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Behaviors of D- and L-Lactic Acids during the Brewing Process of Sake (Japanese Rice Wine)

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The amounts of D- and L-lactic acids during the brewing process of sake were determined by capillary electrophoresis using 2-hydroxypropyl-β-cyclodextrin as a chiral selector. Because L-lactic acid, which prevents the growth of nonuseful microorganisms, is a raw material of sake, the ratio of L-lactic acid to total lactic acid is almost 1.0 at the initial stage of sake brewing. During brewing, the ratio decreased gradually and finally reached 0.39. Yeast (*Saccharomyces cerevisiae*) for sake brewing produced D-lactic acid, but not L-lactic acid in a culture medium. These results suggest that the decrease in the ratio of L-lactic acid to total lactic acid during sake brewing resulted in D-lactic acid production by yeast. The ratios in 18 brands of sake obtained commercially ranged from 0.23 to 0.78. The levels of D-lactic acid in sake (140−274 mg/L) were in a narrower range than those of L-lactic acid (61−461 mg/L). Although the D-lactic acid level in sake did not correspond to total lactic acid level, the L-lactic acid level correlated well with total lactic acid level ($R^2 = 0.867$). These results suggest that the ratio of L-lactic acid to total lactic acid in sake reflected the amount of L-lactic acid added at the initial stage of sake brewing.

KEYWORDS: Enantiomer separation; lactic acid; sake; food analysis; capillary electrophoresis

INTRODUCTION

Sake, Japanese rice wine, is an alcoholic beverage which, like grape wine, is brewed by yeast (*Saccharomyces cerevisiae*). Because rice does not have an abundance of mono- and oligosaccharides and organic acids that are present in grape, the brewing process of sake is significantly different from that of wine. The brewing process of sake is shown in **Figure 1**. The characteristic features of brewing sake are the use of a mold called rice *koji* that saccharifies the rice starch during fermentation by the yeast in parallel. *Koji* produces diastatic enzymes which convert starch to sugar, proteolytic enzymes which break down proteins, and more than fifty other enzymes that are responsible for the flavor and taste of sake. Sake has a good flavor due to the alcoholic and esteric compounds contained therein. Another feature is the use of lactic acid, which prevents abundant growth of nonuseful microorganisms when producing *shubo*, a yeast mash. Whereas naturally occurring lactic acid bacteria are used in the traditional brewing of sake, the use of lactic acid has recently been predominant in order to simplify sake brewing. Lactic acid in sake, as well as in wine, is a major organic acid (*1*) and is thought to have a great influence on the taste. Lactic acid exists in two enantiomeric forms that can be present in various organisms as a metabolite of carbohydrate metabolism. However, there have not been any reports on changes in the levels of L- and D-lactic acids during the sake brewing process despite the significance of lactic acid.

A number of investigations on the analysis of L- and D-lactates have been conducted by using enzymatic reactions specifically catalyzed by L- and D-lactate dehydrogenases (*2, 3*), high performance liquid chromatography (*4*−*9*), thin-layer chromatography (*10*), and capillary gas chromatography (*11, 12*). Capillary electrophoresis (CE) is a recently developed powerful analytical technique with a wide range of applications. The availability of many chiral selectors make CE an important tool for chiral analysis as described in several reviews (*13*−*17*). Recently we have found that the aliphatic α-hydroxy acids (lactic, 2-hydroxybutyric, 2-hydroxy-3-methylbutyric, and 2-hydroxyisocaproic acids) could be included in the cavity of 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD) and were enantioseparated without derivatization by CE using 2HP-β-CD (*18*). Subsequently, we analyzed D- and L-lactic acids in food products such as wine, sake, and yogurt by the CE method using a sample stacking technique successfully (*19*).

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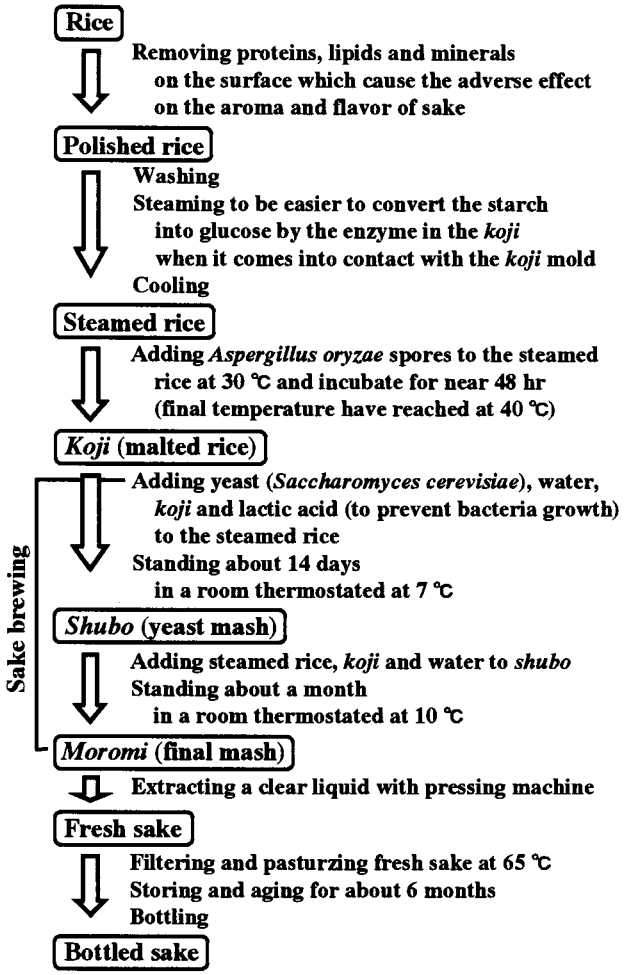

Figure 1. Flow diagram of the steps used to make sake.

The object of the present study is to analyze D- and L-lactic acids during the brewing process of sake by the CE method and to clarify the reason for the changes in concentrations of these acids.

MATERIALS AND METHODS

Chemicals. 2-Hydroxypropyl-β-cyclodextrin (average degree of substitution, 7) and DL-lactic acid lithium salt were obtained from Sigma Chemical Co. L-Lactic acid lithium salt and other chemicals were purchased from Wako Pure Chemical Industries Ltd.

CE Apparatus. Electrophoretic experiments were carried out using a Capillary Electrophoresis System G1600A (Agilent Technologies). Direct chiral resolution of lactic acid in sake was performed as previously reported (*19*). Briefly, the separations were performed in a poly(vinyl alcohol) (PVA)-coated bubble cell capillary of 50 μm i.d. $\times$ 64.5 cm (effective length 56 cm). The capillary was kept at 16 °C. Samples were injected by pressure (50 mbar, for 200 s). The power supply was operated in the constant-voltage mode, at -30 kV. D- and L-Lactic acids migrated toward the positive pole and were detected at 200 nm.

Sake Making Procedure. Sake was made by Fukumitsu-ya, a sake manufacturer, essentially as shown in **Figure 1**. In this process, appropriate amounts of *Aspergillus oryzae* spores were mixed with 15 kg of steamed rice for 52 h. The resulting product, *koji* (malted rice) was mixed with steamed rice (30 kg), L-lactic acid for sake brewing grade (210 g), yeast (*Saccharomyces cerevisiae*, 1.5×10^{11} viable cells), and water (50 L) in a 3-kL tank, and the mixture was allowed to stand for 14 days in a room thermostated at 7 °C. The resulting product, *shubo* (yeast mash), was mixed with steamed rice (520 kg), *koji* (135 kg), and water (1050 L) and was allowed to stand for 36 days in a room thermostated at 10 °C, to yield *moromi* (final mash).

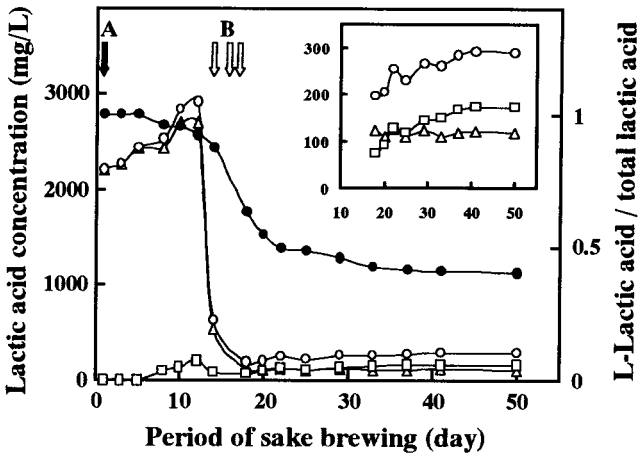

Figure 2. Changes in levels of D- and L-lactic acids during sake brewing. A, Yeast, water, *koji*, and L-lactic acid were added to steamed rice, and sake brewing was started; B, after *shubo* was obtained, steamed rice, koji, and water were added to *shubo*. ○, Total lactic acid; △, L-lactic acid; □, D-lactic acid; ●, the ratio of L-lactic acid to total lactic acid. The inset shows part of the same data with an expanded *y*-axis.

Culture Conditions for Yeast. Yeast was cultured in media (pH 4, 5, 6, and 7) of Yeast Nitrogen Base (Difco, pH 4−7) at 30 °C for 7 days. Then, the medium obtained was centrifuged at 3000 rpm for 5 min, and the supernatant was applied to CE.

Buffer and Sample Preparation. The background electrolyte (BGE) in the electrophoretic experiments was 90 mM phosphate buffer (pH 6.0) containing 240 mM 2-hydroxypropyl-β-cyclodextrin and was filtered with a 0.22-μm filter before use. Purified water was prepared using a Toray Ultrapure Water System.

Stock solutions of 100 mM DL-lactic acid and 100 mM L-lactic acid were individually prepared in purified water, stored at 4 °C and diluted to 1 mM (90 mg/L) and 0.5 mM (45 mg/L), respectively, before use.

Samples obtained at various steps of sake brewing were centrifuged at 3000 rpm for 10 min, and the supernatants were stored at −20 °C. They were thawed before use and diluted the appropriate number of times with water. Then, the diluted samples were applied to CE.

Eighteen brands of sake, whose labels did not show any added acidulants, were purchased from a local market. Sake samples obtained commercially were diluted 10- or 30-fold by water, and the diluted samples were applied to CE.

Levels of Ethanol and α-D-Glucose during Sake Brewing. A sample was distilled, and the distillate was made up to the same volume with water, then ethanol was determined with a hydrometer. α-D-Glucose was measured by HPLC with polarized photometric detection (*20*).

RESULTS AND DISCUSSION

General Features during Sake Brewing Process. Sake is brewed by converting starch to glucose, which is catalyzed by enzymes from *Aspergillus oryzae*, and which occurs in parallel with fermentation of yeast. This is performed at low pH by addition of a large amount of lactic acid. Yeasts are well-known to grow and survive at low pH levels. The pH levels during the sake brewing process in this study were in the range of 3.1 to 4.4. The α-D-glucose level was initially 180 g/L and gradually decreased to 20 g/L at the final stage. As the glucose level decreased, the ethanol level increased, reaching 17.2% (v/v) at the final stage.

Changes in the Levels of D- and L-Lactic Acids during the Sake Brewing Process. The levels of D- and L-lactic acids at each brewing step of sake were analyzed by capillary electrophoresis with 2HP-β-CD as a chiral selector (**Figure 2**). Sake brewing was started by adding yeast, water, *koji*, and L-lactic acid to the steamed rice (**Figure 2**; arrow A). The levels

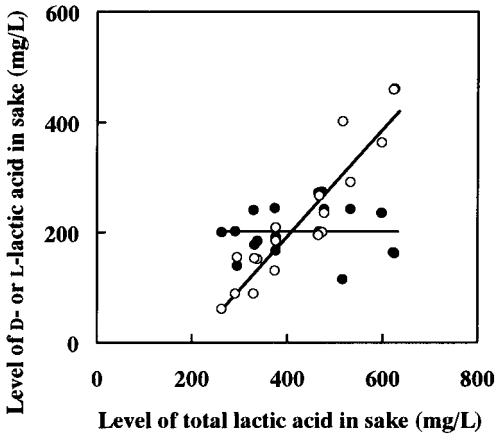

Figure 3. Relationship between the level of total lactic acid and the levels of L- or D-lactic acids in sake obtained commercially: ○, L-lactic acid; ●, D-lactic acid.

of both D- and L-lactic acids gradually increased up to day 12. After steamed rice, *koji*, and water were added three times (at days 14, 16, and 17) to *shubo* (a yeast mash), D- and L-lactic acids were significantly decreased by the dilution (**Figure 2**; arrow B). The D-lactic acid level then showed a small gradual increase up to day 50, whereas the L-lactic acid level remained constant. Because the concentrations of D- and L-lactic acids changed in the above manner during sake brewing, the ratio of L-lactic acid to total lactic acid changed drastically. We used L-lactic acid for sake brewing as a raw material. As D-lactic acid could not be detected at less than 3% in total lactic acid in the CE method, D-lactic acid was not detected until day 4. The ratio decreased significantly from day 14 to day 22 and decreased gradually up to day 50.

Levels of D- and L-Lactic Acid Produced by *Koji* and Yeast. D- and L-Lactic acids in *koji* (a malted rice), which was obtained by mixing the spores of *Aspergillus oryzae* into the steamed rice and allowing it to sit at room temperature (30 °C) for 2 days, were determined by CE. The levels of L- and D-lactic acids were at 5.0 mg/kg and 10.5 mg/kg, respectively. Thus, the ratio of L-lactic acid to total lactic acid was 0.32. The total amount of *koji* used during sake brewing was 150 kg and the levels of L- and D-lactic acids contained in *koji* were 0.75 and 1.5 g, respectively. Therefore, the levels of L- and D-lactic acids contained in *koji* were too low to affect those in sake.

We also studied the production of lactate by yeast using a culture medium without lactate. Only D-lactic acid was detected, with a level of 59.4 mg/L. The detection of only D-lactic acid and its level were unaffected when the pH of the medium was varied over the range 4 to 7. Hara (*21*) reported that yeast for sake brewing produced significantly more D-lactic acid than L-lactic acid and could consume L-lactic acid.Although D-lactic acid increased gradually up to day 50, it was not observed that L-lactic acid decreased during the final step (days 18−50) of sake brewing. These results suggest that the changes in D-lactic acid level during the final step were attributed to D-lactate production by yeast.

Determination of D- and L-Lactic Acid in Sake. D- and L-Lactic acids in 18 brands of sake obtained commercially were determined (**Figure 3**). The ratio of L-lactic acid to total lactic acid in 18 brands of sake ranged from 0.23 to 0.78. The levels of D-lactic acid in sake (140−274 mg/L) were in a narrower range than those of L-lactic acid (61−461 mg/L). The regression equation for the relationship between the amount of total lactic acid and that of the D-lactic acid enantiomer in sake was $y = 0.016x + 196$ with a correlation coefficient (R^2) of 0.002. Unlike the D-lactic acid level, the L-lactic acid level corresponded well with the total lactic acid. The regression equation for the relationship between the amount of total lactic acid and that of the L-lactic acid enantiomer in sake was $y = 0.984x - 196$ with a correlation coefficient (R^2) of 0.867. If it is postulated that all of these commercial brands of sake were essentially made by the procedure shown in **Figure 1**, the amount of L-lactic acid in sake can be attributed to the L-lactic acid added at the initial stage of brewing, and the amount of D-lactic acid can be attributed to the D-lactic acid production by yeast during sake brewing. If these assumptions are correct, the ratio of L-lactic acid to total lactic acid in sake reflects the amount of L-lactic acid initially added to make *shubo*.

The ratio of L-lactic acid to total lactic acid varied among commercial brands of sake. However, it remains unclear how the amount of L-lactic acid added at the start of brewing affects the quality of sake. Considering lactic acid is a major organic acid, it seems that lactic acid can have a great effect on the taste of sake. In our preliminary study, solutions of 500 mg/L D- and L-lactic acids (pH 4.2) were individually prepared in purified water. As a result, we judged that D-lactic acid in water has a different sour taste from L-lactic acid. Sensory studies are needed to relate the effects of D- and L-lactic acids to the organoleptic properties of sake.

CONCLUSION

Direct chiral resolution of lactic acid in sake was performed by CE using 2-HP-β-CD. During the sake brewing, the ratio of L-lactic acid to total lactic acid decreased gradually. This suggests that this decrease depended on the amount of D-lactic acid production by yeast. The levels of D-lactic acid in sake obtained commercially (140−274 mg/L) were in a narrower range than those of L-lactic acid (61−461 mg/L). The L-lactic acid level, but not D-lactic acid level, correlated well with the total lactic acid level ($R^2 = 0.867$). These results suggest that the amount of L-lactic acid in sake can be attributed to the L-lactic acid added at the initial stage of brewing, and that the amount of D-lactic acid can be attributed to the D-lactic acid production by yeast during sake brewing.

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