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Original Research Article

Anti-Cancerous/Anti-Bacterial Activities of Allicin Generated *In Situ* from Diastereo Pure Alliins by Alliinase

.

ABSTRACT

Aims: To facilitate allicin generation *in-situ* from pure diastereomers of alliin by enzymatic reaction of alliinase and assess its anti-cancerous/anti-bacterial activities.
Study design: Chemical synthesis and *in-vitro* assay of anti-cancerous/anti-bacterial activities.
Place and Duration of Study: Protein Research Laboratory, Research Resources Center, University of Illinois at Chicago, between February 2014 and February 2015.

Methodology: Cancer MTT cell viability assay, bacterial plate-diffusion growth inhibition assay, and flow cytometry cell cycle analysis have been used to demonstrate the anti-cancerous/anti-pathogen activities of the *in-situ* allicin. Diastereomers of alliin are produced by H_2O_2 oxidation of deoxyalliin, which is prepared by mixing L-cysteine and allyl bromide. Deoxyalliin and diastereomers of alliin are purified to high purity with repeated fractional crystallization. In addition, fluorenylmethyloxycarbonyl (Fmoc) protected alliin and alliin methyl ester are synthesized and purified with RP-HPLC to test the importance of amino and carboxyl groups of alliin in alliinase enzymatic reaction. Alliinase is produced by a simple and effective method from an aqueous garlic extract

Results: Results from spectrophotometric alliinase activity assay indicate that (+)-L-alliin is more reactive toward alliinase than (-)-L-alliin, and both amino and carboxyl groups of the cysteine portion of alliin are critical in alliinase enzymatic reaction. Results from cancer MTT cell viability, bacterial plate-diffusion growth inhibition, and flow cytometry cell cycle analysis confirm that *in-situ* allicin is as active as allicin purified from aqueous garlic extract or allicin synthesized chemically in a dose-dependent manner.

Conclusion: We describe here facile pathways to synthesize diastereomerically pure alliins and isolate allinase. The *in-situ* allicin conversed from alliin by allinase is very active. The data obtained here provide useful information on the design of the *in-situ* allicin strategy.

Keywords: Allium sativum; alliin; allicin; alliinase; anti-cancerous; anti-bacterial.

1. INTRODUCTION

Allicin protects garlic from pests, insects, and microorganisms [1-3]. It has lipid-lowering, anti-blood coagulation, anti-hypertension, anti-thrombotic, anti-inflammatory, anti-oxidant, anti-cancerous, anti-viral, anti-microbial, and anti-parasitic activities with low toxicity towards human [2-4]. Not surprisingly, garlic is the best-selling herbal supplements in the United States and allicin has been explored as a potential lead compound for drug design [5,6]. Allicin is formed by the action of the enzyme alliinase on alliin. Purified allicin is unstable with a characteristic smell of garlic [7-9]. It decomposes into biologically less active oil-soluble organosulfur compounds (OSCs) such as diallyl sulfide, diallyl disulfide, diallyl trisulfide (allitridi), dithiins, and ajoene and some water-soluble OSCs such as S-allyl cysteine and S-allyl mercaptocysteine [10-12]. Allicin reacts rapidly with free thiol groups

UNDER PEER REVIEW

[13,14]. As soon as allicin is taken up by the body or is in the circulatory system, it reacts with accessible thiol groups, penetrating biological membranes and decomposing to other compounds. Therefore it disappears from the circulation within a few minutes after injection [13-15]. This is why the majority of the potent anti-cancerous/anti-bacterial activities effects of allicin were demonstrated in vitro and makes a pharmaceutical application of allicin implausible [4,8,16].

However, the usage of allicin in medical areas mentioned above remains an attractive endeavor. In recent years there has been a growing interest of circumventing the instability of allicin by use a binary system consisting of the plant enzyme alliinase and its substrate alliin to generate allicin *in situ* [4,17-19]. In this binary system, the two inactive stable components generate an active compound that exhibit biological activity *in situ*. In one proof-of-concept study, alliinase is conjugated to an anti-CD20 antibody rituximab which successfully inhibits the proliferation of CD20+ B chronic lymphocytic leukemia via allicin produced from alliin *in situ* [18]. It is therefore possible to initiate biological activity in a controlled, effective manner. Apart from circumventing the instability of allicin, this binary system has additional advantages, such as low toxicity of its individual components and site-directed selective activation. Furthermore, alliinase is able to react besides alliin other S-alk(en)yl-L-cystene solfoxides such as dihydroalliin, S-isopropyl-L-cysteine sulfoxide, and S-butyl-L-cysteine sulfoxide [20]. In general, substrates of allinase reaction contain the following structural features: (1) They must be derivatives of cysteine. (2) The sulfur atom of the cysteine derivative must link to alk(en)yl group. (3) The amino group of the cysteine portion of the compound must not be blocked. (4) The sulfur atom of the cysteine derivative must be a sulfoxide. These observations pave the way to a range of novel binary systems with specific chemical and biochemical properties.

In situ allicin has shown promising applications *in vivo*. However, more information on reagent preparation and reaction conditions are needed to realize the biomedical potential of *in situ* allicin strategy. For example, there is lack of information on the efficacy of *in situ* allicin systems produced from pure diastereomers or derivatives of alliin. Here we developed a facial synthesis for mass production of diastereo pure alliins to alleviate the need of purchasing expensive alliin, an effective method of purifying alliinase, and simple methods of assessing the efficacy of the *in situ* allicin. In addition, the inactivity of fluorenylmethyloxycarbonyl (Fmoc) protected alliin and alliin methyl ester in alliinase enzymatic reaction enable us to conclude that the amino and carboxyl moieties of alliin are critical for alliinase reaction. Results indicate that the length of alliin in cancer cell culture before addition of alliinase is most effective within 3 days and ineffective after 7 days as a substrate of the enzymatic reaction of alliinase. In addition, allinase reacts less with (-)-L-alliin than with (+)-L- alliin. Cancer MTT cell viability assay, alliinase assay, bacterial plate-diffusion growth inhibition assay, and flow cytometry cell cycle analysis are effective in validating the anti-cancerous/anti-pathogen activities of the *in-situ* allicin.

2. MATERIAL AND METHODS

2.1 Materials

All reagents and solvents were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA) with the highest purity available and used without further purification. Allicin was prepared from either aqueous garlic extract or by H_2O_2 oxidation of allyl disulfide with HPLC purification as previously described [8, 9]. Double distilled water was used throughout the experiments. Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 6538™) and Escherichia coli (Migula) Castellani and Chalmers (ATCC® 8739™) were from ATCC

UNDER PEER REVIEW

(Manassas, VA, USA). PD10 desalting column packed with Sephadex G-25 medium (GE Healthcare Biosciences, Piscataway, NJ, USA) was used to separate low-molecular weight (< 1000 Da) from high-molecular weight (> 5000 Da) biomolecules. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker FT-NMR spectrometer (Billerica, MA, USA) at 900 and 225 MHz, respectively. The nmr spectra were measured in D_2O or CD_3CN at 25 ^{o}C and the ^{1}H and ^{13}C chemical shift values (δ) were reported in parts per million (ppm) relative to the TSP (δ 0.0 ppm) and DMSO (δ 39.5 ppm), respectively. Coupling constants (J values) were given in Hz. Multiplicities are reported as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, td = triplet of doublets, tt = triplet of triplets, q = quartet, dq = doublet of quartet. Accurate mass measurements (ESI-HRMS) were recorded on a LC-MS IT-TOF (Ion Trap-Time of Flight) mass spectrometry (Shimadzu Scientific Instruments, Columbia, MD, USA). Resolution was set between 5000 and 10000. It delivered high mass accuracy and high mass resolution independent of MS mode with a dual source and a suitable external calibrant. Thin-layer chromatography was carried out on aluminum-backed 0.25-mm silica gel plates with fluorescence indicator (Dynamic Adsorbents, Norcross, GA, USA) and were developed with n-butanol:glacial acetic acid:water (2:1:1, v/v/v). Substance spots on the chromatographic plate were detected by illumination with UV light (254 nm), KMnO4 staining, or 0.5% ninhydrin solution spraying. Reaction products of Fmoc protected alliin and alliin methyl ester were purified using either a Perseptive Biosystems (Life Technologies, Carlsbad, CA, USA) or an Agilent (Agilent Technologies, Santa Clara, CA, USA) HPLC system with a Phenomenex (Torrance, CA, USA) Kinetex Reversed-Phase C18 150 x 2.1 mm or 250 x 4.6 mm column. Gradient elution was run using solvents A and B (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in acetonitrile) from 100% A for 5 min and then to 100% B for another 20 min by linearly increasing solvent B. UV absorbance at 220 nm or 260 nm was used as detection. The progress of the reaction was monitored by RP-HPLC. ^{1}H NMR spectrum in an appropriate deuterium solvent of the resultant reaction solution confirmed completion of the reaction. The products were confirmed by TLC, melting point, and spectroscopic (^{1}H,^{13}C 1-D or 2-D NMR and HRMS-ESI) analysis. Yields refer to chromatographic and spectroscopic pure compounds.second level heading.

2.2 Preparation of aqueous garlic extract

Garlic extract was prepared from garlic cloves as in previously described protocol with modifications [8,9]. Briefly, garlic (The Garlic Company, Bakersfield, CA, USA) was purchased from the Costco Wholesale Corporation at Naperville (Naperville, IL, USA) and the aqueous garlic extract (AGE) was prepared from peeled garlic cloves in hepes buffer (50 mM, pH 7.2) containing 10% glycerol, 1 mM CaCl2, 1 mM MnCl2, 1 mM MgCl2, 20 uM pyridoxal 5'-phosphate, and 0.25 M NaCl at 1g/mL. A 900W high-torque motor blender (NutriBullet 900, Los Angeles, CA) was used to crush and pulverize the garlic cloves using three 5-second pulses with 2-minute breaks between pulses at 4 ^{o}C. The garlic puree was strained through 3 layers of cheesecloth. The filtrate was kept overnight at -20 ^{o}C. The following day, AGE was spun at 9878 x g for 4 minutes at 4 ^{o}C. Finally, the supernatant was filtered through a sterilized 0.22µm syringe-driven filter (Becton Drive, Franklin Lakes, NJ, USA) to remove any residual precipitate and to sterilize the AGE. The clear supernatant was aliquoted and stored at -80 ^{o}C until use.

2.3 Alliinase purification and characterization

The enzyme alliinase was purified to at least 95% homogeneity using PD10 and gel-filtration columns. Briefly, 2.5 mL of AGE was loaded onto a PD10 column. High-molecular weight (> 5000 Da) biomolecules eluted into the first 3.5 mL fraction and low-molecular weight

UNDER PEER REVIEW

compounds into next 5 mL fraction with hepes buffer (50 mM, pH 7.2) containing 10% glycerol, 1 mM CaCl2, 1 mM MnCl2, 1 mM MgCl2, 20 uM pyridoxal 5'-phosphate, and 0.25 M NaCl. Subsequently, 2 mL of the first 3.5 mL PD10 protein fraction was injected onto Phenomenex (Torrance, CA, USA) Yarra 3 µm gel-filtration 300 x 7.8 mm HPLC column and the column was developed using hepes buffer at a flow rate of 1 mL/min and 280 nm detection. Pure alliinase fraction was collected at 8 min which was verified by alliinase enzyme activity assay and mass spectrometry. Concentration of alliinase was measured by 1-D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) band intensity using BSA as standard. The alliinase solution displayed a single protein band (~52 kDa) on 1-D SDS-PAGE containing 4-12 % (w/v) polyacrylamide (Invitrogen, Carlsbad, CA) with MES running buffer and stained with Coomassie blue.

The enzyme activity assay of alliinase was determined by the spectrophotometric assay using 4-mercaptopyridine (4-MP) method in a cell free system according to Miron et al. with modifications [21]. The assay is based on the assessment of the production rate of allicin using alliin as subtract. One enzyme unit is defined as the amount of enzyme necessary to convert alliin into 1 µmol pyruvate per minute. The molar consumption of 4-MP in its reaction with allicin is equivalent to the production of pyruvic acid. One mL of the reaction solution contained 4-MP (10^{-4} M) in 50 mM sodium phosphate, 2 mM EDTA, 0.02 mM pyridoxal 5'-phosphate, 200 µg alliin (1.12 mM), and 20 µg alliinase (0.36 µM) , pH 7.2. Enzymatic activity started upon addition of 20 µL alliinase (1mg/mL solution in hepes buffer, 18 µM). Enzymatic activity was monitored at room temperature by the initial rate of the decrease in UV light absorption at 324 nm over a period of 5 min using quartz cuvettes of 1 cm in path length and a Spectronic Genesys UV/Vis spectrometer (Thermo Spectronic, Rochester, NY, USA). One unit of enzyme activity was defined as releasing 1 µmol pyruvate per minute and calculated using molar absorptivity ε_{324} = 19,800 M^{-1} cm^{-1}.

2.4 Synthesis of (R_C)-(-)-S-2-propenyl-L-cysteine (3) (L-deoxyalliin)

The synthesis of compound 3 is a modified literature procedure which is based on earlier work by Iberl et al. with precise temperature control throughout the reaction [22,23]. L-cysteine hydrochloride monohydrate (20g, 0.11 mol) was dissolved in 20 mL of water and stirred in an ice bath; then, 20 mL of an aqueous solution of sodium hydroxide (16 g, 0.4 mol) was added drop wise. Next allyl bromide (10.8 mL, 0.12 mol) was gradually added to the solution and the reaction mixture was stirred for 14 hours at room temperature, cooled in an ice bath, and glacial acetic acid was slowly added to neutralize the reaction solution to pH 5. The solution was filtered and the precipitate was washed three times with absolute ethanol. The white solid was crystalized using water to produce purified compound **3**. This was done by dissolving the crude L-deoxyalliin in a minimum volume of warm 60 ^{0}C water. After complete dissolution, the solution was cooled to room temperature and was kept at 4 ^{0}C overnight. The L-deoxyalliin crystals were separated, washed with cold ethanol and then dried under vacuum. The yield was 76% (14.2 g) with purity of higher than 97%. The characterization data of the product were consistent with the literature results [22, 24-26]. TLC: R_f 0.68; $[\alpha]^{25}_D$ = - 10.4° (*c*=1, H_2O); HRMS: (ESI, MH^+) calcd for $C_6H_{11}NO_2S$, 162.0589, found 162.0584; ^{1}H NMR (900 MHz, D_2O): δ = 2.97 (dd, J = 7.6, 14.9 Hz, 1 H, =CHCH$_2$SC$\underline{H}_2$), 3.07 (dd, J = 4.2, 14.9 Hz, 1 H, =CHCH$_2$SC$\underline{H}_2$), 3.22 (dd, J = 7.3, 13.8 Hz, 1 H, =CHC$\underline{H}_2$S), 3.24 (dd, J = 7.1, 13.8 Hz, 1 H, =CHC$\underline{H}_2$S), 3.91 (dd, J = 4.3, 7.7 Hz, 1 H, SCH$_2$C$\underline{H}$N), 5.21 (ddd, 1 H, J = 1.3, 2.2, 9.9 Hz, C$\underline{H}_2$=CH), 5.23 (ddd, 1 H, J = 1.3, 2.8, 17.1 Hz, C$\underline{H}_2$=CH), 5.84 (dddd, 1 H, J = 7.1, 7.3, 9.9, 17.1 Hz, CH2=C$\underline{H}$-S); ^{13}C NMR (DEPTQ, 225 MHz, D_2O): δ = 30.05 (1 C, S$\underline{C}$H$_2$CHN), 33.30 (1 C, =CH$\underline{C}$H$_2$S), 52.88 (1 C, CH$_2$=$\underline{C}$H), 117.81 (1 C, $\underline{C}$H$_2$=CH), 132.91 (1 C, SCH$_2\underline{C}$HN), 172.32 (1 C, O$\underline{C}$=O).

UNDER PEER REVIEW

2.5 Synthesis of (2R)-2-amino-3-[(S)-prop-2-enylsulfinyl]propanoic acid (4) [(+)-L-alliin]

S-2-propenyl-L-cysteine sulfoxides were prepared by oxidation of the corresponding S-2-propenyl-L-cysteines with hydrogen peroxide with quantitative yield following a procedure described previously which was based on earlier work by Yu et al. [22,27] with modifications. To a 200 mL aqueous solution of compound **3** (7.67 g, 0.047 mol) at 0 ^{o}C, 5.4 mL of 30% H_2O_2 (0.047 mol) was added drop wise. The mixture was stirred at 20 ^{o}C for 14 h and lyophilized. The crude diastereo mixture was determined to be 42 % (+)-L-alliin by NMR. A cascade fractional crystallization was used to produce diastereomericaly pure alliins. Crystallization was done using warm aqueous ethanol. This was achieved by adding minimum amount of 50 ^{o}C water to dissolve alliin, followed by addition of ethanol until solution turned slight turbid. The mixture was then heated to yield a clear solution. This solution was kept overnight at 4 ^{o}C to complete the crystallization. The crystals and the supernatant were separated and analyzed by NMR. Each crystallization supernatant contained more (-)-L-alliin while crystals were enriched in (+)-L-alliin. The crystals were subjected to fractional crystallization multiple times to isolate (+)-L-alliin (**4**) (1.0g, 13% yield, > 97% pure). The characterization data of the product were in accordance with published data [23, 26, 28, 29]. TLC: R_f 0.48; $[\alpha]^{25}_{D}$ = 59° (c=1, H_2O); HRMS: (ESI, MH^+) calcd for $C_6H_{11}NO_3S$, 178.0532, found: 178.0534; ^{1}H NMR (900 MHz, D_2O): δ = 3.13 (dd, J = 7.7, 14.0 Hz, 1 H, =CHCH$_2$SOC$\underline{H_2}$), 3.34 (dd, J = 6.2, 14.0 Hz, 1 H, =CHCH$_2$SOC$\underline{H_2}$), 3.55 (dd, J = 8.1, 13.3 Hz, 1 H, =CHC$\underline{H_2}$SO), 3.75 (dd, J = 7.0, 13.3 Hz, 1 H, =CHC$\underline{H_2}$SO), 4.11 (dd, J = 6.2, 7.7 Hz, 1 H, SOCH$_2$C$\underline{H}$N), 5.39 (d, 1 H, J = 17.2 Hz, C$\underline{H_2}$=CH), 5.46 (d, 1 H, J = 10.2 Hz, C$\underline{H_2}$=CH), 5.82 (dddd, 1 H, J = 7.0, 8.1, 10.2, 17.2 Hz, CH2=C$\underline{H}$-SO); ^{13}C NMR (DEPTQ, 225 MHz, D_2O): δ = 51.59 (1 C, SO$\underline{C}$H$_2$CHN), 52.73 (1 C, SOCH$_2\underline{C}$HN), 57.08 (1 C, =CH$\underline{C}$H$_2$SO), 126.31 (1 C, CH$_2$=$\underline{C}$H), 127.33 (1 C, $\underline{C}$H$_2$=CH), 173.34 (1 C, O$\underline{C}$=O). In addition, 2-D ^{1}H, ^{1}H correlation spectrometry (COSY) has confirmed the ^{1}H NMR assignments and 2-D ^{1}H, ^{13}C heteronuclear multiple quantum coherence (HMQC) spectrum, which contains a peak for each unique proton attached to the carbon being considered, has confirmed the ^{13}C NMR assignments (δ 51.59 and δ 57.08 have been wrongfully assigned to =CH$\underline{C}$H$_2$SO and SO$\underline{C}$H$_2$CHN in previous publication [29]).

2.6 Synthesis of (2R)-2-amino-3-[(R)-prop-2-enylsulfinyl]propanoic acid (5) [(-)-L-alliin]

Compound **5** was synthesized using the same procedure as for the synthesis of compound **4**. After completion, the supernatant was subject to fractional crystallization in warm aqueous ethanol and the process was repeated a number of times to produce pure compound **5** (0.9 g , 12% yield, > 95% pure). The characterization data of the product were consistent with the literature results [23, 26, 28, 29]. TLC: R_f 0.48; $[\alpha]^{25}_{D}$ = - 61° (c=1, H_2O); HRMS (ESI, MH^+): m/z calcd for $C_6H_{11}NO_3S$: 178.0532; found: 178.0534; ^{1}H NMR (900 MHz, D_2O): δ = 3.28 (dd, J = 8.5, 14.5 Hz, 1 H, =CHCH$_2$SOC$\underline{H_2}$), 3.32 (dd, J = 3.3, 14.5 Hz, 1 H, =CHCH$_2$SOC$\underline{H_2}$), 3.59 (dd, J = 7.9, 13.3 Hz, 1 H, =CHC$\underline{H_2}$SO), 3.74 (dd, J = 7.1, 13.3 Hz, 1 H, =CHC$\underline{H_2}$SO), 4.15 (dd, J = 3.3, 8.5 Hz, 1 H, SOCH$_2$C$\underline{H}$N), 5.41 (d, 1 H, J = 17.2 Hz, C$\underline{H_2}$=CH), 5.46 (d, 1 H, J = 10.2 Hz, C$\underline{H_2}$=CH), 5.82 (dddd, 1 H, J = 7.1, 7.9, 10.2, 17.2 Hz, CH2=C$\underline{H}$-SO); ^{13}C NMR (DEPTQ, 225 MHz, D_2O): δ = 51.34 (1 C, SO$\underline{C}$H$_2$CHN), 52.10 (1 C, SOCH$_2\underline{C}$HN), 56.55 (1 C, =CH$\underline{C}$H$_2$SO), 126.24 (1 C, CH$_2$=$\underline{C}$H), 127.40 (1 C, $\underline{C}$H$_2$=CH), 173.21 (1 C, O$\underline{C}$=O). In addition, 2-D ^{1}H, ^{1}H COSY has confirmed the ^{1}H NMR assignments and 2-D ^{1}H, ^{13}C HMQC spectrum has confirmed the ^{13}C NMR assignments.

2.7 Synthesis of Fmoc-(2R)-2-amino-3-[(S)-prop-2-enylsulfinyl]propanoic acid (6) ([Fmoc-(+)-alliin])

UNDER PEER REVIEW

Compound **6** was synthesized following a procedure with modifications [30-33]. Alliin (3.3 mg, 1 equiv) was dissolved in 660 µL of water and $NaHCO_3$ (6.2 mg, 4 equiv), Na_2CO_3 (3.9 mg, 2 equiv), and 50 µL of 1,4-dioxane was added in sequence. Subsequently, *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) was added drop wise to this clear alliin solution, which became turbid at first but cleared after stirring overnight. Fmoc-OSu solution was prepared by dissolving Fmoc-OSu powder (7.5 mg, 1.2 equiv) in 165 µL of 1,4-dioxane. Fmoc-alliin was purified by RP-C18 HPLC and dried as a white powder (6.7 mg, 90 % yield). The purity of the product was higher than 97% determined by NMR. The characterization data of the product were as follows. TLC: R_f 0.75; HRMS (ESI, MH^+): m/z calcd for $C_{21}H_{21}NO_5S$: 400.1213; found: 400.1210; RP-HPLC (t_R, min): 18; ^1H NMR (900 MHz, CD_3CN): δ = 3.55 (dd, J = 7.2, 13.7 Hz, 1 H, =CHCH$_2$SOC$\underline{H}_2$), 3.81 (dd, J = 5.8, 13.7 Hz, 1 H, =CHCH$_2$SOC$\underline{H}_2$), 3.93 (dd, J = 7.8, 13.2 Hz, 1 H, =CHC$\underline{H}_2$SO), 4.08 (dd, J = 7.1, 13.2 Hz, 1 H, =CHC$\underline{H}_2$SO), 4.67 (dd, J = 6.7, 7.0 Hz, 1 H, SOCH$_2$C$\underline{H}$N), 4.79 (dd, J = 6.5, 16.3 Hz, 1 H, CHC$\underline{H}_2$OCONH), 4.81 (dd, J = 7.0, 16.3 Hz, 1 H, CHC$\underline{H}_2$OCONH), 4.97 (dd, J = 6.5, 7.0 Hz, 1 H, C$\underline{H}$CH$_2$OCONH), 5.83 (d, 1 H, J = 17.1 Hz, C$\underline{H}_2$=CH), 5.87 (d, 1 H, J = 10.0 Hz, C$\underline{H}_2$=CH), 6.29 (dddd, 1 H, J = 6.7, 7.1, 10.0, 17.1 , 7.2 Hz, CH2=C$\underline{H}$-SO), 7.77 (dd, J = 7.2, 7.4 Hz, 2 H, C$\underline{H}$CH=CCHCH$_2$OCONH), 7.85 (dd, J = 6.7, 6.9 Hz, 2 H, C$\underline{H}$=CHCH=CCHCH$_2$OCONH), 8.08 (dd, J = 6.3, 6.7 Hz, 1 H, C$\underline{H}$=CCHCH$_2$OCONH), 8.25 (dd, J = 6.0, 7.2 Hz, 1 H, C$\underline{H}$CH=CHCH=CCHCH$_2$OCONH); ^{13}C NMR (DEPTQ, 225 MHz, D_2O): δ = 47.27 (1 C, SOCH$_2$$\underline{C}$HN), 49.47 (1 C, $\underline{C}$HCH$_2$OCONH), 51.98 (1 C, SO$\underline{C}$H$_2$CHN), 55.06 (1 C, =CH$\underline{C}$H$_2$SO), 67.26 (1 C, CH$\underline{C}$H$_2$OCONH), 120.52 (1 C, =$\underline{C}$HCH$_2$SO), 124.78 (1 C, $\underline{C}$H$_2$=CH), 125.79 (2 C, $\underline{C}$HCH=CHCH=CCHCH$_2$OCONH), 125.67 (2 C, $\underline{C}$H=CCHCH$_2$OCONH), 127.80 (2 C, $\underline{C}$HCH=CCHCH$_2$OCONH), 128.37 (2 C, $\underline{C}$H=CHCH=CCHCH$_2$OCONH), 141.50 (2 C, $\underline{C}$H=CHCH=CHCH=CCHCH$_2$OCONH), 144.28 (2 C, CH=$\underline{C}$CHCH$_2$OCONH), 157.02 (1 C, O$\underline{C}$ONH), 172.53 (1 C, HO$\underline{C}$=O).

2.8 Synthesis of methyl (2R)-2-amino-3-[(S)-prop-2-enylsulfinyl]propanoate (7) ([(+)-L-alliin methyl ester]) and methyl (2S)-2-amino-3-[(S)-prop-2-enylsulfinyl]propanoate (8) ([(+)-D-alliin methyl ester])

Compound **7** and **8** were synthesized as a mixture following a procedure described with modifications [34,35]. Five mg of alliin was dissolved in 1.2 M HCl in anhydrous methanol (700 µL) and mixed overnight. Seventy mg of NH_4HCO_3 was slowly added to neutralize the solution and the solution was lyophilized. The white powder containing 59% of (+)-L-alliin and 41% (+)-D-alliin methyl ester was dissolved in aqueous 50% acetonitrile and HPLC was used to isolate the (+)-DL-alliin methyl ester. Alliin methyl ester was eluted at 4.6 min and dried as a white powder with 91% yield. The purity of the product was higher than 97% determined by NMR. The characterization data of the product were as follows. TLC: R_f 0.46; HRMS (ESI, MH^+): m/z calcd for $C_7H_{13}NO_3S$: 192.0689; found: 192.0682; RP-HPLC (t_R, min): 4.7; ^1H NMR (900 MHz, D_2O): δ [(+)-L-alliin methyl ester] = 3.33 (dd, J = 5.9, 14.1 Hz, 1 H, =CHCH$_2$SOC$\underline{H}_2$), 3.42 (dd, J = 6.4, 14.1 Hz, 1 H, =CHCH$_2$SOC$\underline{H}_2$), 3.57 (dd, J = 8.0, 13.3 Hz, 1 H, =CHC$\underline{H}_2$SO), 3.75 (dd, J = 8.2, 13.3 Hz, 1 H, =CHC$\underline{H}_2$SO), 3.77 (s, 3 H, COOC$\underline{H}_3$), 4.61 (dd, J = 6.2, 6.2 Hz, 1 H, SOCH$_2$C$\underline{H}$N), 5.40 (d, 1 H, J = 17.2 Hz, C$\underline{H}_2$=CH), 5.47 (d, 1 H, J = 10.1 Hz, C$\underline{H}_2$=CH), 5.82 (dddd, 1 H, J = 8.0, 8.2, 10.1, 17.2 Hz, CH2=C$\underline{H}$-SO); ^{13}C NMR (DEPTQ, 225 MHz, D_2O): δ = 50.74 (1 C, SO$\underline{C}$H$_2$CHN), 50.80 (1 C, SOCH$_2$$\underline{C}$HN), 55.96 (1 C, COO$\underline{C}H_3$), 57.02 (1 C, =CH$\underline{C}H_2$SO), 125.96 (1 C, CH$_2$=$\underline{C}$H), 127.63 (1 C, $\underline{C}$H$_2$=CH), 170.26 (1 C, O$\underline{C}$=O).

^1H NMR (900 MHz, D_2O): δ [(+)-D-alliin methyl ester] = 3.36 (dd, J = 8.3, 14.6 Hz, 1 H, =CHCH$_2$SOC$\underline{H}_2$), 3.40 (dd, J = 4.1, 14.6 Hz, 1 H, =CHCH$_2$SOC$\underline{H}_2$), 3.60 (dd, J = 7.8, 13.4 Hz, 1 H, =CHC$\underline{H}_2$SO), 3.76 (dd, J = 8.0, 13.3 Hz, 1 H, =CHC$\underline{H}_2$SO), 3.79 (s, 3 H, COOC$\underline{H}_3$), 4.58 (dd, J = 4.2, 8.3 Hz, 1 H, SOCH$_2$C$\underline{H}$N), 5.41 (d, 1 H, J = 17.2 Hz, C$\underline{H}_2$=CH), 5.47 (d, 1

UNDER PEER REVIEW

H, J = 10.1 Hz, C$\underline{H}_2$=CH), 5.82 (dddd, 1 H, J = 7.8, 8.0, 10.1, 17.2 Hz, CH2=C$\underline{H}$-SO); ^{13}C NMR (DEPTQ, 225 MHz, D_2O): δ = 50.30 (1 C, SOCH$_2\underline{C}$HN), 50.44 (1 C, SO$\underline{C}$H$_2$CHN), 56.14 (1 C, COO$\underline{C}$H$_3$), 56.69 (1 C, =CH$\underline{C}$H$_2$SO), 125.96 (1 C, CH$_2$=$\underline{C}$H), 127.66 (1 C, $\underline{C}$H$_2$=CH), 170.45 (1 C, O$\underline{C}$=O).

2.9 MTT cell viability assay of *in situ* allicin

The viability of the *Mus musculus* colon carcinoma CT26.WT cells after treatment with *in situ* allicin was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Wallert and Provost Laboratory, Minnesota State University, Moorhead, MN) following a procedure described previously with modifications [8]. This assay is based on the conversion of MTT to MTT-formazon by mitochondrial enzymes. MTT assay was performed as follows: first, cells were plated at a density of 7,500 cells/well (144 µL/well) in 96-well plates and incubated at 37°C in a humid environment containing 5% CO_2 overnight. Then, 8 µL of alliin at varying concentrations was added to each well. After 1-7 days of incubation, 8 µL of alliinase at varying concentrations was mixed into each well. The following day, 30 µL of MTT solution (5 mg/mL in PBS) was added to each well. After incubation for 3.5 h, the medium was aspirated and 150 µL of MTT solvent (4 mM HCl and 0.1% NP-40 in isopropanol) was added to each well. After mixing well on an orbital shaker for 20 min, absorbance of the maximum at 545 nm with a reference filter of 630 nm was read by a multimode microplate reader SpectraMax M2 (Molecular Devices, Sunnyvale, CA). The absorbance readings revealed the percentage of the cell viability. The half maximal inhibitory concentration (IC_{50}) was calculated using a four-parameter logistic curve of SigmaPlot (Systat Software, Richmond, VA, USA). A control group (PBS, alliin, or alliinase treated cells) or blank group (water) was also included and the absorbance was considered to be 100% cell viability. Experiments were performed in triplicates. Data are presented as means ± standard deviations. Where appropriate, one-way analysis of variance (ANOVA) or student's paired t test was performed using SigmaPlot. P values less than 0.05 were considered as statistically significant. The CT26.WT cell line was used because it can be easily inoculated into BALB/c mice for later in vivo studies and there were reports that garlic consumption is beneficial for the suppression of colon cancer.

2.10 Antimicrobial activity of *in situ* allicin

A bacterial plate-diffusion growth inhibition assay was used to assess the efficacy of *in situ* allicin on eliminating human pathogens [36-39]. Gram-positive Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 6538™) and gram-negative Escherichia coli (Migula) Castellani and Chalmers (ATCC® 8739™) were used as test microorganisms. Bacteria in log phase growth in LB medium (Difco laboratories, Detroit, MI, USA) were grown at 37 ^{o}C oven to an OD_{560} = 0.18 and diluted 10 times with LB medium. 0.5 mL of the bacterium solution was spread on a 10 cm diameter petri dish with agar, which was prepared with 15 mL of a sterilized LB agar aqueous solution (40 g/liter). Ten mm diameter holes (wells) were punched out and filled with 100 µL PBS solution containing 20 µL of various concentrations of alliin, alliinase, allicin, AGE, a mixture of alliin and alliinase, or PBS. Plates were then incubated overnight at 37 ^{o}C and the diameter of the inhibition zone was measured. A dilution series of the *in situ* allicin was used to establish the proportionality of the relationship between the amount of active allicin and diameter of inhibition zone.

2.11 Flow cytometric analysis of cell cycle using propidium iodide DNA staining

UNDER PEER REVIEW

Mus musculus colon carcinoma CT26.WT cells were plated at a density of 1 x 10^5 cells/well in 6-well plates, permitted to adhere and grown for 48 h in RPMI-1640 with 10% FBS. Wells were treated separately with 100 µL PBS solution containing 20 µL of alliin, alliinase, allicin, AGE, a mixture of alliin and alliinase, or PBS at a range of concentrations for 12h. The cells (~ 3.6 x 10^5) were then harvested by trypsin-EDTA treatment into sterilized 15 mL of Falcon tubes. After washing twice with 2 mL PBS, centrifuging at 300 x g for 5 minutes, and decanting the buffer from the pelleted cells, cells were fixed in 70% cold ethanol at 4℃ overnight. The 70% cold ethanol was added drop wise to the pellet while vortexing to ensure fixation of all cells and minimize clumping. Before analysis, cells were washed twice with PBS, suspended in 500 µL of PBS containing 0.5% BSA, and treated with 200 µg/mL RNase A (Roche Diagnostics, Indianapolis, IN) and 40 µg/mL propidium iodide (PI; Sigma Corp., St. Louis, MO). Propidium iodide binds to double stranded DNA by intercalating between base pairs. After incubation for 30 min at 37 ℃ and single cell suspension with Falcon cell-strainer (Corning Inc., Durham, NC, USA), the cells were subjected to DNA content analysis. The PI fluorescence was analyzed by using a Cyan ADP flow cytometer with 488 nm line of argon-ion laser excitation (Beckman Coulter, Pasadena, CA). Flow cytometer was cleaned with bleach once and with water twice before it was used. In all the experiments, cells were gated on forward and side scatter to eliminate dead cells and debris. Data from at least 10,000 cells were analyzed with Summit software (Beckman Coulter, Pasadena, CA). Each DNA histogram was analyzed for peak position and for the percentage of cells in different histogram regions. Cell cycle distributions were obtained by deconvolution of histograms using Peakfit software (Systat Software, Richmond, VA, USA). This technique measures DNA content of 5,000 cells per second and trace the dynamic changes in cell proliferation cycle. Data show percentages of cell in different phases like sub-G_1, G_1, S, and G_2/M [40].

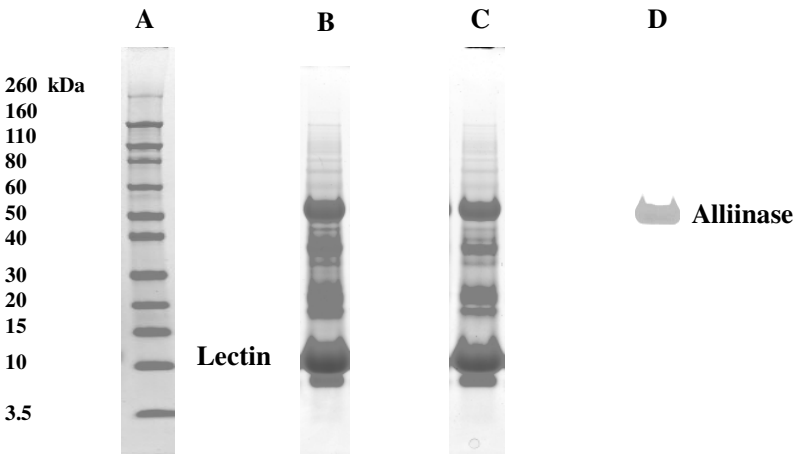
3. RESULTS AND DISCUSSION

Aqueous garlic extract (AGE) was prepared from garlic clove according to with modification in hepes buffer (50 mM, pH 7.2) containing 10% glycerol, 1 mM CaCl2, 1 mM MnCl2, 1 mM MgCl2, 20 µM pyridoxal 5'-phosphate (PLP), and 0.25 M NaCl at a concentration of 1g/mL [8, 9]. Glycosylated dimeric alliinase, which is used to convert bioinactive alliin to bioactive allicin, is purified to homogeneity from AGE. Alliinase is the major protein in AGE, and NaCl is required to preserve the enzymatic activity of alliinase because it can stabilize the alliinase dimer [41]. The inclusion of PLP and glycerol in hepes buffers is necessary to stabilize the enzyme during purification [42,43]. Our results from cancer MTT cell viability assay, bacterial plate-diffusion growth inhibition assay, and flow cytometry cell cycle analysis confirm the importance of buffer on the activity of alliinase (data not shown). Alliinase is completely inactive if prepared in water, semi-active if prepared in PBS, and most active if prepared in the hepes buffer. However, there is no detrimental effect if AGE is prepared in water because garlic itself contains the necessary compounds to stabilize the alliinase. It is the subsequent purification steps which require the hepes buffer.

Many protocols have been used to prepare alliinase from AGE using gel-filtration, ion-exchange, hydroxyapatite, and/or ConA-Sepharose 4B affinity columns with varying enzymatic activities and protein purities obtained at low yield [41,44-46]. Reports on recombinant expression of alliinase are scarce [47-49]. Here PD10 and high-resolution gel-filtration columns were used to produce highly purified alliinase with high yield. Many different kinds of columns such as ion-exchange, hydroxyapatite, hydrophobic, and ConA-Sepharose columns do not perform well in this study. A PD10 column is used to separate high-molecular weight (> 5000 Da) biomolecules from low-molecular weight compounds. All purification steps are done at 4℃ in this facile procedure. Analysis of samples in every steps of the alliinase preparation by gel electrophoresis is shown in **Figure 1**. Data reveal that the

UNDER PEER REVIEW

AGE contains two major protein bands at 52 and 12 kDa together with several minor bands. LC/MS/MS data on trypsin in-gel digestion of these two protein bands together with MassMatrix and X! Tandem database searches have confirmed that the 52 kDa protein is the enzyme alliinase, which is a mannose rich glycoprotein, and the 12 kDa protein is lectin, which is specific for mannose (data not shown). This observation points to possible interaction between these two proteins [44]. Concentration of alliinase was determined to be 1.5 mg/mL by SDS-PAGE band intensity using BSA as standard. SDS-PAGE after purification on PD10 (**Fig. 1**, Lane A) shows a similar protein profile to AGE and purification on high-resolution gel-filtration (**Fig. 1**, Lane B) reveals that alliinase is the only protein stained with Coomassie blue. Eighty percent of the alliinase in AGE is recovered with this protocol.



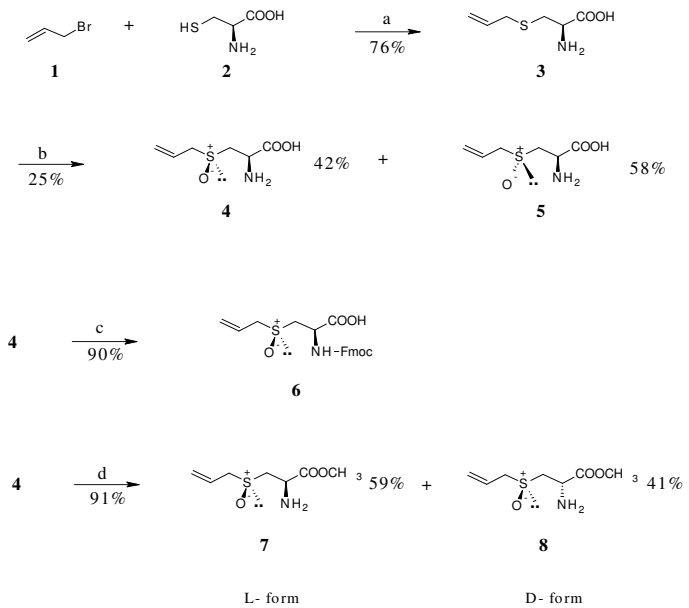
Lane A, protein molecular weight markers; Lane B, aqueous garlic extract (AGE);
Lane C, protein fraction of PD10 purification of AGE;
Lane D, alliinase purified on high-resolution HPLC size exclusion chromatography

Fig. 1. SDS-PAGE analyses of protein samples

The enzyme alliinase contains one PLP cofactor per subunit and belongs to the class I of PLP-dependent enzymes. It is able to cleave the C-S bond of sulfoxide derivatives of cysteine to produce allicin. The enzyme activity assay of alliinase was determined by the spectrophotometric assay using 4-mercaptopyridine (4-MP) method using pure (+)-L-alliin in a cell free system according to Miron et al. with modifications [21]. One unit of enzyme activity was defined as releasing 1 µmol pyruvate per minute. The final pure alliinase fraction prepared in this study shows an activity of 12 units/mg which is similar to the allinase purified in Miron et al. and much higher than the allinase purified in Mallika et al. [21,46]. It is interesting to note that some of the partially purified allinase fractions containing lectin show higher enzyme activity than the pure allinase fraction (data not shown), suggesting that lectin might enhance the activity of allinase.

UNDER PEER REVIEW

The course of the synthesis of (+)-L-alliin, (-)-L-alliin, Fmoc-(+)-alliin or (+)-DL-alliin methyl ester is illustrated in Schematic 1. The synthesis started with the formation of L-deoxyalliin (**3**) from the commercially available allyl bromide (**1**) and hydrochloride of L-cysteine (**2**) under alkaline conditions. A white precipitate was formed after the reaction solution was neutralized with acetic acid. Compound **3** was purified from the precipitate by crystalization in warm water and it does not show NMR signal for methyl group. It is interesting that proton coupling patterns of compound **3** between 2.97 and 3.07 and between 3.22 and 3.24 require non-first-order correction just as in the case of deoxyisoalliin, isoalliin, or allicin [8,9,22,50].



a) NaOH, H_2O, 20 °C, 14h, RP-HPLC; b) 30% H_2O_2, H_2O, 20 °C, 14 h, repeated fractional crystallization;
c) $NaHCO_3$, Na_2CO_3, 1,4 dioxane, Fmoc-OSu; d) $NaHCO_3$, Na_2CO_3, 1,4-dioxane, Fmoc-OSu;
d) HCl, anhydrous MeOH, neutralize with NH_4HCO_3 and lyophilized

Scheme 1. Chemical syntheses of (+)-L-alliin, (-)-L-alliin, (+)-Fmoc-L-alliin, and (+)-L,D-methyl alliin ester

(+)-L-alliin (**4**) and (-)-L-alliin (**5**) were prepared by oxidation of the corresponding L-deoxyalliin (**3**) with hydrogen peroxide in H_2O. The reaction is very efficient with a high yield. By careful control of temperature and concentration, further oxidation to sulfate is avoided. This oxidation reaction converts the divalent sulfur of compounds **4** and **5** to trivalent sulfur, which becomes a chiral center. Because the oxygen can be introduced from either side of the sulfur, a mixture of separable diastereomers is produced. Although many papers have reported synthesis of compounds **4** and **5,** these methods produce either low yield or low purity [20, 28, 29]. Solvent extractions and multiple fractional crystallization are used to separate and purify compounds **4** and **5**, which are dried as white powders with 13% and 12% total yield of > 97% purity, respectively. ^{1}H, ^{13}C NMR chemical shifts are quite similar between (+) and (-) isomers except for ^{1}H NMR chemical shift of $=CHCH_2SOC\underline{H_2}$. 2-D ^{1}H, ^{1}H correlation spectrometry (COSY) has confirmed the ^{1}H NMR assignments and 2-D ^{1}H, ^{13}C Heteronuclear Multiple Quantum Coherence (HMQC) spectrum, which contains a peak

UNDER PEER REVIEW

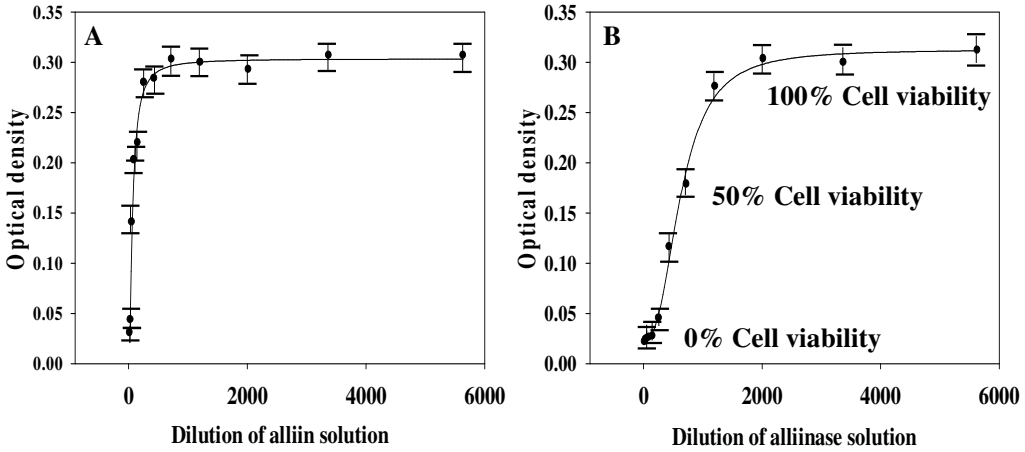
for each unique proton attached to the carbon being considered, has confirmed the ^{13}C NMR (δ 51.59 and δ 57.08 have been wrongfully assigned to =CH<u>C</u>H_2SO and SO<u>C</u>H_2CHN for Compound **4** in previous publication[29]). The spin coupling pattern between δ 3.13 and δ 3.34 and between δ 3.54 and δ 3.75 of compound **4** can be understood by non-first-order pattern resulting from the ratio between the chemical shift difference in Hz and the spin coupling of 14.0 (Δ v / J = 195.7/14.0) and 13.6 (Δ v / J = 182.7/13.4), respectively. The spin coupling pattern between δ 3.28 and δ 3.32 and between δ 3.59 and δ 3.74 of compound **5** can be understood by non-first-order pattern resulting from the ratio between the chemical shift difference in Hz and the spin coupling of 2.5 (Δ v / J = 36.6/14.5) and 10.1 (Δ v / J = 133.8/13.3), respectively. Alliinase activity measurement using **5** as the substrate reveal that the enzyme possess an activity of 4.6 units/mg compare to 12 units/mg by using **4** as the substrate.

Fmoc-(+)-alliin (**6**) and (+)-DL-alliin methyl ester have also been synthesized to test the roles of amino and carboxyl groups in alliinase reaction. Compound **6** was synthesized by mixing (+) alliin with FmocOSu. (+)-DL-alliin methyl ester was synthesized by mixing (+) alliin with anhydrous methanol in acidic conditions. The (+)-alliin methyl ester produced contains both (+)-L-alliin methyl ester (**7**) and (+)-D-alliin methyl ester (**8**) due to racemization [51, 52]. Instead of fractional crystallization in 95% ethanol, RP-HPLC is used to purify compounds **6** and (+)-DL-alliin methyl ester. Results show that compounds **6** and (+)-DL-alliin methyl ester are eluted at 18 and 4.6 min, respectively. Compound **6** and (+)-DL-alliin methyl ester are dried as a white powder with 76% yield of > 97 % purity. Non-first-order correction is not needed to explain the pattern of the couplings for compounds **6** and (+)-DL-alliin methyl ester. The alliinase activity assay results of compounds **6** and (+)-DL-alliin methyl ester show that both compounds are inactive in alliinase enzymatic reaction. Alliinase belongs to the class of carbon-sulfur lyases named S-alk(en)yl-L-cystene solfoxide lyase and catalyzes the cleavage of carbon-sulfur bond of S-allyl-L-cysteine sulfoxide in a beta-elimination/deamination reaction involving an aminoacryl bound PLP-amino acrylate (Schiff's base) reaction intermediate [45,47,53,54]. Alliinase provides acidic and basic groups in the active site of the enzyme, inducing polarization of the S=O group of the substrate, which initiates the carbon-sulfur bond breakage and produces an acrylamide bound PLP and allyl sulfenic acid. This catalytic mechanism is similar to that of cystathionine β-lyase [53]. Subsequently, the schiff's base of acrylamide bound PLP is spontaneously hydrolyzed and decomposed to pyruvate and ammonia. Simultaneously, condensation of two allyl sulfenic acids yields allicin. In agreement with this mechanism, it is plausible that the Fmoc group in Fmoc-alliin is blocking PLP cofactor binding and the inability to form carboxylate anion in (+)-alliin methyl ester inhibits the alliinase reaction. It is interesting to know that optimal pH value for alliinase catalytic activity is 6.5, while the optimal temperature is 33 ^{o}C.

The in vitro cytotoxicity and anti-cancer effect of *in situ* allicin against *Mus musculus* colon carcinoma cells CT26.WT was determined by MTT assay [8]. **Figure 2** shows the IC_{50} dose-response curve of *in situ* allcin on viability of *Mus musculus* colon carcinoma cells CT26.WT. Results show clear evidence of cytotoxicity of the *in situ* allicin against the CT26.WT in a dose dependent manner, compared to the controls (PBS, alliin, or alliinase). Shrinkage, granulation of cytoplasm, and detachment were visible in allicin treated cells. The half maximal inhibitory concentration (IC_{50}) is the dilution of either the alliin or allinase solution to produce fifty percent inhibition of cells *in vitro*. The IC_{50} of 11.24 mM (+)-L-alliin solution is calculated to be 1:60 dilution measured by mixing diluted alliin with 0.58 μM alliinase. The IC_{50} of 11.54 μM alliinase solution is calculated to be 1:630 dilution measured by mixing diluted alliinase with 0.56 mM (+)-L-alliin. Since 2 mol of alliin produces 1 mol of allicin in the allinase enzymatic reaction, the IC_{50} in term of allicin is 93.67 μM. Comparable results were obtained with AGE, allicin, and *in situ* allicin [8, 9]. Time course results have revealed that (+)-L-alliin in culture medium is fully active for 3 days, but is then gradually declines until it is

UNDER PEER REVIEW

inactive after 7 days. Data also reveal that with high (+)-L-alliin concentrations, less alliinase is needed to reach the 50% viable cells and vice versa. The IC_{50} of 11.24 mM (-)-L-alliin solution is calculated to be 1:19 dilution. In addition, anti-cancer effect has been tested with Fmoc-(+)-alliin and (+)-DL-alliin methyl ester with negative results, which confirm that both compounds are inactive in alliinase enzymatic reaction.



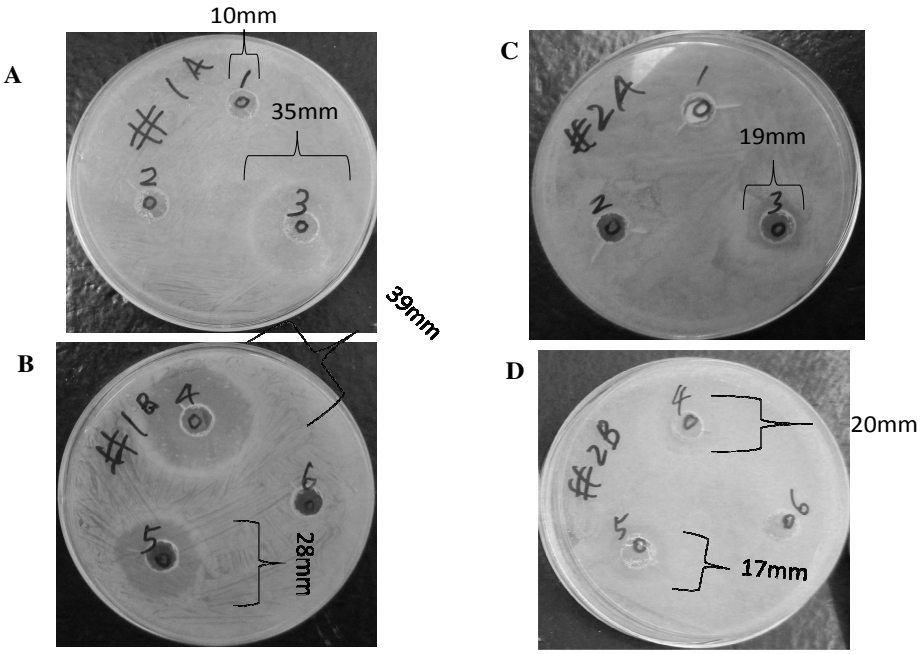
A, *in situ* allicin solution generated from different alliin concentrations with 0.58 µM alliinase;
B, *in situ* allicin solution generated from different alliinase concentrations with 0.56 mM alliin

Fig. 2. Dose–response curve of viability of *Mus musculuscolon* carcinoma cells CT26.WT assessed by MTT assay

Experimental data have indicated that allicin is responsible for the antimicrobial activity of AGE against a broader spectrum of microorganisms [2,4,55,56]. Allicin is also reported to be active against methicillin resistant human pathogen Staphylococcus aureus (MRSA) [38]. The fact that diallyl disulfide has a significantly lower antimicrobial activity indicates that the thiosulfinate group plays an important role in allicin's activity. In this study, the antibacterial activities of AGE, allcin, and *in situ* allcin against gram-positive Staphylococcus aureus subsp. aureus Rosenbach and gram-nagative Escherichia coli were measured using bacterial plate-diffusion growth inhibition assay (**Figure 3**). Data show that the maximum zone of inhibition (39 mm) was observed in Staphylococcus aureus and the minimum (17 mm) was observed for Escherichia coli. PBS was used as a negative control. The lowest concentration at which there is no growth was reported as minimum inhibitory concentration (MIC). Results from the dose–response study of allicin (data not shown) show that MICs were in the ranges of 56–140 and 280–560 µM in Staphylococcus aureus and Escherichia coli, respectively. The *in-situ* allicin is as active as allicin purified from AGE or allicin synthesized chemically in a dose-dependent manner. It has been reported by Miron et al.

UNDER PEER REVIEW

that allicin readily diffuses across the phospholipid membranes [14]. The basis for antimicrobial activity of the majority of antibiotics, allicin and the non-plant-derived antibiotics penicillin, citrinin, gliotoxin, clavacin and pyocyanines is their ability to react with cysteine. When allicin penetrates the cell, it reacts with free thio groups indiscreetly [13-15]. In addition, it is known that the antimicrobial efficacy of AGE is time and temperature dependent [7-9,57]. This explains why allicin is effective *in situ* because alliin and alliinase are more stable and less reactive toward free thio groups [58].



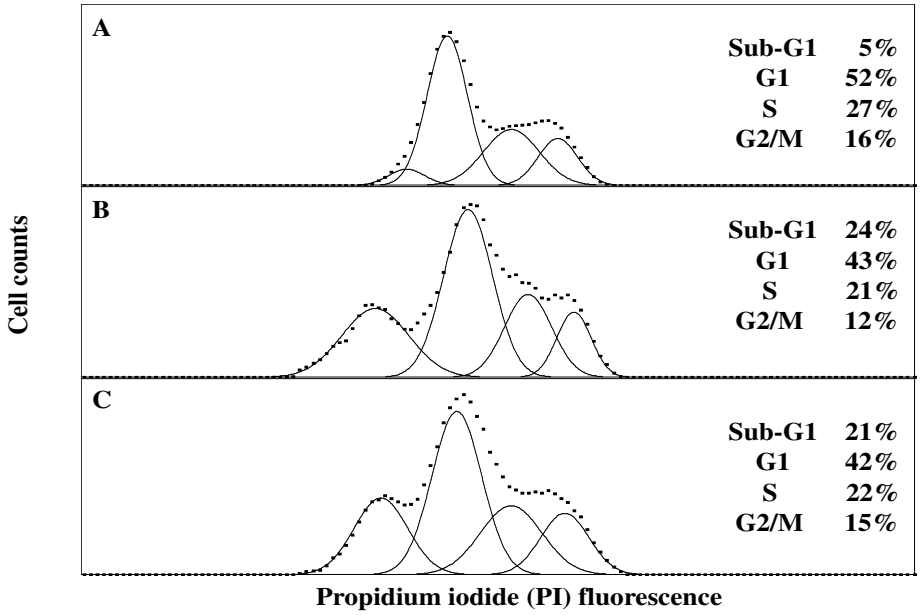
phylococcus aureus was grown in the presence of (1): alliin, (2): alliinase, or (3): mixture of alliin and alliinase;
phylococcus aureus was grown in the presence of (4): AGE, (5): allicin, or (6): PBS;
C, Escherichia coli was grown in the presence of (1): alliin, (2): alliinase, or (3): mixture of alliin and alliinase;
D, Escherichia coli was grown in the presence of (4): AGE, (5): allicin, or (6): PBS

Fig. 3. Effect of alliin, alliinase, allicin, AGE, mixture of alliin and alliinase, or PBS on the growth of gram-positive Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 6538™) or gram-nagative Escherichia coli (Migula) Castellani and Chalmers (ATCC® 8739™) by a bacterial plate-diffusion growth inhibition assay

Flow cytometric univariate analysis of cellular DNA content allows discrimination among sub-G_1, G_1, S, and G_2/M phase cells and is the simplest and most commonly used method to reveal the frequency of cells in these cell cycle phases [40]. The cells are fixed or permeabilized with a detergent and then stained with a nucleic acid-specific fluorophore such as propidium iodide (PI). Results of fluorescence measurements are displayed as a cellular DNA content frequency histogram, which shows the proportions of cells in particular phases of the cycle based on the fluorescence intensity. The content of cellular DNA in G_2 and M phases have twice as much DNA as G_1 phase cells and S phase cells have intermediate DNA content. Flow cytometry can measure DNA content of 5000 cells per second and trace the dynamic changes in cell cycle. During apoptosis, a portion of nuclear DNA may be lost from the cell in shedding apoptotic bodies and some fragmented DNA may be extracted during fixation and staining. Consequently, apoptotic cells are often

UNDER PEER REVIEW

distinguished on frequency histograms by a deficit in DNA content (sub-G_1 cells). The actual percentages of G_1, S, G_2/M, and sub-G_1 cells are obtained by deconvolution of the histograms using a PeakFit program. **Figure 4** shows DNA content frequency histograms of the effect of PBS, *in situ* allicin, or AGE on the cell cycle of *Mus musculuscolon* carcinoma cells CT26.WT. The data show a notable increase in sub-G_1 populations of the cell cycle of the cells treated with allicin, indicating the induction of apoptosis. This agrees with previous finding that allicin induces apoptosis in cancer cells [59]. Again the results show that *in-situ* allicin is as active as allicin purified from AGE or synthesized chemically.



A, 8 µL of PBS was added into 200 µL cell culture;
B, 1.5 µL of alliin (56.18 mM) and 8 µL of alliinase (11.54 µM) were added into 200 µL cell culture;
C, 8 µL of AGE was added into 200 µL cell culture

Fig. 4. Flow cytometric analyses of the effect of PBS, *in situ* allicin, or aqueous garlic extract (AGE) on the cell cycle of *Mus musculuscolon* carcinoma cells CT26.WT

4. CONCLUSION

We describe here a facile pathway to synthesize diastereomerically pure alliins and their conversion to allicin by enzymatic reaction of alliinase. Cancer MTT cell viability assay, bacterial plate-diffusion growth inhibition assay, and flow cytometry cell cycle analysis have been used to demonstrate the anti-cancerous/anti-pathogenic activities of the *in-situ* allicin. Protocols developed are simple and effective. Results demonstrate that the *in-situ* allicin is as active as allicin purified from AGE or allicin synthesized chemically in a dose-dependent manner. The data obtained here provide useful information on the design of the *in-situ* allicin strategy. Further research in modifying alliin structure (other substrates) and alliinase conjugated to a tumor targeting antibody against mouse cancer cells are also in progress.

UNDER PEER REVIEW

CONSENT (WHERE EVER APPLICABLE)

The author hereby declares all experiments have been examined and approved by the appropriate ethics committee.

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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ABBREVIATIONS

AGE, aqueous garlic extract; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.