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THE YOLK POLYPEPTIDES OF A FREE-LIVING RHABDITID NEMATODE

CARLOS EDUARDO WINTER

Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, 05508 São Paulo, Brasil Tel.: (011) 813-6944; Fax: (011) 813-0845

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Abstract—1. The yolk proteins of hermaphrodite *Dolichorhabditis* sp. (Nematoda, Rhabditida) are composed of at least three polypeptides: VT1, VT2 and VT3 with molecular masses of 175.2, 107 and 82 kDa respectively.

2. All three yolk polypeptides make up at least one native protein complex which can be resolved by PAGE.

3. The yolk proteins are glycosylated and can be isolated by chromatography in Con A–Sepharose.

4. Partial chymotryptic hydrolysis shows that VT2 is different from its *C. elegans* homologue, YP115.

5. The main polypeptides synthesized by whole animals are the yolk components which are actively secreted in the incubation medium.

INTRODUCTION

The accumulation of reserves in the growing oocytes of oviparous animals has been termed vitellogenesis (Wahli *et al.*, 1981). One characteristic of this phenomenon is the uptake by the oocytes of a huge amount of protein synthesized in extraovarian sites (Wallace, 1985). These accumulated proteins belong to a class of lipoproteins known as vitellogenins (Wahli, 1988). The vitellogenin synthesis is frequently restricted to the females of the species at a given time in their development or physiological stage (Wahli, 1988).

Nematodes have been shown to synthesize a group of yolk protein precursors in their intestinal cells (Kimble and Sharrock, 1983). In *Caenorhabditis elegans* the vitellogenins consist of four polypeptides (YP170A, $M_r = 188$ kDa; YP170B, $M_r = 186$ kDa; YP115, $M_r = 109$ kDa, and YP88, $M_r = 83$ kDa) which are arranged in two complexes exhibiting different sedimentation velocities (Sharrock *et al.*, 1990). Although the genomic organization and complete amino acid sequence of all these yolk polypeptides are known (Heine and Blumenthal, 1986; Spieth *et al.*, 1991), a thorough comparative analysis of other nematode species is necessary to gain a better understanding of how they evolved in this group of animals (Byrne *et al.*, 1989).

Here we describe for the first time the characterization of the yolk polypeptides of a free-living nematode different from *C. elegans* but belonging to the same family. This species was isolated by us at S. Paulo, Brazil and has been provisionally put in the genus *Dolichorhabditis* (L. Carta, personal communication, 1991). The population we cultivated in the laboratory (strain B6/D6) was composed exclusively of hermaphrodite animals which makes easier the isolation and study of yolk proteins. Recent results have shown that this species is sufficiently distant from *C. elegans* to be distinguished at the DNA level (Winter and Blumenthal, unpublished results) although they are morphologically very similar.

Abbreviations—VT, vitellin; YP, yolk polypeptide; Con A, concanavalin A; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl)amino-methane; NP-40, nonidet P-40; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular mass; Da, dalton.

MATERIALS AND METHODS

Animals

Dolichorhabditis sp. hermaphrodites were initially isolated from the soil at São Paulo and subsequently grown in a monoxenic medium basically as described by Brenner (1974). Isogenic strains were obtained by starting the cultures with single individuals. The strain used in this work is termed B6/D6. The adult size of this strain is approximately 1.0 mm long with 12 chromosomes in the diploid cells (Dergovics and Winter, in preparation). *Caenorhabditis elegans*, Bristol strain (N2), was given by Dr. Thomas Blumenthal from a stock maintained at the Department of Biology, Indiana University, Bloomington, IN.

Maintenance of stocks

Both *C. elegans* (strain N2) and *Dolichorhabditis* sp. (strain B6/D6) were grown in solid support (NGM Agar; Brenner, 1974) with a lawn of *E. coli* strains NA 22 (for *C. elegans*) or MI 178 (CGSC–5680; *carA*, *relA*, *metB*, *thr*, Hfr) (for *Dolichorhabditis* sp). The animals were transferred with a sterile platinum wire from one plate to the other. *Dolichorhabditis* sp. does not form dauer larvae and if left to themselves the plates have no living worms 10 days after the bacteria are exhausted.

Mass liquid culture

The techniques developed for *C. elegans* (Sulston and Brenner, 1974) were used with some modifications for *Dolichorhabditis* sp. and *C. elegans*. Instead of agitating the culture in a shaker, aeration was obtained with a small quarium compressor.

Egg isolation

Dolichorhabditis sp. apparently lays its eggs just after fertilization. As a consequence the laid eggs of this species take longer to hatch and accumulate to great numbers in the plates. We first resuspended in S buffer (Brenner, 1974) the contents of plates just before the bacteria were exhausted. Filtering this suspension with a 30 μm nylon net, a large amount of eggs were obtained with very few adults and L1s. The eggs obtained by this procedure were dissolved in sample buffer and analyzed by SDS–PAGE.

Homogenization of animals

The worms were homogenized by two different procedures; one yielded the homogenate used for affinity chromatography purification and the other the homogenate used on PAGE electrophoresis.

(a) *For affinity chromatography*. The worms grown in a liquid medium as described above were first concentrated under gravity. These worms were then packed by brief centrifugation at low speed. Four grams of packed worms (adults plus larvae and eggs) of *Dolichorhabditis* sp. strain B6/D6 were resuspended in 30 ml of homogenization buffer A 2 × (1.2 M NaCl; 2 mM $CaCl_2$; 2 mM $MnCl_2$; 1 mM PMSF; 2 mM iodoacetamide; 40 mM Tris pH 7.4) containing 1 mM PMSF and 1 mM iodoacetamide. The worms were disrupted by intermittent sonication at 4°C until no intact animal was detected by phase microscopy (approximately 10 min).The homogenate was centrifuged at 750 ***g*** for 10 min at 4°C. The supernatant was then centrifuged at 12,000 ***g*** for 10 min at 4°C. The supernatant of the second centrifugation (S10) was diluted twice with 1 × homogenization buffer A and immediately applied to a Con A–Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). Homogenization of *C. elegans* grown in bulk quantities was done exactly as described by Krishna *et al.* (1988), starting with 5–7 g of a mixed worm population with no dauer larvae.

(b) *For PAGE*. The homogenization was done as described by Sharrock (1983) with some modifications. Three to four grams of a mixed population of *Dolichorhabditis* sp. strain B6/D6 were resuspended in homogenization buffer B (5 mM $MgCl_2$; 5 mM $CaCl_2$; 250 mM sucrose and 50 mM sucrose and 50 mM Tris pH 8.0). The suspension was then dropped slowly over liquid nitrogen in a porcelain mortar and the frozen pellets were then reduced to a powder. The frozen powder was allowed to melt in a ice bath and then centrifuged for 10 min at 1500 ***g*** at 4°C. The precipitate was washed twice with homogenization buffer, resuspended in 10 ml of the same buffer and NP-40 was added to 0.1% (v/v) final concentration. This solution, which was rich in yolk proteins (Sharrock *et al.*, 1990), was then ultracentrifuged for 4 hr at 112,500 ***g*** at 4°C. The supernatant, which contained the solubilized yolk proteins was submitted to discontinuous cylinder gel electrophoresis.

Con A–Sepharose affinity chromatography

This was carried out as described by Krishna *et al.* (1988) with some modifications. All steps were done at 4°C. Approximately 30 ml of worm homogenate was applied to a Con A–Sepharose column with a gel bed of 7.8 ml (1 cm diameter) at 10 ml/hr. After all the homogenate was applied the column was washed overnight at 5 ml/hr with 1 × homogenization buffer until $A_{280\,nm}$ was near zero. The proteins bound to the column were eluted with homogenization buffer containing 100 mM each of α-methyl mannoside and α-methyl glucoside. Fractions containing $A_{280\,nm}$ above 0.05 were analyzed by SDS–PAGE.

SDS–PAGE

Protein samples were dissolved in sample buffer and submitted to SDS–PAGE in slabs according to Winter *et al.* (1985). Protein staining was done with Coomassie Brilliant Blue R (Winter *et al.*, 1977) or by the silver-nitrate method (Merril *et al.*, 1983).

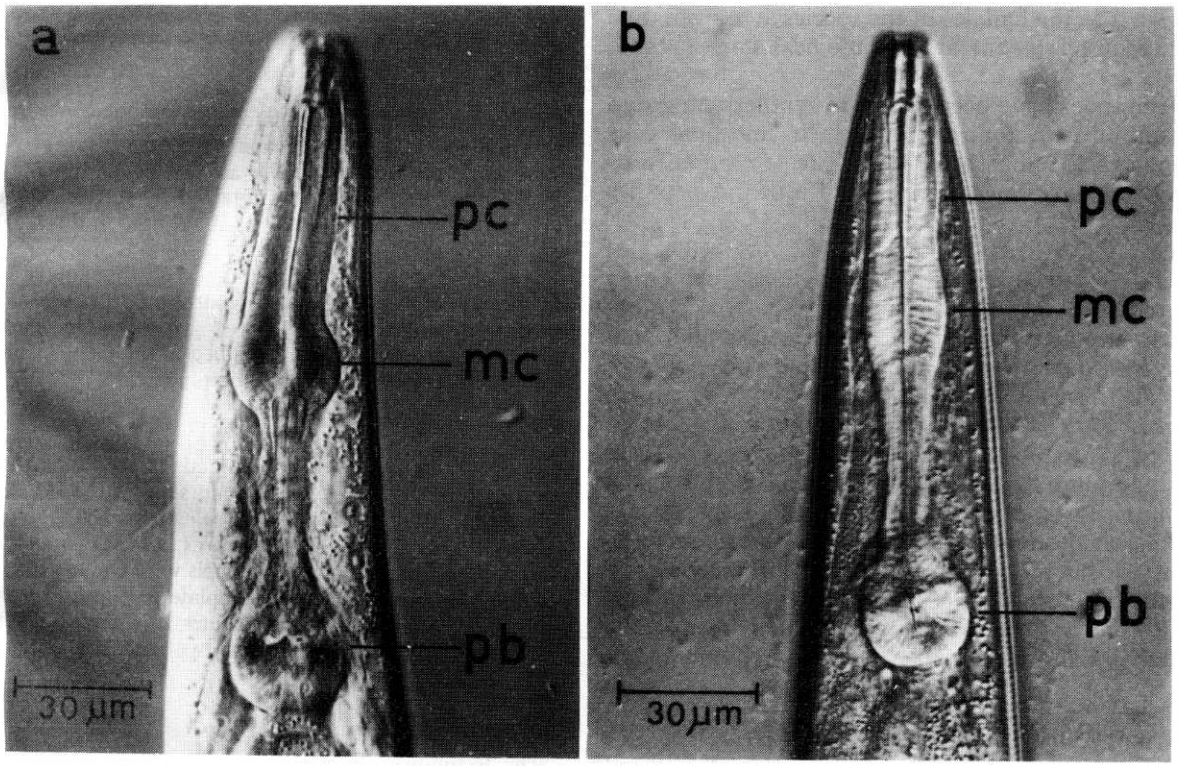

Fig. 1. Micrographs of the anterior portion of *Dolichorhabditis* sp. and *C. elegans*. The animals were anesthetized with 0.01% NaN_3, put over a thin 4% agarose pad and covered with a coverslip. The micrographs were made with Nomarski optics. (a) Anterior portion of *C. elegans*; (b) anterior portion of *Dolichorhabditis* sp. pc = procorpus; mc = metacorpus; pb = posterior bulb.

Discontinuous gel electrophoresis

This was done in glass tubes according to Davis (1964) using the same solutions used for SDS–PAGE without SDS. The runs were made in 7% gels according to Winter *et al.* (1985). All samples were run in duplicates. After electrophoresis the gels were removed from the glass tubes and one of them was frozen at −20°C while the other was stained with Amido Black (Panyims and Chalkley, 1969). The frozen gel was sliced into 1.7 mm fractions. The proteins in these fractions were eluted in SDS sample buffer at 100°C and submitted to SDS–PAGE.

Protein molecular mass estimative

The molecular mass was estimated by SDS electrophoresis according to Lambin *et al.* (1976), utilizing linear polyacrylamide gradients or according to Plikaytis *et al.* (1986) utilizing gels of single polyacrylamide concentration ($T = 10\%$). The molecular mass markers were: human erythrocyte α-spectrin (240 kDa) and β-spectrin (220 kDa); myosin heavy chain (200 kDa); β-galactosidase (166 kDa); phosphorylase *b* (92 kDa); bovine serum albumin (66 kDa); catalase (60 kDa); egg white albumin (45 kDa); lactate dehydrogenase (36 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20 kDa). Except for the human

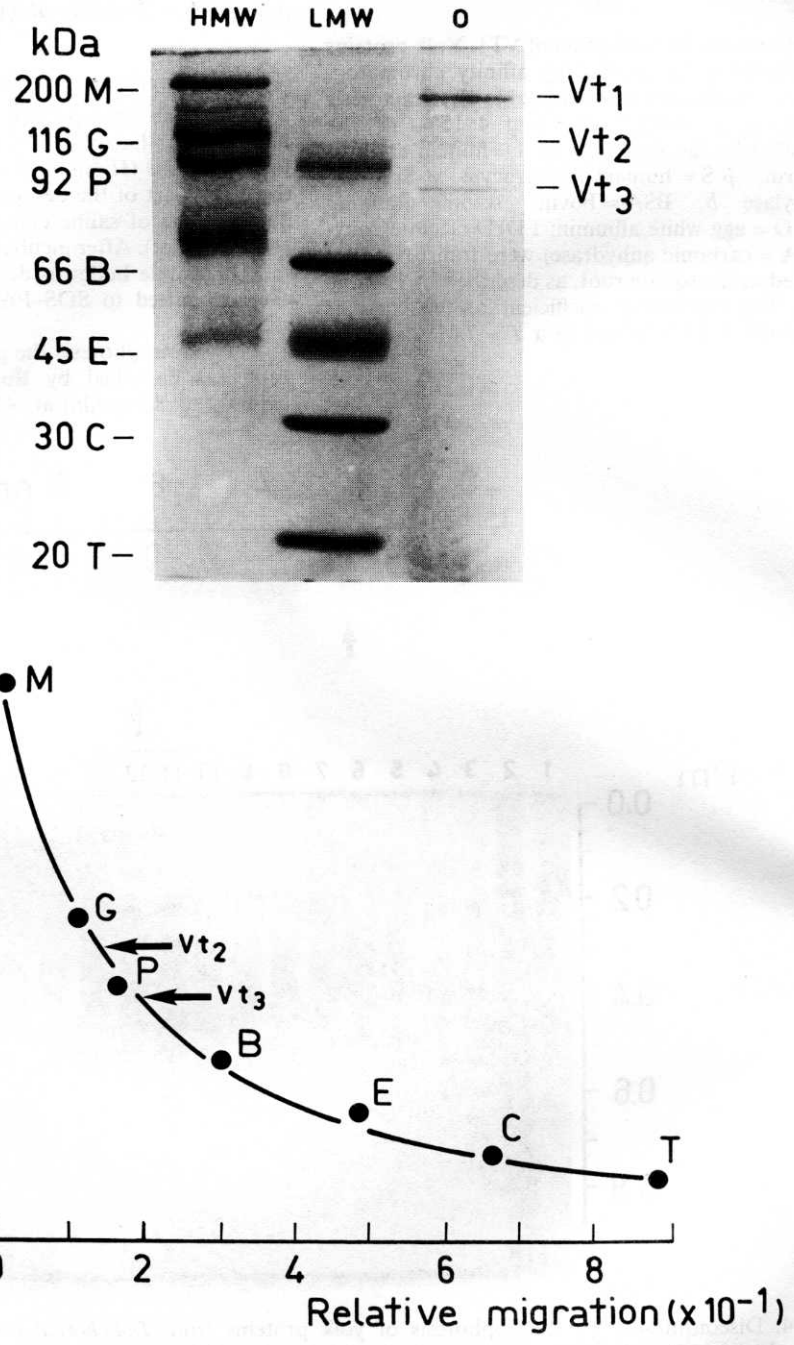

Fig. 2. Analysis of yolk proteins from eggs of *Dolichorhabditis* sp. The photograph shows a SDS–PAGE of proteins from *Dolichorhabditis* sp. eggs and molecular mass markers. The electrophoresis was run in a 10% polyacylamide gel slab and stained with Coomassie Blue R. The molecular mass curve at the bottom of the figure was obtained using the data shown in the photograph adjusted according to Plikaytis *et al.* (1986). M = Myosin heavy chain; G = β-galactosidase; P = phosphorylase *b*; B = bovine serum albumin; E = egg albumin; C = carbonic anhydrase; T = soybean trypsin inhibitor; HMW = high molecular mass markers; LMW = low molecular mass markers; O = egg proteins.

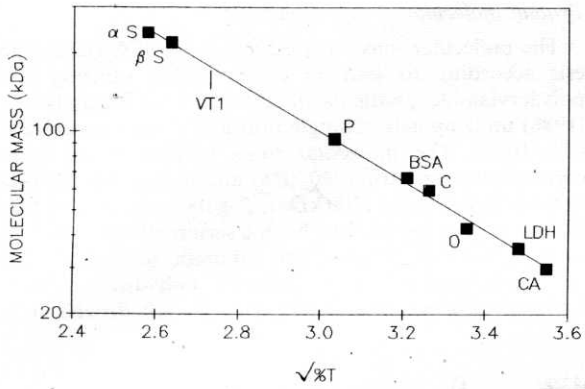

Fig. 3. Molecular mass determination of VT1. Yolk proteins from *Dolichorhabditis* sp. purfied by affinity chromatography in Con A–Sepharose were subjected to electrophoresis in SDS–PAGE in a linear gradient of 4–15% *T*. The distance migrated by the standards (α S = human erythrocyte α-Spectrin; β S = human erythrocyte β-Spectrin; P = phosphorylase *b*; BSA = bovine serum albumin; C = catalase; O = egg white albumin; LDH = lactate dehydrogenase; CA = carbonic anhydrase) were transformed in %*T* and plotted as the square root, as described by Lambin *et al.* (1976). The correlation coefficient of the curve is $r = 0.9984$. VT1 migrated to a $T = 7.41\%$.

erythrocyte spectrins (which were given by Dr. Julio Pudles, Department of Parasitology—ICB USP) all other molecular mass markers came from commercial sources (Pharmacia Fine Chemicals and Bio-Rad, Richmond, CA).

Chymotrypsin A partial digestion

Yolk proteins from both *C. elegans* and *Dolichorhabditis* sp., purified by Con A–Sepharose chromatography were subjected to SDS–PAGE in a $T = 10\%$ gel. The gels were rapidly stained and dried. The appropriate bands were cut and subjected to Chymotrypsin A partial digestion and fractionated in a $T = 15\%$ SDS–PAGE as previously described (Winter *et al.*, 1985). This gel was silver-stained as described by Merril *et al.* (1983).

^{3}H-Leu incorporation and fluorography

^{3}H-Leucine incorportion was made as described by Kimble and Sharrock (1983) in a saline solution developed for *C. elegans* (Hirsh *et al.*, 1976). Ten worms were cut at the distal part of the oesophagus and incubated for 1 hr at 20°C in 5 μl of saline containing 25 μCi of L-(4,5-^{3}H)-leu (145 Ci/mmol). After incubation, the animals were dissolved in SDS sample buffer as described above. This sample was then submitted to SDS–PAGE in a 10% polyacrylamide slab gel.

After electrophoresis the gels were processed for fluorography as described by Bonner and Laskey (1974) and exposed to X-ray film at −70°C.

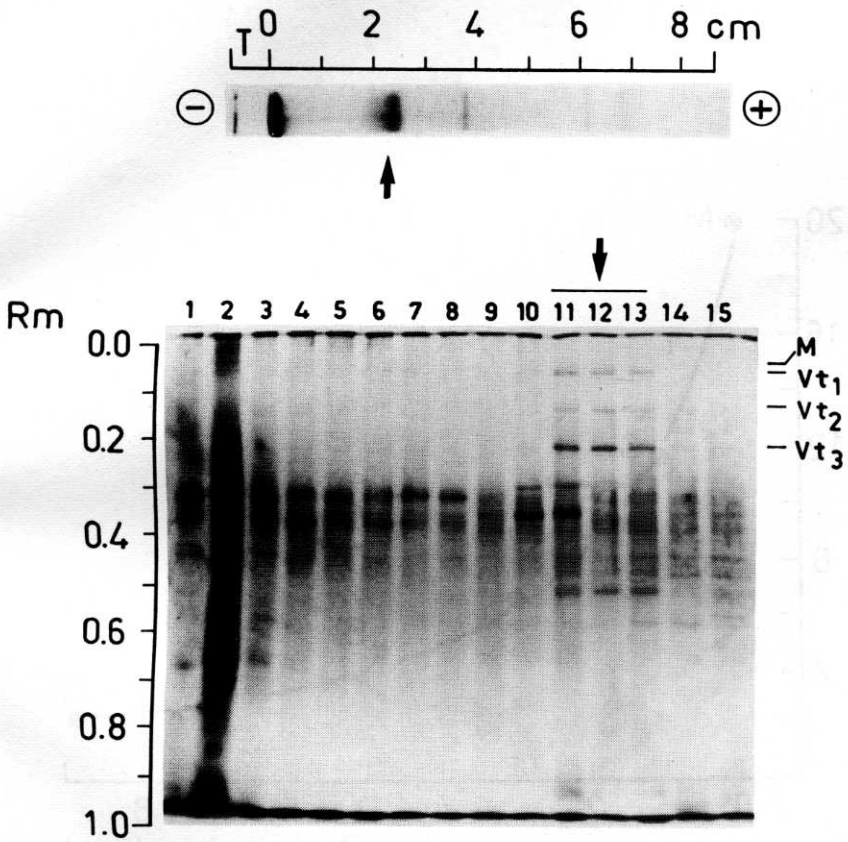

Fig. 4. Discontinuous gel electrophoresis of yolk proteins from *Dolichorhabditis* sp. Proteins of the particulate fraction of a worm homogenate (see Material and Methods) were submitted to discontinuous electrophoresis in a gel cylinder at a polyacrylamide concentration of 7%. This gel was stained with Amido Black and the result is shown at the top of the figure. An identical cylinder was fractionated and fractions 1–15 (each one with 1.7 mm) were submitted to SDS–PAGE in a 10% gel slab. This gel was silver-stained and the result of this second electrophoresis is shown at the bottom of the figure. At the left of the SDS–PAGE is shown the relative migration rate (R_m) scale. At the right is shown the position of the yolk polypeptides (VT1, VT2 and VT3) and the myosin heavy chain (M). The arrow in both electrophoreses shows the position of the native yolk proteins; *T* = stacking gel.

RESULTS

Morphology

Adult hermaphrodites of *Dolichorhabditis* sp. are almost the same size as *C. elegans*. The only difference which can be readily seen at the light microscopy level is the oesophagus morphology. The oesophagus of *Dolichorhabditis* sp. does not show the conspicuous bulb-like metacorpus which is clearly observed in *C. elegans* (Fig. 1a,b).

Egg proteins

The yolk proteins *Dolichorhabditis* sp. were first identified in eggs. Eggs obtained as described in Material and Methods were dissolved in sample buffer and submitted to SDS–PAGE. The main egg polypeptides observed after staining were labeled VT1, VT2 and VT3 (Fig. 2). The molecular mass of VT2 and VT3 were obtained from a 10% gel and shown to be 107 and 83 kDa respectively (Fig. 2). The molecular mass of VT1, which could not be obtained from this gel, was calculated as 175.2 kDa (Fig. 3) from a gradient gel (not shown). All these values for the Mr of VT1, VT2 and VT3 are approximately the same obtained for YP 170A + B, YP115 and YP88 from *C. elegans* yolk (Sharrock *et al.*, 1990).

Native yolk polypeptide complexes

Worm homogenate, obtained as described in Material and Methods, was submitted to discontinuous electrophoresis with SDS. After the run the cylinder was fractionated and the polypeptides present in each fraction were analyzed by SDS–PAGE. Figure 4 shows the results obtained. The cylinder electrophoresis show a prominent band approximately 2 cm from the origin. This region contains the three polypeptides which were found in the eggs. This shows that they are part of the same protein or of proteins which have nearly the same electrophoretical behavior. In Fig. 2 VT3 is more stained than the other yolk polypeptides. This is due to its greater affinity for silver salts. The reason for this phenomenon is not known but has already been noticed with other proteins (Merril *et al.*, 1983).

Affinity chromatography of the yolk

After homogenization as described in Material and Methods *Dolichorhabditis* sp. proteins were submitted to affinity chromatography in Con–A Sepharose. Figure 3 shows the results obtained with this procedure. Part of the protein of the homogenate is retained in the column and is eluted with α-methyl

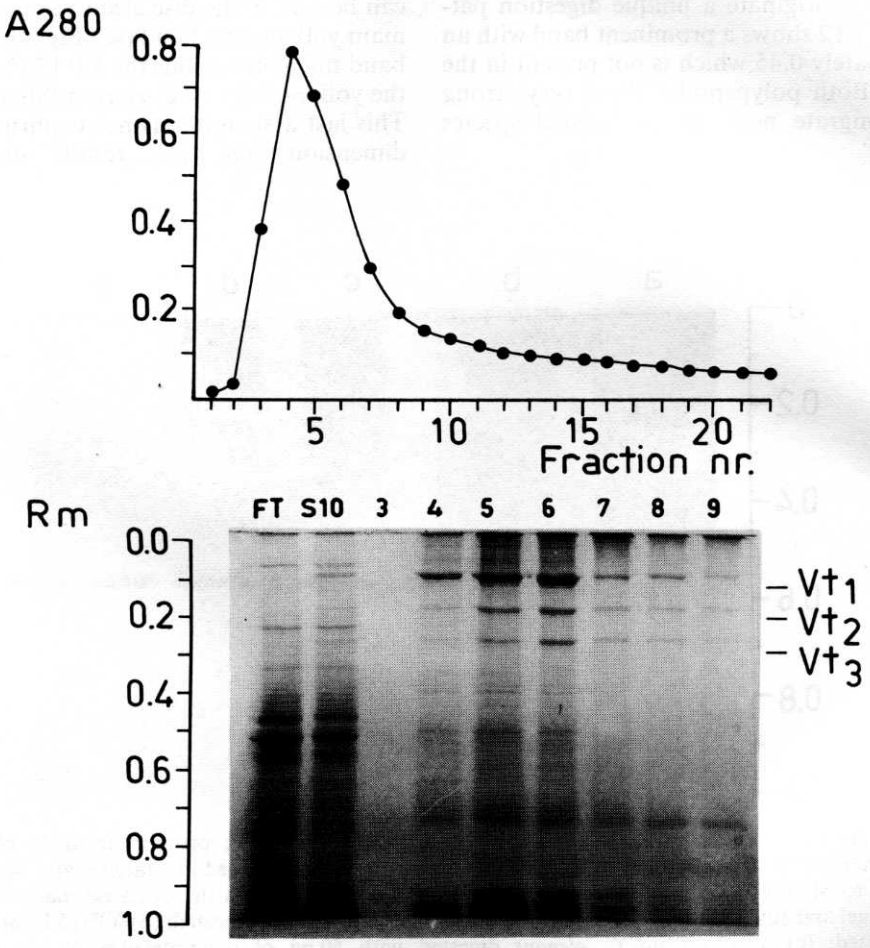

Fig. 5. Purification of *Dolichorhabditis* sp. yolk proteins by Con A–Sepharose affinity chromatography. The graphic on the top of the figure shows the absorbancy profile of the proteins eluted from the column with α-methyl mannoside and α-methyl glucoside. The homogenate was applied to the column and washed with 10 volumes of homogenization buffer. The bound proteins were then eluted with α-methyl glucoside and α-methyl mannoside as described in Material and Methods. The photograph at the bottom shows the polypeptides present in fractions 3–9. FT corresponds to the proteins present in the flow-through and S10 to those present in the homogenate applied to the column.

mannoside and α-methyl glucoside. This fraction contains, among other polypeptides, those that make up the native yolk proteins (Fig. 5). Moreover, all the yolk polypeptides present in the initial homogenate (Fig. 5, S10) are retained in the column. This can easily be seen when we analyze the polypeptides present in the flow-through (Fig. 5, FT) which is completely devoid of VT1, VT2 and VT3.

VT2 and YP115 comparison

The fact that *Dolichorhabditis* sp. and *C. elegans* are so similar at the morphological level promped us to analyze the peptide map of VT2 of *Dolichorhabditis* sp. and YP 115 of *C. elegans*. Both YP115 and YP88 are originated by the cleavage of a common 193 kDa precursor (Sharrock *et al.*, 1990). This precursor is coded by the *vit*-6 gene (Spieth and Blumenthal, 1985) which is the only autosomal vitellogenin gene from *C. elegans* (Heine and Blumenthal, 1986) being located at linkage group IV.

Figure 6 shows the results of the partial digestion of VT2 and YP115 by chymotrypsin. Without chymotrypsin treatment the polypeptides show only a smear below them (Fig. 6a,b). When they are partially digested with chymotrypsin we can see that both polypeptides originate a unique digestion pattern (Fig. 6c,d). VT2 shows a prominent band with an R_m of approximately 0.45 which is not present in the YP115 profile. Both polypeptides show very strong bands which migrate near the undigested species (R_m 0.12).

Yolk polypeptides synthesis

When animals whose anterior parts were sectioned were incubated in a medium containing ^{3}H-Leu we could see that the main proteins synthesized by the worms were the yolk components (Fig. 7). The analysis of the incubation medium by SDS–PAGE and fluorography shows that VT1 (and probably also the other yolk peptides) is actively secreted, as occurs with *C. elegans* (Kimble and Sharrock, 1983).

DISCUSSION

We have shown that the yolk proteins of *Dolichorhabditis* sp. are composed of at least three polypeptides: VT1 (175.2 kDa), VT2 (107 kDa) and VT3 (82 kDa). These yolk polypeptides have molecular masses which are very similar to those calculated for the *C. elegans* yolk polypeptides (Sharrock *et al.*, 1990).

As in *C. elegans* the yolk polypeptides of *Dolichorhabditis* sp. form native multimers. In *Dolichorhabditis* sp. this was shown by two-dimensional electrophoresis. Contrary to what has been shown in *C. elegans* (Sharrock *et al.*, 1990) we did not detect more than one native protein complex with different polypeptide composition. Nevertheless, a faint band can be seen in the disc electrophoresis just before the main yolk protein band (see Fig. 4). This could be the band that corresponds the YP 170A dimer detected in the yolk proteins of *C. elegans* (Sharrock *et al.*, 1990). This last assumption is not confirmed by the second dimension run. The results obtained by Con

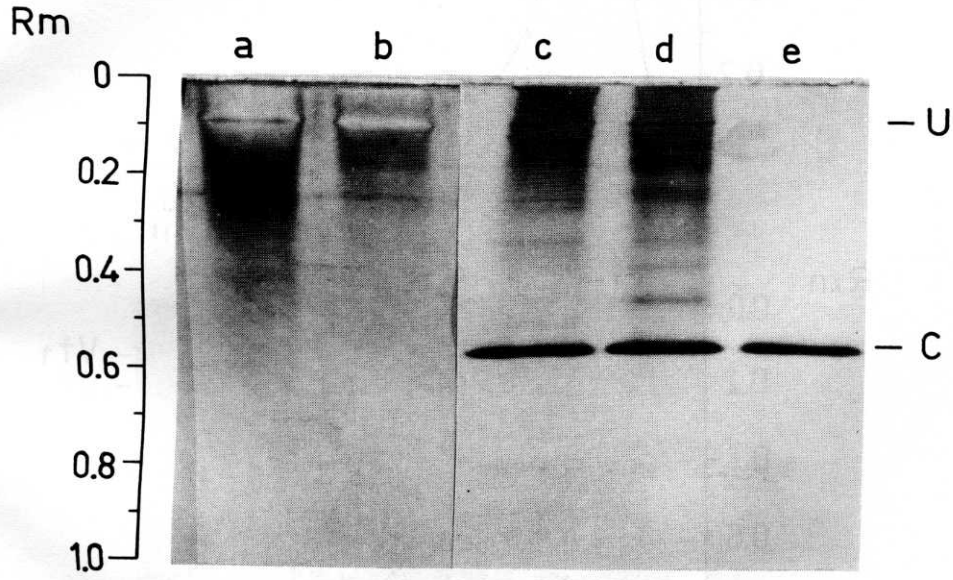

Fig. 6. Chymotrypsin A partial digestion of YP115 and VT2. Yolk proteins from *C. elegans* and *Dolichorhabditis* sp. were purified by affinity chromatography as described in Material and Methods and subjected to SDS–PAGE in a 10% gel. YP115 and VT2 were cut from the dried gel, partially digested in a 15% gel and silver-stained. (a) VT2 from *Dolichorhabditis* sp. not digested; (b) YP115 from *C. elegans* not digested; (c) YP115 from *C. elegans* digested with 50 ng of chymotrypsin A; (d) VT2 from *Dolichorhabditis* sp. digested with 50 ng of chymotrypsin A; (e) chymotrypsin A (50 ng) alone. (a) and (b) were obtained in a different gel. The band that runs at R_m 0.24 in (a) and (b) probably corresponds to a contaminant from the running buffer and can only be seen in these wells because this gel was overdeveloped in the silver-stain. That is also why the undigested VT2 and YP115 bands appear white, instead of black. At the left side of the photos is shown the R_m (relative migration rate) scale taking an arbitrary point below the chymotrypsin band as 1.0. On the right is shown the position of the undigested VT2/YP115 band (U) and the chymotrypsin band (C).

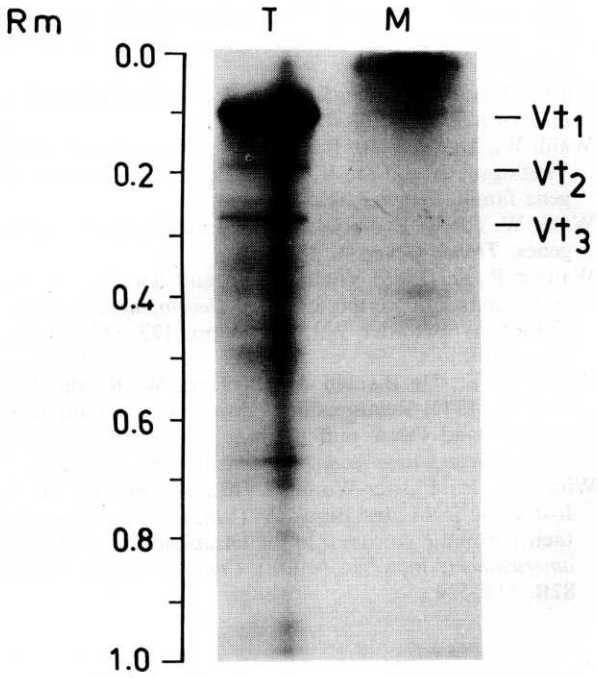

Fig. 7. Synthesis of yolk polypeptides by whole animals. Ten worms were incubated in L-[4,5-^{3}H]Leu as described in Material and Methods. The labeled proteins were then fractionated by SDS–PAGE in a 10% polyacrylamide gel slab. The photograph shows the fluorography of this gel. On the left is shown the relative migration rate. On the right is shown the position of VT1, VT2 and VT3. T corresponds to the labelled proteins in the whole worms and M corresponds to proteins secreted to the medium.

A–Sepharose show that some (if not all) the yolk polypeptides of *Dolichorhabditis* sp. are glycosylated. The native yolk proteins isolated by this procedure are composed of all the yolk polypeptides detected in the eggs and worm homogenate.

The VT1 band is clearly broader than other bands of similar M_r composed of single polypeptides. If VT1 is homologous to the *C. elegans* high molecular mass yolk polypeptides it could be composed of two polypeptides similar to YP170A and YP170B (Sharrock, 1983). Nevertheless, the detection of only one native complex of yolk polypeptides (see Fig. 4) could be an indication that *Dolichorhabditis* sp. does have only one polypeptide at the VT1 region. The results obtained so far cannot distinguish between those two possibilities.

Although VT2 and YP115 have similar molecular masses they can be distinguished by chymotrypsin A partial digestion (Fig. 6). These results can be interpreted as an indication that *Dolichorhabditis* sp. have vitellogenin genes which are homologous, but not identical in sequence, to the *C. elegans* proteins (Spieth *et al.*, 1991). The vitellogenins from chicken, frog and *C. elegans* have been compared at the sequence level. These proteins have been shown to have very few absolutely conserved sequences. They can still be aligned as belonging to a single gene family (Spieth *et al.*, 1991). The fact that *Dolichorhabditis* sp. and *C. elegans*, which belong to the same family, have yolk proteins with different partial digestion profiles supports the idea that what is conserved in the vitellogenins is the amino acid composition (Spieth *et al.*, 1991).

The yolk polypeptides of *Dolichorhabditis* sp. are actively synthesized and secreted in the incubation medium by whole adult animals. The synthesis of VT1 accounts for more than 50% of the proteins synthesized by the whole animal (Fig. 7). This is in accordance with data obtained in *C. elegans* which show that the vitellogenin genes transcripts are among the most abundant mRNAs in the whole worm (Blumenthal *et al.*, 1984).

The results presented herein have shown that the yolk proteins of *Dolichorhabditis* sp. and *C. elegans* have many similarities in chemical behavior and structure. However, when compared by partial digestion, differences could be shown among *Dolichorhabditis* sp. VT2 and *C. elegans* YP115. This last result is in accordance with preliminary data obtained on the sequence of one of the vitellogenin genes of *Dolichorhabditis* sp. (Winter and Blumenthal, unpublished results).

Like *C. elegans*, *Dolichorhabditis* sp. can be easily maintained in the laboratory. This characteristic and its morphological and biochemical similarities to *C. elegans* make it a useful organism or which to carry out comparative studies on nematodes.

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