# Oocyst wall formation and composition in coccidian parasites

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*The oocyst wall of coccidian parasites is a robust structure that is resistant to a variety of environmental and chemical insults. This resilience allows oocysts to survive for long periods, facilitating transmission from host to host. The wall is bilayered and is formed by the sequential release of the contents of two specialized organelles - wall forming body 1 and wall forming body 2 - found in the macrogametocyte stage of Coccidia. The oocyst wall is over 90% protein but few of these proteins have been studied. One group is cysteine-rich and may be presumed to cross-link via disulphide bridges, though this is yet to be investigated. Another group of wall proteins is rich in tyrosine. These proteins, which range in size from 8-31 kDa, are derived from larger precursors of 56 and 82 kDa found in the wall forming bodies. Proteases may catalyze processing of the precursors into tyrosine-rich peptides, which are then oxidatively crosslinked in a reaction catalyzed by peroxidases. In support of this hypothesis, the oocyst wall has high levels of dityrosine bonds. These dityrosine crosslinked proteins may provide a structural matrix for assembly of the oocyst wall and contribute to its resilience.*

Key words: Coccidia - oocyst - dityrosine crosslinking - *Toxoplasma* - *Eimeria*

Coccidian parasites, which include the genera *Toxoplasma, Neospora, Hammondia, Isospora, Sarcocystis* and *Eimeria,* amongst others, share many features. For example, like all Apicomplexa, their lifecycle includes asexual stages (sporozoites and merozoites) that possess a phylum-defining feature, the apical complex of organelles, associated with cell invasion. An additional defining feature of the Coccidia is the oocyst; coccidian parasites are transmitted from host to host by accidental ingestion of oocysts that contaminate food or water. Oocysts are remarkably hardy and able to persist in the environment for prolonged periods of time. The "soft-bodied" parasites are safely encapsulated inside a unique structure, the oocyst wall.

The oocyst wall is extremely robust. It is resistant to mechanical and chemical damage; breaking oocysts for laboratory studies requires prolonged, high-speed vortexing with glass beads and oocysts are routinely cleaned with bleach and stored in the harsh oxidant, potassium dichromate, or the mineral acid, sulphuric acid (Dubey et al. 1970, Shirley 1995, Schares et al. 2005). The wall is also resistant to proteolysis and impermeable to water-soluble substances, including many detergents and disinfectants (Monné & Hőnig 1954, Ryley 1973). This contributes to the difficulty experienced in attempting to exclude oocysts from, for example, poultry houses. The oocyst wall is, however, permeable to some small molecules and lipid+soluble substances; ammonia and methyl bromide being two of the best known (Monné & Hőnig 1954, Ryley 1973, Kuticic & Wikerhauser 1996).

The oocyst wall is essentially consistent in structure across different species of coccidian parasites (Belli et al. 2006), but it is the oocyst wall of *Eimeria* that has been best studied, largely because of the relative ease of acquiring large numbers of oocysts of the parasites of this genera.

## Oocyst wall biogenesis

The oocyst wall is formed from the contents of two specific organelles, wall forming bodies Type 1 (WFB1) and 2 (WFB2), found exclusively in the sexual, macrogamete stage of coccidian parasites (Scholtyseck et al. 1969, Scholtyseck 1973, Ferguson et al. 1975, 2003, Pittilo & Ball 1980, Belli et al. 2006). In the early stages of macrogamete biogenesis, the macrogametocyte contains a centrally-located nucleus and numerous spherical deposits of electron dense material within swollen regions of the rough endoplasmic reticulum; these represent the early WFB2. Shortly after this, in the mid-stage of macrogamete development, spherical electron dense granules appear in the cytoplasm. These secretory-like granules arise from material that traffics from the Golgi bodies and represent the early WFB1 (Ferguson et al. 2003). Also present in the early/mid and mid-stage of development are an additional group of membrane bound vacuoles termed the veil forming bodies (Ferguson et al. 2000, 2003).

The veil forming bodies vary in appearance between species, being electron dense in *Toxoplasma gondii* (Ferguson et al. 2000) and electron lucent in *Eimeria maxima* (Ferguson et al. 2003). They are secreted during macrogamete maturation and are responsible for the formation of a veil on the outer surface of the maturing macrogamete and oocyst (Ferguson et al. 2000, 2003).

Financial support: Australian Research Council's Discovery and Linkage Projects funding schemes (DP0664013, LP0454145), Abic Ltd (Israel), Rural Industries Research and Development Corporation's Postgraduate Scholarship Scheme, ARC/NHMRC research Network for parasitology (RN0460368)
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Received 16 October 2008
Accepted 4 December 2009

The outer veil of *E. maxima* stains positively with antibodies that are specific for the apple domains of *T. gondii* microneme protein 4, a protein associated with oocyst wall formation in this parasite. This suggests that the veil contains MIC4-like proteins and that the structure of the veil is conserved across the Coccidia (Ferguson et al. 2000, 2003). The veil is lost when oocysts are excreted from the host (Ferguson et al. 1975, Pittilo & Ball 1980); therefore it is thought that the outer veil plays no role in protection of parasites during passage out of the host or in the external environment (Belli et al. 2006). Its precise role is still unknown, though it seems possible that it provides a temporary "frame" under which the oocyst wall is assembled. This idea is supported by the fact that the veil remains in place throughout the entire intracellular development phase of the oocyst (Pittilo & Ball 1980).

WFB1 in mature macrogametocytes are large, spherical, membrane bound structures with electron dense contents (Pittilo & Ball 1979, Ferguson et al. 2003, Belli et al. 2006). They are located in the periphery of the parasites and have long been thought to contain mucoproteins, mucopolysaccharides and glycoproteins (McLaren 1969, Scholtyseck 1973). The size of WFBs is species-specific. For example, WFB1 are larger than WFB2 in *E. maxima*, *Eimeria tenella, Eimeria stieda* and *Eimeria perforans,* whereas in *Eimeria bovis*, *Eimeria falciformis* and *T. gondii*, the reverse is true (Scholtyseck 1973). WFB2 are less electron dense than WFB1, have a less distinct structure (Ryley 1973, Scholtyseck 1973) and appear as spherical dilations enclosed within the rough endoplasmic reticulum (Ferguson et al. 1975, 2003, Pittilo & Ball 1979). In the mature macrogametocyte, WFB1s locate in the periphery of the parasite inter-mixed with WFB2. A large number of polysaccharide granules, containing amylopectin and some lipid droplets are also seen in the cytoplasm of the mature macrogametocyte (Ferguson et al. 1975, Pittilo & Ball 1980).

Once fertilized by a microgamete, a macrogamete develops into a zygote. The oocyst wall starts to form shortly afterwards (Pittilo & Ball 1980), with WFB1s aligning beneath the limiting membrane of the zygote cytoplasm. A recent study (Ferguson et al. 2003) of oocyst wall formation in *E. maxima,* using immunocytochemistry with antibodies specific for WFBs, adds compelling evidence to support the mechanism of oocyst wall formation originally proposed by Pittilo and Ball in 1980. Thus, Ferguson et al. (2003) showed that oocyst wall formation is a sequential release of the contents of: (i) the veil forming bodies, (ii) WFB1 and (iii) WFB2. It is thought that this mechanism may be controlled at the level of the rough endoplasmic reticulum/ Golgi apparatus. Thus, in the mature macrogametocyte or zygote, after the veil has been laid down, WFB1 relocate to the periphery of the parasite. WFB1s quickly disaggregate and appear to fuse together at the surface of the parasite, ultimately forming the outer layer of the oocyst wall. Shortly after WFB1 form the outer layer, the WFB2s, located in the rough endoplasmic reticulum are transferred via the Golgi body and secretory granules to the surface where they fuse to create the inner layer of the oocyst wall.

The mature oocyst wall is bilayered. Initially, the outer layer may be as thick as 500-600 nm but, as the development of the wall progresses, the outer layer is compacted to 200 nm or less. An inner zone of approximately 40 nm separates the outer and inner layers, the latter layer also ultimately being ~40 nm thick (Ferguson et al. 2003). The electron dense outer layer is variable in size across different genera; for example, it can be ~200 nm thick in *E. maxima* (Ferguson et al. 2003) but is only 20-40 nm thick in *T. gondii* (Ferguson et al. 1975, 2000). The electron lucent inner layer, formed from the contents of WFB2, is more consistent, being around 40 nm thick in most genera studied (Belli et al. 2006). The two layers do not seem to be tightly fused and can be separated relatively easily (Monné & Hőnig 1954); bleach treatment, for instance, usually strips the outer layer away from the inner layer.

## Biochemical composition of the oocyst wall

The first serious microscopic and chemical examination of the oocyst wall (of *E. maxima*) was conducted by Monné and Hőnig (1954), who used a number of destructive treatments that led them to conclude that the outer layer of the oocyst wall contained mainly quinone-tanned proteins without lipids, since the outer layer reacted with ammoniacal silver nitrate solution (an indication of quinones). They also noted that the outer layer was stripped off upon exposure to sodium hypochlorite, whereas the structure of the inner layer remained unchanged, leading them to conclude that the inner layer consisted of a lipid-protein matrix; they believed that lipids were bound firmly to proteins, thus protecting the inner layer against disintegration by sodium hypochlorite.

The first true biochemical examination of the oocyst wall was carried out by Ryley in 1973 using *E. tenella.* Ryley (1973) also noted that the outer layer was removed by sodium hypochlorite and found that it contained carbohydrates and proteins, with high proline content, whereas the inner layer consisted of 1.5% carbohydrates, 30% lipids and 70% proteins. The lipid in the inner layer was extractable in chloroform and appeared to be a mixture of "waxes" containing very small amounts of nitrogen and phosphorus. However, there are some limitations in this report: first, it did not show detailed analyses of the experimental work and, second, it did not document the metabolites detected in the inner wall.

The most recent biochemical analysis of the oocyst wall was conducted by Stotish et al., in 1978, using unsporulated oocysts isolated from the caeca of chickens infected with *E. tenella*. These researchers proposed that lipids were concentrated in the outer layer whereas the inner layer was composed of mainly glycoproteins. In addition, compositional analysis of the oocyst wall by gas-liquid chromatography and SDS-PAGE indicated that it was 19% carbohydrates, 14% lipids and 67% protein (Stotish et al. 1978). Lipids identified from the walls included a number of fatty alcohols, steroids and fatty acids such as docosanol, tetracosanol, hexacosanol, myristate, palmitate, stearate, oleate, linoleate and phospholipids. Protein content in the oocyst wall consisted of a repeating subunit of ~10 kDa. Four carbohydrates

- mannose, galactose, glucose and hexosamine - were detected. However, some doubts surround the validity of these conclusions because Stotish et al. (1978) noted that sodium hypochlorite treatment did not affect the structure of the oocyst walls from *E. tenella* unsporulated oocysts and this was a major basis for their conclusion that lipids are concentrated in the outer layer of the wall (since lipids would be expected to give some protection against stripping by sodium hypochlorite). In direct contradiction to the observations of Stotish et al. (1978), it has been shown that sodium hypochlorite treatment does have an effect on the structure of the oocyst wall in both *E. maxima* and *E. tenella* - the outer layer is stripped off (Monné & Hőnig 1954, Nyberg et al. 1968, Nyberg & Knapp 1970, Ryley 1973, Belli et al. 2006). Therefore, the biochemical compositions assigned to the two layers of the oocyst wall of *E. tenella* by Stotish et al. (1978) could be erroneous. We have, therefore, reinvestigated the biochemical composition of the oocyst wall using more reliable sensitive modern methods.

We isolated oocyst walls from intact *E. maxima* and *E. tenella* oocysts as published previously by Stotish et al. (1978). Oocyst walls (equivalent to the wall preparations isolated from 2 x $10^6$ oocysts) were then resuspended in 100 µL of distilled water and treated with alpha-amylase to eliminate polysaccharide granules according to the protocol from Ryley (1973), with slight modification. Briefly, alpha-amylase was added to a final concentration of 0.5 mg/mL and 1 mg/mL for comparison and samples were incubated at 20ºC overnight. On the next day, the samples were centrifuged at 10,000 *g* for 10 min at 4ºC, the supernatant was discarded and the pellet was washed in distilled water. The washing step was repeated three times. The effect of alpha-amylase on elimination of polysaccharide granules was investigated by microscopic examination and transmission electron microscopy (TEM) as published previously by Ferguson et al. (2003), with a minor modification. Briefly, each sample of oocyst wall fragments was centrifuged and the pellets were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer. The pellets were post-fixed in 1% osmium tetroxide in phosphate buffer, dehydrated in absolute ethanol, treated with propylene oxide and embedded in Spurr's epoxy resin. Thin sections of oocyst walls were then mounted on copper grids and stained with uranyl acetate and lead citrate for routine electron microscopy. In addition, some sections were mounted on formvar-coated gold grids and stained with silver methenamine. The sections were floated on drops of a freshly prepared and filtered mixture containing silver nitrate and hexamethylene tetramine in a borax-based buffer (pH 9.2). Sections were stained in the dark at 40°C for 60 min and washed in water prior to examination. In both cases, sections were examined in a Jeol 1200EX TEM.

In the absence of treatment with alpha-amylase, the purified oocyst walls appeared to be heavily contaminated with polysaccharide granules (Fig. 1A, B). When alpha-amylase was added to the oocyst walls, the number of polysaccharide granules was reduced markedly, as shown in the sample treated with 0.5 mg/mL alpha-amylase (Fig. 1C) and, more especially, in the sample treated with 1 mg/mL alpha-amylase (Fig. 1D). Examination by light and UV microscopy (Fig. 1E, F, respectively) confirmed the characteristic blue autofluorescence of the purified oocyst walls after alpha-amylase treatment, indicating that the basic chemical structure of the walls was not altered significantly by the treatment.

The oocyst walls prepared from *E. tenella* and *E. maxima* sporulated and unsporulated oocysts were examined by TEM to confirm the presence of the inner and/or outer layers. The outer layer of the oocyst walls (both *E. tenella* and *E. maxima*), appeared electron-dense with a roughened appearance on the outer surface, and the inner layer was electron-lucent (Fig. 2A-C, E, G), observations that are consistent with those of Ferguson et al. (2003). The oocyst walls from unbleached samples remained intact (retaining a bi-layered structure) whereas in bleached samples, generally only the inner layer was seen (Fig. 2D, F, H), confirming the observations that bleaching does strip the outer layer of oocyst walls (Monné & Hőnig 1954, Nyberg et al. 1968, Nyberg & Knapp 1970, Ryley 1973, Belli et al. 2006). There was a single exception to this pattern; walls prepared from a sample of bleached *E. tenella* sporulated oocysts remained intact as a bi-layered structure (Fig. 2B). This was not always observed and the reason for this exception is not known. However, this exceptional sample proved very useful in subsequent comparisons of wall composition.

The compositional analysis of bleached oocyst walls was carried out as published previously by McConville et al. (1990), with a minor modification (unbleached samples were not analysed due to the presence of various contaminants, including residual amylopectin, which would distort the analyses). Briefly, the oocyst walls

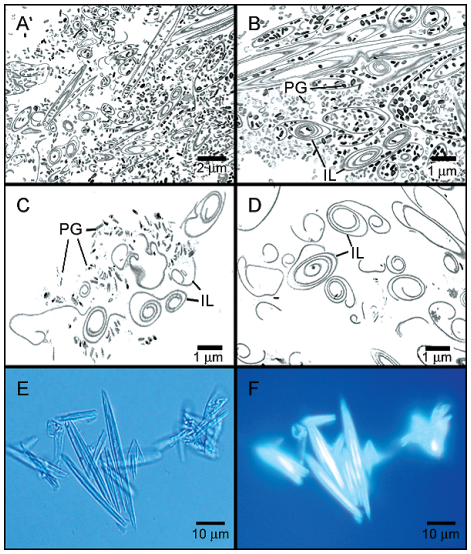


Fig. 1: micrographs of alpha-amylase treated oocyst walls prepared from *Eimeria maxima* bleached unsporulated oocysts. Each sample contained oocyst walls equivalent to 2 x $10^6$ *E. maxima* unsporulated oocysts in the absence of alpha-amylase (A, B) or treated with alpha-amylase to a final concentration of 0.5 mg/mL (C) or 1 mg/mL (D). E: purified, alpha-amylase treated oocyst walls under bright field; F: purified, alpha-amylase treated oocyst walls under UV light; IL: inner layer of oocyst walls; PG: polysaccharide granules.

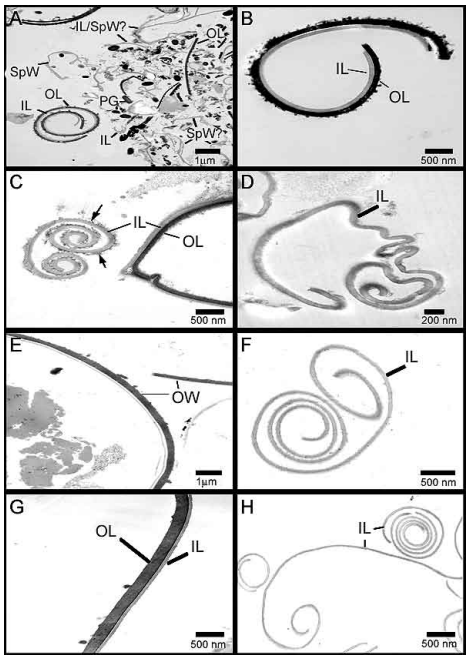


Fig. 2: electron micrographs showing the effect of bleach on oocyst wall (OW) preparations from *Eimeria tenella* and *Eimeria maxima*. A, B: *E. tenella* unbleached and bleached sporulated OW, respectively; C, D: *E. tenella* unbleached and bleached unsporulated OW, respectively [arrows in C indicate hydrolyzed polysaccharide granules (PG) attached to the OW]; E, F: *E. maxima* unbleached and bleached sporulated OW, respectively; G, H: *E. maxima* unbleached and bleached unsporulated OW, respectively; IL: inner layer of OW; OL: outer layer of OW; SpW: sporocyst walls.

were resuspended in 50 µL of 1:1 chloroform:methanol and transferred into a clean glass capillary tube with one end flame sealed. Heptadecanoic acid (C17:0; 10 nm) was added to the capillary tube as an internal standard for subsequent quantification of metabolites. The sample was then dried *in vacuo* at 50ºC. This step was repeated again in the presence of methanol to enable the complete removal of $H_2O$. Methanolic HCl (0.5 M HCl in methanol; 50 µL) was added to each capillary tube and sealed under vacuum. The sample was then incubated at 80ºC overnight. Following methanolysis, the sample was cut open and the acid was neutralized by the addition of 10 µL pyridine. The neutralized solution was then transferred to a GC-MS vial insert and dried *in vacuo*. Trimethylsilyl (N-methyl-N-trifluroacetamide + 1% trimethylchlorosilane; 50 µL) reagent was added to the tube, which was gently flicked to ensure the sample was well mixed. The sample was then transferred into an insert vial of autosampler vials for compositional analyses of carbohydrates and lipids by gas chromatography (GC; Agilent 6890N GC) and mass spectrometry (MS, Agilent 5973 Mass Selective Detector).

Oocyst wall samples were also hydrolyzed in 6N HCl as published previously (McConville et al. 1990), with a minor modification. Briefly, the oocyst walls were resuspended in 100 µL of 1:1 chloroform:methanol and transferred into GC-MS vial inserts containing 1 nm of *scyllo*-inositol as an internal control for subsequent quantification of metabolites. The sample was dried *in vacuo* at 55º. The vial inserts were then transferred into reaction vials containing 200 µL 6N HCl, followed by evacuation under nitrogen. The samples were capped immediately to prevent oxidation by air and incubated at 110ºC over night. The vial inserts were then placed into GC-MS vials and the residual HCl was evaporated under nitrogen. Vial contents were then incubated for 2 h at rt in 20 µL pyridine to allow resuspension and neutralization of HCl contaminants. N,O-bis[trimethylsilyl] trifluoroacetamide + 1% trimethylchlorosilane (20 µL) was added to enable trimethylsilyl derivatisation and the reactions were carried out at 80ºC for 30 min. The amino acid composition of the oocyst wall proteins was analyzed by GC-MS.

Finally, oocyst wall proteins were extracted using the method described by Meyers et al. (1998). Oocyst walls (from 2 x $10^6$ oocysts) were resuspended in 100 µL of 1 M NaOH, followed by boiling for 10 min to solubilize the wall proteins. The lysate was then cooled down at rt for 10 min. Twenty microliters of 5M HCl was added to neutralize the lysate, which was then brought up to 1 mL by the addition of 880 µLPBS. Absorbance at 230 nm and 260 nm was measured using a spectrophotometer (GeneQuant, Amersham Biosciences) and protein concentration was calculated using the following equation: $\{[\text{Protein}] = (183 \times A_{230}) - (75.8 \times A_{280})\}$.

Our analyses revealed remarkable similarities between all the oocyst wall samples (Table I). Thus, the relative levels, on a percentage w/w basis of carbohydrate, lipid and protein were contained in relatively narrow ranges of 0.6-2.0% carbohydrate, 1.4-7.6% lipid and 90.4-98.3% protein, for unsporulated or sporulated oocysts, from *E. tenella* or *E. maxima*. It is perhaps noteworthy, however, that the lower values for carbohydrates and lipids were seen in the sporulated oocyst samples from both *E. tenella* and *E. maxima*. This indicates that the single bilayered oocyst wall sample (the sporulated oocysts of *E. tenella*) did not exhibit a distinctly different composition than the single layered sporulated oocyst wall sample from *E. maxima*. In contrast to these results, the percentages of carbohydrate, lipid and protein reported by Stotish et al. (1978) for unsporulated oocysts of *E. tenella* are quite different, with only 67% protein and 19% carbohydrate and 14% lipid being recorded.

Analysis of 6N HCl hydrolysates of the oocyst walls of *E. tenella* and *E. maxima* revealed that essentially all twenty amino acids are present in the walls of sporulated and unsporulated oocysts. A limited qualitative comparison of five of the more abundant amino acids, isoleucine (Ile), aspartic acid (Asp), valine (Val), proline (Pro) and arginine (Arg) was possible by assigning Ile a standardized value of 1.0 and presenting the other four amino acids as a ratio of this. It is important to not over interpret this data; it provides a limited snapshot of the relative order of abundance of these five amino acids but the ratio values probably do not very precisely reflect their abundance. When examined in this way, the relative abundance of the amino acids is similar in all the samples, whether *E. tenella* or *E. maxima,* unsporulated or sporulated oocysts, and is in the order Ala>Pro>Val>Asp>Ile. The only slight exception to this is the sporulated oocyst

sample of *E. maxima,* where Asp and Val are reversed, but marginally. The results of Stotish et al. (1978) are in accord with these observations.

Sporulated oocysts of both species contained a number of fatty acids (saponifiable lipids) including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), behenic acid (C22:0), lignoceric acid (C24:0) (Table II). Non-saponifiable lipids such as cholestadiene, cholestane and cholesterol were also detected. Most of these were present in a similar w/w percentage in the two species except that the bilayered *E. tenella* sample was comprised of 44.7% oleic acid whereas the *E. maxima* sample was only 19.5% oleic acid. The *E. tenella* sample also had higher quantities of palmitic acid (13.2% versus 5.9% in *E. maxima).* In apparent compensation of these differences, walls from *E. tenella* sporulated oocysts had lower relative percentages of linoleic acid (2.1% in *E. tenella* versus 8.2% in *E. maxima*), lignoceric acid (1.8% versus 5.4%) and, especially, cholestane (3.5% versus 11%) and cholesterol (12.1% versus 24.2%) than the *E. maxima* sporulated oocyst walls.

The levels of lipids in unsporulated oocysts of *E. tenella* and *E. maxima* were quite similar to each other but differed from the sporulated wall samples in that they totally lacked linoleic acid. The relative levels of oleic acid in the unsporulated oocyst samples resembled that seen in the *E. maxima* sporulated oocyst sample and, so, were also different from the levels apparent in the bilayered *E. tenella* sporulated oocyst sample. The data from Stotish et al. (1978) for lipid composition of unsporulated oocyst walls of *E. tenella* did not resemble the observations made here using GC-MS. First, the samples analysed by Stotish et al. (1978) contained no behenic acid, lignoceric acid, cholestadiene or cholestane. Second, Stotish et al. (1978) reported the presence of myristate, as well as several fatty alcohols (docosanol, tetracosanol, hexacosanol, octacosanol and triacosanol) not detected here; Stotish et al. (1978) reported hexacosanol and phospholipids as being especially abundant.

The carbohydrate content of *E. tenella* sporulated oocyst walls was very similar to that of sporulated oocyst walls of *E. maxima*, the carbohydrate content being made up of 4.3-5% mannose, 33.7-37.4% galactose and 58.3-61.3% glucose (the latter possibly reflecting residual contamination with amylopectin). Likewise, the carbohydrate profile of unsporulated oocyst walls of *E. tenella* was similar to that of unsporulated oocyst walls of *E. maxima* but quite different to that of the sporulated walls, being dominated by galactose (62.3%-67.6%), followed by glucose (26-27.9%) and mannose (6.4-9.8%). The carbohydrate composition of unsporulated oocysts of *E. tenella* reported by Stotish et al. (1978) was, however, very different; they reported that the carbohydrate profile of the wall was 79.6% glucose and 11% hexosamine (which was not detected in the GC-MS analysis reported here) and only 7.2% galactose and 2.2% mannose.

Thus, to summarise, GC-MS analyses of oocyst walls of *E. tenella* and *E. maxima* indicates that they are primarily made up of protein (greater than 90%) with relatively small amounts of lipid and carbohydrate. The composition of the oocyst walls of both species was very

TABLE I

The biochemical composition of the oocyst wall of *Eimeria*

| *Eimeria tenella* oocyst walls % of metabolites (w/w) | | | |
|---|---|---|---|
| | Metabolite[a] | | |
| Sample | Carbohydrate | Lipid | Protein |
| Walls from S/O | 0.6 | 1.6 | 97.8 |
| Walls from U/O | 1 | 4.4 | 94.6 |
| *Eimeria maxima* oocyst walls % of metabolites (w/w) | | | |
| | Metabolite[a] | | |
| Sample | Carbohydrate | Lipid | Protein |
| Walls from S/O | 0.3 | 1.4 | 98.3 |
| Walls from U/O | 2.0 | 7.6 | 90.4 |

*a*: relative levels of carbohydrate, lipid and protein in oocyst walls prepared from $10^6$ sporulated (S/O) or unsporulated oocysts (U/O).

TABLE II

The lipid composition of the oocyst wall of *Eimeria*

| *Eimeria tenella* oocyst walls % of metabolites[a] (w/w) | | |
|---|---|---|
| | Sample | |
| Lipid | Walls from S/O | Walls from U/O |
| Palmitic acid (C16:0) | 13.2 | 13.3 |
| Stearic acid (C18:0) | 13.7 | 22.2 |
| Oleic acid (C18:1) | 44.7 | 16.1 |
| Linoleic acid (C18:2) | 2.1 | 0 |
| Behenic acid (C22:0) | 4.5 | 9.8 |
| Lignoceric acid (C24:0) | 1.8 | 2.9 |
| Cholestadiene | 4.4 | 7.2 |
| Cholestane | 3.5 | 16.7 |
| Cholesterol | 12.1 | 11.8 |
| *Eimeria* maxima oocyst walls % of metabolites[a] (w/w) | | |
| | Sample | |
| Lipid | Walls from S/O | Walls from U/O |
| Palmitic acid (C16:0) | 5.9 | 11.7 |
| Stearic acid (C18:0) | 14.4 | 21.4 |
| Oleic cid (C18:1) | 19.5 | 18.4 |
| Linoleic acid (C18:2) | 8.2 | 0 |
| Behenic acid (C22:0) | 6.4 | 9.6 |
| Lignoceric acid (C24:0) | 5.4 | 10.9 |
| Cholestadiene | 5.0 | 6.4 |
| Cholestane | 11.0 | 7.0 |
| Cholesterol | 24.2 | 14.6 |

*a*: relative levels of lipids prepared from $10^6$ sporulated (S/O) or unsporulated oocysts (U/O).

similar, whether sporulated or unsporulated, though there was perhaps slightly more protein (and, resultingly, proportionally less carbohydrate and lipid) in the sporulated oocyst walls. This may reflect the presence of sporocyst walls, which were occasionally seen in the sporulated oocyst wall samples (Fig. 2A) and, if so, indicates that the composition of the sporocyst wall is slightly different to that of the oocyst wall. Surprisingly, the presence of both the outer and inner layer of the oocyst wall (as in the *E. tenella* sporulated oocyst wall sample) did not greatly affect the overall composition of the wall, indicating that both layers are chemically similar, even though their relative electron densities are different, as indicated by the TEM images.

All of the above results are at odds with those of Stotish et al. (1978), who reported much lower protein content and much higher quantities of carbohydrate and lipid in the unsporulated oocyst wall of *E. tenella.* These discrepancies can almost certainly be ascribed to contamination of the walls with the internal contents of the oocyst, as the TEM images presented here document the presence of large numbers of amylopectin granules in the samples prepared following the method described by Stotish et al. (1978). The high levels of glucose reported by Stotish et al. (1978) - much higher than detected by the GC-MS analyses reported here - support this contention as amylopectin is a polymer of glucose monomers joined by alpha-1,4 linkages (Ryley et al. 1969, 1973).

The lipid compositions of the oocyst wall samples analysed in this study are also different to those reported by Stotish et al. (1978), who failed to detect several of the lipids detected by the sensitive GC-MS methodology used here. Ironically, Stotish et al. also detected several fatty alcohols and phospholipids, as well as hexosamine, none of which were detected in this study. This could reflect the higher stringency of the analyses conducted in our study - GC-MS rules out false positives very effectively - but also probably betrays the fact that the wall samples prepared by Stotish et al. (1978) were contaminated with internal contents of the oocysts.

## Proteins of the oocyst wall

The observation that the oocyst wall is dominated by protein is an important one as it implies that understanding the structure and characteristics of oocyst wall proteins - the major building blocks of the oocyst wall - will provide an insight into how the oocyst wall is formed and, potentially, identify vulnerabilities for attack by chemical or immunological agents either within the host and prior to oocyst wall formation or in the environment (e.g., floor litter of poultry houses).

There are only a small number of oocyst wall proteins that have been identified and characterized, mostly from *Eimeria* species. Stotish et al. (1978) believed that the protein content of the oocyst wall was predominantly, if not solely, a repeating subunit of ~10 kDa, based on their detection of only a single band on SDS-PAGE gels. Karim et al. (1996) made similar conclusions when describing a monoclonal antibody to a single 12 kDa protein band of the oocyst wall of *E. tenella;* the antibody reacted with macrogametocytes, as well as the inner wall of oocysts, and crossreacted with *E. maxima*. Eschenbacher et al. (1996) discovered 14 kDa oocyst wall proteins in *E. tenella* and *Eimeria acervulina,* noting that these proteins were characterized by an abundance of amino acids that contain hydroxyl groups in their side-chains, especially serine, tyrosine and threonine. Based on this, they resurrected the idea, first proposed by Monné and Hőnig (1954), and reitereated by Pittilo and Ball (1980), that the oocyst wall contains quinone-tanned protein.

Studies on the macrogametocyte stage of *Eimeria* have yielded valuable information about oocyst wall proteins. Mouafo et al. (2002) reported that a monoclonal antibody raised against macrogametocytes of *E. tenella,* reacted with three proteins of 23, 25 and 30 kDa in the inner layer of the oocyst wall, in addition to an antigen of 51 kDa in the macrogametocytes. In parallel, detailed studies on the 56 and 82 kDa proteins from the macrogametocytes of *E. maxima* (EmGam56 and EmGam82) demonstrated (by N-terminal amino acid sequencing and Western blotting) that a series of oocyst wall proteins of 8, 10, 12 and 31 kDa were all derived from these two precursor proteins (Belli et al. 2003). Like the 14 kDa proteins described by Eschenbacher et al. (1996), these proteins are tyrosine-rich (it seems likely that the 10, 12 and 14 kDa proteins described by Stotish et al. 1978, Eschenbacher et al. 1996, Mouafa et al. 2002, are essentially the same as the 8, 10 and 12 kDa proteins uncovered by Belli et al. 2003 using methods that enabled separation of an apparent single band on an SDS-PAGE gel into three bands). An additional protein of ~29 kDa was also noted but its composition was not defined.

Antibodies to EmGam56 and EmGam82 and to affinity purified gametocyte antigens (APGA) were subsequently used to definitively map the relocation of proteins from WFB1 to the outer layer of the oocyst wall and proteins from WFB2 to the inner layer of the oocyst wall of *E. maxima* (Ferguson et al. 2003). We have recently obtained similar results with both *E. tenella* and *E. acervulina,* demonstrating the conservation of these proteins and the process of wall formation (SI Belli et al., unpublished observations). Recently, Krücken et al. (2008) have confirmed the presence of two *E. tenella* homologues of EmGam56 and, additionally, reported the discovery of a 22 kDa antigen in the macrogametocytes of *E. tenella*. Like the other proteins so far characterized, antibodies to this protein react with WFB2 and the inner layer of the oocyst wall. Although this 22 kDa protein does contain some tyrosine residues, it is dominated by histidine and proline residues. It is notable for another reason, namely that its gene is present in extremely high copy number (in contrast to the single copies of the genes for, for example, EmGam56 and EmGam82), indicating that it may be important in oocyst wall formation via a mechanism distinct from that of the tyrosine-rich proteins. As yet, no information is available about whether this 22 kDa protein is processed into smaller polypeptides nor how it is incorporated into the oocyst wall, though its involvement in stabilizing the oocyst wall via cross-links between histidine and catechols, as seen in insect cuticles (Christensen et al. 1991, Xu et al. 1997, Kerwin et al. 1999), is a distinct possibility (Krücken et al. 2008).

The only other oocyst wall proteins to be documented thus far are the members of the multigene *Cryptosporidium* oocyst wall protein (COWP), a family of large (174-190 kDa), cysteine-rich proteins that localise to WFB1 of macrogametocytes and the inner wall of the *Cryptosporidium* oocyst (Spano et al. 1997, Templeton et al. 2004). Furthermore, the protein also localizes to a single large vesicle within immature macrogametocytes, indicating that it is a potential early indicator of sexual stage development.

It is thought that COWP forms extensive disulphide bridges and matrices within the oocyst wall (Spano et al. 1997). This would make it quite distinct from the other oocyst wall proteins described for *Eimeria* but this fits with the fact that the *Cryptosporidium* oocyst wall does not autofluoresce blue under UV light, in contrast to those of *Eimeria, Toxoplasma, Neospora, Sarcocystis, Isospora* and *Cyclospora,* which all display the blue autofluorescence characteristic of dityrosine bonds (Belli et al. 2006).

An exhaustive analysis of the *Cryptosporidium parvum* genome sequence revealed eight putative paralogues of COWP1, all displaying varying degrees of conservation of the Type I and Type II repeats (Templeton et al. 2004). While all nine members of this gene family appeared to contain multiple copies of the cysteine-rich, Type I repeats, only COWP1, 2 and 3 were observed to contain the Type II repeats. Furthermore, characteristic histidine-rich domains were observed in all *C. parvum* COWP sequences with the exception of COWP4 and COWP9. Semi-quantitative RT-PCR analyses revealed up-regulation in the transcription of all nine genes in parasite stages associated with sexual stage development and oocyst formation in *C. parvum*, supporting the hypothesis that these COWP proteins are involved in the formation of the oocyst wall.

The study by Templeton et al. (2004) also uncovered a homologue of COWP6 in the *T. gondii* genome sequence (referred to as *TgOWP1*), potentially extending this wall-forming hypothesis to other coccidians. This hypothesis was also validated to some degree by the observation that no homologue to any of the COWP proteins could be found in the non-coccidian, apicomplexan, *Plasmodium falciparum*. Very recently, we have found two apparent homologues in *Eimeria* (Walker et al., unpublished observations). The role of these proteins in oocyst wall formation in *Toxoplasma* and *Eimeria* has yet to be investigated.

## Proteases, peroxidases, dityrosine bonds and oocyst wall formation

A model for how the tyrosine-rich wall proteins are incorporated into the oocyst wall of *Eimeria* has been proposed by Belli et al. (2006), based on two well characterised gametocyte proteins, EmGam56 and EmGam82. Briefly, the model proposes that EmGam56 and EmGam82 are precursors that are processed into small tyrosine-rich wall proteins, perhaps by macrogamete-specific proteases. The model goes on to propose that the tyrosine-rich proteins are oxidized, by peroxidase(s), and crosslinked via their tyrosine residues to form a matrix that subsequently becomes dehydrated ("tanned") and leads to hardening of the oocyst wall, with its accompanying, notorious resilience. This model generates several testable hypotheses: first, precursor proteins found in WFBs will be processed by gametocyte-specific proteases into smaller, tyrosine-rich peptides that are found in the oocyst wall, second, peroxidases will be specifically located in WFBs and will catalyse cross-linking of proteins via their tyrosine residues and, third, dityrosine bonds will be present in the oocyst wall. There is some evidence that all these hypotheses are correct.

As mentioned above, it has been shown that EmGam56 and EmGam82 are processed into small tyrosine-rich wall proteins of 8, 10, 12 and 31 kDa (Belli et al. 2003). Thus, antibodies to EmGam56 and EmGam82 also react with these smaller oocyst wall proteins and N-terminal sequencing maps the location of these proteins within EmGam56 and EmGam82. It is easy to envisage that this processing is the result of proteolysis by an, as yet, unidentified protease or proteases. If proven, and the proteases that are responsible for the processing of these two gametocyte proteins are identified, then a totally new class of anticoccidial drug - a specific protease inhibitor (or inhibitors) - is not hard to imagine. Thus, future work directed at identifying proteases from the sexual stages of *Eimeria* is well warranted. Indeed, it has already commenced and our recent work has detected several gametocyte-specific proteases (Katrib et al., unpublished observations). Included amongst these are several subtilisins, which are particularly interesting with regard dityrosine bond formation because of their known role in the formation of the cuticle of nematodes. Thus, the assembly of cuticlins and collagens to form the cuticle involves a number of catalytic pathways: (i) collagens are synthesised as proproteins that are cleaved at the N-terminus by a subtilisin-like protease prior to cuticle formation (Thacker et al. 1995, 2000, 2006, Thacker & Rose 2000b), (ii) the collagens (and cuticlins) are held together by di- and tri-tyrosine crosslinks (Page & Winter 2003) and (iii) dual oxidase is the oxidative enzyme responsible for the generation of the tyrosine crosslinks (Page & Winter 2003). Mutations at any one of these steps, results in a deficiency of di and tri-tyrosine in the cuticle, the formation of a structurally defective cuticle and parasite death (Page & Winter 2003). Our model of oocyst wall formation predicts a very similar picture.

It has also been shown that the macrogametocytes of *E. maxima* have high levels of activity of peroxidases and that this activity is exquisitely focused within the WFBs and developing oocyst wall of the parasite (Belli et al. 2003, 2006). An endogenous peroxidase has not yet been isolated from the WFBs of coccidian parasites but we have been able to demonstrate that exogenous peroxidases from horseradish or *Arthromyces* can induce crosslinking of EmGam56 in vitro, with accompanying formation of dityrosine bonds, as detected by HPLC with UV and visible detectors (Mai et al., unpublished observations).

Dityrosine bonds are readily demonstrable in the oocyst wall. Thus, oocysts of all coccidians are well known to autofluoresce blue under UV light in the excitation wavelength range of 330-385 nm, a characteristic

of dityrosine bonds (Belli et al. 2006). Furthermore, the oocyst wall of *E. maxima* contains remarkable levels of dityrosine and, to a lesser extent, 3,4-dihydroxyphenylalanine (DOPA), another derivative of tyrosine (Belli et al. 2003). These levels are much higher than many normal physiological situations and imply that the generation of dityrosine bonds in the oocyst wall is the result of a deliberate enzymatic process engineered by the parasite (Belli et al. 2006).

The hypothesis that dityrosine crosslinking of proteins is a key feature of oocyst wall formation and structure helps to explain the incredible environmental resilience of coccidian oocysts - quinone tanning and dityrosine crosslinking, leading to sclerotization, are widespread in the animal kingdom, and beyond, in association with the construction of structural matrices. Examples include such structures as insect cuticular resilin (Andersen 1964), exoskeletons of the nematodes, *Haemonchus contortus*, *Caenorhabditis elegans* and *Ascaris suum* (Fujimoto 1975, Fetterer & Rhoads 1990, Lassandro et al. 1994), ascospore walls from the yeast, *Saccharomyces cerevisiae* (Briza et al. 1986), cell walls of *Candida albicans* (Smail et al. 1995), and the fertilization membrane of sea urchin eggs, *Stronglycocentrotus purpuratus* (Foerder & Shapiro 1977). The presence of dityrosine in vertebrate animal proteins is also seen in chicken aorta elastin, collagen and connective tissues from bovines, rats and cats, and in tissues of normal and diseased humans (LaBella et al. 1968, Amado et al. 1984, Davies et al. 1999). DOPA-containing proteins are also widely distributed in nature and are involved in the synthesis of extraorganismic structural materials such as helminth worm and mosquito eggshells, egg capsules, cocoons, mussel byssal threads and various biological glues and cements (Waite 1990, Huggins & Waite 1993). In addition, phenol oxidase activity (catalyzing oxidation of DOPA to dopachrome) is detected only in homogenates of female *Trichuris suis* (a parasite inhabiting the intestines of pigs), not males, suggesting that the enzyme is likely to be associated with hardening of eggshell and that the eggshell proteins might consist of DOPA-containing proteins (Fetterer & Hill 1993). Understanding this process in oocysts may well lead to novel strategies to limit the transmission of coccidian parasites. In fact, there is probably already an outstanding example of this, in the form of a subunit vaccine against coccidiosis in chickens, CoxAbic®.

CoxAbic® is the only commercially available subunit vaccine against a protozoan parasite. It has been used around the world in trials involving 177 million broiler chickens and the results from all trials clearly demonstrate that, in all aspects of parasite control, broiler growth, flock health and mortality, the performance of CoxAbic® broilers was similar to that of medicated or live vaccine control groups. Indeed, in most instances, CoxAbic® broilers performed slightly better than flocks reared on prophylactic drugs or live vaccines (M Wallach et al., unpublished observations). CoxAbic® is composed of an APGA preparation from *E. maxima,* dominated by EmGam56 and EmGam82 (Wallach 2002). Immunization of broiler breeder hens with the vaccine stimulates the production of IgG (sometimes referred to as IgY) antibodies that are transferred to the developing broiler chick via the egg yolk. The results presented in this paper, together with previous studies on oocyst wall formation (Belli et al. 2003, Ferguson et al. 2003), are consistent with the hypothesis that the protective antibodies prevent formation of oocysts by either: (i) protecting the full length versions of EmGam56 and EmGam82 from degradation/proteolysis into smaller tyrosine-rich polypeptides destined for incorporation into the oocyst wall or (ii) interfering with the formation of dityrosine crosslinks between the tyrosine-rich polypeptides. Whether or not, this knowledge can be applied to control of other parasites remains to be seen.

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