Effective production of S-allyl-L-cysteine through a homogeneous reaction with activated endogenous γ -glutamyltranspeptidase in garlic (*Allium Sativum*)

Xiaobian Xu · Yelian Miao · Jie Yu Chen · Qimei Zhang · Jining Wang

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Abstract S-allyl-L-cysteine (SAC) is a bioactive compound in garlic (*Allium sativum*). A novel process including soaking and homogeneous reaction was applied for the effective production of SAC with endogenous γ -glutamyltranspeptidase (γ -GTP, EC 2.3.2.2) in garlic. The effects of temperature and CaCl_2 concentration on γ -GTP activity in soaking, and the relationship of SAC production with γ -GTP activity in homogeneous reaction were investigated, using fresh garlic as raw material. The experimental results showed that the γ -GTP in fresh garlic was activated by soaking. The yield rate and the final content of SAC increased linearly with increasing initial γ -GTP activity in the homogeneous reaction at 37 °C. The final SAC content reached 606.3 $\mu\text{g/g}$ (i.e. 32 times higher than that in fresh garlic) after soaking for 72 h in a 10-mM CaCl_2 solution at 10 °C, and the homogeneous reaction for 8 h at 37 °C. SAC was produced effectively through the homogeneous reaction with activated endogenous γ -GTP in garlic.

Keywords Garlic · Soaking · Homogeneous reaction · S-allyl-L-cysteine · γ -glutamyltranspeptidase

Introduction

S-allyl-L-cysteine (SAC, $\text{C}_6\text{H}_{11}\text{NO}_2\text{S}$) is a colorless, odorless, water-soluble, stable and bioactive organosulfur compound existing in garlic (*Allium sativum*). It has protective effects against oxidation, free radicals, cancer, cardiovascular diseases and neuronal degeneration diseases (Kim et al. 2001; Amagase 2006; Wang et al. 2010; Rojas et al. 2011). The toxicity of SAC is less than 4% of that of allicin and diallyl disulfide (DADS), as shown in a test of the oral 50% lethal dose of typical garlic compounds in mice by the U.S. National Cancer Institute (Amagase 2006). A pharmacokinetic study on the oral administration of garlic supplement containing SAC to human volunteers indicates that the SAC from garlic consumption is rapidly absorbed from the gastrointestinal tract, and its half-life and excretion time are more than 10 h and 30 h, respectively (Kodera et al. 2002). SAC is detected in the blood, and its concentration in blood and other pharmacokinetic parameters are well-associated with doses of orally administered SAC in animal studies. Because SAC accounts for a main portion of garlic's biological activities, it is suggested to use SAC as a chemical marker for the standardization of garlic preparations (Kim et al. 2001; Amagase 2006).

In garlic, SAC is derived from γ -glutamyl-S-allyl-L-cysteinyls (GSAC, $\text{C}_{11}\text{H}_{18}\text{N}_2\text{O}_5\text{S}$) through an enzymatic transformation with the catalysis of γ -glutamyltranspeptidase (γ -GTP, EC 2.3.2.2) (Amagase 2006). So far, aging is considered as a typical process to accumulate SAC in garlic. By aging of fresh garlic bulbs or cloves for 40 days in a thermo-hydratostat chamber controlled at 65–80 °C in temperature and 70%–80% in relative humidity, the SAC content reached 98–194 $\mu\text{g/g}$, i.e. 4.5–8 times higher than that in fresh garlic (Wang et al. 2010; Bae et al. 2012). When peeled garlic cloves were frozen at –25 °C, then crashed and aged for 15 days at 40 °C, the SAC content increased to 4.6 times higher than that in fresh garlic (Park et al. 2010). In addition, SAC accumulation was different in dormant and dormant-terminated garlic bulbs. An

X. Xu · Y. Miao (✉) · Q. Zhang
College of Food Science and Light Industrial Engineering, State Key Laboratory of Materials-Oriented Chemical Engineering, Nanjing University of Technology, Nanjing City, Jiangsu 211816, China
e-mail: ylmiao@njut.edu.cn

J. Y. Chen
Faculty of Bioresource Science, Akita Prefectural University,
Akita City, Akita 010-0195, Japan

J. Wang
School of Economics and Management, Nanjing University of
Technology, Nanjing City, Jiangsu 211816, China

increase of SAC content from 0.3 mg/g-dry matter to 5.3 mg/g-dry matter in dormant-terminated garlic bulbs was achieved by aging for 12 days at 45 °C, while this increase was from 0.2 mg/g-dry matter to 3.2 mg/g-dry matter in dormant garlic bulbs by aging for 14 days at 55 °C (Yamazaki and Okuno 2008). In dried fresh garlic bulbs, however, the γ -GTP activity is very low. Furthermore, γ -GTP is bounded on cell membranes Martin and Slovin (2000), and its substrate GSAC locates in vacuole (Jones et al. 2004), so that it is difficult for γ -GTP to act on GSAC. In these cases, the enzymatic transformation of GSAC to SAC is limited.

In the present study, a novel process including soaking and homogeneous reaction was applied for the effective production of SAC with the endogenous γ -glutamyltranspeptidase (γ -GTP, EC 2.3.2.2) in garlic. The effects of temperature and CaCl_2 concentration on γ -GTP activity in soaking, and the relationship of SAC production and γ -GTP activity in homogeneous reaction were investigated, using fresh garlic as raw material.

Materials and methods

Garlic Fresh garlic (*Allium sativum*) bulbs produced in the suburb of Xuzhou City in 2011 were used as the test material for soaking and homogeneous reaction. The fresh garlic bulbs were sun-dried to an equilibrium moisture content of 63.3%, and then stored at the room temperature until use.

Soaking Fresh garlic bulbs (300 g) were soaked in a dilute CaCl_2 solution for 72 h in order to improve its γ -GTP activity with Ca^{2+} . The soaking temperature was set at 5, 10, 20, 30, 40 °C, while the CaCl_2 concentration of soaking solution was set at 0 (i.e. deionized water), 4, 8, 10, 12, 16 mM, respectively. Changes in the moisture content, the SAC content and the γ -GTP activity of garlic were investigated during soaking. The moisture content was determined by slicing and drying a 5-g peeled garlic clove sample for 2 h at 105 °C, and expressed on the wet basis of peeled garlic cloves.

Homogeneous reaction After the soaking, the garlic bulbs were peeled, 150-g garlic cloves were mashed with a mortar and pestle, and transferred into a 500-mL Erlenmeyer flask together with 150-mL deionized water. Homogeneous reaction of the mashed garlic was performed to produce SAC with its endogenous γ -GTP on a shaker at 37 °C and 100 rpm. During the homogeneous reaction, samples of 20 mL in each were taken from the flask for analyzing of SAC.

Measurement of γ -GTP activity 50-mM Tris-HCl buffer (pH 8.0) containing 6-aminocaproic acid at a concentration of 5 mM was used as the extracting solution. For the extraction of γ -GTP, 25-g peeled garlic cloves were mashed, and

mixed with 40-mL extracting solution in a 500-mL Erlenmeyer flask at the room temperature for 4 h. After the extraction, the mixture was centrifuged for 40 min at $7,000\times g$ and 4 °C to remove the solid fraction. The supernatant containing γ -GTP was transferred into a 50-mL volumetric flask, and the Tris-HCl buffer was added in capacity for measuring of γ -GTP activity (Li et al. 2008; Zhao and Qiao 2009).

γ -GTP activity of the enzyme solution was measured with the colorimetric method Martin and Slovin (2000). 20-mL enzyme solution was mixed with 0.5 mL of 5-mM L- γ -glutamyl-p-nitroaniline monohydrate (AR, TCI Shanghai, China), 0.5 mL of 0.1-M N-glycylglycine (purity $\geq 99\%$, Aladdin reagent Co., Ltd., USA) and 1.5 mL of 50-mM Tris-HCl buffer solution (pH 8.0) in a test tube (25 mm $\times$ 200 mm). The enzymatic reaction was performed for 30 min at 37 °C, and terminated by boiling for 10 min on a thermostated water bath. After cooling to the room temperature and filtering with filter paper (GB/T 1914–2007), the absorbance of reaction solution at 410 nm was determined using a UV-Vis spectrophotometer (752S, Shanghai Lengguang Technology Co., Ltd.). P-nitroaniline (purity $\geq 99\%$, Aladdin reagent Co., Ltd., USA) was used as the reference material in the measurement.

The unit of γ -GTP activity was defined as the amount of enzyme which produces 1- μmol p-nitroaniline in 1 min from γ -glutamyl-p-nitroaniline under optimum conditions: temperature of 37 °C, and pH of 8.0. The γ -GTP activity of garlic, E (U/g), was calculated with Eq. (1):

$$E = \frac{C_N \times V_N \times n}{t \times m_G} \quad (1)$$

where C_N is the p-nitroaniline concentration of reaction solution ($\mu\text{mol/mL}$), V_N is the volume of reaction solution ($V_N = 22.5$ mL), n is the volume ratio of total enzyme solution to used

Fig. 1 Change of moisture content in garlic during soaking in deionized water at different temperatures

Fig. 2 Change of γ -GTP activity in garlic during soaking in deionized water at 10 °C

enzyme solution ($n=2.5$), t is the reaction time ($t=30$ min), and m_G is the dry mass of garlic used in the measurement (g).

Analysis of SAC For the garlic bulb samples taken during the soaking process, their endogenous enzymes were inactivated by heating for 60 s using a microwave oven under the condition of a microwave power output at 400 W and a microwave frequency at 2,450 MHz. After the enzyme inactivation, 25-g peeled garlic cloves of each sample were mashed, transferred into a 150-mL Erlenmeyer flask together with 40-mL deionized water, and then extracted for 2 h over a 90 °C water bath (Wang et al. 2010; Cui et al. 2007). For the mixture samples taken during homogeneous reaction, 20-mL deionized water was added each, and then extraction was performed for 2 h over a 90 °C water bath. After the extraction, the mixture was centrifuged at $7,000\times g$ for 40 min to remove the solid fraction. The supernatant was transferred to a 50-mL volumetric flask, and the Tris-HCl buffer was added in capacity for analyzing of SAC.

Fig. 3 Effect of soaking temperature on γ -GTP activity in the garlic soaked for 72 h in deionized water

Fig. 4 Effect of CaCl_2 concentration on γ -GTP activity in the garlic soaked for 72 h at 10 °C

SAC in the extracts was analyzed using a high-performance liquid chromatograph (Summit, Dionex, USA) equipped with a variable-wavelength UV detector (UVD-170U, Dionex, USA) and a Zorbax SB-Aq column (4.6 mm $\times$ 250 mm, Agilent, USA). The extract samples were filtered through Sep Pak C18 filters (Millipore) and thus injected into the chromatograph under these conditions, i.e. a column temperature of 25 °C, a methanol-water mixture as mobile phase at a flow rate of 0.8 mL/min, a detecting wavelength of 220 nm, and an injection volume of 20 μ L (Kodera et al. 2002; Li et al. 2010). Methanol and water fractions in the mobile phase were changed as (1) 0 to 5.5 min at 3% (v/v) methanol+97% (v/v) water, (2) 5.5 to 8 min from 3% (v/v) methanol+97% (v/v) water to 12% (v/v) methanol+88% (v/v) water (linear gradient), (3) 8 to 10 min from 12% (v/v) methanol+88% (v/v) water to 20% (v/v) methanol+80% (v/v) water (linear gradient), and (4) 10 to 20 min at 20% (v/v) methanol+80% (v/v) water. SAC (purity $\geq$ 98%, Tokyo Kasei Kogyo Co., Ltd., Japan) was used as the reference material in the analysis.

Fig. 5 Change of SAC content in garlic during soaking in deionized water at 10 °C

Fig. 6 Effect of soaking temperature on SAC content in the garlic soaked for 72 h in deionized water

The SAC content of garlic, M_S (%), was calculated with Eq. (2):

$$M_S = \frac{C_S \times V_S}{m_G} \times 100\% \quad (2)$$

where C_S is the concentration of SAC solution (g/mL), and V_S is the volume of SAC solution ($V_S=50$ mL).

The measurement of moisture content, γ -GTP activity and SAC content was conducted in duplicate, and the data presented here are the average results.

Results and discussion

Effect of soaking on γ -GTP activity in garlic The fresh garlic had a moisture content of 63.3% before soaking. During soaking, the moisture content in garlic increased due to the water absorption (Fig. 1). The increase rate and the final

Fig. 7 Effect of CaCl_2 concentration on SAC content in the garlic soaked for 72 h at 10 °C

Fig. 8 Increase of SAC content in garlic during the homogeneous reaction with an initial γ -GTP activity of 7.5 mU/g (reaction temperature: 37 °C)

moisture content were affected by the soaking temperature. At the temperatures of 5, 10, 20, 30 and 40 °C, the moisture content increased to 69.2, 70.4, 71.1, 71.2 and 71.6% in 36–72 h, respectively.

The fresh garlic had a γ -GTP activity of 0.4 mU/g before soaking. The γ -GTP activity increased with soaking time in 48 h as shown in Fig. 2. The increase of γ -GTP activity was caused by the activation of γ -GTP, due to the synthesis of plant hormones, growth regulators and mRNA (Gaspar et al. 1996; Du et al. 2011). After 48 h, however, the γ -GTP activity turned to decrease slightly because of the decomposition of γ -GTP by proteinase. The optimal soaking temperature for increasing of γ -GTP activity was 10 °C (Fig. 3). On the other hand, moulds were observed on the surface of garlic bulbs soaked at 40 °C. It was consistent with the report that γ -GTP expression in garlic was induced by cold storage, but suppressed by storage at 20 °C (Cho et al. 2012).

The effect of CaCl_2 concentration on γ -GTP activity in the garlic soaked for 72 h at 10 °C is shown in Fig. 4. When the CaCl_2 concentration increased from 0 mM to 10 mM, the

Fig. 9 Changes of SAC yield rate and final SAC content with initial γ -GTP activity in homogeneous reaction (reaction temperature: 37 °C)

γ -GTP activity increased from 7.5 mU/g to 9.4 mU/g. In the range of CaCl_2 concentration higher than 10 mM, however, the γ -GTP activity decreased with increasing CaCl_2 concentration. It was also reported that the activity of γ -GTP from *Lentinus edodes* and *Bacillus Subtilis* could be improved by adding a proper amount of Ca^{2+} to the enzyme solution (Yi et al. 2009; Song et al. 2011). γ -GTP is a dimeric of two subunits with a different molecular weight each. Ca^{2+} helps to enhance the connection of subunits (Yi et al. 2009; Keillor et al. 2005). However, too much Ca^{2+} might destroy the secondary structure and active center of γ -GTP, resulting in a decrease in activity (Dai et al. 2012).

Effect of soaking on SAC content in garlic The fresh garlic had a SAC content of 19.0 $\mu\text{g/g}$ before soaking. It was comparable to the reported value (Wang et al. 2010; Kodera et al. 2002; Bae et al. 2012). The SAC content increased with soaking time and became constant after soaking for 60 h (Fig. 5). The increase of SAC content was related to the increase of γ -GTP activity in the garlic.

The effects of soaking temperature and CaCl_2 concentration on SAC content are shown in Figs. 6 and 7, respectively. The garlic was soaked for 72 h. Under the optimum condition of a soaking temperature at 10 °C and a CaCl_2 concentration at 10 mM, the SAC content increased to 76.7 $\mu\text{g/g}$, i.e. 4 times higher than that in fresh garlic.

SAC production in homogeneous reaction After soaking for 72 h under different conditions of temperatures and CaCl_2 concentrations, the garlic bulbs were peeled and mashed. Homogeneous reaction of the mashed garlic was performed at 37 °C to produce SAC with its endogenous γ -GTP. When the initial γ -GTP activity was 7.5 mU/g, the SAC content increased from 58.2 $\mu\text{g/g}$ to 482.9 $\mu\text{g/g}$ in 8 h (Fig. 8). The increase of SAC content was due to the easy contact of γ -GTP with its substrate GSAC.

In the homogeneous reaction, both the yield rate and the final content of SAC increased linearly with increasing initial γ -GTP activity as shown in Fig. 9. The SAC yield rate was defined as the average increment in the first 2 h of homogeneous reaction. The relationship of SAC yield rate (R_S , $\mu\text{g}/(\text{g}\cdot\text{h})$) and initial γ -GTP activity (E_0 , mU/g), and the relationship of final SAC content (M_{SF} , $\mu\text{g/g}$) and initial γ -GTP activity (E_0 , mU/g) could be expressed with Eqs. (3) and (4):

$$R_S = 16.0E_0 + 9.7 \quad (R^2 = 0.965) \quad (3)$$

$$M_{SF} = 55.6E_0 + 64.9 \quad (R^2 = 0.960) \quad (4)$$

The SAC yield rate was 12.1 $\mu\text{g}/(\text{g}\cdot\text{h})$, and the final SAC content was 49.3 $\mu\text{g/g}$ for the fresh garlic with a γ -GTP activity of 0.4 mU/g. The SAC yield rate increased to 170.5 $\mu\text{g}/(\text{g}\cdot\text{h})$, and the final SAC content increased to 606.3 $\mu\text{g/g}$

(i.e. 32 times higher than that in fresh garlic) for the garlic with an initial γ -GTP activity of 9.4 mU/g, when the garlic was previously soaked for 72 h in a 10 mM CaCl_2 solution at 10 °C. SAC was produced effectively through the homogeneous reaction with activated endogenous γ -GTP in garlic.

It is obvious that the novel process proposed in the present study has advantages over the conventional aging process in activating the γ -GTP and facilitating the enzymatic reaction in garlic. The SAC-enriched garlic may be used as a health promoting ingredient for the production of designer foods, or functional foods.

Conclusion

The present study presented the data on the effective production of S-allyl-L-cysteine (SAC) through homogeneous reaction with the activated endogenous γ -glutamyltranspeptidase (γ -GTP) in garlic. The γ -GTP activity of fresh garlic increased from 0.4 mU/g to 9.4 mU/g, and the SAC content increased from 19.0 $\mu\text{g/g}$ to 76.7 $\mu\text{g/g}$ after soaking for 72 h in a 10-mM CaCl_2 solution at 10 °C. During homogeneous reaction at 37 °C, the yield rate and the final content of SAC increased linearly with increasing initial γ -GTP activity. The final SAC content reached 606.3 $\mu\text{g/g}$ (i.e. 32 times higher than that in fresh garlic) after the soaking for 72 h in 10-mM CaCl_2 solution at 10 °C and the homogeneous reaction for 8 h at 37 °C.

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