Characterization of Major Stable Proteins in Chardonnay Wine

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Chardonnay wine produced in the conventional manner contained 69.8 to 108 mg/L soluble proteins including nondialyzable polypeptides; with an average of 87.2 mg/L. Proteins were separated by precipitation with ammonium sulfate, and fractionated into two fractions, F1 (mainly invertase and proteoglycans) and F2 (glycoproteins), by Sephadex G-100 column chromatography. The fractions were further separated by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) [isoelectric focusing and SDS-polyacrylamide gel electrophoresis]. More than 160 and 150 protein spots were detected on the 2-D PAGE maps of F1 and F2, respectively. The major proteins or polypeptides were determined to be fruit proteins, such as thaumatin- and osmotin-like proteins, invertase, lipid transfer protein (LTP), and their hydrolysis products. This is first report on the existence of LTP (or its hydrolysis product) and the hydrolysis products of major grape proteins (invertase, osmotin-like protein, and thaumatin-like protein) in wine.

Keywords: wine, protein, grape, hydrolysis

Introduction

Stable soluble proteins present in wines after clarification and fining are considered to contribute significantly to wine taste and quality (Fukui *et al.* 2002). Although the soluble proteins in wine are derived from grape juice, seed, skin and yeast, the major source of most soluble proteins is juice (approximately 90% of total protein content) (Fukui *et al.* 2003, Yokotsuka *et al.* 1994). Experimental and commercial white and red wines produced in Japan contain 29.8 to 107.1 mg/L soluble proteins, and very stable soluble proteins in the range of 13.6 to 36.4 mg/L remain in the wines even after severe clarification and fining (Fukui *et al.* 2003). Fukui *et al.* (2002) reported potential taste reduction of catechin and grape seed dimeric phenols (for example, astringency or bitterness reduction) by wine proteins (all of which are glycoproteins), and the interaction of polysaccharides or polymeric tannins with the proteins to form complexes or similar entities, thereby stabilizing the soluble proteins in the wines.

However, the roles of proteins in wine on its quality are still not fully understood because most studies on wine proteins have focused on heat-unstable soluble proteins (Hsu and Heathebell 1987a, 1987b; Hsu *et al.* 1987c; Waters *et al.* 1991; Yokotsuka *et al.* 1991; Yokotsuka and Singleton 1997) which are responsible for protein-associated turbidity in white wine. As we believe that heat-stable soluble proteins are more important contributors to wine quality than heat-unstable proteins, more research on them is needed. We reported on the separation and char-

acterization of proteins from Semillon grape juice and from the wine produced from it (Fukui 2003) because, among the wines tested, it had the highest protein content. Recently, Chardonnay wine has become popular in Japan, where its production has increased. At present, Chardonnay, a European variety, is the most important variety of white wine in Japan, and is comparable to the Koshu variety, which is native to Japan. Therefore, in this study, stable soluble proteins were separated by ammonium sulfate precipitation from white table wine produced from Chardonnay grapes harvested in Japan, and fractionated and purified by Sephadex G-100 chromatography followed by 2-D PAGE. Then, the N-terminal amino acid sequences of the purified proteins or polypeptides were determined.

Materials and Methods

Production of Chardonnay wine Chardonnay grapes were harvested in Kofu (1994, 2000, and 2002) and Katsunuma (1997 and 2001), Yamanashi, Japan. Common white table wines were produced from the grapes in the conventional manner at the Institute of Enology and Viticulture, University of Yamanashi, Kofu, and at the Fujicco Winery Co., Ltd., Katsunuma. The wines were stabilized by bentonite fining (0.01% $\times$ 2 or 0.001% $\times$ 3) and chilling at -5.5°C for one week. The concentration of free SO_2 in the wines was adjusted to approximately 30 mg/L immediately before bottling using potassium metabisulfite and the wines were then stored in an air-conditioned underground cellar at 15°C until use.

Separation of soluble proteins from wine Chardonnay wine was centrifuged at $14,000 \times g$ at 4°C for 15 min, and filtered through a $0.45\mu\text{m}$ membrane filter. The filtrate

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was concentrated to approximately one-tenth of its original volume by rotary evaporation at 40°C. Ammonium sulfate (80% saturation) was added to each concentrate and mixed well, and the mixture was allowed to stand overnight at 4°C. The precipitate formed was collected by centrifugation and homogenized in a small volume of water using a glass homogenizer. The homogenate was centrifuged at 40,000×g at 4°C for 30 minutes. The supernatant was dialyzed three times against 100 volumes of deionized water using a dialysis membrane (Viskase Sales) at 4°C for 2 days, and the retentate was lyophilized. The precipitate formed during dialysis was removed by centrifugation.

The lyophilisate containing 50 mg of proteins was dissolved in 15 mL of 10 mM HCl and centrifuged. The supernatant was applied to a Sephadex G-100 column (2.6 i. d.×100 cm; Pharmacia) equilibrated with the same solution. The column was eluted with 10 mM HCl at room temperature at a flow rate of 2.0 mL/minute. Fractions of 5 mL each were collected and peaks were determined by measuring the absorbance at 280 nm. Fractions corresponding to the peaks were collected, dialyzed against deionized water as mentioned above, and lyophilized.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) Protean IEF Cell and ReadyPrep™ 2-D Starter Kit (Bio-Rad) were used. Proteins were dissolved at 15 mg/mL in ReadyPrep™ rehydration/sample buffer containing 10 mL of 8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte® 3/10 ampholyte, and bromophenol blue (trace). The protein solution (10 µL) was loaded by passive overnight rehydration on ReadyStrip™ IPG pH 3–6 strip. Isoelectric focusing was conducted at 10 kVh in a focusing tray at 20°C according to the ReadyStrip™ IPG strip instruction manual (catalog # 163–2099). The strips were then equilibrated in 375 mM Tris-HCl (pH 8.8) containing 6 M urea, 20% glycerol, 2% SDS, and 2% DTT in order to reduce disulfide bridges (20 minutes with stirring), followed by alkylation upon addition of 2.5% iodoacetamide for 10 minutes with gentle stirring. SDS-PAGE analysis was performed in 10% (for F1 proteins) or 15% (for F2 proteins) polyacrylamide gels using a vertical slab-gel apparatus (160×160×1 mm, type RAPIDAS AE-6200, ATTO Corp., Tokyo). The electrophoretic run was conducted at a constant current of 30 mA per gel until the bromophenol blue tracking dye reached the edge of the separation gel. The spots were stained with Coomassie Brilliant Blue R-250.

Blotting and N-terminal amino acid sequencing of proteins After 2-D PAGE analysis, proteins were blotted onto PVDF membrane using an electroblotting method, as described previously (Okuda *et al.* 1999). Briefly, proteins on 2-D PAGE gel were blotted onto a PVDF membrane with a semi-dry-type electroblotting apparatus (AE-6675; ATTO, Tokyo). Blotted proteins were visualized by CBB staining. Protein spots were cut out from the membrane, and the N-terminal amino acid sequences of the proteins were determined with a protein sequencer (Model 491; Applied Biosystems, a division of Perkin-Elmer, Foster City, CA).

Analyses Analyses were carried out to determine the general composition of three bottles of wine manufactured in the same year and analytical values were obtained from the average of the three samples. The lyophilisates separated from wine by ammonium sulfate precipitation and the protein fractions fractionated by Sephadex G-100 column chromatography were analyzed for proteins (micro-Kjeldahl method), neutral sugars (phenol-sulfuric acid method), and total phenols (Folin-Ciocalteu method), as described previously (Fukui *et al.* 2003, Yokotsuka and Singleton 1997).

Results and Discussion

General composition of Chardonnay wines and protein fractions Some characteristics of the Chardonnay white wines produced from grapes harvested in 1994, 1997, 2000, 2001 and 2002 were determined (Table 1) so that the results of the protein analysis could be better understood. These wines are considered average among Japanese wines in terms of composition except for the slightly higher pH, lower acidity and higher total phenol content. The content of soluble proteins in the Chardonnay wines was in the range of 69.8 to 108 mg/L, with an average of 87.2 mg/L (Table 1). The amount of proteins in the precipitates (lyophilisates) separated from 1 L of the wines by ammonium sulfate precipitation was in the range of 15.9 to 35.8 mg. The percentage of recovered proteins from all the soluble proteins present in the wines ranged from 22.2 to 48.2%, with an average of 29.7%. The yield did not depend on the aging period, and the variance in the yield may be attributable to the processes involved in the concentration of wines and the separation of proteins by ammonium sulfate precipitation. However, the variance did not affect the electrophoretic patterns.

Proteins (500 mg) in the lyophilisate obtained by ammonium sulfate precipitation were fractionated by Sephadex G-100 column chromatography. A typical elution pattern of the proteins isolated from the wine made in 2002 is shown in Fig. 1.

Two protein peaks, F1 and F2, and one phenol peak (F3, not protein) were obtained for all the wines made in five years. The percentage of neutral sugars in the lyophilisates for F1 from the five wines was 28 to 55% (Table 1), which was considerably higher than that for F2 at 3 to 5%. The lyophilisates for F1 contained mainly invertase and proteoglycans, while those for F2 contained glycoproteins. These results are very similar to those obtained with Muscat Bailey A red wines (Yokotsuka and Singleton 1997). For 2-D PAGE analysis, F1 and F2 from the 2002 wine were used.

The total protein fraction obtained by ammonium sulfate precipitation, and F1 and F2 were subjected to one-dimensional SDS-PAGE analysis (data not shown). The electrophoretic patterns of the wine samples were almost the same except for a thin 9 kDa band which was detected in F2 from 2002. Comparison of the electrophoretic pattern of the total protein fraction with those of F1 and F2 showed that the migration distances of all the bands in the patterns of F1 and F2 corresponded to those in the

total protein fraction pattern. Thus, there was no evidence of the presence of degradation products, such as small polypeptides, from the total protein fraction during chromatographic fractionation.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of wine proteins Thaumatin- and osmotin-like

Fig. 1. Separation of protein fraction obtained from Chardonnay wine by ammonium sulfate precipitation and fractionation by Sephadex G-100 column chromatography.

proteins were detected as the major proteins in Semillon wine in our previous study (Fukui *et al.* 2003). We also reported on the considerable amount of invertase in 14 grape cultivars (Fukui *et al.* 2003, Nakanishi and Yokotsuka 1991), and on the N-terminal amino acid sequence of an invertase from Semillon wine (Fukui *et al.* 2003). The invertase from Semillon wine was fractionated and purified by Superdex® 75HR column chromatography. However, the chromatography gave poor resolution of the protein peaks because of the presence of many kinds of proteins (or polypeptides). Anion exchange HPLC was used to provide better separation; however, the protein recovery was very low. Therefore, two-dimensional PAGE analysis (IEF analysis in the first dimension, and SDS-PAGE analysis in the second dimension) was used here.

Two-dimensional PAGE analysis of F1 and F2 generated more than 160 and more than 150 protein spots, respectively (Fig. 2). The major protein spot in F1 had a molecular weight (MW) of 60 kDa, while several other spots in the 22 to 24 kDa range were also observed. The isoelectric points (pIs) of the protein spots from F1 were between 3.0 and 6.0, with a few exceptions.

The protein content of F2 was ninefold higher than that of F1. The MWs and pIs of most of the protein spots from F2 were in the 25 to 30 kDa range and approximately 4.5, respectively. Spots with pIs above 6.0 were also obtained. The MWs obtained were similar to those of proteins fractionated from Semillon wine in our previous study (Fukui *et al.* 2003).

The 2-D PAGE analysis of F1 and F2, 13 (A to M in Fig. 2-A) and 17 (a to v in Fig. 2-B) major protein spots (large or visibly dense spots) were obtained, respectively. The

Table 1. Composition of protein fractions from Chardonnay wines produced during the period from 1994 to 2002.

	Year Produced				
	1994	1997	2000	2001	2002
pH	3.56	3.75	3.45	3.78	3.65
Alcohol (v/v)	11.8	12.4	12.0	12.2	11.8
Free SO ₂ (mg/L)	16	10	20	44	17
Titrate acidity (g/L, as tartaric acid)	4.74	6.02	5.64	6.06	5.25
Total nitrogen (mg/L, microkjeldahl N)	230	275	251	300	329
Proteins (mg/L, microkjeldahl N × 6.25)	74.1	69.8	86.9	108	97.4
(NH ₄) ₂ SO ₄ precipitation					
Lyophilisate of precipitate separated by (NH ₄) ₂ SO ₄ precipitation (mg/L)	72.9	23.9	37.8	41.0	28.9
Proteins in lyophilisate of precipitate separated by (NH ₄) ₂ SO ₄ precipitation (mg/L)	35.9 (49) ¹	15.9 (66)	24.7 (66)	29.1 (71)	21.8 (75)
Sephadex G-100 column chromatography					
F1Lyophilisate (mg/L)	5.24	4.88	4.46	4.76	4.36
Proteins	2.86 (55) ²	1.39 (28)	1.57 (35)	1.36 (29)	2.08 (48)
Neutral sugars	1.48 (28)	2.54 (52)	1.98 (44)	2.43 (51)	2.08 (48)
Phenols	0.33 (6)	0.25 (5)	0.32 (7)	0.25 (5)	0.39 (9)
Total	4.67 (89)	4.18 (85)	3.87 (86)	4.04 (85)	4.56 (105)
F2Lyophilisate (mg/L)	24.7	21.7	22.8	18.8	27.2
Proteins	19.3 (78)	14.5 (67)	17.8 (78)	12.6 (67)	18.2 (67)
Neutral sugars	0.8 (3)	1.1 (5)	0.7 (3)	1.0 (5)	1.3 (5)
Phenols	1.6 (6)	2.7 (12)	2.4 (11)	1.8 (10)	2.9 (11)
Total	21.7 (87)	18.3 (84)	20.9 (92)	15.4 (82)	22.4 (83)

¹ Values in parentheses are the percentage of proteins in the lyophilisate obtained by (NH₄)₂SO₄ precipitation.

² Values in parentheses are the percentage of the weight of the lyophilisate (F1 or F2).

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis of F1 (A) and F2 (B) obtained from Sephadex G-100 column chromatography.

N-terminal amino acid sequences of these proteins were determined.

N-terminal amino acid sequences of major wine proteins Protein spots on 2-D PAGE gels were transferred onto PVDF membranes, and their N-terminal amino acid sequences were determined. The results are shown in Table 2.

Invertase The N-terminal amino acid sequences of three proteins, spots A, B, and C from F1, with MWs of approximately 60 kDa (Nakanishi and Yokotsuka 1991, Porntaveewat *et al.* 1994, Takayanagi *et al.* 1995, Kwon 2004) were the same as or very similar to that of invertase. However, the pIs of these proteins at 3.75, 4.23, or 5.16, respectively, were different, perhaps due to the difference in their amino acid sequences. The N-terminal amino acid sequence of these proteins corresponded to residue 100 from the initiation Met residue, upon comparison with the amino acid sequence deduced from the nucleotide sequences of the vacuole invertase gene (GIN1) which was cloned from cDNAs of mature grape berries (Davies and Robinson 1996).

Protein spots E, F, and L (Fig. 2) had the same sequence as invertase but smaller MWs of 39, 29, and 38 kDa, respectively. Furthermore, protein spots J and n, and G and M had N-terminal amino acid sequences starting at residues

333 and 489, respectively, of the invertase sequence deduced from the cDNA encoding the invertase (Davies and Robinson 1996). The data demonstrated that the mature invertase protein was undoubtedly hydrolyzed to several polypeptide fragments during vinification. In our previous paper (Fukui *et al.* 2003), we reported the presence of invertase with MW of approximately 60 kDa in Semillon white wine. In this study, we obtained for the first time evidence for the existence of hydrolyzed invertases.

Osmotin-like protein The N-terminal amino acid sequences of 13 spots (Fig. 2) were very similar to the sequence deduced from the cDNA of VVOSM1 (osmotin-like protein) (Davies and Robinson 2000, Fukui *et al.* 2003, Loulakis 1997, Monteiro *et al.* 2003, Salzman *et al.* 1998, Singh *et al.* 1987) (Table 2). Of these 13 proteins, the amino acid sequences of 12 started at residue 25 of the deduced sequence and that of one started at residue 106. It is likely that proteins u and v, which had the largest MW (30 kDa) in relation to VVOSM1 (Table 2) and were found to be prominent in the 2-D PAGE map (Fig. 2-B), were hydrolyzed to smaller polypeptides, as in the case of invertase.

Thaumatococin-like protein The N-terminal amino acid sequences of major protein spots a, b, and c in Fig. 2-B corresponded to that of the deduced sequence of VVTL1 (thaumatococin-like protein) (Davies and Robinson 2000, Fukui 2003, Tattersall *et al.* 1997, Waters *et al.* 1996). These proteins are considered to be derived from grape juice because the thaumatococin-like protein is known to accumulate in grapes at the onset of ripening (Davies and Robinson 2000, Kwon 2004, Monteiro *et al.* 2003, Tattersall *et al.* 1997). The sequence near the N-terminal amino acid region of spot h was the same as those of spots a, b and c, but its MW was very different from those of the latter three. Therefore, spot h was considered to be a hydrolysis product of VVTL1.

Lipid transfer protein (LTP) The N-terminal amino acid sequence of spot s (Fig. 2-B) corresponded to that of the deduced amino acid sequence of the LTP gene (Acc. No. AF467945) in Pinot noir grapes. Spot s seems to be a hydrolysis product of LTP because its MW (9.6 kDa) was smaller than that estimated from the cDNA sequence (11.6 kDa). A number of possible functions for LTP have been proposed (Otera *et al.* 2001, Samuel *et al.* 2002, Trevino and O'Connell 1998), including involvement in epicuticular wax or cuticle biosynthesis (Pyee *et al.* 1994, Sterk *et al.* 1991), as well as in defense against pathogens (Cammue *et al.* 1995, Segura *et al.* 1993). The role of this protein in grapes has not been reported sufficiently (Salzman *et al.* 1998), but considering the amount of spot s, this protein should have an important role in the grape berry.

The N-terminal amino acid sequences of the other protein spots in Fig. 2 could not be determined because of their small amount, the presence of contaminants, or perhaps, the blocking of the N-terminal amino acid residues (for chitinase, a major grape protein). In our previous reports, we indicated that the major source of soluble proteins in wine is juice, with approximately 90% of the

Table 2. N-terminal amino acid sequences of Chardonnay wine proteins.

Spot	Sequence	M.W.	pI
Invertase ^a	YNWTTAMLAWQRTAFHQPEK	—	—
A	YXXTTAMFAWQRTAFHQPEK	60	3.8
B	YXXTTAMFAWQRTAFHQPEK	60	4.2
C	YGETTAMFAWQRTAFHQPEK	60	5.2
E	YXXTTAMFAWQRTAFHQPEK	39	5.2
F	YGETTAMFAWQRTAFHQPEK	29	4.0
L	YXXTTAMFAWQRTAFHQPEK	38	4.0
Invertase ^b	VIYNCSTSAGAAGRGALGPF	—	—
G	VIYNXSTSAGAAGRGALGPF	24	3.8
M	VIYXESTSAGAAGRGA	22	4.1
Invertase ^c	ASMDNRRHDYYALGEYDPM	—	—
J	ASMDNRRHDYAALGADLPS	19	5.2
n	ASMDNRRHDYAALGADLPS	14	5.4
VVOSM1 ^d	ATFNIQNHCSYTVWAAAVPG	—	—
d	ATFNIQNHXS	28	5.3
e	ATFNIQN	27	5.4
f	ATFNIQN	27	3.9
g	ATFNIQN	18	4.0
j	ATFNIQNHXS	27	5.0
l	ATFNIQN	18	4.9
m	ATFNIQN	22	5.2
o	ATFNIQN	21	5.3
p	ATFNIQN	20	5.4
t	ATFNIQNHXS	27	4.9
u	ATFNIQNH	30	4.8
v	ATFNIQN	30	5.1
VVOSM1 ^e	AEYALNQFNLDYDISLVD	—	—
k	AEFALNQFSNLDFDISLVD	14	4.0
VVTL1 ^f	ATFDILNKCTYTVWAAASPG	—	—
a	ATFDILNKCTYTVWAAASPG	28	4.8
b	ATFDILNK	30	4.9
c	ATFDI	28	5.1
h	ATFDILN	18	4.1
LTP ^g	TITCGQVASA	—	—
s	TITXGQVASA	9.6	5.1
Not Specified			
I	APVIKHVS	22	5.8
q	AFNPTQNIST	14	5.3

^a From residue 100 of deduced amino acid sequence of GIN1 (Acc. No. 2214476A)^b From residue 489 of deduced amino acid sequence of GIN1 (Acc. No. 2214476A)^c From residue 333 of deduced amino acid sequence of GIN1 (Acc. No. 2214476A)^d From residue 25 of deduced amino acid sequence of VVOSM1 (Acc. No. Y10992)^e From residue 106 of deduced amino acid sequence of VVOSM1 (Acc. No. Y10992)^f From residue 25 of deduced amino acid sequence of VVTL1 (Acc. No. AJ237999)^g From residue 28 of deduced amino acid sequence of *Vitis vinifera* lipid transfer protein cDNA (Acc. No. AF467945)

total protein content (Fukui *et al.* 2003, Yokotsuka *et al.* 1994). The difference in elution profiles of grape juice and wine proteins on GPC-HPLC (Superdex® 75HR; Pharmacia) (Fukui *et al.* 2003) indicated that proteins in grape juice were modified during vinification. The study revealed that hydrolysis of specific positions of the proteins occurred during vinification because white wines contain several proteins (or polypeptides), which have the same N-terminal amino acid sequence but different MWs. It should be noted that wine yeasts are not capable of transporting proteins across the cell membrane or degrading them into amino acids outside their cells (Boulton *et al.* 1996, Dizy and Bisson 2000). One possibility is that grape juice proteins are hydrolyzed by proteases released by the autolysis of yeast cells because grape juice does not exhibit appreciable protease activity (no protease activity was detected with casein as substrate in our study), or juice proteins may be resistant to protease or

peptidase activity in juice (Waters *et al.* 1992). The hydrolyzed protein fragments may be more soluble because of their low MWs, and can be utilized by yeast as an important source of nitrogen. Further research is needed to determine the contribution of the hydrolysis products of juice proteins to wine quality, particularly the taste of wine.

Conclusions

1. Chardonnay wine contained 69.7 to 108 mg/L soluble proteins or peptides. More than 310 protein or polypeptide fractions were separated from the wine by ammonium sulfate precipitation, Sephadex G-100 chromatography, and 2-D PAGE analysis.
2. Thaumatin- and osmotin-like proteins, invertase, lipid transfer protein and their hydrolysis products were found to comprise soluble wine proteins and polypeptides.
3. It is conceivable that juice proteins were enzymatically

hydrolyzed to smaller polypeptides during vinification. The MWs and pIs of the proteins or polypeptides were in the ranges of 14 to 60 kDa and 3.75 to 5.76, respectively.

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