GLUTATHIONE AND CYSTEINE INHIBITION OF ALLYL ISOTHIOCYANATE ANTIMICROBIAL ACTIVITY AGAINST *Escherichia coli* O157:H7

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Introduction

Escherichia coli was first isolated in 1855 from children's feces by the German bacteriologist Theodor Escherich and was recognized as a commensal bacteria from the human gastrointestinal tract. In 1982, two hemorrhagic colitis outbreaks were found to be due to a new *E. coli* strain called O157:H7. The first outbreak occurred in Oregon and three months later 21 patients were diagnosed with the same problem in Michigan. Both outbreaks were related to undercooked hamburger consumption from the same fast food chain (Buchanan and Doyle, 1997). To date human infections attributed to *E. coli* O157:H7 have been reported in over 30 countries. Most outbreaks have been associated with the consumption of undercooked meats, especially ground beef or hamburger patties (Nadarajah et al., 2005). As an alternative to reduce pathogens in foods, natural antimicrobials have been studied. Allyl isothiocyanate (AIT) results from hydrolysis of glucosinolates by endogenous myrosinase in cruciferous plants, including mustard and horseradish. Although the antimicrobial activity of AIT varies widely, this volatile compound has been shown to limit the survival of *E. coli* including serotype O157:H7 in meat (Delaquis and Sholberg, 1997; Nadarajah et al., 2005). AIT is also an electrophilic compound and can react with a wide rage of nucleophiles such as amines, thiols, hydroxyls and sulfites (Cejpek et al., 2000), and it is suggested that AIT inhibits enzymes and can disrupt the microbial cell membrane, leading to bacterial death (Ahn et al., 2001). However, little work has been conducted to understand how AIT acts against bacteria and whether the reaction of AIT with intrinsic components of food products can occur, reducing its antimicrobial activity.

Material and Methods

Bacterial strains. Six strains of *E. coli* O157:H7 numbered 7110 (pathogenic), 1840, 0628, 0627, 3581 and 0304 (non-pathogenic) were used. The cultures were grown at 37°C for 16 h in Luria broth prior to the experiments. The overnight cultures were inoculated into fresh medium. Cells in mid-exponential phase (optical density ~ 0.6, at 600 nm) were used.

Antioxidants and AIT. Fresh Luria broth was prepared (pH 7.4) containing either 10mM glutathione (GSH) or 10mM ascorbic acid. The mixtures were filter-sterilized through a 0.22 mm membrane (Millipore). Then, 10 ml aliquots were added to screw capped tubes, followed by 0.1 ml *E. coli* O157:H7 inoculum and 100 mg/ml of AIT. Standards containing antioxidant + inoculum and AIT + inoculum were also prepared. Cell density was measured after 16 h (600 nm) at 37ºC and samples were inoculated on Luria agar for bacterial enumeration at 37ºC for 24 h. Cysteine (10 mM) and glycine (10mM) were also used following the procedure described previously to verify the interaction of AIT and other thiol and amine-containing substances, respectively.

Measurement of free thiol content in AIT-GSH solution. Equimolar concentrations (0.1 mM) of AIT and GSH were dissolved in deionized water and kept at 37ºC with 200 rpm agitation for 30 min, 1, 2, 4, 6, 12 and 24 h. Standards containing either 0.1mM GSH or 0.1 mM AIT were prepared and kept at the same conditions to determine the behavior of these compounds alone. The thiol content was determined using Ellman's method (1959). Briefly, 1 ml sample of the reaction or standard solutions were transferred to a photometer cuvette and 33 µl of a 10mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution was added. Color developed within 2 min, and the absorbance was measured at 412 nm.

HPLC and LC-MS analyses. Time course HPLC analyses were performed using a C18 reverse-phase column (250mm X 4.6 mm). Elution was carried out for 40 min at a flow rate of 0.5mL/min, using a solvent system containing 40% acetonitrile (0.1% acetic acid) and 60% water (0.1% of acetic acid). The eluate was monitored simultaneously at 220 and 254 nm. Electrospray mass spectrometry was used to determine the byproducts of the reaction between AIT and GSH.

Statistical analyses. The data reported are the average values from a minimum of three experiments. Differences between treatments were analyzed by Tukey's test. A *P* value of 0.05 was used as the cut-off for statistical significance.

Results and Discussion

Addition of antioxidants to the medium: There was no significant difference in broth optical density when the medium with AIT contained ascorbic acid. However, the addition of glutathione completely reversed the AIT antimicrobial activity. These results were confirmed through bacterial count. In addition, when cysteine and glycine were added to broth containing AIT, only the tubes containing cysteine showed bacterial growth. These results suggest that AIT is more likely to react with free thiol group-containing substances in such a way that it loses its antimicrobial activity (Figure 1). Moreover, meat and meat derivates are a good source of free thiol-containing compounds, and this could make it necessary to use higher doses of AIT to achieve similar results as with thiol free foods.

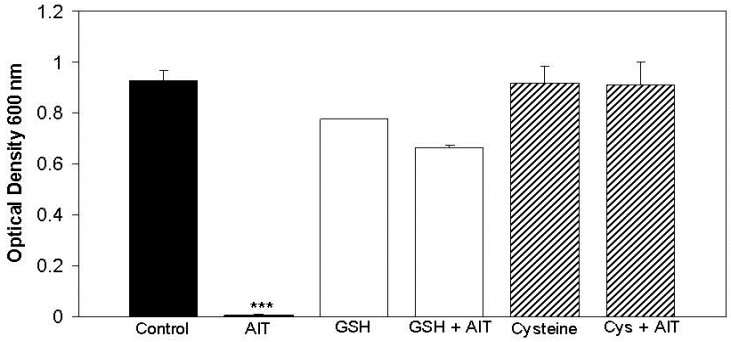

Figure 1. Effect of glutathione (10 mM) and cysteine (10mM) on AIT (100 mg/mL) antimicrobial activity against *E. coli* O157:H7 #0304. AIT significantly decreased microbial growth at 16 h (***P>0.001). Glutathione and cysteine almost completely reversed the growth inhibition. All other strains gave similar results.

AIT and glutathione reaction: A time-course study revealed that AIT reacted with glutathione in aqueous environments, forming a substance or substances that did not contain a free SH group. The reaction was more pronounced after 24 h, when the glutathione initially caused a high intensity of yellow color, but the color intensity subsequently faded. This result suggests that glutathione's thiol group is involved in the reaction, and probably forms a disulfide bond by attacking the AIT sulfur atom.

Chemical analyses: HPLC results confirmed that AIT and glutathione were reacting and forming one main byproduct. The molecular weight of the reactant by LC-MS analysis was ~406, coinciding with the molecular weight of an AIT-GSH conjugate.

Conclusions

This study demonstrated that AIT appears to react with thiol-containing substances and loses its antimicrobial activity. This is an important observation, since AIT has been proposed as an agent which can enhance safety of meat and meat products. Meat can contain up to 1% of non-proteic sulfhydryl substances by weight, and glutathione accounts for approximately 76% of this (Faustman and Cassens, 1991). Further studies are under way to confirm that this reaction occurs, and to develop techniques to prevent it from happening.

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