Purification and Characterization of Isoamyl Alcohol Oxidase ("Mureka"-Forming Enzyme)

Nobuo YAMASHITA, Toru MOTOYOSHI, and Akira NISHIMURA

Research and Development Department, Hakutsuru Sake Brewing Co. Ltd., 4-5-5, Sumiyoshiminamimachi, Higashinada-Ku, Kobe 658-0041, Japan

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Isoamyl alcohol oxidase (IAAOD) was purified to apparent homogeneity on SDS-PAGE from ultrafiltration (UF) concentrated sake. IAAOD was a glycoprotein, a monomeric protein with an apparent molecular mass of 73 and 87 kDa, by SDS-PAGE and gel filtration on HPLC, respectively.

IAAOD showed high substrate specificity toward C₅ branched-chain alkyl alcohol (isoamyl alcohol), and no activity toward shorter (C₃-C₄) or longer (C₇-C₆₇) alkyl alcohols tested. IAAOD was stable between pH 3.0-6.0 at 25°C. The optimum pH was 4.5 at 35°C. Heavy metal ions, p-chloromercuribenzoate (PCMB), hydrazine, and hydroxylamine strongly inhibited the enzyme activity, and an anti-oxidant like 1-ascorbate did also.

Isovaleraldehyde was produced markedly in pasteurized sake by adding purified IAAOD, therefore, we conclude that it was the enzyme that causes formation of mureka, an off-flavor of sake, the main component of which is isovaleraldehyde.

Key words: alcohol oxidase; off-flavor enzyme; mureka; Aspergillus oryzae

An unfavorable and characteristic smell is often formed in non-pasteurized sake (nama-shu) during storage and commercial distribution at room temperature. This smell is called mureka and its main component is isovaleraldehyde (3-methyl-1-butanal).[^1] It is important to prevent mureka formation in sake quality control, because it deteriorates the quality of sake remarkably. Formation of mureka is usually prevented by ultrafiltration (UF) or pasteurization (hi-ire) of sake,[^2-3] suggesting that this compound is formed enzymatically.

We already reported that isovaleraldehyde in sake was produced from isoamyl alcohol (3-methyl-1-butanol) by isoamyl alcohol oxidase (IAAOD) of a koji mold, Aspergillus oryzae.[^4]

On fungal primary alcohol oxidase, many reports have been published. Primary alcohol oxidase of methanol-using yeast (Hansenula polymorpha)[^5], Candida sp.[^6-11] and Pichia sp.[^12,13] have been most studied at the enzymatic or molecular genetic level. These yeast primary alcohol oxidases show high activity toward relatively short chain alkyl alcohols like methanol or ethanol and less activity with increasing length of the alkyl chain. In addition, primary alcohol oxidases, which show a wide range of substrate specificity toward aliphatic primary alcohols from C₄ to C₁₈, were found in yeasts or filamentous fungi.[[^14-17]]

Compared with these known primary alcohol oxidases, IAAOD showed a completely different substrate specificity in the results of examination with its partial-purified preparation.[^6] It showed the highest activity toward isoamyl alcohol among some alcohols tested. Because of its unique substrate specificity, it was suggested to be a novel alcohol oxidase.

However, little is known about its enzymatic properties or physiological function, because the purified enzyme was not obtained. In this paper, we describe the purification and some properties of IAAOD as a novel alcohol oxidase that is responsible for forming the off-flavor of sake.

Materials and Methods

Chemicals. SP-Toyopearl, butyl-Toyopearl, and Toyopearl HW55S were from Tosoh Co., Tokyo. Leuco patent blue violet (LPBV) and horseradish peroxidase (POD) were from Sigma Co. All the other chemicals were of special grade.

Enzyme assays. We employed one of two assay methods for measuring the enzyme activity of IAAOD according to the purpose of the experiment, as follows.

Assay of isovaleraldehyde producing activity (IVP assay). The reaction mixture (1 ml) contained 200 mM sodium acetate buffer (pH 4.5), 40 mM isoamyl alcohol (added as DMSO solution), and the enzyme solution. The reaction mixture was incubated in a 10-ml vial at 35°C for 2 h. The reaction was stopped by heat treatment (100°C, 2 min), and 10 ppm (final concentration) of 3-pentanol was added as an internal standard. The amount of isovaleraldehyde produced was measured by head space gas chromatography (HSGC). One unit was defined as the amount that produced 1 μmole of isovaleraldehyde in 60 min.

[^1]: To whom correspondence should be addressed. Nobuo YAMASHITA, FAX: +81-78-822-4832; E-mail: yamashita@hakutsuru.co.jp
[^2]: Abbreviations: IAAOD, isoamyl alcohol oxidase; UF, ultra filtration; A, Aspergillus; DMSO, dimethyl sulfoxide; SP, sulfopropyl; IVP assay, assay of isovaleraldehyde producing activity; LPBV, leuco patent blue violet; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride, GC-MS, gas chromatography and mass spectrometry.
Characterization of Novel Alcohol Oxidase

Assay of alcohol oxidase activity (LPBV assay). Alcohol oxidase activity was measured by a slight modification of the LPBV/POD method of Nishimura et al.\(^2\) The reaction mixture (1 ml) contained 50 mM PIPES-NaOH buffer (pH 6.5), 0.2 mM LPBV, 50 U POD, 40 mM isovaleryl alcohol (added as DMSO solution), and the enzyme solution. The reaction mixture was incubated at 35°C for 1 h, then the color produced was measured spectrophotometrically at 639 nm. One unit was defined as the amount that produced 1 μmole of H₂O₂ in 60 min. Protein was measured using a Bio-rad protein assay kit (Bio-rad Co., Ltd) with bovine serum albumin as a standard.

Electrophoresis. SDS-PAGE was done with a slab of 5–20% gradient polyacrylamide gel by the method of Laemmli.\(^3\) The gel was stained for protein bands using Coomassie brilliant blue R-250. Isoelectric focusing (IEF) was done on a Ampholine PAGplate (Amersham Pharmacia Biotech) using an electrophoresis apparatus Multiphor II (Amersham Pharmacia Biotech). Protein bands were stained as described for SDS-PAGE.

Enzyme purification. All steps of the purification were done at 5°C except for Step 1. The enzyme activities were monitored by an IVP assay.

Step 1. Crude enzyme preparation. The UF concentrated and cut off sake fraction was prepared by an actual scale ultrafiltration machine used in the sake brewery. This fraction was further concentrated to approximately 400-fold of freshly brewed sake and its solvent (sake) was changed to 20 mM sodium acetate buffer (pH 4.5) by a pilot scale ultrafiltration machine (SHINKO PANTEC Co., Ltd, Hyogo) with a UF module FS03-AFC-FUS0353 (Cut-off molecular mass 30,000, DAICEL CHEMICALS, Tokyo). By these process, we obtained a concentrated fraction containing the proteins with molecular masses larger than 30,000. This fraction was used as a crude enzyme preparation.

Step 2. SP-Toyopearl 550C cation exchange column chromatography. The crude enzyme preparation was put on a SP-Toyopearl 550C column (5.0×26 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.5). Elution was done by a linear gradient of NaCl (0–0.2 M) at a flow rate of 2.5 ml/min.

Step 3. Butyl-Toyopearl 650M hydrophobic interaction column chromatography. The active fractions from the previous step were collected and saturated by addition of ammonium sulfate to 40% saturation and put on a butyl-Toyopearl 650M column (2.5×42 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.5) containing 40% saturation of ammonium sulfate. Elution was done by a linear gradient of ammonium sulfate (40–0% saturation) at a flow rate of 1.5 ml/min.

Step 4. SP-Toyopearl 650S cation exchange column chromatography. The active fractions obtained in step 3 were collected and dialyzed against 20 mM sodium acetate buffer (pH 4.3) for 16 h under gentle stirring. The dialyzed solution was put on a SP-Toyopearl 650S column (2.5×54 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.3). Elution was done by a linear gradient of NaCl (0–0.2 M) at a flow rate of 1.2 ml/min (Fig. 1).

Step 5. Toyopearl HW55S gel filtration column chromatography. The most active fraction No. 62 (15 ml) obtained in step 4 (Fig. 1) was concentrated to 2 ml by ultrafiltration, and put on a Toyopearl HW55S column (2.5×93 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.5) containing 0.2 M NaCl. Gel filtration was done with the same buffer at a flow rate of 0.7 ml/ min (Fig. 2).

Molecular mass estimation. Molecular mass was estimated by SDS-PAGE (described above) and by gel filtration on HPLC. The purified enzyme solution (20 μl con-
taining 40 µg protein) was put on a HPLC system (600E, Waters) on a G-3000SWXL protein column (0.78φ × 30 cm, Tosoh). Elution was done with 50 mm potassium phosphate buffer (pH 7.0) containing 0.3 m NaCl at a flow rate of 0.7 ml/min. The absorbance of the effluent was recorded at 280 nm.

**Deglycosylation treatment.** Purified IAAOD was deglycosylated with a GLYCOPRO DEGLYCOSYLATION KIT (Prozyme Inc.) containing peptide N-glycosidase, endo-

**Km calculation.** The Km of the enzyme for isomyl alcohol was calculated using Lineweaver-Burk plots. The enzyme activity was measured by an IVP assay with various concentrations of isomyl alcohol.

**HSGC analysis.** A Shimazu GC-17A gas chromatograph with a flame ionization detector (FID) and a 0.52 mm i.d. × 30 m fused silica capillary column coated with DB-5 (J&W SCIENCE, INC.) was used. The column temperature was programmed to keep at 35°C for 15 min, then rise to 240°C at 25°C/min. The temperatures of the injection port and detector were 120°C and 280°C respectively. Helium was used as a carrier gas, flowing at a rate of 0.47 ml/min, and the split ratio was 1/10. The sample was injected after heating at 90°C for 15 min.

**GC-MS analysis.** A Hewlett-Packard 5970 GC-MS spectrometer was used. The ionizing voltage was 70 eV. The GC conditions were almost the same as those described by Nishimura et al. 1)

**N-Terminal amino acid sequence analysis.** Purified IAAOD (approximately 100 pmol) was transferred to a PVDF membrane using ProSorb (PE Applied Biosystems) and the N-terminal amino acids were sequenced with a gas-phase protein sequencer (PPSQ-10, Shimadzu, Kyoto).

**Results and Discussion**

**Purification of IAAOD**

IAAOD was produced by a koji mold, A. oryzae. Therefore, we examined that the culture supernatant of A. oryzae strain, Takadiastase and rice-koji extract were used as crude enzyme sources besides UF-concentrated sake. UF-concentrated sake had a high specific activity and protein concentration (Table 1). This shows that a large amount of rice-koji, approximately 100 kg, is required to obtain the identical total activity of IAAOD contained in 1 l of UF-concentrated sake. This indicates that although very little IAAOD is produced by A. oryzae during koji-making, it is likely to remain prominent among various proteins in sake and is highly concentrated with fractionating from relative small proteins by the UF process. As a result, we adopted UF-concentrated sake as the enzyme source because of the advantage of convenience in collecting large amounts of IAAOD.

IAAOD was purified 34-fold with a yield of 1.2% from the crude enzyme preparation as described in Materials and Methods (Table 2). The purified IAAOD had a specific activity of 229 munits/mg of protein on the IVP assay. The purified preparation showed a single band on SDS-PAGE as well as IEF, indicating an apparent homogeneity of the protein (Fig. 3A, lane 2; Fig. 4, lane 2).

**Molecular mass, carbohydrate content and isoelectric point**

The molecular mass of IAAOD was estimated to be 73 kDa on SDS-PAGE, and it was shifted to 59 kDa by deglycosylation (Fig. 3A). Therefore, the carbohydrate content of the purified IAAOD was estimated to be about 20% on the average. This value corresponded to the value measured by the phenol-sulfuric method with mannose as a standard. Complete deglycosylation was observed only by the cooperative treatment with 3 types of glycosidases (peptide N-glycosidase, endo-O-glycosidase, and sialidase A), suggesting N-linked and O-linked sugar chains both are associated with IAAOD.

Although we tried to remove the sugar chain from native purified enzyme to examine the changes in some enzymatic properties by deglycosylation, it resulted in failure. Strong denaturing treatment was necessary to remove the sugar chains. (In this treatment condition, IAAOD was completely inactivated.) But in general, high glycosylation of enzyme protein increases water solubility or pH stability. 20,21) The sugar chains associated with IAAOD may assist the increase of solubility in sake and pH stability in acid region.

### Table 1. Evaluation of Enzyme Sources for Purification

<table>
<thead>
<tr>
<th>Resource</th>
<th>mU/ml Ratio</th>
<th>Protein activity (mg/ml)</th>
<th>Specific activity (mU/mg)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>0.01</td>
<td>1</td>
<td>0.02</td>
<td>0.50</td>
</tr>
<tr>
<td>5% Takadiastase solution</td>
<td>0.04</td>
<td>4</td>
<td>3.36</td>
<td>0.01</td>
</tr>
<tr>
<td>Rice-koji extract</td>
<td>0.12</td>
<td>12</td>
<td>0.15</td>
<td>0.80</td>
</tr>
<tr>
<td>UF-concentrated sake</td>
<td>61.51</td>
<td>6151</td>
<td>6.39</td>
<td>9.62</td>
</tr>
</tbody>
</table>

* Enzyme activity was measured by IVP assay as described in Materials and Methods.

### Table 2. Summary of IAAOD Purification

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (mU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (mU/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>97820</td>
<td>12580</td>
<td>6.8</td>
<td>1</td>
</tr>
<tr>
<td>SP-Toyopearl 550C</td>
<td>26934</td>
<td>1978</td>
<td>26</td>
<td>3.82</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650M</td>
<td>12171</td>
<td>125</td>
<td>97</td>
<td>14.3</td>
</tr>
<tr>
<td>SP-Toyopearl 650S</td>
<td>1970</td>
<td>12</td>
<td>164</td>
<td>24.1</td>
</tr>
<tr>
<td>Toyopearl HW 55S</td>
<td>1145</td>
<td>5</td>
<td>229</td>
<td>33.7</td>
</tr>
</tbody>
</table>

* Enzyme activity was measured by IVP assay described in Materials and Methods.
Characterization of Novel Alcohol Oxidase

Gel filtration on HPLC showed that the native molecular mass of the enzyme was 87 kDa (Fig. 3B). These findings indicate that IAAOD was a monomeric protein and show that the usual UF process (cut off molecular mass, 30,000) is effective for removing IAAOD from non-pasteurized sake.

Primary alcohol oxidases of methanol-using yeasts are large molecules with subunits structure, for example, the identical octameric subunits structure for Hanse nula polymorpha,6 and Candida boidinii,7 or the identical tetrameric subunits structure for Pichia sp.8 These yeast alcohol oxidases are all intracellular enzymes. In contrast, IAAOD was considered to be an extracellular enzyme because of the probability of N-linked sugar chain association (described above) and the result of the liquid culture experiment on the A. oryzae industrial strain. In this experiment, IAAOD activity of the culture supernatant was already detected at logarithmic phase and was maximum at stationary phase. In addition, total and specific activities of the culture supernatant both were much higher than those of the cell-free extract throughout the culture period (data not shown). This result indicates that IAAOD is a secretory protein.

Figure 4 shows the isoelectric point of IAAOD was 4.61, so this enzyme is considered to be an acidic protein.

Substrate specificity

Table 3 shows the substrate specificity of purified IAAOD. All alkyl alcohols tested were added to the reaction mixture of LPBV assay as DMSO solution to 40 mm (final concentration). The purified IAAOD showed high substrate specificity toward C1 branched-chain alkyl alcohol (isoamy alcohol) and very weak activity toward C2 normal-chain (n-amyl alcohol), C6 normal-chain (n-hexanol), and C6 branched-chain (i-hexanol) but no activity toward more shorter (C1–C4) or longer (C7–C10) length alkyl alcohols tested.

Yeast primary alcohol oxidase shows high activity toward short chain alkyl alcohols like methanol or...
Table 3. Substrate Specificity of IAAOD

<table>
<thead>
<tr>
<th>Substrate (40 mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>0</td>
</tr>
<tr>
<td>ethanol</td>
<td>0</td>
</tr>
<tr>
<td>n-propanol</td>
<td>0</td>
</tr>
<tr>
<td>i-propanol</td>
<td>0</td>
</tr>
<tr>
<td>n-butanol</td>
<td>0</td>
</tr>
<tr>
<td>i-butanol</td>
<td>0</td>
</tr>
<tr>
<td>n-amyl alcohol</td>
<td>4.1</td>
</tr>
<tr>
<td>i-amyl alcohol</td>
<td>100</td>
</tr>
<tr>
<td>n-hexanol</td>
<td>4.9</td>
</tr>
<tr>
<td>i-hexanol</td>
<td>5.9</td>
</tr>
<tr>
<td>n-heptanol</td>
<td>0</td>
</tr>
<tr>
<td>n-octanol</td>
<td>0</td>
</tr>
<tr>
<td>n-nonanol</td>
<td>0</td>
</tr>
<tr>
<td>n-decanol</td>
<td>0</td>
</tr>
</tbody>
</table>

ethanol and a fairly wide range of substrate specificity. On the other hand, IAAOD showed a very narrow substrate specificity. We have ever guessed that the physiological function of IAAOD is mainly detoxification by oxidative degradation of middle chain alkyl alcohols produced during koji-making. But the narrow substrate specificity of IAAOD is partially inconsistent with our speculation, because IAAOD showed no activity toward isobutyl alcohol (C₄) which also is produced like isoamyl alcohol during koji-making. Anyway, further study is need to clarify the reason for the unique substrate specificity of IAAOD.

In addition, the Kₘ for isoamyl alcohol was 30 mM. Freshly brewed sake generally contains 2.5–3.5 mM isoamyl alcohol, so suggesting that the velocity of enzyme reaction increase with increasing isoamyl alcohol concentration in sake if other conditions are almost the same.

Effects of pH on IAAOD and thermal stability

Figure 5A shows the effects of pH on the enzyme activity. The optimum pH of IAAOD was 4.5. Figure 5B shows IAAOD was stable between pH 3.0–6.0, and significantly unstable in the alkaline region. Almost all the alcohol oxidases ever reported had optimum pH within the neutral or alkaline regions and were unstable in the acid region. In contrast, the pH characteristics of IAAOD were unique for alcohol oxidases. These findings indicate that IAAOD is hardly inactivated and able to act in sake the pH of which is 4.2–4.5.

IAAOD was completely inactivated by treatment at 70°C for 15 min (Fig. 6). This result supports the usual conditions for the pasteurization of sake are effective for inactivation of IAAOD.

Effects of metal ions and chemical reagents

The enzyme activity was strongly inhibited by Cu²⁺, Ag⁺, and Cd²⁺ and completely by Hg²⁺. It also was strongly inhibited by PCMB, hydrazine and hydroxylamine. (Table 4) These findings suggest that sulfhydryl and carbonyl groups exist in the catalytic site of the enzyme and are important in the enzyme reaction. Strong inhibition by anti-oxidants like 1-ascorbate or methabisulfate directly indicate that the purified enzyme is exactly an oxidase.

Presumption on the prosthetic group of IAAOD

The absorption spectrum of purified enzyme had two absorption maxima at 354 and 438 nm (Fig. 7), suggesting that IAAOD is a flavoprotein. Although we tried to liberate of flavin compounds with denaturing enzymes, this resulted in failure. This suggests that flavin compounds bind covalently to the protein, but further study was need to clarify the identity of the prosthetic group of IAAOD.
Characterization of Novel Alcohol Oxidase

Table 4. Effects of Metal Salts and Chemical Reagent on IAAOD Activity

<table>
<thead>
<tr>
<th>Material (1 mM)</th>
<th>Relative activity (%)</th>
<th>Reagent (1 mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>LiCl</td>
<td>105</td>
<td>PCMB</td>
<td>1.9</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>103</td>
<td>DTTN²</td>
<td>100</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>100</td>
<td>Iodoacetate</td>
<td>106</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>189</td>
<td>NaN₃</td>
<td>83</td>
</tr>
<tr>
<td>FeCl₂</td>
<td>26</td>
<td>o-Phenanthroline</td>
<td>97</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>77</td>
<td>EDTA</td>
<td>65</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>0.7</td>
<td>L-Ascorbate</td>
<td>0</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>27</td>
<td>Metabisulfate</td>
<td>12</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>4.9</td>
<td>Hydrazine</td>
<td>7.6</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0</td>
<td>Hydroxylamine</td>
<td>8.9</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>2.2</td>
<td>PMSF²</td>
<td>100</td>
</tr>
</tbody>
</table>

The enzyme was measured by LPBV assay after incubation with a metal salt or a reagent for 5 min.

Fig. 6. Effects of Temperature on Stability of IAAOD.
The residual enzyme activity was measured by IVP assay after incubation of portions of the enzyme in 200 mM sodium acetate buffer (pH 4.5) at 20-70°C for 15 min. The concentration of enzyme in reaction mixture was 0.01 units/ml on IVP-assay.

Fig. 7. Absorption Spectra of IAAOD.
Solid line: native purified IAAOD (0.4 mg/ml); broken line: native purified IAAOD (0.4 mg/ml) + 50 mM sodium hydrosulfite.

Fig. 8. Production of Isovaleraldehyde in Sake by Purified IAAOD.
A, B: Chromatogram of extract from stored sake. A, Extract of sake stored at 30°C for 4d after addition of purified IAAOD; B, Extract of sake stored with no addition (negative control).
C, D: Mass spectra. C, peak 1 in Fig. 6A; D, authentic isovaleraldehyde (3-methyl-1-butanal).

Formation of isovaleraldehyde in sake by purified IAAOD

The purified IAAOD solution (50 µl, 0.025 units) was added to 1 ml of pasteurized sake containing 18% ethanol, and stored steriley at 30°C for 4 d. A negative control with no addition of the purified enzyme solution was done in the same manner. After storage, volatile compounds in sake were extracted with ethyl acetate and put into a GC-MS system. In the test sample, a very large peak at the retention time of 8.76 min (Fig. 8A, peak 1) was observed, but it was only a trace in the negative control sample (Fig. 8B). Figure 8C shows the mass spectra of peak 1. It was the same pattern as that of authentic isovaleraldehyde shown in Fig. 8D. This indicates that isovaleraldehyde in sake increased markedly during storage upon addition of the purified enzyme, therefore we concluded the purified protein (IAAOD) is the enzyme that causes formation of mureka, the main component of which is isovaleraldehyde.

N-Terminal amino acid sequence of IAAOD

The N-Terminal sequence obtained from the purified
IAAOD was Ala-Asp-Ser-Ser-Ser (ADSSS). We searched for a similar sequence in a non-redundant protein data base bank accessed by the BLAST service at NCBI, but no similar sequence was found.

Cloning and analysis of IAAOD gene will enable us to obtain further detailed information on this enzyme. These studies are now being undertaken.

Acknowledgments
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References