The vagina has reducing environment sufficient for activation of *Trichomonas vaginalis* cysteine proteinases

J F Alderete, Daniele Provenzano

**Background:** *Trichomonas vaginalis*, a worldwide distributed sexually transmitted protozoan, is remarkable for synthesis of numerous, distinct cysteine proteinases, the significance of which is evidenced by the presence in *in vivo* of soluble proteinases in secretions and antiproteinase antibody in serum of patients with trichomonosis. These proteinases purportedly play a role in host parasitism and immune evasion.

**Objective:** It is known that for cysteine proteinases to be functional, they must be activated by disulphide reducing reagents. Whether or not the host vaginal environment has the reducing environment essential for activation of the trichomonad cysteine proteinases is unknown. Our goal, therefore, was to determine whether or not vaginal secretions had sufficient reducing power to activate the trichomonad proteinases.

**Methods:** 48 vaginal washes (VWs) from patients were assayed for reducing equivalents and a score in dithiothreitol (DTT) reducing equivalents was assigned to each VW. Activation of trichomonad cysteine proteinases was then tested under the range of reducing equivalents detected from VWs. The possible protective effect of hydrogen peroxide, an oxidising agent produced by some *Lactobacillus* species, on proteinase activity was also determined.

**Results:** Nine of 48 VWs (18.7%) possessed < 10 μM DTT reducing equivalents, four VWs (8.3%) had from 20 μM DTT to 40 μM DTT reducing equivalents, and most (50%) were between 10 μM to 15 μM. Overall, the range in VWs was from ∼10 μM to 40 μM reducing equivalents. Importantly, data suggest differential proteinase activation over this in vivo range of reducing level. Only two *T vaginalis* cysteine proteinase activities were stimulated at 2-5 μM DTT in contrast with all proteinase activities present at 40 μM DTT, albeit quantitatively diminished compared with the activity at 1 mM DTT, the concentration routinely used in vitro. Finally, hydrogen peroxide reversibly neutralised all trichomonad proteinases.

**Conclusions:** These results show that the vagina of women has a reducing environment adequate for activation of trichomonad proteinases. The data underscore that the host environment plays a role in the host-parasite interrelation. Finally, hypotheses can now be formulated to help explain resistance and susceptibility to infection commonly reported among women and between men and women with trichomonosis.

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**Keywords:** *Trichomonas vaginalis*; vagina; cysteine proteinase

**Introduction**

The host-*Trichomonas vaginalis* interrelation is exceedingly complex. Infection with this sexually transmitted parasite often leads to unpredictable outcomes in symptomatology that range from a carrier state to severe foul smelling discharge, irritation, and discomfort associated with inflammation and tissue cytopathology. It is now appreciated that patients with trichomonosis (vaginitis) are at higher risk for HIV seroconversion for adverse outcomes during pregnancy and, possibly, for cervical cancer. The identity of factors, either of host or parasite origin, responsible for the variations in host responses to *T vaginalis* infection remains unknown.

Recently, trichomonad cysteine proteinases have been recognised as virulence factors. The parasites generate numerous cysteine proteinases, many of which are secreted during normal cultivation in complex medium. The in vivo relevance of the proteinases is affirmed by the detection of proteinases and antiproteinase antibody in vaginal secretions and in sera of women with trichomonosis, respectively, but not women who are uninfected or who have other sexually transmitted diseases. This enzymes probably contribute to numerous virulence properties of *T vaginalis* organisms, such as acquisition of nutrients, subversion of host immune responses and, through unknown mechanisms, parasite cytoadherence.

Substrate degradation by cysteine proteinases requires breakage of disulphide bonds, as occurs under reducing conditions. In this study, we have attempted to address the question of whether the vaginal environment of patients has sufficient reducing levels to activate trichomonad cysteine proteinases. This is important because all studies performed to date have activated the cysteine proteinases by addition of reducing reagents such as DTT. Equally important, it has been hypothesised that the host environment may play a role in regulating, both qual-
tatively and quantitatively, the numerous tri-
chomonal proteinases thereby contributing to a
chronic, non-self limiting infection and mini-
mal manifestation of tissue cytopathology.
In this report we show that vaginal secre-
tions obtained from patients with trichomon-
sis do indeed possess an environment ade-
quate for activation of the cysteine pro-
teinases. It is noteworthy that the reducing
levels produced differential activation or quan-
titatively distinct amounts of proteinases.
These data reinforce the idea that variations in
reducing levels in vaginal secretions play a role
in infection by T. vaginalis and possibly in dis-
ease outcome. The observations made in this
report point to future questions that must be
answered to fully understand this complex
host-parasite interaction and to meaningfully
develop future infection and/or disease inter-
vention strategies.

Materials and methods
CULTURES
T. vaginalis isolates IR78, NYH 286, T048,
and T068-II were grown at 37°C in tryp
ticase yeast extract maltose (TYM) supplemen-
ted with 10% heat inactivated horse serum
(HHIS), as described before.2324 To optimally
visualise for trichomonal cysteine proteinase
activities in substrate gels, as described below,
iso
tate T068-II was also grown under low iron
conditions in the presence of 30 μM 2,2-
diprydil (2,2-DP).2526 Organisms (2 x 107)
were collected at mid to later logarithmic
ph
ase of growth23 by centrifugation at 500 x g for 5
minutes after washing three times in ice cold
phosphate buffered saline (PBS). Cells were
either used immediately or frozen at −70°C
until needed.16 Identical proteinase patterns
were obtained with lysates derived from either
fresh or frozen parasites, as before.16

DETERMINATION OF REDUCING LEVEL IN
HUMAN VAGINAL WASHES
To maximise detection of reducing ability,
vaginal washes (VWs) were obtained from the
source previously reported by us and
processed as described before.1112 Briefly, a
volume of 5 ml of a VW was obtained in sterile
PBS by rinsing the vaginal wall numerous
times. To this VW a large swab, which was
utilised to scrape the vaginal wall, was then
immersed in the VW from the same patient.
VW was then clarified as previously described
by centrifugation at 500 x g to remove large
acellular debris followed by centrifugation at
17 500 x g to remove additional insoluble
debris. The VWs were kept stored at −70°C
until utilised. Upon thawing and just before
use, VWs were filter sterilised through 0.22 μm
acrodisc filters (Gelman Sciences, Ann Arbor,
MI, USA).

The reducing potential of patient VWs was
determined spectrophotometrically at 412 nm
by comparing readings with a standard curve
derived from a buffer comprised of known
concentrations of DTT in Ellman's reagent
(5.5'-dithio-bis(2-nitrobenzoic acid; DTNB))28
(100 mM TRIS·HCl, pH 8.0, 100 mM
EDTA, 10 mM DTNB, 50 mM NaPO4, pH
7.0, 10% sodium dodecylsulphate (SDS)).
Reducing values of VWs diluted in DTNB
were obtained from the average of triplicate
determinations, and values were expressed as
μM DTT equivalents. Reducing equivalents
≤ 10 μM DTT were below the detection limit
of DTNB and, therefore, represented as ≤ 10.

DETECTION OF TRICHOMONAD CYSTEINE
PROTEINASE ACTIVITY
Protocols for preparation of parasite lysates
and for substrate gel electrophoresis have been
extensively described by us and others.161922
Pelleted parasites were solubilised in 200 μl
electrophoresis dissolving buffer29 (125 mM
TRIS·HCl, pH 6.8, 4% SDS, 20% glycerol,
and 10% bromophenol blue) in the absence of
β mercaptoethanol.16 Samples (1 μl) were
electrophoresed at 100 V in a minigel apparatus
(BioRad Laboratories, Hercules, CA, USA)
on 7% acrylamide copolymerised with 1.5 mg
gelatin, as before.191822

After SDS-PAGE, trichomonad proteinases
were activated in proteinase activation buffer
(PAB) (100 mM sodium acetate and 2-5%
Triton-X 100, pH 5-5) prewarmed at 37°C
containing various concentrations of DTT. In
this case, preparative slab gels were cut so that
identical amounts of trichomonad protein
were incubated separately in PAB containing
specific amounts of DTT. Negative controls
were incubated in PAB without any addition.
PAB with hydrogen peroxide (10 μM) was
also tested to determine the effect on
proteinase activity.1031 Activation of proteinases
was performed by placing gels on a shaker for 2
hours at 37°C with one change of buffer, and
proteinase activity was seen after staining in
Coomassie brilliant blue overnight followed by
destaining for 2 hours in a solution of 7%
acetic acid and 5% methanol.

DENSITOMETRIC ANALYSIS OF SUBSTRATE GELS
FOR DETERMINATION OF RELATIVE PROTEINASE
ACTIVITY
All gels were digitalised with a flatbed scanner,
and pixel intensity of each clear area repre-
senting a proteinase degradation band was
quantified and graphed with the NIH Image
software version 5.17. Percentage degradation
for each band was calculated as follows:
100 ÷ ((no pixels scanned from gels activated
at 1 mM DTT) x (no pixels scanned from
gels activated at < 1 mM DTT)).

Results
APPROXIMATION OF REDUCING LEVELS IN VWs
We did not know whether it would be possible
to measure any reducing ability in VWs obtained
and processed as described in Materials and
methods. We further appreciated that any value
of reducing level detected may not reflect that
found at a specific site in the vagina parasitised
by T. vaginalis. None the less, the question
regarding whether the vagina possessed an envi-
ronment sufficient to activate trichomonad pro-
teinases was important to attempt determination
of in vivo reducing levels.
The vagina has reducing environment sufficient for activation of Trichomonas vaginalis cysteine proteinases

between 15 μM and 20 μM and four VWs (8-3%) had 20 μM to 40 μM DTT equivalents. This range (< 10 μM to 40 μM) was far lower than the 1 mM amount of DTT normally employed to examine the trichomonad cysteine proteinases in vitro.\(^\text{18,19,22}\) None the less, these data show that VWs from patients do indeed possess reducing ability.

**Are VW reducing levels sufficient to activate trichomonad cysteine proteinases?**

We next wanted to determine whether cysteine proteinases were detected when activation was performed using concentrations of DTT within the range found in the VWs. In addition, because the manner in which the VWs were obtained and processed could yield lower values than those of undiluted vaginal secretions, we evaluated reducing levels from 10 μM up to 1 mM for comparative purposes.

Figure 2 illustrates proteinase profiles of lysates corresponding to 1 × 10^8 T. vaginalis isolate TO68-II organisms where gels were treated with decreasing amounts of DTT (lanes 1–9). Lysate of this parasite density was used because, by substrate gel electrophoresis, individual proteinase band activities were best resolved for accurate visualisation and comparisons.\(^\text{15}\) This amount of lysate also gives reproducible total proteinase patterns among different T. vaginalis isolates that were examined identically as presented here and for which similar results were obtained. Equally important, the number of parasites is relevant to that found in vivo.\(^\text{32}\) Finally, that the degrading activities being analysed are cysteine proteinases has been confirmed using specific inhibitors.\(^\text{16-18,22}\)

Amounts of DTT above 100 μM (lane 2) gave patterns similar to 1 mM (lane 1). By contrast, beginning at 100 μM (lane 2), there was a decrease in total proteinase activity, especially in the lower M₅s (lane 1 versus lanes 3). Concentrations of DTT as low as 2.5 μM still gave detectable proteinase activity above background, as seen in lane 9 (greater than the negative control without addition of any reducing agent).

We had noticed that any basal level of proteinase activity observed in the negative controls could be readily neutralised by addition of 10 μM hydrogen peroxide in the activation buffer (lane 10). We, therefore, thought it would be important to determine whether hydrogen peroxide neutralised the activity of DTT pretreated cysteine proteinases. Hydrogen peroxide is known to reverse the effect of DTT,\(^\text{30,31}\) and, as recently hypothesised, hydrogen peroxide producing *Lactobacillus* might therefore afford protection from *T. vaginalis* infection.\(^\text{33-35}\)

Identical experiments as above were performed in substrate gels that were activated in 40 μM DTT, the minimum concentration found in VWs that gave good activation of the trichomonad cysteine proteinases (fig 2), except that activation buffer incorporated varying amounts of H₂O₂. At 200 μM H₂O₂, no activation of proteinases was detectable,
Protease bands F and G were greatly decreased by 100 μM DTT (table) and were reduced in activity by 90% at 50 μM DTT. Prominent substrate degradation by protease bands D and E were also strongly affected by a decline in DTT concentration; a ~50% reduction in proteolysis occurred by 100 μM DTT, and ~80% activity was diminished by 50 μM DTT. Band C was decreased 50% in activity by 50 μM DTT, while band regions A and B approached a 50% reduction in activity by 20 μM DTT. Therefore, when evaluating the level of DTT at which a 50% reduction in activity is detected, differences are apparent among the protease band regions.

**Discussion**

The interrelation between the host and *Trichomonas vaginalis* is exceedingly complex, and some of the many factors that contribute to such complexity include the multiple cysteine proteases that potentially contribute to host cytopathology. We have previously hypothesised that the in vivo synthesis of the proteases must somehow be under the control of environmental cues so as to modulate the number and amount of proteases needed at any particular moment of infection. In this scenario only that quantity of proteases necessary for parasite survival and which provoke minimal host damage would be both present and active. Recent evidence shows the induction by iron of proteases with specific functions, such as degradation of complement C3 to avoid lysis through the alternative pathway. In addition to regulatory paths that control the synthesis of the trichomonad proteases, we now present the possibility that host reducing levels may also regulate the activity of the proteases. This would provide another mechanism of control over the many proteases produced by *T. vaginalis*.

Of importance was the finding that VWs indeed possess reducing ability. To our knowledge, this may be the first time that the question regarding whether, during infection, in vivo conditions are suitable to activate the trichomonad proteases has been addressed. In this report, we show that VWs of patients with trichomonosis have reducing ability for activation of the cysteine proteases, albeit the levels detected by us were at 20-fold lower concen-

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**Figure 3** Densitometric scanning of the protease bands obtained after activation at the designated dithiothreitol (DTT) concentrations. The surface under each peak was analysed as described in Materials and methods for quantitative presentation in the table. The dotted line was used to align each peak.

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**The effect of reducing level on relative activity of *T. vaginalis* cysteine proteases**

<table>
<thead>
<tr>
<th>In vivo range</th>
<th>DTT (μM)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<tr>
<td>-</td>
<td>1000-0</td>
<td>1-00</td>
<td>1-00</td>
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<td>1-00</td>
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<tr>
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<td>0.17</td>
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<tr>
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<td>0.08</td>
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<tr>
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<tr>
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<td>0.00</td>
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</table>

*Relative activity was calculated as described in Materials and methods.

†Band designations were from the individual areas of protease activity as presented in figures 2 and 3.

‡Minus and plus signs refer to the levels of reducing ability in DTT equivalents as determined from the evaluations of vaginal washes as shown in figure 1 and described in Materials and methods.
trations than those used in vitro. It is conceivable that the lower amounts of reducing levels represent a dilution resulting from obtaining and/or processing the VV. Regardless, it is clear that lower amounts of reducing ability produce qualitatively and quantitatively decreased activity of parasite proteinases when compared with 1 mM DTT. Finally, it might have been predicted that all the proteinase activities would decrease equally as particular reducing level. This was not the case reafirms the distinctness among the numerous cysteine proteinases, as suggested earlier.

It is essential that we begin to understand the host factors and parameters within the site of infection that contribute to individual susceptibility and resistance to infection. The hypothesis that women with higher vaginal reducing ability may be more susceptible to parasitism and cytopathology seems reasonable, especially given the relation between qualitative and quantitative proteinase activities and differential reducing levels. This idea is plausible since proteinase activity is necessary for survival of *T. vaginalis* through nutrient acquisition, cytoadherence, and immune evasion.

If the hypothesis presented above is correct, then relative resistance by men to infection by *T. vaginalis* may be related to the presence of oxidising reagents that prevent activation of the trichomonad cysteine proteinases essential for survival. In fact, reactive oxygen species produced by human spermatozoa have been described, and this oxidative environment would not allow for host parasitism and establishment of a non-self limiting infection.

We showed the action of hydrogen peroxide in neutralising the reducing ability of DTT, which is a prerequisite for activation of the cysteine proteinases. Our results reinforce the notion that hydrogen peroxide producing *Lactobacillus* may confer some measure of defence against *T. vaginalis*. This is only possible, however, if sufficient amounts of hydrogen peroxide are produced and bathe the site of infection. The idea to test for neutralisation of trichomonad proteinases resulted from the known reversible effect by hydrogen peroxide on systems requiring reducing environments.

Furthermore, the idea may have merit because of the reports that the host may be protected from other sexually transmitted diseases by hydrogen peroxide producing *Lactobacillus*. Although reversible, demonstration of hydrogen peroxide neutralisation of the activation power of DTT on trichomonad proteinases suggests that an approach for intervention of *T. vaginalis* infection and cytopathology may result from generation of irreversible proteinase inhibitors or, alternatively, upon inhibition of the ability of the parasite to alter the normal protective flora of patients.

Based on the hypothesis proposed above, numerous questions require attention. (1) Does the level of vaginal reducing ability vary during menses, and, if so, are certain times during the cycle more favourable to proteinase activation, which then promotes host parasitism? (2) Does the relative reducing level in the vagina vary among different ethnic groups, thus explaining higher rates of trichomoniasis among African American women? (3) Does behaviour that predisposes women to *T. vaginalis* infection, such as smoking, alter the vaginal reducing environment? (4) Is there a relation between the level of reducing ability, parasite growth and multiplication in vivo, and symptomatology? (5) This is the absence of symptomatology and the self limiting nature of infection among males correlate with absence of reducing ability at those sites? Our results provide a foundation from which testable hypotheses can be formulated and that may, ultimately, help us understand those important host factors pertinent to infection by this sexually transmitted agent.

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J F Alderete and D Provenzano

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