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Model Studies on Precursor System Generating Blue Pigment in Onion and Garlic

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Reactions involved in blue-green discoloration in a mixture of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) were investigated. Vivid-blue color was successfully reproduced by using a defined model reaction system comprising only *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (1-PeCSO) from onion, *S*-allyl-L-cysteine sulfoxide (2-PeCSO) from garlic, purified alliinase (EC 4.4.1.4), and glycine (or some other amino acids). Four reaction steps identified and factors affecting the blue color formation were in good agreement with those suggested by earlier investigators. When crude onion alliinase was used in place of garlic alliinase, less pigment was formed. This result was explained by a difference in the amount of thiosulfinates, colorless intermediates termed color developers, yielded from 1-PeCSO by these enzymes.

KEYWORDS: Allium; garlic; onion; greening; pinking; thiosulfinate; alk(en)yl-L-cystein sulfoxides; alliinase.

INTRODUCTION

During our investigation on blue-green discoloration that occurred in mixed purees of garlic (*Allium sativum* L.) and onion (*Allium cepa* L.), we found that heat-treating onion bulbs before pureeing would produce blue color significantly stronger than that produced with unheated bulbs. Intrigued by this observation, we attempted to elucidate the reason for the difference by examining chemical components and reaction steps involved in the blue color formation.

Discoloration phenomena known as "greening" of garlic and "pinking" of onion have been studied by several investigators. Shannon et al. (*1*) investigated the reactions involved in formation of pink pigment in onion extensively and proposed a three-step reaction scheme comprising: (i) formation of colorless, ether-soluble substance termed color developer (CD) by the catalytic action of alliinase on unknown precursors (UP) in the neutral plus basic amino acid fraction; (ii) formation of a colorless, ether-insoluble pigment precursor (PP) from the CD and an amino acid such as glycine; and (iii) formation of a pink pigment from PP and a naturally occurring carbonyl (NOC) such as formaldehyde. They also discussed, though inconclusively, that the likely candidate for UP was 1-PeCSO (*2*), a substrate for alliinase and the major precursor for the characteristic flavor and lachrymator of onion. Yamaguchi et al. (*3*) speculated that a blue pigment-forming "carbonyl" was present in garlic, and when reacted with PP in place of NOC from onion, blue color would form. Subsequently, Shannon et al. (*2*) reported that the blue pigment-forming "carbonyl" would be derived from 2-PeCSO (alliin), another substrate for alliinase and the major precursor of garlic flavor. Later, Lukes (*4*) found that storage of garlic bulbs at or below 12 °C increased the amount of 1-PeCSO, the presence of which in garlic had not been well-known before, and by adding small amounts of 1-PeCSO to nongreening, nonheated garlic puree, he found a positive correlation between the amount of added 1-PeCSO and the degree of greening measured as absorbance at 590 nm. Luke's findings suggest that the same reactions may be responsible for "greening" of garlic alone and of the mixture of garlic and onion. Furthermore, Lee et al. (*5*) reported a positive correlation between thiosulfinate concentration and pink pigment formation, and Kubec et al. (*6*) confirmed the major CD compound to be 1-propenyl-containing thiosulfinates. Recently, Bai et al. (*7*) studied the mechanism of the green color formation in the traditional homemade Chinese "Laba" garlic, in which the greening is desirable and required. They reported that alliinase and acetic acid were required for the color formation and that the decrease in the total thiosulfinates in garlic cloves was closely associated with the pigment formation.

Although chemical constituents proposed by earlier investigators for the discoloration reactions are generally in agreement, the detailed reaction mechanisms and the chemical structures of the pigments or those of the intermediates have not been fully elucidated to date. We, therefore, attempted to resolve those remaining issues by establishing a model reaction system that comprised only well-defined, highly purified constituents. In this paper, we report identifications of the substances involved in the pigment formation and various conditions that affected the rate and the extent of the color development.

MATERIALS AND METHODS

Materials. All chemicals used were purchased from Wako Pure Chemical (Osaka, Japan) or Kanto Chemical (Tokyo, Japan) unless

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otherwise noted. All solvents used were chromatography grade. Yellow onion and garlic were purchased from a local store.

Apparatus. High-resolution fast atom bombardment (HRFAB) mass spectra were recorded in positive mode on a VG Auto Spec-Q (Micromass, Manchester, U.K.) by using thioglycerol as the matrix and Xe for atom bombardment. The NMR measurements were performed on a JNM-A 500 spectrometer (JEOL, Tokyo, Japan). Absorbance at 590 nm for color formation and absorption at 515 nm for thiosulfinate formation were obtained with a UV-3100 PC (Shimadzu, Kyoto, Japan).

Preparation of Juices. Onion and garlic juices were prepared by homogenizing one part by weight of peeled bulbs with one part by volume of distilled water in a kitchen blender, and the homogenate was centrifuged (4700*g* × 15 min) to remove the pulp. For preparation of heat-treated juices, whole bulbs were cooked in a 600 W microwave oven until endogenous alliinase was inactivated before homogenizing. The cooking time was 5 min per 350 g bulbs.

Isolation of an Active Compound from Heat-Treated Onion Juice. Onion juice prepared from heat-treated onion bulbs was passed through a column of Amberlite IR-120B cation-exchange resin (Rohm & Haas, U.S.) saturated with H^+, and adsorbed amino acids and peptides were eluted with 1 M NH_4OH. The eluate was adjusted to pH 3.3 with HCl and subjected to preparative medium-pressure liquid chromatography (MPLC) on a YFLC system (Yamazen, Osaka, Japan), equipped with a reverse-phase LiChroprep RP-18 column (310 mm × 25 mm i.d. 40−60 μm, Merck, Darmstadt, Germany) and eluted with distilled water adjusted to pH 3.3 with trifluoroacetic acid (TFA) at 8.5 mL/min. The ability to form blue color (measured as absorbance at 590 nm) was tested by adding unheated garlic juice to each fraction and incubating the fraction at 37 °C for 1−2 days. From 1.5 kg of fresh onion bulbs, 353 mg dry weight of the active compound was obtained as a white powder. The identity of the active compound was confirmed as 1-PeCSO by HRFAB mass spectral data and by comparisons of NMR data with published data (*8*).

Isolation of an Active Compound from Unheated Garlic Juice. Garlic juice prepared from unheated bulbs was extracted with ether three times. The ether extract was applied as a streak to a preparative silica gel TLC plate (silica gel 60 GF_{254}, 20 cm × 20 cm, 0.5 mm thick, Merck) and developed with *n*-hexanes−ethyl acetate (3:1 v/v). Silica gel from ultraviolet light (UV 254 nm) absorbing regions were scraped off and desorbed with ether. Fractions thus obtained were evaporated to dryness and redissolved in distilled water. The ability to form blue color was determined by adding unheated onion juice supplemented with glycine to each fraction and incubating the fraction at 37 °C for 1−2 days. The active compound was found at R_f of ca. 0.5 on the TLC plate and exhibited strong UV absorption at 254 nm. The identity of the active compound, obtained as a colorless oil, was confirmed as allicin by NMR analyses. The spectra obtained were consistent with the published data (*8*, *9*).

Purification of 2-PeCSO from Garlic Juice. From the heat-treated garlic juice, 2-PeCSO was isolated as a white powder by using the same procedure used for isolation of 1-PeCSO described above. The molecular structure of the isolate was confirmed as 2-PeCSO by comparisons of NMR data with published data (*10*).

Quantitation of 1-PeCSO and 2-PeCSO. Concentration of 1-PeCSO and 2-PeCSO was determined by HPLC (LC-10A, Shimadzu, Kyoto, Japan) equipped with a TSK-gel ODS-80TM column (250 mm × 4.6 mm i.d. 5 μm, TOSOH, Tokyo, Japan) and a UV detector (230 nm). The mobile phase was distilled water adjusted to pH 3.3 with TFA. Flow rate and temperature were 0.7 mL/min and 30 °C, respectively.

Preparation of Crude Onion Alliinase. Yellow onion bulbs were homogenized and filtered in a kitchen juicer. The onion juice was combined with an equal volume of 0.05 M potassium phosphate buffer (pH 6.5) containing 10% glycerol and 20 μM pyridoxal-5′-phosphate. The diluted onion juice was chilled to 0 °C on an ice bath and made 80% saturation with ammonium sulfate. The resultant precipitate was collected by centrifugation at 5 °C and redissolved in the same buffer.

Preparation of Crude Garlic Alliinase. Unheated garlic juice chilled to 5−10 °C was adjusted to pH 4.0 with HCl. The precipitate was collected by centrifugation at 5 °C and redissolved in 0.05 M potassium phosphate buffer (pH 6.5) containing 10% glycerol and 20 μM pyridoxal-5′-phosphate.

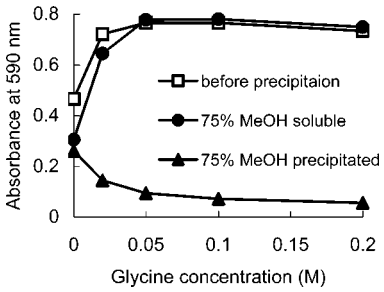

Figure 1. Effect of glycine, added to a mixture of 1-PeCSO and unheated garlic juice, on the solubility of blue color formed after incubation at 37 °C for 3 days.

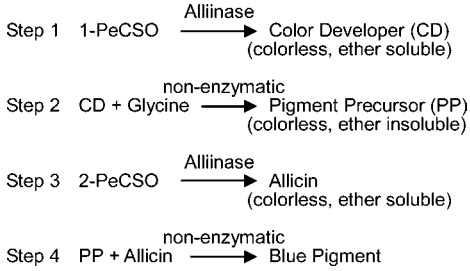

Figure 2. Reaction steps for blue pigment formation.

Purification of Onion and Garlic Alliinase. Purified alliinase was prepared by the procedure of Nock and Mazelis (*11*) with some modifications. All steps were carried out at 5−10 °C. Crude alliinase preparation was adsorbed onto hydroxylapatite (batchwise treatment), and the hydroxylapatite was washed with 0.05 M potassium phosphate buffer (pH 6.5) containing 10% glycerol and 20 μM pyridoxal-5′-phosphate. The hydroxylapatite was collected by centrifugation, and alliinase was eluted with the 0.5 M potassium phosphate buffer (pH 6.5) containing 10% glycerol and 20 μM pyridoxal-5′-phosphate. After centrifugation, eluate was placed on a concanavalin A-Sepharose 4B (Amersham Biosciences, U.S.) column, and eluted with 0.05 M potassium phosphate buffer (pH 6.5) containing 10% glycerol, 20 μM pyridoxal-5′-phosphate, and 100 mM methyl α-D-mannopyranoside.

Alliinase Activity Assay. Alliinase activity was determined by pyruvate formation based on the coupling assay involving oxidation of NADH to NAD by lactate dehydrogenase (LDH), as described by Schwimmer and Weston (*12*). The assay solution contained 0.06 μmol pyridoxal-5′-phosphate, 0.5 μmol NADH, 0.5 μg LDH, and 5.6 μmol 1-PeCSO in 3 mL of 0.1 M potassium phosphate buffer (pH 6.5). The pyruvate formation was monitored as a decrease in absorbance at 340 nm. One unit of the activity was defined as that capable of producing 1 μmol pyruvate from 1-PeCSO in 1 min at room temperature.

Preparation of Color Developer and Allicin. The color developer (CD) and allicin used in the model reaction system were prepared by treating isolated 1-PeCSO and 2-PeCSO, respectively, with purified garlic alliinase at 37 °C for 1 min. The CD and allicin produced was extracted with ether, evaporated to dryness, and redissolved immediately in distilled water. CD and allicin were prepared freshly before use.

Determination of Thiosulfinates. Spectrophotometric determination of thiosulfinates was carried out according to Lee et al. (*5*). The reagents were added in the following order: 0.1 mL of ether extract sample, 0.3 mL of 0.05 M *N*-ethylmaleimide in isopropyl alcohol, 0.3 mL of 0.25 M KOH, and 0.45 mL of 10 g/L ascorbic acid in distilled water. After mixing, the absorbance at 515 nm was measured immediately.

Effect of the Free Amino Acids or Amines on the Blue Color Formation. The solution containing CD and allicin was prepared by extracting and redissolving (in 60 mL of 0.1 M acetate buffer, pH 5.6) an ether-soluble fraction from a mixture of 1-PeCSO (30 mg) and 2-PeCSO (60 mg) treated with crude garlic alliinase. To 2 mL of the solution, 0.16 mL of 0.2 M amino acid or amine solution in the same buffer was added, except for Tyr, Cystine, Trp, and Glu, for which

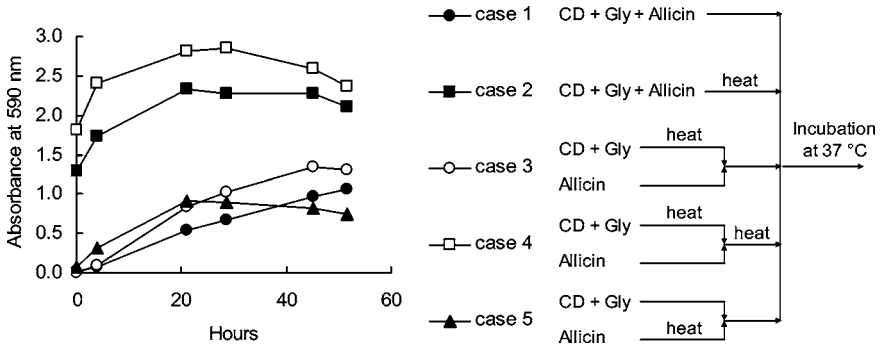

Figure 3. Increased blue color formation observed by heating components of the reaction system at 100 °C (in boiling water) for 10 min before incubation at 37 °C.

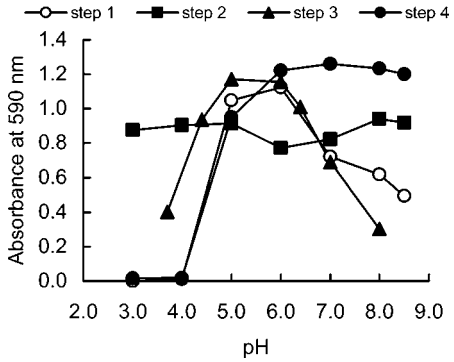

Figure 4. Effect of pH on blue color formation observed by varying pH for each reaction step while keeping the other steps at a constant pH of 5.6. Temperature and time for reaction were: step 1, 37 °C 1 min; step 2, 100 °C 10 min; step 3, 37 °C 1 min; step 4, 37 °C 2 days.

saturation concentrations were used because of their limited solubility in the buffer. Prepared solution was incubated at 37 °C for 3 days and the absorbance at 590 nm was measured.

Difference in CD Produced by Crude Alliinase from Garlic and Onion. After treatment of 1-PeCSO (0.25 mg) with crude alliinase (3.6 U) at 37 °C for 1 min, CD was extracted with 0.5 mL ether, and 0.1 mL of the extract was used for a *N*-ethylmaleimide reaction to detect thiosulfinates.

RESULTS AND DISCUSSION

Establishment of the Model Reaction System. While purees of onion and garlic had been used in our initial investigations, after confirming that vivid-blue color could be produced easily by mixing juices from heated onion and unheated garlic (data not shown), we used juices rather than purees as the starting materials. When an aqueous solution of isolated 1-PeCSO at a concentration equivalent to heat-treated onion juice (1.6 mg/mL) was added to unheated garlic juice, approximately 80% of the blue color developed in the mixture of onion and garlic juices was obtained (data not shown).

Addition of glycine to the mixture not only increased the total and the 75% v/v methanol (MeOH) soluble fraction of the blue pigment, but also decreased the fraction that precipitated with 75% v/v MeOH (**Figure 1**). These results suggested that some 75% v/v MeOH-insoluble substances, presumably proteins in the garlic juice, participated in the pigment formation and that they were less reactive than 75% v/v MeOH-soluble free amino acids, such as glycine.

The active compound that yielded blue color when combined with unheated onion juice was isolated from unheated garlic juice and was identified as allicin derived from 2-PeCSO by the action of alliinase.

Finally, we confirmed that vivid-blue color could be produced by using a highly defined model reaction system comprising only isolated 1-PeCSO, 2-PeCSO, pure glycine, and purified garlic alliinase. The optimal molar ratio of 1-PeCSO/2-PeCSO/glycine for the maximum blue color formation was determined as approximately 1:2:1 by, first, varying the relative amount of 2-PeCSO to 1-PeCSO in the presence of crude garlic alliinase to determine the ratio that produced the strongest color formation, and second, by adding varying amount of glycine to the solution containing the thus-determined combination of 1-PeCSO and 2-PeCSO (data not shown). The blue color formed in our model reaction system showed a maximum absorption at about 580 nm, and its absorption spectrum agreed well with the spectrum reported in Yamaguchi et al. (*3*) for the pigment formed by mixing purees of onion and garlic.

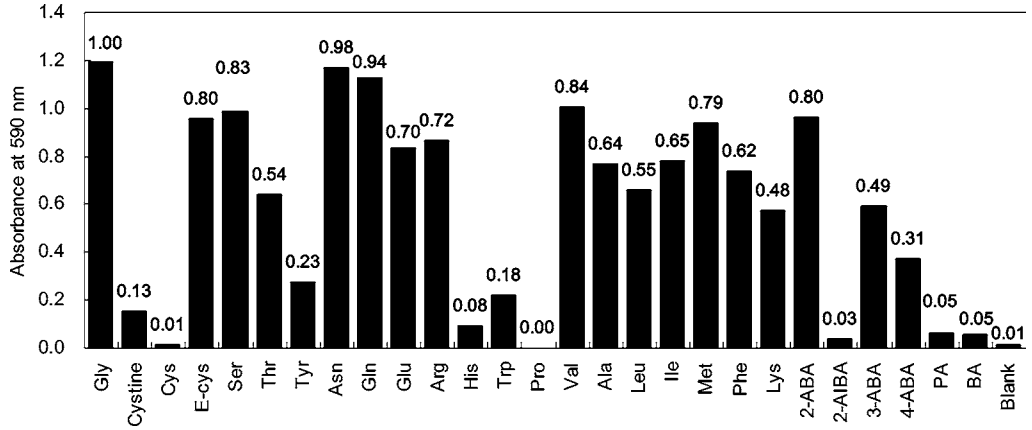

Figure 5. Blue color formation with various free amino acids or amines added to a solution containing CD and allicin. The value shown above each column represents the color intensity relative to that obtained with glycine.

In contrast to Kubec et al.(*6*), who reported pigment formation when glycine was allowed to react with synthetically prepared 1-propenyl-containing thiosulfinates such as *S*-allyl (*E*)-1-propenethiosulfinate, we observed only very faint pink coloration when glycine alone was added to thiosulfinates prepared from 1-PeCSO. Although the chemical identity of the thiosulfinates was not determined because of their instability, the most dominant thiosulfinate derived from 1-PeCSO should be *S*-1-propenyl (*E*)-1-propenethiosulfinate. Thus, we concluded that, in addition to the 1-propenyl-containing thiosulfinates from 1-PeCSO, allicin was an essential component in the blue pigment formation.

From the above observations, at least four reaction steps were established (**Figure 2**). If allicin in step 4 is replaced with formaldehyde, steps 1, 2, and 4 were identical to the three-step reaction scheme proposed by Shannon et al. (*1*) for pinking of onion.

Conditions Affecting Reactions Involved in the Blue Color Formation. Since steps 2 and 4 were supposed to proceed nonenzymatically, the effect of heating on the color development was investigated by using color developer (CD) and allicin prepared individually from isolated 1-PeCSO and 2-PeCSO, respectively (**Figure 3**).

Heating the mixture of CD, glycine, and allicin in boiling water for 10 min accelerated the color forming reactions dramatically, and most of the blue color developed within 10 min (case 2). Heating the mixture of CD and glycine alone prior to mixing with allicin, however, did not enhance the color formation significantly (case 3), although Shannon et al. (*1*) reported acceleration of this reaction by heating. Finally, reheating the mixture after addition of allicin produced the highest color formation (case 4). These results suggested that, in addition to step 2, step 4 representing the reaction between pigment precursor (PP) and allicin would be greatly accelerated by heating and that it was the primary rate-limiting step in the formation of blue pigment.

As shown in **Figure 4**, the effect of pH on the color formation was studied by varying pH for each step with McIlvaine buffer, while keeping the other steps at pH 5.6 with 0.1 M acetate buffer. The optimal pH of 5−6 found for steps 1 and 3 was close to the optimal pH of 6.5 reported for garlic alliinase activity (*13*). While step 2 was more or less independent of pH, the pigment-forming reaction in step 4 appeared to be hindered by pH lower than 5.

As shown in **Figure 5**, a varying degree of blue pigment formation was observed when glycine in the reaction step 2 was replaced with other free amino acids or amines. Among the free amino acids and amines tested, glycine (Gly), asparagine (Asn), and glutamine (Gln) were most effective in developing the blue color, while with cysteine (Cys), histidine (His), proline (Pro), 2-amino isobutyric acid (2-AIBA), propylamine (PA), and butylamine (BA), little or no pigment was developed. These results agreed very well with the results reported by Shannon et al. (*1*) for pinking in onion.

Effect of the Source of Alliinase Preparation on the Blue Color Formation. By using our model reaction system comprising 1-PeCSO, 2-PeCSO, glycine, and alliinase, we found that crude onion alliinase was less efficient in blue color formation than crude garlic enzyme showing equal alliinase activity. Moreover, the amount of pigment formation did not increase if the activity of crude onion alliinase added to the model reaction system was increased by about 2-fold (**Figure 6**). HPLC analyses of residual PeCSO revealed that, in all cases, more than 85% of 1-PeCSO and 90% of 2-PeCSO initially

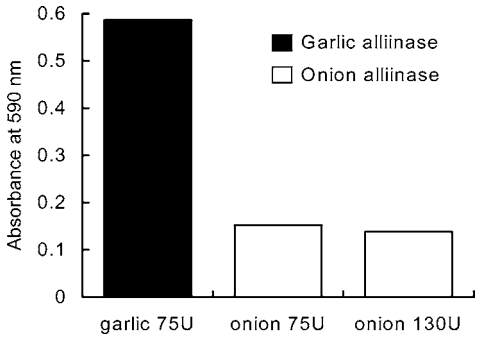

Figure 6. Blue color formed in solutions containing 1-PeCSO, 2-PeCSO, glycine, and crude alliinase from either garlic or onion, after incubation at 37 °C for 1 day.

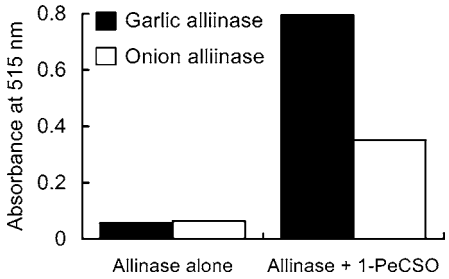

Figure 7. Difference in the amount of CD produced from 1-PeCSO by crude alliinase of garlic and onion.

present in the reaction mixture had been hydrolyzed by the alliinase activity. Thus, the difference in the amount of pigment formed could not be explained by the amount of alliinase activity in the reaction system.

Since Lee et al. (*5*) and Kubec et al. (*6*) suggested that CD could be one or more of the thiosulfinates derived from 1-PeCSO by the action of alliinase, we determined the amount thiosulfinates formed from 1-PeCSO colorimetrically by using the *N*-ethylmaleimide reaction. As shown in **Figure 7**, the crude onion alliinase yielded only about half the amount of thiosulfinates produced by the equal amount of crude garlic alliinase activity.

We also confirmed that use of purified onion alliinase instead of crude onion alliinase increased the amount of pigment formation in the model reaction system. After treating 1-PeCSO (2.5 mg) and 2-PeCSO (5.0 mg) in 3 mL of 0.1 M acetic acid buffer (pH 5.6) with 100 units of either purified onion alliinase or garlic alliinase for 1 min at 37 °C, CD was extracted with ether, evaporated to dryness, and redissolved in the same buffer (5 mL). Glycine (0.1 M solution, 0.5 mL) was added to each CD solution and allowed to form pigments for 2 days at 37 °C. The amount of pigment formation, as measured by absorbance at 590 nm, was nearly the same, i.e., 1.33 and 1.39, respectively, from purified onion alliinase and purified garlic alliinase.

These results were explained by the presence of lachrymatory factor (LF) synthase in the crude onion alliinase preparation (*14*). When the crude onion alliinase was used, the LF synthase converted a considerable part of 1-promenylsulfinic acid, the first intermediate produced from 1-PeCSO by the alliinase activity, into lachrymatory factor (propanethial-*S*-oxide), reducing the amount of the color-developing thiosulfinate (CD) and the pigment formation.

Thus, the model reaction system proved to be a useful tool for studying the mechanisms of discoloration in onion and garlic, as well as for elucidating chemical structures of the pigments and related compounds (*15*).

ABBREVIATIONS USED

1-PeCSO, *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide; 2-PeCSO, *S*-allyl-L-cysteine sulfoxide; CD, color developer; UP, unknown precursor; PP, pigment precursor; NOC, naturally occurring carbonyl; TFA, trifluoroacetic acid; NADH, nicotinamide adenine dinucleotide (reduced); NDA, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; MeOH, methanol; E-cys, ethyl-cystein; ABA, aminobutyric acid; AIBA, aminoisobutyric acid; PA, propylamine; BA, butylamine; MPLC, medium-pressure liquid chromatography; LF, lachrymatory factor (propanethial-*S*-oxide).

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