JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

2-(1*H*-Pyrrolyl)carboxylic Acids as Pigment Precursors in Garlic Greening

DAN WANG, HUSILE NANDING, NA HAN, FANG CHEN, AND GUANGHUA ZHAO*

College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China

Six model compounds having a 2-(1*H*-pyrrolyl)carboxylic acid moiety and a hydrophobic R group were synthesized to study their effects on garlic greening, the structures of which are similar to that of 2-(3,4-dimethyl-1*H*-pyrrolyl)-3-methylbutanoic acid (PP-Val) (a possible pigment precursor for garlic greening). The puree of freshly harvested garlic bulbs turned green after being soaked in solutions of all these compounds, and with both increasing concentrations and incubation time the green color of the puree became deeper. In contrast, neither pyrrole alone nor pyrrole combined with free amino acids had the ability to discolor the puree. The compounds exhibited a good relationship between structure and activity of garlic greening, namely, the smaller the size of the R group, the larger the contribution. Also, it was found that the unidentified yellow species can be produced by reacting the model compounds with pyruvic acid at room temperature (23–25 °C). Moreover, blue species were formed by incubation of the model compounds with di(2-propenyl) thiosulfinate at room temperature. On the basis of these observations, a pathway for garlic greening was proposed.

KEYWORDS: 2-(1*H*-Pyrrolyl)carboxylic acids; pigment precursor; garlic greening; pyruvic acid; di(2-propenyl) thiosulfinate; thiosulfinate

INTRODUCTION

Garlic greening is a major concern during garlic processing because it limits commercial utilization and reduces economic value (*1*), but it is required and desirable for the preparation of "laba" garlic, a traditional homemade Chinese food product (*2, 3*). Despite having been studied for about 50 years, garlic greening is still poorly understood. On the basis of previous results, it is established that the green discoloration of garlic purees occurs only with garlic which has a high enough content of *S*-(1-propenyl)-L-cysteine sulfoxide (1-PeCSO) and is similar to the pink discoloration of onion, which is a multistep process (*4–8*). The first step corresponds to the production of an ether-soluble organosulfur compound, which is also called "color developer", under the action of alliinase on 1-PeCSO. Recent studies showed that the color developer could be di(1-propenyl) thiosulfinate (*9*) or 1-propenyl-containing thiosulfinate (*10*). Support for this conclusion comes from previous results showing that the amino acid 1-PeCSO was necessary for the development of the garlic green color (*11*). Also, consistent with this finding, recent results obtained by Kubec et al. indicated that, at pH 5.5, a dark blue pigment with a maximal absorbance at 582 nm was formed in a system containing 1-PeCSO, *S*-(2-propenyl)-L-cysteine sulfoxide (2-PeCSO), glycine, and alliinase (*8*). The second step corresponds to the formation of pigment precursor(s) by a reaction between thiosulfinates and amino acids; evidence has been presented that thiosulfinate consumption in the pickling solution of laba garlic is proportional to the formation of the pigments (*2*). The third step represents the formation of pigment via a reaction between the pigment precursor(s) and unidentified compound(s). Recently, Imai et al. prepared 2-(3,4-dimethyl-1*H*-pyrrolyl)-3-methylbutanoic acid (PP-Val) and 2-(3,4-dimethyl-1*H*-pyrrolyl)propanoic acid (PP-Ala) (**Figure 1**) by reacting di(1-propenyl) thiosulfinate with L-valine and L-alanine, respectively, and found that the PP-Val can react with di(2-propenyl) thiosulfinate into pigments at 100 °C in vitro (*9*), so PP-Val and its derivatives with 2-(1*H*-pyrrolyl)carboxylic acid moiety were considered to be the pigment precursor(s). Although PP-Val and its analogues might act as pigment precursors to garlic greening, more evidence is needed to verify this idea because only in vitro results based on model systems do not yet adequately support the above mechanism.

Lee et al. tried to isolate and purify a green pigment from a garlic homogenate, but they were not successful as judged from the reported ^{13}C NMR spectrum (*12*). Recent studies from two other groups suggested that the green pigment(s) responsible for garlic greening are composed of yellow and blue species (*2, 10*). It has been well-characterized that 2 mol of *S*-alk(en)yl-L-cysteine sulfoxides was catalyzed by alliinase into 1 mol of corresponding thiosulfinates, 2 mol of pyruvic acid, and 2 mol of ammonia (*13*), demonstrating that pyruvic acid was produced accompanied by the formation of thiosulfinates. Therefore, during the formation of PP-Val from the reaction of di(1-propenyl) thiosulfinate with L-valine (*9*), a certain amount of pyruvic acid must be produced. This opens the question of

* Corresponding author (telephone +86-10-62737645; fax +86-10-62737434-11; e-mail gzhao318@yahoo.com.cn).

10.1021/jf073025r CCC: $40.75
Published on Web 01/16/2008

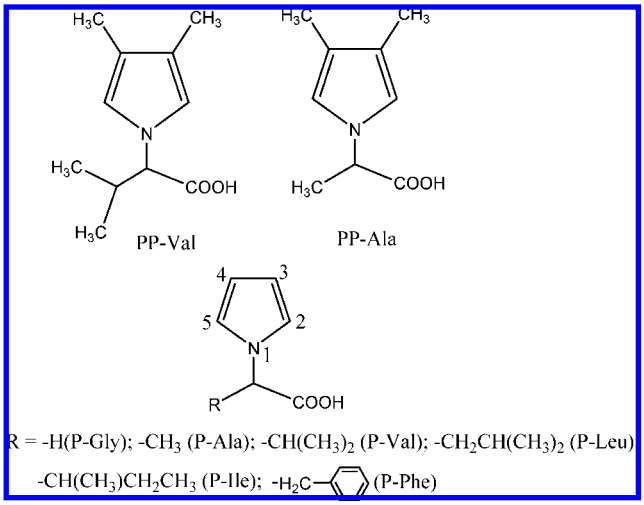

Figure 1. Structures of pigment precursors 2-(3,4-dimethyl-1*H*-pyrrolyl)-3-methylbutanoic acid (PP-Val) and 2-(3,4-dimethyl-1*H*-pyrrolyl)propanoic acid (PP-Ala) and their six model compounds, 2-(1*H*-pyrrolyl)carboxylic acids.

whether pyruvic acid could react with PP-Val or its analogues to produce pigment(s).

In this study, a series of pyrrole derivatives similar to PP-Val and PP-Ala in structure were prepared to obtain insights into the mechanism of garlic greening. At the same time, the possible pathways of the formation of yellow and blue species in which these pyrrole derivatives were involved were also explored. It was found that all of them can facilitate garlic greening but to different degrees upon their addition to garlic puree prepared from freshly harvested garlic. Moreover, these model compounds can react with pyruvic acid and di(2-propenyl) thiosulfinate to form unidentified yellow and blue pigments, respectively.

MATERIALS AND METHODS

Chemicals. L-Glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-phenylalanine, and pyrrole were purchased from Xinjingke Biotechnological Co. (Beijing, China). 2,5-Dimethoxytetrathydrofuran and diallyl disulfide were obtained from Fluka (Beijing, China). Methanol, hydrochloric acid, acetic acid, sodium acetate anhydrous, ethyl acetate, sodium sulfate anhydrous, ethanol, and potassium hydroxide were purchased from Beijing Chemistry Co. (Beijing, China). Pyruvic acid was obtained from Sinopharm Chemical Reagent Co. (Beijing, China). All solvents/chemicals used were of analytical grade or purer.

All 2-(1*H*-pyrrolyl)carboxylic acids containing 2-(1*H*-pyrrolyl)acetic acid (P-Gly), 2-(1*H*-pyrrolyl)propanoic acid (P-Ala), 2-(1*H*-pyrrolyl)-3-methylbutanoic acid (P-Val), 2-(1*H*-pyrrolyl)-4-methylvaleric acid (P-Leu), 2-(1*H*-pyrrolyl)-3-methylvaleric acid (P-Ile), and 2-(1*H*-pyrrolyl)-3-phenylpropanoic acid (P-Phe) were synthesized by reacting corresponding amino acids with 2,5-dimethoxytetrahydrofuran as previously described (*14, 15*) except for purification procedures. Briefly, sodium acetate anhydrous (2.0 g) and corresponding amino acids (27.2 mmol) were dissolved in 50 mL of glacial acetic acid. 2,5-Dimethoxytetrahydrofuran (27.2 mmol) was added to the above solution. The mixture was stirred under reflux for 40 min and then was poured into 300 mL of ice–water. The resultant solution was extracted with ethyl acetate. After the solvent was removed by rotary evaporation, the resulting residual was dissolved in water, and its pH was adjusted to 7.0. The resulting solution was acidified by hydrochloric acid (2.0 M) to pH in the rage of 1.0−2.0. The resulting precipitate or oil was collected for ^{1}H NMR and MS measurements. The identity and purity of the synthesized compounds were checked by ^{1}H NMR and MS. The di(2-propenyl) thiosulfinate was synthesized as previously described (*16*).

Instrumentals. UV−visible spectra were recorded with a Cary 50 UV–vis spectrophotometer (Varian Co.). ^{1}H NMR spectra were obtained on a dpx-300 MHz NMR spectrometer (Brucker Co.). DMSO was used as a solvent with tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained by using LC-MS/MS (Alliance2695/Quattro Micro API, Waters Co.), and detection was performed in the negative mode.

Plant Materials. Freshly harvested (May 2007) garlic bulbs were obtained from a local market at China Agriculture University, stored at room temperature, and used immediately for the following experiments.

Preparation of Garlic Purees and Measurement of Their UV−Vis Absorption or Spectra. After the garlic bulbs had been cracked, shriveled, damaged, and small cloves were discarded; the remaining cloves were peeled and rinsed with distilled water three times. Garlic (420 cloves) was homogenized in a blender. The resulting materials were equally divided into 14 parts. Eight parts were soaked in 20 mL of 2.5 mM acetic acid solution, 2.5 mM pyrrole solution, and 2.5 mM pyrrole combined with six free amino acids (glycine, alanine, valine, leucine, isoleucine, and phenylalanine, 2.5 mM) solution as controls, respectively. The remaining six parts were immersed in 20 mL of P-Gly, P-Ala, P-Val, P-Ile, P-Leu, and P-Phe, respectively, at four different concentrations (1.0, 2.5, 5.0, and 10.0 mM) for up to 15 days of incubation at room temperature (23–25 °C). For P-Ile, P-Leu, and P-Phe, only two different concentrations, 1.0 and 2.5 mM, were used to pickle garlic puree due to limited solubility in water. The final pH for all samples was in the range of 4.0−5.0. All experiments were performed in triplicate.

After 1 day of incubation, all resultant mixtures were centrifuged at 10000*g* for 10 min at 4 °C. The residues produced were kept for further use, and the resulting supernatant was collected, 1 mL of which was placed into a quartz cuvette for UV−vis spectral measurement. After UV–vis measurement, the 1 mL aliquot was recombined with the supernatant and the residual for further incubation. The same procedure was repeated every day at the same time as above until the 16th day.

Reactions between 2-(1*H*-Pyrrolyl)carboxylic Acids and Pyruvic Acid. Both pyruvic acid (0.284 mM) and P-Gly (0.284 mM) in methanol were mixed thoroughly with a volumetric ratio of 1 to 1. The resulting mixture was allowed to stand at room temperature (23–25 °C) for 5 min, and 1 mL of the reaction solution was put into a quartz cuvette for UV−vis spectral measurement. The reactions of other compounds containing P-Ala, P-Val, P-Ile, P-Leu, or P-Phe with pyruvic acid were carried out with the same procedure used for P-Gly. All of the chemicals had good solubility in methanol.

Reactions between 2-(1*H*-Pyrrolyl)carboxylic Acids and Di(2-propenyl) Thiosulfinate. Di(2-propenyl) thiosulfinate (0.246 mM) and P-Gly (0.246 mM) in methanol were mixed with a volumetric ratio of 1 to 1 followed by standing at room temperature (23–25 °C) for 5 min. Then, 1 mL of the reaction solution was placed into a quartz cuvette for UV−vis spectral measurement. Other reactions between other compounds containing P-Ala, P-Val, P-Ile, P-Leu, P-Phe, and di(2-propenyl) thiosulfinate were carried according to the same procedure as used for P-Gly. All compounds are soluble in methanol.

Preparation of Green Garlic Pickling Solution and Separation of Yellow and Blue Species. Freshly harvested garlic bulbs were stored at 4 °C for 2 months. After the garlic bulbs had been cracked, garlic cloves were peeled, rinsed with tap water, and then triple rinsed with distilled water three times. The peeled cloves (500 g) were pickled in 500 mL of acetic acid solutions (5%, v/v) at pH 2.0 for 4 days. The resulting green pickling solution was filtered and passed through an Amberlite CG-50 cation exchange column (2.6 × 50 cm) eluted with ethanol/acidic water (1:9 v/v) at 5 mL/min. Eluent (70–80 mL) with a green color was collected and concentrated to 5 mL by ultrafilitration. The resulting solution was subjected to an LH-20 column (1.6 × 75 cm), which was eluted with methanol at 0.3 mL/min. The resulting yellow and blue fractions were collected and concentrated to 2 mL, respectively, for UV−vis spectral measurements.

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 significance of differences between means.

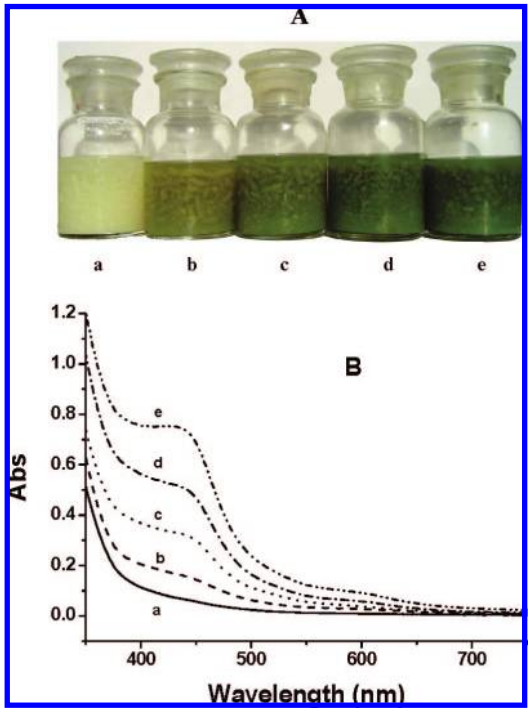

Figure 2. (**A**) Photographs and (**B**) UV–vis spectra of green color formation of puree of freshly harvested garlic cloves immersed in 2-(1*H*-pyrrolyl)acetic acid (P-Gly) for 7 days at various concentrations (b, 1 mM; c, 2.5 mM; d, 5 mM; e, 10 mM). No color was formed when the freshly harvested garlic was soaked in 2.5 mM acetic acid solution, 2.5 mM pyrrole, or 2.5 mM pyrrole plus 2.5 mM six free amino acids for 7 days (a), respectively.

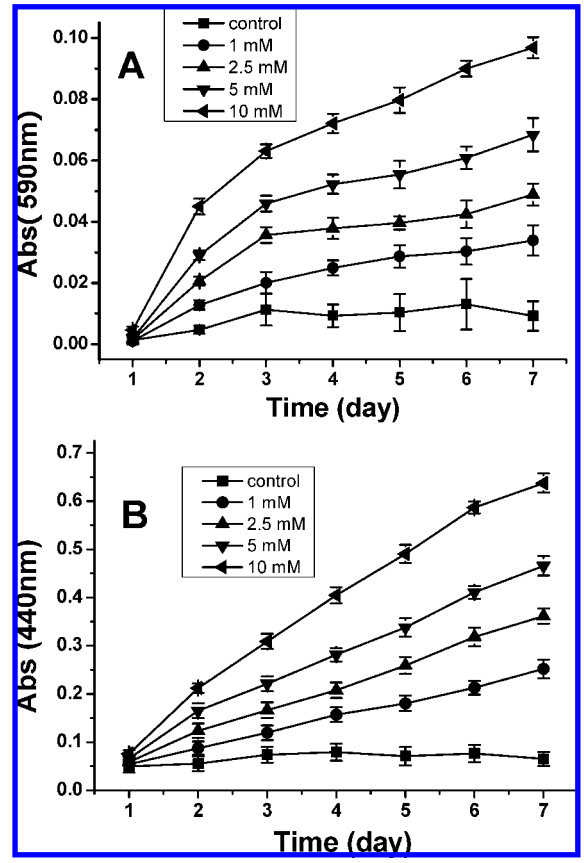

Figure 3. Effects of P-Gly at four different concentrations (1.0, 2.5, 5.0, and 10.0 mM) on the green discoloration of puree made from freshly harvested garlic. The garlic greening was detected at both 590 nm (**A**) and 440 nm (**B**). Each point represents a separate sample, and its value is the average of three independent measurements. Vertical bars represent the standard deviation.

RESULTS

On the basis of their ^{1}H NMR spectra and MS data (Supporting Information), the structures of six model compounds were identified as 2-(1*H*-pyrrolyl)carboxylic acids. Melting points of P-Gly and P-Phe were 84–87 and 90–93 °C, respectively, which are nearly identical to those (P-Gly, 85–88 °C; P-Phe, 90–93 °C) reported in the literature (*14, 15*), confirming the purity of these synthesized compounds. All of them have a structure similar to that of PP-Val (**Figure 1**) and just lack two methyl groups located on the pyrrole moiety as compared to PP-Val. In addition, these model compounds are soluble in water and methanol, but P-Leu, P-Ile, and P-Phe have relatively lower solubility in water with respect to P-Gly, P-Ala, and P-Val. Therefore, only two concentrations, 1.0 and 2.5 mM, were used for making P-Leu, P-Ile, and P-Phe samples throughout this study.

PP-Val and their derivatives with 2-(1*H*-pyrrolyl)carboxylic acid moiety could be pigment precursors during the discoloration of onion and garlic (*9*). If PP-Val were a pigment precursor, freshly harvested garlic puree would also turn green upon addition of these model compounds. First, P-Gly was chosen to add to puree of freshly harvested garlic because it has the simplest structure among the six compounds. As expected, the garlic purees became green after being immersed in P-Gly solution at four different concentrations (1.0, 2.5, 5.0, and 10.0 mM), and with increasing P-Gly concentration, the green color became deeper and deeper after 7 days incubation (**Figure 2A**). Their corresponding UV–vis spectra are displayed in **Figure 2B**. It was observed that there are two maximum absorptions at ca. 440 and 600 nm, which are in good agreement with the UV–vis absorptions (440 and 590 nm) of pigment(s) responsible for garlic greening reported in the literature (*2, 8, 10*). Similarly, puree of freshly harvested garlic also became pronouncedly green when P-Ala and P-Val were used instead of P-Gly. In contrast, the degree of garlic greening became less when the other three compounds were used instead under the same experimental conditions (data not shown). However, neither pyrrole alone nor the mixture of pyrrole and free amino acids had the ability to discolor the puree under the same conditions (data not shown), indicating that both pyrrole and carboxylic moieties were necessary for the structure of the pigment precursor(s).

The above results strongly suggested that a compound including the 2-(1*H*-pyrrolyl)carboxylic acid unit is involved in garlic greening. This finding encouraged further investigation into the relationship between these pyrrole derivatives and garlic greening. Therefore, the kinetics of garlic greening was studied using UV–vis spectrophotometry upon addition of these compounds at four different concentrations (1.0, 2.5, 5.0, and 10.0 mM). Consistent with the above observation, garlic greening became stronger and stronger with time upon addition of P-Gly to garlic puree. In addition, it was evident that the degree of garlic greening is a function of its concentration (**Figure 3**). A similar profile was observed with P-Ala and P-Val, whereas the other compounds, P-Ile, P-Leu, and P-Phe, turned garlic puree green to a lesser extent (data not shown). These results suggest that these compounds might exhibit a different effect on garlic greening.

To clarify their contributions to garlic greening, garlic puree was immersed in a series of solutions containing 2.5 mM concentrations of the six model compounds, respectively (**Figure**

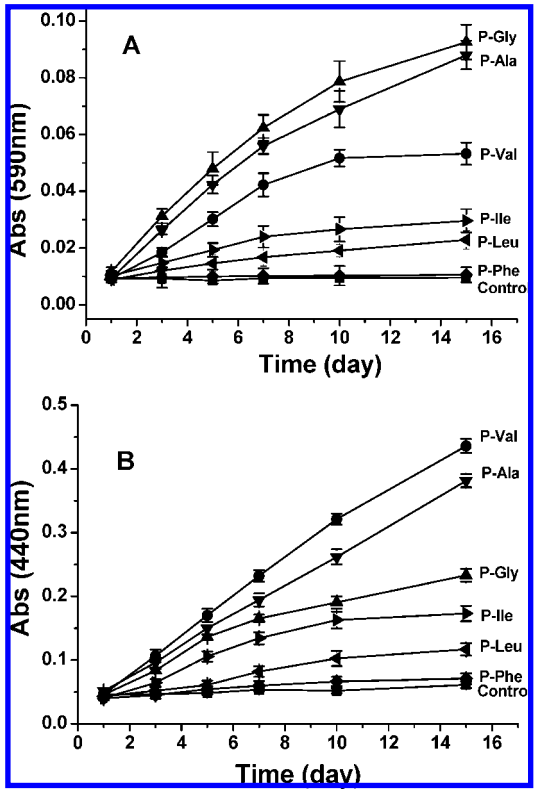

Figure 4. Effects of six 2-(1*H*-pyrrolyl)carboxylic acids (2.5 mM) on the greening discoloration of puree made from freshly harvested garlic. Garlic greening was detected at both 590 nm (**A**) and 440 nm (**B**). Each point represents a separate sample, and its value is the average of three independent measurements. Vertical bars represent the standard deviation.

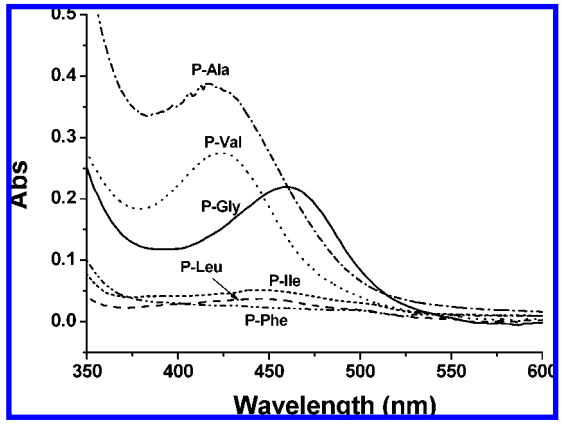

Figure 5. UV–vis difference spectra of the formation of yellow species by a reaction between six synthesized compounds (P-Gly, P-Ala, P-Val, P-Ile, P-Leu, and P-Phe) and pyruvic acid. The concentrations of both the six compounds and pyruvic acid equal 0.284 mM in methanol.

4). Because pigments related to garlic greening have two maximum absorptions at 440 and 590 nm, the two absorptions were used as detection wavelengths (*2, 8*). Interestingly, there is a good relationship between garlic greening and structure, namely, garlic greening correlates with molecular size (**Figure 1**). With increasing size of the R group from H− (P-Gly), CH_3− (P-Ala), $(CH_3)_2CH$− (P-Val), $CH_3CH_2CH(CH_3)$− (P-Ile), or $(CH_3)_2CHCH_2$− (P-Leu) to benzyl (P-Phe), the absorption at 590 nm generally decreased (**Figure 4A**), indicating that the compounds with larger R groups made less of a contribution to the production of blue pigments, which are involved in garlic

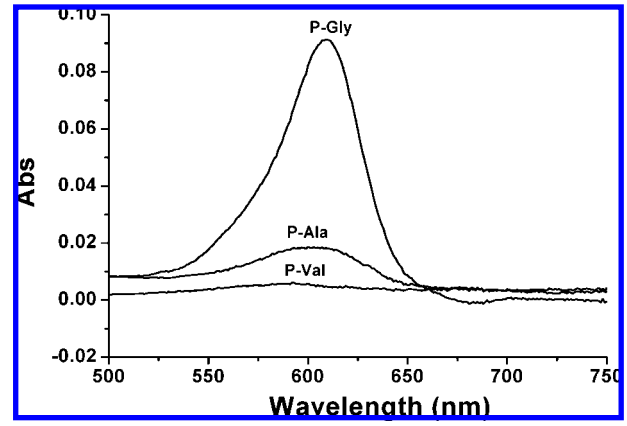

Figure 6. UV–vis difference spectra of the formation of blue species by a reaction between three synthesized derivative compounds (P-Gly, P-Ala, and P-Val) and di(2-propenyl) thiosulfinate. The concentrations of both the six compounds and di(2-propenyl) thiosulfinate are 0.246 mM in methanol.

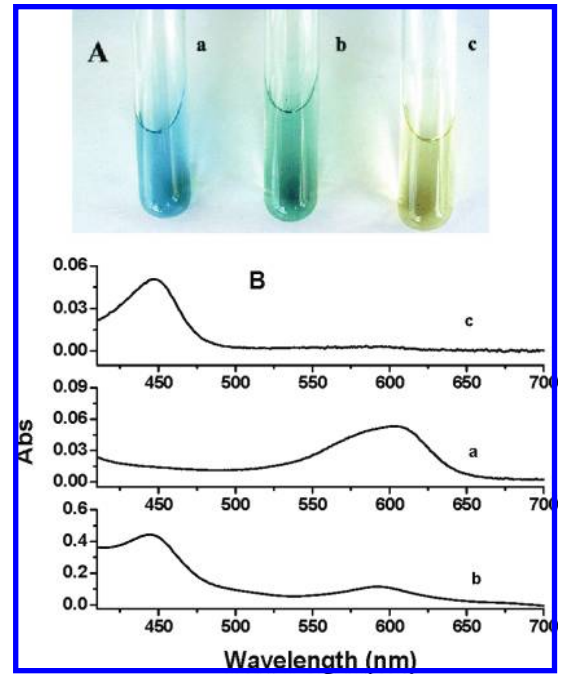

Figure 7. (**A**) Photograph of two solutions containing blue (a) and yellow (c) species separated from green pickling solution (b) of "Laba" garlic. (**B**) UV−vis spectra of the three solutions (a, b, and c). Green garlic-pickling solution (b) was diluted 10 times prior to UV−vis measurement.

discoloration. A similar result was also found with the absorption at 440 nm, which characterized yellow species also related to garlic greening (*2*) except that P-Val and P-Gly switched their positions in the ability to make garlic puree yellow (**Figure 4B**). Thus, among the compounds, P-Gly makes the largest contribution to the formation of the blue species, whereas P-Val has the largest effect on the production of the yellow species.

Recent studies have shown that green pigments responsible for garlic greening might consist of yellow and blue species (*2, 10*). To explore the pathway of the formation of yellow pigment(s), reactions between these PP-Val model compounds and pyruvic acid [a product accompanied with the formation of di(1-propenyl) thiosulfinate from 1-PeCSO by alliinase] were studied. It was found that these compounds can react with pyruvic acid to produce unidentified yellow species with a maximum UV–vis absorption in the range of 418−460 nm. The order of UV–vis absorption intensity of the yellow species at 440 nm is as

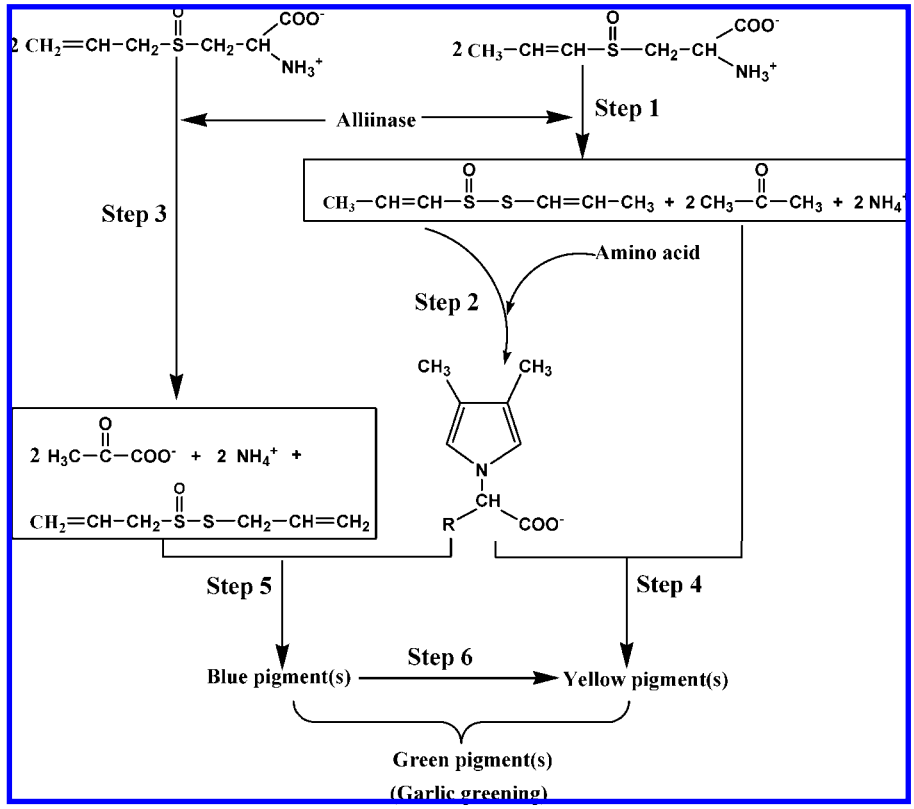

Figure 8. Proposed six-step pathway responsible for garlic greening.

follows: P-Ala > P-Val > P-Gly > P-Ile > P-Leu > P-Phe (**Figure 5**), generally consistent with the order of these compounds indicative of their contributions to the absorption at 440 nm related to garlic greening (**Figure 4B**). This result supports the proposed pathway of the yellow species production.

Likewise, the possible pathway correlated with the formation of the blue species was also studied. The UV–vis spectra of the reaction solution of the six compounds and di(2-propenyl) thiosulfinate are shown in **Figure 6**. It was found that a reaction solution of P-Gly and di(2-propenyl) thiosulfinate exhibits the deepest blue color, the maximum absorption of which is at 608 nm (**Figure 6**). A reaction solution of di(2-propenyl) thiosulfinate and P-Ala also showed a blue color with a maximal UV–vis absorption at 602 nm, whereas the mixture of di(2-propenyl) thiosulfinate and P-Val had a very weak blue color with an absorption at about 594 nm (**Figure 6**). In contrast, no color change was observed with P-Ile, P-Leu, or P-Phe upon incubation with di(2-propenyl) thiosulfinate under the same conditions. These results suggested that only P-Gly, P-Ala, and P-Val can react with di(2-propenyl) thiosulfinate to form the blue species under this condition.

Recent studies suggested that green pigments are a mixture consisting of yellow and blue species (*2, 10*). Agreeing with this idea, yellow and blue pigments were obtained from the green pickling solution of "laba" garlic (*2*) as shown in **Figure 7**. A photograph of solutions containing yellow pigment(s) and blue pigment(s) separated from green garlic pickling solution is displayed in **Figure 7A**. Corresponding UV−vis spectra of the three different solutions are given in **Figure 7B**. It was observed that a solution containing yellow species only have one visible absorption at about 440 nm in the visible region, whereas a solution containing blue species exhibited a visible absorption at about 590 nm, demonstrating that green pigments responsible for garlic greening are a mixture of yellow and blue species (*2, 10*).

DISCUSSION

A large body of work demonstrated that garlic greening occurs only with aged garlic, which is typically stored at low temperature (10 °C or lower) for more than 1 month (*1–3, 11, 17*). Later, it was found that this kind of storage can markedly increase the amount of 1-PeCSO depending on storage time. Moreover, 1-PeCSO was necessary for the development of garlic greening on the basis of the observation that the degree of garlic greening was proportional to the concentration of 1-PeCSO (*11, 17*). Support for this conclusion came from a recent paper showing that a dark blue pigment with a maximal absorbance was formed in a system containing 1-PeCSO, 2-PeCSO, glycine, and alliinase; pink and magenta pigments were produced, respectively, in a model system consisting of 1-PeCSO and alliinase and in another system of 1-PeCSO, glycine, and alliinase (*8*). Only 1-propenyl-containing thiosulfinates can react with amino acids to form pigments (*10*). All of these results emphasize the importance of 1-PeCSO for garlic greening. However, the pathways in which 1-PeCSO participates in the formation of green pigment(s) are still poorly understood.

Di(1-propenyl) thiosulfinate, a product from the catalytic conversion of 1-PeCSO by alliinase, can react with L-valine or L-alanine in vitro to produce 2-(3,4-dimethyl-1*H*-pyrrolyl)-3-methylbutanoic acid (PP-Val) and 2-(3,4-dimethyl-1*H*-pyrrolyl)propanoic acid (PP-Ala) (**Figure 1**), and PP-Val can react with di(2-propenyl) thiosulfinate, another thiosulfinate, to form pigment(s) (*9*). This finding raises the possibility that a compound containing the 2-(1*H*-pyrrolyl)carboxylic acid moiety could act as a precursor to the pigment(s) involved in garlic greening.

The present in vivo study confirms the above proposed pathways of the pigment precursor formation, namely, that 1-PeCSO was first converted into di(1-propenyl) thiosulfinate by alliinase and the produced thiosulfinate subsequently reacted with amino acids in the absence of enzyme to form a compound

containing a 2-(1*H*-3,4-dimethyl-1*H*-pyrrolyl)carboxylic acid unit called the pigment precursor (PP). If a compound containing the 2-(1*H*-pyrrolyl)carboxylic acid moiety were not PP, it would not turn puree (which was made from freshly harvested garlic bulbs) green. This was not the case, and the addition of the synthesized model compounds to the garlic puree, from freshly harvested garlic bulbs, resulted in garlic greening to a different extent depending on their structures. It seems there is a good relationship between the structure and activity of these compounds, that is, the smaller the side chain size of the model compounds, the stronger the garlic greening (**Figures 3** and **4**). This result suggests that the synthesized compound with a smaller size side chain could contribute more to the formation of the final pigment.

Because of the formation of the pigment precursor by the reaction of di(1-propenyl) thiosulfinate with amino acids (*9*), the present study also provided some information on the contribution of these amino acids to garlic greening. The amino acids with a smaller size side chain such as glycine, alanine, and valine make a bigger contribution to garlic greening as compared with other analogues with a larger size side chain including leucine, isoleucine, and phenylalanine. Agreeing with this observation, glycine, alanine, and valine have been found to be involved in the formation of blue and red pigments (*8, 9*). Further support comes from recent studies showing that a species produced from reacting glycine or alanine or valine with 1-propenyl-containing thiosulfinate has a stronger color than that from a reaction of leucine or isoleucine or phenylalanine with 1-propenyl-containing thiosulfinate (*10*). However, it is possible that blue pigment might be produced by reaction between P-Ile, P-Leu, and P-Phe and di(2-propenyl) thiosulfinate under other conditions including increasing the incubation time or the temperature.

In addition, the present study proposed a possible pathway for garlic greening, which likely contains six steps as depicted in **Figure 8**. Step 1 was described in a previous paper and corresponds to the catalytic conversion of 1-PeCSO under the action of alliinase into di(1-propenyl) thiosulfinate (*9, 13, 16*). Step 2 represents the formation of the pigment precursors by the reaction of the produced di(1-propenyl) thiosulfinate with amino acids. The role of the pigment precursors was proven by the fact that 2-(1*H*-pyrroly)carboxylic acids can turn garlic puree (which was prepared from freshly harvested garlic bulbs) green. Step 3 is a reaction similar to step 1, corresponding to the production of di(2-propenyl) thiosulfinate from 2-PeCSO catalyzed by alliinase. The yellow species was produced in step 4 by the reaction of the pigment precursor and pyruvic acid, another product from step 1. In parallel, a reaction between the pigment precursor and di(2-propenyl) thiosulfinate representing step 5 also occurs accompanying step 4 to form unidentified blue pigment(s). Step 6 corresponds to another possible pathway related to the production of yellow pigment(s) (*2*). Different from the yellow species produced in step 4, yellow species formed through step 6 stem from the degradation of blue species produced in step 5 (*2*). The mixing of both yellow and blue pigments finally leads to garlic greening. In accordance with this idea, yellow and blue pigments were obtained from the green pickling solution of laba garlic (**Figure 7**). This conclusion is also in accordance with recent observations (*2, 10*). Although the above six-step pathway was proposed, other pathways associated with garlic greening also probably coexist because garlic greening is a very complex process and many compounds occurring in garlic may attend this process in a different way (*8, 10*). However, it is likely that there is a predominant pathway related to garlic greening which needs to be determined in future study.

Supporting Information Available: ^{1}H NMR spectra and MS data of six compounds including P-Gly, P-Ala, P-Val, P-Leu, P-Ile, and P-Phe. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Adams, J. B.; Brown, H. M. Discoloration in raw and processed fruits and vegetables. *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 319–333.

(2) Bai, B.; Chen, F.; Wang, Z.; Liao, X.; Zhao, G.; Hu, X. Mechanism of the greening color formation of "Laba" garlic, a traditional homemade Chinese food product. *J. Agric. Food Chem.* **2005**, *53*, 7103–7107.

(3) Bai, B.; Li, L.; Hu, X.; Wang, Z.; Zhao, G. Increase in the permeability of tonoplast of garlic (*Allium sativum*) by monocarboxylic acids. *J. Agric. Food Chem.* **2006**, *54*, 8103–8107.

(4) Joslyn, M. A.; Sano, T. The formation and decomposition of green pigment in crushed garlic tissue. *Food Res.* **1956**, *21*, 170–183.

(5) Shannon, S.; Yamaguchi, M.; Howard, F. D. Precursors involved in the formation of pink pigments in onion purees. *J. Agric. Food Chem.* **1967**, *15*, 423–426.

(6) Shannon, S.; Yamaguchi, M.; Howard, F. D. Reactions involved in formation of a pink pigment in onion purees. *J. Agric. Food Chem.* **1967**, *15*, 417–422.

(7) Imai, S.; Akita, K.; Tomotake, M.; Sawada, H. Model studies on precursor system generating blue pigment in onion and garlic. *J. Agric. Food Chem.* **2006**, *54*, 848–852.

(8) Kubec, R.; Hrbáčová, M.; Musah, R. A.; Velíše, J. *Allium* discoloration: precursors involved in onion pinking and garlic greening. *J. Agric. Food Chem.* **2004**, *52*, 5089–5094.

(9) Imai, S.; Akita, K.; Tomotake, M.; Sawada, H. Identification of two novel pigment precursors and a reddish-purple pigment involved in the blue-green discoloration of onion and garlic. *J. Agric. Food Chem.* **2006**, *54*, 843–847.

(10) Kubec, R.; Velíšek, J. *Allium* discoloration: the color-forming potential of individual thiosulfinates and amino acids: structural requirements for the color-developing precursors. *J. Agric. Food Chem.* **2007**, *55*, 3491–3497.

(11) Lukes, T. M. Factors governing the greening of garlic puree. *J. Food Sci.* **1986**, *51*, 1581–1582.

(12) Lee, E. J.; Cho, J. E.; Kim, J. H.; Lee, S. K. Green pigment in crushed garlic (*Allium sativum* L.) cloves: purification and partial characterization. *Food Chem.* **2007**, *101*, 1677–1686.

(13) Imai, S.; Tsuge, N.; Tomotake, M.; Nagatome, Y.; Sawada, H.; Nagata, T.; Kumagai, H. An onion enzyme that makes the eyes water. A flavoursome, user-friendly bulb would give no cause for tears when chopped up. *Nature* **2002**, *419*, 685.

(14) Gloede, J.; Poduška, K.; Gross, H.; Rudinger, J. Amino acids and peptides. LXXIX. α-Pyrrolo analogues of α-amino acids. *Collect. Czech. Chem. Commun.* **1968**, *33*, 13071314..

(15) Sircar, I.; Winters, R. T.; Quin J., III; Lu, G. H.; Major, T. C.; Panek, R. L. Nonpeptide angiotensin II receptor antagonists. 1. Synthesis and in vitro structure-activity relationships of 4-[[[(1*H*-pyrrol-1-ylacetyl)amino]phenyl]methyl] imidazole derivatives as angiotensin II receptor antagonists. *J. Med. Chem.* **1993**, *36*, 1735–1745.

(16) Shen, C.; Parkin, K. L. *In vitro* biogeneration of pure thiosulfinates and propanethial-*S*-oxide. *J. Agric. Food Chem.* **2000**, *48*, 6254–6260.

(17) Ichikawa, M.; Ide, N.; Ono, K. Changes in organosulfur compounds in garlic cloves during storage. *J. Agric. Food Chem.* **2006**, *54*, 4849–4854.

Received for review October 13, 2007. Revised manuscript received December 10, 2007. Accepted December 15, 2007. This project was supported by the National Natural Science Foundation of China (30570181), the Ministry of Education of the People's Republic of China, Specialized Research Fund for the Doctoral Program of Higher Education (20070019004), and the National Key Technology R&D Program (2006BAD06B02).

JF073025R