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Original Research

Molecular detection and in vitro antioxidant activity of S-allyl-L-cysteine (SAC) extracted from Allium sativum

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Abstract: It is well known that Allium sativum has potential applications to clinical treatment of various cancers due to its remarkable ability in eliminating free radicals and increasing metabolism. An allyl-substituted cysteine derivative - S-allyl-L-cysteine (SAC) was separated and identified from Allium sativum. The extracted SAC was reacted with 1-pyrenemethanol to obtain pyrene-labelled SAC (Py-SAC) to give SAC fluorescence properties. Molecular detection of Py-SAC was conducted by steady-state fluorescence spectroscopy and time-resolved fluorescence method to quantitatively measure concentrations of Py-SAC solutions. The ability of removing 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical using Py-SAC was determined through oxygen radical absorbance capacity (ORAC). Results showed the activity of Py-SAC and Vitamin C (VC) with ORAC as index, the concentrations of Py-SAC and VC were 58.43 mg/L and 5.72 mg/L respectively to scavenge DPPH, and 8.16 mg/L and 1.67 mg/L to scavenge •OH respectively. Compared with VC, the clearance rates of Py-SAC to scavenge DPPH were much higher, Py-SAC could inhibit hydroxyl radical. The ability of removing radical showed a dose-dependent relationship within the scope of the drug concentration.

Key words: S-allyl-L-cysteine, antioxidant activity, Allium sativum, scavenging free radicals.

Introduction

Allium sativum is a perennial herb of allium liliaceae, which is rich in nutrition (1). It has been widely used as food and flavouring for more than 3,000 years in Asia, Europe and North America (1,2). Currently, it is considered that the main garlic bioactive substances are sulfur compounds. Garlic contains 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 (4-10). All these functions can be attributed to the sulfur compounds in garlic.

The substituted sulfides are the main composition of sulfur compounds in garlic. 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 garlic, and the non protein amino acid derivative having the highest content in thick garlic. SAC and ACSO are main flavor substances in garlic. γ-L-glutamyl-S-allyl-L-cysteine (GSAC), which does not have obvious pungent odor, currently people have limited understanding on its biological functions, some scholars believe that the function of GSAC may be the biological reserves of S and N elements. 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 garlic (11-18).

Currently, the research on anti-oxidant activities of garlic extracts has been conducted, moreover the obtained data showed that the extracts had a significant antioxidant activity in vivo and in vitro. Previous studies on antioxidant activity of garlic sulfide mainly related to the lipid soluble sulfide allicin (allyl sulfur sulfinic acid ester), sulfur ether, ahold ene, and water soluble sulfides such as SAC and ACSO (7,8,10-12,14,16,19-25). The anti-glycation activity and inhibition of hepatic stellate cell proliferation activity of GSAC were also reported in the literatures (13,15,17-21). SAC, ACSO and GSAC are derivatives of amino acid with cysteine structure, the reduced type of glutathione (GSH) also contains cysteine with reduced type of thiol, which is different with a glycine of GSAC structure (16,19,23,24). However, to the best of our knowledge, the antioxidant activities of modified GSAC have not yet been reported. In this study, the most commonly investigated water soluble sulfur SAC was separated and identified from Allium sativum, SAC was reacted with 1-pyrenemethanol to generate pyrene-labelled SAC (Py-SAC). GSH was used as a reference to determinate and compare the free radicals removing ability and ferrous ion chelating ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH) to evaluate antioxidant activities of Py-SAC and Vitamin C (VC). This study provides a method of separation and identification for SAC to obtain the information of SAC structure, and reveal the antioxidant activity to further investigate and develop a variety of drugs from the water soluble sulfides.

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Materials and Methods

Materials

1-Pyrenemethanol, *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), DPPH and VC were purchased from Aldrich. Fresh Allium sativums - products of Yongnian Town of Hebei Province, were purchased from Hebei Agricultural University farmer's market. All other chemicals and solvents were purchased from Beijing Chemical Reagent Co. and used as received.

Instruments

The steady-state fluorescence spectra of the Py-SAC 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 at 346 nm and emission was monitored form 350 to 450 nm. The fluorescence decays of the Py-SAC solutions were acquired on a Perkin-Elmer time-resolved fluorometer equipped with a nanoLED light source. The solutions were excited at 344 nm and the emission was monitored at 375 nm. The fluorescence decays were acquired with a 370 nm cut-off filter to prevent stray light scattering from reaching the detector. The absorption spectra were acquired on a Beckman DU640 UV-Vis spectrophotometer.

Pretreatment of garlic samples with separation of sulfide ACSO

The garlics were placed at 0-4 °C in a freezer for 2 months to fully break dormancy. Garlic (600 g) was added in 400 mL anhydrous alcohol, the mixture was loaded into a boiling water bath for 10 min followed by cooling. The heterogeneous solution was transferred into a beater and mixed at a high speed for 10 min. After filtration, mashed garlic was again extracted with adding another 400 mL anhydrous ethanol. The combined filtrate was evaporated to about 200 mL at 60 °C. Hydrochloric acid (2.5 mol/L) was added to adjust the 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 discoloured. The solution was fully oscillated, filtered by 0.35 μm membrane, then storaged in a ultra-low temperature refrigerator for overnight to yield white powder as the product. SAC was placed in the ultra-low temperature refrigerator for use.

Determination of free radical scavenging capacity of DPPH

DPPH (12.36 mg) was weighed and dissolved in ethanol in a 10mL brown flask, a known volume of solution was read from the scale. The solution was placed at 4 °C in dark. The test solution was diluted with anhydrous ethanol diluted to 2.5×10^{-5} mol/L before use. VC (12.25 mg) was dissolved in ethanol in a 10 mL volumetric flask, known volume of solution was read from the scale after shaking, the required concentrations were obtained by dilution before use.

The sample solution (0.1 mL) at different concentrations and the DPPH free radical solution at concentration of (0.1 mL) were added into a hole plate. The commonly used antioxidant VC was used as a positive control to prepare solutions with different concentrations to conduct the same experiments. At the same time, every concentration of the sample solution without DPPH free radical (DPPH free radical was replaced by 0.1 mL anhydrous ethanol) was used as a control to eliminate the interference of color from sample itself on the test results. DPPH free radicals were set as the negative control (the sample was replaced by 0.1 mL anhydrous ethanol). The hole plate was loaded into a microplate reader and stored in this condition (in dark at room temperature) after oscillation for 5 min. The absorbance value A was determined after 1 h at the wavelength of 517nm. The free radical scavenging rate was calculated according to the following equation:

$$\text{free radical scavenging rate (\%)} = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\% \quad (1)$$

where A_0 is the absorbance of DPPH negative control group; A_1 is the absorbance of sample group; A_2 is the absorbance of the sample solution plus the absorbance of anhydrous ethanol control group.

Determination of free radical scavenging capacity of hydroxyl free radical (•OH) solution

The preparation of •OH solution and determination of the free radical scavenging rate were referred to the previous section, as the same as DPPH.

Results

Synthesis of Py-SAC

SAC (0.85 g, 5.0 mmol) and 1-pyrenemethanol (1.16 g, 5.0 mmol) were dissolved in 10 mL of $CHCl_3$, followed by the addition of DCC (1.03 g, 5.0 mmol) and DMAP (0.06 g, 0.5 mmol). The reaction mixture was stirred at 0 °C for 24 h. After filtration, the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography using CH_2Cl_2 as eluent to yield 1.06 g (~40% yield) of Py-SAC. The resulting product was dried overnight in a vacuum oven at 25 °C. The reaction scheme was shown in Figure 1.

Steady-state fluorescence of Py-SAC

Steady-state fluorescence measurement was applied to study the Py-SAC. The steady-state fluorescence spectra of the Py-SAC sample at different concentrations are shown in Figure 2.

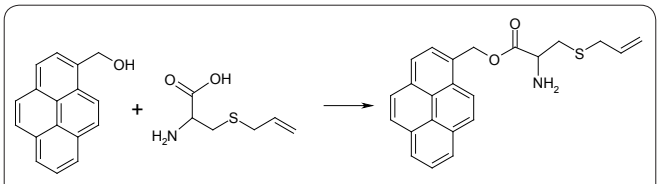

Figure 1. Reaction scheme for the preparation of Py-SAC.

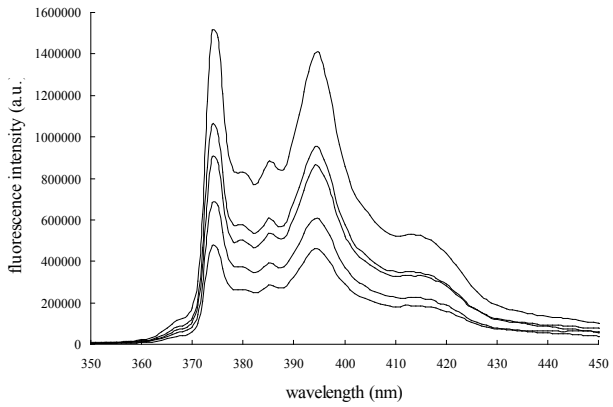

Figure 2. Fluorescence spectra of Py-SAC at concentrations of (from top to bottom) 98.62 mg/L, 47.38 mg/L, 36.19 mg/L, 25.33 mg/L and 10.66 mg/L. The steady-state fluorescence spectra of the Py-SAC solution showed the characteristic peaks of the pyrene monomer between 370 and 400 nm, which is a typical characterization of pyrene and its derivatives. As expected, the fluorescence intensity decreased as a decrease in sample concentration.

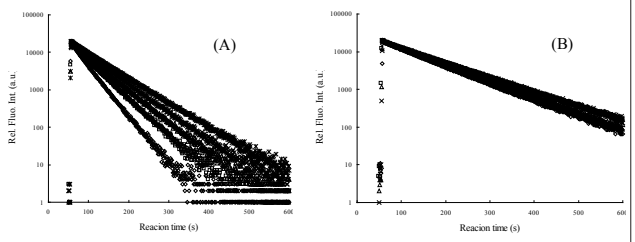

Figure 3. The fluorescence decay curves with different concentrations of Py-SAC solution (A) (from top to bottom) 98.62 mg/L, 47.38 mg/L, 36.19 mg/L, 25.33 mg/L and 10.66 mg/L, and VC (B) (from top to bottom) 86.45 mg/L, 43.26 mg/L, 22.57 mg/L and 11.82 mg/L.

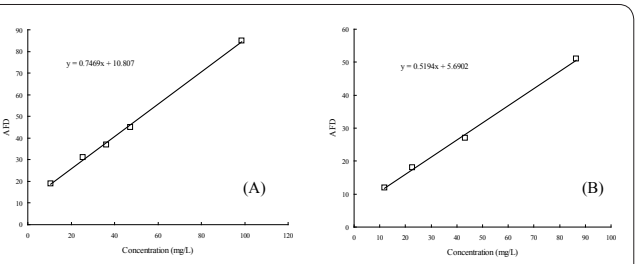

Figure 4. The linear relationship between AFD and Py-SAC (A), and VC (B).

Free radical scavenging capacity of DPPH by Py-SAC and VC

The free radical scavenging capacity of DPPH by Py-SAC and VC was estimated using time-resolved fluorescence measurement. The fluorescence decay curves of the different concentrations of Py-SAC and VC were shown in Figures 3A and 3B. According to the method of area calculation under the fluorescence decay curve, the area under fluorescence decay curves (AFD) of Py-SAC and VC solution at different concentrations can be calculated, the results were listed in Table 1. From the analysis, it can be seen that there is a good linear relationship between AFD and concentration as the change of solution concentration, as shown in Figure 4A and 4B. At the same time, the ORAC values of Py-SAC and VC are 58.43 mg/L and 5.72 mg/L, respectively. Therefore, the ability of the Py-SAC to remove DPPH is much stronger than that of VC.

Free radical scavenging capacity of •OH by Py-SAC and VC

The data describing inhibition effect of Py-SAC and VC on •OH were listed in Table 2. In the range of used concentrations, the inhibition effect of a certain concentration of Py-SAC is higher than the effect of the VC control group for •OH. The inhibition rate of VC is very small, and the rate is almost no change in a certain range of concentrations. However, the inhibiting ability of •OH release by PY-SAC is weaker than that of VC.

Discussion

DPPH in solution generates a stable nitrogen radical. The solution displays a color of typical purple, which has strong absorption at 517 nm within the region of UV-visible light. When antioxidants are added into DPPH solution, the purple solution fades due to its free radical scavenging effect, resulting in absorption intensity decreases with increase of the amount of antioxidants (16,17). The free radical scavenging effect can be calculated by the change of absorbance before and

Table 1. The area under the fluorescence decay curves (AFD) of Py-SAC and VC.

Py-SAC concentration (mg/L)	AFD	VC concentration (mg/L)	AFD
98.62	74.25	86.45	76.24
47.38	68.76	43.26	73.18
36.19	60.44	22.57	70.67
25.33	53.18	11.82	67.52
10.66	42.09		

Table 2. Inhibiting activity of Py-SAC and VC against •OH.

Py-SAC		VC	
Mass concentration (mg/L)	Inhibiting rate against •OH (%)	Mass concentration (mg/L)	Inhibiting rate against •OH (%)
826.15	8.65±0.48	50	16.28±0.57
1836.75	13.86±0.52	100	17.12±0.61
2796.55	18.27±0.57	150	17.88±0.73
3816.08	26.13±0.62	200	18.45±0.75
4828.458.64	35.45±0.74	250	19.36±0.82

after addition of antioxidants. In this study, the DPPH scavenging effect by Py-AC at different concentrations was investigated. In the concentration range of 27.84 to 520.36 mg/L, the scavenging effect increased significantly with increase of Py-SAC concentration. When Py-SAC concentration was greater than 168.52 mg/L, the DPPH scavenging effect of Py-SAC is stronger than that of VC.

The basic principle of ORAC method is similar to DPPH scavenging method. The experiments showed that the area under the fluorescence decay curves increased with an increase of sample content, and exhibited a good linear relationship with the concentration.

Free radical scavenging capacity of •OH system is similar to the ORAC method, that is, antioxidants competes with adjacent dinitrogen phenanthrene and combines the hydroxyl free radical (·OH). Due to the presence of the antioxidant, the oxidation of the hydroxyl free radical (·OH) to phenanthroline red complexes was inhibited (18-21). The experimental results showed that the ability of inhibiting hydroxyl radical (·OH) is enhanced with the increase of the content of Py-SAC in the range of 932.57 to 6082.19 mg/L. But the inhibition effect of half-clearance rate was weaker than that of VC.

Obviously, the Py-SAC has stronger ability to scavenge DPPH free radicals, and it also has the ability of scavenging superoxide anion radical and inhibiting hydroxyl radical. Furthmore, in the experimental concentration ranges, the concentration of Py-SAC and free radical scavenging rate exhibits a good dosage-dependent relationship. The free radical half-clearance rates of corresponding Py-SAC are different, which might result from the difference of specific monomer having different reaction to different free radicals, while the Py-SAC in each monomer compounds may vary.

In conclusion, the pyrene modified SAC still remains good antioxidant activity, Py-SAC may be used as a natural antioxidant to scavenge active free radicals such as hydroxyl free radicals and superoxide anion produced by normal metabolism of human body to avoid a variety of diseases induced by imbalance of free radicals. This research provides a theoretical fundamental for development of SAC and other sulfides extracted from Allium sativum.

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