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Fasciola gigantica : Eggshell solubility and permeability properties

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Abstract

The cross-linkages contained within the proteinaceous opercular cement seal holding the egg operculum of *Fasciola gigantica* solubilised in 0.1N saturated NaOH, concentrated HCl, H_2SO_4 and H_3PO_4, whereas the quinone tanned eggshell solubilised only in concentrated HNO_3 and NaOCl. In TCA, H_3BO_3 and CH_3COOH, the cross linkages of proteinaceous opercular cement and the quinone tanned eggshell remained unaffected. This study has shown that the cross linkages of the opercular cement, the operculum and the quinone-tanned eggshell differ in their permeability and solubility properties.

Keywords: *Fasciola gigantica*, eggshell, permeability and solubility in acids, alkali and chemicals

Introduction

The formation of the eggshell in helminths has fascinated and challenged parasitologists (Smyth & Clegg, 1959; Ramalingam, 1972, 1973; Cordingley, 1987). It is mainly due to the fact that a large proportion (20- 30%) of the total energy budget of helminth parasites is devoted to eggshell formation (Wharton, 1983; Fairweather *et al.,* 1999) and to the pathological complications caused by the entrapment of helminth eggs in host tissues and blood capillaries (Malek, 1980). The helminth eggshell consists of sclerotin or quinone tanned proteins (Ramalingam, 1972, 1973; Cordingley, 1987), which involves the conversion of DOPA (3,4- dihydroxy phenyl-L-alanine) residues to unstable O-quinones by the action of phenol oxidase (phenolase or catechol oxidase) and hence react with free amino or sulfhydryl or disulphide groups on adjacent protein molecules to bind the shell materials together. The phenolic tanned proteins form a very stable, tough, water proof and highly resistant barrier to environmental variables and bacterial infection and form a protective capsule around the developing embryo (Smyth & Halton, 1983; Fairweather *et al*., 1999). Information on the permeability and solubility properties of the eggshell of the digenean trematode *Fasciola gigantica* is limited. Hence, the present investigation attempts to elucidate the permeability and solubility properties of tanned eggshell. Various chemicals, detergents, acids and alkali used here are known to interfere/ affect the cross linkages of eggshell proteins which in turn determine the nature of the permeability/ solubility of the cross linkages of tanned eggshell and possible interference with the life cycle of *F. gigantica*.

Materials and methods

Isolation of Fasciola gigantica eggs

F. gigantica were removed from condemned fresh buffalo livers at the Perambur slaughter house, Chennai, India. The flukes were briefly rinsed and transported back to the laboratory in sterilised saline (0.9% NaCl). The uterus was identified in the anterior region close to the ventral sucker where the eggs are identical and matured. The upper integumental layer was carefully removed exposing the uterine tubules which were dissected out and the matured eggs were removed and transferred to a microfuge tube using a micropipette (Fig.1).

Light Microscopic observations

Approximately 100 eggs were transferred to a cavity slide and subjected to 22 different treatments involving either an acid, an alkali or a chemical as detailed in Table 1. The permeability/solubility of the eggshells were periodically observed over time intervals of 0, 1, 12 and 18 hrs by using the light microscope.

Scanning electron microscopic observations

Freshly isolated acid/base/chemical treated and untreated eggs were processed for scanning electron microscopy (SEM). The eggs were fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2 containing 3% sucrose for 2 h at 4°C. The eggs were buffer washed and osmicated in 1% aqueous Osmium tetroxide for 1 h, briefly washed in distilled water, dehydrated in an ascending series of acetones, dried in a critical-point dryer, sputter coated with gold/palladium and examined in a JEOL JSM 6400 scanning electron microscope operating at 10keV.

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Table 1. Fasciola gigantica: Eggshell cross-linkage stability in acids, alkali and chemicals (Solubility and permeability)

Chemicals Treated	0-5 minutes	1 hr	12 hr	18 hr
1N Sodium hydroxide (1N NaOH)	No apparent change	Seal of operculum breaks open	Rest of the quinone tanned eggshell remains normal	
0.1N Sodium hydroxide (0.1N NaOH)	No apparent change	Seal of operculum breaks open	Rest of the quinone tanned eggshell remains normal	
Sodium hypochlorite	Seal of operculum opened		Covalent cross-links of quinone-proteins were broken and the reactive groups were exposed and the eggshell rendered soluble	
Ammonium hydroxide (NH_3OH) (Saturated)	No apparent change			
1% Sodium nitroprusside + 30% Ammonium hydroxide	No apparent change			
Urea (Saturated)	Shrinkage of eggshell the cross-links of proteins were affected	Relapsed to normal size and shape		
Sodium Azide (Saturated)	Shrinkage of eggshell the cross-links of proteins were affected	Relapsed to normal size and shape		
0.75% Sodium thiosulphate	No apparent change			
10% Leadacetate	Shrinkage of eggshell the cross-links of proteins were affected			
Ferric chloride (Saturated)	Shrinkage of eggshell the cross-links of proteins were affected			
Concentrated Nitric acid (HNO_3)	Seal of operculum breaks open		Covalent cross-links of quinines / S-S bonds involved in stabilisation of eggshell were cleaved the reactive groups were exposed and the eggshell rendered soluble	
0.1N Nitric acid	No apparent change, eggs appeared brown in colour			
Hydrochloric acid (HCl) (35% concentrate)	Shrinkage and relapsed to normal size and shape	Seal of operculum breaks open cell contents released	Remainder of quinone tanned eggshell remains normal	
2N Hydrochloric acid (2N HCl)	No shrinkage of eggshell, contents of the egg appeared dark brown			
90% Concentrated Sulphuric acid (H_2S0_4)	Seal of operculum breaks open – rest of the quinone tanned eggshell remains normal, granules dark pink			
1N Sulphuric acid	No shrinkage, contents of the egg-appeared dark brown			
Orthophosphoric acid	Shrinkage of eggshell and opaque	Relapsed to normal size and shape	Seal of operculum breaks open, pinkish in colour	
10% Trichloro acetic acid (10% TCA)	Shrinkage of eggshell and opaque			
TCA concentrated	Shrinkage of eggshell and opaque			
Boric acid (Saturated)	No apparent change			
Acetic acid	Shrinkage of eggshell and opaque			
β-mercaptoethanol	No shrinkage	Opaque		
Triton X-100	Shrinkage of eggshell and opaque			

Results

F. gigantica is flattened with an average length of 4-5 cm when compared to its smaller relative *F. hepatica* (2-3cm). In *F. gigantica* the uterus tubules and Mehlis' gland are clearly visible. The *F. gigantica* eggs exposed to various acids, alkalis and chemical treatments and the results are set out in the Table 1. Untreated eggs are transparent, brown to golden yellow in colour, oval in shape and contain vitelline globules within the egg (Fig. 1a). The proteinaceous opercular cement was dissolved in 0.1 N and saturated sodium hydroxide (NaOH), concentrated hydrochloric acid (HCl), sulphuric acid (H_2SO_4) and orthophosphoric acid (H_3PO_4), hence the operculum of the eggs were opened and their contents were released (Fig. 1b-d, 2a-c). Trichloro acetic acid (TCA), boric acid, acetic acid (CH_3COOH) did not affect the quinone tanned eggshell as well as the operculum cement and thus the eggshell of *F. gigantica* was found to be extremely resistant to action of the above acids. Small molecules (organic, inorganic, polar and non-polar molecules) pass through the eggshell matrix rapidly while the larger molecules penetrate the eggshell matrix slowly. There are no organised pores through which the molecules could be permeable. However, permeability could occur in the spaces between the adjacent protein molecules.

There were no apparent changes in the morphology of egg of *F. gigantica* treated with Ammonium hydroxide, 1% Sodium nitroprusside in 30% Ammonium hydroxide (no apparent change), Saturated Boric acid and β-mercaptoethanol and these observation may indicate the absence of permeability of the eggshell with these chemicals.

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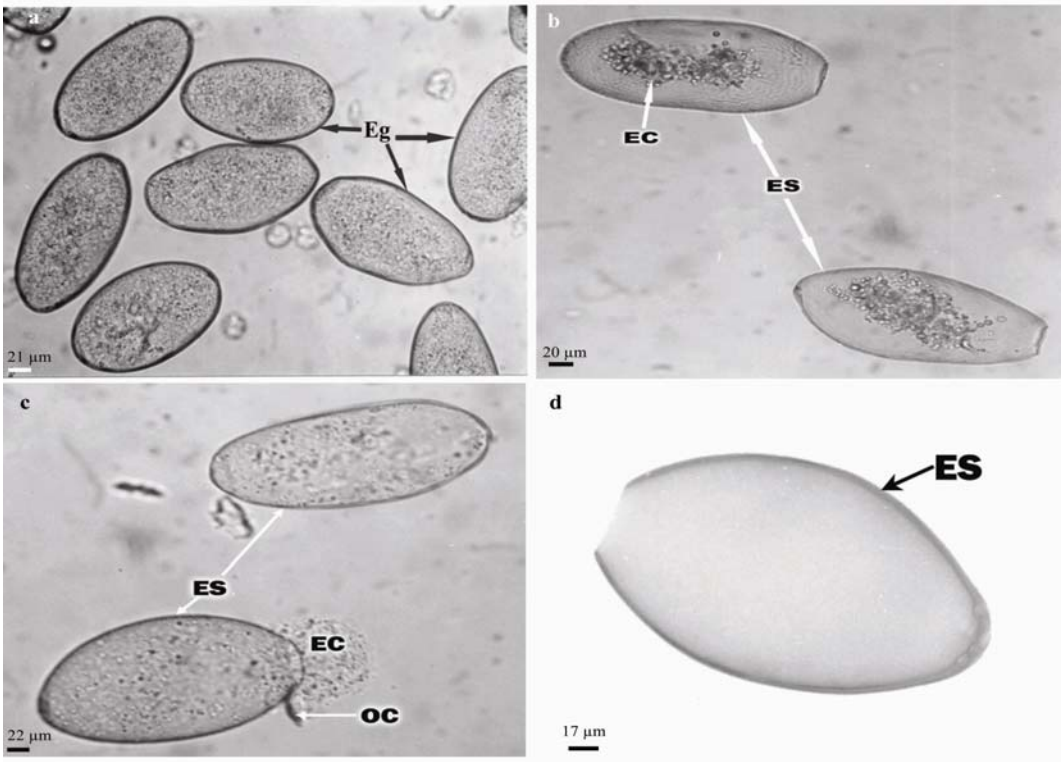
Fig.1a-d. Eggs of F. gigantica were subjected to various acids, alkalis and chemicals. SEM of egg. a) Normal eggs; b) HCl treated eggs show the opened opercular region and he egg contents (EC) present inside the eggs; ES- eggshell; c) 0.1N NaOH treated egg shows the opened operculum with released egg contents. OC- operclum; d) H2SO4 treated eggs show the dark pinkish brown colour with opercular opening



SEM observations clearly show that the opened operculum and opercular cement treated with 0.1N NaOH (Fig. 2c). Treatment with H_2SO_4 gave a dark pinkish coloration to the eggshell (Fig. 1d). Treatment with the detergent Triton X-100, sodium sulphite, CH_3COOH, 10% lead acetate and TCA caused the eggshell to shrink initially and then apparently returned to the presumed normal shape and opaqueness (Fig. 2c). The cross linkages of the tanned eggshell and the opercular cement remained apparently normal even after prolonged periods of exposure indicating that the water molecules from the egg-capsules are attracted towards the solute into the medium.

Concentrated nitric acid (HNO_3) cleaved the quinone and S-S linkages and softened the eggshell exposing the reactive groups of the stabilised eggshell proteins rendering them soluble. In sodium hypochlorite (NaOCl), the golden yellow colour of the eggshell was bleached and on prolonged exposure, it dissolves the cross linkages of the eggshell proteins.

Discussion

Solubility and permeability tests indicated that the eggshell of *F. gigantica* consists of quinone tanned proteins but the proteinaceous opercular cement has been shown to be chemically different from the rest of the eggshell. The opercular cement dissolved in strong mineral acids such as HCl, H_2SO_4 and H_3PO_4 and alkalis such as NaOH, while the rest of the eggshell remained unaffected indicating that the quinone cross-linkages were not disturbed or altered. In addition, with H_2SO_4 and H_3PO_4 the eggshell turned a dark pinkish colour indicating the presence of a keratin type protein as occurs in the eggshell of *Diplodiscus mehrai* (Madhavi, 1968). Anantaraman and Ravindaranath (1973) showed that the hooks of acanthocephalans were highly resistant to dilute as well as concentrated mineral acids like HCl and H_2SO_4. The eggshell and operculum were highly resistant to the dilute mineral acids HCl, H_2SO_4 and alkalis KOH and NaOH whereas concentrated acids weekend the junction between the eggshell and the operculum and the operculum was easily removed. However, concentrated alkalis dissolved the eggshell of *D. mehrai* (Madhavi, 1968). In *F. gigantica* the opercular cement was dissolved even in mild alkaline 0.1N NaOH while the rest of the quinone tanned eggshell remained unaffected.

Concentrated HNO_3 dissolved the cross linkages of the eggshell and the opercular proteinaceous cement of *F. gigantica*. Anantaraman and Ravindaranath (1973) showed that the hooks of *Moniliformis moniliformis* were resistant for about one hour in concentrated HNO_3 before disintegrating. When the eggshell of *F. gigantica* was treated with the detanning agent sodium hypochlorite, the golden yellow colour was bleached. On prolonged treatment for 24 h, the eggshell was dissolved indicating the possible presence of a quinone bond (Stephenson, 1947; Smyth, 1954). Anantaraman (1969) showed that the concentrated hydrochloric acid and sulphuric acid dissolved the inner laminated layer of the cuticle of *Acanthosentis oligospinus.* In this respect, the eggshell of *F. gigantica* exhibits similar changes to those observed in the hooks and outer cuticle layer of the acanthocephalans. *Diplodiscus* eggshell is stabilised by S-S linkage due to the presence of keratin based proteins whereas, *Fasciola* eggshell is made up of quinone tanned

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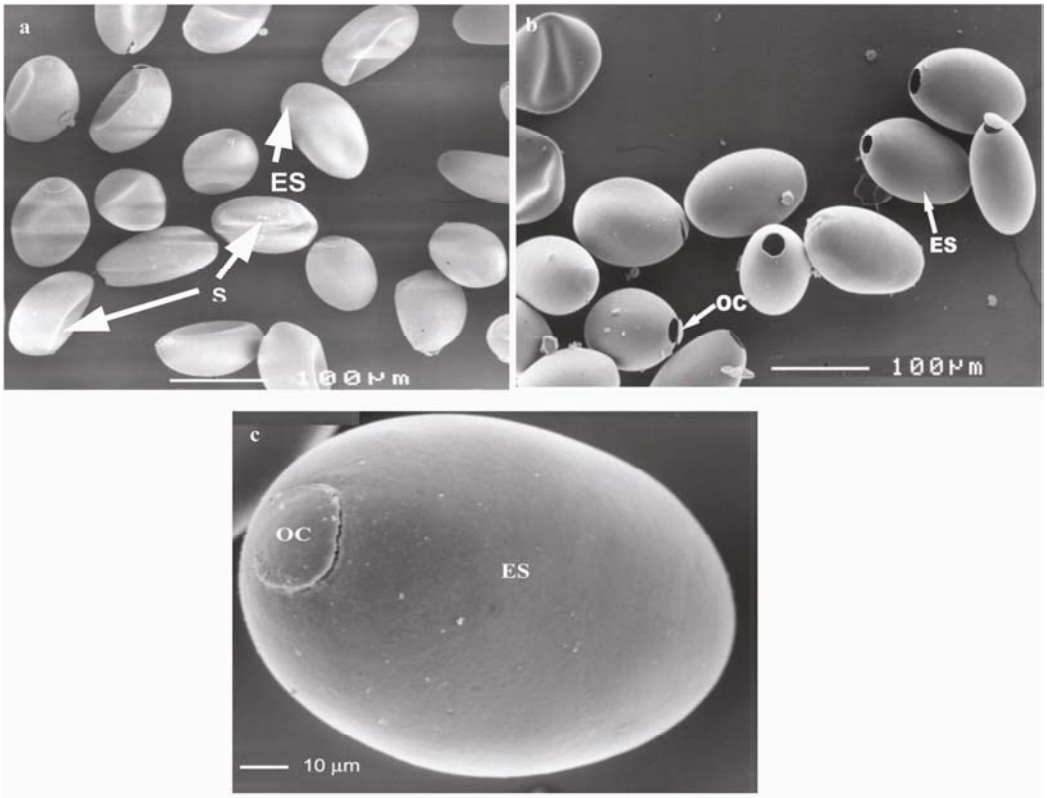
proteins. These studies on the solubility of the eggshell thus give an insight to the understanding of the nature of the cross-links that determine the three dimensional architecture of structural proteins. The present study concludes that the properties of the cross linkages of the cement of the operculum are different from the rest of the quinone tanned eggshell and the counter parts of the trematode paramphistome eggshell and opercular cement also different from *Fasciola*. Further studies are needed to develop potential applications to interrupt the life cycle of *F. gigantica*.

Fig.2a-c. Eggs of F. gigantica were subjected to various acids, alkalis and chemicals. SEM of egg. a) Triton-X 100 treated eggs show the shrinkage; b) H_2SO_4 treated eggs show the opercular opening and rest of the eggshells remain unaffected; c) 0.1N NaOH treated egg shows the opening of operculum with opercular cement. ES- eggshell; OC- opercclum; S-shrinkage



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