilution into electrophoresis sample buffer and heating for 2 min at 100°C. Samples were electrophoresed directly.

Chemical Methods

Protein concentrations were estimated by a modification of the method of Lowry et al. (17) using bovine serum albumin as a standard. Proteins were precipitated from SDS solutions and washed twice with 90% acetone before analysis.

Neutral sugar content was estimated by the phenol-sulfuric acid assay of Dubois et al. (18) using D-glucose as a standard. Lyophilized cuticle samples were hydrolyzed for 2 h in 1.5 NH_2SO_4 at 100°C before analysis. Amino sugars were quantified using D-glucosamine as a standard, as described by Gatt and Berman (19).

Samples for amino acid analysis were hydrolyzed in evacuated sealed tubes at 110°C for 24 h in 6 NHCl and processed on a Durrum D-500 amino acid analyzer. Cysteine was determined as cysteic acid on samples that had been oxidized with performic acid (20). No corrections were made for amino acids destroyed during hydrolysis.

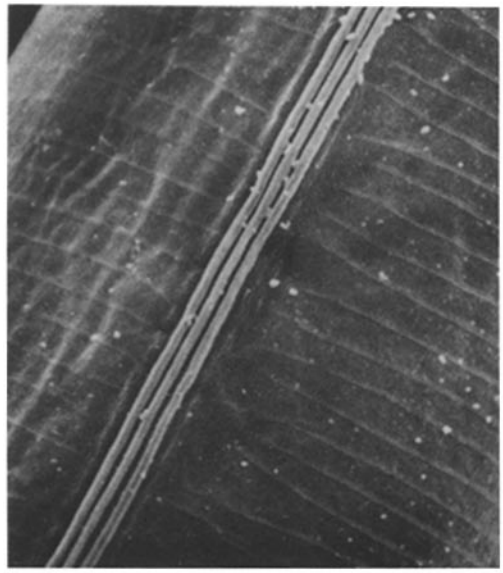

FIGURE 1 Surface features along a lateral side of an adult cuticle seen in the scanning electron microscope. Many annuli are visible in regular transverse rows perpendicular to single tri-lobed ala. The orientation of the annuli and alae in a cuticle are sketched in Fig. 3. Field width, 30 μm.

RESULTS

Adult Cuticle Structure

C. elegans adults are ~1,300 μm long and 60 μm wide. Except for small openings in the pharynx, anus, excretory pore, and vulva, the entire animal is encased within an extracellular cuticle that is ~0.5 μm thick. The structure of the cuticle is basically similar throughout most of the length of the animal. The most prominent surface features of the cuticle are the alae, which run along the lateral sides of the animal and are composed of three closely spaced treadlike projections upon which the animal lies when moving (Figs. 1 and 2 *C*). The cuticle is indented circumferentially at regular 1-μm intervals, creating pleated-appearing annuli (Figs. 1 and 2 *A*). The path of each annulus is continuous around the worm except at the lateral ridges where it is interrupted by the alae.

The internal architecture of the cuticle is complex, consisting of two main layers of about equal thickness separated by a clear space that is probably fluid-filled (Fig. 2). Columnar structures, which we term "struts", join one layer to the other and are most often seen lying beneath the annular furrows. Struts are not observed beneath the alae. The inner or basal cuticle layer is comprised of three sublayers: an amorphous layer adjoining the hypodermis and two layers of tightly organized fibers that spiral around the animal in opposite directions. The outermost fiber layer spirals around the animal in a clockwise direction from head to tail, whereas the inner fiber layer spirals in a counterclockwise direction. Small patches of fibers are also present in the outer or cortical cuticle layer underlying the three alae ridges. The cortical cuticle layer is

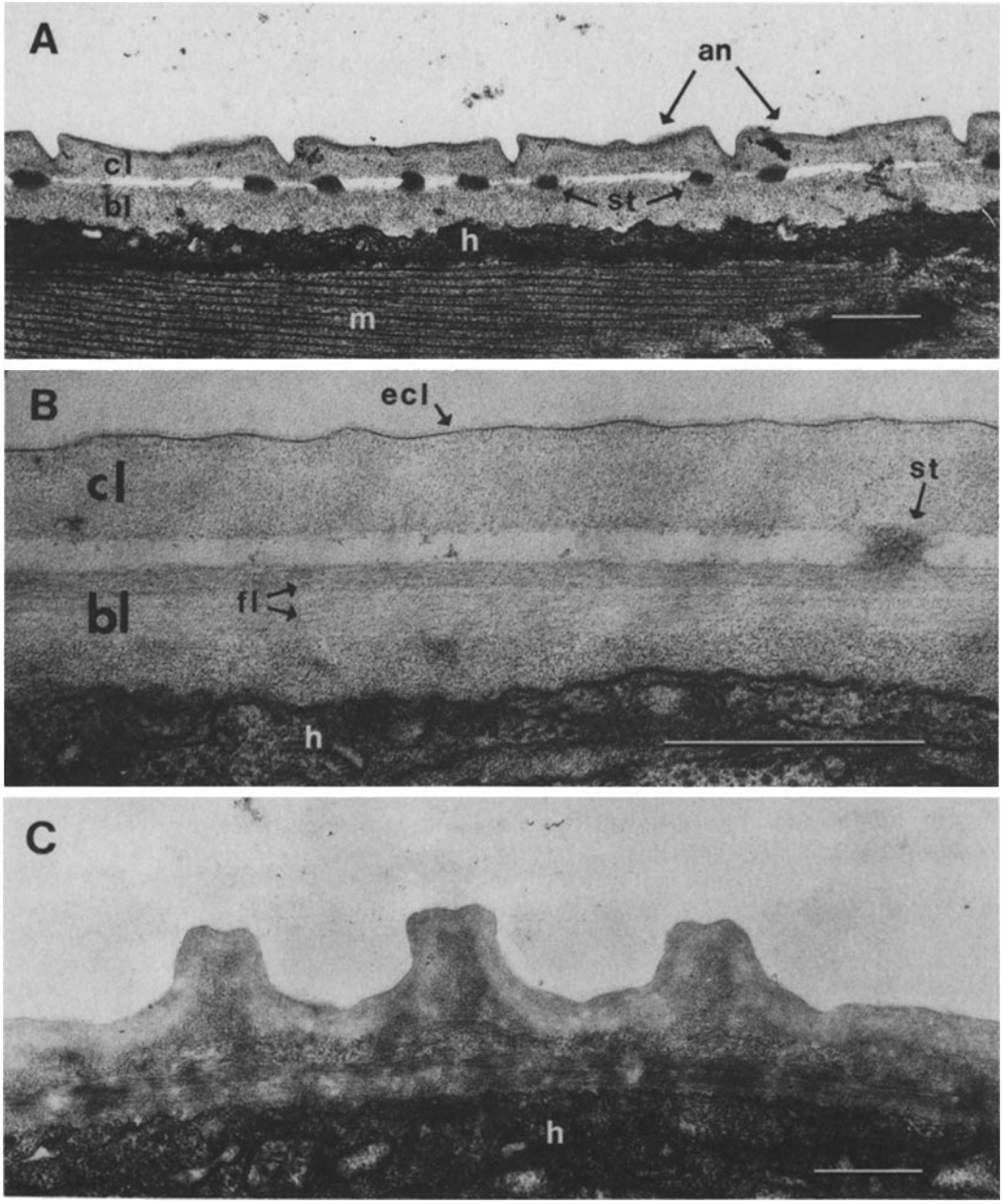

FIGURE 2 Transmission electron micrographs of the adult cuticle. A, longitudinal section showing the organization of the basal (*bl*) and cortical (*cl*) cuticle layers, struts (*st*), and annuli (*an*). The hypodermis (h) and a muscle cell (m) are also visible. × 23,400. B, higher magnification transverse section showing the detailed fine structure of the cuticle. The two fiber layers (*fl*) and external cortical layer (*ecl*) are indicated. × 70,300. C, transverse section of the lateral alae. × 29,100. Bar, 0.5 μm.

bounded by a thin, very osmiophilic layer that is referred to as the external cortical layer (21). The remainder of the cortical layer appears devoid of any regular structure, although at least two layers of different electron density can be distinguished. The most dense of these layers, which directly apposes the external cortical layer, becomes tapered toward each of the annular furrows.

Variations in the thickness and morphology of the cortical layer give rise to the surface structures of the cuticle, the alae and annuli. The morphology of the basal layer, in contrast, appears uniform throughout the worm. A diagram summarizing these features of cuticle morphology is presented in Fig. 3.

Morphological Characteristics of Isolated Cuticles

The cuticle can be isolated largely intact by sonication and brief treatment with 1% SDS at 100°C, as described in Materials and Methods. SDS treatment is necessary to remove tightly adhering muscle fragments and other remains of the body wall. Cuticle architecture is not readily observable by light microscopic observation of living animals. The cuticle is transparent and obscured by underlying tissues. Isolated cuticles, as observed by phase-contrast microscopy, generally maintain a rigid, tubelike appearance even after cellular material has been removed (Fig. 4). Struts appear as small, round, dense bodies and can be clearly seen to be organized into regular transverse rows on either side of the annular indentations (Fig. 4 *B*). To a much lesser extent, struts are also present singly in an apparently random arrangement between the annular furrows. The cuticle linings of the pharynx, anus, excretory pore, and vulva are also preserved by these treatments (see Fig. 4 *A*). The general cuticle surface topography is apparently little affected by these treatments (Fig. 4 *C*), as judged by comparison to surface anatomy of animals prepared for SEM (Fig. 1) or living animals coated with fluorescein-conjugated goat anti-rabbit antibodies (reference 6).

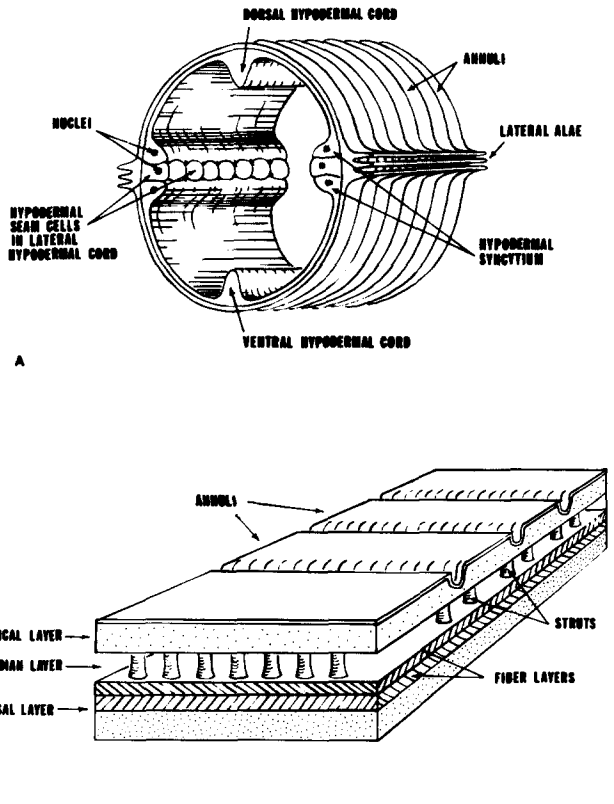

FIGURE 3 Diagrammatic sketch of the adult cuticle. *A*, cross section of an adult nematode showing the general organization of the cuticle and underlying hypodermis. *B*, magnified view of the internal anatomy of the cuticle. Neither figure is drawn to scale.

Transmission electron micrographs of purified cuticles (Fig. 5) verify that cellular material, including muscle bundles and basement lamina, have been removed by SDS. The basal and cortical layers are easily distinguished in these cuticles and appear similar to the corresponding structures of *in situ* cuticles (compare to Fig. 2 *B*). The two cuticle layers are usually separated from each other but joined by the struts beneath the annular furrows. Although struts are not evident in the electron micrographs presented in Fig. 5, they were usually observed in thicker sections. The two fiber layers appear more disorganized in SDS-treated cuticles and the annuli are also less sharply defined. The lateral alae typically display a buckled appearance (Fig. 5 *B*), allowing clear visualization of the fibrous elements that underlie these structures. The electron microscopy evidence indicates that whereas sonication and SDS treatment may have partially denatured portions of the cuticle (e.g., the fiber layers) or solubilized some structural components, these treatments leave cuticles intact with many morphological features still preserved.

Purified cuticles exhibit a weak but characteristic birefringence when viewed with plane-polarized light (Fig. 6). Using compensators, we have determined that the direction of birefringence is transverse to the long axis of the worm (negative birefringence), opposite to that of the muscles. Along two narrow lines corresponding to the lateral alae, cuticle birefringence is positive rather than negative. The cuticle linings of the anus and pharynx also appear birefringent.

Biochemical Composition of Isolated Cuticles

As a gross measure of composition and regional differentiation, we examined the susceptibility of purified cuticles to digestion by the various enzymes listed in Table I. The effects of these enzymes on the two visible cuticle layers and the struts were monitored by phase-contrast microscopy. Sample results are shown in Fig. 7. Within 2 h, the inner cuticle layer and struts are destroyed by bacterial collagenase or pronase. The struts dissolve first, allowing the inner and outer cuticle layers to separate from one another and causing the cuticles to appear as "double bags." The outer cuticle layer becomes faint after treatment with these enzymes, but is still present after 18 h of incubation. The cortical layer material resistant to these enzymes exhibits no detectable birefringence. The cuticle lining of the pharynx is not visibly digested by collagenase but is digested by pronase. Elastase digests both the inner and outer cuticle layers, but not the pharynx cuticle. These experiments indicate that protein is primarily responsible for the integrity of the cuticle and that differences exist in the primary structures of the protein components of the inner and outer cuticle layers and the pharynx cuticle.

Addition of sulfhydryl reducing agents (BME or dithiothreitol) to purified cuticles in the presence or absence of denaturing agents causes them to lose most of their structure. 69% of the cuticle by weight is dissolved (Table II). By phase-contrast microscopy, the struts and inner cuticle layer are observed to dissolve, leaving an outer cuticle layer that collapses upon itself in pleats (Fig. 4 *D*, Table I). The cuticle lining of the pharynx

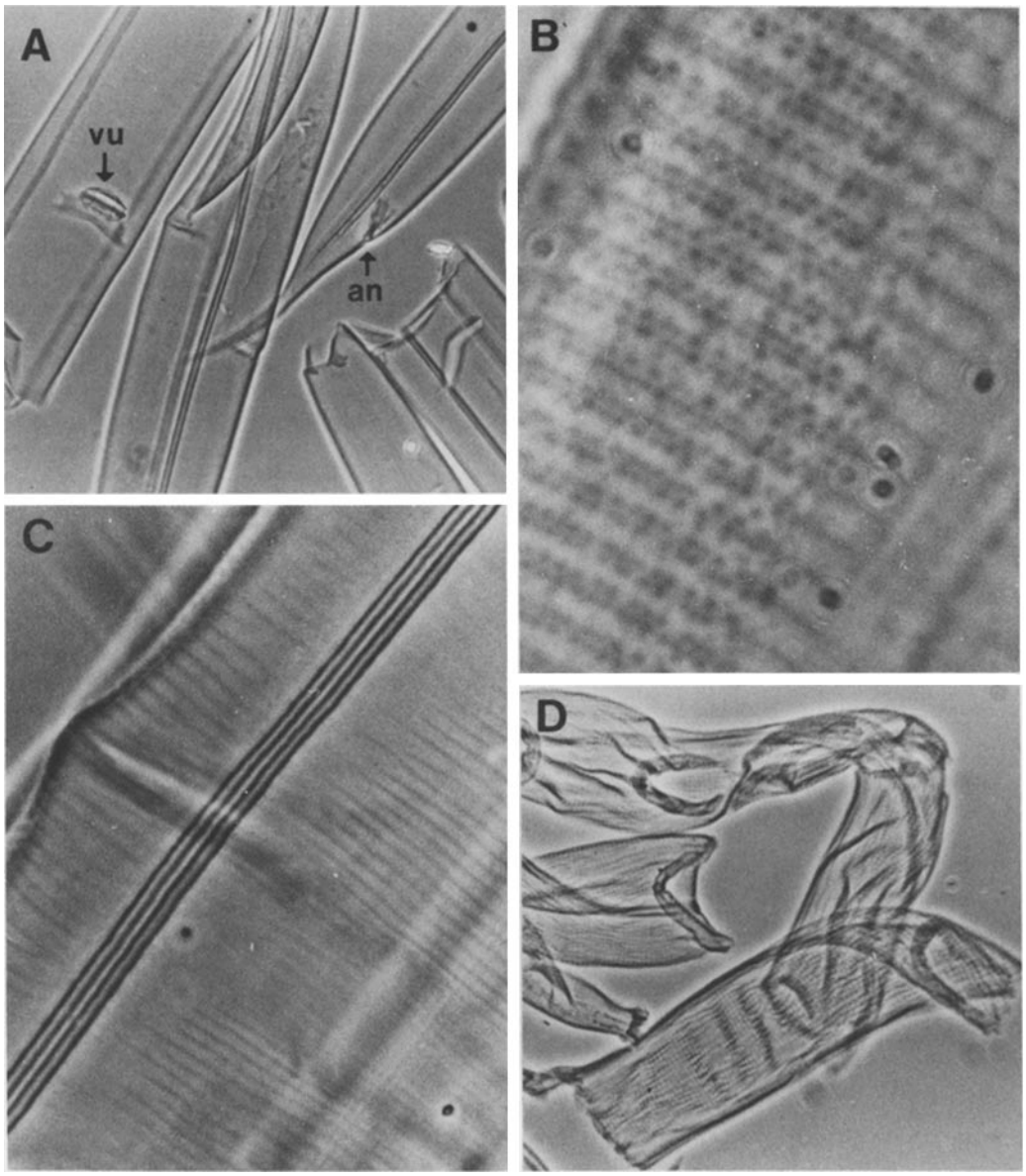

FIGURE 4 Phase-contrast micrographs of SDS-purified cuticles. *A*, low magnification micrograph showing the rigid, tubelike appearance of purified cuticles. Arrows point to the cuticle lining of the vulva (*vu*) and anus (*an*). × 300. *B*, higher magnification view showing the ordered array of the struts beneath the annular furrows. × 5,000. *C*, annuli and lateral alae of purified cuticles. × 2,000. *D*, cuticle material remaining after treatment with BME. × 300.

is also solubilized. The insoluble cortical layer material is no longer birefringent and the demarcations of the annuli and alae are no longer distinct in these cuticles. Electron micrographs of the insoluble cuticle material (Fig. 5) confirm that the basal layer and struts are dissolved by BME and indicate further that most of the internal cortical layer has also been solubilized. Only the external cortical layer and a small amount of adhering internal cortical layer material are left intact after treatment with BME.

As a further measure of chemical differences, amino acid compositions were determined for the protein components of the BME-soluble and insoluble cuticle fractions (Table III). The compositions of both groups of proteins are unusual. The soluble cuticle proteins have an amino acid profile characteristic of collagens: these proteins contain large amounts of glycine (26%), alanine (11%), proline (11%), and hydroxyproline (12%), and are rich in the acidic amino acids and poor in the basic and aromatic amino acids. Hydroxylysine was not detected. These proteins also contain significant amounts of cysteine (3%).

The insoluble cuticle proteins have a composition similar to, but distinct from that of, the soluble cuticle proteins. Most notably, the insoluble proteins contain decreased amounts of glycine (22%) and the basic amino acids and increased amounts of alanine (19%) and valine. Although the total imino acid content (21%) of the insoluble cuticle proteins is similar to that of the soluble proteins, many fewer of the proline residues are hydroxylated.

Both cuticle fractions contain a small amount of carbohydrate: the insoluble cuticle material contains both neutral and amino sugars, whereas only neutral sugars were detected in the soluble cuticle material (Table II).

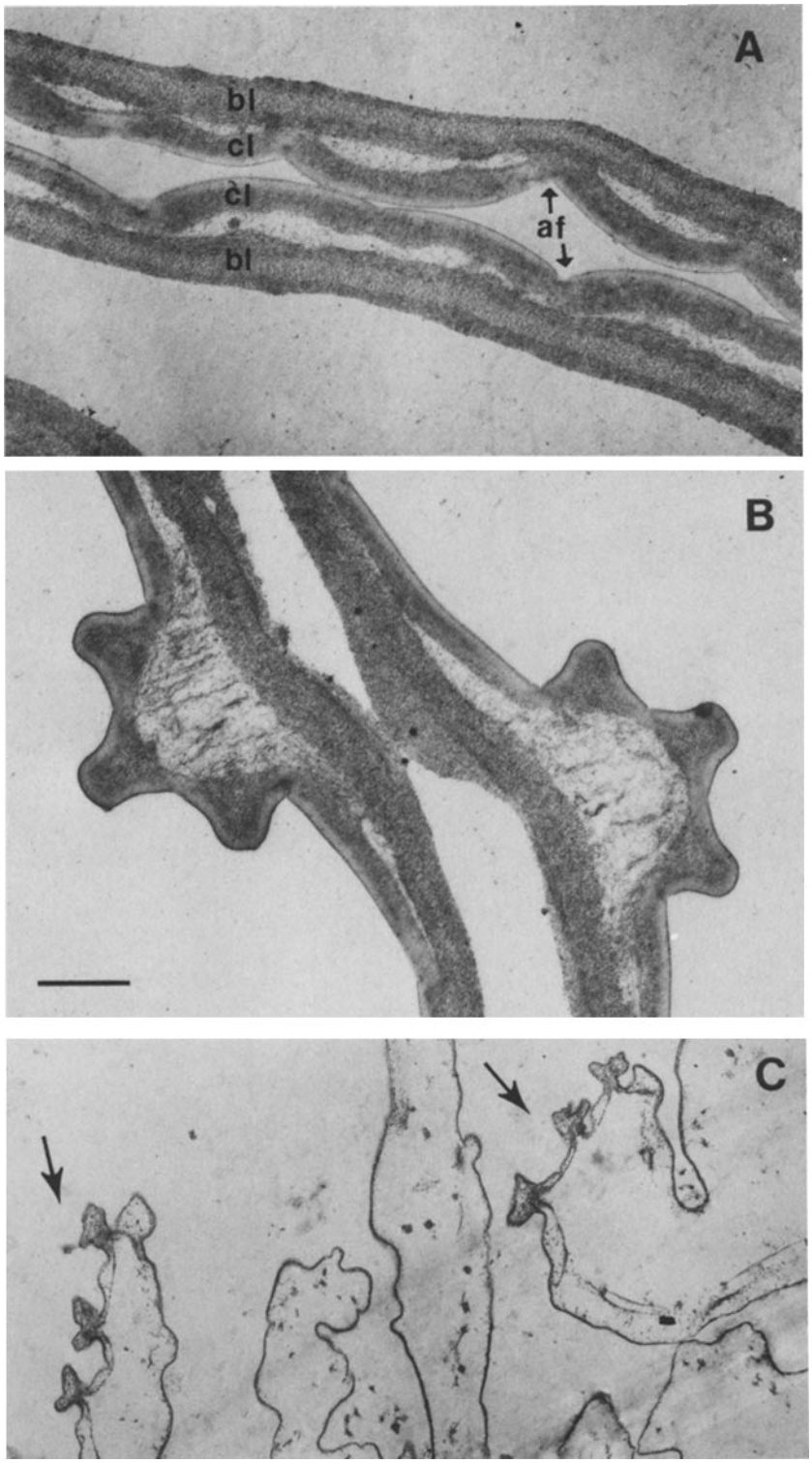

FIGURE 5 Transmission electron micrographs of purified adult cuticles before and after treatment with BME. *A* and *B*, SDS-purified cuticles. The three sublayers of the basal layer (*bl*) and varying electron-dense zones of the cortical layer (*cl*) are clearly visible in the cuticles shown in *A*. Only one of the two fiber layers appears fibrillar in these cuticles, due to the orientation of the section. Annular furrows (*af*) are indicated. *B*, buckled appearance of the lateral alae after treatment with SDS. Note the fibrillar material underlying the alae. *C*, cuticle material remaining after treatment with BME. Arrows point to the remains of the lateral alae. All micrographs × 12,200. Bar, 1 μm.

Characterization of Soluble Cuticle Proteins by SDS PAGE

The proteins released from purified cuticles by BME were analyzed on SDS-acrylamide gels of various percentages. On 6% gels (Fig. 8*A*), these proteins are separated into eight major components of approximately 55,000, 70,000, 90,000, 105,000, 140,000, 170,000, 200,000, and 210,000 mol wt. These major proteins have been named cpA through cpH in order of increasing molecular weight (Table IV). Several minor proteins

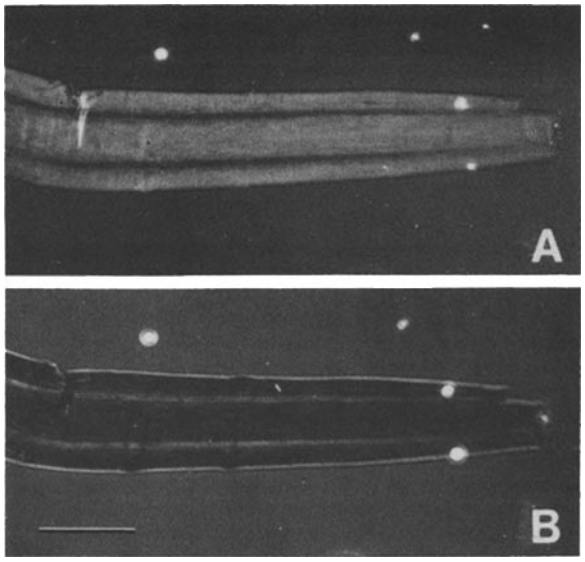

FIGURE 6 Birefringence of the adult cuticle. Note that the lateral alae have a birefringence opposite to that of the rest of the cuticle. Specimen stage in *B* is rotated 45° with respect to *A*. Bar, 50 μm.

interspersed among the major proteins (e.g., between A and B and between D and E, Fig. 8*A*) were occasionally but not always observed. Many minor proteins of very high molecular weight (>220,000) are routinely observed on these gels. Their absence on gels of material treated with pepsin at low temperature suggests that they are cross-linked aggregates of lower

TABLE I

Effects of Enzymes and β-Mercaptoethanol on Adult Cuticle Structure *

Treatment	Basal cuticle layer	Cortical cuticle layer	Pharynx cuticle
Collagenase	+	0	0
Elastase	+	+	0
Pronase	+	0	+
Chitinase	0	0	0
Lipase	0	0	0
β-Mercaptoethanol	+	0	+

* SDS-purified adult cuticles were incubated with the above enzymes or β-mercaptoethanol, as described in Materials and Methods, and the effects of these treatments on the two visible cuticle layers and pharyngeal cuticle material were monitored by phase-contrast microscopy. (+) indicates complete digestion, whereas (0) indicates no or only partial digestion.

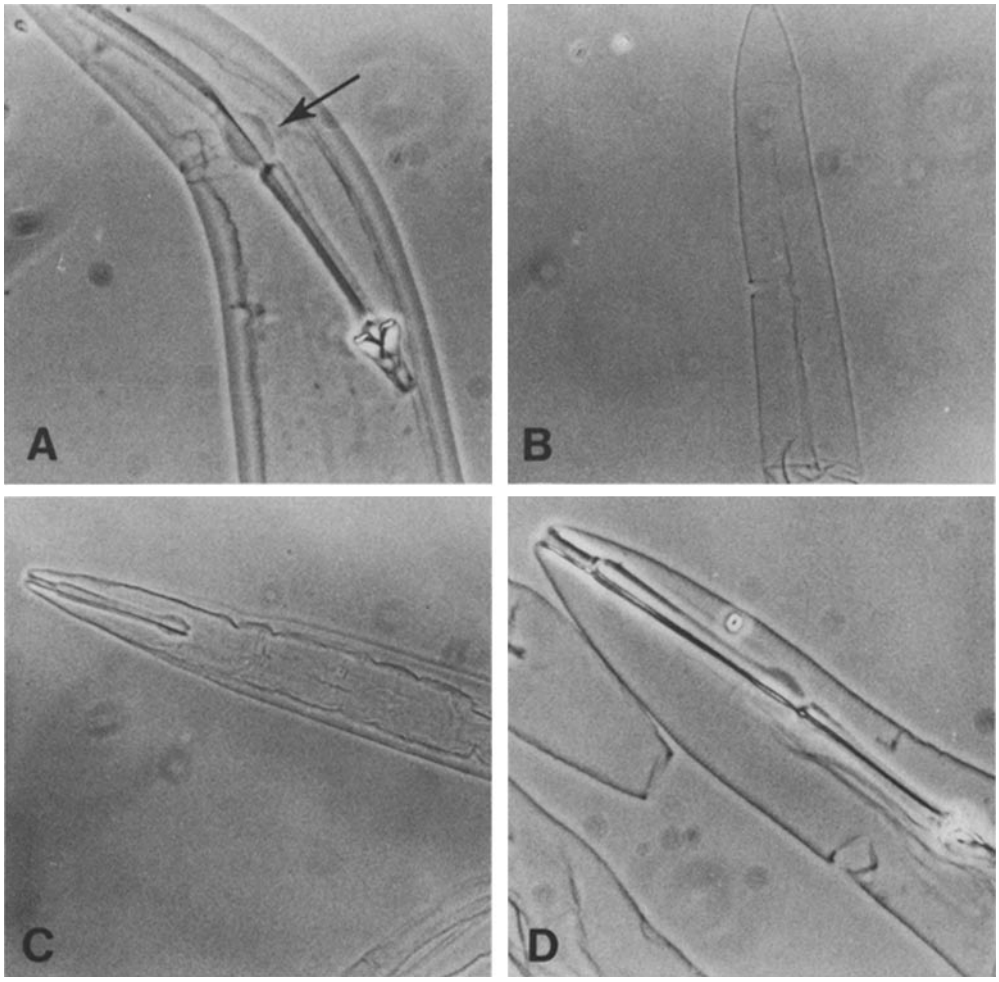

FIGURE 7 Effects of pronase and collagenase on purified adult cuticles. SDS-purified adult cuticles were washed once with an appropriate buffer and incubated at 22°C for the times indicated with either pronase or collagenase as described in Materials and Methods. *A*, pronase, 45 min. Unlike untreated controls (Fig. 4*A*), pronase-treated cuticles appear as "double bags" due to separation of the outer and inner cuticle layers. Arrow points to the cuticle lining of the pharynx. × 600. *B*, pronase, 4 h. The inner cuticle layer has been completely digested and the outer cuticle layer appears faint. × 250. *C*, Collagenase, 1.3 h. Cuticles appear as double bags. × 280. *D*, collagenase, 4 h. The inner cuticle layer has been completely digested and the outer cuticle layer appears faint. The cuticle lining of the pharynx is still present and does not appear to be digested. × 600.

molecular weight material (unpublished results). Four additional minor proteins of low molecular weight (15–40,000) are detected when soluble cuticle proteins are electrophoresed on 10% gels (Fig. 8 *B*). Three of the major proteins, cpF, cpG, and cpH, show increased apparent molecular weights on 10 vs. 6% gels, whereas cpB exhibits a slightly decreased molecular weight (by ~10–15%) on 10 vs. 6% gels.

cpC and cpD account for the major portion (76%) of total soluble cuticle protein in bands below 220,000, and these two proteins are present in a weight ratio of ~2:3, respectively. The proportion of total soluble cuticle protein present in the other major bands is given in Table IV. A variable amount (~20%) of total soluble cuticle protein is present in bands >220,000, with most of this material barely entering a 6% gel.

The banding patterns observed on these gels are highly reproducible in terms of the mobilities and relative amounts of the various protein species. Identical gel patterns are observed

TABLE II

Dry Weight and Carbohydrate Composition of the Adult Cuticle *

	Dry weight	Neutral sugar	Amino sugar
	μg/10³ nematodes	%	%
Adult nematodes	469		
Purified cuticles	21.4		
Insoluble cuticle material	6.6	0.2	0.9
Soluble cuticle material	14.8‡	1.7	0

* Data are for 28-h postdauer *fer-1* adults and are the means for at least three determinations. Dry weights were determined on samples that had been rinsed several times with distilled water and lyophilized. Sugar content is given as percentage dry weight.

‡ Derived by substracting insoluble cuticle dry weight from purified cuticle dry weight.

TABLE III

Amino Acid Composition of β-Mercaptoethanol-soluble and -insoluble Adult Cuticle Proteins *

Amino Acid	Soluble proteins	Insoluble proteins
	Residues/1000 total amino acids	
Cysteine‡	30	25
Hydroxyproline	120	75
Aspartic acid§	80	59
Threonine	22	26
Serine	38	44
Glutamic acid§	100	110
Proline	113	134
Glycine	263	224
Alanine	112	195
Valine	19	34
Methionine	2.5	2.1
Isoleucine	10	9.2
Leucine	14	14
Tyrosine	5.2	7.5
Phenylalanine	9.6	8.8
Histidine	11	10
Hydroxylysine	0	0
Lysine	23	11
Arginine	30	14

* Averages of four 24-h hydrolyses on three different cuticle preparations obtained from *fer-1* adults.

‡ Determined as cysteic acid in one sample that was oxidized with performic acid.

§ Includes the amidic forms.

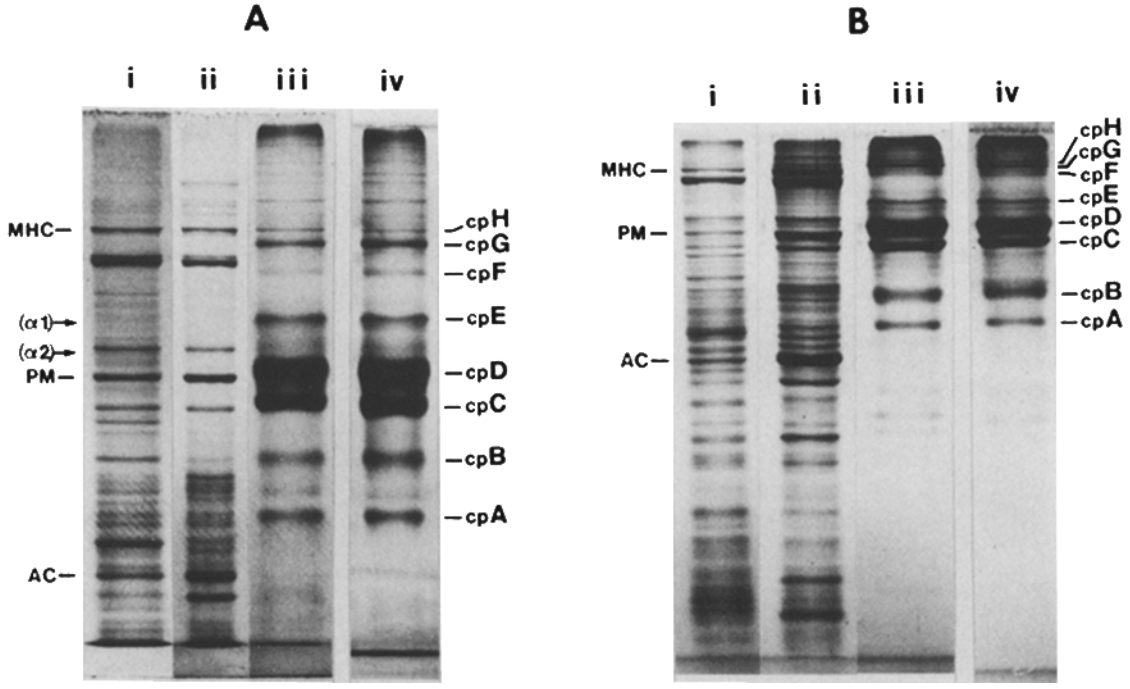

FIGURE 8 6% (*A*) and 10% (*B*) SDS-polyacrylamide gel electrophoresis of wild-type (N2) and *fer-1* soluble cuticle proteins. Proteins released during sonication of wild-type adult nematodes and by treatment of cuticles with SDS are displayed in lanes i and *ii*, respectively. The muscle proteins, myosin heavy chain (MHC, 210,000 mol wt), paramyosin (PM, 92,500 mol wt), and actin (AC, 42,000 mol wt), are indicated. Arrows point to the positions where the α1 and α2 chains of rat-tail tendon collagen migrate in our gel system. *iii*, soluble cuticle proteins of N2 adults. *iv*, soluble cuticle proteins of *fer-1* adults. The major cuticle proteins are labelled cpA through cpH. The approximate molecular weights of these proteins are given in Table IV. Each gel lane contains 30 μg (*A*) or 50 μg (*B*) of protein.

TABLE IV

Soluble Proteins of the Adult Cuticle

Protein	Apparent molecular weight*	Total Soluble Cuticle Protein‡
		%
cpA	54,000	4.5 ± 1.9
cpB	70,000	6.1 ± 0.7
cpC	91,000	28.6 ± 2.6
cpD	106,000	47.4 ± 2.7
cpE	138,000	5.2 ± 2.2
cpF	171,000	1.8 ± 0.6
cpG	197,000	5.6 ± 1.3
cpH	212,000	1.4 ± 0.8

* Molecular weights were determined on 6% SDS gels.

‡ The percentage of total soluble cuticle protein in each band was determined by scanning Coomassie Blue-stained gels at 595 nm and weighing cutouts of the individual peaks. The amounts of protein applied to the gels varied between 20 and 60 μg. Proteins present in bands >220,000 are not included in these calculations because of nonlinearity. This material represented 3 and 25% of total soluble cuticle protein when 20 and 60 μg of cuticle protein, respectively, were analyzed. Data are the means ±1 SD for six determinations using four different cuticle protein preparations.

when cuticle proteins are carbamidomethylated with iodoacetamide before electrophoresis (22) or when gels are run under reducing conditions (in the presence of 10 mM thioglycolate); therefore, it is highly unlikely that any of these proteins are aggregation products resulting from the reoxidation of cysteine residues during electrophoresis. All of the soluble cuticle proteins described are seen in cuticle protein extracts prepared from both wild type (N2) nematodes and a temperature-sensitive fertilization-defective mutant (*fer-1*, see Fig. 8); therefore, none of these proteins can be contaminants arising from eggshells, which copurify with cuticles (see Materials and Methods). Soluble cuticle proteins from males have also been examined. No differences were detected between these and the proteins isolated from hermaphrodites.

A comparison of the gel lanes in Fig. 8 indicates that none of the major soluble cuticle proteins are present among the proteins released from the nematodes by sonication or from cuticles by SDS treatment. Although some cuticle proteins appear to comigrate with proteins released by SDS (e.g., cpH and MHC in Fig. 8), other results (discussed in the following paragraphs) indicate that the cuticle proteins are distinct from the SDS-soluble proteins. First, only the cuticle proteins are sensitive to collagenase digestion. Second, Coomassie Brilliant Blue R-250 stains cuticle proteins pink and all other proteins blue. The absence of the major muscle proteins, myosin, paramyosin, and actin, from these soluble cuticle protein preparations confirms the effectiveness of our isolation methods.

Identification of Soluble Cuticle Proteins as Collagens

To determine which of the major soluble cuticle proteins were collagens, we treated these proteins with *Clostridial* collagenase for various lengths of time at various enzyme to substrate ratios as described in Materials and Methods and analyzed the products by SDS-PAGE. *Clostridial* collagenase specifically hydrolyzes the X-Gly bond in the sequence Pro-X-Gly-Pro-Y commonly found in collagens (23). Seven noncollagen proteins normally used as molecular weight markers were also treated in parallel incubations. As shown in Fig. 9, all of the major soluble cuticle proteins, as well as all of the minor proteins present in bands >220,000, are extensively degraded by collagenase. None of the noncollagen control proteins are affected by this enzyme, indicating that the digestion observed with the soluble cuticle proteins is specific and not the result of contaminating proteases. When cuticle protein digestion products are electrophoresed on 10% gels, no distinct bands are observed, but rather a new large smear appears in the 15–25,000-mol-wt range. This material may represent incomplete digestion products or noncollagen portions of soluble cuticle protein polypeptide chains.

Several studies (24, 25) have demonstrated that collagens from a wide variety of vertebrate sources exhibit metachromatic staining with the dye Coomassie Blue R-250. Noncollagen proteins stain blue with this dye, whereas all collagens examined stain pink. All of the major soluble cuticle proteins also stain pink with this dye. On the same gels, the SDS-soluble and buffer-soluble proteins stain blue, as expected for noncollagen proteins.

DISCUSSION

This report offers the first detailed description of the ultrastructure and protein composition of the *C. elegans* adult cuticle. Although the cuticle has a complex morphology, it appears to be comprised primarily of a limited number of protein components. Our isolation procedure (sonication followed by incubation with SDS) provides cuticles that appear free of contamination by cellular material. Although the morphology of these cuticles is largely similar to that of *in situ* cuticles, as judged by both light and electron microscopy, it is possible that some structural cuticle proteins are removed by SDS.

The major portion of the cuticle, corresponding to the entire basal layer, struts, and internal cortical layer, is solubilized by reducing agents, indicating that the integrity of these structures in vivo must derive from disulfide cross links between the component proteins. The finding of 3% half-cysteine residues in these proteins is consistent with the notion of extensive disulfide cross-linking within the cuticle. The proteins that comprise these structures are resolvable into eight major and several minor components in the 50–220,000 molecular weight range by SDS-PAGE. By several criteria (amino acid composition, collagenase sensitivity, and metachromatic staining with Coomassie Blue R-250), the eight major components appear to be collagens. In more recent studies (M. Kusch, unpublished results), we have found that these proteins are largely resistant to degradation by pepsin, a further indication of their collagenous nature. Because collagens migrate anomalously on SDS gels (26), the molecular weights determined for these proteins should, at present, be considered only tentative.

It is not possible at this time to say whether the different molecular-weight collagen species represent unique collagen chains or whether some are cross-linked aggregates or specific post-translational cleavage products of other collagen molecules. It is probable that most of the minor proteins of molecular weight >220,000 observed on our gels are cross-linked aggregates of the lower molecular-weight species. It is unlikely that the heterogeneity in molecular weight of the major collagen species is due to variability in associated carbohydrate; little carbohydrate is associated with the soluble cuticle proteins and, furthermore, the apparent molecular weights of these proteins, except for cpB, do not decrease appreciably when these proteins are electrophoresed on higher percentage gels (i.e., 10 vs. 6%) as would be expected for glycoproteins (27). The generation of antibodies to specific adult cuticle proteins and the analysis of mutants lacking specific cuticle proteins should aid in determining the uniqueness of these molecules

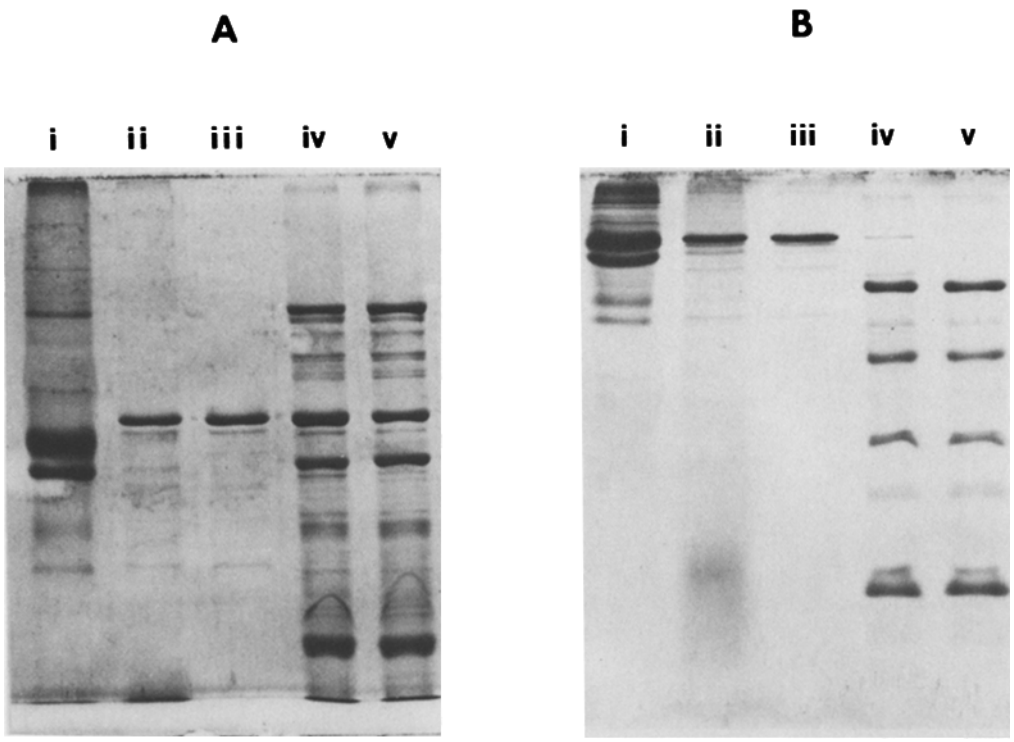

FIGURE 9 Collagenase sensitivity of soluble cuticle proteins. Soluble cuticle proteins and noncollagen proteins (molecular weight markers) were incubated with *Clostridial* collagenase for 5 h at 37°C at an enzyme-to-substrate ratio of 1:10, as described in Materials and Methods. Control samples were treated in parallel incubations minus enzyme. Digestion products are displayed on 6% (*A*) and 10% (*B*) SDS polyacrylamide gels. Lane i, soluble cuticle proteins (40 μg) incubated without collagenase; *ii*, soluble cuticle proteins (40 μg) incubated with collagenase; *iii*, collagenase (4 μg); *iv*, noncollagen proteins (10 μg) incubated with collagenase (note that collagenase comigrates with one of the noncollagenase proteins in gel *A* and is obscured); *v*, noncollagen proteins (10 μg) incubated without collagenase.

and in localizing them to specific regions of the cuticle.

The triple helical structure of collagen requires that glycine be present in every third position; therefore, the low bulk glycine content (26%) of the soluble cuticle proteins suggests that they can contain on the average a maximum of 78% collagen helix structure and that globular, nonhelix regions must comprise significant portions of these polypeptide chains. In this respect, the structure of these molecules may be similar to that of vertebrate procollagens or basement-membrane collagens (28).

The insoluble portion of the *C. elegans* cuticle appears to consist primarily of the external cortical layer and a small amount of internal cortical layer material. The nature of the covalent cross links responsible for the integrity of these structures is not known. From electron micrographs, these structures do not seem to correspond to 31% of the cuticle's volume. One possible reason for this discrepancy is that the insoluble cuticle material is more densely packed than the rest of the cuticle. The insensitivity of this material to digestion by *Clostridial* collagenase suggests that it is comprised primarily of noncollagen proteins. However, certain annelid cuticle collagens are not readily digested by this enzyme (29, 30); therefore, until further studies are performed, we cannot rule out the possibility that the insoluble cuticle material is comprised of collagen forms distinct from those of the soluble cuticle layers. We consider this proposal unlikely, however, because the corresponding insoluble material of the *Ascaris* cuticle, which also appears to derive from the external cortical layer, does not give an x-ray diffraction pattern typical of collagen (31).

The weak negative birefringence of the cuticle appears to be due to ordered material in the basal cuticle layer, because removal of this layer with enzymes or BME causes the cuticle to lose its birefringence. With *Ascaris*, the birefringence of the adult cuticle has been shown to be due largely to the sublayers of spiraling fibers present in the basal cuticle layer (32, 33). The fiber layers of the *Ascaris* cuticle spiral around the animal in opposite directions, each at an angle of 70° relative to the worm's long axis. The fiber layers, therefore, cross each other at an angle of ~140°. This orientation would produce a negative birefringence similar to that observed for the *C. elegans* cuticle. These observations suggest that the two fibrillar layers of the *C. elegans* cuticle and those of the *Ascaris* cuticle are organized in a similar manner. The opposite, positive birefringence of the alae suggests that the fibrillar material beneath these structures is organized longitudinally.

Comparison of the cuticle of *C. elegans* and that of *Ascaris lumbricoides* and *Panagrellus silusiae*, the only other nematodes for which both biochemical and ultrastructural data are available, reveals many similarities and a number of striking differences. In contrast to the large number of different molecular-weight collagen species found in the *C. elegans* cuticle, the *Ascaris* cuticle appears to be comprised of only three different collagen chains, each with a molecular weight of 52,000 (34). This difference is surprising in that the internal architecture of both cuticles is basically similar (32). The *Panagrellus* cuticle, which has a structure quite different from that of *C. elegans*, also appears to be comprised of a large number of protein components, of which at least four appear to be collagens (10, 35).

Although the cuticles of these three nematodes appear to be

chemically similar, significant differences are apparent when the amino acid compositions of the cuticle proteins of these nematodes are compared (9, 10, 31). Most striking is the difference in levels of hydroxylated proline residues. The ~1:1 ratio of hydroxyproline to proline in the *C. elegans* soluble cuticle proteins is similar to the ratio found in many vertebrate collagens; however, it is much greater than the ratios of these two amino acids observed in the corresponding proteins of the *Ascaris* (1:20) or *Panagrellus* (1:8) cuticles. Furthermore, hydroxyproline is completely absent from the insoluble proteins of the *Ascaris* cuticle. This latter difference may indicate that some collagens are cross-linked into the insoluble material of the *C. elegans* cuticle. Our amino acid composition of the *C. elegans* cuticle proteins (Bristol strain) differs in several respects from that reported for the Bergerac strain (8). However, the analyses are not directly comparable because these authors used a different method (hot trichloroacetic acid extraction) to obtain the cuticle proteins. Additionally, the purified adult cuticles shown in their report have a morphology quite different from that determined by us for the Bristol strain.

Our studies of the *C. elegans* cuticle have raised several questions concerning its assembly. First, the apparently large number of collagen species in the cuticle raises the question of whether the different proteins serve distinct or overlapping functions. The different collagen chains identified in humans to date all show distinct tissue and structural specificities (36). It is an attractive notion that the morphological complexity of the cuticle derives from the use of a number of structurally different proteins in its assembly. If this is the case, then it should be possible to localize the different proteins to specific parts of the cuticle, such as the struts and fiber layers. These studies are currently in progress. A more general question raised by this and other studies of extracellular structures (36, 37) is to what extent the various component proteins determine the structural organization of the cuticle? From ultrastructural studies of molting nematodes (3, 21), it is clear that the hypodermis, through its pattern of secretion, plays a major role in determining the organization of the surface features of the cuticle, the annuli and alae. Whether the internal architecture of the cuticle is similarly derived remains to be determined.

The studies on the adult cuticle reported here are intended to lay the groundwork for comparative developmental studies of the cuticles of the various juvenile forms and for analyses of putative cuticle mutants. As we have noted, some of the juvenile forms have cuticles of distinctive morphology, and our preliminary observations (unpublished) indicate that the patterns of soluble proteins isolated from the cuticles of the various juvenile stages are also distinctive. To date, we have identified mutations in >30 genes that affect the structure of the cuticle (6; unpublished results) and have now begun to extend the phenotypic characterization of these mutants to the ultrastructural and biochemical levels. At least one of these mutants appears to be lacking specific adult cuticle proteins. Thus, we believe that the formation of the *C. elegans* cuticle offers promise as a complex developmental process amenable to both genetic and biochemical analyses.

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