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Relationship between γ-Glutamyl Transpeptidase Activity and Garlic Greening, As Controlled by Temperature

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It was established that storage at low temperature (less than 10 °C) was required for garlic greening occurring either during processing or in the course of "Laba" garlic preparation while storage at high temperature (higher than 20 °C) inhibited its occurrence. However, the reason for this observation is unclear. To obtain insights into a tie connected between storage temperature and garlic greening, it was detected if the γ-glutamyl transpeptidase (GGT) activity correlated with garlic greening because the activity of this enzyme is very sensitive to storage temperature. Results showed that garlic puree (which was prepared from fresh garlic) turned green upon addition of GGT but the color of garlic puree remained unchanged when either water or heat-treated GGT (which has no activity due to heat treatment) was used, a result giving a positive answer to the above proposal. Subsequently, to further clarify the relationship between the GGT activity and garlic greening, the GGT activity, the degree of garlic greening, and the concentration of total thiosulfinates in garlic bulbs were determined respectively after the garlic bulbs had been stored at 4 °C for up to 59 days followed by storage at 35 °C for up to 22 days. It was found that cold storage facilitated the GGT activity whereas warm storage inhibited the activity of this enzyme, just like the effect of storage temperature on greening, indicating that the increase of GGT activity could be a direct factor resulting in garlic greening. Consistent with this conclusion, the concentration of total thiosulfinates (the color developers) in garlic purees likewise exhibited a reversible change by moving garlic bulbs from one low storage temperature to a higher one; namely, it increased with increasing storage time during storage at 4 °C while decreasing as storage time increased during storage at 35 °C. The present study provided direct evidence that the GGT is involved in garlic greening.

KEYWORDS: Garlic; γ-glutamyl transpeptidase; greening; storage; thiosulfinates

INTRODUCTION

Garlic has been widely cultivated in the world for more than 4000 years due to its variety of pharmacological benefits (*1*). It was processed in various forms such as powder, granules, puree, minced paste, and oleoresin. During this processing, a green discoloration is a major concern because it limits commercial utilization and reduces economic value (*2, 3*). Green pigments corresponding to greening are considered to be secondary metabolites in garlic. As garlic bulbs are stored for several months after harvest to ensure year-round supplies for customers, the storage conditions are important to prevent loss of shelf life and quality, such as surface discoloration, moisture loss, sprouting, and rooting. Previous studies showed that storage at low temperature favors garlic greening whereas storage at high temperature inhibits the discoloration (*3*). Consistent with this observation, an aging process that garlic was stored at low temperature is also required for the formation of the green pigment of "Laba" garlic, a homemade Chinese garlic product, which was prepared by immersing the aged garlic in vinegar for about 1 week (*4, 5*). Effects of temperature on garlic greening are ascribed to the proposal that storage at low temperature favors the breaking of dormancy and sprout and root development of the garlic bulbs while storage at high temperature prolongs the process of their dormancy (*6–8*). As a consequence, certain chemical/biochemical changes in garlic bulbs were caused by the breaking of their dormancy. However, the changes which are related to garlic greening are poorly understood.

γ-Glutamyl transpeptidases (GGT) ([5-L-glutamyl]-peptide: amino acid 5-glutamyltransferase; EC 2.3.2.2) are ubiquitous enzymes found in bacteria and animals as well as plants where they catalyze the hydrolysis of the uniquely linked N-terminal

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Glu from the reduced glutathione (GSH), oxidized glutathione (GSSG), and glutathione *S*-conjugates, as well as from a number of dipeptides and other tripeptides having an N-terminal γ-linked Glu. The Glu moiety is transferred to either water (hydrolysis) or to an acceptor amino acid, dipeptide, or tripeptide, including GSH (transpeptidation), resulting in a new amide bond with an N-terminal, γ-linked Glu (*9, 10*).

In plants, enzymes with GGT activity are believed to be involved in secondary metabolism. Although GGT catalyze the synthesis of a range of γ-glutamyl dipeptides, which are formed during fruit ripening and accumulate in storage tissues such as seeds or bulbs in certain plants (*11–14*), GGT occurring in onion catalyzes the last step in the formation of volatile compound precursors by cleaving the γ-glutamyl moiety off γ-glutamyl alk(en)yl-Cys sulfoxides (*15, 16*). Previous studies showed that the activity of GGT was greatly improved upon germination and sprouting of onion bulbs. For example, no GGT activity can be detected in the fleshy layers of mature dormant onion bulbs, but sprouted onions are capable of releasing *p*-nitroaniline (PNA) from γ-L-glutamyl-*p*-nitroaniline (GPNA), an artificial substrate (*17, 18*).

On the basis of the above results and the fact that both garlic and onion belong to the *Allium* genus, it opens interesting questions as to whether the breaking of the dormancy of garlic by cold storage likewise causes an increase of the activity of GGT in garlic; on the contrary, does the prolongation of the dormancy process induced by warm storage prevent the activity of GGT? If GGT do have an effect on garlic greening, what is the relationship between GGT and garlic greening? To answer the above questions, the activity of GGT in garlic was measured during storage at both low (4 °C) and high (35 °C) temperature, respectively. In parallel, the content of garlic greening was also determined under the same conditions. It was found that the activity of garlic GGT was controlled by temperature and that the increase of the garlic GGT results in garlic greening. All results suggested that there is a close relationship between the GGT activity and garlic greening.

MATERIALS AND METHODS

Chemicals. All solvents/chemicals used were of analytical grade or purer. γ-Glutamyl-*p*-nitroanilide (GPNA) and phenylmethanesulfonyl fluoride (PMSF) were purchased from Research Organics (Beijing, China). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), $NaNO_2$, ammonium sulfamate, naphthylethylenediamine dihydrochloride, and L-cysteine were purchased from Sigma Chemical Co. (Beijing, China). Solutions of L-cysteine (6.0×10^{-4} M) and DTNB (3.0×10^{-4} M) were both freshly prepared in sodium phosphate buffer, and DTNB was stored in a brown reagent bottle at 4 °C after preparation.

Plant Materials. Garlic bulbs were obtained from a local market of China Agriculture University. They were harvested in May 2006.

Preparation of Garlic GGT. GGT in garlic was purified on the basis of previous methods with slight modifications (*15, 16*). Briefly, sprouting garlic cloves were peeled off, rinsed with tap water, and then triple rinsed with distilled water three times. The cleaned cloves (400 g) were homogenized in a blender with 500 mL of chilled 50 mM Tris-HCl (pH 7.5) including 1 mM PMSF and 5 mM 6-aminohexanoic acid and filtered through cheesecloth. The filtrate was centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was saturated with approximately 70% $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation at 12000*g* for 30 min at 4 °C. The precipitate was resuspended in 20 mL of 50 mM Tris-HCl (pH 7.5). The resulting solution was applied to a phenyl-Sepharose column (2.5 × 12 cm) followed by a concanavalin A−Sepharose column (1.6 × 3 cm). The enzyme concentration was determined by the Lowry method with bovine serum albumin as standard (*19*). One unit of GGT activity is defined as that amount of enzyme which will liberate 1 μM *p*-nitroaniline in 1 min under standard assay conditions. Results here are reported as milliunits.

Preparation of Garlic Purees and Measurement of Their UV/Vis Spectra. After cracking fresh garlic bulbs, shriveled, damaged, and small cloves were discarded; the remaining cloves were peeled off and rinsed with distilled water three times. Garlic cloves (30) were homogenized in a blender with 30 mL of ddH_2O. The resulting materials were equally divided into three parts. The first was mixed with 100 μL of GGT solution including 500 milliunits. The second was combined under stirring with 100 μL of GGT solution which had been heated at 100 °C for 10 min. The third was mixed just with 100 μL of 50 mM Tris-HCl (pH 7.5) as control. All resultant mixtures were allowed to stand at room temperature overnight and filtrated by filter paper. The filtrate was collected and concentrated by ultrafiltration to 1 mL, which was placed into a quartz cuvette for UV/vis spectral measurement.

Determination of Garlic GGT Activity, Total Thiosulfinate Concentration, and Content of Garlic Greening. After garlic cloves was stored for 0, 15, 22, 27, 32, 37, 42, 47, 52, and 59 days at 4 °C followed by storage for 0, 5, 12, 17, and 22 days at 35 °C, respectively; ~150 g of cloves was taken out and homogenized in a blender with 150 mL of ddH_2O. Freshly prepared garlic puree was used for the determination of the garlic GGT activity and the concentration of total thiosulfinates. In contrast, the resulting garlic puree was allowed to stand overnight at room temperature and then used for measurement of greening degree.

The garlic GGT activity was measured as previously described with some modifications (*20*). Basically, around 6 g of garlic puree was filtered by cheesecloth and centrifuged at 10000*g* for 10 min at 4 °C, and resulting solution was saturated with ~70% $(NH_4)_2SO_4$. The produced precipitate was dissolved in 3 mL of 50 mM Tris-HCl (pH = 7.5). The resulting solution was used as the raw GGT enzyme after removing residual $(NH_4)_2SO_4$ by dialysis against 50 mM Tris-HCl (pH 7.5). The final volume of the raw enzyme was still kept in 3 mL. One milliliter of the enzyme solution was added to 0.5 mL of assay solution including 4 mM GPNA and 100 mM L-methionine in Tris-HCl (pH = 9) and incubated at 37 °C, and the assay was terminated by adding 2 mL of 5 M HOAc. The releasing *p*-nitroaniline was diazotized to form a pink dye by sequential addition of 1 mL of 0.1% $NaNO_2$, 1 mL of % ammonium sulfamate, and 1 mL of naphthylethylenediamine dihydrochloride solution. Absorbance at 540 nm was determined.

To determine the concentration of total thiosulfinates in garlic, about 5 g of garlic purees was lyophilized for 12 h and was powdered with a mortar and pestle. Freeze-dried and powdered garlic samples (~0.1 g) were stirred into 100 mL of 50 mM sodium phosphate buffer (pH 6.9), containing 1 mM EDTA. After 10 min, the solutions were filtrated, and total thiosulfinates were quantified spectrophotometrically with Cary 50 spectrophotometer (Varian) according to a previous method (*21*).

The degree of garlic greening was determined as follows: about 10 g of garlic puree was allowed to stand overnight, then filtered by cheesecloth, and centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was concentrated to 1 mL of solution, which was placed into a quartz cuvette for absorbance measurement at 590 nm (*4*).

Determination of Garlic GGT Activity at Different Storage Temperatures. The method used was as above described except that garlic cloves were stored for 50 days at four different temperatures, 4, 12, 23, and 35 °C, respectively.

Statistical Analysis. Statistics on a completely randomized design were determined using SAS 9.0 for Windows. Duncan's multiple-range test ($p < 0.05$) was used to determine the significance of differences between means.

RESULTS AND DISCUSSION

To determine whether GGT plays a role in garlic greening, GGT, heat-treated GGT (100 °C for 10 min), and buffer were added to equal amounts of garlic puree prepared from fresh garlic bulbs, respectively. After the resulting materials were allowed to stand overnight at room temperature and filtrated, UV/vis spectra of filtrate were obtained by scanning and displayed in **Figure 1**. It was found that the color of garlic puree

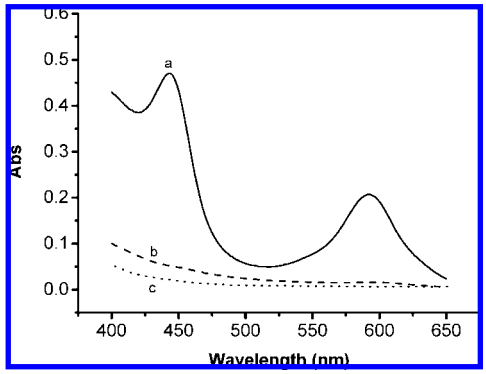

Figure 1. UV/vis spectra of water extracts from fresh garlic puree treated with and without γ-glutamyl transpeptidases (γ-GT) on garlic greening. Water extracts from (**a**) fresh garlic puree + γ-GT, (**b**) fresh garlic puree + heat-treated γ-GT, and (**c**) fresh garlic puree alone, respectively.

turned green upon treatment with GGT. A solution from the garlic puree exhibited two maximal absorbances at 440 and 590 nm (**Figure 1**, spectrum a), which are characteristic of garlic greening (*4, 22, 23*). In contrast, when the same volume of buffer was used instead of GGT, no change was observed with its color as indicated by the UV/vis spectrum (**Figure 1**, spectrum c). This result suggested that GGT was involved in garlic greening. Consistent with this proposal, when the same enzyme was used but it completely lost the activity due to treatment at 100 °C for 10 min, the color of garlic puree likewise remained unchanged (**Figure 1**, spectrum b).

Many studies showed that fresh garlic can be converted into aged garlic through an aging process corresponding to around 1−4 month storage at low temperature and that garlic greening only occurs in the aged garlic rather than the fresh one after they were either crushed or soaked in vinegar (*3, 4, 23–25*). The most likely reason for greening of the aged garlic was ascribed to its dormancy termination (*6–8, 24, 25*). Previous studies indicated that the dormancy termination will result in a significant chemical/biochemical change in the plant. For example, the activity of GGT was improved greatly after the germination and sprouting of onion (*17, 18*). Therefore, this raises a question as to whether the breaking of the garlic dormancy by cold storage (4 °C) may change the activity of the garlic GGT. To answer this question, the GGT activity was determined with fresh garlic puree, and corresponding results were shown in **Figure 2A**. A plot of GGT activity against storage time exhibits a sigmoidal curve, indicating that the effect of storage time on the activity of GGT consists of three phases. The first is an initial phase, and the activity of garlic GGT was slowly growing as storage time increased from 0 to 25 days. Beyond about 25 day storage, the activity of GGT was rapidly growing with an increase of the storage time until the 40th day. After 40 day storage, the increase of the GGT activity became slow again.

To elucidate the relationship between the GGT activity and garlic greening, garlic greening was first monitored as a function of storage time after freshly prepared garlic puree was allowed to stand overnight at room temperature, and results were shown in **Figure 2B**. Being in good agreement with the change of the GGT activity shown in **Figure 2A**, the change of garlic greening also exhibited a sigmoidal curve with increasing storage time. The curve is composed of three phases, namely, the initial slow phase followed by the fast growing phase and the third phase. This result suggested that the GGT activity correlated positively related with garlic greening. Thus, it is reasonable to believe that the dormancy termination of garlic by low-temperature

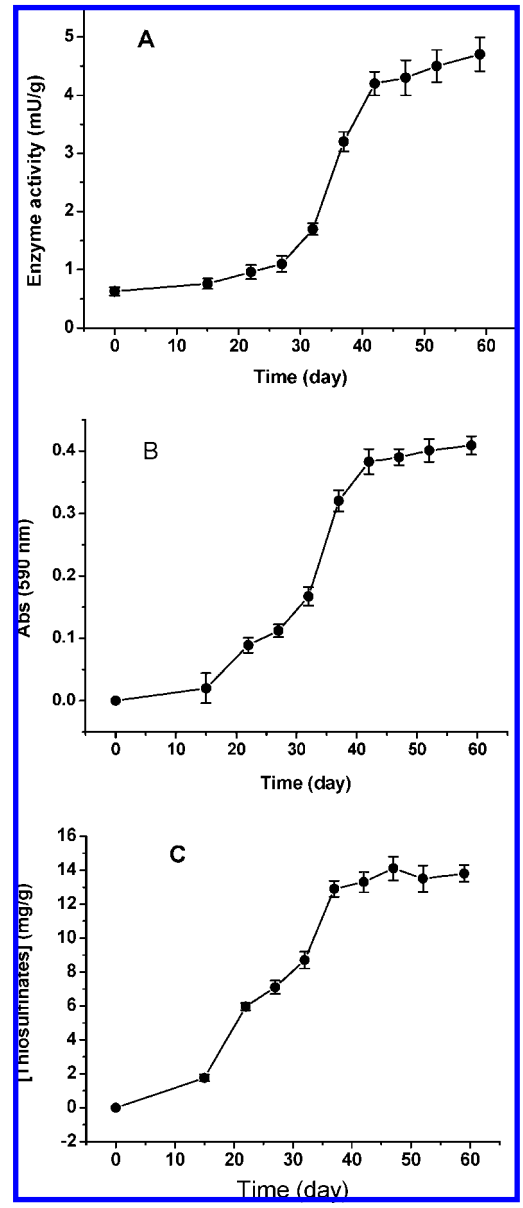

Figure 2. Effects of storage at low temperature (4 °C) on the activity of γ-glutamyl transpeptidases (GGT) (**A**), garlic greening (**B**), and the concentration of thiosulfinates (**C**) in garlic. Each point represents a separate sample, and its value is the average of three independent measurements. Vertical bars represent the standard deviation.

storage caused the increase of the GGT activity which triggered a series of chemical reactions, resulting in garlic greening.

Similar to GGT separated from onion (*17, 18*), the present studies show that garlic GGT also has the ability to catalytically hydrolyze the amide bond of γ-L-glutamyl-*p*-nitroaniline (GPNA) to release *p*-nitroaniline (PNA). It is likely that the garlic GGT is also capable of hydrolyzing dipeptides contained in garlic such as γ-glutamyl-*S*-alk(en)yl-Cys sulfoxides (*26*) to produce *S*-alk(en)yl-Cys sulfoxides. If this were the case, resulting *S*-alk(en)yl-Cys sulfoxides would convert to their corresponding thiosulfinates catalyzed by another enzyme, alliinase released from vacuole due to crush during the preparation of garlic purees. It has been well established that thiosulfinates participate in the formation of green pigment(s) responsible for garlic greening (*4, 5, 22, 27–29*). Possible pathways have been proposed that the thiosulfinates, the color developers (*22, 29*), would react with amino acid to form a pyrrole compound, ultimately resulting in the formation of the green pigments (*27, 28*).

To confirm the above idea that the garlic GGT activity correlates with greening, the concentration of thiosulfinates was measured as a function of storage time in parallel using the same batch of garlic puree and the same method as used for the measurement of the GGT activity (**Figure 2C**). It was observed that the profile of the concentration of thiosulfinates varying with storage time is pretty similar to that of the GGT activity change and also similar with that of the change of garlic greening versus storage time. This finding demonstrated that the increase of the garlic GGT activity resulted in hydrolysis of γ-glutamyl-*S*-alk(en)yl-Cys sulfoxides into corresponding *S*-alk(en)yl-Cys sulfoxides which were catalyzed into the thiosulfinates by alliinase, finally resulting in the formation of green pigment(s). Thus, the increase of the garlic GGT activity is indeed involved in garlic greening as suggested by the results shown in **Figure 1**.

In contrast, storage at high temperature was reported to inhibit garlic greening ascribed to the extension of the dormancy process (*3, 6–8, 24, 25*). However, the reason for inhibition is still poorly understood. To obtain insights into the clue related to the inhibition for greening by warm storage, the same batch of garlic bulbs already stored at 4 °C for up to 59 days for the above experiments was moved and further stored at 35 °C over a period of 22 days, and then garlic greening, the activity of the GGT, and the concentration of thiosulfinates were determined, respectively, as functions of storage time using the same methods as above (**Figure 3**). As expected, the degree of garlic greening became lighter and lighter with an increase of storage time, and greening almost disappeared on the 22nd day (**Figure 3B**) as suggested by the absorbance at 590 nm decreasing from 0.41 $\pm$ 0.02 to 0. This result suggested that garlic greening is a reversible process controlled by storage temperature. Support for this conclusion came from the previous observation that garlic bulbs were switched from greening to nongreening and back again several times by moving them from one storage temperature to another (*3*). Therefore, it is convenient that, by the regulation of storage temperature, garlic greening can be either inhibited during garlic processing (*2*) or facilitated for the preparation of the "Laba" garlic (*4*).

To further confirm the above observation that the GGT was closely related to garlic greening, the garlic GGT activity likewise was monitored during storage at 35 °C. Results showed that the GGT activity decreased much faster (**Figure 3A**) as compared to the increase of its activity due to cold storage (**Figure 2A**). By comparison, it was found that the profile of the decrease of the GGT activity was similar to that of the decrease of greening degree, suggesting that the inhibition of garlic greening during warm storage may be derived from the decrease of the GGT activity. This result supports the foregoing conclusion that there is a close relationship between the GGT activity and garlic greening.

Subsequently, to find a tie connecting the GGT activity with garlic greening, the concentration of thiosulfinates contained in the same batch of garlic bulbs was measured at various storage times, and results were shown in **Figure 3C**. Like the GGT activity, the concentration of thiosulfinates also declined as storage time increased. The results indicated that the decrease of the GGT activity induced by high-temperature storage led to the decrease of its hydrolyzing products, *S*-alk(en)yl-Cys sulfoxides, thereby decreasing the production of thiosulfinates and ultimately attenuating greening. Consistent with the present observation, Lukes found that there is a linear relationship between the depth of greening and the amount of *S*-(1-propenyl)cysteine sulfoxide (PRECSO), which is a precursor

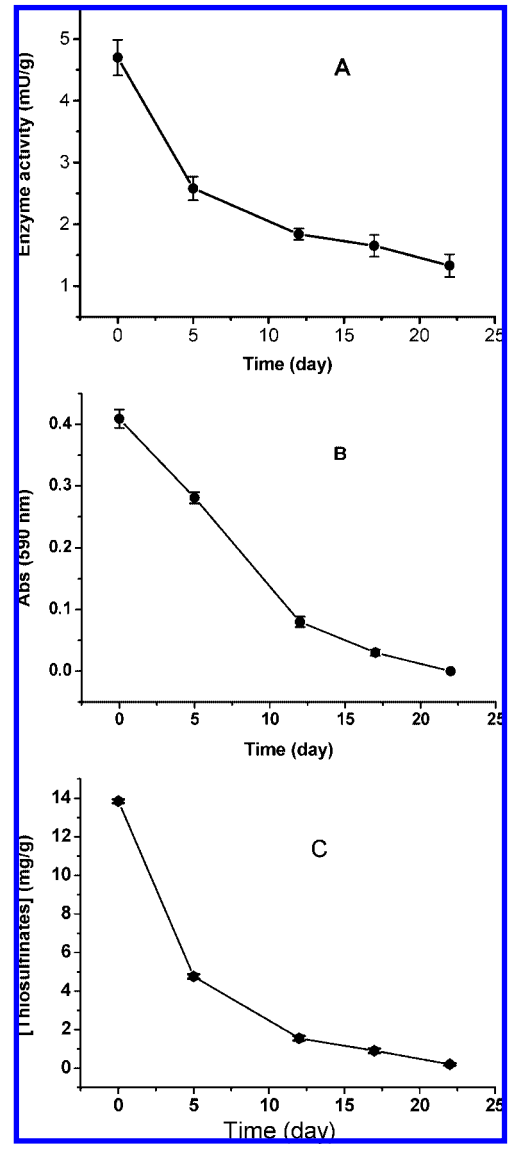

Figure 3. Effects of storage at high temperature (35 °C) on the activity of γ-glutamyl transpeptidases (GGT) (**A**), garlic greening (**B**), and the concentration of thiosulfinates (**C**) in garlic. Each point represents a separate sample, and its value is the average of three independent measurements. Vertical bars represent the standard deviation.

of thiosulfinates, and that cold storage facilitates the formation of this compound in garlic bulbs (*3*).

All of the above results suggested that the GGT activity has a great effect on garlic greening, but its activity seems to be controlled by storage temperature just like PRECSO (*3*). To further confirm this observation, the GGT activity was detected after garlic bulbs were stored for 50 days at four different temperatures including 4, 12, 23, and 35 °C, respectively (**Figure 4**). Consistent with the above result, the activity of GGT markedly decreased as storage temperature increased from 4 to 35 °C. Consequently, the GGT possessed the strongest activity (4.2 $\pm$ 0.7 milliunits/g) upon storage at 4 °C whereas it exhibited the lowest activity (0.17 $\pm$ 0.09 milliunits/g) after storage at 35 °C. It was reported that cold storage induced the dormancy termination of garlic bulbs and *vice versa* (*6–8, 24*). Accompanied by the breaking of the dormancy caused by cold storage, many chemical/biochemical changes occurred in garlic bulbs just as in other plants, but the increase of the GGT activity represents a direct factor which is involved in garlic greening; in other words, it corresponds to an early step for garlic greening.

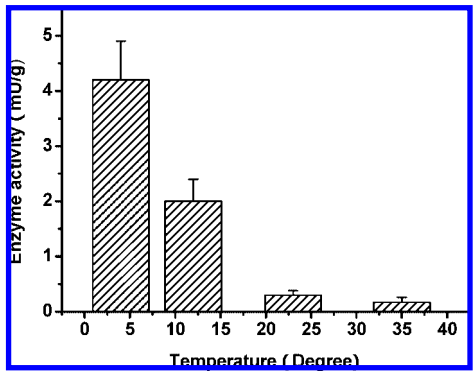

Figure 4. Effect of different temperatures on the activity of garlic γ-glutamyl transpeptidases (γ-GT). Garlic bubs were stored for 50 days at 4, 12, 23, and 35 °C, respectively, prior to measurement. Each value represents the average of three independent measurements. Vertical bars represent the standard deviation.

In summary, the GGT naturally occurred in garlic bulbs. The present study showed that the activity of the enzyme increased during cold storage whereas it declined during warm storage. This enzyme exhibited a very similar profile to the degree of garlic greening as a function of storage temperature, demonstrating that GGT correlated with garlic greening. Agreeing with above conclusion, the change of thiosulfinate concentration in garlic bulbs also showed a similar profile to the change of the GGT activity with storage temperature. Since garlic greening really depends on the GGT activity, the present study provides an easy way to diagnose the batch of garlic bulbs where greening potentially occurs.

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