Characterization of a 14 kDa oocyst wall protein of *Eimeria tenella* and *E. acervulina*

K.-H. ESCHENBACHER[1], P. EGGLI[2], M. WALLACH[3] *and* R. BRAUN[1]*

[1] *Institut für Allgemeine Mikrobiologie, Universität Bern, Baltzerstrasse 4, CH-3012 Bern, Switzerland*
[2] *Anatomisches Institut, Medizinische Fakultät, Universität Bern, Bühlstrasse 26, CH-3012 Bern, Switzerland*
[3] *ABIC Ltd Pharmaceutical and Chemical Industries, Industrial Zone, Kiryat Nordau, Natanya, P.O.B. 8077, Israel*

(*Received 19 April 1995; revised 16 August 1995; accepted 16 August 1995*)

SUMMARY

We have extracted a protein of 14 kDa from purified oocyst walls of several *Eimeria* species. Polyclonal antibodies were raised in rats against the 14 kDa proteins of *E. acervulina* and *E. tenella*. On immunoblots these antisera reacted in a highly specific manner with the homologous 14 kDa antigens, but not with heterologous antigens. In addition, specific binding of the two antisera to oocyst wall fragments of *E. acervulina* and *E. tenella* was demonstrated by immunofluorescence. Partial amino-terminal sequences comprising 20 amino acid residues were obtained from the 14 kDa oocyst wall proteins of *E. acervulina* and *E. tenella*. They are characterized by an abundance of amino acids containing hydroxyl groups in their side chains (serine, tyrosine, threonine). Binding of the oocyst wall protein of *E. tenella* by peanut agglutinin indicates the presence of *O*-linked carbohydrates.

Key words: Apicomplexa, *Eimeria*, oocyst wall protein, polyclonal antibodies, N-terminal sequence, peanut agglutinin.

INTRODUCTION

Members of the apicomplexan genus *Eimeria* cause disease in poultry, sheep, cattle, and other farm animals. Transmission of *Eimeria* spp. occurs by oocysts which are excreted with the faeces to become ingested by another host animal. The oocysts are ruptured mechanically (or enzymatically) and the sporocysts are set free. Under the influence of bile and of pancreatic enzymes, the infective sporozoites are released from the sporocysts (Levine, 1982). The shape, dimensions, and colour of the oocysts are useful diagnostic characteristics in describing different species of *Eimeria*. However, it is generally not possible to rely on these characters alone for species identification, since they can be affected by several factors (Jeffers & Shirley, 1982; Long & Joyner, 1984).

The wall surrounding *Eimeria* oocysts has some remarkable features. It provides a protective barrier which enhances the chances for survival under adverse conditions. The oocyst wall is resistant to several harsh chemicals, such as treatment with sulfuric acid, sodium hydroxide, sodium hypochlorite and potassium dichromate. It is, however, permeable to many small, uncharged and hydrophobic molecules, e.g. gases or organic solvents (Wang, 1982). A chemical analysis of purified oocyst walls of *Eimeria tenella* indicated that they are composed of 67% peptide, 14% lipid, and 19% carbohydrate. All carbohydrate was found to be associated with the protein. Electron microscopy revealed that oocyst walls have an electron-dense outer layer (about 10 nm thick) and a less dense inner layer (90 nm thick). It was proposed that the lipid fraction, which contains long-chain alcohols, phospholipids, sterols and triglycerides, constitutes the outer layer, whereas the inner layer consists of glycoprotein (Stotish, Wang & Meyenhofer, 1978; Wang, 1982).

Little is known about the biosynthesis of this complex wall matrix. Electron microscopical observations indicate that it is derived from 2 distinct types of granules, called wall-forming bodies 1 and 2 (WF 1, WF 2), which are present in the macrogametocytes. The WF 1 contain electron-dense material and are thought to give rise to the outer layer of the oocyst wall. Correspondingly, the WF 2, which appear to contain proteinaceous material, are apparently involved in the formation of the inner layer of the oocyst wall (Chobotar & Scholtyseck, 1982; Wang, 1982).

Stotish *et al.* (1978) demonstrated the presence of one or several polypeptides of about 10 kDa in oocyst walls of *Eimeria tenella* after solubilization by boiling in 8 M urea for several hours. In this study, we have used comparatively mild conditions to extract a protein of low molecular weight from purified oocysts walls. In this way we were able to characterize wall proteins of 2 *Eimeria* species, *E.*

* Reprint requests to Dr R. Braun, Institut für Allgemeine Mikrobiologie, Universität Bern, Baltzerstrasse 4, CH-3012 Bern, Switzerland.

acervulina and *E. tenella*, by N-terminal amino acid sequencing, and by raising polyclonal antibodies.

MATERIALS AND METHODS

Parasites

Eimeria oocysts were obtained from the following sources: Hoffmann-La Roche Company, Basel (*E. acervulina*, *E. tenella*); BIOPHARM Research Institute of Biopharmacy and Veterinary Drugs, Prague, Czech Republic (*E. maxima*, *E. media*, *E. stiedai*); Institute for Animal Health, Compton, England (*E. tenella*, Houghton strain); Institut für Parasitologie, Universität Zürich, Switzerland (*E. tenella*, Houghton strain). Oocysts were purified from chicken faeces by salt flotation and sodium hypochlorite treatment. The oocysts were fully sporulated; in the case of *E. tenella*, unsporulated oocysts were also used.

Purification of oocyst walls

The protocol described by Stotish *et al.* (1978) was used in a slightly modified form. About 10^8–10^9 oocysts were pelleted in a 50 ml conical plastic centrifuge tube and washed twice with water. Centrifugation was done at 2000 *g* for 5 min at room temperature using a swinging bucket rotor. The final pellet was resuspended in a small volume (ca. 1 ml) of wash solution (1 mM EDTA, pH 8, 0·1 % Triton X-100). An equal volume of glass beads was added (0·75–1 mm in diameter), and the mixture was vortexed in a glass centrifuge tube at full speed for 2–3 min until most of the oocysts were disrupted, as seen by microscopical examination. By repeatedly adding wash solution and swirling, the suspension was transferred to a 50 ml conical tube, leaving the glass beads behind. For further disruption of the oocysts, the suspension was sonicated for 1 min in intervals of 10 s (MSE Soniprep, 3 mm tip, power setting at 18 microns). The sonicate was centrifuged as before, and washed twice with 50 ml of wash buffer. The pellet was resuspended in 50 ml of 1 M sucrose (in wash solution) and centrifuged for 10 min at 2500*g* in a swing-out rotor. This sucrose centrifugation step removes amylopectin granules and debris, and it was repeated until the supernatant was clear. After discarding the supernatant, the pellet containing the oocyst walls was washed 3 times with wash solution. The purified oocysts walls were either stored as frozen pellets, or resuspended directly in sample buffer.

Protein electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by standard procedures using 12·5 % or 8–15 % gradient gels. The gel dimensions were 7·5 × 14 × 0·75 cm. The pelleted oocysts walls were resuspended in a double volume of SDS sample buffer (1·5 % SDS, 2·5 % β-mercaptoethanol, 31 mM Tris, pH 6·8, 5 % glycerol, 0·01 % bromophenol blue) and incubated in a boiling water bath for 3 min. Aliquots containing approximately 5×10^6 oocyst walls were loaded onto each 6 mm wide gel slot. For isolating larger amounts of protein for immunization, preparative gels (1·5 mm thick) were run and stained without fixation. For protein sequence analysis, samples were run on 13 % gels using a Tris/Tricine buffer system (Schägger & von Jagow, 1987).

Protein blotting

Electrophoretic transfer of proteins onto nitrocellulose membranes was accomplished by semi-dry blotting using a Multiphor II electrophoresis unit (LKB Pharmacia). The transfer buffer contained 25 mM Tris, 192 mM glycine, 20 % methanol, 0·05 % SDS, and was pH unadjusted. The presence of SDS markedly enhanced the effectiveness of the transfer of the 14 kDa protein. Transfer was performed for 30 min at 2 mA/cm^2. Protein bands were stained on the blots with Ponceau S. For immunodetection, blots were blocked for 30 min in Tris-buffered saline (TBS; 10 mM Tris pH 7·4, 150 mM NaCl) containing 5 % powdered skimmed milk and then incubated with the first antibody (diluted 1 : 10000 in blocking buffer) for at least 1 h, followed by 3 washes (10 min each) with TBS–T 0·1 % (Tween 20 in TBS). Peroxidase-conjugated rabbit anti-rat antibody (dil. 1 : 2000) was used as second antibody (30 min). After repeated washing, bands were visualized on X-ray films by chemiluminescence (ECL detection system, Amersham). Exposure times were between 10 and 30 s. All incubations were at room temperature.

Amino acid sequencing

Proteins were transferred from SDS–PAGE/Tricine gels to a polyvinylidene difluoride (PVDF) membrane (ProBlott, Applied Biosystems) as described above, except that a solution containing 50 mM borate, 20 % methanol, 0·05 % SDS (at pH 9·0) was used as transfer buffer. Transfer time was 1 h at 2 mA/cm^2. After transfer, the proteins were stained on the blot by immersing it in Coommassie blue solution (0·25 % in 45 % methanol, 9 % acetic acid) for 2 min, followed by destaining in 40 % methanol, 10 % acetic acid. Membrane pieces containing the 14 kDa band doublet were cut out and subjected to Edman degradation using a model 477 A protein sequencer (Applied Biosystems). Protein sequencing was performed by a service unit of the Biochemistry Department of the University of Berne.

Immunizations

Bands containing the 14 kDa oocyst wall protein were cut out from preparative gels. The protein was recovered from the gel by electroelution and emulsified with adjuvant using a glass-Teflon homogenizer. A BIOTRAP elution chamber (Schleicher und Schuell) was used according to the supplied protocols. Three rats were immunized with the *E. acervulina* antigen, and 2 with the *E. tenella* antigen. Freund's complete adjuvant was used for primary immunizations, and Freund's incomplete adjuvant for the 2 booster injections. Each immunization dose contained about 10 μg of protein. The injections were administered intramuscularly at time-intervals of 2 weeks. The animals were bled 1 week after the second boost and serum was prepared (Harlow & Lane, 1988).

Indirect immunofluorescence

Purified oocyst walls were air-dried onto microscope slides and processed with or without fixation with acetone/methanol (1:1). Samples were blocked with bovine serum albumin (3% in TBS–T) and then incubated for 1 h with the primary antibody, which was diluted 1:300 (*E. acervulina*) or 1:100 (*E. tenella*) in blocking buffer. Fluorescein isothiocyanate (FITC)-labelled rabbit anti-rat IgG (DAKO) was used as second antibody (diluted 1:200). Each antibody-binding step was followed by 3 washes with TBS–T (10 min each). The specimens were mounted in a 2·5% solution of 1,4-diazobicyclo[2,2,2]octane (DABCO) in glycerol. The slides were observed under a Zeiss Axiovert 135 fluorescence microscope equipped with filter set no. 23. Colour slide films (Kodak EPH 1600X) or negative films (Kodak Ektar 1000) were used for photography (Harlow & Lane, 1988).

Lectin binding

A Glycan Differentiation Kit (Boehringer Mannheim) was used according to the manufacturer's protocol. This method employs a set of digoxigenin-labelled lectins for on-blot detection of carbohydrate moieties of glycoproteins. The lectins are *Galanthus nivalis* agglutinin (GNA), *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), peanut agglutinin (PNA) and *Datura stramonium* agglutinin (DSA). Bound lectins were visualized by an alkaline phosphatase-conjugated anti-digoxigenin antibody and subsequent colour reaction with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates. For competition experiments, D-galactose or lactose (both from Merck, Darmstadt) were added to the lectin incubation solution at a concentration of 100 mM.

Scanning electron microscopy

Oocyst suspensions were processed as described by Kirschner (1978). Formvar-coated aluminum support disks were placed on moist filter paper in Petri dishes. One drop of oocyst suspension (approx. 25 μl) was placed onto each disk at 4 °C and allowed to settle for 2 min. The samples were fixed by adding 50 μl of cold glutaraldehyde (1% in 0·15 M sodium cacodylate buffer, pH 7·3, 425 mOsm). Immediately, glutaraldehyde and oocyst suspension were gently mixed and fixation was continued for 17 h at 4 °C. After washing in phosphate-buffered saline, the samples were dehydrated in a graded series of ethanol, dried in a critical-point drier, coated with a layer of gold and examined in a Philips XL 30 FEG scanning electron microscope.

RESULTS

The purification protocol yielded oocyst wall preparations which contained only small amounts of contaminating particles, as judged by light microscopy. No intact oocysts, sporocysts, or sporozoites were present. The disrupted oocyst walls had a needle-like appearance (Fig. 1B). Scanning electron microscopy revealed that the broken oocyst walls rolled up into tube-like structures (Fig. 1C). Triangular or spread-out shapes were also observed (Figs 1B and 4B).

When purified oocyst walls were subjected to SDS–PAGE under reducing conditions, a major protein of 14 kDa was observed, which migrated as a doublet on most gels. Proteins of this size were present in oocyst walls of *E. acervulina*, *E. tenella* (2 strains), *E. maxima* (Fig. 2), as well as in *E. praecox*, *E. media* and *E. stiedai* (not shown). No difference was seen when samples from unsporulated and sporulated oocysts were compared (Fig. 2, lane T_1 and T_3). The 14 kDa protein was not present in supernatants that contained soluble material released from the disrupted oocysts, sporocysts and sporozoites (Fig. 2, lane T/s). An additional minor protein species migrated in the 30 kDa range. This band was missing in extracts obtained from unsporulated oocysts (Fig. 2, lane T_3). It was found that this protein has a high content of proline (about 20% of all residues; data not shown) but it has not been analysed further.

Polyclonal antibodies were generated against the 14 kDa protein isolated from oocyst walls of *E. acervulina* and *E. tenella*. Since resolution of the doublet bands could not be achieved with preparative gels, the antigen preparations used for the immunization contained both bands of the doublet. The produced antisera are referred to as anti-Ea14 (*E. acervulina*) and anti-Et14 (*E. tenella*), respectively. On Western blots, these antisera produced strong

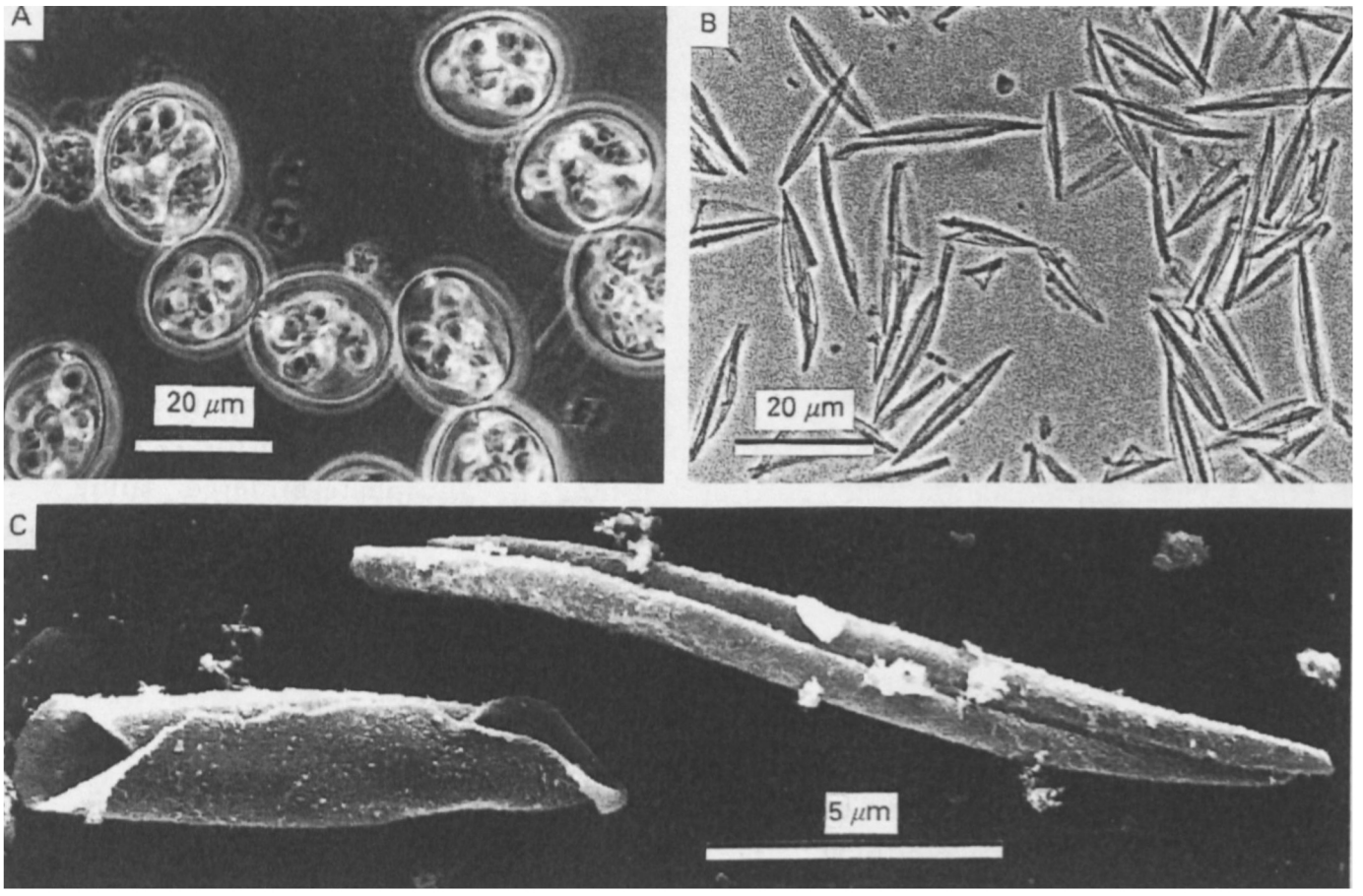

Fig. 1. (A) Intact sporulated oocysts of *Eimeria tenella* (phase contrast). (B) Broken oocyst walls of *E. tenella* after purification (phase contrast). (C) Same, scanning electron micrograph.

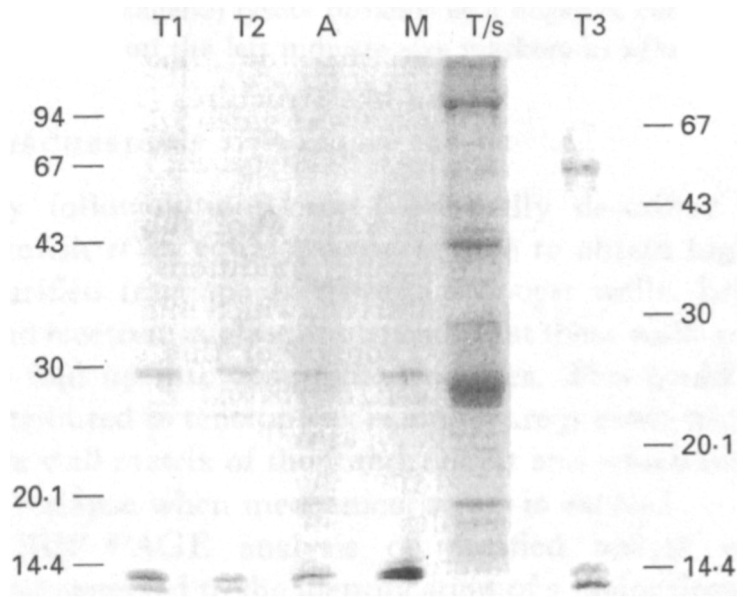

Fig. 2. SDS-PAGE of oocysts wall proteins under reducing conditions using an 8–15 % gradient gel (lanes T_1–T/s) or a 12·5 % gel (lane T_3). Samples in lanes T_1–T/s were from sporulated oocysts. T_1, *Eimeria tenella* (Houghton strain); T_2, *E. tenella* (Roche strain); A, *E. acervulina*; M, *E. maxima*; T/s, supernatant of *E. tenella* (Roche strain) oocysts after disruption; T_3, *E. tenella*, unsporulated oocysts (Houghton strain). The amount of protein loaded onto each lane corresponds to about 5×10^6 oocysts. Numbers indicate the apparent molecular weights in kDa.

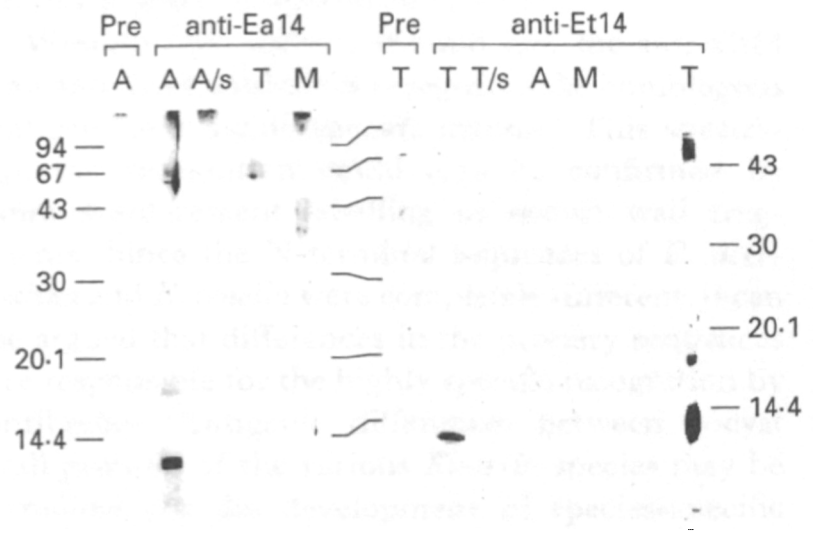

Fig. 3. Western blot of oocyst wall proteins probed with anti-Ea14 and anti-Et14 sera. Lanes labelled 'Pre' were incubated with the corresponding pre-immune sera. (A) *Eimeria acervulina*; T, *E. tenella*; M, *E. maxima*; A/s, T/s, supernatants from disrupted oocysts of *E. acervulina* and *E. tenella*, respectively. Numbers indicate apparent molecular weights in kDa. Antisera were used at a dilution of 1:10000, except for lane T on the extreme right side (1:5000).

signals with the homologous 14 kDa antigens (Fig. 3). When the antiserum was used at a higher concentration, additional weaker bands appeared at 17 kDa and at about 50 kDa (Fig. 3, lane T on the extreme right side). The anti-Ea14 serum did not react with oocyst wall proteins of *E. tenella*, and vice versa. Neither of the two antisera reacted with oocyst wall proteins of another species, *E. maxima*. In addition, protein supernatants of disrupted oocysts from *E. tenella* and *E. acervulina* did not react with the respective antisera (Fig. 3, lane A/s and T/s).

Oocyst wall fragments could be stained by immunofluorescence using the antibodies directed against the 14 kDa proteins (Fig. 4). It was possible to distinguish between oocyst walls of *E. acervulina* (Fig. 4 A) and *E. tenella* (Fig. 4 B) by specific binding

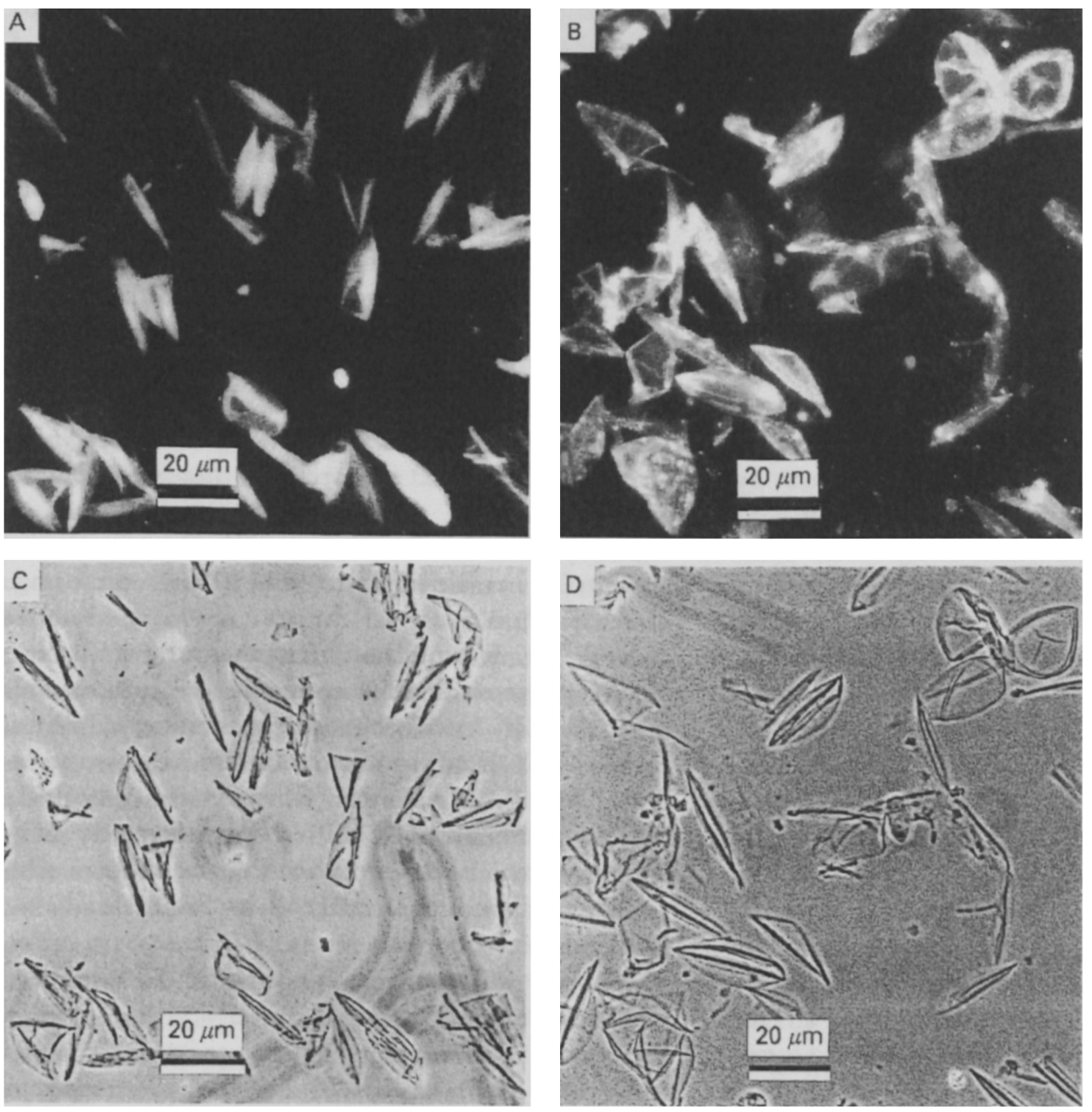

Fig. 4. Immunofluorescent staining of oocyst wall fragments of *Eimeria acervulina* (A) and *E. tenella* (B) by anti-Ea14 (A) and anti-Et14 (B). The corresponding phase-contrast images are shown in (C) and (D). With pre-immune serum only autofluorescence was observed. This was also the case when *E. acervulina* oocysts were incubated with anti-Et14 as primary antibody, and vice versa (not shown).

	1	11
A)	GWASPYTRYY	SSYSYYTPYS
	1	11
B)	FSSSFYPAFH	PYTYSSSYAL

Fig. 5. Amino-terminal amino acid sequences of the 14 kDa oocyst wall protein of *Eimeria acervulina* (A) and *E. tenella* (B). The *E. tenella* sequence was determined twice from 2 different strains (Houghton and La Roche) and was found to be identical in both cases.

to anti-Ea14 and anti-Et14, respectively. Intact oocysts or sporocysts did not stain (not shown). The staining of the oocyst walls was essentially uniform, although it was somewhat stronger at the edges of the folded structures. Fixation of the oocyst wall fragments with acetone/methanol prior to the antibody incubation did not affect the staining pattern (not shown).

Amino-terminal sequencing of the 14 kDa oocyst wall proteins of *E. acervulina* and *E. tenella* resulted in the identification of the first 20 amino acid residues of each protein (Fig. 5). Both N-terminal sequences contain large amounts of amino acids with hydroxyl groups in their side-chains. These 3 amino acids (serine, tyrosine, threonine) constitute 70% of all amino acids in the *E. acervulina* partial protein sequence, and 55% in the *E. tenella* sequence. Aromatic amino acids (phenylalanine, tyrosine, tryptophan) amount to 40% (*E. acervulina*) or 35% (*E. tenella*) of all residues. Remarkably, a sequence motif consisting of 3 serine residues flanked by aromatic amino acids (F/Y-S-S-S-F/Y) is repeated twice within the sequence of the *E. tenella* protein.

To determine if the 14 kDa protein of *E. tenella* is glycosylated, we performed a lectin-binding test on the blotted protein band. A positive signal was obtained with peanut agglutinin (PNA; Fig. 6, lane 1). None of the 4 other lectins tested (GNA, SNA, MAA, DSA; see Materials and Methods section) reacted with the 14 kDa band (not shown). PNA did not bind to bacterial proteins, which were used as a negative control (Fig. 6, lane 3).

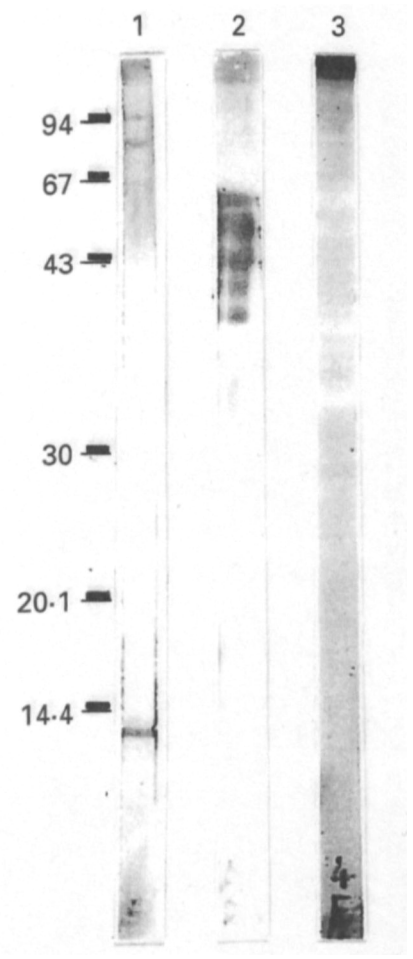

Fig. 6. Western blots probed with digoxigenin-labelled peanut agglutinin (PNA). Lane 1, oocyst wall proteins of *Eimeria tenella*. Lane 2, desialylated fetuin as a positive control protein. Lane 3, *Escherichia coli* (XL1-Blue, Stratagene) lysate proteins as a negative control. Numbers on the left indicate size markers in kDa.

DISCUSSION

By following a protocol originally described by Stotish *et al.* (1978), we were able to obtain highly purified fractions of disrupted oocyst walls. Light and electron microscopy reveals that these walls tend to fold up into tube-like structures. This could be attributed to tension forces which are present within the wall matrix of the intact oocyst and which make it collapse when mechanical stress is exerted.

SDS–PAGE analysis of purified oocyst wall fragments led to the identification of a major protein of 14 kDa and only a few additional minor bands. Likewise, in the related coccidian parasite, *Cryptosporidium parvum*, only a limited number of protein bands were found when fractions enriched for oocyst shells were analysed (Lally *et al.* 1992). The 14 kDa band was found in all Eimeria species so far, including 2 species of rabbit *Eimeria* (*E. media*, *E. stiedai*; not shown), and the proteins obtained from the various *Eimeria* species show only very little variation in size. This suggests that the occurrence of this small protein species is probably common to all Eimerian oocyst walls.

Based on its presence in oocyst walls and on its apparent molecular weight, we assume that the 14 kDa protein described here might be related to the 10 kDa fraction that has been reported earlier by Stotish *et al.* (1978). However, this fraction appeared as a rather broad band on an SDS–PAGE gel and probably contained degradation products as well, due to prolonged solubilization in hot urea (Stotish *et al.* 1978). We preferred the more gentle solubilization by SDS (for only 3 min), although it is less efficient (Stotish *et al.* 1978). To avoid possible contamination with sporocyst walls, we used unsporulated oocysts in our initial experiments. Since the 14 kDa protein was present in oocyst wall extracts from both unsporulated and sporulated oocysts, however, we decided to use sporulated oocysts which are easier to obtain.

The isolation of the 14 kDa protein from pure fractions of oocyst walls, and its absence from sporozoite extracts, suggested that it is a component of the wall. This could clearly be demonstrated by immunofluorescence using oocyst wall fragments and antisera raised against the 14 kDa proteins of *E. acervulina* and *E. tenella*. Intact oocysts could not be stained, which could be due to the outer lipid layer which might render the antigenic sites inaccessible to antibodies. Immunofluorescence microscopy was complicated by the strong autofluorescence produced by the oocyst walls (Russel & Sinden, 1981). However, this problem could be overcome by using a filter set which makes the emitted autofluorescence appear red in colour so that it can be distinguished from the green fluorescence of FITC.

Western blot analysis showed that the anti-Ea14 and anti-Et14 antibodies recognized the homologous antigens in a highly specific manner. This species-specific recognition could also be confirmed by immunofluorescent labelling of oocyst wall fragments. Since the N-terminal sequences of *E. acervulina* and *E. tenella* were completely different, it can be argued that differences in the primary sequences are responsible for the highly specific recognition by antibodies. Antigenic differences between oocyst wall proteins of the various *Eimeria* species may be exploited for the development of species-specific diagnostic tests. The identification of *Eimeria* species still remains a laborious and difficult task (Long & Joyner, 1984; MacPherson & Gajadhar, 1993; Stucki, Braun & Roditi, 1993).

The binding of peanut agglutinin to the 14 kDa oocyst wall protein of *E. tenella* (and also of *E. acervulina*; not shown) suggests the presence of *O*-linked carbohydrate residues. PNA recognizes the disaccharide galactose-$\beta(1 \rightarrow 3)$*N*-acetylgalactosamine which usually forms the core unit of *O*-glycans. However, complex-type glycan moieties often contain sialic acid residues in their terminal regions (Alberts *et al.* 1989) which prevent PNA from binding unless they are removed by treatment with neuraminidase (e.g. in the case of fetuin which was used as a positive control in Fig. 6). Since binding of PNA to the 14 kDa protein occurred without pre-treatment with neuraminidase or exoglycosidases, the carbohydrate moiety is probably not of the complex type (Lotan *et al.* 1975; Sueyoshi,

Tsuji & Osawa, 1988; Boehringer Mannheim DIG Glycan Differentiation Kit, product information). Since PNA might also have other binding specificities, further experiments will be needed to determine the nature of the carbohydrate residue(s). Binding of PNA to the 14 kDa protein was inhibited in the presence of the disaccharide lactose, and – less efficiently – the monosaccharide galactose (data not shown). This observation is in agreement with the data reported by Lotan *et al.* (1975) and Russel & Sinden (1981). The notion that the 14 kDa protein might be *O*-glycosylated is also supported by the finding that the N-terminal sequences are rich in serine and threonine, which are target sites for this type of modification. However, since excessive glycosylation would have interfered with Edman degradation, we assume that the degree of modification is probably low, at least in the N-terminal region. Post-translational modifications of various kinds could also be responsible for the appearance of the 14 kDa protein as a band doublet. Microheterogeneity was also found to occur in the 10 kDa oocyst wall protein described by Stotish *et al.* (1978).

The observation that FITC-labelled PNA binds to the surface of glutaraldehyde-fixed oocysts of *E. acervulina* and *E. tenella* (Russel & Sinden, 1981) is also in agreement with the results presented here. In addition, PNA-binding glycoproteins have also been found in sporulated oocysts and sporozoites of *E. tenella*, suggesting that *O*-glycosylation is quite frequent in *Eimeria* spp. (Michalski *et al.* 1993). The nature of the carbohydrate component of the oocyst wall proteins, as well as its function in the wall matrix, remain to be determined. It has been suggested that mannitol or mannitol phosphate might become incorporated into the oocyst wall (Schmatz, 1989).

At present, only 1 genomic sequence coding for a coccidian oocyst wall protein has been reported (of *C. parvum*; Lally *et al.* 1992; Ranucci *et al.* 1993). The putative amino acid sequence of this *Cryptosporidium* protein, however, bears no similarities to the partial N-terminal sequences we obtained for *Eimeria* oocyst wall proteins. The N-terminal sequences of the 14 kDa proteins of *E. tenella* and *E. acervulina* constitute only about 18% of the total length of the polypeptide (assuming a molecular weight of 14 kDa), and interpretation of these data is therefore speculative.

A striking feature of the partial amino acid sequences is the high frequency of amino acids which contain hydroxyl groups in their side chains. By cross-linking individual proteins via these hydroxyl groups, a complex polymeric wall matrix may be formed. For example, cross-linking by tyrosine residues is involved in the hardening of the fertilization envelope of sea urchin eggs (Hall, 1978) and in the synthesis of the yeast ascospore wall (Briza *et al.* 1986). Tanning processes utilizing tyrosines and other phenolic compounds also play a crucial role in insect cuticle sclerotization (reviewed by Hopkins & Kramer, 1992) and in eggshell formation in *Schistosoma mansoni* (Seed & Bennett, 1980), *Fasciola hepatica* (Waite & Rice-Ficht, 1987) and *Trichuris suis* (Fetterer & Hill, 1994). The putative amino acid sequence of an eggshell protein of *S. mansoni* shows a high content of tyrosine residues (Johnson, Taylor & Cordingley, 1987). Monné & Hönig (1954) proposed that the formation of the coccidian oocyst wall could occur by a tanning process. The observation that the anti-14 antisera weakly recognize bands in the higher molecular weight range could be explained by cross-links between protein monomers. The 14 kDa protein would then represent the monomer. It should also be noted that additional polypeptide species might participate in the formation of the wall like, for example, the 30 kDa proline-rich protein which was not recognized by the anti-14 antibodies. In addition, the identification of a mannitol cycle in *E. tenella* has led to the speculation that during oocyst formation mannitol could create an osmotic pressure within the oocyst until the hardening of the shell is completed (Schmatz, 1989).

It remains to be established whether the 14 kDa wall proteins can be localized within the wall-forming bodies of the gametocytes. Since gametocyte antigens have been reported to induce a protective immune response in chickens (Wallach *et al.* 1989), the 14 kDa proteins might also be useful for vaccination trials.

We wish to thank J. Eckert (University of Zurich), P. Bedrnik (BIOPHARM, Prague), G. Weber (Hoffmann-La Roche, Basel) and F. Tomley (Institute for Animal Health, Compton) for providing oocysts, and I. Roditi for helpful discussions and for critically reading the manuscript. This work was supported by the Bundesamt für Bildung und Wissenschaft, Bern (Project: Coccidiosis, COST 89 & 820) and the Swiss National Science Foundation.

REFERENCES

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K., WATSON, J. D. (1989). *Molecular Biology of the Cell*, 2nd edn. New York: Garland Publishing.

BRIZA, P., WINKLER, G., KALCHHAUSER, H. & BREITENBACH, M. (1986). Dityrosine is a prominent component of the yeast ascospore wall. *Journal of Biological Chemistry* **261**, 4288–94.

CHOBOTAR, B. & SCHOLTYSECK, E. (1982). Ultrastructure. In *The Biology of the Coccidia* (ed. Long, P. L.), pp. 101–165. London: University Park Press.

FETTERER, R. H. & HILL, D. E. (1994). Localization of phenol oxidase in female *Trichuris suis*. *Journal of Parasitology* **80**, 952–9.

HALL, H. G. (1978). Hardening of the sea urchin fertilization envelope by peroxidase-catalyzed phenolic coupling of tyrosines. *Cell* **15**, 343–55.

HARLOW, E. & LANE, D. (1988). *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory. New York: Cold Spring Harbor Laboratory Press.

HOPKINS, T. L. & KRAMER, K. J. (1992). Insect cuticle sclerotization. *Annual Review of Entomology* **37**, 273–302.

JEFFERS, T. K. & SHIRLEY, M. W. (1982). Genetics, specific and infraspecific variation. In *The Biology of the Coccidia* (ed. Long, P. L.), pp. 63–100. London: University Park Press.

JOHNSON, K. S., TAYLOR, D. W. & CORDINGLEY, J. S. (1987). Possible eggshell protein gene from *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **22**, 89–100.

KIRSCHNER, R. H. (1978). High resolution scanning electron microscopy of isolated cell organelles. In *Principles and Techniques of Scanning Electron Microscopy* (ed. Hayat, M. A.), pp. 278–296. New York: Van Nostrand Reinhold Company.

LALLY, N. C., BAIRD, G. D., McQUAY, S. J., WRIGHT, F. & OLIVER, J. J. (1992). A 2359-base pair DNA fragment from *Cryptosporidium parvum* encoding a repetitive oocyst protein. *Molecular and Biochemical Parasitology* **56**, 69–78.

LEVINE, N. D. (1982). Taxonomy and life cycles of coccidia. In *The Biology of the Coccidia* (ed. Long, P. L.), pp. 1–33. London: University Park Press.

LONG, P. L. & JOYNER, L. P. (1984). Problems in the identification of species of *Eimeria*. *Journal of Protozoology* **31**, 535–41.

LOTAN, R., SKUTELSKI, E., DANON, D. & SHARON, N. (1975). The purification, composition and specifity of the anti-T lectin from peanut (*Arachis hypogaea*). *Journal of Biological Chemistry* **250**, 8518–23.

MACPHERSON, J. M. & GAJADHAR, A. A. (1993). Differentiation of seven *Eimeria* species by random amplified polymorphic DNA. *Veterinary Parasitology* **45**, 257–66.

MICHALSKI, W. P., PROWSE, S. J., BACIC, A. & FAHEY, K. J. (1993). Molecular characterization of peanut agglutinin binding glycoproteins from *Eimeria tenella*. *International Journal for Parasitology* **23**, 985–95.

MONNÉ, L. & HÖNIG, G. (1954). On the properties of the shells of the coccidian oocysts. *Arkiv för Zoologi* (Uppsala) **7**, 251–6.

RANUCCI, L. R., MULLER, H. M., LAROSA, G. L., RECKMANN, I. R., GOMEZ MORALES, M. G., POZIO, E. P., SPANO, F. S. & CRISANTI, A. A. (1993). Characterization and immunolocalization of a *Cryptosporidium* protein containing repeated amino acid motifs. *Infection and Immunity* **61**, 2347–56.

RUSSEL, D. G. & SINDEN, R. E. (1981). The role of the cytoskeleton in the motility of coccidian sporozoites. *Journal of Cell Science* **50**, 345–59.

SCHÄGGER, H. & VON JAGOW, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166**, 368–79.

SCHMATZ, D. M. (1989). The mannitol cycle – a new metabolic pathway in the coccidia. *Parasitology Today* **5**, 205–8.

SEED, J. L. & BENNETT, J. L. (1980). *Schistosoma mansoni*: phenol oxidase's role in eggshell formation. *Experimental Parasitology* **49**, 430–41.

STOTISH, R. L., WANG, C. C. & MEYENHOFER, M. (1978). Structure and composition of the oocyst wall of *Eimeria tenella*. *Journal of Parasitology* **64**, 1074–81.

STUCKI, U., BRAUN, R. & RODITI, I. (1993). *Eimeria tenella*: Characterization of a 5S ribosomal RNA repeat unit and its use as a species-specific probe. *Experimental Parasitology* **76**, 68–75.

SUEYOSHI, S., TSUJI, T. & OSAWA, T. (1988). Carbohydrate-binding specificities of five lectins that bind to *O*-glycosyl-linked carbohydrate chains. Quantitative analysis by frontal-affinity chromatography. *Carbohydrate Research* **178**, 213–24.

WAITE, J. H. & RICE-FICHT, A. C. (1987). Presclerotized eggshell protein from the liver fluke *Fasciola hepatica*. *Biochemistry* **26**, 7819–25.

WALLACH, M., MENCHER, D., YARUS, S., PILLEMER, G., HALABI, A. & PUGATSCH, T. (1989). *Eimeria maxima*: identification of gametocyte protein antigens and their possible role in protective immunity. *Experimental Parasitology* **68**, 49–56.

WANG, C. C. (1982). Biochemistry and physiology of the coccidia. In *The Biology of the Coccidia* (ed. Long, P. L.), pp. 167–228. London: University Park Press.