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Inhibition of streptococcal pyrogenic exotoxin B using allicin from garlic

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ABSTRACT

Streptococcal pyrogenic exotoxin B (SpeB) is an important virulence factor of group A streptococci (GAS) and inactivation of SpeB results in the significantly decreased virulence of the bacterium. The protein is secreted as an inactive zymogen of 40 KDa (SpeBz) and undergoes proteolytic truncation to result in a 28 KDa mature active protease (SpeBm). In this study the effect of allicin on the proteolytic activity of SpeBm was evaluated using azocasein assay. Allicin neutralized the SpeBm proteolytic activity in a concentration dependent manner (IC50 = 15.71 ± 0.45 μg/ml). The loss of activity was completely reversed by subsequent treatment with a reducing agent, dithiothreitol (DTT; 10 mM final concentration), suggesting that allicin likely inhibits the SpeBm by forming a disulfide linkage with an active thiol group in its active site. This mechanism of action was further confirmed with the fact that DTT did not reverse the SpeBm activity in the presence of E–64, a cysteine protease-specific inhibitor, which works specially by forming a thioether linkage with free sulfhydryl groups in enzymes active site. The MIC of allicin against GAS was found to be 32 μg/ml. Exposure of GAS culture to allicin (25 μg/ml) inhibited maturation of SpeBz to the SpeBm.

In conclusion, the results of this study suggest that allicin inhibits the maturation of SpeBz and proteolytic activity of SpeBm and could be a potential therapeutic agent for the treatment of GAS infections.

1. Introduction

Streptococcus pyogenes (group A streptococci [GAS]) is one of the most common and significant human bacterial pathogens among 5–15 years old children worldwide [1]. It is responsible for a wide variety of infections ranging from mild and self-limiting infections such as pharyngitis and pyoderma to invasive infections such as necrotizing fasciitis or rapidly fulminating streptococcal toxic shock syndrome [1]. Group A streptococci infections can also lead to serious nonsuppurative complications including rheumatic fever/rheumatic heart disease and acute post streptococcal glomerulonephritis (APSGN) [2]. The GAS has a large armamentarium of virulence factors responsible for this broad range of human diseases. The abilities of strains differ to express a wide range of virulence factors. However, some virulence factors are highly conserved and expressed by almost all GAS strains. The streptococcal pyrogenic exotoxin B (SpeB) is among such factors. The SpeB is chromosomally encoded and essentially produced by 100% of GAS strains [3,4].

SpeB, which functions as cysteine protease, is produced as a 40 KDa precursor (SpeBz) and subsequently cleaved to a 28 KDa active molecule (SpeBm) [5]. Several lines of evidence have been accumulated through both *in vitro*, *in vivo* and clinical studies indicate the potential role of SpeB in the pathogenesis of streptococcal infections [6]. In vitro, SpeB has broad proteolytic activity and degrades or activates several biologically important host proteins. It has been shown that SpeB cleaves fibronectin and vitronectin two abundant connective tissue proteins [7]. It also degrades human immunoglobulins and complement components [8]. The enzyme activates human interleukin 1β [9] and matrix metalloproteinases [10], and releases biologically active kinins from H-kininogen [11]. Additionally, SpeB regulates streptococcal proteins by degrading or releasing them from the bacterial surface [6]. *In vivo* experiments have shown that intravenous injection of purified SpeB causes myocardial necrosis and death in animals [12]. In addition inactivation of *speB* gene significantly decreases lethality to mice and ability to disseminate into the body [13]. Human studies have shown that patients with low antibody levels to SpeB are more likely to succumb to severe GAS infections than are

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individuals with high antibody levels [14] suggesting that such antibodies are protective against severe diseases. This is supported by the fact that passive immunization of mice with anti-SpeB antibodies protects mice against lethal challenge with GAS [15]. Some studies indicated the presumptive role of SpeB in kidney diseases as high titer of anti-SpeB antibodies were found in APSGN patient sera and SpeB was detected in kidney biopsies from APSGN patients [16]. It has been supported by the fact that nephritis associated strains preferentially release the SpeB protein in the extracellular products when compared to non-nephritis associated strains [17].

The data indicate that SpeB is an important virulence factor in streptococcal infection, making it an attractive therapeutic target.

Garlic (*Allium sativum*) has been used as a medicine since ancient times and has long been proven to have antimicrobial activity against wide variety of microorganisms [18]. Antimicrobial properties of garlic are primarily attributed to the most abundant thiosolfinat molecule known as allicin [19]. Allicin's biological activities were suggested to be due to its thiol–disulfide exchange reactions with the sulfhydryl enzymes [20]. The objective of present study was to evaluate the *in vitro* efficacy of allicin to inhibit proteolytic activity of GAS extracellular cysteine proteases, SpeB, and transformation of SpeBz to the mature enzyme.

Microbial SH-containing enzymes so far shown to be inhibited by allicin include: *Entamoeba histolytica* cysteine protease, GAS streptolysin O, pneumococcal pneumolysin O and *Proteus mirabilis* urease [21–24].

2. Materials and methods

2.1. Bacterial strains, chemicals and media

Two GAS strains, AP1 of serotype M1 and AL1, were used in this study. These were kindly gifted by Professor L. Bjorck, Department of Clinical Science, Division of Infection Medicine, Lund University, Lund, Sweden. The GAS strain AL1 is an isogenic derivative of strain AP1 lacking active SpeB production (25). Bacteria were cultured on sheep blood agar plates for daily use and stored at −80 °C in a trypticase soy broth with 15% glycerol, for subsequent uses. Epoxysuccinylleucylamido(4-guanidino)butane (E–64) and azocasein were purchased from Sigma (St Louis, MO). All other chemicals and media were purchased from Merck (Darmstadt, Germany) if not noted otherwise.

2.2. The antimicrobial activity assay

The antimicrobial activity assay of allicin against GAS (AP1 and AL1) was performed using a microdilution method according to procedures recommended by the Clinical Laboratory Standards Institute guidelines document M7-A7 [26], except that C-medium (CM) was used in this study. Briefly, 2-fold serial dilutions of allicin were prepared in sterile CM for a testing concentration range of 2–1024 μg/ml. Then 100 μL from each dilution was pipetted into the wells of a microtiter plate and inoculated with a fixed amount (5 μL) of the standardized (1.5×10^7 CFU/mL) cell suspension. Plates were incubated at 37 °C overnight, and the lowest concentration of the allicin showed no visible growth was considered as the MIC. Following MIC test, minimum bactericidal concentration (MBC) was determined by sub-culturing 10 μL aliquots of broth from each well that showed no visible growth onto sheep blood agar plates. The lowest concentration of allicin that killed the majority (99.9%) of the original bacterial inoculum was considered as MBC.

2.3. Allicin preparation and quantification

Allicin (purity ≥95%) was purified from garlic extract using the semi-preparative HPLC method and quantified by analytical HPLC as described in detail by authors elsewhere [19,27].

2.4. SpeBm production and proteolytic activity assay

For SpeB production, 50 μL of overnight culture of GAS strains in todd hewitt broth were transferred into 45 mL sterile CM consisting of 0.5% (w/v) Bacto Peptone No. 2 (Difco, Detroit, MI) and 1.5% (w/v) yeast extract (Hi media, India) dissolved in CM buffer (10 mM K_2PO_4, 0.4 mM $MgSO_4$, and 17 mM NaCl pH 7.5) [25]. Cultures were grown for approximately 24 h at 37 °C under a 5% CO_2 atmosphere without agitation. When culturing strain AL1, CM was supplemented with 150 μg/ml of kanamycin (Biobasic Canada) for selective pressure. The GAS cultures were centrifuged for 15 min (4 °C) and the resulting supernatants used for SpeB activity assay. Protease activity of SpeB was measured using a quantitative chromogenic azocasein hydrolysis assay as described elsewhere with minor modifications [28]. Briefly, 250 μl of GAS culture supernatant was added to 400 μl of reaction mixture [2.7 mg/ml of azocasein in 50 mM Tris–HCl (pH 8.0)] which had been prewarmed at 37 °C. After incubation at 37 °C for 2 h, the reaction was terminated by adding 100 μl of 30% ice-cold trichloroacetic acid. The reaction mixture was held on ice for 15 min and then centrifuged, and an equal volume of 0.5 M NaOH was added to the supernatant. The absorbance at 450 nm of the samples were measured with a microplate reader (BioTek 120, USA), to determine the amount of azopeptides not precipitated with trichloroacetic acid. One unit of enzyme activity was defined as the amount of enzyme that resulted in an increase in absorbance of 0.1 units after 2 h incubation under the assay conditions described and reported in terms of protease activity per milliliter. The specificity of protease activity was confirmed using cysteine protease-specific inhibitor E–64 at a final concentration of 40 μM.

2.5. Inhibition of SpeBm proteolytic activity

To examine the inhibitory effect of allicin on SpeBm, 250 μl culture supernatant of strain AP1 were preincubated with various concentrations of allicin [0.5–12 μg (4–48 μg/ml)], for 30 min at ambient temperature and then SpeB activity was measured according to the method described above. The positive control for the assay was prepared in the same manner, but without the allicin. Activity without the allicin was considered to be 100%, and the residual activity at each concentration of allicin was determined relative to this value. In parallel, to ensure that the color change was not due to allicin or azocazein itself, negative controls with and without allicin were included.

2.6. Effects of a reducing agent on SpeBm re-activation

An experiment was conducted to elucidate whether the reducing agent DTT could restore the proteolytic activity of SpeBm, which was lost after incubation with allicin. A total of 250 μl of AP1 strain culture supernatant in CM medium was pre-incubated with 20 μg of allicin for 30 min at room temperature and then the mixture was further incubated with 10 mM DTT (final concentration) in the same condition. SpeBm activity was measured as described above and residual activity was expressed relative to allicin free control. In order to elucidate the molecular mechanism of SpeBm inhibition, the same experiment was conducted on SpeBm inhibited by E–64.

2.7. Effect of allicin on converting the SpeBz to SpeBm

The effect of allicin on maturation of SpeBz into the active