Photochemical Coloring Reaction of Sake

Presence of Kynurenic Acid as Photosensitizer in Sake

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Coloring reaction of sake caused by exposure to sun-light was investigated. There is one of the coloring reactions in which deferri-ferrichrysin participate. The reaction was named Reaction I.

Deferri-ferrichrysin, tyrosine, Mn^{2+} and unknown nitrogenous compound named conveniently compound X were essential for Reaction I and citric acid was stimulative.

Compound X was purified by using Amberlite IR 120 (H type) column, active carbon column, silicic acid column and alumina column, and crystallized from methanol-water (1 : 1).

The crystals decomposed at $288\sim 290^{\circ}C$. The ultraviolet and infrared spectra of the compound X were essentially identical with those of authentic kynurenic acid.

From these results the compound X was identified as kynurenic acid.

Since kynurenic acid alone did not cause the color development and riboflavin could be substituted for kynurenic acid in Reaction I, kynurenic acid may act as photosensitizer in Reaction I.

In the series of studies on changes in color of sake caused by exposure to sun-light,^{1,2)} it was found that one of the coloring reactions proceeded as the following scheme:

DFCY: Deferri-ferrichrysin formed by *Aspergillus oryzae* during rice koji making.³⁾

Compound X: Unknown nitrogenous compound contained in sake.

Organic acids: Hydroxy or keto acids, which stimulate the reaction.

The reaction was named Reaction I.

The present investigation was undertaken to purify compound X and clarify its properties.

MATERIALS AND METHODS

Measurement of the activity of compound X in reaction I. Basal reaction mixture was as follows; $MnCl_2$ (100 μM), citrate buffer (5 mM, pH 4.6) and tyrosine (500 ppm). Solution A was prepared by adding the test solution to the basal reaction mixture, to measure the activity of compound X.

Another solution (solution B) was prepared by the addition of DFCY (50 ppm) to the solution A. Ten ml of the solution A or B were exposed to sun-light in a 50 ml Erlenmeyer flask stoppered with aluminium foil.

After exposure to sun-light for 3 hr, the increases of optical density of the solution A at 420 $m\mu$ in 10 mm cell ($\Delta O.D._{420}^{420}(A)$) and the solution B ($\Delta O.D._{420}^{420}(B)$) were measured. The activity of compound X in Reaction I was expressed as $\Delta O.D._{420}^{420}(DF)$ which was defined according to the following equation:

$$\Delta O.D._{420}^{420}(DF) = \Delta O.D._{420}^{420}(B) - \Delta O.D._{420}^{420}(A)$$

1) S. Sato, K. Nakamura, M. Tadenuma, T. Hasuo, T. Yao, K. Motegi and H. Shigehuji, *Nippon Jozo Kyokai Zasshi*, **64**, 541 (1969).

2) S. Sato, K. Nakamura and M. Tadenuma, *ibid.*, **65**, 255 (1970).

3) M. Tadenuma and S. Sato, *Agr. Biol. Chem.*, **31**, 1482 (1967).

DFCY was prepared from sake or rice koji extract by the method described previously.³¹

Ion-exchange resin. Amberlite IR 120 (H type) was employed for adsorption of compound X from sake. The resin was packed in a column (inner diameter, 60 mm).

Active carbon. Granular active carbon CPG (Pittsburgh Activated Carbon Co., Ltd.) was used for separation of tyrosine and compound X.

Silicic acid column chromatography. Silicic acid (20 g) column was employed for the purification of compound X. Solvent system was water saturated chloroform-ethanol.³¹ Silicic acid (100 mesh, analytical grade) was obtained from Mallinkrodt Chemical Works, New York.

Alumina column chromatography. Aluminium oxide standardized (for chromatography, E. Merck) was treated with 2N H₂SO₄ for 24 hr, and washed with water to neutral pH, and dried at 105°C for 24 hr. The acid treated alumina (20 g), after suspended in methanol, was packed in a column (inner diameter, 22 mm). Solvent system was methanol-water.

Thin-layer chromatography. Thin-layer chromatography using Avicel SF (Microcrystalline Cellulose) was employed for the detection of impurities. Avicel SF was obtained from Funakoshi Pharmaceutical Co., Ltd.

Solvent systems were as follows:

- Isopropanol: ammonia water (28%): water (8:1:1).
- n*-Butanol: acetic acid: water (4:1:2).
- Benzene: acetic acid: water (2:2:1).

Ultraviolet spectra. The Ultraviolet spectra were obtained with a type EPS-2U recording spectrophotometer, Hitachi Ltd.

Infrared spectrum. The infrared absorption spectrum was obtained in Nujol Mull by a Hitachi model EPI-G2 grating infrared spectrophotometer.

RESULTS

1) Purification of compound X

Sake (20 liters) was passed through the Amberlite IR 120 (H) column and the column was washed with water. Compound X was

eluted with 2N NaOH (1.8 liters). The effluent was adjusted to pH 4.6 with conc. HCl.

The effluent from the Amberlite IR 120 column was passed through the active carbon column (200 g). The column was washed with water (10 liters), 15% ethanol (10 liters) and 60% ethanol (10 liters) to remove tyrosine. Compound X was eluted subsequently with 10 liters of pyridine: methanol: water (1:1:4) and the eluate was concentrated *in vacuo*. The residue was suspended in a small volume of water. The suspension was adjusted to pH 4.6 and rechromatographed on the active carbon column (50 g) and eluted with the same solvent system as above mentioned. In the pyridine fraction only a trace amount of

TABLE I. EFFECT OF EACH FRACTION ISOLATED BY ACTIVE CARBON COLUMN CHROMATOGRAPHY ON REACTION I

Fraction added to reaction mixture ^{a)}	$\Delta O.D._{10}^{420} (DF)$
Water and ethanol fraction	0.007
Pyridine fraction	0.013
Water and ethanol fraction + Pyridine fraction	0.130
Tyrosine ^{b)} + Pyridine fraction	0.262

^{a)} Reaction mixture: Mn²⁺ (100 μ M), Citrate buffer (5 mM, pH 4.6) and DFCY (50.0 ppm).

^{b)} 624 ppm.

FIG. 1. Silica Gel Column Chromatography of Pyridine Fraction.

The basal reaction mixture is the same as described in "Material and Methods."

FIG. 2. Alumina Column Chromatography of Compound X.

The basal reaction mixture is the same as described in "Material and Methods."

tyrosine was detected by thin-layer chromatography.

The effect of pyridine fraction on Reaction I is shown in Table I. It is clear that the pyridine fraction is essential for Reaction I.

The pyridine fraction was submitted to the silica gel column (inner diameter, 22 mm) chromatography. Each solvent used was the 100 ml. As shown in Fig. 1 compound X was eluted in fractions of CHCl_3 of 75% to 50%. These fractions were combined and concentrated *in vacuo*. The residue was dissolved in 2 ml of methanol.

The methanolic solution of compound X was adsorbed on the top of the acid treated alumina column (inner diameter, 22 mm) and eluted with methanol (100 ml), 90% methanol (100 ml), 80% methanol (200 ml), 60% methanol (100 ml), 30% methanol (100 ml) and water (100 ml). Compound X was eluted with 90~60% methanol as shown in Fig. 2. These fractions were pooled and concentrated to dryness *in vacuo*.

2) Crystallization of compound X

The purified compound X was dissolved in

20 ml of 0.1 N NH_4OH . The solution was adjusted to pH 2 with 12 N H_2SO_4 . After allowing to stand overnight in a refrigerator, crude yellowish crystals were obtained. The crude crystals were recrystallized from methanol-water (1 : 1) as prism like crystals (22.3 mg); mp 288~290°C (decomp.).

Elementary analyses were made with Yanagimoto C. H. N. coder. *Anal.* Found: C, 58.51; H, 3.94; N, 6.82. Calcd. for $\text{C}_{10}\text{H}_7\text{NO}_3\text{H}_2\text{O}$: C, 57.97; H, 4.38; N, 6.76%.

3) Properties of compound X

This compound is somewhat sensitive to Photo-decomposition, so it is not always homogeneous in thin-layer chromatography, unless chromatography is carried out in a

TABLE II. *R_f* VALUES ON THIN-LAYER CHROMATOGRAM OF COMPOUND X AND KYNURENIC ACID

Solvent system	Compound X	Kynurenic acid
<i>n</i> -BuOH : AcOH : H ₂ O (4 : 1 : 2)	0.49	0.49
Isopropanol : NH ₄ OH : H ₂ O (8 : 1 : 1)	0.33	0.33
Benzene : AcOH : H ₂ O (2 : 2 : 1)	0	0

Adsorbent: Avicel SF.

Detection: UV ray.

FIG. 3. UV Spectra of Compound X.

Solid line: in N/10 HCl, broken line: in N/10 NaOH. Concentration of compound X is 5.0 ppm.

FIG. 4. IR Spectra of Kynurenic Acid (A) and Compound X (B) in Nujol Mull.

dark chamber. The spot on the thin-layer showed the violet fluorescence under UV lamp. During the exposure to UV lamp, violet fluorescence changed into yellowish green fluorescence. The spot of compound X was made visible with bromophenol blue, but did not react with Folin's reagent, ferricyanide, ninhydrin, 2,4-dinitrophenylhydrazine and Fe^{2+} . The R_f values of compound X and kynurenic acid are shown in Table II with three kinds of solvents.

Absorption spectra of compound X in N/10 HCl and N/10 NaOH are shown in Fig. 3. These spectra suggest that compound X has a quinoline or an acridine ring structure.⁴⁾ Absorption spectra of compound X and authentic kynurenic acid were completely identical.

Infrared spectrum of compound X in Nujol Mull (Fig. 4) shows the presence of $-\text{CO}-$ group and from the results of the color reactions (2,4-dinitrophenylhydrazine reaction negative and bromophenol blue reaction positive) compound X seems to have a carboxyl group and the spectrum is essentially identical with that of kynurenic acid.

By heating compound X with acetic anhydride at 140°C , a reddish violet color appeared. This color reaction has been reported to be a characteristic of kynurenic acid.⁵⁾

4) "Heterocyclic Chemistry, An Introduction" A. Albert, The Athlone Press, Translated by K. Nakanishi and J. Yamasaki, Maruzen, 1963, p. 249.

4) Photochemical properties of compound X

In order to ascertain the photochemical properties of compound X, irradiation test in

TABLE III. IRRADIATION TEST OF MODEL SYSTEMS

	O.D. ₁₀ ⁴²⁰
Complete	0.173
DFCY omitted	0.007
Compound X omitted	0.001
Mn ²⁺ omitted	0.022
Tyrosine omitted	0
Citrate omitted	0.120

Complete reaction mixture: DFCY (50 ppm), compound X (0.5 ppm), Mn^{2+} (100 μM), tyrosine (500 ppm) and citrate buffer (5 mM, pH 4.6).

FIG. 5. Comparison of Compound X with Kynurenic Acid in the Coloring Reaction.

Solid line: kynurenic acid, broken line: compound X.

5) "Beilsteins Handbuch der Organischen Chemie," Vol. XXII, ed. by Deutschen Chemischen Gesellschaft, Springer Verlag, Berlin, 1935, p. 231.

the model systems shown in Table III was carried out. Table III suggests that DFCY, compound X, Mn^{2+} and tyrosine are essential for Reaction I and that citrate was stimulative.

The relation between the concentration of compound X or kynurenic acid and $\Delta O.D._{30}^{420}$ (DF) is shown in Fig. 5. Compound X and kynurenic acid had essentially an identical effect on Reaction I.

From these results cited above, compound X was conclusively identified as kynurenic acid.

DISCUSSION

From the results above mentioned and previous reports,^{1,2)} it is concluded that DFCY, kynurenic acid, Mn^{2+} , tyrosine, organic acids and oxygen participate in Reaction I.

Kynurenic acid alone did not cause the color development and so the function of kynurenic acid was investigated. Various heterocyclic compounds (5 ppm, as for kynurenic acid; 0.4 ppm) were added to the basal reaction mixture and the effects on Reaction I were compared between them. Table IV shows that quinoline, acridine and flavin compounds with the exception of 8-hydroxyquinoline and xanthurenic acid having a hydroxy group at the position of 8 of quinoline ring were able to be substituted for kynurenic acid.

The relation between the wavelength of light source and the degree of coloring reaction in the model system was studied by using a Jasco Concavegrating Mount Irradiator model CRM-50. The pattern of the wavelength for the activation of Reaction I (Fig. 6) is in good agreement with the ultraviolet absorption spectra of kynurenic acid as seen in Fig. 3.

Furthermore, the pattern of the wavelength for the activation of Reaction I by riboflavin exhibits characteristic peaks at 200~250 $m\mu$ and 450 $m\mu$ and a shoulder at 375 $m\mu$ (Fig. 7). These wavelengths of peaks and a shoulder are essentially identical with those of absorp-

tion maxima of riboflavin (260, 375 and 450 $m\mu$).

Kynurenic acid may act as photosensitizer in Reaction I, because riboflavin is known as one of photosensitizers and kynurenic acid shows the same effect as riboflavin in Reaction I.

TABLE IV. EFFECT OF VARIOUS HETEROCYCLIC COMPOUNDS ON REACTION I

Compound	$\Delta O.D._{30}^{420}$ (DF) ^{a)}
Indole	0.002
Skatole	0.005
5-Hydroxyindole	0.003
Tryptophol	0.003
Indole-3-aldehyde	0.019
Indoleacetic acid	0.003
5-Hydroxyindoleacetic acid	0.009
Indolepropionic acid	0.004
Indolebutyric acid	0.003
Indolelactic acid	0.001
Indoleacrylic acid	0.002
Indoxylacetate	0.003
Tryptophan	0.002
N-Acetyltryptophan	0.001
5-Hydroxytryptophan	0.006
Tryptamine	0.003
Serotonin	0.003
Kynurenine	0.006
Pyridine	0.001
Picolinic acid	0.002
Quinolinic acid	0.001
Pyridine-2,6-dicarboxylic acid	-0.002
Quinoline	0.162
Isoquinoline	0.148
8-Hydroxyquinoline	0.001
Quinaldic acid	0.198
Kynurenic acid	0.178
Xanthurenic acid	0.007
Acridine	0.273
Rivanol	0.006
Riboflavin	1.110
Folic acid	0.052
Nicotinamide	0.002
Pyridoxine	0.011
Thiamine	0

^{a)} Optical density was measured by 30 mm cell at 420 $m\mu$. Concentration of heterocyclic compounds was 5.0 ppm (kynurenic acid; 0.4 ppm).

FIG. 6. Relation between Wavelength of Light Source and the Degree of the Coloring Reaction.

The basal reaction mixture is the same as described in "Material and Methods."

Concentration of kynurenic acid is 0.5 ppm.

Since the rate of Reaction I to the whole photochemical coloring reactions in sake is 22.5~15%,^{1,2)} other reactions may take part in the coloring reaction in sake.

In the course of sake-making, kynurenic acid could be detected only in moromi mash and could not be detected in steamed rice or rice koji. These facts indicate that kynurenic acid in sake may be formed by yeast in the alcoholic fermentation.

Kynurenic acid is one of metabolites of

FIG. 7. Relation between Wavelength of Light Source and the Degree of the Coloring Reaction. (Riboflavin was substituted for kynurenic acid).

The basal reaction mixture is the same as described in "Material and Methods."

Concentration of riboflavin is 0.25 ppm.

tryptophan^{6~8)} and it seems that kynurenic acid in sake will be produced from tryptophan by way of kynurenine by yeast; this is under investigation.

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6) Y. Kotake, *Z. Physiol. Chem.*, **195**, 139 (1931).

7) M. Mason and C. P. Berg, *J. Biol. Chem.*, **188**, 783 (1951).

8) I. L. Miller, M. Tsuchida and E. A. Adelberg, *ibid.*, **203**, 205 (1953).