Journal of Chromatography B, 879 (2011) 3259–3267

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

High-performance liquid chromatography analysis of naturally occurring D-amino acids in sake☆

Yoshitaka Gogami, Kaori Okada, Tadao Oikawa*

Department of Life Science and Biotechnology, Faculty of Chemistry, Materials, and Bioengineering, Kansai University, 3-3-35 Yamate-Cho, Suita, Osaka-Fu 564-8680, Japan

ARTICLE INFO

Article history:
Received 29 November 2010
Accepted 4 April 2011
Available online 13 April 2011

Keywords:
D-Amino acid
Sake
Chiral derivatization
HPLC
Lactic acid bacteria
Yeast

ABSTRACT

We measured all of the D- and L-amino acids in 141 bottles of sakes using HPLC. We used two precolumn derivatization methods of amino acid enantiomer detection with *o*-phthalaldehyde and *N*-acetyl-L-cysteine, as well as (+)-1-(9-fluorenyl)ethyl chloroformate/1-aminoadamantane and one postcolumn derivatization method with *o*-phthalaldehyde and *N*-acetyl-L-cysteine. We found that the sakes contained the D-amino acids forms of Ala, Asn, Asp, Arg, Glu, Gln, His, Ile, Leu, Lys, Ser, Tyr, Val, Phe, and Pro. We were not able to detect D-Met, D-Thr D-Trp in any of the sakes analyzed. The most abundant D-Ala, D-Asp, and D-Glu ranged from 66.9 to 524.3 μM corresponding to relative 34.4, 12.0, and 14.6% D-enantiomer. The basic parameters that generally determine the taste of sake such as the sake meter value (SMV; "*Nihonshudo*"), acidity ("*Sando*"), amino acid value ("*Aminosando*"), alcohol content by volume, and rice species of raw material show no significant relationship to the D-amino acid content of sake. The brewing water ("*Shikomimizu*") and brewing process had effects on the D-amino acid content of the sakes: the D-amino acid contents of the sakes brewed with deep-sea water "*Kaiyoushinosousui*", "*Kimoto yeast starter*", "*Yamahaimoto*", and the long aging process "*Choukijukusei*" are high compared with those of other sakes analyzed. Additionally, the D-amino acid content of sakes that were brewed with the adenine auxotroph of sake yeast ("*Sekishoku seishu kobo*", *Saccharomyces cerevisiae*) without pasteurization ("*Hiire*") increased after storage at 25 °C for three months.

1. Introduction

Sake is a naturally brewed alcoholic beverage from Japan. Sake is similar to beer and wine, but it is made from rice [1]. The origins of sake go back thousands of years [2]. The sake brewing process is unique because rice starch undergoes simultaneous fermentation by "*Koji*" fungi (*Aspergillus oryzae*) [3] and "*Shubo*" yeast (*Saccharomyces cerevisiae*) [4], while beer [5] and wine [5] go through two separate processes. Today Japanese food is being integrated into Western diets for its exotic taste and healthy benefits [6]. Sake is one type of Japanese food that is being integrated into diets all over the world [7].

D-Amino acids have been detected in many foods, including vegetables [8], fruits [8], milk [9], orange juice [10], beer [11], wine [12], and cocoa [13], using high-performance liquid chromatography (HPLC) [14], gas chromatography (GC) [15], and capillary electrophoresis (CE) [16]. Recently, D-serine was found to play a significant role in the mammalian central nervous system, and D-aspartate was found to have a role in endocrine and/or neuroendocrine organs [17,18]. In addition, D-alanine is located in the islets of Langerhans in the rat pancreas and therefore might participate in the regulation of mammalian blood-glucose levels [19]. Accordingly, we have focused a great deal of attention on detecting D-amino acids in foods to help clarify their effects on human health [20].

In this study, we succeeded in measuring all of the D- and L-amino acids in sake using HPLC with a protocol that combined two precolumn derivatization methods of amino acid enantiomer detection, OPA-NAC (*o*-phthalaldehyde and *N*-acetyl-L-cysteine) and FLEC/ADAM ((+)-1-(9-fluorenyl)ethyl chloroformate/1-aminoadamantane), and one postcolumn derivatization method with OPA-NAC. We describe the quantity and distribution of D-amino acids in 141 sake products and speculate on the possible D-amino acid production mechanisms in sake.

2. Experimental

2.1. High-performance liquid chromatography

High-performance liquid chromatography analysis was performed with a Shimadzu HPLC prominence system (Simadzu,

☆ This paper is part of the special issue "Analysis and Biological Relevance of D-Amino Acids and Related Compounds", Kenji Hamase (Guest Editor).

* Corresponding author. Tel.: +81 6 6368 0812; fax: +81 6 6388 8609.
E-mail address: oikawa@kansai-u.ac.jp (T. Oikawa).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.04.006

Kyoto, Japan) equipped with an RF-10A$_{XL}$ fluorescence detector. The D- and L-amino acids in sake were measured with precolumn derivatization with OPA and NAC according to the method of Asward [21]. The column used was a Develosil ODS-UG-5 (ϕ6.0 × 250 mm, Nomura Chemical Co., LTD. Seto, Japan) maintained at 40 °C. The mobile phase A was 50 mM sodium acetate buffer, and the mobile phase B was methanol. The HPLC program used for elution of D- and L-amino acids was as follows: (0 min, B% = 0); (16 min, B% = 24); (24 min, B% = 24); (29 min, B% = 40); (50 min, B% = 40); (69 min, B% = 67). Elution was carried out at a flow rate of 1.2 ml/min and monitored with fluorescence detector at excitation and emission wavelengths of 340 and 450 nm, respectively.

Enantiomers of asparagine, histidine, lysine, and proline in sake were measured with two elution modes of precolumn derivatization with FLEC and ADAM according to a modified method of Einarsson and Josefsson [22]. Asparagine, histidine, and lysine were separated by gradient elution mode, and proline was by isocratic elution mode. Each sample (10 μl) was mixed with a 0.5 M borate buffer, pH 9.0 (10 μl), and then a 1 mM FLEC-acetone solution (20 μl) was added. The mixture was incubated at 40 °C for 30 min allowing the reaction to reach completion. A 40 mM ADAM solution (25 μl) was added to the reaction mixture to remove excess FLEC. After 7 min, a 50 mM sodium acetate buffer, pH 4.0 (35 μl), was added to stop the derivatization reaction. A portion of the reaction mixture (5 μl) was subjected to HPLC analysis. Elution was carried out at a flow rate of 0.75 ml/min for both elution modes and monitored with fluorescence detector at excitation and emission wavelengths of 263 and 313 nm, respectively.

The cysteine in sake was measured as DL-cysteine by the postcolumn derivatization method of amino acid with OPA and NAC according to Ishida et al. [23].

An Shim-pac amino-Na column (ϕ6.0 × 100 mm, Simadzu, Kyoto, Japan) was used as a main column for separation of DL-cysteine and an Shim-pack ISC-30 (ϕ4.0 × 50 mm, Simadzu, Kyoto, Japan) was used as a guard column to remove the ammonia in mobile phase. The mobile phase A was a 20 mM sodium citrate buffer (pH 3.2) containing 7% ethanol, and the mobile phase B was 600 mM sodium citrate containing 200 mM borate (pH 10.0), and the mobile phase C was 200 mM sodium hydrate. The amino acid eluted from a main column was reacted with the reagent buffer containing 384 mM sodium carbonate, 216 mM boric acid, 108 mM potassium sulfide, and subsequently with the OPA reagent solution containing 0.08%(w/v) OPA, 1.40%(v/v) ethanol, 0.04%(w/v) Brij-35, 0.10%(w/v) NAC. Elution was carried out at a flow rate of 0.6 ml/min with fluorescence detector at excitation and emission wavelengths of 340 and 450 nm, respectively.

Quantification of amino acid enantiomers was carried out with external standard calibrations. We made a calibration curve for each derivatization method to eliminate the effect of different fluorescence intensities between three derivatization methods. Sample was diluted at least 100 times with ultrapure water (MilliQ) and almost all background noise derived from interfering substances disappeared. Each sample was measured at least three times, and the replicates were averaged. The limits of quantitative detection of D-amino acids using HPLC ranged from 0.006 μM to 20 μM for the OPA-NAC precolumn derivatization method, 0.01 μM to 20 μM for the FLEC/ADAM precolumn derivatization method, and 1.5 μM (DL-Cys) for the OPA-NAC postcolumn derivatization method. The relative amounts of the D-amino acids were calculated according to Eq. (1):

$$\%D = 100 \times \frac{A_D}{A_D + A_L}, \quad (1)$$

where %*D* is the relative amount of the D-enantiomer and A_D and A_L are the peak areas of the D- or L-enantiomer, respectively.

Table 1
The differences between specific classes of sakes.

Specific class	Ingredient	Polishing ratio	Use of rice koji
"*Daiginjo*"	Rice, rice koji, alcohol	50% or less	15% or more
"*Junmai Daiginjo*"	Rice, rice koji	50% or less	15% or more
"*Ginjo*"	Rice, rice koji, alcohol	60% or less	15% or more
"*Junmai Ginjo*"	Rice, rice koji	60% or less	15% or more
"*Special Honjozo*"	Rice, rice koji, alcohol	60% or less	15% or more
"*Special Junmai*"	Rice, rice koji	60% or less	15% or more
"*Honjozo*"	Rice, rice koji, alcohol	70% or less	15% or more
"*Junmai*"	Rice, rice koji	Not specified	15% or more
"*Futuu*"	Not specified	Not specified	Not specified

2.2. Reagents and chemicals

2.2.1. Sakes

Sakes (141 bottles produced at 51 prefectures in Japan) were purchased from randomly chosen manufacturers. The specific classes of sakes purchased were "*Daiginjo* (*n* = 9)", "*Junmai Daiginjo* (*n* = 14)", "*Ginjo* (*n* = 9)", "*Junmai Ginjo* (*n* = 42)", "*Special Honjozo* (*n* = 2)", "*Special Junmai* (*n* = 9)", "*Honjozo* (*n* = 15)", "*Junmai* (*n* = 35)", and "*Futuu* (*n* = 6)". The differences between these specific classes of sakes are summarized in Table 1. After three months stored at 25 °C, all of the sakes were reanalyzed by the methods described above.

2.2.2. Chemicals

(+)-1-(9-Fluorenyl) ethyl chloroformate (FLEC) and 1-aminoadamantane (ADAM) were purchased from Sigma–Aldrich (St. Louis, MO, USA). *o*-Phthalaldehyde (OPA) and *N*-acetyl-L-cysteine (NAC) were purchased from Wako Chemical Co., LTD. (Osaka, Japan). HPLC-grade sodium acetate, methanol, acetonitrile, and tetrahydrofuran were purchased from Kanto Chemical Co., LTD. (Tokyo, Japan). D- and L-amino acids were purchased from Wako Chemical Co., LTD. and Watanabe Chemical Co., LTD. (Hiroshima, Japan). All of the other reagents used were of the best grade commercially available. Ultrapure water (MilliQ) produced by Synergy UV from Nihon Millipore K.K. (Tokyo, Japan) was used throughout the course of this study.

3. Results and discussion

We succeeded in measuring all of the D- and L-amino acids in sake using HPLC analysis with two precolumn derivatization methods, OPA-NAC (Fig. 1A-b) and FLEC/ADAM (Fig. 1B-b, C-b), and one postcolumn derivatization method with OPA-NAC (Fig. 1D-b).

We found that the sakes contain D-amino acid enantiomers of Ala, Asn, Asp, Arg, Glu, Gln, His, Ile, Leu, Lys, Ser, Tyr, Val, Phe, and Pro. Interestingly, we were not able to detect D-Met, D-Thr or D-Trp in any of the sakes analyzed. The relative rates of the numbers of sakes that contained each D-amino acid to the total number of sakes analyzed ($R_D\% = 100 \times n/N$; *n*, number of sakes that contained each D-amino acid; *N* = 141, total number of sakes analyzed) were D-Asp (97.8%), D-Ala (96.4%), D-Arg (82.9%), D-Pro (71.4%), D-Glu (66.4%), D-Val (65.7%), D-Lys (60.7%), D-Phe (59.9%), D-Ile (58.4%), D-His (52.1%), D-Asn (51.4%), D-Tyr (51.1%), D-Gln (33.8%), D-Leu (4.4%), D-Ser (2.5%), D-Met (0%), D-Thr (0%), and D-Trp (0%). In contrast, all of the sakes contained almost all of the L-amino acids. The relative rates of numbers of sakes that contained each L-amino acid to the total number of sakes analyzed ($R_L\% = 100 \times n/N$; *n*, number of sakes that contained each L-amino acid; *N* = 141, total number of sakes analyzed) were L-Ala (100%), L-Asn (100%), L-Arg (100%), L-Asp (100%), L-His (100%), L-Met (100%), L-Thr (100%), L-Phe (99.3%), L-Val (99.3%), L-Ser (99.3%), L-Gln (99.2%), L-Pro (99.2%), L-Glu (99.0%), L-Ile (97.8%), L-Lys (97.1%), L-Leu (88.3%), L-Tyr (75.9%), L-Trp (99.3%) and DL-Cys (75.6%). All of the sakes contained Gly.

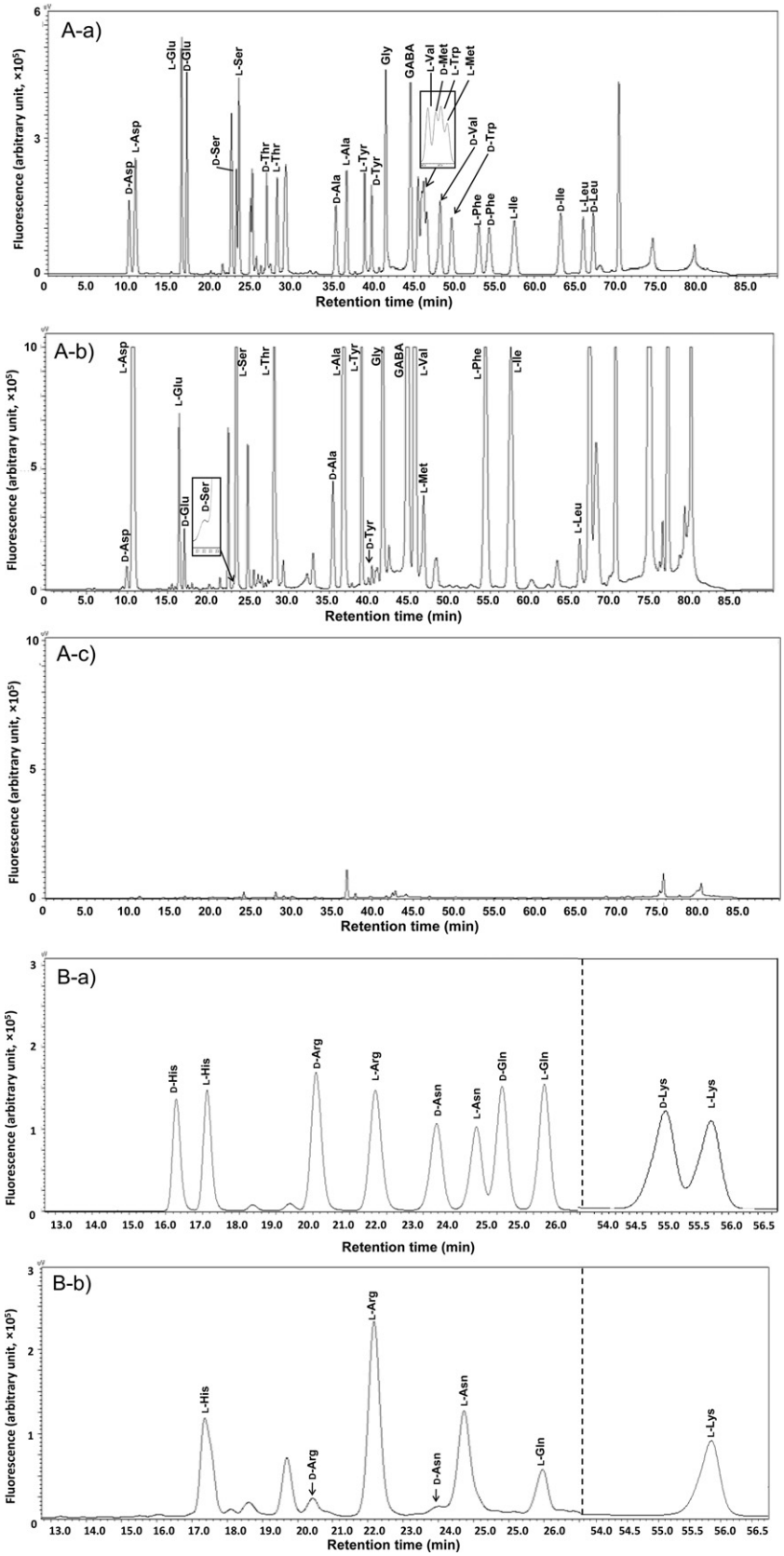

Fig. 1. Detection of D- and L-amino acids in sake using HPLC. The example analysis was done with sample: No. 4401, 5 μl. (A) Detection of D- and L-forms of Ala, Asp, Glu, Ile, Leu, Phe, Ser, Thr, Trp, Tyr, Val, and Gly with the *o*-phthalaldehyde and *N*-acetyl-L-cysteine precolumn derivatization methods. (a) 10 μM standard, (b) sample, and (c) blank. (B) Detection of D- and L-forms of Arg, Asn, Gln, His, and Lys with the (+)-1-(9-fluorenyl)ethyl chloroformate/1-aminoadamantane precolumn derivatization method. (a) 25 μM standard, (b) sample, and (c) blank. (C) Detection of D- and L-Pro with the (+)-1-(9-fluorenyl)ethyl chloroformate/1-aminoadamantane precolumn derivatization method. (a) 25 μM standard, (b) sample, and (c) blank. (D) Detection of D- and L-Cys with the *o*-phthalaldehyde and *N*-acetyl-L-cysteine postcolumn derivatization method. (a) 100 μM standard, (b) sample, and (c) blank.

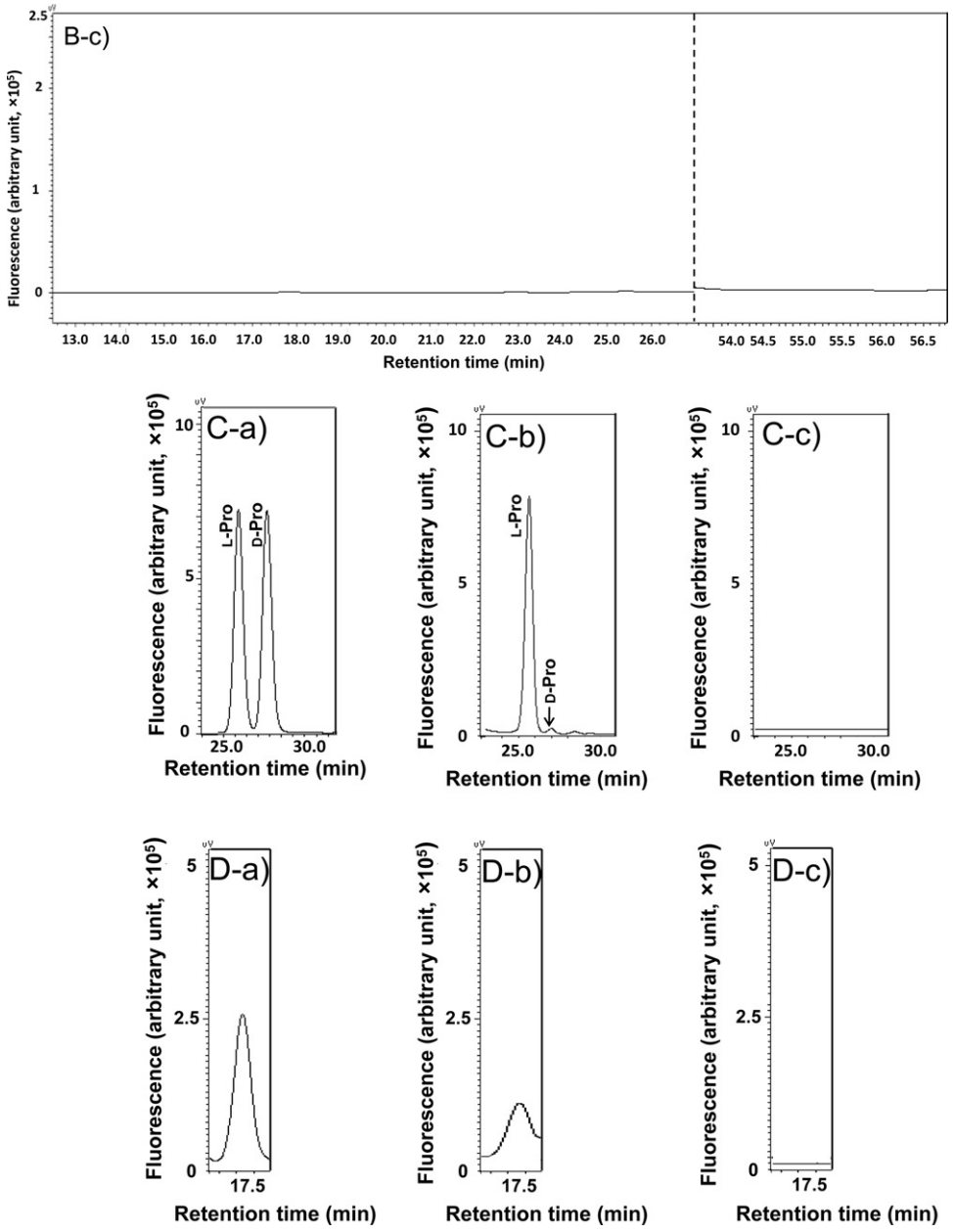

Fig. 1. (*Continued*)

To determine the factors that affect the D-amino acid content of sake, we compared the basic properties of the twenty sakes with the highest and the lowest amounts of D-amino acids (Tables 2 and 3). As shown in Table 2, the sakes that contain high concentrations of D-amino acids are not in the same class. The basic parameters that generally determine the taste of sake such as the sake meter value (SMV; "*Nihonshudo*"), acidity ("*Sando*"), amino acid value ("*Aminosando*"), polishing ration ("*Seimaibuai*"), alcohol content by volume, and rice species, have no significant correlations with the D-amino acid contents of sake. However, the brewing water and brewing process did have effects on the D-amino acid content of the sakes. The D-amino acid contents of the sakes brewed with deep-sea water "*Kaiyoushinsousui*" (No. 2903, No. 3901), "*Kimoto yeast starter*" (No. 4401, No. 0204, No. 3303, No. 4701), "*Yamahaimoto*" (No. 2204), and the long aging process "*Choukijukusei*" (No. 0802, No. 1401, No. 1303) are higher than those of the other sakes analyzed.

Table 4 shows the amino acid content of various sakes brewed with deep-sea water. Compared with the D-amino acid content of sakes brewed without deep-sea water, the sakes brewed with deep-sea water contained higher amounts of D-Tyr. Hard water that contains a large concentration of minerals, like deep-sea water, is known to enhance the fermentation process [24]. Deep-sea water was reported to increase the transcript levels of several genes involved in amino acid metabolism in yeast during sake brewing [24]. We analyzed deep-sea water used as brewing water of sample No. 2903, but we were not able to detect any D-amino acid (data not shown). Hence, deep-sea water is effective in increasing the content of D-amino acids, especially D-Tyr, in sake. The biosynthetic pathway of D-Tyr in microorganism has not been clarified in details, but some microorganism is known to produce D-amino acids during wine brewing [25]. The minerals in deep-sea water may stimulate growth of microorganism.

Table 2
The basic properties of the twenty sakes with the highest amount of D-amino acids.

Rank	Area of production	No.	Specific class	SMV value "*Nihonsyudo*"	Acidity "*Sando*"	Amino acid value "*Aminosando*"	Polishing ratio (%) "*Seimaibuai*"	Alcohol content (%)	Rice species	Remark
1	Chiba	4401	"*Junmai*"	−38	8	4.9	90–93	6–15	"*Koshihikari*"	"*Kimoto yeast starter*"
2	Toyama	2903	"*Honjozo*"	5	1.3	1.2	55	15–16	–	Deep-sea water
3	Mie	0204	"*Junmai Ginjo*"	2	1.7	1.4	53	16–17	"*Yamadanishiki*"	"*Kimoto yeast starter*"
4	Ishikawa	3901	"*Honjozo*"	2	1.3	–	68	14–15	"*Gohaykumangoku*"	Deep-sea water
5	Tottori	3303	"*Junmai*"	6.5	2.3	–	60	16.2	"*Yamadanishiki*"	"*Kimoto yeast starter*"
6	Oita	3403	"*Honjozo*"	−1	1.2	–	65	15–16	–	–
7	Ehime	2105	"*Junmai Ginjo*"	4.5	1.9	–	60	15–16	"*Shizukuhime*"	–
8	Wakayama	4503	"*Junmai Ginjo*"	4	1.7	2.3	55–60	18	"*Yamadanishiki*"	"*Kyoukai Kobo* (No. 9)"
9	Ibaraki	0802	"*Special Honjozo*"	5	1.5	–	55	15.5	"*Miyamanishiki*"	"*Choukijukusei*"
10	Nagasaki	3502	"*Junmai*"	0	1.3	1.2	60	15–16	–	Subsoil water in Saga
11	Gifu	1401	"*Junmai Ginjo*"	10	1.4	1.1	45	15–16	"*Miyamanishiki*"	"*Choukijukusei*"
12	Oita	3402	"*Junmai*"	−2	1.4	–	60	15–16	–	–
13	Wakayama	4502	"*Junmai Ginjo*"	0	1.5	2	55–60	18	"*Bizenomachi*"	"*Kyoukai Kobo* (No. 9)"
14	Ehime	2104	"*Junmai Ginjo*"	−61	2	–	65	9.5	–	"*Sekishoku Seishu Kobo*"
15	Osaka	4701	"*Special Junmai*"	8	2.1	1.3	70	18–19	"*Yamadanishiki*"	"*Kimoto yeast starter*"
16	Yamanashi	2502	"*Junmai*"	5	1.7	–	60	15–16	"*Gyokuei*"	–
17	Saitama	1303	"*Junmai*"	6	1.6	–	55	15–16	"*Gohyakumangoku*"	"*Choukijukusei*"
18	Fukushima	4901	"*Junmai*"	−50	8	2.5	60	8.2	"*Gohyakumangoku*"	"*Sekishoku Seishu Kobo*"
19	Aomori	2003	"*Special Junmai*"	3	1.5	–	55	15–16	"*Hanafubuki*"	–
20	Shimane	2204	"*Junmai Ginjo*"	3	1.6	–	55	15–16	"*Yamadanishiki*"	"*Yamahaimoto*"

Table 3
The basic properties of the twenty sakes with the lowest amount of D-amino acids.

Rank	Area of production	No.	Specific class	SMV value "*Nihonsyudo*"	Acidity "*Sando*"	Amino acid value "*Aminosando*"	Polishing ratio (%) "*Seimaibuai*"	Alcohol content (%)	Rice species	Remark
121	Ibaraki	4101	"*Junmai*"	4	1.4	1.2	60	15–16	"*Hitachinishiki*"	Subsoil water in Tsukuba
123	Hokkaido	0701	"*Daiginjo*"	5	1.2	1.1	40	15–16	"*Ginpu*"	–
124	Mie	0207	"*Junmai Ginjo*"	3	1.4	1.1	55	16–17	"*Yamadanishiki*"	–
125	Tochigi	4301	"*Junmai Ginjo*"	4	1.4	0.9	55	16–17	"*Wakamizu*"	–
126	Tottori	3302	"*Junmai*"	9.5	2.1	1.3	80	ND	"*Gouriki*"	–
127	Tokyo	1102	"*Junmai Ginjo*"	1	1.6–1.8	1.2–1.4	55	15–16	"*Gohyakumangoku*" "*Miyamanishiki*"	–
128	Siga	0501	"*Junmai Daiginjo*"	5	1.4	1	50	16–17	"*Yamadanishiki*"	"*Sekishoku Seishu Kobo*"
129	Nagano	0303	"*Ginjo*"	−2	1.6	1.1	55	18	"*Miyamanishiki*", 80% "*Hitogokochi*"	"*Kyoukai Kobo* (No. 7)"
130	Gifu	1402	"*Junmai Daiginjo*"	13	1.5	0.8	40	15–16	"*Yamadanishiki*"	–
131	Fukushima	0901	"*Junmai Daiginjo*"	2	1.3	1.1	50	15		"*Kimoto yeast starter*"
132	Tochigi	4302	"*Daiginjo*"	3	1.3	0.8	35	17–18	"*Yamadanishiki*"	–
133	Hiroshima	0603	"*Futuu*"	1.5	1.2	1.2	–	15–16	"*Hattannishiki*"	Barreled sake
134	Mie	0202	"*Daiginjo*"	3	1.2	0.9	40	16–17	–	–
135	Hiroshima	0602	"*Ginjo*"	2	1.3	1.2	60	15–16	–	–
136	Hiroshima	0601	"*Daiginjo*"	3.5	1.2	1	38	16–17	"*Yamadanishiki*" "*Sennbonnishiki*"	–
137	Hyogo	0101	"*Junmai Ginjo*"	4.5	1.5	1.2	60	16	"*Yamadanishiki*" "*Nihonbare*"	–
138	Ibaragi	0803	"*Honjozo*"	3	1.4	1.4	70	15–16	–	–
139	Siga	0503	"*Junmai*"	5	1.4	1.1	70	15–16	"Ancient black rice"	–
140	Saitama	1301	"*Junmai Ginjo*"	6	1.6	–	50	16–17	"*Yamadanishiki*"	"*Kyoukai Kobo* (No. 7)"
141	Hiroshima	0604	"*Honjozo*"	3	1.3	1.2	69	14–15	"*Hattannishiki*"	"*Namasake*"

Table 4
Amino acid content of various sakes brewed with deep-sea water.

No.	2903			2905			3801			3901			4001			4002		
AA	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D
Asp	275	46.9	14.6	178	4.2	2.3	116	1.7	1.4	376	15.1	3.9	196	2.2	1.1	195	2.3	1.2
Glu	568	20.9	3.5	395	3.1	0.8	231	ND	–	676	9.8	1.4	549	2.6	0.5	466	1.6	0.3
Asn	275	5.9	2.1	297	9.8	3.2	293	ND	–	1770	5.2	0.3	457	6.5	1.4	386	3.5	0.9
Ser	375	6.6	1.7	253	ND	–	235	ND	–	726	3.2	0.4	560	6.9	1.2	622	0.7	0.1
Gln	10	ND	–	21	ND	–	11	0.7	5.9	69	0.8	1.1	308	ND	–	161	4.4	2.7
Thr	165	ND	–	106	ND	–	533	ND	–	604	ND	–	831	ND	–	353	ND	–
Gly	308	–	–	231	–	–	250		0.0	332		0.0	140		–	253		0.0
His	216	ND	–	179	ND	–	221	ND	–	431	ND	–	223	ND	–	177	7.2	3.9
Ala	1000	524.3	34.4	922	75.3	7.6	780	75.3	8.8	1130	171.8	13.2	1122	93.5	7.7	1048	84.3	7.4
Arg	933	4.9	0.5	801	21.8	2.6	743	16.9	2.2	268	64.7	19.5	894	10.2	1.1	724	24.7	3.3
Tyr	186.4	54.9	22.8	148.5	59.1	28.5	127.1	35.5	21.8	391.3	23.4	5.6	177.5	44.3	20.0	132.4	106.8	44.6
Val	13	3.1	19.3	24	ND	–	20	ND	–	9	ND	–	55	ND	–	25	ND	–
Met	35	ND	–	40	ND	–	28	ND	–	28	ND	–	23	ND	–	7	ND	–
Trp	32.1	ND	–	16.9	ND	–	17.9	ND	–	15.9	ND	–	55.1	ND	–	35.1	ND	–
Phe	133	ND	–	111	ND	–	77	ND	–	382	ND	–	166	ND	–	114	ND	–
Ile	80	ND	–	27	ND	–	13	ND	–	220	ND	–	32	ND	–	38	ND	–
Leu	10	ND	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND	–
Lys	187	0.4	0.2	165	1.4	0.9	11	ND	–	445	0.6	0.1	9	1.0	10.0	41	ND	–
Cys	ND	–	–	ND	–	–	ND		–	ND		–	26		–	18		–
Pro	961	ND	–	608	ND	–	660	ND	–	1020	1.1	0.1	871	ND	–	709	ND	–

Table 5
Amino acid content of various in sakes brewed with "*Kimoto yeast started*", "*Yamahaimoto*", and "*Chokizyukusei*".

No.	4401			0204			3303			4701			2204			0802			1401			1303		
AA	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D	L-AA (μmol/l)	D-AA (μmol/l)	%D
Asp	695	18.1	2.5	490	66.9	12.0	650	6.8	1.0	906	7.7	0.8	298	33	10.0	555	33.8	5.74	278	21	7.02	619	9.4	1.50
Glu	771	132.0	14.6	927	38.3	4.0	715	11.4	1.6	809	33.3	4.0	612	16	2.5	1232	ND	–	588	9	1.51	736	11.7	1.56
Asn	1298	6.0	0.5	316	ND	–	663	7.4	1.1	204	3.9	1.9	762	7.5	1.0	536	2.8	0.52	468	ND	–	319	ND	–
Ser	1228	11.6	0.9	1554	ND	–	1183	ND	–	815	0.7	0.1	465	1	0.2	775	ND	–	493	ND	–	730	ND	–
Gln	958	ND	–	26	ND	–	42	ND	–	176	7.2	3.9	106	ND	–	40	ND	–	33	ND	–	–3	ND	–
Thr	966	ND	–	907	ND	–	1011	ND	–	895	ND	–	183	ND	–	906	ND	–	405	ND	–	959	ND	–
Gly	406	–	–	607	–	–	358	–	–	514		0.0	339	–	–	771	–	–	467	–	–	344	–	–
His	527	ND	–	152	ND	–	449	ND	–	438	ND	–	87	ND	–	131	ND	–	220	ND	–	324	6.5	1.95
Ala	1228	367.1	23.0	1554	315.1	16.9	1183	78.2	6.2	1328	83.8	5.9	1015	7.8	0.8	1807	179	9.01	1046	133.6	11.33	1452	107.7	6.91
Arg	1877	49.9	2.6	156	ND	–	862	133.1	13.4	1287	11.5	0.9	246	49.0	16.6	1636	10.3	0.63	355	ND	–	754	ND	–
Tyr	504	31.4	5.9	510.3	1.4	0.3	454.4	17.5	3.7	468.4	12.1	2.5	332.9	13	3.8	426.6	ND	–	302.2	9.5	3.05	367	10.4	2.76
Val	340	ND	–	1099	29.1	2.6	21	ND	–	755	ND	–	460	13.7	2.9	189	ND	–	510	19.9	3.76	802	22.3	2.71
Met	33	ND	–	57	ND	–	45	ND	–	81	ND	–	38	ND	–	871	ND	–	51	ND	–	29	ND	–
Trp	134.5	ND	–	90.7	ND	–	12.3	ND	–	75.3	ND	–	34.5	ND	–	41.4	ND	–	55.4	ND	–	19.3	ND	–
Phe	984	ND	–	363	ND	–	581	ND	–	500	19.9	3.8	234	31.5	11.9	398	ND	–	208	13.3	6.01	304	8.2	2.63
Ile	660	ND	–	209	ND	–	341	ND	–	832	8.3	1.0	346	3.8	1.1	196	ND	–	250	3.2	1.26	529	7.1	1.32
Leu	0.5	ND	–	836	ND	–	1045	ND	–	624	ND	–	270	ND	–	1042	ND	–	600	ND	–	1141	15.5	1.34
Lys	410	ND	–	366	0.8	0.2	495	2.0	0.4	ND	528	–	20	2.2	9.9	670	ND	–	267	2.8	1.04	421	4.9	1.16
Cys	157	–	–	75	–	–	147	–	–	1683		–	1350		–	ND	–	–	ND	–	–	3	–	–
Pro	3808	2.4	0.06	1292	0.8	0.06	1788	0.2	0.01	232	–	–	65	–	–	1216	ND	–	1084	0.5	0.05	1691	0.3	0.02

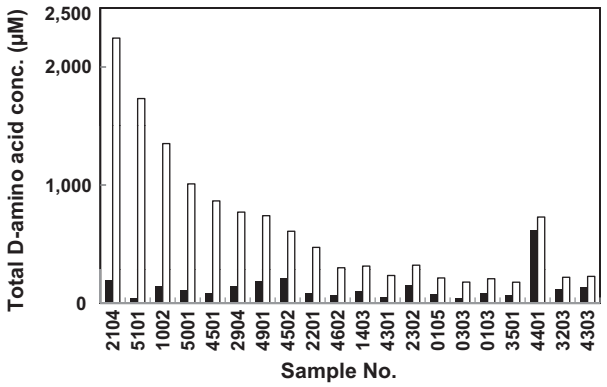

Fig. 2. Increase in total D-amino acid concentration of sakes after three months of storage at 25 °C. Black bar represents the concentration measured immediately after opening a bottle. White bar represents the concentration measured after storage at 25 °C for three months.

Table 5 shows the amino acid content of various sakes brewed with "*Kimoto yeast starter*", "*Yamahaimoto*", and "*Choukijukusei*". The sakes brewed with "*Kimoto yeast starter*" and "*Yamahaimoto*" contain high amounts of diverse D-amino acids, such as Asp, Glu, Ser, Ala, Arg, Tyr, Val, and Pro. The "*Kimoto yeast starter*" is a traditional mother yeast culture for sake brewing. The natural microflora of lactic acid bacteria that usually live in a sake factory "*Kura*" help suppress other contaminating bacteria from growing in the yeast preparation. It usually takes a month to prepare this kind of mother sake yeast starter. An improvement has been made by the "*Kimoto yeast starter*" that eliminates the labor intensive work of "*Yamaoroshi*", called "*Yamahaimoto*". Therefore, the sakes brewed with "*Kimoto yeast starter*" and "*Yamahaimoto*" contain sufficient amounts of acids and amino acids that give a characteristic flavor from lactic acid fermentation. The amount of free amino acids produced by "*Kimoto yeast starter*" and "*Yamahaimoto*" is about three times higher than the amount produced by *Sokujo-moto* [26], which is probably one of the reasons that sake brewed with "*Kimoto yeast starter*" and "*Yamahaimoto*" contains high amounts of several D-amino acids. The relationship between lactic acid bacteria and D-amino acids in foods was reported previously in orange juice [10] and beer [11]. Of the sakes analyzed, sake No. 4401 contains especially high amounts of D-amino acids sake No. 4401 was brewed with rice with a polishing ratio of 90 to 93, which is similar to that of table rice. In general, brown rice contains about 4–6 nmol amino acids per g [27], and most of the amino acids are removed during polishing. Therefore, in addition to the brewing method, the polishing ratio of the rice probably affects the D-amino acid content of sake. The long aging process "*Choukijukusei*" is also effective in increasing the D-amino acid content of sakes, but the mechanism of this increase is still unknown. The D-amino acid concentrations of balsamic vinegar and beer also increase notably during the course of the long aging processes [28].

After three months of storage at 25 °C, we reanalyzed the amino acid content of all of the sakes. Table 6 shows the basic properties of the twenty sakes that contained the highest amounts of D-amino acids after three months storage at 25 °C. After storage, the basic parameters of sake such as the SMV, acidity, amino acid value, alcohol content by volume, and rice, show no significant correlation to the D-amino acid increase of sake. Interestingly, four sakes brewed with the adenine auxotroph of sake yeast ("*Sekishoku Seishu Kobo*", *Saccharomyces cerevisiae*) [29] without pasteurization ("*Hiire*") contained high amounts of D-amino acids and were ranked in the top ten of sakes based on D-amino acid content in Table 6. In general, sake yeast including the adenine auxotroph does not produce D-amino acids because the gene encoding the

Table 6
Basic properties of the twenty sakes that contained the highest amounts of D-amino acids after three months storage at 25 °C.

Area of production	No.	Specific class	SMV value "*Nihonsyudo*"	Acidity "*Sando*"	Amino acid value "*Aminosando*"	Polishing ratio (%) "*Seimaibuai*"	Alcohol content (%)	Rice species	Remark
Ehime	2104	"*Junmai*"	−61	2	–	65	9.5	–	"*Sekishoku Seishu Kobo*"
Shimane	5101	"*Futuu*"	−13.5	2.3	–	70	14–15	–	"*Sekishoku Seishu Kobo*"
Yamaguchi	1002	"*Junmai Ginjo*"	6	1.2	–	50	15–16	"*Yamadanishiki*"	"*Okayama Hakutou Kobo*"
Okayama	5001	"*Junmai*"	−50	3.2	1.4	65	13	"*Akebono*"	"*Sekishoku Seishu Kobo*"
Wakayama	4501	"*Junmai Ginjo*"	−3	1.4	1.4	55	19	–	–
Toyama	2904	"*Junmai*"	5	1.3	1.2	50	15–16	–	–
Fukushima	4901	"*Junmai*"	−50	8	2.5	60	8.2	"*Gohyakumangoku*"	"*Sekishoku Seishu Kobo*"
Wakayama	4502	"*Junmai Ginjo*"	0	1.5	2	55,60	18	"*Bizenomachi*"	"*Kyoukai Kobo* (No. 9)"
Shimane	2201	"*Junmai Daiginjo*"	4	1.6	–	45	16–17	"*Yamadanishik*"	–
Shizuoka	4602	"*Junmai Ginjo*"	2	1.3	–	55	16–17	"*Homarefuji*"	–
Gifu	1403	"*Junmai Daiginjo*"	11		–	45	15–16	"*Miyamanishiki*"	–
Tochigi	4301	"*Junmai Ginjo*"	4	1.4	0.9	55	16–17	"*Wakamizu*"	–
Kanagawa	2302	"*Junmai*"	16	2.14	–	70	19–20	"*Yamadanishiki*"	–
Hyogo	0105	"*Honjozo*"	3.5	1.4	–	70	15	–	–
Nagano	0303	"*Ginjo*"	−2	1.6	1.1	55	18	"*Miyamanishiki*" 80% "*Hitogokochi*" 20%	–
Hyogo	0103	"*Futuu*"	1	1	0.8	78	12.5	–	–
Nagano	3501	"*Honjozo*"	1	2.1	1.4	60	14	–	–
Chiba	4401	"*Junmai*"	−38	8	4.9	90–93	6–15	Organic rice	–
Tokushima	3203	"*Ginjo*"	5	1.7	–	58	18.5	–	–
Tochigi	4303	"*Junmai Ginjo*"	4	1.4	0.9	50	16–17	"*Bizenomachi*"	–

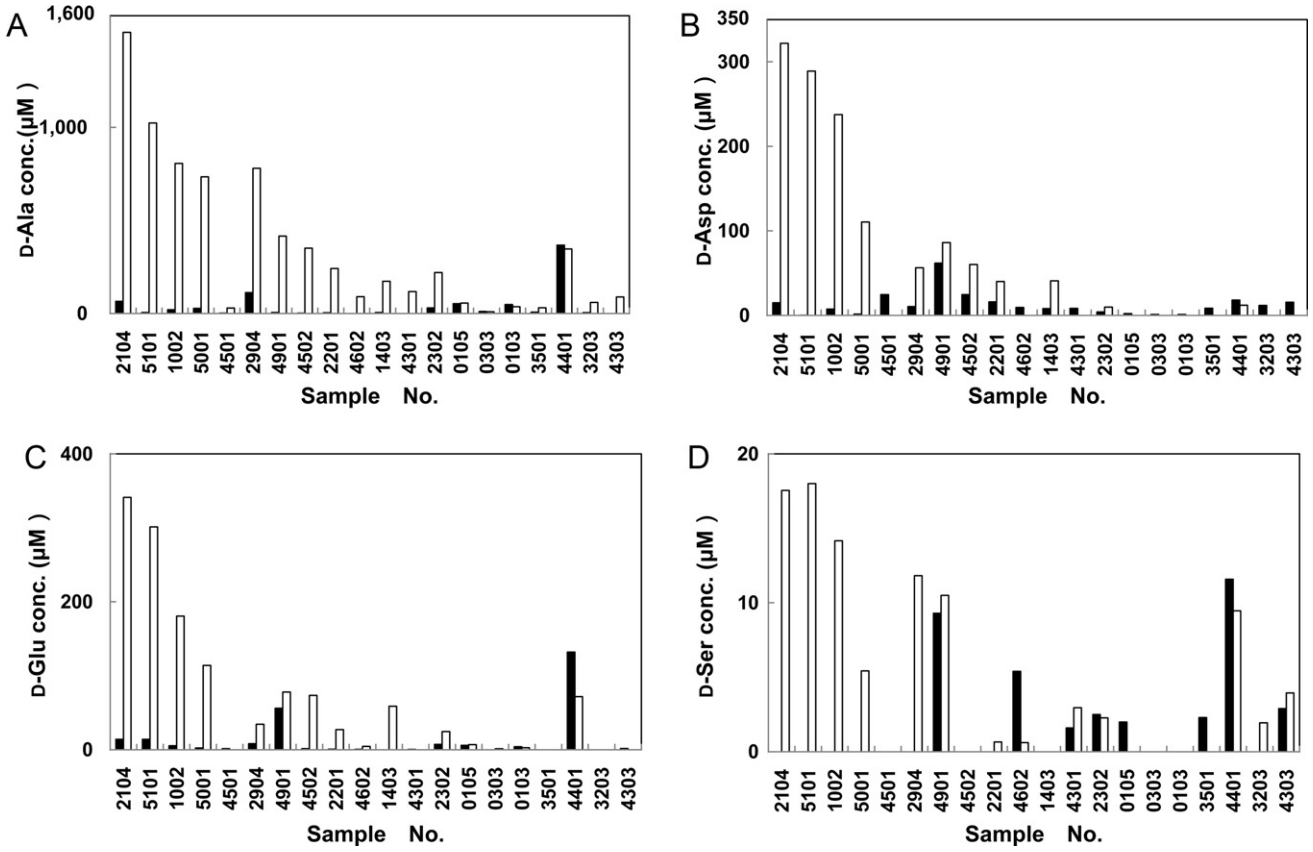

Fig. 3. Change in D-amino acid concentration of sakes stored at 25 °C for three months. (A) D-Ala concentration. (B) D-Asp concentration. (C) D-Glu concentration. (D) D-Ser concentration. Black bar represents the concentration measured immediately after opening a bottle. White bar represents the concentration measured after storage at 25 °C for three months.

amino acid racemases is not in the *Saccharomyces cerevisiae* genome (SGD: http://www.yeastgenome.org/). The adenine auxotroph of the sake yeast cannot produce as high of a concentration of alcohol as its wild-type strain, and the SMV value of sake brewed with the auxotroph is lower than that of the other sakes analyzed. In addition, the sakes brewed with the auxotroph are not pasteurized by heat treatment (called "*Namasake*"), and several bacteria were alive in these sakes (data not shown). Fig. 2 shows the increase in the D-amino acid concentrations of sakes stored at 25 °C for three months. The maximum total D-amino acid concentration increase was observed in sample No. 2104; an approximately 11.9-fold higher total D-amino acid concentration was detected after three months storage at 25 °C. Specifically, concentrations of D-Ala (Fig. 3A), D-Asp (Fig. 3B), D-Glu (Fig. 3C) and D-Ser (Fig. 3D) increased after three months of storage at 25 °C (Fig. 3). These results suggest that the increases in D-amino acid concentration observed after storage are primarily derived from the long-term exposure of the microorganisms in the sakes to an optimum growth temperature that can produce D-amino acids.

4. Conclusions

For the first time, we report that sake contains high amounts of several D-amino acids. The brewing water, brewing process, polishing ratio of the rice, kind of sake yeast, and the microorganisms existing in the sake affect the D-amino acid concentration of the sake. By optimizing these factors, we may be able to produce D-amino acid-enriched sakes in the near future. Since ancient times, sake has been said to be the best of all medicines in Japan. The D-amino acids in sake probably show some physiological functions, but further studies must be performed to clarify them with various biochemical assay systems.

Acknowledgments

This work was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN).

References

[1] K. Kitamoto, Adv. Appl. Microbiol. 51 (2002) 129.
[2] N. Urasato, Petrotech 26 (2003) 653.
[3] M. Machida, O. Yamada, K. Gomi, DNA Res. 15 (2008) 173.
[4] S. Izawa, K. Ikeda, T. Miki, Y. Wakai, Y. Inoue, Appl. Microbiol. Biotechnol. 88 (2010) 277.
[5] K. Yoshizawa, Jpn. J. Taste Smell Res. 7 (2000) 211.
[6] Y. Mogi, Agric. Economy 72 (2006) 15.
[7] N. Ishida, Jpn. J. Brew. Soc. 104 (2009) 570.
[8] H. Brückner, T. Westhauser, Amino Acids 24 (2003) 43.
[9] S. Rubio-Barroso, M.J. Santos-Delgadoa, C. Martín-Olivara, L.M. Polo-Díeza, J. Dairy Sci. 89 (2006) 82.
[10] H. Brückner, M. Lüpke, Chromatographia 31 (1991) 128.
[11] T. Erbe, H. Brückner, J. Chromatogr. A 881 (2000) 81.
[12] H.S. Ali, R. Pätzold, H. Brückner, Amino Acids 38 (2010) 951.
[13] R. Pätzold, H. Brückner, Amino Acids 31 (2006) 63.
[14] R.H. Buck, K. Krummen, J. Chromatogr. 387 (1987) 255.
[15] R. Pätzold, A. Schieber, H. Brückner, Biomed. Chromatogr. 19 (2005) 466.
[16] M.C. Waldhier, M.A. Gruber, K. Dettmer, P.J. Oefner, Anal. Bioanal. Chem. 394 (2009) 695.
[17] Z. Long, J.A. Lee, T. Okamoto, N. Nimura, K. Imai, H. Homma, Biochem. Biophys. Res. Commun. 276 (2000) 1143.
[18] J.P. Mothet, L. Pollegioni, G. Ouanounou, M. Martineau, P. Fossier, G. Baux, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 5606.
[19] A. Morikawa, K. Hamase, T. Ohgusu, S. Etoh, H. Tanaka, I. Koshiishi, Y. Shoyama, K. Zaitsu, Biochem. Biophys. Res. Commun. 355 (2007) 872.

[20] Y. Gougami, K. Ito, T. Oikawa, Trace Nutr. Res. 23 (2006) 1.
[21] D.W. Aswad, Anal. Biochem. 137 (1984) 405.
[22] S. Einarsson, B. Josefsson, P. Möller, D. Sanchez, Anal. Chem. 59 (1987) 11191.
[23] Y. Ishida, T. Fujita, K. Asai, J. Chromatogr. 204 (1981) 143.
[24] M. Sami, H. Sugiyama, H. Uwagami, M. Nakao, R. Katoh, H. Uehigashi, Deep Ocean Water Res. 5 (2004) 7.
[25] S. Kato, T. Ishihara, H. Hemmi, H. Kobayashi, T. Yoshimura, J. Biosci. Bioeng. 111 (2011) 104.
[26] H. Akiyama, Nippon Nogeikagaku Kaishi 31 (1957) 913.
[27] M. Araki, Y. Matsue, A. Kaneko, Jpn. J. Soil Sci. Plant Nutr. 70 (1999) 19.
[28] T. Erbe, H. Brückner, Eur. Food Res. Technol. 207 (1998) 400.
[29] N. Nishitani, J. Iimura, T. Iki, T. Yamashita, Y. Ishikawa, J. Brew. Soc. Jpn. 79 (1984) 691.