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Original Research

Characterization of antioxidant activity of sulfur compounds in black garlic

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Abstract: Different types of sulfur compounds, namely S-allyl-L-cysteine (SAC), S-allyl-L-cysteine sulfoxide (ACSO) and a synthetic γ-L-glutamyl-S-allyl-L-cysteine (GSAC) were extracted from black garlic and their inhibition to the advanced glycation end-products (AGEs) were investigated. Upon addition of inhibitor, the amount of produced fructosamine was determined by UV visible spectroscopy. The change of pentosidine and fluorescent AGEs during reaction was detected by fluorescence method and the change of carboxymethyl lysine (CML) was detected by high performance liquid chromatography. It was found that the inhibitory effects of SAC and ACSO are stronger to the early and mid non-fluorescent products in glycosylation reaction, and GSAC has an obvious inhibitory effect on the later reaction products. All these three inhibitors can effectively inhibit advanced glycation reaction. Although their effects on glycation products are different due to different chemical structures, they have similar inhibitory effects on fluorescent products.

Key words: S-allyl-L-cysteine; S-allyl-L-cysteine sulfoxide; γ-L-glutamyl-S-allyl-L-cysteine antioxidant activity; Black garlic.

Introduction

Allium sativum, or garlic possesses many physiological functions, such as lowering blood glucose, prevention and treatment of cardiovascular and cerebrovascular diseases, antibacterial, regulating immunity, anti-tumor, removing free radical and anti-oxidation etc (1-4). Black garlic is prepared from allium sativum through fermentation and some processing procedures. After fermentation, all garlic cloves turn into black. In recent years, black garlic is very popular all over the world due to significant anticancer effects. The fermentation process will allow garlic to have stronger antioxidant activity than ordinary garlic, black garlic has been widely applied in various types of health food.

All the functions of garlic products can be attributed to the sulfur compounds. Among these compounds, the substituted sulfides are the main compositions in black garlic(5-18). There are three important sulfides. S-allyl-L-cysteine (SAC), which is the water-soluble cysteine derivatives in garlic and the oxidation precursor of alliin. S-allyl-L-cysteine sulfoxide (ACSO) or alliin is the most important sulfide in black garlic, and the non protein amino acid derivative having the highest content in thick garlic. SAC and ACSO are main flavor substances in black garlic. γ-L-glutamyl-S-allyl-L-cysteine (GSAC), which does not have obvious pungent odor. GSAC is the precursor of SAC and ACSO in the process of secondary metabolism of garlic. SAC, ACSO and GSAC are the sources of the transformation of the other sulfur compounds in garlic. There are balanced proportion and mutual transformation presented between the sulfides, the contents of which is often changed due to the different growth states and storage methods of black garlic.

Maillard reaction (19, 20) is a non-enzymatic glycation process that exists widely in food industry, the reaction taking place between carbonyl compounds (mainly reducing sugar) and amino compounds (amino acid and protein) produces a large number of black and brown macromolecular substances (melanoid). The reaction also generates intermediate molecules with many different odor, these products play significantly important role on the formation of food color, fragrance and taste. Maillard reaction occurs not only in high temperature process such as cooking but also during storage at room temperature. It involves a series of complicated reaction process with various products. Advanced glycation end-products (AGEs), one of the reaction products, is a hotspot in the current medical research. AGEs can affect protein structure and stability, induce protein cross-linking polymerization, alter protein functional characterization, AGEs are related to the physiological changes of diabetes and aging related pathology, and cause atherosclerosis, kidney disease, cataract, Alzheimer's disease, Parkinson and amyotrophic lateral sclerosis and other diseases (21-25).

It has been found that fresh garlic extracts had good inhibition effects on non-enzymatic glycosylation also, especially in the process of food manufacturing and storage. The sulfur compounds in fresh garlic during food processing under higher temperature can efficiently inhibit glycosylation reaction, although the mechanism is not very clear yet (9,11,14,15,17). The current study focuses on SAC, ACSO and synthetically GSAC as inhibitors to investigate inhibition of sulfur-containing compounds based on the structure of AGEs with different substitution comparison of black garlic, and provide fundamentals for in-depth study of inhibition mechanism of sulfur compounds in black garlic on gly-

cation products generated during food processing.

Materials and Methods

Materials

Monometallic sodium orthophosphate, sodium dihydrogen phosphate, sodium acetate, hydroxylamine hydrochloride, nitroblue tetrazolium and ethanol were purchased from Aldrich. Fresh black garlics – local products of Jiangsu Province, were purchased from Xuzhou Institute of Technology farmer's market. All other chemicals and solvents were purchased from Beijing Chemical Reagent Co. and used as received.

Instruments

The absorption spectra were acquired on a Beckman DU640 UV-Vis spectrophotometer. The steady-state fluorescence spectra were obtained with a Photon Technology International (PTI) LS-100 steady-state fluorometer, which was equipped with an Ushio UXL-75Xe Xenon arc lamp and a PTI 814 photomultiplier detection system. A quartz cuvette with a 10 mm path length was used to measure the fluorescence spectra. Samples were excited and emission was monitored from 250 to 500 nm.

Pretreatment of garlic samples

The black garlics were placed at 0-4 °C in a fridge for 8-10 weeks in order to fully break dormancy. Garlic sample (500 g) was added in 500 mL anhydrous alcohol, the mixture was loaded into a boiling water bath and heated for 10 min followed by cooling. The yielded heterogeneous solution was transferred into a beater and stirred for 20 min. After filtration, mashed garlic was extracted again after addition of another 500 mL anhydrous ethanol. The combined filtrate was evaporated to about 100 mL at 60 °C. Hydrochloric acid (2.5 mol/L) was added to adjust pH value to 4.5. The solution was placed in the refrigerator for overnight at 4 °C followed by filtration with a 0.35 μm membrane.

SAC obtained by reduction of ACSO

Sodium iodide and acetyl chloride were added into ACSO obtained from previous procedure to reduce the sulfoxide groups of ACSO. The generated iodine was reduced by stannous chloride until the brown yellow solution discolored. The solution was fully oscillated, filtered by 0.35 μm membrane, then stored in an ultra-low temperature refrigerator for overnight to yield white powder as the product. SAC and ACSO samples were placed in the ultra-low temperature refrigerator for use.

Determination of biological inhibitors concentrations

Three concentrations 0.010mol/L, 0.015mol/L, and 0.020mol/L were used for SAC, ACSO and GSAC, respectively. The glycosylation reaction system was added and the sample without inhibitor was used as control group, all samples were reacted within incubator at a constant temperature, the reaction solution after 4h reaction was taken for 280 - 500 nm wavelength scanning, the inhibitory effect at different concentration of inhibitors was compared to determine the best inhibitory concentration of inhibitors.

Determination of early glycation products

Reaction solution (0.5mL) was taken at 1 h, 2 h, 3 h and 4 h respectively in a 50mL flask, sodium acetate (10 g/L, 1.0mL) and hydroxylamine hydrochloride (1 g/L, 2 mL) were added and heated in water bath for 20 min. The volume was fixed with distilled water after cooling, the absorbance was determined at a wavelength of 250 nm.

Lactulose was determined according to nitro blue tetrazolium (NBT) reduction method. Reaction solution (50 μL) was taken at 1 h, 2 h, 3 h and 4 h respectively, NBT solution (0.025 mol/L, 1 mL) was added and heated in water bath for 5 min. The reaction was terminated with 10% acetic acid solution, the absorbance was determined at a wavelength of 540 nm.

Determination of fluorescent AGEs and glycosylation products CML

The fluorescent AGEs products exhibit spontaneous fluorescence properties at an excitation wavelength of 370nm and show a maximum peak an emission wavelength of 440nm. The reaction sample (0.1 mL) at 1 h, 2 h, 3 h and 4 h were taken respectively and loaded in a small test tube followed by 30 times dilution using PBS buffer to measure the fluorescence behaviors.

CML is an important product of nonenzymatic glycation, it is one of the most abundant products in AGEs without any fluorescence properties. In this study, HPLC method was used to detect the production of CML in glycation reaction system. The reaction samples were taken at 4 h, filtered by 0.35 μm filter membrane.

Statistical method

SPSS 13.0 software was used for data processing. The experimental results were expressed by mean±SD. Single-factor Analysis of Variance (ANOVA) was used to compare the difference between the control group and the experimental group, $p<0.05$ indicates that there are no significant differences between the data.

Results and Discussion

Determination of inhibitors optimum concentration

The inhibitory effect of all three inhibitors increased with concentration according to the change in concentration of three inhibitors. Figure 1 shows the UV-vis spectra of SAC, ACSO and GSAC scanning from 250 nm to 450 nm at different concentration in non-enzyma-

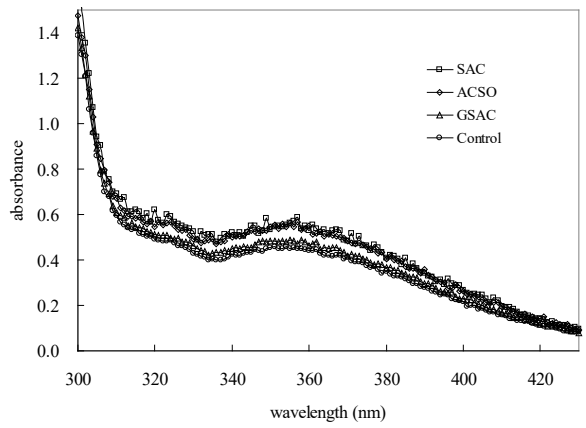

Figure 1. UV-visible spectra of three inhibitors and control sample at different concentrations.

tic glycation reaction system at 60 °C for 5 h. Considering the amount of extraction and separation, the intermediate concentration of 0.15 mol/L was selected as the optimum inhibitory concentration for the inhibitors.

Inhibitory effect on glyoxal

Glyoxal is an intermediate product in glycosylation reaction, its production can be expressed by absorbance at 233 nm. Because absorption reflects how much glyoxal was created in the reaction, the capacity of three inhibitors can be compared by absorption. The yield results were shown in Figure 2, there are no inhibitory effects of SAC and ACSO on glyoxal, these two inhibitors even involved in the reaction of carbonyl compounds to release more glyoxal. The inhibitory effect of GSAC on glyoxal is significant ($P < 0.05$). Glyoxal is an intermediate product, suggesting that SAC and ACSO have poor inhibitory effects on products formed at the middle stage of glycation reaction, while GSAC has a good inhibitory effect on intermediate products.

Inhibitory effect of inhibitors on lactulose

Fructose as an early product of glycation significantly influences glycation. Through determination of fructose by reduction method, the production of fructose during reaction can be expressed by absorption, further reflecting the difference in inhibition of the early glycation reaction. As can be seen from Figure 3, the formation of fructose decreases gradually with time then increased rapidly in different reaction systems, suggesting that fructosamine generated in early stage was consumed by Maillard reaction at the final stage of reaction. The absorption decreases with reaction time, fructosamine generation rate increases, and accumulates at final stage to increase the absorption. To the inhibition system, the inhibitory effect of SAC is significant ($P<0.05$) at the start, and almost no effect was observed at the final stage. ACSO exhibits a good inhibitory effect at the final stage ($P<0.05$). While GSAC always maintains a fixed inhibition rate with no obvious change during the reaction time. These observations suggest that SAC is involved in the inhibition of fructosamine reaction from the beginning, but significantly consumed with reaction time and not able to inhibit fructosamine generation at the final stage. On the contrary, ACSO might be involved in the inhibition with very slow release rate, the internal structure changes with reaction time to significantly increase the inhibition rate.

Inhibitory effects on pentosidine

The amount of pentosidine production can be used to assess protein damage during glycation, therefore it can be used as a reference to measure the inhibitory effect of inhibitors on AGEs. Figure 4 shows that the inhibitory effect of SAC in the whole reaction process is relatively constant with an inhibition rate of around 28%, while ACSO and GSAC have a similar inhibitory effect within the early stage of reaction (within 2 hours), ACSO has a better inhibitory effect at the final stage ($P<0.01$). Thus, the inhibition of the pentosidine content shows that three inhibitors have similar inhibitory activity.

Inhibition of fluorescent AGEs

AGEs causes protein denaturation and results in structural and functional damage. Since most AGEs products contain spontaneous fluorescent behavior, level of AGEs can be determined by measuring its fluorescence. Figure 5 shows that the inhibitory effect of SAC remains unchanged and the inhibition effect of ACSO is always better than GSAC comparing the control group. Although early inhibition effect of GSAC is poor, it was significantly enhanced at final stage ($P<0.05$). Thus, all sulfur compounds have certain inhibitory effects on fluorescent AGEs. AGEs represent a general term of glycosylation end products, including some fluorescent products and some non-fluorescent substances. The reaction pathways of the two substances are different,

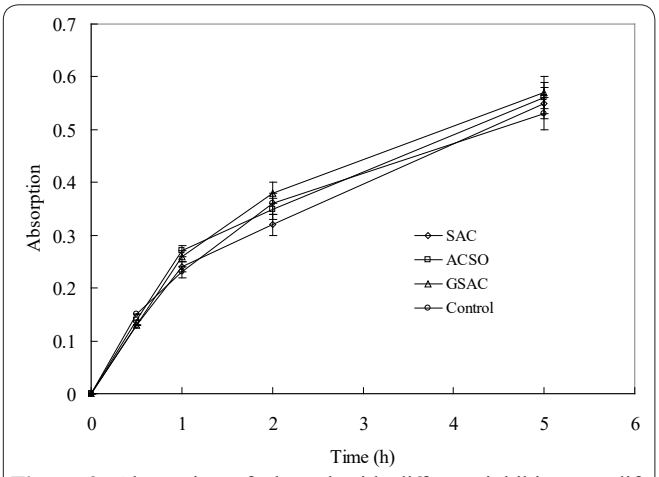

Figure 2. Absorption of glyoxal with different inhibitors at different inhibitory time.

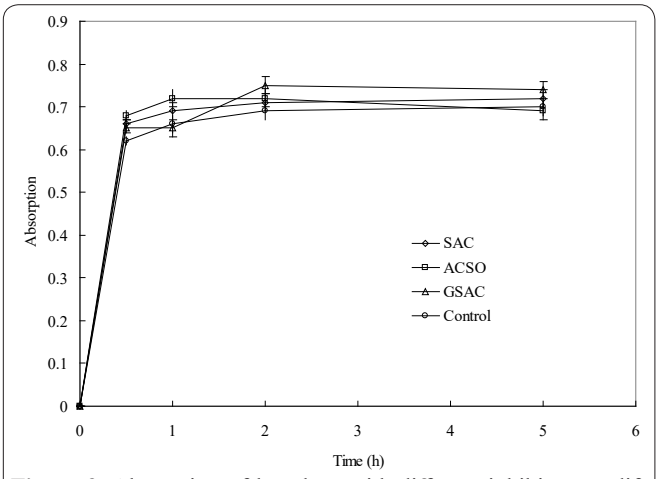

Figure 3. Absorption of lactulose with different inhibitors at different inhibitory time.

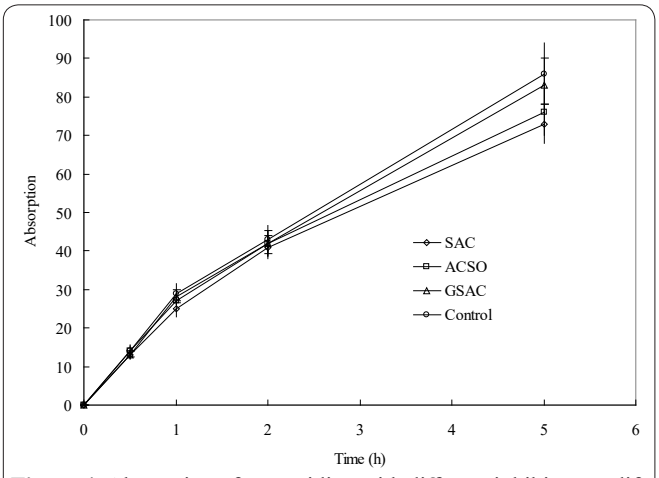

Figure 4. Absorption of pentosidine with different inhibitors at different inhibitory time.

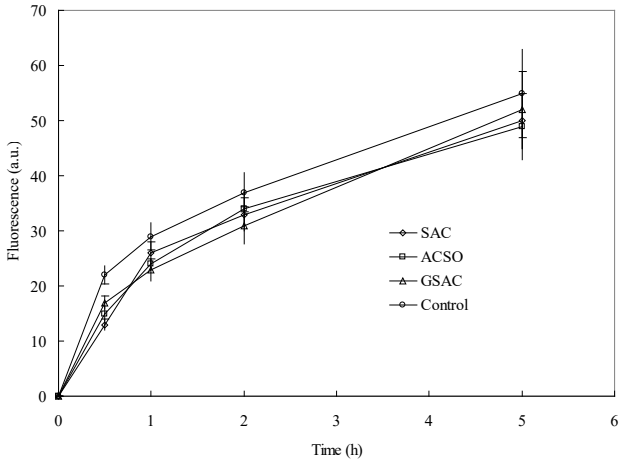

Figure 5. Fluorescence intensity of AGEs with different inhibitors at different inhibitory time.

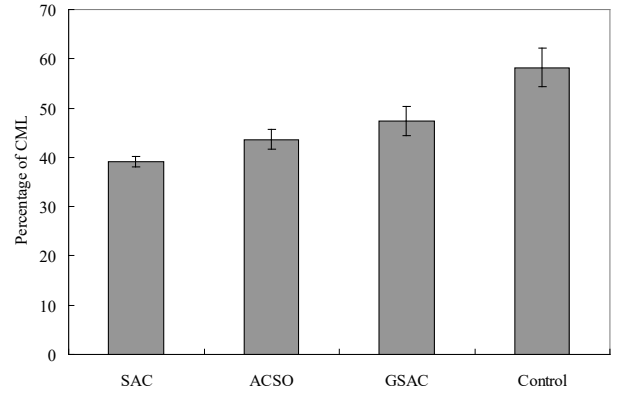

Figure 6. Percentage of CML in different reaction systems detected by HPLC.

and their structures are very different. The experimental detection of early reaction product of fructosamine and glyoxal is formed by Maillard products and a variety of glycolysis products formed by condensation, further reaction between these products yields non-fluorescent final product, such as CML or pyrraline. While fluorescent AGEs is mainly crosslinked products of amino acids, such as pentosidine. The results show the inhibition ability of these sulfur compounds towards fluorescent products. The inhibition effects of all inhibitors on pentosidine and fluorescence AGEs are consistent, suggesting that the fluorescent substances from glycosylation reaction products contain similar structure.

Inhibitory effects of inhibitors on CML

Figure 6 shows the change of CML content in the main product after 4 hours, the results are expressed with the percentage of CML. CML content in the blank control group is 58.26% (column A). As shown in Figure 6, CML content in SAC system is the least and in GSAC system is the most, indicating that the inhibitory effect of SAC on CML production is most significant ($P<0.05$). According to previous results, the inhibitory effect of GSAC on glyoxal is most significant, thus the inhibition of SAC on CML is mainly due to the inhibition of intermediate product.

In this study, the inhibitory effects of organic sulfur compounds on glyoxal and fructosamine, fluorescent product pentosidine, AGEs and non-fluorescent product CML were analyzed and discussed, results show that all three sulfur compounds can inhibit glycosylation. Further investigation has found that SAC and ACSO have better inhibitory effect on the early and intermediate glycation products, while the inhibition of GSAC is limited. Different inhibitory effects from three inhibitors indicate that different chemical structures will affect the inhibitory.

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