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Analysis of the Proteinases of Representative *Trichomonas vaginalis* Isolates

K. A. NEALE AND J. F. ALDERETE*

Department of Microbiology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7758

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Isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with acrylamide copolymerized with gelatin (substrate-SDS-PAGE) were combined to evaluate the proteinases of both long-term-grown and fresh isolates of *Trichomonas vaginalis*. This two-dimensional substrate-SDS-PAGE resolved as many as 23 distinct proteinase activities in several isolates, and proteinases had relative molecular masses between 23 and 110 kilodaltons (kDa). Isoelectric points (pI) of proteinases ranged from 5.7 to 7.0. Overall, the various representative proteinase profiles were similar among those of long-term-grown and fresh isolates, although heterogeneity existed among several cysteine proteinase activities. Pattern changes were detected in fresh isolates passaged over several weeks, showing the ability of proteinases to be differentially expressed and to undergo phase variation. The two-dimensional proteinase patterns were very reproducible for isolates analyzed over a certain period of time before expression of some proteinases varied. The heterogeneity and differential expression of certain proteinases were not coordinated with phenotypic variation of already characterized immunogens and adhesins. Data suggesting that a 43-kDa proteinase resided on the parasite surface were obtained on the basis of removal of activity following pronase or proteinase K treatment of live organisms. Finally, immunized experimental animals produced antibody to many *T. vaginalis* proteinases, which indicates the immunogenic nature of trichomonad proteinases.

Evidence has been obtained that some of the proteinase activities of *Trichomonas vaginalis* may be involved with the virulence properties of host cell adherence (13). Parasitism of host cells and tissues by this sexually transmitted protozoan is dependent upon the expression of adhesin proteins on the cell surface (3, 6), and four proteins have been identified as putative adhesin molecules in this organism (8). Another study showed that *T. vaginalis* proteinase activity mediated the autolysis of a parasite surface immunogen (11). Additionally, a cell-detaching factor with trypsinlike (16, 30) activity which is active in an acidic pH range has been previously described.

Studies by others show that nearly all the proteinase activity of *T. vaginalis* is thiol activated and detectable at an acidic pH (24, 28). At least 11 different cysteine proteinases are present in *T. vaginalis*, as demonstrated by one-dimensional electrophoretic analysis in hemoglobin gels (14), by gelatin-polyacrylamide gels (27), and by inhibitor and activator sensitivities (14, 24). Some proteinases were found to be secreted into culture medium (26, 27), and a heterogeneity among some of the proteinases for various isolates was described previously (27).

One goal of our laboratory is to identify the proteinases involved in virulence and pathogenesis, such as cytadherence as mentioned above (13). This, however, requires that the extent of heterogeneity and the complexity of the total cysteine proteinase repertoire be clearly delineated among *T. vaginalis* isolates. We, therefore, characterized trichomonad cysteine proteinases by a two-dimensional electrophoresis system combining isoelectric focusing (IEF) with substrate-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (substrate-SDS-PAGE) in which acrylamide is copolymerized with gelatin (17). This technique resolved common and different proteinases of *T. vaginalis*, and proteinases were identified which ranged in size from 23 to 110 kilodaltons (kDa). Isoelectric points also varied between 5.7 and 7.0. At least 23 distinct activities were identified. Fresh isolates possessed profiles which were also similar to long-term cultures, which shows the usefulness of common laboratory isolates in the characterization of the diversity of proteinase patterns. Importantly, data from analysis of fresh isolates grown in vitro showed that some proteinases were differentially expressed and underwent phase variation. The immunogenic nature of proteinases was also demonstrated, and these results are discussed in terms of vaccine potential and their importance to trichomonal virulence and pathogenesis.

MATERIALS AND METHODS

Cell culture, iodination, and enzyme treatments. Previously described *T. vaginalis* isolates NYH286, RU375, IR78, and AL20W (1, 4, 10) were passaged daily in Diamond's Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-maltose medium (15) supplemented with 10% horse serum (1, 7). Fresh isolates were obtained from the Obstetrics-Gynecology Department of the University of Texas Health Science Center and were analyzed by substrate-SDS-PAGE immediately upon axenization as described previously (4).

Surface radioiodination of washed, live *T. vaginalis* organisms was accomplished as described previously (9, 12).

Pronase (Calbiochem-Behring, La Jolla, Calif.), papain, proteinase K, trypsin, and chymotrypsin (all from Sigma Chemical Co., St. Louis, Mo.) were prepared as 10-fold stock solutions, and amounts, each containing the equivalent of 5 mg of enzymes, were added to samples of 4×10^6 live, radioiodinated *T. vaginalis* isolates. The final volume was adjusted to 500 μl with phosphate-buffered saline (6), and samples were then incubated for 15, 30, 45, or 60 min at 37°C. The reactions were stopped by 5-min incubations at

* Corresponding author.

room temperature with addition of inhibitor (antipain for papain, phenylmethylsulfonyl fluoride for pronase and proteinase K, and soybean trypsin inhibitor for trypsin and chymotrypsin). Organisms were then washed twice with phosphate-buffered saline and examined microscopically to insure that the treatments did not affect parasite viability and motility (6). Cells were then pelleted and prepared for substrate-SDS-PAGE and autoradiography.

One- and two-dimensional SDS-PAGE for proteinase detection. Analysis of proteinase activity combined IEF and substrate-SDS-PAGE. IEF (29) was performed with the Bio-Rad mini 2-D cell (Bio-Rad Laboratories, Richmond, Calif.). Gels consisting of 9.2 M urea, 4% acrylamide, 2% (wt/vol) Nonidet P-40, and 2% Ampholines (pI, 3.5 to 10) (Pharmacia-LKB) were cast in 60-mm lengths with capillary tubes (1 by 75 mm). Samples were prepared by adding IEF buffer (9.5 M urea, 2.0% Nonidet P-40, 5% 2-mercaptoethanol, 1.6% Ampholines [pI, 5 to 8], 0.4% Ampholines [pI, 3.5 to 10]) directly to cell pellets or to material to be analyzed (7). Fifty-microliter volumes of IEF buffer were added to 2×10^6 cell equivalents. Samples were triturated, incubated for 30 min at 37°C, and then centrifuged to remove insoluble material. Fifty microliters of clarified samples was immediately loaded onto IEF gels and overlaid with 50 μl of overlay buffer (9.0 M urea, 0.8% Ampholines [pI, 5 to 8], 0.2% Ampholines [pI, 3.5 to 10], 0.0025% bromophenol blue). Anode and cathode buffers consisted of 20 mM sodium hydroxide and 10 mM phosphoric acid, respectively. Electrofocusing was carried out at 500 V for 10 min and then at 750 V for 3.5 h, and focused gels were then used immediately for substrate-SDS-PAGE as described below. The IEF gels were sealed onto preparative stacking gels with 1% agarose in IEF equilibration buffer (0.625 M Tris hydrochloride [pH 6.8], 2.3% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol). Electrophoresis was conducted in 25 mM Tris–192 mM glycine buffer (23) with mini Protean II minigels (1 by 75 by 60 mm). Second-dimension slab gels were prepared by copolymerizing acrylamide with 0.2% gelatin as substrate for proteinases (17, 27).

After the gels were electrophoresed, they were soaked in 2.5% (wt/vol) Triton X-100 for 1 h to remove SDS. Proteinases were activated by incubation for 2 h in 100 mM sodium acetate buffer (pH 4.0) containing 1 mM dithiothreitol at 37°C with one change of buffer (14, 17). The gels were stained for 30 min in 0.275% (wt/vol) Coomassie brilliant blue R prepared in 40% (wt/vol) methanol–10% (vol/vol) acetic acid and destained. Cleared regions represented gelatin digestion by proteinases.

The majority of the proteinase activities of *T. vaginalis* belong to the thiol or cysteine proteinase family (14, 24–28). On the basis of these earlier studies, we chose to analyze cysteine proteinases capable of digesting gelatin. This system allowed for obtaining reproducible patterns with both one- and two-dimensional substrate-gel electrophoresis. Isolates were analyzed no less than five times within a 2-day period, and identical patterns were obtained that showed the highly reproducible one- and two-dimensional patterns of proteinase activities.

Antisera, immunofluorescence, and fluorescence-activated cell sorting. Mouse antisera against NYH286 and IR78 were obtained as previously described (1, 2) from mice inoculated subcutaneously with live parasites. Rabbit antisera were generated by immunizing rabbits with *T. vaginalis* in Freund adjuvant as previously described (1).

Monoclonal antibody C20A3 was used to monitor the fluorescence phenotype of all isolates by indirect immuno-

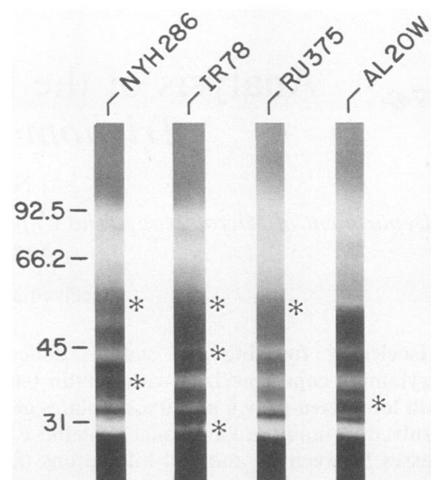

FIG. 1. One-dimensional substrate-SDS-PAGE analysis of long-term-in vitro-grown *T. vaginalis* isolates. Cleared regions of the gel represent proteinase digestion of gelatin copolymerized with acrylamide. Variant proteinase activities are indicated by asterisks.

fluorescence (4, 9, 10, 12). Fluorescence-activated cell sorting was used in order to separate fluorescent and nonfluorescent trichomonads from the heterogeneous NYH286 parent isolate (10).

Immunoprecipitation. Immunoprecipitation has been previously described by us (1). Briefly, approximately 2×10^6 parasites were solubilized in 200 μl of 0.05% Zwittergent 3-12 (Calbiochem-Behring) prepared in phosphate-buffered saline. Primary antibody incubation was carried out for 2 h at 37°C. Immune-complexed proteins were precipitated by addition of protein A-bearing *Staphylococcus aureus* followed by solubilization of immunoprecipitated, radiolabeled immunogen in SDS-dissolving buffer for 30 min at 37°C (1).

RESULTS

Identification of *T. vaginalis* proteinases. Figure 1 depicts the one-dimensional proteinase patterns of four long-term-grown isolates of *T. vaginalis*. Up to 11 regions of activity were evident, which was consistent with the reports of others (14, 24, 27). Each gel showed activities common among all isolates in addition to unique proteinase activities (Fig. 1, asterisks). These patterns were representative of many other isolates which we have described previously (4–13). This method of proteinase analysis, however, did not clearly resolve the proteinases present in the high-molecular-mass region of ≥65 kDa.

Two-dimensional substrate-SDS-PAGE. Because one-dimensional analysis as presented in Fig. 1 may not be representative of the overall complexity of the proteinase composition of *T. vaginalis*, we performed two-dimensional analysis of proteinases. Up to 23 distinct proteinase activities could be resolved by two-dimensional substrate-SDS-PAGE (Fig. 2). The overall commonality of the total proteinase patterns was evident, and activities were included in three prominent gel regions which were identified for comparative analysis among isolates. Region I represented the group of high-molecular-mass activities of >60 kDa with pIs between 6.1 and 7.2. Region II included proteinases with molecular masses between 40 and 60 kDa and with pIs of 6.0 to 6.5. Region III contained all proteinase activities detected with molecular masses of <40 kDa.

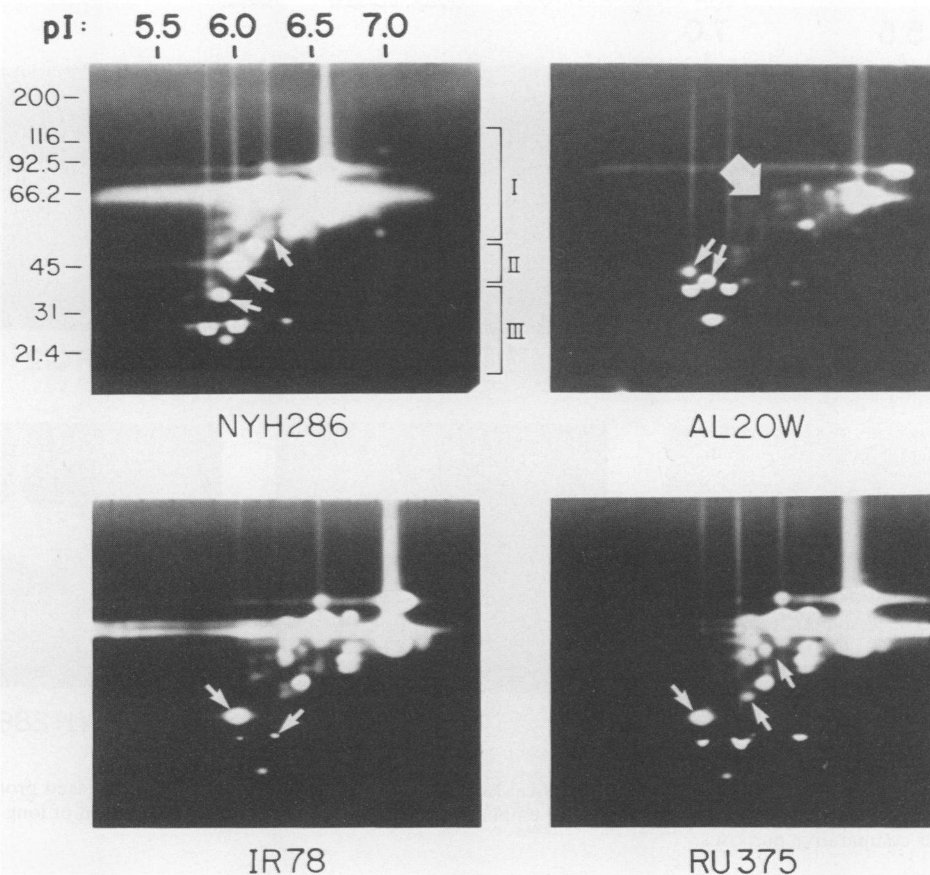

FIG. 2. Two-dimensional substrate-SDS-PAGE analysis of long-term-in vitro-grown *T. vaginalis* isolates. Activities were divided into three arbitrary regions representing high (I), middle (II), and low (III) M_r activities. Variant activities are denoted by arrows. Note the lack of region I and region II activities in AL20W, as indicated by the large arrow.

Examination of each region among the different isolates, however, showed that isolates contained dissimilarities in the patterns of proteinase activities, as evidenced by both qualitative and quantitative differences. For example, although proteinases of region III were present in every isolate, Fig. 2 illustrates the variations that were observed in electrophoretic mobilities, pIs, and overall sizes of areas of proteinase gel clearing. Some examples are illustrated in Fig. 2 by arrows. Furthermore, it is noteworthy that many of the region I proteinases (those with molecular masses between 65 and 90 kDa and pIs of 6.0 to 6.6) and nearly all region II activities were absent from the gel pattern of the long-term-grown isolate AL20W (Fig. 2, large arrow).

Proteinase activities of fresh isolates. We next compared proteinase patterns of long-term-grown isolates (Fig. 2) with proteinase patterns of parasites that were obtained from patients who had trichomoniasis but that had been cultivated for ≤3 days. The same quantitative and qualitative differences that existed in the long-term-grown isolates were also evident among the fresh isolates studied. Four patterns representative of fresh isolates are presented in Fig. 3. This shows that our commonly studied isolates yield patterns similar to organisms present during human infections.

Importantly, continued in vitro passage of fresh isolates resulted in a change in proteinase profiles of the same isolates. For example, after 6 weeks of in vitro daily passage, isolate T005 changed dramatically in proteinase activities with molecular masses from 65 to 80 kDa (pI, ~7.0) (region I) and in the addition of proteinase with molecular masses between 40 kDa (pI, 6.0) (region II) and 23 kDa (pI, 5.8) (region III). Other fresh isolates, like T005, which have been examined similarly, also demonstrated conversions during in vitro cultivation. These results indicate that some proteinases may indeed be differentially expressed.

Relationship between established fluorescence phenotypes and proteinase patterns. The differential proteinase expression of isolates like T005 (Fig. 3) prompted us to test whether proteinase activities were coordinated with phenotypic variation of the surface expression of immunogens (9, 12, 14) and adhesin proteins (4, 10) previously reported by us. Isolate NYH286 was fractionated by flow cytofluorometry with monoclonal antibody C20A3 (10), and fluorescent and nonfluorescent subpopulations were examined by substrate-SDS-PAGE. No specific proteinase patterns correlated with fluorescence or nonfluorescence phenotypes, however, as both fractionated subpopulations gave proteinase profiles identical to those of the parent population (data not shown).

Localization of proteinase activities to surface membranes. Recently, it has been suggested that some proteinase or proteinases were expressed on trichomonal surfaces (13). Radioiodinated parasites were treated with various proteinases as has been described previously (8), and treatment of live organisms with pronase and proteinase K was effective in eliminating the 43-kDa proteinase (Fig. 4, asterisk). We also showed the removal of iodinated surface proteins by these enzymes as a control, as described in our earlier published results (8). Controls, consisting of untreated cells

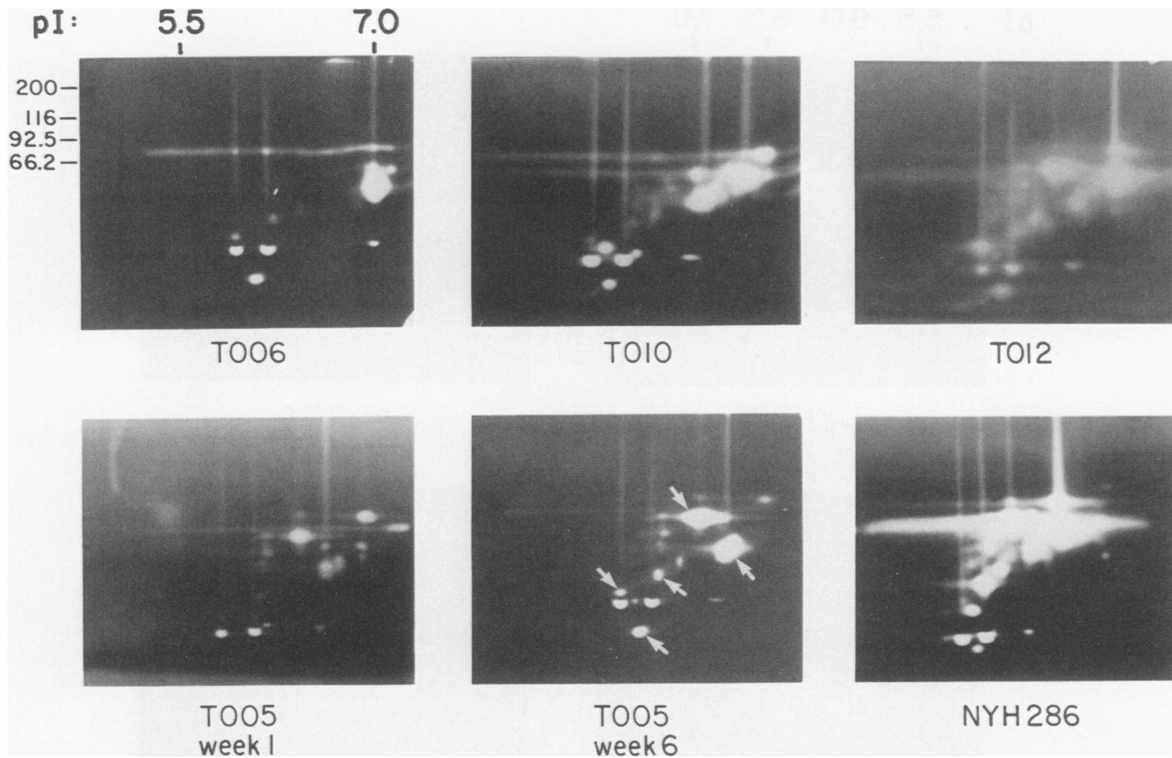

FIG. 3. Two-dimensional substrate-SDS-PAGE of fresh clinical isolates of *T. vaginalis*. Differentially expressed proteinase activities of fresh isolate T005 after 6 weeks of daily in vitro cultivation are emphasized with arrows. The proteinase pattern of long-term-grown isolate NYH286 is included for comparative purposes.

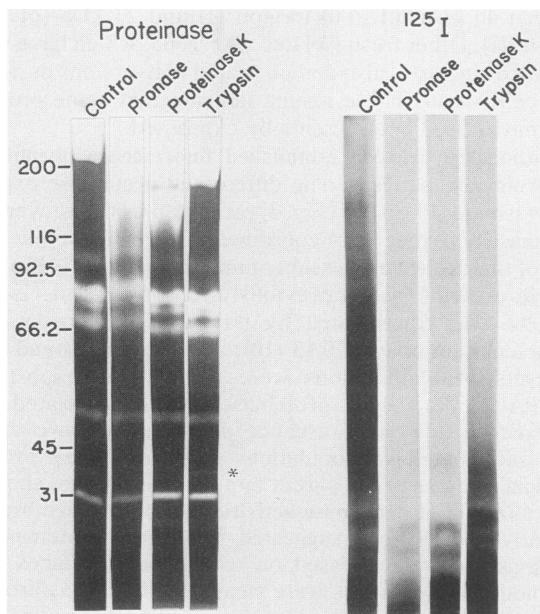

FIG. 4. Localization of a 43-kDa proteinase activity (asterisk) to the parasite surface. Activity was removed by proteinase treatment of live *T. vaginalis* organisms prior to processing for substrate-SDS-PAGE. The corresponding autoradiograph shows the removal of numerous iodinated proteins by treatment with the various proteinases, as has been previously described (5).

and cells treated with inhibitors, retained the full complement of proteinase activities and iodinated surface proteins.

The immunogenic nature of *T. vaginalis* proteinases. Finally, we tested the immunogenic nature of *T. vaginalis* proteinases by the radioimmunoprecipitation assay used earlier in antigen analysis studies (1). Figure 5 depicts the reactions obtained with mouse and rabbit antisera (1, 2) against detergent extracts of two *T. vaginalis* isolates. Each antisera possessed antibodies that reacted equally well against proteinases of both isolates NYH286 and IR78. The identical reactions of both antisera suggest that each isolate expressed the same repertoire of proteinases. Immunoprecipitations of the 81-, 35-, and 31-kDa proteinases were retained by using antisera diluted up to 1:500, which indicates the highly immunogenic property of these particular proteinases.

DISCUSSION

This analysis of *T. vaginalis* shows the complexity and heterogeneity of the cysteine proteinases in this sexually transmitted protozoan. Such careful analysis is necessary for the discussion of the roles of these important biofunctional molecules, especially with regard to differentiation of the role of proteinases in properties such as cytadherence (13), cytopathogenicity (16, 19, 21, 22, 30), or other considerations, such as nutrition (18). Our combined use of IEF and substrate-SDS-PAGE allowed us to discriminate among activities that comigrate in a one-dimensional electrophoresis system (Fig. 1) (14) and to compare and contrast the patterns of trichomonad proteinases among isolates. Our analysis revealed a complex pattern of *T. vaginalis* cysteine proteinases when compared with those seen by others with one-

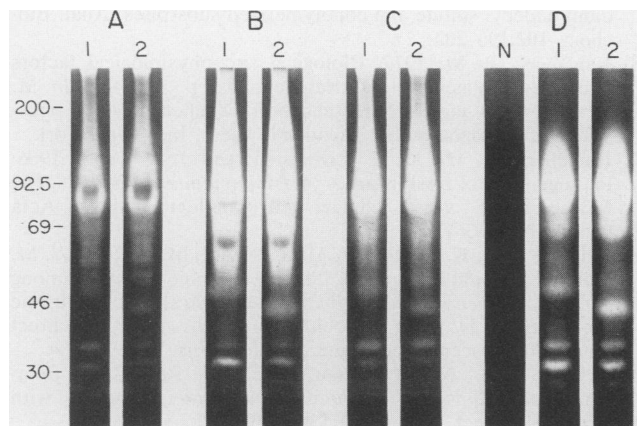

FIG. 5. Immunoprecipitation-one-dimensional substrate-SDS-PAGE analysis of *T. vaginalis* proteinases with mouse and rabbit antiserum diluted 1:100. Detergent extracts of isolates NYH286 (lanes 1) and IR78 (lanes 2) were incubated with mouse (A) (9) or rabbit antiserum against isolate NYH286 (B) (1, 2) and mouse antiserum against isolate IR78 (C) (9). (D) Lane N shows a representative immunoprecipitation of *T. vaginalis* detergent extract of either isolate with negative control serum. Detergent extracts of NYH286 and IR78 are also shown in panel D, lanes 1 and 2, respectively. The identical proteinase patterns produced when the extract of an isolate was immunoprecipitated with antisera against either of the isolates NYH286 or IR78 indicate that both isolates produce the same repertoire of proteinases, albeit at different levels (Fig. 1).

dimensional gels (14, 27). While up to 23 distinct activities could be detected by two-dimensional substrate-SDS-PAGE, we recognize the possibility that some of these spots could be fragments of larger proteinases and that the number of enzymes could be lower than our estimate. However, this total number of proteinases may be reasonable, since earlier reports indicate that little autolysis of proteinases appears to occur in cell homogenates under the conditions used in this study (27).

This technique allowed for the identification of the proteinases of all isolates, and fresh isolates were similar to long-term-grown isolates in displaying common as well as heterogeneous proteinase patterns. A clear understanding of the activities common to all isolates and to those found in only some isolates may help in the dissection of the trichomonad factors responsible for the spectrum of symptomatologies. Also, differences in pathogenic factors displayed by trichomonal isolates as previously described by numerous investigators may be resolved (3–8, 13, 18–22). For example, examination of isolates not expressing all the proteinases in vivo, such as T005 (as shown in Fig. 3), but which still produce symptoms among patients with trichomoniasis, may elucidate which proteinases are important or necessary in cytadherence or cytopathogenicity (13) or in other aspects of the biology of the organism or both. Clearly, the study of these molecules may be key to assigning virulence roles and establishing correlations between proteinase activities and the pathobiochemistry of trichomoniasis.

Of particular interest and possible significance were the dramatic qualitative and quantitative changes seen upon in vitro culture (Fig. 2 and 3), which indicates that at least some of the proteinases might be differentially expressed. This phenomenon was not coordinated with the changes seen in previously described immunogens and adhesins, which are known to undergo phenotypic variation (4, 9, 10, 12, 14). In fact, isolate T005 (Fig. 3) is composed of a homogeneous population of trichomonads which do not undergo phase variation for immunogens and adhesins (type I) (10). This study, therefore, shows another family of molecules involved in phase variation. This report also illustrates that *T. vaginalis* is possibly capable of responding to changing environmental conditions of the host, further contributing to the complexity of the host-parasite interrelationship. It is not unreasonable to suppose that expression of these proteinases may be under environmental regulation mechanisms, since differentially expressed proteinases of fresh isolates, such as T005, were found only after in vitro cultivation.

Importantly, our data indicate that many proteinases are immunogenic. Cross-reactivity among the proteinases of the two isolates examined was readily observed, and this suggests that isolates are similar in their in vitro expression of the total proteinase repertoire. A more careful evaluation of the immunogenicity of these molecules in patients with trichomoniasis will be important for reinforcing the idea that proteinases may be potential vaccine candidates. Furthermore, these types of data, with sera from patients, may delineate which of the many proteinases may be the most important in the pathobiochemistry of trichomoniasis.

Previous data have suggested that proteinases of *T. vaginalis* reside on the surface of the parasite (13). We were unable to detect iodinated proteinases by autoradiography (Fig. 4 and 5), and this may be a quantitative issue, as very few cell equivalents were required to detect proteinase activity by substrate-SDS-PAGE. Our results, however, indicate that a 43-kDa proteinase may be surface exposed, as it is readily removed by treatment of live organisms with specific enzymes. The other trichomonad proteinases may either be integral membrane proteins, reside intracellularly, or be shed into the culture medium as has been previously described (5, 26, 27).

Finally, the establishment of these proteinase repertoire fingerprints among trichomonal isolates appears necessary in order to allow us to determine the proteinases involved in virulence properties, such as cytadherence (3, 6, 8, 13) and cytotoxicity (10, 13, 16, 21, 30). The direct involvement of proteinases in establishment of infection is supported by our recent discovery that proteinase action is prerequisite for cytadherence (13). Therefore, any data defining more precisely important proteinases of *T. vaginalis* are prerequisite to ultimately devising reagents for pharmacologic intervention and control of trichomoniasis.

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