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(u) Synthesis of Stable Microcapsules from Trematode Eggshell Components

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The trematode Fasciola hepatica produces a unique protein eggshell or microcapsule the components of which are the subject of the current study. The shell is composed of three major proteins, vpA, vpB and vpC each of which contains unusual post-translational modifications of tyrosine residues to dihydroxyphenylalanine (DOPA); the DOPA residues permit enzymatic crosslinking of the shell proteins to produce a hard quinone tanned microcapsule with unusual properties.

The focus of this project is to i) characterize the protein components of the microcapsule, ii) characterize the enzymes involved in the crosslinking process, iii) define the chemical crosslinks involved in shell maturation, iv) survey the role of metals in the crosslinking and stability of the shell and v) isolate the genes encoding the structural components in order to mimick aspects of microencapsulation in vitro.

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PROGRESS REPORT DATE: JUNE 30, 1990

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PRINCIPLE INVESTIGATORS: Allison R. Ficht, J. Herbert Waite, Barbara L. Doughty, and W. Michael Kemp.

CONTRACTOR: Texas A & M University

CONTRACT TITLE: Synthesis of Stable Microcapsules from Trematode Eggshell Components

START DATE: 1 May 1987

RESEARCH OBJECTIVE: To examine the process and materials of eggshell construction at the biochemical and molecular levels with the long-term goal of using the products and processes for synthetic microencapsulation. The short term goals include characterization of all structural proteins and enzymes used in the natural process. To date three major structural proteins have been purified from the vitellaria and are designated vitelline proteins A, B,and C (vpA, vpB, and vpC). The analysis includes by necessity a study of the corresponding genes from which a primary sequence will be determined and from which recombinant protein will be derived for protein cross-linking studies in vitro.

PROGRESS (YEAR 3): In the past year major progress has been made in several areas including i) a detailed study at the protein and gene levels of the microheterogeneity observed in the major eggshell proteins (vpB group), ii) extensive peptide analysis of vpB demonstrating the identity of both the vpB1 and vpB2 gene copies with purified vpB, iii) further analysis of a polymeric protein which copurifies with vpB, iv) localization of transcripts encoding vpB and the polymeric protein indicating the tissue of origin for the proteins and v) isolation of putative gene copies encoding vpC.

I. A detailed study of the microheterogeneity of vpB at the protein and gene levels. In order to understand the natural microencapsulation process it is necessary to determine which proteins are present and their relative importance in the overall shell structure. Initial studies of the most abundant protein in the microcapsule, vpB, indicated the presence of a single homogeneous protein; later studies with the vpB cDNAs and with vpC proteins revealed heterogeneity and suggested a re-examination of vpB. It is now apparent that vpB consists of a family of approximately 20 proteins whose heterogeneity is based on variations in post-translational modification of tyrosine to DOPA as well as differences in primary sequence. Digestion of vpB in borate buffers has permitted the isolation of many additional tryptic peptides for amino acid sequence analysis and allowed analysis of post-translational modification at individual sites within the proteins. DOPA modifications range from 95% to 50%, and although there is no absolute consensus sequence for modification, DOPAs are most frequently flanked by Lys, Ser and Asp on the N-terminal side and Gly or Asp on the C-terminal side. All of the electrophoretic variants fall within a narrow pH range (30-33kDa) and a pI range of 8.0-8.8. The majority of variants share a single amino terminal sequence up to residue 28 based on amino acid sequence (Waite and Rice-Ficht, 1990).

Complete analysis of two cDNAs representing vpB indicate the copies differ significantly in primary amino acid sequence through a central 85 amino acid region (30% divergence); however, the copies share complete identity through the aminoterminal 100 amino acids and the carboxyterminal 85 amino acids (Rice-Ficht and Waite, 1990). Both contain the predicted amino terminal 28 amino acids derived from protein sequencing. Analysis of genomic DNA copies homologous to vpB reveals that only one of 6-7 possible genomic copies is associated with the predicted N-terminal amino acid sequence based on hybridization with an oligomer (30-mer) homologous to vpB1 and vpB2 termini. Since the cDNAs clearly represent two different transcripts each bearing the same terminus, the mechanism by which each copy acquires the N-terminus in under study. RNA splicing (cis or trans) is likely responsible and is being pursued. From the standpoint of this project the important point is the number and type of proteins incorporated into the shell; peptide sequence indicates the presence within the purified vpB protein population of numerous tryptic peptides corresponding to the sequence predicted by the vpB1 and vpB2 cDNAs (Waite and Rice-Ficht, 1990). There are three peptides not accounted for in the two cDNA sequences which suggests the existence of at least one additional structural vpB gene not yet located. Polymerase chain reaction (PCR) using RNA as starting template is being employed to "count" the transcripts containing the predicted N-terminus. Beyond this the heterogeneity may be based largely on post-translational modification.

II. Analysis of the primary sequence of vpB in relation to post-translational processing. The modification status of a majority (64%) of the tyrosines within the primary sequences of vpB1 and vpB2 has been determined through sequencing of tryptic peptides. Yields during amino acid sequencing suggest that specific residues are either completely modified to DOPA or completely unmodified; of the 23 residues observed 19 were completely modified and 3 completely unmodified. An exception to this observation occurs at residue 39 in which 50% of the tyrosine is unmodified. Analysis of the primary sequences flanking DOPA residues for a possible consensus has lead to the general observation that N-terminal flanking residues are frequently Lys, Ser or Asp while C-terminal sequences are frequently Gly or Asp, but no absolute consensus has appeared. Computer analysis of exposed vs. buried regions of the molecule based on primary DNA sequence alone suggests that modification may be surface-dependent although the correlation between modification and surface probability is not absolute.

III. Analysis of a polymeric protein which copurifies with vpB. A family of cDNAs has been isolated which appear to encode a polymeric protein of approximately 65kDa (monomer) as determined by immunoblot analysis via SDS-PAGE. Northern blot analysis agrees with this observation since the mature mRNA migrates at approximately 1850bp and could potentially encode a protein of 60-75kDa. The coding region of both transcripts analyzed (cDNA) is composed of a 63 nucleotide repeating sequence which gives rise to two open reading frames of dramatically differently amino acid composition. One reading frame is extremely rich in glutamine having the consensus sequence (Q-X)$_n$ LKI and is recognized by antibody raised against the native trematode protein. This is the likely reading frame expressed in the parasite although expression of the second reading frame cannot yet be ruled out. Peptides have been generated representing each of the reading frames and high titre polyvalent rabbit antisera raised against each. The sera are currently being used in whole worm sections and in immunoblot and immunoprecipitation studies with protein extracts to determine which of the reading frames is actively

translated.The closest GENEBANK homology to this protein is involucrin, a component of epidermal cells involved in cornification. This protein also undergoes crosslinking although quite chemically distinct from quinone tanning. In situ hybridization indicates transcription to occur in a layer of subtegumental cells near but not within the vitelline glands; this suggests that the protein may not be directly involved in shell formation. Antibody localization of the final product in the worm will confirm its role either in shell production or tegument structure.

IV. Localization of vpB transcripts and protein using monoclonal antibody and in situ hybridization. An important facet of the project which complements the biochemical studies outlined is the localization of products to specific areas of the worm in order to determine compartmentation of products and the timing of their synthesis. Several suggestions have been made in the literature that the catechol oxidase cross-linking enzyme is stored as an emulsion with its substrate, the vitelline DOPA containing proteins, within vitelline globules. The location of vpB within the vitelline globules has now been established at the light and EM levels with both monoclonal and polyvalent antisera. In addition, in situ hybridization has indicated that the vpB transcript is most abundantly produced in the vitelline glands and is present in reduced quantity in the proximal vitelline ducts; the message diminishes as the vitelline cells approach Mehlis' gland where it finally becomes undetectable (Rice-Ficht and Waite, 1990). These types of studies are informative in understanding eggshell deposition.

V. Isolation of gene copies encoding vitelline protein C. A number of putative gene copies representing vitelline protein C have been isolated using an oligomer (29-mer) representing the N-terminus of the protein (Waite and Rice-Ficht, 1989). Inosine was substituted at the most degenerate positions and tetramethylammonium chloride salts were employed during hybridization to create a base composition independent hybridization. A number of cDNAs 500-600bp in length have been isolated and 6 of these sequenced, locating a number of extremely histidine-rich coding sequences but as yet no sequence encoding the exact amino acid sequence of the vpC protein. Additional isolates are being examined with an application of asymmetric PCR which circumvents subcloning and permits a survey of numerous isolates simultaneously.

WORK PLAN FOR YEAR 4:

The project focus for the next year will be:

1) Characterization of cDNAs encoding the 17kDa proteins, vpC.

2) Isolation of the catechol oxidase enzyme and its corresponding gene.

3) Preliminary survey of the metal composition of eggshells using electron probe microanalysis.

4) Analysis of shell cross-links via solid state NMR (in collaboration with Dr. J. Schaefer, Washington University).

5) Use of bifunctional cross-linking agents to study protein-protein interactions.

6) Isolation of cDNAs encoding vpA to determine primary sequence.

INVENTIONS (YEAR 3): The DNA sequences of the shell proteins vpB1 and VpB2 as well as the polymeric protein may be patentable if they prove to have unique materials application.

PUBLICATIONS AND REPORTS (YEAR 3):

1. Waite, J. H. and Rice-Ficht, A. C. (1990) Eggshell Precursor Proteins of Fasciola hepatica: Microheterogeneity in Vitelline Protein B. (to be submitted to Mol. Biochem. Parasit.)

2. Rice-Ficht, A. C., Dusek K. A., Kochevar, G. J. and Waite, J. H. (1990) Eggshell Precursor Proteins of F. hepatica; Structure and Expression of Vitelline Protein B. (to be submitted to Mol. and Biochem. Parasit.)

TRAINING ACTIVITIES: At the present time there is one technician, one graduate student and one postdoctoral enrolled in the study.

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Non-citizens - 1