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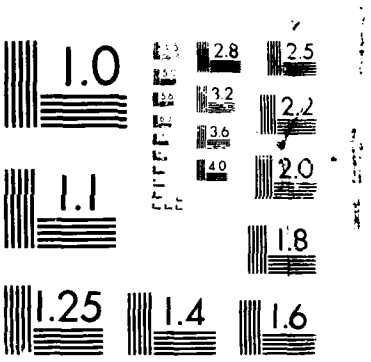

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Four Dopa-containing proteins have been isolated from Fasciola hepatica and purified to homogeneity (72kd, 31kd, 21kd, 12kd); the proteins are synthesized in the worm vitellaria and are strongly implicated as precursors of the quinone-tanned eggshell. Amino acid composition of the four proteins has been determined and tryptic fragments of the 31kd and 12kd have been sequenced. All proteins are similar in solubility and isoelectric point and are extremely rich in glycine and dihydroxyphenylalanine. Sequence data (31kd, 12kd) indicate that modified tyrosine residues are flanked by glycine (gly-dopa-gly) and that large regions of polyglycine are present in the protein. Rabbit antisera has been utilized to isolate putative gene copies of the 20kd and 31kd genes from newly synthesized cDNA libraries. In addition, a battery of monoclonal antibodies has been raised against the 31kd protein as a tool to probe production and modification of the eggshell proteins in vivo and in vitro. Monoclonal antibody developed against components of Mehlis' gland, uterus, ootype and vitellaria are also being evaluated.

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Synthesis of Stable Microcapsules from Trematode Eggshell Components

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CONTENTS

SUMMARY

Four Dopa-containing proteins have been isolated from Fasciola hepatica and purified to homogeneity (72kd, 31kd, 21kd, 12kd): the proteins are synthesized in the worm vitellaria and are strongly implicated as precursors of the quinone-tanned eggshell. Amino acid composition of the four proteins has been determined and tryptic fragments of the 31kd and 12kd have been sequenced. All proteins are similar in solubility properties and are extremely rich in glycine and dihydroxyphenylalanine. Sequence data (31kd, 12kd) indicate that modified tyrosine residues are flanked by glycine (gly-dopa-gly) and that large regions of polyglycine are present in the protein. Rabbit antisera has been utilized to isolate putative gene copies of the 20kd and 31kd genes from cDNA libraries. In addition, a battery of monoclonal antibodies has been raised against the 31kd protein as a tool to probe production and modification of the eggshell proteins in vivo and in vitro. Monoclonal antibody developed against components of Mehlis' gland, uterus, ootype and vitellaria are also being evaluated.

I. Dopa Protein Purification

The major dihydroxyphenylalanine containing proteins of Fasciola hepatica (72kda, 31kd, 21kd and 12kd) have been purified to homogeneity from adult worms largely by virtue of their solubilities at neutral and acid pH. The 31kd protein is the most abundant DOPA containing protein and has been extensively characterized (1); fractionation of 31kd eggshell precursor protein on a borate affinity column indicates that i) protein bound tyrosine residues serve as precursor to DOPA residues and ii) a gradient of posttranslational modification (tyrosine -> DOPA) exists in the population of precursor molecules. Amino acid analysis on all four highly purified proteins has also been carried out (Table I) and suggests that glycine and DOPA are major constituents by weight of all four precursor proteins. Three of the proteins are also extremely aspartate/asparagine rich with a histidine predominance in the fourth protein (12kda). Sequencing of tryptic Dopa-rich peptides derived from the 31kda protein and also from the 12kda protein has provided valuable insite as to the site specificity of tyrosine modification; in all cases documented, modified tyrosine residues are flanked by glycine. Peptide sequence has also provided a basis for synthesizing oligomeric DNA probes employed in isolating genes and/or confirming the identity of cloned sequences.

II. Probe Isolation/Application

A. Oligomeric DNA probe - In addition to the previously constructed probes specific for the 31kd protein we have constructed a probe representing a sequence within the 12kd gene. The previously constructed oligomeric probes were extremely degenerate due to the presence of numerous glycine residues and as a result were not extremely useful in hybridization studies; in contrast the peptide sequence of the 12kd protein can be "back-translated" into a DNA sequence with a limited degeneracy. This probe is currently being employed for hybridization selection of the 12kd protein coding sequence directly from a cDNA library. The peptide sequence is shown below along with the corresponding DNA and probe sequence:

N	HIS	HIS	TRP	ASP	GLY	DOPA	GLY	DOPA	C	peptide
5′	CAT	CAT	TGG	GAT	GGG	TAT	GGG	TAT	3′	"sense" strand
	C	C		C	A	C	A	C		
					T		T			
					C		C			
3′	GTA	GTA	ACC	CTA	CCC	ATG	CC	5′		probe
	G	G		G	T	A				
					A					
					G					

B. Polyvalent antisera probes - Polyvalent rabbit antisera were raised against purified 31kd protein and a mixture of 20kd and 31kd protein. These antisera were raised by Cambridge Research Biochemicals and their reactivity confirmed via Western Blot analysis. The antibody has been used to successfully screen an adult Fasciola hepatica expression library and is also being used to localize the proteins in adult and juvenile worm sections (2).

C. Monoclonal antibody probe - One of the objectives of this proposal involves the study of the biological processes of eggshell formation in the trematode flukes S. mansoni and F. hepatica. We have elected to use monoclonal antibodies against purified eggshell precursor proteins to begin to determine points of crosslinking and assist us in purifying large quantities of these proteins for further study. Our first objective was to produce and characterize a battery of monoclonal antibodies reactive against eggshell components. We have currently in our inventory 43 monoclonal hybrids reactive against a purified 31kda eggshell precursor protein (Table II). These monoclonal antibodies are being cloned by limiting dilution and rescreened by ELISA, Western blot and immunoelectron microscopy. Additional monoclonal antibodies produced after immunization with Mehlis' gland, uterus ootype and vitellaria are also currently being evaluated. Additional human monoclonal antibodies reactive with S. mansoni eggs have been characterized to be cross-reactive with F. hepatica. One of these monoclonal antibodies, an IgM antibody, is reactive against two bands of molecular weight range 30-40 kda in a Western blot using a crude homogenate preparation of F. hepatica. Other monoclonal antibodies are now being evaluated by immunofluorescence and immunoelectron microscopy. Electron micrographs of the Dopa-rich vitellarial granules of juvenile and adult F. hepatica are shown in Figures 1A and 1B, respectively.

III. Gene Isolation

A. Library construction - A cDNA library was recently constructed using a lambda gt11 expression vector and mRNA derived from adult flukes. This library contained approximately 1 x 10^6 original clones eighty percent of which carried fasciola DNA inserts. Three libraries were constructed using size fractionated RNA in the following ranges i) 500-1000 bp, ii) 1000-2000 bp and iii) >2000 bp. Parallel libraries were also constructed in the vector lambda gt10 to facilitate hybridization screening. (3, 4) Previous expression libraries were constructed using genomic DNA and mung bean nuclease; these proved to express an extremely limited set of genes and were not useful for eggshell gene isolation.

B. Isolation of putative 20kd and 31kd gene coding sequences - An antibody screening technique (2) was used to select a number of isolates from the expression library containing 500-1000 bp inserts. The isolates fell into three groups as follows: Group I contained a single 1000 bp insert; Group II contained a single 400 bp insert. Group III contained a 1000 bp insert bisected by the cloning enzyme EcoRI releasing a 540 and a 500 bp fragment. Probing of these inserts with an oligomer corresponding to the female-specific 20kd protein of zurita (5) suggests that Group II encodes a 20kd protein. The 1000 bp DNA's presumably encode the 31kd protein since the antisera used for selection recognize only a 20kd and 31kd protein. Subcloning and DNA sequencing of the genes is proceeding to confirm the identity of these sequences.

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TABLE I

PUTATIVE FASCIOLA EGGSHELL PRECURSOR PROTEINS

PROTEIN	APPARENT M W	MAJOR AMINO ACIDS
A	70kD	Asx 21% Gly 11 Dopa 4 Tyr 9 Arg 8
B	31kD	Asx 14 Gly 16 Dopa 10 Lys 13
C*	21kD	Asx 16 Gly 12 Lys 12 Tyr 9
D	12kD	Gly 30 Dopa 15 His 14

* from Zurita et al., 1987

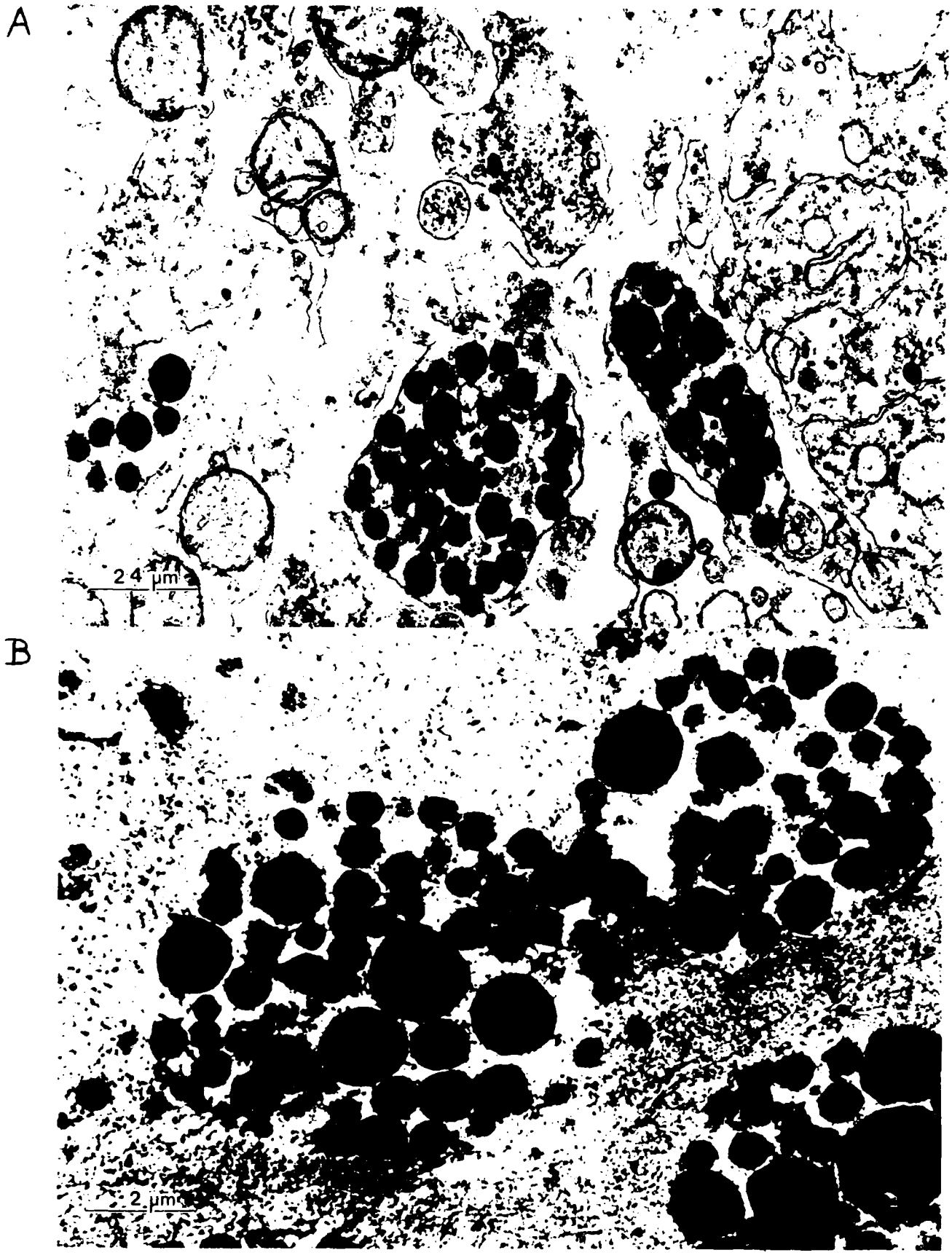
TABLE II

MONOCLONAL ANTIBODIES REACTIVE WITH 31 kda EGGSHELL PRECURSOR FROM F. HEPATICA

MONOCLONAL ANTIBODY	E/C OD AGAINST 31 kda
ID1	1.457
AG5	0.966
FB7	0.519
GG12	0.447
GF12	0.414
FF5	0.406
HC12	0.383
BC11	0.379
FF9	0.348
FB12	0.345
BB10	0.345
FF6	0.294
FC12	0.268
CD9	0.273
FA12	0.283
CA12	0.250

FIGURE 1A: Transmission Electron Micrograph of the vitellaria of the juvenile F. hepatica fluke after excystment and one week of growth under in vitro tissue culture conditions. 10,000 X

FIGURE 1B: Transmission Electron Micrograph of an adult F. hepatica fluke showing a more highly developed vitelline granule within the vitelline cell. 20,000 X


A
2.4 µm
B
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