Proteomic analysis of the *Schistosoma mansoni* surface membranes

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Abstract

Adult schistosomes can survive in the hostile environment of the mammalian blood system for decades and must therefore display effective strategies to evade the host immune responses. The surface of the worm is covered by a living syncytial layer, the tegument, which is bound by a multilaminate surface comprised of a plasma membrane overlain by a secreted membrane-like bilayer, the membranocalyx. The recent sequencing of the *S. mansoni* genome and transcriptome has enabled the use of proteomics to study this organism. Proteins can be separated, digested, and subjected to mass spectrometry. The spectra are used to match entries in the genome/transcriptome database, from which putative identities can be sought based on their similarity to characterised proteins from other species.

The aim of this thesis is to identify the protein constituents of the schistosome surface, and determine the relative locations of these proteins within the tegument membrane complex. The tegument was removed from worm bodies and the surface membranes were purified. Approximately 200 proteins integral to, or associated with, the tegument apical membranes were identified by shotgun proteomics. More refined methods were then used to label the exposed molecules on live worms, and isolate the membranocalyx, before protein identification by mass spectrometry. Components of the host immune and blood system were identified on the parasite surface, as well as many schistosome proteins, including membrane enzymes, nutrient transporters, structural proteins and numerous components with unknown functions. The identification of Sm29 in different membrane preparation/labelling experiments, together with its signal sequence, makes this protein a potential component of the membranocalyx. All other schistosome proteins are probably situated below this secreted bilayer, protected from the host immune system. The reproducible identification of key membrane or secreted proteins warrants the investigation into their specific functions at the schistosome surface.

Contents

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Declaration

All the work presented in this thesis is my own, with the following exceptions:

The Venn diagram in Chapter 2 was calculated and drawn by Rachel Curwen and Peter Ashton.

Meg Stark performed the sectioning for electron microscopy (Chapter 3 and 5).

Martha Truscott performed the haemagglutination assay in Chapter 5.

All rat handling (Chapter 5) was performed by Pat Coulson and Alan Haigh.

Abbreviations

1-DE	One-dimensional electrophoresis
2-DE	Two-dimensional electrophoresis
ACh	Acetycholine
AChE	Acetylcholinesterase
AChr	Acetylcholine receptor
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CF	Cationised ferritin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
CRP	Complement regulatory protein
CTL	Cytolytic T-lymphocytes
DAF	Decay accelerating factor
EST	Expressed sequence tag
GeLC	1-DE separation of proteins followed by digestion and liquid chromatography
GlyR	Glycine receptor
GP	Gradient pellet
GPI	Glycophosphatidylinositol
HRP	Horse radish peroxidase
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
LC	Liquid chromatography
LDL	Low-density lipoprotein
MALDI	Matrix assisted laser desorption ionisation
MBL	Mannan-binding lectin pathway
MCP	Membrane cofactor protein
MDLC	Multidimensional liquid chromatography
MHC	Major histo-compatibility complex
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification technology
nr	Non-redundant
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
pI	Isoelectric point
pH	Potential of hydrogen
PMF	Peptide mass fingerprint
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
SDS	Sodium dodecyl sulphate
SB 3-10	N-decyl-N,N-dimethyl-3-ammonio-1-propane sulphate
SGTP	Schistosome glucose transporter protein
Strep	Streptavidin
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TNF	Tumour necrosis factor
ToF	Time of flight

Chapter 1

General introduction

1.1 Schistosomiasis

Approximately 200 million people are infected with schistosomiasis, the debilitating and potentially fatal disease caused by trematodes of the genus *Schistosoma* (Chitsulo et al., 2004). The disease is endemic in 74 countries and 500-600 million people worldwide are at risk of infection, making schistosomiasis a parasitic disease of major concern, second only to malaria in terms of mortality and morbidity in the tropics and subtropics (Savioli et al., 1997). Five species are capable of infecting humans, but *S. mansoni* is generally the most widely studied and is the subject of research in this thesis. It is prevalent in many parts of Africa, the Middle East, South America and some Caribbean islands.

1.2 Parasite life cycle

The *S. mansoni* life cycle is complex and the parasite relies on two hosts, an aquatic snail and a mammal, for its survival. Eggs, excreted from the mammalian host *via* the faeces, hatch when in contact with fresh water and release the first larval stage, the miracidia. These free-swimming larvae infect the snail of the genus *Biomphalaria* and transform into mother sporocysts, where germinal cells divide and differentiate, giving rise to secondary sporocysts. Upon maturation, these migrate to the digestive glands of the snail, and a second phase of asexual reproduction occurs. Vast numbers of free-living cercariae are produced and shed from the snail into water, where they spend a brief life prior to infection of a mammalian host. They directly penetrate the skin of their host with the aid of proteolytic secretions (McKerrow and Salter, 2002). Their bifurcated tails and glycocalyx are lost during the infection process, and the cercariae transform into schistosomula. After a period of 2-5 days in the skin (Crabtree and Wilson, 1985), the schistosomula enter the vascular or lymphatic system of the host and migrate *via* the heart to the pulmonary capillary beds of the lungs, where they must elongate and squeeze through the capillaries to reach the pulmonary vein (Wilson et al., 1978). The blood circulation then carries the larvae through the left side of the heart and distributes them to organs throughout the body, where they reach and must pass through further capillary beds, and are eventually transported to the liver. Parasites first appear in this organ after approximately eight days, and continue to accumulate until about 21 days post infection (Miller and Wilson, 1980). Between 28-35 days post-infection, male and female worms pair and start reproducing. The mature female is cylindrical in shape and measures approximately 1-2 cm long and 0.1 mm in diameter. In contrast, the male is shorter and more muscular, and his flattened body curves ventrally to form a gynaecophoric canal that

surrounds the female, so that only her head and tail are exposed. The pair travel up the hepatic portal vessels to oviposition sites in the mesenteric veins and may produce as many as 300 eggs per day (Wakelin, 1996), which pass into the lumen of the intestine and are subsequently defecated outside the body, thus completing the life cycle.

1.3 Pathology

At 3-6 weeks post infection, an acute reaction is sometimes observed, particularly in patients from non-endemic areas (de Jesus et al., 2002). Symptoms include a high temperature, abdominal pains and diarrhoea, and this clinical condition is sometimes known as Katayama fever. Despite this, adult schistosomes living in the heptatic portal vein cause no ill effects to their mammalian host. The severe pathology associated with schistosome infection commences with the onset of egg-laying. Not all the eggs traverse into the lumen of the intestines to be excreted with the faeces; instead many are swept downstream and become lodged in the pre-sinusoidal tissues of the liver. The trapped eggs produce secretions which provoke granulomatous inflammation consisting of macrophages, eosinophils, and CD4+ cells (Wynn et al., 2004). Fibroblasts are recruited and collagen is deposited around the granulomas, eventually killing the egg but leaving a fibrotic scar in the liver (Fallon et al., 2000). The immune factors that serve to destroy the eggs also play a role in the host's demise. The formulation of granulomas results in the blockage of blood vessels and subsequent portal hypertension. The continued accumulation of eggs in the liver causes an extreme build up of pressure, eventually resulting in the formation of porta-caval anastomoses, as well as hepatomegaly and splenomegaly (McHugh et al., 1987).

1.4 Combating schistosomiasis

The control of schistosomiasis is a challenging task for endemic countries, and has been the focus of concerted efforts including snail control, improved sanitation, and health education to high-risk communities (Ross et al., 1997). The high costs and ecological consequences associated with mollusc management, together with the snails' diverse habitats make their widespread control daunting. Likewise, the construction and maintenance of separate water supplies and sewage is an enormous task, especially in poor communities. The treatment of schistosomiasis is almost solely dependent on the chemotherapeutic agent praziquantel ((2-cyclohexylcarbonyl)-1,2,3,6,7,11b-hexa-hydro-2H-pyrazino(2,1a)isoquinolin-4-one) (Fenwick et al., 2003). Treatment with this drug is

rapid, safe, cost-effective and has few side effects, but has several key disadvantages. Firstly, drug treatment does not prevent schistosome re-infection, requiring the continued treatment of patients in endemic areas. Secondly, symptoms of disease are sometimes not evident for several years post-infection, by which time severe damage may have occurred that cannot be remedied by drug treatment. This problem is exacerbated by the lack of a quick, simple and sensitive diagnostic test for *S. mansoni* infection (Rabello, 1997). Finally, the reliance on a single drug, together with the requirement for its repeated use, will almost certainly lead to the development of drug resistance (Doenhoff et al., 2002). In the light of these limitations, the requirement for the developments of new drugs and/or a schistosome vaccine is highly desirable. The ideal control of schistosomiasis is a vaccine that could be administered quickly and cheaply to children before they come into contact with infected water, providing a long-lasting high degree of protection. As studies of humans and animal models provide evidence for the acquisition of immunity (Wilson and Coulson, 1999), the prospect of induced immunity by vaccination is plausible. To date, numerous schistosome antigens have been submitted as vaccine candidates, but none of those tested have provided sterile immunity (Bergquist et al., 2002).

1.5 The schistosome surface

The adult schistosome life in the blood vasculature is fraught with danger, as the parasites are constantly exposed to host immune defence systems. That schistosomes can live in their mammalian host for up to 30 years (Harris et al., 1984) testifies to their possession of extremely effective mechanisms to counter host immunity. Schistosomes are not protected by a hard, inert cuticle, such as that of nematodes, but are covered by a soft living syncytium, suggesting a more sophisticated method of self-preservation. The method(s) of immune evasion at the schistosome surface has been the focus of much research to understand how the parasite prevails against the host. The following sections describe the physiology of the schistosome surface, its role in nutrient uptake, its interaction with the host, and some of the parasite/host proteins that have been reported at this location.

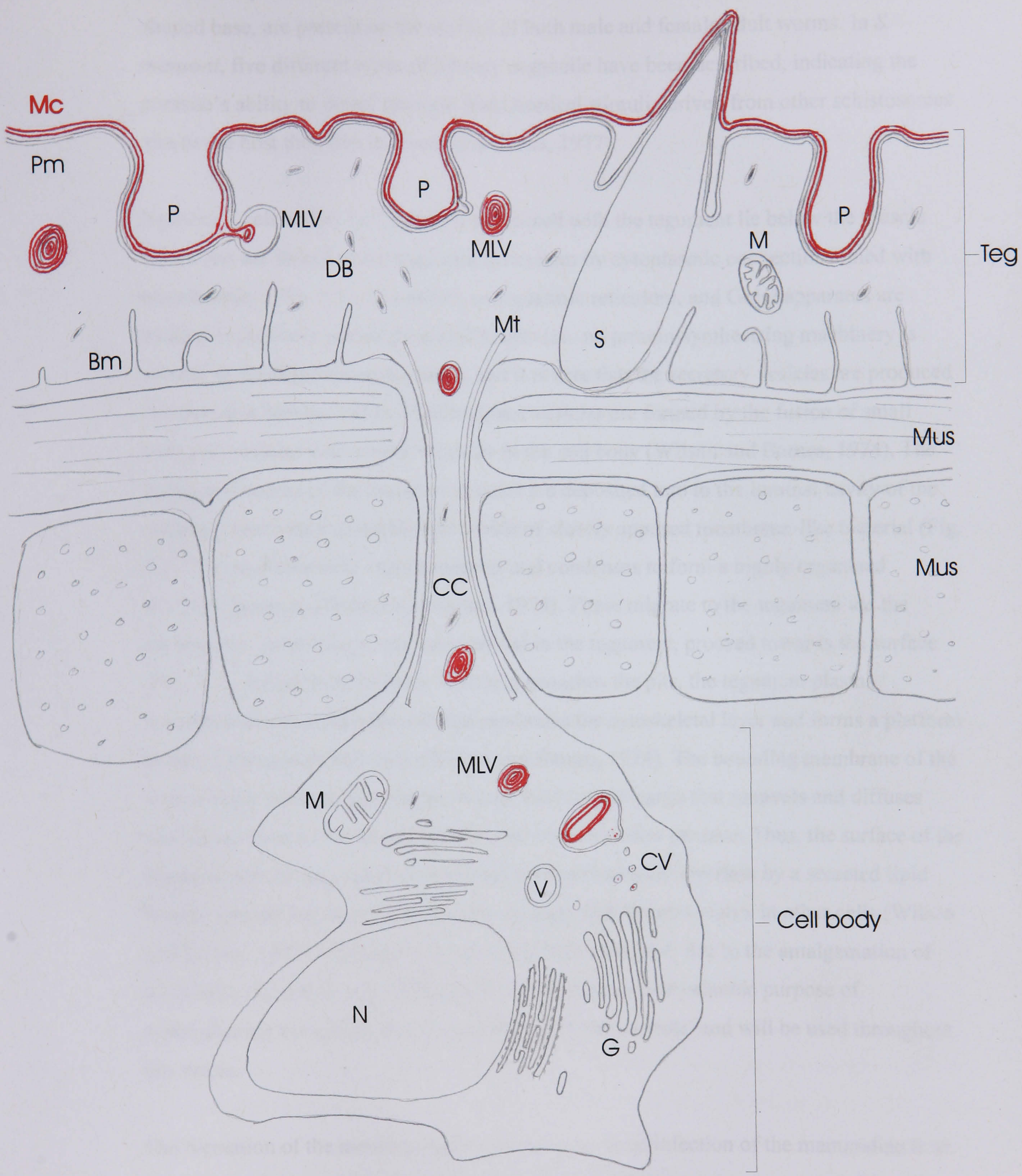
1.5.1 The *S. mansoni* tegument

The tegument is a 0.9 - 3.0 μm thick syncytial layer that covers the entire adult worm (Wilson and Barnes, 1974), and its surface forms the host-parasite interface (Fig 1.1). The tegument cytoplasm is made up of an electron-dense matrix, and is contained by a plasma and basal membrane (Fig. 1.1). The latter separates the syncytium from the underlying musculature, and is regularly cast into thin invaginations that project upwards into the cytoplasm of the tegument (Wilson and Barnes, 1974b). Three types of membrane-bound inclusion are found within the *S. mansoni* tegument, as follows. Small mitochondria are present in low numbers throughout the tegument indicating the requirement for energy within the syncytium (Wilson and Barnes, 1974). Discoid bodies are the most common inclusion within the tegument. They are bound by a lipid bilayer and contain carbohydrate-rich material (Wilson and Barnes, 1974). Their fate is not fully understood, but it has been proposed that they either degenerate to form the tegument matrix, or fuse with the surface plasma membrane and release their contents to the extracellular vicinity of the parasite (McLaren, 1980). The third inclusion, the multilaminate vesicle (sometimes referred to as the multilamellate or membranous vesicle), is larger than the discoid body but is outnumbered by approximately 15 to 1 (Wilson and Barnes, 1974). These vesicles contain concentric layers of membrane-like material, which are thought to contribute to the tegument surface bilayers (described below).

Large spines are located within the tegument of both male and female worms (Fig. 1.1), particularly on the dorsal surface (Morris and Threadgold, 1968). They are firmly attached to the basal membrane, and their protrusions are always covered by the apical membranes of the tegument (Hockley and McLaren, 1973). The spines, comprised of paracrystalline actin (Cohen et al., 1982), are used to help anchor the worms within the blood vessels of their mammalian host, and play a role in the female's migration to oviposition sites (Crabtree and Wilson, 1980). Further cellular architecture is evident directly beneath, and adherent to, the plasma membrane of the tegument (Hockley and McLaren, 1973; Wilson and Barnes, 1974). This electron-dense layer lines deep pits and invaginations within the tegument, and may provide the molecular scaffold to maintain the surface structure of the tegument. This highly organised cytoskeleton can be destroyed by incubating worms in cytochalasin B, implicating actin as a key component in tegument structure (Wilson and Barnes, 1974). Dynein light chains, myosin and paramyosin may also contribute to the

Figure 1.01. Diagrammatic representation of the *S. mansoni* adult tegument and associated cell body. Cell bodies are located beneath the muscle layers (Mus), and contain the nuclei (N), endoplasmic reticulum (ER) and Golgi bodies (G). Carrier vesicles (CV) bud off from the Golgi, and fuse with vacuoles (V), releasing their contents that self-assemble into multilaminate sheets. The vesicles condense to become multilaminate vesicles (MLV), which migrate to the tegument (Teg) *via* cytoplasmic connections (CC) lined by microtubules (Mt). Upon reaching the tegument, the multilaminate vesicles progress towards the surface pits (P), where they fuse with the plasma membrane (pm) and release their membrane-like material. This unravels within the lumen of the pit and diffuses laterally over the tegument to form the membranocalyx (Mc). Discoid bodies (DB) are also made in the cell body and are transported to the tegument. The bottom of the tegument is bound by a basement membrane (Bm) which formulates several infoldings into the syncytium. Small mitochondria (M) are present in both the cell body and the tegument syncytium.

This figure is based on diagrams and electron micrographs in publications by Hockley and McLaren (1973), Wilson and Barnes (1974), and McLaren (1980), and from my own observations using transmission electron microscopy. (This diagram is not to scale.)


Mc
Pm
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Mt
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CC
MLV
M
CV
V
N
G
Cell body

tegument's cytoskeleton and provide pathways for the movement of secretory vesicles (reviewed by Jones et al., 2004). Sense organs, often with cilia extending from their dome-shaped base, are present on the surface of both male and female adult worms. In *S. mansoni*, five different types of sensory organelle have been described, indicating the parasite's ability to detect physical and chemical stimuli derived from other schistosomes and/or the host they live in (Senft and Gibler, 1977).

Numerous cell bodies (or "cytons") associated with the tegument lie below the muscle layers and are linked to the tegument syncytium by cytoplasmic connections lined with microtubules (Fig. 1.1). The nuclei, endoplasmic reticulum, and Golgi apparatus are located exclusively within these cell bodies (*i.e.* no protein-synthesising machinery is present in the tegument syncytium), and it is here that the secretory vesicles are produced (Wilson and Barnes, 1974). Multilaminate vesicles are formed by the fusion of small transport vesicles with empty vacuoles in the cell body (Wilson and Barnes, 1974). The granular contents of the transport vesicles are deposited into to the luminal cavity of the vacuole, where they assemble into whirls of closely apposed membrane-like material (Fig. 1.1). The multilaminate vesicle matures and condenses to form a highly organised secretory package (Wilson and Barnes, 1974). These migrate to the tegument *via* the cytoplasmic connections, and upon arrival in the tegument, proceed towards the surface (Fig. 1.1). As the multilaminate vesicle approaches the pits, the tegument plasma membrane forms a side channel that penetrates the cytoskeletal layer and forms a platform to which the vesicle can dock (Wilson and Barnes, 1974). The bounding membrane of the vesicle fuses with the plasma membrane, releasing its cargo that unravels and diffuses laterally to form a continuous lipid bilayer over the entire parasite. Thus, the surface of the tegument can be interpreted as a normal plasma membrane overlain by a secreted lipid bilayer, termed the *membranocalyx* by analogy with the glycocalyx in other cells (Wilson and Barnes, 1977). Although this name has been criticised, due to the amalgamation of two distinct scientific terms (McLaren, 1980), it serves the valuable purpose of distinguishing the apical bilayer from the plasma membrane, and will be used throughout this thesis.

The formation of the membranocalyx commences upon infection of the mammalian host. Schistosomula recovered 30 minutes after skin penetration possess large quantities of multilaminate vesicles within their tegument, but their surfaces still consist of a single

membrane bilayer (Hockley and McLaren, 1973). After 3 hours, the new parasite surface is complete, consisting of the plasma membrane overlain by the membranocalyx (Hockley and McLaren, 1973). The rapidity with which this surface is synthesised suggests that the membranocalyx plays a key role in the parasites' survival during their subsequent migration to, and life in, the host blood system. The membranocalyx is able to defend the schistosome against host immune responses by two possible mechanisms. Firstly, it may act as an immunologically inert layer, shielding underlying antigens from antibody detection. The remarkable ability of the membranocalyx to acquire host molecules (discussed below), may further disguise the parasite from antibody recognition. Secondly, upon binding of immune complexes, the membranocalyx could be rapidly shed and replaced. Indeed, under certain conditions *in vitro*, the membranocalyx turnover was reported to take approximately four hours (Wilson and Barnes, 1977). Despite a capacity for rapid turnover rate, studies using "natural" markers of the membranocalyx, such as host molecules, report a 10-day or two-week period before the surface is replaced (Ruppel and McLaren, 1986; Saunders et al., 1987). Such a slow turnover argues against immune evasion by the continual replacement of the parasite surface.

The fate of the membranocalyx is also not fully understood. Wilson and Barnes (1977) studied the pattern of membrane turnover using cationised ferritin, a marker of negatively charged cell surfaces that can be observed by electron microscopy. The authors noted a pattern of membranocalyx movement from the tegument pits to natural protrusions (*e.g.* tips the spines), where the bilayer was shed into the surrounding medium. Thus, it was proposed that the membranocalyx moves in a conveyor belt-like fashion over the plasma membrane, and is continually being replaced by contents of the multilaminate vesicles (Wilson and Barnes, 1977). Indeed, that the membranocalyx covers the spines of the tegument, a sight where multilaminate vesicles cannot fuse with the plasma membrane (Fig. 1.1), suggests that this membrane-like secretion must be able diffuse over the plasma membrane. Therefore, it is plausible that the membranocalyx is continually being shed into the surrounding media, as described by Wilson and Barnes (1977). A mechanism by which the two lipid bilayers stay so firmly attached to each other, yet allows the lateral movement of the membranocalyx over the plasma membrane, is not known. It is possible that phospholipid-binding proteins act as the molecular 'glue' between the two membranes, or maybe they are held together simply by hydrophobic interaction or electrostatic charge (discussed further in Chapter 5).

Some workers argue against the sloughing of tegument membranes (Brouwers et al., 1999). The rate of phospholipid turnover was studied by pulse-labelling adult worms with radioactive-labelled palmitic acid (Brouwers et al., 1999). After 20 hours culture *in vitro*, the radioactive signal was lost from the surface of the worm, but was also absent from the surrounding medium. Brouwers and others proposed that the loss of label was due to deacylation/reacylation, not sloughing of the tegument membranes. However, if a more stable label was used, and the incubation times were extended beyond 20 hours, perhaps Brouwers et al. would have been able to observe the sloughing of the membranocalyx. The possible recycling of tegument membranes by endocytosis offers an alternative hypothesis for the fate of the membranocalyx. A study using hydrophilic styryl dye has shown the internalisation of tegument membranes by vesicle formation (Ribeiro et al., 1998). However, Wilson and Barnes (1977) observed no evidence of membrane recycling using cationised ferritin as a marker. Similarly, labelled human LDL, that attached to the surface of schistosomula, was not internalised by endocytosis (Bennett and Caulfield, 1991).

1.5.2 Tegument surface proteins

Methods to isolate the tegument and purification of the surface membranes were developed over twenty years ago (Bennett and Seed, 1977; Oaks et al., 1981; Roberts et al., 1983). Although the protein components could be separated by electrophoresis or liquid chromatography, little progress was made in identifying the individual components. Since then, several proteins have been described at the tegument surface, either by immunocytochemical, histochemical or functional studies. A brief summary of some of the well-characterised tegument surface protein follows.

Nutrient transporters

The adult schistosomes living in the mesenteric blood vessels of their vertebrate host are surrounded by a plethora of food, served in the form of erythrocytes as well as all the newly absorbed nutrients from the host's gut. The mature parasite acquires most of its nutritional needs by the digestion of red blood cells within its gut, with an estimated consumption of 39,000 and 330,000 cells per hour in male and female worms, respectively (Halton, 1997). However, the tegument serves as a secondary source of nutrients: a system that the parasite relies upon during its migration to the hepatic portal vein (Fripp, 1967). The highly invaginated surface of the tegument provides a large surface area for

nutritional absorption (Fig 1.1). Glucose transporters have been extensively studied at the parasite's surface; a group of three proteins that share homology with glucose transporters in mammalian cells have been cloned, characterised and named *Schistosome Glucose Transporter Protein (SGTP) 1, 2,* and *4* (Skelly et al., 1994). SGTP1 and SGTP4 are functional, facilitated-diffusion transporters and are both present in the tegument, but their distribution is asymmetric (Jiang et al., 1996). Using specific antibodies, Jiang and others reported SGTP1 to be present at the basal membrane of the tegument, and located SGTP4 to the secretory vesicles (both the discoid body and multilaminate vesicle) and both the apical membranes (*i.e.* the plasma membrane *and* the secreted membranocalyx). Thus, it seems that SGTP4 enables glucose uptake from the blood into the tegument, and SGTP1 transports the nutrient from the syncytium to the underlying tissues.

Amino acid (Asch and Read, 1975), purine and pyrimidine (Levy and Read, 1975) transport have also been reported to occur at the tegument surface. The enzymatic activity of aminopeptidases has been localised to the tegument surface, and it was speculated that they digest proteins into free amino acids for uptake into the tegument (Fripp, 1967). More recently schistosome permease 1 light chain (SPRM1lc) has been localised to the tegument surface by immunolocalisation, as well as being present in several other tissues throughout the life cycle (Skelly et al., 1999). This transporter is capable of the uptake of neutral and dibasic amino acids, and is likely to be involved in the absorption of these nutrients from the host blood system and their subsequent distribution throughout the parasite body.

The transport of nutrients across the tegument surface is problematic, given that the particle must traverse two lipid bilayers. As the membranocalyx is a secretion, resulting from the luminal contents of the multilaminate vesicles, is it unlikely to contain membrane-spanning proteins. If any proteins are components of the membranocalyx, only those with a leader sequence and no transmembrane domains are likely candidates. It is possible that non-protein-based pores, or discontinuities in the membranocalyx, allow the diffusion of nutrients through to be transported across the underlying plasma membrane (discussed in more detail in Chapter 4).

Alkaline phosphatase

Activity of the glycosylphosphatidylinositol- (GPI) anchored membrane protein, alkaline phosphatase, has long been associated with the surface of the intramammalian schistosome (Cesari, 1974). Anti-alkaline phosphatase antibody is able to bind specifically to live adult worms (Pujol and Cesari, 1990), and incubation of the parasites in media containing phosphatidylinosytol-specific phospholipase C, an enzyme that cleaves GPI anchors, results in the release of the membrane protein (Espinoza et al., 1988). Furthermore, patients infected with *S. mansoni* have a marked antibody response to alkaline phosphatase (Pujol and Cesari, 1990). Although internal membranes also possess alkaline phosphatase activity (Halton, 1967), approximately 70 % of the enzyme has been localised to the tegument surface (Cesari, 1974). As a result, this protein is often used as a marker of the plasma membrane during isolation procedures (Roberts et al., 1983; Pujol and Cesari, 1993). By homology with alkaline phophatase in other organisms, its function on the tegument surface may be to hydrolyse organic phosphates and enable de-phosphorylated molecules to cross the plasma membrane.

Ecto ATP Diphosphydrolase

A second enzyme capable of hydrolysing organic phosphates, diphosphohydrolase, has been located to the tegument surface by immunolocalisation, and is also expressed in all other stages of the parasite life cycle (DeMarco et al., 2003). The ability to hydrolyse ATP and ADP at the parasite surface may serve as a protective mechanism against immune attack mechanisms. Activated platelets and T-lymphocytes release ATP, and the hydrolysis of this nucleotide may prevent cytotoxic attack against the parasite (Torres et al., 1998).

Calpain

Calpain is a calcium-activated neutral protease that is recognised by antibodies of *S. mansoni*-infected humans (Andresen et al., 1991), and has been localised to the tegument syncytium as well as the underlying muscle layers (Siddiqui et al., 1993). Using protease inhibitors, this enzyme was shown to have a functional role in the synthesis of the apical membrane complex (Siddiqui et al., 1993). As the structure of schistosome calpain differs in to that of host calpain (Andresen et al., 1991), the protein has been promoted as a vaccine candidate. However, the vaccination trials had mediocre results; a baculovirus-expressed large subunit of calpain induced only 29-39% protection in mice (Hota-Mitchell

et al., 1997), and a gene gun delivery approach elicited 60% immunity following three vaccinations using a DNA construct encoding the same subunit (Hota-Mitchell et al., 1997). Why this normally cyotsolic protein is eliciting an immune response is somewhat of a mystery. Even though calpain binds to phospholipids in the presence of calcium, it is restricted to the cytoplasmic side of the plasma membrane (Molinari and Carafoli, 1997), and should therefore be protected from antibody recognition.

Sm23

This 23 kDa tetraspanin protein was identified as a target of antibodies from mice immunised with membrane extracts of mechanically-transformed schistosomula (Harn et al., 1985). Using immunolocalisation, Sm23 was detected on the surface of cercariae, schistosomula, and adult worms (Harn et al., 1985). Its *S. japonicum* homologue, Sj23, was also immunolocalised to the adult stage surface (Wright et al., 1991). When mice were immunised with a DNA plasmid construct of Sm23, a significant level (21-44%) of protection was observed (Da'dara et al., 2001).

Sm13

Abath et al. (2000) successfully cloned this 13 kDa protein, and identified it as a principle target of antibodies from mice protectively vaccinated with a membrane preparation from adult worms. Immunolocalisation revealed that the protein is present in the tegument of 21-day worms and adults, but was not detected in skin-stage parasites (Abath et al., 2000). Circulating antibodies against the *S. haemotobium* homologue, Sh13, are currently under investigation as a diagnostic marker of the parasite infection (F. Mutapi, personal communication).

200 kDa Surface protein

The "200 kDa surface protein" is proposed to be present at the surface of the tegument (Sauma et al., 1991). The action of praziquantel against schistosomes in mice is immune-dependent, as the anti-helminth effect of the drug is diminished in schistosome-infected mice with immunodeficiencies (Brindley and Sher, 1987). Within minutes of incubation with praziquantel, schistosomes sustained tegument damage *in vitro* (Becker et al., 1980), which leads to the exposure of parasite antigens (Harnett and Kusel, 1986). The transfer of monoclonal anti-"surface protein" antibodies to immunodeficient mice infected with *S. mansoni*, followed by praziquantel treatment, resulted in the death of the worms (Hall et

al., 1995). Therefore, it appears that this 200 kDa GPI-anchored protein is exposed on the schistosome surface by praziqantel treatment.

Acetylcholinesterase and Acetylcholine receptor

Acetylcholinesterase (AChE) is abundant at the schistosome surface as well as in the musculature of the parasite (Levi-Schaffer et al., 1984; Tarrab-Hazdai et al., 1984), and its activity is inhibited by the anti-schistosome drug metrifonate (Bloom, 1981). The relative concentrations of surface AchE differs between schistosome species; *S. haemotobium* possesses approximately 20x more activity than *S. mansoni* (Camacho et al., 1994). The species' respective sensitivity to metrifonate reflects the amount of enzyme on their surface, suggesting that the drug targets external AChE rather than the muscular homologue. Futhermore, it highlights the important function of this surface enzyme for the survival of the schistosome. Whereas the best known role of AChE is to hydrolyse the neurotransmitter acetylcholine, its purpose at the schistosome surface is not known. One possibility is to increase the availability of nutrients for absorption, as differing concentrations of acetycholine affects glucose uptake *in vitro* (Camacho and Agnew, 1995). A nicotinic acetylcholine receptor has been located on the dorsal surface of the adult male, and its expression is increased when the parasites pair and become sexually active (Camacho et al., 1995). The extra requirement for glucose at this stage provides more evidence that acetylcholine may play an important role in nutrient uptake *via* the tegument.

TGFβ receptor

Davies and Pearce (1995) successfully biotinylated a surface protein with kinase activity; a diagnostic marker for many cell surface receptors. This protein was reported as a serine threonine receptor kinase (SmRK-1), and may have an important role in mediating morphogenesis and development of the worm (Davies et al., 1998). It has similarities with receptors to transforming growth factor β (TGFβ), and Beall and Pearce (2001) have provided evidence that it is capable of transmitting signals in response to human TGFβ. In support of this, two genes encoding intracellular proteins involved in the downstream signal pathway initiated by TGFβ, termed *Smad*, were identified in *S. mansoni* (Beall et al., 2000). Both these proteins are able to interact with the intracellular domain of SmRK-1 *in vitro*.

Low-density lipoprotein receptor

The presence of low-density lipoprotein (LDL) has been reported at the tegument surface of newly-transformed schistosomula (Rumjanek et al., 1983). Worms incubated in media containing human serum expressed a protein on their surface that was capable of interaction with LDL. The possible LDL receptor protein could be labelled with radioiodination, and purified from parasite extracts using immobilised LDL (Rumjanek et al., 1985). The *S. mansoni* transcript for this protein has since been sequenced, and has been highlighted as a potential vaccine candidate (Verjovski-Almeida et al., 2003). Using dye-labelled LDL, the lipoprotein was shown to bind specifically and saturatably to the worm's surface (Bennett and Caulfield, 1991). The bound LDL may serve to block anti-schistosomal antibody access to the surface membranes (Chiang and Caulfield, 1989). Moreover, some of the LDL was ingested by the parasite and absorbed into the internal tissues (Bennett and Caulfield, 1991). Thus, host LDL may serve not only as a protective guise on the parasite's surface, but also as a source of lipid for the developing worm.

Tumour necrosis factor α receptor

Severe combined immunodeficient (SCID) mice that lack B and T lymphocytes (but have normal macrophages) infected with cercariae were able to support the maturation and mating of schistosomes, but there was a noticeable lack of granulomas in the livers (Amiri et al., 1992). Although the SCID mice had more worm pairs than the controls, the egg production was reduced by >70%, and no eggs were found in the faeces (*i.e.* they all went to the liver). When the mice were treated with tumour necrosis factor α (TNFα), the egg production increased in a dose-dependent manner. The same was also noted for control mice. Therefore, it was suggested that TNFα can play a crucial role in egg production and granuloma development. How the schistosome responds to TNFα levels has not been determined, but a receptor on the tegument surface may provide an explanation. In rebuttal, it has been reported that the reduced egg production of schistosomes in SCID mice was only a temporary phenomenon, and worm fecundity soon reached normal levels regardless of TNFα levels in the host blood (Cheever et al., 1999). Furthermore, there are no significant sequence matches to a conventional TNFα receptor in the *S. mansoni* genome or EST database.

Alpha-2 macroglobulin

Immunocytochemical studies by Damian et al. (1973) and Kemp et al. (1976) suggested the presence of alpha-2 macroglobulin on the surface of adult worms perfused from mice and rhesus monkeys. Damian and others reasoned that as the same protein was detected in worms derived from different mammals argues against host antigen absorption, and supports the theory of parasite synthesis of alpha-2 macroglobulin. Furthermore, the *S. mansoni* database contains an mRNA encoding this protein (Sm07745), as well as a matching gene prediction (glimmer04895). Later work used 1- and 2-D electrophoresis, followed by Western blots probed with specific antibody, to detect alpha-2 macroglobulin in material shed from the tegument membranes into fresh culture medium (Gearner and Kemp, 1994). The protein inhibits endoproteases by steric hindrance (Enghild et al., 1989), forming a 1:1 complex with the protease and trapping the enzyme, but the role of this protease inhibitor at the schistosome surface is still not fully understood.

1.5.3 The tegument and host molecules

A variety of host skin and blood molecules have been reported at the tegument surface of both schistosomula and adult worms of *S. mansoni*. The mechanism(s) by which they are absorbed onto the parasite surface is not understood, although several theories have been proposed. A freeze-fracture and transmission electron microscope study has reported neutrophil plasma membrane fusion with the membranocalyx of schistosomula preincubated in anti-schistosome serum and Complement (Caulfield et al., 1980). However, Caulfield and others acknowledge that this may be an artefact of sample preparation. Pearce et al. (1990) suggested that the lipophillic properties of the parasite surface may facilitate the fusion of small "blebs" from host cells. Alternatively, the acquisition of host GPI-anchored proteins (*e.g.* decay accelerating factor, see below) could be "flipped" onto the surface of the membranocalyx (Pearce et al., 1990). Further hypotheses for the method of acquisition of host antigens by the parasite are discussed in Chapter 3. Whatever the mechanism of transfer is, the process seems to be selective towards certain molecules, some of which have been characterised and are described in the following paragraphs.

Erythrocyte glycolipids

Pioneering studies by Smithers et al. (1969) showed that when adult schistosomes were transferred from mice into monkeys that had been previously immunised against mouse

antigens, the worms were killed by immunological attack directed at the tegument surface, as revealed by electron microscopy. In the control experiment, worms placed in naïve monkeys were not killed by the host, despite a brief period of reduced egg production (Smithers and Terry, 1967). When worms were transferred to naïve monkeys, and then transferred into anti-mouse monkeys seven days later, the worms survived (Smithers et al., 1969). Thus, it appears host molecules are acquired by the surface of adult worms, and can be replaced when the parasites are transferred to a different host. Further work revealed that these antigens were similar to erythrocyte glycolipids (Clegg et al., 1970; Perez and Terry, 1973). The mechanism by which the glycolipids are transferred from the erythrocyte to the parasite surface appears to be passive, as even dead worms incubated *in vitro* are able to attain antigens from red blood cells (Dean, 1974). Furthermore, light-microscope studies have localised host antigens to the parasite surface, not to internal tissues, which suggests that they are acquired rather than synthesised by the schistosome (Goldring et al., 1977). Indeed, the structure of the glycolipid is believed to be conducive to uptake into a lipid bilayer, as Lewis blood group antigens in serum are embedded into the surface of erythrocytes (Marcus and Cass, 1969). However, it does not explain how glycolipids are removed from the erythrocyte membrane, rotated 180°, and then incorporated into the schistosome membrane. Nonetheless, it is appealing to believe that the acquisition of host glycolipids enables the worm to disguise itself as host material, thereby evading the immune response.

Immunoglobulins

Worms of varying ages recovered from their mammalian host were found to have antibody bound to their surface, with the exception of lung-stage worms (McLaren et al., 1978). Specific anti-antibody labelling together with electron microscopy has revealed the presence of IgG1, IgG2, IgG3, IgA and IgM at the surface of adult worms perfused from mice (Kemp et al., 1978). Moser et al. (1980) were able to label skin- and lung-stage worms with a hapten (trinitrophenyl; TNP), that does not affect the parasites' viability, but serves as a way to make the worms appear as 'foreign' when transferred to mice immunised with TNP. The skin-stage worms were rapidly destroyed. Conversely, the lung-stage parasites were resistant to the anti-TNP antibody-mediated destruction, despite the antibody binding in similar quantities as it did to the skin-stage somules. Thus, the tegumental changes between the two stages seem to play a vital role in parasite survival, and the acquisition of erythrocyte glycolipids is not the sole mechanism of immune

evasion (Moser et al., 1980).

It is intriguing that bound antibodies do not cause the destruction of the schistosome, and several different hypotheses concerning this phenomenon have been put forward. The attachment of immunoglobulins to the parasite may not be the result of antigen-binding, but by non-specific binding, or *via* Fc receptors at the schistosome surface. Kemp et al. (1977) immunised infected mice with three different antigens (human red blood cells, bovine serum albumin and horseradish peroxidase). When the worms were retrieved, washed, and incubated with a series of different antigens, only the specific antigen (to which the mouse was immunised) was localised on the parasite surface. Similar results were observed when red blood cells adhered to schistosomes that had been pre-incubated in media containing anti-erythrocyte antibody (Torpier et al., 1979). The ability to absorb antibodies specific to another molecule suggests that these imunoglobulins are attached to the parasite *via* their Fc region (allowing their Fab regions free to bind antigens). Whether the antibodies are attached by an Fc receptor or non-specific binding is not known. More recently, the cytoskeletal protein paramyosin was shown to bind non-specific antibodies, and was touted as the illusive Fc receptor on both *S. mansoni* and *S. japonicum* (Loukas et al., 2001). Specific anti-paramyosin antibodies interfered with Fc reception by the protein, and this was interpreted by Loukas et al. (2001) as a possible mechanism by which mice immunised against paramyosin showed 26-33% resistance to schistosome infection (Pearce et al., 1988). The presence of this myofibrillar protein at the surface of the parasite is a conundrum, as the entire tegument is devoid of muscle and paramyosin has neither a transmembrane domain nor a signal sequence. In some reports paramyosin has been localised to the tegument surface (Matsumoto et al., 1988; Deng et al., 2003), but other researchers were unable to label the protein with impearmeant biotin on live worms (Skelly and Shoemaker, 1996). Moreover, paramyosin was only biotinylated if the parasites' surface membranes had been previously permeabilized with Triton X-100 (Davies and Pearce, 1995).

Complement and Complement regulators

Complement is a complex group of serum proteins that eliminate pathogens by cell lysis and facilitating phagocytosis. It can be activated by three pathways, termed the classical, alternative and mannan-binding lectin (MBL) pathway (Morgan and Harris, 1999). Various proteins of the Complement pathway have been reported at the surface of

schistosomes (Skelly, 2004). Complement component C3 was localised to the tegument pits using antibodies specific to the protein, but the parasites showed no evidence of Complement-induced damage (Rasmussen and Kemp, 1987). However, after a three-hour incubation in media alone, a step which Rasmussen and Kemp believed effectively removed all host material from their surface, the worms became prone to attack by naïve mouse serum, mediated by the Complement alternative pathway. This damage could be blocked by the incubation of schistosomes in serum obtained from immunised mice, and the authors suggested that the binding of schistosome-specific IgG may protect the parasite from Complement by binding to, and therefore blocking, Complement activation sites on the tegument surface (Rasmussen and Kemp, 1987). More recently, a putative C3 binding protein was localised to the surface pits as well as the multilaminate vesicles within the tegument (Silva et al., 1993). The schistosome-induced binding of this potentially lethal protein does seem puzzling at first, but the receptor may also act to inactivate C3, thus restricting damage by all three Complement pathways (Morgan and Harris, 1999). Other proposed receptors for specific Complement components have since been reported on schistosomes: a C2 receptor (Inal et al., 2003), and paramyosin (a putative C8 and C9 receptor; (Deng et al., 2003)).

Sophisticated mechanisms exist on the surface of host membranes to restrict Complement activation to non-self bodies (Morgan and Harris, 1999). Complement inhibitor 1 (C1 inh), Factors H and I, C4 binding protein (C4 bp), S-protein, clusterin, carboxypeptidase and anaphylatoxin inactivator are all Complement regulatory factors found in the blood plasma. Other regulating proteins are found in the plasma membrane of host cells, attached either by a GPI-anchor or a transmembrane domain: decay accelerating factor (DAF, CD55), membrane co-factor protein (MCP, CD46), Complement receptor 1 (CR1), homologous restriction factor (HRF), CD59 and Complement related receptor protein (Crry; only found in rats and mice). In schistosomes, DAF has been localised to the parasite's surface using anti-DAF antibodies (Pearce et al., 1990). When the worms were treated with phospholipase C, a proteolytic enzyme that cleaves GPI-anchored proteins from the lipid bilayer, the binding of anti-DAF was significantly reduced and the schistosomes became susceptible to damage inflicted by the Complement alternative pathway. Thus, DAF acquired from the host is able to protect the parasite, but its method of incorporation into the apical membrane remains unknown. Other factors may be involved in the protection of schistosomes from Complement. For example, the release of

serpin by schistosomula (as identified by proteomics, R. Curwen, personal communication) may inhibit the Complement attack pathway (Morgan and Harris, 1999).

Major histo-compatibility complex

Major histo-compatibility complex (MHC) glycoproteins have also been reported on the surface of young schistosomes raised in mice (Sher et al., 1978), as well as the small subunit of the MHC class I molecule, beta-2 microglobulin (Torpier et al., 1979). Worms were able to exchange these alloantigens when transferred to allogeneic mice in a period of 3-4 days (Sher et al., 1978). Products of the *K* and *I* regions were localised to the tegument by immunofluorescence, and were also recognised by alloreactive cytolytic T-lymphocytes (CTL). Despite prolonged incubation with CTL adhering to their surface, the parasites remained fully viable (Butterworth et al., 1979). More recently, host DNA sequences coding for MHC domains were reported in schistosome genes (Iwamura et al., 1995). These DNA sequences were also found in cercariae, and it was proposed that schistosomes retain host DNA throughout the life cycle (Imase et al., 2001). Despite this, no EST matching MHC class I is found in the *S. mansoni* database, although a homologue to MHC class II beta chain is present (accession number Sm16347).

In summary, a large variety of parasite and host proteins have been proposed to be present at the schistosome surface, some of these by convincing antibody or histochemical experiments, others by mere speculation. *Proteomics* offers an alternative and comprehensive way to identify proteins at the parasite-host interface, as described in the following sections.

1.6 Proteomics

The term *proteomics* describes the identification, quantification, interaction, activity, and function of all the proteins produced by the genes of a particular cell, tissue, or organism (Wasinger et al., 1995). With recent advances in mass spectrometry and bioinformatics, coupled to more established methods such as electrophoresis and liquid chromatography, proteomics provides a powerful tool for the study of post-genomic processes. The use of proteomics in this thesis is largely restricted to protein separation and identification, which will be discussed in the following paragraphs.

1.6.1 Two-dimensional electrophoresis

Due to the large numbers of proteins in any biological sample, a method of their separation is crucial in a proteomic study. Two-dimensional electrophoresis (2-DE) is traditionally the tool of choice, and was first reported 30 years ago (O'Farrell, 1975). The proteins are solubilised using solvents of low ionic strength, and then separated according to their individual isoelectric points (pI; defined as the pH at which the net charge on the protein is zero) by isoelectric focusing (IEF). This technique involves the application of high voltages to proteins within pH gradients. The large potential difference causes the proteins to migrate towards either the cathode or anode until they reach the pH corresponding to their respective pI. At this point, the protein's net charge is zero, therefore it will cease to migrate, and the protein is referred to as being "focused". The strip containing the focused proteins is then transferred to the top of a normal SDS-PAGE gel, and the proteins are separated by their molecular weight. The result is a protein map comprising spots which can be visualised by a number of techniques, most commonly with Coomassie (Meyer and Lamberts, 1965), silver (Switzer et al., 1979), or fluorescent stains (Steinberg et al., 1996; Kang et al., 2003). Isoelectric focusing became more reproducible with the development of immobilised pH gradients (IPG), eliminating the possibility of the pH gradient degradation (Bjellqvist et al., 1982). As a result, 2-DE now facilitates the accurate comparison of protein expression between two or more samples. Software designed to detect, match and calculate the volumes of spots between gels, enables quantitative and statistical analyses. Protein spots of interest can be excised from the 2-D gel, digested, and the peptides subjected to mass spectrometry (MS) to identify the parent protein (as described in the following section). The limitations of 2-DE-based proteomics, and alternative methods of protein separation, are discussed in sections 1.6.3 to 1.6.5.

1.6.2 Protein identification by mass spectrometry

Until recently, the identification of a protein mostly relied upon antibody recognition, substrate activity, or Edman sequencing. Antibodies are a very useful tool, but their binding to a given protein is not necessarily proof that it is the product of a specific gene, as cross-reactivity can result in the antibody binding unrelated proteins (Rappsilber and Mann, 2002). Furthermore, identification depends upon the availability of specific antibodies, and can be a very expensive and time-consuming way to analyse the entire protein constituents in a study. The use of substrates to identify proteins is even more

unreliable than the use of antibodies, as many different protein species have similar active sites and domains that are capable of the same catalytic activities. Edman sequencing depends upon the stepwise cleavage of amino acids from the N-terminus of a protein (Edman and Begg, 1967). Often no peptides that were sufficiently long enough to provide protein-specific identification could be sequenced, or the N-terminus was acetylated or otherwise blocked, preventing the Edman reaction (Steen and Mann, 2004).

In the 1990s, mass spectrometry became the tool of choice for protein identification, providing a far quicker, cheaper and more sensitive alternative to Edman degradation (Wilm et al., 1996). Moreover, mass spectrometry does not require the complete purification of proteins prior to their identification, and it can process proteins containing modified amino acids. All protein identification in this thesis is based upon matrix-assisted laser desorption/ionisation – time of flight (MALDI-ToF), and therefore will be the focus of this section. However, it should be noted that electrospray ionisation provides a valuable alternative method to MALDI-ToF. Peptides (*e.g.* from a digested 2-D gel protein spot) are dried together with an acidic UV-absorbing analyte (termed the "matrix") onto a metal target plate. The matrix provides a vehicle for ions to be created from polar or charged peptides, as follows. The sample is irradiated with a pulsed laser, and the UV light energy is absorbed by the matrix and transferred to the peptides, causing their desorption and ionisation into a gas phase, usually in the form of singly charged ions (Karas and Hillenkamp, 1988). These ions are allowed to cool briefly, and then accelerated through an electric field into flight tube. As all ions have the same kinetic energy ($e = {}^1/_2mv^2$), the time taken for a given peptide to travel through the flight path is dependent on its mass, with the smaller molecules travelling faster than the larger ones (Ashton et al., 2001). The time of flight is measured by an ion detector at the far end of the flight path, from which the mass of each peptide can be calculated with an accuracy better than ±0.5 Da (Ashton et al., 2001). The flight path can be increased by the incorporation of an ion mirror (or reflectron) at the end of the flight tube, reversing the direction of the ions and reflecting them to a detector, serving to resolve multiple ions of similar masses.

The masses of peptides resulting from a protein digested by a residue-specific protease provide a "peptide mass fingerprint" (PMF) specific to that protein (Cottrell, 1994). Proteolysis with trypsin, for example, results in cleavage at the carboxyl side of lysine and arginine. Since the peptide masses depend on their amino acid sequence, they are

characteristic of the parent protein. By matching the observed peptide masses obtained by MS with the predicted masses from theoretical digests of known proteins, it is possible to identify a protein by its PMF alone. Specialised software has facilitated searching for matches in a protein database, and mass deviations such as those caused by the possible modification of amino acids or missed cleavages can be automatically calculated. A score is given based on the probability that a match between the observed and theoretical peptide masses is not random. Although just three or four peptide peaks can be used to identify a protein, many researchers rely on the percentage coverage of the theoretical protein by peptides observed by mass spectrometry. Typically a coverage of >20% can be interpreted as a significant match (Barrett et al., 2005). Although PMF is a useful tool for the identification of protein spots from a 2-D gel, false positives often arise from a more complicated mixture of peptides (*e.g.* from a 1-D gel band or in-solution digest, see sections 1.6.4 & 1.6.5) due to peptide mass redundancy (two or more peptides of differing amino acid sequences may have very similar masses).

If a protein cannot be identified by its PMF alone, primary sequence information on the peptides is required. This can be obtained by tandem MS, so called because it couples two stages of MS, as follows. A selected peptide ion is isolated and collided with an inert gas (usually argon, helium or nitrogen; Steen and Mann, 2004), causing it fragment. A MS/MS spectrum is generated from the resulting peptide fragments, called a fragmentation spectrum. The peptide breakage occurs through the lowest energy route, which is predominantly along the peptide backbone (Steen and Mann, 2004). This leads to the formation of b-ions or y-ions when the charge is retained on the amino- or carboxy-terminal fragment, respectively. Therefore, each of the fragments will contain a different number of amino acids, resulting in the spectrum bearing peaks of differing masses. By measuring the distance between the peaks, it is possible to determine the amino acid sequence of the fragmented peptide. Several algorithms are available to interpret fragmentation spectra (Mann and Wilm, 1994; Yates et al., 1995; Perkins et al., 1999). MASCOT, the search engine used by York proteome researchers, calculates the theoretical mass fragmentation patterns for all peptides in a database in "top-down" fashion using the most intense y- and b-ions (Perkins et al., 1999). A score is given on the probability basis that the spectrum match is not random, called "probability based matching". The fragmentation spectra of one or more peptides, together with support from PMF analysis, provide a quick and definitive way to identify proteins by MS.

1.6.3 The limitations of 2-DE-based proteomics

Two-dimensional gels have several limitations, must notably in their failure to separate proteins of extreme pH, size, and hydrophobicity (Santoni et al., 2000). Generally, proteins are only amenable to 2-DE if their pI is between pH 3 and 10, their molecular weight is between 5-200 kDa, and they can be solubilised in a buffer of low ionic strength. Although some membrane proteins can be solubilised in chaotropic agents such as urea and thiourea (Molloy et al., 1998), the presence of detergents is invariably required. Zwitterionic detergents such as CHAPS and ASB14 (Henningsen et al., 2002), or organic solvents such as trifluoroethanol and chloroform (Zuobi-Hasona et al., 2005) aid the solubility of some proteins without compromising their separation by IEF. However, the requirement of powerful ionic detergents such as sodium dodecyl sulphate (SDS) to solubilise multi-membrane-spanning proteins is not compatible with 2-DE, as the SDS-solubilised proteins adopt a negative charge and therefore cannot be resolved by IEF. Furthermore, membrane proteins are generally in lower abundance than cytosolic proteins, so their detection by electrophoresis is often limited. Although pre-fractionation of samples prior to electrophoresis is a valuable way to overcome the problems of detecting low abundant proteins, membrane proteins are still generally under-represented by 2-DE (Righetti et al., 2005).

1.6.4 1-D gel-based methods of membrane protein identification

As isoelectric focusing is the biggest obstacle to overcome in the identification of membrane proteins by 2-DE, many laboratories have developed strategies to use alternative methods in protein separation, bypassing the IEF step altogether. The simplest solution involves the use of a 1-D SDS-PAGE gel coupled with mass spectrometry for protein identification (Galeva and Altermann, 2002). Proteins are first separated by their molecular weight, each gel band is excised, the proteins digested and subjected to MS. However, due to the potentially large numbers of proteins in a single gel band and therefore an increased complexity of peptides produced by the digestion step, identification by MS may be restricted to only the most abundant proteins. This limitation can be overcome in two ways: firstly, the proteins can be fractionated prior to SDS-PAGE using methods such as differential solubility (McCarthy et al., 2005), differential centrifugation (Wigge et al., 1998), liquid chromatography (Neverova and Van Eyk, 2005), affinity purification (Peirce et al., 2005) or blue native gel electrophoresis (Brookes et al., 2002). Secondly, following 1-D SDS-PAGE, the peptide mixture can be separated

by a reversed-phase liquid chromatographic step. The latter is called *GeLC-MS* (Li et al., 2003), and will be referred to as such in this thesis.

1.6.5 Non gel-based proteomics

Non gel-based methods have provided an alternative and effective approach in protein/peptide separation. Proteins are digested or chemically cleaved in-solution prior to their separation by liquid chromatography and analyses by MS. This methodology has distinct advantages over gel-based techniques. Firstly, it does not involve time-consuming handling steps such as IEF, SDS-PAGE, staining/destaining, spot/band excision, and in-gel digestion. Secondly, as the proteins are digested in-solution, the protease activity is not obstructed by the gel, and the resultant peptides do not have to be extracted from the gel piece: a process that is far from efficient (Katayama et al., 2001). However, as with 2-DE, the insolubility of membrane proteins is a major difficulty to overcome in non-gel based proteomics. Because the proteins are digested in solution, the buffer used must be compatible with the enzyme of choice. Even the more robust proteases such as trypsin are only functional in mildly denaturing conditions (Blonder et al., 2004). The use of ionic detergents also has adverse effects on downstream analysis by reversed-phase chromatography and mass spectrometry (Blonder et al., 2004). The use of a cation-exchange chromatography serves as a good way to remove SDS from peptides samples, and it also provides a way to separate peptides in a complex mixture prior to reversed-phase chromatography-MS. The use of two chromatographic steps followed by MS is often referred to as multi-dimensional liquid chromatography (MDLC) or multi-dimensional protein identification technology (MudPIT; Washburn et al. 2001).

The use of chemicals to cleave proteins at specific sites in their polypeptide chain is a valuable alternative to proteases, especially in the analysis of membrane proteins. Washburn et al (2001) solubilised yeast membrane in 90% formic acid, and cleaved the proteins using cyanogen bromide (CNBr). As this chemical cleaves a protein at methionine residues, thereby generating peptides that are typically too large for MS/MS analysis (>4000 kDa), a second chemical/enzyme is often required to cleave/digest the peptides further (Washburn et al., 2001). The use of organic solvents to solubilise proteins prior to their digestion is an alternative to strong detergents and hazardous chemicals (*e.g.* CNBr) which are unsuitable for everyday laboratory use (Blonder et al., 2004). Blonder and others used trypsin in the presence of 60% methanol to digest membrane proteins.

In contrast to the efforts devoted to solubilising hydrophobic proteins prior to their digestion, a new technique has been developed to identify and analyse membrane proteins still within their lipid bilayer (Wu et al., 2003). Rat brain homogenate was incubated in a high pH carbonate buffer, a condition that promotes sealed membrane vesicles to form into membrane sheets (Howell and Palade, 1982). These sheets were then pelleted, and the hydrophilic domains of the membrane proteins were digested using Proteinase K, an enzyme that non-specifically cleaves protein into dipeptides under optimal conditions. At high pH, however, this protease typically produces peptides of a size suitable for MS (6 to 20 amino acids), and overlapping peptide sequences enable the identification of the membrane proteins. As the proteins are in their native state throughout the digestion procedure (*i.e.* they are still in the lipid bilayer), the peptides provide valuable information about protein topology (such as hydrophilic loops) and may therefore provide insights to protein structure, as well as receptor-binding and catalytic domains (Wu et al., 2003).

1.7 Schistosome genome and EST databases

As identification by MS relies heavily on sequence conservation, cross-species matches cannot be obtained easily, hindering proteomics studies on organisms that have not yet been sequenced. The *S. mansoni* genome sequence is near completion thanks to the efforts of the Wellcome Trust Sanger Institute (Cambridge, UK) and The Institute for Genomic Research (TIGR; Rockville, MD, USA). It is estimated to contain 270Mb, encoding between 14,000 and 20,000 genes (Franco et al., 1995; Verjovski-Almeida et al., 2003). Furthermore, the recent sequencing of 125,000 ESTs from six *S. mansoni* life cycle stages (Verjovski-Almeida et al., 2003), together with the previously available 14,000, has greatly facilitated proteomic studies on the parasite. Although not all entries in the database have been fully assembled and annotated, they still provide a database to which MS database can be searched (Wilson et al., 2004). Putative identities and functions can be obtained using BLAST (Altschul et al., 1997) to search for matches to annotated proteins in other organisms, and the physical properties of the proteins, such as transmembrane domains, signal sequences and glycosylation sites, can be predicted using specific algorithms.

1.8 Aims / thesis overview

The aim of the work presented in this thesis is to identify proteins at the adult schistosome surface. Only by determining the molecular components of the tegument surface can we gain insight of how the parasite prevails over the host immune system. Moreover, proteins identified in the surface complex may provide valuable clues to realise the cellular mechanisms at the tegument apex. How do schistosomes mask themselves in host molecules? Are there really receptors to host cytokines and immunological factors? What are the protein constituents, if any, of the membranocalyx? The near-completion of the *S. mansoni* transcriptome and genome has finally enabled the in-depth proteomic analysis of the parasite, which may provide answers to some of these questions.

Chapter 2 describes the isolation of the membranes of the worm surface, and the use of 2-DE- and liquid chromatography-based proteomic techniques to produce an inventory of the proteins integral to, or associated with, the tegument's apical lipid bilayers. The identities are divided in sub categories according to their function, and inferences are made about their role within the surface membranes or the underlying tegument.

Chapter 3 builds on the foundation of proteins identified in the previous chapter, and significantly adds to the list by using a larger starting preparation and more sensitive techniques of protein identification. Several host proteins are discovered and a mechanism for their acquisition by the schistosome surface is postulated.

Chapter 4 details the use of impermeant biotinylation reagents to label the most exposed proteins on live worms. The tagged proteins are purified using affinity precipitation and identified by tandem mass spectrometry. Two forms of label are used, with differing penetration power, to provide insights of the surface-exposed proteins and their relative accessibility to the external environment.

In chapter 5, two methods of membranocalyx isolation are developed. The first employs cationised ferritin to initiate the sloughing of the secreted bilayer, and the second uses the acquired host red blood cell antigens to tag the apical membrane using anti-erythrocyte antibodies. The labelled membrane is then isolated with affinity beads. The content of both the purified samples are then examined using proteomic methods compatible with the analysis of hydrophobic membrane proteins.

Chapter 2

The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: a proteomic analysis after differential extraction

The chapter comprises a paper, as published in the journal *Proteomics*.

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RESEARCH ARTICLE

The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: A proteomic analysis after differential extraction

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The blood fluke *Schistosoma mansoni* can live for years in the hepatic portal system of its human host and so must possess very effective mechanisms of immune evasion. The key to understanding how these operate lies in defining the molecular organisation of the exposed parasite surface. The adult worm is covered by a syncytial tegument, bounded externally by a plasma membrane and overlain by a laminate secretion, the membranocalyx. In order to determine the protein composition of this surface, the membranes were detached using a freeze/thaw technique and enriched by sucrose density gradient centrifugation. The resulting preparation was sequentially extracted with three reagents of increasing solubilising power. The extracts were separated by 2-DE and their protein constituents were identified by MS/MS, yielding predominantly cytosolic, cytoskeletal and membrane-associated proteins respectively. After extraction, the final pellet containing membrane-spanning proteins was processed by liquid chromatographic techniques before MS. Transporters for sugars, amino acids, ions and other solutes were found together with membrane enzymes and proteins concerned with membrane structure. The proteins identified were categorised by their function and putative location on the basis of their homology with annotated proteins in other organisms.

Keywords:
Cytoskeleton / Immune evasion / Membrane enzymes / Schistosome / Transporter

1 Introduction

Schistosomiasis is an important parasitic disease, caused by trematode worms of the genus *Schistosoma*, affecting approximately 200 million people primarily in developing countries [1]. Threadlike male and female *S. mansoni* worms, approximately 1 cm long, inhabit the blood vessels of the hepatic portal system, where they can survive for decades in the face of the immune response. The entire worm surface is covered by a syncytial layer, the tegument, which is linked by narrow cytoplasmic connections to underlying nucleated cell bodies. The protein export machinery, consisting of ribosomes, ER and Golgi apparatus, is situated in the cell bodies, producing two types of inclusion, discoid bodies and multilaminate vesicles, for transport to the tegument cytoplasm. The apical membranes of the tegument form the parasite–host interface where immune evasion mechanisms must reside, and ultrastructural studies in the 1970s revealed its heptalaminate appearance [2, 3]. This structure was interpreted as a normal plasma membrane overlain by a secreted bilayer termed as membranocalyx, by analogy with the glycocalyx of eukaryotic cells [4]. The membranocalyx is formed when multilaminate vesicles fuse with the tegument plasma membrane, releasing their contents over its surface; it is

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Abbreviations: EB, extraction buffer; **GeLC**, SDS-PAGE separation followed by LC; **MDLC**, multidimensional LC; **SB 3-10**, *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulphate; **TM**, transmembrane

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continually replaced with a half-life of 5 days *in vivo* [5]. Proteins with diverse functions are believed to be present at the tegument surface, including transporters for sugars [6] and amino acids [7], enzymes [8] and receptors [9, 10]. Some of these are known to provoke antibody production in infected hosts but the resident parasites are apparently unaffected by the specific circulating antibodies.

In order to investigate the molecular basis of immune evasion, methods were devised in the 1980s to strip off the surface membranes and examine their composition [11]. Using alkaline phosphatase as a diagnostic marker, density gradient procedures achieved a considerable enrichment of surface membranes over the total worm body. Unfortunately, with the tools available at the time, little progress was made in characterising the constituents or dissecting the molecular architecture of the surface. With the advent of proteomic techniques it is now possible to identify individual proteins and link them to their encoding DNA [12]. To date three comparative studies of the schistosome proteome have been published: the soluble proteins across four life cycle stages [13]; male *versus* female proteins of *S. japonicum* [14]; an inventory of tegument syncytium proteins relative to those of stripped worm bodies [15]. We report here a proteomic study that focuses on the surface membranes of the tegument, obtained using isolation techniques previously developed in our laboratory [11]. Initial results revealed that cytosolic and cytoskeletal constituents were associated with the membranes, requiring the development of differential extraction protocols. These produced soluble fractions suitable for 2-DE separation, plus a Final Pellet processed by two different methods prior to LC separation of peptides. Proteins in the various fractions were identified by MS/MS, their function and distribution were evaluated and related to tegument surface organisation.

2 Materials and methods

2.1 Parasite maintenance and worm recovery

A Puerto Rican strain of *S. mansoni* was maintained using albino *Biomphalaria glabrata* snails and NMRI mice as laboratory hosts. To provide sufficient adult worms for a membrane preparation, 40 mice were each exposed to 350 cercariae and parasites were retrieved 6-wk postinfection by portal perfusion [13]. The male and female worms (no attempt was made to separate the sexes) were washed three times in RPMI 1640 medium (Gibco, Paisley, UK), transferred to plastic tubes, snap frozen in liquid nitrogen and stored at −80°C.

2.2 Tegument removal and surface membrane enrichment

The tegument was removed by a freeze/thaw method and surface membranes enriched by sucrose-gradient centrifugation as previously described [11]. The frozen worms were slowly thawed to 4°C, 3 mL of ice-cold RPMI medium plus protease inhibitors (Protease inhibitor cocktail; Sigma, Poole, Dorset, UK) were added and the tegument was removed by ten 1-s vortex pulses at maximum speed. The supernatant was collected, and the membranes were pelleted by centrifugation at 100 × *g* for 30 min at 4°C. The pellet was resuspended in 0.5 mL of 10 mM Tris, loaded onto a continuous 20–70% sucrose gradient and centrifuged at 100 000 × *g* for 40 min. The sucrose gradient was separated into 24 × 0.5 mL fractions, and an alkaline phosphatase assay (an apical membrane marker [11]) was performed on each fraction using BluePhos substrate (Kirkegaard & Perry Laboratories, Washington, USA) in a microtitre format. Three 0.5 mL fractions, which contained the highest activity, were pooled, diluted ten-fold in 10 mM Tris, pH 7.4, and centrifuged at 100 000 × g for 1 h to produce a pellet highly enriched in surface membranes, termed the Gradient Pellet [11].

2.3 Differential extraction of the Gradient Pellet

Proteins were sequentially extracted from the Gradient Pellet using a three-step process with reagents of increasing solubilising power (based on Molloy *et al.* [16]) as follows. Extract 1: the Gradient Pellet was suspended in 200 µL of 40 mM Tris, pH 7.4, (Sigma) at 4°C, vortexed for 2 min and allowed to stand on ice for 20 min after which the insoluble material was pelleted by centrifugation at 100 000 × g for 1 h and the supernatant removed. The whole process was repeated twice and supernatants containing the Tris-soluble proteins were pooled. Extract 2: the pellet was then subjected to a similar process at 25°C using three extractions of 5 M urea (BDH, VWR International, Dorset, UK), 2 M thiourea (BDH) in 40 mM Tris, pH 7.4, (Extraction Buffer 2; EB2) to yield a supernatant and a pellet. Extract 3: the residual pellet was treated three times with Extract Buffer 3 (EB3; EB2 plus 4% CHAPS (Sigma) and 2% *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulphate (SB 3-10; Sigma), pH 7.4) to yield a third supernatant and a Final Pellet.

2.4 2-DE of Extracts 1, 2 and 3

Extracts 1–3 were concentrated to 50 µL using a 3000 Da cutoff centrifuge filter (Millipore, Watford, UK), added to 300 µL of EB3 containing 65 mM DTT and 0.8% carrier ampholytes (Resolytes; BDH) and used to rehydrate 17 cm, pH 3–10 linear, IPG strips (ReadyStrip; BioRad, Hemel Hampstead, UK) for 12 h at 25°C. IEF was carried out at 300 V (Rapid) for 1 min, 3500 V (slow) for 3 h, 4000 V (rapid) for 3 h and 8000 V (rapid) for a total of 64 000 Vh at 25°C, at a maximum current of 50 µA/strip in a Protean IEF Cell (BioRad). During equilibration between the first and second dimension separations, proteins were reduced and alkylated according to Gorg *et al.* [17], with the buffer pH changed from 6.8 to 8.8. SDS-PAGE was performed across 9–16% gradient gels (18 × 18 × 0.1 cm), at 40 mA *per* gel for 5 h, in a Protean II MultiCell tank (BioRad). Gels were stained with Sypro Ruby (BioRad) according to the manufacturer and

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imaged using a Molecular Imager FX (BioRad) before the analysis using Phoretix 2-D Evolution software (Nonlinear Dynamics, Newcastle, UK). Spots were automatically detected, followed by minimal manual editing to remove obvious artefacts (*e.g.* Sypro Ruby speckles) and delete, split and redraw poorly assigned spots. Automatic image warping and spot matching were performed; again, minimal intervention was necessary to correct these parameters. To account for inequalities in the amounts of protein loaded on each gel (the total Extract was separated in each case), normalised spot volumes were calculated. The volumes of detected spots on a single gel were expressed as percentages of the total spot volume, after background subtraction.

2.5 In-gel digestion

Gels were restained using BioSafe CBB (BioRad) prior to excision of prominent spots for their tryptic digestion 'in-gel', briefly described as follows. Gel pieces were washed three times in 20 mM NH_4HCO_3/50% ACN for 20 min followed by a single 100% ACN wash for 15 min. The supernatant was removed and the gel pieces were dried in a Speed-Vac (Savant, Thermo Lifescience, Basingstoke, UK) for 30 min before being reswollen with 10 μL of 0.02 μg/μL sequencing grade modified trypsin (Promega, Southampton, Hampshire, UK) in 20 mM NH_4HCO_3 for 30 min, then covered with 60 μL of 20 mM NH_4HCO_3 and incubated overnight at 37°C. The resulting peptides were extracted three times from gel pieces in a solution of 50% ACN/0.1% TFA, pooled and concentrated to ~10 μL in a Speed-Vac. A 1 μL aliquot of each sample was spotted onto a MALDI target plate and 0.5 μL of matrix (a saturated solution of CHCA in 50% ACN and 0.1% TFA, diluted to half strength in the same solvent) was added to each sample before it had dried completely.

2.6 Processing of the Final Pellet

The Final Pellet was split in half and fractionated for MS analysis in two ways. The first involved 1-D electrophoretic separation followed by trypsinisation and LC fractionation (GeLC-MS/MS (GeLC–SDS-PAGE separation followed by LC)), and the second involved digestion of the sample in solution, followed by two different LC steps (MDLC-MS/MS (MDLC–multidimensional LC)).

2.6.1 GeLC-MS/MS

The Final Pellet was solubilised in 1× lithium dodecyl sulphate sample buffer and reducing agent (Invitrogen) before SDS-PAGE was performed using NuPAGE 4–12% Bis-Tris gels (Invitrogen), followed by Sypro Ruby staining and image capture, under the conditions described in Section 2.4 **■Check section no.■**. The gel lane was sectioned into 16 equal slices with a scalpel. Each slice was then divided into 1 mm cubes, before the proteins it contained were reduced and alkylated according to Shevchenko *et al.* [18]. The gel pieces were washed, dehydrated and reswollen with enough trypsin solution (0.02 μg/μL) to saturate them (typically 60–90 μL) before being covered with 20 mM NH_4HCO_3 and digested overnight as described above. The resulting peptides from each gel slice were extracted, concentrated and a 5 μL of aliquot was injected onto a Monolith RP column (LC Packings, Dionex, Sunnyvale, CA, USA) on a nanoLC instrument (Dionex). Following the wash step (2% ACN, 0.1% TFA for 2 min), peptides were eluted over a 10 min 2–50% ACN gradient. The eluate was combined directly with 0.5 μL of matrix, and 80 5-s fractions were spotted onto a target plate using a Probot spotting robot (LC Packings). Each of the gel slices was processed and spotted separately onto a MALDI plate, creating a total of 16 plates for MS/MS analysis.

2.6.2 MDLC-MS/MS

The Final Pellet was solubilised in 200 μL of 0.1% SDS, 1% Triton X-100 in 20 mM NH_4HCO_3, and digested overnight in-solution with trypsin at an enzyme:substrate ratio of ~1:20. The digested material was adjusted to pH 3 with 800 μL of 20 mM KH_2PO_4, and injected onto a POROS cation exchange column (Applied Biosystems, Framingham, MA, USA) using an AKTA 10 FPLC instrument (Amersham Bioscience, Uppsala, Sweden); peptides were eluted off the column with an increasing concentration of potassium chloride pH 3 (0–1 M) in 10 mM KH_2PO_4/25% ACN. The flow-through was collected, followed by ten fractions each representing 10% increments of elution buffer. The peptides in each of these 11 fractions were desalted by washing on an RP column (Source 5RPC ST 4.6/150, Amersham Pharmacia), before rapid elution into a single collection. Each desalted fraction was concentrated by evaporation to ~5 μL and an equal volume of 0.1% TFA added before 5 μL of aliquots were separated on the Monolith RP column and collected directly onto a MALDI plate as described in Section 2.6.1; a total of 11 plates were produced for MS/MS analysis.

2.7 MALDI TOF MS and MS/MS

A 4700 Proteomics Analyser with TOF-TOF Optics (Applied Biosystems) was used to obtain MS and MS/MS spectra in reflector positive mode. The machine was calibrated using CalMix2 (Applied Biosystems) according to the manufacturer. In addition, where present, two trypsin autolysis peaks were used as an internal calibrant for MS spectra. Where MALDI targets resulted from digestion of individual gel spots, the 15 most intense peaks from each MS spectrum were automatically selected for fragmentation, ignoring masses in the 'exclusion list' (*e.g.* common trypsin autolysis peaks and keratin contamination [19]). For MALDI targets prepared after LC separation, an MS spectrum was collected for each plate position and up to six peptides automatically selected for MS/MS. However, where peptides were common

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to more than one position, only the most intense was chosen for fragmentation, meaning ~300 peptides were fragmented *per* plate.

2.8 Database searching

MS/MS data from each in-gel digest were processed by GPS Explorer software v2.0 (Applied Biosystems) to provide peak lists, which were then submitted to MASCOT v1.9 [20] (Matrixscience, London, UK) and searched against the NCBI and clustered *S. mansoni* ORESTES databases. An in-house database, containing all the clustered ESTs and gene predictions obtained from *S. mansoni* GeneDB (http://www.schistodb.org), was also searched but no new identities were obtained. Mass tolerance was set to ±0.1 Da for the precursor ion and ±0.15 Da for product ion spectra. Search parameters allowed for a maximum of one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine. Where product ion spectra were produced following LC separation, data from all plates were searched together using GPS Explorer as described above, except the mass tolerance that was set to ±0.3 Da for both precursor and product ion spectra. A protein was considered to be identified when two or more peptides, each with a GPS-generated confidence interval of above 99%, were positively matched in the database. Putative functions were assigned using BLAST [21] against the GenBank database; scores with an expect value $<1e-16$ were regarded as a significant match. Subcellular localisation was predicted using Proteome Analyst Specialized Subcellular Location Server v1.00 [22] while transmembrane (TM) domains were identified by HMMTOP (http://www.enzim.hu/hmmtop) and PSORT II [23].

3 Results

3.1 Analysis of crude Gradient Pellet

Separation of the Gradient Pellet, containing the isolated and enriched tegument surface membranes, by SDS-PAGE revealed a banding pattern (data not shown) closely resembling that of a previous study using the same isolation method (illustrated in Roberts *et al.* [24]). Initial analysis of 20 bands excised from the gel identified 21 different proteins, with up to three proteins found in a single band. Five of these proteins possessed TM domains, but cystosolic and cytoskeletal proteins were prominent, revealing that the membrane preparation contained significant nonmembrane components after the sucrose-gradient enrichment step.

3.2 Differential extraction of Gradient Pellet

The differential solubilisation procedure enriched the membrane proteins further, the amount of material visibly reducing in size with each successive extraction. 2-D gels were produced of the IEF-compatible Extracts 1–3 (Fig. 1A–C). These gels are considerably less complex than ones from similar extracts of whole adult schistosome material (~750 spots detected in the three gels compared to ~1500 in a whole worm extract, see Curwen *et al.* [13]). There are also many unique spots, illustrating the success of the freeze/thaw method plus density gradient centrifugation in the enrichment of the tegument surface.

Analysis with Phoretix 2-D Evolution software provided a quantitative and qualitative comparison between the gels. Gels A and B show both the greatest complexity (274 and 316 spots detected, respectively, *cf.* 164 in Gel C) and similarity of spot pattern (112 spots are shared between them) (Fig. 2). The increased solubilising power of the chaotropic agents in EB2 is evident as many of the spots common to both Gels A and B are enriched in the latter, for example the protein spots 52–55 in the low molecular weight region of the gels (Fig. 1). Gel C had the most distinct spot pattern, sharing only 48 spots with Gel B and 32 with Gel A. Notably, the unique spots in the third extract (Gel C) account for almost two-thirds of its normalised volume, whereas approximately only one-third of Extracts 1 and 2 (Gels A and B) is unique (Fig. 2).

MS analysis of the 100 most abundant spots in 2-D gels of Extracts 1–3 resulted in the identification of 51 different species and can be viewed in Table 1; a protein that is identified in a given extract is represented by a dot (●). For example, enolase was identified in Extract 1 only, whereas heat shock protein 70 was found in Extracts 1–3 as well as in the Final Pellet by MDLC-MS/MS. The percentage of the spots identified reduced from 59 to 37 to 12 in Gels A–C respectively. Proteins were categorised by subcellular location according to the results of Proteome Analyst and PSORT II analysis. The cytosolic, mitochondrial and cytoskeletal categories contained the most entries, whereas comparatively few ER, vesicular or membrane components were identified. No nuclear, ribosomal or Golgi proteins were found. Five entries from Gels A–C represent matches to *S. mansoni* proteins that do not have known homologues in other species, and to which, in the absence of full-length coding sequences, subcellular location could not be assigned. Glycolytic (*e.g.* triosephosphate isomerase and enolase) and detoxifying (*e.g.* thioredoxin peroxidase and glutathione S-transferase 26) enzymes dominated the cytosolic proteins identified. The proteins with dynein light chain homology, Sm21.7 and Sm22.6, were found and ascribed a cytoskeletal function.

3.3 Analysis of the Final Pellet by two methods

The insoluble pellet remaining after the differential extraction procedure, the Final Pellet, was split equally and processed in two ways. Firstly, GeLC-MS/MS was performed on half of the material. After tryptic digestion of the 16 gel slices and chromatographic separation of the released peptides by RP, 26 different proteins were identified from the MS/MS spectra (Table 1, Pellet 4a). Of these, 24 had not been found

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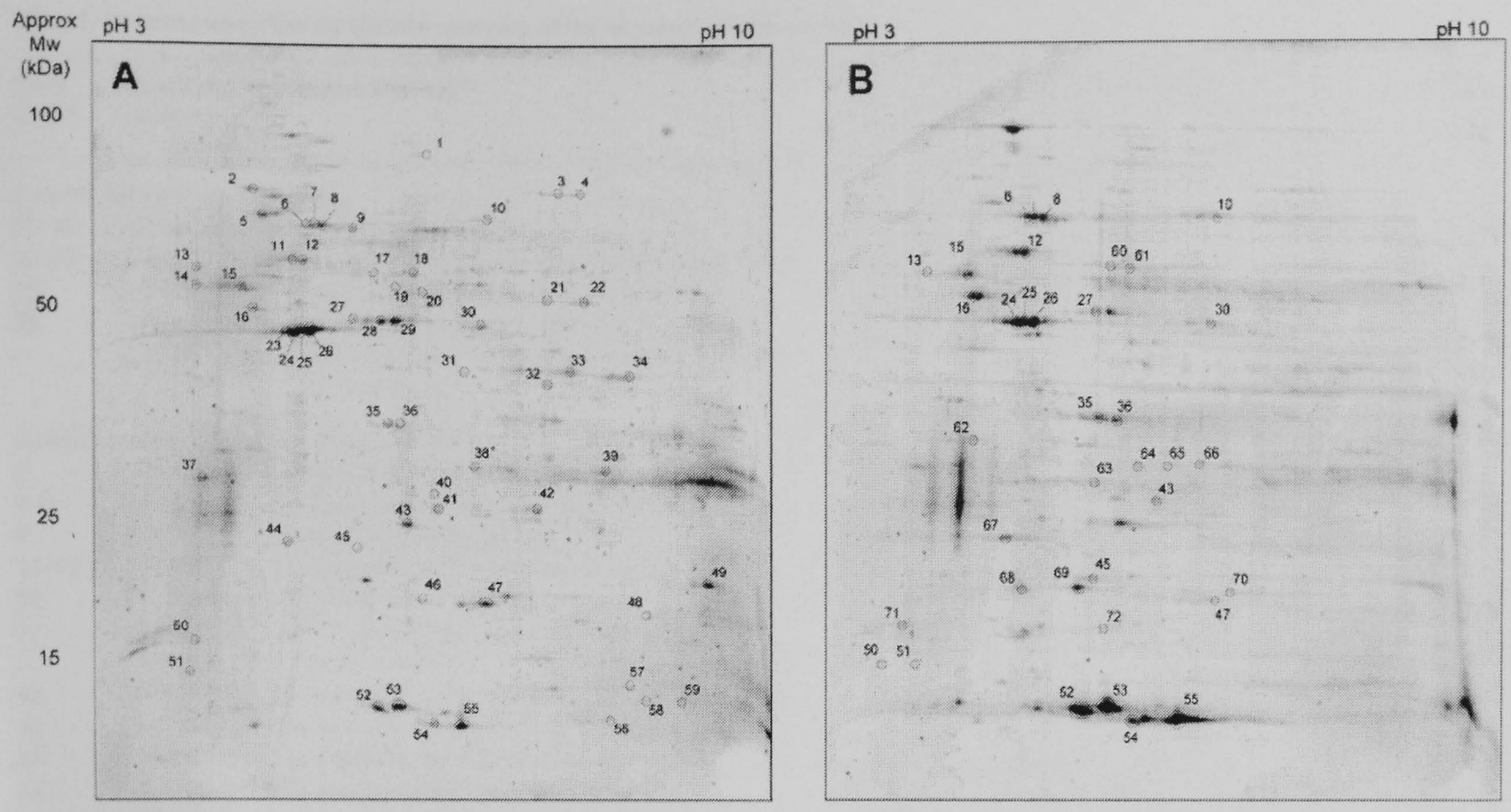

Figure 1. 2-D electrophoresis of the tegument Gradient Pellet proteins solubilised in the differential extraction procedure. (A) Extract 1: Proteins soluble in Tris; (B) Extract 2: Proteins soluble in urea and thiourea; (C) Extract 3: proteins soluble in urea, thiourea, CHAPS and SB 3-10. Proteins that were positively identified by MS/MS are circled, numbered and their corresponding details shown in Table 1.

previously in the 2-D gels of Extracts 1–3, 15 of which had TM domains. Secondly, MDLC-MS/MS revealed 38 proteins (Pellet 4b, Table 1), 7 of which were common to Extracts 1–3 and 20 to the GeLC-MS/MS separation. The 12 proteins unique to the MDLC analysis were classified as one cytosolic, three mitochondrial (two of which have TM domains), two membrane-spanning vesicular proteins, three plasma membrane and three with no homology.

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Table 1. Proteins identified by MS/MS analyses of the tegument Gradient Pellet

Spot ID(s)[a]	Accession number[b]	Putative identity[c]	Extract Pellet[d]					TM domains[e]
			1	2	3	4a	4b	
Cytosol energy metabolism								
28–29	C606843.1	Enolase (*S. mansoni*)	●	–	–	–	–	–
32–33	C610811.1	Fructose-bisphosphate aldolase (*S. mansoni*)	●	–	–	–	–	–
38	C604844.1	Phosphoglycerate mutase (*S. japonicum*)	●	–	–	–	–	–
39	C603032.1	Triosephosphate isomerase (*S. mansoni*)	●	–	–	–	–	–
–	C610807.1	Glyceraldehyde 3-phosphate dehydrogenase (*S. mansoni*)	–	–	–	–	●	–
Cytosol stress response and chaperones								
2	C607734.1	Heat shock protein 90 (*B. pahangi*)	●	–	–	–	●	–
5–8	C611944.1	Heat shock protein 70 (*S. mansoni*)	●	●	●	–	●	–
9	C605472.1	Mortalin (*R. norvegicus*)	●	–	–	–	–	–
11–12	C607350.1	Heat shock protein 60 (*S. mansoni*)	●	●	–	–	–	–
31	C603239.1	HSP20 dimer (P40) (*S. mansoni*)	●	–	–	–	–	–
37	C604582.1	14-3-3 protein homologue 1 (zeta isoform) (*S. mansoni*)	●	–	–	–	–	–
40	C606260.1	Thioredoxin perioxidase 3 (*S. mansoni*)	●	–	–	–	–	–
42	C603345.1	Glutathione s-transferase 26 (*S. mansoni*)	●	–	–	–	–	–
45	C606067.1	Thioredoxin peroxidase (*S. mansoni*)	●	●	–	–	–	–
46	C607402.1	Superoxide dismutase (*S. mansoni*)	●	–	●	–	–	–
49	C605171.1	Cyclophilin (*S. mansoni*)	●	–	–	–	–	–
58–59	C603749.1	Heat shock protein 10 (*M. musculus*)	●	–	–	–	–	–
62	C605378.1	T-complex protein (*H. sapiens*)	–	●	–	–	–	–
Cytosol other								
48	C608935.1	Islet brain protein 1 (*S. japonicum*)	●	–	–	–	–	–
50	C600256.1	Calmodulin (*M. senile*)	●	●	–	–	–	–
57	C601665.1	Fatty acid binding protein Sm14 (*S. mansoni*)	●	–	–	–	–	–
81	C609593.1	Calcium binding protein SmE16 (*S. mansoni*)	–	–	●	–	–	–
Cytoskeleton								
23–24	C604453.1	Actin (*G. fascicularis*)	●	●	–	–	–	–
25–26	C607807.1	Actin 2 (*C. gigas*)	●	●	–	●	–	–
27	C608638.1	Severin (*E. granulosus*)	●	●	–	–	–	–
43	C611999.1	Tegument antigen Sm22.6 (*S. mansoni*)	●	●	–	–	●	–
51, 71	C608222.1	Myosin light chain (*S. mansoni*)	●	●	–	–	–	–
52	C601934.1	Dynein light chain (*C. elegans*)	●	●	–	–	–	–
53–56	C602817.1	Dynein light chain 2 (*D. melanogaster*)	●	●	–	–	–	–
80	C606840.1	Antigen Sm21.7 (*S. mansoni*)	–	–	●	–	–	–
Mitochondrial matrix								
1	C601558.1	Oxoglutarate dehydrogenase (*M. musculus*)	●	–	–	–	–	–
3–4	C609269.1	Aconitate hydratase (*A. gambiae*)	●	–	–	–	–	–
10	C606723.1	Succinate dehydrogenase (*X. laevis*)	●	●	–	–	–	–
17–20	C600731.1	Aldehyde dehydrogenase (*R. norvegicus*)	●	–	–	–	–	–
21–22	C604097.1	Glutamate dehydrogenase (*O. mykiss*)	●	–	–	–	–	–
30	C611824.1	Citrate (SI)-synthase (*A. gambiae*)	●	●	–	–	–	–
34	C608337.1	Aspartate aminotransferase (*C. elegans*)	●	–	–	–	–	–
44	C609596.1	Malate dehydrogenase (*N. lapillus*)	●	–	–	–	–	–
63	C612017.1	Prohibitin (*M. musculus*)	–	●	–	–	–	–
Mitochondrial membrane								
15–16	C610735.1	ATP synthase beta subunit (*B. taurus*)	●	●	–	–	–	1
72	C608531.1	Cytochrome C reductase (*S. mansoni*)	–	●	–	–	–	–
79	C606032.1	ATP synthase alpha subunit (*M. musculus*)	–	–	●	–	–	–
–	C603833.1	Electron transfer flavoprotein (*X. laevis*)	–	–	–	–	●	–
–	C606671.1	Voltage dependent anion channel (*G. gallus*)	–	–	–	●	●	–

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Table 1. Continued

Spot ID(s)[a]	Accession number[b]	Putative identity[c]	Extract Pellet[d]					TM domains[e]
			1	2	3	4a	4b	
–	C609818.1	Fatty acid coenzyme A ligase 5 (*C. elegans*)	–	–	–	–	●	1
–	C610367.1	ADP/ATP translocase 1 (*T. brucei brucei*)	–	–	–	●	●	2
–	C610408.1	ADP/ATP Translocase 3 (*S. cerevisiae*)	–	–	–	–	●	6
ER								
13–14	C607707.1	Calreticulin precursor (*S. mansoni*)	●	●	–	–	–	–
60–61	C603288.1	Protein disulphide isomerase (*S. mansoni*)	–	●	–	–	–	–
Vesicle								
–	C605207.1	Membrane protein of lysosome/endosome (*G. gallus*)	–	–	–	–	●	1
–	C606467.1	Secretory carrier membrane protein 3 (*R. norvegicus*)	–	–	–	●	–	4
–	C603436.1	Hydrogen-transporting two-sector ATPase (*A. gambiae*)	–	–	–	–	●	7
Plasma membrane transporters								
–	C607755.1	Amino acid transporter type B (*O. cuniculus*)	–	–	–	●	●	1
–	C604128.1	Plasma membrane calcium ATPase (*C. elegans*)	–	–	–	●	●	10
–	C609225.1	Glucose transport protein (SGTP1) (*S. mansoni*)	–	–	–	●	●	11
–	C603642.1	Copper ion transporter (*D. melanogaster*)	–	–	–	–	●	3
–	C601172.1	Na/K transporting ATPase beta subunit (*D. melanogaster*)	–	–	–	–	●	6
–	C612608.1	Aquaporin 9 (*H. sapiens*)	–	–	–	●	●	6
–	C602634.1	SNaK1 alpha subunit (*S. mansoni*)	–	–	–	●	●	7
Plasma membrane enzymes								
73–76	C607243.1	Alkaline phosphatase (*M. musculus*)	–	–	●	–	●	–
–	C606156.1	Calpain (*D. melanogaster*)	–	–	–	●	●	–
77–78	C606073.1	Phosphodiesterase 5 (*M. musculus*)	–	–	●	–	●	1
–	C608449.1	Ecto-ATP diphosphohydrolase (*H. sapiens*)	–	–	–	●	●	1
Plasma membrane structure								
68–69	C602490.1	Sorcin (*S. japonicum*)	–	●	–	–	●	–
–	C604664.1	Dysferlin (*M. musculus*)	–	–	–	●	●	1
–	C603955.1	Tetraspanin A (Sj25) (*S. japonicum*)	–	–	–	●	●	4
–	C606057.1	Tetraspanin B (TE736) (*S. japonicum*)	–	–	–	●	●	4
–	C609892.1	Tetraspanin C (Sm23) (*S. mansoni*)	–	–	–	●	●	4
–	C612391.1	Annexin (*S. mansoni*)	–	–	–	●	●	–
Membrane–other								
–	C608019.1	Tyrosinase (*B. taurus*)	–	–	–	–	●	1
–	C606248.1	Sm29 (*S. mansoni*)	–	–	–	●	●	–
Unknown								
35–36, 64–66	C610230.1	No homology	●	●	–	–	–	–
35–36, 64–66	C610230.1	No homology	●	●	–	–	–	–
41	C601775.1	No homology	●	–	–	●	●	–
47	C606251.1	No homology	●	●	–	–	–	–
67	C604178.1	No homology	–	●	●	–	–	–
70	C602542.1	No homology	–	●	–	–	–	–
–	C610255.1	No homology	–	–	–	●	–	–
–	C610686.1	No homology	–	–	–	●	–	–
–	C602694.1	No homology	–	–	–	●	–	–
–	C600951.1	No homology	–	–	–	●	–	–
–	C611493.1	No homology	–	–	–	●	●	1
–	C605163.1	No homology	–	–	–	●	●	2
–	C608350.1	No homology	–	–	–	●	●	1

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Table 1. Continued

Spot ID(s)[a]	Accession number[b]	Putative identity[c]	Extract Pellet[d]					TM domains[e]
			1	2	3	4a	4b	
–	C603834.1	No homology	–	–	–	●	●	–
–	C608364.1	No homology	–	–	–	–	●	–
–	C609117.1	No homology	–	–	–	–	●	3
–	C608784.1	No homology	–	–	–	–	●	–

a) Spot ID equating with spot number on Fig. 1. Where no number is given (–), the identity was not found in any of the 2-D gels, but was derived from an LC-based analysis.
b) Accession number from the *S. mansoni* ORESTES database (http://cancer.lbi.ic.unicamp.br/schisto6/).
c) Putative identity gained by BLAST analysis against the GenBank database. Organism with the closest match is shown in brackets.
d) ● Records presence of a protein in Extract 1–3 and LC analyses of Final Pellet by GeLC-MS/MS (4a) and MDLC-MS/MS (4b).
e) Number of TM domains as predicted by HMMTOP and PSORT II.

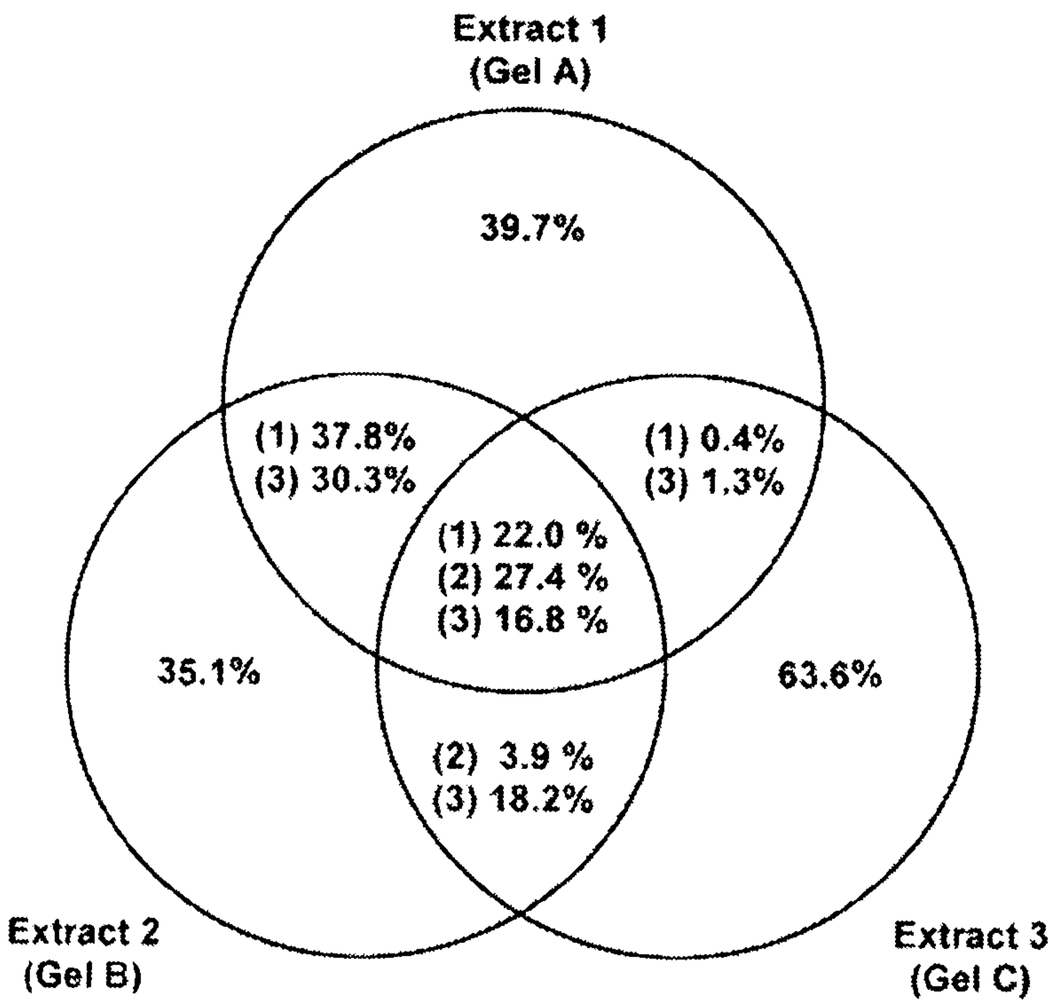

Figure 2. Distribution of protein spots in the gels of Extracts 1–3 (Fig. 1A–C). Normalised spot volume of each gel (100%) is divided to show the extent of overlap (spot matching) between preparations as determined by analysis with Phoretix 2-D Evolution software (Nonlinear Dynamics). Where more than one gel is in a sector, the number in brackets indicates the extract to which the percentage pertains.

3.4 Effectiveness of the enrichment process

Proteins identified in the crude Gradient Pellet were approximately evenly distributed between cytosolic, cytoskeletal and plasma membrane categories (data not shown). By contrast, the differential extraction procedure resolves the complexity of the protein mixture. Cytosolic proteins were the major constituents of Extract 1 (Gel A) whereas cytoskeletal components were preferentially represented in Extract 2 (Gel B). Furthermore, phosphodiesterase, a single membrane-spanning protein, and the GPI-anchored enzyme, alkaline phosphatase, were only identified in Gel C. No proteins with multiple TM domains were present in any of the extracts compatible with IEF. Both methods used to analyse the Final Pellet revealed that approximately two-thirds of the identified proteins were integral to, or associated with, membranes; around double the percentage of membrane proteins were identified from the crude Gradient Pellet. The changing predominance of a specific subcellular location between the fractions is evident as a 'drift' across the columns in Table 1. The extraction procedure has not only successfully achieved the desired goal of membrane protein enrichment, but it has also subdivided nonmembrane proteins.

Increasing numbers of proteins with no homology were found in each successive fraction. By assembling these proteins according to the fraction in which they were identified, another drift is apparent (bottom of Table 1). This allows inferences to be drawn about the subcellular location of proteins of unknown function. For example, C606251.1 was only found in Extracts 1 and 2 and is therefore likely to be cytosolic. Conversely, C611493.1 was in the Final Pellet alone (identified by both LC methods) and is more probably of membrane origin. With this in mind, we can tentatively assign the 16 proteins with unknown identity to a subcellular location, namely cytosolic, 3; cytoskeletal, 2; and membrane, 11. The ability of the complete fractionation protocol to categorise proteins in the tegumental membrane preparation is summarised in a series of pie charts (Fig. 3).

To analyse the detailed protein composition of the tegument surface preparation we combined the information from the four separate extracts. Homology searches were performed with the NCBI protein database to supplement the annotation in the schistosome databases and arrive at a classification of function (Table 1). Two major types of cytosolic proteins were evident. The glycolytic pathway was represented by 5 enzymes, whilst 13 proteins which functioned as chaperones and/or in a stress response were prominent, leaving 4 with no particular affiliation. Eight pro-

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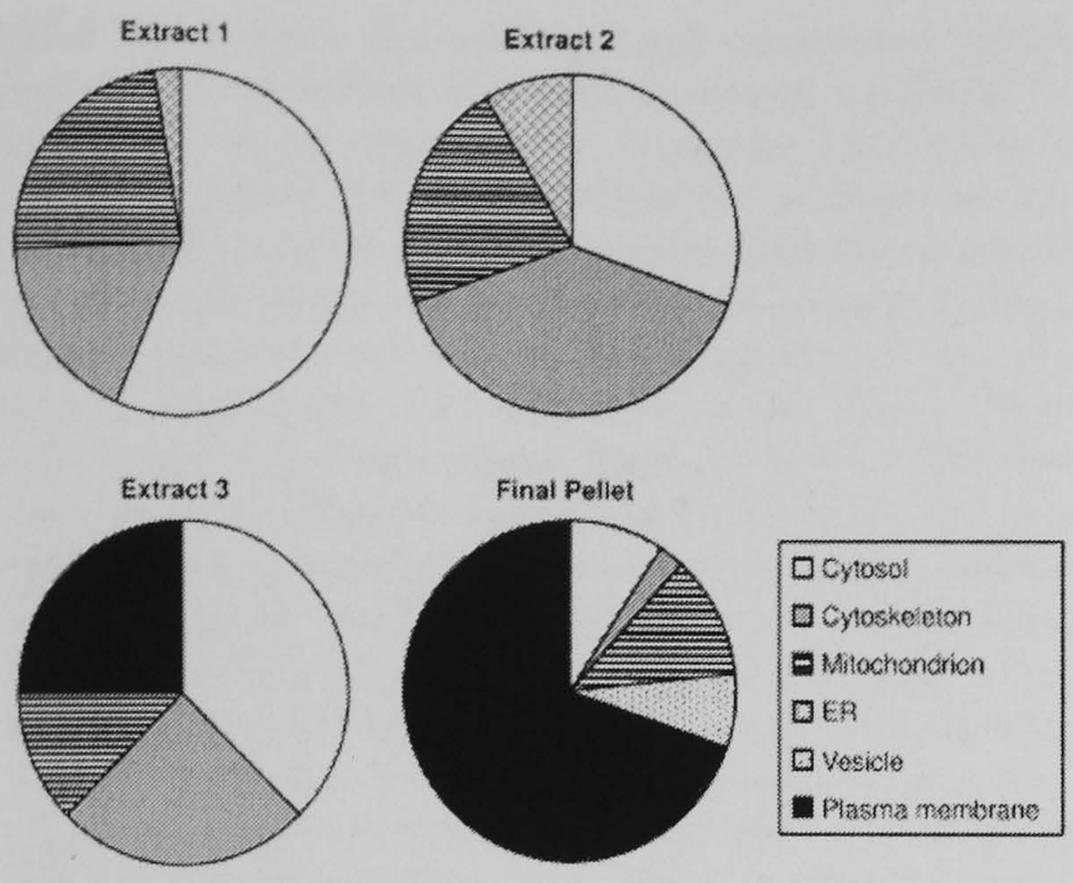

Figure 3. Distribution of the proteins identified in Table 1 according to their subcellular location as determined by Proteome Analyst [22] or PSORT II [23]. Final Pellet data obtained by the GeLC-MS/MS and MDLC-MS/MS were combined for the purpose of this diagram.

teins with putative cytoskeletal functions were preferentially extracted by the urea/thiourea mixture. Their volume represented not less than 25% of protein amenable to separation by 2-DE. Contamination of the preparation with mitochondria was apparent by the presence of eight membrane and nine matrix proteins from that organelle.

Proteins of the export pathway were represented by two with a putative location in the ER and three associated with secretory vesicles (Table 1). The fractionation procedure revealed a group of plasma membrane proteins mostly in the Final Pellet. Of these, seven were involved in the transport of molecules, including sugars, ions, amino acids, metals and solutes across the plasma membrane. Four known membrane-associated enzymes were found, as well as six proteins involved in membrane structure. Two additional membrane proteins, Sm29 and tyrosinase, were detected by LC-MS/MS. The former transcript encodes a leader sequence but no membrane-spanning regions and possesses three putative *N*-glycosylation sites.

4 Discussion

The purpose of this study was to characterise the protein composition of the apical membranes of the schistosome tegument. We used a freeze/thaw method, originally developed in this laboratory [11], to detach the tegument and recovered the membranes by density gradient centrifugation. This process generated sheets of membrane material, leaving the underlying worm bodies relatively intact. The method compares favourably with two other techniques for tegument detachment, namely incubation of live worms in 0.3 M calcium chloride [25] or in 0.2% Triton X-100 [26]. In our hands the former gave a poor yield, whilst the latter extracted the plasma membrane marker alkaline phosphatase, precluding tracking of the membranes during purification. Furthermore, after 1-D separation of the Gradient Pellet we obtained protein banding patterns similar to those reported in previous work with the preparation [24]. However, the first attempts at protein identification by MS revealed greater amounts of cytosolic and cytoskeletal protein constituents than the original authors presumed, since the isolation procedure removes spines and large amounts of tegument ground substance [11]. This outcome highlighted the need for selective removal of associated proteins from the membrane preparation.

The differential extraction technique, modified from Molloy *et al.* [16], was used to remove components sequentially from the surface membranes according to the strength of their association. Although the scheme of enrichment involved several steps before differential extraction commenced, three treatments with Tris buffer still recovered quantities of glycolytic enzymes, stress response proteins and antioxidant enzymes. While at least two glycolytic enzymes, triosephosphate isomerase [27] and glyceraldehyde-3-phosphate dehydrogenase, have been reported as expressed on the surface of larval schistosomes [28], this is probably a special case related to the shedding of surface membranes after skin penetration [29], and it seems very unlikely that these essentially cytosolic proteins are exposed at the external surface of adult worms. However, there is evidence from other cell systems that both glycolytic enzymes [30, 31] and various heat shock proteins [32, 33] can bind to cytoskeletal elements such as actin, microfilaments and microtubules (see below). The presence of glycolytic enzymes immediately beneath the tegument surface could utilise incoming glucose for ATP production required for functions such as nutrient or solute uptake, or for changes in cytoskeletal conformation. It is unclear whether the various chaperones are present beneath the surface for a specific purpose or simply due to their protein-binding properties. However, the presence of antioxidant enzymes (superoxide dismutase, thioredoxin peroxidase) as well as GST (which participates in the detoxification of reactive electrophilic compounds) immediately below the plasma membrane would be of obvious benefit to the parasite, given the need to withstand attack by free radicals generated during an immune response.

After removal of the Tris-soluble proteins the preparation was treated with the chaotropic agents, urea and thiourea, to recover more firmly but noncovalently bound material, the bulk of which could be classified as cytoskeletal. Indeed, once the identities of the small group of five cytoskeletal proteins had been established, it was apparent that their combined spot volumes on Gels A–C represented not less than a quarter of the Gradient Pellet proteins. Early ultrastructural investigations revealed an electron dense layer immediately beneath, and adherent to, the surface membranes [2, 3]. The highly pitted appearance of the tegument syncytium indi-

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cated the existence of a well-organised cytoskeleton, whilst the collapse of surface structures in worms incubated in cytochalasin B, an actin disrupter, implicated this protein as a key component [34]. Our identification of actin and the myosin light chain in Extract 2 confirms their strong association with the surface membranes. The presence of severin, which organises actin filaments, is suggestive of dynamic activity. Furthermore, the extraction of two dynein light chains plus the dynein-related proteins, Sm21.7 [35] and Sm22.6 [36], is indicative of molecular motor activity near the surface. It is tempting to conclude, purely on the basis of electron density and protein abundance, that the layer immediately beneath the plasma membrane is largely composed of dynein homologues plus actin, in a macromolecular complex. As well as providing structural scaffolding for the tegument, the cytoskeletal proteins are likely to function in the shuttling of vesicles to the surface plasma membrane. Microtubules normally form an important part of the cytoskeleton in eukaryotic cells, but have never been described in ultrastructural studies of the tegument syncytium, only in the cytoplasmic tubules that connect it to the cell bodies. Furthermore, we found no trace of the diagnostic α and β tubulins. A further cytoskeletal protein, SCIP1, recently identified as paramyosin [37], reported at the tegument surface of adult worms by immunofluorescence staining [38], was not detected here by MS/MS analysis.

A series of proteins identified by MS may be present in the Gradient Pellet by virtue of their association with organelles that have copurified with the surface membranes. The two ER proteins, calreticulin and protein disulphide isomerase, have C-terminal HDEL and KDEL sequences respectively, and so should have been retained within the cisternae of the organelle in the tegument cell bodies. There is no other evidence in the form of ribosomal or nuclear proteins for contamination from the cell bodies, so these proteins may have reached the tegument within secretory vesicles. In this context, two proteins associated with vesicle membranes (SCAMP 3 and membrane protein of lysosome/late endosome) were identified, plus a hydrogen transporter involved in vesicle acidification (we are not aware of lysosomes being described in the tegument syncytium). The principle organelle contaminants of the Gradient Pellet were mitochondria, as revealed by the nine matrix and eight membrane protein components detected, the former in Extracts 1 and 2, and the latter largely in the Final Pellet. Electron micrographs depict numbers of small mitochondria within the tegument syncytium [2, 3], and we must assume that they occupy the same position as the membranes on the sucrose density gradient. When cytochrome oxidase was used as a marker in the development of the original Gradient Pellet preparation [11], some enzymatic activity was detected providing biochemical evidence for the presence of mitochondria.

The principal targets of our investigations, the integral membrane and membrane-associated proteins, were present largely in the Final Pellet or to a lesser extent in Extract 3. A total of four plasma membrane enzymes were identified, of which three (phosphodiesterase, ecto-diphosphohydrolase and alkaline phosphatase) are completely capable of hydrolysing organic phosphate compounds and are likely by analogy with other cells to be situated on the external leaflet of the plasma membrane. In such a location they would be shielded from antibodies by the overlying membranocalyx, which would nevertheless need to possess pores of sufficient size to permit transit of organic phosphates from the external environment for hydrolysis. All three hydrolases have been previously reported as constituents of the tegument surface by immunostaining [39–42], which provides strong confirmatory evidence for the authenticity of our tegument surface preparation. The fourth enzyme calpain, a cysteine protease, is by analogy with other cells most likely to be associated with the cytoplasmic leaflet of the plasma membrane [43]. In this situation it is involved in the constant remodelling of the cytoskeleton, which we have already suggested, and is particularly well developed beneath the tegument surface. In the context of schistosomes, the β subunit of calpain has been investigated as a vaccine candidate [44]. By analogy, our proposed more extrinsic location of the three other plasma membrane enzymes should implicate them as vaccine targets, but only the diphosphohydrolase has been cloned [42] and none has been tested for protection.

Our analysis revealed seven distinct transporter proteins in the membrane preparation. Two of these, the facilitated glucose transporter SGTP1 [45] and the B-type amino acid transporter [46], confirm the occurrence of active nutrient uptake at the tegument surface [7]. With respect to the glucose transporter our findings differ from the cytochemical observations of Zhong *et al.* [45], who reported it at the basal but not the apical surface of the adult tegument. The other well-characterised glucose transporter reported to be at the tegument surface, SGTP4 [47], was not found in this study but has since been detected in our current work (manuscript in preparation). The presence of the B-type amino acid transporter confers the ability to take up cysteine, neutral and dibasic amino acids, but it is distinct from the SPRM1 amino acid permease whose light chain has been localised at the tegument surface by immunocytochemistry [48]. The flux of inorganic ions across the tegument surface appears to be a prominent function, with the identification of SNaK1, a sodium–potassium ATP-driven antiporter, but no symporter yet discovered that might be coupled to sodium influx. We have also found a calcium ATPase which probably functions to maintain calcium homeostasis by expelling the cation from the tegument cytoplasm. It is distinct from the voltage-gated calcium channel which is the putative target of the antischistosome chemotherapeutic agent Praziquantel [49]. Its presence may be particularly important in this respect due to the lack of ER in the tegument cytoplasm, which could serve as a calcium store, and its activity may be linked to the role of calpain in cytoskeletal organisation. The identification of aquaporin indicated the diffusional transfer of water and

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noncharged solutes such as purines and pyrimidines across the surface membrane [50]. Lastly, the presence of a copper ion transporter implies a significant requirement for this metal ion, presumably acquired from ceruloplasmin, transcuprein or albumin in host blood [51]. A high requirement for copper may be linked to the action of copper-containing polyphenol oxidase (tyrosinase) in eggshell formation [52, 53].

A group of six proteins can be tentatively assigned a role in membrane structure. Three distinct tetraspanins A, B and C were present, two closely related and one (C) more distant, representing a small subset of the 25 or more encoded by the *S. mansoni* genome (http://www.schistoDB.org/). All three are paralogues, having diverged within the schistosomes, so it is difficult to assign a specific function. However, tetraspanins of other animals associate with specific proteins in a molecular network, the tetraspanin web, largely within the plasma membrane, rather than with extracellular ligands [54, 55]. Thus, they may provide the spatial organisation for other tegument plasma membrane proteins. Tetraspanin C (Sm23) has been investigated as a vaccine candidate [56]. The three other proteins involved in membrane structure have calcium-binding properties related to their function. Sorcin is a membrane-associated protein that regulates the fluxes mediated by the calcium ATPase [57]. Dysferlin has a C-terminal membrane anchor with several C2 cytoplasmic calcium-binding domains and may function in calcium-dependent vesicle fusion with the plasma membrane [58], potentially to repair damaged surfaces. The annexins are a family of lipid-binding proteins, usually present at the cytoplasmic surface, but on occasion found in an extracellular location [59]. Although the tegument annexin lacks a signal sequence or TM domains, it nevertheless appears to be firmly associated with the apical surface.

Extensive analysis of the *S. mansoni* transcriptome has revealed approximately 55% of genes with no assignable function [60]. The fact that in our analysis of the tegument proteome we were able to assign identities or putative functions to 71 out of 87 MS hits (82%) is therefore surprising. The most obvious explanation is that proteome analysis is less sensitive than transcriptome analysis, and so fails to detect scarce proteins for which corresponding transcripts can be found. The assumption implicit in such a conclusion is that abundant proteins are intrinsically more likely to have had their functions characterised than scarce proteins. In this respect, the amount of material available may limit the scope of parasite proteomic analysis.

In this study we have successfully used a combination of membrane isolation and MS/MS to explore the composition of the surface of the schistosome tegument, the most important parasite–host interface for the evasion of immune responses. Our study differs markedly in approach from that of van Balkom *et al.* [15] who treated the tegument as a single entity. They produced an inventory of proteins associated with the tegument, the stripped bodies, and those shared between the two. Although van Balkom *et al.* listed 43 proteins as unique to the tegument, this does not take into consideration the fact that all tegumental proteins originate in the cell bodies which lie beneath the musculature of the body wall and will therefore also be present in the stripped bodies (quite possibly at lower concentrations). The differences in approach limit the comparison that can be made between the two studies. It is not surprising that out of the 87 proteins identified in our tegument membrane preparation, 50 were found by van Balkom *et al.* in both their tegument and stripped body fractions. We identified 11 proteins in our tegument membrane preparation that were detected only in the tegument by van Balkom *et al.*, and 10 proteins deemed to be specific to the worm body, while 16 were unique to our study. Notably, all the plasma membrane proteins that were common to both studies were proposed to be present in the tegument by van Balkom *et al.*, with the exception of tetraspanin C (Sm23) which was only found in their stripped body fraction. Although the two studies differ in their aims and techniques, it is reassuring to observe that many proteins can be reproducibly identified as constituents of the schistosome tegument.

The variety of structural proteins that we found within and immediately underneath the plasma membrane attests to the morphological and functional compartmentalisation of the parasite surface. The presence of the calcium pump together with several calcium-dependent proteins suggests a central role for this cation in the regulation of tegument surface function. We have identified several transporters responsible for nutrient uptake and solute flux, and others will undoubtedly be added to the list. The absence of any receptors or host proteins among the surface components is surprising in view of the proposed ability of schistosomes to interact with host cytokines such as TGF-β [61] and TNF-α [62], or immune factors such as IgG and complement [63], but this could reflect their low abundance. As yet, we cannot determine if any of the proteins identified are components of the membranocalyx, *i.e.* the contents of the tegumental multilaminate vesicles. However, the salient features of Sm29 make it the most likely candidate for secretion outside the plasma membrane, and hence associated with the membranocalyx. We need to develop methods for extrinsic labelling of surface proteins to determine the extent to which individual molecular species are exposed to the external environment. We also require a method for enrichment of the membranocalyx, separate from the underlying plasma membrane, if we are to solve the riddle of immune evasion.

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Chapter 3

A liquid chromatography-mass spectrometric-based analysis of the tegument surface membrane proteins

3.1 Introduction

The protein identifications presented in Chapter 2 provided the first step towards the characterisation of the tegument surface proteome, revealing membrane proteins as well as cytoplasmic constituents that were proposed to have affiliations with the tegument apical bilayers. Based on the identifications by tandem MS/MS, it was assumed that the Tris and urea/thiourea extracts contained primarily cytosolic and cytoskeletal proteins, and thus the chaotropic agents were incapable of solubilising membrane proteins. However, results from preliminary experiments using biotin to label proteins on the adult schistosome surface (presented in Chapter 4) revealed that multispanning membrane proteins were solubilised by urea and thiourea and could be identified by LC-MS/MS. Furthermore, this extract also contained proteins that had not been identified in Chapter 2, some of which significantly added to our understanding of schistosome surface composition and hence its interaction with the host immune system. Thus, it seemed that several proteins in the 2-D gels of Extract 1-3, and possibly in the Final Pellet too (Chapter 2), had not been identified. Indeed, many of the spots in the 2-D gels had no identity, especially in Extract 3.

A recent proteomic study by van Balkom *et al.* (2005) identified over 700 proteins belonging to the *S. mansoni* tegument, stripped body, or both fractions. These authors proposed that 222 of these proteins were derived from the tegument, 43 of which were not found in the worm body. As argued in the discussion of Chapter 2, *all* proteins within the tegument originate from the cell bodies that lie below the musculature of the body wall. Thus, no proteins will ever be tegument-specific. Nonetheless, the fact that these proteins were identified in the tegument fraction, and not the stripped body, might reflect their relative concentrations within the two fractions. Within these "tegument-specific" proteins, several membrane proteins were identified, such as SGTP4 and "22K surface membrane protein" [Sm25], that were not reported in Chapter 2.

The failure to identify all the proteins in the Gradient Pellet (Chapter 2) could be due to the lack of starting material or the method of their separation prior to identification (the limitations of 2-D gels are discussed in the general introduction). It was therefore believed necessary to alter both these parameters to gain more coverage of the protein constituents; the amount of starting material was tripled, and protein identification was based upon the LC fractionation of peptides prior to tandem mass spectrometry. The methods of tegument

membrane recovery and purification are the same as those used in the previous chapter. Therefore, this work can be viewed as an appendix to Chapter 2, but it has been separated into a chapter of its own due to the large number of proteins identified and the extensive discussion of their relevance at the schistosome surface.

3.2 Materials and Methods

3.2.1 Parasite culture and tegument membrane isolation

One hundred and twenty mice, each infected with 350 *S. mansoni* cercariae, were perfused to obtain six-week-old worms. The tegument was removed by the freeze/thaw/vortex method and the surface membranes were purified by density centrifugation, as described in Chapter 2. However, due to the large amount of material involved, the sample was split into four equal amounts, and each was loaded onto a separate sucrose gradient. The alkaline phosphatase-rich material was collected from each gradient and pooled to yield the starting material, termed the Gradient Pellet (GP).

3.2.2 Transmission electron microscopy

A subset of 3-4 denuded worms was examined by transmission electron microscopy to examine the efficiency of the tegument removal process. The specimens were fixed in 4% formaldehyde, 2% glutaraldehyde mixture in 100 mM phosphate buffer overnight. After two washes in phosphate buffer, they were post-fixed in 1% osmium tetroxide in 100 mM phosphate buffer for two hours, washed in water, and fixed in 0.5% uranyl acetate in distilled water for four hours. The parasites were embedded in resin and sectioned transversely through the middle of the worm using a Leica Ultracut. Sections, approximately 70 nm thick, were examined using a FEI Tecnai G^2 transmission electron microscope (Tecnai, FEI company, Hillsboro, Or, USA).

3.2.3 Differential extraction of the Gradient Pellet

Proteins were sequentially extracted in solvents of increasing strength, using the same methods as described in Chapter 2, to yield four samples soluble in Tris (Extract 1), urea/thiourea (Extract 2), CHAPS/SB3-10/urea/thiourea (Extract 3), and SDS (Extract 4).

3.2.4 GeLC- and MDLC-MS/MS

The protein constituents of each extract were separated by one-dimensional SDS-PAGE,

stained with Sypro Ruby and imaged using a FX Imager (BioRad). Each gel lane was cut into 10 sections of equal size and the slices were then diced into 1 mm cubes with a scalpel. The gel pieces were digested with trypsin, and the peptides were separated by reversed-phase liquid chromatography, as described in Chapter 2. The proteins solubilised in the final step of the differential extraction procedure (using 2% SDS) were also analysed by multidimensional liquid chromatography (MDLC). These proteins were digested in-solution, and the tryptic peptides were separated by two phases of liquid chromatography (Chapter 2). The peptides from both the GeLC and MDLC separations were collected directly onto MALDI plates and subjected to tandem MS/MS, as described in Chapter 2.

3.2.5 Database searching

The fragmentation spectra generated from each peptide was used to search the *S. mansoni* EST and genomic databases, as well as the NCBInr database, using the parameters set in Chapter 2. A protein was considered to be identified if two or more peptides with a GPS-generated confidence interval of above 99% were positively matched to a sequence in the database. However, if a protein was represented by only one peptide, its fragmentation spectrum was checked for MS/MS quality, and only if >75% of the predicted peptide sequence could be matched to y- or b-ion peaks, was it considered a positive identification.

3.3 Results

3.3.1 Tegument removal and density gradient membrane purification

Transmission electron microscopy was used to examine the effect of the freeze/thaw/vortex process on the tegument of the adult worm. The micrographs revealed that the tegument had been almost completely detached, with the exception of a few spines which remained firmly attached to the basement membrane (Fig. 3.01). The process removed the tegument from both male and female worms without any notable difference. The underlying muscle layer had lost some of its integrity, as the muscle fibres were not as neatly packed as seen in an untreated worm. Nonetheless, the basement membrane of the tegument appeared intact, trapping most of the muscle material within the denuded worm carcass.

The released tegument membranes were collected, pelleted by low-speed centrifugation,

and purified by a continuous sucrose gradient. Two distinct isolates of material were visible after the density centrifugation, the first consisting of a milky-coloured band two-thirds of the way down the gradient, and the second consisting of a dark brown pellet at the bottom of the centrifuge (represented diagrammatically in Fig. 3.02a). The gradient was profiled for alkaline phosphatase activity and this enzyme was used as a marker of membrane content. Activity was highest in the fractions that corresponded with the milky-coloured band in the sucrose gradient (Fig. 3.02b). These fractions were pooled to yield the starting material (Gradient Pellet; GP).

3.3.2 Differential extraction and first dimension separation

The GP was treated to a four-step differential extraction process to remove proteins according to their solubility. Each extract was separated by 1-D SDS-PAGE and visualised by Sypro Ruby staining. The gel revealed banding patterns unique to each extract (Fig. 3.03). Extract 1 contained the most diverse range of protein sizes, with bands ranging from approximately 250 to12 kDa. One very prominent band was evident at ~37 kDa, otherwise the staining intensity remained relatively uniform throughout the sample. Extract 2 also contained a wide variety of protein sizes, with dominant bands at approximately 140, 75, 40, 25 and 12 kDa (Fig. 3.03). Especially towards to the lower molecular weight end of the gel, Extract 2 was quite smeared. Extract 3 contained notably fewer protein bands than the previous two, with only 11 prominent members (Fig. 3.03). Similarly, the final extract was even less complex in its staining pattern, but had several bands in common with Extract 3.

Half of Extract 4 (SDS fraction) was digested in solution and the peptides separated by Strong Cation Exchange chromatography. The UV absorbance of the material eluted off the column revealed a large flow-through followed by four major peaks throughout the increasing salt gradient (Fig. 3.04). Two further peaks were observed as the salt concentration reached 0.5 and 1.0 M, respectively. As the peptides were not eluted in a constant manner, the ten fractions collected were not based on uniform timepoints, but on the relative peptide content (Fig. 3.04).

3.3.3 Reversed-phase chromatography and tandem mass spectrometry

The peptides from each gel piece and salt cut were separated by reversed-phase

chromatography and spotted onto a MALDI plate prior to analyses by tandem MS. The first peptides started to elute off the column when the acetonitrile concentration reached ~12%, and subsequent peaks were typically observed up until the gradient reached 45% acetonitrile (Fig. 3.05). As the monolith column allowed a high flow-rate (3.0 µl/min), a whole cycle was complete within 20 minutes. Even with this relatively steep gradient, the peptides were adequately separated for MS, with typically no more than five peptides in each well of a MALDI plate (Fig 3.06). Tandem mass spectra were obtained from each peptide with a signal/noise ratio greater than 30. In total, approximately 30,000 peptides were fragmented, and their peak lists submitted to search the *S. mansoni* and NCBI databases. An example of a typical fragmentation spectrum can be seen in Figure 3.07.

3.3.4 Protein identification

A complete list of the proteins identified is shown in Table 3.1. To aid the reader, the proteins have been grouped according to their putative location or function, as determined by their homology to characterised proteins in other species. The extract(s) in which the protein was identified is shown in the columns, and the number within each cell represents the number of different peptides that were identified from that protein. For example, just one peptide from *integrin alpha 2* was found in Extract 3, whereas *Sm29* was identified in all extracts by either 1 or 2 peptides.

A total of 196 proteins were identified within the GP, many of which were shared between two or more different extracts (Table 3.1). Extract 1 provided the largest number of proteins (95), and also contained the highest percentage of proteins that were unique to a single extract (Fig. 3.08). Extracts 2 and 3 contained progressively fewer proteins, and the proportion of identities that were unique to each extract reduced in a similar manner (Fig. 3.08). Eighty-seven different proteins were found in the SDS extraction (Extract 4), 55 and 78 of which were found by the GeLC- and MDLC-MS/MS approaches, respectively (Fig. 3.09). Out of all the proteins identified by GeLC-MS/MS, only 9 were not found by MDLC-MS/MS. Conversely, 32 proteins were uniquely identified by MDLC-MS/MS (Fig. 3.09).

3.3.5 Protein identites

A total of eight mouse proteins was identified in the GP, as determined by their specific matches to sequences in the NCBInr database. Integrin alpha 2 was represented by a single

peptide in Extract 3, but there was no evidence of a possible beta chain that it may once have been associated with. Complement component C4 and a Complement regulatory protein, Crry, were found in Extract 4 by the MDLC-MS/MS process. Fibrinopeptide B, a constituent of the blood clotting process, and chemokine ligand 11 (with a C-X-C motif) were identified in Extracts 2 and 4, respectively. Three host receptors were present in this study, namely a glycine receptor, acetylcholine receptor and CD44 (a hyaluronate receptor). Besides the Complement C4, no other constituents of the mouse immune system were detected, neither were any erythrocyte-specific proteins.

Proteins were ascribed as secreted based on their possession of a signal sequence without any subsequent transmembrane domains. Two *S. mansoni* proteins fitted these criteria, both of which have been fully sequenced and named Sm13 and Sm29, based on their approximate molecular weight. Both these proteins were represented in each extract of the GP, with relatively constant peptide counts in each (Table 3.1).

Thirteen distinct proteins involved in the transmembrane trafficking of solutes were identified in the study: two well-characterised tegument sugar transporters (SGTP1 and 4), an amino acid transporter, the beta and alpha subunit of SNaK1, three isoforms of calcium transporting ATPase, a further cation ATPase, two pore-forming proteins (aquaporin and voltage dependent anion channel), a protein involved in serotonin transport, three isoforms of phospholipid-transporting ATPase, and an aminophospholipid transporter. The peptide distribution of each transporter protein was generally skewed towards Extract 4, although 46% of these proteins were represented by at least one peptide in Extracts 1-3 (Table 3.1).

Five tetraspanins were identified, three of which had been fully characterized and named Sm23, TE716, and CD63-like protein (in this thesis they are referred to as tetraspanin B, C, D, respectively). Similar to the transporter proteins, the majority of peptides from the tetraspanins were found in the Extract 4, with the exception of Tetraspanin D which was represented by seven peptides in Extract 1. Further proteins involved in the structure and/or maintenance of phospholipid bilayers included dysferlin, two forms of annexin, sorcin, scramblase, syntenin and several proteins involved in phospholipid transport (Table 3.1).

Three membrane phosphate hydrolases were present, namely alkaline phosphatase, phosphodiesterase, and ATP-diphosphohydrolase. All three were identified by large numbers of peptides in Extracts 3 and 4. Two further membrane enzymes, Calpain B and acetylcholinesterase, were identified; the former in every extract, and the latter only in Extract 3 (Table 3.1). An additional five membrane proteins, whose function did not fall into any of the above categories, were also found. Two of these, Sm25 and 200 kDa "Surface protein", are fully sequenced *S. mansoni* proteins. Three other schistosome proteins were found, which bear significant homology to Fibronectin, Delta B and the GTP-binding Rap-1b.

Components of the secretory pathway, either constituents of the vesicle membrane, or involved in vesicle docking or recycling, were represented by eight proteins. Those with multiple membrane spanning domains, such as the secretory carrier membrane protein, were found in Extracts 3 and 4. Those with no predicted hydrophobic domains, such as endophilin, were discovered only in the Extract 1. Only two ER and four nuclear proteins were identified in this study. Conversely, a relatively large number (26) of mitochondrial proteins were identified.

Cytoskeletal constituents of the GP were abundant, particularly in Extract 1. Eleven proteins made up this category, only two of which were specific to *S. mansoni* (Sm20.8 and 22.6). Thirty-seven cytosolic proteins were identified, and in a similar manner to the cytoskeletal proteins, they were found mostly in Extract 1 (Table 3.1). Three different proteases were identified: two forms of cathepsin and a schistosome haemoglobinase. All these were identified in Extract 1.

More than a quarter (27%) of the positive matches to sequences in the *S. mansoni* database were to EST or gene predictions that had no significant homology to any entry in the GenBank database. Thus, they were classed as schistosome-specific proteins with an unknown function or location. Proteins within this category were found throughout all the extracts, and it is noticeable that a large proportion of them are represented by a single peptide.

3.3.6 Patterns within each extract

The distribution of the proteins' putative locations from each extract can be summarised as a chart (Fig. 3.10). Several trends can be observed throughout the differential extraction process. Probably the most notable is the progressive increase in membrane proteins identified from Extract 1 through to Extract 4. Cytosolic proteins are the dominant constituent of Extract 1, whereas mitochondrial proteins are most commonly found in Extract 2. Putative components of the cell bodies (*i.e.* nuclear and ER proteins) and gut are only found in the first 3 extracts. The percentage of secreted, cytoskeletal, vesicular and unknown proteins remained roughly constant across all the extracts (Fig. 3.10).

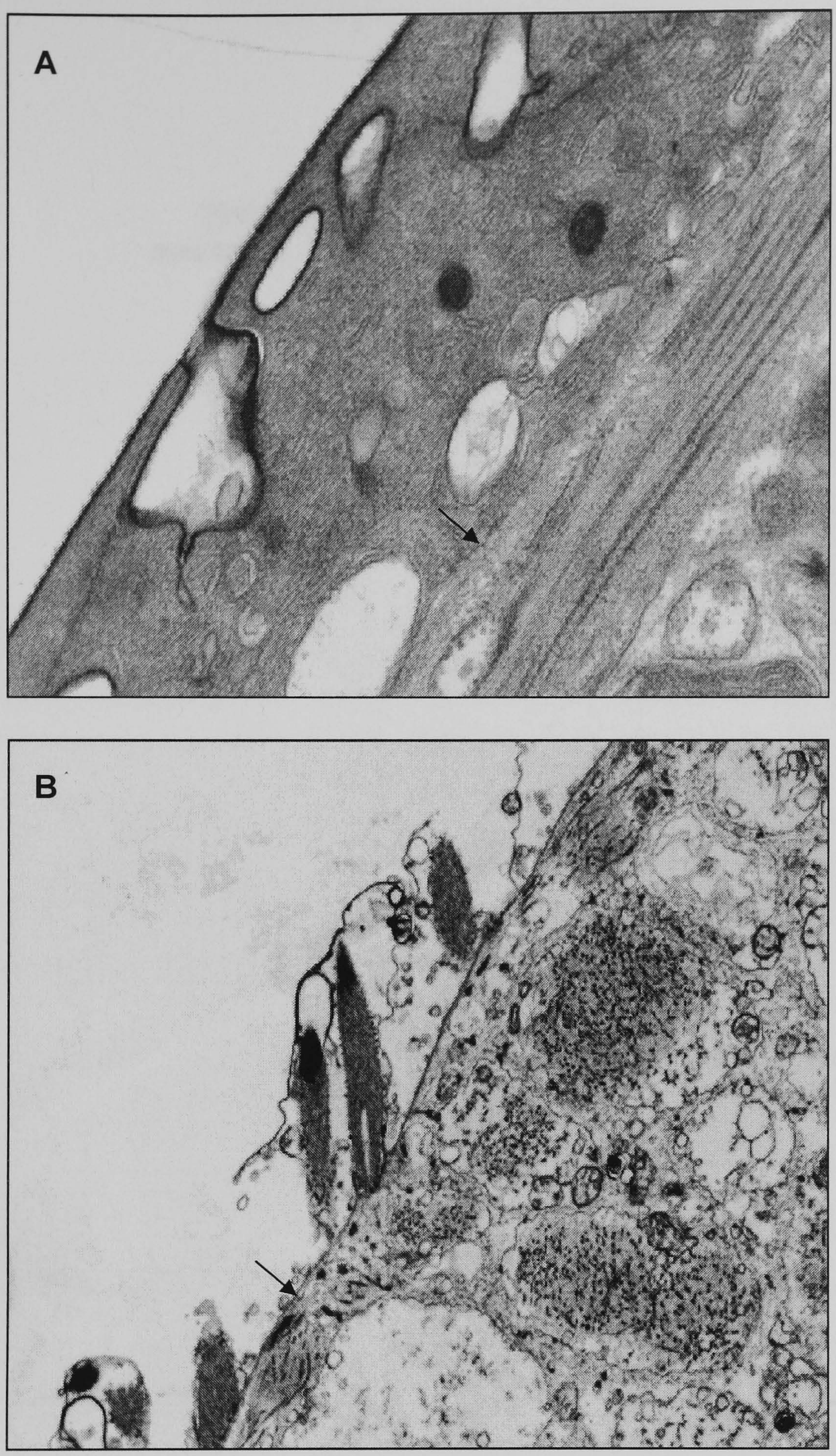
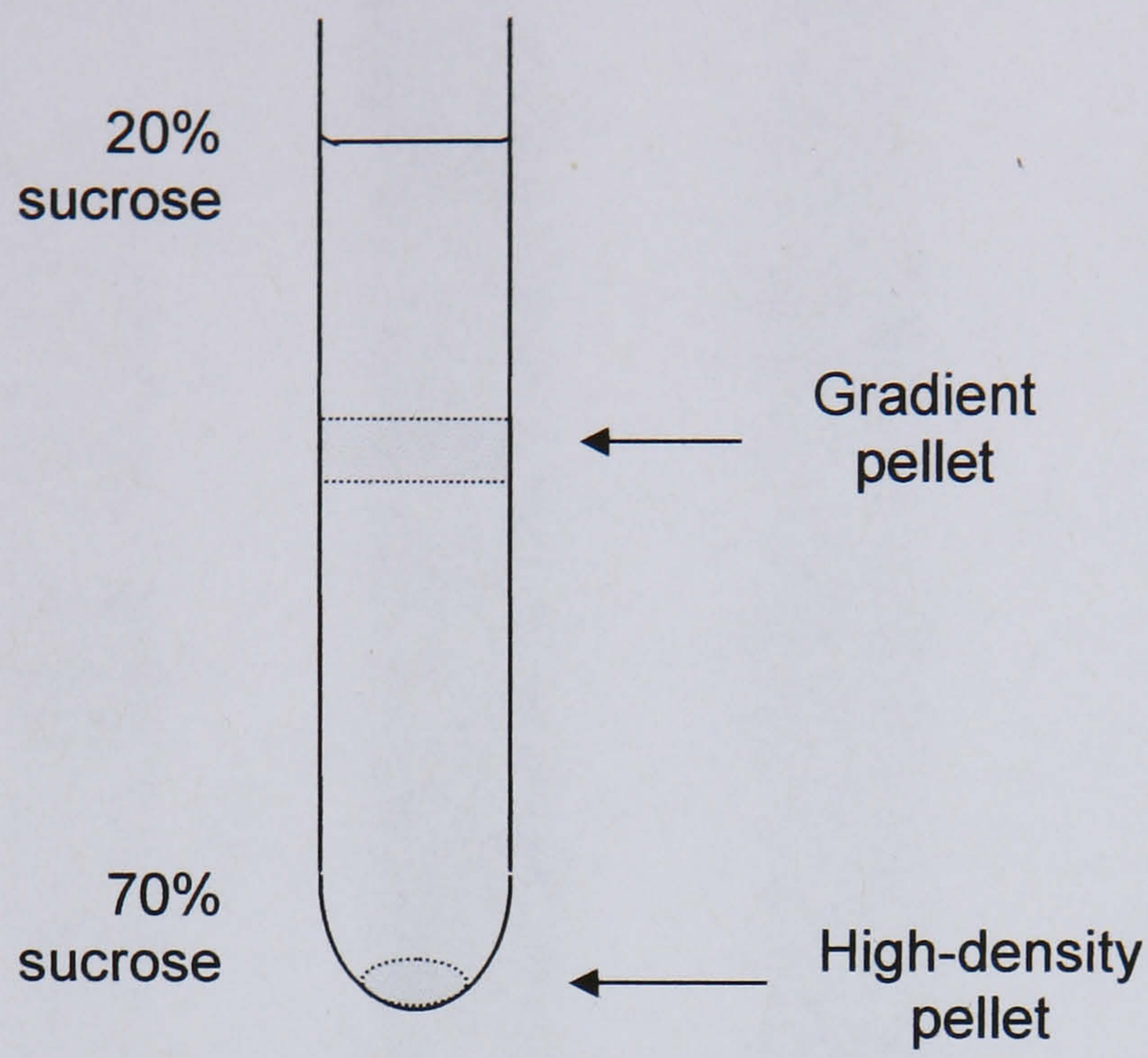


Figure 3.01. Transmission electron micrographs of a normal adult tegument (A) and a worm denuded by the freeze/thaw/vortex process (B). The arrows point to the basement membrane of the tegument.

A



B

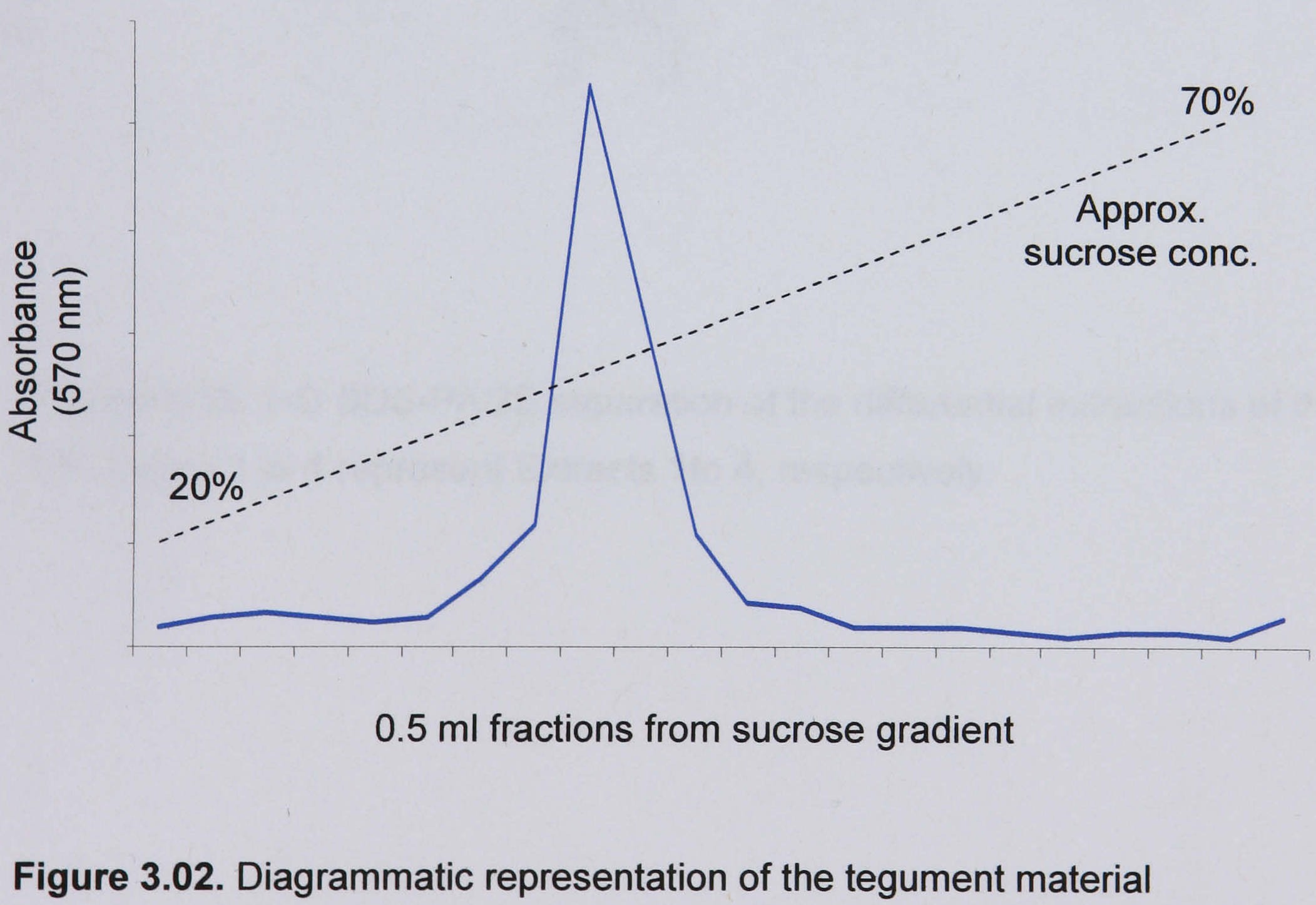


Figure 3.02. Diagrammatic representation of the tegument material separated by sucrose gradient centrifugation (**A**). Alkaline phosphatase assay of 0.5 ml fractions taken from the sucrose gradient (**B**). Those with the highest activities were pooled to yield the starting membrane preparation.

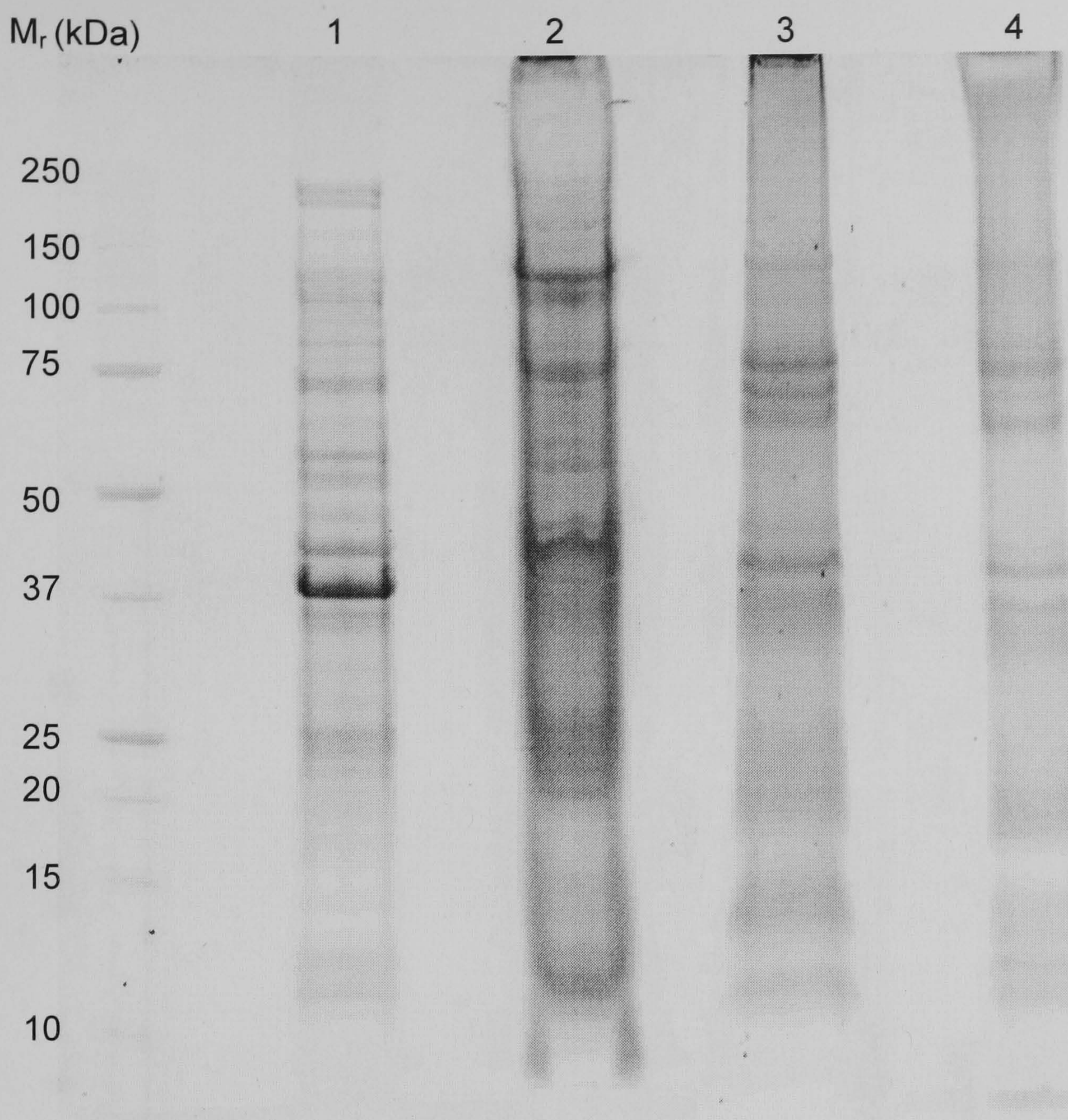


Figure 3.03. 1-D SDS-PAGE separation of the differential extractions of the GP. Lanes 1 to 4 represent Extracts 1to 4, respectively.

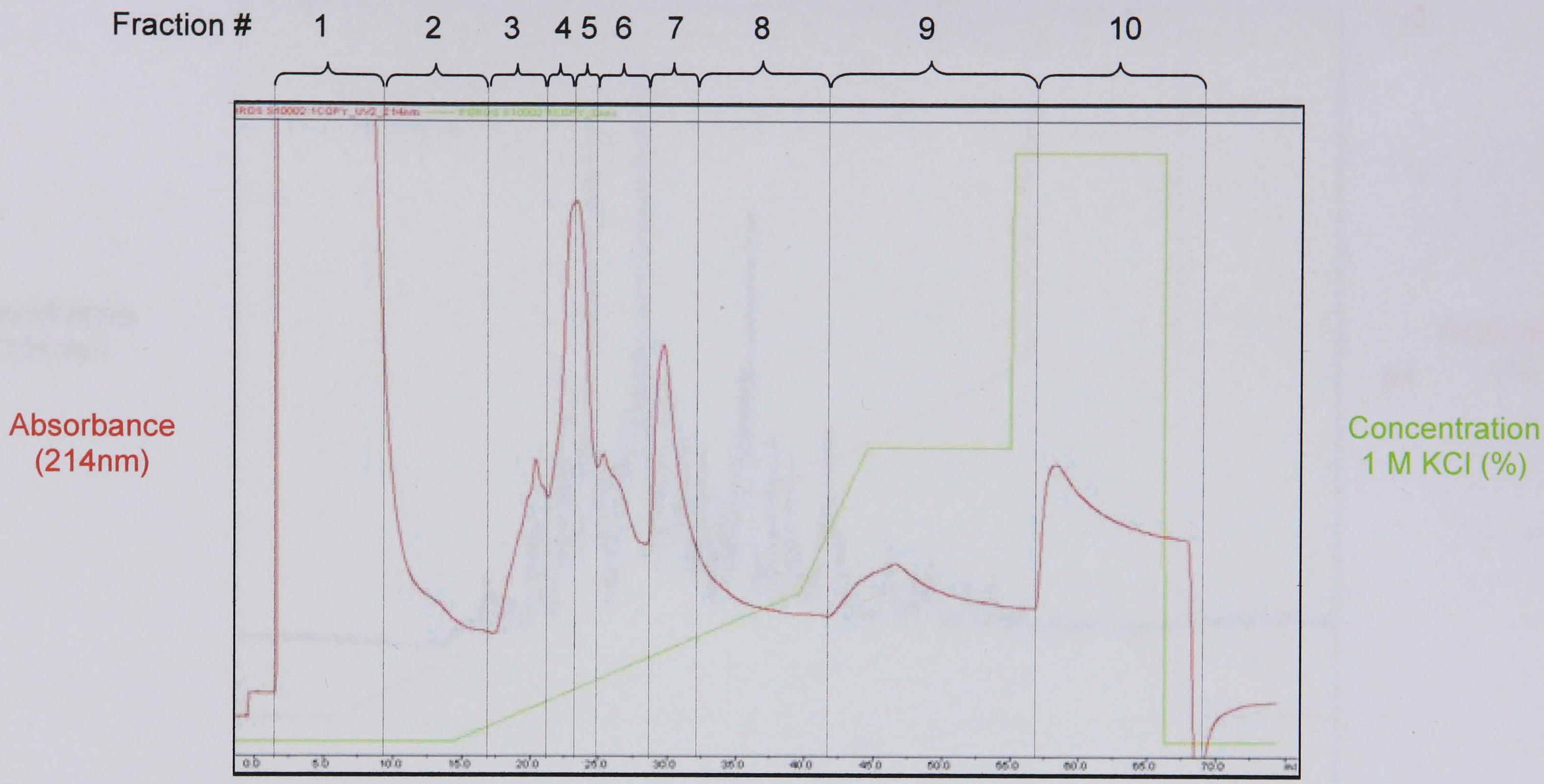


Figure 3.04. UV trace of the Strong Cation Exchange separation of tryptic peptides in Extract 4. Each fraction was then desalted and subjected to reversed-phase chromatography followed by tandem mass spectrometry.

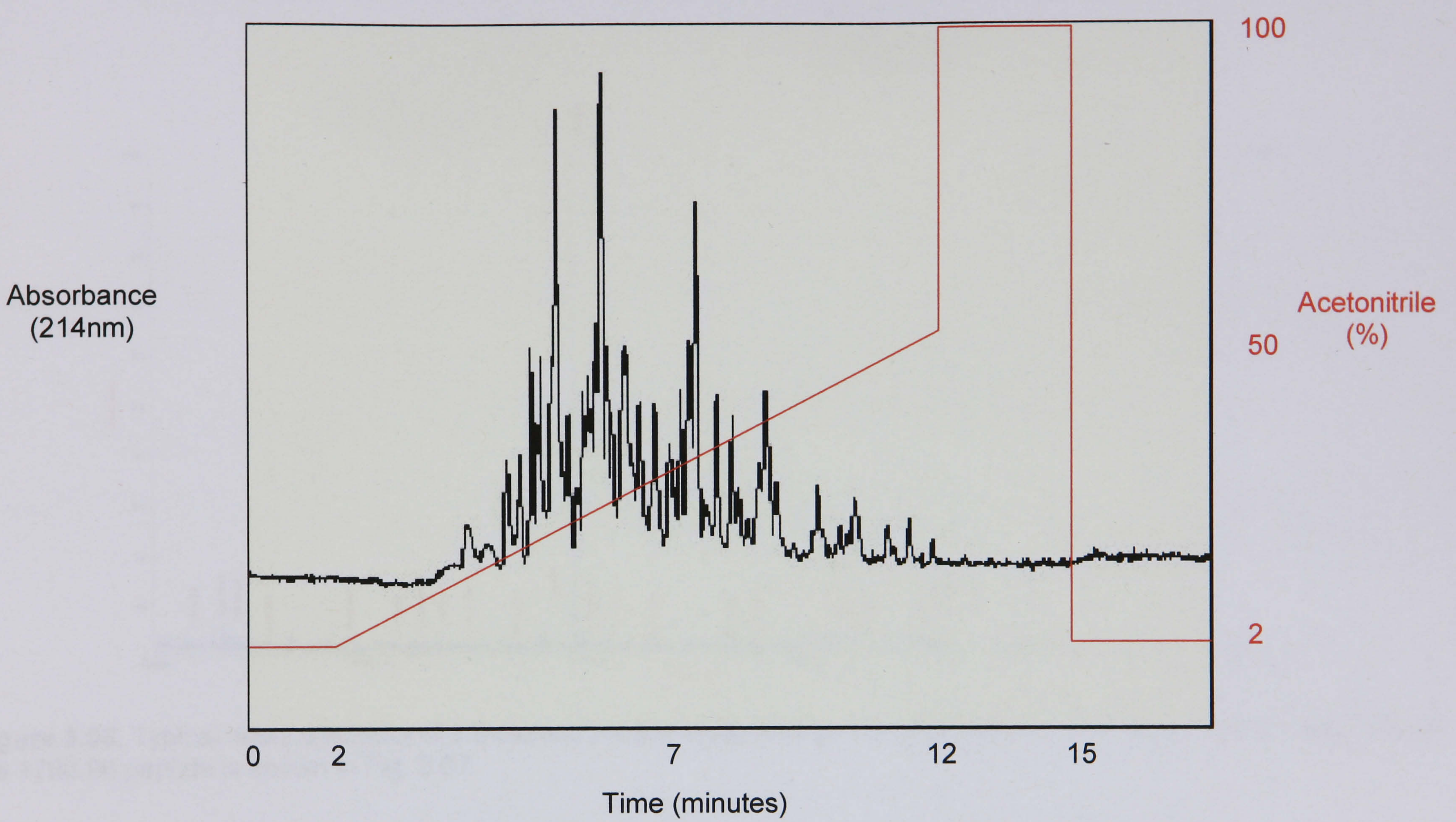


Figure 3.05. A typical UV trace of peptides separated by reversed-phase chromatography on a Monolith column.

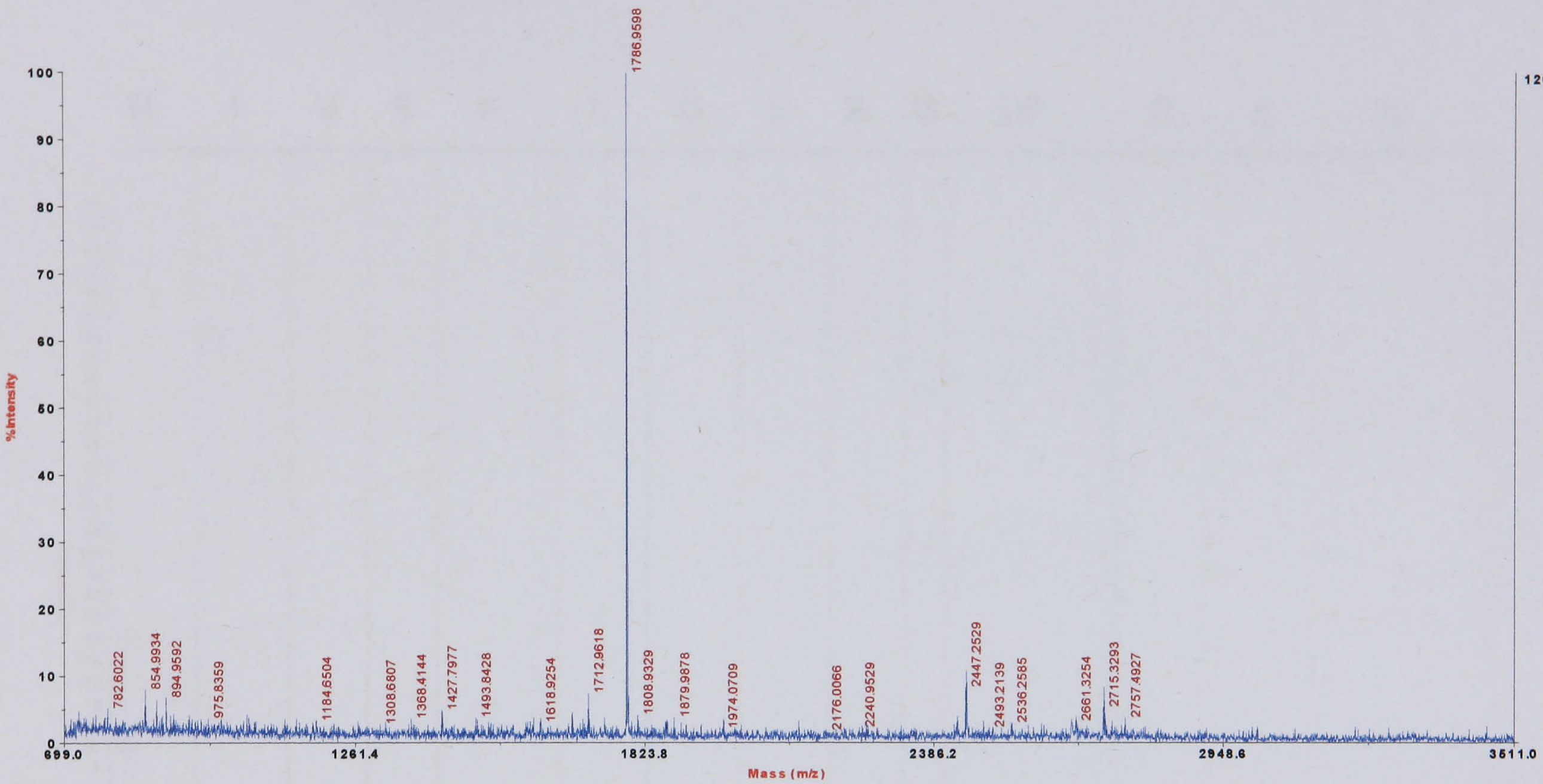


Figure 3.06. Typical mass spectrum of a 5-second fraction eluted off the Monolith column. The fragmentation spectrum of the 1786.96 peptide is shown in Fig. 3.07.

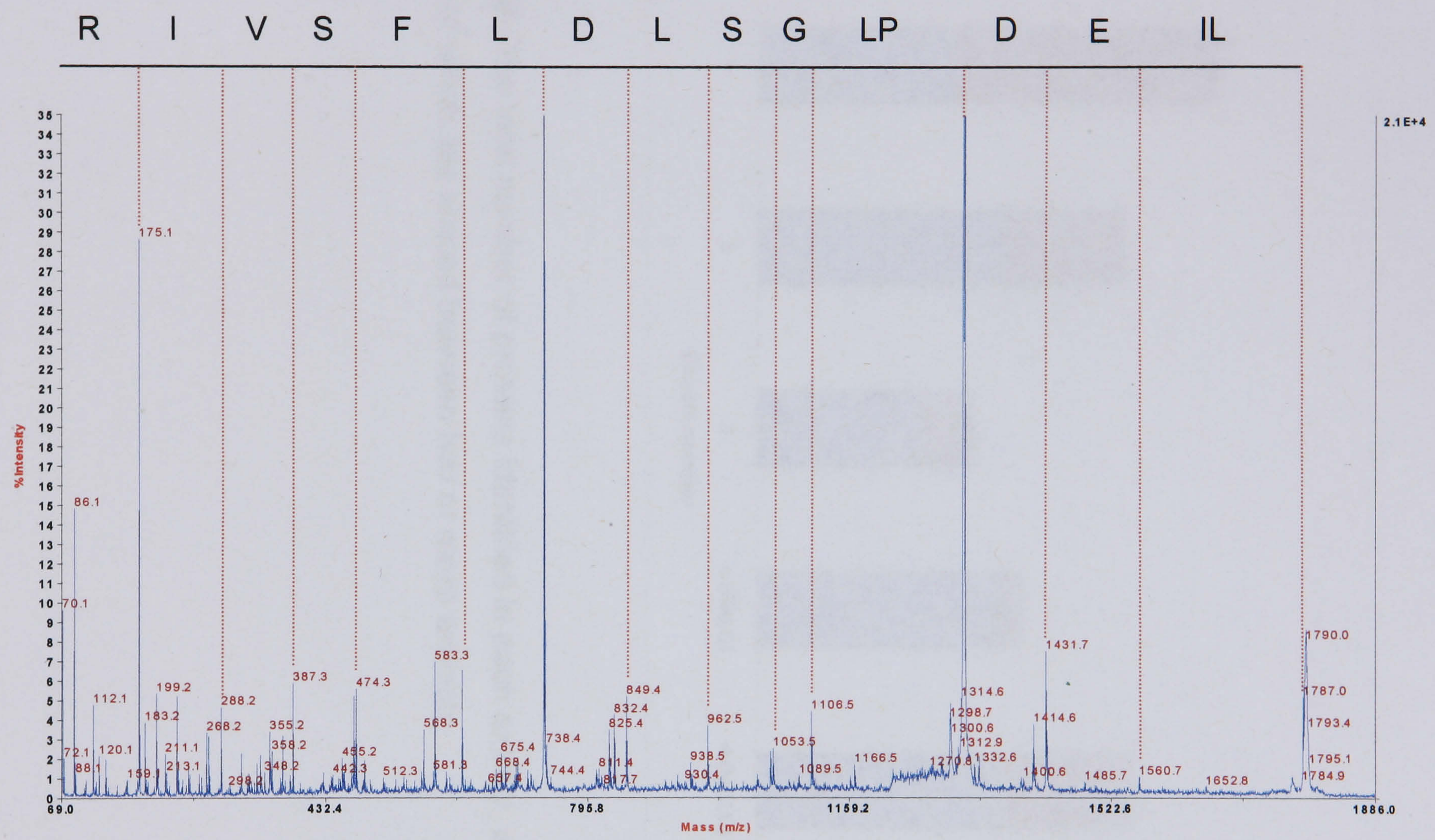


Figure 3.07. Fragmentation spectrum of the peptide LIEDPLGSLDLFSVIR from ATP-diphosphohydrolase 1. The Y-ion series are represented by the red dotted lines.

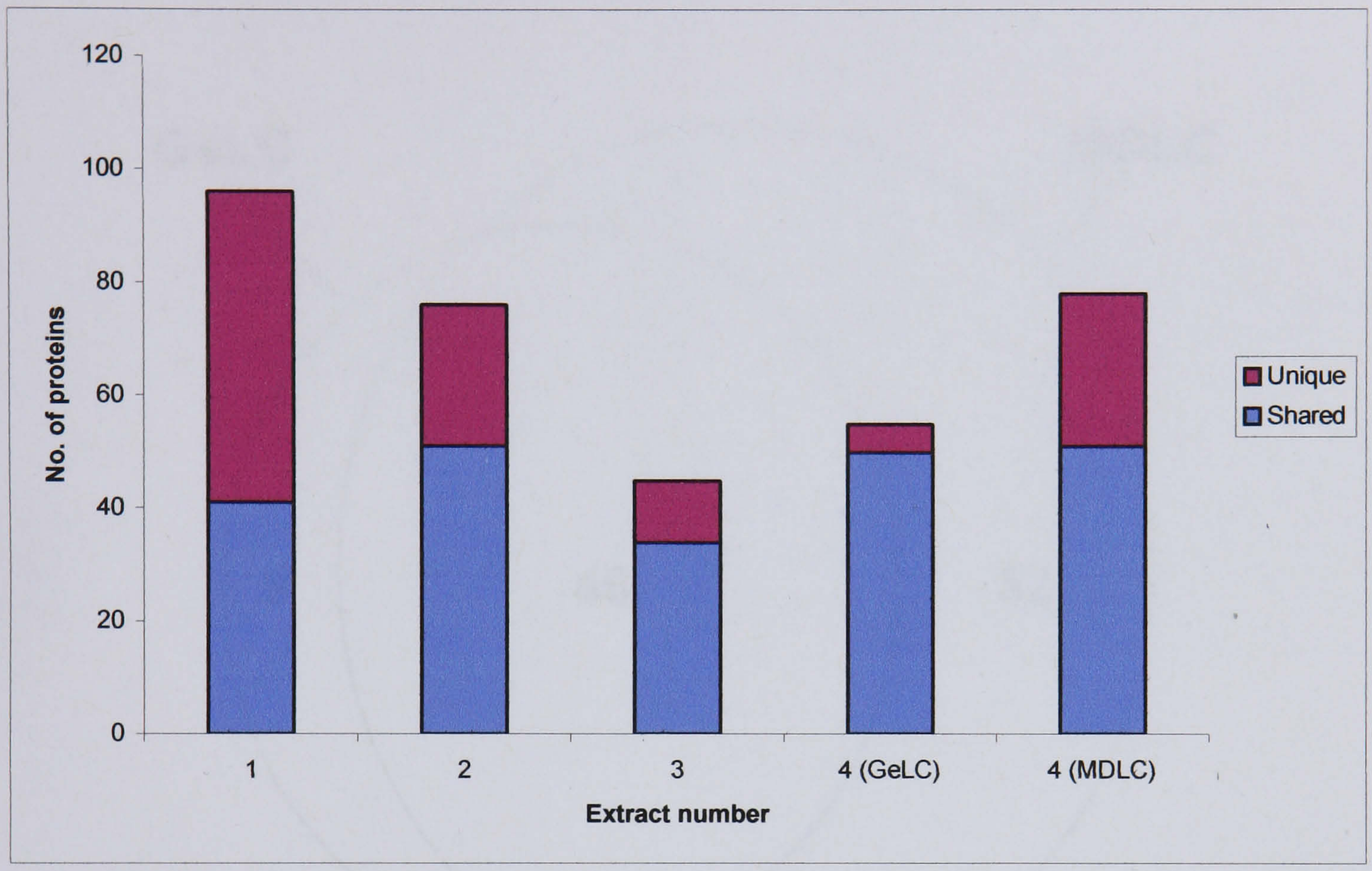


Figure 3.08. The total number of proteins identified in each extract, and the proportion of which are shared between two or more extracts.

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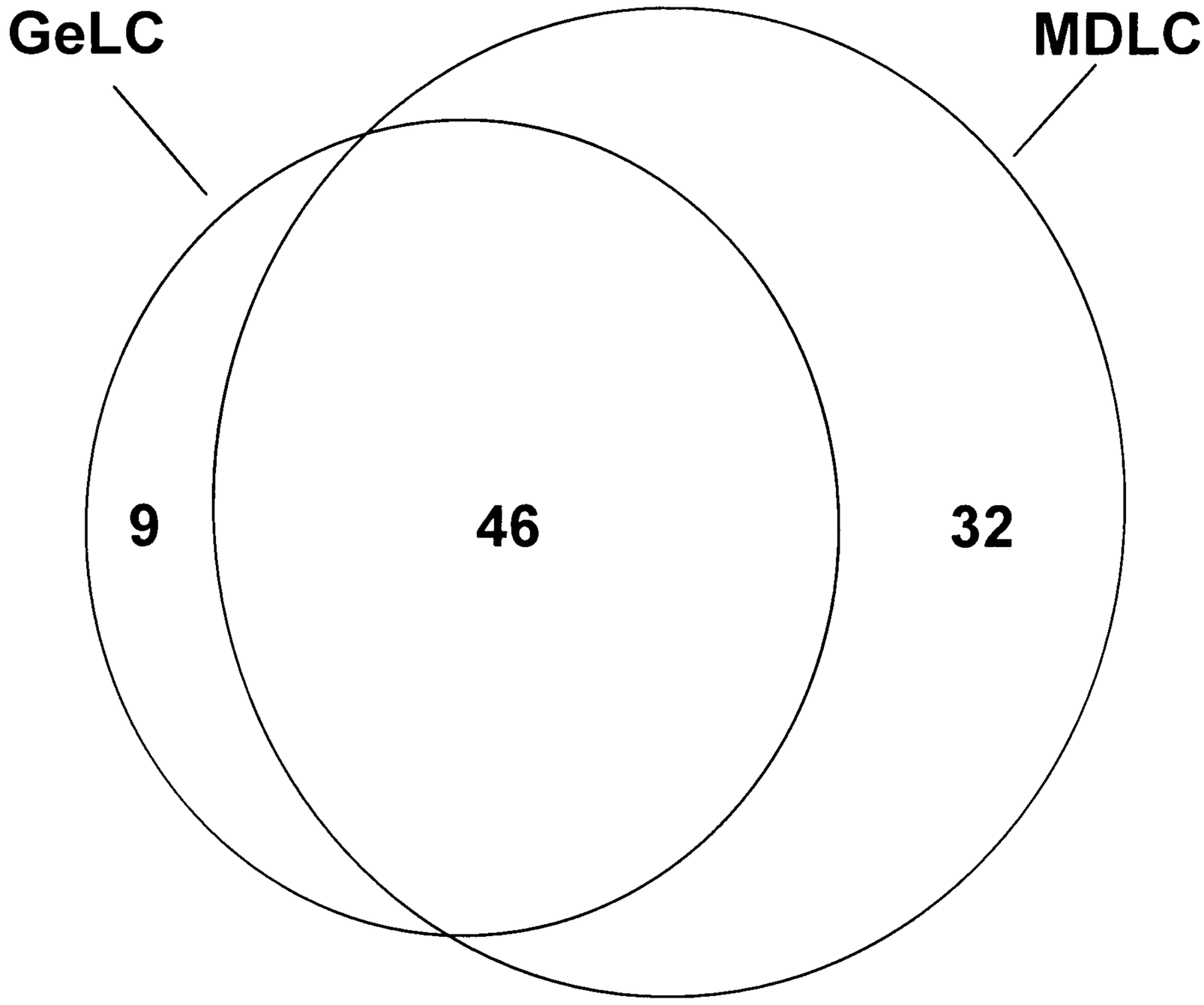

Figure 3.09. Venn diagram showing the overlap of proteins identified in Extract 4 by the two different LC-based methods. A total of 86 proteins were identified, 9 and 32 of which were discovered only by GeLC- or MDLC-MS/MS, respectively.

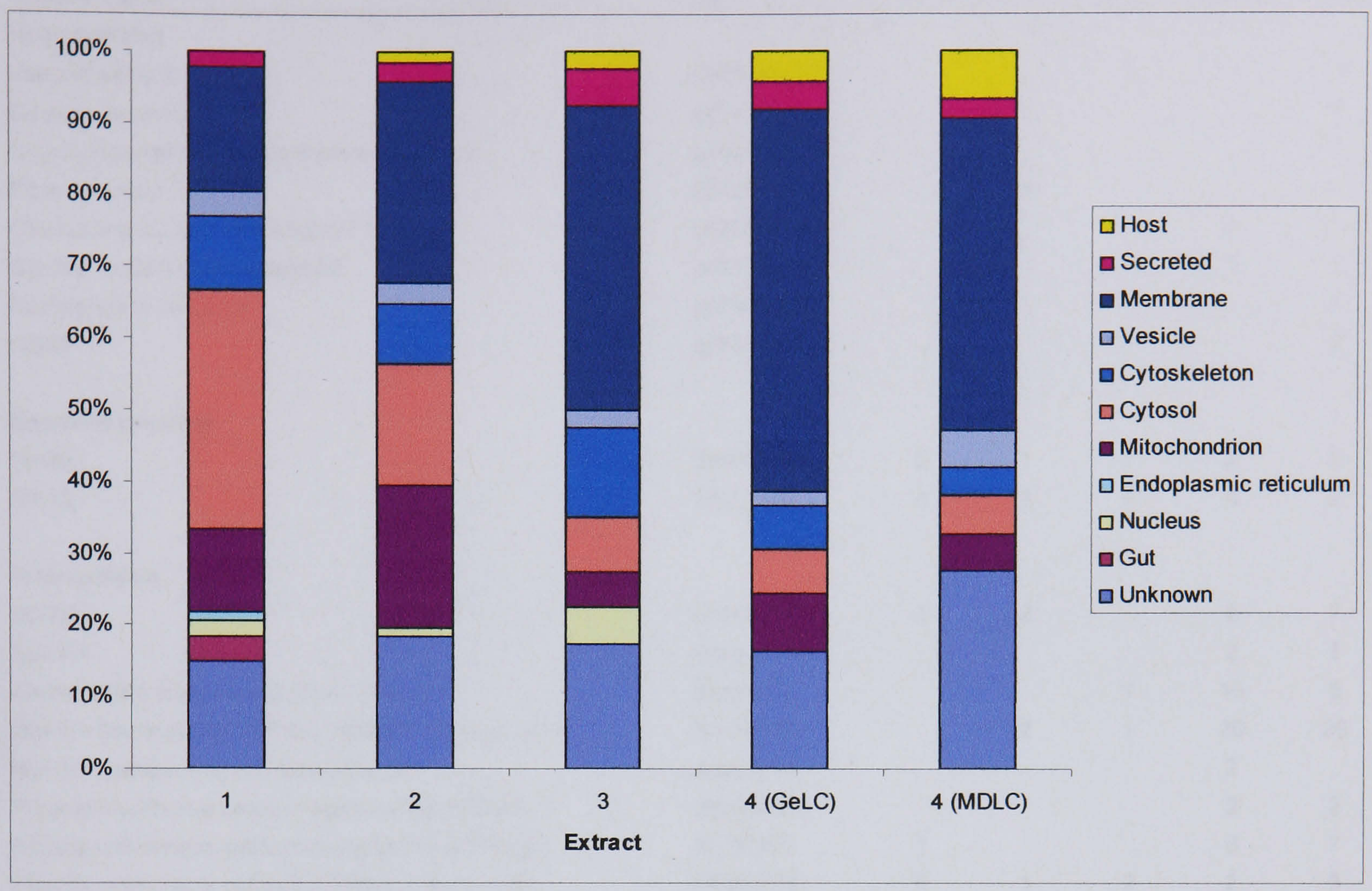

Figure 3.10. Distribution of the putative location of proteins identified in the different extracts of the GP.

Table 3.1. Protein identities of the GP

Protein name	Accession no.	No. of peptides Ex 1	Ex 2	Ex 3	Ex 4a	Ex 4b
Host proteins						
Integrin alpha 2	gi|41054731			1		
C4 complement	gi|387438					1
Crry complement regulatory protein Chain B	gi|42543136					1
Fibrinopeptide B	gi|229334		1			
Chemokine (C-X-C motif) ligand 11	gi|33636734				1	
Glycine receptor betaZ subunit	gi|11322386				1	1
Acetylcholine receptor	gi|191613					1
CD44	gi|53674					2
Secreted proteins						
Sm29	Sm09193	2	1	1	2	2
Sm13	Sm12315	4	3	4	4	2
Transporters						
SGTP1	Sm11978	1	2		2	7
SGTP4	snap43116				2	1
Amino acid transporter SLC3A1 (rBAT)	Sm01225			1	10	8
Na+/K+ transporting ATPase (SNaK1) alpha subunit	Sm07319		2	1	20	26
Na+/K+ transporting ATPase beta 1a	Sm00144				2	
Plasma membrane calcium-transporting ATPase 2	Phat02905				2	2
Plasma membrane calcium-transporting ATPase 3	Sm02430	1			5	7
Plasma membrane calcium ATPase 3 isoform 3b	Phat08744	6	1	2	2	9
Cation-transporting ATPase 3	Sm27215					3
Voltage-dependent anion channel	Sm00707		4	1	5	7
Copper transporter	Sm03357					2
Serotonin transporter 4	snap28998					1
Aquaporin 3	Sm03693				5	7
Membrane structural proteins						
Tetraspanin A	Sm12172		1		1	1
Tetraspanin B (Sm23)	Sm02886				2	1
Tetraspanin C (TE736)	Sm04463				2	2
Tetraspanin D (CD63-like protein Sm-TSP-2)	Sm12366	7	3	4	6	6
Tetraspanin E	Sm07392				2	1
Dysferlin	Phat08157	8	10	15	14	14
Annexin 11a, isoform 2	Sm00204	3	5	3		2
Annexin VI	Sm03987	12	10	15	10	14
Sorcin	Sm12950		3		1	2
Phospholipid scramblase 1	Sm12654	1	1	1	2	3
Phospholipid-transporting ATPase IA	Phat05022					2
Phospholipid-transporting ATPase IG	snap28221		1			
Phospholipid-transporting ATPase IIB	Sm19989		1		1	2
Aminophospholipid transporter ATPase, type 8A, member 1	Sm00376					1
Multidrug resistance protein 2	snap04278					1
Syndecan binding protein (Syntenin)	Sm12683	1	1			

Protein name	Accession no.	No. of peptides				
		Ex 1	Ex 2	Ex 3	Ex 4a	Ex 4b
Membrane enzymes						
ATP-diphosphohydrolase	Sm12745		1		6	9
Phosphodiesterase	snap05486			9	6	8
Alkaline phosphatase	Sm00962		1	6	6	10
Calpain-B	snap25325	1	1	1	1	1
Acetylcholinesterase	snap16831			1		
Amidase	Phat07749					3
Membrane other						
Gp18-22 (Sm25)	Sm04760	1	2	2	2	2
200 kDa Surface protein	Sm03865	15		3		
Fibronectin type III	snap20471	1				
DeltaB	glimmer04896	10				
Ras-related protein Rap-1b (GTP-binding protein smg p21B)	Sm04550	3	2			
Vesicular pathway						
Vacuolar-type H(+)-ATPase	Sm08744			1		1
Secretory carrier membrane protein 2	snap23630					1
Synaptotagmin II	snap28168					1
SV2 related protein	snap19043					1
Synaptobrevin	Sm11347		1			
Endophilin	Sm00115	1				
Ras-related protein Rab-27B	Phat08796	1	1		1	
Sm50 protein	snap19425	1				
Endoplasmic reticulum						
RTN4-N	Sm03117	2				
Long-chain-fatty-acid--CoA ligase 5	Sm02502	1				
Cytoskeleton						
Actin	Sm01276	14	3			
Alpha Spectrin	snap34399	2				
Myosin head (motor domain)	Sm00173	36	1			
Myosin heavy chain	Sm12294	21	1	2	2	2
Dynein light chain 1	Sm00765	3	3	2		
Dynein light chain 2	Sm00371	4				
Sm20.8	snap26948	3	3	1	1	2
Sm22.6	Sm12876	3	3	1		
Severin	Sm04123	8				
Twitchin	snap13575			1		
Rac GTPase	Sm06722				1	1
Cytosolic						
Glutathione S-transferase 26 kDa	snap31662	3				
Glutathione S-transferase 28 kDa	Sm02267	5				
Glutathione S-transferase omega	snap11466	1				
HSP4	snap28164	1				
HSP10	Sm00296	6	1			

Protein name	Accession no.	No. of peptides				
		Ex 1	Ex 2	Ex 3	Ex 4a	Ex 4b
Cytosolic (continued)						
HSP60	snap11876		1			
HSP70	glimmer11688	4		1		
HSP86	Sm19149	2				
HSP90beta	Sm01524	9				
Major egg antigen (P40)	Sm01604	2				
Fructose-bisphosphate aldolase	Sm05684	8				
Enolase	Sm12193	3				
Glycogen phosphorylase	Sm12571	2				
Polyubiquitin	snap31919	1		1	3	4
Thioredoxin peroxidase 1	glimmer09070	2	1			
Thioredoxin peroxidase 2	snap13087	2				
Thioredoxin peroxidase 3	Sm00674	4	3			
Triosephosphate isomerase (TIM)	Sm00999	4				
Gamma-glutamyltransferase 4 precursor	snap02165				1	3
Aldehyde dehydrogenase	Sm03220					1
Lactate dehydrogenase-like protein	Sm11814	2				
Fatty long (79.0 kD)	snap04412			2	2	4
Casein kinase II, alpha chain	Sm01487	1	1			
14-3-3 protein homolog 1	Sm12452	6				
Calcium binding protein P22	Sm03738		1			
Dimethylargininase	snap14559		1			
Major vault protein	Sm01538	5				
Peptidyl-prolyl cis-trans isomerase	Sm12777	2	1			
Phosphoenolpyruvate carboxylase	snap05285	3				
Phosphoglycerate mutase	Sm03441	2				
Carbonate dehydratase	snap23233		1			
Carbonic anhydrase 4	Sm04975					1
Recombination protein recU	glimmer11810	1				
SH3 domain GRB2-like protein B1	Sm04606	4	3			
Methionine aminopeptidase	Sm12792				1	
Similar to Drosophila melanogaster ctp (Fragment)	Sm05446	1	1			
Phospholipase C-like 2	glimmer01136				1	
Valosin-containing protein	snap34604	6				
Islet brain 1 (IB1) protein	Sm04825	2	2			
Gut proteases						
Cathepsin B endopeptidase precursor	Sm01349	2				
Cathepsin B1 isotype 2 precursor	Sm12907	1				
Sm32 Hemoglobinase precursor	Sm00989	2				
Mitochondrial						
Citrate Synthase	snap21539	7				
Aconitate hydratase	Sm07278	7				
Aspartate aminotransferase, mitochondrial	Sm03863	5				
ATP synthase beta chain, mitochondrial precursor	Sm00685		2			
glycerol-3-phosphate dehydrogenase	Phat09808			1	2	3
NADH dehydrogenase	Sm05963		1			
Cytochrome c	Sm06370		1			

Protein name	Accession no.	No. of peptides				
		Ex 1	Ex 2	Ex 3	Ex 4a	Ex 4b
Mitochondrial (continued)						
Lon protease homolog, mitochondrial precursor	Sm10045	2				
Ornithine aminotransferase	Sm01046	1				
Malate dehydrogenase	Sm00493	7	1			
Isocitrate dehydrogenase	snap25132	2				
Oxoglutarate dehydrogenase	Sm04501	4				
Porin 31HM	Sm03202					1
H(+)-transporting ATP synthase beta chain	gi|23100430		1			
Pyruvate/2-oxoglutarate dehydrogenase	gi|23126923		1			
Succinate dehydrogenase Ip subunit	Sm03958		2			
Succinate-Coenzyme A ligase	snap04740	2				
Suclg1 protein	Sm00415	2				
ATP synthase F1, beta subunit	gi|39995224		1			
Glucose/ribitoldehydrogenase	Phat11699		1			
NADH-cytochrome b5 reductase	Sm12752		4	2	2	9
Procollagen-lysine, 2-oxoglutarate 5- dioxygenase 2, short	Sm06485		1			
Metalloprotease 1	Sm04516	5				
Glycerol-3-phosphate dehydrogenase 2	Sm08702		1		2	
ADP/ATP carrier	Sm01758		4		2	8
Prohibitin protein Wph	Sm05391		3			
Nuclear						
TBP-associated factor 172	Sm06265			1		
SMC4 protein	snap41477			1		
Transposase	snap30562		1			
Elongation factor	Sm05188	2				
***S. mansoni* specific**						
Unknown	snap14504					1
Unknown	Sm03376					1
Unknown	Sm12238		1			
Unknown	Sm01352	3	1		2	4
Unknown	Sm02396	4	6			
Unknown	Sm11042	8	3	4	2	2
Unknown	Sm13096	4	5		7	8
Unknown	Sm13027	2		3	4	5
Unknown	Sm03630	2	1			1
Unknown	Sm00749	1	1		2	1
Unknown	Sm11921				2	4
Unknown	snap20271		4			
Unknown	snap06914	5	1	1		
Unknown	Sm00310	2				
Unknown	Sm05431	1				
Unknown	Sm00215	1			1	
Unknown	snap38731			2		
Unknown	Sm00745					4
Unknown	snap14644					2
Unknown	Sm03911		1		1	
Unknown	snap30245		1			

Protein name	Accession no.	No. of peptides				
		Ex 1	Ex 2	Ex 3	Ex 4a	Ex 4b
***S. mansoni* specific (continued)**						
Unknown	Sm02627		1			1
Unknown	snap07691	1				1
Unknown	Sm14946	1				
Unknown	snap09038	1		1		
Unknown	snap27117	1				
Unknown	snap09428	1				
Unknown	glimmer10923	1		1	1	1
Unknown	Sm25602	1				
Unknown	snap03363		1			
Unknown	snap36217		1			
Unknown	Sm03716		1	1		
Unknown	Phat08522		1			
Unknown	Sm00364		1	1		
Unknown	snap34981		1	1		
Unknown	Sm23574					1
Unknown	Sm22301					1
Unknown	Sm14376					1
Unknown	snap16387					1
Unknown	snap05600				1	
Unknown	snap04788				1	1
Unknown	snap29745			1		
Unknown	snap21379				1	1
Unknown	Sm13630			1		
Unknown	glimmer04832	1				
Unknown	glimmer16638		1			
Unknown	Sm09712			1		
Unknown	Sm19579			1		
Unknown	snap16125			1		
Unknown	Sm12256				1	1
Unknown	snap01199					1
Unknown	snap41466					1
Unknown	Sm12884	3				

Extracts 4a and 4b represent the SDS-soluble proteins, identified by GeLC- or MDLC-MS/MS, respectively. The numbers in each cell represent the numbers of peptides identified from a given protein.

3.4 Discussion

The purpose of this work was to delve deeper into the protein constituents of the tegument surface membranes using a larger starting preparation and more sensitive identification techniques than in Chapter 2. Particular attention was given to the protein constituents in Extracts 1-3, as it was believed that many of these were not identified in the previous chapter. The amount of starting material was increased and 2-D gels were not used as a means of protein separation prior to mass spectrometry. Instead, all extracts were processed by LC-based methods to gain better coverage of the different protein species. Discussion of the tegument removal and membrane isolation by the freeze/thaw/vortex procedure and sucrose gradient, respectively, is covered in Chapter 2, and therefore is not dealt with further in this chapter. Similarly, as expected there is a large degree of overlap with the proteins identified in both chapters, so the reader will be referred to the previous chapter when necessary, to avoid repetition.

3.4.1 Shotgun proteomics to identify tegument membrane proteins

More than double the number of individual protein identities was obtained in this study compared to the previous chapter. This was particularly evident in Extract 3, where the number of proteins identified rose from 8 to 45 between the two studies. It is intriguing that Tris and urea/thiourea were able to solubilise so many transmembrane proteins, a phenomenon that was not seen in Chapter 2. Although many of the identities may have arisen from soluble peptides derived from proteolytically-cleaved membrane proteins, several cases (*e.g.* Tetraspanin D) were identified in Extracts 1 and 2 by a range of peptides throughout the protein sequence. It is possible that membrane proteins were solubilised by Tris, urea and thiourea (without detergents) in Chapter 2 but they failed to be resolved by 2-D electrophoresis, probably due to precipitation problems during IEF (Lescuyer et al., 2003).

Shotgun approaches using LC-MS/MS provide an efficient alternative to 2-D gels in the identification of hydrophobic proteins (Wu and Yates, 2003), but suffer from their limitations in the ability to quantity proteins. The chromatographic traces generated by the separation of peptides are often irreproducible, extremely complicated, and each peak may contain more than one peptide, making the comparison of two samples very difficult. Likewise, each band on a 1-D gel is often comprised of several different proteins, leaving any change in banding pattern impossible to interpret in terms of differential protein

concentration. Although protein quantification is not the priority of this research, it would be interesting to know which of the proteins identified on the schistosome surface are the most abundant. Indeed, an anti-schistosomal drug would presumably be more efficient if its target was plentiful on the parasite surface (Camacho et al., 1994). The number of peptides identified from each protein in the various extracts could reflect their relative concentrations, but such an interpretation must be made with caution due to the differences in sequence length and distribution of tryptic cleavage sites. Furthermore, the extent of ion suppression is unlikely to remain constant between samples, especially in complicated peptide mixtures (Cohen and Chait, 1996). New methods involving the differential labelling of protein or peptide mixtures with stable isotopes have provided a way to compare protein concentrations without the use of 2-D gels (Gygi et al., 1999; Ross et al., 2004; Beynon et al., 2005). One of these quantification techniques is currently being employed in this laboratory to determine the concentration of several carefully selected tegument membrane proteins, and is described in more detail in the Concluding Discussion (Chapter 6). But for now, no valid comments can be made about the relative quantities of the proteins identified in this chapter, and they will be discussed solely on their potential function at the schistosome surface.

The analysis of Extract 4 using two LC-based methods of separation (GeLC and MDLC) provides a way to compare the benefits and deficits of both techniques: a study that, to my knowledge, has not been published elsewhere. In terms of protein coverage, the in-solution digestion followed by MDLC separation of peptides proved to be more sensitive, identifying ~30% more proteins than by the GeLC method. Perhaps the denatured proteins in solution are more efficiently digested, or the limited recovery of peptides after in-gel digestion may be the major drawback in the GeLC protocol. Indeed, a more efficient method to enhance peptide retrieval from polyacrylamide gels by the addition of n-octyl glucoside to the extraction buffer has been reported (Katayama et al., 2001). Unfortunately, when this detergent was used in our laboratory, it interfered with reversed-phase chromatography, and caused the newly spotted samples to merge with each other on the MALDI plate, defeating the purpose of peptide separation. Despite a lower identification capacity, separation by GeLC has one extremely useful advantage over MDLC: the electrophoresed proteins can be visualised prior to digestion, from which inferences can be made about their molecular mass. This can be an extremely important tool in the interpretation of the status of the protein identified, as discussed for

Complement component C4 and CD44 (below). Ideally, a combination of GelC and MDLC separation would be used for every sample. Alas, this comes with high financial and time costs.

3.4.2 Proteins identified in the Gradient Pellet

3.4.2.1 *Host proteins*

Proteins of mouse origin were expected to be present in this study, based on the results of biotinylation experiments in which several immunoglobulins and Complement component C3 were discovered at the tegument surface (presented in Chapter 4). A similar array of proteins with immune functions were anticipated and perhaps a few more, due to the larger amount of starting material. Therefore, it was surprising that the only protein identified within this category was Complement component C4. Furthermore, the discovery of host "civilian" proteins, some of which have transmembrane domains or GPI anchors, on the schistosome surface was even more perplexing.

How do transmembrane host proteins transfer to the parasite surface?

The direct transfer of intact transmembrane proteins from a host cell to the schistosome surface is problematic due to the hydrophobic domain(s) of the protein. Wilson and Barnes (1974) speculated that the acquired surface molecules of red blood cells might be the direct result of ingested erythrocyte material that had been transported from the worm's gut to the tegument surface. Although this hypothesis was very attractive, no such transportation mechanism has been demonstrated. Furthermore, erythrocyte antigens can be transferred onto the surfaces of dead worms (Dean, 1974). Thus, it seems the schistosome plays no active part in the acquisition of host molecules. The fusion of leukocytes plasma membrane with the membranocalyx (Caulfield et al., 1980), or the acquisition of "blebs" from host cells (Pearce et al., 1990) have also been postulated as a mechanism by which the parasite covers itself in host molecules. I favour the views of Pearce et al. (1990), and with the aid of advances in cell biology, the following two paragraphs offer a more refined hypothesis on this remarkable transfer of transmembrane proteins from host to parasite.

Ectosomes (also known as microparticles or microvesicles) are small membrane-bound vesicles that are released from platelets, endothelial cells, vascular smooth muscle cells, leukocytes, lymphocytes and erythrocytes *in vitro*. The cytoskeleton of the parent cell is

disrupted by calpain (Miyoshi et al., 1996), and small (<0.1 μm in diameter) vesicles are budded off into the extracellular surrounding. Thus, ectosomes represent small patches of the plasma membrane (proteins included) of the cell from which they originated, sometimes specifically enriched in various molecules. Indeed, ectosomes released from human erythrocytes have a higher concentration of acetycholinesterase and decay accelerating factor relative to a normal erythrocyte surface (Butikofer et al., 1989) but are devoid of cytoskeletal components such as spectrin (Lutz et al., 1977). Their functions *in vivo* are still not fully understood, although they have been associated with coagulation, inflammation and vascular (dys-)function (VanWijk et al., 2003). Their release can be stimulated by bacterial antigens (Satta et al., 1994), activated Complement components (Pilzer et al., 2005), or induced by apoptosis (VanWijk et al., 2003).

The ectosomes released from host cells are likely to come into close proximity with the adult schistosome in the blood system, and it is plausible that these microvesicles fuse with the membranocalyx, rather like a bubble landing in soapy water. Thus, the parasite surface can be interpreted as a secreted bilayer which contains intermittent patches of intact plasma membrane from host cells. This model provides a possible explanation for the acquisition of host erythrocyte, endothelial, and leukocyte antigens by the parasite *in vivo*, and it allows for the acquisition of these molecules by dead worms (Dean, 1974). These intact "pieces" of host membrane would benefit the parasite in two ways; firstly, it would provide the worm with a disguise (albeit a patchy one), and secondly, the acquisition of fully functional membrane proteins such as Complement inhibitors and pore-forming complexes would protect the worm against immune attack and facilitate the transport of nutrients across the membranocalyx, respectively.

Complement component C4 and complement inhibitor Crry

Complement component C4 is a plasma protein that is activated by Ig-bound C1s (classical pathway) or carbohydrates on the pathogen surface (mannan-binding lectin pathway; MBL). The removal of C4a from C4 exposes a thioester that is able to bind covalently with amine or hydroxyl groups on the surface of the pathogen's cell membrane (Morgan and Harris, 1999). In this study, C4 was represented by a single peptide originating in the C4 alpha chain of the protein (not to be confused with C4a). Unfortunately, as this peptide was separated by MDLC rather than by an electrophoretic method, it is impossible to estimate the molecular weight of the protein from which it

originated; it may have come from the C4 precursor, the cleaved product (C4b), a complex of C2 and C4b (C4b2 or C4b2a) or from the inactivated C4b (iC4b). Because the protein was only detected in the SDS extract of the GP, one could argue that C4 had been activated and was firmly attached to the schistosome surface. If this is correct, it means that both the classical and MBL pathway could be responsible for the presence of Complement components on the tegument surface (the alternative pathway does not utilise C4). The absence of immunoglobulins in this study argues for the Complement activation by the MBL pathway. Indeed, lectin has been shown to attach to the adult schistosome surface *in vitro*, and is capable of Complement activation *via* the MBL pathway (Klabunde *et al*., 2000). However, further proteomic studies on the schistosome surface have revealed the presence of IgG and IgM (Chapter 4), arguing for Complement fixation *via* the classical pathway.

It is intriguing that the most abundant Complement protein, C3, was not found in this study. Peptides originating from C2 were also absent. If the Complement pathway ran to completion, C2 would bind to C4b to form the C3 convertase, C4b2a. C3 would then be cleaved, bind to the parasite surface, and initiate the lytic pathway. However, it is possible that Complement regulatory proteins (CRP) inhibit this attack process at several points along the pathway. One such protein was identified in this study, and it is referred to as Crry. This CRP is specific to rats and mice, and is found on all their endothelial, epithelial and all circulating cell surfaces (Morgan and Harris, 1999). Functional studies revealed that Crry has decay accelerating factor (DAF) and membrane cofactor protein (MCP) properties (Li et al., 1993). DAF interrupts the Complement pathway by the regulation of C3 convertases in two different ways. Firstly, the association between C4b and C2 is inhibited, and secondly, it promotes the dissociation of C2a from the C4b2a complex. MCP works together with *Factor I* and *Complement receptor 1* to promote the inactivation of C4b. Thus, Crry functions to thwart the Complement cascade after the C4b has bound to the membrane, and may explain why any downstream components of the Complement pathway were not found in this study. However, a fragment of C3 on the adult worm was identified by tandem MS (Chapter 4), suggesting that Crry does not completely block the Complement attack pathway.

Acetylcholine receptor, Glycine receptor and acetylcholinesterase

Two host neurotransmitter receptors were identified at the *S. mansoni* surface, both with opposing functions in the regulation of membrane potential. Upon binding of its ligand, acetylcholine receptor (AChR) briefly forms a channel in the membrane to allow the passage of cations (Na^+ and Ca^{2+}) through the bilayer. In a postsynaptic cell, where AChR has been extensively studied, the influx of cations depolarizes the cell, allowing the generation of an action potential. The glycine receptor (GlyR) is an inhibitory neurotransmitter, and opens anion (Cl^-, HCO_3^-) channels that lead to hyperpolarisation, thus preventing neural firing (Cascio, 2004).

AChR has long been documented in schistosomes (Hillman and Gibler, 1975) and was later localized to the tegument surface of the parasites using snake venom α-bungarotoxin, conjugated to fluoroscein isothiocyanate, and confocal microscopy (Camacho et al., 1995). As the snake venom binds to all nicotinic AChR, Camacho and others were unable to determine whether the receptor was derived from the parasite or the host. The *S. mansoni* genome does encode a sequence for the AChR, and its expression is increased when worms pair and become sexually active (Camacho and Agnew, 1995), which coincides with a notable increase in glucose uptake (Cornford and Fitzpatrick, 1987). *S. haemotobium* worms incubated in an increased concentration of acetylcholine results in the higher uptake of glucose *in vitro* (Camacho and Agnew, 1995). These authors therefore speculated that AChR may have a key role in nutrient uptake across the tegument surface. Mononuclear leukocytes, platelets and endothelial cells are potential sources of blood plasma ACh (Chuang et al., 1976) (Ikeda et al., 1994). If the proposed model of host membrane incorporation into the membranocalyx (above) is correct, then it is possible that host receptors, such as those found by proteomics in this study, which have been acquired by the surface of the parasite are still fully functional. It is appealing to consider that these host receptors could form pores in the membranocalyx, thus allowing the transport of ions through to the plasma membrane.

The change in membrane potential caused by the influx of ions through the AChR or GlyR may also play an important role in the attachment of the membranocalyx with the plasma membrane (discussed further in Chapter 5). Moreover, the affinity of glucose transporters (Parent et al., 1992) and Na K ATPase (Vasilets and Schwarz, 1993) can be influenced by changes in membrane potential. The functions of SGTP4 and SNaK1 may therefore be

affected by host AChR and GlyR within the membranocalyx. Needless to say, the acquisition of these host proteins could just be an artefact caused by the properties of the membranocalyx, and they serve no purpose at the schistosome surface. T-lymphocytes and erythrocytes possess AChR on their surface and may provide a likely source of the receptor within the mammalian host blood system (Richman and Arnason, 1979; Bennekou, 1993).

Acetylcholinesterase (AChE) is an enzyme responsible for hydrolyzing acetylcholine in synaptic clefts, and a GPI-anchored homologue has been localised at the surface as well as the musculature of adult worms (Camacho et al., 1996). It appears that one gene codes both proteins, but they serve different functions at their respective locations. The muscle AChE has a normal synaptic role, whereas the tegument version is proposed to modulate the interaction between host acetylcholine and the "schistosome" AChR (Camacho et al., 1995). It is puzzling that the parasite should acquire host AChR and GlyR, but produce its own AChE. This schistosome protein is GPI-anchored and is likely to have a large extracellular domain. Indeed, AChE has been proposed as a target for anti-schistosomal therapies such as metrifonate. This drug has been successfully used to treat *S. haematobium* and *S. bovis*, but its effect on *S. mansoni* was less successful, presumably because of the relative abundance of AChE on different parasite strains (Camacho et al., 1994).

Chemokine CXC ligand 11 (CXCL11)

The chemokine CXC ligand 11 (interferon-inducible t-cell a chemoattractant; ITAC) is secreted by monocytes and lymphocytes within the blood system, and is capable of binding CXC-receptor3 (CXCR3; Booth et al. 2004). This receptor is found on cytotoxic T cells, T helper 1 cells, activated T cells (Rojo et al., 1999), and it causes the release of calcium ions in activated T cells. Whether CXCL11 has been randomly acquired by the parasite, or has been captured by a specific receptor remains unknown. Given the identification of other host receptors at the parasite surface (above), it is also possible that host CXCR3 is present on the tegument, but at a concentration below the level of detection by tandem MS.

CD44

CD44 is a transmembrane glycoprotein found on numerous cell surfaces including endothelial cells and leukocytes, and plays a role in cell adhesion, migration and invasion (Cichy and Pure, 2003). CD44 also functions to control cell-cell and cell-matrix interactions *via* its affinity for hyaluronan, osteopontin, collagens and metalloproteinases in the extracellular matrix. A soluble form of CD44 has been discovered in serum and it is thought to be a secreted variety of the protein without a transmembrane domain. Alternatively, its detachment from the cell surface could be due to proteolytic cleavage. The two peptide hits to this protein were obtained by MDLC-MS/MS, so the size of the protein that they originated from could not be determined. It is also possible that the cleavage of CD44 from the host cell surfaces is caused by released schistosome proteases. Alternatively, this protein may have been acquired when the worms come into close proximity of host endothelium. The migratory stages through the tight capillaries of the lung, or the deposition of eggs in the mesenteric venules are two possible scenarios.

Integrin 2a

Integrins are a family of cell surface proteins that are involved in binding to extracellular matrix components and signal transduction. They are made up of two transmembrane proteins, termed the alpha and beta chains, which act together in the binding of ligands. The numerous combinations of the 17 different alpha and 8 beta chains enable the binding of specific proteins. As only the alpha 2 chain was identified in this study, the integrin on the schistosome surface could have originally served to bind collagen or laminin.

Fibrinopeptide B

Fibrinopeptides are ~20 residue-long fragments that are discarded from fibrinogen when it is activated to form fibrin during the blood clotting process. Their fate *in vivo* is still not fully understood, but it has been suggested that fibrinopeptide B may function to make thrombin more efficient in its clotting action (Blomback and Bark, 2004). Fibrinogen binds fibronectin (discussed below) as well as other proteins within the blood system, and this may explain why a fragment of this soluble plasma protein has been identified at the schistosome surface. Other researchers, where unable to detect fibrinogen in material release from adult worms over a four hour incubation (Gearner and Kemp, 1994).

3.4.2.2 Schistosome proteins

Membrane and secreted proteins

As well as searching for host proteins on the schistosome surface, this study sought to extend the coverage of membrane proteins of parasite origin. A total of 23 transmembrane or membrane-associated proteins were found in this study that had not been detected in Chapter 2. It was reassuring to identify SGTP4, the tegument's apical sugar transporter (Jiang et al., 1996), as well as other well-documented proteins at the tegument surface such as Sm13 (Abath et al., 2000), Sm25 (Wright et al., 1988), and 200 kDa surface protein (Sauma et al., 1991). Several other proteins of interest were discovered in this study that had not been previously reported to be constituents of the tegument surface. These included two addition tetraspanins (D and E), making a total of five different species of this protein superfamily identified, and providing further evidence for a key role, either collectively or individually, at the schistosome surface (discussed in Chapter 2). Tetraspanin D has been previously named "CD63-like protein" or "TSP-2" (Smyth et al., 2003), but its function is as yet unknown. The other tetraspanin new to this study bears the characteristic CCG motif and four transmembrane domains of the superfamily, but its purpose at the schistosome surface will only be determined by functional studies.

Phospholipid transporters

A series of proteins involved in phospholipid transport were identified in this study that had not been previously detected, and they may have important functions in membrane dynamics at the tegument surface. Flippases act as ATP-dependent transporters that "flip" phospholipids from the extracellular (or ER lumenal) leaflet to the cytosolic leaflet, whereas floppases facilitate the reverse action. Scramblases are activated by Ca^{2+} and permit phospholipids to flow down their concentration gradient to form a symmetrical bilayer (Daleke, 2003). Multidrug resistance protein 2 (MDR2) also has an essential role to transport phospholipids from the internal to external leaflet of the plasma membrane in mouse liver cells. The excess phospholipids in the outer leaflet cause them to be secreted into the bile duct where they form an essential part of bile (Smit et al., 1993). Increased expression of the *S. mansoni* MDR2 homologue (SMDR2) offers no protection to the antischistosomal drugs hycanthone or oxamniquine (Bosch et al., 1994), suggesting that this protein has an alternative role than drug resistance. Similar to MDR2 in mouse liver cells, perhaps SMDR2 functions to generate lipid asymmetry at the plasma membrane of

the tegument. However, instead of being shed into the host blood stream, perhaps the excess phospholipids are incorporated into the membranocalyx.

Delta B

The identification of Delta B at the schistosome surface has not been previously reported. This transmembrane protein acts as a ligand for Notch, and the two proteins play an important role in cell-cell interaction. Upon binding to Delta, the intracellular domain of Notch is cleaved and translocates to the nucleus where it stimulates the transcription of Notch, and down-regulates the expression of Delta (Iso et al., 2003). The relevance of Delta B on the schistosome surface is not clear, but it may serve to bind Notch on host cells. Furthermore, *S. mansoni* Delta B shares ~20% homology with mouse alpha-2 macroglobulin. This host protein has been detected at the schistosome surface using alpha-2 macroglobulin antibodies (see general introduction for details). Could it be that these antibodies were binding to a homologous domain of Delta B? It should be noted, of course, that this Delta B might not be located at the parasite surface, but could reside in the basal membrane of the tegument, and interact with cells below the tegument syncytium.

Fibronectin

The detection of fibronectin at the schistosome surface is supported by immunohistochemical studies using sera from rabbits that were immunised with human fibronectin (Ouaissi et al., 1984). However, Ouaissi and others suggested that the fibronectin was derived from the host, as the antisera employed also cross-reacted with hamster fibronectin. Based on the results of my proteomic experiments, it seems that tegumental fibronectin is synthesised by the parasite and not the host. Another study, using anti-fibronectin antibodies to probe Western blots of "eluted" material after a 4-hour incubation step in media, was unable to detect this protein (Gearner and Kemp, 1994). That the protein was not eluted into the surrounding media in a similar manner to immunoglobulins, suggests that it is firmly anchored to the parasite. These results give strength to the hypothesis that fibronectin is of parasite origin, and is not acquired from the host. Its role at the parasite surface is not yet known, but it may aid the schistosome's adhesion to the endothelium of the blood vessels or bind host Complement (Ouaissi et al., 1984). Similar to Delta B, fibronectin could have an internal location within the tegument. Indeed, Ouaissi and others perfused the hamsters with phosphate buffered saline: a

medium that damages the tegument surface (A. Wilson, personal communication). Thus, it is possible that specific antibodies employed by Ouaissi et al. were in fact binding to fibronectin below the apical bilayers.

Gut proteins and other non-membrane contamination

The identification of cathepsin B and Sm32 is cause for concern, as these proteases are almost certainly derived from the schistosome gut (Caffrey et al., 1997; Sajid et al., 2003). Although cathepsin L has been localized to the tegument in adult worms (Brady et al., 1999), there have been no such reports for cathepsin B. It is likely that gut contents could have leaked from adult worms during the vigorous actions of the freeze/thaw/vortex procedure, and co-purified with the membrane fraction on the sucrose gradient. Other potential sources of contamination include the underlying muscle layer and subtegumental cell body. Indeed, proteins representing muscle constituents were found, such as twitchin (Benian et al., 1996), and nuclear and ER proteins signified cell body constituents.

As argued in Chapter 2, the large number of cytoskeletal proteins identified in this "membrane preparation" reflects the high degree of intracellular organization at the tegument surface (Wilson and Barnes, 1974), and the strong associations of this cytoskeleton with the plasma membrane. Likewise the mitochondrial constituents of the GP reveal the co-purification of this organelle with the surface membranes, as seen in Chapter 2.

Schistosome specific proteins

Approximately 27% of all the matches to EST or genomic sequences in the *S. mansoni* database were to sequences that share no homology to any protein in the NCBInr database, a figure that is remarkably close to the 28% of *S. mansoni*-specific tegument proteins identified in another proteomic study (van Balkom et al., 2005). The proportion of schistosome-unique proteins has increased substantially from the previous chapter, where only 18% of the proteins identified were unmatched. As stated in Chapter 2, the more abundant proteins are more likely to have been characterised. Thus, the starting amount of protein in this LC-based study has been raised, and it seems that less abundant proteins are being identified in this study. The fact that approximately 55% of *S. mansoni* genes have no assignable function (Verjovski-Almeida et al., 2003) suggests that only when this

percentage is matched by proteomic data, can we claim to have the complete protein coverage of the organism.

3.4.3 Conclusions

The research presented in this chapter adds to the list of tegument proteins reported in Chapter 2 and by van Balkom et al. (2005), and has allowed valuable insights into the membrane constituents of the schistosome surface. The LC-based proteomics has proved invaluable for the identification of membrane proteins, and offers a valuable alternative to 2-D gels in terms of membrane protein coverage, despite the limitations in quantification. The identification of host proteins testifies to the high level of interaction between the two organisms, and argues for the activation (and subsequent inhibition) of Complement attack against the parasite. Moreover, the discovery of "civilian" host proteins on the schistosome surface provides evidence for the random acquisition of host antigens, possibly as a result of ectosome fusion with the membranocalyx. The schistosome membrane proteins identified in this chapter reveal the sophistication of the tegument surface, with proteins involved in nutrient transport, membrane structure and maintenance, vesicle fusion and cytoskeletal remodeling. However, there are likely to be many more proteins present at the tegument apex that have not been identified in this or previous studies. Likewise, there is no conclusive evidence to state which of the schistosome proteins discovered (if any) are components of the membranocalyx, or which are the most exposed on the parasite surface. Chapters 4 and 5 cover the labelling of these exposed proteins, and strategies to isolate the membranocalyx, respectively, prior to protein identification by tandem mass spectrometry.

Chapter 4

Proteins exposed at the adult schistosome surface revealed by biotinylation

This Chapter comprises a paper, as published in the journal *Molecular & Cellular Proteomics*

Proteins Exposed at the Adult Schistosome Surface Revealed by Biotinylation*[S]

Simon Braschi‡ and R. Alan Wilson

The human blood-dwelling parasite *Schistosoma mansoni* can survive in the hostile host environment for decades and must therefore display effective strategies to evade the host immune responses. The surface of the adult worm is covered by a living syncytial layer, the tegument, bounded by a complex multilaminate surface. This comprises a normal plasma membrane overlain by a secreted bilayer, the membranocalyx. Recent proteomic studies have identified constituents of the tegument, but their relative locations remain to be established. We labeled the most exposed surface proteins using two impermeant biotinylation reagents that differed only in length. We anticipated that the two reagents would display distinct powers of penetration, thereby producing a differential labeling pattern. The labeled proteins were recovered by streptavidin affinity and identified by tandem mass spectrometry. A total of 28 proteins was identified, 13 labeled by a long form reagent and the same 13 plus a further 15 labeled by a short form reagent. The parasite proteins included membrane enzymes, transporters, and structural proteins. The short form reagent additionally labeled some cytosolic and cytoskeletal proteins, the latter being the constituent of the intracellular spines. Only a single secreted protein was labeled, implying a location between the plasma membrane and the membranocalyx or as part of the latter. Four host proteins, three immunoglobulin heavy chains and C3c/C3dg, a fragment of complement C3, were labeled by both reagents indicating their exposed situation. The presence of the degraded complement C3 implicates inhibition of the classical pathway as a major element of the immune evasion strategy, whereas the recovery of only one truly secreted protein points to the membranocalyx acting primarily as an inert protective barrier between the immune system and the tegument plasma membrane. Collectively the labeled parasite proteins merit investigation as potential vaccine candidates. ***Molecular & Cellular Proteomics 5:•••–•••, 2006.***

The trematode *Schistosoma mansoni* is a long lived parasite of the human hepatic portal system, infecting millions of people in Africa and South and Central America (1). Infection of the mammalian host occurs by direct cercarial penetration through the skin followed by transformation into schistosomula. These then undertake a protracted intravascular migration to the hepatic portal vein where they begin to feed on blood, mature, and pair. The male carries the female up the mesenteric blood vessels to the gut wall where she begins to deposit eggs. A proportion of these pass through the tissue and reach the gut lumen to continue the life cycle, whereas others are washed downstream to the liver where they initiate the granulomatous inflammation that characterizes the disease. That schistosomes can survive for 30–40 years in humans (2) attests to their possession of an effective immune evasion strategy.

The mature worm is covered by a syncytial cytoplasmic layer, the tegument, attached to underlying cell bodies by narrow cytoplasmic connections. The nuclei, ribosomes, endoplasmic reticulum, and Golgi apparatus are located in these cell bodies, and their vesicular products, the discoid bodies and multilaminate vesicles, traffic to the tegument syncytium via the connections. Early ultrastructural studies showed that the apical surface of the tegument has a complex multilaminate appearance (3) that has been interpreted as a plasma membrane overlain by a trilaminate secretion, which was termed a membranocalyx by analogy with the glycocalyx of eukaryotic cells (4). The membranocalyx is formed when the bounding membrane of the multilaminate vesicle fuses with the apical plasma membrane of the tegument to release its sheetlike contents. These unfold and flow laterally across the plasma membrane. The membranocalyx has some unusual properties including the ability to sequester glycolipids from host erythrocytes (5). Although initial *in vitro* observations suggested a rapid turnover, *in vivo* studies using erythrocyte antigens as a marker indicated a half-life of approximately 5 days (6). Given a slow turnover, it is plausible that the membranocalyx provides a physical barrier protecting the underlying plasma membrane, which possesses normal cellular functions, from immune attack.

Methods were devised more than 30 years ago to detach the tegument surface complex for compositional analysis (7–9). However, despite separation of the protein constituents by electrophoresis (10, 11), little progress was made in obtaining their identities. The advent of proteomics has provided the tools to link proteins with their encoding cDNA. Simultaneously the generation of a large *S. mansoni* expressed sequence tag database (12) and release of the draft genome sequence (www.Schistodb.org) allow us to assign putative functions to many of the transcripts/genes. Two recent studies have reported on tegument protein composition. In the first, an inventory was compiled of proteins present in the

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whole tegument syncytium relative to the worm body (13), but the approach provided no information about the position of the constituents within the complex structure. In the second, we reported on the composition of a surface membrane preparation highly enriched by density gradient centrifugation and then subjected to a differential extraction procedure. We were able to identify many components and classify them into cytosolic, cytoskeletal, membrane, and secreted categories (14). However, it was not possible to determine the location of individual constituents within the surface complex and hence to say which are potentially accessible to the external environment of the parasite (including the host immune system). We report here the use of impermeant biotinylation reagents to label live adult worms *in vitro*. We then recovered the labeled proteins by streptavidin affinity for identification by tandem MS from which we inferred their relative accessibility. The results were interpreted in the context of immune evasion.

EXPERIMENTAL PROCEDURES

Biological Material—Details of schistosome isolate, life cycle maintenance, and recovery of 7-week-old adult worms were as described previously (14).

Surface Labeling of Live Worms—The scheme from parasite labeling through recovery of biotinylated proteins for MS is set out as a flow chart (Fig. 1). We first established the reaction conditions using BSA as a model protein before applying them to the parasite. Worms were washed five times in Hanks' balanced salt solution (Invitrogen), and any that showed damage were removed under 10× magnification. Two sulfo-NHS[1]-biotin reagents, differing in the length of their spacer arms (Table I), were used to label worms, namely sulfo-*N*-succinimidyl-6-(biotinamido) hexanoate (long form reagent; EZ-Link™ sulfo-NHS-LC-biotin) and sulfo-*N*-succinimidobiotin (short form reagent; EZ-Link sulfo-NHS-biotin; Pierce). For each experiment the parasite load from 40 mice, ~2000 worm pairs (no attempt was made to separate male and female worms), was incubated in 15 ml of Hanks' balanced salt solution containing 890 μM labeling reagent for 30 min at 4 °C with gentle agitation (15). The labeling solution was removed, and any unbound reagent was quenched using RPMI 1640 medium (Invitrogen) containing free amino acids. Worms were then washed three times in RPMI 1640 medium, plunged into liquid nitrogen, and stored at −80 °C with protease inhibitors (protease inhibitor mixture, Sigma). Uniformity of labeling and integrity of the surface membrane was examined by exposing a sample of live, labeled worms in RPMI 1640 medium to streptavidin-conjugated FITC (Sigma) for 30 min at room temperature. After incubation the worms were washed three times in RPMI 1640 medium and optically sectioned using a Zeiss LSM 510 meta confocal microscope (Zeiss UK Ltd., Welwyn Garden City, Herts, UK).

Tegument Removal and Differential Extraction—The tegument was removed from labeled worms by the freeze/thaw/vortex method (7), and the released membrane material was pelleted at 1000 × *g* for 30 min at 4 °C to yield the starting material. The pellet was subjected to five sequential cycles of extraction with 200 μl of different solvents of increasing strength followed by centrifugation at 100,000 × *g* for 1 h.

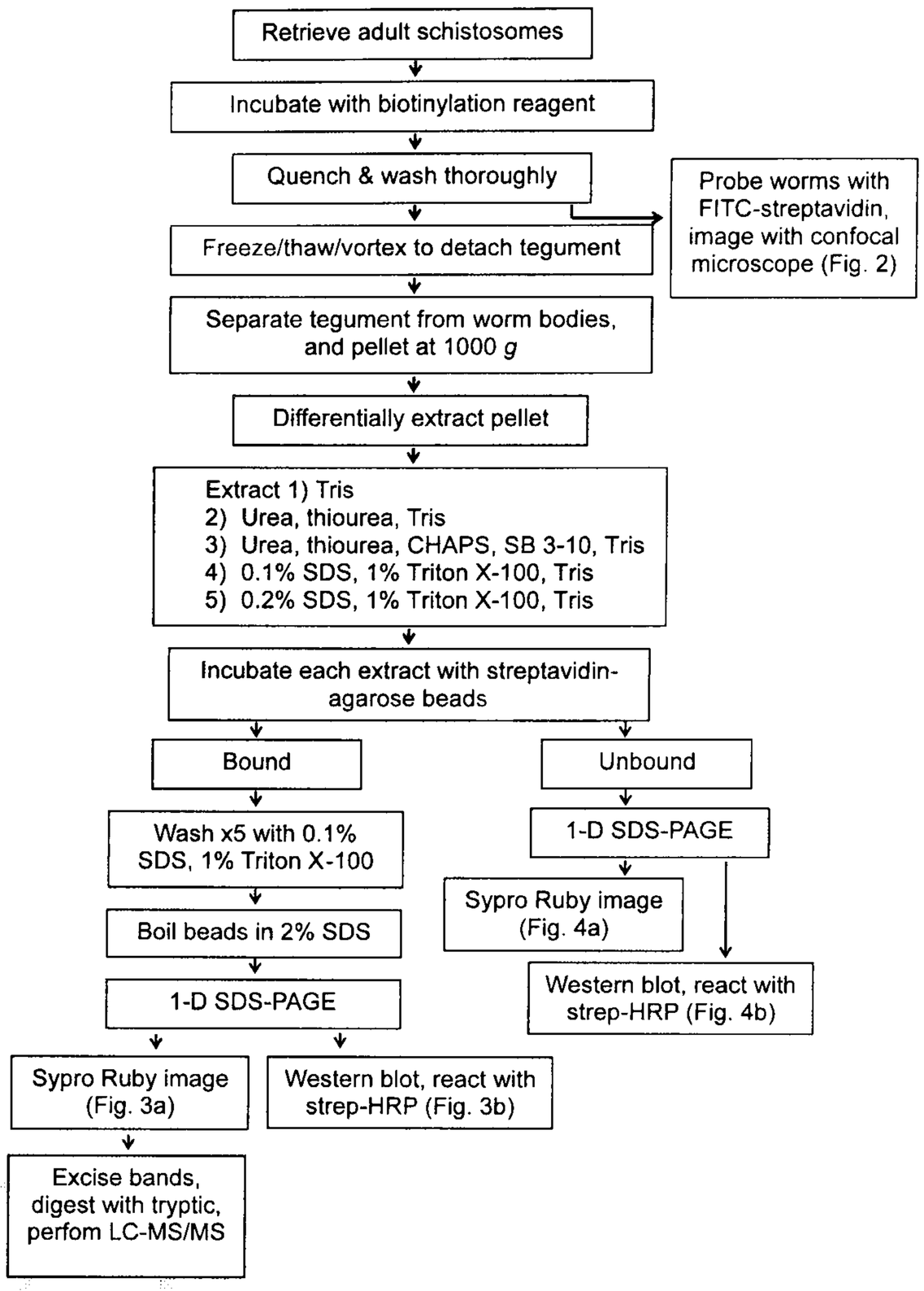


FIG. 1. **Experimental workflow.**

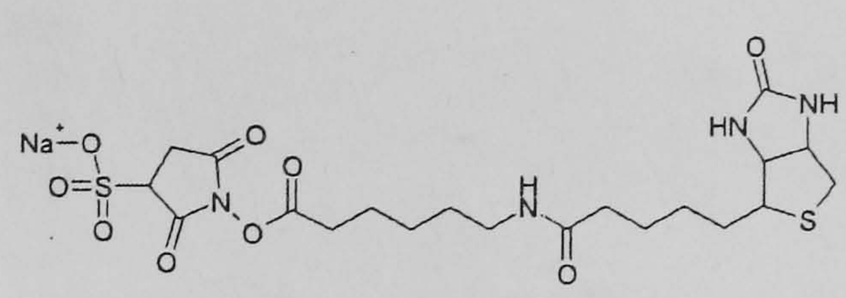
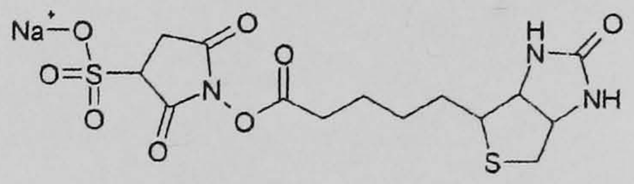
Each cycle of the extraction procedure was performed three times, and the resulting supernatants were pooled to give a sample volume of 600 μl. The solubilizing regimes were: Extract 1, 40 mM Tris, pH 7.4, vortexed for 1 min and allowed to stand for 20 min on ice; Extract 2, 5 M urea (BDH, VWR International, Dorset, UK), 2 M thiourea (BDH, VWR International) in 40 mM Tris, pH 7.4, 25 °C; Extract 3, 5 M urea, 2 M thiourea, 4% CHAPS (Sigma), 2% *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfate (SB 3-10; Sigma) in 40 mM Tris, pH 7.4, 25 °C; Extract 4, 0.1% SDS, 1% Triton X-100 in 40 mM Tris, pH 7.4, 25 °C; Extract 5, 0.2% SDS, 1% Triton X-100 in 40 mM Tris, pH 7.4, 25 °C. Protease inhibitor mixture (Sigma) was added to each pooled supernatant prior to isolation of the extracted labeled molecules. AQ: E AQ: F

Processing the Biotinylated Proteins—The biotinylated material in each of the five extracts was isolated by incubation with 60 μl of a prewashed slurry of streptavidin immobilized onto agarose beads (ImmunoPure® immobilized streptavidin, Pierce) for 2 h at room temperature with head-over-head mixing. The streptavidin-bound complex was pelleted by centrifugation for 1 min at 3000 × *g*, and the supernatant (unbound material) was recovered. The beads were then washed for 5 min in 500 μl of 0.1% SDS, 1% Triton X-100 in PBS by head-over-head mixing at room temperature to remove any further unbound material and pelleted as before. This wash procedure was repeated four times. The bound material was recovered from the washed streptavidin beads by addition of 100 μl of 2% SDS, heating to 90 °C for 10 min, vortexing at maximum speed for 2 min, and pelleting of denuded beads by centrifugation as above. The supernatant containing the biotinylated macromolecules was recovered,

[1] The abbreviations used are: NHS, *N*-hydroxysuccinimide; 1-D, one-dimensional; CRP, complement-regulatory protein; SB 3-10, *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfate; strep-HRP, streptavidin conjugated to horseradish peroxidase; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

TABLE I

Properties of the two biotinylation reagents used to label the S. mansoni surface

Name	M_r (Da)	Spacer arm length (A)	Structure
Long-form			
Sulfo-NHS-LC-biotin	556.59	22.4	
Short-form			
Sulfo-NHS-biotin	433.42	13.5	

the procedure was repeated, and supernatants were combined to yield 200 μl for each of the five extracts.

SDS-PAGE and Western Blotting—Proteins from each of the five extracts were precipitated with 10% TCA, 80% acetone and solubilized in SDS sample buffer plus reducing agent (Invitrogen). They were separated by 1-D SDS-PAGE using 7-cm NuPAGE 4–12% bis-Tris gels (Invitrogen) followed by SYPRO Ruby (Bio-Rad) staining and image capture using Molecular Imager FX (Bio-Rad). The biotin content of the bound and unbound material after the incubation with streptavidin-agarose beads was examined by Western blotting. An aliquot of the samples was separated by 1-D SDS-PAGE and transferred onto a PVDF membrane using an XCell II blot module (Invitrogen). The membrane was blocked in 4% casein, probed with streptavidin conjugated to horseradish peroxidase (strep-HRP; Amersham Biosciences), and TMB peroxidase substrate (Kirkegaard & Perry Laboratories) was added to visualize labeled bands.

LC-MS/MS and Database Searching—The SDS-PAGE gels of the bound material were restained with Coomassie BioSafe (Bio-Rad), and each protein band was excised, digested with trypsin, and subjected to LC-MS/MS as described previously (14). Sections of gel lanes where no protein staining was visualized were also analyzed for potential protein content. Briefly peptides were separated on a reversed-phase Monolith column (LC Packings, Dionex, Sunnyvale, CA) using an Ultimate nanoflow HPLC system (LC Packings) and spotted directly onto a MALDI target plate using a Probot (LC Packings). A 4700 Proteomics Analyzer with TOF-TOF optics (Applied Biosystems, Framingham, MA) was used to obtain fragmentation spectra, which were processed by GPS Explorer software version 2.0 (Applied Biosystems) to provide peak lists. These were submitted to MASCOT version 1.9 (16) (Matrix Science, London, UK) and searched against the National Center for Biotechnology Information non-redundant (NCBInr) database as well as the *S. mansoni* genome and transcriptome databases (www.SchistoDB.org/and cancer.lbi.ic.unicamp.br/schisto6/, respectively). Mass tolerance was set to ±0.3 Da for both the precursor and product ion spectra, and search parameters allowed for a maximum of three missed tryptic cleavage sites, the carbamidomethylation of cysteine, the possible oxidation of methionine, and the possible modification of lysine and N-terminal residues by the biotinylation reagents. A protein was considered to be identified when two or more peptides, each with a GPS-generated confidence interval of above 99%, were positively matched in the database. Putative functions were assigned using BLAST (17) against annotated proteins in the GenBank™ database; scores with an expect value $<1e^{-16}$ were regarded as a significant match. Potential signal sequences and glycosylation sites were predicted using SignalP version 3.0 and NetNGlyc/NetOGlyc version 1.0, respectively (www.cbs.dtu.dk/services/), and transmembrane domains were predicted using HMMTOP (www.enzim.hu/hmmtop/). AQ: K

FIG. 2. **Optical section of an adult male schistosome labeled with impermeant biotinylation reagent and probed with streptavidin conjugated to FITC.**

RESULTS

The Biotin Reagents Uniformly Label the Worm Surface—When the BSA was subjected to the complete procedure from labeling to tandem MS and compared with unmodified BSA, no dramatic alterations in peptide mass fingerprints were observed. From the labeled BSA, we identified 14 peptides, six of which (43%) contained a biotinylated lysine residue (peptide details can be found in the supplemental table). The extent of labeling of live adult worms was assessed using FITC-conjugated streptavidin. A sharply demarcated line of surface staining was observed on whole mounts (Fig. 2). F2

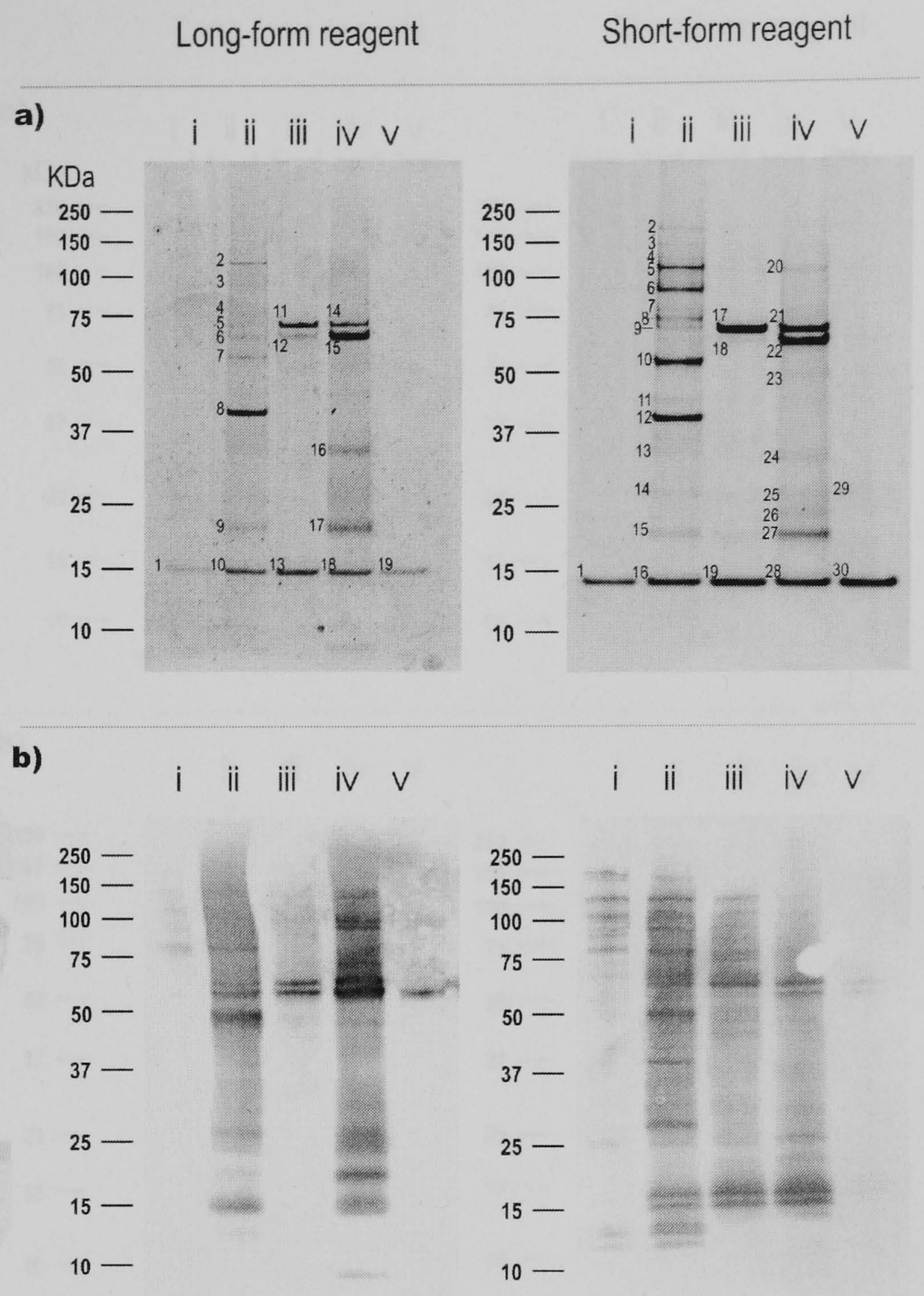


FIG. 3. **Bound material.** *a*, labeled proteins recovered by affinity precipitation, separated by SDS-PAGE, and stained with SYPRO Ruby. Proteins bands that were positively identified by tandem MS are *numbered*, and their details are shown in Table II. *b*, corresponding blots probed with strep-HRP. *Lanes i–v* represent extracts 1–5, respectively.

Optical sectioning through the worm body confirmed that the pattern of staining was confined to the surface with no penetration into internal tissues. No biotin reagent or streptavidin had entered the gut during incubation.

Streptavidin Beads Recover Biotinylated Proteins from the Differential Extracts—The labeled tegument surface, detached from worm bodies, was extracted to recover proteins according to their differential solubility. Labeled proteins were then retrieved from each of the five extracts using streptavidin-agarose beads, and the bound material was separated by SDS-PAGE. The SYPRO Ruby-stained images revealed a sharp pattern of bands with strong similarities between the patterns labeled by the two biotin reagents (Fig. 3*a*). Overall more proteins were retrieved after labeling with the short form rather than the long form reagent. In both instances, the Tris and 0.2% SDS, 1% Triton X-100 extracts (*lanes i* and *v*, respectively) appeared devoid of protein with the exception of a single band of ~15 kDa, which was also present in other extracts. Conversely the other three extractions (*lanes ii–iv*) recovered more complex mixtures of labeled material. Thus, after urea/thiourea treatment (*lane ii*), nine bands were visible in the long form-labeled extract, and 15 were visible in the short form-labeled extract. After urea/thiourea/CHAPS/SB 3-10 treatment (*lane iii*) only three bands were present in both long and short form extracts; the ~70-kDa species were absent from *lane ii*. After 0.1% SDS, 1% Triton X-100 treatment (*lane iv*), five bands were present in the long form and nine were present in the short form extracts; apart from the ~15- and ~70-kDa species, the remainder were unique.

To verify that the proteins recovered from the beads were biotinylated, an aliquot was separated by SDS-PAGE, blotted onto a PVDF membrane, and then probed with strep-HRP.

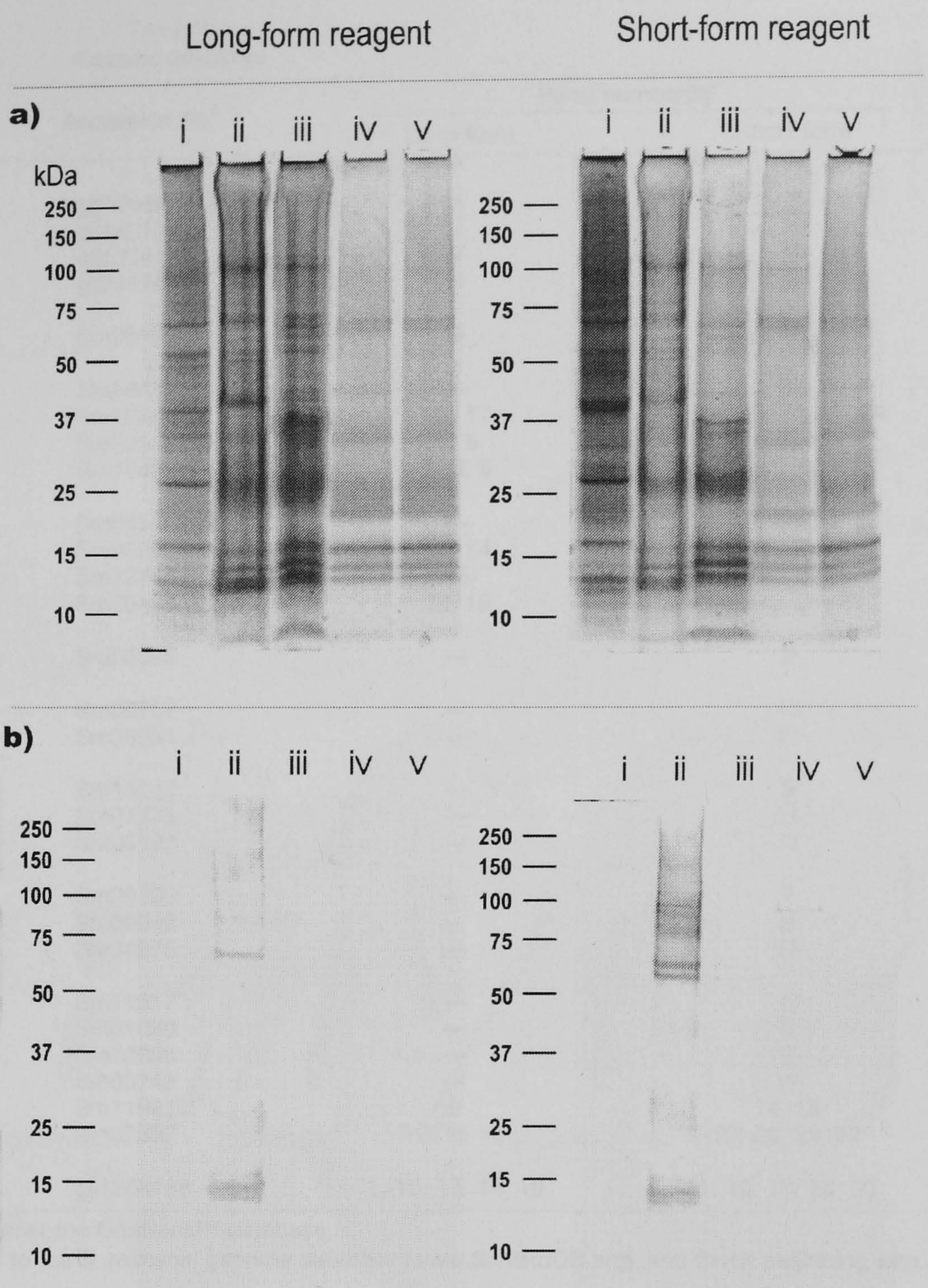


FIG. 4. **Unbound material.** *a*, SYPRO Ruby-stained 1-D SDS-PAGE separation of proteins that did not co-precipitate with the streptavidin beads. *b*, the corresponding Western blots probed with strep-HRP. *Lanes i–v* represent extracts 1–5, respectively.

Each protein band visualized by SYPRO Ruby could be detected as a matching band on the Western blot (Fig. 3*b*) with the exception of the aforementioned ~15-kDa protein, which was absent from several lanes. Conversely additional bands of labeled material were detected only on the Western blots. In particular, after short form labeling, a whole array of new bands appeared, especially noticeable in the high molecular weight region of Tris extract (*lane i*), whereas the blot of long form-labeled material more closely resembled its corresponding SYPRO Ruby-stained gel (Fig 3*b*). These data indicate that the sensitivity of biotinylated protein detection by blotting and enzymatic amplification of the signal is much greater than staining with SYPRO Ruby.

The Unbound Material Is Almost Exclusively Non-biotinylated—The supernatant recovered after each of the five extracts that had been incubated with streptavidin beads (*i.e.* the unbound material) was profiled for both protein and biotin content by SDS-PAGE and Western blotting, respectively. The SYPRO Ruby-stained 1-D gel revealed large numbers of protein bands, reducing in complexity from extract 1 to extract 5 (Fig. 4*a*, *lanes i–v*). The blotted material showed traces of biotinylation in experiments with both reagents (Fig. 4*b*) that was more evident when the short form was used. In both cases the labeled material was almost exclusively confined to the urea/thiourea extract (*lane ii*) with only the faintest traces visible in 0.1% SDS, 1% Triton X-100 extract (*lane iv*). Notably the few biotinylated protein bands in the unbound material were also detected in the gels and blots of the bound material (Fig. 3).

Both Parasite Surface and Host Proteins Are Biotinylated—Bands detected on 1-D separations of fractions recovered from the agarose beads were excised and trypsinized, and the

TABLE II
Proteins identified

Protein name[a]	Accession no.[b]	Band number(s)[c]	
		Long form	Short form
Host proteins			
IgM heavy chain	gi|70048	4	7
IgG1 heavy chain	gi|440121	7	10
IgG3 heavy chain	gi|1304160	7	10
Complement C3 (C3c/C3dg fragment)	gi|28175786	6	9
Secreted protein			
Sm29	Sm09193	8	12
Membrane structural proteins			
Tetraspanin B (TE736)	Sm04463	—	20, 22, 25
Tetraspanin D (CD63-like tetraspanin)	Sm12366	16, 17	14, 20, 24, 27, 29
Annexin	Sm03987	4, 8	4, 7, 12
Dysferlin	Sm10433	2, 3, 5	3, 5, 6, 8
Membrane enzymes			
Calpain	Sm08542	—	7, 11, 22, 23, 26
Alkaline phosphatase	Sm00962	11, 14	9, 17, 21, 23
ATP-diphosphohydrolase	Sm12745	15	20, 22
Phosphodiesterase	Sm03458	12, 15	18, 20, 21, 22
Membrane (other)			
200-kDa Surface protein	Sm03865	—	2
Transporters			
Voltage-dependent anion channel	Sm00707	—	13
Sodium/potassium transporter (SNaK1)	Sm08331	—	21
Cytoskeletal proteins			
Fimbrin	Sm13240	—	8
Actin	Sm01276	—	11
Severin	Sm04123	—	11
Cytosolic proteins			
Glycerol-3-phosphate dehydrogenase 2	Sm08702	—	7
Heat shock protein 70	Sm09042	—	8
Carbonic anhydrase 4	Sm04975	—	23
No homology			
Unknown	Sm11517	—	6
Unknown	Sm01030	—	8
Unknown	Sm13096	—	10
Unknown	Sm00749	—	17
Unknown	Sm11921	9	14, 15
Unknown	Sm07392	15	13, 20, 21, 22
Protein from agarose beads			
Streptavidin	gi|1364156	1, 10, 13, 18, 19	1, 16, 19, 28, 30

[a] The putative identity gained by BLAST analysis against the GenBank™ database.

[b] Accession numbers beginning with Sm correspond to the *S. mansoni* genome database (www.SchistoDB.org), and those beginning with gi correspond to the NCBInr database.

[c] Band number equating with protein bands in Fig. 3*a*. Where no number is given (—), the identity was not found in that sample.

resulting peptide mixture was fractionated by reversed-phase chromatography before identification by tandem MS. In total, 117 peptide fragmentation spectra were matched to genome/expressed sequence tag reads, but only one revealed the presence of a modified lysine residue (peptide details can be found in the supplemental table). A total of 28 identities was obtained plus streptavidin in all five extracts (Table II). These comprised 13 proteins in samples recovered after use of the long form reagent and the same 13 plus a further 15 after use of the short form reagent. It should be noted that almost invariably a single gel band contained two or more proteins, making it impossible to draw inferences about the relative amounts of individual constituents in the preparation. Furthermore some proteins were identified in several gel bands of different molecular weights.

Four host proteins, three distinct immunoglobulin heavy chains and complement component C3, were consistently recovered from SYPRO Ruby-stained gels after surface biotinylation. The heavy chains for IgG1 and IgG3 were present in a single band of an apparent molecular mass of 55 kDa. The four peptide hits for IgG3 originated in constant domains C_H1, C_H2, and C_H3 (two peptides), whereas six peptides for IgG1 originated in the variable domain framework regions V_H1 and V_H3 and the constant domains C_H2 and C_H3 (two in each). The heavy chain for IgM was present in a gel band of apparent molecular mass 75 kDa with peptide hits originating in the constant domains C_H2 and C_H4. Complement component C3 was identified in a gel band of apparent molecular mass 69 kDa, and the two peptide hits both originated in the α chain C3dg region; the apparent molecular mass is consistent with the polypeptide recovered being the partial degradation product of the C3 molecule comprising C3c/C3dg.

The 24 schistosome proteins could be grouped according to their putative functions (Table II). The long form reagent labeled one protein, Sm29, apparently secreted on the basis of SignalP analysis of the amino acid sequence. Three membrane enzymes, alkaline phosphatase, phosphodiesterase, and diphosphohydrolase, capable of hydrolyzing organic phosphates were recovered as well as three structural membrane proteins, annexin, dysferlin, and the tetraspanin D (CD63-like tetraspanin). The short form reagent retrieved a 200-kDa membrane-bound protein, termed "Surface protein" in the original description, plus one further tetraspanin (B; tetraspanin TE736) involved in membrane structure. A membrane protease, calpain, was also labeled, as were two transporters, the sodium/potassium transporter (SNaK1) and a voltage-dependent anion channel. The short form reagent also appeared to have greater penetrating power, labeling three cytoskeletal proteins, actin, fimbrin, and severin, and three cytosolic proteins, carbonic anhydrase 4, heat shock protein 70, and glycerol-3-phosphate dehydrogenase 2. A further four schistosome proteins of unknown function were also labeled by the short form reagent (Table II).

DISCUSSION

To determine the external accessibility of tegument surface proteins, we incubated live adult schistosomes with impermeant biotinylation reagents. The two reagents used label proteins with exposed amine groups either at lysine residues or at the N terminus. Because of their sulfo group, they are water-soluble and therefore excluded by the lipid bilayer, preferentially labeling secreted proteins and membrane proteins with extracellular loops or domains. It was necessary to keep the duration of the reaction short (30 min) because labeling had to take place in an amino acid-free balanced salt solution rather than culture medium, increasing the possibility of worm degradation. We first optimized the methods for labeling, recovery of proteins, and their identification by tandem MS using purified BSA. The retrieval of biotinylated molecules from agarose-immobilized streptavidin is challenging because of the exceptionally high binding constant of the complex ($K_d \sim 10^{-15}$ M (18)). We found that boiling in 2% SDS was the best method, recovering >90% of the bound protein (some streptavidin was also detached from the agarose). Although tandem MS revealed that 43% of peptides derived from labeled BSA contained biotinylated lysines, there were also numerous unmodified lysines. This suggests either an incomplete reaction or the inaccessibility of some lysines to the reagent. (Similar results were obtained with a second model protein, bovine alkaline phosphatase.[2]) The biotinylation of a lysine residue prevents cleavage by trypsin at that site, altering the composition of the peptide mixture generated for tandem MS, which could result in peptides too long (>4000 Da) for satisfactory fragmentation. This did not create a problem for the identification of BSA (or tegumental proteins) because of the partial biotinylation; furthermore we recovered the intact labeled proteins rather than individual biotinylated peptides.

We utilized two forms of sulfo-NHS-biotin for parasite labeling in the expectation that they would have different physical properties and labeling capabilities, and this proved to be the case. The fact that the only cytosolic and cytoskeletal proteins identified were labeled uniquely by the short form reagent reveals its greater penetration of the tegument surface. The different patterns of protein labeling produced with the long and short form reagents enable us to make inferences about the putative location of individual proteins. Because the long form reagent did not react with any cytoplasmic proteins, we assume that all 13 proteins detected lie within or external to the plasma membrane (Fig. 5). However, as we have no means of separating the plasma membrane from the overlying membranocalyx, we cannot deduce with certainty whether any of the 13 proteins are located in the latter structure. Nine parasite proteins (and four host proteins, discussed below) were identified and placed in secreted, membrane structure, membrane enzyme, and no homology categories. Of the three enzymes, two are membrane-spanning, whereas the third, alkaline phosphatase, is glycosylphosphatidylinositol-anchored. All three hydrolyze organic phosphate substrates, possess large extracellular domains, and, by analogy with other organisms, are likely to be located on the external leaflet of the plasma membrane, features entirely consistent with their labeling. Such a location implies the existence of water-filled "pores" in the membranocalyx to allow the ingress of substrates (and the labeling reagent). However, we surmise that such pores would not be large enough to give antibodies access to the protein domains situated immediately external to the plasma membrane. Similarly it seems unlikely that secreted proteins lying between the two layers would escape via such pores. As plasma membrane enzymes, the three proteins would traffic to the surface anchored in the bounding membrane of the multilaminate vesicle, not its contents.

The long form reagent labeled the three proteins involved in membrane structure. However, these included only one of the three tetraspanins (tetraspanin B) identified in our compositional study (14). It is a member of a large family of proteins encoded by the *S. mansoni* genome and likely to form part of the intramembranous web that provides a scaffold for other components (19). The second structural protein, annexin, normally binds to phospholipids at the cytoplasmic face of membranes, but its labeling by the long form reagent implies an extracellular location. Such an occurrence has been reported in other organisms (20), although the annexin gene does not encode a signal peptide or transmembrane domains. Given its lipid binding properties, it is tempting to speculate that it provides the molecular "glue" that sticks the membranocalyx to the underlying plasma membrane (Fig. 5). Alternatively its

[2] ■. Borges, personal communication

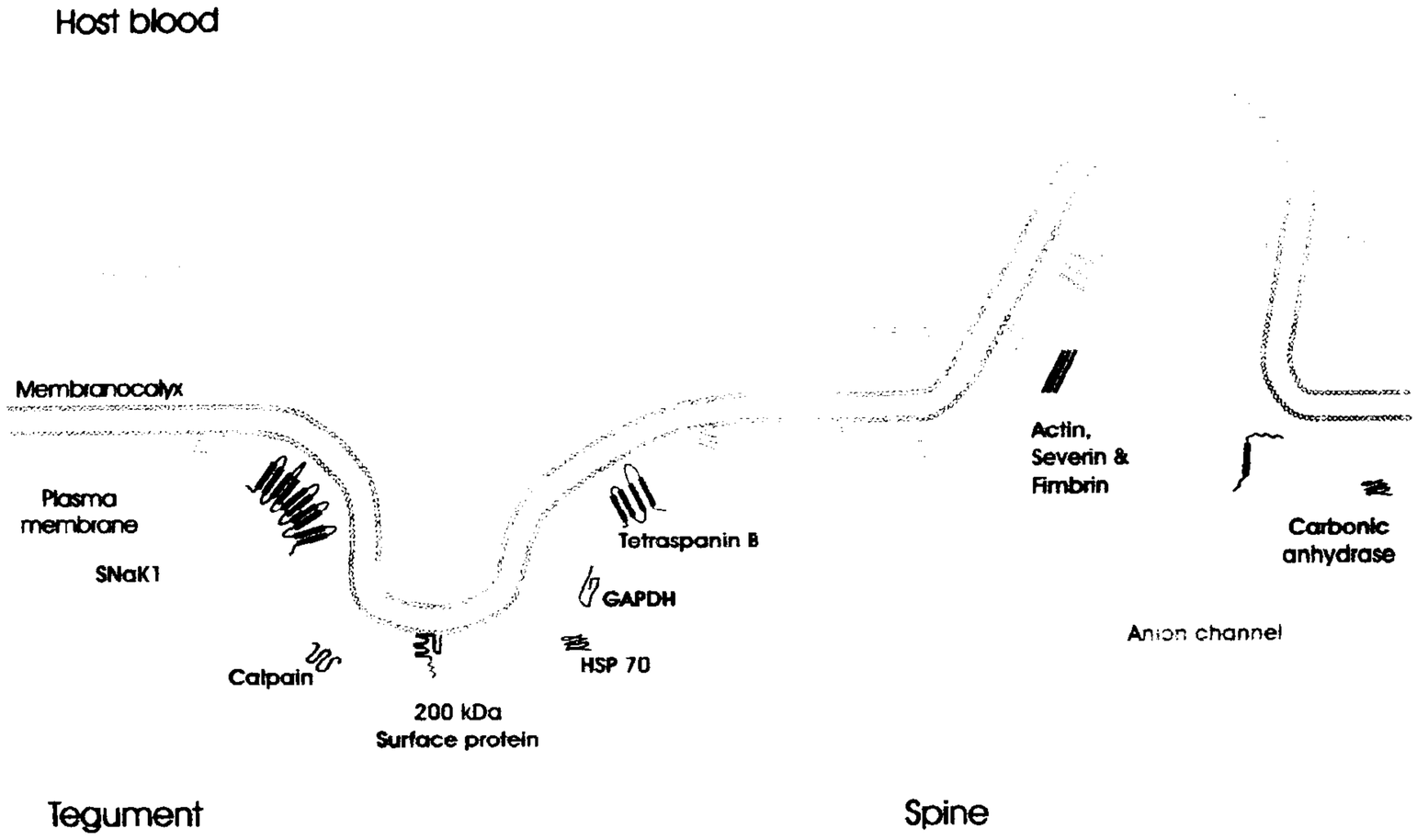


FIG. 5. **Diagrammatic representation of the tegument and the proposed locations of the proteins identified.** Those proteins shown in *blue* are labeled by both the long form and short form biotinylation reagents. The proteins in *red* are labeled only by the short form reagent and are therefore likely to have a more intrinsic location. The schistosome proteins with no homology are not represented as their full-length sequence is unknown.

GG

capacity to assemble into molecular sheets (21) could provide a scaffold for the relatively fluid membranocalyx. The third membrane structural protein, dysferlin, facilitates vesicle fusion and repair of the plasma membrane in other organisms (22). Only one true secreted protein, Sm29, was labeled by the long form reagent and would thus be exported in the multilaminate vesicle contents, making it a possible constituent of the membranocalyx or the space between the two lipid bilayers. Given our argument about the size of the pores in the membranocalyx, Sm29 would be unlikely to pass through this layer. Indeed there seems to be little evidence for rapid secretion of proteins from the tegument surface, although the slow turnover of the membranocalyx (6) implies that if it contains significant protein constituents these must enter the bloodstream environment. Both the proteins of unknown function, labeled by the long form reagent, bear membrane-spanning domains and so are most likely to reside in the plasma membrane, not the membranocalyx, because of its origin as the multilaminate vesicle contents (4).

Three of the six intracellular proteins labeled by the short form reagent, actin, fimbrin, and severin, have a cytoskeletal role. Paracrystalline actin is a major constituent of tegumental spines (23), whereas severin and fimbrin are likely determinants of spine structure (Fig. 5), the former cross-linking actin (24) and the latter capping growing filaments (25). The tight apposition of the plasma membrane to the underlying spines (26) would require minimal penetration of reagent to label the three proteins. By inference, the other three cytosolic proteins, carbonic anhydrase, glycerol-3-phosphate dehydrogenase, and HSP70 are likely to be located in the cytoplasm immediately beneath the plasma membrane. The last two are abundant in the cytosol (27), but other proteins such as enolase, aldolase, or glutathione *S*-transferase, which are equally abundant, were not labeled, and so were presumably not so accessible. In this context, the short form labeling of the calcium-activated protease calpain, which functions in cytoskeleton remodeling, is in accord with its location in other organisms on the inner leaflet of the plasma membrane (28). The labeling of tetraspanin B only by the short form reagent implies a more cryptic location in the intramembranous tetraspanin web than for tetraspanin D. It is notable that only two transporters, SNaK1 and voltage-dependent anion channel, were biotinylated; presumably this reflects the relative accessibility or lysine content of their extracellular loops compared with other transporters identified previously in the tegument (14). The failure of the long form reagent to label the 200-kDa Surface protein is enigmatic, given that it possesses a large extracellular domain. However, its inaccessibility to antibodies prior to praziquantel treatment of worms does indicate a relatively protected location (29). The four proteins with no known homology, labeled by the short form reagent, were identified from partial sequences in the database. Two of them possess predicted transmembrane domains, suggesting a plasma membrane location, but nothing can be inferred about the remaining two. The detection of more bands on Western blots by strep-HRP than in the corresponding gels by SYPRO Ruby staining raises the issue of sensitivity, particularly for the short form reagent. It appears that the amplification step in Western blotting, whether using enzyme-labeled streptavidin or antibody, significantly enhances sensitivity, and a proportion of these proteins are detected at concentrations below the threshold for identification by tandem MS. This may explain why we failed to detect the glucose transporter SGTP4 when others using a similar protocol to biotinylate the worm surface and recover tagged proteins were able to locate it on a blot using specific antibody (30). We also

AQ: S AQ: T AQ: U AQ: V AQ: W

failed to detect SmRK-1, a member of the transforming growth factor-β family of receptor kinases, reported previously as labeled by sulfo-NHS-LC biotin (15, 31), and SCIP-1 (paramyosin), proposed both as an Fc receptor (32) and as an inhibitor of complement C9 polymerization (33).

We recovered four host proteins with immunological functions after labeling with both long and short form reagents implying an extrinsic location (Fig. 5). These were the immunoglobulin heavy chains of IgM, IgG1, and IgG3 plus complement component C3. All were present after thorough washing of worms; from this we infer that they were not simple contaminants, particularly as we did not recover albumin, a more abundant plasma protein. We conclude that all three Ig heavy chains were intact from their respective molecular weights on gels and the distribution of peptide hits throughout the molecules. Antibodies have been detected previously at the tegument surface by immunocytochemical methods (34–36). The principle question is whether they bind via their Fc or Fab regions. Fc receptors have been reported at the tegument surface (37) that are capable of binding heterospecific antibody, but we did not detect such host proteins or potential schistosome homologues; the latter do not appear to be encoded in the *S. mansoni* genome (www.Schistodb.org). Thus, if schistosomes possess endogenous Fc receptors they will only be identified by functional studies. Indeed the muscle protein paramyosin has been proposed as an Fc receptor in *Schistosoma japonicum* (32) and reported at the tegument surface by immunofluorescent staining (33) but was not detected in this or our previous study on the *S. mansoni* surface (14). The presence of IgM at the surface, for which no Fc receptor has been described in schistosomes, may indicate binding to specific antigens via the Fab region. Furthermore the fixation of complement C3, which requires a free Fc region on immunoglobulin to initiate the classical pathway, also argues for binding to antigen via the Fab region.

The molecular weight and location of peptide hits on complement component C3 is consistent with it being the α chain C3c/C3dg fragment. This means that C3 has been activated by C3 convertase and subsequently inactivated by the complement-regulatory protein (CRP) factor I in combination with membrane cofactor protein or factor H. At least one CRP, decay-accelerating factor, has been identified at the schistosome surface by immunocytochemistry (38), but it acts too early by accelerating the destruction of C3 convertase to explain the presence of the C3c/C3dg fragment in our study. However, although we did not detect any CRPs after biotinylation, we did find complement receptor-related protein gene y (Crry), which possesses both decay-accelerating factor and membrane cofactor protein activity (39), in an LC-based tandem MS analysis of purified tegument membranes.[3] The inactive form of C3 argues for inhibition of the complement pathway as a major element of the parasite's immune evasion strategy by the recruitment of CRPs. The presumed hydrophobic lipid nature of the membranocalyx may also be important in this context and could also shield the underlying plasma membrane against the assembly of membrane attack complex should the complement pathway progress that far. A list of other host proteins associated with the surface has been proposed, including α_2-macroglobulin (40) and major histocompatibility complex (41), but we failed to find either of them.

The surface of the schistosome tegument has long been a fascination as the main site of immune evasion in these macroscopic, long lived, intravascular parasites. It can be speculated that the relatively few parasite proteins biotinylated represent the subset most accessible to host immune attack and therefore merit investigation as vaccine candidates. (So far only calpain has received serious attention (42)). Our data suggest the majority of labeled parasite proteins appear to be associated with the plasma membrane rather than the membranocalyx. It must be emphasized that the latter originates as a secretion within the lumen of the multilaminate vesicles where the bilayer structure assembles during vesicle maturation in the tegument cell body (26). Consequently proteins translated from genes encoding a leader sequence, but not transmembrane domains, are the most likely candidates for inclusion. The detection of only one protein in this category, Sm29, suggests that the membranocalyx contains relatively few proteins. It would thus function primarily as an inert barrier between the host immune system and the vulnerable plasma membrane with its assortment of enzymes, transporters, and structural proteins. Our task is to devise new strategies to isolate the membranocalyx and analyze its composition.

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Chapter 5

The isolation and proteomic characterisation of the membranocalyx

5.1 Introduction

The membranocalyx provides the outermost surface of the blood-dwelling stages of the schistosome, and its structure and formation are described in the general introduction. In Chapters 2 and 3, protein identities were sought from both the plasma membrane and membranocalyx that were detached and purified together by the freeze/thaw/vortex process and sucrose gradient. Although protein candidates of the membranocalyx were proposed based on their predicted lipid binding properties and signal sequences, no conclusive evidence was gathered for their specific location. The biotinylation studies presented in Chapter 4 also pointed to several possible constituents of the outer bilayer, but the suggested pores in this membrane would permit the transmission of the biotinylation reagents through to label the proteins of the plasma membrane. It was therefore decided that a method to isolate the membranocalyx would provide the basis from which the undisputed protein constituents of the membranocalyx could be discovered.

5.1.2 Isolation techniques of the membranocalyx

The isolation of the membranocalyx from the tegument has been the focus of much work over the past thirty years. The development of a method has proved to be a difficult task, because the *in vivo* collection of sloughed membrane material would not be possible, and to remove the membranocalyx selectively from worms *in vitro* would require disrupting the outer bilayer without affecting the plasma membrane underneath. Thus, a very sensitive and carefully controlled approach is required. The following paragraphs review some published methods of membranocalyx isolation/enrichment.

5.1.2.1 The use of detergents

Kusel (1970; 1972) pioneered the use of digitonin and saponin to remove the surface bilayers of the *S. mansoni* schistosomula and adult worms. Over ten years later, techniques were developed to remove the membranocalyx followed by the plasma membrane from the tegument of mature worms (McDiarmid et al., 1983). These authors first labelled the live parasites with diazotised (^{125}I)-iodosulfanilic acid (DISA) and used the label as a marker of the membranocalyx. Alkaline phosphatase, Na^+ and Mg^{2+} ATPase activity was used as an indicator of the plasma membrane. An optimal incubation time of 2x 6 minutes in 0.1% digitonin resulted in two 80% pure fractions of the membranocalyx and plasma membrane. However, Saunders et al. (1988) demonstrated that DISA was not a good

marker of the membranocalyx because of it poor binding properties, and its ability to penetrate the lipid bilayer and label proteins on the plasma membrane. Pilot experiments (using just 10 mice worth of worms; data not presented) tested the use of digitonin as a method to isolate the membranocalyx. TEM was used to examine the effect of the detergent on the membranocalyx over different time points, and LC-based proteomics to identify proteins released in the incubation steps. Unfortunately, my results did not replicate those of McDiarmid et al. (1983); the tegument appeared damaged even after just two minutes in digitonin, and >95% of the proteins identified at this time point were predicted to have a cytosolic location. As a result, the use of detergents was not pursued in this thesis.

5.1.2.2 Polylysine- and lectin-coated beads

Polylysine-coated acrylamide beads were successfully used to isolate membranes from erythrocytes and HeLa cells (Cohen et al., 1977; Jacobson and Branton, 1977). The negatively-charged cell surface binds "tenaciously" to the positively-charged beads. When the bound cells are detached by vortexing, large patches of plasma membrane remain on the bead. Cesari et al. (1983) and Payares and Evans (1987) used this technique to remove surface proteins and antigens from adult *S. mansoni* worms. The beads readily attached to the surface of the parasite, and their removal left small round scars on the tegument surface. Electron microscopy of the beads revealed the presence of membrane-like sheets, but there was also evidence of other tegument material such as spines (Payares and Evans, 1987). Preliminary observations (produced by TEM; data not presented) revealed poor yields of membrane material when I tested this method. Furthermore, the two lipid bilayers of the tegument surface were not separated; the bound material appeared to consist of the membranocalyx, plasma membrane, vesicles, mitochondria, spines and ground substance. In short, this technique provided no more than a convoluted and unrewarding procedure to remove large chunks the tegument.

A more delicate surface membrane isolation method using lectin-coated beads was employed by Pujol and Cesari (1993). This approach takes advantage of the tegument surface's high affinity for plant-derived proteins such as concanavalin A (Bennett and Seed, 1977) and wheat germ agglutinin (Murrell et al., 1978) (Simpson and Smithers, 1980) (Linder and Huldt, 1982). Pujol and Cesari (1993) used Ca^{2+} ATPase, alkaline phosphatase and phosphodiesterase activity as marker for membrane content. Surface

material captured on lectin-coated beads had significantly more activity of these three enzymes than worm homogenates. Although this method may provide an enriched membranocalyx, the presence of alkaline phosphatase and phosphodiesterase suggests that the plasma membrane (and quite possibly syncytial material too) has also become attached to the lectin beads.

5.1.2.3 Induced sloughing of the membranocalyx

Rabbit antibodies against mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA and IgM were reported to bind to adult worms that had been perfused from mice (Kemp et al., 1980). Using fluoresceinated *Staphylococcus aurues*, which binds to the Fc region of the rabbit antibody, Kemp and others showed that the interaction of the rabbit and mouse antibodies induced the shedding of this complex from the parasite surface within 20 minutes at 37 °C. Moreover, the induction of shedding is only caused by the specific binding to mouse immunoglobulin by the rabbit antibody and can be inhibited by sodium fluoride, caffeine, cytochalasin B and D, 2-deoxy-D-glucose or reduced temperatures (Kemp et al., 1980). Whether the membranocalyx is shed together with the mouse antigens was not discussed by the authors. I toyed with a similar experimental design using sera from rats immunised against mouse erythrocyte ghosts. The antibodies attached to the surface of the adult worms, as visualised by goat anti-rat alexa-480 antibody and fluorescent microscopy, but the binding of the secondary antibody did not induce the release of the complex even after 24 hours (data not shown). Likewise, transmission electron microscopy revealed no difference in the state of the membranocalyx at various time points after the binding of the antibodies.

Wilson and Barnes (1977) were able to coat the surface of adult worms with cationised ferritin (CF), which promoted the sloughing of the membranocalyx. Within four hours, the labelled lipid bilayer had been completely shed into the surrounding media. The CF binds to the parasite by the same principles as the polycationic beads; the membranocalyx is negatively charged and interacts with positively charged molecules. The use of CF has three distinct advantages over the beads. Firstly, as the CF is only ~10 nm in diameter, it is able to label the entire surface of the worm, including all the pits and invaginations of the tegument. The beads are big and cumbersome, and their diameter is approximately half that of the female worm. As a result, only a limited number of beads can bind to a single worm, and large surfaces of the bead do not interact with the tegument surface. Secondly,

the CF binds to the worms under physiological conditions, whereas the attachment of beads required pH 5 acetate buffer, which rapidly killed the parasites. Finally, the CF selectively induces the release of the membranocalyx and does not the damage the plasma membrane or tegument syncytium (Wilson and Barnes, 1977). This method could be easily replicated and is used as a template for one of the membranocalyx isolation procedures presented in this chapter.

5.1.3 Markers of the membranocalyx

Whilst markers of the tegument plasma membrane are plentiful (*e.g.* alkaline phosphatase, phosphodiesterase, Ca^{2+} ATPase (Pujol and Cesari, 1993)), a specific indicator for the membranocalyx is less definite. As mentioned above, early studies used diazotised [^{125}I]-iodosulfanilic acid to label the surface bilayer, but this method was later refuted (Saunders et al., 1988). Hayunga et al. (1979) recommended the use of Bolton-Hunter reagent to radiolabel the adult schistosome's surface. This procedure iodinates free amino groups and does not damage the parasite. However, the authors acknowledge that the label is capable of penetrating human erythrocyte membranes (Hayunga et al., 1979), and it is also likely to have a similar affect on schistosome membranes. Concavalin A has also been used to label the membranocalyx via specific binding sites (Bennett and Seed, 1977; Wilson and Barnes, 1977; Oaks et al., 1981; McDiarmid et al., 1983), but the label had a harmful effect on the tegument by interfering with the mechanism of vesicle docking with the plasma membrane (Wilson and Barnes, 1977).

The acquisition of host molecules by the membranocalyx makes them an ideal candidate for a marker of the schistosome's most peripheral surface. Host antibodies and Complement components have been well documented at the schistosome surface (reviewed in Chapter 1). Similarly, the presence of erythrocyte antigens provides a possible starting point for the isolation of the membranocalyx. Initial trials using anti-mouse erythrocyte ghost antibodies revealed their specific binding of the tegument surface of live worms, and that the antigen-antibody complex was not shed by the parasite (data not shown). Furthermore, the use of solvents (*e.g.* 20% ethanol) did not remove these antigens from the worm's surface. Thus, it seems that erythrocyte ghost components are firmly bound to, and provide a useful marker for, the membranocalyx.

5.1.4 Aims of this chapter

In order to fully understand the mechanisms of immune evasion by the membranocalyx, it is necessary to be able to isolate it. In this chapter, two methods are utilised to remove the lipid bilayer before protein compositional analyses by electrophoresis, liquid chromatography and tandem mass spectrometry. The first method recreates the experiments of Wilson and Barnes (1977) using of cationised ferritin to promote the sloughing of the membranocalyx. The second makes use of the acquisition of mouse erythrocyte glycolipids by the membranocalyx. Antibodies were generated against these murine antigens, and used to label the surface of live adult worms. The labelled membranocalyx was then captured using magnetic beads coated with anti-rat antibody.

5.2 Methods

Part 1: Induced membranocalyx sloughing using cationised ferritin

A flowchart summarising the experimental workflow of membranocalyx isolation using cationised ferritin (CF) is shown in Fig. 5.01.

5.2.1 Labelling the adult worms with cationised ferritin

Forty mice were each infected with 350 cercariae *via* the abdomen. After six weeks, they were perfused in Eagle's medium (Invitrogen) containing 10 mM hepes and heparin (5 units/ml) at room temperature, as previously described (Curwen et al., 2004). The adult worms were washed three times in heparin-free Eagle's medium, and damaged worms and debris were removed under 10x magnification. No attempt was made to separate the male and female worms. The worms were transferred to a flat-bottomed 30 ml conical flask and labelled with 1.1 mg/ml CF (ICN Biomedicals, Cambridge, UK) in 15 ml Eagle's medium at 37 °C for 30 minutes with intermittent gentle agitation (Wilson and Barnes, 1977); this period was termed the "pulse" incubation. The worms were then washed three times in fresh Eagle's medium to remove the excess label, and incubated in 15 ml Eagle's medium at 37 °C in 5% CO_2.

5.2.2 Chase incubations

Four hours after the labelling procedure, the supernatant containing the sloughed membranes was carefully decanted from the parasites. The worms were washed once in fresh 10 ml Eagle's medium, and the wash supernatant was combined with the initial supernatant to yield the "4 hour chase material". The worms were replaced in 15 ml fresh medium and incubated for another four hours under the same conditions. After this second chase period, the sloughed membranes were harvested as before to yield the "8 hour chase material". Finally, the schistosomes were incubated for a further 16 hour period in fresh Eagle's medium to examine the long-term viability of the labelled parasites.

5.2.3 Transmission electron microscopy

Following the pulse and three chase steps, a sample of 4-5 worms from each time point were selected, and the quantity and distribution of CF labelling, as well as the integrity of the tegument, was examined by transmission electron microscopy. The specimens were

fixed in a formaldehyde/glutaraldehyde mixture, and then post-fixed in osmium tetroxide followed by uranyl acetate, as described in Chapter 3.

5.2.4 Processing the sloughed material

The sloughed material collected from the 4 and 8 hour chase incubations was pelleted by centrifugation at 100,000 *g* for 1 hr at 4 °C in a Beckman L8 Ultracentrifuge using a SW41Ti swing-out rotor. The pellets were solubilised in LDS sample buffer/ reducing agent (Invitrogen) and heated to 80 °C for 10 minutes prior to electrophoresis. One-dimensional SDS-PAGE was carried out using a pre-cast NuPAGE 4-12% Bis-Tris 1 mm thick gel (Invitrogen), run at a constant potential difference of 200 V for 40 minutes using MES buffer (Invitrogen). The gel was stained overnight using Sypro Ruby (BioRad) and imaged using a FX Imager (BioRad).

5.2.5 Tryptic digestion, LC-MALDI and database searching

The protein bands were visualised by Coomassie staining (BioSafe; BioRad) according to the manufacturer's instructions, and both gel lanes were sectioned into ten 10x10x1 mm slices with a scalpel. Each slice was subjected to tryptic digestion, the resultant peptides were separated by reversed-phase chromatography and identified by tandem mass spectrometry and database searching, as previously described (Chapter 2).

Part 2: Membranocalyx isolation using antibody labelling and affinity bead purification

A flowchart summarising the experimental workflow of antibody production, worm labelling, and membranocalyx purification using affinity beads is shown in Fig. 5.07.

5.2.6 Immunisation of rats with mouse red blood cell ghosts

Erythrocytes were obtained by the cardiac puncture of two naïve NMRI mice using a heparinised syringe, and the red blood cells were pelleted by centrifugation at 1000 *g* for 30 minutes at 4 °C. The supernatant was discarded, and the erythrocytes were resuspended in ice-cold 50 ml phosphate buffer saline (PBS) and pelleted as before. This wash procedure was repeated twice before red blood cell ghosts were produced, as follows (Marchesi and Palade, 1967). Packed cells were lysed in 10 volumes of 5 mM Tris, 1 mM EDTA, pH 7.0 (low-salt buffer), mixed for 20 minutes, and centrifuged at 25,000 *g* for 30 minutes. The supernatant was discarded, and the procedure was repeated twice. The erythrocyte membranes were then washed three times in a high-salt buffer (50 mM Tris, 0.5 M NaCl, 1 mM EDTA) for twenty minutes, and centrifuged as before. The sample, now faint pink in colour, was washed three more times in the low-salt buffer to yield a milky white preparation of erythrocyte ghosts.

The erythrocyte ghosts were resuspended in 600 µl PBS, divided into eight equal aliquots and stored at -80 °C. Two WISTAR rats each received 150 µl of an emulsion consisting of equal volumes of the erythrocyte ghost suspension and TiterMax Gold adjuvant (Sigma) mixed according to the manufacturer's instructions. Each rat was given two subcutaneous injections to the shoulder region and the process was repeated three weeks later. Bleeds were taken from the lateral tail vein two weeks before the immunisation (naïve bleed), two weeks after the initial immunisation (pre-boost bleed), and two weeks after the boost by cardiac puncture (terminal bleed). The blood was allowed to clot at room temperature for 4 hours, the top of the clot was detached from the side of the tube using a 25-gauge needle, and the blood was centrifuged at 2000 *g* for 1 hour at 4 °C. The serum was collected, aliquoted into 20 µl fractions and stored at -20 °C.

5.2.7 Measurement of antibody titre: haemagglutination

A haemagglutination assay was performed to measure the activity of anti-mouse erythrocyte antibodies in serum from the immunised rats. 50 µl PBS were added to each well of a 96-well round bottomed plate, and 50 µl sera (diluted 1/10 in PBS) from the naïve bleed, pre-boost bleed, and final bleed were added to column 1 (rows A-C for Rat 1, and D-F for Rat 2). A doubling dilution was performed across the plate so that column 12 contained sera at a concentration of 1/40960. A 50 µl 3% suspension of mouse red blood cells was added to each well and mixed with the sera by gentle pipetting. The plate was left at room temperature for 1 hour for the haemagglunitation reaction to take place.

5.2.8 Labelling live worms with anti-mouse erythrocyte antibody

Six-week old adult worms from 40 mice (approximately 2000 worm pairs) were washed three times in RPMI media and incubated in 15 ml rat anti-mouse RBC serum diluted 1/300 in RPMI for 30 mins at room temperature. Naïve rat serum was used on a small subset of worms as a negative control. The worms were washed 5 times in 50 ml RPMI and five worms were selected to examine antibody binding using fluorescent microscopy, whilst the rest were processed to isolate the membranocalyx.

5.2.9 Examining antibody labelling with fluorescence microscopy

The rat antibody-labelled worms were incubated with 1/2000 goat anti-rat IgG Alexafluor 488 antibody (Molecular Probes) for 30 minutes in the dark. Worms were washed 5 times as before and whole mounts were examined using a Nikon Labophot microscope (Nikon Corp, Tokyo, Japan) with a 10x objective lens. Digital photographs were taken with a Nikon Coolpix 995 camera (Nikon Corp).

5.2.10 Removing the labelled tegument

The worms labelled with rat antibody were treated to a freeze/thaw/vortex procedure to remove the tegument, as previously described (Chapter 2). The supernatant was recovered, centrifuged at 1000 *g* for 30 minutes to pellet the membranes, protease inhibitors were added and the sample stored on ice.

5.2.11 Affinity bead isolation of membranocalyx

A 500 µl slurry of sheep anti-rat IgG 450 µm magnetic beads (Dynal Biotech, Bromborough, UK) was washed three times in ice-cold PBS. A MPC-E magnet (Dynal

Biotech; a generous loan from Dr Jack Ford, York, UK) was used to isolate the beads between each wash step. The magnet causes the iron-containing beads to accumulate at the side of the eppendorf, which facilitates the removal of unbound material (including insoluble matter) with a long-form pipette tip. The beads were blocked in 4% bovine serum albumin for 1 hour at room temperature (to minimise non-specific binding), washed three times, divided into six equal fractions and each was made up to 500 µl with PBS. The labelled tegument pellet (from section 5.2.10) was dispersed in 3 ml of PBS and 500 µl aliquots were added to each of the six eppendorfs containing the Dynal beads. The tubes were continuously agitated by head-over-head mixing for 2 hours at room temperature. After this incubation step, the bead-membranocalyx complex was captured using the magnet as described above, the unbound material was removed and recovered (termed the flow-through), and the beads were washed seven times in PBS for 20 minutes with head-over-head mixing. Each wash supernatant was stored for further analyses. Finally, the bound material was eluted from the beads with the addition of 500 µl 100 mM ammonium acetate pH 2.0 for 20 minutes with head-over-head mixing. The process was repeated and the eluted material was pooled.

5.2.12 Electron microscopy of magnetic beads

After the wash steps, a small sample of beads was selected for inspection by transmission electron microscopy. The beads were fixed and sectioned as described in Chapter 3. The iron content of the beads did not pose a problem for sectioning, as the metal made up only a small percentage of the bead, and was disturbed evenly throughout the sphere, rather than comprising a solid core.

5.2.13 Dot-blots

A dot-blot approach was used to examine the contents of the flow-through, wash, and eluted material. A 20 µl aliquot of each sample was diluted to 200 µl in PBS, and equal volumes were pipetted over three wells onto a methanol-pre-soaked PVDF membrane (BioRad) using a 96 well Bio-Dot Apparatus (BioRad). A vacuum was applied to suck the samples onto the membrane, and an additional 200 µl PBS was added to each well to flush through any lose material. The PVDF membrane was blocked in 100 ml 4% bovine serum albumin (Sigma) for 4 hours at room temperature, washed three times for 20 minutes in PBS containing 0.1% Tween-20 (PBSt), and cut into three strips. Strip 1 was incubated in rabbit anti-mouse RBC antibody (Cedarlane laboratories Ltd, Hornby, Canada) diluted

1000-fold in PBSt for 1 hour at 37 °C, washed as before, and then incubated in goat anti-rabbit HRP antibody (1/1000 in PBSt). Strip 2 was reacted with goat anti-rat HRP antibody (1/1000 in PBSt). Strip 3 was incubated in rhesus serum (1/800 in PBSt) that had been infected with *S. mansoni* and had eliminated the worms after 18 weeks. This strip was then reacted with rabbit anti-monkey HRP antibody (1/1000 PBSt). Each strip was then washed for 3x 20 minutes in PBSt, and reacted with TMB peroxidase membrane substrate (KPL) until colour appeared (typically after 30-60 seconds). The membranes were then washed in water to quench the reaction, and scanned at 300 dpi using an Expression 1600 Pro Scanner (Epson, UK).

5.2.14 1-D SDS-PAGE, glycoprotein and protein staining

Aliquots of 100 µl of the flow-through material and each of the seven washes were concentrated to 20 µl using Ultrafree-MC Microfuge 5 kDa cut-off filters (Millipore). The eluted material was concentrated to 20 µl using a Speed-Vac. (A spin column was only necessary for the unbound and wash material because of their PBS content; as the bound material was eluted in ammonium acetate, the volume could be reduced by evaporation.) The samples were separated by SDS-PAGE as previously described (section 5.2.4). The gel was first stained for glycoproteins using Pro-Q Emerald 300 (Molecular Probes) according to the manufacturer's instructions, illuminated under ultra violet light and imaged by a digital camera (1 second exposure). The gel was then washed in 40% methanol 10% acetic acid overnight, and assayed for protein content by Sypro Ruby staining.

5.2.15 GeLC MS/MS and database searching

The gel lane containing the bound material was sectioned into 10 pieces, and each piece was digested with trypsin and subjected to LC-MS/MS as previously described (Chapter 2). Identities were sought using the tandem mass spectra to search the *S. mansoni* genome and EST databases, as well as the NCBInr database.

5.3 Results part 1 – membranocalyx isolation using cationised ferritin

5.3.1 Cationised ferritin (CF) induces the shedding of the membranocalyx

The adult worms incubated in media containing CF for 30 mins became coated in an evenly distributed layer of the label approximately 60-90 nm thick (Fig. 5.02). The CF bound to the membranocalyx over the entire worm, including the surface of the tegumental pits and invaginations. No label had penetrated the apical membranes, and the underlying tegument appeared undamaged by the labelling and washing procedures. After 4 hours in CF-free medium, the amount of bound label had greatly reduced, and was mostly restricted to the apical membranes surrounding the spines (Fig. 5.03). At natural protrusions, the membranocalyx had started to peel away from the tegument, leaving behind an exposed, but seemingly intact plasma membrane. After 8 hours in CF-free medium, the label was almost entirely lost from the worms' surface (Fig. 5.04). Only a few patches remained on the dorsal surfaces of the worm. Large sheets of the membranocalyx appeared to be peeling away from the plasma membrane and shedding into the surrounding medium. Even after 8 hours, the underlying tegument looked healthy and there were no signs of damage to the plasma membrane. The sub-tegumental cell bodies continued to produce discoid bodies and membranous vesicles, and the latter could be seen in close proximity to the surface pits. There was no visible evidence of CF bound to the tegument surface 24 hours after the initial labelling process (Fig. 5.05). The labelled membranocalyx appeared to have been completely shed from the worms' surface and replaced by a new, unlabelled lipid bilayer (Fig. 5.05 inset). A fault line running parallel to the tegument basal membrane could be seen at the 24 hour time point, otherwise the syncytium appeared to be intact, with no obvious signs of degradation such as blebs or large vacuoles (Fig. 5.05). Furthermore, after this time >95% of the worms were still paired and wriggling. There was no evidence of the label being internalised by endocytosis, as no endosomes containing CF were visible at any of the time points. Other tissues with surfaces potentially exposed to CF, such as the oesophagus and gut, showed no signs of labelling.

5.3.2 More material is released after the 8-hour chase incubation

The shed surface membranes from both time points were collected by high-speed centrifugation. The pelleted 4 hour chase material resembled a very small, opaque pellet that dispersed easily with gentle pipetting. The 8 hour chase yielded visibly more material,

almost twice that of the initial 4 hour chase. Furthermore, this pellet had a slightly darker colour (a light brown tinge), and was harder to dislodge from the centrifuge tube. Both pellets were solubilised in SDS and separated by 1-D SDS-PAGE (Fig. 5.06). Protein staining by Sypro Ruby revealed differences in the composition of the material released after four and eight hours. The former resembled a smear throughout the gel, with only a few distinguished bands at ~220, 40 and 20 kDa (Fig. 5.06). The 40 and 20 kDa bands were also present when a sample of CF alone was run down a 1-D gel (Fig. 5.06). In contrast, the banding pattern of the material released after eight hours contained many distinct bands ranging in size from 150 to 10 kDa (Fig. 5.06). Due to the smudged appearance of the gels, it is very difficult to compare the two samples with any exactness.

5.3.3 Membrane and secreted proteins are shed into the medium

The proteins in both gel lanes were digested and their identities were sought by LC-MS/MS and database searching. Table 5.1 displays the positive matches to the *S. mansoni* EST, genomic or NCBInr databases. A combined total of 27 different proteins were identified from both the time points, and putative identities were ascribed based on homologies with known proteins in the GenBank database. It was reassuring to identify the labelling component, horse ferritin, at both time points. Nine other proteins were common to both time points, 56% of which were classified as membrane spanning, membrane bound or secreted according to PSORT II and PENCE analyses. These comprised the schistosome sugar transporter, SGTP1; two tetraspanin proteins, B (Sm23) and D (CD63-like protein); the secreted protein, Sm29; and the phospholipid-binding proteinase, calpain. The intracellular proteins that were common to both chase incubations consisted of the structural components actin, alpha and beta tubulin, as well as the omnipresent heat shock protein 70.

Seven proteins were unique to the material released after the 4 hour incubation in CF-free medium (Table 5.1). The tegumental antigen, Sm13, a putatively secreted schistosome protein was notable in this category, as well as three more heat shock proteins, HSP 90, 86 and 60. Two nuclear proteins (histone 4 and 2a) were identified, together with two schistosome proteins that bore no homology to entries in the GenBank database. The 8 hour chase released nine proteins that were not identified in the material released after four hours. Two of these were membrane spanning proteins, SNaK1 and dysferlin, as well as the phospholipid-binding protein, annexin. Two further proteins, Sm22.6 and T complex

protein-10, both involved in cytoskeletal functions were identified at this time point, plus the cytosolic enzyme GAPDH and three uncharacterised proteins unique to *S. mansoni*. It should be noted that no proteins derived from the host were identified in either sample. Likewise, there was no evidence for parasite proteins belonging to the mitochondria, endoplasmic reticulum, or Gogli apparatus, and no components involved in signal transmission/reception were identified.

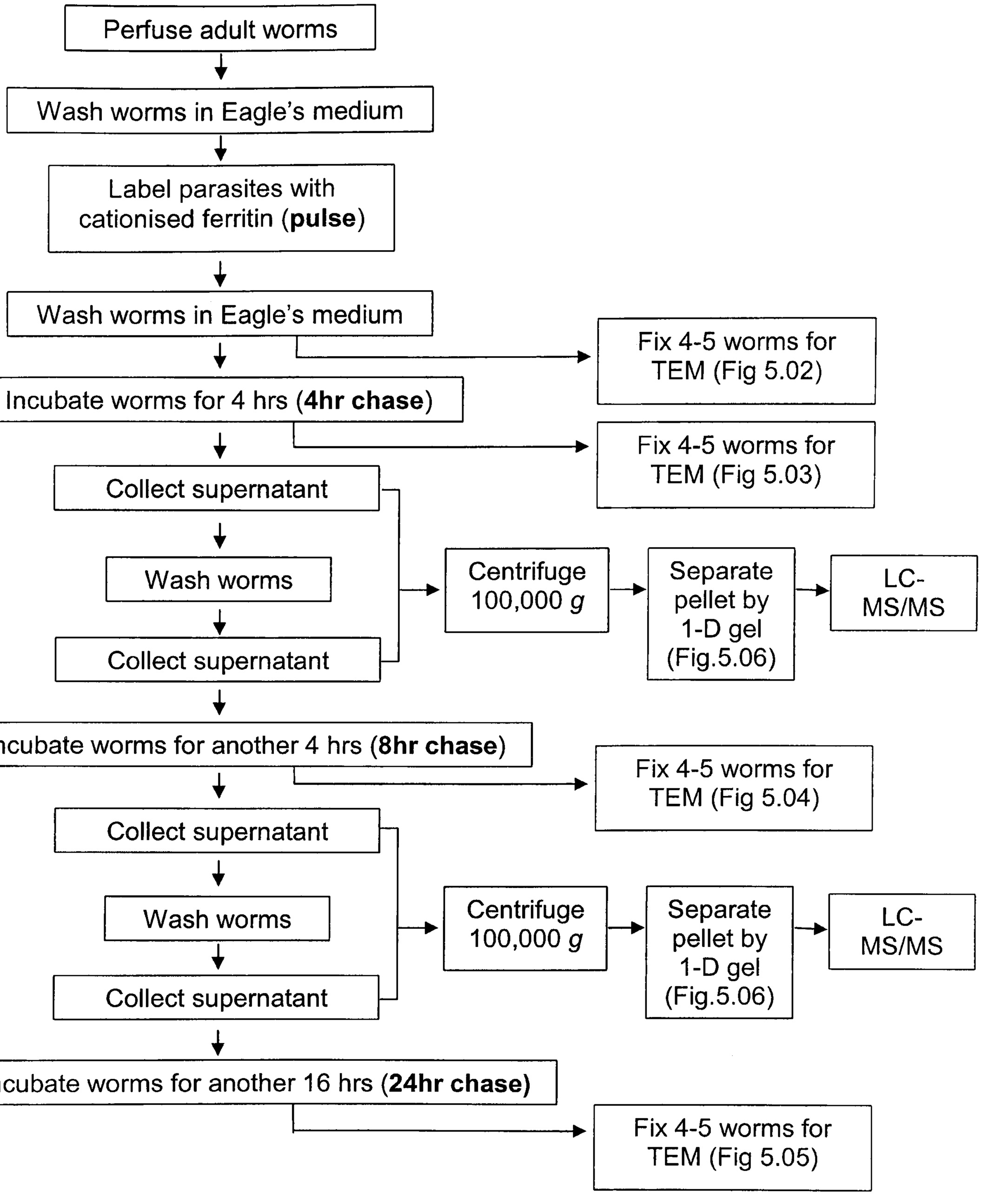

Figure 5.01. Experimental workflow of membranocalyx isolation using cationised ferritin.

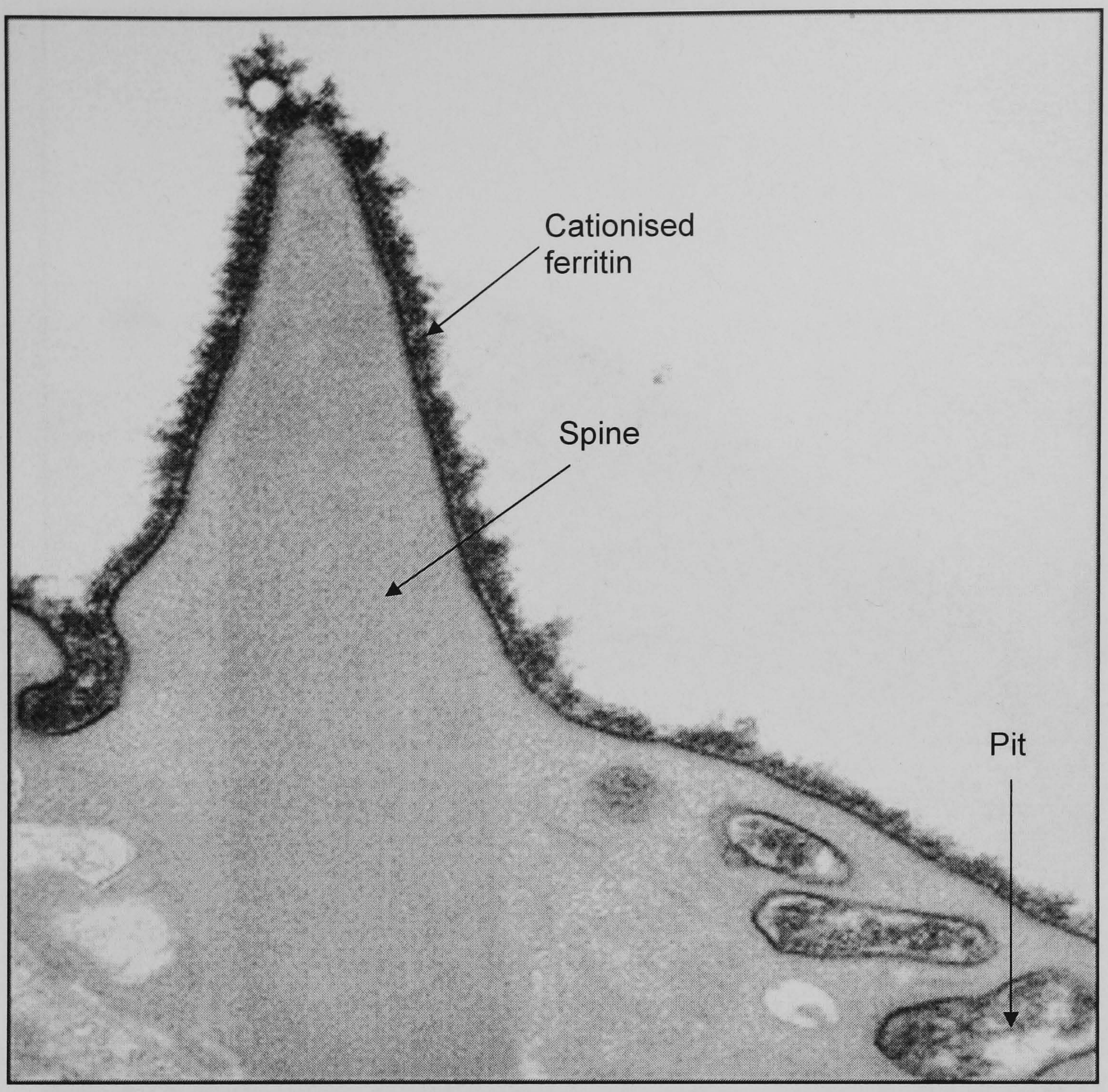

Figure 5.02. Transmission electron micrograph showing the dorsal surface of an adult male worm after a 30 minute incubation in cationised ferritin. The label has attached to all the exposed surfaces of the parasite including the tegumental pits.

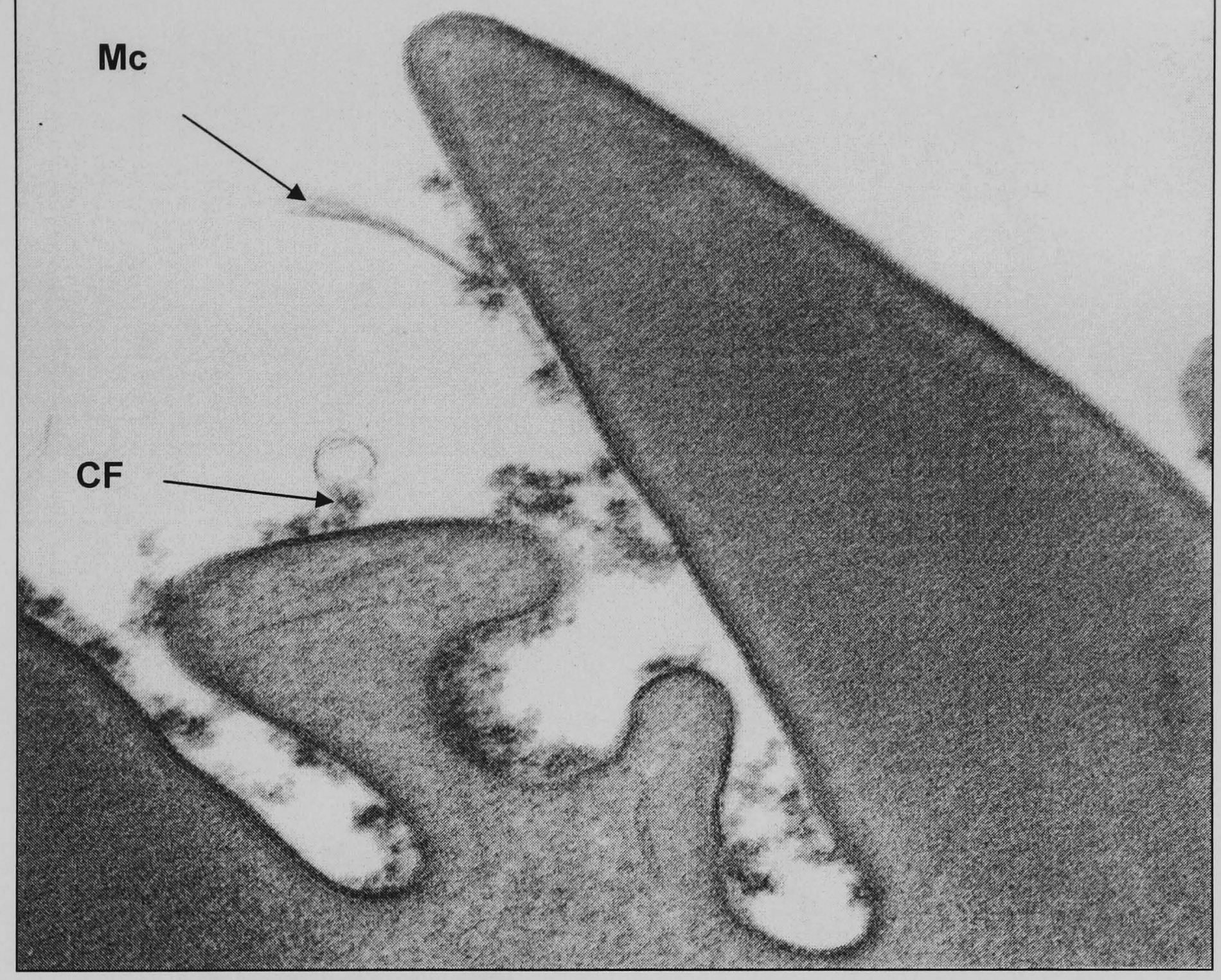

Figure 5.03. The surface of the tegument 4 hours after it was initially labelled with cationised ferritin (CF). The label is visible in patches, especially in the vicinity of the spines. At some sites, the membranocalyx (Mc) appears to be peeling away from the plasma membrane.

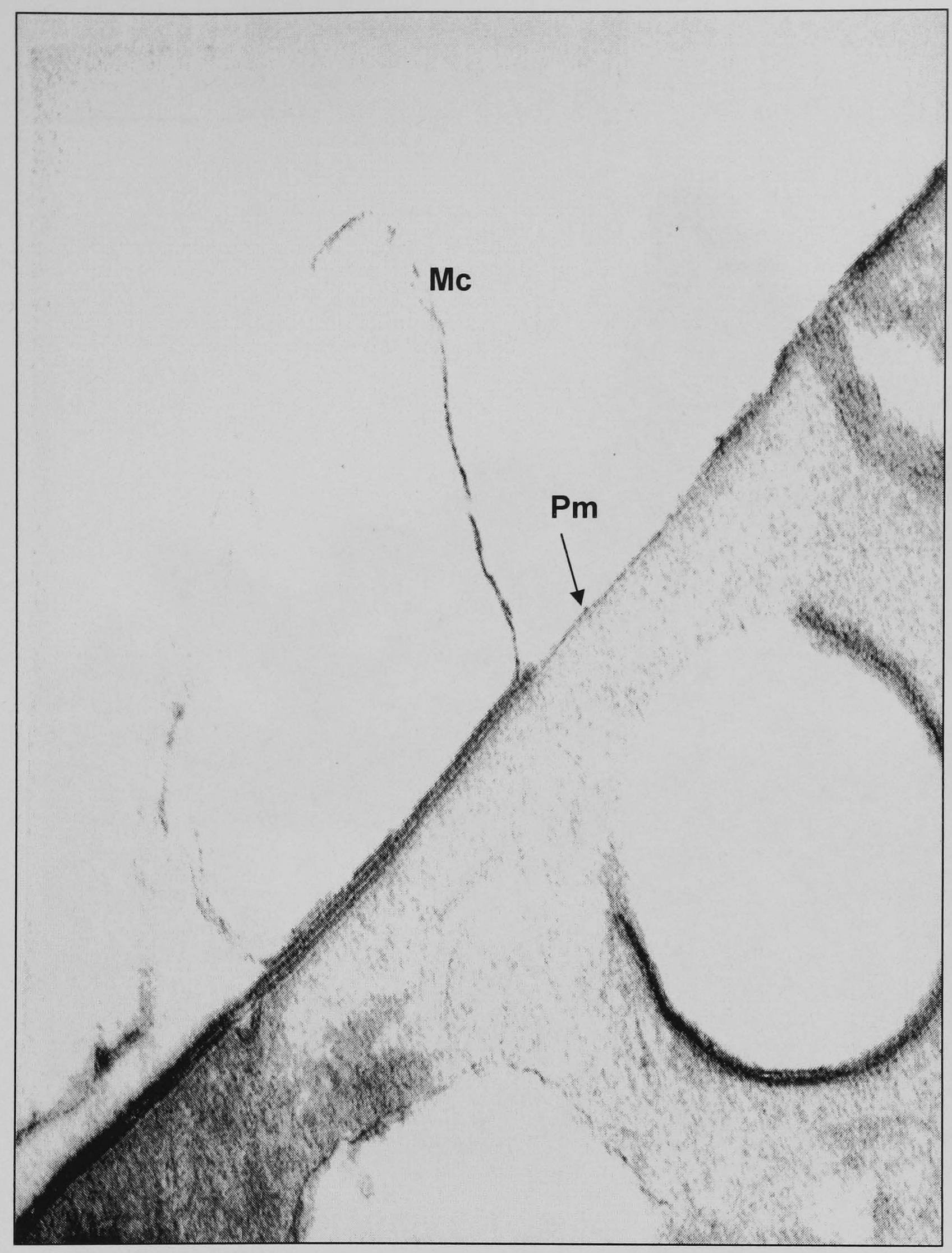

Figure 5.04. TEM of the tegument surface 8 hours after labelling with cationised ferritin. The label has been virtually cleared from the parasite surface, and the membranocalyx (Mc) can be seen peeling away from the plasma membrane (Pm).

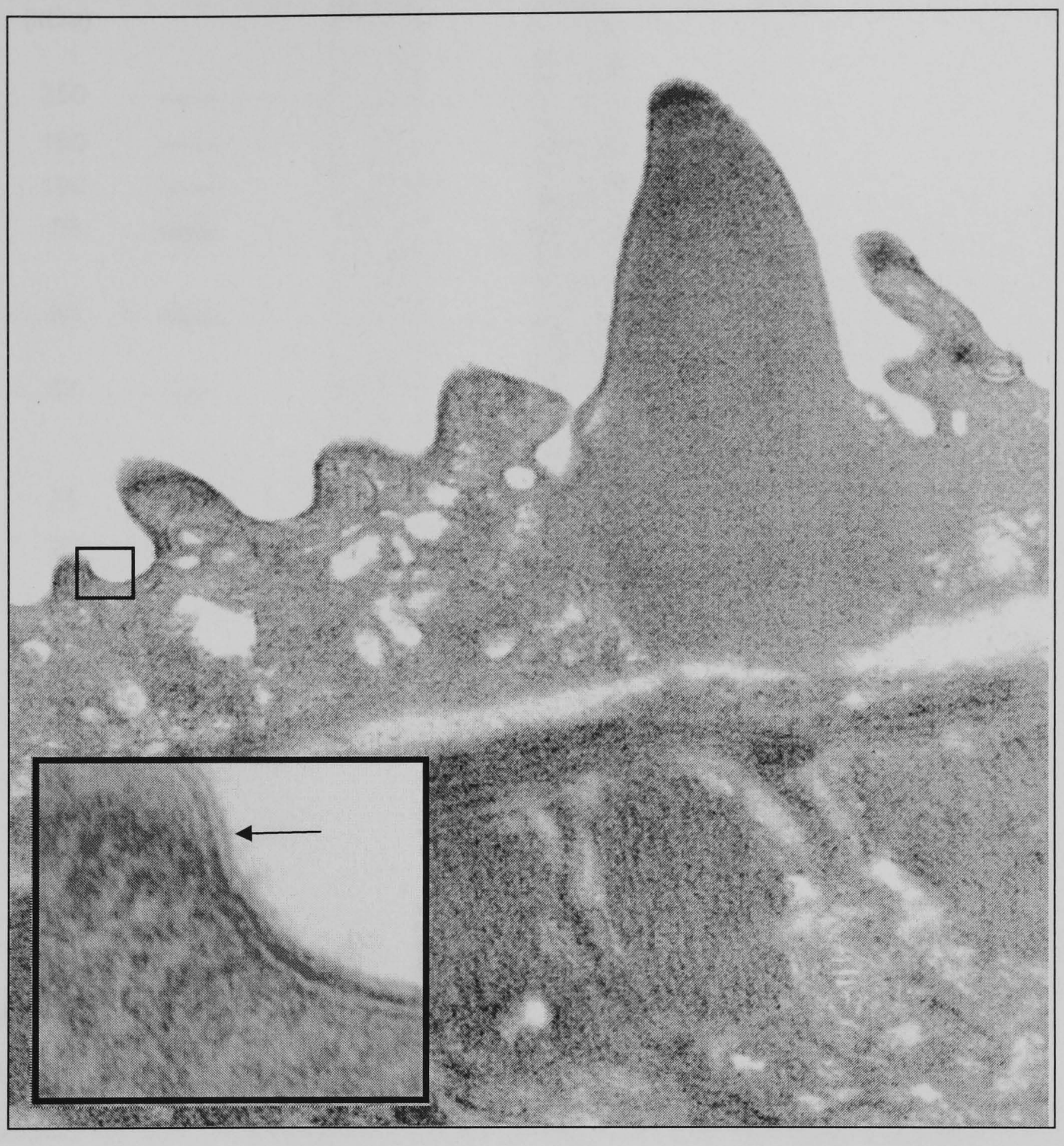

Figure 5.05. 24 hours after the labelling procedure, the tegument surface has been restored to its native state. There is no evidence of cationised ferritin bound to the apical membranes. The tegument appears intact, and the characteristic double bilayer surface of the tegument is visible (arrow).

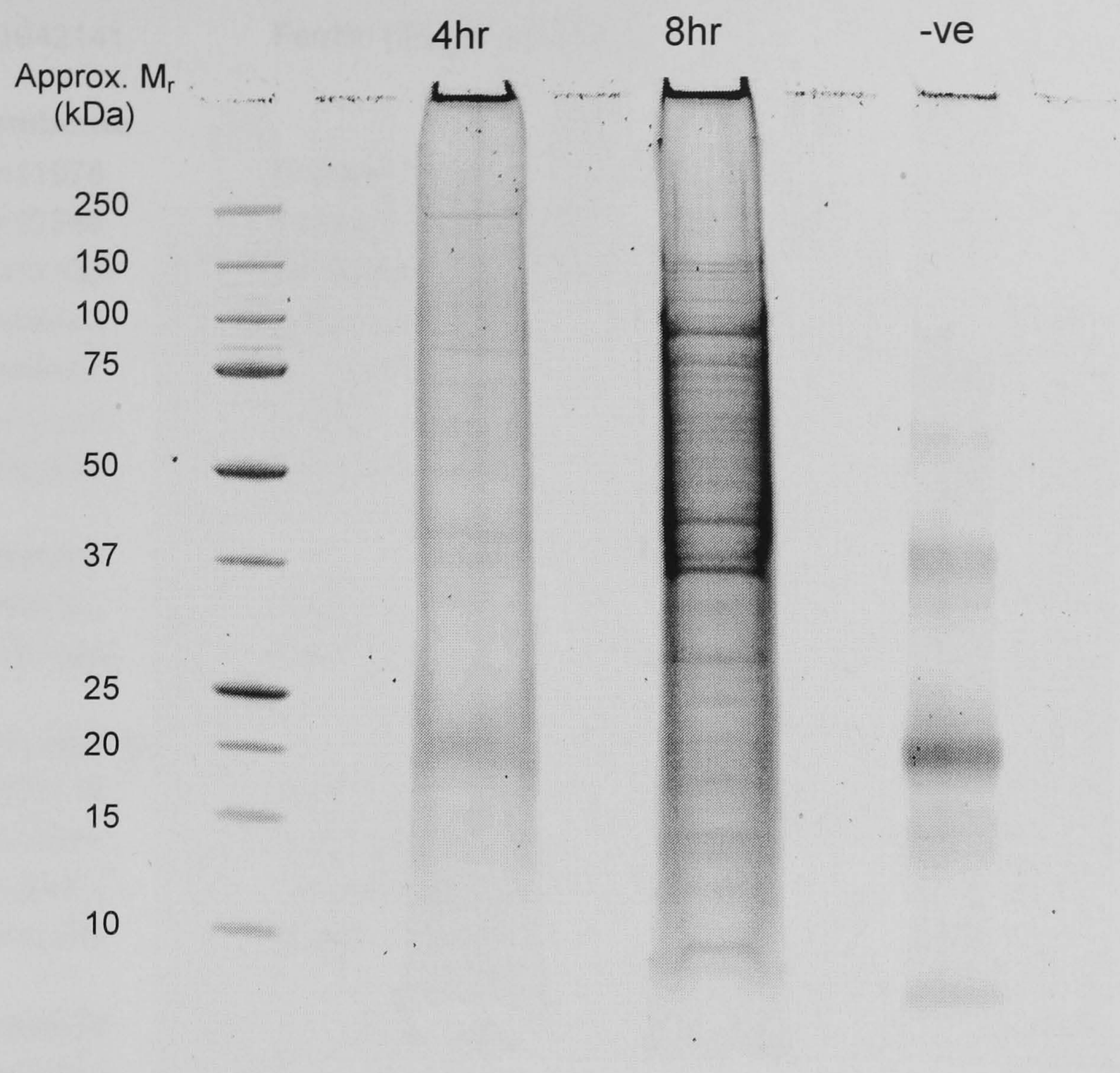

Figure 5.06. Sypro Ruby-stained 1-D separation of the insoluble material released four hours (**4hr**) and eight hours (**8hr**) after parasite labelling with cationised ferritin. The negative control (**-ve**) is 5 µl of the label by itself.

Accession #	Protein name	Time point 4hr	8hr
Label			
gi|1942141	Ferritin (*Equus caballus*)	♦	♦
Membrane			
Sm11978	Glucose transport protein SGTP1	♦	♦
Sm02886	Tetraspanin C (Sm23)	♦	♦
Sm12366	Tetraspanin D (CD63-like protein)	♦	♦
Sm08542	Calpain-B	♦	♦
Sm03987	Annexin		♦
Sm07319	SNaK1		♦
Sm02186	Dysferlin		♦
Secreted			
Sm09193	Sm29	♦	♦
gi|1519552	Sm13	♦	
Cytoskeletal			
Sm01276	Actin	♦	♦
Sm00654	Tubulin alpha chain	♦	♦
Sm06624	Tubulin beta chain	♦	♦
Sm12876	Sm22.6		♦
Cytosolic			
Sm09042	Heat shock protein 70	♦	♦
Sm01524	Heat shock protein 90	♦	
gi|320985	Heat shock protein 86	♦	
Sm01537	Heat shock protein 60	♦	
gi|201736	T complex protein-10		♦
Sm14536	GAPDH		♦
Nuclear			
Sm08070	Histone 2a	♦	
Sm06454	Germinal histone H4 gene	♦	
No homology			
Sm11358	Unknown		♦
Sm08994	Unknown	♦	
Sm03716	Unknown	♦	
Sm09966	Unknown		♦
Sm05086	Unknown		♦

Table 5.1. Proteins releases from live worms 4 and 8 hours after labelling with cationised ferritin.

Results part 2 – membranocalyx isolation with magnetic beads

5.3.4 Haemagglutination

A haemagglutination assay was performed to determine the antibody titre of the sera from rats immunised against mouse erythrocytes ghosts. The naïve-bleed sera of both rats did not agglutinate the RBCs; all cells precipitated into tight buttons at the bottom of the wells (Fig. 5.08). The pre-boost serum from rat 1 caused the erythrocytes to form rafts until the concentration of serum was diluted to 1/160 (Fig. 5.08). The equivalent serum from rat 2 did not prevent erythrocyte sedimentation. The rat antibodies from the terminal bleed were able to agglutinate the mouse RBCs until the sera reached a dilution of 1/320 and 1/640 in rats 1 and 2, respectively (Fig. 5.08).

5.3.5 Anti-mouse RBC antibody reacts with the surface of *ex-vivo* worms

The extent of antibody labelling of the membranocalyx was studied using a secondary antibody conjugated with a fluorescent tag. The worms incubated with naïve rat serum (negative control) showed no signs of reaction with the secondary antibody (Fig. 5.09), although a small amount of auto-fluorescence was observed, especially from the eggs and vitellaria of the female worms. The worms labelled with rat anti-mouse RBC ghost antibodies exhibited high reactivity with the secondary antibody (Fig. 5.09). All the exposed surfaces of the worm, from the oral sucker to the tail on worms of both sexes, glowed with equal fluorescence intensity. Only those areas of the worm that were shielded from antibody binding, the gynaecophoric canal of the male for example, showed no evidence of labelling. Similarly, there was no sign of antibody ingestion into the oesophagus or gut of the parasites.

5.3.6 1-D SDS PAGE – glycoprotein staining

The flow-through, wash, and eluted material from the magnetic beads were separated by 1-D SDS-PAGE. The gel was first stained for glycoprotein content (Fig. 5.15). The flow-through contained at least 30 distinct bands (lane 1), and the wash steps displayed progressively decreasing amounts of staining (lanes 2-8). The eluted sample showed extremely high reactivity with the Pro-Q Emerald glycoprotein stain, especially evident at the 20-25 kDa range (lane 10). Bands of lesser fluorescent intensity were also evident at approximately 75, 50, 30 and 12 kDa.

5.3.7 1-D SDS-PAGE – protein staining

After staining for glycoproteins (5.3.6), the gel was destained and treated with Sypro Ruby to detect protein bands (Fig. 5.16). The flow-through contained the largest amount of protein with clear bands ranging from >250 to <10 k Da (lane 1). The protein-stained material removed from the beads by the washing procedure also exhibited a broad diversity of proteins. After three washes (lane 4), there was no protein staining was visible. The eluted material contained approximately 10 bands of distinct molecular weights (lane 9). Blurred staining was evident throughout this lane, but was especially prominent between 37 and 20 kDa.

5.3.8 Electron microscope study

Transmission electron microscopy was used to examine the material that had bound to the beads after the 2 hour incubation and wash steps. The anti-mouse RBC antibody labelled material became attached to the magnetic beads in a patchy manner (Fig. 5.11 & 5.12). Lipid bilayers could be observed at the surface of the beads, sometimes as a single bilayer, and sometimes as multilaminate sheets that had folded up on themselves (Fig. 5.11). Small blobs (approximately 30 nm in diameter) could be seen attached to the membranes, but whether they were of parasite or host origin, or merely contaminants from the separation procedure, could not be determined (Fig. 5.11B). In some of the samples examined by TEM, it was evident that the tegument syncytial material was still attached to the apical bilayers (Fig. 5.13). In these cases, tegument organelles such as the multilaminate vesicles could be observed. The tegument membrane material derived from worms that had been incubated in naïve rat serum did not bind to the magnetic beads (negative control; Fig. 5.14).

5.3.9 Dot-blots

Dot-blots were used to analyse the contents of the flow-through, wash and eluted material, to assess the efficiency of the membranocalyx isolation procedure. The rabbit anti-mouse RBC antibody reacted with the flow-through of rat antibody-labelled tegument and the negative control (Fig. 5.10, strip 1). However, only the eluted material derived from the rat antibody-labelled worms reacted with the rabbit antibody (Fig. 5.10, strip 1 +ve). Goat anti-rat antibody was only reactive with the eluted, and to a lesser extent, the flow-through and wash steps of the tegument material that had been incubated with immunised rat serum (Fig. 5.10, strip 2); the negative control showed no presence of rat antibody (Fig.

5.10, strip 2 -ve). The rhesus anti-schistosome antibodies bound to the material in the flow-through, wash and elution steps of the labelled tegument, revealing the presence of numerous parasite antigens (Fig. 5.10, strip 3 +ve). The primate antibody also bound to the flow-through and wash samples, but not to the eluate, of the negative control (Fig. 5.10, strip 3 -ve).

5.3.10 Protein identities

A total of 21 different proteins were identified in the material eluted from the magnetic beads (Table 5.2). (No attempt was made to characterise the proteins in the flow-through or wash steps.) Five membrane proteins were detected, three of which (Sm25, Tetraspanin D (CD63-like protein) and SNaK1) have transmembrane domains, whilst annexin and calpain both have phospholipid-binding properties. No membrane-spanning enzymes or receptors were present. A single secreted protein was identified, Sm 29. Of the secreted and membrane proteins, SNaK1, Sm25 and Sm29 have N-glycosylation sites (predicted by NetNGlyc 1.0 Server). Six components of the cell cytoskeleton were identified, namely the tubulin alpha and beta chains, actin, calponin, and Sm20.8 & 22.6, the latter two possess a calcium-binding EF-hand motif. A further four intracellular proteins were identified: HSP 86, methyltransferase, and 14-3-3 of the cytosol, and histone H4 of the nucleus. The tandem mass spectra also matched three gene-predictions or ESTs in the *S. mansoni* database that possessed no homology to any protein in the GenBank database. The heavy and light chains of the labelling rat antibody were identified, but no other rat proteins were found by proteomics. Furthermore, no mouse erythrocyte, blood plasma or immunological proteins were identified.

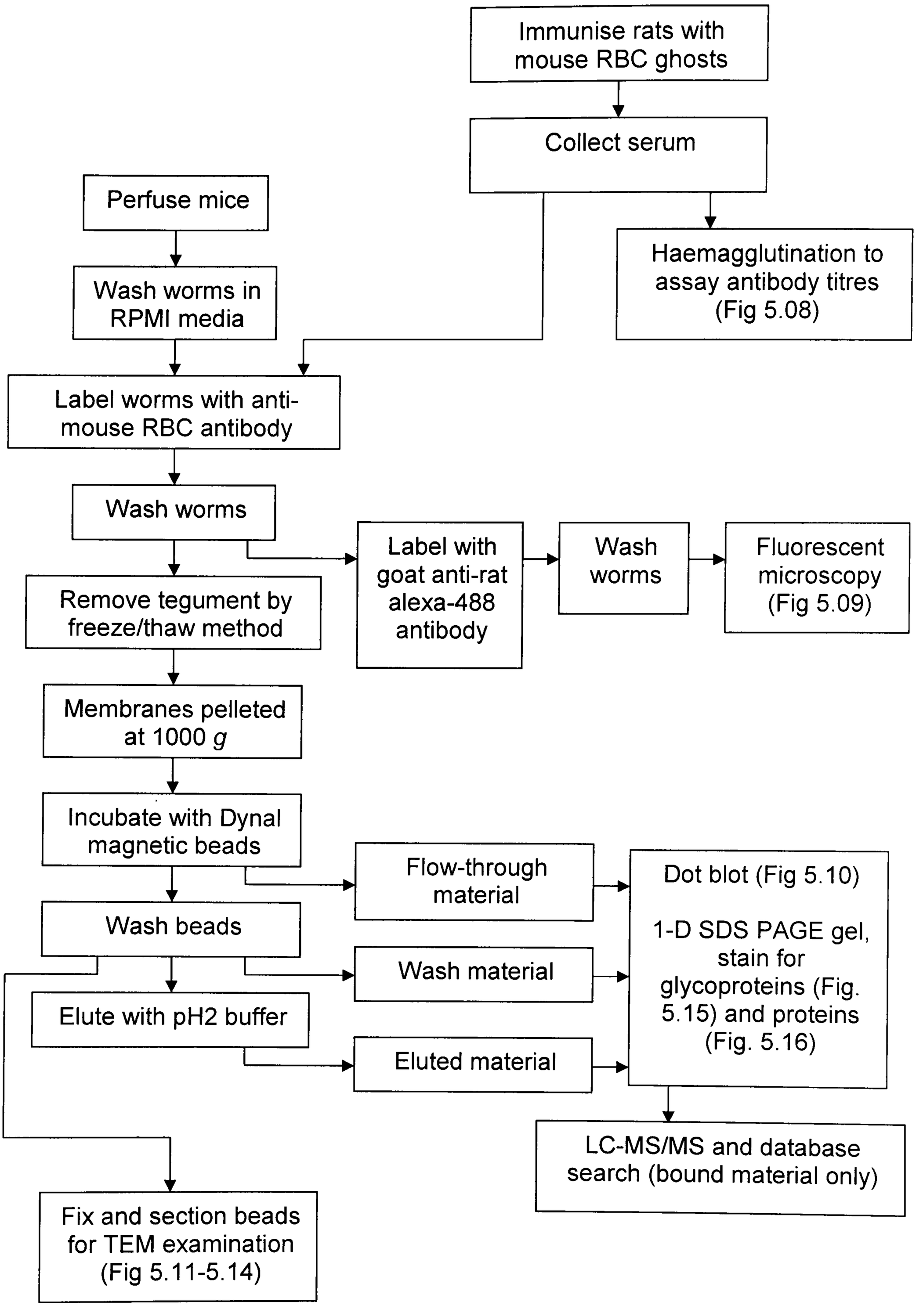

Figure 5.07. Experimental workflow of membranocalyx isolation using antibody labelling and magnetic beads.

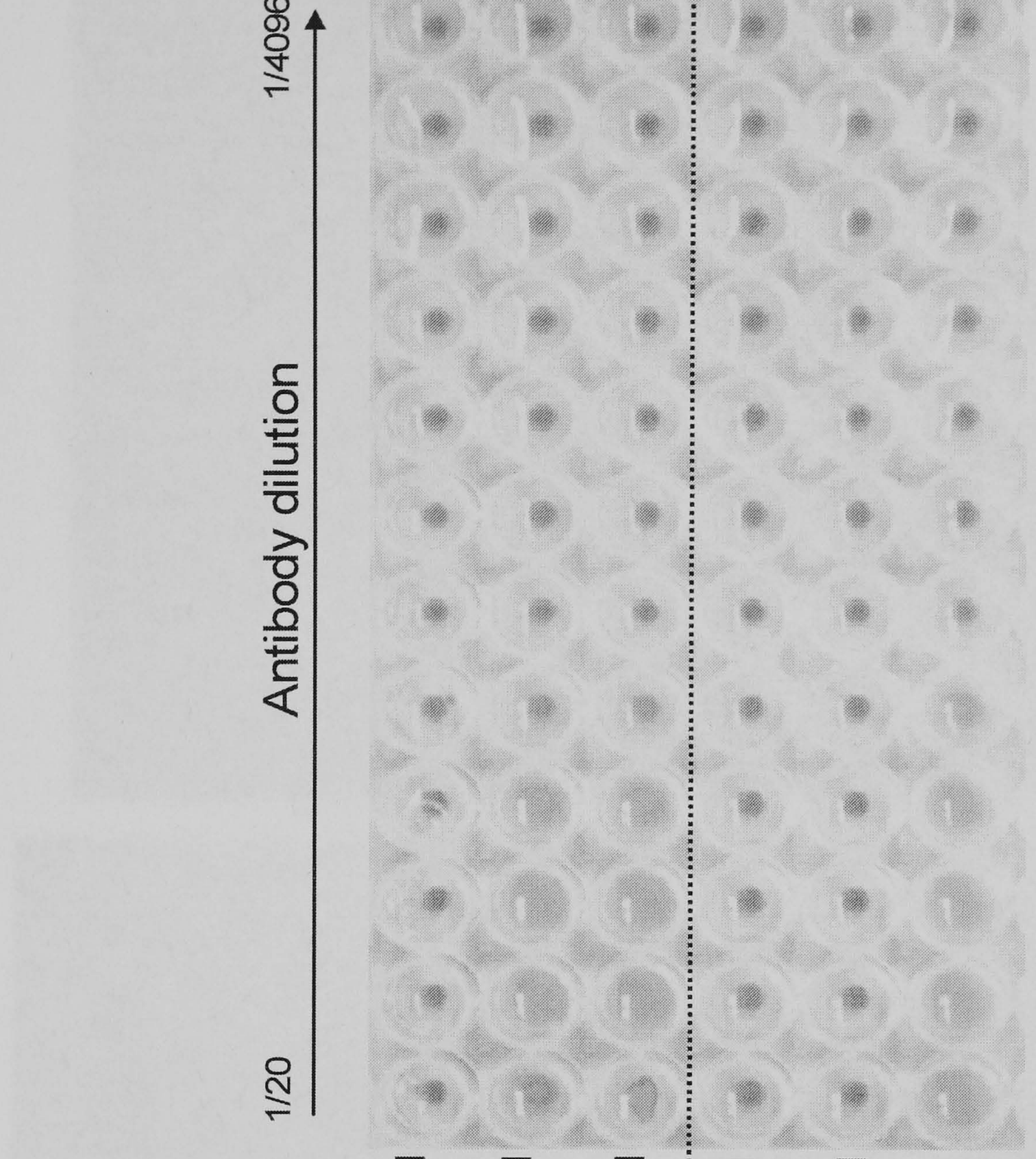

Figure 5.08. Haemagglutination of mouse red blood cells in sera obtained from naïve, pre-boost, and terminal bleeds of rats immunised with mouse erythrocyte ghosts.

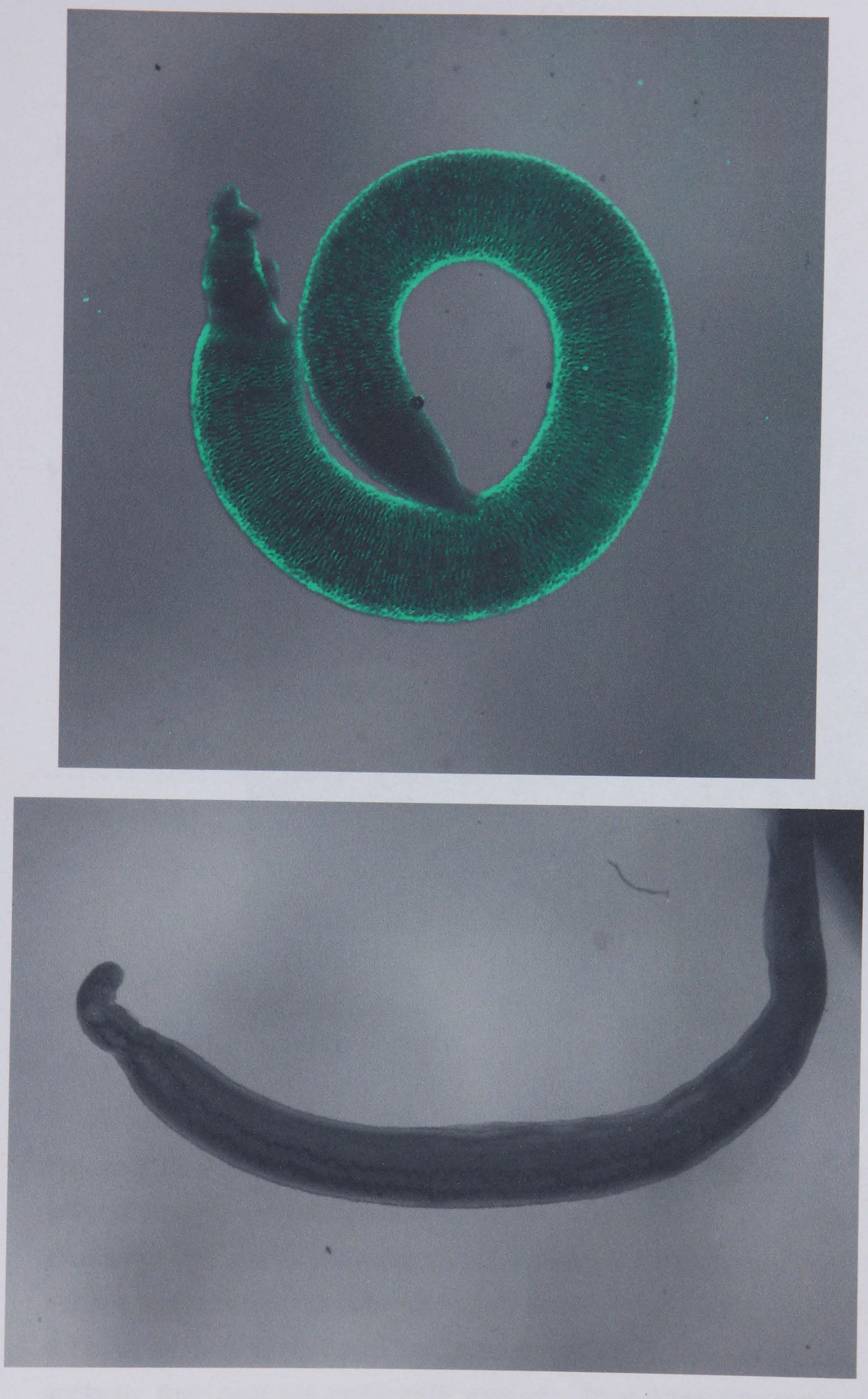

Figure 5.09. Adult male worms incubated with rat anti-mouse RBC ghost serum (top), or naïve rat serum (bottom). Antibody binding was visualised using Alexafluor goat anti-rat antibodies.

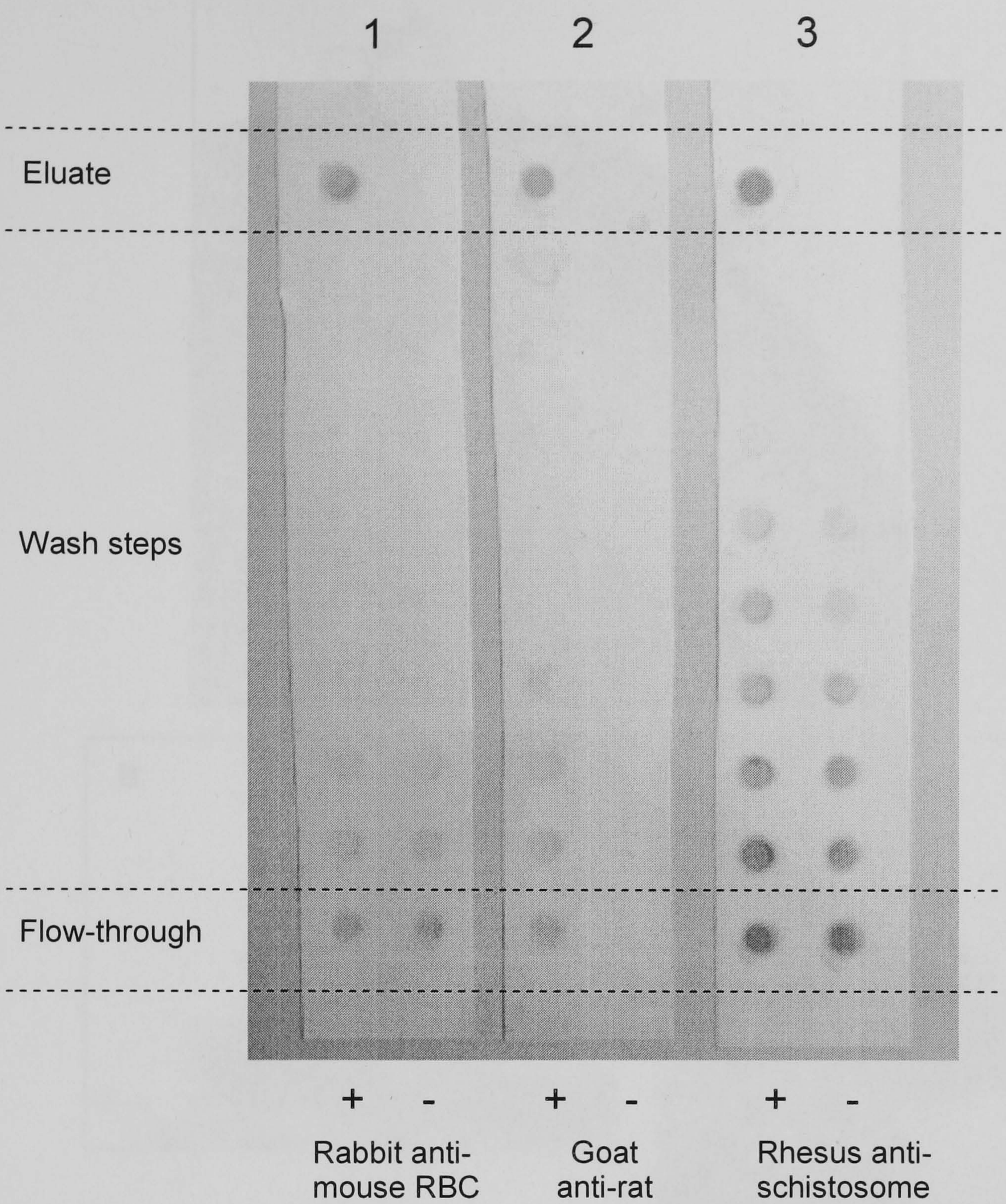

Figure 5.10. Dot-blot analyses. The stripped tegument that had been labelled with rat anti-mouse RBC ghost antibody (**+**) or naïve rat serum (**-**) was incubated with anti-rat magnetic beads. An aliquot of the flow-through, wash steps and eluate was transferred to a PVDF membrane and tested for specific antibody binding: **Strip 1** was first reacted with rabbit anti-mouse RBC antibody, then goat anti-rabbit HRP antibody. **Strip 2** was incubated with goat anti-rat HRP antibody. **Strip 3** was reacted with rhesus anti-schistosome antibody, then rabbit anti-monkey HRP antibody. The bound antibody in each strip was visualised by the addition of peroxidase substrate.

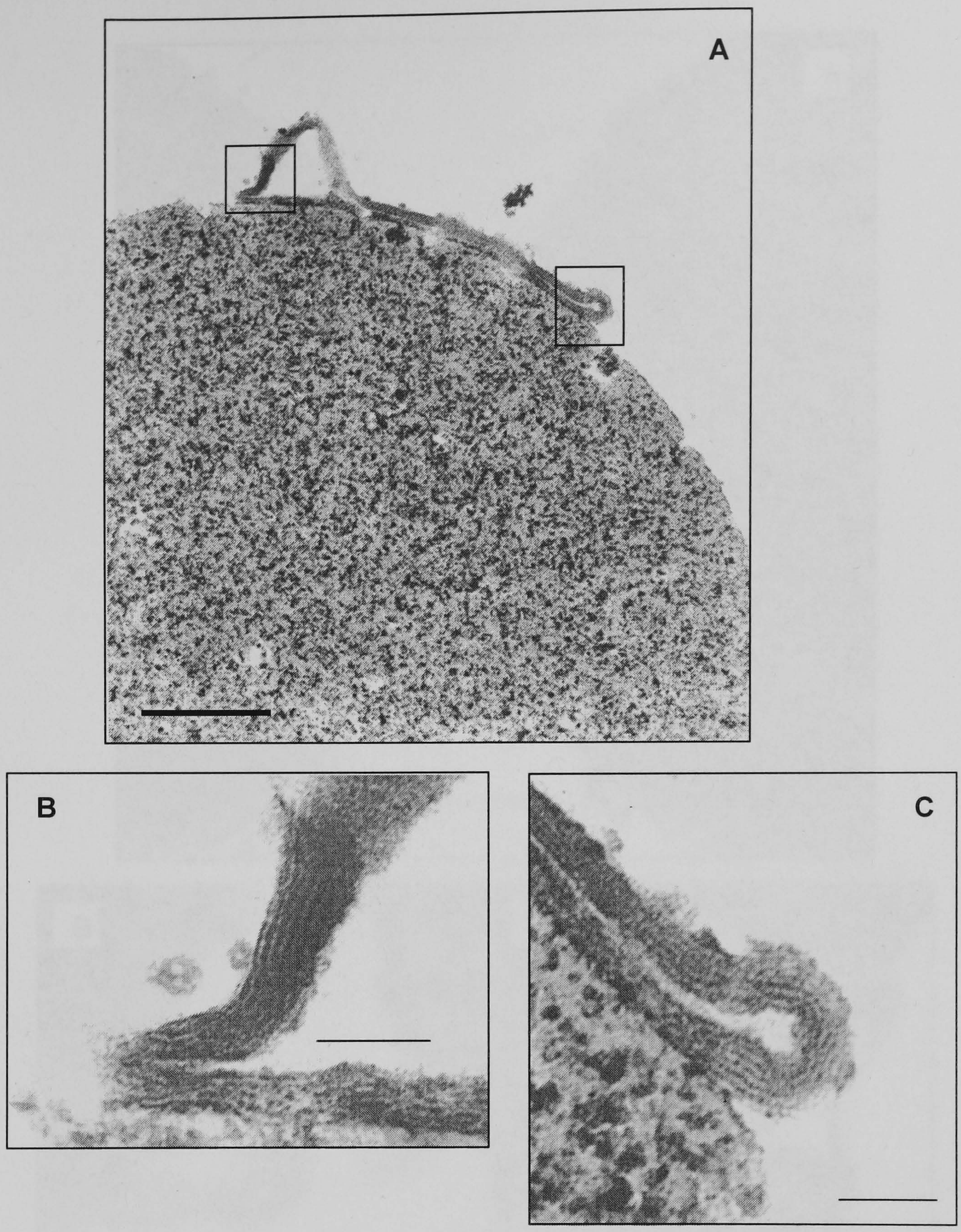

Figure 5.11. TEM pictures of membranous material bound to magnetic beads. Boxes **B** and **C** show high magnification images of the indicated areas in **A**. Bar equals 500, 80 and 100 nm in **A**, **B**, & **C**, respectively.

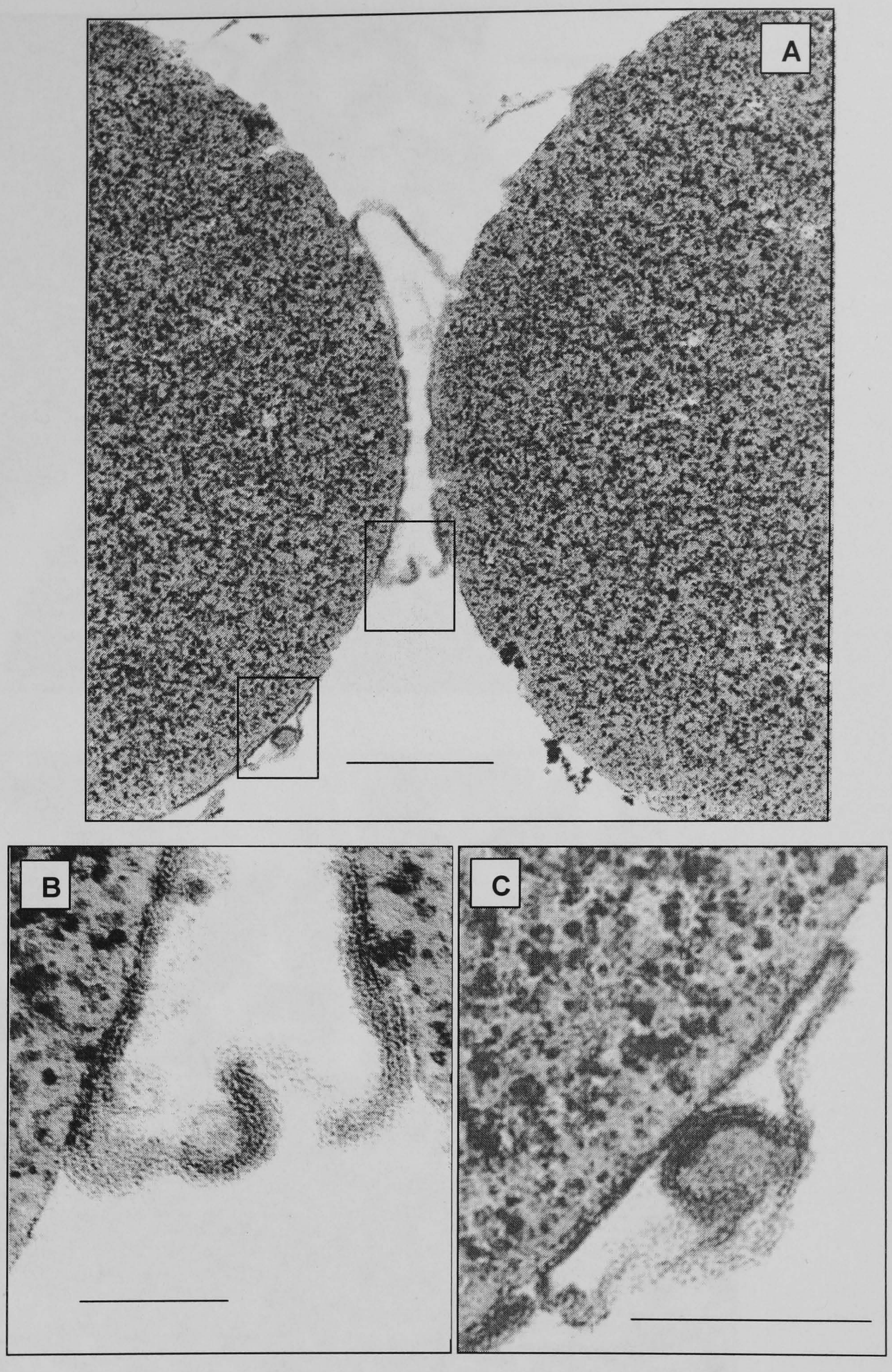

Figure 5.12. TEM images of Dynal beads attached to apical membranes of the tegument. **B** and **C** show a magnified view of the boxed areas in **A**. Bar equals 600, 130, 230 nm in **A**, **B** & **C**, respectively.

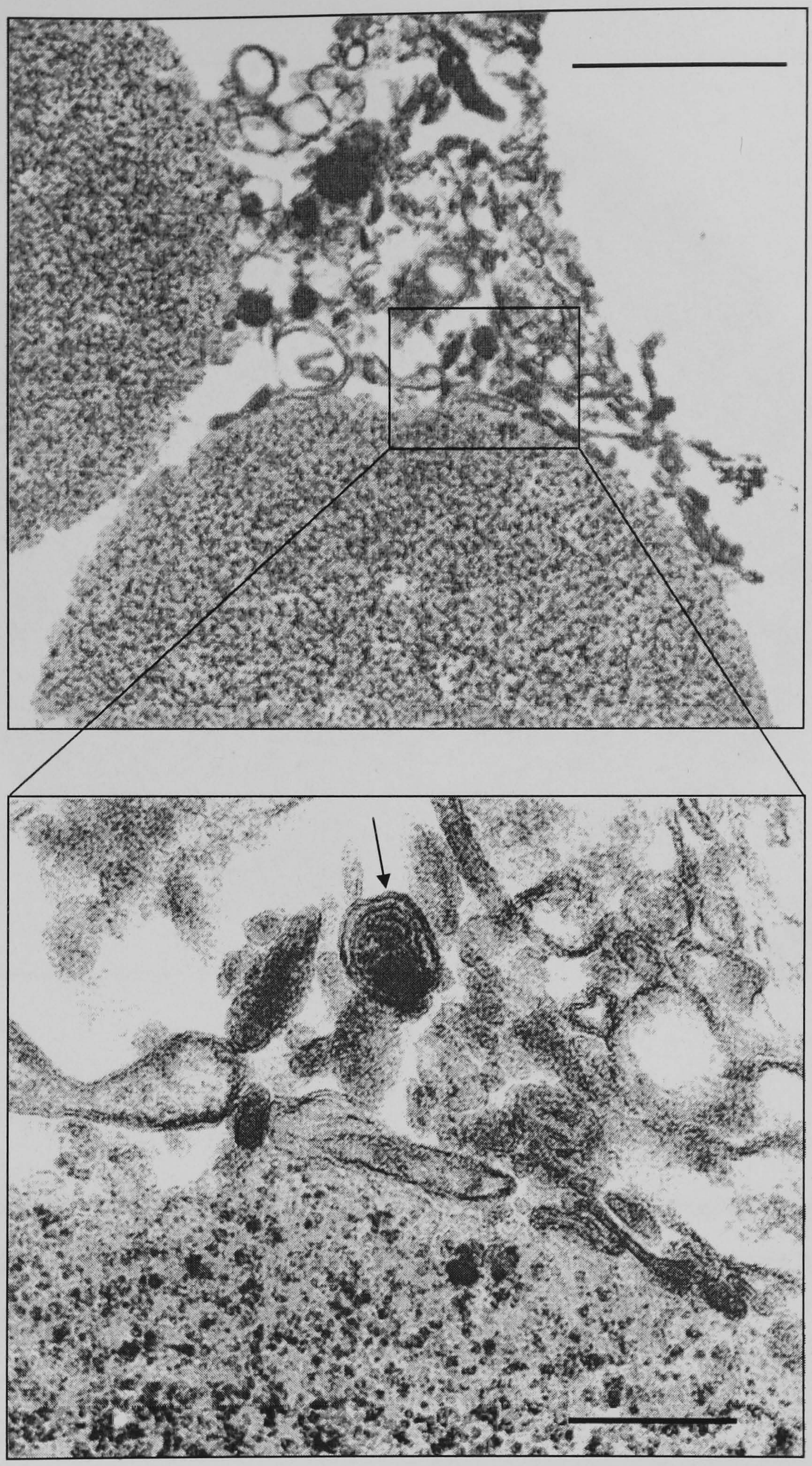

Figure 5.13. TEM image of internal tegument material attached to a magnetic bead. Arrow points to a multilaminate vesicle. Bar equals 1.4 µm (top) and 230 nm (bottom).

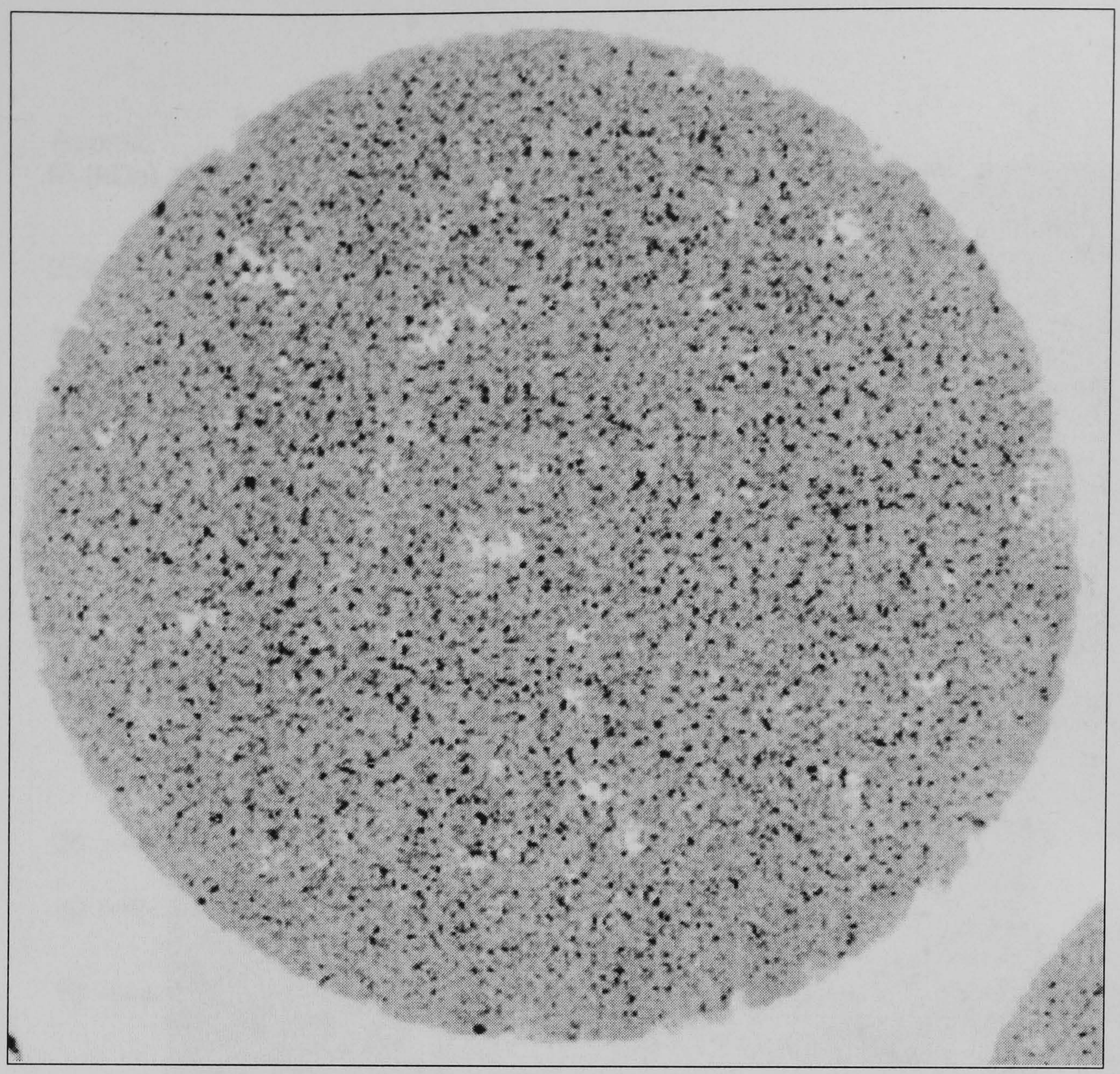

Figure 5.14. Negative control. TEM image of a Dynal bead that was incubated with tegument material derived from worms that were incubated in naïve rat serum.

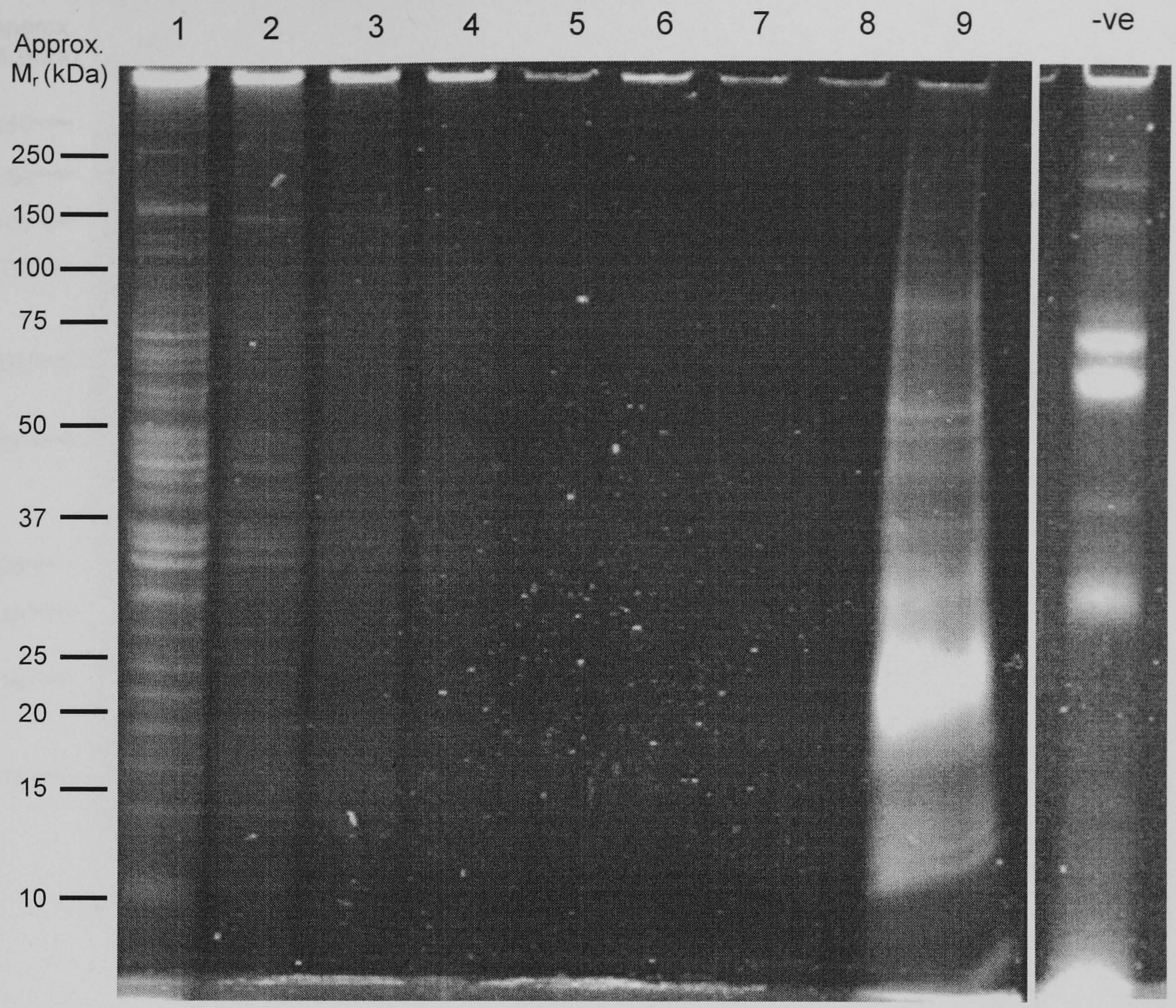

Figure 5.15. 1-D separations of flow-through (lane 1), wash (lanes 2-8) and eluted material (lane 9) from the magnetic beads, stained with Pro Q Emerald to identify glycan residues. The negative control (-ve) comprises the eluted material from beads incubated with rat anti-mouse RBC serum.

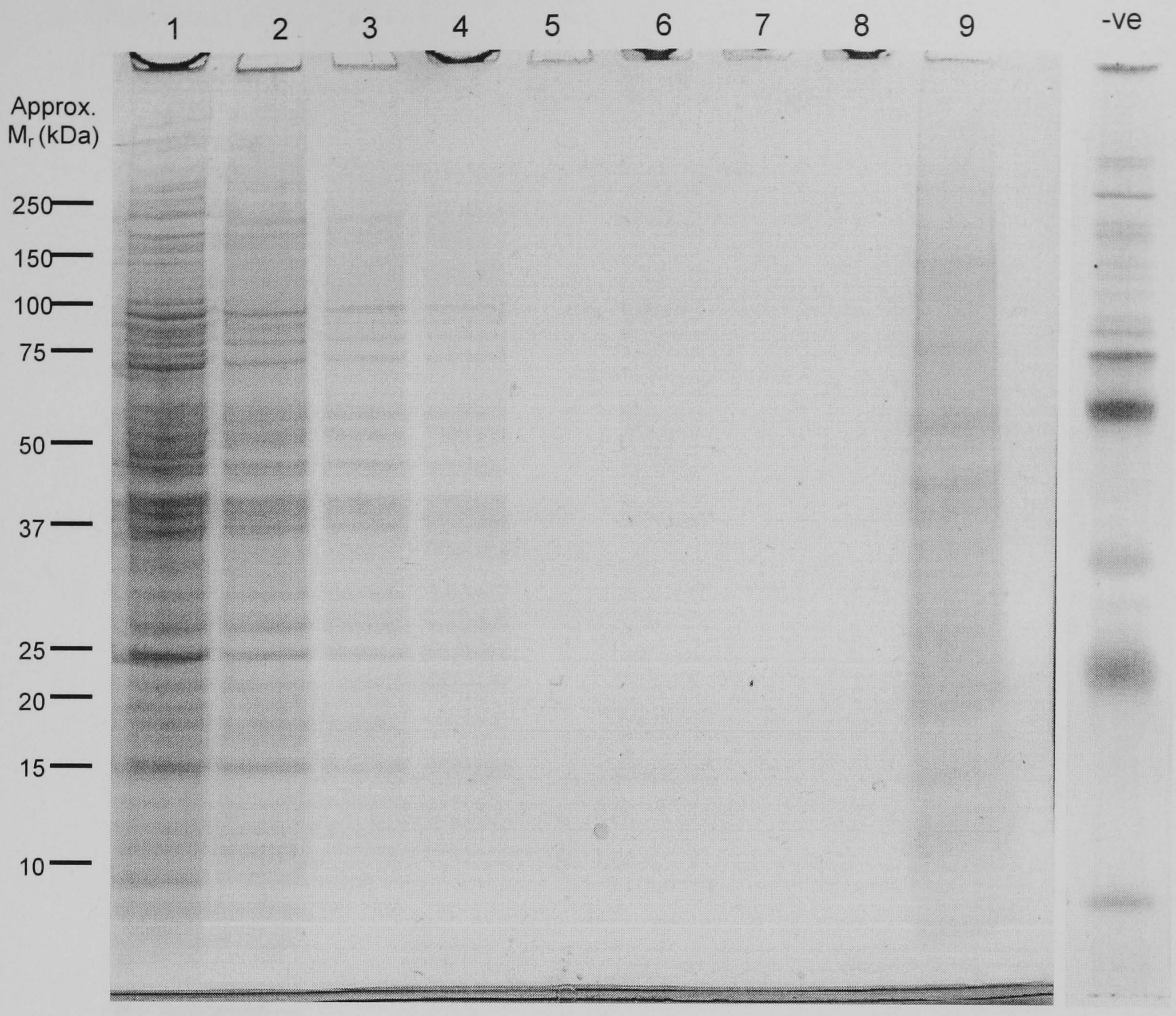

Figure 5.16. 1-D separations of flow-through (lane 1), wash (lanes 2-8) and eluted material (lane 9) from the magnetic beads, stained with Sypro Ruby to visualise proteins. (This is the same gel that was previously stained with Pro Q Emerald; Fig. 5.15.)

Accession #	Name
Membrane	
Sm04760	Glycoprotein 18-22 (Sm25)
Sm12366	Tetraspanin D (CD63-like protein)
Phat05159	SNaK1
Sm03987	Annexin
Sm08542	Calpain
Secreted	
Sm09193	Sm29
Cytoskeleton	
Sm00654	Alpha tubulin
glimmer09102	Beta tubulin
glimmer06193	Actin 1
Sm09837	Calponin homolog
Sm00101	Sm20.8
Sm12876	Sm22.6
Cytosol	
Sm10462	Heat shock protein 86
Sm04020	Methyltransferase
Sm12452	14-3-3 homolog
Nuclear	
glimmer11104	Histone H4
No homology	
Sm03716	Unknown
Sm02757	Unknown
snap03009	Unknown
Labelling proteins	
GI:663228	Rat IgG heavy chain
GI:38571753	Rat IgG light chain

Table 5.2. Eluted proteins from Dynal beads identified by tandem MS.

5.4 Discussion

Part 1 - The use of cationised ferritin to promote sloughing of the membranocalyx

This experiment set out to induce the sloughing of the membranocalyx and examine the protein constituents of this secreted bilayer. CF has a positively charged tertiary amino group and was originally used to label the negatively charged plasma membrane on erythrocytes (Danon et al., 1972). The cationic label also bound firmly and uniformly to the surface of the membranocalyx, indicating that anionic sites are available over the entire surface of the bilayer. This technique was first used on the schistosome by Wilson and Barnes (1977) to measure the rate of membranocalyx turnover. The authors estimated that the secreted bilayer has a half-life of 2-3 hours under the *in vitro* conditions of the experiment, but these results are not consistent with those observed in this study, where the label took approximately 8 hours to be removed from the tegument surface. Nonetheless, the pattern of sloughing was the same in both studies; the newly formed unlabelled membranocalyx appears to originate from the base of the pits, diffuse laterally over the surface of the plasma membrane, and replace its CF-labelled predecessor that is discarded into the surrounding medium. Furthermore, the absence of internalised CF in the tegument or cell bodies argues against membranocalyx recycling by endocytosis.

It is intriguing that under these *in vitro* conditions a labelled worm is able to replace its membranocalyx with such rapidity, since *in vivo* this process is proposed to take several days (Saunders et al., 1987). Saunders and others used host erythrocyte antigens as an indicator for the outer bilayer, a natural constituent of their surface. Perhaps the unfamiliar CF on the surface of the worm provokes the schistosomes to increase the rate of membranocalyx shedding. On the other hand, the cationic label may create a change in the properties of the membranocalyx to facilitate its removal from the underlying plasma membrane. The mechanism by which the two lipid bilayers remain so strongly attached to each other is not understood, but if electric charge plays a role, it is plausible that CF may have a detrimental affect on this interaction. The manner in which the CF-labelled membranocalyx peels from the tegument, leaving behind a vulnerable plasma membrane exposed to attack, is different to the way I would expect the surface membrane to turnover *in vivo*. In my opinion, the newly formed membranocalyx continually pushes the old one over the plasma membrane. When it reaches the tip of the spines, the membranocalyx

breaks away from the plasma membrane and is jettisoned off into the blood stream. Thus, the plasma membrane is never exposed to the outside environment.

Only a small number of membrane proteins were detected by tandem MS in the study. The reproducible identification of SGTP1, a glucose transporter that has been well-documented to be present at the basal tegument membrane and not at the apical membranes (Zhong et al., 1995), is perplexing. It seems very unlikely that the basal membrane could have contributed to the preparation unless the worms were severely damaged. Such extensive injury would have been visible by electron microscopy and would have undoubtedly led to the identification of many tegument syncytial proteins such as enolase or aldolase (Chapter 2). Boyle et al. (2003) were successfully able to reduce SGTP1 gene expression in sporocysts using RNA interference (RNAi). They observed 40% reductions in the uptake of exogenous glucose by the RNAi-treated larvae, suggesting that the transporter is responsible for sugar transport into the parasite. Therefore, if SGTP1 is present at the surface membranes of the larval parasites, it may have a similar location on the adult worms.

The failure to detect SGTP4 at either of the time points in this study conflicts with published data. This schistosome glucose transporter has been located to the tegument surface membranes using specific antibody staining and fluorescence microscopy (Jiang et al., 1996). If the CF promotes the release of the membranocalyx into the surrounding then SGTP4 would be one of the proteins expected to be identified by proteomics. Although SGTP4 has been identified by tandem mass spectrometry in previous studies (van Balkom et al. 2005; Chapter 3), a theoretical tryptic digest of the transporter protein yields only four peptides that are of a suitable mass (between 1000 and 3000 Da) for protein identification by MALDI-ToF/ToF. If the starting peptide mixture is complex, it is possible that other peptides may suppress the detection of weaker signals. In this case, one could argue that SGTP4 is a less abundant protein in the starting preparation. Analysis of the material by Western blot followed by probing with specific anti-SGTP4 antibody would provide a more sensitive method to detect the presence of the glucose transporter in the CF-induced released material.

Two tetraspanins, Sm23 and CD63-like protein, were identified at both time points. Sm23 has been reported at the schistosome surface and was promoted as a vaccine candidate

(Lee et al., 1995; Da'dara et al., 2001), but its function has yet to be determined. The second tetraspanin shares 39% homology with human CD63 and was originally selected as a protein of interest by searching cDNAs for transmembrane or secreted proteins (Smyth et al., 2003). Similar to Sm23, neither its function nor its ligand have been ascertained. The identification of these tetraspanins, together with SNaK1 and dysferlin, is difficult to explain given that they are unlikely candidates of the membranocalyx due to their transmembrane domains. These proteins are probably highly represented on the plasma membrane and may interact with the membranocalyx. Other transmembrane or membrane-anchored proteins, such as alkaline phosphatase, phosphodiesterase and diphosphohydrolase were not found in this study; these proteins were deemed to be very abundant constituents of the plasma membrane (Chapter 2), but may not have any affiliations with the membranocalyx. The identification of annexin in this study strengthens our argument for its potential association with the membranocalyx (Chapter 4), and it is possible that this lipid binding protein is removed together with the membranocalyx during sloughing.

Two potentially secreted proteins of unknown function were identified in this study; both have been cloned and named Sm13 (Abath et al., 2000) and Sm29 (Hancock and Tsang, unpublished data), based on their molecular weight determined by SDS-PAGE. Sm13 is a principle antigen recognised by mice protectively vaccinated with a preparation of tegument surface membranes (Simpson et al., 1990). Although proposed to be a membrane spanning protein (Abath et al., 2000), SOSUI (Hirokawa et al., 1998) and PSORT II (Horton and Nakai, 1997) analyses detected only a putative signal sequence. Pearce et al. (1990) suggest that Sm13 could be anchored to a lipid bilayer by the post-translation modification of cysteine by palmatic acid. Therefore, Sm13 may be transported to the tegument surface in the lumen of the secretory vesicles, possibly attached to the multilaminate whirls that will form the membranocalyx. Once again, the identification of Sm29 at the schistosome surface provides further evidence for its potential involvement as part of the membranocalyx.

The difference in protein concentration released in the two chase incubations may reflect the pattern of CF-induced membranocalyx turnover, starting off slowly and finishing with gusto over the eight-hour post-labelling incubation. Alternatively the increased amount of material may reflect worm damage over time as proteins leak out into the medium.

Furthermore, the contamination of this membrane preparation by worm vomit, eggs and sperm is a genuine cause for concern. Although there was no proteomic evidence for gut contents in this study (no digestive enzymes were identified), the light brown colour of the 8 hour chase pellet could represent haematin pigment from worm vomit (Hall, personal communication). The presence of histones is puzzling given the lack of nuclei in the tegument. Nuclear proteins are present in the muscle layers and cell bodies beneath the syncytium, but severe damage to the worms would be necessary to release these constituents. A more likely source of nuclear proteins is from sperm ejaculated by male worms during the chase incubations, and this hypothesis is currently under investigation in our laboratory. The presence of sperm in the preparation would also explain the detection of alpha and beta tubulins, as these two proteins are major constituents of flagella (Mohri, 1993).

In attempts to isolate the surface membranes, there has been a recurring problem of contamination from cytoskeletal and cytosolic proteins. The presence of actin in both the 4 and 8 hour chase may originate from several sources, such as sperm (discussed above) or tegumental structures (*e.g.* spines). The origin of Sm22.6 is less ambiguous, as this protein is expressed in the tegument cell bodies (Jones et al., 2004). Although there is a strong immune response to Sj22.6 (the *S. japonicum* homologue) in infected animals, vaccination with a recombinant protein expressed in bacteria provided no protection in mice (Li et al., 2000). This protein also shares homology with Sm20.8 and Sm21.7, and possesses an EF hand motif (Shabaan et al., 2003). Thus, it is likely that Sm22.6 has an intracellular location and functions as a structural protein in the tegument syncytium. The release of Sm22.6 by a damaged or dying worm *in vivo* is a likely target for antibody attack, and the identification of Sm22.6 in this study suggests a similar level of injury *in vitro*. The cytosolic heat shock proteins and GAPDH provide more evidence of worm damage throughout the chase incubations.

Host proteins are notably absent from this study. If the mouse antibodies are attached as the result of antigen binding, I would expect them to be firmly adhered to the membranocalyx surface even after it had been sloughed from the tegument. Similarly, when Complement C3 is converted to its active state by C3 convertase, it forms a covalent link with the pathogen surface. Even after C3 is de-activated by regulatory factors, a small section of Complement protein (C3dg) still resides on the cell membrane that it once

sought to destroy (Morgan and Harris, 1999). Despite this, Rasmussen and Kemp (1987) discovered that when *ex vivo* worms were incubated in serum-free media for three hours, host components were lost from the worm surface. The four-hour incubation step after the CF labelling would therefore provide ample time for a similar loss of mouse proteins. The supernatant that was collected from the labelled worms was spun at 100,000 *g* and only the pellet was profiled for proteins putatively originating from the membranocalyx. If the supernatant had also been subjected to tandem MS analyses, it might have yielded some soluble host proteins that were once attached to the membranocalyx.

Part 2 - Discussion on membranocalyx isolation by affinity beads

This research took advantage of mouse erythrocyte glycolipid acquisition by the membranocalyx. Assuming that this antigen is present only on the surface membrane of the worm (and maybe also within the lumen of the gut), the generation of antibodies against mouse RBC ghosts provides a way to tag and purify the membranocalyx. Haemagglutination assays revealed that both rats immunised with murine RBC ghosts produced specific antibodies against mouse erythrocytes. The antibodies were also able to bind uniformly to the schistosome surface, confirming the acquisition of erythrocyte antigens by the parasite. Whether this serves to disguise the worm and circumvent host immune attack, or is just an artefact of the membranocalyx's lipophilic properties, remains unknown. Nonetheless, these mouse antigens provided a suitable handle for the isolation of the membranocalyx.

In preliminary experiments, antibody-labelled live worms were incubated with goat anti-rat magnetic beads. This resulted in a very poor yield of membranocalyx isolation, as revealed by electron microscopy (data not shown). Therefore it was necessary to detach the whole tegument by freeze/thaw/vortex, and incubate it with the Dynal beads with constant agitation. This method proved to be more efficient in membranocalyx recovery, although the dot-blots revealed that there were still mouse RBC antigens that had not bound to the beads. Likewise, the presence of rat antibodies in the flow-through and wash steps suggested that they had not been efficiently captured by the goat anti-rat beads. A longer incubation step, or a pre-fractionated starting preparation might have helped to overcome this inefficiency.

Electron microscopy of the isolated material on the magnetic beads provides a novel way to observe the properties of membranocalyx. In some cases, areas of the bead surface were covered with just a single bilayer, but multiple layers of membranes were frequently observed stacked up on each other, resembling a cross-section through a myelin sheath. This phenomenon is sometimes witnessed on the surface of fixed worm sections (Hockley and McLaren, 1973) and has also been noted in surface membrane preparations (Roberts et al., 1983). The aggregation of multiple layers of membranocalyx reveals the high self-affinity of the bilayer, and could be the result of simple hydrophobic interaction or a more sophisticated mechanism by which the membranocalyx remains so tightly bound to the

plasma membrane of the tegument. The understanding of this self-affinity may also unveil the secrets of membranocalyx formation within the multilaminate vesicles.

The co-purification of tegument syncytial material with the membranocalyx requires careful interpretation of the proteomic data. The seven wash steps in PBS were not enough to remove all non-membranocalyx material completely. This highlights the strong attachment of the apical bilayer to the underlying plasma membrane and tegument cytoskeleton. Stronger wash solvents such as urea, thiourea or a high pH buffer (e.g. carbonate extraction) could not be used, as this would disrupt the antibody-antigen bond (Dynal Biotech, personal communication). Similarly, the use of detergents would result in the disruption of the membranocalyx, and might lead to the solubilisation of the erythrocyte glycolipids, thus loosing our only marker of this bilayer.

Out of the 21 proteins identified by tandem MS, five are either membrane spanning or phospholipid binding. The glycoprotein Sm25 is an antigenic membrane protein of the *S. mansoni* tegument, and is the target of host protective immune responses (Wright et al., 1988; Smithers et al., 1989). The protein spans a lipid bilayer in the conventional manner, contains a very short hydrophilic domain, and the C-terminus is covalently modified by palmitic acid to permit membrane anchorage (Pearce et al., 1991). Furthermore, Sm25 is available for tryptic digestion at the surface of intact adult worms (Pearce et al., 1991). The authors suggest that the protein may be a constituent of the outer membrane, and is attached to the inner membrane *via* the palmatic acid modification of the C-terminus; the short hydrophilic region would represent the gap between the two lipid bilayers. However, Pearce et al. (1991) failed to mention that Sm25 could span the plasma membrane and attach to the membranocalyx by the palmitoylation of the C-terminus; such an orientation has been proposed (Karcz et al., 1988). Although this conformation does not explain the digestion of Sm25 at the schistosome surface, it does provide a possible mechanism of protein attachment to the membranocalyx by palmitoylation.

The identification of Tetraspanin D (CD63-like protein) provides further evidence for its involvement in the tegument surface membranes. Likewise, the phospholipid-binding properties of annexin make this protein a potential candidate for membranocalyx anchorage, as discussed in Chapter 4. Once again, Sm29 is discovered as a protein constituent of partially purified membranocalyx material. This secreted protein was

identified from the large smear at approximately 37-25 kDa on the Sypro Ruby stained 1-D gel. The intense staining of this region relative to the whole gel alludes to the relatively high concentration of Sm29 in the preparation, but definitive quantification is not possible from 1-D electrophoresis due to the high probability of two or more proteins co-migrating to the same molecular weight.

A large proportion of the proteins identified (~30%) had putative roles as cytoskeletal components. Actin, Sm20.8 and Sm22.6 are well-documented members of the tegument syncytium (Jones et al., 2004), but the presence of both alpha and beta tubulin are more difficult to explain. Tubulin is present in the tegument as a component of the microtubules that connect the cell body with the syncytium (Duvaux-Miret et al., 1991), but these are unlikely to become detached in large quantitites by the freeze/thaw/vortex procedure. Sperm may provide a source of the tubulin and histones discovered in this study, as discussed above. Another potential source of tubulin is from the ciliated sense organs which occur all over the surface of male and female worms (Senft and Gibler, 1977). The cilia protrude from dome-shaped elevated bases, and are in a susceptible location to suffer injury from the perfusion process. The three cytosolic proteins identified in this study, HSP86, methyltransferase, and 14-3-3 homologue, confirm that the membranocalyx preparation was not pure. However, 14-3-3 proteins have been extensively studied in other organisms (Mhawech, 2005), and the schistosome homologue may play important roles in protein folding, cellular signalling, vesicular tracking and cytoskeletal organisation within the tegument.

Mouse erythrocyte proteins were not identified by proteomics, nor were any other mouse serum proteins such as immunologulins or Complement components. The absence of abundant RBC intracellular constituents, such as haemoglobin, testifies to the clean preparation of adult worms prior to tegument removal. It is interesting that no erythrocyte membrane proteins, such as glycophorin or Band 3, were detected in the 1-D gel. This suggests that only glycolipids are transferred from the erythrocytes to the membranocalyx. However, the failure to identify mouse immunological proteins in this study argues that their concentration was below the threshold of detection by MS/MS, and a similar argument could be made for erythrocyte membrane proteins. Transferring the sample onto a membrane by Western blotting, and probing with specific antibodies would provide a more sensitive approach to search for individual proteins.

The Pro-Q Emerald stained 1-D gel revealed the high concentrations of carbohydrate material attached to the Dynal beads. The host erythrocyte glycolipids are likely to constitute a significant proportion of this material. In addition, three of the schistosome membrane and secreted proteins identified have predicted n-glycosylation sites (Sm25, Sm29 and SNaK1). Indeed, the large smear between 37 and 20 kDa of the protein-stained 1-D gel may be due to the carbohydrate content of Sm25 and Sm29. A Western blot of the 1-D gel, probed with anti-mouse erythrocyte antibodies would have provided a simple way to distinguish the mouse from the parasite material. Mannose, glucose, galactose, N-acetylglucosamine, N-acetyl-galactosamine and sialic acid are likely carbohydrate candidates exposed on the adult schistosome surface (Simpson and Smithers, 1980). Antigenic glycans on the surface of schistosomula are the targets of lethal antibody attack (Langley and Dunne, 1992), but the carbohydrates on adult worms are largely refractory to antibody binding (Murrell et al., 1978; Simpson and Smithers, 1980). These inert glycans, together with host-derived carbohydrates, may serve to camouflage the parasite against immune attack – a process inventively named "glycomimesis" (Schmidt, 1995).

Conclusion

The work in this chapter sought to isolate the membranocalyx using two different methods, and identify the protein components of this membrane-like secretion. Both techniques effectively served to enrich the membranocalyx, but its complete isolation remained elusive; cytoplasmic contaminants were identified in both cases. It is my opinion that the only way to isolate the membranocalyx lies in the understanding of the mechanism by which it attaches to the plasma membrane, either by electronic charge, protein, lipid or glycan interactions. Nonetheless, the proteins identified in this chapter are likely to represent those at the tegument periphery. The relatively low number of proteins discovered gives testament to the nature of the membranocalyx. It seems that this lipid bilayer is almost devoid of proteins, and functions as an inert barrier to protect the vulnerable plasma membrane. However, the consistent identification of Sm29, CD63-like protein and annexin, together with their salient characteristics, implicates these proteins as integral to, or heavily associated with, the membranocalyx.

Chapter 6

Concluding Discussion

This chapter summarises the rationale behind the work described in this thesis, the procedures used to identify proteins at the schistosome surface, the significance of the results, the limitations of this work and future directions worth pursuing.

The aim of this study was to characterise the proteins of the *S. mansoni* tegument surface. Methods had been developed over twenty years ago to isolate the tegument, and purify the surface membranes, but little was progressed beyond separating the protein constituents by electrophoresis or liquid chromatography. During the last two decades, schistosome protein identification has been largely based on the use of antibodies and histochemical assays, searching for a specific antigen or family of proteins. As a result, the literature is filled with reports providing glimpses of the tegument surface protein composition. With recent advances in mass spectrometry-based proteomics, together with the sequencing of the *S. mansoni* transcriptome and genome, it has become possible to compile a much more complete inventory of proteins at the tegument surface.

My work commenced with established methods to remove the tegument and enrich the surface membranes. Tandem mass spectrometry was employed to produce fragmentation spectra that could be searched against the *S. mansoni* databases. Preliminary results suggested that this surface membrane preparation contained many intracellular contaminants, so a differential extraction procedure were employed to remove cytosolic and cytoskeletal proteins, generating a highly enriched membrane fraction. Two-dimensional electrophoresis and liquid chromatography were used to separate the soluble and hydrophobic proteins, respectively, and a list of proteins loosely associated with, or integral to, the tegument apical membranes was compiled. However, many previously described tegument surface proteins were not identified, so the procedure was repeated using more starting material together with liquid chromatography-based separation techniques. Approximately 200 proteins were identified, but it was not possible to infer their relative locations within the multilaminate tegument membranes.

Subsequent work sought to label the exposed proteins using impermeant biotin tags. Two forms of biotinylation reagent were employed, differing only in their length. The short form was able to penetrate deeper into the surface complex of the tegument, allowing inferences to be made about the relative exposure of proteins at the worms' surface. Finally, methods were developed to isolate the membranocalyx from the underlying

tegument. Cationised ferritin promoted the sloughing of the apical bilayer without damaging the underlying plasma membrane, and anti-mouse RBC antibodies were used to label the membranocalyx, which allowed the purification of this lipid bilayer using affinity beads. The identified components of the enriched membranocalyx material, together with the biotinylated molecules are likely to represent the most peripheral proteins at the schistosome surface.

The combined results of all the proteomic experiments presented in this thesis allow the insertion of protein identities into a two-dimensional model of the *S. mansoni* tegument, and my opinion on the composition of the parasite surface, as well as the structure and function of the tegument, is described in the following paragraphs.

It appears that the membranocalyx is almost completely devoid of schistosome proteins and serves to shield the underlying tegument from host immune attack. Only the characteristics of Sm29, together with its reproducible identification in the membranocalyx purification and biotinylation experiments, point to this protein as being a component of the secreted bilayer. No other schistosome proteins reported in this thesis match all the requirements to occupy such a location. Conversely, many host proteins, some of them membrane spanning, litter the surface of the membranocalyx. Components of the immune system, such as immunoglobulins and Complement proteins, and other constituents of the blood system were identified in different surface preparations. As no conventional Fc receptors were identified, I propose that the antibodies identified at the schistosome surface are bound to antigens on the membranocalyx and cause the fixation of Complement components. However, the Complement attack pathway either does not run to completion or it is unable to destroy the parasite. The double bilayer of the schistosome may have evolved to counteract Complement-mediated attack, as assembly of membrane attack complexes in the membranocalyx would not necessarily result in the lysis of the tegument. However, as no proteins of the membrane attack complex (C5 to C9) were identified, defence against this form of immune attack is probably mediated by other mechanisms. Indeed, the detection of an inactivated fragment of C3 suggests that host Complement attack is regulated by specific proteins. Astonishingly, it appears that the acquisition of host proteins is at least partly responsible for the protection of the parasite; the mouse Complement regulatory protein, Crry, was identified firmly attached to the parasite surface.

The method of host protein acquisition by the parasite is a contentious issue, and cannot be resolved by proteomics alone. I think that the transfer of these molecules is the result of host ectosomes fusing with the membranocalyx. If this mechanism occurs, the acquired proteins would be correctly orientated and fully functional. Although these may serve to disguise the parasite as host material, I feel that they have may benefit the parasite in other ways. The protective properties of the membranocalyx also cause problems for the parasite's ability to uptake nutrients from the blood stream, as the molecules have to traverse two lipid bilayers. Pores must exist to allow the influx of solutes, and the biotinylation experiments suggest that these pores facilitate the transfer of molecules with a mass of at least 500 Da. Schistosome-synthesised pore-forming proteins in the membranocalyx are unlikely, as this secreted bilayer originates from the luminal contents of the secretory vesicles, not their bounding membrane. Therefore, I propose intact host proteins acquired by the membranocalyx may serve as pores in this outer bilayer; only functional studies will determine if this is the case.

No proteins specific to endocytosis were identified in the tegument surface preparations. Furthermore, the failure to observe cationised ferritin being internalised strengthens the argument against membranocalyx recycling by the parasite. It is my opinion that this secreted bilayer is sloughed into the surrounding environment and is continually replaced by the contents of the multilaminate vesicles. Although the parasite is capable of turning over its surface bilayer with relative rapidity, I believe that the timeframe *in vivo* is generally slow. The inert nature of the membranocalyx, together with its acquisition of functional host proteins, means that the parasite has no need to replace this bilayer with haste.

The intimate association of the membranocalyx with the plasma membrane is integral to the parasite's survival in the turbulent blood flow within the hepatic portal system. The secreted bilayer must remain tightly attached to the tegument in order to protect it, but the association has to allow the bilateral movement of the membranocalyx over the plasma membrane. Although the hydrophobic properties of the membranocalyx may aid its attachment to the plasma membrane, I think that other mechanisms are involved in this surface complex. The repeated identification of annexin, coupled with its phospholipid-binding properties, makes this protein an ideal candidate for anchoring the

membranocalyx. Again, MS-based proteomics alone cannot determine such functions; only by knocking out the annexin gene, or diminishing its expression by RNA interference, and observing the phenotype of the parasite, can we fully understand the role of this protein at the tegument surface.

In contrast to the membranocalyx, the plasma membrane contains a plethora of proteins, and the layer in-between the two membranes is a protein-rich environment, containing all the extracellular domains of the transmembrane and lipid-anchored components of the plasma membrane. It seems highly likely that membrane-bound enzymes, whose active sites reside between the two layers, function in a similar manner to their homologues in other species. For example, the three enzymes involved in hydrolysing phosphates, alkaline phosphatase, phosphodiesterase and diphosphohydrolase, are responsible for the dephosphorylation of molecules to allow their diffusion across the plasma membrane.

The numerous transporters identified, involved in the trafficking of sugars and amino acids, are integral to the plasma membrane and confirm the uptake of nutrients from the host blood system. Indeed, the surface architecture of the tegument is typical of an adsorptive tissue, with folds and pits that dramatically enhance its surface area. Although the main source of food comes from the ingestion of red blood cells, the tegument offers an alternative source of nutrients. However, there are some inconsistencies with the transport proteins identified in this thesis and those reported by other studies. For example, the repeated identification of SGTP1 at the tegument surface contests its described location at the basal membrane of the tegument. Similarly, the amino acid transporter SPRM1lc was not identified by proteomics, instead another B-type transporter, SLC3A, was discovered.

Regulated ion movement across the plasma membrane is also evident. Calcium, sodium, potassium and copper ion transporting proteins were reproducibly identified in different tegument surface preparations. Aquaporins are also likely to facilitate the movement of solutes across the plasma membrane. The uptake of calcium, for example, is crucial for the operation of many tegument proteins, such as calpain and annexin, and this ion seems to play an integral role in the functioning of the syncytium. Indeed, the antischistosomal drug, praziquantel, is thought to disrupt calcium homeostasis, which leads to damage of the tegument.

The repeated identification of tetraspanins in different surface membrane preparations points to these proteins as abundant components at the tegument periphery. By analogy with tetraspanins in other organisms, it is tempting to speculate that schistosome tetraspanins play a structural role in the plasma membrane. Their ability to interact with themselves and other proteins may provide an organised scaffold within the lipid bilayer. Similarly, their extracellular loops may provide platforms for other proteins or glycans that may interact with the membranocalyx. As some tetraspanins are identified more readily than others, they are likely to be present at differing concentrations or locations within the tegument surface complex. Again, only functional studies will determine the precise function of these proteins at the schistosome periphery.

Situated immediately below the plasma membrane is an extensive cytoskeletal layer, comprising many protein species including actin, dynein light chain, Sm22.6 and Sm 20.8. This layer provides the foundation which dictates the contours of the tegument surface including the folds and pits. However, this structure is not rigid, but acts as a shock absorber to external buffeting, thus minimising the damage caused by mechanical forces within the host blood system. The parasites' migration through the lung capillaries, and the female's movement to oviposition sites in the mesenteric venules, are possible situations when the cushioning properties of the tegument protect the surface membranes from damage. Further cytoskeletal components may facilitate the transport of secretory vesicles through the tegument syncytium, and proteases such as calpain are necessary for the breakdown of the peripheral cytoskeleton, to facilitate the docking of multilaminate vesicles at the plasma membrane. Other proteins, including dysferlin and annexin may aid the fusion of these vesicles with the plasma membrane, but the mechanism by which the multilaminate whirls of membranes unravel in the lumen of the pits remains unknown.

The detection of specific peptides provides irrefutable evidence for the identification of a given protein, and the identification of several proteins supports many previous investigations into the composition of the adult schistosome surface. The confirmation of host immune components attached to the tegument, for example, will no doubt please the researchers who first reported them. Nonetheless, perhaps this thesis' greatest contribution to schistosome research is provided by the identification of proteins that have not been previously reported. For example, the components involved in membrane structure and maintenance, such as annexin, dysferlin, phospholipid transporters and the assortment of

tetraspanins, allow the greater understanding of membrane dynamics at the parasite surface. Similarly, the many "unknown" proteins that do not bear homology to characterised proteins from other organisms are of particular interest for several reasons. As argued by van Balkom et al (2005), these proteins may be involved in processes unique to the tegument, such as the formation of multimlaminate vesicle, the interaction of the membranocalyx with the underlying plasma membrane, or even the regulation of the host immune system. Furthermore, their novel sequence and structure make them excellent targets for antischistosomal drugs that would have minimal effects on the host.

Many proteins that have been postulated to be integral to the schistosome surface were not identified; perhaps most notable is the absence of schistosome receptors for host cytokines. Of course, the concentration of these receptors may be below the threshold of detection by tandem mass spectrometry, and the failure to detect them by the methods in this thesis provides no grounds to rebut previous studies. Furthermore, such receptors may not bear homology to conventional receptors found in other organisms. Thus, their presence will never be detected by mass spectrometry alone. Indeed, proteomics should be viewed more as a technique than a discipline, and future studies should integrate it with other research tools such as genomics, bioinformatics, molecular interactions and cytometry, if a more complete understanding of the research field is to be achieved.

The reproducible identification of several proteins by the different methods reported in this thesis substantiates their location at the tegument periphery (a brief summary is presented in Table 6.1), and warrants the investigation into their specific functions. Indeed, if I were to choose proteins for further examination, possibly leading to vaccination trials, I would pick those presented in Table 6.1. Given adequate funding, together with the skills of young and enthusiastic researchers, perhaps these proteins will receive the attention that they deserve.

Protein name	Chapter 2 GP	Chapter 3 GP (LC)	Chapter 4 Long biotin	Chapter 4 Short biotin	Chapter 5 CF 4 hr	Chapter 5 CF 8 hr	Chapter 5 Beads
Sm29	♦	♦	♦	♦	♦	♦	♦
Tetraspanin D (CD63-like)		♦	♦	♦	♦	♦	♦
Annexin	♦	♦	♦	♦		♦	♦
Dysferlin	♦	♦	♦	♦		♦	
Tetraspanin B (Sm23)	♦	♦			♦	♦	
Tetraspanin C (TE736)	♦	♦		♦			
ATP-diphosphohydrolase	♦	♦	♦	♦			
Phosphodiesterase	♦	♦	♦	♦			
Alkaline phosphatase	♦	♦	♦	♦			
Unknown (Sm07392)		♦	♦	♦			
Unknown (Sm11921)		♦	♦	♦			
Calpain-B	♦	♦		♦	♦	♦	♦
SGTP1	♦	♦			♦	♦	
SNaK1	♦	♦		♦		♦	♦
200 kDa Surface protein		♦		♦			
Unknown (Sm03716)	♦	♦			♦		♦

Table 6.1. Summary of some schistosome proteins that have been repeatedly identified throughout this thesis (represented by ♦). They are listed in the order of my interest, based on the experiments in which they were found and their predicted secreted/transmembrane domains.

Future directions

The work in this thesis has illuminated many exciting corridors of future research. The possibilities for the continued isolation of specific components of the tegument surface are numerous. For example, one study is currently underway to liberate GPI-anchored proteins from live worms using phosphatidylinosytol-specific phospholipase C. Another option includes digesting exposed proteins on the surface of live worms with trypsin or Proteinase K. Such an experiment would be relatively straightforward and may provide valuable insights of the topology of parasite membrane proteins, as well as the identification of more host proteins.

One of the major limiting factors of conventional LC-base proteomic methods is the inability to quantify proteins, either within or across samples. Unfortunately, the traditional method of assaying relative protein concentrations (*i.e.* by 2-D electrophoresis) is incompatible for studying membrane proteins, due to the requirement of ionic detergents. The use of stable isotopes has become a valuable tool in protein quantification, and one such method is being employed in this laboratory to quantify tegument membrane proteins, as follows. The recent technique, known as QCAT (Beynon et al., 2005), utilises an artificial gene made up of a string of peptides, each from carefully selected proteins (*e.g.* schistosome membrane proteins). The gene is inserted into a vector and expressed as a single protein in *E. coli* raised in medium containing $^{15}NH_4Cl$ as the only nitrogen source. Thus, the so-called "Q protein" consists of a series of heavy peptides, all at an equal concentration. The Q protein is digested and added to the sample (*e.g.* a schistosome surface membrane preparation). The heavy and light peptides are readily identified by the mass difference between the two peaks, correlating to the number of nitrogen atoms in the peptide. As the peak height is directly proportional to the concentration of the peptide, the amount of a specific protein in a sample can be calculated. Moreover, the relative concentrations of different proteins can be compared.

The mouse antibodies identified at the parasite surface by biotinylation confirm previous reports of their affinity for the tegument surface, yet their targets remain unknown. The schistosome-bound antibodies could be biotinylated, removed, and purified by streptavidin beads, as in Chapter 4. After a non-reducing elution from the beads, the antibodies could be used to probe 1- or 2-D Western blots of surface membrane extracts. The targets,

assuming that they are proteins, could be identified directly from the Western blot by tryptic digestion and mass spectrometry (Lamer and Jungblut, 2001).

It is possible that proteins are not the targets of the host antibodies, but glycans may provoke the immunogenic response. Indeed, the presence of carbohydrates at the schistosome surface has been demonstrated by previous studies (Bennett and Seed 1977; Schmidt et al. 1995). Although Schmidt et al. (1995) reported that the schistosome surface carbohydrates are similar to mammalian glycans, and are therefore unlikely to be the target of antibody attack, it might be valuable to know which carbohydrates are exposed on the parasite. This could be achieved by gas chromatography to separate the carbohydrates, followed by mass spectrometry (Robinson et al., 2005). Such a study may also determine which host glycans are acquired by the membranocalyx.

The techniques used in this thesis are currently being applied to study the surface of the lung-stage parasite. The immunisation of mice with radiation-attenuated cercariae induces high levels of protection against challenge infections (reviewed by Coulson, 1997). As parasite elimination occurs in the lungs and is T-cell mediated, antigens exposed on, or secreted by, the lung-stage schistosome are a likely target of the immune system (Harrop et al., 1999). Some of the soluble proteins of lung-stage parasites have been identified by 2-D electrophoresis and mass spectrometry (Curwen et al., 2004), but the surface proteins are unlikely to have been represented, as only Tris-soluble proteins were studied. The use of strong solubilising agents, such as SDS, followed by separation techniques compatible with hydrophobic membrane proteins, would permit the study of the lung-stage worm tegument membranes. The comparison of surface-exposed proteins between the adult and lung-stage worms may provide valuable insights into the target of the immune responses during the migratory stages of the schistosome. However, it should be remembered that the mechanically-transformed schistosomula do not have the opportunity to acquire host antigens, and are therefore likely to possess a different surface composition to that of the skin-penetrated parasite.

In conclusion

The work presented in this thesis has produced a wealth of data on the protein constituents of the tegument surface, and their relative locations within this surface complex. It has significantly added to our knowledge of the *S. mansoni* tegument and the parasite's interaction with the host blood system. The use of proteomics has proved to be a powerful tool with which to determine the protein composition at this parasite-host interface, but it is clear that many more constituents have yet to be identified. The development of improved techniques in protein identification, together with novel methods to isolate specific components of the tegument, will no doubt add to the identities presented in this thesis and further our understanding of this truly remarkable biological entity.

7 References

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Appendix

From genes to vaccines via the proteome

This appendix comprises a review on schistosome proteomics, as submitted to the journal *Memorias do Instituto Oswaldo Cruz*. I contributed to the section entitled 'What does proteomics reveal about schistosomes'.

From Genomes to Vaccines Via the Proteome

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An effective vaccine against schistosomiasis mansoni would be a valuable control tool and the high levels of protection elicited in rodents and primates by radiation-attenuated cercariae provide proof of principle. A major obstacle to vaccine development is the difficulty of identifying the antigens that mediate protection, not least because of the size of the genome at 280Mb DNA encoding 14,000 to 20,000 genes. The technologies collectively called proteomics, including 2D electrophoresis, liquid chromatography and mass spectrometry, now permit any protein to be identified provided there is extensive DNA data, and preferably a genome sequence. Applied to soluble (cytosolic) proteins from schistosomes, proteomics reveals the great similarity in composition between life cycle stages, with several WHO vaccine candidates amongst the most abundant constituents. The proteomic approach has been successfully applied to identify the secretions used by cercaria to penetrate host skin, the gut secretions of adult worms and the proteins exposed on the tegument surface. Soluble proteins can also be separated by 2D electrophoresis before western blotting to identify the full range of antigenic targets present in a parasite preparation. The next step is to discover which target proteins represent the weak points in the worm's defences.

Key words: *Schistosoma mansoni* - vaccine - proteomics - mass spectrometry - antigen

STRATEGIES FOR A HUMAN SCHISTOSOME VACCINE

Most current viral and microbial vaccines were developed empirically, but in the knowledge that first exposure to the pathogen generated a strong immunity to re-infection. For parasites the situation is altogether more complex, not least because they have evolved efficient mechanisms to evade host immune responses. In the case of schistosomiasis mansoni, the result is a chronic debilitating infection that may persist for more than 30 years (Harris et al. 1984). In these circumstances the development of a schistosome vaccine was always going to be a difficult task. In what might be termed the classical approach, the strategy is to identify protected individuals in an endemic population. The immune mechanisms that such people deploy to limit or prevent establishment of invading cercariae should form the basis of a successful vaccine. In the last two decades great progress has been made in characterising human responses to schistosomes (Dunne & Mountford 2001) but no immune mechanisms or specific antigens *strongly* associated with a protected status have been identified. Indeed, in virtually all re-infection studies after curative chemotherapy, prepubertal children show little evidence of protection; these are the individuals with the highest intensities of infection who would benefit most from a vaccine.

MODELS OF PROTECTION

If the investigation of human responses to infection has not provided any obvious avenues to a vaccine, are there any model systems whose analysis might provide better pointers? The attenuated cercarial vaccine has long provided the gold standard for schistosome vaccine development. Its success in rodents and primates underlines the fact that protective immunity against schistosomes is a feasible prospect. The attenuated larvae must migrate a sufficient distance within the host to prime the immune system, without establishing a patent infection, and high levels of protection ensue (> 85% in baboons and mice; Mountford et al. 1996, Kariuki et al. 2004) It is important to stress that "protection" means reduction in worm burden, not proportion of hosts protected, as would be the case for microbial or viral vaccines. However, sterile immunity is probably not essential because a substantial reduction in worm burden would diminish both clinical disease and transmission.

The immunity induced by the radiation-attenuated vaccine in mice has been the most thoroughly explored (Coulson 1997). A single exposure of C57BL/6 mice generates a predominantly cell-mediated protection, dependent on CD4+ T cells recruited to the lungs. These cells orchestrate a Th1-type focal inflammation against challenge larvae that effectively blocks their onward migration through the lung capillaries. We believe that the larvae trapped in the lung eventually expire, and certainly they do not succumb immediately to a lethal hit e.g. by nitric oxide from macrophages. With multiple exposures of mice to attenuated larvae, the antibody-mediated component of protection becomes more prominent. The timing of challenge elimination is again early after exposure, in either the skin or lungs, but neither the complement pathway nor Fc receptors seem to be essential (P Coulson, unpublished data), so the mechanism of antibody-mediated protection remains enigmatic.

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For primates, multiple exposures to attenuated cercariae are necessary to achieve a high level of protection and IgG levels at challenge provide the best correlation. There is no information on the site of parasite elimination or mechanism of protection but larvae again seem the likely targets.

In addition to the attenuated vaccine, there are at least two other schistosome-host interactions that might form the basis for a vaccine, both involving hosts where the parasites establish but are then eliminated in a self-cure response. In the laboratory rat this occurs around four weeks after a primary infection when the worms are in the portal veins of the liver, coincident with rising IgE levels in the circulation and hepatic mast cell degranulation (Newlands et al. 1995, Cutts & Wilson 1997). Whilst such a mechanism might be difficult to replicate in a vaccine, the antigens that trigger the protective response should represent a chink in the parasite's armour. The rhesus monkey is an altogether more promising model where our recent observations suggest that parasite elimination, occurring between 12 and 18 weeks after infection, correlates with IgG level. As a result of immune pressure, the mature worms in the portal system first cease egg laying, then blood feeding and eventually starve to death (P Coulson, unpublished data).

THE NEED TO IDENTIFY PROTECTIVE ANTIGENS

If any of the above models is to serve as the basis for a vaccine, then it will be necessary to identify the relevant antigens, clone and express them as recombinant proteins (or insert them in a DNA vaccine vector) and formulate them for delivery to induce the desired immune response. Irrespective of the model, the proteins secreted by the parasite or expressed on its exposed epithelial surfaces are the obvious targets. (Glycan epitopes might also mediate protection but, whilst their characterisation is not an insuperable obstacle, would add a layer of complexity to the task of antigen identification.) For protein antigens the classical approach is to raise antisera against e.g. the secretions of cercariae or lung worms, and use these to screen cDNA expression libraries constructed with mRNA from the appropriate life cycle stage. However, when this approach was tried with the larval stages it yielded a very meagre harvest of novel antigens (Harrop et al. 1999, 2000). Instead, the sera detected the same panel of abundant, highly immunogenic, cytosolic or cytoskeletal antigens reported in other library screens; these incidentally included some of the vaccine candidates promoted by WHO (Bergquist et al. 2002). Our conclusion is that a radically new approach is needed to identify the antigenic targets of protection in any of the models described above.

THE APPLICATION OF PROTEOMICS TO ANTIGEN IDENTIFICATION

The proteome can be defined as the total protein complement of an organism, tissue, cell or subcellular organelle/complex. The technologies that have been developed over the last few years permit any protein to be identified, provided that an extensive cDNA or genomic database is available. The first step in the process is to separate the proteins in a complex mixture. For cytosolic or other soluble fractions this is readily achieved by 2D electrophoresis, with isoelectric focusing in the first dimension and SDS-PAGE in the second. Sensitive stains with a wide, linear dynamic range, such as Sypro Ruby (Molecular Probes, Oregon) make possible the visualisation of very small amounts of protein in the gel. The combination of a fluorescence imager to capture the gel image, and analysis software, allows spot patterns to be compared between gels to pinpoint differences in composition.

Less soluble proteins, such as many cytoskeletal components, can also be made compatible with 2D electrophoresis by solubilisation in chaotropic agents and zwitterionic detergents (Molloy et al. 1998). After gel mapping, spots are excised and subjected to proteolytic digestion e.g. by trypsin that cuts peptide bonds C-terminal to lysine or arginine residues to create a diagnostic peptide mixture. After differential extraction the final fraction, containing integral membrane proteins, can only be solubilised in ionic detergents that are not compatible with isoelectric focusing; for such proteins a combination of 1D SDS-PAGE electrophoresis and/or liquid chromatographic (LC) separations must be used (Washburn et al. 2001).

MASS SPECTROMETRIC IDENTIFICATION OF PROTEINS

Mass spectrometry (MS) is now the method of choice for the identification of proteins (Ashton et al. 2001). It is possible, even with very small amounts of starting material, to confidently make a link between a protein spot on a gel and its encoding DNA sequence. For protein spots that have been excised from 2-D gels, the usual approach is to generate a peptide mass fingerprint (PMF). In theory, this should be diagnostic of a particular protein and searching a database of full-length protein sequences for the organism under investigation will lead to identification if the sequence for that protein is present in the database. However, with the increasing size of protein databases, it is now common to get false positive hits to e.g., very large and unrelated proteins. In analyses where some form of LC precedes the mass spectrometry, it is not even possible to produce a PMF, since the peptides from each of the proteins in the mixture will be separated into several different fractions. In both these cases, it is necessary to obtain identifications based not on the masses of the peptides from a single protein, but on the basis of individual peptides. This is achieved using tandem mass spectrometry.

In a tandem mass spectrometer, there are two separate stages of mass analysis. The first stage allows a single peptide to be selected from a complex mixture. This peptide is then typically allowed to enter a collision cell containing an inert gas, where it is fragmented. The second stage then measures the masses of the fragments that are produced. Again this data can be used to search against a sequence database, but it is not necessary to have the full-length sequence of the protein under investigation since the technique is just as good at finding matches from EST data, or even from unassembled genomic sequences. All that is necessary is that some of the (partial)

sequences in the database contain the region of the relevant gene, which encodes the peptide. Since the masses of the peptide fragments depend on the sequence of amino acids, this type of search is much more discriminating. In practice, identifications are usually based on the fragmentation patterns of several peptides, even if these are spread across multiple LC fractions. At York, we perform most of our mass spectrometry on a 4700 Proteomics Analyser (Fig. 1, Applied Biosystems). This instrument is capable of generating a peptide mass fingerprint and 10 peptide fragmentation spectra from a single protein in ~ 5 min, and can easily do this for up to 200 samples (or LC fractions) in a single run.

THE SCHISTOSOME GENOME RESOURCE

The *S. mansoni* genome contains 270 Mb of DNA encoding not less than 14,000 genes (Verjovski-Almeida et al. 2003), and perhaps as many as 20,000 (Franco et al. 1995). As of March 2004, it has been sequenced to > 9x coverage, but not yet assembled or annotated. However, the 2.9 million reads themselves provide a database against which MS spectra can be searched. Sequencing of the *S. japonicum* genome is also underway in China. A second and crucial resource, both for proteomics and gene finding is the expressed sequence tag (EST) database. Prior to 2003, approximately 14,000 ESTs had been deposited in the public domain, but a further 125,000 ESTs from six life cycle stages were added in October as a result of the efforts of a sequencing consortium in the state of São Paulo, Brasil, led by Dr Sergio Verjofski-Almeida, increasing the number of sequences that could be searched for a match by an order of magnitude (Verjovski-Almeida et al. 2003). Again, a Chinese project has produced ~ 45,000 ESTs from *S. japonicum* adult worms and eggs (Hu et al. 2003). It has been estimated that the São Paulo data has hits to ~ 92% of *S. mansoni* genes, but ~ 70% of the sequences have no homology to known proteins in other organisms, probably a reflection of the distinct phylogenetic position of schistosomes. Furthermore, it seems that on average around 7000 genes are expressed in each life cycle stage, about 1000 of which may be stage-specific; this information is very relevant for proteomic analysis.

WHAT DOES PROTEOMICS REVEAL ABOUT SCHISTOSOMES?

A 2D separation of the most soluble proteins from any schistosome life cycle stage has two striking features. First, the preparation contains a complex mixture of several thousand spots (Fig. 2). Secondly, the spot pattern is remarkably similar, irrespective of the stage examined (Curwen et al. 2004). The greatest similarities were observed where the life-cycle stages were developmentally adjacent to one another. For example, lung schistosomula shared 90% of their analysed protein with adults. Eggs were the least similar to any other stage, probably as a result of their containing a free-living miracidium.

Peptide mass fingerprinting of the 40 most abundant spots in soluble cercarial, lung worm, adult and egg extracts revealed that to a large extent, the dominant proteins were indeed shared. With the exception of one protein resident in the endoplasmic reticulum, all species identified by PMF were cytosolic in origin, as would be expected for a highly soluble cell fraction. Proteins with catalytic activity, particularly glycolytic enzymes, were dominant, along with several calcium binding proteins. Actin and other muscle components were also abundant, understandable given the predominance of this tissue throughout all life-cycle stages. The majority of these abundant proteins were originally identified by virtue of their immunological properties, either as antibody targets in previously infected hosts or as putative vaccine candidates.

Why should the different stages show such a similar composition and what are the implications? Schistosomes possess tissues differentiated into rudimentary organs such as gut, nervous system, musculature and excretory system. However, as members of the Phylum Platyhelminthes, they have a solid body plan from which these individual organs cannot be dissected out. Simple extraction of the whole parasite body therefore averages for composition across the various tissues so that constituents common to all, such as glycolytic enzymes, are dominant. It is also striking that several of the vaccine candidates promoted by WHO are dominant within the cytosolic and cytoskeletal fractions.

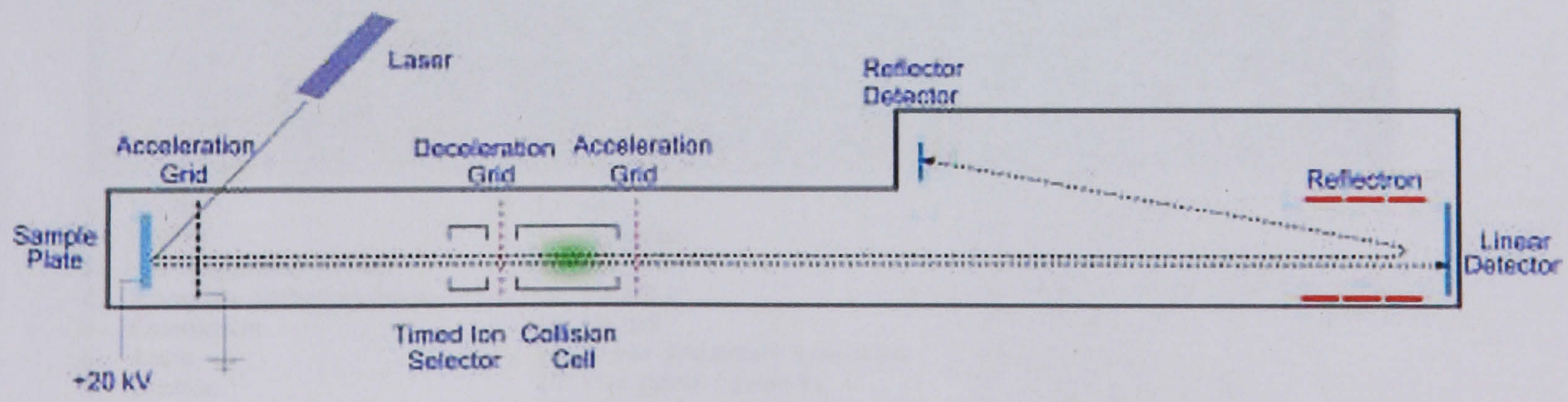
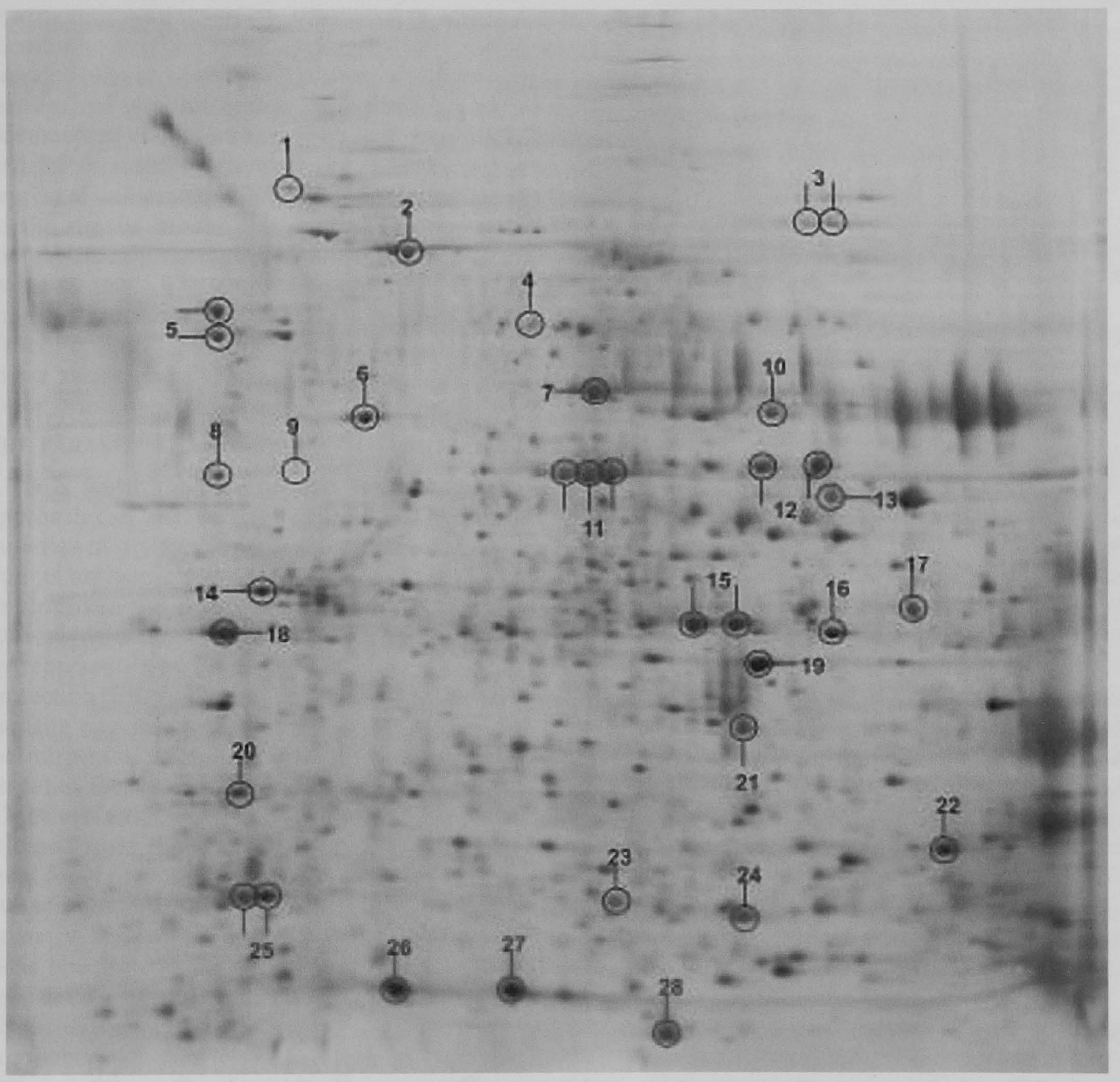


Fig. 1: the 4700 Proteomics Analyser with ToF-ToF optics (Applied Biosystems). Protein digests, together with a UV-absorbing matrix, are applied to the sample plate on the left hand side. The peptides are desorbed from the plate into the vacuum by the laser and then accelerated through the first time-of-flight (ToF) stage. The timed ion selector allows a single peptide ion to pass through into the collision cell where it is fragmented. The fragments are then accelerated and their masses measured by the second ToF analyser. The peptide will tend to fragment at its most labile bond, the peptide bond; the resulting fragments will then differ by the mass of individual amino acid residues, allowing the sequence of the peptide to be deduced, or retrieved from a database.

Given our stated objective of identifying secreted or surface-exposed proteins as potential vaccine candidates, how do we circumvent the problems of complexity and similarity in the parasite fractions? The solution is targeted proteomics; the parasite samples to be characterised need a simpler composition. This can be achieved e.g., by collecting the secretions released from a specific larval stage. However the strategy carries with it the cost of much lower protein yields, but hopefully the material obtained will be of much greater relevance to vaccine development.

With these caveats in mind we have developed an in vitro scheme for analysing the proteins released by the cercaria as it transforms to the schistosomulum, and by the larvae in culture up to 8-10 days, by which time they are fully developed to the lung stage. The cercarial secretions are the most complex, in part due to the holocrine nature of secretion from the acetabular glands. (The entire contents of the gland are squeezed out, so that both cytosolic and vesicular proteins are present.) Both cercarial elastase (Newport et al. 1988) and Sm16 (Rao & Ramaswamy 2000), two previously characterised cercarial proteins, are dominant in the preparation, plus a number of other novel proteins. These, together with the mol-



1	HSP86	11	p40	21	Myosin Light Chain
2	HSP70	12	Aldolase	22	Cycophilin
3	ATP:Guanidino Kinase	13	GAPDH	23	Superoxide Dismutase
4	Adenylate Dehydrogenase	14	14-3-3 e	24	Fatty Acid Binding Protein (Sm14)
5	Calreticulin	15	GST28	25	SME16
6	Actin	16	Triose Phosphate Isomerase	26	Thioredoxin
7	Enolase	17	Elongation Factor 1a	27	Dynein Light Chain
8	Tropomyosin	18	14-3-3 homolog 1	28	Ubiquitin
9	Serpin-like	19	GST26	29	Adenylate Kinase
10	Phosphoglycerate kinase	20	Calpain		

Fig. 2: the major soluble proteins of *Schistosoma mansoni* identified by peptide mass fingerprinting, illustrated on an annotated 2D gel of material from eggs. Modified from Curwen et al. 2004.

ecules secreted by the skin and lung stage larvae (~ 20 in total) will, when fully characterised, cloned and expressed, form a panel of new antigens to be tested in vaccination experiments designed to emulate the attenuated cercarial vaccine.

We are developing a similar approach to characterise the gut secretions of adult worms, where at present only 2-3 proteases have been identified. Apart from the parasite eggs, these gut secretions represent the major source of material released by the adults into the host bloodstream and hence a likely source of antigens relevant to the rhesus and rat self-cure models.

The only other major source of antigens that could serve as vaccine candidates is the surface tegument of both larvae and adults. This syncytial layer, which envelops the entire worm surface, is bounded by a normal plasma membrane overlain by an apparently inert and lipophilic secreted bilayer, or membranocalyx, which provides a barrier against immune attack. The plasma membrane has documented transport functions and enzyme activities, and the exposed portions of the proteins involved may represent vulnerable points for attack, especially relevant to the rhesus model. It is also unclear whether the membranocalyx contains proteins that could serve as immune targets. The proteomic approach will allow the molecular architecture of the parasite surface to be established, defining both the extent of possibilities for immune attack and the nature of the very successful immune evasion mechanism.

THE PARASITE GLYCOME

It is worth mentioning that the mass spectrometric approach can also be applied to characterise the glycan moieties of glycoproteins and glycolipids that can function as antigenic epitopes, provoking antibody production. Given the potent response that primates make to such glycans, we have questioned whether this reactivity represents a smokescreen rather than a component of protection (Eberl et al. 2001). This is especially true of the parasite egg that seems to advertise its presence by releasing proinflammatory secretions to provoke a response essential for its transit through tissues to the gut lumen (Doenhoff et al. 1986). The glycan residues must first be released from the parent protein(s) by PNGase F treatment for N glycans and PNGase A treatment, plus reductive elimination, for the O glycans, prior to mass spectrometry. Empirical structures can be predicted from the mass spectra obtained but structure assignments then need to be confirmed by further rounds of exo- and endoglycosidase digestions, and MS. Our preliminary studies in collaboration with Professor Anne Dell of Imperial College, London on the glycoproteins released by cercariae during skin penetration, and mature eggs in culture, reveal a rich mixture of both N and O glycans. Moreover, the majority of cercarial N glycans appear to be shared with eggs, but the latter contain a lot of glycans structures not present in the cercarial secretions. The fact that immunisation with eggs fails to elicit protection is further evidence that the cross-reactive N glycans are a smokescreen. Assigning definitive structures and biological functions to the various glycans will be a major task.

THE IMMUNOME

The immunome can be defined as that subset of the proteome that reacts with the immune system, most easily demonstrated where proteins act as targets for antibody. Clearly, in vaccine development, where protection is demonstrably mediated by antibodies it is desirable to pinpoint the precise proteins and even epitopes that mediate binding. An important caveat is that the presence of an antigenic epitope on a protein does not make it protective. Indeed, most reactivities will be spurious clues, and the stringent use of non-protective control sera is essential to eliminate them.

Western blotting is the method of choice to identify reactive proteins. It is necessary to run duplicate 2D gels of a protein preparation, one of which is then blotted onto a PVDF membrane before gel and blot are stained with Sypro Ruby (using different protocols) hopefully to reveal identical patterns of proteins; the blot is then probed to visualise the targets. Careful matching of blot to gel pinpoints the protein subset to be excised, digested and subjected to MS to obtain identities. (N.B. It is not possible to get protein identities directly by excision of blot spots because the presence of the two antibodies, the enzyme label and the blocking protein swamps other signals.)

In the context of schistosomiasis we are currently exploring the reactivity of sera from mice given multiple exposures of the attenuated cercarial vaccine, and rhesus monkeys that have undergone self-cure. Not only have the conventional soluble antigen preparations from different life cycle stages (SCAP, SLAP, SWAP, SEA) proved very similar in composition (Curwen et al. 2004) but their cytosolic (GST, Sm14) or cytoskeletal (paramyosin) components promoted as vaccine candidates are also the most immunoreactive proteins. The rational explanation is that such proteins possess immunogenicity because they have not been subject to immunological pressure during evolution due to their internal location. Consequently the ~ 20% protection often reported after vaccination with such antigens may be their maximum potential. Our principal task is to pinpoint the reactive antigens within the preparations highly enriched in secretory and membrane proteins that we are developing, since these should include the proteins that represent the parasite's Achilles heel. We also need to consider the contribution made to reactivity by glycan epitopes, especially in view of the degree of glycan-sharing between proteins, which results in cross-reactivity.

With respect to the immunome, there are two technical problems that still need to be solved before the full power of the approach can be realised. The inability to separate membrane proteins by 2D electrophoresis is one limitation. The only way in which such proteins can be blotted is after a 1D separation, which provides inadequate resolution of the mixture, such that a reactive band may contain one to several proteins and it will be unclear which is the real antibody target. The second is the difficult task of screening a protein preparation for T cell reactivity at the level of individual constituents that can be identified by MS.

CONCLUSIONS

It has been possible for 20 years to perform 2D separations of complex protein mixtures, but only recently have technical developments made such gels reproducible. The parallel developments in the mass spectrometry of proteins have also made it possible to identify the many proteins present, provided the DNA sequence data exists. Thus the scene is set, given adequate funding, to identify the complete subset of secreted and surface-exposed schistosome proteins from which the possible vaccine candidates will emerge. In our estimation the targeted proteomic approach will reduce the potential candidates from the not less than 14,000 proteins encoded by the genome, to a maximum of 100-200 dominant proteins in the secretory and membrane preparations. This is a much more manageable number, and the information should be available within in a time frame of 2-3 years.

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