The molecular basis for oocyst wall formation in *Eimeria maxima*

by

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CERTIFICATE OF AUTHORSHIP

I certify that this thesis has not previously submitted for any degree and is not being submitted as part of candidature for any other degree. I also certify that the thesis has been written by me and that any help that I have received in preparing this thesis, and all sources used, have been acknowledged in the thesis.

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Kelly Mai

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LIST OF ABBREVIATIONS

aa	amino acids
AGRF	Australian Genome Research Facility
APGA	affinity purified gametocyte antigens
APx	*Arthromyces* peroxidase
3'AT	3'-amino-1, 2, 4-triazole
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BSTFA	*N, O-bis*(trimethylsilyl)trifluoroacetamide
bp	base pairs
BSA	bovine serum albumin
CD	circular dichroism
COWP	*Crptosporidium* oocyst wall protein
DAPI	4, 6-diamidino-2-phenylindole
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DOPA	3, 4-dihydroxyphenylalanine
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EmGam56	56 kDa gametocyte antigen from *E. maxima*
EmGam82	82 kDa gametocyte antigen from *E. maxima*
ExPASy	Expert Protein Analysis System
g	centrifugal force (gravity)
GC	gas chromatography
GLC	gas-liquid chromatography
hr	hour(s)
HPLC	high-pressured liquid chromatography
HRPO	horseradish peroxidase
I (or IL)	inner layer of oocyst walls

IPTG	isopropyl-b-thiogalactopyranoside
IUPs	intrinsically unstructured proteins
kDa	kilodalton
LB Amp medium	Luri-Bertani medium containing 100 μg/ml ampicillin
LB Amp plate	Luri-Bertani plate containing 100 μg/ml ampicillin
LB medium	Luria-Bertani medium
lpm	litre per minute
M	molar
mA	milliampere(s)
min	minute(s)
ml	milliliter
μl	microlitre
μm	micrometre
mM	millimoles per litre
MPx	myeloperoxidase
MS	mass spectrometry
NCS	normal chicken serum
1D-NMR	one-dimensional nuclear magnetic resonance
O (or OW)	oocyst walls
OD	optical density
O (or OL)	outer layer of oocyst walls
O/N	over night
PAGE	polyacrylamine gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PEG	poly(ethylene glycerol)
pETEmGam56.102-225	gene encoding for a truncated form of EmGam56
pETEmGam56.102-225	a truncated form of EmGam56
PG	polysaccharide granules
PH	phenylhydrazine hydrochloride

ppm	parts per million
PVDF	polyvinylidene fluoride
RIA	radioimmunoassay
rpm	revolutions per minute
RNN	recursive neural network
RT	retention time
r56	gene encoding for a recombinant form of EmGam56
r56	a recombinant form of EmGam56
S	sporocyst walls
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polacrylamide gel electrophoresis
SHA	salicylhydroxamic acid
SpW	sporocyst walls
S/O	sporulated oocysts
TBE	tris borate/EDTA
TBS	tri-buffered saline
t-BHP	*tertiary*-butylhydroperoxide
TCA	trichloroacetic acid
TE	tris/EDTA
TEM	Transmission Electron Microscopy
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
Tris	tris[hydroxymethyl]aminomethane
U/O	unsporulated oocysts
UV	ultraviolet
W or WFB	wall forming bodies
W1 or WFB1	wall forming bodies type 1
W2 or WFB2	wall forming bodies type 2

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ABSTRACT

Eimeria is a genus of protozoa within the Apicomplexa, a phylum that includes *Plasmodium* species (which cause malaria) and *Toxoplasma gondii* (which cause foetal abnormalities and encephalitis), and many other species of parasites. *Eimeria* belongs to the subclass Coccidia, and a defining characteristic of this group is their transmission from host to host via oocysts that contaminate food and water. The resilient oocyst wall protects the parasites as they are excreted in the host's faeces and in the outside world, allowing them to survive for several months between hosts. It is formed from the contents of specialised organelles – wall forming bodies – found in the macrogametocyte stage of the parasites.

Two proteins, EmGam56 and EmGam82, from the wall forming bodies of *Eimeria maxima* have been studied intensively in recent years (see Belli *et al.,* 2006). Both are processed and/or degraded into smaller tyrosine-rich polypeptides (from 8 to 33 kDa) and incorporated into the oocyst wall. The tyrosine richness of these proteins and the presence of dityrosine in the oocyst wall has led to the proposal that dityrosine cross-linking of these proteins forms a matrix that is crucial component for oocyst wall formation (Belli *et al.,* 2006). The aims of this thesis are to:

[1] deduce the biochemical composition of the oocyst walls using gas chromatography (GC) and mass spectrometry (MS);

[2] determine the structural features of EmGam56 using bioinformatics, circular dichroism (CD), and one-dimensional nuclear magnetic resonance (1D-NMR);

[3] demonstrate that peroxidase-catalysed dityrosine crosslinks can be induced to form between truncated forms of EmGam56.

GC and MS revealed that the *Eimeria* oocyst wall is composed mainly of proteins (>90%) with small amount of lipids (1.4-7.6%) and carbohydrates (0.3-2.0%). There is little difference between the unsporulated and sporulated oocyst walls of *E. tenella* and *E. maxima*. Thus, the structure of proteins like EmGam56 is key to understanding how oocyst walls are constructed.

Bioinformatic analyses indicated that EmGam56 is an intrinsically unstructured protein (IUP), dominated by random coils (52-70%), with some α-helices (28-43%) but few β-sheets (1-11%); this was confirmed by CD and 1D-NMR. Furthermore, the structural integrity of the protein under extreme temperatures (boiling for 40 minutes) and pH (pH 1.3-11) indicated its IUP nature. The intrinsic lack of structure in EmGam56 could facilitate its incorporation into the oocyst wall in two ways: first, IUPs are highly susceptible to proteolysis, explaining the several differently-sized oocyst wall proteins derived from EmGam56; and, second, the flexibility of IUPs could facilitate cross-linking between these tyrosine-rich derivatives.

Peroxidases are key to the formation of dityrosine bonds (see Belli *et al.,* 2006 for a review). An *in vitro* cross-linking assay was developed using a recombinant 42 kDa truncation of EmGam56. The protein was exposed to various peroxidases and peroxides, the formation of polymers was followed by Western blotting, and the formation of dityrosines was determined by HPLC. Peroxidases from plants or fungi, but not mammals, catalysed rapid formation of polymers. No peroxidase has yet been found in the incompletely annotated *E. tenella* genome database but peroxidase activity has been detected in the wall forming bodies (Belli *et al.,* 2006). Therefore, future searches for *Eimeria* peroxidases should focus on plant-like homologous.

The results presented in this thesis support the proposal that dityrosine bonding between proteins is an important factor in the formation of the oocyst wall of coccidian parasites and are consistent with the hypothesis that antibodies stimulated by vaccination with EmGam56 and related proteins could prevent formation of oocysts.