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Comprehensive analysis of dipeptides in alcoholic beverages by tag-based separation and determination using liquid chromatography/electrospray ionization tandem mass spectrometry and quadrupole-time-of-flight mass spectrometry

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ABSTRACT

Fermented foods and beverages contain several different types of dipeptides, which are believed to be important components for taste. To date, however, a method for the comprehensive analysis of dipeptides in these products has not yet been established. In this study, comprehensive analysis of dipeptides in alcoholic beverages was performed by a high-resolution separation method based on the structural characteristics of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)-derivatized dipeptides as well as dipeptide quantification and structural estimation using ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) and UHPLC-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOFMS), respectively. Dipeptide content was found to differ considerably among Japanese sake, beer, and wine; UHPLC–MS/MS analysis revealed that many types of dipeptides are present in sake. Dipeptide quantification analysis identified 32 types of dipeptides within the concentration range of 1.1–97.2 μM in sake. The analysis was validated by dipeptide recovery of 64.0–107.2% (2.5 μM of standard) with a relative standard deviation of ≤33.2% from an actual alcoholic sample. Furthermore, UHPLC-Q-TOFMS analysis suggested the existence of more than 35 types of dipeptides in sake. Thus, by the combined analysis methods, we discovered that more than 60 dipeptides are present in sake. This research is the first report of dipeptide profiling of fermented alcoholic beverages by comprehensive analysis.

1. Introduction

Oligopeptide consists of short amino acid sequences containing a number of residues ranging from 2 to 20 amino acids. Oligopeptides have recently attracted attention in various fields including food chemistry, physiology, molecular biology, pharmacology, and other areas, because of their importance in pharmacologic materials, hormones, and nutrients as well as their role in conferring properties such as taste [1].

Large numbers and many types of oligopeptides are reportedly present in fermented foods such as cheese [2,3], soy sauce [4–6], *miso* [5,6], and *katsuobushi* [1] as well as in beverages including Japanese sake [7] and wine [8]. Their presence is attributed to protein degradation that occurs via microorganism protease reaction. Oligopepetides consisting of large numbers of dipeptides are present in some fermented foods [8,9].

In mammals, dipeptides are actively consumed by proton-coupling oligopeptide transporter 1 (PEPT1) in small intestinal epithelial cells [10]. PEPT1 can utilize the proton gradient that forms from the outer microclimate layer toward the inner epithelial cells; therefore, oligopeptide uptake is more rapid and efficient than amino acid uptake [11,12]. For this reason, the possibility of industrial application of dipeptides has been explored in health foods, and as supplements for athletes, babies, and the elderly. In addition, dipeptides are believed to be important determinants for taste such as sweetness, umami, and kokumi [2,3] synergistically with amino acids. However, their physiological importance of dipeptides and their profiling in fermented beverages have not yet been elucidated.

By counting the number of possible combinations of 20 types of α-amino acids, we can conclude that more than 400 types of

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dipeptides exist. Thus, analysis of each dipeptide in terms of profiling, structural estimation, and quantification is a considerable challenge. To date, several analytical studies of dipeptides have been reported [13–15]. Up to 10 types of dipeptides were detected in these reports, indicating that dipeptides can be quantitatively determined in some cases. Nevertheless, the number of dipeptide species that can be determined is still small, particularly in the case of fermented alcoholic beverages that contain many matrices. Therefore, it is highly desirable to establish a new and comprehensive analytical method that achieves three important goals: (1) molecular separation, (2) specific and quantitative detection, and (3) estimation of the peptide structure.

For molecular pre-treatment, pre-column derivatization reagents such as *o*-phthalaldehyde (OPA) [16] and phenylisothiocyanate (PITC) [17], which specifically attack the amino group, have been widely used to analyze amino acids. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [18,19] is particularly popular as such a pre-column derivatization reagent because (1) it reacts rapidly with both amino and imino groups ($t_{1/2} < 1$ s), (2) derivatized amino acids are stable at 4 °C for a long period, (3) excess reagents are hydrolyzed with water at a considerably slow rate to form 6-aminoquinoline (AMQ), which does not interfere with separation and detection, and (4) it is commercially available.

Many types of detection methods have been used for amino acid analysis including photodiode array (PDA), fluorescent detector, mass spectrometry (MS), tandem MS, time-of-flight mass spectrometry (TOFMS), and their combinations. In particular, the precursor ion scanning method and the multiple-reaction monitoring (MRM) method, both available on triple quadrupole mass spectrometers, are effective tools for accurate determination of amino acid [15,20,21]. Combining these methods with a high-resolution separation system can strongly facilitate reproducible and reliable detection. Ultra-high-pressure liquid chromatography (UHPLC) has rapidly been developed as a method for compound separation since its first use in 2004 [22]. UHPLC is extremely effective for high-resolution separation when used with high-pressure tolerance columns such as sub 2-μm columns, monolith columns, and core–shell-type columns [23].

In this study, we accomplished the comprehensive analysis of dipeptides in alcoholic beverages. First, we performed high-resolution separation based on the structural characteristics of AQC-derivatized dipeptides. Then, we performed dipeptide quantification using UHPLC–tandem mass spectrometry (UHPLC–MS/MS) and structural estimation using UHPLC-quadrupole-TOFMS (UHPLC-Q-TOFMS). This is the first report that describes the dipeptide profiling of fermented alcoholic beverages by comprehensive analysis.

2. Experimental

2.1. Materials

Japanese sake samples were either brewed at the National Research Institute of Brewing from polished rice (Japanese cultivar; *Yamadanishiki*) and cerulenin-resistant sake yeast (Kyokai-1801) [24], or purchased commercially. Malt beer produced by Japanese brewer was purchased commercially. Red wine samples were purchased commercially. Samples were dispensed and preserved at −30 °C until use.

Dipeptides AP, FF, LF, FL, GG, WG, KG, AE, AG, and EG were purchased from Kokusan Chemical (Tokyo, Japan); EE, GF, GP, and HL were purchased from Peptide Institute, Inc. (Osaka, Japan); GS, DG, LG, and PG were purchased from Sigma–Aldrich (St. Louis, MO, USA); GI, GL, GY, GW, and GV were purchased from the Tokyo chemical industry (Tokyo, Japan); and KD, VD, SE, VV, AD, VG, VA, GE, and VE were purchased from Ajinomoto (Tokyo, Japan).

Both AccQ-Tag™ Eluent A concentrate and AccQFluor™ reagent kit were purchased from Waters (Milford, MA, USA). The Poroshell 120 EC-C18 column (2.7 μm, 3.0 mm internal diameter (ID), 150 mm length) and the Ascentis Express RP-amide column (2.7 μm, 3.0 mm ID, 150 mm length) were purchased from Agilent Technologies (Palo Alto, CA, USA) and Supelco (St. Louis, MO, USA), respectively.

Formic acid, acetonitrile (LC/MS grade), and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Sample pre-treatment for (U)HPLC–MS/MS analysis

All standard dipeptides except LF and FL were dissolved in MilliQ water (18.2 MΩ) to prepare 1 mM dipeptide samples. Hydrophobic dipeptides such as LF and FL, were dissolved in 0.1% hydrochloric acid. Standard dipeptide mixtures (25 μM) were prepared by mixing 32 types of 1 mM dipeptide samples and diluting with water.

All alcoholic beverage samples were deproteinized by passing through a Microcon YM-3 filter device (Millipore; Billerica, MA, USA) at 4 °C before derivatization treatment (see Fig. 1A).

Dipeptide derivatization with AQC was performed according to the manufacturer's directions. A standard dipeptide mixture or an alcoholic beverage sample (10 μL) was mixed with AccQ-Tag Ultra borate buffer (70 μL), then 5 mM of the AccQ-Tag reagent (20 μL) previously dissolved in the AccQ-Tag Ultra reagent diluent (2.0 mL) was added. The reaction proceeded at room temperature for 1 min, and then at 55 °C for 10 min.

All derivatized samples were stored at 4 °C in a dark place and analyzed within two weeks.

2.3. Dipeptide separation and MS/MS detection by triple quadrupole mass spectrometry

Liquid chromatographic analysis was performed on a Waters Acquity UPLC system, equipped with a binary solvent manager, an autosampler, a column heater, and a PDA detector interfaced to a tandem quadrupole detector. The separation columns—the Poroshell 120 EC-C18 column (120 Å pore size) followed by the RP-amide column (90 Å pore size)—were connected in tandem. To minimize the size of the system, tubes with ID of 0.0025 in. were selected. The autosampler and the column heater were set at 4 and 37 °C, respectively. Mobile phase A consisted of 0.1% (v/v) formic acid in MilliQ water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The mobile phase flow rate was maintained at 0.3 mL/min. The gradient conditions (B%) were as follows: 0–5 min = 5%, 5–40 min = 30% (linear), 40–45 min = 100% (maintaining for 5 min), 50–52 min = 5% (linear), 52–57 min = 5%. The injection volume was 3 μL. The PDA detector, which was inserted between the UHPLC and the MS, was set to monitor 255–265 ± 1.2 nm with a UV blocking (<210 nm) filter.

MS detection was performed with a Waters Quattro Premier™ XE tandem quadrupole detector via an electrospray ionization (ESI) probe following the PDA detector. MassLynx software 4.1. (Waters) was used to control these instruments. The ESI source was operated in positive ion mode at 130 °C with a desolvation temperature of 400 °C, a desolvation gas flow rate of 900 L/h, and a capillary voltage of 3.0 kV. The extractor and radiofrequency voltages were fixed at 2.0 and 0.0 V, respectively. The cone voltage was fixed at 28 V with a cone gas flow rate of 50 L/h. Argon was used as the collision gas at a flow rate of 0.15 mL/min, and collision energy of 20 eV was selected. The parameters for mass-spectrum sensitivity and selectivity, LM Resolution 1 and 2, were set to 10.0 and 12.0, respectively.