

EFFECTS OF PROTEOLYTIC ACTIVITY ON THE CHARACTERISTICS OF AROMATIC RED RICE WINE

BY YUJI TERAMOTO, NORIAKI SAIGUSA, AND SEINOSUKE UEDA

(Department of Applied Microbial Technology, Kumamoto Institute of Technology, Ikeda 4-22-1, Kumamoto 860)

AND KIYOSHI YOSHIKAWA

(Faculty of Brewing Science, Tokyo University of Agriculture, Sakuragaoka 1-1-1, Setagaya-ku, Tokyo 156, Japan)

Received 24 August 1993

The quality of rice wine made from uncooked, unpolished aromatic red rice grain was improved by use of a commercial preparation of acid protease from *Aspergillus niger* during ethanol fermentation. The fermentation rate of the mash which contained the acid protease was much higher than that of mash that did not contain the preparation of acid protease. The rice wine made from uncooked, polished aromatic red rice, which usually had a less acceptable aroma, was improved by use of the preparation of acid protease, and large amounts of isobutyl alcohol, *n*-propyl alcohol and ethyl acetate were detected in the resultant rice wine. By contrast, the quality of rice wine made from the bran fraction of aromatic red rice was not improved by the preparation of acid protease. The polished rice fraction of aromatic red rice was affected by the acid protease and the aromatic quality of the rice wine was improved. The aromatic characteristics of red rice wine made from cooked, unpolished aromatic red rice grains, which was rather inferior in terms of both aroma and color, were also improved by the addition of the preparation of acid protease during ethanol fermentation. Thus acid protease has beneficial effects on the production of aromatic red rice wine.

Key Words: Aromatic red rice (*Oryza sativa* var. *Indica* Tapol), red rice wine brewing, ethanol fermentation without cooking, protease preparation produced by *Aspergillus niger*, effects of protease on rice wine brewing

INTRODUCTION

Red rice wine can be made from aromatic red rice (*Oryza sativa* var. *Indica*, Tapol) by use of an economical, uncooked ethanol-fermentation system¹⁰.

It has been reported previously that the quality of red rice wine made with a commercial preparation of glucoamylase was much better than that of red rice wine made without such a preparation¹¹.

Various hydrolytic enzymes are considered to play important roles during ethanol fermentation. Proteases have synergistic effects on the saccharification of starchy materials⁵ and the activity of β -glucosidase⁶ is closely related with aroma formation during ethanol fermentation of *shochu moromi*.

In Japanese sake brewing, the starch and protein in the mash are important factors in the fermentation process. The nature of these components of the mash is intimately associated with the characteristics of the resultant sake. Hydrolysis of protein^{3,4}, saccharification of starch and the characteristics of the fermentation process are all interrelated^{7,8}.

In this study the effects of proteolytic activity on red rice wine brewing and, in particular, on aroma formation in aromatic red rice wine has been investigated.

MATERIALS AND METHODS

Rice grains

Aromatic red rice (*Oryza sativa* var. *Indica*, Tapol) was used for the fermentation test. Unpolished and polished rice grains were ground to a particle size of 2-3 mm in diameter. Bran from aromatic red rice grains was also used for the fermentation test.

Preparation of enzymes

Sumizyme, a preparation of glucoamylase produced by *Rhizopus* sp. (Shinnihon Kagaku Kogyo Co. Ltd., Anjo) was used as the saccharifying agent for ethanol fermentation. Sumizyme AP, a preparation of acid protease produced by *Aspergillus niger* (Shinnihon Kagaku Kogyo Co. Ltd.) was used as an additive in the ethanol fermentation process.

Procedure for brewing red rice wine

i) Ethanol fermentation with and without the preparations of glucoamylase and acid protease

Thirty grams of uncooked, unpolished aromatic red rice, 3 g of compressed baker's yeast, and 100 ml of tap water were dispensed into a 300-ml Erlenmeyer flask with a gas trap¹¹. Ethanol fermentation was performed with 0.2 g of the preparation of glucoamylase, Sumizyme having an acid protease activity (1.2×10^6 U/g Sumizyme) (run 1), with 0.2 g of preparation of glucoamylase, Sumizyme plus 0.1 g of the preparation of acid protease, Sumizyme AP (2.9×10^7 U/g Sumizyme AP) (run 2), with 20 g of glucose (run 3), and with 20 g of glucose plus 0.1 g of the preparation of acid protease, Sumizyme AP (run 4).

The pH of the initial mash was adjusted to 4.0 with 1 N HCl and 1 N NaOH. Ethanol fermentation was performed at 30°C. The decrease in weight of the entire Erlenmeyer flask and its contents, as a result of the evolution of CO₂ gas, was recorded at 24-h intervals.

ii) Ethanol fermentation of uncooked, polished aromatic red rice and uncooked aromatic red rice bran with and without the preparation of acid protease

Twenty-one grams of uncooked, polished aromatic red rice (runs 5 and 6) or 9 g of uncooked aromatic red rice bran (runs 7 and 8), 20 g of glucose, 3 g of compressed baker's yeast, and 100 ml of tap water were dispensed into a 300-ml Erlenmeyer flask with a gas trap. Ethanol fermentation was performed with 0.1 g of the preparation of acid protease,

Sumizyme AP (runs 6 and 8) and without this preparation (runs 5 and 7) according to the procedure described above.

iii) *Ethanol fermentation of cooked and uncooked, unpolished aromatic red rice with and without the preparation of acid protease*

Thirty grams of unpolished aromatic red rice and 60 ml of tap water were dispensed into a 300-ml Erlenmeyer flask and autoclaved at 121°C for 15 min (runs 9 and 10). After cooling, 0.2 g of the preparation of glucoamylase, Sumizyme, 40 ml of tap water, and 3 g of compressed baker's yeast were added to the cooked mash in a 300-ml Erlenmeyer flask.

For comparison, ethanol fermentation of uncooked, unpolished aromatic red rice was also performed (runs 11 and 12).

Ethanol fermentation was performed with 0.1 g of the preparation of acid protease, Sumizyme AP (runs 10 and 12) and without the preparation of acid protease (runs 9 and 11) according to the procedures described above.

Assays of enzymatic activities

Glucoamylase activity was measured as previously described¹¹. Proteolytic activity was measured by the method of Tsuru *et al.*^{2,9}.

Ethanol and aroma analysis

Ethanol and volatile aromatic components of rice wines were analyzed with a Shimadzu model GC-14A gas-chromatograph equipped with a 3.1-m PEG-HT column (Gasukuro Kogyo Inc., Tokyo), as previously described¹¹.

General analytical procedures

Reducing sugars were quantitated by the method of Bertrand¹. Acidity was measured by titrating 10 ml of rice wine with 0.1 N NaOH¹¹. Organoleptic tests were also performed to evaluate the various rice wines¹¹.

RESULTS

Ethanol fermentation with and without the preparations of glucoamylase and acid protease

Fermentation rates of the mash that contained the preparation of acid protease (runs 2 and 4) were higher than those of the mashes that did not contain the acid protease (runs 1 and 3) (Fig. 1). The final amount of CO₂ generated from the mash that contained the acid protease (runs 2 and 4) also was larger than those of the mashes that did not contain acid protease (Table I). The final consumption of starchy material in runs 2 and 4 was not affected by the acid protease. Acidity of the filtrates of fermented mashes, namely, the rice wines made with the preparation of acid protease was a little higher in runs 2 and 4 than in other runs.

The amount of ethyl acetate in the rice wine made with the preparation of acid protease (runs 2 and 4) was very much higher than that in the rice wine made without this preparation (runs 1 and 3) (Table II). Organoleptic tests revealed that the aromatic characteristics of the rice wine were slightly improved by the use of the preparation of acid protease. The quality of rice wines made with the glucoamylase preparation that contained acid protease activity (1.2×10^6 U/g) was also a little higher than that of rice wines made without glucoamylase.

Ethanol fermentation of uncooked, polished aromatic red rice and uncooked aromatic red rice bran with and without the preparations of acid protease

Except for run 5, the fermentation rate was the same for all runs (Figure 2). The final amount of CO₂ generated from the mashes that contained the preparation of acid protease (runs 6 and 8) was relatively high (Table III). The acidity of the rice wine made with the preparation of acid protease

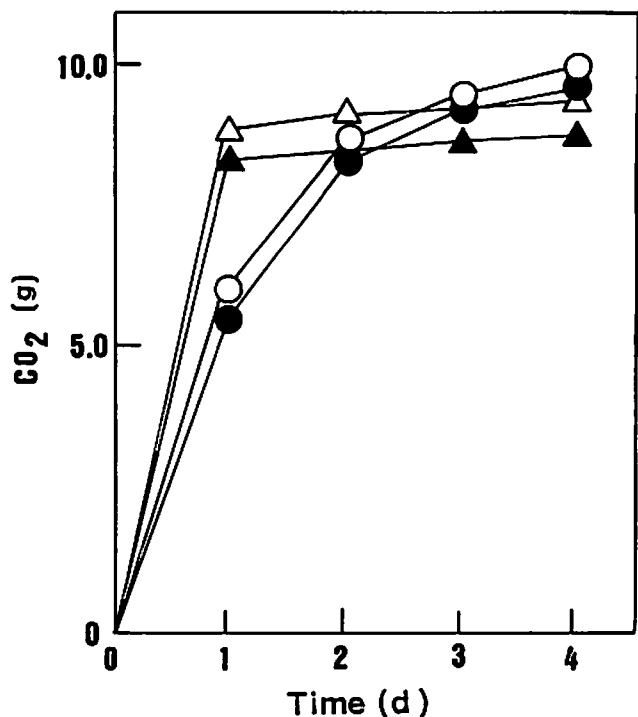


Fig. 1. Time courses of fermentation of the mash with and without the preparations of glucoamylase and acid protease.

Symbols: ●, 0.2 g of glucoamylase preparation (run 1); ○, 0.2 g of glucoamylase preparation and 0.1 g of acid protease preparation (run 2); ▲, 20 g of glucose (run 3); △, 20 g of glucose and 0.1 g of acid protease preparation (run 4).

TABLE I. Characteristics of rice wines made with and without the preparations of glucoamylase and acid protease

Runs	1	2	3	4
Glucoamylase preparation	○ ^{a)}	○	— ^{b)}	—
Glucose	—	—	○	○
Acid protease preparation	—	○	—	○
CO ₂ output (g)	9.7	9.9	8.9	9.3
Total glucose in feed (g)	19.4	19.4	39.4	39.4
Total glucose in fermented mash (g)	0.7	0.7	0.7	0.5
Consumption of glucose (%)	97	97	97	98
pH	3.8	4.0	3.8	4.1
Acidity (ml)	8.3	8.9	7.8	8.0

a) ○, Added to the initial mash.

b) —, Not added.

was higher than that of the rice wine made without this preparation.

Amounts of isobutyl alcohol, *n*-propyl alcohol, ethyl acetate, and acetaldehyde in the rice wines made with the preparation of acid protease were larger than those in the rice wines made without the preparation of acid protease (Table IV). Organoleptic testing indicated that rice wines made from uncooked, polished aromatic red rice and glucose were improved to some extent by the acid protease. By contrast, the aromatic characteristics of rice wines made from uncooked aromatic red rice bran and glucose were superior and were not improved by use of the preparation of acid protease.

TABLE II. Aroma analysis of rice wines made with and without the preparations of glucoamylase and acid protease

Runs	1	2	3	4
Glucoamylase preparation	○ ^{a)}	○	— ^{b)}	—
Glucose	—	—	○	○
Acid protease preparation	—	○	—	○
Ethyl alcohol (% v/v)	10.8	11.0	9.5	10.4
Isobutyl alcohol (ppm)	293	221	115	193
Isoamyl alcohol (ppm)	453	352	322	324
<i>n</i> -Propyl alcohol (ppm)	41	41	8	14
Ethyl acetate (ppm)	298	428	70	268
Ethyl lactate (ppm)	1	1	1	1
Isoamyl acetate (ppm)	3	4	ND ^{c)}	1
Acetaldehyde (ppm)	108	102	79	111
Organoleptic test ^{d)}	++++	++++	+	++

a) ○, Added in the initial mash.

b) —, Not added.

c) ND, Not detected.

d) Organoleptic test: +++++, excellent; ++, good; +, tolerable.

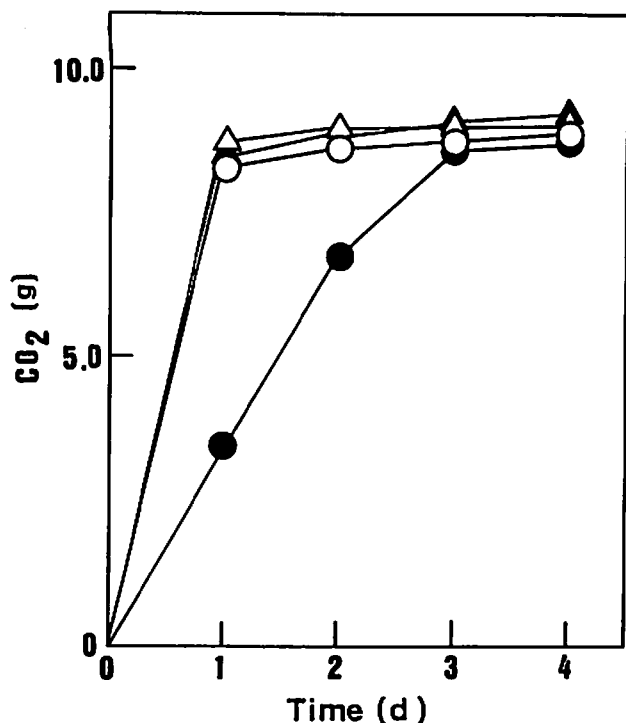


Fig. 2. Time courses of fermentation of mashes composed of uncooked, polished aromatic red rice or uncooked aromatic red rice bran with and without the preparation of acid protease.

Symbols: △, uncooked, polished aromatic red rice (run 5); ▲, uncooked, polished aromatic red rice and 0.1 g of acid protease preparation (run 6); ○, uncooked aromatic red rice bran (run 7); ●, uncooked aromatic red rice bran and 0.1 g of acid protease preparation (run 8).

Ethanol fermentation of cooked and uncooked, unpolished aromatic red rice with and without the preparation of acid protease

Fermentation rates of mashes composed of cooked, unpolished aromatic red rice were much higher than those of mashes composed of uncooked, unpolished aromatic red rice (Figure 3). Fermentation rates of mashes that contained the preparation of acid protease were higher than those of mashes that did not contain the acid protease. The acidity

TABLE III. Characteristics of rice wines made from uncooked, polished aromatic red rice and uncooked aromatic red rice bran with and without the preparation of acid protease

Runs	5 Polished rice (21 g)	6 Rice bran (9 g)	7 Rice bran (9 g)	8 Rice bran (9 g)
Acid protease preparation	— ^{a)}	○ ^{b)}	—	○
CO ₂ output (g)	8.7	9.2	8.7	9.0
Total glucose fed in the mash (g)	20	20	20	20
Total glucose in rice wine (g)	0	0	0.2	0.2
Consumption of glucose (%)	100	100	99	99
pH	3.8	4.2	3.7	3.9
Acidity (ml)	4.9	6.7	8.0	8.2

a) ○, Added to the initial mash.

b) —, Not added.

TABLE IV. Aroma analysis of rice wines made from uncooked, polished aromatic red rice and uncooked aromatic red rice bran with and without the preparation of acid protease

Runs	5 Polished rice (21 g)	6 Rice bran (9 g)	7 Rice bran (9 g)	8 Rice bran (9 g)
Acid protease preparation	— ^{a)}	○ ^{b)}	—	○
Ethyl alcohol (% v/v)	9.3	10.1	9.2	9.4
Isobutyl alcohol (ppm)	170	227	174	206
Isoamyl alcohol (ppm)	392	296	455	369
<i>n</i> -Propyl alcohol (ppm)	16	33	12	18
Ethyl acetate (ppm)	114	273	132	157
Ethyl lactate (ppm)	1	1	ND ^{c)}	ND
Isoamyl acetate (ppm)	1	1	ND	ND
Acetaldehyde (ppm)	74	252	120	139
Organoleptic test ^{d)}	+	++	++	++

a) ○, Added to the initial mash.

b) —, Not added.

c) ND, Not detected.

d) Organoleptic test: ++ good; +, tolerable.

of the rice wine made with the acid protease was larger than that of the rice wine made without the preparation of acid protease (Table V).

The amount of ethyl acetate in the rice wine made with the preparation of acid protease was much larger than that in the rice wine made without this preparation (Table VI), in the case of fermentation both without cooking and with cooking. Organoleptic testing revealed that the aroma of the rice wine made with the preparation of acid protease was improved. The quality of rice wine made from cooked, unpolished aromatic red rice was lower, but it was improved by the preparation of acid protease to some extent.

DISCUSSION

In aromatic red rice wine brewing, inclusion of a preparation of acid protease had various positive effects: promotion of fermentation rates; reinforcement of aromatic components; and improvement of the quality of the aromatic red rice wine.

The promotive effects of the acid protease on the fermentation may be ascribable to synergistic effects of the acid protease and various other hydrolytic enzymes on the degradation of ground rice particles.

The mechanism of aroma formation in a mash composed

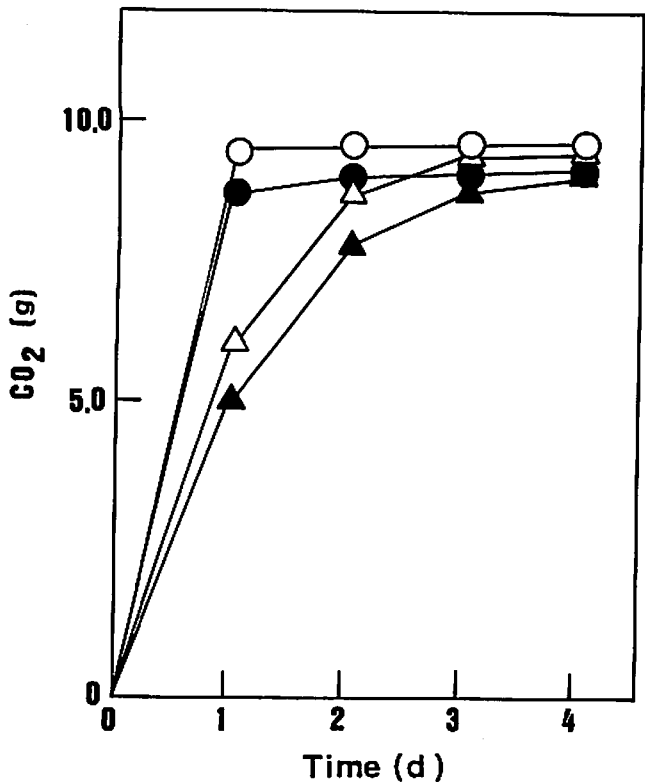


Fig. 3. Time courses of fermentation of mashes composed of cooked and uncooked, unpolished aromatic red rice wine with and without the preparation of acid protease.

Symbols: ▲, 30 g of cooked unpolished aromatic red rice (run 9); ●, 30 g of cooked unpolished aromatic red rice and 0.1 g of acid protease preparation (run 10); △, 30 g of uncooked unpolished aromatic red rice (run 11); ○, 30 g of uncooked unpolished aromatic red rice and 0.1 g of acid protease preparation (run 12).

TABLE V. Characteristics of rice wines made from cooked and uncooked aromatic red rice wine with and without the preparation of acid protease

Runs	9		10		11		12	
	With cooking		Without cooking		With cooking		Without cooking	
Acid protease preparation	— ^{a)}		○ ^{b)}		—		○	
CO ₂ output (g)	9.1	9.7	9.7	9.9	9.7	9.7	9.9	9.9
Total glucose in feed (g)	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5
Total glucose in fermented broth (g)	0.8	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Consumption of glucose (%)	96	96	96	96	96	96	96	96
pH	3.9	4.0	3.8	4.0	3.8	3.8	4.0	4.0
Acidity (ml)	6.8	8.6	8.3	8.9	8.3	8.3	8.9	8.9

a) ○, Added to the initial mash.
b) —, Not added.

of aromatic red rice and the preparation of acid protease is still unclear. However, amino acids produced by the acid

TABLE VI. Aroma analysis of rice wines made from cooked and uncooked aromatic red rice wine with and without the preparation of acid protease

Runs	9		10		11		12	
	With cooking		Without cooking		With cooking		Without cooking	
Acid protease preparation	— ^{a)}		○ ^{b)}		—		○	
Ethyl alcohol (% v/v)	9.8	10.1	10.1	10.9	10.1	10.1	10.9	10.9
Isobutyl alcohol (ppm)	182	271	293	221	293	293	221	221
Isoamyl alcohol (ppm)	442	384	435	352	435	435	352	352
n-Propyl alcohol (ppm)	22	33	41	41	41	41	41	41
Ethyl acetate (ppm)	152	390	269	428	269	269	428	428
Ethyl lactate (ppm)	1	ND ^{c)}	1	1	1	1	1	1
Isoamyl acetate (ppm)	1	2	3	4	3	3	4	4
Acetaldehyde (ppm)	102	205	108	102	108	108	102	102
Organoleptic test ^{d)}	—		+		++++		++++	

a) ○, Added to the initial mash.

b) —, Not added.

c) ND, Not detected.

d) Organoleptic test: +++++, excellent; +, tolerable; —, bad.

protease or the acid protease itself may be connected directly or indirectly with aroma formation.

Unpolished rice grains were fractionated to yield polished rice and rice bran and used for fermentation tests. The beneficial effects of the acid protease on the aroma, and on the formation of ethyl acetate, in particular, were evident when polished rice was used as the raw material. Thus the polished rice fraction of aromatic red rice grains was affected by the acid protease and the quality of the aromatic red rice wine was improved. However, improvement of the aromatic characteristics of the aromatic red rice wine originated not only in the formation of ethyl acetate but also in other pleiotropic effects.

Acknowledgments. The authors are grateful to the Kumamoto Agricultural Research Institute for generously providing aromatic red rice and to Shinnihon Kagaku Kogyo Co. Ltd. for generously providing the preparations of glucoamylase and acid protease. The authors also thank Bishonen Shuzo Co. Ltd. for support during this study.

REFERENCES

- Bertrand, G. *Bulletin de la Société Chimique de France*, 1906, 35, 1285.
- Fukumoto, J., Tsuru, D. & Yamamoto, T. *Agricultural and Biological Chemistry*, 1967, 31, 710.
- Ishikawa, T. & Nunokawa, Y. *Bulletin of the Agricultural Chemical Society of Japan*, 1970, 44, 21.
- Kuruma, K. & Nunokawa, Y. *Bulletin of the Agricultural Chemical Society of Japan*, 1968, 42, 319.
- Nunokawa, Y., Shiinoki, S., Iwano, K. & Saito, K. *Journal of Brewing Society of Japan*, 1981, 76, 350.
- Ohta, T., Shimojo, H., Hashimoto, K., Kondo, H., Samuta, T. & Ohba, T. *Journal of Brewing Society of Japan*, 1991, 86, 536.
- Takeuchi, I., Shimada, K. & Nakamura, S. *Bulletin of the Agricultural Chemical Society of Japan*, 1968, 42, 33.
- Takeuchi, I., Shimada, K. & Nakamura, S. *Bulletin of the Agricultural Chemical Society of Japan*, 1968, 42, 40.
- Tsuru, D., Yamamoto, T. & Fukumoto, J. *Agricultural and Biological Chemistry*, 1966, 30, 651.
- Ueda, S., Ueki, T., Ohba, R., Teramoto, Y. & Yoshizawa, K. *Journal of Fermentation and Bioengineering*, 1990, 70, 326.
- Ueda, S., Teramoto, Y., Saigusa, N., Ueki, T., Ohba, R. & Yoshizawa, K. *Journal of Fermentation and Bioengineering*, 1991, 72, 173.