Parasitol Res (1998) 84: 800–805

ORIGINAL PAPER

Philip F. Jemilohun

Isolation and characterization of flagella from *Trichomonas vaginalis*

Received: 12 December 1997 / Accepted: 5 June 1998

Abstract As a first step in the biochemical and biomedical analyses of flagella from *Trichomonas vaginalis* the flagella were isolated, purified, and analyzed. The flagella were detached by mechanical shearing and a crude flagellar preparation was obtained by low-speed differential centrifugation. The crude flagellar preparation was subjected to further purification by discontinuous sucrose density-gradient centrifugation. Electron micrographs (EM) of the purified flagella showed the typical 9 + 2 axonemal arrangement. The structural integrity and the flagellar membrane were not destroyed by the deflagellation method or the purification scheme employed. The flagellar preparations were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified preparation contained many flagellar enriched proteins ranging from 20 to 120 kDa. Three major proteins of 65 kDa and a doublet of about 50–58 kDa were observed. The protein patterns and EM appearance of the fractions were highly reproducible.

Introduction

Trichomonas vaginalis is a flagellated protozoan parasite. Its life cycle has a single stage known as a trophozoite and its only known habitat is the urogenital tract of humans. The parasite has four anterior flagella and a recurrent flagellum that forms an undulating membrane at the posterior end of the parasite (Honigberg et al. 1984; Warton and Honigberg 1979). It is the causative agent of trichomoniasis (vaginitis and urethritis), one of the world's most common sexually transmitted diseases (Heine and McGregor 1993; Krieger et al. 1990; Thomason and Gelbart 1989). It is estimated that about 180 million people are infected worldwide annually (Krieger et al. 1990; Thomason and Gelbart 1989). Trichomoniasis in women is not only extremely common but is the source of considerable itching and stress and may result in marked suffering and incapacitating illness (Sobel 1992).

The increasing incidence of trichomoniasis (Heine and McGregor 1993) has led to renewed interest in the study of subcellular structures of the pathogen for potential immunogens and as possible targets for antiparasitic drugs (Bagnara et al. 1996; Katiyar and Edlind 1994; Mathews et al. 1987). As a result of these novel approaches, preparations of subcellular components with the highest possible degree of purity are important. Attempts in this direction have been initiated in some other parasitic protozoa (Ismach et al. 1989; Piras et al. 1981; Ruiz et al. 1986; Saborio et al. 1989; Segura et al. 1977; Warburg et al. 1989). This approach has little, if any, application to *T. vaginalis* at present because of the difficulty in isolating and purifying subcellular structures of this organism without contaminants.

Of the many subcellular components of *T. vaginalis*, the flagellum is important and relevant for adhesion and elicitation of an immune response because it is the first subcellular structure of the parasite to make contact with its targets. Adhesion of *T. vaginalis* and *Tritrichomonas foetus* to a polystyrene substrate and to the surface of epithelial cells via the anterior flagella and some regions of the cell body has been reported (Corbeil et al. 1989; Silva Filho et al. 1988). In view of these reports and the universally increasing incidence of trichomoniasis it is significantly important that isolation and elucidation of the flagellar structure of *T. vaginalis* be the first step in attempts to understand the roles of flagella in the adhesion and pathogenesis of the parasite.

In this study, flagella of *T. vaginalis* were isolated, purified, and microscopically analyzed before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Details regarding the detection and isolation of flagellar immunogens and adhesins of *T. vaginalis* will be the subject of a separate communication.

P.F. Jemilohun
Department of Biology, Jackson State University,
1400 J.R. Lynch Street,
Jackson, MS 39217, USA
Fax: +1-601-974-5853

Materials and methods

Parasite strain and cultivation

Trichomonas vaginalis strain ATCC 30235 was kindly provided by Dr. William Lushbaugh (University of Mississippi Medical Center, Jackson, Miss., USA). The parasite was cultured (axenically at 37 °C in Diamond trypticase-yeast extract-maltose (TYM) medium (pH 6.0) supplemented with 10% heat-inactivated horse serum (Diamond 1957). For each flagellar isolation experiment we used 1.5 l of the parasite culture in tissue-culture flasks (Falcon), at the mid-logarithmic phase.

Preparation of flagella

Cells from 1.5 l of the culture were harvested, washed twice with phosphate-buffered saline (PBS), and suspended in 15 ml buffer A [0.25 *M* sucrose, 10 m*M* TRIS-HCl (pH 7.2), 1 m*M* ethylene diaminetetraacetic acid (EDTA), 4 m*M* $MgCl_2$, 1 m*M* ethylene glycol tetraacetic acid (EGTA), 1 m*M* dithiothreitol (DTT)]. The cell suspension was cooled on ice for 30 min and phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 m*M*. The cell suspension was deflagellated by mechanical shearing (vortexing for 3 min at full speed; Rosebaum and Child 1967). Deflagellation was monitored by phase-contrast microscopy.

The deflagellated suspension was diluted four times with buffer B [0.25 *M* sucrose, 10 m*M* TRIS-HCl (pH 7.2), 4 m*M* $MgCl_2$, 1 m*M* EDTA] and centrifuged at 252 g for 10 min to remove the cell bodies. The supernatant containing the flagella was collected. The pellet was resuspended, diluted four times again with buffer B, and centrifuged as described above. The second supernatant was collected and added to the first. The combined supernatant was centrifuged first at 252 g for 5 min and then at 10 000 g for 15 min to pellet the flagella. The flagellar pellet was suspended in 4 ml buffer B with 1 m*M* PMSF, and an aliquot of 2 ml was layered on a 6-ml cushion of 1.5 *M* sucrose solution [1.5 *M* sucrose, 10 m*M* TRIS-HCl (pH 7.2), 4 m*M* $MgCl_2$,1 m*M* EDTA] and then centrifuged at 16 000 g for 15 min to remove subcellular contaminants. The band forming at the interface of 1.5 *M* sucrose was collected and discarded. The remaining supernatant and the pellet were gently mixed and the resulting suspension was referred to as "crude flagella." The crude flagellar preparation was further purified by discontinuous sucrose density-gradient centrifugation (Jemilohun et al. 1996; Pereira et al. 1977; Piras et al. 1981; Russell et al. 1983). The preparation was layered on top of a sucrose density gradient (1.4, 1.6, 1.80, and 2.0 *M* sucrose in buffer B). The gradient was centrifuged at 112 500 g for 2 h. The bands forming between the 1.4/1.6- and 1.6/1.8-*M* sucrose interfaces containing the flagella were carefully removed, diluted, and centrifuged at 10 000 g for 15 min. The flagellar pellet was carefully suspended in buffer B, PMSF was added to a 1-m*M* final concentration, and the preparation was stored at −20 °C until used. This was designated "purified flagella."

Gel electrophoresis

The flagellar preparations were solubilized in 50 m*M* TRIS-HCl (pH 8.2), 5 m*M* EDTA, 1% SDS, and 1 m*M* PMSF (Adoutte et al. 1980). Protein concentration was determined (Bradford 1976) using a commercially available kit (Bio-Rad Laboratories, Hercules, Calif., USA). Bovine serum albumin was used as the protein standard. SDS-PAGE was performed in a minigel apparatus (Bio-Rad Laboratories) using a 4% stacking and 12% separating polyacrylamide gel systems. Samples were prepared from purified flagella, crude flagella, and whole cell homogenates. The samples were solubilized under denaturing conditions in 2X sample buffer (20% glycerol, 10% 2-mercaptoethanol, 2% SDS, 125 m*M* TRIS-HCl, pH 6.8; Adoutte et al. 1980). The samples were boiled for 5 min prior to their addition to the gel wells. Protein separation was accomplished at 100 constant volts (Laemmli 1970). Gels were stained with 0.1% Coomassie blue R-250 in a solution containing 40% methanol and 10% acetic acid. The gels were destained in a solution containing 40% methanol and 10% acetic acid. The following broad-range molecular-weight standards (Bio-Rad Laboratories) were used: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (31.0 kDa).

Results

Figure 1 represents a summary of the fractionation scheme. Many of the classic deflagellation and deciliation methods previously used by other investigators for eukaryotic systems (Adoutte et al. 1980; Dentler 1980; Gualtieri et al. 1986; Hyams 1992; Pereira et al. 1977; Piperno and Luck 1976; Rosebaum and Child 1967; Satir et al. 1976; Witman 1986; Witman et al. 1972) were initially employed, but none worked for *Trichomonas vaginalis* with the exception of mechanical shearing (Rosebaum and Child 1967). Flagella from the pH-sucrose method (Rosebaum and Child 1967) became swollen with vigor. *T. vaginalis* cells treated with up to 50 m*M* dibucaine (Dentler 1980; Satir et al. 1976; Witman 1986) did not lose their flagella. The cells remained motile and active. The detergent method (Hyams 1992; Piperno and Luck 1976) often solubilized the flagella and cellular membranes, leading to contamination of the flagellar preparation. The ethanol plus calcium method (Gualtieri et al. 1986; Piperno and Luck 1976; Witman et al. 1972) did not result in deflagellated cells. Observation by phase-contrast microscopy showed that when deflagellation was performed by mechanical shearing, most of the cells remained intact while the flagella were detached.

Cellular and most subcellular contaminants were removed from the preparation by differential centrifugation (Fig. 2). This resulted in a flagellum-rich preparation with few subcellular contaminants. Further purification of the flagellar preparation was achieved by discontinuous sucrose density centrifugation. The flagella sedimented at the 1.4/1.6- and 1.6/1.8-*M* sucrose interfaces because of the heterogeneity in density of the flagella due to differences in length and complex formation. As a result the majority of shorter flagella always sedimented at the 1.4/1.6-*M* sucrose interface, whereas most of the longer ones formed a band at the 1.6/1.8-*M* sucrose interface.

Examination of the crude flagellar preparation with the light microscope indicated that it consisted mainly of flagella with few subcellular contaminants (Fig. 2). Most of the flagella were straight, with some being spirally shaped. In the electron micrographs of purified flagella (Figs. 3, 4) the flagellar sheath (membrane) remained intact without being affected by the method of deflagellation or the purification schemes. There was little, if any, apparent disruption of the flagella since the majority of them remained intact. Occasionally, bare axoneme and empty vesicles were observed (Fig. 4a). The

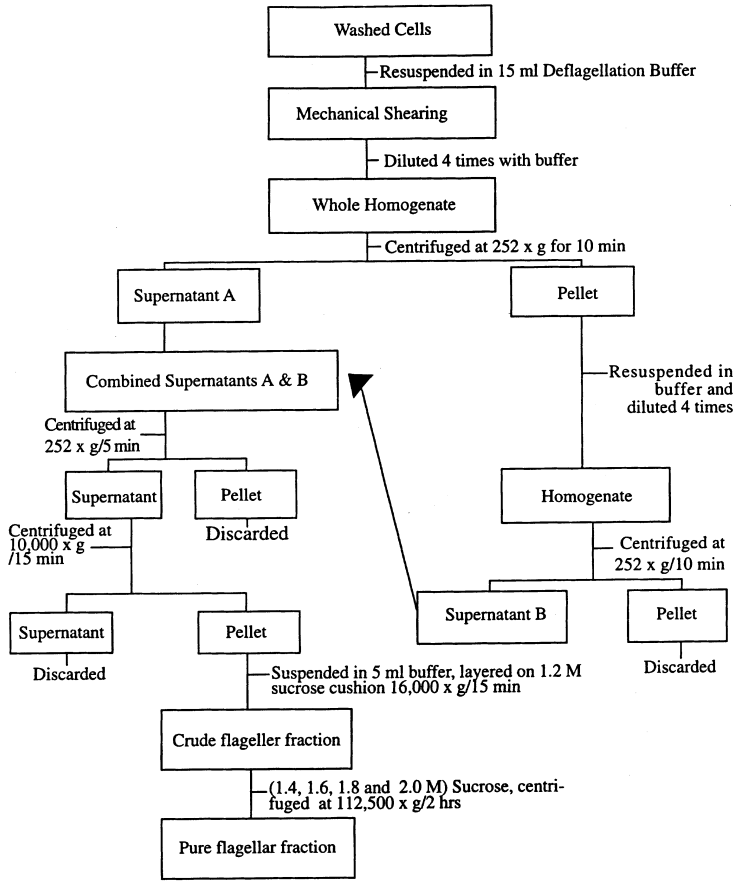

Fig. 1 Schematic diagram showing all steps used for the isolation and purification of the flagella of *Trichomonas vaginalis*

classic 9 + 2 axonemal arrangement was not affected by the preparation schemes.

In addition to microscopic analyses, SDS-PAGE was used to monitor and analyze the samples from the flagellar preparation. Figure 5 shows the analysis of whole cell lysate and of crude and purified flagellar fractions on 12% SDS-PAGE. Highly reproducible gel patterns were obtained from gel analysis of different flagellar preparations. The whole flagella showed a complex pattern of polypeptides with a molecular weight of about 20–120 kDa. In the purified flagellar sample (Fig. 5, lane 4), many low- and high-molecular-weight protein bands were present. A prominent protein of 65 kDa was consistently observed in crude and purified flagellar preparations. Several flagellar enriched polypeptides ranging in size from 20 to 120 kDa were observed. A doublet protein of about 50–58 kDa stained heavily in crude and purified flagellar preparations.

Discussion

Although the primary function of flagella is locomotion, the flagella of *Trichomonas vaginalis* have been implicated in the adhesion of the organism to vaginal epithelial cells (Corbeil et al. 1989; Silva Filho et al. 1988). This linkage makes it worthwhile to study the flagella of this parasite. The main objective of this study was to develop a method for the isolation and purification of flagella of *T. vaginalis* and to provide microscopic and electrophoretic analyses of the flagella. Such a flagellar preparation may be used for biomedical studies.

On the other hand, the flagella purified as described in the present study, may be used for structure and biochemical studies. All of the flagella of *T. vaginalis* are not the same. The four anterior flagella are free, whereas the recurrent flagellum extends to the posterior end of the parasite to form an undulating membrane (Honigberg et al. 1984; Warton and Honigberg 1979). Besides the difference in structural location, the anterior flagella have particles that are arranged into rosettes known as flagellar rosettes (Bardele 1983) and the recurrent flagellum is not normally free (Honigberg and King 1964). The deflagellation method employed in this study effectively detached free flagella with little, if any, impact on the recurrent flagellum, which is not free. Therefore, it was expected that minimal numbers of recurrent flagella, if any, would be present in the flagellar preparations.

Of all the various deflagellation and deciliation methods initially employed, mechanical agitation was found to be effective, reproducible, and efficient with minimal cell lysis. In addition, flagella of high purity and

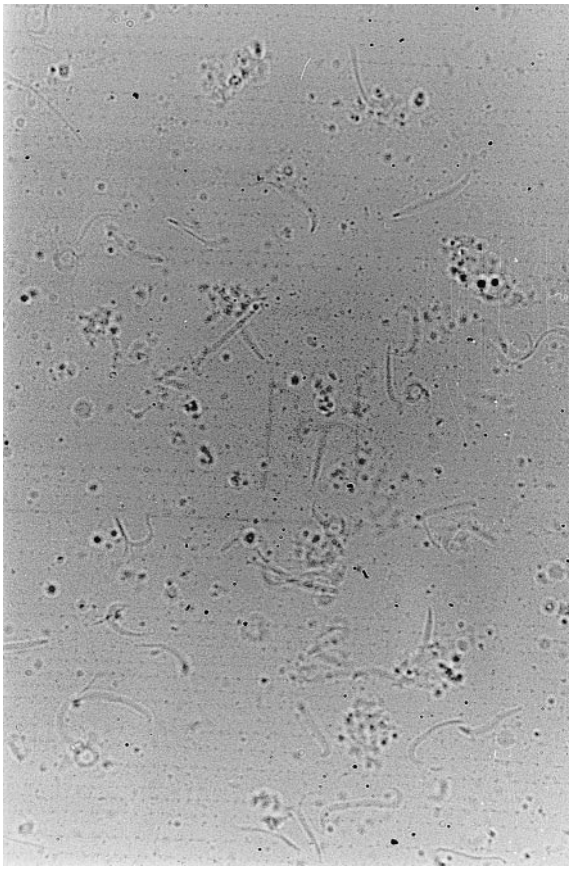

Fig. 2 Light micrograph of the crude flagellar preparation, showing flagella and some subcellular contaminants

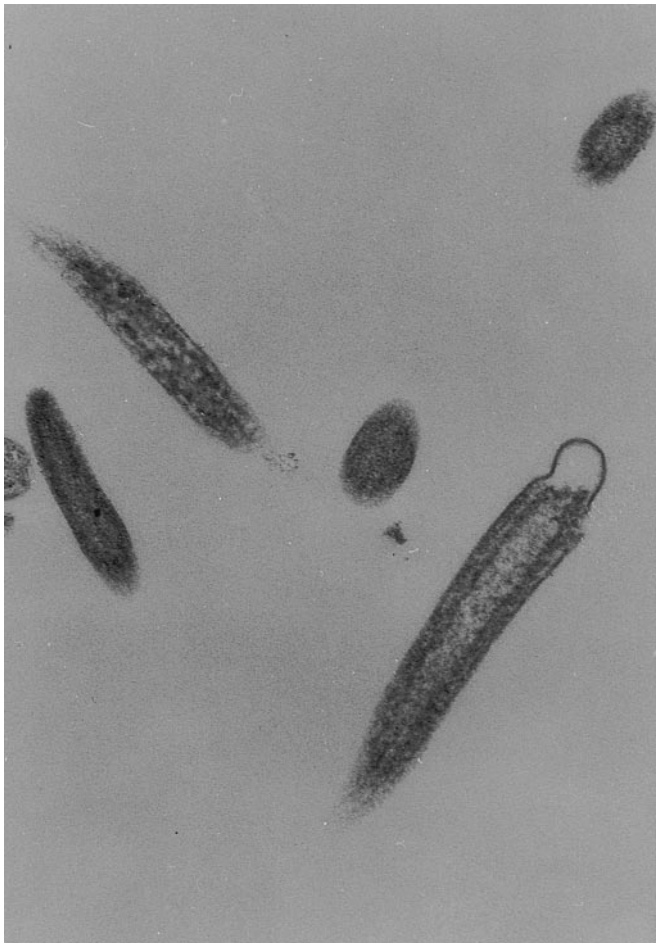

Fig. 3 Electron micrograph of a longitudinal section of purified flagella, showing intact flagella with densely stained matrix

integrity in moderately good yield were obtained with this method. The duration of the mechanical shearing was critical to the achievement of a clean preparation with full-length flagella. Some of the flagella had detached from the cells as kinetosome-flagellum complexes (Monteiro-Leal et al. 1993). This condition, coupled with the lack of homogeneity with regard to the length of the flagella, might have offset the normal equilibrium of the flagella in the sucrose density gradient (Monteiro-Leal et al. 1993). Despite these obstacles, the flagellar preparation was consistently of high purity as judged by electron microscopy (Figs. 3, 4). The purity of the flagellar preparation was comparable with other protozoan flagellar preparations that have previously been reported (Pereira et al. 1977; Piras et al. 1981; Segura et al. 1977; Silva et al. 1989; Warburg et al. 1989).

In most flagellar preparation methods (Gualtieri et al. 1986; Ismach et al. 1989; Pereira et al. 1977; Piras et al. 1981; Rosebaum and Child 1967; Ruiz et al. 1986), calcium ions are added to the buffer systems. In the present study we noticed that the addition of calcium ions, even at a very low concentration, consistently led to aggregation of vesicles and other subcellular structures, which affected the purification schemes.

As judged from the electron micrographs of the flagellum, the classical 9 + 2 axonemal arrangement was preserved. The electron micrographs showed that the flagella were intact and well preserved, with the flagellar membrane not being affected by the isolation method or the purification schemes. The cross section of the flagella also revealed some axonemes lacking the central doublet (Fig. 4a). This morphology (Piccinni and Mammi 1978) is typical of the proximal portion of flagella and has been observed in flagella isolated from *Euglena gracilis* (Gualtieri et al. 1986).

The results of the SDS-PAGE analyses show that the flagella of *T. vaginalis* contain many proteins. Among them a protein of 65 kDa and a doublet protein of 50–58 kDa were very prominent (Fig. 5, lane 4). The 65-kDa protein and the doublet protein (50–58 kDa) stained heavily in purified and crude flagellar fractions but not in the whole cell sample. Other minor flagellar enriched proteins (Fig. 5, arrows) of 26, 35, 42, 48, 78, and 120 kDa were detected. Some of these flagellar enriched polypeptides (65, 42, and 35 kDa) have molecular weights similar to those reported for adhesins of *T. vaginalis* by other investigators (Alderete et al. 1995; Arroyo et al. 1992; Fiori et al. 1993; Rappelli et al. 1995). However, the 65-kDa protein detected in this study may not have functional similarity to the previ-

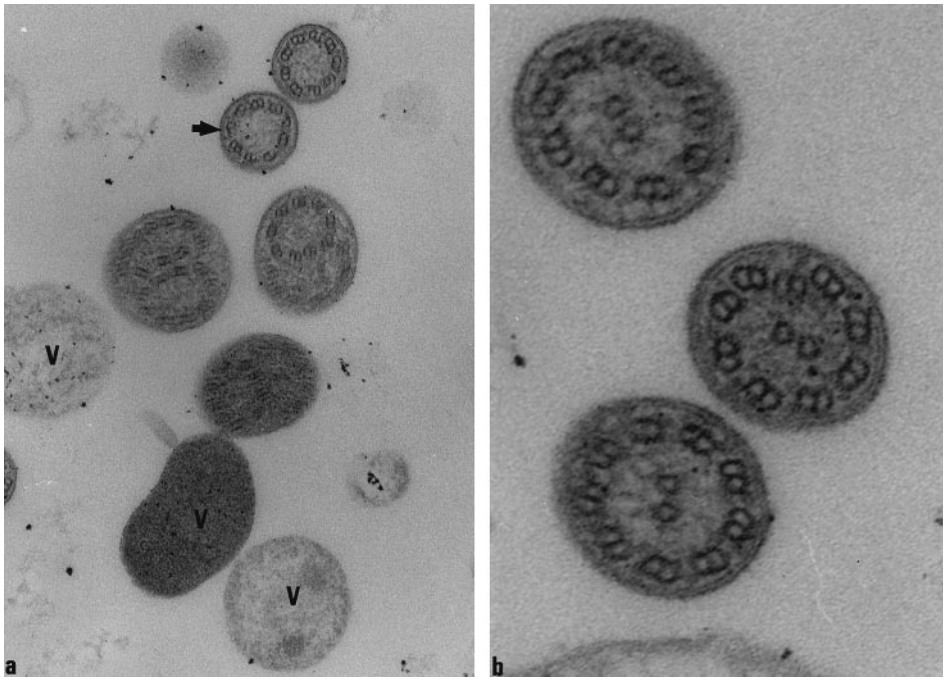

Fig. 4a, b Electron micrographs of cross sections of purified flagella. **a** Transversal section of flagella, showing membrane vesicles (*V*) and a flagellar axoneme lacking a central doublet (*arrow*). **b** Cross section of purified flagella, showing the flagellar axoneme and the central doublet. In both **a** and **b** the flagellar membrane is intact

ously reported AP65 because of its localization as a flagellar protein as oppose to the association of AP65 with malic enzyme found in the hydrogenosomes of the parasite. The abundance, based on intense staining of the 65-kDa protein, suggests that it may be an important structural protein or may be involved in host-parasite interactions.

In conclusion, this study set forth the methodology for the isolation and purification of flagella from *T. vaginalis*. This is a fundamental step in the establishment of the structural and biochemical analyses of flagella of the parasite. The isolation and purification method is also of paramount importance in the investigation of the roles of flagella from *T. vaginalis* in pathogenesis and host-parasite interactions.

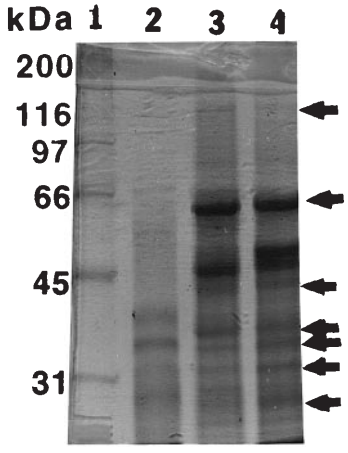

Fig. 5 SDS-PAGE analysis of flagellar fractions from different purification steps (*1* Molecular-weight markers with sizes shown in kDa, *2* whole cell homogenate, *3* crude flagellar sample, *4* purified flagellar sample). The *arrows* indicate flagellar enriched proteins

Acknowledgements I thank Dr. William Lushbaugh for providing the strain of *T. vaginalis* and for helping in the initial stages of parasite culture. I also thank Dr. John C. Meade for suggestions and for critical reading of the manuscript. The author thanks Drs. Fred Nelson and Arthur Jones for their supports and encouragement. I thank Dr. Francis Chigbo for the materials he donated toward this study. I am thankful to those people who assisted me in this study, especially to Dr. Raji Abayomi for assistance in literature search and acquisition and provision of some chemical reagents. This work was partly supported by the Department of Biology, Jackson State University.

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