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# Fractionation and Properties of Antibacterial Substance from Grapes and Wines*

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An antibacterial substance was obtained from various grape juices and wines by Dowex 50-X2 chromatography followed by treatment with charcoal. The substance had distinct antibacterial activity against many bacteria, but the degree of the antibacterial activity varied with the strains of bacteria. The activity was considerably affected by other wine components, particularly organic acids. However, heating of the substance at various pH resulted in no loss of the antibacterial activity. It was found that the antibacterial substance was not, in fact, the materials such as alcohols, phenol compounds, $SO_2$, organic acids, amino acids, peptides, and metal salts.

The antibacterial properties of grape juices and wines have commonly been known for long.[1,2] The properties seem to have been attributed to phenolic compounds.[3,4] Few studies, however, have been reported on the antibacterial activity of wine components other than phenolic compounds. This report describes the isolation and fractionation of antibacterial activity of a non-phenolic substance obtained from grape juices and wines, and its properties.

## Materials and Methods

**Grape juices and wines** The grape varieties used were Koshu, Delaware, Neomuscat, Muscat Bailey A, and Cabernet Sauvignon, grown in the Institute Vineyard in 1975-1980. Three white wines and two red wines were made from the above grapes in the usual way at the Institute Experimental Winery.

**Culture medium for growth of bacteria** Glucose nutrient broth (pH 6.6) was used as growth medium for bacteria. The bacteria used in this experiment were listed in Table 4.

**Assay of antibacterial activity** Glucose nutrient broth-agar medium seeded with the bacteria tested were poured into Petri-dishes, and small filter paper discs (Toyo, 8 mm in diameter, 0.7 mm in thickness) soaked in samples were placed on the surface of the agar. Incubation was carried out at 30°C for 18 hr. The diameters of clear inhibitory zones occurred around the discs were measured.

## Results and Discussion

Grape juice or wine (2 ℓ) was diluted twice with water. The sample was applied to a column of Dowex 50-X2 ($H^+$type, 2.2×52.6 cm) and the column was washed with water. The adsorbed fraction was eluted with 2 ℓ of 10 % pyridine-acetic acid buffer (pH 5.6). The eluate was dried *in vacuo* and the residue obtained was dissolved in water. The antibacterial activity in the sample solution was investigated by the paper disc method using *B. subtilis, E. coli, P. aeruginosa, and S. aureus*. All of the sample solutions from the grape juices and wines used showed clear inhibitory zones against all of the bacteria. However, it seems plausible to

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confirm that the inhibitory zones occurred by substances such as pyridine or acetic acid used as eluting solution, pyridinium acetate, or the eluate from Dowex 50 resin, which were not potentially present in grape or wine. To confirm the above statement, a model wine (pH 3.0), consisting of 14 % of ethanol and 2 % of tartaric acid, was applied to the column of Dowex 50-X2 instead of the juice or wine. The eluate from the column with 10 % pyridine-acetic acid buffer (pH 5.6) was obtained by the same procedure as stated above, and was evaporated to dryness. Antibacterial activity of the residue was examined, but no activity was obtained. This shows that the antibacterial activity was due to grape juice or wine components.

When 4 N $NH_4OH$, 0.1 N NaOH, 0.2 M $Ba(OH)_2$, and mixtures of these solutions and some organic solvents were used as eluting solution instead of 10 % pyridine-acetic acid buffer (pH 5.6), the residues of the eluates did not show any activity. The concentrates of the wines (pH 6.6) also showed no activity. From these findings, the antibacterial activity was eluted with the pH 5.6 buffer after grape juice or wine was applied to a column of Dowex 50-X2 and the column was successively washed with water, 4N $NH_4OH$, and water to remove amino acids and peptides.

Charcoal (50 $\mu$g to 350 $\mu$g) was added to 4 mℓ of the aqueous solution including the antibacterial activity, which was obtained from 80 mℓ of juice or wine, to remove phenolic compounds. The mixture was vigorously stirred and filtered through a Toyo No. 5c filter paper. The charcoal on the filter was washed with 4 mℓ of water and the two filtrates were combined. Absorbance at 400 nm, phenol content, and antibacterial activity of the filtrate were examined. The absorbance became about 0 by the addition of 300 mg of charcoal, and no phenol content was observed. However, no antibacterial activity was lost. The filtrate was lyophilized and this was used as crude antibacterial substance. The yield of the substance was 135 mg from 1 ℓ of Koshu wine.

Table 1 shows the antibacterial activity of the substance against the four bacteria. The substance had higher activity against *E. coli*, *B. subtilis*, and *P. aeruginosa* than it did against S. aureus. The activity of the substance obtained from the juices was higher than that from the wines.

Table 1. Antibacterial activities of the crude substances obtained from various grape juices and wines.

| Grape juices or wines | Diameters of inhibitory zones against *B. subtilis* | *E. coli* | *P. aeruginosa* | *S. aureus* |
|---|---|---|---|---|
| | | (mm) | | |
| Grape juices | | | | |
| Koshu | 23.3 | 24.7 | 21.3 | 20.1 |
| Neomuscat | 20.1 | 21.7 | 18.5 | 10.4 |
| Muscat Bailey A | 22.7 | 26.0 | 20.8 | 11.1 |
| Wines | | | | |
| Koshu | 14.7 | 18.7 | 15.8 | 16.4 |
| Neomuscat | 17.5 | 24.7 | 18.2 | 10.5 |
| Muscat Bailey A | 11.5 | 17.7 | 14.2 | 10.4 |
| Delaware | 10.5 | 18.0 | 14.2 | 10.4 |
| Cabernet Sauvignon | 15.2 | 21.0 | 17.3 | 10.3 |

The crude antibacterial substance (270 mg) from 2 ℓ of Koshu wine was dissolved in water. The sample solution was applied to a column of Dowex 50-X2 ($H^+$type, 2.2 × 52.6 cm) and the column was eluted with 2 ℓ of 10 % pyridine-acetic acid buffer (pH 5.6) at a flow rate of 60 mℓ/hr. Fractions of 50 mℓ were collected. The substance contained a large amount of $Na^+$ and $K^+$ and a small amount of $Zn^{++}$. The concentrations of these cations were determined

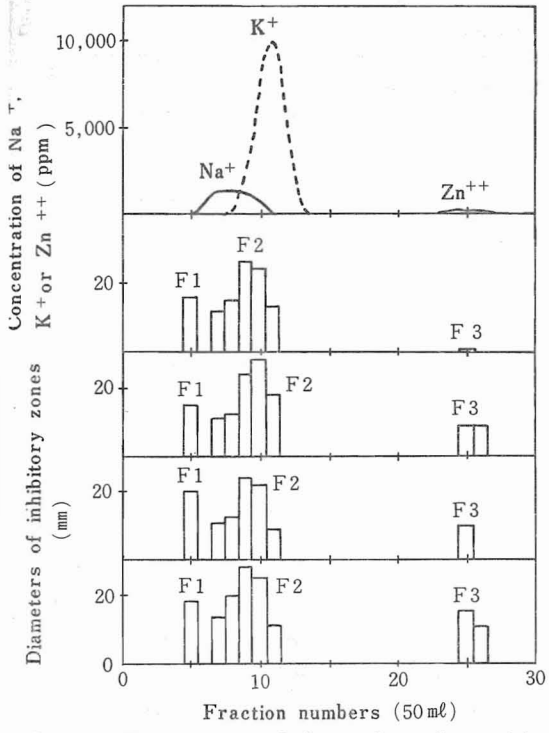


Fig. 1. Fractionation of the crude antibacterial substance on Dowex 50-X2 chromatography.

with a Hitachi, Model 170-30, atomic absorption spectrophotometer. Figure 1 shows the elution patterns of the cations and antibactrial activity. $Na^+$ was eluted in the fractions from No. 5 to No. 11, $K^+$ in the fractions from No. 8 to No. 17, and $Zn^{++}$ in the fractions from No. 23 to No. 28. The antibacterial activity in each fraction was examined. The activity was observed in the fraction of No. 5, the fractions from No. 7 to No. 11, and the fractions of No. 25 and No. 26. The degree of the activity against *P. aeruginosa* was smaller than those against the other three bacteria. The fraction No. 5 (F 1), the fractions from No. 7 to No. 11 (F 2), and the fractions of No. 25 and No. 26 (F 3) were collected, concentrated by rotary evaporation, and lyophilized. F 1, F 2, and F 3 were rechromatographed on Dowex 50-X2. The column sizes and fraction volumes collected were 1.8 × 7.9 cm and 5 mℓ for F 1, 2.2 × 44.7 cm and 40 mℓ for F 2, and 1.5 × 5.7 cm and 2.5 mℓ for F 3. Each sample was dissolved in water to give a final concentration of 0.1 M of each cation, and applied to each column equilibrated with 1.6 % pyridine-acetic acid buffer (pH 3.1). Stepwise elution was carried out with the pH 3.1 buffer and 10 % pyridine-acetic

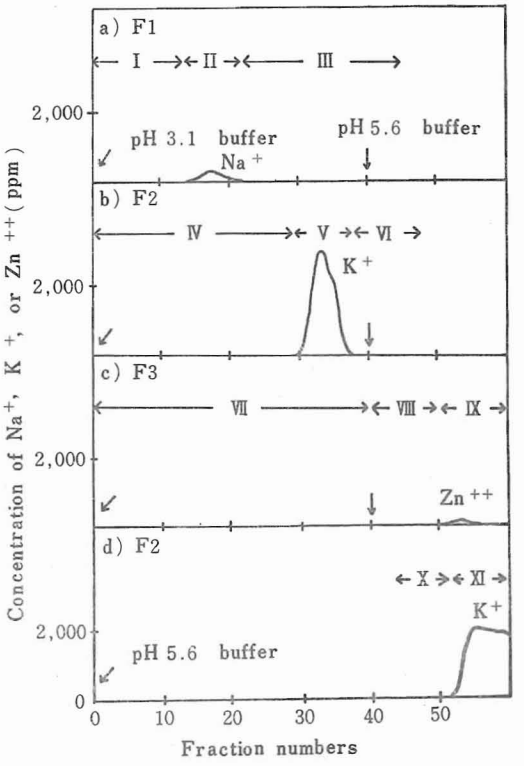


Fig. 2. Fractionation of the antibacterial fractions on Dowex 50-X2 (a-c) and Dowex 50-X8 (d) chromatographies.

acid buffer (pH 5.5). Fractions indicated by I to IX in Fig. 2 were collected separately and antibacterial activity of the fractions was investigated. The activity was observed in the fractions I, V, and IX (Fig. 2-(a)-(c)). The fraction F 2 was also rechromatograped on Dowex 50-X8. The column size and the flow rate were 2.2 × 26.3 cm and 60 mℓ/hr, respectively, with 10 % pyridine-acetic acid (pH 5.6) as eluting buffer. Fractions of 10 mℓ were collected. In Fig. 2-(d), the antibacterial activity (fraction X) and $K^+$ could be separated from each other on this chromatography.

Thus, the crude antibacterial substance contained at least three different activities, and two of these were metal ion-free. Table 2 shows the activities of the fractions obtained.

Table 2. Antibacterial activities of the fractions obtained by Dowex 50-X2 and Dowex 50-X8 chromatographies.

| Fractions * | Diameters of inhibitory zones against (mm) | | | |
|---|---|---|---|---|
| | *B. subtilis* | *E. coli* | *P. aeruginosa* | *S. aureus* |
| I | 16 | 11 | 12 | 13 |
| II | 0 | 0 | 0 | 0 |
| III | 0 | 0 | 0 | 0 |
| IV | 0 | 0 | 0 | 0 |
| V | 14 | 10 | 14 | 25 |
| VI | 0 | 0 | 0 | 0 |
| VII | 0 | 0 | 0 | 0 |
| VIII | 0 | 0 | 0 | 0 |
| IX | 20 | 13 | 10 | 11 |
| X | 14 | — | — | — |
| XI | 0 | — | — | — |

* See Fig. 2.

Figure 3 represents the relationship between the amounts of the crude antibacterial substance and the diameters of the inhibitory zones against the four bacteria graphically. The range of linearlity was between at least 50 μg and 400 μg of the antibacterial substance. The relative activities in Tables 3 and 4 were obtained using these standard curves.

As stated earlier, the Koshu wine concentrate did not show antibacterial activity. We assumed that the antibactrial substance was bound to some components in the wine, and the complexes formed did not have any activity. To elucidate this assumption, the following experiments were carried out. Koshu wine (1 ℓ) was passed through a column of Dowex 50-X2 ($H^+$ type, 3×28 cm), The non-adsorbed fraction was passed through a column of Amberlite IR-4B ($OH^-$ type, 3 × 28 cm). The fraction not adsorbed on this column was designated as a neutral fraction (1.5 ℓ). The adsorbed fraction was eluted with 1 ℓ of 2N $NH_4OH$. The eluate was evaporated to dryness at 40°C, and to the residue, 500 mℓ of water was added. The solution was passed through a column of Amberlite IR-120 ($H^+$ type, 3 × 28 cm) to remove $NH_3$ and the column was washed with water. The non-adsorbed fraction (1.5 ℓ) was obtained and was designated as an acidic fraction. The crude antibacterial fraction (13.5 mg) was dissolved in 300 mℓ of

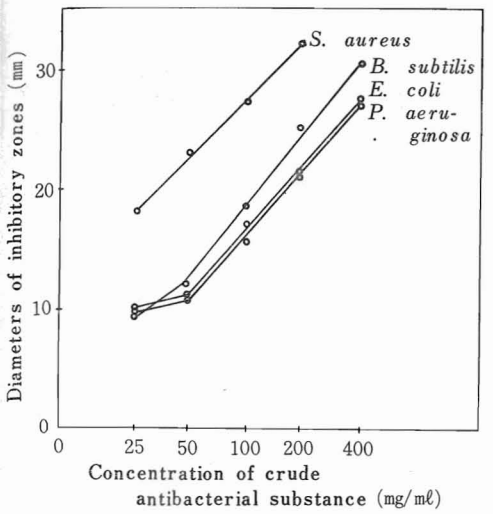


Fig. 3. Standard curves of the crude antibacterial substance for four bacteria.

the neutral fraction or the acidic fraction. Each fraction was adjusted to pH 3.0 and was allowed to stand for 1 hr at room temperature. After concentration to dryness, 1.25 mℓ of water was added to the residue. The solution was adjusted to pH 6.6 and was subjected to assay of the antibacterial activity.

In Table 3, the activity of the crude antibacterial substance decreased considerably or completely by the addition of the neutral fraction or the acidic fraction. The neutral fraction contained glucose, and the acidic fraction contained organic acids. Therefore, the effects of organic acids and glucose on the activity were examined. The activity decreased considerably by the addition of some organic acids, but increased slightly by glucose. The addition of metal salts gave no effect.

Table 3. Effects of wine components on antibacterial activity of the crude substance.

| Wine components added | Relative activity [2] | | | |
|---|---|---|---|---|
| | *B. subtilis* | *E. coli* | *P. aeruginosa* | *S. aureus* |
| None | 100 | 100 | 100 | 100 |
| Neutral fraction [1] | | | | |
| (a) | 100 | 36 | 68 | 108 |
| (b) | 86 | 36 | 55 | 92 |
| (c) | 86 | 36 | 61 | 100 |
| (d) | 73 | 42 | 55 | 92 |
| (e) | 86 | 42 | 68 | 85 |
| Acidic fraction [1] (a)–(e) | 0 | 0 | 0 | 0 |
| Acetic acid (0.16g) [3] | 60 | 82 | 77 | 59 |
| Lactic acid (0.02g) | 30 | 18 | 27 | 72 |
| Malic acid (1.46g) | 21 | 18 | 52 | 53 |
| Tartaric acid (0.67g) | 21 | 18 | 52 | 53 |
| Citric acid (0.02g) | 17 | 30 | 25 | 72 |
| Glucose (0.18g) | — | — | 113 | — |
| (0.35g) | — | — | 106 | — |
| (0.53g) | — | — | 114 | — |
| (1.75g) | — | — | 114 | — |
| NaCl (1.0g) | — | — | 105 | — |
| KCl (1.3g) | — | — | 100 | — |

1) The crude antibacterial substance (13.5mg) from Koshu wine was added to 300mℓ of the neutral fraction or the acidic fraction from Koshu wine, and the mixture was adjusted to pH 2.0 (a), pH 4.0 (b), pH 6.0 (c), pH 8.0 (d), or pH 10.0 (e). The mixture was allowed to stand for 1 hr at room temperature, then adjusted to pH 6.6.

2) Relative activity was obtained by use of the standard curves in Fig. 3.

3) The values in Parentheses are the amounts of the organic acids added. The composition of organic acids in Koshu wine was analyzed by the methods of Yamashita et al. [5, 6] Each organic acid was added to the aqueous solution of the crude antibacterial substance on the basis of the contents of organic acids which had been determined above.

Table 4. Minimum inhibitory concentration of crude antibacterial substance against various bacteria.

| Bacteria tested | Minimum inhibitory concentration (mg/mℓ) | Bacteria tested | Minimum inhibitory concentration (mg/mℓ) |
|---|---|---|---|
| *Bacillus brevis IAM* 1031 | 1.8 [1] | *Escherichia coli IAM* 1239 | 1.7(0.8) |
| *Bacillus cereus IAM* 1029 | 3.2 | *Eschericia coli (M) Cost & Chalm NIHJ JC*-1 | 1.7(0.8) |
| *Bacillus cereus* var. *mycoides IAM* 1190 | 1.7 | *Proteus mirabilis OM*-1 | 1.7(0.8) |
| *Bacillus circulans IAM*1140 | 3.2(1.7) [2] | *Pseudomonas aeruginosa IAM*1095 | 0.8 |
| *Bacillus firmus IAM* 1188 | 3.2(1.7) | *Pseudomonas decunhae IAM* 1048 | 0.8 |
| *Bacillus licheniformis IAM* 11054 | 3.2 | *Pseudomonas diminuta IAM* 1513 | 0.8(0.3) |
| *Bacillus macerans IAM* 1243 | 0.8(0.2) | *Pseudomonas ovalis IAM* 1002 | 0.8 |
| *Bacillus megaterium IAM* 1166 | 1.7 | *Pseudomonas putrefaciens AJ*2065 | 0.3 |
| *Bacillus polymyxa IAM* 1210 | 1.7 | *Pseudomonas putrefaciens AJ*2261 | 0.8 |
| *Bacillus sphaericus IAM* 1244 | 1.7(0.8) | *Pseudomonas rubescens NRRL*1551 | 0.5(0.3) |
| *Bacillus subtilis IAM* 1064 | 3.2 | *Pseudomonas taetrolens IAM* 1653 | 0.8(0.5) |
| *Bacterium ammoniagenes IAM*1641 | 1.7(0.8) | *Staphylococcus aureus IAM* 1011 | 3.2(0.8) |

1) Bacteriocidal action
2) Bacteriostatic action

Table 4 shows the minimum inhibitory concentration of the crude antibacterial substance against various bacteria. The substance showed bacteriocidal action against all of the bacteria tested above the concentration of 3.2 mg/mℓ, and bacteriostatic action against *B. macerans* at the concentration of 0.2 mg/mℓ and against *P. diminuta* and *P. rubescens* at the concentration of 0.3 mg/mℓ. Here, we defined the bacteriocidal action as being in a state of no bacterial growth for one week after inoculation. The bacteriostatic action was defined as being in a state of no growth for one day after inoculation and good growth after that.

The effects of pH and temperature on the activity and stability of the antibacterial substance were examined. The substance was dissolved in water at the concentration of 1.35 g/ℓ and adjusted to pH 2.0–10.0 with dilute HCl or NaOH. The samples were allowed to stand in boiling water for 1 hr, cooled in running water, adjusted to pH 6.6, and then subjected to assay of the antibacterial activity. The activity never changed before and after the above treatment. However, most of the activity was lost after the aqueous solution of the substance was stored at 4°C for several months.

From these results, the antibacterial substance was not the materials such as alcohols, phenol compounds, $SO_2$, organic acids, and metal salts, which have been known as antibacterial substance. The substance present in grapes and wines was the inactive form which was bound to some grape or wine components.

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