# Inhibition of streptolysin O by allicin – an active component of garlic

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Streptolysin O (SLO) is a potent cytolytic toxin produced by almost all strains of group A streptococci and is considered an important virulence factor for this organism. In this study we investigated the effect of allicin and aqueous garlic extracts on the haemolytic activity of SLO. All tested materials potentially inhibited the SLO haemolytic activity. Allicin neutralized SLO in a dose- and time-dependent manner. A 15 min incubation of SLO with 35 µg allicin totally inhibited the haemolytic activity of SLO [IC<sub>50</sub> (concentration necessary to reach half maximum inhibition)=5.97 µg]. The inhibitory activity of an old extract of garlic was equipotent to pure allicin (IC<sub>50</sub>=6.27 µg; P<0.05). In contrast, fresh extract of garlic inhibited the SLO haemolytic activity at lower concentrations (IC<sub>50</sub>=1.59 µl; 1.9 µg allicin). The inhibitory effect of the allicin was restored by addition of reducing agent DTT at 2 mM, suggesting that allicin likely inhibits the SLO by binding to the cysteine residue in the binding site. These results indicate a new activity for allicin and allicin may be a potential alternative drug against streptococcal diseases.

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# INTRODUCTION

Streptococcus pyogenes (group A streptococci; GAS) is a highly versatile pathogen that causes a wide variety of important human diseases, ranging from localized infections, such as pharyngitis, erysipelas and cellulites, to lifethreatening invasive diseases like streptococcal toxic shock syndrome and necrotizing fasciitis (Martin & Green, 2006; Steer et al., 2007). GAS infections are associated with nonsuppurative immune-mediated complications such as poststreptococcal glomerulonephritis, acute rheumatic fever and post-streptococcal reactive arthritis (Martin & Green, 2006; Steer et al., 2007). This organism has a large armoury of virulence factors responsible for this broad range of human diseases (Cunningham, 2008). The strains differ in the expression of this wide range of virulence factors. However, some virulence factors are highly conserved and expressed by almost all strains. The potent streptococcal cytolytic toxin streptolysin O (SLO) is among such factors (Muller-Alouf et al., 1997; Shiseki et al., 1999). SLO contributes to the pathogenesis of streptococcal infections by direct toxic effects on human cell types, and by inducting the production and release of pro-inflammatory factors.

In vitro studies demonstrated that SLO kills a variety of

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human cell types by disruption of the biological membranes, at lethal concentrations (Alouf & Palmer, 1999), and recently it has been reported that SLO triggers the apoptotic death of human leukocytes, at sublethal concentrations (Timmer et al., 2009). Purified SLO elicits high amounts of pro-inflammatory cytokines by immune cells and potentiates inflammatory responses, in vitro and in vivo (Hackett & Stevens, 1992; Shanley et al., 1996). In parallel, isogenic SLO-deficient mutants showed a reduced ability to elicit the production of pro-inflammatory factors compared to parent strains (Ruiz et al., 1998). Administration of SLO in animal models produces severe pathophysiological effects. In rabbits, intravenous administration of SLO caused blood vessel contraction, increased capillary permeability, massive intravascular thrombosis, dermal necrosis, cardiotoxicity and death (Alouf, 1980). Limbago and colleagues reported that SLO-deficient GAS was attenuated and mice infected with the stable slo mutant exhibited a significant decrease in mortality rates compared to mice infected with wild-type GAS (Limbago et al., 2000). The data indicate that SLO has great potential as a drug target for the treatment of streptococcal infections.

SLO belongs to a family of pore-forming toxins referred to as thiol-activated toxins or recently as cholesterol-dependent cytolysins (Palmer, 2001). All toxins in this family consist of a single polypeptide chain, the length of which ranges from 471 amino acid residues with pneumolysin (Walker *et al.*, 1987) to 571 amino acid residues with SLO

Abbreviations: GAS, group A streptococci; HU, haemolytic unit;  $IC_{50}$ , concentration necessary to reach half maximum inhibition; RBC, red blood cell; SLO, streptolysin O.

(Kehoe et al., 1987). A single cysteine residue in these toxins plays an essential role in activity. In every case, the single cysteine residue lies in an 11 amino acid sequence common to each toxin (Kehoe et al., 1987). These toxins are reported to be active only in the reduced state, and thiol-group blocking agents inhibit their activity (Bernheimer & Avigad, 1970; Geoffroy & Alouf, 1982). Owing to this structure, SLO is expected to be neutralized by allicin, an active component of garlic. Allicin is the most abundant thiosulfinate molecule found in garlic extract. Allicin's main biological properties were suggested to be due to thiol-disulphide exchange reactions with the thiol containing proteins (Rabinkov et al., 1998). Microbial SHcontaining enzymes so far shown to be inhibited by allicin include malarial and intestinal parasite Entamoeba histolytica cysteine proteases (Ankri et al., 1997; Coppi et al., 2006). In the present study we evaluated the in vitro efficacy of allicin to neutralize the haemolytic activity of streptococcal SLO.

## **METHODS**

**Bacterial strains, chemicals and medias.** A total of four strains of GAS were used in this study: strains AP1, AP4 and AP12, kindly gifted by Professor L. Bjorck, Department of Clinical Science, Division of Infection Medicine, Lund University, Lund, Sweden, and ARUMS, a clinical isolate that was cultured from a patient with pharyngitis. The bacteria were cultured in Todd–Hewitt Broth medium (THB) (Sigma-Aldrich), stored at 4  $^{\circ}$ C for daily use and at -70  $^{\circ}$ C, along with 15 % glycerol, as a stock culture. Pure allicin was obtained from LKT Laboratories and all the other chemicals were from Merck except where noted.

Preparation of aqueous garlic extract. The garlic root bulbs were purchased from a local market in Ardabil city, Iran. Prior to extraction, the dead auxiliaries were removed and the bulbs divided in cloves. The cleaned cloves were washed in running tap water followed by rinsing in distilled water. To extract the juice, 20 g garlic cloves were crushed manually using a mortar and pestle, homogenized using a homogenizer (Silentcrush M; Heidolph) and sonicated for 5 min continuously at 100 % amplitude, using an ultrasonicator (UP200H; Hielscher Ultrasonics), in 60 ml distilled water on ice. The obtained mash was squeezed through five layers of cheesecloth and the suspension transferred into a 50 ml Falcon tube and centrifuged (with an Eppendorf 5810R centrifuge) at 1258 g at 4 °C for 20 min in order to separate the remaining debris from the liquid. The supernatant was transferred into a second sterile 50 ml Falcon tube and sealed. The resultant extract was either used immediately or stored at 4 °C until use within 5 months. A small fraction was stored at room temperature for stability analysis.

**Quantitative analysis of allicin.** The allicin content of garlic extracts was quantitatively determined by HPLC as reported elsewhere (Arzanlou & Bohlooli, 2010). Briefly, 100 µl internal standard [150 µg ethylparaben (ethyl-para-hydroxybenzoate) ml<sup>-1</sup> in mobile phase] solution (final concentration 15 µg ml<sup>-1</sup>) was added to 10 µl extracts and the final volume was adjusted to 1 ml by mobile phase, vortexed and centrifuged at 15 294 *g* for 10 min. A 20 µl volume of supernatant was injected onto the HPLC system (Jasco liquid chromatography system) equipped with a C18, Nucleosil 100 ODS (5 µm) analytical column with a size of 4.6 mm × 150 mm (Alltech Grom). The mobile phase was methanol–water (50:50, v/v) with flow rate of 1 ml min<sup>-1</sup>. The allicin in the effluents was detected

by measuring the absorbance at 220 nm and quantified by comparing the peak area produced by garlic extracts with that of standard allicin.

**Stability of allicin in aqueous garlic extract.** For stability analysis of the allicin in garlic extract in this study, samples stored at 4 and 22  $^{\circ}$ C were taken at particular time intervals, and the chemical stability of allicin was assessed using HPLC and quantitatively determined as mentioned before.

Screening of strains for production of SLO. Ten microlitres of overnight cultures of strains in THB was transferred into tubes containing 12 ml sterile THB and further incubated overnight at 37 °C without shaking. The cells were pelleted by centrifugation at 1258 g for 15 min. The haemolytic activity was determined with 1 ml serial dilutions of culture supernatants in PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4)]. Washed human red blood cells (RBCs) of type O were added to the tubes to yield a final concentration of 2 %. All tubes were incubated at 37 °C for 30 min. The remaining intact erythrocytes were removed by gentle centrifugation at 805 g for 2 min. The absorbance of released haemoglobin was determined at 541 nm using a spectrophotometer (UV/VIS spectrometer T80+; PG Instruments). The amount of toxin that produced 50 % haemolysis was defined as one haemolytic unit (HU). Controls containing 2% erythrocytes and de-ionized water, which was considered as 100% haemolysis, were used to determine the percentage of haemolysis. Confirmation of the haemolysis due to SLO was carried out with a control incubated with water-soluble cholesterol 'a specific inhibitor of SLO' at a final concentration of  $0.5 \text{ mg ml}^{-1}$  before the addition of the erythrocytes. In the other reaction mixture, DTT, a reducing agent, as the activator of SLO was added at a final concentration of 2 mM and the haemolytic activity was determined as above. All experiments were conducted in triplicate.

Inhibition of SLO. The SLO inhibition experiments were carried out in the same manner as the haemolytic activity assay. Before initiation of the reaction, the mixtures [culture supernatant (1 HU) + PBS] were pre-incubated with various concentrations of pure allicin, freshly prepared and old (stored at 4 °C, ≥10 days) extract of garlic in separate tubes for 15 min at ambient temperature. Washed RBCs were added to yield a final concentration of 2%. All tubes were incubated at 37 °C for 30 min. To remove the intact RBCs, tubes were centrifuged gently at 805 g for 2 min. The absorbance of the released haemoglobin was determined at 541 nm. The positive control for the assay was prepared in the same manner but without the test materials. Activity without the inhibitor was considered to be 100%, and the residual activity at each concentration of inhibitors was determined relative to this value. In parallel, to ensure that the lysis was not due to the test materials, negative controls consisting of test materials without culture supernatant were included.

**Time-dependent inhibition.** Before the reaction initiation, tubes containing reaction mixture [culture supernatant (1 HU) + PBS] in a final volume of 1 ml were incubated with constant concentration (35 µg ml<sup>-1</sup>) of allicin for particular time intervals. Then substrate (RBCs) was added and haemolytic activity was determined as described above. The residual activity was described as fraction of time.

**Inhibition of SLO in the presence of a protecting agent.** An experiment was conducted to elucidate whether the reducing agent DTT could restore the haemolytic ability of SLO, which is lost as a sequela of incubation with allicin. This was conducted in the same manner as the inhibition study. Before initiation of the reaction, the mixtures were incubated with DTT in a final concentration of 2 mM for 30 min at room temperature. The allicin was included in the

minimum concentration that fully inhibited the SLO as obtained from above-mentioned experiments.

**Statistical analysis.** Experiments were performed in triplicate. The data were presented as means  $\pm$  SD. Statistical significance was assayed by Student's *t*-test for unpaired data and the differences were considered to be significant at the *P*<0.05 level. IC<sub>50</sub> (concentration necessary to reach half maximum inhibition) values were calculated by fitting data to the Hill equation using SigmaPlot (version 11.0) software (Systat Software).

## **RESULTS AND DISCUSSION**

The principal goal of this study was to explore the potential capacity of pure allicin and aqueous extracts of garlic (with defined allicin content) to inhibit the haemolytic activity of SLO. The effect of the reducing agent DTT on the restoration of the blocked haemolytic ability of SLO was also investigated. To select an efficient strain for the production of SLO, four different strains were screened and a strain that exhibited potent haemolytic activity was selected for further studies. In this study, the stability of allicin in garlic extract at 4 °C and 22 °C was also examined.

## Screening of strains for production of SLO

The results showed that all examined strains were able to produce SLO with various potentials (Table 1). AP12 and AP4 produced the most abundant SLO versus AP1 and ARUMS strains. AP12 and AP4 produced up to  $2.84\pm0.12$ and  $2.41\pm0.03$  HU ml<sup>-1</sup> in the non-activated form and  $6.11\pm0.18$  and  $6.04\pm0.14$  HU ml<sup>-1</sup> in the presence of 2 mM DTT, respectively. AP12 was used for the inhibition studies. We used culture supernatant as SLO sources so the contribution of other virulence factors may be assumed. To rule out the possible contribution of other virulence factors to the haemolysis of erythrocytes, we used a control incubated with water-soluble cholesterol, a specific SLO inhibitor. The results showed that cholesterol completely inhibited the haemolysis of erythrocytes so haemolysis

Table	1. SLC	D activity	in	culture	supernatants	from	four
differer	nt strains	s of GAS					

Strain	Haemolytic activity o	Haemolytic activity of SLO (HU ml <sup>-1</sup> )				
	Without DTT	With DTT*				
AP1†	$0.42 \pm 0.05$	$0.75 \pm 0.01$				
AP4	$2.41 \pm 0.03$	$6.04 \pm 0.14$				
AP12	$2.84 \pm 0.12$	$6.11\pm0.18$				
ARUMS†	$0.55 \pm 0.03$	$0.62\pm0.02$				

\*DTT was added at a final concentration of 2 mM ml<sup>-1</sup>.

†AP1 and ARUMS strains produced SLO at less than 1 HU; activation increased the activity but did not reach higher than 1 HU.

caused by culture supernatant in our experiment condition may be mainly attributed to SLO.

## Inhibition of SLO

Various concentrations of pure allicin and two preparations of fresh and old aqueous garlic extracts were tested for their abilities to inhibit the haemolytic activity of SLO. To the best of our knowledge the data presented in this report showed for the first time that allicin and garlic extract are potent SLO inhibitors. Previously it has been shown that allicin can disable enzymes containing free thiol groups (a cysteine residue) in their active sites (Wills, 1956). Results indicated that all test materials decreased SLO haemolytic activity in a dose-dependent manner and showed different degrees of inhibition.

Allicin inhibited SLO haemolytic activity at low concentrations; 35  $\mu$ g (0.2  $\mu$ M) (IC<sub>50</sub>=5.97  $\mu$ g) of allicin completely inhibited the haemolytic activity of SLO (Fig. 1). This is comparable with previous studies where allicin inhibited E. histolytica cysteine proteases, malarial cysteine proteases and triose phosphate dehydrogenase at 10, 25 and 50 µmol, respectively (Ankri et al., 1997; Coppi et al., 2006; Wills, 1956). In the study by Wills (1956) it was demonstrated that 500 µmol allicin inhibited the activity of all thiol enzymes tested, including: succinate dehydrogenase, urease, papain, xanthine oxidase, choline oxidase, hexokinase, cholinesterase, glyoxylase and alcohol dehydrogenase. The differences in the inhibitory concentrations of allicin toward different enzymes may be due to inconsistency in the concentration of enzymes used in the inhibition studies, and under the same conditions the molecular structure of the enzymes may make it hard for the inhibitor to access the active site. Despite these facts,



**Fig. 1.** Inhibitory effects of the pure allicin ( $\blacksquare$ ) and the old extract of garlic ( $\blacklozenge$ ) on the haemolytic activity of SLO. Before the initiation of the reaction, bacterial culture supernatant (fixed to 1 HU ml<sup>-1</sup> in PBS) was pre-incubated for 15 min with various concentrations of pure allicin (2.5, 5, 10, 15, 20, 25, 30 and 35 µg ml<sup>-1</sup>) and/or volumes of old extracts of garlic with amounts of allicin adjusted to those used for pure allicin. Residual activity was measured and expressed as the mean ± sD.

the inhibitory concentration of allicin toward SH-containing enzymes is relatively low. This is may be due to the high affinity for allicin to free thiol groups (Rabinkov *et al.*, 1998) and characteristics such as its simple structure and low molecular mass that make it easier to enter the substrate binding pocket as well as to avoid unfavourable steric interactions with amino acid residues in its vicinity.

The inhibitory activity of old extract of garlic (day 49; 4 °C) was the same as that of pure allicin (IC<sub>50</sub>=6.27  $\mu$ g; P < 0.05). An extract of 31.25 µl containing 25 µg allicin completely inhibited the haemolytic activity of the SLO (Fig. 1). In contrast to the last two materials fresh extract of garlic inhibited the SLO haemolytic activity more strongly  $(IC_{50}=1.59 \ \mu l; 1.9 \ \mu g \ allicin)$ . The SLO activity was fully inhibited in the presence of 4.5  $\mu$ l (5.4  $\mu$ g allicin) of fresh extract of garlic (Fig. 2). This means that the contribution of other components in fresh extract of garlic must be considered. These substances may be degraded or transformed to other molecules during the storage period. The exact chemical nature of the extra inhibitory factors in fresh extract of garlic remains to be elucidated but most probably they are steroids and other steroidal molecules. It is well known that cholesterol and other steroids inhibit SLO at very low (nanomolar) concentrations (Alouf & Palmer, 1999). Garlic contains small amounts of steroids such as sitosterol (Al-Khatib et al., 1987) and plenty of steroidal saponins that are structurally similar to steroids (Lanzotti, 2006). It has previously been demonstrated that plant steroids strongly inhibit SLO haemolytic activity, as does cholesterol (Alouf & Palmer, 1999).



**Fig. 2.** Inhibitory effects of fresh extract of garlic on the haemolytic activity of SLO. Before the initiation of the reaction, bacterial culture supernatant (fixed to 1 HU ml<sup>-1</sup> in PBS buffer) was preincubated for 15 min with various volumes ( $\mu$ l ml<sup>-1</sup>) of extract containing known amounts of allicin (the stock solution contained 1.2 mg allicin ml<sup>-1</sup>). Residual activity was measured and expressed as the mean ± SD.

## Effect of a protecting agent on the reaction

The effect of DTT on the inhibitory activity of allicin on SLO haemolytic activity was investigated. Bacterial culture supernatant (fixed to 1 HU ml<sup>-1</sup> in PBS) was incubated for 15 min with 35 µg allicin ml<sup>-1</sup>; before the initiation of the reaction the mixture was further incubated with 2 mM ml<sup>-1</sup> DTT, and then the haemolytic activity was assayed as described in Methods. The residual activity was calculated as mean  $\pm$  sD in comparison to the allicin-free control (100 % residual activity) and the allicin (35 µg ml<sup>-1</sup>) (-DTT) control (0 %±0 residual activity). The results of this experiment indicated that incubation of allicin-treated SLO with 2 mM DTT completely restored the haemolytic activity of SLO (99.95 %±0.05 residual activity).

The proposed mode of action for allicin is the formation of disulphide bond with a cysteine residue in the active site of enzymes, which inhibits the catalytic activity of thiol enzymes (Rabinkov et al., 1998). A well-known structural feature of SLO is the presence of a reactive cysteine residue in a conserved 11 amino acid sequence domain (Kehoe et al., 1987), so allicin is suggested to inhibit the SLO by binding to this cysteine residue. No enzymic activity for haemolytic effects of SLO and other related toxins have been identified. They make pores on cholesterol-rich membranes by binding and oligomerization on it. Cysteine groups in the conserved 11 amino acid sequence domain may be responsible for the direct binding of the toxin to the target membrane or for the maintenance of the proper conformation for activity (Bhakdi et al., 1985). The lytic and lethal properties of these toxins are suppressed by thiol-group blocking agents and restored by thiols and other reducing agents (Bernheimer & Avigad, 1970; Geoffroy & Alouf, 1982). As the inhibitory effect of allicin was restored by DTT, the result of this study can explain the proposed mode of SLO inhibition by allicin.

#### **Time-dependent inhibition**

The time-dependent inhibitory activity of allicin is shown in Fig. 3. The results show that the haemolytic activity of SLO decreases with the increase of pre-incubation time. Inhibition of SLO with allicin was rapid and more than 50% of activity was abolished after 1 min pre-incubation. Maximal inhibition was achieved on 15 min pre-incubation time.

#### Stability of allicin in garlic extract

From the physiological perspective, the main problem with the systemic use of allicin is its instability in complex biological fluids (Freeman & Kodera, 1995). However, attempts to develop externally used formulations of allicin are progressing (Cutler *et al.*, 2009) and its chemical stability in different solvents has been examined in several studies.

The amount of allicin contained in a freshly prepared garlic extract was determined to be  $1.2 \text{ mg ml}^{-1}$ . As shown in



**Fig. 3.** Time-dependent inhibition of the haemolytic activity of SLO by allicin. Bacterial culture supernatant (fixed to 1 HU ml<sup>-1</sup> in PBS) was pre-incubated with 35  $\mu$ g pure allicin ml<sup>-1</sup> for various time intervals. Residual activity was measured and expressed as mean  $\pm$  SD.

Fig. 4, allicin content decreased with time and temperature. The loss of allicin activity at 4 °C was very low compared to allicin stored at room temperature (22 °C). Its chemical half life at 4 °C was determined to be more than 150 days, while at room temperature it took around 20 days. Lawson (1996) reported a 30–40 days half-life for allicin in aqueous solution at 23 °C. Recently, Fujisawa *et al.* (2008) demonstrated the more stable nature of allicin in aqueous solution at 4 °C. They estimated the half-life of allicin to be about a year and 6.5 days at 4 °C and 23 °C, respectively. The results of our stability experiments are in agreement with these findings showing the half-lives of allicin to be more than 150 days and 20 days at 4 and 22 °C, respectively. Inconsistency may arise from differences in the concentration of allicin in the extracts. It has been



**Fig. 4.** Stability of allicin in garlic extract upon storage at 4  $^{\circ}C(\spadesuit)$  and 22  $^{\circ}C(\blacksquare)$ . Samples of extracts were taken at particular time intervals; allicin content was determined by HPLC.

reported elsewhere that the stability of allicin increases in diluted aqueous solution (Lawson, 1996).

In conclusion, because of the multilateral contribution of SLO in the pathogenesis of GAS infections, the blocking of SLO activity by allicin presents a new 'virulence-based therapy' approach to the treatment of GAS infections and may explain a reported finding of the superiority of garlic extract over penicillin against throat infections (Fortunatov, 1952). Now allicin, beside its broad-spectrum classical antimicrobial property, is considered as a virulence-blocking agent, and has introduced a new approach to the treatment of infectious disease. Study of the effect of allicin on other SH-containing virulence factors is suggested.

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