Isolation and Identification of Phenolic Acids in Rice Vinegar

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The fractions obtained from the ether soluble part of rice vinegar were separated by means of cellulose column and of Amberlite XE-64 ion exchange resin. The following compounds were identified: *p*-hydroxyphenylethanol, *p*-hydroxyphenylacetic acid, *p*-hydroxyphenyllactic acid, *p*-hydroxybenzoic acid and phenyllactic acid.

There has been no report published on the existence of phenolic acids in rice vinegar. However, the tyrosol found in sake^{1,9)} and other compounds can be considered to arise from the carbohydrates and proteins of the raw materials by the action of bacteria or yeasts during the fermentation^{2-7,9-11)}.

The experiment presented here was carried out with a sample of rice vinegar whose raw materials and manufacturing processes were known and from which all kinds of antiseptics were excluded at all manufacturing stages. After some fractions of phenolic acids were separated by column chromatography, five compounds were identified.

EXPERIMENTAL

Preparation of Samples.

Rice vinegar used was a commercial product named, "Fundokin-Komesu", manufactured by Kotegawa Soy Sauce Manufacturing Co., Oita, Japan, under the

supervision of one of the authors (Kodama) in Oct. 1962.

Twenty liters of the rice vinegar was concentrated under reduced pressure, ca. 30~40 mmHg, until about 500 ml of syrup was obtained. The concentrate was extracted with ether. The ether solution was shaken with 1N sodium bicarbonate solution until carbon dioxide evolution ceased. The sodium bicarbonate solution of about 300 ml was washed three times with 100 ml ether, and then acidified with 5N sulfuric acid. The acidified solution was reextracted with ether continuously. After the ether solution was dried with anhydrous sodium sulfate for a few days as usual, the ether was distilled, and moreover the residual acetic acid in the solution was evaporated as much as possible on a water bath. The residue was dissolved in dilute aqueous ammonia. After removed the excess ammonia, the resulting solution was used as a sample. The yield was about 2 g.

Methods.

(a) Sample Fractionation on Cellulose Columns.

Column: cellulose powder (90% of 100~200 mesh powder and 10% of 300 mesh powder) was packed into 2×50 cm column.

Developing solvent: *n*-butanol saturated with 1.5N aqueous ammonia (butanol was shaken with 1.5N ammonia for 20 minutes at room temperature and allowed to stand still overnight).

(b) Column Chromatography by Ion Exchange Resin.

Resin: Amberlite XE-64, 100~200 mesh, H⁺ form.

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Column: 0.65×95 cm.

Developing solvent: methylethylketone : acetone : 0.2N HCl=2:1:6. The resin was prepared in accordance to the procedure followed by Moore and Stein¹⁴⁾. The flow rate was 2½ drops/min. and every gram was collected by a fraction collector.

RESULTS

About 0.5 g of sample was applied on the top of cellulose column (a) and 2 ml fractions collected. From each fraction 0.5 ml was withdrawn and after 0.5 ml of water was added, the solvent was evaporated in a water bath to remove butanol. The dried eluates were dissolved in water and checked by Pauly's diazo reaction. The results are shown in Fig. 1.

(1) Purification and Identification of Fraction 1 (Fig. 1).

The effluent corresponding to fraction 1 was collected and evaporated to dryness at 80°C. Small amounts of crystals were obtained. The crystals were dissolved in ether and reprecipitated with petroleum ether, in order to obtain slightly yellowish plate-like crystals, m.p. 98°C. Physical properties of this compound, the R_F value of paper chro-

matography, purple color reaction with diazotized *p*-nitroaniline, blue coloration by ferric sulfate and potassium ferricyanide, ultraviolet spectrum, and the odor indicated that the compound was *p*-hydroxyphenylethanol (tyrosol). Finally it was identified by means of the mixed melting point determination with an authentic sample of tyrosol.

(2) Purification and Identification of Fraction 3 (Fig. 1).

The eluate of fraction 3 (Fig. 1) was collected and rechromatographed with ion exchange column (b). Each fraction was evaporated to dryness in water bath to remove solvent, and then dissolved in 4 ml of water. The optical density at 260 $m\mu$ was measured with the results shown in Fig. 2 (The maximum absorption may differ each fraction, but the measurement was carried out at 260 $m\mu$ for the sake of convenience).

(2-1) Identification of fraction 3-(4), (peak 4 in Fig. 2).

Evaporation of the solvent of this fraction on a water bath, yielded a slightly yellowish oily material. After standing for a few days at room temperature the residue crystallized.

FIG. 1. Fractionation of Sample by Cellulose Column.

2×50 cm column.

Every 2 ml was collected.

Solvent; *n*-BuOH sat. with 1.5N aq. NH_4OH .

Visualized by diazo reaction (● Red, ○ Yellow).

14) S. Moore, and W.H. Stein, *J. Biol. Chem.*, **211**, 893 (1954).

FIG. 2. Rechromatography of Fraction 3.

Amberlite XE-64, 100~200 mesh H⁺ form 0.65×95 cm.
Solvent; Me-CO-Et : Me-CO-Me : 0.2 N HCl=2 : 1 : 6.

The crystal obtained was pressed on a clay plate, and then recrystallized from water. Finally about 15 mg of colorless needle-shaped crystal (m.p.* 124.5~125°C) was obtained. Probably the crystal contained water of crystallization, since became opaque at 100~105°C. This material gave a yellow color with 1% phenol-5% ferric chloride solution (Uffelman's test), and showed acid reaction with bromophenol blue or bromocresol green, and did not react with an aqueous bromine solution. In the molten state, the crystal smelled like of cinnamic acid. The ultraviolet spectrum is shown in Fig. 3 and the infra-red spectrum also shown in Fig. 4 (curve 3).

Those facts showed that the compound was β -phenyllactic acid. The final confirmation was carried out by using the mixed melting point method with synthetic L- β -phenyllactic acid (124~125°C) or D- β -phenyllactic acid

FIG. 3. UV-Spectrum of Fraction 3-(4).

(124~125°C).

Both isomers were synthesized from D- and L-phenylalanine by means of nitrous acid. The IR-spectra are shown in Fig. 4 (curves 1, 2). Differences between the D-form and L-form in IR-spectra seems to consist in absorption bands at 1000 cm⁻¹, 1035 cm⁻¹, and at 880 cm⁻¹, 910 cm⁻¹. The mixed melting point of D- β -phenyllactic acid and the material under investigation was 98°C. On the other hand, that of L- β -phenyllactic acid and of the material was 124.5~125.5°C and no depression was observed when the mixture was melted. Therefore, fraction 4-(4) was identified as L- β -phenyllactic acid. The IR-spectrum of this fraction was also the same as that of L- β -phenyllactic acid. The odor was removed by repeated recrystallization, but cinnamic acid-like odor returned when allowed to stand for a long time at room temperature in a closed container or when heated in aqueous solution. Hence the acid presumably plays an important part in the aroma of rice

* All melting points were measured by a micro melting point measuring apparatus.

15) W. Grassmann, K. Hanning and M. Plöckl, *Zeits. Physiol. Chem.*, **299**, 258 (1955).

FIG. 4. IR Spectra of β -Phenyllactic Acid.

1) D-form, 2) L-form, 3) Isolated material.

FIG. 5. Paper Ionogram of Fraction 6.

pH. 3.9, 600 V, 2 hrs.
Visualized with diazo reaction.

FIG. 6. Rechromatogram of Fraction 6.

vinegar.

Fractions 3-(6) and 3-(7) also had a pleasant aroma, but further studies were difficult because of very small quantities available.

(3) Separation, Fractionation and Identification of Fraction 6 (Fig. 1).

By evaporating the solvent of fraction 6 on a water bath, a brown oily substance was obtained, which formed brown needle-shaped crystals when left at room temperature. The crystals were applied to the paper ionophoresis as follows: electrolyte; pH 3.9, acetic acid-pyridine buffer¹⁵, 600 volts, 2 hours. The result is shown in Fig. 5. The ionogram was visualized by means of Pauly's diazo reaction. As shown in the Fig. 5, the crystalline mixture was separated into three substances. Consequently, fraction 6 was reapplied to an ion exchange column (0.65×80 cm), and 1 ml fractions were collected and tested for the presence of phenolic acids. The elution curve is shown in Fig. 6.

(3-1) Identification of fraction 6-(1), (peak 1 in Fig. 6).

After the fraction was evaporated in a sulfuric acid desiccator under reduced pressure, slightly brown needle-shaped crystals (about 300 mg) were obtained. The crude crystals

were recrystallized from ethyl acetate, producing colorless long needles which became opaque at 100~105°C and melted at 171.5~172°C. The crystals showed the following color reactions: blue with ferric sulfate-potassium ferricyanide, blue with phosphomolybdic acid-ammonia, reddish purple with Pauly's diazo reaction, purple with diazotized *p*-nitroaniline-sodium carbonate, and yellow with

Uffelman's test. Considering the infra-red spectrum, cf. Fig. 7, this compound was very probably *p*-hydroxyphenyllactic acid.

L-p-Hydroxy- β -phenyllactic acid was prepared by treating *L*-tyrosine with sodium nitrite and hydrochloric acid. This acid melted at 171.5~172.5°C, and the color reactions and other properties were all identical with the material separated from vinegar. The mixed

FIG. 7. IR Spectra of *L-p*-Hydroxy- β -phenyllactic Acid.

4) *L-p*-Hydroxy- β -phenyllactic acid, 5) Isolated material.

FIG. 8. IR Spectra of *p*-Hydroxyphenylacetic Acid.

6) *p*-Hydroxyphenylacetic acid, 7) Isolated material

FIG. 9. Rechromatogram of Fraction 7.

acid.

(3-2) Identification of fraction 6-(2), (peak 2 in Fig. 6).

The eluates of the column were evaporated in a sulfuric acid desiccator under reduced pressure. The residual material was rechromatographed and about 7 mg of colorless plate-like crystals were obtained. Melting point was 149~150°C. The crystalline product showed the following color reactions, red in Pauly's diazo reaction, purple with diazotized *p*-nitroaniline-sodium carbonate, and negative in ferric chloride and Uffelman's test. The infra-red spectrum shown in Fig. 8 and other physical properties, inferred that the crystalline substance was *p*-hydroxyphenylacetic acid. The IR-spectrum for authentic sample was exactly the same and the result of the mixed melting test was 149~150°C. The fraction 6-(2) was then identified as *p*-hydroxy-

FIG. 10. IR Spectra of *p*-Hydroxybenzoic Acid.
8) *p*-Hydroxybenzoic acid, 9) Isolated material.

melting point was undepressed, 171.5~172°C. The infra-red spectrum of an authentic material was also the same as that of the sample from vinegar. The material of fraction 6-(1) was identified as *L*-*p*-hydroxy- β -phenyllactic

phenylacetic acid.

(4) Segregation and Identification of Fraction 7, (peak 7 in Fig. 1).

The fraction 7 of the eluates from the cellulose column afforded a slightly brown oily

material after evaporation of the solvent on a water bath. However, the material crystallized on standing. The crystals were purified further with column (b), and the elution curve obtained is shown in Fig. 9. Although the crystals obtained by recrystallization from water melted above 195°C, the material seemed to be still impure. The crystals were sublimed in a sealed tube under reduced pressure and about 10 mg of spindly crystals which melted at 215.5~216°C were obtained. This material showed the following color reactions: yellow in Pauly's diazo reaction, blue with ferric sulfate-potassium ferricyanide, and pink with diazotized *p*-nitroaniline. The IR spectrum is shown in Fig. 10 and the crystals were thought to be *p*-hydroxybenzoic acid. The spectrum of the fraction was the same as that of authentic *p*-hydroxybenzoic acid. Moreover, the mixed melting point was undepressed, 215.5~216°C. Consequently, the material of fraction 7 was identified as *p*-hydroxybenzoic acid.

DISCUSSION

p-Hydroxy- β -phenyllactic acid has been reported to be formed besides tyrosol by the degradative action of *Bact. lactis aerogenes* on tyrosine⁷⁾, and tyrosine was also proven to be degraded through *p*-hydroxyphenyllactic acid into *p*-hydroxyphenylacetic acid, and finally into *p*-cresol by the action of some kind colitis germs⁵⁾.

p-Hydroxyphenylacetic acid has been identified as a metabolite of *Hypochnus sasakii*

*shiraii*²⁾ and it has been reported that *p*-hydroxyphenylacetic acid was also formed from tyrosine by the action of *Trichophyton gypseum*⁶⁾. On the other hand, some bacteria which produce *p*-hydroxyphenylacetate by the oxidation of phenylacetic acid have been reported¹¹⁾.

β -Phenyllactic acid seems to originate from phenylalanine, but no papers on the subject have been found.

It has been reported that small quantities of *p*-hydroxybenzoic acid are contained in cheese³⁾, and that some plants, e.g. an Indian bean¹²⁾, and a broad bean¹³⁾, contain a considerable amount of *p*-hydroxybenzoic acid.

There are many reports on the existence of tyrosol in sake¹⁾, matured "moromi" (unrefined sake or soy) and cider^{9,10)} or autolyzed yeast⁸⁾.

The fractions obtained from the ether-soluble fraction of rice vinegar and soluble in aqueous sodium bicarbonate were separated by cellulose columns and Amberlite XE-64 ion exchange resin column and shown to consist of at least 18 compounds. Among them the following five compounds were identified: *p*-hydroxyphenylethylalcohol, *p*-hydroxyphenylacetic acid, *p*-hydroxy- β -phenyllactic acid, phenyllactic acid, and *p*-hydroxybenzoic acid.

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