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Presclerotized Eggshell Protein from the Liver Fluke *Fasciola hepatica*†

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ABSTRACT: Trematode parasites protect their eggs with a tough tanned eggshell. Eggshell precursor proteins are synthesized and stockpiled within the extensive vitellaria of the animal. A major eggshell precursor protein with an apparent molecular weight of 31 000 and p*I* of 7.4 was isolated from the vitellaria of *Fasciola hepatica*. This protein, which represents 6–7% of the total protein in mature *Fasciola*, is unique in containing rather high levels of the amino acid 3,4-dihydroxyphenylalanine (DOPA), i.e., 110 residues per 1000. Other prominent amino acids are glycine, aspartic acid, and lysine. A prominent DOPA-containing tryptic peptide derived from eggshell precursor protein has the sequence Gly-Gly-Gly-DOPA-Gly-Gly-DOPA-Gly-Lys. DOPA residues disappear during the maturation of the eggshell and by treatment in vitro with mushroom polyphenol oxidase. This disappearance may be related to the formation of cross-links in the eggshell protein.

The liver fluke *Fasciola hepatica* is a parasitic trematode that occurs throughout the world and causes "liver rot" in infected sheep and milder cirrhotic complications in cattle, goats, and humans. Immature *Fasciola* migrates through the liver parenchyma, consuming copious amounts of tissue. Upon maturation, *Fasciola* moves from the parenchyma to the bile ducts of the liver where it feasts on blood, leaving a trail of eggs and fecal material. A mature liver fluke produces an average of 2500 eggs daily (Björkman & Thorsell, 1963). Some of these eggs find their way into the gut, through which they are passed to continue their life cycles. The entrapped eggs, however, eventually decompose, setting up serious inflammatory and fibrotic reactions in surrounding tissues (Pantelouris, 1965; Malek, 1980).

To protect the eggs from the host as well as the external environment, the liver fluke encapsulates the eggs with a mechanically tough and chemically resistant scleroprotein eggshell. The stability of this scleroprotein has been variously ascribed to quinone tanning (Stephenson, 1949; Nollen, 1971), dityrosine cross-links (Ramalingam, 1972), and keratin (Smyth & Halton, 1982) but has never been adequately investigated. Eggshell precursor proteins are synthesized and stockpiled by vitelline cells in the extensive vitellaria of *Fasciola* (Smyth, 1954; Björkman & Thorsell, 1963; Irwin & Threadgold, 1972). Mature vitelline cells sequester eggshell precursors in mottled globules that contain two immiscible substances. The discontinuous "polka dot" substance strongly binds heavy metals (Björkman & Thorsell, 1963), basophilic dyes such as malachite green (Johri & Smyth, 1958), and di- and tetrazotized salts and other reagents with affinities for polyphenols (Stephenson, 1949; Smyth, 1954; Bogitsch, 1984). These tests suggest the presence of a cationic protein rich in orthodiphenolic groups (Smyth & Clegg, 1959). The continuous

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substance, on the other hand, appears to contain the enzyme polyphenol oxidase, also called catechol oxidase, phenolase, and tyrosinase (Smyth, 1954).

Actual formation of the eggshell begins following passage of the ovum, sperm, and about 30 vitelline cells through a structure known as Mehli's gland. By an unknown mechanism this gland induces the vitelline cells to release their globules and encircles the cells with a delicate lipoprotein membrane (Clegg, 1965). The interfacial tension between this membrane and the migrating globules is such that the latter spread spontaneously on contact with the membrane (Threadgold & Irwin, 1970), forming a uniformly dense homogeneous shell. The polyphenolic and cationic histochemical properties of the eggshell diminish rapidly as the egg proceeds through the uterus. By the time the egg reaches host tissue, the eggshell has become a histochemically inert capsule (Smyth & Clegg, 1959).

To date only Rainsford (1972) has reported isolation of eggshell precursor proteins from a trematode, namely, *Fasciola*. He described two proteins staining with a tetrazotized dye (Fast Red B) and having molecular weights of 6000 and 12000, respectively. Although these proteins vaguely resembled the eggshell in their amino acid composition, no polyphenolic and few basic amino acids were detected to account for their unusual staining properties. Rainsford's explanation for this apparent paradox was that the stains were probably not as specific as they were thought to be.

In his first detailed histochemical investigation of eggshell formation in *Fasciola*, Smyth (1954) compared the vitelline globules to granules in the so-called "phenol gland" of the marine mussel *Mytilus edulis*. The phenol gland contains precursors for the byssus, which is a quinone-tanned attachment structure. Precursor protein from the phenol gland has recently been purified and characterized (Waite et al., 1985; Waite, 1983). In agreement with Smyth's histochemical predictions, mussel precursor protein is highly cationic and polyphenolic with more than 200 and 130 residues per 1000 of lysine and 3,4-dihydroxyphenylalanine (DOPA), respectively. In light of these results, we have reexamined the biochemistry of the purified eggshell precursor protein of *Fasciola*. Indeed, the eggshell precursor protein is enriched in lysine and DOPA, and it is DOPA in particular that undergoes drastic reduction during quinone tanning in vitro.

Experimental Procedures

Isolation of Fasciola Eggshells. The anterior portions of about 60 mature *Fasciola* were dissected in cold 0.15 M NaCl to expose the uterus. Each uterus, which is usually packed with hundreds of eggs, was teased apart with microforceps, thereby releasing the eggs into the medium. When stained directly with molybdate–nitrile (Waite & Benedict, 1984), a specific reagent for *o*-diphenols, these eggs are typically in various stages of sclerotization, with those in the proximal uterus staining intensely and those in the distal uterus showing little if any staining. The eggs were harvested from the saline medium by centrifugation for 2 min with an Eppendorf microfuge followed by two additional wash–spin cycles. The pellet was resuspended in 5% acetic acid with 8 M urea and disrupted in a tightly fitting glass tissue grinder (Kontes, Vineland, NJ) with ten strokes. The homogenate was stirred at 25 °C for 6 h and then spun in the microfuge for 2 min. This pellet was resuspended in 5% acetic acid with 2 M urea and 0.01% (w/v) pepsin and digested overnight. Insoluble residue was harvested in the microfuge as before and washed with two wash–spin cycles of 0.1 M borate, pH 8.0, with 2 M urea. Trypsin (0.01%) was added to the third buffer change, and the digestion was carried out overnight at 25 °C. The insoluble residue was collected by centrifugation for 2 min and rinsed with four wash–spin cycles using double-distilled water. Upon microscopic examination the insoluble residue was found to contain only empty eggshells. These were hydrolyzed in 6 M HCl with 5% phenol in vacuo at 110 °C for 24 and 48 h and subjected to amino acid analysis.

Extraction and Purification of Eggshell Precursor Protein. Liver flukes (*F. hepatica*) were removed from condemned bovine livers at the abattoir in Sealy, TX. The flukes were briefly rinsed in buffered saline (0.9% NaCl) and frozen in lots of 30 in liquid nitrogen. The vitellarial portions of the flukes were dissected on blocks of dry ice and homogenized on ice in 50-mL ground glass tissue grinders with neutral salt buffer [0.15 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 10 mM *N*-ethylmaleimide, 25 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM potassium cyanide]. The homogenate was centrifuged for 10 min at 5000*g*, and the pellet was redispersed in more neutral salt buffer. The second pellet was rehomogenized in cold 5% (v/v) acetic acid with 4 M urea and centrifuged for 1 h at 35000*g*. The supernatant was freeze-dried, resuspended in a small volume of 5% acetic acid, dialyzed against 200 volumes of the same, and concentrated under 40 psi of nitrogen by using a PM-10 membrane (Amicon Corp.). The freeze-drying step was absolutely necessary to prevent degradation of the eggshell precursor protein presumably by acid proteases that abound in *Fasciola* (Simpkin et al., 1980). The concentrate was adjusted to 5.5% guanidine hydrochloride and 0.001% (v/v) Triton X-100 and applied to a column of preequilibrated sulfoethyl- (SE-) Sephadex C-50 (1 cm × 10 cm). The column was washed with 10 volumes of 5.5% guanidine hydrochloride, 5% acetic acid, and 0.001% Triton X-100, and protein elution was achieved with a linear gradient of 5.5–20% guanidine hydrochloride (Waite, 1983). Fractions were assayed at 280 nm and specifically for DOPA with nitrite–molybdate reagent (Waite & Benedict, 1984). Peak DOPA-containing fractions were pooled, concentrated under nitrogen, and reapplied for gel filtration to a column (3 × 60 cm) of Sephadex G-150 eluted with 0.2 M acetic acid. Fractions of peaks at 280 nm were again assayed for DOPA, and those with highest DOPA contents were pooled, concentrated under nitrogen, and further purified by reversed-phase high-performance liquid chromatography (HPLC) using a C-8 silanized silica (Aquapore RP-300, Brownlee) with acetonitrile as the mobile phase. When this system is used, DOPA-containing proteins rather characteristically elute at 27–29% acetonitrile (Waite et al., 1985). Peak fractions (280 nm) were freeze-dried.

Preparation of Tryptic DOPA Peptides. Purified eggshell precursor protein containing 9.5–11% DOPA was selected for trypsinization. Protein (1–5 mg) was dissolved in 5–10 mL of deaerated 0.1 M Tris-HCl, pH 7.8, with 0.1 mM $CaCl_2$ and 2 M urea. The protein is not initially soluble in this solution. Trypsin (Boehringer Mannheim) was added at an enzyme to protein ratio of 1:100, and digestion was carried out for 12 h at 25 °C under 40 psi of nitrogen. Trypsinization was terminated by passage of the digest through a 5-mL bed of phenyl boronate on an affinity column (Glyco-Gel B, Pierce Chemical Co.). Trypsin and peptides not containing DOPA were washed through with 0.1 M Tris-HCl, pH 7.8, and 0.001% sorbitol (Hawkins et al., 1986). DOPA peptides were eluted with 0.2 M acetic acid and further resolved by C-8 reversed-phase chromatography using a shallow (5–8%) gradient of acetonitrile in water and 0.1% trifluoroacetic acid

Table I: Purification of *Fasciola* Eggshell Precursor Protein Based on the DOPA-to-Protein Ratio

step	vol (mL)	DOPA[a] (mg)	protein[a] (mg)	DOPA/protein	*x*-fold	yield (%)
neutral salt homogenate	400	7	700	0.01	1	100
acetic acid extract	100	6	150	0.04	4	86
SE-Sephadex peak	10	4	40	0.10	10	57
Sephadex G-150, DOPA-rich	20	3	25	0.12	12	43
HPLC peak, leading edge	3	2	12.5	0.16	16	30

[a] DOPA and protein were quantified by amino acid analysis following hydrolysis.

(Waite et al., 1985). Fractions with peaks at 280 nm were freeze-dried, and a small aliquot (5–10%) was hydrolyzed for amino acid composition.

Composition and Sequencing. Amino acid analysis was performed on a Beckman 6300 analyzer following hydrolysis in 6 M HCl with 5% phenol at 110 °C for 24 h in vacuo (Waite & Benedict, 1984). Standard L-DOPA was from Sigma. Peptide sequencing and amino-terminal analysis were done with a Beckman 890C sequenator with the 0.1 M Quadrol program in the presence of polybrene and glycylglycylglycine, which was subjected to three precycles of Edman degradation (Ozols & Heinemann, 1982). Cleavage of the anilinothiazolinone derivatives was done with anhydrous heptafluorobutyric acid. Conversion of these to phenylthiohydantoin (PTH) derivatives was carried out in methanolic HCl, and the PTH derivatives were identified by HPLC on a C-18 silane column (Waters μBondapak) using a 5–45% linear gradient of methanol in aqueous 0.1% acetic acid. Identification of amino acids was confirmed by amino acid analysis following hydrolysis of PTH derivatives in HI vapor (Ozols et al., 1976). Two amino acids, threonine and serine, are usually destroyed by PTH derivatization. These are identifiable as alanine and α-aminobutyric acid, respectively, following HI back-hydrolysis. The PTH-DOPA standard coelutes with PTH-alanine on C-18 HPLC, but the two are distinct following HI hydrolysis (Waite et al., 1985).

Electrophoresis. Routine electrophoresis was done on 5% polyacrylamide gels in 5% acetic acid with 2 M urea (Waite & Benedict, 1984). This system was convenient here because it lends itself readily to staining either for protein or DOPA. Molecular weights were determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel (10% acrylamide) electrophoresis using the method described in detail in the Hoefer Scientific catalog. Isoelectric focusing was performed with ampholytes in the pH range 6–9 (Servalytes) according to a procedure of Miller and Elgin (1974) except that the urea was decreased from 8 to 2 M.

UV Difference Spectroscopy. UV difference spectroscopy as applied to DOPA proteins is based on the differential absorptivity and wavelength shift of *o*-diphenols in borate buffers at pH around 8 vis-à-vis non-borate-containing buffers at similar pH (Waite, 1984). Typically, a DOPA-containing protein is dissolved in 0.1 M borate, pH 8.2, and placed in the sample cuvette; an identical amount of protein is dissolved in distilled water or 0.1 M acetic acid and placed in the reference cuvette. The latter two buffers were necessitated by the fact that the eggshell precursor protein precipitated in 0.1 M phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and Tris at pH 6–9.

Cross-Linking in Vitro. Eggshell precursor protein with at least 9.5% DOPA content was mixed with mushroom polyphenol oxidase (Sigma, T7755) at an enzyme to eggshell protein ratio of 1:10 by weight. This high ratio was necessitated by the rather sluggish reaction rate. The mixture was reacted at 25 °C in 0.05 M sodium phosphate (pH about 7.8) with 0.001% (v/v) Triton X-100 under constant stirring. The Triton X-100 was added to retard the tendency of eggshell precursor to precipitate in the buffered pH range 6–9. Aliquots (10–20 μL) were removed at various time points in the course of the reaction. These were evaluated by amino acid analysis following hydrolysis for 24 and 48 h. A control reaction was run using boiled (2 min) enzyme.

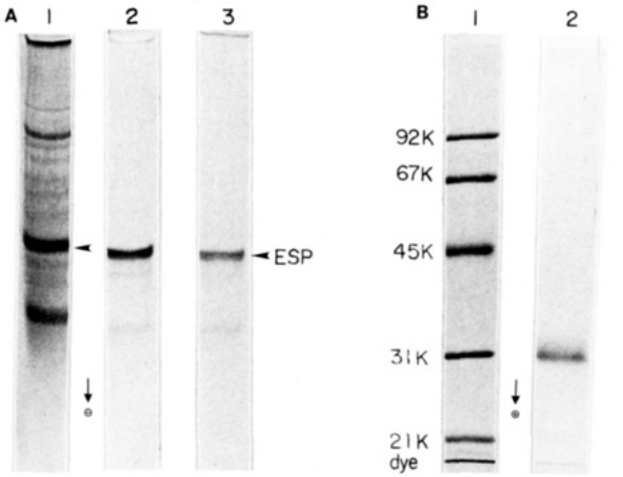

FIGURE 1: Polyacrylamide gel electrophoresis of eggshell precursor proteins. (A) Acid–urea gels: 70 μg of acetic acid extracted protein from *Fasciola* vitellaria stained with Coomassie blue R-250 (lane 1); 20 μg of eggshell precursor purified by SE-Sephadex stained with Coomassie blue (lane 2); 40 μg of eggshell precursor (ESP) stained for DOPA by molybdate–nitrite reagent (lane 3). (B) SDS gels: 5 μg each of phosphorylase *a* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400), which coelutes with the tracker dye bromphenol blue (lane 1); 15 μg of eggshell precursor protein purified by C-8 HPLC (lane 2). Both gels are stained with Coomassie blue R-250. Vertical arrows denote orientation of polarity during electrophoresis.

RESULTS

Purification of DOPA Protein. An unusual DOPA-containing protein, which we shall call an eggshell precursor protein, was isolated from *Fasciola* by a combination of selectively extractive and chromatographic procedures (Table I). This protein was found to be easily adsorbed to surfaces and to be insoluble in the pH range 6.0–9.0. Considerable care was required in order to formulate a plan of purification that was not fraught with significant protein loss: working at a pH of about 3.0 is particularly important. The DOPA-to-protein ratio is a convenient index for assessing the purity of protein after each purification step (Table I). The DOPA/protein value of 0.01 in the first neutral salt homogenate suggests that eggshell precursor protein(s) may represent 6–7% of the total protein in *Fasciola*. This estimate is based on the observation that, in purified eggshell precursor, DOPA represents about 16% of the weight of the protein. It should be noted here, however, that the molybdate–nitrate stained gels in Figure 1A suggest that more than one protein from *Fasciola* may contain DOPA; only the DOPA-to-protein ratio of the major protein is known. DOPA-to-protein ratios in other DOPA proteins or in incompletely hydroxylated versions of the precursor described here may be more or less than 0.16. In general, the

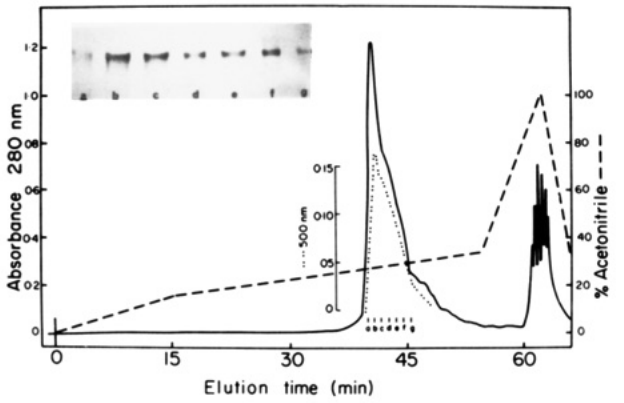

FIGURE 2: Elution profile of *Fasciola* eggshell precursor on a C-8 reversed-phase column. Fractions were assayed by absorbance at 280 nm, molybdate–nitrite reactivity at 500 nm, and acid–urea gel electrophoresis. Inset shows results of gel electrophoresis of fractions a–g.

Table II: Amino Acid Composition of the Major Eggshell Precursor Protein of *Fasciola hepatica* in Residues per Thousand

amino acid	purified[a]	oxidized (6 h)[a]	eggshell[a] immature	eggshell[a] mature
Asx	140 ± 4.3	161 ± 2.1	185 ± 1.1	192[b]
Thr	18 ± 3.1	24 ± 1.0	19 ± 0	21
Ser	52 ± 4.8	60 ± 2.3	59. ± 2.7	45
Glx	83 ± 3.7	101 ± 3.4	83 ± 0.8	83
Pro	16 ± 0.9	24 ± 1.0	28 ± 0.6	22
Gly	165 ± 4.6	183 ± 4.3	194 ± 0.9	194
Ala	69 ± 2.4	81 ± 3.2	48 ± 0.6	47
$^1/_2$-Cys	0	0	< ± 0.5	48
Val	9 ± 2.1	11 ± 0.2	13o ± 0.8	16
Met	23 ± 4.2	23 ± 2.3	16 ± 0	9
Ile	5 ± 1.0	11 ± 0.8	8 ± 0.5	29
Leu	38 ± 1.4	44 ± 3.1	32 ± 0.8	19
DOPA	106 ± 9.8	23 ± 5.2	61 ± 1.5	nd
Tyr	21 ± 5.0	28 ± 2.4	35 ± 0.8	12
Phe	38 ± 3.1	39 ± 2.5	32 ± 0.2	39
His	45 ± 3.7	35 ± 3.7	49 ± 0.8	76
Lys	120 ± 6.5	86 ± 8.1	75 ± 2.0	67
Arg	60 ± 3.2	65 ± 1.1	63 ± 0.5	63

[a] Ser, Thr, DOPA, and Tyr corrected for losses due to hydrolysis. $N = 5$. [b] Rainsford (1972).

highest purification of the precursor protein is achieved following C-8 reversed-phase HPLC (Figure 2).

Characterization of DOPA Protein. The amino acid composition of the leading edge of the peak in Figure 2 is shown in Table II (colu nn 1) and highlights primarily four amino acids: aspartic acid/asparagine, glycine, DOPA, and lysine. Met content is also quite high for this usually scarcer amino acid; cysteine (or cysteic acid), on the other hand, appears to be absent. Curiously, the peak obtained by HPLC is never symmetric (Figure 2). Amino acid analysis of other hydrolyzed fractions under the curve reveals that these vary in the extent of the tyrosyl to DOPA conversion (Figure 3). All fractions under the curve in Figure 2 contain proteins having identical mobilities on acid urea gels (inset, Figure 2). This implies that the protein is modified posttranslationally in the vitellaria and that a continuum of modification exists at any particular time. The apparent molecular weight of eggshell precursor is 31 000 ± 2000 as determined by SDS–polyacrylamide gel electrophoresis (Figure 1B). Isoelectric focusing of the precursor protein over a fairly narrow pH gradient resulted in a broad band (staining for both DOPA and protein) having a p*I* of about 7.3–7.5. Two-dimensional gel electrophoresis was not possible owing to the irreversible binding of the eggshell precursor protein to polyacrylamide following isoelectric focusing.

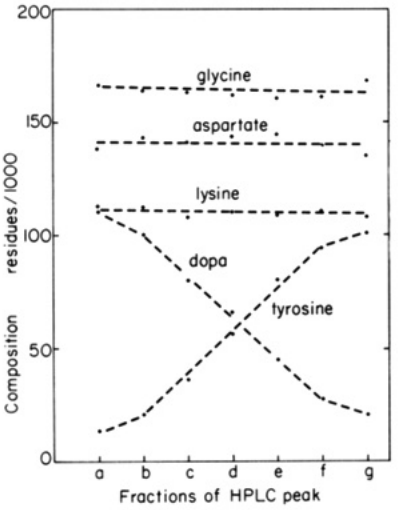

FIGURE 3: Distribution patterns for the amino acids Asx, Lys, Gly, DOPA, and Tyr following acid hydrolysis in vacuo of HPLC fractions a–g.

Preparation of Fasciola Eggshells. The preparation of eggshells from *Fasciola* uteri offers a convenient alternative to their isolation from host tissues or feces in that they can easily be obtained in large numbers without the problem of adsorbed and contaminating host proteins. We prefer to refer to these eggshells as "immature" since they have not yet been released from the uterus. The amino acid composition of immature *Fasciola* eggshells resembles that of the 31-kDa precursor protein, although Asx and Gly levels are higher and Lys and DOPA levels are significantly lower in the eggshells (Table II). For comparison, the composition of a "mature" eggshell preparation as reported by Rainsford (1972) has been included in Table II. We must caution, however, that in our hands the alkaline extraction method used by Rainsford leads to artifactually low Tyr values, the complete destruction of DOPA, and a new peak coeluting with cystine (data not shown).

Oxidation of DOPA Protein by Mushroom Polyphenol Oxidase. In vitro oxidation of the 31-kDa protein with mushroom polyphenol oxidase also results in a composition with decreased DOPA and Lys and correspondingly higher Asx and Gly (Table II). Notably, Tyr does not show any decreasing tendency with reaction time. Gel electrophoretic analysis of aliquots of the in vitro oxidation suggests a rapid disappearance of the 31-kDa protein with no clear *incremental* trend to higher or lower molecular weight (results not shown). Pigmented protein aggregates accumulate on the walls of the reaction vessel.

Characterization of DOPA-Containing Peptides. A number of tests aimed at demonstrating that the amino acid DOPA was associated with the protein in a covalent manner were carried out. As already shown above, it is not removed from the eggshell precursor by neutral salt extraction and migrates with the protein during acid and SDS gel electrophoresis as shown by nitrite–molybdate treatment of gels (Figure 1). DOPA can also be detected spectrophotometrically in eggshell precursor by borate difference spectra. The molar absorptivity constant for difference spectra of DOPA in proteins is 3200 M^{-1} cm^{-1} (Waite, 1984); tyrosyl and phenylalanyl residues do not interfere significantly. The most direct method for demonstrating DOPA in the primary sequence of the eggshell precursor is to sequence DOPA-containing peptides. This was achieved following digestion of DOPA-enriched eggshell precursor protein with trypsin, isolation of DOPA-containing peptides using a boronate affinity column, and purification of

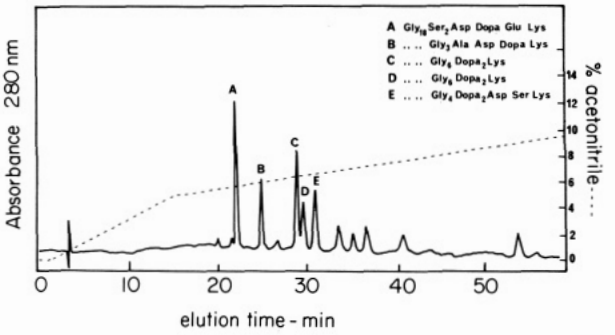

FIGURE 4: Elution profile of DOPA-containing tryptic peptides following C-8 reversed-phase HPLC. Peptides were isolated by affinity chromatography on boronate gel. Fractions were assayed by absorbance at 280 nm and amino acid analysis.

A NH_3^+-Ser-Asp-Gln-Dopa-Gly-Gly-Gly-Gly-Gly-

B NH_3^+-Gly-$\begin{matrix}\text{Asp}\\\text{Gly}\end{matrix}$-$\begin{matrix}\text{Gly}\\\text{Asp}\end{matrix}$-Ala-Dopa-Gly-Lys-COOH

C NH_3^+-Gly-Gly-Gly-Dopa-Gly-Gly-Dopa-Gly-Lys-COOH

E NH_3^+-Gly-Gly-Gly-Dopa-Asp-Ser-Dopa-Gly-Lys-COOH

Me NH_3^+-Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys-COOH

Mc NH_3^+-Ile-Thr-Tyr-Hyp-Hyp-Thr-Dopa-Lys-Hyp-Lys-COOH

FIGURE 5: Amino acid sequence of four DOPA-containing tryptic peptides derived from eggshell precursor protein. Sequence of (A) is partial only. Included for comparison are consensus peptides from the polyphenolic proteins of the mussels *Mytilus edulis* (Me) and *M. californianus* (Mc).

the peptides by reversed-phase C-8 HPLC (Figure 4). The justification for selection of the DOPA-enriched fractions deserves comment. Suppose the eggshell precursor contains a tryptic peptide with a full potential of two DOPA groups in it. Since the precursor extracted from *Fasciola* seems to reflect a continuum of tyrosine-to-DOPA processing, then this hypothetical tryptic peptide can be expected to be present as four variations in the extracted protein, namely, X-DOPA-Y-DOPA-Z-Lys, X-DOPA-Y-Tyr-Z-Lys, X-Tyr-Y-DOPA-Z-Lys, and X-Tyr-Y-Tyr-Z-Lys. Selection of DOPA-enriched precursor simplifies purification of the tryptic peptides since it is more likely to contain sequences with a fully realized potential of DOPA groups. The six major peaks purified by HPLC are all enriched in glycine and DOPA and presumably have C-terminal lysines. Amino acid compositions are shown above the peaks in Figure 4. Sequences for four of the peptides are listed in Figure 5. Two are nonapeptides with DOPA in the fourth and seventh positions, one is a hexapeptide with a single DOPA and, finally, the largest has 20 amino acids, 1 of which is DOPA. Other peaks in the profile have yet to be sequenced.

Discussion

We have characterized a prominent protein from the vitellaria of *Fasciola* that represents 6–7% of the total prtein in the animal. This protein is rich in Asx, Lys, Gly, and DOPA and has an apparent molecular weight of 31 000 and an average p*I* of 7.4 ± 0.1. The similarity between the amino acid composition of this protein and the eggshell, particularly with regard to DOPA, suggests that it is a presclerotized precursor of the eggshell. It is not necessarily the only eggshell precursor since other DOPA-containing proteins are detectable following acid–urea–polyacrylamide gel electrophoresis (Figure 1). Recent sequence of a cDNA clone from the female genital complex of *Fasciola* coding for a Tyr-rich 21-kDa protein (Zurita et al., 1987) lends credence to the existence of other precursors; it is noteworthy that the putative protein of Zurita

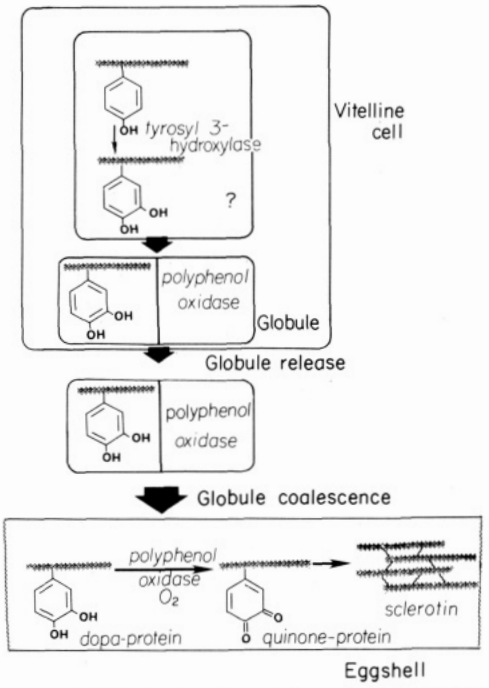

FIGURE 6: Model illustrating the origin and fate of DOPA in the eggshell precursor protein of *Fasciola*. This amino acid might be formed by the action of a putative tyrosine 3-hydroxylase on a protoeggshell precursor protein. The cellular compartment of this hydroxylation is unknown. The DOPA-containing eggshell precursor is then stockpiled along with polyphenol oxidase in the biphasic vitelline globules. Vitelline globules are released from vitelline cells and coalesce during eggshell formation, during which polyphenol oxidase acts on the DOPA residues, converting them into cross-linking *o*-quinones.

shares a pre-DOPA sequence (GGGYDSYGK) with the 31-kDa protein described here. The 6- and 12-kDa eggshell precursors of Rainsford (1972), however, are questionable since he took no precautions to inhibit protease activity in the extracts; the presence of DOPA was not shown in these precursors. The abundance of the 31-kDa precursor in *Fasciola* reflects the emphasis on reproduction and associated activities in the energy budget of helminths (Calow, 1981; Wharton, 1984). In the cestode *Hymenolepis diminuta*, roughly a third to half of the animal's energy budget is earmarked for reproductive activities; an estimated third of the reproductive allotment is invested in eggshell formation (Wharton, 1984). In a similar vein, 10% of the mRNA in mature female *Schistosoma mansoni* was found to code for a putative eggshell precursor protein (Bobek et al., 1986).

The presence of DOPA in the primary sequence of a protein is unusual, though not without precedent. The consensus repeats from adhesive DOPA proteins obtained from two mussel species are shown in Figure 5. Notably, these bear little resemblance to the eggshell sequences beyond the presence and relative proximity of DOPA and Lys. Presumably the DOPA is derived from tyrosyl groups by a protein-directed tyrosine 3-hydroxylase. At present nothing is known about such an enzyme. As illustrated in Figure 6, the enzyme is presumed to act posttranslationally on tyrosyl groups of the protein while it is in the vitelline cells. This is supported by the fact that a DOPA/Tyr gradient exists in HPLC-purified precursor protein (Figure 3). Features of enzyme specificity are reflected in the sequence of tryptic peptides: DOPA is always found flanked on at least one side by Gly. Frequently, Gly flanks DOPA on both sides, and when substitutions occur, these are amino acids with small side chains, e.g., Ser and Asp. It is noteworthy that most of the tyrosines in cDNA clones coding for the putative eggshell precursors of *Schistosoma mansoni*

described by Bobek et al. (1986) and Johnson et al. (1987) occur in similar environments, although in the latter the bulkier Lys is also found flanking some of the tyrosines. A better understanding of hydroxylase sequence preference awaits purification of the responsible enzyme.

The function of DOPA in the eggshell precursor is still somewhat unclear. The decrease in DOPA from hydrolysates of eggshells and eggshell precursor protein treated with mushroom polyphenol oxidase probably reflects its conversion to an *o*-quinone. However, no new peak was detected by amino acid analysis. Lysine also decreases in hydrolysates of eggshells and enzyme-treated precursor protein vis-à-vis the untreated precursor. Quinones and polyphenol oxidase have long been suspected as playing a role in eggshell sclerotization. Polyphenol oxidase activity has been detected in the eggshells and vitelline cells of *Fasciola* (Smyth, 1954; Thangaraj et al., 1982) and *Schistosoma* (Bennett et al., 1978; Wang et al., 1986), where it appears to be localized in the vitelline globules, presumably side by side with the eggshell precursor proteins (Smyth, 1954) (Figure 6). There is some suggestion that while in the globules the enzyme is present in latent form (Thangaraj et al., 1982); upon secretion, the enzyme probably acts preferentially on proximal DOPA groups, converting them to *o*-quinones.

o-Quinones are notoriously unstable and, in principle, can undergo Michael-type nucleophilic addition reactions with amine, imine, imidazole, and sulfhydryl groups in proteins (Dryhurst et al., 1982; Schaefer et al., 1987). A progressive sclerotization of the eggshell could result from the formation of cross-links involving DOPA–quinone and lysyl side chains. A reaction linking DOPA and Lys would be in agreement with the general observation that eggshells lose their polyphenolic *and* cationic properties as they advance down the uterus (Smyth & Clegg, 1959). Such cross-links have yet to be characterized, however.

Biochemical characterization of choriogenesis in invertebrates is fragmentary at best and limited to a few insects and sea urchins. In the insects, existing gene and protein sequences of chorion proteins show some remarkable similarities in primary structure. Silkworm (Regier et al., 1983; Eickbush et al., 1985), cockroach (Pau, 1984), and fruit fly (Levine & Spradling, 1985; Wong et al., 1985) chorion proteins all have regions rich in Gly *and* Tyr, even though the Tyr residues are not converted to DOPA in these organisms. Stability of these proteins is derived from conformation, i.e., β-pleated sheet, and disulfide bonds (Hamodrakas et al., 1984), although quinone tanning has also been reported in some mantid and cockroach eggcases (Brunet, 1967; Kawasaki & Yago, 1983). In the latter case, the *o*-quinones are enzymatically derived from low molecular weight *o*-diphenols such as 3,4-dihydroxybenzoic acid and *N*-acyldopamine. In sea urchins, the eggshell precursor protein has not been well characterized, but it is known to be stabilized by dityrosine cross-links formed by an ovoperoxidase (Hall, 1978; Deits et al., 1984).

How much diversity there is in the primary sequence and stabilization of eggshell proteins in the *Platyhelminthes* is not currently known. Smyth and Halton (1982) surveyed a number of histochemical studies on eggshell formation in mono- and digenetic trematodes and concluded that with the exception of one family (Paramphistomatidae), all showed qualitative evidence of having polyphenolic protein precursors and the enzyme phenoloxidase. These two features also characterize eggshell formation in cestodes (Smyth & Clegg, 1959) and the free-living *Platyhelminthes* (Bunke, 1972; Ishida & Teshirogi, 1986).

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The Canine Renal Parathyroid Hormone Receptor Is a Glycoprotein: Characterization and Partial Purification†

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ABSTRACT: Covalent labeling of the canine renal parathyroid hormone receptor with [^{125}I]bPTH(1–34) reveals several major binding components that display characteristics consistent with a physiologically relevant adenylate cyclase linked receptor. Through the use of the specific glycosidases neuraminidase and endoglycosidase F and affinity chromatography on lectin–agarose gels, we show here that the receptor is a glycoprotein that contains several complex N-linked carbohydrate chains consisting of terminal sialic acid and penultimate galactose in a β1,4 linkage to *N*-acetyl-D-glucosamine. No high mannose chains or O-linked glycans appear to be present. The peptide molecular weight of the deglycosylated labeled receptor is 62 000 [or 58 000 if the mass of bPTH(1–34) is excluded]. The binding of [^{125}I]bPTH(1–34) to the receptor is inhibited in a dose-dependent fashion by wheat-germ agglutinin, but not by either succinylated wheat-germ agglutinin or *Ricinus communis* lectin, suggesting that terminal sialic acid may be involved in agonist binding. A combination of lectin affinity chromatography and immunoaffinity chromatography affords a 200-fold purification of the covalently labeled receptor.

It is generally agreed that most of the effects of parathyroid hormone (PTH)[1] on its major targets (kidney and bone) are mediated, at least in part, by an intrinsic membrane-bound receptor which is catalytically linked to adenylate cyclase through a stimulatory guanine nucleotide regulatory protein (G_s) (Goltzman et al., 1978; Nissenson, 1982; Teitelbaum et al., 1982; Habener et al., 1984). Whereas the biological properties of this receptor have been characterized extensively, little information on its structure has been forthcoming since its initial identification by photoaffinity radiolabeling techniques (Coltrera et al., 1981; Draper et al., 1982). It has been necessary to covalently label the receptor in order to detect its presence after detergent solubilization, because disruption of the receptor–G_s complex shifts the receptor to a very low affinity state for agonist binding (Goltzman et al., 1978; Teitelbaum et al., 1982), and no ligands (either PTH agonists or antagonists) of sufficient affinity to be useful for detection of the low-affinity state of the PTH receptor are currently

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[1] Abbreviations: PTH, parathyroid hormone; b, bovine; h, human; VIP, vasoactive intestinal peptide; HSAB, *N*-succinimidyl 4-azidobenzoate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid trisodium salt; GTP, guanosine 5′-triphosphate; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; WGA, wheat-germ agglutinin; S-WGA, succinylated wheat-germ agglutinin; RCA_I, *Ricinus communis* agglutinin I; Con A, concanavalin A; PNA, peanut agglutinin; UEA-F, *Ulex europaeus* agglutinin I; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-(+)-galactose; Man, methyl α-D-mannopyranoside; GTP, guanosine 5′-triphosphate; Gpp(NH)p, guanylyl imidodiphosphate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); App(NH)p, adenylyl imidodiphosphate; G_s, stimulatory guanine nucleotide regulatory component of adenylate cyclase; Iodo-Gen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Triton, Triton X-100; kDa, kilodaltons; DMSO, dimethyl sulfoxide; Ig, immunoglobulin.

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