A Study of an Aroma Extraction Method and Evaluation of the Aroma Extract Contribution to the Palatability and Reinforcement Effect of Dried Bonito Using Mice

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Summary Japanese cuisine has provided satisfying meals by fully utilizing the characteristic aroma and taste of katsuodashi (dried bonito broth), though it is not rich in sugars or fats. Katsuodashi is a very basic and indispensable element in Japanese cuisine, and is a hot water extract of katsuobushi (dried bonito). It has been reported that a dextrin solution containing natural dried bonito broth has a significant reinforcement effect, and has been suggested that the olfactory stimulation is important for the reinforcement effect. We examined various source materials for broth and identified an optimal method of aroma extraction by two-bottle choice and conditioned place preference tests in mice. By two-bottle choice tests, a solution containing arabushi (a type of katsuobushi) aroma extract obtained by a supercritical CO2 extraction method showed a significantly high preference. The conditioned place preference test showed the dashi-taste solution with arabushi supercritical CO2 extract had a reinforcement effect. Our results suggest that the arabushi extract obtained by supercritical CO2 extraction contains components responsible for preference and reinforcement effects in mice; it could become conducive to making Japanese cuisine more satisfying and palatable.

Key Words: dried bonito, difference in aroma, extraction method, palatability, reinforcement effect

Humans have a strong preference for sugars and fats, and they recognize dishes and sweets rich in sugars and fats as palatable food. Rodents also have a strong preference for sugars and fats. Two-bottle choice tests using sugars and fats have been reported to show not only their high palatability but also a reinforcement effect (1, 2). Conditioned place preference (CPP) tests have been widely used to determine the reinforcement effect of a given sample (3).

Tastes considered palatable by humans are not limited to sugars and fats. For example, broths, which contain taste components such as flavors and distinctive aromas extracted from plants and animals, are indispensable for enhancing the taste of dishes. Dashi (broth) made from katsuobushi (dried bonito) is a very basic and indispensable element in Japanese cuisine. Although it is not rich in sugars or fats, Japanese cuisine has provided satisfying meals by fully utilizing the unique aroma and taste of katsuodashi (dried bonito broth). Therefore, it is important to re-evaluate the value of Japanese cuisine that provides satisfaction with low calories.

Katsuobushi (dried bonito) is made from katsuo (Skipjack tuna, Katsuwonus pelamis) by the following processes: cutting raw katsuo into pieces, boiling-aging, roasting-drying (smoking and drying over a smoking wood fire), molding, mold inoculation, and sun drying. In this process, the intermediate product from the roasting-drying step is called arabushi. Because tar from the roasting-drying step is deposited on the surface of the arabushi, the surface is shaved during the molding step and the powdery shavings are called G powder. The product from the molded arabushi that is subjected to repeated cycles of mold inoculation and sun drying steps is called karabushi. Both arabushi and karabushi are widely marketed as katsuobushi. A hot water extract of shavings of arabushi or karabushi is called katsuodashi (dried bonito broth), an indispensable broth in Japanese cuisine. Other fishes including maguro (yellowfin tuna, Thunnus albacares), goma-saba (spotted mackerel, Scomber australasicus), soude-katsu (bullet tuna, Auxis rochei), and katakuchi-iwashi (Japanese anchovy, Engraulis japonicus) are processed in similar ways for use in making broths.

Kawasaki et al. and Ackroff et al. found a preference for dried bonito broth in two-bottle choice tests using mice (4, 5). Furthermore, Kawasaki et al. performed CPP tests and found a significant reinforcement effect for a dextrin solution containing natural dried bonito broth (BD), but not for a dextrin solution alone (6). In addition, they analyzed taste components in natural

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328
dried bonito broth and prepared a NaCl/IMP/GMP/amino acid solution (dashi-taste solution) reproducing taste components in dried bonito broth. Although a dextrin solution containing dashi-taste solution (AD) did not show a significant reinforcement effect, AD supplemented with katsuos aromatics did show such an effect. BD did not show a significant reinforcement effect in mice with olfactory deprivation. These results suggest that the olfactory stimulation is important for the reinforcement effect of BD (6). Given that citral, vanillin, or menthol flavored ADs did not elicit a reinforcement effect, the observed reinforcement effect is not a non-specific response to any smell (6). Specific characteristics of aromatic compounds in natural dried bonito broth are important for the reinforcement effect, but no investigation to reveal what are the responsible aroma components for its palatability has proceeded yet.

To narrow the range of candidate aromatic compounds responsible for the palatability and the reinforcement effect of dried bonito broth, we evaluated aromatic compounds extracted from katsuobushi with different extraction methods in two-bottle choice and CPP tests in this study. We also studied preference for broth ingredients other than katsuuo in ethological experiments.

MATERIALS AND METHODS

Animals. Eight week old BALB/cCr male mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan), for each experiment. The mice were housed in cages maintained at 23±2°C under a 12 h/12 h light/dark cycle (lights on 6:00-18:00). Food (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided ad libitum. All studies were conducted in accordance with the ethical guidelines of Kyoto University Animal Experimentation Committee.

Dashi samples. Powdered dried bonito broth, a powdered katsuobushi extract, was obtained from Shimaya Co., Ltd. (Yamaguchi, Japan). Arubushi, magurobushi, and niboshi (boiled and dried Japanese anchovy) were purchased from Maruhachi Muramatsu, Inc. (Yaizu, Japan). Karebushi was purchased from Shintou Corporation (Tokyo, Japan). G powder, the powdery shavings of the tar layer on the surface of arubushi, was purchased from Nissei Kyoei Co., Ltd. (Tokyo, Japan).

Preparation of dashi-taste solution. The dashi-taste solution was prepared according to analytical data (4). In brief, NaCl (Nacalai Tesque, Inc., Kyoto, Japan), IMP (MP Bio Japan K.K., Tokyo, Japan), GMP (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 14 amino acids (L-Asparagine, L-glutamic acid, L-serine, glycine, L-histidine, L-arginine, L-threonine, L- alanine, L-proline, L-tyrosine, L-valine, L-methionine, L-isoleucine, and L-leucine) were mixed similarly to the reported analytical ratio of dried bonito broth, and diluted with deionized water. The concentration of the dashi-taste solution was equal to a 1% powdered dried bonito broth solution. All amino acids were purchased from Nacalai Tesque, but L-methionine was from Wako Pure Chemical Industries.

Aroma extraction methods.

Steam distillation extraction: Ground arabushi in a flask was subjected to steam distillation under normal pressure by using a water bath. The distillate was saturated with NaCl, and was poured into a column filled with synthetic adsorbents (DIAION™ HP20, Mitsubishi Chemical Corporation, Tokyo, Japan) for the separation of aromatic compounds. The absorbents were washed with water followed by washing with 95% ethanol to extract the aromatic compounds. The yield of the arubushi steam distillation extract was 5.0%.

Aqueous ethanol extraction: Approximately 75% ethanol was prepared by diluting 95% ethanol with water. Ground arubushi was extracted with diluted ethanol (80±2°C, 2 h) in the flask. After extraction, the extract was filtered and the filtrate was evaporated under reduced pressure. The yield of the arubushi aqueous ethanol extraction extract was 65%.

Supercritical carbon dioxide (sCO₂) extraction: A series of extractions was carried out with a supercritical CO₂ extraction system (JASCO Corporation, Tokyo, Japan). The extraction column was filled with ground samples (arubushi, karebushi, G powder, magurobushi and niboshi). The extraction temperature was 40°C, the CO₂ flow rate was 10 ml/min and the pressure was 25 MPa. The yields of the supercritical CO₂ extracts were 1.3, 1.0, 6.0, 1.2, and 2.0%, respectively.

Two-bottle choice test procedure. Two-bottle choice tests were done in the dark cycle. Mice were housed individually and were deprived of chow and water for 40 min before every test. All test solutions were placed in a 50 mL plastic tube fitted with a stainless steel spout. Mice were trained three times by administering water and 0.89% NaCl water solutions for 30 min. After training, mice were administered dashi-taste solution and dashi-taste solution with aroma extract, and their preferences for the test solutions were tested. The deprivation conditions and test time were same as in the training period. Preference values (%) for test solutions were obtained as the value of each solution intake divided by the total intake and multiplied by 100.

CPP test procedure. We used the same CPP test apparatus and procedures as previously described (6). In brief, the test schedule consisted of 10 consecutive days in the light cycle. Day 1 to day 3 were for measurements of basal preferences for the conditioning places, namely light and dark boxes, for 20 min. Day 4 to day 9 were for conditioning. Mice were offered the test solution or water for 30 min in the light or dark box. Conditioning for the test solution was in the light box while that for water was in the dark box, and it was repeated 3 times on alternate days. Day 10 was for the measurement of reinforcement of the conditioning place. The time spent in each box was measured for 20 min without offering the test solution or water. The time spent in the light box (conditioning place) on day 10 was compared to the measured time spent in the light box on day 3. It was concluded that the test solution had a reinforcement effect when the time spent on day 10 was extended significantly compared to that on day 3.
Olfactory nerve transection. Mice were divided into a sham treated (Sham) group and olfactory nerve transected (ONX) group. The olfactory nerve fibers projecting through the cribriform plate to the olfactory bulb were transected by the methods described in the previous reports (6–8). The sham group mice received similar surgical treatments, except the nerve transection. Both groups of mice were pre-exposed to potato chips (Calbee, Inc., Tokyo, Japan) before surgery and made to learn its odor. Three to five days after surgery, mice were tested for their olfactory capabilities. A small piece of potato chip was buried under fresh nesting paper chips in a plastic cage. Mice, deprived of chow for 20 h before testing, were allowed 3 min to find the buried potato chip in the cage. The Sham group mice that found the potato chip within 3 min were regarded as olfactory normal and were used in further experiments. The ONX group mice that couldn’t find the potato chip within 3 min were regarded as anosmic and were used in further experiments.

Statistical analyses. All experiments used a criterion of \( p<0.05 \) for statistical significance. The data were analyzed by a t-test. The preference values for the dashi-taste solution with aroma extract from different dried fish products were analyzed by one-way ANOVA and Tukey’s multiple-comparison test as a post-hoc test. Values given in the figures are means±SE. All statistical analyses were performed with GraphPad Prism ver. 5 for Mac (GraphPad Software, Inc., San Diego, CA).

RESULTS

Experiment 1. Preference for katsuobushi extracts prepared by different methods

The three kinds of extracts were prepared from katsuobushi by steam distillation, aqueous ethanol extraction, or supercritical carbon dioxide (sCO\(_2\)) extraction, as described in the experimental section. These extracts were added to the dashi-taste solution and then subjected to two-bottle choice tests of dashi-taste solution vs. dashi-taste solution with extract according to the method described in the experimental section (Fig. 1). Dashi-taste solution constituents were those described in the experimental section. The extracts prepared by the three methods were tested at 0.05%, 0.30%, and 0.05%, respectively. The aroma intensities of each extract added to water were evaluated by well-trained flavorists from T. Hasegawa Co., Ltd. On the basis of the evaluation, the flavorists decided the additive rates of each aroma extract to make the intensities equivalent to one another. A significant difference for the katsuobushi sCO\(_2\) extract compared to the dashi-taste solution without the extract was observed among the test groups.

Experiment 2. Preference for sCO\(_2\) extracts from different katsuobushi products and other dried fish products

In addition to an katsuobushi specimen, karebushi, G powder, magurobushi (dried tuna), and niboshi were subjected to sCO\(_2\) extraction, and the extracts in dashi-taste solution were evaluated in two-bottle choice tests (Fig. 2). All extracts were tested at 0.05%.

Significant differences were observed in groups that received the extracts from katsuobushi, karebushi, magu-
robust, and niboshi against only the dashi-taste solution (Fig. 2A). In particular, the amount of intake of the extract containing dashi-taste solution was greatest for the arabisu. Calculated preference values for dashi-taste solution with aroma extract are shown in Fig. 2B. The one-way ANOVA showed a significant difference in the preference values [F(4, 35) = 4.056, p < 0.01]. A post hoc analysis revealed that the preference value for arabisu was significantly greater than that for karebushi, magurobushi, and G powder.

Experiment 3. Preference for arabisu sCO2 extract in olfactory-deprived mice

Using mice olfactory deprived by olfactory nerve transection as described in the methods section and sham operated mice, we conducted a two-bottle choice test of dashi-taste solution vs. dashi-taste solution with the arabisu sCO2 extract (Fig. 3). The concentration of the added sCO2 extract was 0.05%. The result showed a significant difference in intake of dashi-taste solution vs. dashi-taste solution with the extract in Sham group mice, whereas ONX group mice showed no significant preference for dashi-taste solution with the extract. Experiment 4. CPP test of the arabisu sCO2 extract

Three groups of mice were conditioned with AD, BD, and AD + arabisu sCO2 extract, respectively. Concentrations of dextrin, natural dried bonito broth, and arabisu sCO2 extract were 21.9%, 1%, and 0.05%, respectively. Mice in the BD group conditioned with the natural dried bonito broth served as a positive control, and the group conditioned with the arabisu sCO2 extract stayed in the conditioned boxes for significantly prolonged periods of time (Fig. 4).

DISCUSSION

In experiment 1, to identify components responsible for preference of dried bonito broth, we prepared extracts from katsuo arabisu, the source material for broth, by three different methods. The extracts obtained were tested as dashi-taste solution in a short-term two-bottle choice test (dashi-taste solution vs. dashi-taste solution with the extract) to determine the optimal extraction method. The result showed a significant preference for the sample containing the extract obtained by sCO2 extraction (Fig. 1). Even if the same source material was used, components in the extract and their compositions vary depending on the method used for extraction (9–12). Steam distillation is a method for obtaining volatile components by which a sample is heated in the presence of water, and volatile components distilled with water vapor are condensed and separated from the water. Although the principle is simple, steam distillation can damage compounds by pyrolysis and hydrolysis (13, 14). Difficulties in obtaining a component with a low boiling point and high volatility or with high water solubility represent additional disadvantages of this extraction method. Although broth is a hot water extract of the source material, aroma vaporized from the surface of the broth and aroma dissolved in the broth are both enjoyed. Given that volatile components dissolved in water are difficult to obtain by steam distillation, it is conceivable that the extract obtained by this method does not replicate the balance among aroma components in natural dried bonito broth, and thus did not lead to a preference. Solvent extraction using an organic solvent such as acetone or hexane, or an aqueous alcohol is also a common extraction method. Because this method uses a large quantity of solvent, the solvent must be evaporated to concentrate the extract. Given that the aqueous ethanol used in our study had a somewhat higher boiling point than that of acetone and hexane, highly volatile components may have been readily lost during solvent evaporation. The sCO2 extraction method utilizes CO2 in a supercritical state under high pressure as an extraction solvent. Oxidation by oxygen is suppressed in extraction by this method because extraction occurs in an inert atmosphere of high-pressure CO2. The critical temperature of CO2 and the extraction temperature used in this experiment were 31°C (15) and
40°C, respectively, and the extraction process did not involve heating above these temperatures. Heat-caused damage in this method is relatively moderate, given that the CO₂ solvent is separated from the extract by releasing the pressure and the extraction temperature is lower than the boiling points of water and organic solvents. Because of these features, sCO₂ extraction is excellent for reproducibility of aromas of source materials (11, 13, 14, 16–18). The observed significant preference for the sCO₂ extract may thus be explained by extraction of katsuobushi components with minimum damage.

Broth is prepared from not only arabushi but also other dried fish products. In experiment 2, we investigated other source materials for broth to address whether only the sCO₂ extract of arabushi has palatability or whether the sCO₂ extract of other broth sources also has palatability. Karebushi (which is made by additional processing of arabushi), G powder (which is a byproduct of this process), magurobushi, and niboshi were subjected to sCO₂ extraction, and the extracts were evaluated for palatability by the two-bottle choice test (Fig. 2). Significant differences for all samples but those of G powder were observed, and the group given the arabushi extract showed a particularly high preference value and the greatest intake of the extract-containing solution. In experiment 3, we evaluated the involvement of olfactory sensing in preference in a short-term two-bottle choice test by examining the effect of olfactory deprivation using mice subjected to olfactory nerve transection. After the olfactory capability test using potato chips, the test mice rested for several days before the two-bottle preference test. Thus the influence of the capability test on the two-bottle test was likely negligible. The Sham group mice showed a significant preference for the extract-containing solution, but the ONX group mice showed no significant preference for this extract (Fig. 3).

The results of experiments 2 and 3 being taken into account, it is suggested that the olfactory sense is primarily involved in preference for sCO₂ extracts in the short-term (30 min) two-bottle choice test and that differences in the aroma of the extract affect the preference.

Niboshi is prepared by boiling and subsequently drying fresh Japanese anchovy without a smoking step, whereas a smoking step is part of the process for katsuobushi as described above. Nishibori and Okamoto performed an aroma analysis of katsuobushi and niboshi and reported that no phenolic compounds were detected in niboshi, suggesting that phenolic compounds deposited during smoking are among the elements characterizing the aroma of katsuobushi (19). G powder, the tar-shaved layer on the surface of smoked katsu, likely contains a high level of phenolic compounds. The observation of preferences for katsuobushi, magurobushi, and niboshi extracts but not for G powder extract suggests the possibility that mice wouldn’t show a significant preference for an aroma which mainly consists of phenolic compounds. Yajima et al. fractionated aromatic compounds of katsuobushi based on pH and analyzed the fractions (20, 21). Ketones and aliphatic aldehydes were detected in the neutral fraction, and a large number of alkyl pyrazines were found in basic fractions. Katsuobushi and niboshi contain large quantities of proteins and a small percent of lipid (22). Oils obtained from bonito, tuna, and sardines contain large quantities of polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (23, 24), and these oils are known to be oxidized rapidly (25). Aliphatic aldehydes and ketones are produced by unsaturated fatty acid oxidation (25), and alkyl pyrazines are produced from amino acids by the Maillard reaction (26). Yajima et al. have speculated that lipids and amino acids undergo oxidation, pyrolysis, and Maillard reactions during the processing steps for katsuobushi and that these reactions produce the aroma (20, 21). Tanimoto et al. performed aroma analysis of niboshi and reported that short-chain aliphatic aldehydes having 3–6 carbons, such as propanal, butanal, and hexanal, make dominant contributions to the niboshi aroma (27). In light of these reports, aromatic compounds produced by oxidation, pyrolysis, and Maillard reactions of lipids and amino acids are likely to be involved in preference for sCO₂ extracts from arabushi, karebushi, magurobushi, and niboshi found in this study, and thus further study is indicated.

In experiment 4, we performed a CPP test and investigated the reinforcement effect of the arabushi sCO₂ extract. In the CPP test, we observed a reinforcement effect with BD comprising natural dried bonito broth added to dextrin in BALB/cCr mice (Fig. 4). Although AD comprising dashi-taste solution, which reproduced taste components of those in dried bonito broth, added to dextrin did not show a reinforcement effect, the effect was observed for AD supplemented with the arabushi sCO₂ extract. This result suggests that, in addition to AD, aromatic compounds in the arabushi sCO₂ extract are required for the reinforcement effect. This finding is consistent with a report by Kawasaki et al. in which BD, but not AD, showed the reinforcement effect (6). Thus, the aroma of the arabushi sCO₂ extract prepared in this study is suggested to contain components that can elicit preference and reinforcements effects similar to those of dried bonito broth. Components contributing to the preference and reinforcement effect may be identified by further detailed analysis of the arabushi sCO₂ extract. As elicited aroma compounds that contribute to the preference for katsuo-dashi, they could become conducive to making Japanese cuisine more satisfying and palatable. The influence of taste and aroma balance on the preference, namely, the effect of the ratio of the taste intensity of dashi-taste solution and the additive rate of sCO₂ extract on the preference, remains to be seen.

In summary, we examined various source materials for broth and identified an optimal method of aroma extraction by two-bottle choice and CPP tests in mice. Our results suggest that the arabushi extract obtained by sCO₂ extraction contains aromatic compounds responsible for preference and contributes to acquisition of the reinforcement effects in mice.
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REFERENCES