Photoisomerization of 2-[3-(2-Thioxopyrrolidin-3-ylidene)methyl]-tryptophan, a Yellow Pigment in Salted Radish Roots

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The photostability of (E)-2-[3-(2-thioxopyrrolidin-3-ylidene)methyl]-tryptophan ((E)-TPMT), the main yellow pigment in salted radish, was studied. First we analyzed the photoproduct generated from (E)-TPMT under longwave UV irradiation. On the basis of NMR spectroscopy, the photoproduct was identified as Z-configurated TPMT, and isomerization from the Z- to the E-form was reversibly induced by Vis-light irradiation. The optimum wavelength for isomerization from the E- to the Z-form was 360–380 nm, and that for isomerization from the Z- to the E-form was 440–460 nm. The E/Z-ratios in the photostationary state under UV- and Vis-light irradiation conditions were approximately 0.95:1 and 26:1 respectively. The (Z)-isomer was more sensitive to light irradiation than the (E)-isomer in the quantum yield measurement. Yellowing was dependent on the ratio of the (Z)-isomer, because the b* and chroma value rose with increases in the (Z)-isomer by the colorimeters. Hence, it is possible that the formation of the (Z)-isomer contribute to the yellow color of takuan-zuke during long salting and fermentation.

Key words: yellow pigment; photoisomerization; tetrahydro-β-carboline derivative; salted radish roots; photochrome compound

Salted radish roots called genboku are utilized as materials in various pickled products, such as takuan-zuke, fukujin-zuke and sakura-zuke. During the approximately 8-month salting and fermentation process, the radish roots turn naturally to a bright yellow. It is empirically known that the pungent taste of raw radish roots is closely related to the yellowing, and that the yellowness strengthens in the summer. To maintain this yellow color, one of characteristic qualities of takuan-zuke, is desirable, but the yellow color fades easily under photoradiation. Hence manufacturers are searching for the best way to stabilize the color to reduce the use of the food coloring additives.

In previous studies, we have found that 4-methylthio-3-butenyl isothiocyanate (MTBI), the pungent principle of radish roots, is a key compound in the yellowing reaction in salted radish.1,2) MTBI, which is unstable in aqueous media, is easily converted into the antimicrobial and antigenotoxic product 2-thioxo-3-pyrrolidinecarboxaldehyde (TPC) in the aqueous phase.3–6) TPC can form 1-(2-thioxopyrrolidin-3-yl)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (TPCC) as a precursor of the yellow pigment, which is formed by Pictet-spengler condensation with L-tryptophan, which is produced by microorganisms during the fermentation process.1,7,8) 1,2,3,4-Tetrahydro-β-carbolines (THβCs) are found in numerous foods, including traditional Japanese foods, such as fermented soybean paste and soy sauce.9,10) THβCs are known to be produced during food processing and storage in the fermentation process.11–13) In our recent study,14) TPCC was found to change at weak acidic or neutral pH to a bright yellow pigment, which was identified as (E)-2-[3-(2-thioxopyrrolidine-3-ylidene)methyl]-tryptophan ((E)-TPMT). In addition, we found that (E)-TPMT is probably the major naturally occurring substance in salted radish roots. Thus, (E)-TPMT is probably a factor contributing to the quality of takuan-zuke.
zuke. Hence, it is important to study the chemical behavior of the yellow pigment under various conditions. The present work aimed to clarify the pH and light stability of (E)-TPMT for the stabilization and effective use of the yellow pigment in salted radish roots.

**Results and Discussion**

The storage stability of (E)-TPMT was studied at various pH levels and temperatures under darkness. As shown in Fig. 1, (E)-TPMT was comparatively stable at both pH 5.0 and 6.0, and 90% of (E)-TPMT remained after 240 h of storage. In contrast, 30–40% of (E)-TPMT was lost at pH 3.0 and 8.0 after 240 h of storage. When (E)-TPMT was maintained at 100 and at 120°C, (E)-TPMT was stable at less than pH 6.0, but was unstable under neutral and basic pH conditions (Fig. 2). In order to clarify the relationship between the pH stability and the ionized states of the carboxyl and amino groups, calculation of pKa and pI was performed. The pKa values were 2.54 and 9.37, and the pI value was 5.47. Since the stable pH condition for (E)-TPMT almost corresponded to the condition for forming zwitterions, it is possible that stability at pH 5.0–6.0 is dependent on the ionized form. In high temperature storage under basic pH conditions, it appeared that the thioxopyrrolidine moiety became rapidly unstable, as did TPC. The pH at which (E)-TPMT was comparatively stable coincided with the pH of the salted radish. Therefore, the subsequent light stability tests for (E)-TPMT were done at pH 5.0.

In light irradiation using a xenon arc lamp under a nitrogen atmosphere at room temperature for 10–30 min, the content of (E)-TPMT decreased to about 60% (data not shown). Continuous exposure for a further 4 h gave no more photoreaction. There was no difference between the photoreaction at low temperature, 7°C, and at room temperature (data not shown). Though light-irradiation was carried out in pH 3 and 7 solutions, no difference during the photoreaction was observed. No thermal and pH effects on light-extinction were observed because of the short period of irradiation. Figure 3 shows typical HPLC chromatograms and UV-Vis spectra of (E)-TPMT solutions before or after light-irradiation. Under the conditions described in “Experimental” below, (E)-TPMT (peak A) was eluted at a retention time of 2.4 min, while the retention time of an unknown photoproduct (peak B) was 6.8 min. It was determined that the content of peak B increased while that of (E)-TPMT decreased under light irradiation. In comparison to light irradiation with and without an optical bandpass filter (UV33DS), which can transmit longwave UV and absorbed visible light, the formation of peak B was observed to accelerate under longwave UV light. Under such conditions, the content of (E)-TPMT decreased to about 50%. From UV-Vis spectral analysis with a photodiode array detector, we observed that the maximum absorption of peak B shifted bathochromically from 380 nm to 400 nm (Fig. 3). LC-MS analysis of peak B indicated that its molecular weight was 315, equal to that of (E)-TPMT. The purified compound corresponding to peak B for structural identification could not be obtained because the compound reverted to (E)-TPMT during the purification procedure. Hence, NMR analysis was carried out in the state of the photoreaction mixture. When TPMT solution was exposed to a long-wave UV lamp (at 366 nm) for 30 and 120 min, four signals, at 7.21 (H-5), 7.34 (H-6), 7.58 (H-7), and 7.71 ppm (H-4), assigned to an indol moiety, shifted upfield to 7.17, 7.31, 7.46, and 7.69 ppm in the ¹H-NMR spectrum (Fig. 4a, b and c). The signal of an olefinic proton at 7.78 ppm (H-6' of (E)-TPMT, triplet, J = 2.7 Hz), indicating a long-range coupling with H-4', was also shifted upfield to 7.03 ppm (H-6' of peak B, triplet, J = 1.9 Hz). From the ¹H-NMR spectral data for the geometrical isomer of 3-methylthiomyethyl-2-thioxopyrrolidine, the geometry of C3'–C6' was assigned as Z. A NOESY correlation between H-6'/H-4'
and H-6′/H-10 of the (Z)-isomer was observed, and hence the conformation of C3″=C2=C6″=C3″ was assigned as a transoid like (E)-TPMT. Thus the photoproduct (peak B) from (E)-TPMT was identified to be the (Z)-configuration of TPMT, as shown in Fig. 5. The above-mentioned observations led us to conclude that this photoreaction was not photolysis but rather photoisomerization. The initial E/Z ratio of TPMT, estimated from NMR analysis, was 96:4. The E/Z ratio changed to 73:27 and 51:49 after UVA irradiation after 30 and 120 min respectively (Fig. 4). No change in the E/Z ratio during irradiation over 2 h was observed. When the UV irradiated solution was exposed to visible light from an incandescent lamp for 30 min, a reverse change to the (E)-form was observed (Fig. 4d). Isomerization from the (Z)- to the (E)-form reached equilibrium under irradiation after 30 min, following which the E/Z ratio was 85:15. These results suggest that the photoreaction of TPMT was photochromic isomerization depending on irradiation wavelength.

For detailed research on the effect of the irradiation wavelength, the monochromatic light of a spectrofluoro-
The spectrophotometer was used. The results presented in Fig. 6a indicate that long-wave UV irradiation promoted conversion to (Z)-TPMT more than short- or middle-wave UV irradiation. The irradiation wavelength for the maximum conversion rate in monochromatic light was 360–380 nm. The photoreaction reached equilibrium within 15 min, within which 60% of (E)-TPMT was photoconverted into (Z)-TPMT. Irradiation at wavelengths longer than 420 nm suppressed the Z-isomerization of (E)-TPMT. To clarify the wavelength dependence in the reverse (Z → E) photoreaction, TPMT solution (E/Z ratio = 0.95:1), pre-irradiated at 370 nm, was exposed to visible light. The E-isomerization rate of the (Z)-form was less than 50% when irradiated at 400–420 nm for 15 min (Fig. 6b). When irradiated for 3 min under monochromatic light of 440–460 nm, 60–70% of the (Z)-form was isomerized to the (E)-form. Further, when it was irradiated for 5 min or more, 90% or more was reconverted to the (E)-isomer. The quantum yields at the optimum wavelength for photoisomerization in the presence of nitrogen were 0.24 (E → Z) and 2.53 (Z → E) respectively. Perhaps the Z to E-isomerization was a chain reaction, because the quantum yield was more than 1.18. These results indicated that the Z-isomer had a higher photosensitivity than the E-isomer, and that the Vis-light induced isomerization (Z → E) rate was faster than the UV-light induced isomerization (E → Z) rate. When quantum yield was measured under bubbling in air, the quantum yields were 0.19 (E → Z) and 1.88 (Z → E). It was confirmed that bubbling in air inhibited E,Z-photoisomerization. That is, the dissolved oxygen acted as a quencher of the photoexcited TPMT. Based on these results, it was suggested that the photoisomerization reaction and the photostationary state of TPMT were strongly dependent on the irradiation wavelength. In the photochemical experiment, we found that there was a difference in the E,Z-isomerization rate due to a combination of the light source, optical filter, and diffraction grating. The monochromatic light of the spectrofluorometer with a bandwidth at 10 nm improved the E,Z-isomerization rate more than when the wideband light source was a black lamp. It was found that the irradiation wavelength in the 400–440 nm range was competed with E ↔ Z-isomerization.

Based on the results of the above-mentioned experiments, we observed under direct vision by naked eye
Table 1. Changes in \( L^*, \ a^*, \ b^*, \ C^*, \) and \( h^* \) Values of TPMT Solution under Light Irradiation

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Concen. (ppm)</th>
<th>Irradiated wavelength</th>
<th>Time (min)</th>
<th>( E/Z ) ratio(^a)</th>
<th>( L^* )</th>
<th>( a^* )</th>
<th>( b^* )</th>
<th>( C^* )</th>
<th>( h^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPMT</td>
<td>100</td>
<td>370 nm</td>
<td>0</td>
<td>26:1</td>
<td>99.7</td>
<td>-5.5</td>
<td>11.2</td>
<td>12.5</td>
<td>116.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.7:1</td>
<td></td>
<td></td>
<td>99.6</td>
<td>-8.0</td>
<td>17.5</td>
<td>19.3</td>
<td>114.7</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.95:1</td>
<td></td>
<td></td>
<td>99.5</td>
<td>-9.6</td>
<td>21.7</td>
<td>23.8</td>
<td>113.9</td>
</tr>
<tr>
<td></td>
<td>440 nm</td>
<td>5.2:1</td>
<td>2(^b)</td>
<td></td>
<td>99.6</td>
<td>-6.8</td>
<td>14.3</td>
<td>15.9</td>
<td>115.4</td>
</tr>
<tr>
<td>Curcumine</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td></td>
<td>98.9</td>
<td>-10.3</td>
<td>30.4</td>
<td>32.1</td>
<td>108.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>—</td>
<td>—</td>
<td></td>
<td>99.8</td>
<td>-1.4</td>
<td>3.7</td>
<td>4.0</td>
<td>110.0</td>
</tr>
<tr>
<td>Tartrazine</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td></td>
<td>99.5</td>
<td>-3.7</td>
<td>10.8</td>
<td>11.4</td>
<td>109.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>—</td>
<td>—</td>
<td></td>
<td>99.9</td>
<td>-0.4</td>
<td>1.1</td>
<td>1.1</td>
<td>111.1</td>
</tr>
</tbody>
</table>

Values were obtained using the 2 mm cell.
\(^a\) The \( E/Z \) ratio was calculated as peak areas detected at 380 nm on HPLC analysis.
\(^b\) After UV irradiation for 17 min, visible light was used for 2 min.

that the yellow color of the TPMT solution became darker under UV irradiation, while the solution discolored under visible light irradiation. Colorimetric measurement was carried out in order to determine the changes in these colors under irradiation. As shown in Table 1, the \( L^* a^* b^* \) values when measuring the concentration of TPMT at 100 ppm were like those obtained in 10 ppm curcumine and tartrazine as reference substances. Ten times the concentration of TPMT was necessary to obtain a color similar to these reference compounds. Under UV irradiation, the values of \( L^* \) and \( a^* \) decreased, while that of \( b^* \) and chroma increased. Under Vis-light irradiation, reversed trends and rates of change in all values were observed. These results were supported by changes in yellow color under macroscopic observation. The appearance of the color depended on the ratio of the \( Z^- \) to the \( E^- \) isomer.

This paper reports for the first time a change in the \( Z^- \) to \( E^- \) TPMT under visible light and the photoisomerization of \( Z^- \)-TPMT caused the partial discoloration of \( Z^- \)-TPMT and the yellow color became pale. TPMT was thus found to be the photochromic compound, while the photoisomerization was both reversible and repeatable. In our previous study, TPMT generated from TPCC as the pigment precursor of the salted radish was mainly of the \( E^- \) form, but quantitative HPLC analysis of commercial products indicated the presence of not only the \( E^- \) but also the \( Z^- \) isomer, and that indicated the average \( E/Z \) ratio was about 6:1 (H. Matsuoka, personal communication). The Vis-light induced isomerization of the \( Z^- \) to the \( E^- \) form agreed with the fading of \( takuan-zuke \) under fluorescent light and sunlight irradiation. Thus, it is possible that \( Z^- \)-configured TPMT plays an important role in the yellow color of \( takuan-zuke \).

Light affects most food ingredients, and in particular, oxidative decomposition and photodynamic actions due to UVA and visible light become a problem in food processing and preservation. In the presence of food pigments such as chlorophylls and carotenoids, lipid oxidation is promoted by a photosensitized action. On the other hand, these pigments are photobleached by sunlight and fluorescent light as well as TPMT.\(^{19,20}\) A bleaching mechanism of carotenoids has been reported to occur through oxidation with radical generation in the presence of oxygen and UV irradiation. Antioxidants such as tea polyphenols showed antidiscoloring activity against \( \beta \)-carotene.\(^{21}\) Since the photobleaching of TPMT under visible light was caused not by oxidative decomposition but rather by photoisomerization, no antioxidizing effect of several antioxidants such as ascorbic acid and catechin on TPMT was observed (data not shown). On the other hand, it has been reported that acylated anthocyanins readily isomerized \textit{in vitro} under UV irradiation, and that inter- and intramolecular stacking, self-association, and co-pigmentation inhibited photoisomerization.\(^{22,23}\) This phenomenon is one of the most important biological functions in living plant cells under sunlight irradiation. The phenomena of yellowing and photobleaching in salted radish are of interest in food processing and preservation. In the present report, we indicate the relation between the isomerization of \( Z^- \)- to \( (E)^- \)-TPMT under visible light and the photobleaching. Furthermore, it is possible that \( E^- \)-isomerization of \( (Z)^- \)-TPMT caused the partial discoloration of the commercial \( takuan-zuke \), but we could not identify the inhibition method of isomerization in the present study. Therefore, the stabilization of \( (Z)^- \)-TPMT should be studied further in detail.

**Experimental**

\textit{Instrumental analyses.} \(^{1}\)H-NMR spectra were obtained in 70% CD\(_3\)OD in D\(_2\)O containing DCI with a JEOL EX-400 instrument using TMS as an internal standard. Analytical HPLC was performed with an Agilent series 1100 equipped with a Mightsysl RP-18.
GP column ($\phi3.0 \times 150\, \text{mm}$, Kanto Chemical, Tokyo) and a photodiode array detector. Chromatographic conditions were as follows: the mobile phase was 18% of acetonitrile in 25 mM phosphate buffer (pH 6.6) kept at a constant flow rate of 0.6 ml/min; the column was thermostatted at 40°C. LC-MS (electrospray ionization, ESI) analysis was done on a Mightysil RP-18 GP column ($\phi3.0 \times 150\, \text{mm}$, Kanto Chemical, Tokyo) using an Agilent HPLC-MSD series 1100. The eluent consisted of 25 mM ammonium acetate buffer (pH 5.2, solvent A) and acetonitrile (solvent B). The gradient elution was programmed as follows: 15% B isocratic for 2 min, then linearly increased to 60% B in 8 min, and then held for 5 min.

**pH and heat stability test of (E)-TPMT.** (E)-TPMT was prepared according to our previously described method. A stock solution of (E)-TPMT (1,000 ppm, 3.17 mM) was prepared in 70% MeOH (containing 3.17 mM HCl). One hundred microliters of this solution was transferred into 1.5-ml screw vials for HPLC and further diluted with 0.1M phosphate buffer (900 µl) set at several pHs (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0). In the pH stability test, these solutions were incubated at 40°C for 240 h in the dark. In the heat stability test, the solution was incubated at 100 and 120°C for 2 h. (E)-TPMT remaining in the mixtures was measured during this period by HPLC.

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**Photosubility test of (E)-TPMT.** A photoisomerization test was conducted in a pH 5.0 phosphate buffer. (E)-TPMT solution (100 ppm) in a quartz cell was irradiated at 9,000 lux using a 500 W Xe lamp (Ushio Electric, Tokyo). Irradiation was carried out for 0–30 min at room temperature, fitted with and without band-pass glass filters (UV33DS, Asahi Techno Glass, Tokyo) with nitrogen bubbling. A 0.1-ml portion of the irradiated solution was transferred into the vial at the stated time intervals of irradiation, and the solution was set in the auto-sampler (4°C) for HPLC analysis.

**Wavelength dependence of photoisomerization of TPMT.** The wavelength dependence measurement was carried out using the excitation monochromator of a Shimadzu RF-1500 spectrophotometer (Kyoto, Japan). All (E)-TPMT solutions (100 ppm) in pH 5.0 buffer were exposed to a 150 W Xe lamp under bubbling nitrogen or air. Spectral irradiation was made at 10–20 nm wavelength intervals in a range from 240 to 460 nm with a 10 nm bandwidth for 5 and 15 min, and the remaining (E)-TPMT was measured by HPLC. After UV irradiation at 370 nm for 17 min, the solution was exposed to visible light in a range from 400 to 480 nm for 3, 5, and 15 min. The concentration of the photo-product, (Z)-TPMT, was calculated on the basis of the decreasing rate of the initial peak area of (E)-TPMT on the HPLC chromatograms. Quantum yield was measured with a chemical actinometer using potassium tris(oxalate) ferrite(III) [K$_3$Fe(C$_2$H$_2$O$_4$)$_3$] as the standard compound. Light-irradiation was done at 370 and 440 nm for 2 min. The photolytic production of Fe (II) was analyzed by the 1,10-phenanthroline method. Ferrioxalate actinometry was conducted using quantum yields of 1.21 and 1.11 respectively for irradiated wavelengths at 370 nm and 440 nm.

**Color measurement of TPMT solution.** The colorimetric change in the (E)-TPMT solution (100 ppm) under UV- and Vis-light irradiation was measured with a Minolta CM-3500d spectrophotometer, with illuminant D65 and, 10° observer. CIE (1976) $L^*a^*b^*$ values were obtained by transmittance color measurement with an SCI (specular component included) system. Chroma ($a^{2} + b^{2})^{1/2}$ and hue angle ($\arctan b^*/a^*$) were calculated. The optical path length of the cells for measurement was 2 mm. The reference samples used were curcumin (1 and 10 ppm in MeOH) and tartrazine (1 and 10 ppm in H$_2$O).

**NMR analysis of TPMT solution during UV and Vis light irradiation.** $^{1}$H-NMR analysis was carried out in order to analyze the photochemical reaction of (E)-TPMT. In $^{1}$H-NMR analysis, a solution of (E)-TPMT (4 mg/ml in 70% CD$_3$OD containing 12.7 µmol DCl) in an NMR sample tube was placed at a distance of 1 cm from the light source. The solution was exposed for 0.5–2 h to 366 nm radiation under a hand-held UV lamp. The UV irradiated solution then was re-exposed to Vis-light using an incandescent lamp (250 W at a distance of 20 cm from light source) for 1–2 h. At set intervals of irradiation time, the solution was monitored by $^{1}$H-NMR.

**pKa calculations for (E)-TPMT.** Empirical calculation of pKa values was done using the pKa plug-in in the Marvin suite academic package (Chemaxon, Budapest, Hungary) on an Apple Mac Book Pro computer running MacOS 10.4.10. (E)-TPMT was constructed with default geometries, and no structural optimization was conducted prior to the calculation of pKa values.

**Acknowledgment**

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