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The Chemical Basis of Green Pigment Formation ('Greening') in Crushed Garlic (*Allium sativum* L.) Cloves

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Abstract The chemical processes involved in the formation of green pigment in crushed garlic cloves were investigated based on the principle of pink pigmentation in macerated onions. Intact greening and non-greening garlic cloves were either left untreated or heated at 90°C for 3 min to inactivate enzyme activities. First, a colorless ether soluble compound referred to as color developer reacted with glycine (among all free amino acids) in garlic to form a second compound insoluble in ether. The latter compound then reacted with formaldehyde to yield the green colored pigment. Alliinase activity was necessary for the production of color developer and for the development of green pigment. In greening garlic that had been heat treated, green pigmentation did not proceed due to the heat-inactivation of alliinase, but the addition of alliinase solution into the garlic homogenates restored the pigmentation. However, this phenomenon was not observed in non-greening garlic with or without heat treatment. Finally, the mechanism of green pigment formation in crushed garlicis similar to that of pink pigment formation in macerated onions.

Keywords: alliinase, amino acid, carbonyl, crushed garlic, flavor precursor, green pigment, greening

Introduction

Garlic is processed into various forms including purees, juice, powder, and oleoresin. Recently, the steady growth in retail sales of fresh, ready-to-use vegetables occurring in direct response to marketplace demands, has lead to minimally processed pre-peeled and chopped garlic products (1). During processing, intensely colored pigments are often formed. The formation of green pigmentation, called greening, represents the phenomenon of discoloration from a cream to green color in crushed garlic cloves. This phenomenon occurs only in mechanically bruised or finely cut tissue, but not in carefully sliced garlic cloves. Recently, the development of green pigmentation similar to the greening during garlic processing has been reported in the traditional homemade Chinese 'Laba' garlic product, which is pickled in 5% acetic acid solution (v/v, pH 2.33). After garlic cloves are soaked in acetic acid (such as vinegar) for 2 days, a green color develops. After 4 days, the pickling solution itself begins to turn green (2). The chemical basis of the greening of crushed garlic cloves has not been well documented. Many people familiar with greening suspect that it is due to pesticides applied during field cultivation. However, there have been no reports supporting this hypothesis. In our other studies, we considered greening to be one of the physiological phenomena caused by long periods of low-temperature storage after harvest. Kubec *et al.* (3) showed that S-alk (en)yl-L-cysteine sulfoxides (ACSOs) and alliinase were involved in this discoloration.

It has been hypothesized that the greening of crushed garlic is a multi-step process similar to that leading to the pink discoloration of onion (3-6). The pinking of macerated onions has been elaborately described as a series of reactions depicted in Fig. 1. There is an essential enzymatic step in the first phase of pigment formation, with at least three steps being required, and a wide range of amino acids may be involved (7). Unknown colorless ether-soluble compounds (also called 'color developers') react with certain amino acids in onion to form a second colorless compound insoluble in ether. The latter compound was then found to react with formaldehyde or naturally occurring carbonyl compounds to form the final pigment (5, 6). Soon after, the essential enzyme was identified as an alliinase.

Studies then began to focus on the activity of ACSOs as flavor precursors, especially S-1-propenyl cysteine sulfoxide (1-PeCSO, isoalliin), an abundant lachrymatory compound in onion. Further work suggested that 1-PeCSO might play a role in pinking (7) and greening (4). This hypothesis appears to be supported by the results of a recent study which showed that, at pH 5.5, a dark blue pigment formed in a system containing isoalliin, alliin, glycine, and alliinase (3). Also, the formation of pink pigment in onion and green pigment in garlic were confirmed to be similar in nature, with 1-PeCSO serving as the primary precursor. Factors such as pH, temperature, heating time, salt, and sulfite content have also been investigated, and all influence the formation of green pigment in garlic tissue macerates (1, 4, 9-11).

The purpose of this study was to investigate the chemical basis of the formation of green pigment in crushed garlic cloves based upon the principle of pink pigmentation in macerated onions.

Materials and Methods

Plant material and heat treatment 'Euisung' garlic (*Allium sativum* L.) cultivar was obtained from its respective cultivating area in Korea. Harvested garlic bulbs

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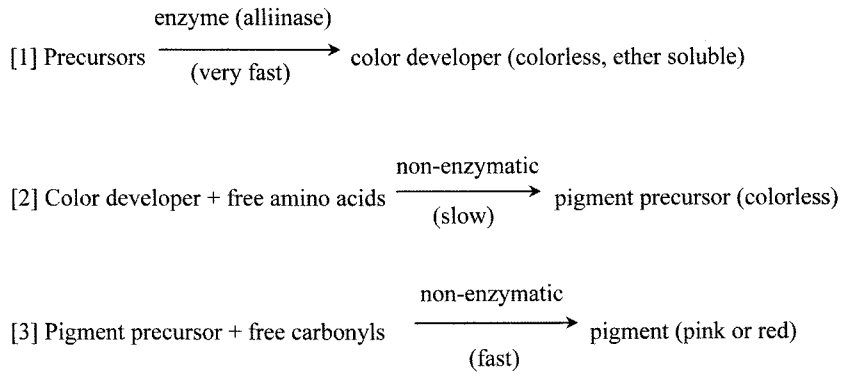


Fig. 1. Predicted reaction involved in pink pigment formation ('pinking' or 'reddening') of macerated onions.

were dried for one month under well-ventilated, shaded conditions until the water content reached about 22-26% of fresh weight. Garlic bulbs were stored at 0, 5, 10, 15 and 20°C for 3 months and observations were made on the formation of green pigment after completely crushing the bulbs. Intact greening garlic ('stored at 0 to 15°C and showing greening after being crushed') and non-greening garlic ('stored at 20°C and not showing greening after being crushed') bulbs were either heated at 90°C for 3 min to inactivate enzyme activity or not heated. The abbreviations use for each garlic sample are shown in Table 1.

Preparation of alliinase solution Garlic cloves without heat treatment were peeled and ground with an equal weight of water in an ice-cold mortar and pestle, and extracted for 5 min at 4°C. The garlic juice was filtered through one layer of cheesecloth and adjusted to pH 3.7 with diluted 0.1 N HCl. After standing several hours, the precipitated material was removed by filtration and the solids were resuspended in pH 6.0 citrate-phosphate buffer containing 1% NaCl, 10% sucrose, 25 mM pyridoxal-5-phosphate, and 0.02% sodium azide (NaN_3) (Sigma, St. Louis, USA). The extract was centrifuged for 30 min at 15,000×g. The supernatant was filtered and dialyzed overnight at 8°C against the same buffer described above. The solution was filtered and the enzyme was found in the cleared filtrate (7, 11).

Preparation of color developer and reaction with free amino acids and carbonyls To remove the color developer compound before crushed garlic cloves turned green, garlic homogenates were repeatedly extracted with various organic solvents. The greening of each solvent extract and solid residue upon standing at room temperature was determined separately. Following the solvent test, color developer was obtained by extracting garlic homogenates with ether (7). The garlic homogenates were shaken with an equal volume of ether immediately after preparation and before the green color developed. After evaporation of the ether, the resulting yellow residue was extracted with 50 mL of citrate-phosphate buffer (pH 5.6) and then filtered to collect the color developer.

The color developer was shaken with aqueous solutions (9 mL) of various D-amino acids at a concentration of 0.1 M (Table 2). Various carbonyls (shown in Table 3) at a

Table 1. Abbreviations for garlic samples

Abbreviation	Garlic	Treatment[1)]
G	greening[2)]	non-heated
GH	greening	heated
N	non-greening[3)]	non-heated
NH	non-greening	heated

[1)]Intact garlic cloves were heated at 90°C for 3 min to inactivate enzyme activity completely.
[2)]Garlic was stored at 0 to 15°C for 3 months and showed greening after being crushed.
[3)]Garlic was stored at 20°C for 3 months and did not show greening after being crushed.

Table 2. Color development following the reaction of various amino acids at 0.1 M with color developer.

D-Amino acid	Color development after reaction
Alanine	yellow
Arginine	yellow
Asparagine	yellow
Aspartic acid	-[1)]
Cysteine	-
Glutamic acid	-
Glutamine	-
Glycine	green
Histidine	yellow
Leucine	-
Lysine	-
Methionine	-
Phenylalanine	yellow
Serine	-
Threonine	-
Tryptophan	-
Tyrosine	-
Valine	yellow

[1)] Resulting product was colorless.

Table 3. Color development following the reaction of various carbonyls at a concentration of 0.3 mM with pigment precursor.

Carbonyl compound	Color development after reaction
Acetaldehyde	purple
Acetone	purple
Diacetyl	pink
Formaldehyde	green
Glycolonitrile	pink
5-Hexen-2-one	pink
Methyl ethyl ketone	pink

concentration of 0.3 mM were also added to the mixtures of color developer and amino acids and then incubated for pigmentation to occur (5). The presence of the color developer in this extract was confirmed by maintaining the mixtures at room temperature for green color development. The color developer, amino acids, and carbonyls were allowed to react at the same time or in a systematic series.

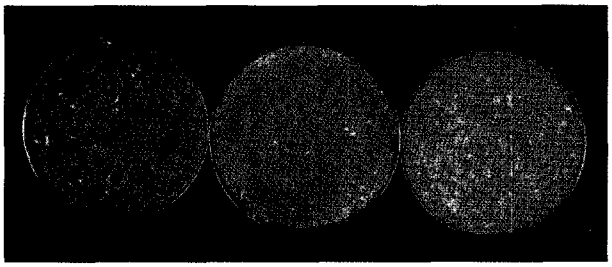
Color developer activity was confirmed on a thin-layer chromatography (TLC) plate (silica gel 60, 5×20 cm, 0.25 mm thickness; E. Merck, Darmstadt, Germany) using the following solvent system: 1-butanol-acetic acid-water (4: 1:1, v/v/v). The active ingredients in the aqueous residue of the ether extraction juice and glycine in water were respectively identified by spotting them very heavily on the TLC plate. After the solvent developed, a suspension of the color developer in citrate-phosphate buffer (pH 5.6) was sprayed onto the aqueous residue. Glycine was detected by spraying the plate with 2% ninhydrin solution.

Ultra-violet spectrometry crude or purified green pigments dissolved in 100% methanol were filtered and placed into a quartz cuvette for spectral measurement (UV 1601; Shimazu, Kyoto, Japan).

Results and Discussion

Effects of heat treatment and alliinase on greening

Garlic stored at 0 to 15°C showed greening after being crushed. Garlic stored at 20°C did not develop greening under any experimental conditions examined. The green color development in crushed garlic cloves was affected by storage at lower temperatures (Fig. 2). In other experiments, greening was only affected by storage temperature, regardless of the cultivar, harvest time, or sprouting (data not shown).



(a) (b) (c)

Fig. 3. Effects of alliinase solution on green pigment formation in crushed garlic cloves. (a) Prepared from heated greening garlic cloves (GH), (b) prepared from non-greening garlic cloves (N), and (c) prepared from heated non-greening garlic cloves (NH). Intact garlic cloves were heated at 90°C for 3 min to completely inactivate enzyme activity.

Heating at 90°C for 2 min inactivated alliinase and destroyed its ability to cause pigment formation in crushed garlic cloves. The activity of alliinase was necessary for the development of green pigment in crushed garlic cloves. The enzyme solution did not turn green by itself, but when it was added to the GH sample homogenates (those that originally showed greening but did not due to heat), the resulting mixture turned green (Fig. 3a). This result indicated that the enzyme plays an important role in green pigmentation (7) as shown in Fig. 1. However, this phenomenon was not observed in both N and NH homogenates prepared from non-greening garlic cloves (Fig. 3b and 3c). Samples G and N appeared to differ depending on whether they contained a precursor acted upon by alliinase. Sample G seemed to have a precursor which alliinase could convert to a color developer necessary for subsequent reactions. It has been suggested that if ACSOs were the precursors, it was most likely a 1-PeCSO (4). 1-PeCSO was necessary for the development of greening, and the extent of greening depended upon the amount of 1-PeCSO added. This hypothesis appears to be supported by the results of a recent study (3) which showed that, at pH 5.5, a dark blue pigment formed in a system containing isoalliin, alliin, glycine, and alliinase. Also, the formation of pink pigment in onion and green pigment in garlic were confirmed to be similar in nature, with 1-PeCSO serving as the primary precursor.

Fig. 2. Features of greening prepared from garlic bulbs stored at 20, 15, 10, 5, and 0°C (from left, respectively) for 3 months.

Color developer and pigment precursor During the course of the color developer investigation, we discovered that ether extraction could greatly reduce the ability of garlic homogenates to green. The garlic homogenates could be repeatedly extracted with ether before the pigment developed, but the aqueous residue left over from extraction could not develop the pigment upon standing. However, if the ether extract was evaporated down and added back to the aqueous residue, then the mixture would green. Neither entity produced color when held separately, but did when combined.

There are at least two compounds involved in the non-enzymatic phase of onion pigmentation (7). One was ether soluble and the other was water-soluble. Although the color developer compound was not identified, it was colorless and did not show the maximum absorption spectrum at 590 nm (Fig. 4). High absorbance at 590 nm was the most important feature of green pigments extracted from crushed garlic cloves (Fig. 4). Purified color developer in onion gave a positive Ehrlich's reagent test for a pyrrole nucleus having a five-membered ring containing one nitrogen atom (5). It was suggested that if an ACSO hydrolysis product, thiosulfinate, were the color developer, then it was most likely 1-propenyl thiosulfinate from 1-PeCSO (7, 9, 12, 13). The color developer immediately reacted with certain free amino acids to produce the pigment precursor. The latter compound then reacted with free carbonyls to form the final pigment.

In our experiments, green pigment compounds were made only in the presence of glycine in the color developer solution prepared from sample G, which was greening garlic stored at 0 to 15°C for 3 months. Glycine was able to form a green color component, namely pigment precursor, when incubated with color developer extracts (Table 2). The color of the pigment formed was dependent on the type of amino acid. The majority of amino acids tested gave the typical yellow to colorless appearance observed when the pigment formed in garlic bulb macerates. Both color developer and glycine were required for green pigment formation, and a slight green coloration occurred in the absence of formaldehyde. The pigment precursor was colorless in the onion pinking reaction, but in our reaction it was green and showed an absorption spectrum and color similar to that of naturally occurring green pigment extracted from crushed garlic cloves (Fig. 5). However, this pigmentation was only observed in greening garlic (G) (Fig. 6).

The addition of formaldehyde to the pigment precursor also increased the green color intensity and rapidly led to the formation of the final green pigment (Table 3). However, the color of pigment formed was a function of the carbonyl involved and some carbonyls inhibited pigment formation. The unsaturated carbonyl formaldehyde only increased the formation of green pigment in crushed garlic cloves.

When the TLC plate was examined to confirm color developer activity, one spot of green color was found at an R_f of approximately 0.29. The green spot was pigment precursor and corresponded in location with that of the amino acid glycine present on the plate (data not shown). This indicated that the ether soluble fraction was reacting with the amino acid in aqueous garlic juice to form

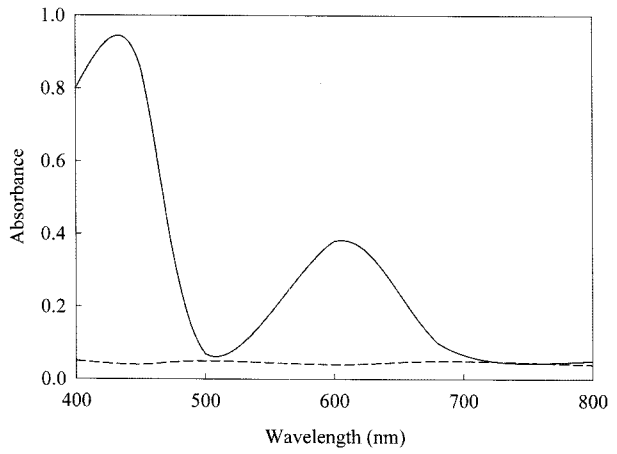


Fig. 4. Absorption spectrum of color developer and naturally occurring green pigment in crushed garlic cloves. Color developer and pigment were extracted with ether and methanol, respectively.

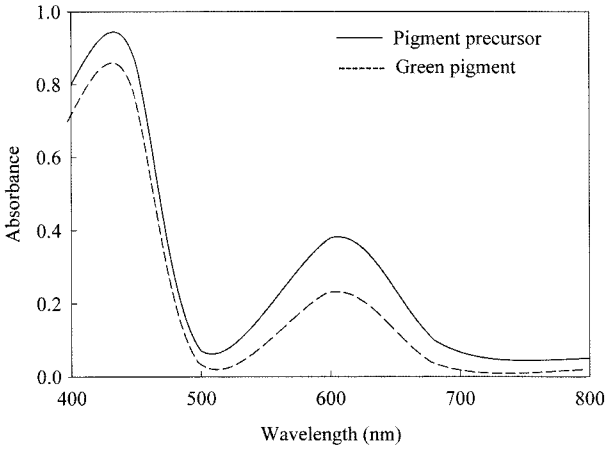
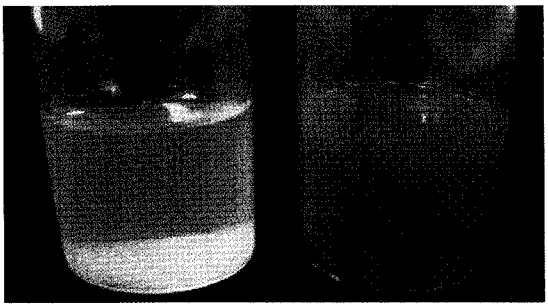
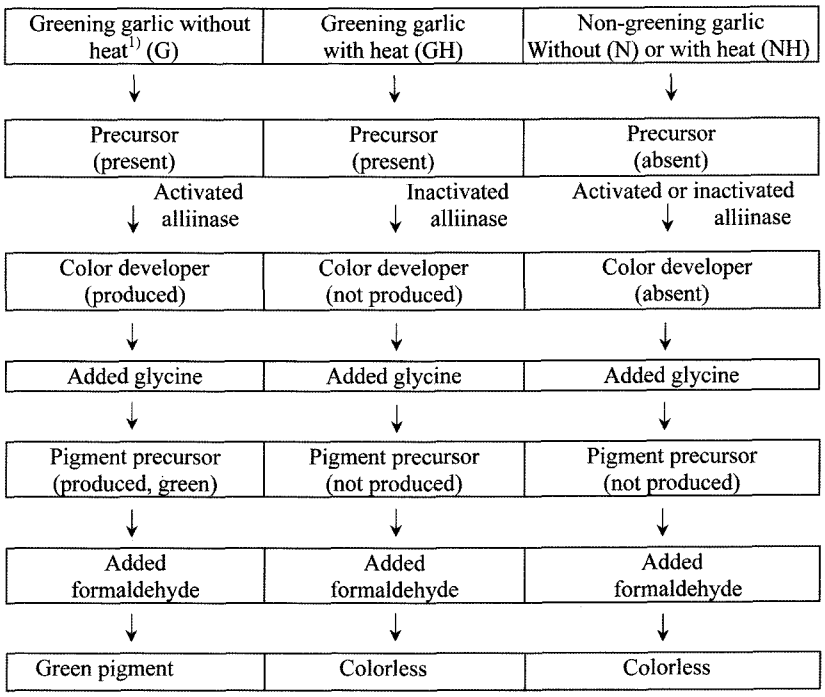


Fig. 5. Absorption spectrum of pigment precursor and naturally occurring green pigment in crushed garlic cloves. Pigment precursor was synthesized by the reaction of color developer with glycine. Green pigment was extracted with methanol.



(a) (b)

Fig. 6. Synthesized pigment precursor from the reaction of color developer with glycine. (a), Prepared from non-greening garlic cloves (N); (b), prepared from greening garlic cloves (B).



[1] Intact garlic bulbs were heated at 90°C for 3 min to completely inactivate enzyme activity.

Fig. 7. Possible reactions involved in green pigment formation ('greening') in crushed garlic cloves

pigment precursor, and also suggested the significant role of glycine in pigmentation. Studies using ^{14}C-labeled glycine and formaldehyde indicated that amino acids and carbonyls were incorporated in the final red pigment molecule in macerated onion. Two molecules of amino acids were involved in the formation of pigment precursors. One amino acid molecule was incorporated in an intact state, while the other was decarboxylated during incorporation (5). The rate and extent of pigmentation and the color of the pigment formed were affected by the kind of amino acid and carbonyl involved.

The predicted reaction resulting in green pigment formation is summarized in Fig. 7. Greening and non-greening garlic cloves were different regarding the presence of precursors acted upon by alliinase. Because non-greening garlic (N, NH) did not have the specific precursor for alliinase to produce the color developer, green pigment was not produced. However, greening garlic (G, GH) has the specific substrate for alliinase to act on, most likely a 1-PeCSO, and thus pigment was non-enzymatically formed by a series of reactions.

Although the reactions in crushed garlic cloves that lead to green pigment formation have not been completely established, green pigment formation is similar to pink pigment formation in chopped onion. Green pigmentation in crushed garlic cloves is thought to be associated with alliinase action on ACSOs and secondary, non-enzymatic reaction products. There is an essential enzymatic step to produce the color developer in the first phase of the pigment formation, when the garlic cells are ruptured. The conversion of a precursor into a colorless intermediate occurs quite rapidly. This then slowly reacts non-enzymatically with glycine and formaldehyde to form the actual green pigment. The color developer resulting from the action of alliinase on the water soluble precursor was ether soluble, but the pigment and its precursor were water soluble. Considering the action of alliinase on its substrate, the color developer is hypothesized to be a species of lipid soluble thiosulfinates from ACSOs. Alliinase activity leading to ACSO hydrolysis and the fate of thiosulfinates in the extracts are important in controlling the extent of greening. Furthermore, it is evident that the identities of the pure thiosulfinates derived from potential ACSOs and the dynamics of these thiosulfinates will allow for the examination of the intrinsic properties of greening. In conclusion, the process of green pigmentation formation in crushed garlic is significantly different from what has been previously reported regarding plant pigment synthesis.

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