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One new pathway for *Allium* discoloration

Yuhui Dong, Dan Wang, Meiliang Li, Xiaosong Hu, Guanghua Zhao *

College of Food Science and Nutritional Engineering, China Agricultural University, Key Laboratory of Fruits and Vegetables Processing, Ministry of Agriculture and Engineering Research Centre for Fruits and Vegetables Processing, Ministry of Education, Qinghuadonglu 17, Haidian District, Beijing 100083, China

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ABSTRACT

(+)-*S*-(1-propenyl)-L-cysteine *S*-oxide (1-PeCSO) is a key compound associated with *Allium* discoloration. The pathway by which 1-PeCSO participates in *Allium* discoloration in the presence of alliinase has been established, but whether 1-PeCSO results in the discoloration in the absence of this enzyme remains unknown. In the present study, one new model reaction system containing 1-PeCSO alone in a phosphate buffer was established to simulate discoloration of white onion tissue and garlic puree. Results showed that the model system generated yellowish-green colour at pH 3.0 and red colour at pH 5.0. Two red species were isolated and identified by high-resolution multistage MS analysis, molecular formulae of which are $C_{24}H_{30}N_2O_5S$ (red species 1) and $C_{27}H_{36}N_2O_6S_2$ (red species 2). Both of them are sulphur-containing pyrrole derivatives and have UV/visible maximum absorbances at 520 (species 1) and 523 nm (species 2). The discoloration reactions occurring in this new system might represent a new pathway correlated with both onion reddening and garlic greening, emphasising that protein amino acids and alliinase are not necessary during the discoloration.

1. Introduction

Onion and garlic have been widely cultivated in the world because of their various pharmacological benefits (Agarwal, 1996; Rahman, 2003). They were processed in various forms, such as powder, granules, puree, minced paste, and oleoresin. During processing, garlic greening and onion reddening are major concerns because they limit commercial utilisation and decrease economic value (Kim, Cho, & Kim, 1999; Lukes, 1986). In contrast, garlic greening is required and desirable for "Laba" garlic, a traditional Chinese food product which is made by soaking aged garlic in vinegar in winter. Eating "Laba" garlic with dumplings is a tradition with a 300-year history to celebrate Chinese New Year in the north of China (Bai, Chen, Liao, Zhao, & Hu, 2005; Bai, Li, Hu, Wang, & Zhao, 2006). Green and red pigments, corresponding to greening and reddening, are considered to be secondary metabolites. *Allium* discoloration, including reddening of onion and greening of garlic, has been studied for many years. It was established that both garlic greening and onion reddening occur in a multistep process only with garlic and onion having high enough amounts of *S*-(1-propenyl)-L-cysteine sulfoxide (1-PeCSO), and are similar to each other in the mechanism of pigment production (Imai, Akita, Tomotake, & Sawada, 2006b; Joslyn & Peterson, 1960; Kubec, Hrbáčová, Musah, & Velíšek, 2004; Lukes, 1986; Shannon, Yamaguchi, & Howard, 1967a, 1967b; Wang, Nanding, Han, Chen, & Zhao, 2008).

It was proposed that there are three steps involved in the formation of red pigments responsible for onion reddening. The first step represents the formation of an ether-soluble organosulfur compound, which is also called a colour developer (CD), under the action of alliinase on 1-PeCSO (Shannon et al., 1967a). Recent studies showed that the colour developer could be di(1-propenyl) thiosulfinate (Imai, Akita, Tomotake, & Sawada, 2006a) or 1-propenyl containing thiosulfinate (Kubec & Velíšek, 2007; Kubec et al., 2004). The second step corresponds to the production of a pigment precursor (PP) by the reaction of the CD and a protein amino acid, such as glycine. This is a non-enzymatic reaction. Subsequently, the PP reacts with a naturally occurring carbonyl (NOC), such as formaldehyde, to form a pink or red pigment, which was believed to be the third step (Shannon et al., 1967a). Recent studies have indicated that a reaction of pigment precursor (possibly a pyrrole derivative) with one of the protein amino acids, such as alanine and valine, might represent the third step for onion reddening (Imai et al., 2006a; Wang et al., 2008).

The first two steps in garlic greening are the same as those in onion reddening. The third step, corresponding to garlic greening, is the formation of allicin from (+)-*S*-(2-propenyl)-L-cysteine *S*-oxide (2-PeCSO) under the catalytic action of alliinase. Resulting allicin reacts with pigment precursor (PP) to produce the final

Abbreviations: 1-PeCSO, (+)-*S*-(1-Propenyl)-L-cysteine *S*-oxide; 2-PeCSO, (+)-*S*-(2-Propenyl)-L-cysteine *S*-oxide; CD, colour developer; PP, pigment precursor; NMR, nuclear magnetic resonance; MS, mass spectroscopy.

* Corresponding author. Tel.: +86 10 62737645; fax: +86 10 62737645 12.
E-mail address: gzhao1000@yahoo.com (G. Zhao).

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green pigment(s), which represents the fourth step for garlic greening (Imai et al., 2006a). Recently, it was found that the reaction of the PP and allicin produced only blue pigments which are unstable and easily decay to yellow pigments. The observed green colour for garlic greening was formed by mixing the formed yellow and un-decayed blue pigments (Bai et al., 2005). The reaction of PP with pyruvic acid might represent another pathway for the formation of the yellow pigment(s) (Wang et al., 2008). These results indicated that garlic greening is more complex than onion reddening.

Much evidence is consistent with the above reaction pathways, showing that protein amino acids, such as glycine and alanine, might be involved in garlic greening and onion reddening (Imai et al., 2006a, 2006b; Kubec & Velíšek, 2007; Kubec et al., 2004; Lukes, 1986; Shannon et al., 1967a; Wang et al., 2008). However, it is uncertain whether other reaction pathways might co-exist, in which these protein amino acids and alliinase are not necessary for *Allium* discoloration. Indeed, in the present study, it was found that the colour of a model reaction system, consisting of only 1-PeCSO in phosphate buffer, turned red at pH 5.0 while it became yellowish-green at pH 3.0. This finding likely represents a new pathway for *Allium* discoloration.

2. Materials and methods

2.1. Chemicals

All solvents/chemicals used were of analytical grade or purer. Acetic acid, phosphoric acid, sodium acetate anhydrous, ammonium acetate, sodium dihydrogen phosphate dehydrate, ninhydrin, butanol and acetone were purchased from Beijing Chemistry Co. (Beijing, PR China). L-Cystine hydrochloride was obtained from Aoboxing Biotechnology Co. (Beijing, China). Allyl bromide was obtained from Beijing Xingjin Chemistry Co. (Beijing, China). Methanol and formic acid, used in HPLC–MS, were of chromatograph grade, and were purchased from Honeywell Burdick and Jackson (SK Chemicals, Korea) and Sigma–Aldrich (Chemie GmbH, Germany) respectively.

2.2. Plant materials

Freshly harvested (May 2008) onion and garlic bulbs were obtained from a local market at China Agriculture University, stored at room temperature, and used immediately for the following experiments.

2.3. Instruments

UV/Visible spectra were recorded with a Cary 50 UV–vis spectrophotometer (Varian Co., USA). ^{1}H NMR spectra were obtained on a dpx-300 MHz NMR spectrometer (Brucker Co., Germany). D_2O was used as a solvent with trace amounts of DDS (3-trimethylsilyl-1-propanesulfonic acid, sodium salt). HPLC–MS/MS analysis was performed by an Alliance 2695 Separations Module (Waters, Milford, MA, USA) coupled to a Waters 2996 photodiode array detector (Waters, Milford, MA, USA) and a Micromass Quattro Micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electro spray ionisation (ESI) source and an ion trap mass analyzer, which were all controlled by MassLynx software. Detection was performed in the positive mode.

2.4. Isolation of (+)-S-(1-propenyl)-L- cysteine S-oxide

(+)-*S*-(1-Propenyl)-L-cysteine *S*-oxide (1-PeCSO) was extracted from commercial yellow onion by a reported method with some modifications (Shen & Parkin, 2000). Typically, 5 kg of onion bulbs were peeled and cut into quarters. Endogenous alliinase was inactivated by cooking the quarters in a microwave oven at 700 W for 1 h. The cooked quarters were cooled by putting them into 4 l of water. Then the mixture was homogenised in a kitchen blender, filtered with cheesecloth, and the filtrate was adjusted to pH 4.0 with acetic acid. The filtrate was kept at 4 °C overnight, followed by centrifugation (9000*g* 20 min) at 4 °C to remove particulate matter.

Half of the supernatant was passed through a column of Dowex 50WX4 (H^+, 5 × 30 cm) (Sigma, USA); the absorbed amino acids and peptides were eluted with 0.2 M sodium acetate (pH 6.5) at 2.5 ml/min. Butanol, acetic acid, water and acetone (24:4:12:20, v/v/v/v) were used as developing solvent in TLC to detect the number of components in the eluate. The 1-PeCSO-containing fractions were combined and passed through a second column of Dowex 50WX4 (H^+, 2.6 × 50 cm) and the column eluted with 0.05 M sodium hydroxide at 2.5 ml/min. The 1-PeCSO-containing fractions were collected and applied to a third column (1.6 × 30 cm) of Dowex 2X8 which was not capable of holding 1-PeCSO. Finally, the fractions containing 1-PeCSO from the third column were loaded onto a fourth column of Dowex 50WX4 (H^+, 2.6 × 25 cm) and eluted with 0.05 M ammonium acetate (pH 5.5). After lyophilisation, 1.5 g of white crystals of 1-PeCSO were obtained and stored in a vacuum desiccator for future use.

2.5. Set-up of model system and isolation of pink pigment

Five equal parts of 1-PeCSO (each containing 60 mg of 1-PeCSO) were dissolved in 10 ml of the same buffers (containing 0.1 M H_3PO_4, 0.1 M Na_2HPO_4, and 0.1 M NaH_2PO_4) but with different values of 3.0, 4.0, 5.0, 6.0, and 7.0 respectively. All resulting solutions were allowed to stand at 40 °C over 25 days, and their UV–visible spectra were recorded every 5 days during standing. Since the pH 5.0 solution produced the deepest colour, among all samples, the resulting red pigments in the solution were isolated. First, the solution was filtered with syringe filter units (0.22 μm) purchased from Hercules (Beijing, China). Secondly, the filtrate was loaded onto the SPE cartridges SUPELCLEAN ENVI-18, and eluted with 5 ml of water, 5 ml of acetone, 5 ml of methanol and 2.5 ml of acidic methanol (0.2% HCl in methanol) in sequence, respectively. Subsequently, the methanol eluate was collected and subjected to HPLC–MS/MS analysis.

2.6. HPLC–MS/MS analysis

A sample (20 μl) was loaded onto a reversed phase ASB-C18 column (250 × 4.6 mm, 5 μm, Venusil, Agela, USA) and eluted with 0.2% formic acid in water (A) and methanol (C). Eluting conditions for the blue pigment sample were as follows: the percentage of methanol was raised linearly from 50% to 90% within 80 min, and then decreased to 50% in the following 10 min. In contrast, eluting conditions for the pink sample were as follows: the percentage of methanol increased linearly from 45% to 70% within the first 40 min, and decreased to 45% during the following 10 min. HPLC conditions were as follows: flow rate, 0.4 ml/min; column temperature, 30 °C; detection wavelength, 250–800 nm; sample size, 20 μl. The MS instrument was operated in the positive ion mode, scanning from *m*/*z* 200 to 800 at a scanning rate of 2.0 s/cycle. The mass parameters were as follows: capillary voltage, 2.5 kV; cone voltage, 5–15 V; source temperature, 110 °C; desolvation temperature, 400 °C; desolvation gas flow, 600 l N_2/h; cone gas flow, 50 l N_2/ h. High-resolution multistage MS analysis was performed by a quadrupole ion-trap time-of–flight mass spectrometer (LCMS-IT-TOF, Shimadzu, Kyoto, Japan). This instrument is based on the ability of a quadrupole ion trap to deliver MS^n capa-

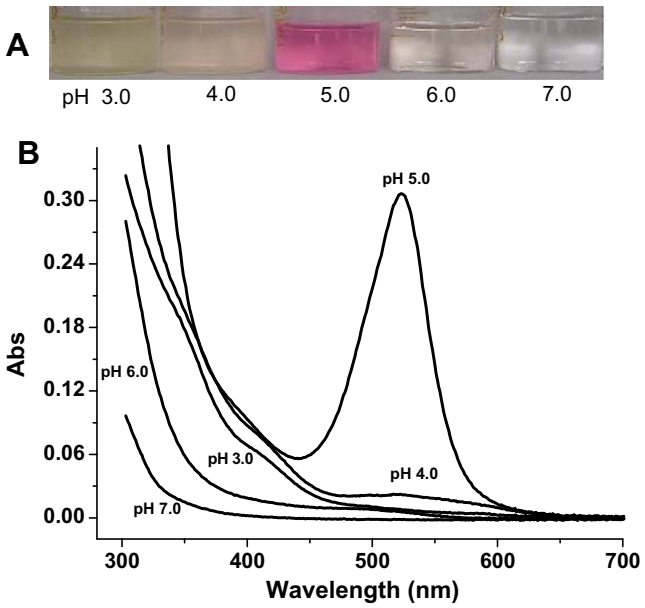

Fig. 1. (A) Photographs and (B) UV–vis spectra of a series of solutions from a model system consisting of 1-PeCSO in H_3PO_4 buffer at different pH values (3.0, 4.0, 5.0, 6.0, and 7.0).

bility and the TOF mass analyzer to support accurate mass measurement.

3. Results and discussion

To elucidate whether these protein amino acids were necessary for *Allium* discoloration, a new model system was made, consisting of 1-PeCSO alone in a phosphate buffer at different pH values (3.0, 4.0, 5.0, 6.0, and 7.0). 1-PeCSO was isolated from yellow onion, and its purity was confirmed by ^{1}H-NMR and MS and was identical to those reported previously (Carson, Lundin, & Lukes, 1966; Shen & Parkin, 2000). After 25 days incubation in 40 °C, it was found that the solution became yellowish-green at pH 3.0 (Fig. 1A), which exhibited a visible absorbance at ~410 nm (Fig. 1B). With increasing pH (to 4.0), the 1-PeCSO solution turned light pink, which is

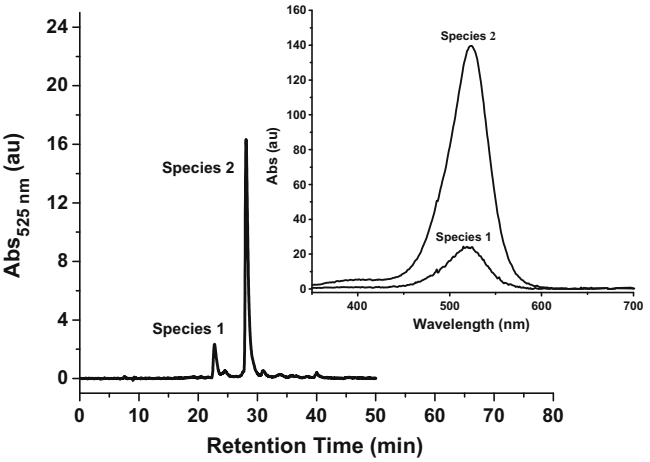

Fig. 2. HPLC chromatogram (at 525 nm detection wavelength) of methanol-eluted fraction after pH 5.0 sample (red solution) is loaded onto C-18 SPE column. Inset: UV–visible spectra of species 1 and 2 in HPLC obtained by a diode array detector. λ_{max} were 520 and 523 nm for species 1 and 2, respectively.

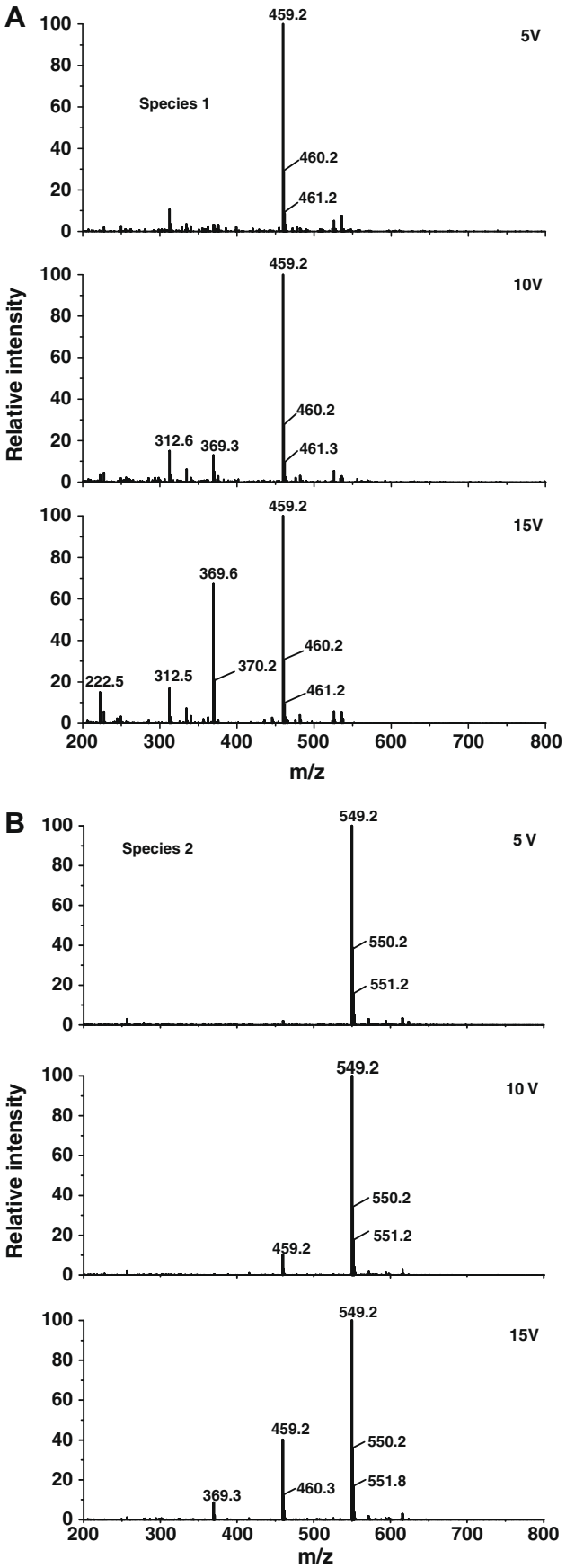

Fig. 3. (A) and (B) MS/MS spectra of the peaks 1 (A), and 2 (B) in HPLC at different cone voltages (5, 10, and 15 V).

characterised by a UV–visible absorbance at ~527 nm (Fig. 1B). At pH 5.0, the solution of 1-PeCSO became red and exhibited a strong UV–visible absorbance at ~523 nm which is nearly identical to λ_{max} (520 nm), usually characteristic of the reddening of onion (Joslyn & Peterson, 1960). In contrast, the discoloration almost disappeared at pH 6.0, and completely disappeared at pH 7.0 (Fig. 1B). Thus, the discoloration of the 1-PeCSO solution is pH-dependent and was strongest at pH 5.0 among all used pH values. The present observation is in perfect agreement with a previous report showing that pH 4.8 was the optimal value for onion reddening (Shannon et al., 1967a). In parallel, when acetic acid was substituted for phosphate as a reaction buffer in the pH range 3.0–7.0, under the same experimental conditions, nearly identical results were obtained (data not shown), indicating that the observed discoloration is not derived from buffer.

Due to the strong absorbance at ~520 nm, the pH 5.0 sample was further analyzed by HPLC–MS/MS coupled with diode array. Prior to the HPLC–MS/MS analysis, the sample was preliminarily purified using the C-18 SPE cartridges, just as described above. Similarly, the methanol-eluted fraction was subjected to HPLC, and the resulting HPLC spectrum is shown in Fig. 2A, which only contained two red species, named 1 and 2, appearing at 22.77

Table 1
Formulae of the red pigments and their fragments observed from high-resolution electrospray ionisation mass spectra.

Species [M + H$^+$]	Formula	Measured (m/z)	Calculated (m/z)	Error (Da)
1	$C_{24}H_{31}N_2O_5S$	459.1914	459.1953	0.0029
2	$C_{27}H_{37}N_2O_6S_2$	549.2045	549.2092	0.0047
Ion with m/z 369.3 (Fig. 4)	$C_{21}H_{25}N_2O_4$	369.1777	369.1809	0.0032

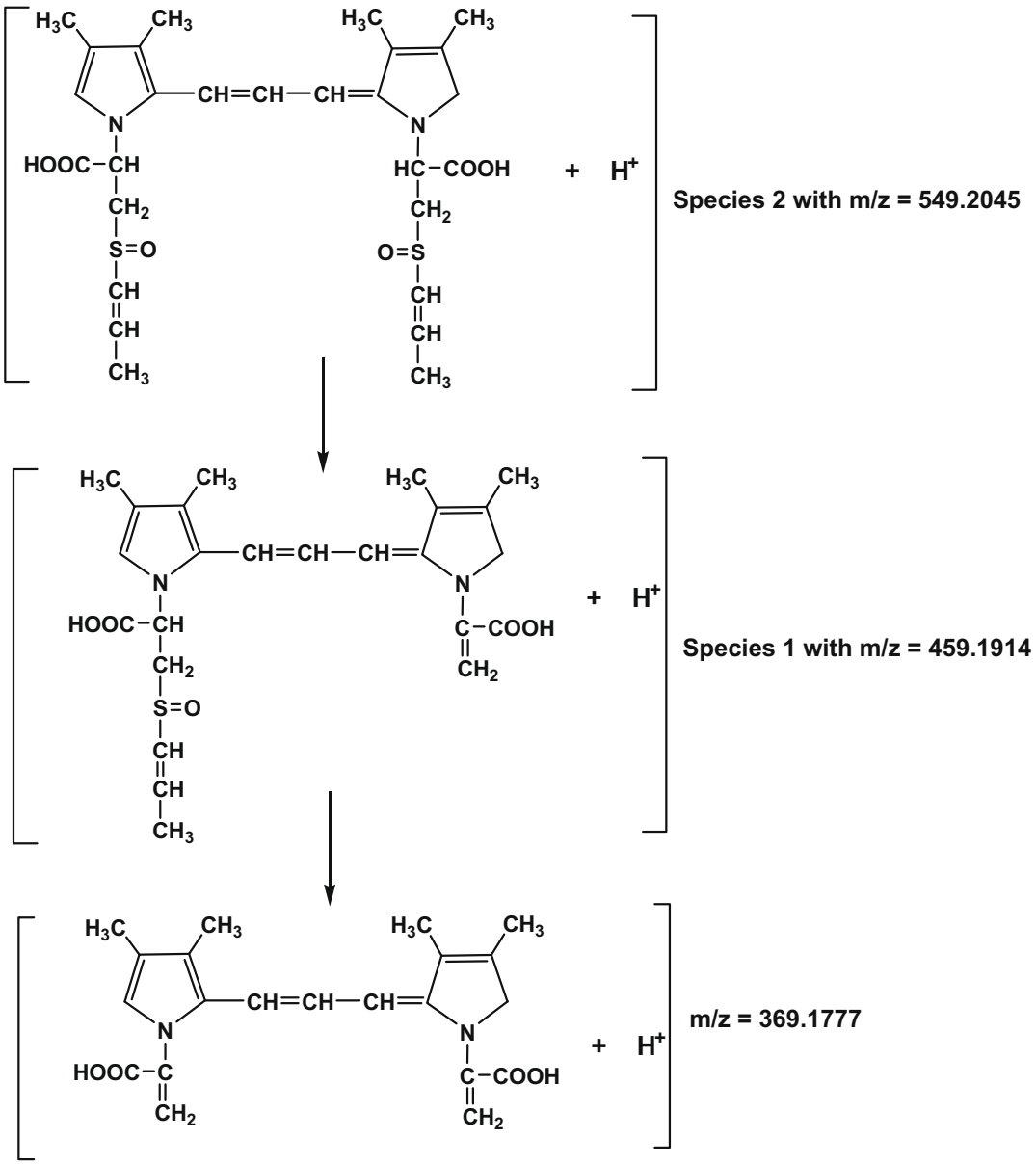

Fig. 4. Proposed possible structure of two red pigments 1 and 2 and the degradation pathway of red pigment 2 at m/z 549.2 [M + H$^+$] in Fig. 4B.

and 28.10 min, respectively. The UV–visible spectra of these two peaks were obtained with a diode array detector (Fig. 2A, inset), showing that red species 1 has a maximum absorbance at around 520 nm while the maximum absorbance of red species 2 is at 523 nm.

ESI MS results, under soft ionisation conditions, are useful in determining relative purity and molecular mass of a compound. A wide mass range scan, with a lower voltage, is typically used to ensure that all components were detected as their protonated adducts. The acquired mass spectra of red species 1 showed a prominent protonated molecular ion peak at m/z 459.2 with two impurity peaks at m/z 312.6 and 535.9 at 5 V of the cone voltage (Fig. 3A). Upon increasing the cone voltage to 10 or 15 V, the 459.4 ion decayed and produced a new ion at m/z 369.2. Unlike red species 1, the mass spectrum of red species 2 exhibited a single protonated molecular ion peak at m/z 549.2 $[M + H]^+$ when the cone voltage was 5 V (Fig. 3B). As the cone voltage increased to 10 V, the mother ion decayed and produced a new ion at m/z 459.2. When 15 V of the voltage was used, the ion at m/z 459.2 became stronger, while another new ion at m/z 369.2 was formed. By comparison of the two mass spectra, of red species 1 and 2, it is found that the daughter ion at m/z 459.2 of the ion at m/z 549.2 $[M + H]^+$ is the same as the molecular ion contained in red species 1. Therefore, the compound in red species 1 with MW 458.2 might be generated from the degradation of the compound with MW 548.2 in red species 2. Consistent with this idea, λ_{max} of the compound contained in red species 2 was a little longer than that of the compound in red species 1. It is possible that red species 1 and 2 are very similar in structure and contain the same chromophoric group, but red species 2 has an extra electron-donating group compared to red species 1.

In contrast with recent studies showing that a reddish-purple pigment from a model system consisting of 2-(3,4-dimethylpyrrolyl)-3-methylbutanic acid (a proposed pigment precursor) and allicin (Imai et al., 2006a) is composed of C, H, O and N elements, the MS/MS results in the present study showed that both mass ions at m/z 459.2 $[M + H]^+$ and m/z 549.2 $[M + H]^+$ have very strong peaks at $X + 2$ (m/z 461.2, and m/z 551.2), which have ~5–9% of the intensities of their corresponding molecular ion peaks. This result suggested that red species 1 and 2 might contain one or two sulphur atoms. Consistent with the present observation, Jedelská, Vogt, Reinscheid, and Keusgen (2008) isolated a sulphur-containing red dye with molecular formula of $C_8H_6N_2S_2$ from *Allium* subgenus *Melanocrommyum*, which had λ_{max} in the visible region at 519 nm (Jedelská et al., 2008). More support came from a recent observation showing that a compound with MW 411, responsible for garlic greening, might contain one sulphur atom; this was isolated from garlic (Lee, Cho, Kim, & Lee, 2007). In addition, based on mass spectra (Figs. 3A and B), it was estimated that both compounds contained even numbers of nitrogen atoms, due to their even nominal mass.

To confirm the above conclusion, the accurate masses of the two ions were determined to be 549.2045 and 459.1914 by high-resolution MS (Table 1), which gives two possible molecular formulae of $C_{27}H_{36}N_2O_6S_2$ (red pigment 2) and $C_{24}H_{31}N_2O_5S$ (red pigment 1). Based on their formulae, the structure of 1-PeCSO and information that Y1 and Y2 have red colours but do not contain metals (data not shown), their possible structures are proposed (Fig. 4). High-resolution MS, in conjunction with MS^n, allows us to obtain accurate molecular weights and their relationship (Table 1 and Fig. 3B). According to these observations, we propose a pathway showing the degradation of mother ion at m/z 549.2 into its daughter ion at m/z 459.2, and finally into an ion at m/z 369.3. This pathway is in agreement with the fact that the stability of the C–S bond in the red pigment 2 is poor. It has been established that alliinase can catalyse the conversion of alk(en)yl-L-cysteine sulfoxides into their corresponding thiosulfinates through cleavage of the C–S bond (Shen & Parkin, 2000). This pathway also explains the production of pigment 2, which stemmed from pigment 1, as suggested by the MS/MS spectra in Figs. 2 and 3.

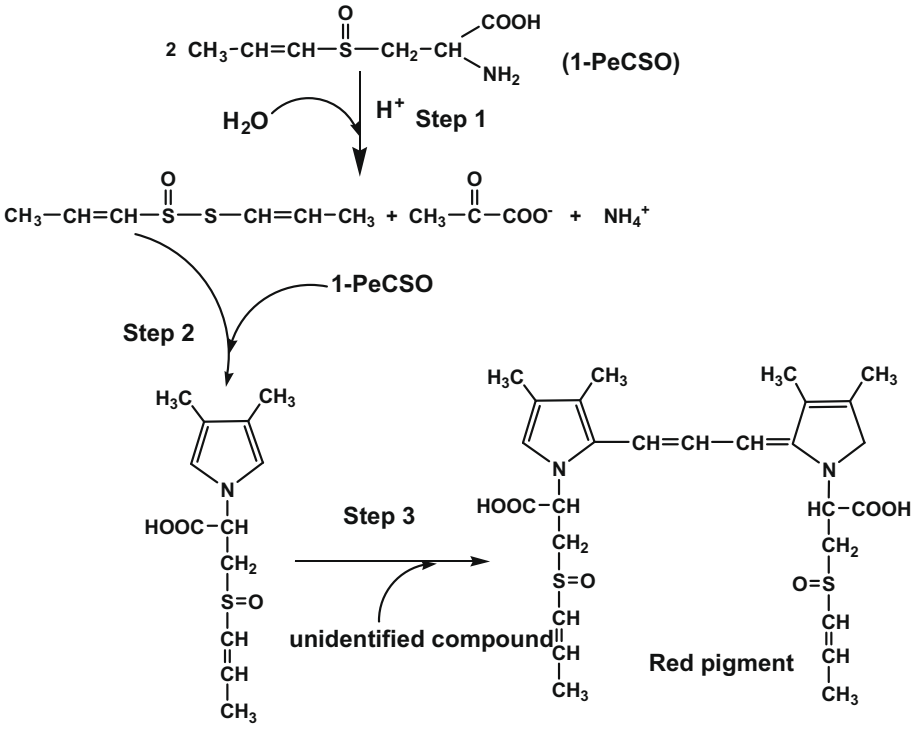

Fig. 5. Proposed three-step pathway of the formation of red pigment related to onion reddening.

Subsequently, a formation pathway of pigment 1 was proposed, as shown in Fig. 5. Step 1 corresponds to the formation of di(1-propenyl) thiosulfinate accompanied with the production of pyruvic acid and ammonium from 1-PeCSO under acidic conditions. This reaction is the same as the catalytic conversion of 1-PeCSO under the action of alliinase (Shen & Parkin, 2000). In agreement with the above proposal, bis(1-propenyl) disulphide was detected as one of the products from a system consisting of 1-PeCSO alone in acetic acid buffer at pH 3.0 by GC–MS, which was most likely produced from its precursor, di(1-propenyl) thiosulfinate (Bai, 2006). Step 2 represents the formation of pigment precursor(PP) by a reaction of di(1-propenyl) thiosulfinate with 1-PeCSO, and PP is a sulphur-containing pyrrole derivative. This reaction is similar to that between di(1-propenyl) thiosulfinate and valine or alanine reported recently (Imai et al., 2006a; Wang et al., 2008). The third step corresponds to the formation of the red pigment by PP reacting with an unidentified compound. Thus, this pigment is generated through a pathway different from that for the reported reddish-purple pigment because di(2-propenyl) thiosulfinate was involved in the production of the reddish-purple species (Imai et al., 2006a). Although we cannot draw a conclusion about the structure of the unidentified compound, we found that the same red pigment was also produced from a model system consisting of 1-PeCSO and formaldehyde (data not shown), a result suggesting that this compound could be formaldehyde. Support for this idea comes from a previous proposal that the PP reacts with formaldehyde to form a red pigment (Shannon et al., 1967a). However, the pathway for the formation of formaldehyde from the system only containing 1-PeCSO remains to be determined. A detailed study of this is underway.

4. Conclusion

The present model system represents a new pathway related to *Allium* discoloration, in which a protein amino acid and alliinase are not required. This is ascribed to the high similarity in structure between 1-PeCSO and protein amino acids so that 1-PeCSO substitutes for the protein amino acids to participate in certain reactions, finally resulting in the discoloration occurring with garlic and onion. In addition, the poor stability of 1-PeCSO at low pH might be important. This new pathway and other known pathways might jointly be responsible for the *Allium* discoloration.

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