

Antimicrobial Properties of Sinigrin and its Hydrolysis Products

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ABSTRACT

Sinigrin, a thioglucoside of Cruciferae plants, may be enzymatically hydrolyzed to yield up to four distinct aglycones when the plants are injured or mechanically disrupted. These aglycones, allyl isothiocyanate (AITC), allyl cyanide (AC), 1-cyano-2,3-epithiopropene (CETP), and allyl thiocyanate (ATC), were tested for their minimum inhibitory concentration (MIC) in broth to 9 species of bacteria and 8 species of yeasts. Sinigrin, AC, and CETP at 1,000 ppm were not inhibitory to any of the bacteria or yeasts tested. The inhibitory activity of ATC was uncertain due to its gradual conversion to AITC. AITC had an MIC of 50 to 1,000 ppm for bacteria and 1 to 4 ppm for nonxerotolerant yeasts, and, against xerotolerant yeasts at 50 ppm, it retarded but did not prevent growth.

Key Words: sinigrin, allyl isothiocyanate, antimicrobial

INTRODUCTION

SINIGRIN IS A GLUCOSINOLATE FOUND IN cruciferous vegetables. It is considered a major precursor of the sulfurous flavors in crucifers. Upon injury or mechanical disruption of plant tissue, sinigrin is hydrolyzed by myrosinase in a reaction that produces up to four distinct compounds (Fig. 1). Allyl isothiocyanate (AITC), allyl thiocyanate (ATC), allyl cyanide (AC), and 1-cyano-2,3-epithiopropene (CETP) are found in some plant homogenates (Table 1). Each compound contributes to the flavor and characteristic aroma of such plants.

The presence of sinigrin hydrolysis products also depends on processing conditions (Bones and Rossiter, 1996; Chew, 1988; Fenwick et al., 1983; Olsen and Sorensen, 1981; West et al., 1977). AITC is usually produced at neutral pH, and AC production occurs at pH 4 (Bones and Rossiter, 1996). CETP is formed as a result of the combined action of ferrous ion and epithiospecifier protein on myrosinase (Springett and Adams, 1988). The formation of ATC is unclear. A mechanism has been proposed (Hasapis and MacLeod, 1982) in which an enzyme causes Z-E isomerization of the aglycone. The E-aglycone then rearranges to form ATC.

Brabban and Edwards (1995) reported that

sinigrin had little effect upon the growth of microorganisms, but its hydrolysis products resulted in inhibition of growth. They did not determine, however, which hydrolysis product(s) was responsible for the antimicrobial activity. AITC isolated from mustard seed and horseradish has long been recognized as a potent antimicrobial substance (Delaquis and Mazza, 1995). Kyung and Fleming (1996) reported that AITC had a minimum inhibitory concentration (MIC) of 50 to 500 ppm for bacteria and 1 to 4 ppm for yeasts.

Our objective was to determine the relative antimicrobial activities of four sinigrin hydrolysis products. A further objective was to test any effects of pH on relative inhibitory activity of AITC and sodium benzoate against *Escherichia coli*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae*. The potential importance of AITC as a food preservative and as a controlling factor in vegetable fermentations was considered.

MATERIALS & METHODS

Chemicals

Sinigrin and benzoic acid were purchased from Sigma Chemical Company (St. Louis, MO). AITC, *m*-Chloroperbenzoic acid, thiourea, hydroquinone, allyl bromide, and AC were obtained from Aldrich Chemical Company (Milwaukee, WI). Sodium sulfate and magnesium sulfate were purchased from Mallinckrodt Specialty Chemicals Company (Paris, KY).

Synthesis of 1-cyano-2,3-epoxypropene

The synthesis of 1-cyano-2,3-epoxypropene was adapted from the general procedures of Hall et al. (1971) for 1-cyano-4,5-epoxybutane.

m-Chloroperbenzoic acid (0.2 mole) was added to dry methylene chloride (200 mL; dried over anhydrous sodium sulfate; with stirring at room temperature). Then, AC (0.2 mole) was added drop-wise to the reaction mixture and stirred overnight (ca 15h) at room temperature. The reaction mixture was vacuum filtered and the precipitate washed with 100 mL cold pentane. The combined filtrates were then concentrated under reduced pressure to yield a white solid. The solid was dissolved in a mixture of methylene chloride and cold pentane and reconstituted at 50°C. The white solid was then distilled under reduced pressure (49°C, 1 mm Hg) to yield 1-cyano-2,3-epoxypropene (4.94g, 29.7%), which was

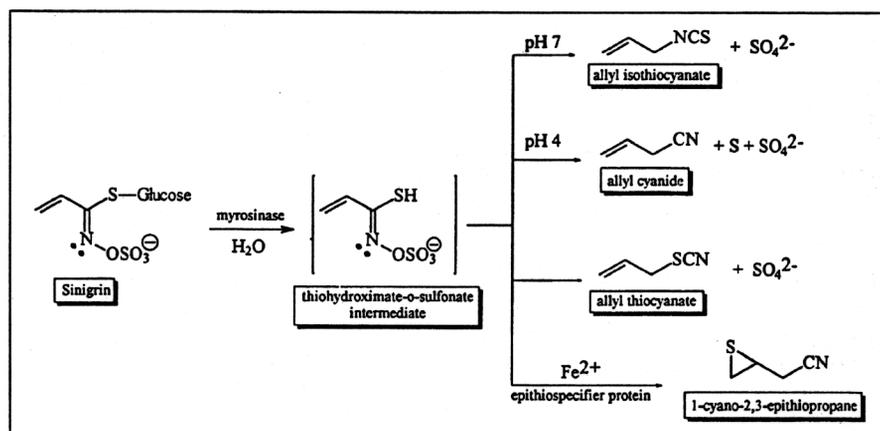


Fig. 1—Sinigrin and its hydrolysis products. Based on the myrosinase-glucosinolate system (Bones and Rossiter, 1996).

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Table 1—Sinigrin hydrolysis products and their occurrence in plants

Compound	Plant sources	Aroma	Reference
AITC	mustard, horseradish, cabbage, cauliflower, Brussels sprouts, turnips, kale, collards	mustard, horseradish	Delaquis and Mazza (1995); Fenwick et al. (1983); Shankaranarayana et al., (1982)
ATC	stinkweed, horseradish	garlic	Fenwick et al. (1983); Shankaranarayana et al. (1982)
CETP	cabbage, Brussels sprouts, crambe seed	sulfurous	Shankaranarayana et al. (1982)
AC	cabbage, cauliflower, Brussels sprouts	mustard, horseradish	Daxenbichler et al. (1977)

found to be >99% pure by HPLC. ^1H NMR (CDCl_3): δ 2.73 (m, 3H), 2.87 (t, $J = 3.7$ Hz, 1H), 3.20 (m, 1H). These nuclear magnetic resonance (NMR) data are: nucleus, ^1H , NMR (solvent, CDCl_3): δ (chemical shift) value (splitting: s = singlet, d = doublet, t = triplet, m = multiplet; J (coupling constant; number of H's in signal).

Synthesis of 1-cyano-2,3-epithiopropene

The synthesis of 1-cyano-2,3-epithiopropene was adapted from the general procedure of Luthy and Benn (1979), where 1-cyano-2,3-epoxypropane (2.89g) was dissolved in 9 mL of acetone. This solution was added drop-wise to a stirred mixture of benzoic acid (4.27g) and thiourea (2.6g) in acetone (22 mL). After 2h, a white precipitate formed, was collected by filtration, and washed with diethyl ether. The air-dried precipitate was dissolved with stirring in 190 mL of aqueous acetone (3:7, acetone-water). Sodium carbonate solution (2g in 10 mL water) was added (5 mL) drop-wise to the solution. The reaction mixture was then extracted with benzene (30 mL). The aqueous phase was treated with more sodium carbonate solution (2 mL) and re-extracted with benzene (30 mL). The combined benzene extracts were washed with water, saturated sodium chloride solution, and dried over magnesium sulfate which was then filtered off and hydroquinone (10 mg) was added to inhibit polymerization. The solvent was concentrated under reduced pressure to yield a yellow liquid. This liquid was distilled under reduced pressure (68°C, 1 mm Hg) (lit. 65.7 ca 0.1 mm) to yield 1-cyano-2,3-epithiopropene (1.36g, 39%), which was >99% pure by HPLC. ^1H NMR (CDCl_3): δ 2.32 (d, $J = 4.8$ Hz, 1H), 2.60 (d, $J = 6.0$ Hz, 1H), 2.80 (d, $J = 5.8$ Hz, 2H), 3.09 (m, 1H).

Synthesis of ATC

The synthesis of ATC was carried out according to the general procedure of Slater (1992). Briefly, a mixture of allyl bromide (30 mmole) in 95% ethanol (10 mL) and sodium thiosulfate pentahydrate (30 mmole) in

water (10 mL) was stirred under reflux for 1h. After cooling to room temperature, the mixture was extracted three times with diethyl ether. The aqueous residue was cooled in ice, and potassium cyanide (30 mmole) was added with stirring during 15 min. After a further 20 min, the cold mixture was extracted four times with pentane, dried over anhydrous sodium sulfate, and concentrated at 0°C to yield only ATC (1.56g, 53%), which was >99% pure by HPLC. ^1H NMR (CDCl_3): δ 3.56 (d, $J = 7.2$ Hz, 2H), 5.40 (m, 2H), 5.93 (m, 1H).

MIC for sinigrin hydrolysis products

The MIC of pure compounds was determined as described by Kyung and Fleming (1996). The antimicrobial activity of test compounds for nonlactic acid bacteria (LAB) was tested in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) with 2% added glucose and for LAB in MRS broth (Difco). The antimicrobial activity of test compounds for yeasts was tested in yeast morphology broth (YMB; Difco). Each test compound was filter-sterilized (0.2 μ , Costar bottle filter, Costar Corp., Cambridge, MA) and dissolved in the appropriate heat-sterilized medium and pipetted into 16 mm \times 150 mm culture tubes with caps. The pH of the medium was confirmed to be the same before and after addition of the filter-sterilized compounds. The final volume in each tube was 10 mL. Initial concentrations of test compounds varied between 100 ppm and 1,000 ppm in increments of 100 ppm. When a test microorganism did not grow at 100 ppm, concentrations varied between 10 and 100 ppm in 10 ppm increments. If no growth occurred at 10 ppm, concentrations varied between 1 and 10 ppm in 1 ppm increments. The media were inoculated with bacteria and yeasts to obtain an initial cell population of $\approx 10^5$ cells/mL and statically incubated. Complete absence of visual turbidity after incubation for 48h at either 30°C or 37°C was regarded as nongrowth. The 48h was found to be sufficient for growth of bacteria and yeasts to produce visible turbidity in the absence of added test compounds. Incu-

bation continued beyond 48h to 96h to observe any changes in those compounds that caused no visible turbidity.

Comparison of MIC values at different pH values

TSB with 2% added glucose was adjusted to the desired pH with 1N HCl for comparing the relative efficacy of AITC and sodium benzoate for inhibition of *E. coli* and *S. aureus*. YMB was adjusted to the desired pH with either 1N HCl or 1N NaOH for comparing the relative efficacy of AITC and sodium benzoate for inhibition of *S. cerevisiae*.

Microorganisms

Bacteria and yeast cultures were maintained in the U.S. Food Fermentation Laboratory culture collection and were obtained from the original sources indicated (Tables 2, 3). Cultures were stored at -84°C in basal media containing 16% glycerol, which included MRS broth for LAB, TSB for other bacteria, and YMB for yeasts.

RESULTS & DISCUSSION

Effect of sinigrin and its hydrolysis products on MIC

Sinigrin and its degradation products (AITC, ATC, CETP, and AC) were tested for antibacterial activity (Table 2). Sinigrin, AC, and CETP at concentrations up to 1,000 ppm were not inhibitory to the growth of bacteria. ATC has been reported to be antimicrobial (Zsolnai, 1966) and appeared to be effective against 3 strains of Gram-negative and 1 strain of Gram-positive bacteria. MIC values ranged between 200 and 400 ppm. Emerson (1971) reported that ATC rearranged to the more stable isomer, AITC, via a quasi 6-membered ring transition state. The low activation energy (23.8 kcal/mol) and large negative energy of activation (-9.4 kcal/mol) suggest that this isomerization is kinetically favored in one direction. The antimicrobial activity of ATC was, therefore, uncertain due to its conversion to AITC, which had consistent activity against Gram-negative and Gram-positive non-LAB, with MIC between 100 and 200 ppm. LAB were more resistant, with MIC between 500 and 1,000 ppm. The MIC values for AITC did not change when incubation time was extended to 96h. This indicated that doubling the incubation time was not sufficient to enable growth of bacteria in the presence of AITC.

Sinigrin, AC, and CETP up to 1,000 ppm were not inhibitory to growth of yeasts (Table 3). ATC was inhibitory only to *Torulopsis etchellsii* and *Torulaspora delbruekii*. AITC was strongly inhibitory to the growth of 5 yeast species, with an MIC of 4 ppm. The apparent activity of ATC against the *Torulopsis* species may have been due to conversion

Table 2—MIC (ppm) of sinigrin and its hydrolysis products against selected bacteria

Strain	Sinigrin	AC	CETP	ATC	AITC
<i>Escherichia coli</i> 33625 ^a	— ^c	—	—	400	200
<i>Escherichia coli</i> NC101 ^b	—	—	—	200	100
<i>Pseudomonas fluorescens</i> MD13 ^b	—	—	—	200	200
<i>Aeromonas hydrophilia</i> 7966 ^a	—	—	—	—	200
<i>Staphylococcus aureus</i> 4220 ^b	—	—	—	200	200
<i>Bacillus subtilis</i> IS75 ^b	—	—	—	—	200
<i>Pediococcus pentosaceus</i> FFL48 ^b	—	—	—	—	1,000
<i>Leuconostoc mesenteroides</i> FFL44 ^b	—	—	—	—	500
<i>Lactobacillus brevis</i> MD42 ^b	—	—	—	—	1,000
<i>Lactobacillus plantarum</i> MOP3 ^b	—	—	—	—	—

^aReceived from American Type Culture Collection, Rockville, MD.

^bReceived from USDA-ARS Food Fermentation Laboratory Culture Collection, Raleigh, NC.

^cMinus sign (-) indicates no detectable inhibition at 1,000 ppm.

Table 3—MIC (ppm) of sinigrin and its hydrolysis products against selected yeasts

Strain	Sinigrin	AC	CETP	ATC	AITC
<i>Saccharomyces cerevisiae</i> MD7 ^b	— ^c	—	—	—	4
<i>Pichia membranefaciens</i> Y1617 ^a	—	—	—	—	2
<i>Torulopsis etchellsii</i> Y6651 ^b	—	—	—	50	1
<i>Hansenula mrakii</i> Y1617 ^b	—	—	—	—	4
<i>Torulaspora delbruekii</i> Y866 ^a	—	—	—	50	4
<i>Saccharomyces rouxii</i> Y2547 ^a	—	—	—	—	—
<i>Zygosaccharomyces globiformis</i> SPY9 ^a	—	—	—	—	—
<i>Zygosaccharomyces bisporus</i> T12626 ^a	—	—	—	—	—

^aReceived from USDA-ARS Culture Collection, Peoria, IL.

^bReceived from USDA-ARS Food Fermentation Laboratory Culture Collection, Raleigh, NC.

^cMinus sign (-) indicates no detectable inhibition at 1,000 ppm.

of a portion to AITC. The MIC values were 50 to 100 times lower for yeasts than for bacteria. These results confirmed Kyung and Fleming (1996), who reported similar MIC values. However, xerotolerant yeasts (*Zygosaccharomyces* and *Saccharomyces rouxi*), grew in the presence of AITC. These are yeasts that grow in the presence of high concentrations of either sugar or salt (Rose, 1987).

Isothiocyanates may be antimicrobial due to their binding to sulfhydryl groups on the active sites of enzymes important to the survival and growth of microorganisms. Tang (1974) proposed such a mechanism to explain the inhibition of papain by benzyl isothiocyanate. Kolm et al. (1995) also supported this hypothesis. Enzymes such as glutathione transferases promote addition of the thiol group of γ -glutamylcysteinylglycine to the electrophilic central carbon of the isothiocyanate group to form dithiocarbamates (Kolm et al., 1995).

pH Effects on antimicrobial activity of AITC

The effects of pH on antimicrobial activity of AITC against *E. coli*, *S. aureus*, and *S. cerevisiae* were studied to assess its potential as a food preservative in comparison with sodium benzoate (Fig. 2). The MIC of AITC varied from 60 to 140 ppm over pH 5 to 7. The MIC of benzoate, however, ranged from 350 to 3,000 ppm over the same pH range.

AITC was, therefore, about 6 to 21 times more inhibitory to the growth of the Gram-negative *E. coli* 33625, than was benzoate. Against *S. aureus* RN4420, the MIC of AITC ranged from 120 to 220 ppm over pH 5 to 7. The MIC of benzoate ranged from 350 to 10,000 ppm over the same pH range. AITC was, therefore, about 3 to 45 times more inhibitory to the growth of the Gram-positive *S. aureus* RN4420 than was benzoate. Against *S. cerevisiae* at different pH values, sodium benzoate was not inhibitory up to 20,000 ppm, but the MIC of AITC was 4 ppm over pH 4 to 7.

The antimicrobial activity of AITC did not vary greatly over pH 4–7 as did benzoate and, in the case of *S. cerevisiae*, no variation was evident. The antimicrobial activity of sodium benzoate depends upon the pH, and it is most effective in the protonated form at lower pH (Chiple, 1993). The fully protonated form of benzoic acid penetrates the cell membrane, dissociates to lower internal cell pH, and thereby stops cell growth. AITC, however, does not have an acidic proton with which it can dissociate and, therefore, may diffuse more readily through the cell at any pH, as has been proposed by others for nonionized compounds (Chiple, 1993; Kyung and Fleming, 1997). This may explain why its activity did not vary over a wide pH range. Methyl methane thiosulfinate also has been shown to be an effective antimicrobial compound over a wide pH range, perhaps for a similar reason

(Kyung and Fleming, 1996).

Results indicated that AITC was the most inhibitory of the sinigrin hydrolysis products. AITC has very strong antimicrobial properties, and is much more inhibitory than sodium benzoate. Factors influencing the generation of AITC may be important in regulating the fermentation of vegetables containing its precursor compound sinigrin. AITC may also function as a preservative in foods where it is naturally present. Further research is needed to show at what level AITC could be inhibitory without adversely affecting flavor quality.

This study involved the antimicrobial activity of sinigrin hydrolysis products in broth culture. There is also considerable interest in the application of gaseous AITC for its antimicrobial activity in packaged foods. Delaquis and Sholberg (1997) found that *Salmonella typhimurium*, *Listeria monocytogenes* Scott A, and *E. coli* 0157:H7 were inhibited when the agar surface on which they were deposited was exposed to 1 ppm AITC/L of gas over the surface. We found MICs of 50 to 1,000 ppm in broth culture for bacteria species. A fuller understanding of the relationship between gaseous and dissolved concentrations

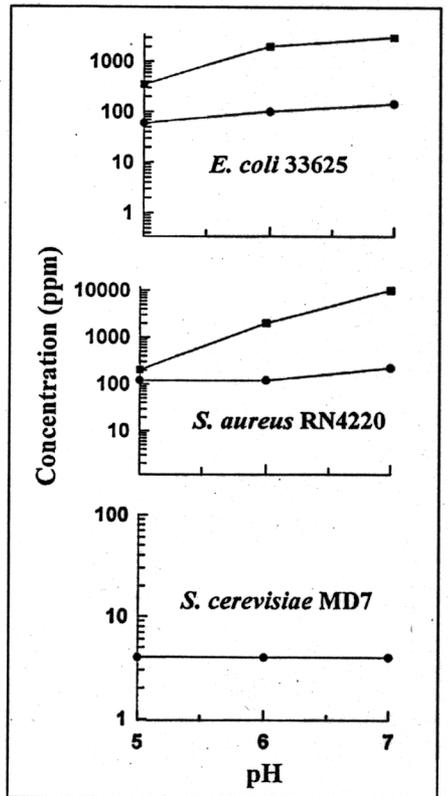


Fig. 2—Minimum inhibitory concentrations (ppm of AITC (●) and sodium benzoate (■) against *E. coli*, *S. aureus*, and *S. cerevisiae*, as influenced by pH. Note: A minimum inhibitory concentration could not be detected for sodium benzoate against *S. cerevisiae* up to 20,000 ppm.

of AITC for antimicrobial activity may lead to more effective applications of this natural food preservative.

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