# Characterization and immunolocalization of a main proteinaceous component of the cell wall of the protozoan parasite *Perkinsus atlanticus*

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SUMMARY

Described in the present study is a major component of the cell wall of two of the most pathogenic parasites of molluscs, *Perkinsus atlanticus* and *P. marinus*. The component is a high-molecular-weight protein (233-kDa), which we have named PWP-1 (for *Perkinsus* wall protein-1). Western blots, using a polyclonal serum generated against purified PWP-1 from *P. atlanticus*, revealed that this protein is expressed by all walled developmental stages of this protozoon. By means of immunogold electron microscopy, labelling for PWP-1 was strong and specifically associated with the cell wall. The label density and distribution pattern was quite different between trophozoites and prezoosporangia. With regard to the structural organization of this protein, PWP-1 is disulphide-linked to other cell wall components and released from the cell wall only following treatment with a sulfhydryl agent. We also report that PWP-1 is a trypsin-resistant protein, both in its native and heat-denatured conformation. In addition, results from the N-terminal microsequence of this protein allow us to define PWP-1 as a novel cell wall protein. Overall, our findings strongly suggest that PWP-1 plays a key role in the organization of the cell wall of these protozoa promoting their survival.

Key words: cell wall, *Perkinsus atlanticus*, *Perkinsus marinus*, *Tapes semidecussatus*, trypsin-resistant protein.

# INTRODUCTION

Protozoa belonging to the genus *Perkinsus* are major disease agents in marine molluscs and have been associated with extensive mortality in bivalves and gastropods worldwide (reviewed by Bower, McGladdery & Price, 1994; Perkins, 1996). Six *Perkinsus* species have been described so far: *P. marinus* (Mackin, Owen & Collier, 1950) in the oyster *Crassostrea virginica*, *P. olseni* (Lester & Davis, 1981) in the abalone *Haliotis ruber*, *P. atlanticus* (Azevedo, 1989) in the clam *Tapes decussatus*, *P. qugwadi* (Blackbourn, Bower & Meyer, 1998) in the scallop *Patinopecten yessoensis*, *P. chesapeaki* (McLaughlin *et al.* 2000) in the clam *Mya arenaria* and, recently, *P. andrewsi* (Coss *et al.* 2001) in the clam *Macoma balthica*. At present, *Perkinsus* species are placed within the recently established phylum Perkinsozoa (Nóren, Moestrup & Rehnstam-Holm, 1999), bridging the groups Apicomplexa and Dinoflagellata, in which *Perkinsus* was previously included (Levine, 1978; Siddall *et al.* 1997).

On the Atlantic and Mediterranean coasts of Europe, parasitism by *P. atlanticus* has been associated with epizootic outbreaks involving large-scale mortalities of commercially valuable venerid clams of the genus *Tapes* (=*Ruditapes* =*Venerupis*), such as the indigenous species *T. decussatus* (Da Ros & Canzonier, 1985; Comps & Chagot, 1987; Azevedo, 1989; Figueras, Robledo & Novoa, 1992; Montes, Durfort & García-Valero, 1996) and the introduced species *T. semidecussatus* (=*T. philippinarum* =*T. japonica*) (Montes, Durfort & García-Valero, 1995*a*; Sagristà, Durfort & Azevedo, 1995). In addition, we have recently reported that *P. atlanticus*-infected clams *T. semidecussatus* from the northern Mediterranean coast of Spain

develop viral and bacterial opportunistic infections, which have detrimental effects on this clam population (Montes, Durfort & García-Valero, 2001).

The life cycle of *P. atlanticus* consists of three main stages: trophozoite, prezoosporangium and zoospore (Azevedo, 1989; Sagristà, Durfort & Azevedo, 1996). Trophozoites are the vegetative form that proliferates in the connective tissue of the host. During incubation in fluid thioglycollate medium, and probably in dying hosts (Auzoux-Bordenave *et al.* 1995), they enlarge substantially and differentiate into prezoosporangia. When this life-cycle form is placed into seawater, it releases biflagellate zoospores, which are the main infective stage of this parasite. Electron microscopic examination of *P. atlanticus* life-cycle stages reveals that both trophozoites and prezoosporangia are characterized by the presence of a well-developed cell wall, which considerably increases in thickness during differentiation of the trophozoite to prezoosporangium (Azevedo, 1989; Sagristà *et al.* 1996). The thickened wall of the prezoosporangia stains blue-black with Lugol's iodine solution, without acid hydrolysis (Ray, 1952).

Little is known, however, about the composition, structure and organization of the cell wall of *Perkinsus* species. Earlier studies by Stein & Mackin (1957), based on cytochemical analyses, suggested that cellulose and hemicellulose constitute the polysaccharidic matrix of the cell wall of *P. marinus*. Recently, we have reported, using lectin histochemistry, that the trophozoite cell wall of *P. atlanticus* shows a low degree of glycosylation (Montes *et al.* 1995*a*). Progress towards understanding the epidemiology and pathogenesis of *Perkinsus* infections requires further knowledge of the surface molecules of this parasite. In this regard, we report the isolation and partial characterization of a main proteinaceous component of the cell wall of *P. atlanticus*.

Polyclonal antibodies have been raised against this protein, allowing us to determine that it is expressed by all walled stages of this parasite. The cross-reactivity of this serum with other *Perkinsus* species is also reported.

## MATERIALS AND METHODS

### *Animals*

Market-sized specimens of non-parasitized and *P. atlanticus*-infected clams, *T. semidecussatus,* were obtained from different clam beds of the Delta of the River Ebro, Tarragona (N.E. Spain), Mediterranean Sea, an area endemic for *P. atlanticus*. Specimens were collected during July-September 1999.

### *Isolation and purification of* P. atlanticus *prezoosporangia*

The prezoosporangia of *P. atlanticus* were purified from isolated parasitized gills following essentially the procedure described by Chu & Greene (1989). Briefly, 425 g of gills (from approx. 2500 clams) were incubated in fluid thioglycollate medium (FTM; Difco Laboratories; Detroit, MI), rehydrated with 0·22 $\mu$m filtered sea water of 25 ‰ salinity and supplemented with 400 U/ml penicillin G (Sigma Chemical Co; St. Louis, MO), 500 $\mu$g/ml streptomycin sulphate (Sigma) and 200 U/ml nystatin (Sigma). Culture was carried out in the dark under anaerobiosis at room temperature. After 72 h of incubation, tissues were chopped and treated with 0·25 % trypsin (Sigma) in seawater for 15 min at 37 °C. The resultant tissue suspension was sequentially filtered through nylon meshes of 300 and 100 $\mu$m to remove gill tissue fragments. Finally, the prezoosporangia fraction was enriched by sequential centrifugation at 825, 200 and 50 ***g*** for 5 min each at 4 °C. Each centrifugation step was repeated 3 times and the

supernatant was always discarded. The purity of the isolation was determined by differential interference contrast microscopy and Lugol's iodine stain.

*Prezoosporangia processing and isolation of antigenic polypeptides*

The enriched fraction of prezoosporangia was resuspended in 4 volumes of lysis buffer (15 mM Tris-HCl (pH 7·4), 150 mM NaCl, 1 mM $MgCl_2$, 1 % Triton X-100, 0·2 % SDS, 1 mM EDTA) containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 $\mu$M leupeptin, 0·3 $\mu$M aprotinin, 28 $\mu$M E-64, 1 $\mu$M pepstatin) and then incubated for 15 min on ice. Thereafter, prezoosporangia were treated by repeated cycles of freezing-thawing and homogenized with a motorized glass-Teflon homogenizer. Prezoosporangium lysates were subsequently sonicated (Vibracell; Sonics & Materials; Danbury, CT) on ice 4 times for 10 s at 50 w at 15 s intervals.

One hundred and thirty-six mg of total protein from prezoosporangium homogenates were electrophoretically resolved on a Protean II vertical slab gel unit (Bio-Rad; Hercules, CA) with 9 % polyacrylamide separating gels and 4 % stacking gels under reducing conditions. Prezoosporangium homogenates were mixed 1:2 with SDS-PAGE sample buffer (62·5 ml Tris-HCl (pH 6·8), 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol), heated at 95 °C for 5 min and clarified by centrifugation. Apparent molecular masses of polypeptides were determined from their relative mobilities compared with a broad molecular weight standard (Bio-Rad). The five major polypeptides of highest molecular weight were selected for rabbit

immunization. After electrophoresis, the selected bands were removed from the gels following the side-strip method (Harlow & Lane, 1988) and kept at -80 °C until injection.

*Immunization and production of antisera*

Specific polyclonal antisera were obtained by direct immunization with SDS-PAGE slices containing the selected polypeptides essentially as described elsewhere (Boulard & Lecroisey, 1982). In brief, young white male New Zealand rabbits, weighing 2-3 kg, were immunized 3 times by intramuscular injections at 2 week intervals with a total of 450 $\mu$g of the purified polypeptides. Immunizations were performed by emulsion of the immunogen in Freund's adjuvant (Sigma), complete for the first injection and incomplete thereafter. The rabbits were bled before the immunizations, and the sera obtained were used for control studies.

Each antiserum was tested following immunoblotting and immunocytochemical techniques by electron microscopy. According to the results, the serum against a polypeptide of an apparent molecular weight of 233 kDa (PWP-1; for *Perkinsus* wall protein-1) was selected for analysis.

*Sample processing and immunoblotting*

The specificity of the serum against PWP-1 was tested by the Western blot technique

in samples from *P. atlanticus* prezoosporangia, and gills from both parasitized and non-parasitized clams, *T. semidecussatus*. Non-parasitized specimens were determined after thioglycollate assay of the two hemibranchs from one side. Gills were homogenized at 4 °C in lysis buffer by 20 strokes in a glass Dounce homogenizer followed by sonication. The reactivity of the serum was also assayed in samples of *P. marinus* isolate LICT-1 acquired from the American Type Culture Collection (ATCC 50508). *Perkinsus marinus* homogenates were obtained as described above for *P. atlanticus* prezoosporangia.

For immunoblotting, denatured samples were separated by either reducing or non-reducing SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell; Dassel, Germany) following Towbin, Staehelin & Gordon (1979). Transfer was achieved at 100 mA overnight at 4 °C in a Mini Trans-Blot apparatus (Bio-Rad). Thereafter, the membranes were presoaked in TBST (50 mM Tris-HCl (pH 7·5), 150 mM NaCl, 0·1 % Tween 20) and treated with blocking buffer (5 % non-fat dried milk in TBST) for at least 30 min. Membranes were incubated with serum against PWP-1 diluted 1:2000 in blocking buffer for 3 h at room temperature. After 3 washes in TBST, peroxidase-conjugated swine anti-rabbit Ig (Dako; Gloustrup, Denmark) was applied for 2 h. Immunoreactive bands were detected using the enhanced chemiluminescence system, and the luminol excitation was imaged on X-OMAT UV films (Kodak; Hemel, UK). For the controls, the specific antiserum was replaced by the matched preimmune serum. In additional controls, incubation with the primary antibody was omitted.

*Trypsin digestion*

To assess the stability of PWP-1 against proteolysis, gill homogenates from heavily *P. atlanticus*-infected clams were treated with trypsin. Gill homogenates were obtained as mentioned before, but the lysis buffer did not contain detergents or protease inhibitors. The assay was carried out as follows: both non-denatured and denatured (95 °C, 5 min) homogenate samples were incubated at 37 °C for 30 min with either trypsin or heat-inactivated trypsin at an enzyme:substrate ratio of 1:1. The final concentration of trypsin was 4 $\mu$g/ml. As a digestion control, the homogenate samples were supplemented with purified rabbit IgGs before the incubation. The reactions were stopped by addition of SDS-PAGE sample buffer and heating at 95 °C for 5 min. Digestion was analysed by immunoblotting with the serum against PWP-1 as described.

*N-terminal amino acid microsequencing of PWP-1*

For Edman microsequencing, prezoosporangium proteins were fractionated by SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane (Schleicher & Schuell) using a Trans blot cell (Bio-Rad). After transfer, the membrane was stained with Coomassie Blue and the band corresponding to PWP-1 was excised. The N-terminal microsequence was determined by automated Edman degradation on a Beckman LF3000 protein sequencer, equipped with a phenylthiohydantoin (PTH)-amino acid analyser System Gold (Beckman Instruments;

Fullerton, CA).

*Immunogold labelling*

Abscesses from *P. atlanticus*-infected gills and isolated prezoosporangia were fixed in 4 % paraformaldehyde, 0·1 % glutaraldehyde in 0·1 M phosphate-buffered saline (PBS) for 2 h at 4 °C. Isolated prezoosporangia were then embedded in 30 % ovalbumin in PBS and polymerized in the same fixative. After washing, all samples were processed for Lowicryl K4M infiltration at low temperature following the manufacturer guidelines (Polysciences LTD; Northampton, UK). Polymerization was induced by UV irradiation at - 35 °C for 2 days. Immunogold labelling was performed as described elsewhere (Montes, Durfort & García-Valero, 1995*b*). After washing and blocking, the grids were incubated with the serum against PWP-1 (1:500) and then bound polyclonal antibodies were visualized following incubation with 10 nm protein A gold (Dr. Slot, Utrecht, The Netherlands). Finally, the grids were washed thoroughly in PBS and double-distilled water, and stained with uranyl acetate and Reynold's lead citrate. Controls were performed by replacing the specific antiserum with matched preimmune serum. Additional controls were carried out by omitting the specific antiserum from the immunolabelling procedure. Observations were carried out with a Hitachi H-600 AB transmission electron microscope.

*Quantitative evaluation*

Label density (LD) was estimated as the number of gold particles per sectioned area of trophozoite and prezoosporangium cell wall. The area parameter was estimated by stereological methods (Weibel, 1979). Values were expressed as mean ± SEM from at least 30 measurements performed in various parasites and significance of mean differences was tested by Student's *t*-test.

# RESULTS

## *SDS-PAGE and immunoblotting*

Under denaturing and reducing conditions, the electrophoretic profile of isolated *P. atlanticus* prezoosporangia consisted of 12 major bands with mobilities ranging from 233 to 42 kDa (Fig. 1A). To obtain a polyclonal serum against a cell wall protein of *P. atlanticus*, the five major bands of highest molecular weight (233, 198, 172, 149 and 102 kDa) were isolated for rabbit immunization. The serum obtained against 233-kDa polypeptide (PWP-1; for *Perkinsus* wall protein-1) showed the highest specificity and was selected for the study.

The immunoreactivity of the serum against PWP-1 was determined by Western blotting. A single band of an expected apparent molecular mass of 233 kDa was detected in the homogenate samples from both *P. atlanticus* prezoosporangia (Fig. 1B, lane 1) and *P. atlanticus* trophozoites-containing gills (Fig. 1B, lane 2). There were no positive signals when this antiserum was assayed in non-parasitized samples (Fig. 1B, lane 3). In addition, using this polyclonal serum, PWP-1 was identified in immunoblots of *P. marinus* isolate LICT-1 (Fig. 1B, lane 4). No apparent differences were observed between these two *Perkinsus* species in molecular weight or in antibody recognition of the polypeptide. In contrast, no protein band was detected on these blots when the preimmune rabbit serum was used as the specific control (data not shown).

To determine whether PWP-1 is linked by disulphide bonds to other cell wall components, denatured (hot SDS) homogenate samples from both *P. atlanticus* prezoosporangia and *P. atlanticus* trophozoites-containing gills were analysed by

immunoblotting under non-reducing conditions (Fig. 2). After incubation with the specific serum, no reactive bands were detected at 233-kDa or at any other mobility (Fig. 2, lanes 2 and 4), indicating that PWP-1 is only released after treatment with a sulfhydryl agent, such as 2-mercaptoethanol (Fig. 2, lanes 1 and 3).

*Trypsin digestion*

The stability of non-denatured and heat-denatured PWP-1 against trypsin proteolysis was probed in *P. atlanticus* trophozoites-containing homogenates as described in the Materials and Methods section. Western blotting using the specific serum showed that trypsin treatment had no detectable effect on the molecular weight or on the recognition of PWP-1, regardless of its conformation (Fig. 3). On the other hand, the degradation of the rabbit IgGs, added to the reaction mixture as internal control, supported the validity of the experimental conditions.

*N-terminal amino acid microsequencing of PWP-1*

N-terminal sequencing of PWP-1 revealed that its first 8 amino acid residues were MEDEGAGG. The comparison of this sequence with the Swiss Prot database did not show homology with any other known protein.

*Immunolocalization*

The immunoreactivity of the serum against PWP-1 was also assayed by immunogold electron microscopy with gill abscesses from *P. atlanticus*-infected clams, *T. semidecussatus*. The resultant labelling was exclusively associated with the trophozoites of *P. atlanticus* (Fig. 4A and B). Neither the capsule nor other host tissues showed affinity for this antiserum. In addition, the lack of labelling after incubation with the preimmune serum demonstrated the specificity of this antiserum (data not shown). Labelling for PWP-1 was entirely confined to the cell wall of the trophozoites (Fig. 4A and B). No significant reactivity was detected in the other trophozoite structures, such as cisternae of endoplasmic reticulum or vacuolar compartment (Fig. 4A).

Labelling of the serum against PWP-1 was strong and distributed throughout the trophozoite cell wall without any regional predominance. The evaluation of the labelling pattern revealed that PWP-1 showed a ordered distribution parallel to the plasmalemma (Fig. 4A). No apparent differences were observed between the maturation stages of the parasite. This ordered distribution was most obvious when the immunogold staining of PWP-1 was analysed in the remnants of mother cell walls originated during parasite proliferation (Fig. 4B).

When immunolocalization of PWP-1 was carried out in the isolated prezoosporangia, the resultant pattern of immunoreactivity closely resembled that obtained in trophozoites, since the prezoosporangium cell wall was the only compartment stained (Fig. 4C and D). However, differences were observed when the labelling pattern for PWP-1 was compared between these two life-cycle forms. Thus,

in the thicker prezoosporangium cell wall, formed during FTM incubation, the immunogold staining was patchy, revealing regional variations in the distribution of this protein. Labelling was usually stronger in the distal than in the proximal areas of the prezoosporangium cell wall (Fig. 4D). In addition, considerable differences were detected when label densities for PWP-1 were compared between both life-cycle forms. Thus, the LD obtained for prezoosporangia (36·7 ± 12·5 gold particles $\mu m^{-2}$) was significantly lower ($p < 0{\cdot}01$) than that obtained for trophozoites (64·0 ± 8·2 gold particles $\mu m^{-2}$).

## DISCUSSION

High-molecular-weight proteins (≥ 200 kDa) are characteristic constituents of the cell wall of unicellular eukaryotes, including apicomplexans (Tilley *et al.* 1990; Bonnin, Dubremetz & Camerlynck, 1991), dinoflagellates (Markell, Trench & Iglesias-Prieto, 1992), diatoms (Kröger, Bergsdorf & Sumper, 1994; Kröger *et al.* 1997) and yeasts (Casanova *et al.* 1992; Mrsa *et al.* 1997). In this regard, the current study shows that a major proteinaceous component of the cell wall of the protozoan parasite *P. atlanticus* is a novel cell wall protein with an apparent molecular weight of 233 kDa, which we have named PWP-1 (for *Perkinsus* wall protein-1).

Western blotting and immunogold electron microscopy using the polyclonal serum generated against purified PWP-1 from *P. atlanticus* revealed that this protein is expressed by all walled developmental stages of this parasite. This finding, together with the fact that PWP-1 is also expressed by *P. marinus*, strongly suggest that PWP-1 plays a key role in either the organization or the physiology of the cell wall of the protozoa of the genus *Perkinsus*.

Previous studies have documented that *Perkinsus* differentiation from the trophozoite to the prezoosporangium stage is accompanied by a change in the staining (Ray, 1952) and antigenic (Choi *et al.* 1991; Montes *et al.* 1995*b*) properties of the cell wall. In this context, we report that these two *P. atlanticus* life-cycle forms differ in both the label density and pattern of labelling for PWP-1. These findings allow us to demonstrate, for the first time, that *Perkinsus* trophozoite to prezoosporangium differentiation not only involves a change in the cytochemical and immunological properties of the cell wall, as mentioned above, but also a substantial modification in

its organization.

As to the structural organization of PWP-1, immunoblotting assays showed that PWP-1 is released from the cell wall only following treatment with a sulfhydryl agent, such as 2-mercaptoethanol. This finding indicates, therefore, that PWP-1 is linked by disulphide bonds to other cell wall constituents of *P. atlanticus*. Likewise, numerous disulphide-bounded protein complexes have been described in the cell wall of other unicellular organisms, including prokaryotes (Newhall *et al.* 1980) and eukaryotes (Chaffin *et al.* 1998; De Stefano *et al.* 1998; Lipke & Ovalle, 1998). It has been suggested that these protein complexes play a structural role, contributing to the stability of the cell wall of these organisms (Chaffin *et al.* 1998; De Stefano *et al.* 1998). Therefore, the presence of disulphide protein complexes in the cell wall of *Perkinsus* may explain the extreme resistance of these protozoa to chemical and mechanical disruption (Saunders, Powell & Lewis, 1993; Krantz, 1994).

On the other hand, there is growing evidence that the trophozoites of *Perkinsus* species survive exposure to lysosomal enzymatic activities after phagocytosis by mollusc haemocytes (La Peyre, Chu & Vogelbein, 1995; Sagristà *et al.* 1995; Perkins, 1996; Bushek *et al.* 1997), so much so that a characteristic feature of mature trophozoites is the presence of host lysosomal membranes embedded in the cell wall (Perkins 1988, 1996). Given that the wall of *Perkinsus* is the cell compartment responsible for direct interaction with host cells, our finding that a major proteinaceous component of this structure is resistant to proteolysis, may partially explain why molluscan phagocytes are not very effective in combatting this disease agent.

In conclusion, we have demonstrated that a high-molecular-weight protein, PWP-1, is a major component of the cell wall of two of the most pathogenic parasites

of molluscs, *P. atlanticus* and *P. marinus*. Our findings strongly suggest that PWP-1 plays a key role in the organization of the cell wall of these protozoa favouring, additionally, their survival.

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CAPTIONS TO FIGURES

Fig. 1. (A) Coomassie Blue-stained electrophoretic profile of isolated *Perkinsus atlanticus* prezoosporangia after SDS-PAGE under denaturing and reducing conditions. The migration positions of molecular mass standards are indicated on the left, and the position of PWP-1 is indicated on the right. (B) Immunoblotting with the rabbit polyclonal serum against PWP-1. Lane 1, isolated *P. atlanticus* prezoosporangia; Lane 2, *P. atlanticus* trophozoites-containing gills; Lane 3, non-parasitized gills; Lane 4, *P. marinus* isolate LICT-1 (ATCC 50508).

Fig. 2. Western blot analysis with the serum against PWP-1 after SDS-PAGE under either reducing (Lanes 1 and 3) or non-reducing (Lanes 2 and 4) conditions, as described in Materials and Methods section. Samples include isolated *Perkinsus atlanticus* prezoosporangia (Lanes 1 and 2) and *P. atlanticus* trophozoites-containing gills (Lanes 3 and 4). The absence of reactivity under non-reducing conditions, irrespective of the life-cycle form, indicates that PWP-1 is disulphide-linked to other cell wall components and, therefore, only released after treatment with a reducing agent.

Fig. 3. PWP-1 resistance to trypsin digestion. Both non-denatured (Lanes 1 and 2) and heat-denatured (Lanes 3 and 4) *Perkinsus atlanticus* trophozoites-containing homogenates were incubated (37 °C, 30 min) with either trypsin (tr) (Lanes 1 and 3) or heat-inactivated trypsin (htr) (Lanes 2 and 4). After incubation, the reaction mixtures were subjected to denaturing reducing SDS-PAGE and Western blotting

analysis with the serum against PWP-1. Prior to incubation, purified rabbit IgGs were added to each reaction mixture as a digestion control that could be visualized by means of the peroxidase-conjugated secondary antibody used in the immunoblotting assay. (IgG HC) Heavy chain of the rabbit immunoglobulin G.

Fig. 4. Localization of PWP-1 by means of immunogold electron microscopy in the two walled life-cycle stages of *Perkinsus atlanticus*. (A and B) *P. atlanticus* trophozoites-containing gill abscesses. (A) Mature trophozoite. Labelling is exclusively associated with the parasite cell wall (w). (cp) Cytoplasm. (v) Vacuole. (c) Capsule. x31000. Bar: 0·5 $\mu$m. (B) Immature trophozoites (T) under division. Both the remnants of the mother cell wall (arrows) and the new developed daughter cell walls (w) are strongly labelled. (c) Capsule. x47000. Bar: 0·25 $\mu$m. (C and D) Isolated *P. atlanticus* prezoosporangia embedded in ovalbumin. (C) The thick cell wall (w) of the prezoosporangia, formed during FTM incubation, shows a strong immunoreactivity. Prezoosporangium cytoplasm (cp) was unlabelled. x23500. Bar: 0·5 $\mu$m. (D) Prezoosporangium cell wall (w) region with a patchy distribution of PWP-1. (cp) Cytoplasm. x42000. Bar: 0·25 $\mu$m