IMMUNOGLOBULIN G PROTEOLYTIC ACTIVITY OF ACTINOBACILLUS ACTINOMYCETEMCOMITANS

Fernanda Akemi Nakanishi; Mario Julio Avila-Campos; Nádia Hizuru Kamiji; Eiko Nakagawa Itano*

1Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES, São Paulo, SP, Brasil; 2Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brasil; 3IC-CPG, Universidade Estadual de Londrina, Londrina, PR, Brasil; 4Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, PR, Brasil

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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).
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 37ºC 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, 4ºC] 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 -80ºC 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 0.15 M 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, Novigilio, Milan, Italy] were sensitized and incubated for 1 h, at 37ºC and overnight at 4ºC. 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 37ºC, 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, 1/128, 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 orthophenylendiamine - Sigma Chemical Co., St. Louis, MO, USA, 10 mL of 0.1 M citrate buffer, pH 4.5 and 5 μL H₂O₂] [100 μL 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 than 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 glicoprotein 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.
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 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...
IgG proteolytic activity of A. actinomycetemcomitans

A. actinomycetemcomitans produces protease activity 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 was 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 eluados 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
REFERENCES


