Note

Quantitative Analysis of Yellow Pigment in *Takuan-zuke* and Their ABTS Radical Cation Scavenging Activity

Asaka TAKAHASHI¹, Hiroki MATSUOKA^{2*}, Emi WATANABE², Atsuko SHINAGAWA², Yoshio OZAWA², Yoichi YAMADA³ and Yasushi UDA⁴

¹ Nagano Prefectural College, 8-49-7 Miwa, Nagano 380-8525, Japan

² Department of Health and Nutrition, Takasaki University of Health and Welfare, 37-1 Nakaorui-cho, Takasaki-shi, Gunma 370-0033, Japan

³ Faculty of Education, Utsunomiya University, 350 Mine-machi, Utsunomiya-shi, Tochigi 321-8505, Japan

⁴ Department of Bioproductive Sciences, Faculty of Agriculture, Utsunomiya University, 350 Mine-machi, Utsunomiya-shi, Tochigi 321-8505, Japan

Received November 5, 2008; Accepted February 7, 2009

2-[3-(2-Thioxopyrrolidin-3-ylidene)methyl]-tryptophan (TPMT) is the main yellow pigment in salted radish roots (*takuan-zuke*). In the present study, we investigated the content of 1-(2-thioxopyrrolidin-3-yl)-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (TPCC) as a precursor of the yellow pigment and TPMT in commercial *takuan-zuke*. The total content of stereoisomers was 0.2–36.4 mg/100 g (2–364 ppm) for TPCC, and 0–6.6 mg/100 g (0–66 ppm) for TPMT. TPCC and TPMT showed radical scavenging activity against ABTS radical cation than the classical antioxidant (ascorbic acid and Trolox). The anti-oxidative activity showed no differences for TPCC stereoisomers, but showed differences for *E/Z* ratio-controlled TPMT solutions.

Keywords: yellow pigment, tryptophan derivative, tetrahydro- β -carboline derivative, salted radish root, radical scavenging activity

Introduction

Takuan-zuke, the salted or dried radish root that is pickled in salty rice bran, is a traditional food in Japan. It was originally defined as the processing radish that is now called *genboku*, a raw material used in various pickled products. *Takuan-zuke* now refers to *genboku* that has been further pickled in seasoning liquid and is classified into *hoshi-takuan* and *shio-oshi-takuan* depending on the initial method of processing the radish. *Hoshi-takuan* is first dried and pickled in salty rice bran paste and then further pickled in seasoning liquid. *Shio-oshi-takuan* is compressed and salted, and then further pickled in seasoning liquid. In addition, *Sato-shibori-daikon* is the one that salted radish was pickled in seasoning liquid containing liquid sugar syrup.

The radish root turns yellow during the several months

of the salting and fermenting processes. Yellowing effects increase particularly in summer, and the generated yellow pigment is related to radish pungent component, 4-methylthio-3-butenyl isothiocyanate (MTBITC) (Ozawa *et al.*, 1990c). MTBITC is mainly converted to 2-thioxo-3-pyrrolidinecarbaldehyde (TPC) in the presence of water (Uda *et al.*, 1990), with 1-(2-thioxopyrrolidin-3-yl)-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (TPCC) formed as a precursor through the Pictet-Spengler reaction with L-tryptophan (Ozawa *et al.*, 1990a, Ozawa *et al.*, 1990b). We previously reported that TPCC is unstable under neutral pH and is easily converted into (*E*)-2-[3-(2-thioxopyrrolidin-3-ylidene)methyl]-tryptophan ((*E*)-TPMT) as the main yellow pigment (Matsuoka *et al.*, 2002). (*E*)-TPMT was isomerized to (*Z*)-TPMT by long wave UV irradiation, and the isomerization from (*Z*)- to (*E*)-form is induced by Vis-light irradiation (Matsuoka *et al.*, 2008). The color of (*Z*)-TPMT is a darker yellow than (*E*)-TPMT and fades easily

*To whom correspondence should be addressed.

E-mail: matsuoka@takasaki-u.ac.jp

by Vis-light irradiation, indicating the important role of (Z)-TPMT in the yellow color of *takuan-zuke*. Figure 1 shows the formation of the yellow pigment from MTBITC.

Tetrahydro- β -carbolines occur in many foods and exhibit a broad range of pharmacological and biological activity (Herraiz, 2000). They can function as neurotransmitters and neuromodulators (Buckholtz, 1980). Recently, Herraiz and Galisteo reported that tetrahydro- β -carbolines in foods and biological systems act as radical scavengers in the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation assay (Herraiz and Galisteo, 2002). L-Tryptophan is an essential amino acid for humans, and as the precursor of the neurotransmitter serotonin, it regulates physiological mechanisms. Several tryptophan derivatives also function as free radical scavengers and antioxidants, stimulating a number of antioxidative enzymes and stabilizing cell membranes (Reiter *et al.*, 1999). Thus, TPCC and TPMT may be applied for uses other than its current use as a pigment. As only mutagenic and antimutagenic properties of TPCC and TPMT have been reported (Ozawa *et al.*, 1999, Matsuoka *et al.*, 2002), we investigated the amount of TPCC and TPMT in commercial *takuan-zuke* and their radical scavenging activities using an ABTS radical cation assay.

Materials and Methods

Materials ABTS and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acids) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). *N* $^{\alpha}$ -(5-Fluoro-2,4-dinitrophenyl)-L-alaninamide (L-FDAA) and (+)-catechin were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Potassium persulfate was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). MTBITC was prepared from radish sprouts according to our previously described method (Uda *et al.*, 1990).

Quantitative analysis of TPCC and TPMT in commercial *takuan-zuke* All *takuan-zuke* samples (*hoshi-takuan*, *shio-oshi-takuan*, and *sato-shibori-daikon*) and *ggaktugi* (cubed radish kimchi) were purchased from a local market; *ggaktugi* is a radish sprinkled with chili peppers and pickled in salt, seasoning, and fish sauce. TPCC and TPMT were extracted from each sample as described previously (Matsuoka *et al.*, 2002) and then analyzed by HPLC with using an eluent of acetonitrile: 25 mM phosphate buffer (pH 6.6) (18:82, v/v) for 6 min; flow rate was kept constant at 0.6 mL/min and the column temperature was 40°C. TPCC and TPMT were detected at 268 nm and 380 nm, respectively.

To separate the TPMT enantiomers extracted from salted radish (*genboku*), derivatization by L-FDAA (Marfey and Ottesen, 1984) was done. The mixture of 50 μ L of 0.5% L-FDAA (in acetone), 100 μ L of 1 M sodium bicarbonate,

Fig. 1. Formation of yellow pigment from MTBITC.

and sample solution (100 μ L) was incubated for 60 min at 40°C. The reaction was terminated by the addition of 100 μ L of 1 M HCl, and the samples were then diluted with 475 μ L methanol. These solutions were analyzed by HPLC and LC-MS. Analytical HPLC was performed with Agilent series 1100 instrument with a photodiode array detector and a Mightysil RP-18 column (150 $\times$ 3 mm I.D., Kanto Chemical Co., Ltd., Tokyo, Japan). The mobile phase was composed of acetonitrile (Solvent A) and 25 mM ammonium acetate buffer (pH 6.6, Solvent B). Elution was performed with a linear gradient from 15% to 35% Solvent A for 30 min. The column temperature was maintained at 45°C and the flow rate was 0.4 mL/min. Derivatives of TPMT were detected at 340 nm.

LC-MS analysis was performed using an Agilent HPLC-MSD series 1100 instrument. MS analysis was carried out by electrospray ionization (ESI) in the positive mode. The desolvation temperature was 350°C, and the capillary volt-

age was 3.5 kV. Nitrogen was used as the desolvation and nebulizer gas.

Preparation of TPCC and TPMT TPCC was synthesized from MTBITC and L- or D-tryptophan according to the method described previously (Ozawa *et al.*, 1999). The diastereoisomeric mixture of (1*S*,3*S*,3'*R*)- and (1*R*,3*S*,3'*R*)-TPCC was subsequently separated by preparative ODS-HPLC. TPMT derived from TPCC (diastereoisomeric mixture) was prepared according to our previously described method (Matsuoka *et al.*, 2002). The enantiomers of synthesized TPMT were analyzed as described above.

ABTS radical cation scavenger test The effect of TPCC and TPMT on the ABTS radical cation (ABTS^{•+}) was estimated according to the method of Re *et al.* (1999). ABTS was dissolved in ultra pure water to a final concentration of 7 mM and the ABTS^{•+} was produced by reacting stock solution with potassium persulfate (final conc., 2.4 mM). The mixture was allowed to stand at room temperature overnight (>12 h) in the dark. The ABTS^{•+} solution was diluted with 8 mM phosphate buffered saline (PBS) to give an absorbance value of 0.70 at 734 nm. The assay was done using a 96-well microplate. After addition of 200 μ L diluted ABTS^{•+} solution to 1 μ L test compounds or Trolox standard in 70% methanol, absorbance was measured using a Corona MTP-800Lab microplate reader 90 sec after initial mixing. All determinations were carried out at least three times. The antioxidant capacity was measured in comparison with Trolox, (+)-cat-

echin, and ascorbic acid as the antioxidant standard.

To compare the radical scavenging activity of geometrical isomers of TPMT, *E/Z* ratio-controlled solutions were also examined. The TPMT solution (100 ppm) in phosphate buffer (pH 5.0) was exposed to visible light at 460 nm for 15 min (Solution A). Solution A was re-irradiated at 370 nm (Solution B). These solutions were prepared with bubbling nitrogen. Irradiation was carried out using the excitation monochromator of a Shimadzu RF-1500 spectrofluorometer (Kyoto, Japan). From the HPLC analysis, the *E/Z* ratio of Solutions A and B was 96:4 and 0.95:1, respectively (Matsuoka *et al.*, 2008). In test tube, the reaction mixture contained ABTS^{•+} solution, Solution A or Solution B (0–23.81 μ M) and 70% methanol at a final volume of 1 mL. The mixture was allowed to react for 15–90 sec after initial mixing. The absorbance was measured using a Hitachi U-2000 double-beam spectrophotometer.

Molecular modeling method Calculation of the lowest energy conformer was done using conformer plug-in in the Marvin suite academic package (version 5.1.03, Chemaxon, Budapest, Hungary) on an Apple MacBook Pro computer running MacOS 10.5.6.

Results and Discussion

The amounts of TPCC and TPMT in *takuan-zuke* samples are presented in Table 1. In our previous study (Matsuoka *et al.*, 2002), we found that salted rad-

Table 1. Amounts of TPCC and TPMT of *takuan-zuke* samples.

Sample	Amounts (mg / 100 g)		
	(1 <i>S</i> , 3 <i>S</i> , 3' <i>R</i>)-TPCC	(1 <i>R</i> , 3 <i>S</i> , 3' <i>R</i>)-TPCC	TPMT ^a
<i>Hoshi-takuan</i> 1	17.13 $\pm$ 0.05	5.21 $\pm$ 0.00	3.43 $\pm$ 0.02
<i>Hoshi-takuan</i> 2	17.17 $\pm$ 0.30	4.89 $\pm$ 0.09	2.94 $\pm$ 0.09
<i>Hoshi-takuan</i> 3	24.85 $\pm$ 0.35	7.31 $\pm$ 0.18	5.58 $\pm$ 0.68
<i>Hoshi-takuan</i> 4	28.29 $\pm$ 0.37	8.13 $\pm$ 0.15	3.43 $\pm$ 0.12
<i>Hoshi-takuan</i> 5	20.06 $\pm$ 0.18	6.86 $\pm$ 0.01	1.34 $\pm$ 0.36
<i>Hoshi-takuan</i> 6	1.71 $\pm$ 0.26	1.52 $\pm$ 0.22	6.55 $\pm$ 0.04
<i>Hoshi-takuan</i> 7	17.67 $\pm$ 0.21	4.71 $\pm$ 0.05	2.73 $\pm$ 0.02
<i>Hoshi-takuan</i> 8	7.86 $\pm$ 0.04	3.36 $\pm$ 0.02	4.96 $\pm$ 0.00
<i>Shio-oshi-takuan</i> 1	9.71 $\pm$ 0.20	2.76 $\pm$ 0.03	0.81 $\pm$ 0.03
<i>Shio-oshi-takuan</i> 2	1.20 $\pm$ 0.03	0.31 $\pm$ 0.00	0.05 $\pm$ 0.00
<i>Shio-oshi-takuan</i> 3	4.85 $\pm$ 0.08	1.77 $\pm$ 0.08	0.38 $\pm$ 0.03
<i>Shio-oshi-takuan</i> 4	2.48 $\pm$ 0.01	0.47 $\pm$ 0.00	0.33 $\pm$ 0.27
<i>Shio-oshi-takuan</i> 5	0.70 $\pm$ 0.04	0.02 $\pm$ 0.03	n.d.
<i>Sato-shibori-daikon</i> 1	1.32 $\pm$ 0.02	0.02 $\pm$ 0.03	n.d.
<i>Ggaktugi</i> 1	0.93 $\pm$ 0.01	0.14 $\pm$ 0.00	n.d.
<i>Ggaktugi</i> 2	1.29 $\pm$ 0.02	0.33 $\pm$ 0.01	n.d.
<i>Ggaktugi</i> 3	2.16 $\pm$ 0.03	1.44 $\pm$ 0.06	n.d.
<i>Ggaktugi</i> 4	0.16 $\pm$ 0.08	n.d.	n.d.

TPCC, 1-(2-thioxopyrrolidin-3-yl)-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid; TPMT, 2-[3-(2-thioxopyrrolidin-3-ylidene)methyl]-tryptophan; n.d.: Not detected.

Values are expressed as means $\pm$ SD.

^aThe total amount of *E*- and *Z*-form.

ish roots (*genboku*) contained about 50 ppm TPCCs ((1*S*,3*S*,3'*R*)-TPCC:(1*R*,3*S*,3'*R*)-TPCC = 4:1) and about 6 ppm TPMT. In the commercial *takuan-zuke*, the content of (1*S*,3*S*,3'*R*)- and (1*R*,3*S*,3'*R*)-TPCCs as precursors of the yellow pigment was 0–8.1 mg/100 g (0–81 ppm) and 0.2–28.3 mg/100 g (2–283 ppm), respectively. The ratio of (1*S*,3*S*,3'*R*)-TPCC to (1*R*,3*S*,3'*R*)-TPCC was about 3:1. TPMT, the main yellow pigment, contained 0–6.6 mg/100 g (0–66 ppm). On average, the amount of TPCCs in *hoshi-takuan* was about 5-fold that in *shio-oshi-takuan*, while the amount of TPMT was 13-fold. Thus, *shio-oshi-takuan* appears to contain scarce amounts of TPCCs and TPMT, most likely because it is manufactured from a desalinated salted radish (*genboku*). In contrast, *ggaktugi* contained only TPCC and no TPMT, indicating that the amount of TPCC is related to the level of fermentation with lactic acid bacteria.

As the L-isomer of amino acids is known to elute prior to the D-isomer, based on an analysis of L-FDAA derivatives (Fujii *et al.*, 1997), we analyzed TPMT as the analogue of tryptophan. TPMT synthesized from MTBITC and L-tryptophan resulted in only the L-isomer. Similarly, only D-TPMT was generated from D-tryptophan. Taken together with previous results (Ozawa *et al.*, 1999), TPCCs gener-

ated from MTBITC and L-tryptophan afford (1*S*,3*S*,3'*R*)- and (1*R*,3*S*,3'*R*)-isomer. In contrast, TPMT extracted from salted radish roots (*genboku*) afforded only the L-form. As commercial *takuan-zuke* is made from salted radish roots (*genboku*), only the L-form of TPMT in commercial *takuan-zuke* is expected to be obtained.

The activity of TPCC and TPMT synthesized from MTBITC and tryptophan as radical scavengers was measured with the ABTS radical cation assay. TPCC and TPMT showed antioxidant activity that was stronger than the classical antioxidant ascorbic acid and Trolox, but weaker than catechin (Fig. 2). Linear curves were obtained with concentrations of the compounds lower than 10 μ M, suggesting that the reaction is not significantly affected by possible side reactions. No difference was observed in the activity of (1*S*,3*S*,3'*R*)- and (1*R*,3*S*,3'*R*)-TPCC or in the activity of either the enantiomers of tryptophan or TPMT. The antioxidant activity of samples was expressed as Trolox equivalent antioxidant capacity (TEAC) (μ M), which represents the concentration (μ M) of Trolox, with the same activity as 1 μ M of the sample. Similar antioxidant values of TEAC ranging from 2.52 to 2.68 μ M were obtained for tryptophan and TPMT. The TEAC value of tryptophan and TPMT was

Fig. 2. ABTS radical cation scavenging activity of TPCC, TPMT, and classical antioxidants. Results are expressed as means $\pm$ SD.

Fig. 3. ABTS radical cation scavenging activity of geometrical isomers of TPMT. ABTS^{•+} solution and TPMT reacted for 15 sec (■), 60 sec (○), 90 sec (▲) after initial mixing. Results are expressed as means $\pm$ SD.

higher than that of ascorbic acid (0.93 μ M) and Trolox, and were 75% of catechin (3.45 μ M). The TEAC value of TPCC was 1.48–1.61 μ M and about 50% of catechin. MTBITC showed hardly any antioxidant activity against the ABTS^{•+}. Tryptophan is converted to kynurenine and *N*-formylkynurenine through the cleavage of indole ring by hydroxyl radical attack or one-electron oxidation (Maskos *et al.*, 1992), and tetrahydro- β -carboline carboxylic acids are decarboxylated to afford the fully aromatic compounds after reaction with the ABTS^{•+} (Herraiz and Galisteo, 2002), suggesting antioxidative ability of TPMT and TPCC.

Most of TPMT prepared from TPCC by our previously described method was the *E*-form. (*E*)-TPMT is isomerized to (*Z*)-TPMT by long wave UV irradiation. To compare the radical scavenging activity of these geometrical isomers, the effect of *E/Z* ratio-controlled solutions (Solutions A and B) on the ABTS^{•+} was examined. The radical scavenging activity of Solution B was higher than that of Solution A; this remarkable activity remained for a short time (Fig. 3). Thus, (*Z*)-TPMT appears to react with ABTS^{•+} earlier than (*E*)-TPMT. Calculation of the lowest energy conformer of TPMT showed that indole and thioxopyrrolidine of the (*E*)-TPMT molecule were a coplanar structure (Matsuoka *et al.*, 2002), and the energy of *E*- and *Z*-forms was calculated to be 56.36 kcal/mol and 59.69 kcal/mol, respectively. These results suggested that the reactivity of (*Z*)-TPMT to ABTS^{•+} is higher than that of (*E*)-TPMT.

The formation of the (*Z*)-TPMT contributes to yellowing of *takuan-zuke*, even though this isomer is more sensitive to light irradiation than the *E*-isomer (Matsuoka *et al.*, 2008). *E*-isomerization of (*Z*)-TPMT under visible light irradiation

may therefore cause partial discolorization of the commercial *takuan-zuke*. Moreover, as some of the β -carbolines are accumulated in human tissues, offering protection against oxidative stress (Pari *et al.*, 2000), TPCC and TPMT might also prevent deterioration in the quality of *takuan-zuke* by oxidation and exhibit antioxidant activity in human tissues.

Acknowledgement We are grateful to Mr. H. Karasawa (Nagano Prefecture General Industrial Technology Center) for his support of the LC-MS analysis.

References

- Buckholtz, N. S. (1980). Neurobiology of tetrahydro- β -carbolines. *Life Sci.*, **27**, 893–903.
- Fujii, K., Ikai, Y., Mayumi, T., Oka, H., Suzuki, M. and Harada, K. (1997). A nonempirical method using LC/MS for determination of the absolute configuration of constituent amino acids in a peptide: elucidation of limitations of Marfey's method and of its separation mechanism. *Anal. Chem.*, **69**, 3346–3352.
- Herraiz, T. (2000). Analysis of the bioactive alkaloids tetrahydro- β -carboline and β -carboline in food. *J. Chromatogr. A*, **881**, 483–499.
- Herraiz, T. and Galisteo, J. (2002). Tetrahydro- β -carboline alkaloids that occur in foods and biological systems act as radical scavengers and antioxidants in the ABTS assay. *Free Radic. Res.*, **36**, 923–928.
- Marfey, P. and Ottesen, M. (1984). Determination of D-amino acids. I. Hydrolysis of DNP-L-amino acid methyl esters with carboxypeptidase-Y. *Carlsberg Res. Commun.*, **49**, 585–590.
- Maskos, Z., Rush, J. D., and Koppenol, W. H. (1992). The hydroxylation of tryptophan. *Arch. Biochem. Biophys.*, **296**, 514–520.

- Matsuoka, H., Honzawa, S., Takahashi, A., Yoshikawa, H., Watanabe, E., Watanabe, T., Ozawa, Y., Yamada, Y., Iizuka, T. and Uda, Y. (2008). Photoisomerization of 2-[3-(2-thioxopyrrolidin-3-ylidene)methyl]-tryptophan, a yellow pigment in salted radish roots. *Biosci., Biotechnol., Biochem.*, **72**, 2262-2268.
- Matsuoka, H., Takahashi, A., Ozawa, Y., Yamada, Y., Uda, Y. and Kawakishi, S. (2002). 2-[3-(2-Thioxopyrrolidin-3-ylidene)methyl]-tryptophan, a novel yellow pigment in salted radish roots. *Biosci. Biotech. Biochem.*, **66**, 1450-1454.
- Ozawa, Y., Kawakishi, S., Uda, Y. and Maeda, Y. (1990a). Isolation and identification of a novel β -carboline derivative in salted radish roots, *Raphanus Sativus L.* *Agric. Biol. Chem.*, **54**, 1241-1245.
- Ozawa, Y., Uda, Y. and Kawakishi, S. (1990b). Generation of a β -carboline derivative, the yellowish precursor of processed radish roots, from 4-methylthio-3-butenyl isothiocyanate and L-tryptophan. *Agric. Biol. Chem.*, **54**, 1849-1851.
- Ozawa, Y., Uda, Y., Matsuoka, H., Abe, M., Kawakishi, S. and Osa-
wa, T. (1999). Occurrence of Stereoisomers of 1-(2'-pyrrolidine-
thione-3'-yl)1,2,3,4-tetrahydro- β -carboxylic acid in fermented
radish roots and their different mutagenic properties. *Biosci.,
Biotechnol., Biochem.*, **63**, 216-219.
- Ozawa, Y., Uda, Y., Ohshima, T., Saito, K. and Maeda, Y. (1990c).
Formation of yellow pigment by the reaction of 4-methylthio-
3-butenyl isothiocyanate with L-ascorbic acid and some dihy-
droxyphenolic compounds. *Agric. Biol. Chem.*, **54**, 605-611.
- Pari, K., Sundari, C. S., Chandani, S. and Balasubramanian, D.
(2000). β -Carbolines that accumulate in human tissues may serve
a protective role against oxidative stress. *J. Biol. Chem.*, **275**,
2455-2462.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and
Rice-Evans, C. (1999). Antioxidant activity applying an im-
proved ABTS radical cation decolorization assay. *Free Radic.
Biol. Med.*, **26**, 1231-1237.
- Reiter, R. J., Tan, D. X., Cabrera, J. and D'Arpa, D. (1999). Mela-
tonin and tryptophan derivatives as free radical scavengers and
antioxidants. *Adv. Exp. Med. Biol.*, **467**, 379-387.
- Uda, Y., Ozawa, Y., Ohshima, T. and Kawakishi, S. (1990). Identi-
fication of enolated 2-thioxo-3-pyrrolidinecarbaldehyde, a new
degradation product of 4-methylthio-3-butenyl isothiocyanate.
Agric. Biol. Chem., **54**, 613-617.