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Isolation and Structural Analysis of Peptides in Koshu White Wine

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Abstract

Peptides in Koshu white wine were desalted by Dowex 50-X2 column chromatography. These peptides were fractionated by ion-exchange chromatography on Amberlite IRC-50 and Amberlite IR-45 and grouped into two fractions. Each fraction was further fractionated and purified by ion-exchange chromatography on Dowex 50-X2, Dowex 1-X2, AG 50-X2 and AG 1-X2, and paper chromatography.

Sixteen ninhydrin-positive peptides were obtained and their amino acid compositions and sequences were determined by the Edman and DNS methods. The sequences of the peptides were Ala-Gly, Tyr-Ala, Arg-His, Gln-Arg, Arg-Glu, Pro-Gly, Lys-Pro, Ser-Asn, Asp-Pro, Pro-Phe, Pro-Ala, Ser-Gly-Arg, Ser-Asn-Asn, Asn-Gly-Gln, Ala-Ala-Ala-Ala and Ile-Arg-Arg-Arg.

Introduction

The main objective of this series of studies has been to determine the relation between the structures of nonvolatile compounds in wines and the color or flavor of the wines. Browning compounds, polyphenols, free amino acids and peptides play important roles in color and flavor production during wine making. In previous publications[1,2] we reported the isolation and characterization of the browning compounds in Koshu white wine and decolorization of the baked wine by cobalt-60 gamma-irradiation. In an accompanying paper,[3] we have described the free amino acids and amino acid compositions of the peptides in the musts and wines from three varieties of Japanese grapes. We, here, describe the isolation of 16 peptides from Koshu white wine and their amino acid sequences.

Materials and Methods

Wine The white wine used was produced from Koshu grapes, grown in Yamanashi prefecture in 1972, at the University Experimental Winery. Results of the general analyses of the wine have been reported in another paper.[3]

Isolation of the peptides from Koshu wine The wine was diluted 2-fold with distilled water, and an aliquot (10 *l*) was passed through a Dowex 50-X2 column (5×48 cm). The column was washed with 5 *l* of distilled water, followed by 3 *l* of 30% pyridine-4% acetic acid to elute the peptides. The effluent, including the peptides, was collected and concentrated to about 50 ml at 40°C under reduced pressure. The concentrate was diluted to a volume of 3 *l* with distilled water and adjusted to pH 3.2 with acetic acid. This solution was passed through an Amberlite IRC-50 column (H^+ type, 5×30 cm). The adsorbed fraction (fraction A) was eluted with 2 *l* of 30% pyridine-4% acetic acid. The non-adsorbed fraction was passed

Chemical Studies on Coloring and Flavoring Substances in Japanese Grapes and Wines (IV)

through an Amberlite IR-45 column (Cl^- type, 5×30 cm). The fraction not adsorbed on this column was designated fraction B. The adsorbed fraction was eluted with 1 *l* of 2 N HCl, but was not studied further because it contained a large amount of free amino acids and some minor peptides.

Fractionation and purification of fractions A and B Fraction A was fractionated on a Dowex 50-X2 column (1.2×120 cm) by linear gradient elution from 1 *l* of 0.2 M pyridine-acetic acid (pH 3.1) to 1 *l* of 2.0 M pyridine-acetic acid (pH 5.0) at room temperature, then by stepwise elution with 8.5 M pyridine-acetic acid (pH 5.6). Fraction B was fractionated on the same type of column with 250 ml of 0.2 M pyridine-acetic acid (pH 3.1), using linear gradient elution with 1 *l* of 0.2 M pyridine-acetic acid (pH 3.1) to 1 *l* of 2.0 M pyridine-acetic acid (pH 5.0) and 8.5 M pyridine-acetic acid (pH 5.6). The flow rate was 15 ml/hr. Fractions of 5 ml were collected and aliquots of 0.25 ml were used to determine the elution profile by the ninhydrin method.[4] Each peak was further fractionated and purified by Dowex 1-X2, AG 1-X2, AG 50-X2 or paper chromatography.

AG 50-X2 chromatography was performed with the aid of a JEOL Model JLC-6AH amino acid analyzer with a column 0.8×65 cm. To recover a large portion of the eluate, a dilution pump was used. The flow rates of the buffer pump, detection pump and dilution pump were 0.84 ml, 0.63 ml and 0.36 ml/min, respectively. On the basis of the flow rates of these three pumps, about 93% of the eluate was recovered in the fraction collector. Peptides were eluted at 50°C with 75 ml of 0.2 M pyridine-acetic acid (pH 3.1), a linear gradient elution from 100 ml of 0.2 M pyridine-acetic acid (pH 3.1) to 100 ml of 1.0 M pyridine-acetic acid (pH 5.0) and finally 50 ml of 2.0 M pyridine-acetic acid (pH 6.5).

AG 1-X2 chromatography was also performed under the above conditions, except that the buffer systems reported by Schroeder[5] were used and the column size was 0.8×30 cm. The column of AG 1-X2 was equilibrated with the pH 9.4 buffer of Schroeder. The mixing chamber contained 30 ml of the same pH 9.4 buffer, into which four solvents, the pH 8.4 buffer (50 ml), the pH 6.5 buffer (70 ml), 0.5 N acetic acid (80 ml) and 2 N acetic acid (80 ml) were successively allowed to flow from their reservoirs.

Ascending paper chromatography was carried out 18 to 20 hr on Toyo No. 50 (19×40 cm) with the solvent system; Butanol-1: acetic acid: water (3 : 1 : 1, v/v).

Amino acid analysis, Edman degradation and the determination of amide residues Edman degradation and amino acid analysis were carried out by methods described previously.[6]

In the determination of the amide residues, the DNS method[7] was used. A solution containing about 0.1 μmole of the peptide was transferred to a small test tube and dried *in vacuo*. This peptide was redissolved in 15 μl of 0.2 M sodium bicarbonate solution, and to this, was added an aqual volume of dansyl chlolide solution (2.5 mg/ml in acetone). The tube was covered with Parafilm and allowed to react at 37°C for 1 hr. The DNS-peptide was subjected to paper electrophoresis at pH 6.5 (0.05 M phosphate buffer, 30 V/cm) for 1.5 hr.

Results and Discussion

Koshu wine could pass through a Dowex 50-X2 column due to the removal of salts, sugars, phenols and acids. The adsorbed fraction, including peptides, was eluted with 30% pyridine-4% acetic acid. Preliminary fractionations of the peptides in the effluent were performed with Amberlite IRC-50 and Amberlite IR-45 chromatography and two peptide fractions, A and B, were obtained.

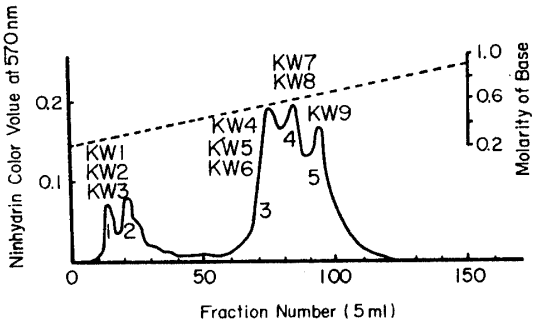

Fig. 1. Elution pattern obtained by chromatography of peptide fraction A from Koshu wine on Dowex 50-X2. Peptides were eluted from a Dowex 50-X2 column (1.2×120 cm) at room temperature with a linear gradient elution from 0.2 M pyridine-acetic acid (pH 3.1) to 2.0 M pyridine-acetic acid (pH 5.0), at a flow rate of 15 ml/hr. The molarity of the base in the buffers is indicated by the dashed line (----).

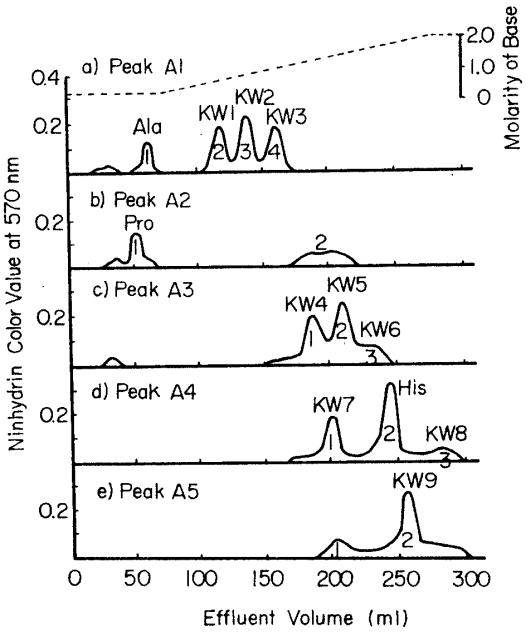

Fig. 2. Elution patterns obtained by AG 50-X2 chromatographies of peaks A1, A2, A3, A4 and A5 (See Fig. 1).

AG 50-X2 chromatography was performed with the aid of a JEOL, (Model JLC-6AH) amino acid analyzer with a column 0.8×65 cm. Peptides were eluted from the AG 50-X2 column at 50°C with 0.2 M pyridine-acetic acid (pH 3.1), a linear gradient elution from 0.2 M pyridine-acetic acid (pH 3.1) to 1.0 M pyridine-acetic acid (pH 5.0) and 2.0 M pyridine-acetic acid (pH 6.5), at a flow rate of 0.84 ml/min. The molarity of the base in the buffers is indicated by the dashed line (----).

Fraction A was fractionated by Dowex 50-X2 chromatography. Its elution profile, shown in Fig. 1, contained five peaks. Each peak was further fractionated and purified by AG 50-X2 chromatography (Fig. 2). Of the peaks obtained, peaks A1-1, A2-1 and A4-2 were free amino acids, alanine, proline and histidine, respectively, and peaks A2-2 and A5-1 contained some minor peptides. The other peaks contained nine peptides (KW1-KW9, Fig. 2).

Fraction B was fractionated by Dowex 50-X2 chromatography. The elution profile is shown in Fig. 3. Amino acid analyses of these peaks indicated that all were heterogeneous. Therefore, each was further fractionated and purified by AG 50-X2 chromatography (Fig. 4), AG 1-X2 chromatography (Fig. 5) and paper chromatography. Peak B1 contained free amino acids, aspartic acid, glutamic acid, leucine, α-aminobutyric acid and monoethanol amine. Peak B2 was separated into three peaks (B2-1-B2-3) by AG 50-X2 chromatography (Fig. 4). Peptides KW10 and KW11 were obtained from peak B2-1 by further fractionation and purification with AG 1-X2 chromatography (Fig. 5) and paper chromatography (Rf: KW10, 0.18; KW11, 0.25). Peptides KW12, KW13 and KW15 were obtained from peaks B2-2, B2-3 and B3-3, respectively, and peptide KW14 was purified from peak B3-1 by paper chromatography (Rf, 0.33).

Peak B4 was separated into five peaks, as shown in Fig. 4. The peaks, other than peak

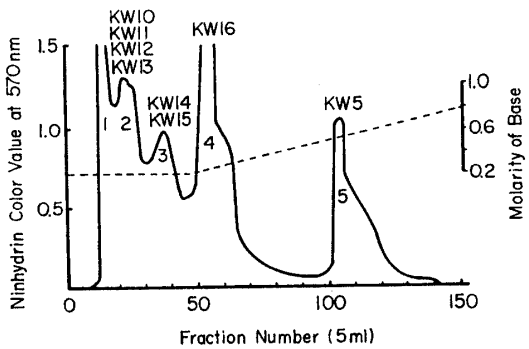

Fig. 3. Elution pattern obtained by the chromatography of peptide fraction B from Koshu wine on Dowex 50-X2.

Peptides were eluted from a Dowex 50-X2 column (1.2×120 cm) at room temperature with 0.2 M pyridine-acetic acid (pH 3.1), then with linear gradient elution from 0.2 M pyridine-acetic acid (pH 3.1) to 2.0 M pyridine-acetic acid (pH 5.0), at a flow rate of 15 ml/hr. The molarity of the base in the buffers is indicated by the dashed line (----).

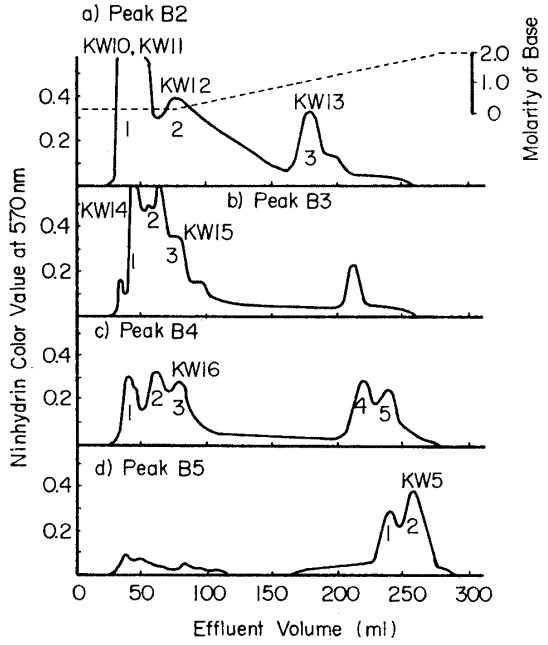

Fig. 4. Elution patterns obtained by AG 50-X2 chromatographies of peaks B2, B3, B4 and B5 (See Fig. 3). Conditions for chromatography were the same as those in Fig. 2.

B4-3, did not contain peptides. They contained free proline, glycine, alanine, tyrosine and phenylalanine. Peptide KW16 was obtained from peak B4-3 by AG 1-X2 chromatography (Fig. 5). Peak B5 was separated into two main peaks (B5-1, B5-2). Peptide KW5 was also obtained from peak B5-2, but peak B5-1 did not contain peptides.

The procedure for fractionating the 16 peptides is summarized in Fig. 6. Their amino acid compositions are given in Table 1.

The purified peptides were subjected to amino acid sequence analysis, using the Edman degradation and DNS methods. Results of one step of the Edman degradation, confirmed that the sequences of peptides KW1, KW3, KW4, KW5, KW6, KW7, KW8, KW10, KW11, KW13 and KW14 were Ala-Gly, Tyr-Ala, Arg-His, Glx-Arg, Arg-Glx, Pro-Gly, Lys-Pro, Ser-Asx, Asx-Pro, Pro-Phe and Pro-Ala, respectively. Only alanine was found in the acid hydrolysate of peptide KW2, but this peptide was eluted at the same position as isoleucine

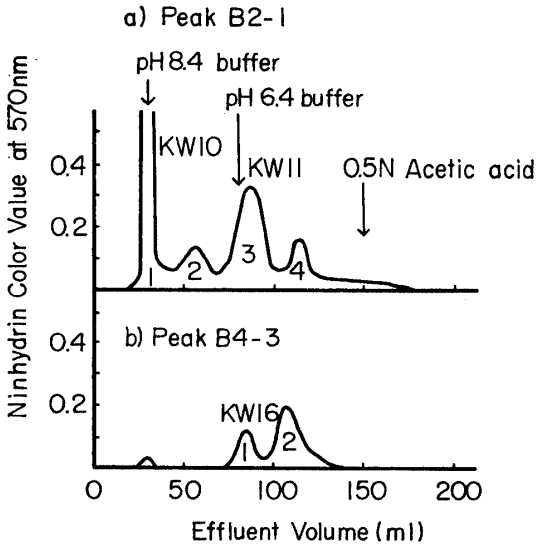

Fig. 5. Elution patterns obtained by AG 1-X2 chromatographies of peak B2-1 and B4-3 (See Fig. 4). A column, 0.8×30 cm, of AG 1-X2 was used. Peptides were eluted at 30°C by gradient elution, using the buffer systems described by Schroeder,[5] at a flow rate of 0.84 ml/min, with the aid of an amino acid analyzer.

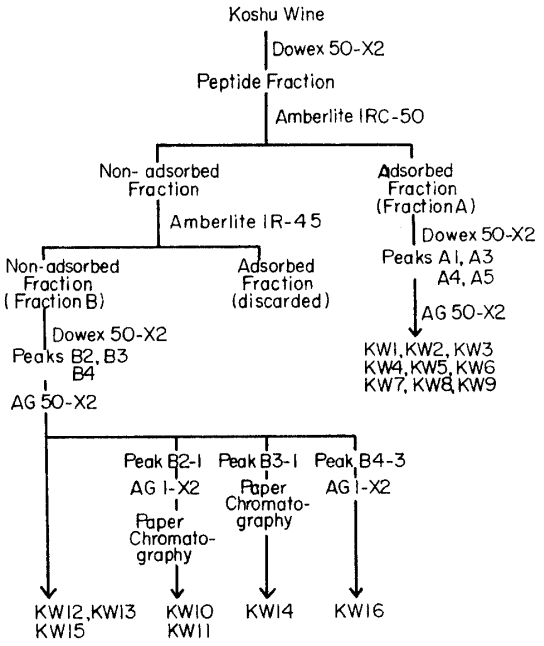

Fig. 6. Scheme for the purification procedure of each peptide from Koshu wine.

Table 1. Amino acid compositions and sequences of the peptides from Koshu wine.

Peptide number	Amino acid[1] composition	Yield (μmole/l)	Sequence
KW 1	Gly 1.00, Ala 1.03	2.56	Ala-Gly
KW 2	Ala	1.09	Ala-Ala-Ala-Ala
KW 3	Ala 1.00, Tyr 0.68	0.07	Tyr-Ala
KW 4	His 1.00, Arg 1.37	2.71	Arg-His
KW 5	Glu 1.00, Arg 0.97	1.16	Gln-Arg
KW 6	Glu 1.00, Arg 0.94	0.39	Arg-Glu
KW 7	Gly 1.00, Pro 0.87	0.56	Pro-Gly
KW 8	Pro 1.00, Lys 1.33	2.62	Lys-Pro
KW 9	Ile 1.00, Arg 3.02	0.15	Ile-Arg-Arg-Arg
KW10	Asp 1.00, Ser 1.20	0.13	Ser-Asn
KW11	Asp 1.00, Pro 1.19	0.20	Asp-Pro
KW12	Ser 0.95, Gly 1.00 Arg 1.07	0.41	Ser-Gly-Arg
KW13	Pro 1.00, Phe 1.27	1.03	Pro-Phe
KW14	Pro 1.00, Ala 0.94	0.15	Pro-Ala
KW15	Asp 2.18, Ser 1.00	0.11	Ser-Asn-Asn
KW16	Asp 0.81, Ser 1.00 Gly 1.00	0.08	Asn-Gly-Gln

1) The values are expressed as molar ratios and amino acids showing a molar ratio below 0.20 are removed.

during amino acid analysis. The ninhydrin color value of the peptide was 1/4 that of its acid hydrolysate and three steps of the Edman degradation showed only alanine as the N-terminal amino acid at each step. Accordingly, the sequence of peptide KW2 is Ala-Ala-Ala-Ala. One step of the Edman degradation of peptides KW9 and KW15 showed

isoleucine and serine as their N-terminal amino acids. Hence, the sequences are Ile-Arg-Arg-Arg and Ser-Asx-Asx, respectively. Two steps of the Edman degradation of peptides KW12 and KW16 established the N-terminal sequences of Ser-Gly and Asx-Gly, respectively. Accordingly, the sequences of these tripeptides are Ser-Gly-Arg and Asx-Gly-Glx.

Peptide KW12:	Ser-Gly-Arg
composition	0.88 0.93 1.00
step 1 (76%)*	**0.40** 0.98 1.00
step 2 (74%)	0.38 **0.67** 1.00
Peptide KW 16:	Asx-Gly-Glx
composition	0.81 1.00 1.00
step 1 (58%)	**0.16** 0.82 1.00
step 2 (76%)	0.11 **0.14** 1.00

Determination of the amide residues of the peptides was performed by the DNS method. The experimental value of the net charge (e) of each DNS-peptide at pH 6.5 was: -0.26 (in conclusion, $e=0$) for peptide KW5; -0.97 ($e=-1$) for KW6; -0.89 ($e=-1$) for KW10; -2.00 ($e=-2$)for KW11; -1.12 ($e=-1$)for KW15 and -1.09 ($e=-1$) for KW16. At this time, the experimental values of the e of arginine, glutamine and glutamic acid were -0.19, -0.92 and -1.83, respectively. Consequently, the Glx or Asx of the dipeptides KW5 and KW10 was, respectively, glutamine or asparagine. All the Glx or Asx of the tripeptides, KW15 and KW16, were the amide type. The complete sequences of the 16 peptides obtained are summarized in Table 1.

Based on these results the characteristics of the peptides in Koshu wine are: (1) The sizes of the peptides are very small. (2) The kinds of peptides are fewer than had been expected from the considerable changes in the amino acids which consitute these peptides during wine making.[3] (3) The main amino acids constituents of the peptides are arginine, aspartic acid (asparagine), glutamic acid (glutamine), glycine, alanine and proline as described in the note.[3] (4) Acidic peptides are few.

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* The yield of the peptide remaining at each step.