Enzyme Inactivation and Quality Preservation of Sake by High-Pressure Carbonation at a Moderate Temperature

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Received May 16, 2007; Accepted September 27, 2007; Online Publication, January 7, 2008
[doi:10.1271/bbb.70297]

The effect of a high-pressure carbonation treatment on the change in quality of sake during storage was investigated. Measurements of the amino acidity and isovaleraldehyde content of carbonated sake (20 MPa pressure at 40, 45 and 50 °C for 7, 21 and 33 min, respectively) as well as of heat-treated sake (reaching temperature of 65 °C and immediately cooled) were almost unchanged during storage at 3 and 20 °C. Glucose in the sake subjected to these treatments was retained at an almost constant under the same storage conditions, except for the sake carbonated at 40 °C and stored at 20 °C. In contrast, the amino acidity, and glucose and isovaleraldehyde contents of non-pasteurized (fresh) sake increased during storage at both temperatures. The sake samples subjected to the carbonation treatment and heat treatment both gave better sensory scores than the fresh sake sample after 6 month of storage at 3 and 20 °C, especially at 3 °C for the flavor. These results suggest that the high-pressure carbonation treatment is an effective new technique for preserving the quality of sake.

Key words: sake; high-pressure carbonation; quality change in storage; inactivation of enzymes; non-pasteurized sake

Materials and Methods

Sake. Non-pasteurized sake was brewed with 50% polished rice at the Food Technology Research Center of the Hiroshima Prefectural Technology Research Institute and was stored at −20 °C until needed.

High-pressure carbonation treatment. Continuous treatment of sake was carried out with the apparatus described in our previous paper12) with a small modification. The temperature of the CO₂-dissolving vessel was maintained at 5 ± 0.5 °C. The sample and CO₂ were
heated to a given temperature by a heating coil, before being retained for a given time in a residence coil. A cooling unit (−20 °C) was attached after a pressure control valve to prevent the volatile components from vaporizing. Three treatment conditions were set based on the results of previous studies12,13 (Table 1). The average residence time was calculated from the flow rate of the sake and CO₂.

**Heat treatment.** Two hundred ml of non-pasteurized sake was put into a 200-ml glass bottle. The bottle was heated in a hot-water bath at 90 °C. When the temperature of sake had reached 65 °C, it was immediately cooled in an ice bath.¹⁹

**Storage of the sake.** Each sample was stored at 3 °C and 20 °C for 6 months in a 200-ml bottle with a tightly fitted screw cap. After the storage, it was frozen at −70 °C until needed for the analyses and sensory evaluation.

**Measurement of the enzymatic activity.** The activities of α-glucosidase and glucoamylase were measured with an α-glucosidase and glucoamylase assay kit. The activity of α-amylase was measured with an α-amylase assay kit, and the activity of acid carboxypeptidase was measured with an acid carboxypeptidase assay kit. All kits were purchased from Kikkoman Corporation (Noda, Japan). The activities of these enzymes, except for α-glucosidase, are expressed as units by the official methods of the National Tax Agency of Japan.¹⁴ One unit of α-glucosidase activity is defined as the amount producing 1 μmole of 4-nitro phenol in 1 min. The activity of isoamyl alcohol oxidase was measured by the method of Yamashita et al.,¹⁴ 100 ml of a sample being concentrated to 1 ml by ultrafiltration (Centriprep YM-10, Millipore Corporation, Massachusetts, USA) for the measurement. One unit of isoamyl alcohol oxidase activity is defined as the amount producing 1 μmole of isovaleraldehyde (3-methyl-1-butanal) in 60 min. The residual activities of these enzymes are defined as the residual percentage of activity after the treatment.

**Measurements of the general components.** Sake meter was measured with a specific gravity meter DA520 (Kyoto Electronics, Kyoto, Japan). The alcohol content, acidity, and amino acidity were measured by the official methods of the National Tax Agency of Japan.¹⁵

<table>
<thead>
<tr>
<th>Experiments</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>50</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Residence time (min)</td>
<td>7</td>
<td>21</td>
<td>33</td>
</tr>
<tr>
<td>CO₂ feeding ratio (g/ml)</td>
<td>0.17</td>
<td>0.14</td>
<td>0.15</td>
</tr>
</tbody>
</table>

All treatments were carried out at 20 MPa.

Analyses of sugars, organic acids, free amino acids and flavor components. Sugars (glucose and isomaltose) were analyzed by HPLC. Separation was carried out by Shim-pack CLC-NH₂ (Shimadzu, Kyoto, Japan), using 75% acetonitrile as the mobile phase at 40 °C. The sugar components were measured by a refractive index detector (RID 6A, Shimadzu). Organic acids were analyzed by capillary electrophoresis as described by Soga et al.,¹⁶ and free amino acids were evaluated with an amino acid analyzer (835, Hitachi, Tokyo, Japan).

Flavor components, except for isovaleraldehyde, were analyzed by the head-space method described in the official method of the National Tax Agency of Japan.¹⁷ Isovaleraldehyde, which is the off-flavor component in sake and produced by isoamyl alcohol oxidase from koji,³⁴ was analyzed by the method described in the previous paper.¹⁸ One hundred ml of a sample was passed through porous polymer beads (Porapak®, type Q, 50–80 mesh, Waters Corporation, Massachusetts, USA) packed in a column. The beads were washed with 30 ml of deionized water, and then isovaleraldehyde was eluted with 60 ml of diethyl ether. After adding 10 μl of a 1% cyclohexanol solution as an internal standard, the eluate was dehydrated on anhydrous sodium sulfate and concentrated to about 200 μl. Isovaleraldehyde in the concentrate was determined by capillary GC.

Sensory evaluation. A sensory evaluation was conducted by a panel of 5 experts. The panel evaluated the flavor, taste and total quality of the sake according to five levels (1, very good; 2, good; 3, normal; 4, bad; 5, very bad).

**Results and Discussion**

Enzymatic activities in sake after high-pressure carbonation

Table 2 shows the enzymatic activities after the treatment by high-pressure carbonation. The activities of five enzymes except α-glucosidase at 40 °C and with a residence time of 33 min (experiment 3), had decreased to less than 6% of the activity in non-pasteurized (untreated) sake. The residual activity of α-glucosidase in the sake in experiment 3 was 29%. We have reported that α-glucosidase in fresh sake was the most stable enzyme against high-pressure carbonation and heat treatment.¹²,¹³ We presume that the enzyme inactivation in sake might be due to irreversible denaturation of the enzyme protein, that is, decomposition of the α-helix resulting from dissolving CO₂ under high pressure, as described in the previous paper.¹²

General components of sake after high-pressure carbonation

Table 3 shows the general components of sake after the treatment by high-pressure carbonation. The measurements of sake meter and alcohol content of the carbonated sake were slightly lower than those of the
heat-treated and non-pasteurized samples. These results suggest that such volatile compounds as ethanol could have been removed along with CO$_2$ gas in the depressurization process. On the other hand, the acidity and amino acidity were unchanged after both the treatments.

**Sugars, organic acids, free amino acids and flavor components of sake after high-pressure carbonation**

The concentrations of the sugars (glucose and isomaltose) in sake were not influenced by the high-pressure carbonation treatment (Fig. 1). In addition, the free amino acids, organic acids (citrate, malate, succinate, pyruvate and lactate) were also unchanged after the high-pressure carbonation treatment (data not shown).

Table 4 shows the concentrations of the flavor components in sake after the high-pressure carbonation treatment. The concentrations of ethyl acetate, isoamyl acetate, and ethyl capronate were decreased to 40–78% of the untreated levels by the carbonation treatment. On the other hand, these compounds were almost unchanged after the heat treatment. Isobutyl alcohol and

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### Table 2. Enzymatic Activities in Sake after Treatment by High-Pressure Carbonation

<table>
<thead>
<tr>
<th>Enzymatic activity (units/ml)</th>
<th>α-Glucosidase</th>
<th>Glucoamylase</th>
<th>α-Amylase</th>
<th>Acid carboxypeptidase</th>
<th>Isoamyl alcohol oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>0.28 × 10$^{-1}$ (3)$^b$</td>
<td>N.D.$^c$</td>
<td>N.D.</td>
<td>0.05 × 10$^2$ (3)</td>
<td>0.02 × 10$^{-4}$ (1)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.49 × 10$^{-1}$ (6)</td>
<td>0.14 (2)</td>
<td>N.D.</td>
<td>0.02 × 10$^2$ (1)</td>
<td>0.05 × 10$^{-4}$ (1)</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>2.47 × 10$^{-1}$ (29)</td>
<td>0.09 (1)</td>
<td>N.D.</td>
<td>0.05 × 10$^2$ (3)</td>
<td>0.01 × 10$^{-4}$ (0)</td>
</tr>
<tr>
<td>Heat treatment$^d$</td>
<td>0.07 × 10$^{-1}$ (1)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.01 × 10$^{-4}$ (0)</td>
<td>3.54 × 10$^{-4}$</td>
</tr>
<tr>
<td>Non-pasteurized sake</td>
<td>8.53 × 10$^{-1}$</td>
<td>9.22</td>
<td>1.09 × 10$^1$</td>
<td>1.52 × 10$^2$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Treatment conditions are shown in Table 1.

$^b$Parentheses indicate the residual activity (%) after the treatment.

$^c$N.D. indicates “not detected.”

$^d$The sake sample was heated to 65°C and then immediately cooled in an ice bath.

### Table 3. General Components of the Sake Samples after the Treatments by High-Pressure Carbonation

<table>
<thead>
<tr>
<th></th>
<th>Sake meter</th>
<th>Alcohol (%)</th>
<th>Acidity (ml)</th>
<th>Amino acidity (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>+5.8</td>
<td>17.1</td>
<td>1.04</td>
<td>0.94</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>+6.0</td>
<td>17.0</td>
<td>1.05</td>
<td>0.96</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>+6.1</td>
<td>16.9</td>
<td>1.05</td>
<td>0.94</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>+6.8</td>
<td>17.4</td>
<td>1.04</td>
<td>0.96</td>
</tr>
<tr>
<td>Non-pasteurized sake</td>
<td>+7.0</td>
<td>17.4</td>
<td>1.04</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Treatment conditions are shown in Table 1.

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**Fig. 1.** Concentration Changes in Sugar Components in Sake Subjected to High-Pressure Carbonation during Storage at 3°C and 20°C.  
A, glucose; B, isomaltose. Symbols: ○, experiment 1; △, experiment 2; □, experiment 3; ●, heat treatment; ■, non-pasteurized sake.  
Treatment conditions are shown in Table 1.
isoamyl alcohol were not influenced by either of these treatments. These results are in accord with the decrease in the measurements of sake meter and alcohol level after the carbonation treatment (Table 3). Further investigations are needed to elucidate this; for example, the recirculation of CO$_2$ in a closed system could lead to a resolution of the problem.

Changes in general components during storage

Figure 2 shows the change in amino acidity of sake during storage at 3 °C and 20 °C. The amino acidity of both the heat-treated and carbonated sake samples remained almost stable during storage at both these temperatures. On the other hand, the amino acidity of the non-pasteurized sake increased during storage. Amino acids are umami compounds and important for the pleasant taste of sake. However, it is considered that an excessive amount of amino acids in sake has a negative effect on the quality. For example, the amino acids cause undesirable tastes such as zatsumi$^{20}$ and amadare.$^{21,22}$ In addition, amino acidity that is negatively correlated with the total quality of sake is shown as one of important quality indices for sake.$^{23,24}$ The increase in amino acidity of the non-pasteurized sake in this study suggests a deterioration in the quality, this increase resulting from the activity of acid carboxypeptidase in the sake (Table 2).

The measurements of sake meter, alcohol level, and acidity of the sake samples subjected to the carbonation treatment were stable during storage at 3 °C and 20 °C, except for the acidity which decreased slightly in every sample during storage at 20 °C (data not shown).

![Fig. 2. Changes in the Amino Acidity of Sake Subjected to High-Pressure Carbonation during Storage at 3 °C and 20 °C. Symbols are the same as those in Fig. 1, and treatment conditions are shown in Table 1.](image)

Changes in sugar components during storage

Figure 1 shows the concentrations of glucose and isomaltose in sake during storage at 3 °C and 20 °C. The glucose content in both the carbonated and heat-treated
sake samples was stable during storage at both these temperatures, except for the sake in experiment 3 stored at 20 °C. The glucose content in the non-pasteurized sake sample increased considerably even during storage at 3 °C. These results were caused by the differences in the activities of α-glucosidase and glucoamylase (Table 2). The isomaltose content in the carbonated sake was stable during storage at 3 °C, but decreased when the sake in experiments 2 and 3 was stored at 20 °C. The isomaltose content in the non-pasteurized sake decreased during storage at both these temperatures. The increase in glucose and the decrease in isomaltose would depend on the activity of α-glucosidase, although enzymes which were not investigated in the present study might have been involved in these effects. An excessive amount of glucose in sake causes an undesirable taste such as amadare.2125 It is thus suggested that the increase in glucose during storage led to a deterioration in its quality.

Changes in free amino acids during storage

Figure 3 shows the contents of total free amino acids, aspartic acid, glutamine, and ammonia in sake during storage at 3 °C and 20 °C. The contents of total free amino acids, aspartic acid, and ammonia in both the carbonated and heat-treated sake samples remained almost unchanged under these storage conditions. The total free amino acids content in the non-pasteurized sake increased during storage at 20 °C, while the aspartic acid and ammonia contents in the non-pasteurized sake significantly increased during storage at both the temperatures. Glutamine in every sample of sake decreased during storage at 20 °C. However, the degree of the decrease of glutamine in the non-pasteurized sake was smaller than that of both the carbonated and heat-treated sake samples. These results are reasonably consistent with the behavior of the amino acidity in each sake sample during storage (Fig. 2). In addition, the quantitative behavior of the free amino acids would have been due to the residual activity of acid carboxypeptidase in each sample (Table 2).

Formation of isovaleraldehyde during storage

Figure 4 shows the formation of isovaleraldehyde in the sake samples during storage at 3 °C and 20 °C. The level of isovaleraldehyde did not increase in either the carbonated or the heat-treated sake sample. On the other hand, it increased considerably in the non-pasteurized sake sample during storage. The formation of isovaleraldehyde was attributable to the activity of isoamyl alcohol oxidase (Table 2). It is consequently suggested that the high-pressure carbonation treatment was able to depress the unacceptable change in the taste and flavor of sake during storage.

Sensory evaluation

Table 5 shows the results of the sensory evaluation of the sake samples before storage. The evaluation of the carbonated sake for flavor and total quality was slightly worse than that of the heat-treated and non-pasteurized sake samples. The evaluation of the taste except for the non-pasteurized sake was almost the same. These results are in accordance with the quantitative measurements of the flavor components, sugars, organic acids, and free amino acids after each treatment (Tables 3 and 4, and Fig. 1). The poorer evaluation of the carbonated sake samples for flavor and total quality is attributed to the loss of volatile flavor components. This problem with the present treatment is mirrored by the results of the sensory evaluation, and further investigation is needed to overcome the limitation.

Table 6 shows the results of the sensory evaluation after 6 months of storage. The flavor and total quality of the non-pasteurized sake was markedly degraded during storage at 3 °C and 20 °C. On the other hand, the flavor...
and total quality of both the carbonated and heat-treated sake samples were retained fairly well during storage at both temperatures, especially at 3°C for the flavor. These results correspond with the levels of sugars, free amino acids, and isovaleraldehyde in each sake sample during storage. It is consequently suggested that the high-pressure carbonation treatment would be an effective new technique for preserving the quality of sake at normal temperatures.

References


Table 6. Sensory Scores for the High-Pressure Carbonated Sake Samples after 6 Months of Storage

<table>
<thead>
<tr>
<th>Storage temperature Evaluation</th>
<th>3°C</th>
<th>20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>2.4 ± 1.1</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>2.2 ± 1.1</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>2.6 ± 1.5</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>2.0 ± 1.0</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Non-pasteurized sake</td>
<td>4.0 ± 1.4</td>
<td>2.8 ± 0.8</td>
</tr>
</tbody>
</table>

| **Taste**                     |     |      |
| Experiment 1                  | 2.4 ± 1.1 | 2.4 ± 0.5 | 2.4 ± 0.9 | 3.0 ± 0.7 | 2.2 ± 0.4 | 2.8 ± 0.8 |
| Experiment 2                  | 2.2 ± 1.1 | 2.2 ± 0.4 | 2.6 ± 0.5 | 2.8 ± 0.4 | 2.4 ± 0.5 | 2.6 ± 0.5 |
| Experiment 3                  | 2.6 ± 1.5 | 3.0 ± 0.7 | 3.0 ± 1.4 | 2.8 ± 0.4 | 2.8 ± 0.8 | 2.6 ± 0.5 |
| Heat treatment                | 2.0 ± 1.0 | 2.0 ± 0.7 | 2.2 ± 0.8 | 2.8 ± 1.3 | 2.8 ± 1.1 | 2.8 ± 1.1 |
| Non-pasteurized sake          | 4.0 ± 1.4 | 2.8 ± 0.8 | 3.8 ± 1.3 | 4.2 ± 1.3 | 3.6 ± 1.1 | 4.0 ± 1.2 |

Treatment conditions are shown in Table 1.
Sensory scores are those defined in Table 5.

