A putative localization of the *ureaplasma urealyticum* IgA1 protease

Felix Melchizedech Mesak, Nana Suhana

Abstrak

Salah satu faktor patogenisitas Ureaplasma urealyticum ialah protease IgA1. Visualisasi aktivitas protease IgA1 dilakukan dengan teknik SDS-PAGE yang memperlihatkan dua fragmen hasil pemecahan rantai berat IgA1 manusia yaitu Fc dan Fab masing-masing berukuran 35 dan 32 kDa. Aktivitas protease hanya terdapat pada sel ureaplasma. Kami tidak dapat menentukan adanya aktivitas dalam supernatan hasil sentrifugasi kultur cair ureaplasma. Produk aktivitas IgA1 baru dapat dideteksi setelah tiga jam inkubasi berdasarkan cara uji yang kami lakukan. Sedangkan ekstraksi dan partisi fase sel ureaplasma dengan Triton X-100 dan Triton X-114 memperlihatkan kemungkinan pendewasaan protease IgA1 di dalam sitosol sebagai protein terlarut dan atau pada akhirnya diekspresi sebagai enzim terikat membran. Dengan memanfaatkan piranti lunak Corel Photo Paint 7, densitas derajat abu-abu dan jumlah piksel pita-pita berbeda hasil SDS-PAGE dapat dihitung dan menghasilkan nilai tertentu yang disebut skor intensitas pita. Skor ini digunakan sebagai pembanding terhadap parameter uji aktivitas protease IgA1 di berbagai fraksi sel ureaplasma dan visualisasinya dalam bentuk grafik linier atau diagram sebar.

Abstract

One of the Ureaplasma urealyticum pathogenicity factors is IgA1 protease. IgA1 protease activity was visualized by SDS-PAGE showing two fragments of digested heavy chain of IgA1 with 35 and 32 kDa in sizes denoted as Fc and Fab, respectively. The protease activity was only found in ureaplasma cells. We could not determine any activity in the supernatant after removing the cells by centrifugation. Under our test condition, the products of the IgA1 activity were detected after three hours. Based on Triton X-100 extraction and Triton X-114 phase partitioning, IgA1 protease is synthesized to mature in the cytosol as a soluble protein and/or to be finally exposed as a membrane bound-enzyme. Employing the advantage of Corel Photo Paint 7 software, the density of grayscales and pixels of distinct bands of the SDS-PAGE were evaluated and these values used to establish a band intensity score. The Score is used as a comparison tool toward testing parameters of the enzyme activity in cellular fractions. They are visualized in linear graphs or scatter diagrams.

Keywords : *IgA1, IgA1 protease, ureaplasma, pixels, Triton X-100, Triton X-114.*

Ureaplasma urealyticum sized about 330 nm in diameter, is one of the smallest self-replicating organisms. This bacterium lacks a cell wall. It is microaerophilic with a growth optimum at 37°C and a pH of 6.0.[1] This microbe mostly colonizes human urogenital tracts of adults. Its presence in these tracts may lead to diseases such as: urethritis and stone formation, prostatitis, infertility, funisitis, endometritis, chorioamnionitis, membrane premature rupture, abortion, chronic respiratory diseases including pneumonia, and meningitis.[2,3] Ureaplasma also causes occasionally respiratory disease in newborn and infants.[3]

Department of Biology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

One of the factors increasing pathogenicity of ureaplasma is an IgA1 protease. Apart from *Neisseria gonorrhoeae*, *N. meningitidis*, *Streptococcus pneumoniae*, *S. oralis*, *S. sanguis*, *S. mitis*, *Haemophilus influenzae*, *H. aegyptius*, *Prevotella* sp, and *Capnocytophaga sp.*[4], *U. urealyticum* produces this enzyme, as well.[5-7] Due to fastidious growth of this very minute cells and the easy loss of its viability the characterization on the IgA1 protease of ureaplasma is less advanced as in the above mentioned bacteria. The detection of ureaplasma colonies is difficult because of their poor growth on agar media. Lately, development of PCR helped to improve clinical assessment.[8,9] Finally, the lack of efficient *in vivo* and *in vitro* gene transfer systems makes genetic studies almost impossible. The situation will improve if the nucleotide sequence of the genome gets published. To be able to use the sequencing data later-on efficiently, we did most of the research with the strain *U. urealyticum*

DKF3 and used for comparison studies also *U. urealyticum* CX8.

We wanted to characterize the ureaplasmal IgA1 protease activity as a basis for enrichment understanding its pathogenicity and later-on for isolation of the enzyme. Initial steps require cultivation, maintenance, and verification of ureaplasma cells. Then we worked out a reliable assay for the IgA1 protease activity based on results from others.[7] Protease activity of human IgA1 can be visualized by cleavage of the heavy chain of human IgA1 as a substrate by SDS PAGE and Coomassie blue or silver staining. Cells were sonicated and fractionated by sonication or Triton X-100 or Triton X-114 extraction. For further characterization, we studied the kinetics of IgA1 protease activity and the localization of the protease within the ureaplasma cells.

Finally to analyze the presence of Fc / Fab fragments after digestion of IgA1 protease activity human IgA1, we also developed semiquantitative measurement based on density of grayscales and pixels employing *Corel Photo Paint 7* software. The resulted scores were tabulated on linear graph or scatter diagram by using *Microsoft Excel 97*.

METHODS

Bacterial strains

U. urealyticum DKF3, *U. urealyticum* CX8 (was a gift from J. Robertson) and *Mycoplasma pneumoniae* M129 (was from the stock collection of R. Herrmann). Unless otherwise stated always *U. urealyticum* DKF3 was used.

Media growth condition and maintenance

Two hundred microliters (µl) of deep-frozen (-80°C) ureaplasma cells in PBS buffer were thawed and diluted serially 1:10 up to 10^{-9} with Bromothymol Blue Broth, pH 6.0 (2.1% PPLO broth without crystal violet (Difco), 0.1% yeast extract, 0.004% bromothymol blue, supplemented with 10% steril normal horse serum pH 6.0, 0.1% GHL solution (Calbiochem) and adjusted pH to 6.0).[10] The tube with the lowest dilution, which just changed its color to green, as the pH started to reach 6.2 as subcultured for the next 24 to 32 hours at 37°C. The tube, which stopped the color change to green at the dilution of 10^{-7}, was defined as 10^7 color changing units. This green color developed in a transparent optical view without any turbidity.

Growth on solid media was achieved by spotting 20 µl of 10^{-4}, 10^{-5}, and 10^{-6} of broth culture into small Petri dishes containing Genital Agar Media (2% PPLO without crystal violet (Difco), 0.75% agar no.1 (Oxoid), 0.1% yeast extract, 1.19% HEPES buffer, supplement with 10 % sterile normal horse serum pH 6.0, 0.025% urea, 0.1% GHL solution (Calbiochem) and adjust pH to 6.0).[11] Microaerophilic environment was simply produced by a candle flame, fading after several minutes inside a tightly closed jar containing the dishes. Then the jar was incubated for 3 days at 37°C. Observation was made using a stereomicroscope with 100-x magnification.

Probe design, labeling, and hybridization to the ureaplasma genome.

A twenty-two –mer oligonucleotide as a specific probe for the ureaplasma species was designed based on DNA of urease sequences from Genbank database with the accession number of X51315. The sequence of the probe GAG GTG TAA ACG GCT TAG TTA A. was selected by employing OLIGO 4.0 software (National Biosciences Inc.). The probe named *UI-ure* was synthesized by DNA Synthesizer model 394A from Applied Biosystems at R. Frank's group at the ZMBH, Heidelberg, Germany. The oligo was purified by ethanol precipitation before it was labelled at the 5' end by polynucleotide kinase ([γ-32-P]ATP).[12]

Ureaplasma DNA isolation followed the method of Sambrook *et al.*[12] The genomic DNA was digested with restriction endonuclease *Eco*RI and the DNA fragmens were separated by agarose gel electrophoresis. Southern transfer of the chromosome to nylon membrane was later on used to hybridize the *UI-ure*. The experiments and its manipulation were done according to Sambrook *et al.*[12] Autoradiography was developed by PhosphoImager (Molecular Dynamics, Inc.).

IgA1 protease activity assay, time course of the assay, Triton X-100 extraction and Triton X-114 phase partitioning

The concentration of total cell protein of ureaplasma was measured by the method of bicinchoninic acid binding and according to Bradford.[13,14] The average value of both methods was used to adjust to 2 mg/ml of total cell proteins. Since protein mixtures are difficult to measure, we applied the two mentioned methods. We found consistently lower result with the bichinchoninic method. Proteins or their cleavage products were visualized by SDS-PAGE and subsequent Coomassie blue or silver staining.[15,16]

Two hundreds ml cultures of 10^7 color changing units were harvested by centrifugation and washed three times with PBS buffer. The final concentration of the proteins was 20 µg/l in aliquots of 100 µl. As much as 300 µg of total protein were mixed with 3-5 µg of human IgA1 (Calbiochem). As a positive control, 1 µg of pure igase from *N. gonorrhoeae* was used, which was kindly provided by S.C. Beck of the Max Planck Institüt für Biologie, Tubingen, Germany. As a negative control, total cell protein of *M. pneumoniae* was used. The sample mixtures were incubated at 37°C overnight. Samples were boiled for two minutes after they had been denatured by protein lysis buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue) and before subjected to SDS-PAGE. The kinetics of the protease activity in total extracts was tested against the time of incubation at hour 1, 3, 6.5, 10, 14.5, and 16.

To localize the protease in cellular fractions, cells were sonicated using BRANSON B15 cell disrupter bursts 8 times 15 seconds with 10 seconds interval. By subsequent centrifugation the suspension was separated into sediment and the supernatant.

Triton X-100 extraction of total protein extract yielded a soluble and an insoluble phase as described.[17] In addition, the Triton X-114 phase partitioning method was also applied with slight modifications of the procedure developed by Bordier.[18,19] For this purpose 400 ml of 10^7 color changing units of ureaplasma culture was harvested taken up in 1 ml, washed three times with PBS, and aliquoted into 200 µl of buffer B (20mM Tris HCl pH 7.5, 150mM NaCl, (1mM PMSF)) and 1% v/v of Triton X-114. It was mixed by vortexing and then incubated at 4°C overnight with mild shaking and several times re-vortexing. Supernatant was transferred to a fresh tube and incubated at 37°C for 30 minutes. Subsequent steps followed as described by Proft and Herrmann.[19] The Triton X-114 phase partitioning and the testing for IgA1 protease activity was also done with frozen (-80°C) up to three months ureaplasmal cells.

Bands analysis using density of grayscales and pixels

Protein bands in SDS-PAGE were scanned and analyzed by using density of grayscales and pixels with *Corel Photo Paint 7* software. The scanned pictures were changed to grayscale (8 bit), starting from value 0 or black until 255 or white. The software can locate bands according their grayscale density in conjunction with the number of located pixels. Level equalization was set automatically. The ruler was also set to in pixel units with tick division 10 per tick. Magic wand mask tool was set into normal with color tolerance mode of 10 (< 80% - 85% of 255) or 3 (> 80 – 85% of 255) and used to locate bands according to their contours. The automatic histogram showed mean, median, and standard deviation of the number of pixels. Pixels in localized bands can be seen at 600x or 1600x magnifications. Measurement was done three times to represent the core, medium, and outer area of bands. The average was calculated as value of the band as describing the score, which was obtained by multiplication of an average of (255-mean), pixels average and normal value used. These semi-quantitative scores can be applied only to a distinct band of the electrophoretic result. Linear graphs or scatter diagrams of the score against the test parameters were done by *Microsoft Excel 97* software.

RESULTS

After inoculation of a fresh medium at the ratio 1:10 with a growing ureaplama culture which just changes color from yellow to green cells, could be reproducibly propagated to 10^7 color changing unit within 48 hours at 37°C. The cultivation on solid agar revealed the formation of colonies after 72 hours in microaerophilic environment at 37°C. Colonies were very minute but distinctively differed from black teint of debris. The colony has dark brown round spots on the yellow background of the agar media. This observation was consistent with Robertson's description.[10,11]

As an additional diagnostic tool, the genomic DNA of cultured ureaplasma cells were probed with the ureaplasma specific oligo *UI-ure* in Southern blot experiments (Fig. 1). As can be seen from lane 4 and 5 from Fig.1B only the ureaplasma DNA cross-hybridized with the specific probe *UI-ure* it was present although at lower concentration than the negative control (Fig. 1A, lane 1-3).

The IgA1 protease partially digested the heavy chain of human IgA1 and produced two fragments with sizes of 35 kDa and 32 kDa (Fig. 2) denoted as Fc and Fab part, respectively. Figure 2 also shows that *M. pneumoniae* does not produce Fc and Fab fragments. The Fc/Fab fragments produced by the ureaplasmal IgA1 protease could be identified only after 3 hours of incubation by SDS-PAGE with Coomassie blue (Fig. 3), after one hour even with the most sensitive method of silver staining, we could not detect any Fc/Fab fragments (data not shown).

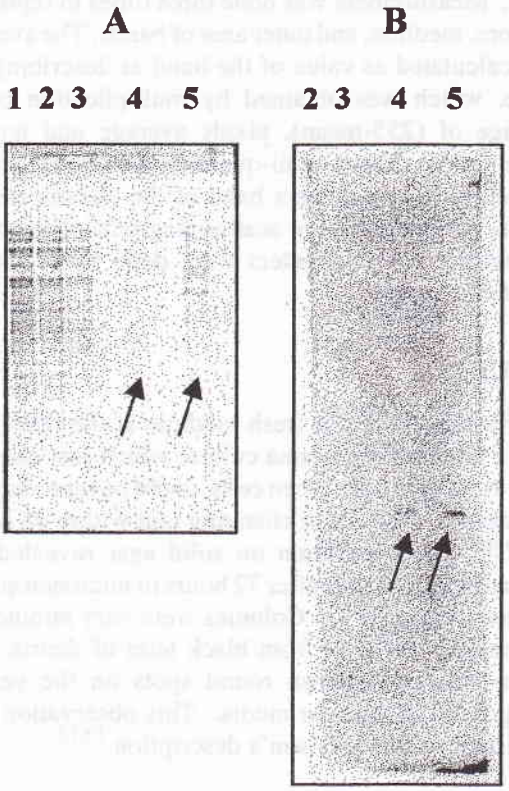

Figure 1. Agarose gel and southern blotting of EcoRI digested genomic DNA. Lanes 1-3 and 4-5 reveal M. pneumonia and U. ureaplasma DNAs (ladder appearance) respectively, visualized by ethidium bromide-stained agarose gel electrophoresis (A). After hybridization with ^{32}P labeled UI-ure oligo, only a single ureaplasmas DNA fragment was recognized (B).

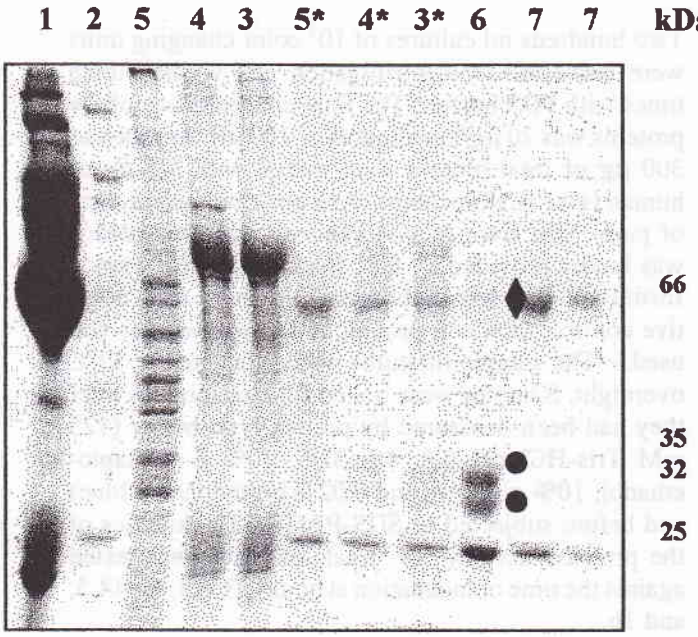

Figure 2. Polyacrylamide gel electrophoresis (SDS-PAGE) for monitoring of IgA1 protease activity. IgA1 protease activities both of ureaplasma strains DKF3 and CX8 produce 35 and 32 kDa of Fc and Fab fragments (•) from the digested heavy chain (◆). Note: (1) horse serum, (2) protein marker, (3) DKF3, (3) + IgA1, (4) CX8, (4*) + IgA1, (5) M. pneumoniae, (5*) + IgA1, (6) igase + IgA1, (7) PBS buffer +IgA1.*

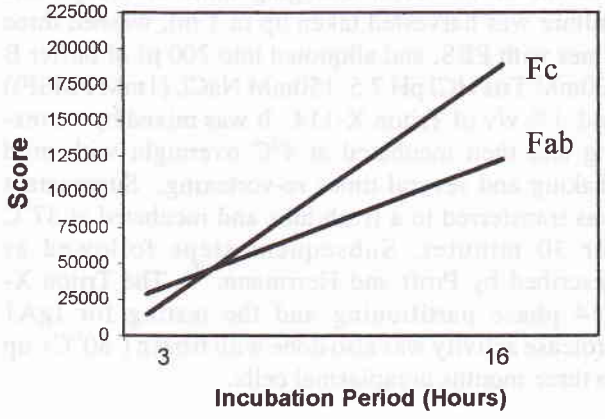

Figure 3. Time course of IgA1 protease activities in cellular fractions. Linear graphs shows growing of Fc and Fab fragments scores in parallel with time or period of incubation. Fc and Fab fragments started to appear at the third hour.

To analyze the localization of the protease activity in the ureaplasmal cells several methods were applied to produce cellular fractions. The phase separation was done by centrifugation of sonicated cells which resulted in two fractions, the pellet and the supernatant. Protease activity was found in both fractions, but as judged by signal strength in SDS-PAGE, the higher activity was detected in the supernatant. In a second approach, we fractioned the proteins according to their solubility in Triton X-100, which provided a soluble and an insoluble fraction. The first one contains all the Triton X-100 soluble protein which includes cytosolic protein and dissolved membrane protein. As shown in Fig. 4B most of the activity is recovered from the soluble fraction. This is in agreement with the data derived from cell fractionation by sonication and centrifugation.

Finally, we applied the Triton X-114 phase partitioning method, which enriches integral membrane proteins and can also be done with cells, which were not pretreated by sonication. The fractionation produced an aqueous phase, a detergent phase, and a detergent insoluble phase (Fig. 5). The detergent phase should contain only the integral membrane proteins. As it turned out, the detergent phase showed the highest activity (Fig. 6) and the insoluble the lowest one. By comparing different sample volumes and their protease activity, we calculated that the detergent phase contained 2.5 times as much activity as the Triton X-114 insoluble fraction.

To test, whether IgA1 protease is also secreted into the medium, we analyzed aliquots from the supernatant after removing the ureaplasmas by centrifugation for 60 min at 20.000xg. Several attempts to show the protease activity failed. Finally we compared the protease activity in the two isolates, *U. ureaplasma* DKF3 and CX8. Both of them showed the same IgA1 protease activity according to our test system.

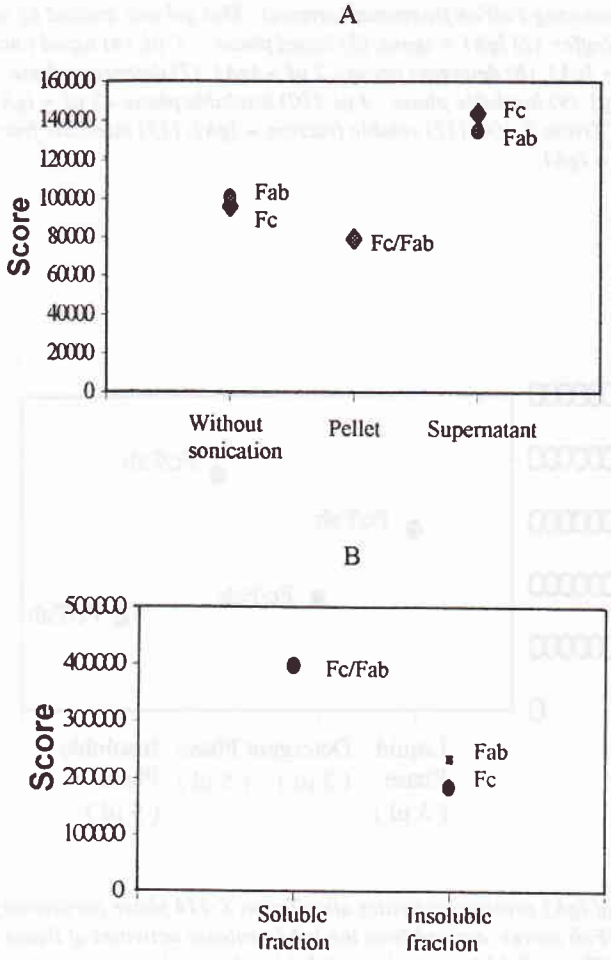

Figure 4. Distribution of IgA1 protease activities in cellular fractions. Scatter diagrams of Fc/Fab scores derived from sonicated cells (A) and Triton X-100 extraction (B). IgA1 protease activities achieve higher scores in soluble fraction of both treatments.

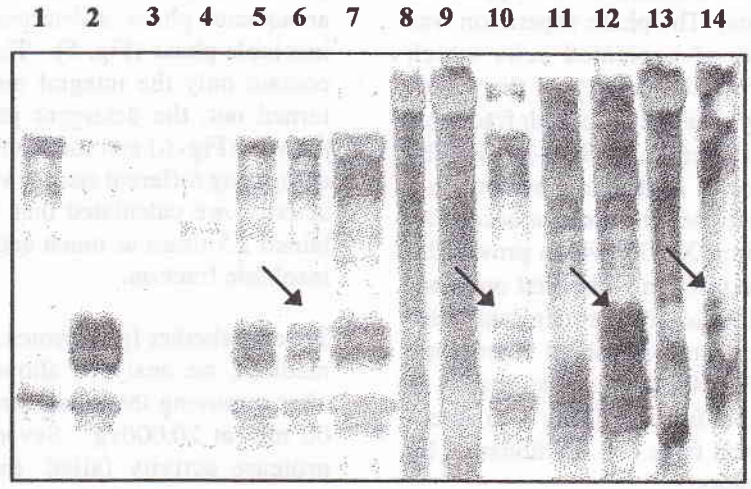

Figure 5. SDS-PAGE for analysis of protease distribution after Triton X extractions. Triton X-114 phase partitioning and Triton X-100 extraction showed that all fractions have IgA1 protease activity, by producing Fc/Fab fragments (arrows). This gel was stained by silver. Note: (1) IgA1 + PBS buffer, (2) IgA1 + igase, (3) liquid phase - 3 µl, (4) liquid phase - 6 µl, (5) liquid phase - 5 µl + IgA1, (6) detergent phase - 2 µl + IgA1, (7) detergent phase - 5 µl + IgA1, (8) insoluble phase - 2 µl, (9) insoluble phase - 4 µl, (10) insoluble phase - 5 µl + IgA1, (11) soluble fraction of Triton X-100, (12) soluble fraction + IgA1, (13) insoluble fraction, (14) insoluble fraction + IgA1.

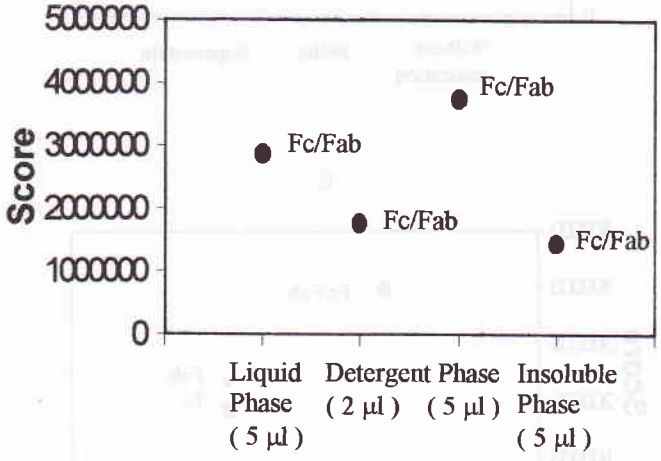

Figure 6. Distribution of IgA1 protease activities after Triton X-114 phase partitioning. Scatter diagram of Fc/Fab scores derived from the IgA1 protease activities of liquid, detergent, and insoluble phases of Triton X-114 partitioning. Soluble phase, which was partitioned into aqueous and detergent fractions, had higher scores than in the insoluble phase.

DISCUSSION

The clear green color of 10^7 color changing units is important to produce maximum health level of ureaplasma culture.[20] Cultivating ureaplasma without urea but with the pH marker bromothymol blue showed the importance of the color changing unit. It was proven that even without any color change due to the lack of urea in the medium, the cells from 200 ml culture could be harvested between 16 and 24 hours to produce 2 mg/ml of total cell protein. Moreover, culture medium with and without urea gave the same signal intensity in the bands with the Fc / Fab fragments. This indicates that IgA1 protease expressed at the same level during those incubating periods regardless of the urea supplementation.

Cultivation on solid media normally is done by subculturing of serial dilution on broth culture.[20,21] Although it needs special skills and a well-equipped microbiology laboratory to assess ureaplasmal colonies, the benefit of colony verification permits the possibility to calculate colony-forming units.[22] Besides that the combination of liquid and solid cultivation is a sensitive methods, which can be done for diagnostic purposes. But the methods of molecular biology are at least as sensitive as cultivation and certainly much faster in the detection of ureaplasma cells. Furthermore, the *UI-ure* specific probe was cross-reacting with the uraplasma genome but not with the genome of *M. pneumoniae*, a non-urea metabolizing mollicutes species. Therefore, we believe that this *UI-ure* oligonucleotide can be used as a specific probe for ureaplasmas within the bacterial class *Mollicutes*.

Digestion of the hinge region of the IgA1 heavy chain depends on its interaction with the catalytic sites of the IgA1 protease at the cell surface of the ureaplasmas. Based on our observation, it seems that ureaplasmal cells tend to aggregate and precipitate in PBS buffer. This was previously assumed to be the reason for the partial digestion of the IgA1 heavy chain. But it turned out that this kind of incomplete digestion appeared in both, supernatant and pellet, and soluble and insoluble fractions. Hence, it is presumed that the activity of IgA1 protease from 300 µg of total ureaplasmal cell protein used is less than 1 µg pure neisserial igase. Nevertheless, one has also to consider that a protein extract might contain protease-inhibiting substances and that further enrichment of the enzyme would result higher protease activity. A reliable assay to test an IgA1 protease activity of ureaplasma can be done by using the soluble part of Triton X-100 extraction and Triton X-114 phase partitioning.

Fc / Fab fragment scores were higher in the soluble than in the insoluble phases. For that reason, it is presumed that the ureaplasmal IgA1 protease is a soluble enzyme, and the proteolysis takes place in the cytoplasm or membrane-bound. Since the detergent phase, which contains the integral proteins, also has an activity against human IgA1, it is most probable that a fraction of this enzyme is tightly bound to the plasma membrane and contains a lipoprotein moiety. Bendjennat *et al.*[23] showed that a nuclease from *M. penetrans* has an activity both in liquid and detergent phases and apparently possesses a lipoprotein precursor component. Meanwhile, Washburn *et al.*[24] reported that surface antigens MAA1 and MAA2 of *M. arthritidis*, which partition into the detergent phase, are integral membrane-bound proteins and lipoproteins. We suggest that ureaplasmal IgA1 protease matures in cytoplasm before inserted into the membrane by a transport apparatus containing protein-like translocase (SecA or SecY), which was identified in *M. pneumoniae*.[25] The essential *secA* gene product recognizes the proteins to be exported and catalyses their movement to the inner cell membrane.[26] Nevertheless, as an alternative, two forms of ureaplasma IgA1 protease may exist either membrane-bound or cytosolic with the same hydrophilic catalytic site. One would expect that the protease activity is surface-exposed or secreted to prevent IgA1 attack.[4] So, if the protease would be a cytosolic enzyme, it should be effective when the cells are lysed.

Acknowledgement

This work was kindly funded, critically reviewed and discussed by Professor R. Herrmann of ZMBH, and Professor H.-J. Freisleben of the Graduate Study Program Biomedical Sciences of UI. This research was part of Felix M. Mesak's doctorate work supported by The Excellent Student Scholarship of the URGE Project.

We thank Dr. R. Frank for synthesis of the oligonucleotide, and E. Pirkl and D. Hofmann for introducing the specific methods of molecular biology to FMM.

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