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Sensory-Guided Analysis of Key Taste-Active Compounds in Pufferfish (*Takifugu obscurus*)

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Abstract

To investigate key taste-active components in *Takifugu obscurus* (*T. obscurus*), twenty-eight putative taste compounds in cooked muscle of *T. obscurus* were quantitatively analyzed and the pivotal components were identified by taste reconstitution, omission and addition tests. Moreover, the role of flavor peptides in overall taste profile of *T. obscurus* were evaluated. Sensory evaluation revealed that glutamic acid, serine, proline, arginine, lysine, adenosine-5′-monophosphate, inosine-5′-monophosphate (IMP), succinic acid, sodium, potassium, phosphates and chlorides, were the core taste-active contributors to *T. obscurus*. Besides glutamic acid, IMP, succinic acid and potassium, the characteristic *T. obscurus*-like umami and kokumi profiles were induced by adding flavor peptides, among which Pro-Val-Ala-Arg-Met-Cys-Arg and Tyr-Gly-Gly-Thr-Pro-Pro-Phe-Val were identified as key substances on the basis of addition test and dose-response analysis. The present data may help to reveal the secret of delicious taste of *T. obscurus* and provide the basis for development of deeper flavor analysis of pufferfish.

Keywords

Taste-active compounds; umami; flavor peptide; taste recombinant; omission test; *Takifugu obscurus*

Introduction

Due to its extremely delicious taste, pufferfish is very popular in Eastern Asia, especially in China and Japan.[1] China is the largest supplier of pufferfish and around 70% of the annual catch are exported overseas.[2] *T. obscurus*, the most commonly consumed pufferfish in China, was had a great market since a legal edible policy was opened in 2016.[3] In particular, umami and kokumi taste characteristics of *T. obscurus* are highly desirable. It can generate palatable, savory, rich continuity, complexity, and palatability.[4,5] Among the non-volatile taste-active compounds, free amino acids, 5′-nucleotides, organic acids, organic bases, inorganic ions and flavor peptides, were reported as the contributors to unique taste of raw as well as cooked meat of pufferfish.[1,4-6] Sodium, potassium, glycine, alanine, arginine, adenosine-5′-monophosphate (AMP), guanosine-5′-monophosphate (GMP) and inosine-5′-monophosphate (IMP) have been identified as key substances determining the typical umami taste of pufferfish.[4] However, the multiple typical taste compounds reported above so far are contradictory in *T. obscurus*. Lately, our research group has also identified several flavor peptides originated from *T. obscurus* by using a series of sensory-guided separation and purification technology processes.[5,6] However, their sensory impact and contribution on overall taste profile in *T. obscurus* remain largely unclear.

Due to complex interactions in foodstuffs, it is difficult to reveal the contribution of each flavor compounds only through quantitative analysis and individual flavor characteristics study.[7,8] The omission experiment, known as removing an individual or

a group of compounds from a mixture content to measure the sensory effect on its taste profile, has bridged the gap between physicochemical analysis and sensory data.[9] Furthermore, complex relationships such as synergistic or masking effects among components can be simplified by omission test, thus allowing a full analysis of the taste contribution of the food extract studied.[8] This method has been widely used to figure out the key taste-active components in food, such as the Yangtze *Coilia ectenes*, crustaceans, the oval squid, tea, tomato and so on.[10,11,12-15]

Based on the above sensory technique, to answer the puzzling question as to which key taste compounds are responsible for the characteristic taste as well as the role of flavor peptides in overall taste profile of *T. obscurus* precisely, the objectives of the present work were (I) to quantitate the putative taste-active compounds in boiled muscle of *T. obscurus*; (II) to identify the key taste contributors and validate their sensory profile by taste reconstitution, omission and addition tests; and finally, (III) to perform dose-response analysis in a simplified taste recombinant to evaluate the taste contribution of flavor peptides in *T. obscurus*.

Materials and Methods

Chemicals. AMP (≥ 99%), GMP (≥ 99%), IMP (≥ 99%), free amino acids [aspartic acid (≥ 99%), glutamic acid (≥ 99%), serine (≥ 99%), glycine (≥ 99%), threonine (≥ 98%), alanine (≥ 99%), proline (≥ 99%), arginine (≥ 99.5%), lysine (≥ 98%), valine (≥ 98%), histidine (≥ 99%), tyrosine (≥ 98%), phenylalanine (≥ 99%), isoleucine (≥ 98%), leucine (≥ 98%), cysteine (≥ 97%), methionine (≥ 98%)], betaine (≥ 99%), trimethylamine N-oxide dihydrate (TMAO, ≥ 99%), trimethylamine (TMA, ≥ 98%),

69 succinic acid (≥ 99.5%) and lactic acid (≥ 98%) (Sigma-Aldrich Corp., Shanghai,
70 China); titanium chloride ($TiCl_3$), trichloroacetic acid (TCA), Reinecke's salt and
71 acetone (Sinopharm Chemical Reagent Corp., Ltd., Shanghai, China); methanol and
72 acetonitrile used for chromatography are HPLC grade (ANPEL Laboratory
73 Technologies Inc., Shanghai, China); the standards of sensory analysis, NaCl (≥
74 99%), monosodium glutamate (MSG, ≥ 99%)), sucrose (≥ 99%) and citric acid (≥
75 99%), were purchased from Sigma-Aldrich Corp (Shanghai, China) while quinine
76 sulfate (≥ 98%) was obtained from Shanghai Yuanye Bio-Technology Corp., Ltd.
77 (Shanghai, China); four flavor peptides, Arg-Pro-Leu-Gly-Asn-Cys (RC-6),
78 Pro-Val-Ala-Arg-Met-Cys-Arg (PR-7), Tyr-Gly-Gly-Thr-Pro-Pro-Phe-Val (YV-8)
79 and Tyr-Lys-Cys-Lys-Asp-Gly-Asp-Leu-Arg (YR-9), which the information of amino
80 acid sequence were originated from our group's previous study, were synthesized by
81 GL Bio-Chem Corp., Ltd. (Shanghai, China).[5,6] The nominal purity of all peptides is
82 higher than 98.5% (*w/w*, the company's Test Reports, SHIMADZU LCMS-2010EV,
83 Kromasil C18, 250 × 4.6 mm, 5 μm). Ultrapure water used for chromatography and
84 sensory evaluation was prepared by a Milli-Q® Reference System (MERCK
85 MILLIPORE, Darmstadt, Germany).

86 **Materials.** Twenty bred *T. obscurus* at two years old were purchased from Dalian
87 Tianzheng Industrial Co., Ltd (Liaoning Province, China). After being caught, *T.*
88 *obscurus* were slaughtered by professional pufferfish operators according to The
89 National Standard Method of China (SC/T 3033-2016), then the dorsal muscle was
90 transported under ice in insulated polystyrene boxes to the laboratory by air transport

(no more than 4 h). Once in the lab, the meat was packaged in aluminum foil bag and stored at -71 °C until further analysis.

Preparation of taste extract. *T. obscurus* muscle extract was prepared according to a process previously described.[5] Frozen muscle fillets were thawed at 4 °C overnight. As shown in Figure 1, 200 g minced meat was mixed with 800 mL of ultrapure water and homogenized at 3 ×1,000 rpm for 2 min with an Ultra Turrax homogenizer (IKA Co., Germany). The homogenate was heated in a 100 °C ultrapure water bath for 3 h prior to filtration through a Whatman.No.54 filter membrane.[5] The cooled filtrate (20 °C) was centrifuged (H1850R, Cence®, China) at 10,000 rpm for 20 min at 4 °C. The supernatant (natural extract) was collected and used directly for the quantitative analysis. The natural extract was diluted with ultrapure water at the ratio of 2:1 (*v/v*), and kept maximum 2 h before sensory evaluation (25 °C).

Quantitative analysis

Quantitation of free amino acids. Free amino acids quantitation was determined by using the method developed by Adeyeye with some modification.[16] The fat in *T. obscurus* was lost during cooking process and filtered out, thus there is no defatting step. The natural extract (10.00 g) was mixed with 15 mL of 0.1 M HCl under ultrasonic extraction for 30 min and centrifuged at 10,000 rpm for 10 min at 4 °C. 10.00 mL of the above supernatant was pipetted into a 50 mL centrifuge tube and 10.00 mL of 10% (w/v) TCA solution was added and mixed. The solution was kept at static stage for 1h to precipitate the potential protein in the sample, and then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and

adjusted to pH 2.0 (pH meter, Five Easy Plus, METTLER TOLEDO, Swiss) with 6 M NaOH and filtered through 0.22 μm water-filter before analysis by amino acid analyzer (L-8800, Hitachi Co., Tokyo, Japan).

Quantitation of the 5′-nucleotides. 5′-nucleotides were quantitated by HPLC (Waters e2695, Waters, US) on a 250 × 4.6 mm, 5 μm, Inertsil ODS-3 column (GL, Japan) using gradient elution with methanol as buffer A and 20.0 mM KH_2PO_4-K_2HPO_4 (*v*/*v*=1:1, pH 5.8) as buffer B at a flow rate of 1 mL/min.[17] The gradient was carried out as following, initial of 0% A for 6 min, linear change to 8% A for 9 min, linear change to 35% A for 5 min, keep for 2 min, linear change to 0% A for 2 min and finally keep for 6 min. The effluent was monitored at 254 nm by a UV detector (Waters 2998 PAD, Waters, US). All analyses were done in triplicate (n=3). The identity and quantity of the nucleotides were determined by comparison with the retention times and peak areas of each 5′-nucleotides standard solution (AMP and GMP respectively: 2.5, 5, 10, 15, 20, 50 mg/100 mL; IMP: 5, 10, 25, 50, 100, 200 mg/100 mL). Specific quantitative parameters are shown in Table S1 of Supplementary Information.

Quantitation of organic acids. As for the contents of succinic acid and lactic acid, the natural extract was filtered by 0.45 μm filters prior to HPLC analysis.[18] The HPLC and column were the same as described in *Quantitation of the 5′-nucleotides*, while the eluting solvents consist of A (methanol) and B (0.05% phosphoric acid). The gradient elution program was as following: initial of 5% A for 10 min, linear change to 15% A for 5 min, linear change to 70% A for 6 min and finally linear change to 5%

A for 4 min. The samples were operated with a flow rate of 1.0 mL/min and monitored at 215 nm. All analyses were done in triplicate (n=3). Quantitative analysis was performed by comparing the peak areas of defined standard solutions (succinic acid: 5, 10, 20, 50, 100, 200 mg/100 mL; lactic acid: 25, 50, 100, 150, 200, 300 mg/100 mL). Specific quantitative parameters are shown in Table S1.

Quantitation of organic bases. The betaine present in the natural extract (50.00 g) was firstly crystallized and precipitated by Reinecke's salt under acidic conditions (pH 1, 12 M of HCl was used). Then the precipitate was dissolved with acetone/water (70/30, *v/v*) and the content was quantitated based on colorimetry at 525 nm using UV-visible spectrophotometer (UV-2450, GL, Japan).[19] The quantitation was performed with a calibration curve, and the standard solution was 4, 8, 12, 16 and 20 mg/100 mL in ultrapure water. TMAO in the samples was reduced to TMA with $TiCl_3$ (1%, *w/v*) and detected by Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent 5977A, Agilent Technologies Inc., US).[20] The natural extract (25.00 g) was mixed with 20.00 mL of 5% (*w/v*) TCA (extraction of TMA from the sample) and centrifuged at 4,000 rpm for 5 min. 2.00 mL of the supernatant was poured into 20 mL headspace vial, and 5.00 mL of 50% (*w/v*) NaOH solution was added and equilibrated 40 min at 40 °C before quantitation of TMA by GC-MS. Operating with a flow rate of 1.0 mL/min, the following gradient was used for temperature program of chromatography: starting 40 °C for 3 min, then increased to 220 °C at the rate of 30 °C/min, keeping 1 min. Using the electron ionization (EI), TMA was identified by the retention time (RT), auxiliary qualifier ion (m/z 59 and m/z 42) and quantitative

ion (m/z 58), while quantitated via peak areas of standard substance (1, 2, 5, 10, 20, 40 mg/mL). The content of TMAO was calculated as the differences in TMA content after and before reduction.[20] Specific quantitative parameters are shown in Table S1.

Quantitation of inorganic ions. Sodium (Na^+) and potassium (K^+) content were determined using flame atomic absorption spectrophotometry (ZEEnit 700, Analytik Jena AG, Germany) according to the method of AOAC 2007.[21] The quantitation was performed with a calibration curve, and the standard solutions for Na^+ and K^+ were 0.574, 0.985, 1.854, 3.076 and 4.010 mg/L as well as 0.481, 1.009, 2.022, 3.000 and 3.992 mg/L in ultrapure water, respectively. Phosphates (PO_4^{3-}) were estimated following the conversion of molybdenum blue and quantitative analysis was carried out by standard solution (0.08, 0.16, 0.24, 0.32, 0.40 mg/L).[22] Average quantitation of chlorides (Cl^-) was measured according the modified method of Carpenter–Volhard.[23] Specific quantitative parameters are shown in Table S1.

Sensory evaluation

General conditions. All the sensory experiments were performed in an air-conditioned sensory lab (25 °C) with suitable ventilation and white light (ISO 8589:2007 E). Fourteen internal panelists (12 females and 2 males, aged from 20 to 26), who had no taste disorders and had half year experience of sensory evaluation, participated in the study after signing consent forms. Nose clips were used to prevent potential interactions with olfactory. The temperature of the samples (poured into plastic cups coded with three random numbers) and mouthwash (ultrapure water) was constant at 25 °C. During evaluation, the panelist placed about 10 mL sample into

mouth, held in mouth for 10 s and then spitted out. Ultrapure water was used for mouth rinsing, in case of any aftertaste. Panelist would take a at least 30 s rest, before evaluating the next sample.

Training sessions. Fourteen panelists were trained during 15 one-hour sessions to learn how to recognize and quantitate the five basic tastes, kokumi and complex situation according to a series of sensory approaches. Firstly, they were trained on five basic tastes to evaluate the aqueous solutions of the following standards in ultrapure water: citric acid (0.430 mg/mL), sucrose (5.76 mg/mL), quinine sulfate (0.0325 mg/mL), NaCl (1.19 mg/mL) and MSG (0.595 mg/mL) for sourness, sweetness, bitterness, saltiness and umami, respectively. Then triangle tests were used to establish the panels' sensitivity of each basic taste at lower concentrations than the above five corresponding standards. Kokumi activity was trained according to the method of Meyer et al. and glutathione (5.0 mM, GSH) was used in this process.[11] Finally, the panelists were trained to rate umami according to a scale composed by different concentrations of MSG (0.24, 0.34, 0.49, 0.70 and 1.00 mg/mL). For experiments with reference sample, the intensities of standard solution using for reference were discussed and agreed within the panel. Then they were trained using standard solutions and asked to memorize its intensity corresponding. The test didn't perform until the panelists' data at stabilized level.

Sensory experiments

Taste profile analysis. Five samples were presented to the panelists, including (1) a freshly prepared natural extract, (2) complete taste recombinant (CTR), (3) simplified

taste recombinant (STR), (4) CTR and (5) STR with 20.0 mg/100 mL of four flavor peptides (RC-6, PR-7, YV-8 and YR-9), respectively. They were asked to scale the intensity of attributes (sourness, sweetness, bitterness, saltiness, umami and kokumi) on a five-point rating scale from 0 to 4 (0, no perceived; 1, weak; 2, average; 3, strong and 4, extremely strong).[14] Intensity of ultrapure water was set as point 0. citric acid (0.430 mg/mL), sucrose (5.76 mg/mL), quinine sulfate (0.0325 mg/mL), NaCl (1.19 mg/mL), MSG (0.595 mg/mL), and GSH (5 mM) were set as point 4 for the five basic tastes and kokumi. Among them, a CTR was prepared according to the following detail: taste compounds range from group I to V (Table 1) were dissolved in ultrapure water in their natural concentrations and diluted with ultrapure water at 2:1 (*v/v*) to reduce fatigue of the panelists. Then the pH of the solution was adjusted to 6.4 (pH of the original natural extract under the same dilution fold) by KOH or HCl (0.1 mM). For the experiments listed below, all the taste recombinants were diluted 1.5 times by volume and the pH were regulated at 6.4 unless otherwise specified.

Omission test. 30 incomplete taste reconstitutions were prepared by omitting single groups or individual taste compounds one by one from the CTR (Table 1). Each of 30 solutions was presented to the panelists who were asked to identify one stimulus out of three (1 incomplete taste reconstitution & 2 CTRs) that is different from the other two by using triangle test.[12,24] The panelists who judged correctly were required to describe the perceived differences in comparison to the CTRs and rate the taste attributes on a five-rating scale as described in *taste profile analysis*.

Addition test. A model solution consisting of 12 taste-active compounds which were

identified by the omission test (refer to *Addition test* in *Results and Discussion*) was prepared firstly, then the 15 components judged as no taste contribution (Table S3) were added to the model solution one by one in their natural concentrations. Each of the sample was presented to the panelists in comparison with two model solutions using a triangle test as mentioned above.

Dose-response analysis. A STR (refer to *Addition test* in *Results and Discussion*) with 20.0 mg/100 mL of PR-7 or YV-8 was prepared as the reference, and its intensity of umami and kokumi was defined as 0. Then the samples were prepared by adding PR-7 or YV-8 to a STR at increasing dose (5.0, 10.0, 20.0, 40.0, 80.0 mg/100 mL), respectively. The panelists were asked to taste the reference solution at first and then to evaluate taste attribute (umami and kokumi) of the samples on an 8 cm unstructured linear scale anchored from "suppress" (value -4) to "contribute" (value +4) based on the reference.[10]

Statistical analysis

The quantitative data were given as averages ± standard deviations (SDs) of three independent replicates. All the sensory evaluations were repeated in triplicate, giving a total of 42 responses in each attribute (14 × 3). The significant difference ($p < 0.05$, $p < 0.01$ and $p < 0.001$) of the triangle tests were performed via a Criteria Table of ISO 4120:2004(E) (Sensory analysis—Methodology—Triangle test).[24] ANOVA was used to determine the difference of individual taste attributes between natural extract, CTR, STR, CTR and STR with 20.0 mg/100 mL of four flavor peptides at a 5% significance level ($p < 0.05$) by SPSS (Version 17.0, SPSS Inc., Chicago, USA).

Results and Discussion

Quantitative analysis

To elucidate the dominant taste-active compounds and construct an artificial solution imitating the natural extract taste, the contents of putative taste compounds needed to be quantitatively determined in a fresh aqueous extract of *T. obscurus* at first. Besides, taste activity values (TAVs) were used to obtain first insights into the taste impact of individual compound. The value was calculated as the ratio between the concentration of compound and its taste threshold.[25,26] As shown in Table 1, the concentrations of all free amino acids in group I were found to be below their taste thresholds. Glutamic acid has a relatively higher TAV of 0.11, whereas TAVs of the others were less 0.1. Among the 5′-nucleotides (group II), quantitative analysis revealed IMP as the prominent umami 5′-nucleotide occurring in *T. obscurus* extract at concentrations of 103.66 ± 0.41 mg/100 mL. In contrast, the concentrations of AMP and GMP were much lower (2.38 ± 0.09 and 5.96 ± 0.07 mg/100 mL, respectively). Simultaneously, IMP has the highest TAV (4.41), followed by GMP (0.73) while AMP presented a lower value (< 0.1). Group III and IV are composed of organic acids and organic bases, respectively. Evaluation of the TAVs of these compounds revealed a rather high value of lactic acid (3.55) and succinic acid (5.32), but much lower values for betaine and TMAO (< 0.1). In group V, TAVs of Na^+, K^+ and Cl^- exceeded 1 except PO_4^{3-} (Table 1). Therefore, IMP, lactic acid, succinic acid as well as Na^+, K^+ and Cl^- may serve as potential taste contributors (TAVs > 1). Although the free amino acids were far below their taste thresholds in water, they were found to enhance the umami

and sweet character as previously described by Chen et al. for crab meat at very low concentrations.[19] So the compounds in *T. obscurus* with a low TAV (< 1) could contribute to taste even at the subthreshold concentration, and the results will be further investigated in the below.

Taste profile analysis of *T. obscurus*

According to quantitative analysis, a complete taste recombinant (CTR) containing the "natural" amounts of 28 taste compounds (Table 1) was prepared, and its taste profiles was compared with a freshly authentic aqueous extract of *T. obscurus* by the five-point rating scale. The sensory data revealed the highest scores of 2.6 and 2.5 for the intensity of umami and kokumi taste in natural extract, respectively. The saltiness was observed with a somewhat lower intensity of 1.1 (Figure 2A). In comparison, the taste attribute of sour, sweet and bitter were rated only with very low intensities (0.3-0.5). Therefore, umami and kokumi were the main body taste characteristics of *T. obscurus*, then was slight salty while the other taste qualities were especially low and could be neglected (Figure 2A). Simultaneously, the taste intensities of CTR were similar to the natural extract (Figure 2B). Sweet and sour were identical to the original, while the umami and kokumi taste just slightly lower intensity than authentic aqueous extract of *T. obscurus* ($p > 0.05$, Table S2). The results confirmed that the typical taste of the natural extract could be effectively reproduced by the 28 taste compounds (Table 1). In other words, key taste components of *T. obscurus* muscle have been successfully identified and quantitated.

Omission test

After completing taste profile analysis, omission tests were carried out to investigate the taste contribution of group and individual component and reveal the key taste substances from Table 1. The first step was group omission test (Table 2). With the exception of group IV, significant taste difference was observed when group I, II, III and V were omitted from the CTR. For example, an extremely significant ($p < 0.001$) reduction of umami and kokumi perception was observed when group I (free amino acids) was removed. Similarly, umami ($p < 0.001$) and kokumi ($p < 0.05$) perceptions were significantly decreased with the exclusion of group II (5′-nucleotides) from the CTR. These results were consistent with previous studies that free amino acids and nucleotides mainly contributed to umami attribute.[10,27] Similar experiments with the omission of group V (inorganic ions) led to a significant ($p < 0.01$) decrease of umami and saltiness. As for group IV, betaine and TMAO have been previously reported in some seafoods and contributed to sweet taste.[11,12] Whereas in this research, the taste profile of *T. obscurus* were mainly umami and kokumi, and taste characteristics of sweet was very low. Thus, the role of group IV (organic bases) in the taste of *T. obscurus* could be neglected.

After identifying the groups responsible for the taste profile of *T. obscurus*, similar omission experiments were prepared by removing individual compound from group I, II, III and V to further reduce the numbers of components that contribute significantly to taste. In the group I (Table 2), the omission of glutamic acid led to a significant ($p < 0.01$) decrease of umami. Glutamic acid is an umami amino acid reported as a taste-active substance in many foods, such as the oval squid, Chinese mitten crab,

311 mushrooms, and tomato, etc.[12,18,26,28,29] In the group II, only the omission of IMP led
312 to a total weakening of sour, salty, kokumi, and especially for umami ($p < 0.05$). The
313 overall acceptability and taste were declined distinctly in the absence of IMP. As
314 such, IMP was a dominant taste-active compound in *T. obscurus* based on the sensory
315 results and determination of TAV. In group III, removing succinic acid resulted in a
316 significantly decrease of umami ($p < 0.01$), which is consistent with quantitative
317 analysis (TAV > 1) and previous reports.[26,30] In the group V, significant taste
318 differences ($p < 0.01$) were observed with the exclusion of the four inorganic ions
319 from the complete model mixture. Omission of K^+ led to a significant increase ($p <$
320 0.05) of sourness and a significant decrease of umami ($p < 0.05$).

321 **Addition test**

322 Combining the results of group and individual omission tests, 12 compounds
323 (glutamic acid, serine, proline, arginine, lysine, AMP, IMP, succinic acid, Na^+, K^+,
324 PO_4^{3-} and Cl^-) were determined as the key taste-active compounds in *T. obscurus*. In
325 order to avoid missing potential core components, addition tests were further
326 implemented to determine whether associations of several components could have an
327 unknown effect on one or more taste properties. However, none of the component was
328 identified at the level of significance ($p < 0.05$) (Table S3).

329 Thus, a simplified taste recombinant (STR) containing the above 12 taste-active
330 compounds was obtained according to the results of the omission and addition tests.
331 In particular, glutamic acid, IMP, succinic acid and K^+, significantly contribute to the
332 umami perception in the taste recombinant of *T. obscurus* ($p < 0.05$, Table 2).

Combined with the results of TAVs, the seven compounds (except for IMP, succinic acid, Na^+, K^+ and Cl^-) were identified as the key taste compounds while their TAVs were less than 1. This means those components induced the characteristic taste of *T. obscurus* at subthreshold level. Although TAVs of lactic acid was higher than 1, it has no significant contribution to the typic taste of *T. obscurus* by a series of sensory evaluation. The reason could be that TAV is a useful value to evaluate the individual taste component, while interaction of compounds in food context may be not taken into consideration, such as synergy or masking effects.[31] Based on the above results, the reported prominent taste components from aquatic products were summarized to explore if there are the common key taste contributors in aquatic products and their correlations with *T. obscurus* (Figure 3, the detail data was presented Table S4). 39 key taste compounds originated from fish, crustaceans, mollusk and *Echinodermata* were found. Ten compounds including glutamic acid, glycine, alanine, arginine, IMP, AMP, Na^+, K^+, Cl^- and PO_4^{3-} were commonly found in most aquatic products (Figure 3), and eight of them served as key contributors to *T. obscurus*. The results indicated that most of the prominent components were similar in aquatic foodstuffs. Those components constituted the basic taste profiles of aquatic foods, while the difference in type of unique compounds and quantitation of shared substances lead to a specific taste for each aquatic food.[11,12]

Since then, taste attributes between the CTR and STR were compared by the triangle test and the five-rating scale as described before. Although a significant difference was found in the single taste attributes through a five-rating scale (Table S2), where

umami and kokumi were stronger ($P < 0.05$) in the CTR, the two solutions could be scarcely discriminated (correct response = 20/42) on the overall taste. MSG-like amino acids (aspartic acid and glutamic acid) and 5′-nucleotides, especially AMP, GMP and IMP, played a dominant role in umami taste, and the synergistic effects of those compounds could further enhance umami-like taste, such as aspartic acid, glutamic acid and IMP, sweet amino acids (serine, glycine and alanine) and IMP.[27,32-34] The lower intensity of umami taste in the STR compared with CTR might be due to the partial absence of those compounds and thus leading to a weaker synergistic effect. Whereas the difference of CTR and STR could have other unknown compounds contributing towards kokumi, such as oligopeptides.[11,35] This part will be further investigated below.

Taste contribution of flavor peptides in *T. obscurus*

To figure out whether the flavor peptides contained in *T. obscurus* contribute to the characteristic umami and kokumi taste of pufferfish, additional taste recombinants were prepared by spiking four flavor peptides (RC-6, PR-7, YV-8 and YP-9) to the CTR and STR, respectively. The four peptides were chosen for their prominent taste of fishy, umami and kokumi during isolation and purification process (the detail taste characteristics of flavor peptides in *T. obscurus* is presented in Table S5).[5,6] When the four peptides were added to the CTR, a significant ($p < 0.05$) increase of umami and kokumi perception was detected by a triangle test (Table 3). Moreover, taste profile analysis of this recombinant presented increased intensities of umami (2.3→2.6) and kokumi (2.3→2.5), which matched very well with the taste profile found in natural

377 extract (Figure 1D). Similarly, an approximate positive result was also observed in the
378 STR. The kokumi and umami intensity score was increased from 2.0 to 2.4 by adding
379 four peptides, respectively (Table S2), and was closer to the taste profile of natural
380 extract (Figure 1E). Therefore, the addition of four peptides mainly bridge the gap of
381 taste difference between the natural extract and artificial imitation recombinant of *T.*
382 *obscurus*.

383 On this basis, omission tests were also carried out to narrow the prominent flavor
384 peptides contribute to taste attribute in *T. obscurus*. Whether in CTR or STR, the
385 omission of PR-7 and YV-8 led to a significant ($p < 0.05$) reductions in umami and
386 kokumi taste (Table 4). However, there was no significant difference ($p > 0.05$) was
387 perceived in both model systems when RC-6 and YP-9 were removed. As a result, it
388 could be concluded that PR-7 and YV-8 were also the key taste-active compounds in
389 *T. obscurus*.

390 **Dose-response analysis.** To further investigate the concentration effects of PR-7 and
391 YV-8 on umami and kokumi taste of *T. obscurus*, dose-response analysis was
392 performed. Positive correlation between the concentrations and umami & kokumi
393 intensity of two peptides were revealed (Figure 4). The Pearson correlation
394 coefficients (r) of umami and kokumi for PR-7 were 0.94 and 0.92, respectively, and
395 they are both 0.98 for YV-8. Therefore, the two flavor peptides have clear umami and
396 kokumi modulation in *T. obscurus* model solution. Due to peptides losing during
397 isolation and purification processes, the amounts of the four flavor peptides (RC-6,
398 PR-7, YV-8 and YP-9) in *T. obscurus* were unknown.[5] Although the concentration

399 (20 mg/100 mL) of four flavor peptides in model solution may be inconsistent with
400 the "natural" concentrations, the results proved that flavor peptides were one of the
401 indispensable components in the characteristic taste for *T. obscurus*. Further research
402 will be necessary to quantitate the concentration of those peptides, so that their
403 contribution to the overall taste of *T. obscurus* could be rigorously elaborate.
404 Collectively, 12 compounds, glutamic acid, serine, proline, arginine, lysine, AMP,
405 IMP, succinic acid, Na^+, K^+, PO_4^{3-} and Cl^- were identified as the key taste-active
406 components in *T. obscurus*. This was similar to the common prominent taste
407 contributors that have been found in aquatic products. More especially, two flavor
408 peptides, PR-7 and YV-8 could significantly increase the typical umami and kokumi
409 taste characteristics of *T. obscurus*. The overall taste profile of *T. obscurus* can be
410 greatly reconstituted by those components. These results, therefore, help for a better
411 understanding of the delicious mystery of *T. obscurus* taste and establish the
412 theoretical foundation for the further development of pufferfish flavor.

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420 **Ethics & Conflict of Interest**

421 The authors declare no competing financial interest.

422 **Supplementary Information**

423 Table S1: Parameters related to quantitative analysis;

424 Table S2: Intensity of taste attribute of natural and synthetic extract;

425 Table S3: Results of addition tests;

426 Table S4: Key taste-active compounds in some aquatic products;

427 Table S5: Taste characteristics of flavor peptides in *T. obscurus*.

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Figure captions

Figure1. Scheme of preparation of natural extract of *T. obscurus*.

Figure 2. Taste attribute of natural and several taste recombinants of *T. obscurus*. A, taste profile of a freshly prepared natural extract; Black dotted line in B, C, D and E represented the taste intensity value which were standardized based on A (six taste attribute values of A were defined as "1"). B, the complete taste recombinant (CTR), containing 28 compounds (Table 1); C, a simplified taste recombinant (STR), containing 12 key taste-active components; D and E, a CTR and STR with four flavor peptides, respectively.

Figure 3. Key taste-active compounds that have been reported in 24 aquatic products. "24" is the result in this study while "1-23" is the literature reports. The 39 prominent components (free amino acids, 5′-nucleotides, organic acids, organic bases, inorganic ions and others) were obtained, which were originated from fish, crustaceans, mollusk and *Echinodermata*. Among them, glutamic acid, glycine, alanine, arginine, IMP, AMP as well as Na^+, K^+, Cl^- and PO_4^{3-} were commonly found in the above foodstuffs. Isoleucine was not contained.

Figure 4. Effects of addition of PR-7 (A) or YV-8 (B) at increased concentrations in a simplified taste recombinant (STR) on each taste attribute (umami and kokumi). Intensity of taste was evaluated by an 8 cm unstructured linear scale anchored from

"suppress" (value -4) to "contribute" (value 4), 0 referring to the taste intensity of standard solution (STR with 20 mg/100 mL PR-7 or YV-8). For each taste attribute, y-axis represents the amount of peptide added was 5.0, 10.0, 20.0, 40.0 and 80.0 mg/100 mL, respectively (bottom to up).

Tables

Table 1 Compositions, contents, taste thresholds and TAVs of non-volatile taste-active compounds in boiled muscles of *T. obscurus* (n=3).

Component	Content (mg/100 mL)	Taste threshold (mg/100 mL)[a]	TAV
Group I: free amino acids			
Aspartic acid	0.68±0.06	53.24	< 0.1
Glutamic acid	1.77±0.15	16.18	0.11
Serine	0.98±0.08	262.75	< 0.1
Glycine	9.47±0.70	187.75	< 0.1
Threonine	1.11±0.10	416.85	< 0.1
Alanine	6.20±0.15	106.92	< 0.1
Proline	1.33±0.12	287.75	< 0.1
Arginine	6.21±0.55	1306.50	< 0.1
Lysine	23.81±2.09	1169.60	< 0.1
Valine	0.59±0.04	351.45	< 0.1
Histidine	0.41±0.03	698.40	< 0.1
Tyrosine	0.39±0.04	72.44	< 0.1
Phenylalanine	0.83±0.08	743.40	< 0.1
Isoleucine	0.39±0.03	131.20	< 0.1
Leucine	0.61±0.05	144.32	< 0.1
Cysteine	ND	24.22[b]	—
Methionine	0.28±0.03	74.60	< 0.1
Group II: 5′-nucleotides			
AMP	2.38±0.09	86.8	< 0.1
GMP	5.96±0.07	8.14	0.73
IMP	103.66±0.41	23.53	4.41
Group III: organic acids			
Lactic acid	239.71±1.55	67.55	3.55
Succinic acid	56.53±5.69	10.63	5.32
Group IV: organic bases			
Betaine	14.95±3.68	234.30	< 0.1
TMAO	0.86±0.05	722.41	< 0.1
Group V: inorganic ions			
Na^+	28.34±2.81	8.97	3.16
K^+	85.31±15.18	50.83	1.68
PO_4^{3-}	77.07±12.46	142.46	0.54
Cl^-	45.09±11.26	13.85	3.26

Taste activity values (TAVs), the ratio between the concentration of taste compounds

and its threshold value; ND, not detected; [a]Taste threshold (mg/100 mL) was determined by a triangle test as reported recently;[11] [b]Taste threshold (mg/100 mL) was taken from the literature.[26]

Table 2 The effect of absence of group or individual compounds on the taste attributes of the complete taste recombinant of *T. obscurus*.

Omission component	NO. of correct identification (n=42)	Level of significance	Degree of taste difference (change in taste intensity)
Group I	**34**	***	**U↓***** (2.3→1.2), **K↓***** (2.3→1.5), S↓, Sa↓
Aspartic acid	19	-	
Glutamic acid	22	**	**U↓**** (2.3→1.9), So↓, Sa↓, K↓
Serine	25	***	S↑, Sa↑, U↓, K↓
Glycine	17	-	
Threonine	19	-	
Alanine	18	-	
Proline	28	***	**U↑**** (2.3→2.6), S↑, Sa↓
Arginine	28	***	S↑, Sa↓, U↑
Lysine	25	***	Sa↓, K↑
Valine	16	-	
Histidine	18	-	
Tyrosine	18	-	
Phenylalanine	16	-	
Isoleucine	15	-	
Leucine	16	-	
Methionine	14	-	
Group II	**36**	***	**U↓***** (2.3→0.9), **K↓*** (2.3→2.0), S↓, Sa↓
AMP	20	*	So↑, Sa↑
GMP	15	-	
IMP	26	***	**U↓*** (2.3→1.7), So↓, Sa↓, K↓, Overall taste faded
Group III	**25**	***	**U↓***** (2.3→1.8), So↓, S↓, Sa↑, K↓
Lactic acid	19	-	
Succinic acid	22	**	**U↓*** (2.3→2.1), So↓, B↑, K↓
Group IV	**12**	-	
Group V	**29**	***	**Sa↓**** (1.3→0.7), **U↓**** (2.3→1.8), So↓,
Na^+	29	***	B↑, Sa↓, U↓
K^+	29	***	**So↑*** (0.5→0.8), **U↓*** (2.3→2.1), Sa↓
PO_4^{3-}	24	**	So↑, Sa↓, U↓

Cl^-	27	***	So↑, B↑, U↓

So, sourness; S, sweetness; B, bitterness; Sa, saltiness; U, umami; K, kokumi; -, Not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3 Effect of four flavor peptides addition on the taste characteristics of a CTR and STR.

addition flavor peptide	NO. of correct identification (n=42)	Level of significance	Degree of taste difference (change in taste intensity)
CTR + peptide	25	***	**U**↑* (2.3→2.6), **K**↑* (2.3→2.5), So↓, Sa↑
STR + peptide	30	***	**K**↑** (2.0→2.4), **U**↑** (2.0→2.4), Sa↑, So↑,

CTR + peptide, a taste recombinant was prepared by adding four flavor peptides (RC-6, PR-7, YV-8 and YP-9) to the complete taste recombinant (CTR) containing 28 taste compounds; STR + peptide, a taste recombinant was prepared by adding four flavor peptides to the simplified taste recombinant (STR) only containing 12 key taste-active compounds; So, sourness; S, sweetness; B, bitterness; Sa, saltiness; U, umami; K, kokumi; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4 Influence of individual flavor peptide omission on the taste attributes of a complete and simplified taste recombinant.

Omission component	NO. of correct identification (n=42)	Level of significance	Degree of taste difference (change in taste intensity)
Complete taste recombinant			
RC-6	13	-	
PR-7	20	*	**U↓*** (2.6→2.4), K↓, Sa↑
YV-8	20	*	U↓, So↓, Sa↓, K↓
YR-9	13	-	
Simplified taste recombinant			
RC-6	18	-	
PR-7	21	*	**K↓*** (2.4→2.1), U↓, Sa↑
YV-8	20	*	**K↓*** (2.4→2.2), U↓
YR-9	18	-	

So, sourness; S, sweetness; B, bitterness; Sa, saltiness; U, umami; K, kokumi; -, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure graphics

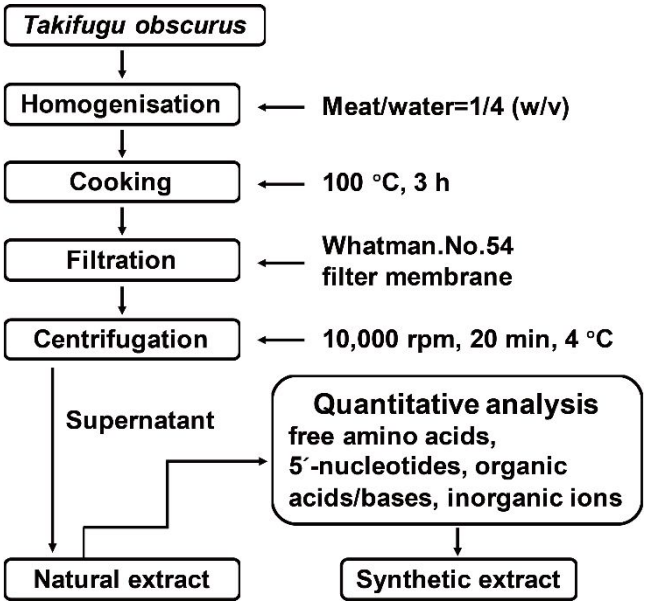
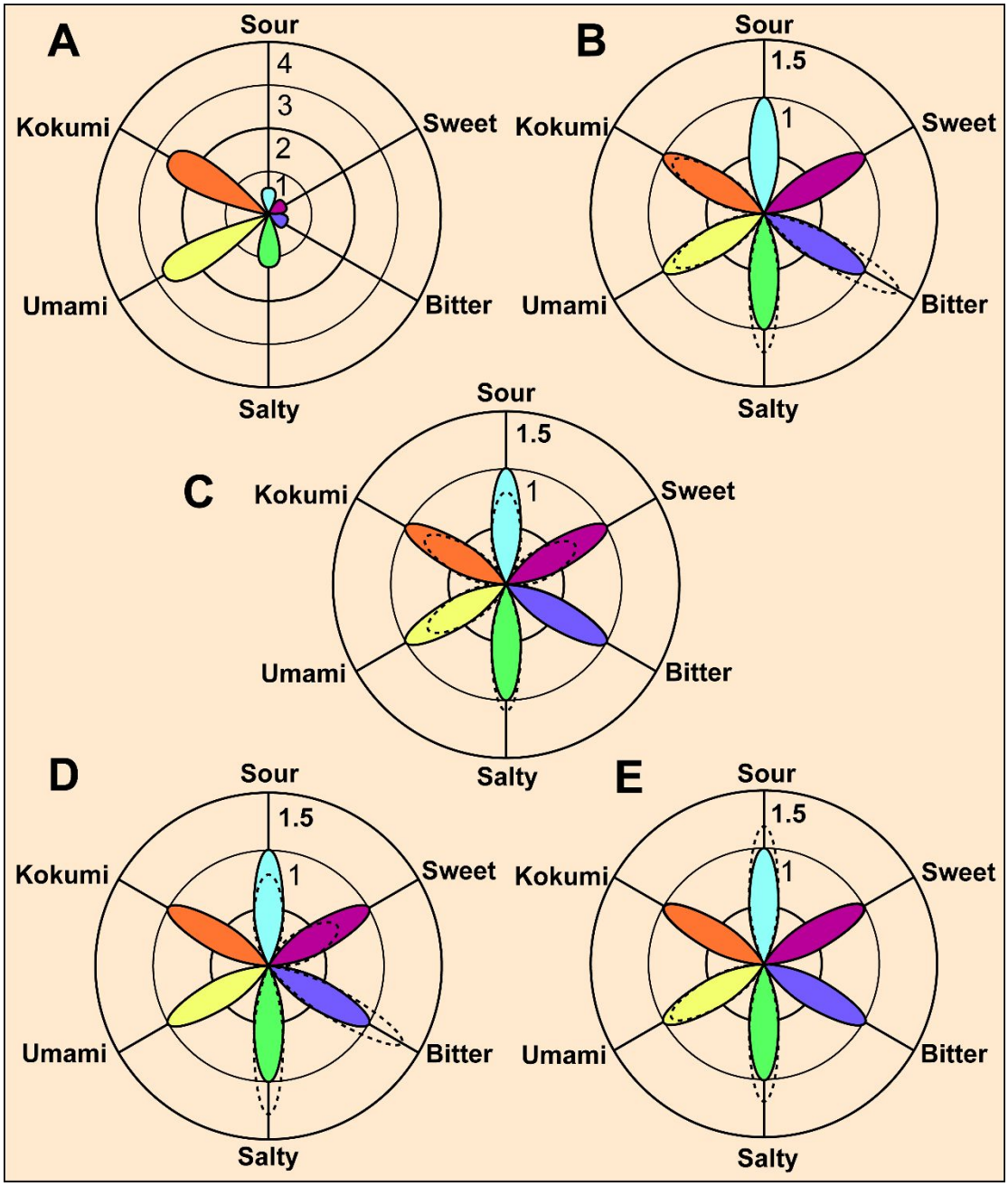


Figure 1


A
Sour
4
3
2
1
Kokumi
Sweet
Umami
Bitter
Salty
B
Sour
1.5
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Kokumi
Sweet
Umami
Bitter
Salty
C
Sour
1.5
1
Kokumi
Sweet
Umami
Bitter
Salty
D
Sour
1.5
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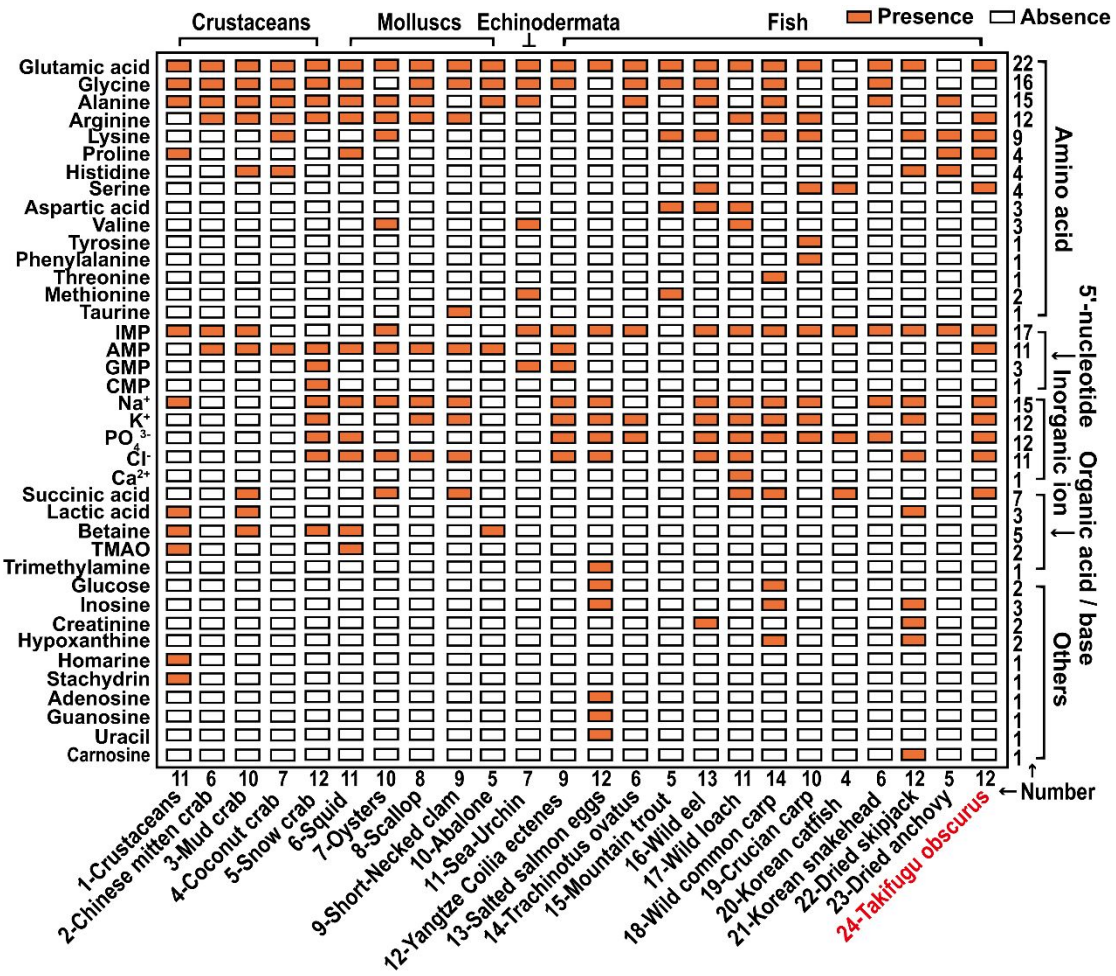
Kokumi
Sweet
Umami
Bitter
Salty

Figure 2


Crustaceans
Molluscs
Echinodermata
Fish
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Absence
Glutamic acid
Glycine
Alanine
Arginine
Lysine
Proline
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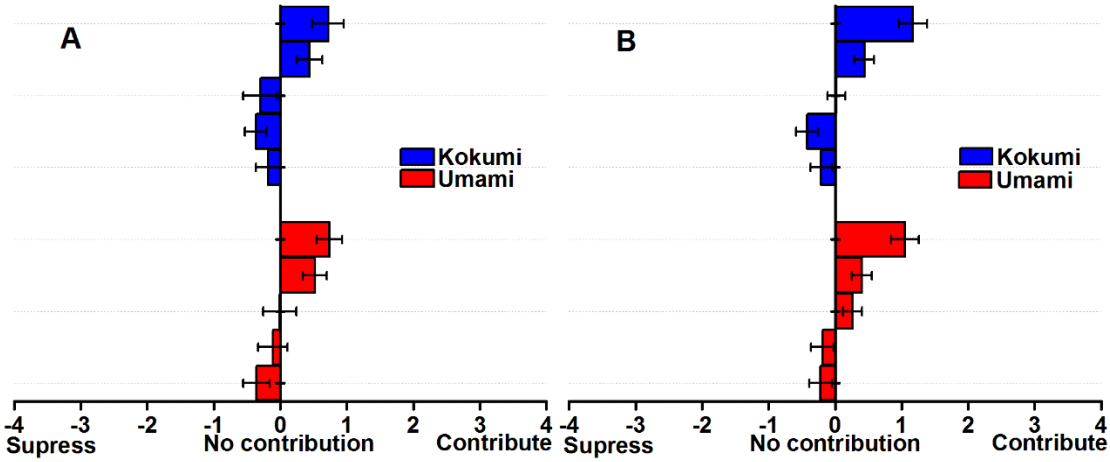
Aspartic acid
Valine
Tyrosine
Phenylalanine
Threonine
Methionine
Taurine
IMP
AMP
GMP
CMP
Na+
K+
PO43-
Cl-
Ca2+
Succinic acid
Lactic acid
Betaine
TMAO
Trimethylamine
Glucose
Inosine
Creatinine
Hypoxanthine
Homarine
Stachydrin
Adenosine
Guanosine
Uracil
Carnosine
22 16 15 12 9 4 4 4 3 3 1 1 1 2 1
17 11 3 1
15 12 12 11 1
7 3 5 2 1
2 3 2 2 1 1 1 1 1
Amino acid
5'-nucleotide
Inorganic ion
Organic acid / base
Others
11 6 10 7 12 11 10 8 9 5 7 9 12 6 5 13 11 14 10 4 6 12 5 12
Number
1-Crustaceans
2-Chinese mitten crab
3-Mud crab
4-Coconut crab
5-Snow crab
6-Squid
7-Oysters
8-Scallop
9-Short-Necked clam
10-Abalone
11-Sea-Urchin
12-Yangtze Coilia ectenes
13-Salted salmon eggs
14-Trachinotus ovatus
15-Mountain trout
16-Wild eel
17-Wild loach
18-Wild common carp
19-Crucian carp
20-Korean catfish
21-Korean snakehead
22-Dried skipjack
23-Dried anchovy
24-Takifugu obscurus

Figure 3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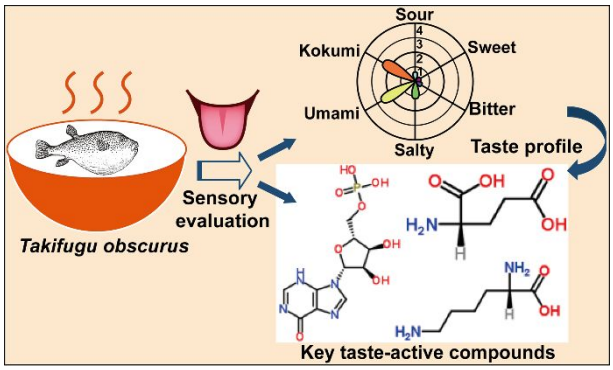
Kokumi
Umami
-4 -3 -2 -1 0 1 2 3 4
Supress
No contribution
Contribute
B
Kokumi
Umami
-4 -3 -2 -1 0 1 2 3 4
Supress
No contribution
Contribute

Figure 4

TOC graphic


Sour
Kokumi
Sweet
Umami
Bitter
Salty
Taste profile
Sensory evaluation
Takifugu obscurus
Key taste-active compounds