

IMMUNOGLOBULIN G PROTEOLYTIC ACTIVITY OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS*

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ABSTRACT

Actinobacillus actinomycetemcomitans produces a protease to human immunoglobulin G that is an important evasion mechanism. In this study, the proteolytic activity of *A. actinomycetemcomitans* strain ATCC 43718 on human immunoglobulin G associated with culture supernatant concentrations, the growth period and the period of incubation with immunoglobulin G were evaluated by an enzyme linked immunosorbent assay. The protease fraction was detected by Sephadex G 150 chromatography. The results showed that *A. actinomycetemcomitans* produced a protease to human immunoglobulin G in the culture supernatant, and the highest activity was achieved when the concentration was 27.5 µg protein/mL, after culturing for 72 hours and incubating with IgG for 24 hours. The molecular mass of the protease active fraction was from 43 to 150 kDa.

Key words: immunoglobulin G, *Actinobacillus actinomycetemcomitans*, ELISA, protease

INTRODUCTION

Actinobacillus actinomycetemcomitans is a Gram-negative coccobacillus, frequently associated with localized aggressive periodontitis [LAP] which is characterized by rapid and marked bone loss around the incisor and molar teeth (1,9,14,20). This organism has been also associated with a variety of non-oral infectious diseases such as endocarditis, osteomyelitis, septicemia, pneumonia, arthritis and abscesses. It has been estimated that approximately 0.6% of infective endocarditis is caused by *A. actinomycetemcomitans*, and it has been detected in 18% of atherosclerotic plaque samples from patients (8).

A. actinomycetemcomitans produces several virulence factors, such as a leukotoxin belonging to the RTX family that destroys neutrophils and monocytes (3,8,18,20), a cytolethal distending toxin (CDT) that causes arrest of the mammalian cell cycle in G2 and a chaperonin 60 with potent leukocyte-activating

and bone resorbing activities (8). It has been shown that this bacteria also produces a protease to collagen type I and fibronectin characterized as serine or metallo protease with 50 kDa (19). The metallocollagenase produces multiple scissions in the collagen molecule, and seems able to degrade native Type I collagen, destroying the periodontal connective tissue (4). *A. actinomycetemcomitans* produces proteins inhibiting osteoblast proliferation and bone collagen synthesis, and a capsular-like polysaccharide that inhibits osteoblast proliferation by inducing apoptosis (8).

Moreover, *A. actinomycetemcomitans* produces a bacteriocin that plays an important ecological role in regulating the intra- and inter-specific oral microbiota (6,10,11). Antibodies produced against these virulence factors may be beneficial to the host, but *A. actinomycetemcomitans* produces proteases that cleave immunoglobulins [Igs] (5) and factors that can inhibit Igs production by human B cells (15) and the lymphoproliferative response (17).

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Considering the sparse data related to the Ig protease of *A. actinomycetemcomitans*, the aim of the present study was to partially characterize the protease produced by *A. actinomycetemcomitans* against human IgG.

MATERIALS AND METHODS

Microorganism

A. actinomycetemcomitans [ATCC 43718] serotype b was grown at 37°C for 24, 48 and 72 h on brain heart infusion broth [BHI] [Biobrás, Montes Claros, MG, Brazil], supplemented with 0.5% yeast extract [Biobrás, Montes Claros, MG, Brazil] under microaerophilic conditions [candle jar] as described by Avila-Campos *et al.* (2). The organisms were harvested by centrifugation [1x] [Hitachi himac CR21, Japan] [10.000 x g, 20 min, 4°C] and the supernatant protein concentrations were determined by Folin method, using bovine serum albumin as standard (12). All samples were adjusted to the same protein concentration [880 mg/mL], and 1.0 mL aliquots were stored at -80°C until use.

Human IgG purification

Human IgG was purified from serum in a Sepharose-protein G column [Sigma Chemical Co., St. Louis, MO, USA]. The IgG was eluted with 0.1 M glycine-HCl, pH 2.8, and immediately neutralized with 2 M Tris, pH 9.0. Fractions [1.0 mL] were collected and read in a spectrophotometer at 280 nm. The fractions with the highest absorbency were mixed. The resulting pool was dialysed against 0.15M phosphate-buffered saline [PBS], and the protein concentration was determined using the Folin phenol method (12).

IgG proteolytic activity of *A. actinomycetemcomitans* ATCC 43718

The IgG proteolytic activity of *A. actinomycetemcomitans* ATCC 43718 supernatants was performed according to Gregory *et al.* (5), with some modifications. Purified human IgG [250 ng well⁻¹] was diluted in 0.1 M carbonate buffer, pH 9.6, and 96-well flat polystyrene plates [Kartell S.P.A, Noviglio, Milan, Italy] were sensitized and incubated for 1 h, at 37°C and overnight at 4°C. The plates were washed 5 times with PBS-0.05% Tween 20 [PBS-T], blocked with PBS-T-5% skim milk [PBS-T-M] for 2 h, at 37°C, and then incubated with *A. actinomycetemcomitans* ATCC 43718 supernatants [24, 48 and 72 h cultures] without dilutions and diluted 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 in a period of 4, 6 e 24 h of incubation. The BHI medium [Biobrás, Montes Claros, MG, Brazil] was used as control. The plates were washed with PBS-T [5x] and incubated with anti-human IgG peroxidase-labeled diluted 1:4000 [Sigma Chemical Co., St. Louis, MO, USA], washed 5 times, and a substrate solution [5mg orthophenylenediamine - Sigma Chemical Co., St. Louis, MO, USA, 10 mL of 0.1 M citrate buffer, pH 4.5 and 5 µL H₂O₂] [100µL well⁻¹] was added. The reaction was halted with 50 µL of 4N H₂SO₄ and absorbency was read in a Titertek Multiskan EIA reader [Labsystems, Helsinki, Finland] at 492 nm. The protease activity was determined in percentage of degradation, considering 100% non-degradation with BHI medium.

[100µL well⁻¹] was added. The reaction was halted with 50 µL of 4N H₂SO₄ and absorbency was read in a Titertek Multiskan EIA reader [Labsystems, Helsinki, Finland] at 492 nm. The protease activity was determined in percentage of degradation, considering 100% non-degradation with BHI medium.

Sephadex G-150 chromatography of *A. actinomycetemcomitans* ATCC 43718 supernatants

A. actinomycetemcomitans ATCC 43718 supernatants [2 mL] were fractionated in a Sephadex G-150 [Sigma, St. Louis, Missouri, U.S.A.] column balanced with 0.15 M PBS pH 7.2. Fractions [1.0 mL] were collected and read in a spectrophotometer at 280 nm, and the active IgG protease fraction was determined by ELISA. For partial characterization, dextran blue [exclusion volume, more than 150 kDa] and 43 kDa glycoprotein from *Paracoccidioides brasiliensis* were applied to the same column.

Statistical analysis

Data were analyzed statistically by using ANOVA F and Tukey's Test [variance analysis] and considered significant if the P value was lower than 0.05 [p<0.05].

RESULTS

IgG proteolytic activity of *A. actinomycetemcomitans* ATCC 43718

The mean value of three trials in triplicate of IgG proteolytic activity of *A. actinomycetemcomitans* ATCC 43718 supernatants determined in percentage of degradation was higher at 1/32 dilution [27.5 µg/mL] than pure and diluted by 1/8, 1/16, 1/64, 1/128, 1/256, 1/512, 1/1024 [p<0.05] and higher at 72 h than at 24 and 48 h of culture [p<0.05]. In relation to IgG incubation time, in a 1/32 dilution the higher activity was observed with 24 h of incubation [p<0.05]. However, in dilutions of 1/128, 1/256 and 1/512 the highest activity was observed after 6 h of incubation [p<0.05] [Figs.1 and 2].

Analysis of IgG protease activity in *A. actinomycetemcomitans* ATCC 43718 fractions

The results of Sephadex G-150 chromatography of *A. actinomycetemcomitans* ATCC 43718 supernatants fraction by ELISA showed IgG protease activity in fractions 13 to 17 [Fig. 3]. The dextran blue eluted in a fraction 9 and 43 kDa glycoprotein in a fraction 19.

DISCUSSION

The human oral microbiota is complex and diverse. It is composed of more than 300 bacterial species (13). The IgG, IgM and IgA antibodies produced against several oral microorganisms have been detected in plasma and crevicular

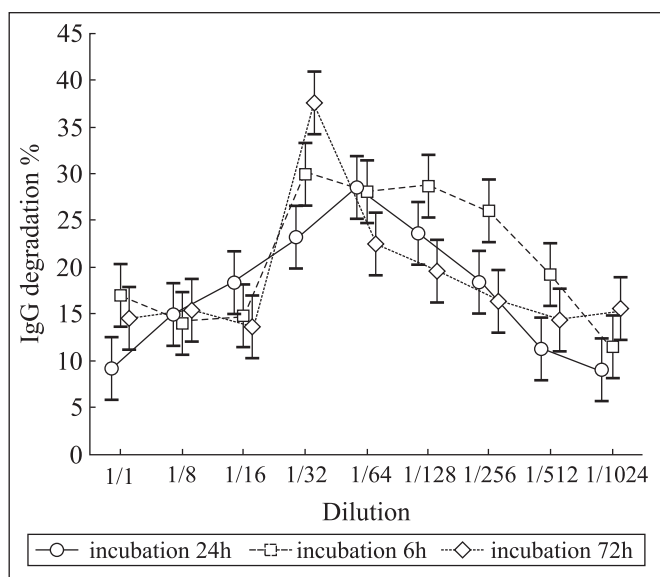


Figure 1. The mean value of three trials in triplicate of human IgG incubated for 4, 6 and 24 h with pure, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 diluted culture supernatant and then incubated with anti-human IgG peroxidase and the absorbency read at 492 nm and the results determined in percentage of degradation, considering 100% non-degradation with BHI medium by ELISA.

1/1,1/8,1/16 x 1/32,1/64 $p < 0.05$;
 1/32, 1/64 x 1/128, 1/256,1/512,1/1024 $p < 0.05$;
 4h x 6h = $p > 0.05$;
 4h x 24h [1/32] = $p < 0.05$;
 6h x 24h [1/32] = $p < 0.05$.

fluid, even in healthy individuals. These antibodies may influence the oral microbiota by interfering with adherence or by inhibiting bacterial metabolism. Moreover, the IgG antibodies may enhance phagocytosis and death of oral microorganisms through activation of the complement or opsonization (13). However, Ig can be degraded by proteases produced by several bacterial species that colonize mucosal and tooth surfaces (13), including periodontal pathogens (4). Gregory *et al.* (5) demonstrated the presence of proteolytic enzymes in the supernatant of cultures of *A. actinomycetemcomitans*, and that these enzymes can cleave IgG, IgA and IgM *in vitro*.

This activity may be the factor which favors the growth of *A. actinomycetemcomitans*. Considering the bacteriocinogenic activity of *A. actinomycetemcomitans* (11), which can regulate intra- and interspecific microbiota, both of these virulence factors may play an important ecological role in the mouth.

Investigation of the factors that may modify the oral microbiota is important, considering the recent evidence suggesting that dental infection may be a predisposing factor

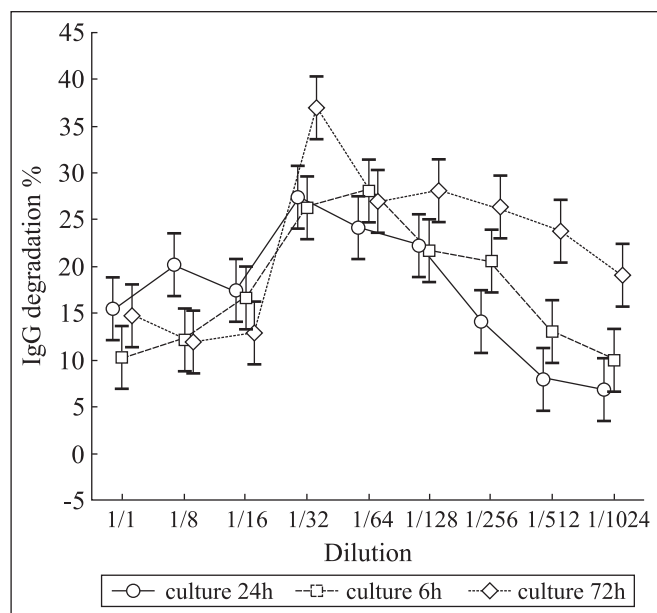


Figure 2. The mean value of three trials in triplicate of human IgG incubated with pure, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 diluted supernatant of 24, 48 and 72 h culture supernatant and then incubated with anti-human IgG peroxidase and the absorbency read at 492 nm and the results determined in percentage of degradation, considering 100% non-degradation with BHI medium by ELISA.

72h x 24h [1/32] = $p < 0.05$;
 72h x 48h [1/32] = $p < 0.05$.

in systemic conditions such as coronary heart disease, diabetes and low birth weight (7).

In order to partially characterize the *A. actinomycetemcomitans* protease to IgG, supernatants of this organism were incubated with human IgG. The IgG degradation was revealed by using an anti-human IgG conjugated with peroxidase. The protease activity in *A. actinomycetemcomitans* strain ATCC 43718 IgG associated with culture supernatant concentrations was dose-dependent; the highest activity was observed when supernatants were diluted 1/32 [27.5 μg protein/mL]. At high concentrations there was low activity, suggesting that some component not studied here may inhibit that activity. Additional studies are necessary to better understand the protease action.

The highest protease activity was observed in the 72 hour culture of the supernatant. This could be explained either by high enzyme production or by accumulation, which may be growth-time-dependent.

The protease activity was observed after 4, 6 and 24 hours of incubation with supernatant culture and human IgG. The highest protease activity in the 1/32 supernatant dilution was observed for the 24 hour incubation. However, in dilutions of 1/128, 1/256

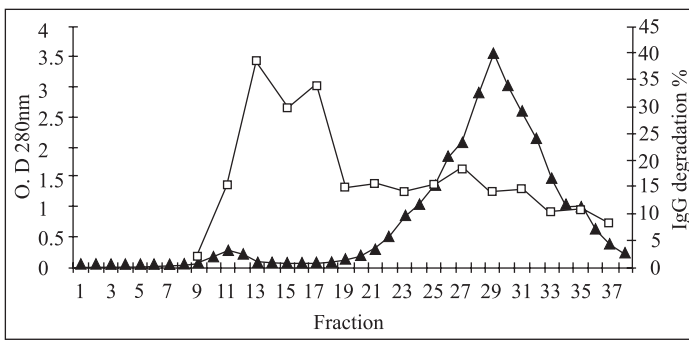


Figure 3. Spectrophotometric profile at 280 nm of fractions obtained from *A. actinomycetemcomitans* ATCC 43718 culture supernatants chromatography in Sephadex G-150 [—▲—] and IgG proteolytic activity by ELISA [—□—]. The fractions [1.0 mL] were collected with an automatic fraction collector, read in a spectrophotometer. The IgG proteolytic activity were determined by three trials in triplicate of human IgG incubated for 24 h with fraction sample [1/32] and then incubated with anti-human IgG peroxidase and the absorbency read at 492 nm. The results of IgG proteolytic activity were determined in percentage of degradation, considering 100% non-degradation with BHI medium.

and 1/512 the highest activity was observed, unexpectedly, after 6 hours of incubation. This result can be explained by the extensive IgG degradation after 24 hours, at these dilutions, and IgG conjugated with peroxidase binding in the ELISA plate, or by other variations including the supernatant culture period, or by some other factor. The use of purified IgG protease may eliminate the many components present in the culture supernatant that may influence the protease activity. This question will be the subject of future investigation.

In addition, to characterize the *A. actinomycetemcomitans* protease to IgG, the culture supernatant was fractionated by gel filtration chromatography and the protease activity was determined by ELISA. IgG protease activity was detected in fractions 13 to 17. The molecular masses [MM] of the components of these fractions were estimated as less than 150 kDa and more than 43 kDa, considering the later elution void volume [150 kDa] and prior to the reference 43 kDa glycoprotein.

The protease activity observed in the five fractions may be due to the participation of proteases with different MMs or to the action of proteases at higher dilutions, as observed in this study. Gathering more precise data concerning the IgG protease MM requires further investigation.

In all of the culture supernatant concentrations, growth periods or incubation times investigated, the supernatant culture results were different from the BHI control, showing a protease activity in the supernatant, even in low degree [data not shown]. This *A. actinomycetemcomitans* IgG protease activity observed

in vitro may also act *in vivo*. Smith and Taubman (16) reported that IgG in dental plaque extracts was extensively degraded as a Fc fragment and Gregory *et al.* (5) observed severe degradation of IgG and IgA with crevice gingival samples from patients with localized aggressive periodontitis.

The *A. actinomycetemcomitans* protease in periodontal tissue may destroy IgG after 4-6 hours of contact, based on the *in vitro* results. Considering that this activity was present even in high supernatant dilutions, it is possible that this enzyme may act externally by diffusion through saliva, periodontal tissue, mucosa or even blood. However, further studies are necessary to confirm this hypothesis.

We concluded that *A. actinomycetemcomitans* produces a protease against human immunoglobulin G with high activity in BHI supernatant cultures at 27.5 µg protein/mL concentration, after 72 hours growth and incubation with IgG for 24 hours. The molecular mass of the active fraction of protease is between approximately 43 and 150 kDa.

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RESUMO

Atividade proteolítica de *Actinobacillus actinomycetemcomitans* sobre imunoglobulina G

Actinobacillus actinomycetemcomitans produz protease ativa sobre imunoglobulina G humana, sendo um dos mecanismos importantes de escape do microrganismo. No presente trabalho, foi analisada a atividade proteolítica de sobrenadante de cultivo de *A. actinomycetemcomitans* ATCC 43718 sobre imunoglobulina G humana em função de concentração, tempo de cultivo do microrganismo e tempo de incubação com IgG, por ensaio imunoenzimático. Adicionalmente, foi determinada a fração com atividade de protease por meio de análise de eluatos de cromatografia em coluna de Sephadex G 150. Os resultados obtidos demonstraram que *A. actinomycetemcomitans* liberou protease ativa sobre imunoglobulina G humana em sobrenadante de cultivo, sendo a sua maior atividade evidenciada na concentração de 27,5 µg proteína/mL, com tempo de cultivo de 72 horas e com 24 horas de incubação com IgG. A massa molecular da fração ativa de protease foi compreendida entre 43 a 150 kDa.

Palavras-chave: imunoglobulina G, *Actinobacillus actinomycetemcomitans*, ELISA, protease

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