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A New Method for Fluorometric Measurement of Proline in Grapes and Wines

KOKI YOKOTSUKA

The Institute of Enology and Viticulture, Yamanashi University, Kofu 400, Japan

A method for the measurement of free proline in grapes and wines containing other amino acids is presented. The method included the removal of phenols and tryptophan by the pre-treatment of samples with activated carbon, the oxidation of proline to the corresponding primary amine with sodium hypochlorite, destruction of other amino acids by oxidation at pH 5.3 using a combination of hydrogen peroxide, ferric chloride, and sodium hypochlorite, and the formation of a fluorescent product from the oxidized proline with *o*-phthalaldehyde in the presence of 2-mercaptoethanol and Brij 35 at pH 5.3. The proline in grapes and wines measured by this method agreed well with the values obtained by the method using an automatic amino acid analyzer with ninhydrin.

Amino acids in grapes and wines are of importance to the growth of yeasts during fermentation, the fermentation rate, and the production of aromatic compounds, and hence, to the quality of the wines. Of the amino acids, proline is the most abundant in most grape juices,[1–3] and the most prominent amino acid in most wines.[1–4] The concentration of proline in grapes increases as the grapes ripen.[5] It also increases rapidly in fermenting grape musts as alcohol is produced.[3] However, there is no distinct relationship between the concentration of proline and the qualities of grapes and wines.

Several colorimetric methods using the ninhydrin reaction are available for the measurement of proline in the presence of other amino acids. Chinard[6] reported the measurement of proline by a photometric method including ninhydrin treatment at pH 1.0, where proline as well as cysteine, ornithine, lysine, and hydroxyproline react with ninhydrin to give red products. Several improved methods based on the same principle were reported by Schweet,[7] Troll and Lindsley,[8] and Lashkhi and Tsiskarishivili.[9] Ough[10] modified the procedure of Lashkhi and Tsiskarishivili and used the modified procedure to measure free proline in grapes and wines.

Previously, Yokotsuka and Kushida[11] reported a method for fluorometric measurement of amino acids including proline and hydroxyproline. The method included the oxidation of proline and hydroxyproline with sodium hypochlorite before the reaction with *o*-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol and a high concentration of Brij 35 for fluorophore formation. In this previous report we suggested that most of the amino acids except for proline and hydroxyproline were destroyed by the oxidation with sodium hypochlorite, and their fluorophore formation did not proceed easily at low pH. The fluorescent compounds of proline and hydroxyproline were selectively formed under limiting conditions by a combination of the oxidation with sodium hypochlorite and/or other oxidants and the reaction with *o*-phthalaldehyde at low pH.

This report describes the fluorometric measurement of proline in the presence of other amino acids in grapes and wines.

Materials and Methods

Reagents A 0.03% solution of sodium hypochlorite (NaClO) and a 0.05% of hydrogen peroxide (H_2O_2) were prepared from 10% NaClO and 31%

H_2O_2, respectively, by diluting with water. The NaClO and H_2O_2 were obtained from Wako and Mitsubishi Gas Kagaku, respectively. The NaClO and H_2O_2 were measured as described in "Official Methods for Analysis of the Association of Official Analytical Chemists."[12] Fresh solutions were prepared daily. The fluorogenic reagent was prepared by mixing 20 ml of 20% Brij 35 (Kao Atlas, 100 g in 500 ml of hot water), 2.5 ml of 16% *o*-phthalaldehyde (OPA, Nakarai, 16% in ethanol), and 0.3 ml of 2-mercaptoethanol (2-ME, Wako), and then filled up to 100 ml with water. Fresh reagent was prepared every week. Deionized and distilled water was used throughout the experiment.

Grape juice and wines Grapes were grown in the Institute experimental vineyard at Yamanashi University in Kofu and harvested at commercial maturity. Juices were obtained from the fresh grapes and stored at −20°C until just before their use. The juices were centrifuged at $25,000 \times g$ for 5 min. Standard procedures were used to make the wines. Activated carbon was added to juice or wine samples to give the final concentration of 1%, stirred and filtered through a Toyo no. 5c filter paper to remove the coloring materials and aromatic amino acids. The filtrate was diluted 5- to 50-fold with water.

Procedure A 0.1 ml of the diluted filtrate, 2 ml of 0.1 M acetate buffer (pH 5.3) containing 0.025 M $CaCl_2$, and 0.1 ml of 0.1 M $FeCl_3$ solution (freshly prepared) were added to a glass test tube (12×100 mm). To the tube, 0.5 ml of 0.05% H_2O_2 and 0.5 ml of 0.03% NaClO were added in that order. The mixture was incubated in a 60°C waterbath for 5 min. The mixture was cooled immediately in tap water for 1 min, followed by the addition of 0.5 ml of the fluorogenic reagent. The mixture was left at room temperature for 60 to 90 min and centrifuged at $1,000 \times g$ for 5 min to remove a yellow precipitate.

The fluorescence of the supernatant was measured within 30 min after centrifugation with a Shimadzu spectrofluorometer (Model RF-500 LC). The band widths were 10 nm. A quartz cell with 10-mm optical path was used. The wavelengths for excitation and emission were 360 nm and 450 nm, respectively. A standard curve was made using proline solutions with 0, 25, 50, 75, 100, 125, and 150 μg/ml in 0.01 M tartaric acid.

Results and Discussion

Conditions for fluorometric measurement of proline The most important point for the fluorometric measurement of proline in grapes and wines in the presence of other amino acids was how amino acids other than proline can be destroyed before the fluorogenic reaction with OPA. Twenty-one amino acids found commonly in grapes and wines produced in Japan[3,4] were used in this study. Sodium hypochlorite used as an oxidant for converting proline to the corresponding primary amine also oxidized most of the amino acids with a primary amino group. When only NaClO was used as an oxidant, the fluorescence of the amino acids other than proline produced by the reactions at pH 5.3 was much smaller than that at pH 10.0 (Compare the conditions C with D in Table 1). pH 10 was the optimum pH for the fluorophore formation in common amino acids,[11] but the fluorescence of lysine under the condition C (with NaClO as an oxidant at pH 5.3) was twice that of proline. Therefore, H_2O_2 plus $FeCl_3$ (condition A) was investigated as a further possible reagent to destroy lysine. Under the condition A, amino acids giving a relatively high fluorescence were proline and tryptophan. Lysine and ammonia gave a slight fluorescence. However, there are very low concentrations of tryptophan and lysine in most grapes and wines except for certain grapes such as the American varieties Concord and Catawba[12] compared to that of proline.[1-5] The pretreatment of samples by activated carbon at the final concentration of 1% completely removed tryptophan from the samples and appreciably reduced tyrosine and phenylalanine. The final procedure (condition A) chosen is described under "Materials and Methods".

Effects of pH The fluorescence intensities of proline and lysine were considerably affected by pH (Fig. 1). Under the condition A, the intensity of proline increased appreciably with increasing pH and the intensity of lysine slightly in the pH range between 5.0 and 6.0. Below pH 5.3, no precipitate was observed and the color of the sample mixture after the oxidation was pale yellow to yellow due to the presence of ferric ion. The fluorescence intensity of proline after the fluorogenic reaction was very low. On

Table 1. Fluorescence intensities[a] obtained from the standard amino acids found in grapes and wines under four different conditions (A, B, C, and D)[b].

Condition for oxidation	A NaClO+ H_2O_2+ $FeCl_3$ pH 5.3[c]	B NaClO+ H_2O_2 pH 5.3	C NaClO pH 5.3	D NaClO pH 10.0
Ala[d]	<1	1	3	173
α-AB[e]	1	1	3	171
β-AB[f]	2	1	6	189
Ammonia	5	7	12	74
Arg	1	1	9	112
Asn	<1	2	3	174
Asp	<1	2	5	138
Cys-Cys	2	3	5	197
Glu	<1	1	<1	65
Gln	<1	3	6	71
Gly	1	<1	8	65
His	<1	<1	6	129
Ile	1	3	3	144
Leu	1	3	2	147
Lys	6	13	190	212
Met	2	2	3	103
Phe	<1	1	2	156
Pro	**100**	**100**	**100**	**100**
Ser	2	2	5	97
Thr	1	2	5	100
Tyr	1	1	16	109
Try	16	27	25	112
Val	<1	2	2	159

[a] Calculated as percentage of the proline present.
[b] The oxidation and fluorogenic reaction were done for 5 min at 60°C using final concentrations of 0.01% H_2O_2 plus 3×10^{-3} M $FeCl_3$ and 0.005% NaClO as oxidative reagents, and for 90 min at room temperature, respectively.
[c] pH for the oxidation and fluorogenic reaction.
[d] The amount of each amino acid used is 10^{-1} μmol.
[e] α-Aminobutyric acid.
[f] β-Aminobutyric acid.

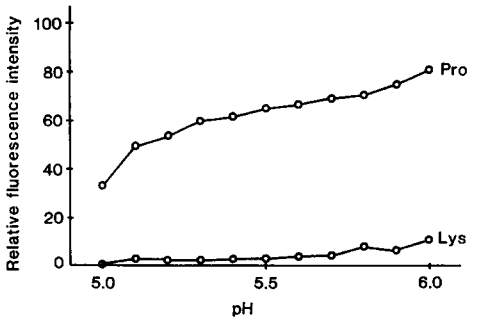

Fig. 1. Effects of the pH of the reaction mixture.

the other hand, at pH 5.3 or above, yellow precipitates occurred during the oxidation and the supernatant obtained after centrifuging the mixture was colorless, and the intensity of proline was higher than that in the colored mixture. The ratio of the intensity of lysine to that of proline was the smallest (about 0.05) at pH 5.3 to pH 5.4. Therefore, pH 5.3 was selected as the reaction pH.

Effects of concentrations of sodium hypochlorite, hydrogen peroxide, and ferric chloride When

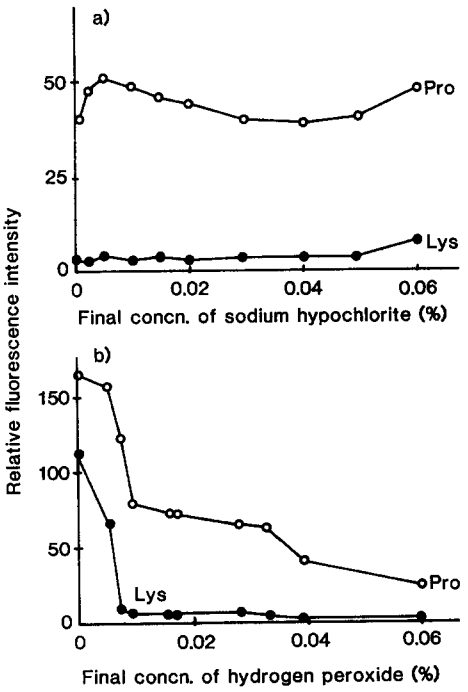

Fig. 2. Effects of the concentrations of sodium hypochlorite and hydrogen peroxide in the oxidative reaction.

the NaClO concentration was changed from 2×10^{-3}% to 6×10^{-2}% in the presence of 1×10^{-2}% H_2O_2 and 3×10^{-3}M $FeCl_3$, the fluorescence intensity of proline was the highest at the final concentration of 5×10^{-3}% NaClO (Fig. 2a). The intensities of proline and lysine decreased sharply with increasing H_2O_2 until 9.4×10^{-3}% in the presence of 5×10^{-3}% NaClO and 3×10^{-3}M $FeCl_3$ and gradually above that concentration, while the intensity of lysine was already very low at the concentration of 7.5×10^{-3}%.

The effects of $FeCl_3$ on the oxidation of proline and lysine were investigated using the final concentration of $FeCl_3$ from 1×10^{-3} to 10×10^{-3}M. The presence of 3×10^{-3}M $FeCl_3$ in the oxidation resulted in considerably reduced relative intensities of lysine and tryptophan (Table 1). The relative intensities of lysine and tryptophan in the presence of $FeCl_3$ were about half of those in the absence of $FeCl_3$. It is well known that amino acids with a primary amino group are oxidized by H_2O_2 and $FeCl_3$ to produce the corresponding fatty acids, carbon dioxide, ammonia and water,[14-16] and by NaClO to produce the corresponding aldehyde and ammonia *via* the formation of the intermediates and carbon dioxide.[17] Under the conditions of the oxidation used here, the amino acids other than proline were mostly destroyed by NaClO and H_2O_2. The small amounts of the amino acids remaining intact were destroyed almost completely by H_2O_2 in combination with $FeCl_3$. During the oxidative reaction, NaClO converted proline to the corresponding primary amine[18] although a part of the proline was also destroyed.

Effects of 2-mercaptoethanol, o-phthalaldehyde, and Brij 35 on fluorophore formation from oxidized proline Increasing amounts (from 0.625 to 3.125 ml) of 16% OPA in absolute ethanol

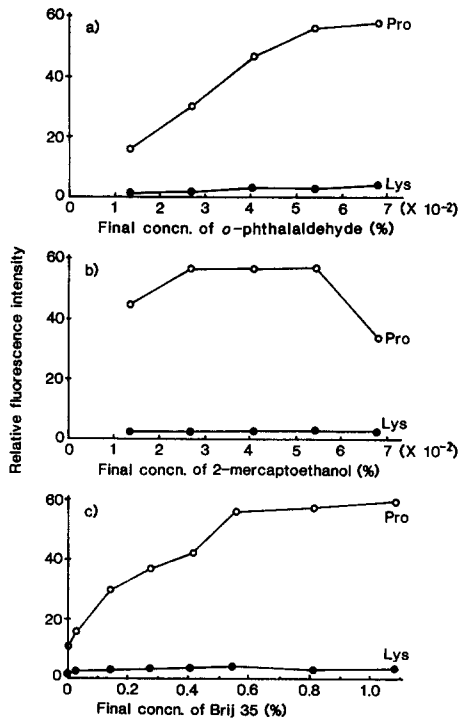

Fig. 3. Effects of the concentrations of *o*-phthalaldehyde (a), 2-mercaptoethanol (b), and Brij 35 (c) in the fluorogenic reaction.

were mixed with 0.4 ml of 2-ME and 15 ml of 20% Brij 35 to make 100 ml with water. One-half ml of this fluorogenic reagent was mixed with 3.2 ml of the reaction mixture after the oxidation. The final concentrations of OPA in 3.7 ml of the mixture were from 0.0135 to 0.068%. The intensity of proline increased as the concentration of OPA increased (Fig. 3a). The optimum concentration of OPA was 0.054%.

When the final concentration of 2-ME increased from 0.014 (0.1 ml 2-ME+20 ml of 20% Brij 35+2.5 ml of 16% OPA/100 ml) to 0.068% (0.6 ml 2-ME+20 ml of 20% Brij 35+2.5 ml of 16% OPA/100 ml), the intensity of proline was almost constant. The maximum was in the range of 2.7×10^{-2} to 5.41×10^{-2}% (Fig. 3b).

Different quantities of a solution of 20% Brij 35 (between 1 and 40 ml) were mixed with 0.4 ml of 2-ME and 2.5 ml of 16% OPA and filled up to make 100 ml with water. The final concentration of Brij 35 ranged from 0.027% to 1.08%. The fluorescence intensity of proline was almost constant between 0.54% and 1.08% of the final concentration of Brij 35 (Fig. 3c). Brij 35 increased the fluorescence intensity of proline even in the fluorogenic reaction at pH 5.3 as well as at alkaline pH. This may be due to the detergent helping to increase the solubility of the fluorescent product of proline in an aqueous solution and thus resulting in the increased fluorescence intensity.

Times for oxidation and fluorogenic reactions, and reaction temperature After mixing the oxidative reagent, the maximum of the fluorescence intensity of proline occurred after 4 min at 60°C. The intensity was stable until about 10 min of incubation. Incubation for more than 10 min led to a slow decrease in the intensity.

The fluorescence intensity of lysine was strongly influenced by time and temperature of the fluorogenic reaction. When the fluorogenic reaction was done at room temperature and if the yellow precipitate produced during the oxidative reaction was not removed until just before the measurement of fluorescence, the intensity of proline and lysine was nearly constant between 40 and 160 min after the fluorogenic reaction had been started. However, the fluorescence intensity of lysine in the supernatant increased gradually after removing the precipitate by centrifugation while that of the proline was constant. The intensity of lysine in the supernatant measured 1 h, 2 h, and 3 h after one hour of the fluorogenic reaction and centrifugation was respectively 1.5, 2, and 3 times the intensity immediately after centrifugation. Therefore, the fluorescence of the supernatant should be measured within 30 min after centrifugation.

Precision and application to juices and wines The relative standard deviations were 3.6% and 4.3% for the standard curves of proline in the concentrations between 1 μg and 15 μg/0.1 ml by this method and an automatic amino acid analyzer,

Table 2. Analysis of variance (ANOVA) table.

Source of variation	Sums of sq.	Degree of freedom	Mean sq.	F
Juice	652841.27	19	34360.07	*243.88
Method	275.63	1	275.62	*1.96
Error	2676.88	19	140.89	
Total	655793.77	39		
Wine	10074656.49	34	296313.43	*270.88
Method	1218.06	1	1218.06	*1.11
Error	37192.94	34	1093.91	
Total	10113067.49	69		

respectively. As little as 0.1 μg of proline could be assayep by the method.

The analysis of variance was made to test for any significance difference in the results of the analysis of 20 juices from 20 different kinds of grape varieties harvested in 1984, 23 wines produced from 23 different kinds of grape varieties in 1984, and 12 wines from 6 different varieties in 1979–81. The ANOVA table is shown in Table 2 using an automatic amino acid analyzer or the fluorometric method. From the results one can see that at the 5% level of probability, there was no significant difference between the two methods of analysis.

A hundred samples pre-treated with activated carbon could be analyzed within 3 h by this method. The average time per sample required for the oxidation and the fluorogenic reaction was about 2 min.

Interference The fluorescence intensities of hydroxyproline, tryptophan, and lysine were 36%, 16%, and 6%, respectively, when these intensities were calculated as percentages of proline. However, since the amounts of these amino acids in grape juices and wines are very small compared to proline,[1-4] and tryptophan could be removed by the pre-treatment of samples with activated carbon, their intensities can be ignored for the measurement of proline. The method is applicable to most grape and wine samples as long as the amount of proline after the pre-treatment with activated carbon is much higher than those of lysine, hydroxyproline, and tryptophan in the samples.

The effects of some components present in grapes and wines on the measurement of proline were investigated with phenolic compounds (10^{-3} M in sample solutions) such as tyrosol, ferulic acid, gallic acid, *p*-coumaric acid, salicylic acid, *p*-hydroxybenzoic acid, caffeic acid, and protocatechuic acid, hydroxy acids (0.2 to 1%) such as tartaric acid and malic acid, sugars (0.4 to 2%) such as glucose and fructose, ethanol (5 to 25%), metal ions (50 mg/*l*) such as Fe^{++}, Cu^{++}, Ca^{++}, Zn^{++}, Mg^{++}, K^{+}, and Na^{+}, and SO_2 (50 mg/*l*). All the components except for hydroxy acids did not affect the measurement. The hydroxy acids in the concentration of the range from 0.2 to 2% reduced the intensity of proline to about 70% of that without the hydroxy acids, but the extent of the reduction did not depend on the concentration. However, when two proline solutions in 0.01 M tartaric acid or in water were used as samples, the fluorescence of the two samples was almost equal when $CaCl_2$ (final concentration of 0.02 to 0.04 M) was added to the pH 5.3 buffer used as the reaction buffer. The reason for the above result is not known.

In Fig. 4, the change in proline concentration was correlated with the change in the sugar concentration during the maturation of grapes. During fermentation, the proline contents increased. An unknown substance other than proline giving fluorescence with OPA under the above conditions was observed in immature grapes, especially in August. The substance decreased as the grapes ripened

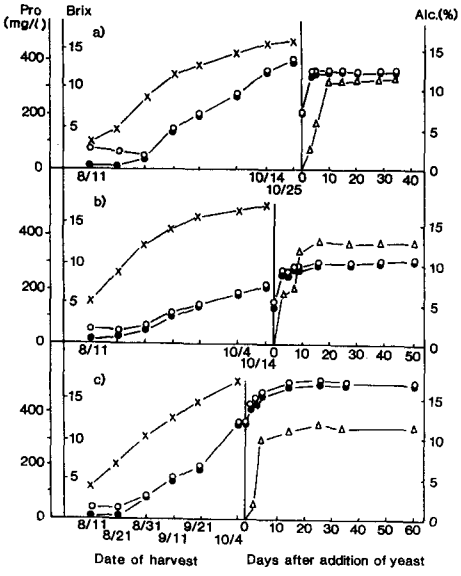

Fig. 4. Changes in the contents of proline: the fluorometric method (○) and an automatic amino acid analyzer (●), sugar (×), and alcohol (△) during maturation of Koshu (a), Muscat Bailey A (b), and Riesling (c) grapes, or fermentation.

and was not formed during the fermentation because the curve of the change in proline during fermentation as measured by the fluorometric method was consistent with that measured by an automatic amino acid analyzer. Therefore, the method is applicable not only for the analysis of the grape juice but also for the analysis of both fermenting and finished wines.

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References

1) Ough, C. S., Bustos, O.: *Wines Vines*, **50**(4), 50 (1969).
2) Ough, C. S.: *Vitis*, **7**, 321 (1968).
3) Yokotsuka, K., Aihara, T., Kushida, T.: *J. Ferment. Technol.*, **59**(8), 631 (1975).
4) Yokotsuka, K., Matsudo, T., Nozaki, K., Kushida, T.: *J. Inst. Enol. Vit., Yamanashi Univ.*, **18**, 51 (1983).
5) Lafon-Lafourcade: *The Biochemistry of Fruits and their Products*, (Hulme, A. C.), Vol. 2, 187, Academic Press, London and New York (1971).
6) Chinard, F. P.: *J. Biol. Chem.*, **199**, 91 (1952).
7) Schweet, R. S.: *J. Biol. Chem.*, **208**, 603 (1954).
8) Troll, W., Lindsley, J.: *J. Biol. Chem.*, **215**, 655 (1955).
9) Lashkhi, A. D., Tsiskarishvili, T. P.: *Vinodel. Vinograd. SSSR*, **27**, 19 (1967).
10) Ough, C. S.: *J. Food Sci.*, **34**, 228 (1969).
11) Yokotsuka, K., Kushida, T.: *J. Ferment. Technol.*, **61**, 1 (1983).
12) Kluba, R. M., Mattick, L. R., Hackler, L. S.: *Am. J. Enol. Vit.*, **29**(2), 102 (1978).
13) *Official Methods of Analysis of the Association of the Official Analytical Chemists*, (Horwitz, W.), 12th. Ed., 87, Association of Official Analytical Chemists, Benjamin Franklin Station, Washington (1975).
14) Dakin, H. D.: *J. Biol. Chem.*, **1**, 171 (1906).
15) Dakin, H. D.: *J. Biol. Chem.*, **4**, 63 (1908).
16) Dakin, H. D.: *J. Biol. Chem.*, **5**, 409 (1909).
17) Langheld, K.: *Ber.*, **42**, 2360 (1909).
18) Waldschmidt-Leitz, E., Akabori, S.: *Z. Physiol. Chem.*, **224**, 187 (1934).

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