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Inhibition of Biofilm By Allicin in *Staphylococcus epidermidis*: The Distance Effect

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Abstract

Background: Biofilm inhibition in *Staphylococcus epidermidis* by an allicin solution in close but not direct contact with bacterial cultures was studied. A similar inhibition effect was observed when bacteria were replaced by papain, an enzyme with a sulfhydryl active center.
Objectives: To explain these effects, a parallel assay was made with papain, a sulfhydryl enzyme, and allicin was placed in a separate well. After 1 hour of exposition time, a substrate was added to the papain solution and proteolytic activity was measured at regular time intervals to study possible enzymatic inhibition by allicin vapors.
Materials and Methods: Growth and biofilm formation were measured according to established methods. These values were then averaged for the wells equidistant to a 3 mM allicin solution, and were related to the distance from the allicin well. Similar assays were performed with a solution of papain. After exposition to allicin vapors, a substrate was added and enzymatic activity was measured.
Results: An inhibition effect was observed both in the bacterial cultures and the enzymatic solutions, and the extent of inhibition depended on the distance from the central well that contained the allicin solution.
Conclusions: Allicin vapor, or some decomposition product of allicin, causes inhibition of bacterial growth and biofilm formation. Parallel enzymatic studies confirmed this inhibitory effect and suggest that sulfhydryl enzymes are involved in biofilm formation in the strain studied.

Keywords: Allicin, Biofilm, Sulfhydryl Enzyme, *Staphylococcus epidermidis*

1. Background

Coagulase-negative staphylococci infections are the subject of increasing interest in medicine because of the ability of these bacteria to form biofilms on medical devices, such as catheters and implants. The most important factor in the pathogenesis of these infections is the formation of microbial biofilms, which often respond inadequately to appropriate antimicrobial therapy (1).

Allicin (diallyl thiosulfinate) is the active principle of garlic (2), with antibacterial (3), antifungal (4), and antiparasitic actions (5), among other medicinal properties. The generally accepted mechanism of action of allicin is the reaction with SH groups from cysteinyl residues of enzymes, resulting in its inactivation (6, 7). In a previous paper (8), we described the ability of allicin to inhibit biofilm production in *Staphylococcus epidermidis*. On the inhibition assays, we observed that control cultures containing no allicin, but that were close to cultures treated with high doses of allicin, demonstrated inhibition of growth and of biofilm formation. These striking results led us to a more systematic study, with concentrated aqueous allicin solutions surrounded by bacterial cultures of *S. epidermidis*. Biofilm density was measured, and the values correlated with distance from the allicin well. The hypothesis of a gaseous substance (allicin vapor or some byproduct) diffusing to neighboring wells was confirmed when papain, an enzyme with a sulfhydryl group at the active center, showed progressive inhibition when its distance from the allicin solution was reduced.

2. Objectives

The experiments described in this article were designed to measure the effect of allicin vapor on cultures near allicin solution. The experiments with the papain enzyme were carried out to reinforce the hypothesis regarding allicin vapor as responsible for the effect observed in bacterial cultures.

3. Materials and Methods

3.1. Bacterial Assays

Bacterial cultures of the biofilm-producing strain *S. epidermidis* ATCC 35984 were grown in a 96-well microtiter plate. A central well was filled with 0.1 mL of 3 mM allicin. The surrounding wells were filled with 0.2 mL of trypticase soy broth inoculated with bacteria. The plate was covered with another sterilized plate and incubated for 24 h at 35°C, after which the optical density at 492 nm was read to measure bacterial growth. The wells were then washed four times with PBS, and the bacterial biofilm was fixed by heating, then stained with crystal violet (8, 9). Excess dye was eliminated with water. The plates were air-dried, then the optical density of each well was measured at 492 nm with an automated microplate reader (Anthos 2020). Five assays were performed and the values were averaged for the wells equidistant to the central allicin-containing well.

3.2. Allicin Synthesis and Determination

Allicin was synthesized by peroxidation of diallyl disulfide with magnesium monoperoxyphthalate and tetrabutylammonium hydrogen sulfate as the phase-transfer reagent (10). Allicin was analyzed by reaction with an excess of cysteine, and the excess was determined by spectrophotometry with 5.5'-dithiobis(2-nitrobenzoic acid) DTNB (Fluka) (11).

3.3. Enzymatic Assays

Allicin (170 mg/L) was placed in a central well in the first column of the plate, and the wells in the same column at distances of 9, 18, and 27 mm were filled with papain (Fluka), 6x10 $^{-6}$ M in 0.1 M phosphate buffer, with a pH of 6.0 (12). A control series was filled with papain and covered with tape (right column in blue in Figure 1A), and the plate was covered with another plate. After 1 hour of exposition time, the allicin solution was aspirated and 25 μL of N-benzoyl-L-arginine 4-nitroanilide (BAPNA) 20 mM (Fluka) in dimethyl sulfoxide and 25 μL of water was added to the enzymatic solutions. The plate was taken to a plate reader (Anthos 2020) and the 405 nm absorbance was measured every 2 minutes to monitor the enzymatic activity.

4. Results

Figure 2 shows stained cultures after 24 hours of incubation with allicin in the central well (marked with "A"). Discoloration of the wells contiguous to the allicin well is apparent, as is the darker color in the wells farther away.

The assay was repeated several times and the values for growth and biofilm formation were averaged for wells equidistant to the central allicin well. These values correlated with distance from the allicin well (Table 1 and Figure 3). We observed no significant change in bacterial growth from the first to the last row of wells. Biofilm formation, however, showed strong inhibition in the wells contiguous to the central well at a 9 mm distance. This inhibition was apparent even at 12.7 mm; biofilm formation then increased rapidly from 18 mm to the farthest wells.

Table 1. Inhibition of Growth and Biofilm Formation Based on Distance from the Allicin Well[a]

Distance, mm	Growth, OD = 490 nm	Biofilm, OD = 490 nm
9	0.47 ± 0.05	0.08 ± 0.02
12.7	0.46 ± 0.05	0.51 ± 0.4
18	0.42 ± 0.04	1.34 ± 0.4
20.1	0.41 ± 0.03	1.45 ± 0.2
25.4	0.45 ± 0.03	1.6 ± 0.1

[a]Values are expressed as mean and SD for n = 5.

Parallel experiments with papain solutions were performed to study the effect of allicin solutions on neighboring wells. Papain is a proteolytic enzyme containing an active cysteinyl residue that can be inactivated by allicin. When in contact with the substrate BAPNA, papain cleaves the molecule to give yellow p-nitroaniline (Figure 4). Enzymatic activity can be followed spectrophotometrically at 405 nm (see material and methods).

A representation of the microtiter plate is depicted in Figure 1A. In the first column are the wells with papain, and the allicin solution is in the central well. The column on the border of the plate was filled with the enzymatic solution and covered with tape; these wells were taken as the controls. After 1 hour of exposition, the substrate was added and enzymatic activity was measured. Figure 1B shows the enzymatic activity curves with a slope variation following the order C > B > A, which indicates increased inhibition of the enzyme as the distance from the allicin well diminishes. Moreover, these curves had a lower slope than those of the sealed wells.

5. Discussion

The most plausible explanation for the results described above is the diffusion of some gaseous product through the space between the culture plates, reaching the neighboring wells and producing both bacterial and enzymatic inhibition. Mass spectrometry analyses at room temperature (HPLC-MS) have confirmed the presence of thiosulfinates in allium odors (13, 14), so allicin vapors may be considered the agent responsible for this effect.

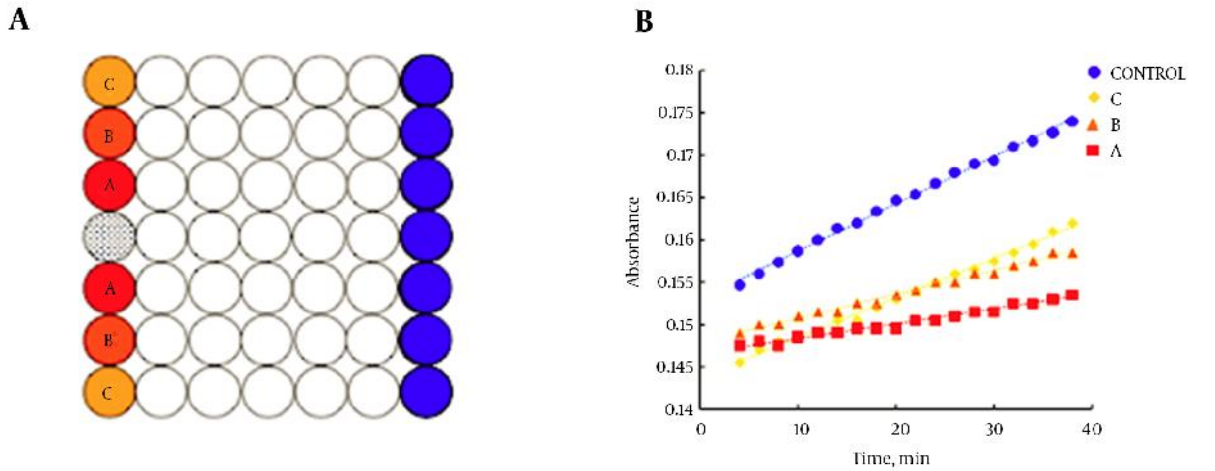

Figure 1. A, disposition of the wells with papain: wells A, B, and C. Blue circles are sealed control wells, the allicin solution is represented by a dotted disc. B, averaged activity of papain in the wells, with allicin in the central position. Trend lines are drawn for comparison of the slopes.

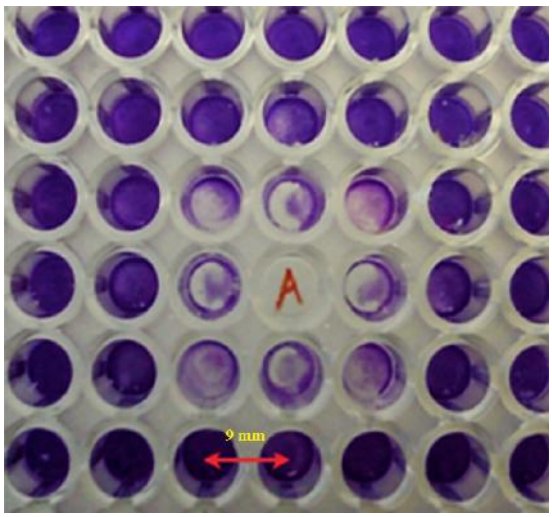

Figure 2. Inhibition of staphylococcal biofilm by allicin located in the central well (marked with "A"). The distance between the centers of two wells (double arrow) was 9 mm; from this value, the distances to other wells were calculated.

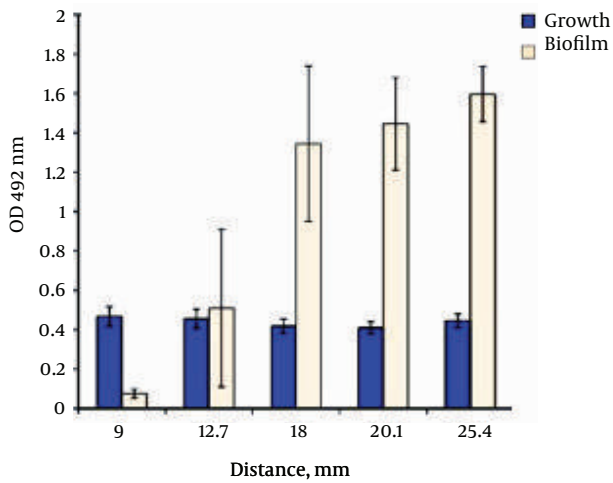

Figure 3. Growth and Biofilm Formation Depending on Distance from the Allicin Well

In bacterial studies of inhibition/distance (Figure 3), we can see that biofilm inhibition is present even at relatively long distances of 18 mm. Since the presence of allicin solution does not seem to affect the normal growth of bacteria in the surrounding wells while biofilm formation is greatly reduced, especially in the wells near the allicin solution, we can imagine a specific inhibition mechanism (not depending on bacterial population) of biofilm production by allicin, which confirms previous results showing biofilm inhibition at subinhibitory concentrations of allicin (8). The close agreement between bacterial cultures and enzymatic assays leads us to ascertain that an inhibition effect is produced by allicin vapors1. These results also suggest that biofilm production is mediated by enzymes with free sulfhydryl groups, in particular those involved in the production of the adherence factor known as polysaccharide intercellular adhesin (PIA) (15).

Infections originating from the adherence of *S. epidermidis* to implants require a long treatment period with antibiotics. The ability of allicin to inhibit biofilms suggests its use alone or in combination with antibiotics in order

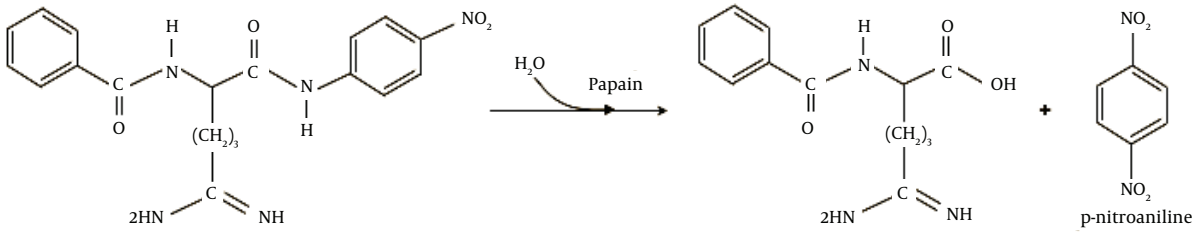

Figure 4. Enzymatic Effect of Papain on the Substrate BAPNA

to prevent these infections; moreover, antibiotic/allicin mixtures could enhance antibacterial action so that lower doses of the antibiotic would be required (16, 17). A recent article (16) describes the use of mixtures of allicin with vancomycin in rabbit models, resulting in inhibition of biofilms on implant surfaces; these in vivo findings allow us to envisage allicin use in hospital practice.

Special care must be taken in assays with high concentrations of allicin, as the vapor effect described here can affect the neighboring wells near the allicin and distort the results (for example, as in the control cultures originally without allicin).

Footnotes

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