*Biosci. Biotechnol. Biochem.*, **75** (7), 1275–1282, 2011

# Isolation and Identification of the Umami Enhancing Compounds in Japanese Soy Sauce

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Received January 17, 2011; Accepted April 15, 2011; Online Publication, July 7, 2011
[doi:10.1271/bbb.110041]

**To clarify the key compounds that account for the umami taste of soy sauce, a typical Japanese soy sauce, *Koikuchi Shoyu*, was separated by preparative chromatography, and the umami enhancing fractions were screened on the basis of an umami intensity of a 6.0 mM monosodium L-glutamate (MSG) solution. Liquid chromatography-time of flight mass spectrometry (LC-TOFMS), 1D/2D nuclear magnetic resonance spectroscopy (NMR) studies of the umami enhancing fractions led to the identification of *N*-(1-deoxy-D-fructos-1-yl)pyroglutamic acid (Fru-pGlu), *N*-(1-deoxy-D-fructos-1-yl)valine (Fru-Val), *N*-(1-deoxy-D-fructos-1-yl)methionine (Fru-Met), pyroglutamylglutamine (pGlu-Gln), and pyroglutamylglycine (pGlu-Gly). Although all the compounds identified were at sub-threshold concentrations in the soy sauce, a taste reconstitution experiment revealed that they contributed part of the umami taste of the soy sauce.**

**Key words:** soy sauce; amadori compound; pyroglutamyl peptide; umami; taste enhancer

Traditionally-brewed soy sauce has been one of the most popular seasonings in Japan for hundreds of years. It is used not only as a sauce for food, but as a seasoning in cooking. Five typical varieties of soy sauce, categorized based on Japan Agricultural Standards, are provincially brewed for traditional dishes. Because of the differences in the materials for brewing, an individual soy sauce has a characteristic aroma and taste. In recent years, a typical Japanese soy sauce, called *Koikuchi Syoyu*, that accounts for about 85% of total soy sauce consumption in Japan is widely consumed all over the world.[1)]

Nearly equal amounts of soy beans boiled to denature the protein and wheat comminuted after roasting are used as starting materials in brewing *Koikuchi Syoyu*, followed by a seed mold called *koji* added to them. This incubated to produce enzymes over several days under high humidity conditions.[1)] After incubation, NaCl solution is mixed in and this is traditionally fermented for 6 to 8 months. After maturation, the brew is compressed to separate the filtrate and residue. The soy sauce is produced by heating the filtrate to deactivate the enzymes and to make a complex flavor.

*Koikuchi Shoyu*, produced through heating and an enzymatic reaction, is a dark-red, clear liquid exhibiting a well-balanced strong salty taste as well as a rich umami taste and the other tastants besides fresh, roast, and sweet aromas.[2–6)] Since the sensory profile of soy sauce is one of the key criteria in judging product quality, some investigations have been done to correlate flavor quality and components, but the data reported to date on the molecules imparting the umami taste of soy sauce are very limited. It is known that the umami taste of soy sauce is due to acidic amino acids, including glutamic acid and aspartic acid from soy protein and wheat gluten enzymatic hydrolysate.[6)] As for the other contributors, acidic peptides exhibiting umami have been isolated from the soy sauce,[7,8)] but the contribution of these peptides to the umami taste of soy sauce was negligible due to low concentrations.[8,9)] Other reports have suggested that low-molecular-weight compounds in soy sauce contribute to the salty and the umami taste,[10)] and that phenylalanine enhanced the umami taste of soy sauce because of its enhancing effect on that of monosodium L-glutamate (MSG),[11)] but the key driver of the umami taste of soy sauce on a molecular level is still unclear.

The objectives of the present investigation were therefore to clarify the umami taste contributors in *Koikuchi Syoyu* using instrumental analyses and sensory analyses in order to isolate and identify the key compounds, and to evaluate their taste impact on the basis of model experiments.

## Materials and Methods

*Chemicals.* Amino acids, organic acids, sugars, minerals, caffeine, and quercetin-3-rutinoside hydrate were purchased from Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemical (Tokyo, Japan), and Tokyo Chemical Industry (Tokyo). Pyroglutamylglutamine (pGlu-Gln) and pyroglutamylglycine (pGlu-Gly) were purchased from Bachem (Bubendorf, Switzerland) (Fig. 1). *N*-(1-deoxy-D-fructos-1-yl)pyroglutamic acid (Fru-pGlu), *N*-(1-deoxy-D-fructos-1-yl)valine (Fru-Val), and *N*-(1-deoxy-D-fructos-1-yl)methionine (Fru-Met) were synthesized following the literature.[12)] All solvents were of HPLC grade (Kanto Chemical, Tokyo). Deuterium oxide was purchased from

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*Abbreviations*: COSY, correlation spectroscopy; DoT, dose-over-threshold; Fru-Met, *N*-(1-deoxy-D-fructos-1-yl)methionine; Fru-pGlu, *N*-(1-deoxy-D-fructos-1-yl)pyroglutamic acid; Fru-Val, *N*-(1-deoxy-D-fructos-1-yl)valine; GPC, gel permeation chromatography; HILIC, hydrophilic interaction chromatography; HMBC, hetero-nuclear multiple quantum coherence; HMQC, hetero-nuclear multiple-bond connectivity; LC-TOFMS, liquid chromatography-time of flight mass spectrometry; MSG, monosodium L-glutamate; NMR, nuclear magnetic resonance spectroscopy; pGlu-Gln, pyroglutamylglutamine; pGlu-Gly, pyroglutamylglycine; RP-HPLC, reverse phase-high performance liquid chromatography

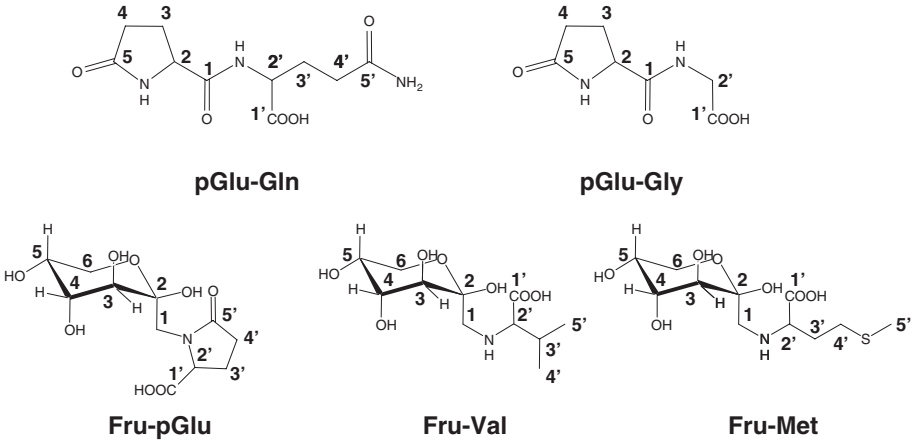


**Fig. 1.** Chemical Structures of Pyroglutamylpeptides and Amadori Compounds ($\beta$-pyranose form).

**Table 1.** Yields of ODS Fractions

| Fraction | Yield from the soy sauce (%) |
|---|---|
| A | 30.5 |
| B | 1.9 |
| C | 1.8 |
| D | 0.7 |
| E | 0.9 |
| F | 0.2 |
| G | 1.9 |
| H | 0.03 |

Euriso-Top (Gif-sur-Yvette, France). *Koikuchi Syoyu* was purchased from Marushinhonke (Yuasa, Japan).

*Preparative separation of soy sauce on reverse phase-high performance liquid chromatography (RP-HPLC).* Two mL of the soy sauce was separated by RP-HPLC using an ODS RP18 column (10 µm, 250 × 20 mm i.d., Fuji Silysia Chemical, Kasugai, Japan). Monitoring the effluent at 214 nm, chromatography was performed with a 0.1% formic acid solution (methanol:water=3:97) at a flow rate of 12.0 mL/min for 5 min, then increasing the methanol content to 15% over 15 min, and finally increasing the methanol content to 100% over a further 10 min. In total, 8 fractions, A to H, were collected individually 100 times, and then the eluates of the corresponding fractions were combined and freeze-dried. The yields of the individual fractions are summarized in Table 1.

*Preparative separation of fraction B on gel permeation chromatography (GPC).* An aliquot (1.0 g) of fraction B was dissolved in distilled water (20 mL) containing 0.1% formic acid, and after filtration, 2 mL of the solution was separated by GPC using a steel column (500 × 22 mm i.d.) filled with an acidic (0.1% formic acid) water slurry of a Sephadex G-10 (55–165 µm, GE Healthcare UK, Little Chalfont, UK). Monitoring the effluent with a refractive index detector, chromatography was performed with a 0.1% formic acid solution at a flow rate of 0.5 mL/min. In total, 4 fractions, B-I to B-IV, were collected individually 10 times, and then the eluates of the corresponding fractions were combined and freeze-dried. The yields of the individual fractions are summarized in Table 2.

*Preparative separation of fraction B-II on hydrophilic interaction chromatography (HILIC).* An aliquot (81 mg) of fraction B-II was taken up in a 90% acetonitrile aqueous solution (acetonitrile:water= 9:1) containing 0.1% formic acid (16.3 mL), and membrane filtered, and then 0.5 mL of the sample was separated by HPLC using an Atlantis HILIC Silica column (5 µm, 250 × 4.6 mm i.d., Nihon Waters, Tokyo). Monitoring the effluent at 214 nm, chromatography was performed with 95% acetonitrile containing aqueous formic acid (0.1% in water) at a flow rate of 1.0 mL/min, then decreasing the acetonitrile content to 91.5% over 16.7 min, and then rapidly decreasing it to 70% over a further 1.3 min. This condition was maintained for 15 min. In total, 20 peaks, B-II-1 to B-II-20, were collected individually 32 times, and then the eluates of the corresponding fractions were combined, evaporated, and freeze-dried.

**Table 2.** Yields of GPC Fractions

| Fraction | Yield (%) | |
|---|---|---|
| | From the soy sauce | From fraction B |
| B-I | 0.1 | 3.2 |
| B-II | 1.4 | 72.9 |
| B-III | 0.3 | 16.5 |
| B-IV | 0.1 | 5.3 |

*Isolation and identification of Fru-pGlu and pGlu-Gly in fraction B-II-6.* An aliquot (6.0 mg) of fraction B-II-6 was taken up in a solution (acetonitrile:water=98:2) containing 0.1% formic acid (0.6 mL) and then 50 µL of the sample was separated by HPLC using an Atlantis HILIC Silica column (5 µm, 250 × 4.6 mm i.d., Nihon Waters, Tokyo). Monitoring the effluent at 214 nm, chromatography was performed with 98% acetonitrile containing aqueous formic acid (0.1%) at a flow rate of 1.0 mL/min over 20 min, then decreasing the acetonitrile content to 86% over 40 min. Fru-pGlu and pGlu-Gly were obtained from 46 min to 48.4 min, and 48.4 min to 52 min respectively. In total, the respective fractions were collected individually 10 times, and then the eluates of the corresponding fractions were combined, evaporated, and freeze-dried.

Fru-pGlu: $^{13}C\{^1H\}$ NMR data and the ratio of the cyclic form at equilibrium in a $D_2O$ solution agreed with the data reported in the literature.[12] $^1H$ NMR (400 MHz, $D_2O$; COSY): $\delta$ 2.13 (*m*, 1H, H-C(3′)), 2.43–2.62 (*m*, 3H, H-C(3′), H-C(4′)), 3.18–4.11 (7H, H-C(1), H-C(3), H-C(4), H-C(5), H-C(6)), 4.56 (*dd*, 0.24H, $^3J = 8.70$ Hz, $^3J = 2.70$ Hz, H-C(2′) in $\alpha$-furanose form), 4.61 (*d*, 0.12H, $^3J = 8.25$ Hz, H-C(2′) in $\beta$-furanose form), 4.67 (*dd*, 0.64H, $^3J = 9.15$ Hz, $^3J = 2.75$ Hz, H-C(2′) in $\beta$-pyranose form).

pGlu-Gly: $^1H$ NMR (400 MHz, $D_2O$; COSY): $\delta$ 2.12 (*m*, 1H, H-C(3)), 2.42 (*m*, 2H, H-C(4)), 2.57 (*m*, 1H, H-C(3)), 4.02 (*s*, 2H, H-C(2′)), 4.37 (*dd*, 2H, $^3J = 4.91$ Hz, $^3J = 8.93$ Hz, H-C(2)); $^{13}C\{^1H\}$ NMR (100 MHz, $D_2O$; HMQC, HMBC): $\delta$ 25.5 [C(3)], 29.6 [C(4)], 41.4 [C(2′)], 57.3 [C(2)], 173.5 [C(1′)], 176.0 [C(1)], 182.8 [C(5)].

*Identification of pGlu-Gln in fraction B-II-13.* pGlu-Gln: $^1H$ NMR (400 MHz, $D_2O$; COSY): $\delta$ 2.01–2.17 (*m*, 3H, H-C(3′), H-C(3)), 2.25 (*m*, 1H, H-C(3′)), 2.39–2.48 (*m*, 4H, H-C(4), H-C(4′)), 2.58 (*m*, 1H, H-C(3)), 4.40 (*dd*, 1H, $^3J = 5.03$ Hz, $^3J = 9.08$ Hz, H-C(2)), 4.44 (*dd*, 1H, $^3J = 5.11$ Hz, $^3J = 9.18$ Hz, H-C(2′)); $^{13}C\{^1H\}$ NMR (100 MHz, $D_2O$; HMQC, HMBC): $\delta$ 25.5 [C(3)], 26.6 [C(3′)], 29.6 [C(4)], 31.6 [C(4′)], 52.7 [C(2′)], 57.2 [C(2)], 175.2, 175.5 [C(1′), C(1)], 178.4 [C(5′)], 182.7 [C(5)].

*Isolation and identification of Fru-Val and Fru-Met in fraction B-II-19.* An aliquot (12.0 mg) of fraction B-II-19 was taken up in distilled

water containing 0.1% formic acid (1.2 mL), and then 50 µL of the sample was separated by HPLC using a Develosil C30-UG column (5 µm, 250 × 4.6 mm i.d., Nomura Chemical, Aichi, Japan). Monitoring the effluent at 214 nm, chromatography was performed with 100% distilled water containing aqueous formic acid (0.1%) at a flow rate of 1.0 mL/min over 15 min. Fru-Val and Fru-Met were obtained from 6.8 min to 7.6 min, and 11.0 min to 11.8 min respectively. In total, the respective fractions were collected individually 20 times, and then the eluates of the corresponding fractions were combined, evaporated, and freeze-dried.

Fru-Val: $^{13}C\{^{1}H\}$ NMR data and the ratio of the cyclic form at equilibrium in a $D_2O$ solution agreed with the data reported in the literature.[12] $^{1}H$ NMR (400 MHz, $D_2O$; COSY): $\delta$ 0.96 (3H, H-C(4′)), 1.04 (3H, H-C(5′)), 2.20 (1H, H-C(3′)), 3.23–3.36 (2H, H-C(1)), 3.47–4.18 (6H, H-C(3), H-C(4), H-C(5), H-C(6), H-C(2′)).

Fru-Met: $^{13}C\{^{1}H\}$ NMR data and the ratio of the cyclic form at equilibrium in a $D_2O$ solution agreed with the data reported in the literature.[12] $^{1}H$ NMR (400 MHz, $D_2O$; COSY): $\delta$ 2.12 (3H, H-C(5′)), 2.17–2.22 (2H, H-C(4′)), 2.61–2.65 (2H, H-C(3′)), 3.27–3.33 (1H, H-C(1)), 3.63–4.20 (6H, H-C(3), H-C(4), H-C(5), H-C(6), H-C(2′)).

*Quantitative analysis.* Amino acids were quantified using an L-8500A amino acids analyzer (Hitachi High Technologies, Tokyo) by ninhydrin detection.[13] Organic acids were quantified by HPLC using a Shim-pack SCR-102H column (7 µm, 300 × 8.0 mm i.d. ×2, Shimadzu, Kyoto, Japan) and detected with a conductivity detector.[14] Sodium, potassium, magnesium, and calcium were quantified by HPLC using a Shim-pack IC-C3 column (7 µm, 100 × 4.6 mm i.d., Shimadzu) detected with a conductivity detector.[15] Chloride was determined by potentiometry.[16] Sugars were quantified by HPLC using an Asahipak NH2P-50 4E column (5 µm, 4.6 × 250 mm i.d., Showa Denko, Tokyo) detected with a refractive index detector. Chromatography was performed under isocratic conditions of 80% acetonitrile at a flow rate of 1.0 mL/min for 40 min. Pyroglutamylpeptides and amadori compounds were quantified by LC-TOFMS using an ACQUITY UPLC BEH C18 column (1.7 µm, 100 × 2.1 mm i.d., Nihon Waters, Tokyo). All quantitative data represent the mean values of triplicate measurements.

*Liquid chromatography-time of flight mass spectrometry (LC-TOFMS).* LC-TOFMS measurements were done with an ACQUITY UltraPerformance LC equipped with an ACQUITY UPLC BEH C18 column (1.7 µm, 100 × 2.1 mm i.d., Nihon Waters) and a Micromass LCT Premier system (Nihon Waters, Tokyo) in $ESI^{+}$ mode. Chromatography was done with a 0.1% formic acid solution (methanol:water=3:97) at a flow rate of 0.5 mL/min for 2 min, then increasing the methanol content to 100% over 3 min. After that, conditions were maintained for 5 min. The TOFMS system was initially calibrated with sodium formate, and analysis was carried out under the following conditions: electrospray positive mode; MS capillary voltage, 3,000 V; sample cone, 50 V; source temperature, 100 °C; cone gas flow, 50 l/h; desolvation gas temperature, 350 °C; desolvation gas flow, 800 l/h; internal reference, leucine enkephalin protonated peak at $m/z$ 556.2771; and scan, $m/z$ 100–1000 at 0.5 s/scan.

*Nuclear magnetic resonance spectroscopy (NMR).* $^{1}H$, $^{13}C$, COSY, HMQC, and HMBC spectroscopic experiments were done using a Bruker AVANCE-400 spectrometer (Bruker Biospin, Yokohama, Japan). $D_2O$ was used as the solvent, and chemical shifts were measured using methanol as internal standard.

*Training of the sensory panel.* Twelve subjects with no history of known taste disorders were trained to evaluate the tastes of aqueous solutions (2 mL each) of the following standard taste compounds in distilled water (pH 6.0) using a triangle test, as described in the literature:[17] sucrose (50 mmol/L) for sweet, lactic acid (20 mmol/L) for sour, NaCl (20 mmol/L) for salty, caffeine (1 mmol/L) for bitter, MSG (8 mmol/L) for umami, and quercetin-3-rutinoside (0.01 mmol/L) for astringency. The assessors had participated at regular intervals for at least 2 years in sensory experiments and were thus familiar with the techniques applied. Sensory analyses were performed in a sensory panel room at 24–26 °C in three different sessions.

*Evaluation of umami taste intensity.* ODS fractions diluted to one-fifth concentration in the soy sauce with distilled water (pH = 4.5) and the five-fold diluted soy sauce with distilled water (pH = 4.5) were presented to the sensory panel, who were asked to score umami taste quality on a scale from 0 (not detectable) to 5 (strong). To achieve this, samples (2 mL) were applied to the tongue with a plastic pipette, briefly swirled around the mouth, and then expectorated. In order to evaluate the potential of the HPLC fractions in enhancing the umami taste of MSG, the umami taste intensity of a 6.0 mM MSG solution containing the fraction diluted to one-fifth concentration in the soy sauce with distilled water was compared to a 6.0 mM MSG solution as control.

*Determination of recognition-threshold concentrations.* The taste thresholds of pyroglutamylpeptides and amadori compounds were determined by triangle test with two samples of distilled water as control. To prevent fatigue, tasting began at a concentration level two steps below the threshold concentration, which had been determined in a preliminary taste experiment. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step. When the panelist selected correctly, the same concentration was presented again as proof for the correctness of the data. The geometric mean of the last and the penultimate concentration was calculated and taken to be the individual recognition threshold. The recognition threshold value of the compound was calculated by averaging the recognition thresholds of the individual panelist.

*Taste reconstitution experiment.* In order to reconstitute the umami taste of the soy sauce, the compounds summarized in Table 3 were dissolved in one-fifth concentrations of the natural source in distilled water, which was adjusted to pH 4.5. The taste intensity of an aliquot (2 mL) of the reconstituted solution was evaluated by a five-point scoring method, and was compared to the taste of the authentic soy sauce.

## Results and Discussion

### *Screening for the umami taste contributors in the soy sauce*

In order to get initial insight into the key drivers of the typical umami sensation imparted by the soy sauce, the sauce was separated into eight fractions by preparative RP-HPLC using an ODS column monitoring the effluent at 214 nm (Fig. 2A). After freeze-drying, an aliquot of each individual fraction was dissolved in distilled water at one-fifth concentration in the soy sauce, and the umami intensity was judged by the trained sensory panel on a scale from 0 to 5. As shown in Fig. 2B, fraction A exhibited the strongest umami taste (1.9) among the ODS fractions, and the other fractions did not exhibit any umami taste. However, the soy sauce and the recombinants of all the ODS fractions (rODS) exhibited much more strongly (3.3 and 2.9). These results suggest that there were umami enhancing compounds in ODS fractions B-H that did not exhibit any umami taste on their own.

To clarify the umami-enhancing effect described above, each ODS fraction was dissolved in an aqueous solution of a 6.0 mM MSG and screened on the basis of the umami intensity of a 6.0 mM MSG solution. As shown in Fig. 2C, fraction A strongly enhanced the umami taste of MSG (1.1 → 4.0), followed by fraction B (1.1 → 2.5), but the other fractions did not enhance the activity of the umami taste of MSG. Because fraction A contained almost all glutamic acid, aspartic acid, Na, and K, which are thought to be the dominant contributors to the umami taste of soy sauce, these compounds appeared to contribute to enhancement of the umami

**Table 3.** Concentrations of Minerals, Organic Acids, Amino Acids, and Sugars in the Soy Sauce

| Compound | Concentration[a] (mmol/L) | One-fifth concentration[a] (mmol/L) | Coefficient of variation[b] (%) |
|---|---|---|---|
| Minerals | | | |
| Na | 2723.0 | 544.7 | 0.1 |
| K | 103.3 | 20.7 | 0.2 |
| Mg | 36.6 | 7.3 | 0.5 |
| Ca | 8.8 | 1.8 | 1.0 |
| Cl | 2576.7 | 515.3 | 1.0 |
| Organic acids | | | |
| phosphoric acid | 61.6 | 12.3 | 0.6 |
| malic acid | 5.5 | 1.1 | 0.8 |
| succinic acid | 1.4 | 0.3 | 3.9 |
| lactic acid | 257.7 | 51.5 | 0.3 |
| acetic acid | 30.5 | 6.1 | 0.8 |
| pyroglutamic acid | 45.6 | 9.1 | 1.0 |
| Sugars | | | |
| glucose | 85.9 | 17.2 | 1.7 |
| fructose | 10.6 | 2.1 | 5.4 |
| Amino acids | | | |
| L-aspartic acid | 30.8 | 6.2 | 0.2 |
| L-threonine | 29.2 | 5.8 | 0.2 |
| L-serine | 52.9 | 10.6 | 0.3 |
| L-glutamic acid | 104.0 | 20.8 | 0.1 |
| glycine | 36.0 | 7.2 | 0.3 |
| L-alanine | 74.1 | 14.8 | 0.3 |
| L-valine | 45.3 | 9.1 | 0.4 |
| L-methionine | 7.7 | 1.5 | 0.3 |
| L-isoleucine | 36.2 | 7.2 | 0.1 |
| L-leucine | 58.0 | 11.6 | 0.2 |
| L-phenylalanine | 28.2 | 5.6 | 0.2 |
| L-lysine | 26.0 | 5.2 | 0.2 |
| L-histidine | 10.6 | 2.1 | 0.2 |
| L-arginine | 8.1 | 1.6 | 0.3 |
| L-proline | 71.4 | 14.3 | 0.3 |

[a]Data are mean value of the triplicate measurement.
[b]The ratio of the standard deviation to the mean.

taste in fraction A (Tables 3 and 4). On the other hand, the umami taste contributors in fraction B, which did not exhibit any umami taste on their own, was still unclear, because fraction B consisted of isoleucine, leucine, methionine, lactic acid, pyroglutamyl acid, and the remaining half of unidentified compounds.

In order to identify the key compounds responsible for the umami-enhancing effect in the fraction B, further separation was performed into four subfractions by preparative GPC (Fig. 3A). After freeze-drying, an aliquot of each individual fraction was evaluated for the umami-enhancing effect of the MSG solution. As shown in Fig. 3B, the presence of fraction B-II resulted in a significant increase in the umami taste intensity of the MSG solution (1.3 → 2.7).

To identify the key drivers of the umami-enhancing effect of fraction B-II, fraction B-II was separated into 20 subfractions by HILIC-HPLC monitoring the effluent at 214 nm (Fig. 4A). After freeze-drying, an aliquot of each individual fraction was evaluated for the umami-enhancing effect of the MSG solution again. As shown in Fig. 4B, the presence of fraction B-II-6, B-II-11, B-II-13, B-II-18, or B-II-19 resulted in a significant increase in the umami taste intensity of the MSG solution.

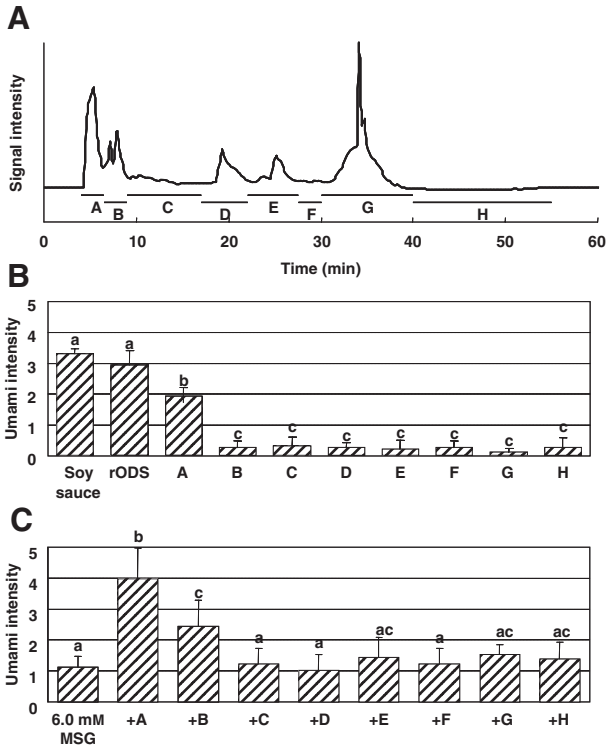


**Fig. 2.** RP-HPLC Chromatogram of the Soy Sauce Monitored at 214 nm (A), Umami Intensity of the Soy Sauce, the Recombinants of All the ODS Fractions (rODS) and the RP-HPLC Fractions (B), and Umami Enhancement Activity of the RP-HPLC Fractions to a 6.0 mM MSG Solution (C) (different letters show significant differences ($p < 0.05$)).

*Identification of pyroglutamylpeptides and amadori compounds from the umami-enhancing fractions*

Because two peaks were detected on the HILIC chromatogram of fraction B-II-6 at 214 nm, further separation was done by HILIC-HPLC. After purification, 1D/2D-NMR and LC-TOFMS measurements led to the identification of Fru-pGlu and pGlu-Gly in fraction B-II-6 (Fig. 1). The $^{13}C\{^1H\}$ NMR spectrum of Fru-pGlu was assigned by comparing the spectroscopic data with a reported reference.[12] The $^1H$ NMR resonance signals at 4.67 ppm, 4.61 ppm, and 4.56 ppm were the H-C(2′) protons of $\beta$-pyranose, $\beta$-furanose, and $\alpha$-furanose respectively, and the ratio of cyclic structures determined by quantifying their intensities was 64% for $\beta$-pyranose, 24% for $\alpha$-furanose, and 12% for $\beta$-furanose (Fig. 5). The resonance signals at 3.18–4.11 ppm were attributed to the D-fructose protons of H-C(1), H-C(3), H-C(4), H-C(5), and H-C(6). The chemical shifts and the assignment of protons H-C(3′) and H-C(4′) were achieved by homonuclear (COSY) and heteronuclear (HMQC, HMBC) correlation experiments. By LC-TOFMS analysis, the exact mass ion $m/z$ 292.1028 (calculated 292.1032) was determined as the pseudomolecular ion. The product ions $m/z$ 274.0913 (calculated 274.0927), 256.0826 (calculated 256.0821), and 238.0708 (calculated 238.0715) were determined due to the loss of molecules of water (Table 5). All spectroscopic data were confirmed by comparison with the synthesized compound. Although Fru-pGlu has been reported in model synthesis,[12] it has not yet been reported from a natural source.

**Table 4.** Concentrations of Minerals, Organic Acids, and Amino Acids in the ODS Fractions

| Compound | Fraction A | | Fraction B | |
|---|---|---|---|---|
| | Concentration[abc] (mmol/L) | Coefficient of variation[d] (%) | Concentration[abc] (mmol/L) | Coefficient of variation[d] (%) |
| Minerals | | | | |
| Na | 2639.4 | 0.1 | tr. | — |
| K | 95.0 | 0.1 | tr. | — |
| Mg | 33.6 | 0.7 | tr. | — |
| Ca | 7.7 | 1.4 | tr. | — |
| Organic acids | | | | |
| phosphoric acid | 56.7 | 0.9 | tr. | — |
| malic acid | 4.1 | 3.4 | tr. | — |
| succinic acid | — | — | tr. | — |
| lactic acid | 182.4 | 0.5 | 31.8 | 0.1 |
| pyroglutamic acid | 29.6 | 2.2 | 12.0 | 0.1 |
| Amino acids | | | | |
| L-aspartic acid | 30.6 | 0.2 | tr. | — |
| L-threonine | 27.1 | 0.1 | tr. | — |
| L-serine | 42.7 | 0.2 | tr. | — |
| L-glutamic acid | 100.6 | 0.1 | tr. | — |
| glycine | 31.8 | 0.1 | tr. | — |
| L-alanine | 70.7 | 0.1 | tr. | — |
| L-valine | 35.3 | 0.2 | 1.2 | 0.2 |
| L-methionine | tr. | — | 6.9 | 0.3 |
| L-isoleucine | 1.0 | 2.6 | 25.4 | 0.3 |
| L-leucine | 3.7 | 0.2 | 12.3 | 0.2 |
| L-phenylalanine | tr. | — | tr. | — |
| L-lysine | 24.5 | 0.2 | tr. | — |
| L-histidine | 9.7 | 0.3 | tr. | — |
| L-arginine | 7.1 | 0.2 | — | — |
| L-proline | 60.3 | 0.2 | tr. | — |

[a]Data are mean values of triplicate measurements.
[b]Data were calculated as the concentration in the soy sauce based on the yields summarized in Table 2.
[c]tr., trace; —, not detected.
[d]The ratio of the standard deviation to the mean.

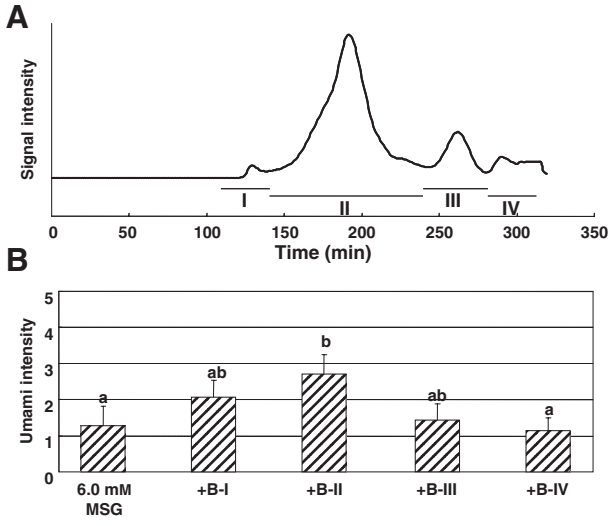


**Fig. 3.** GPC Chromatogram of the Fraction B Monitored by a Reflective Index Detector (A) and the Umami Enhancement Activity of the GPC Fractions to a 6.0 mM MSG Solution (B) (different letters show significant differences ($p < 0.05$)).

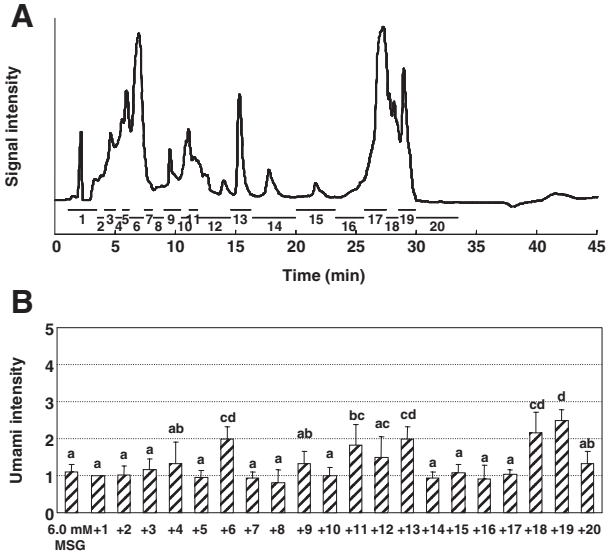


**Fig. 4.** HILIC-HPLC Chromatogram of the Fraction B-II Monitored at 214 nm (A) and the Umami-Enhancement Activity of the HILIC Fractions to a 6.0 mM MSG Solution (B) (different letters show significant differences ($p < 0.05$)).

The other compound in fraction B-II-6 was identified as pGlu-Gly by 1D/2D-NMR and LC-TOFMS measurements. $^1$H NMR showed a resonance signal at 4.37 ppm for the H-C(2) proton with double doublet coupling as part of the amide moiety, and a signal at 4.02 ppm for the H-C(2′) proton with doublet coupling as part of the carbamoyl moiety. The chemical shifts and the assignment of protons H-C(3) and H-C(4) were done in homonuclear (COSY) and heteronuclear (HMQC, HMBC) correlation experiments. Carbonyl carbons,

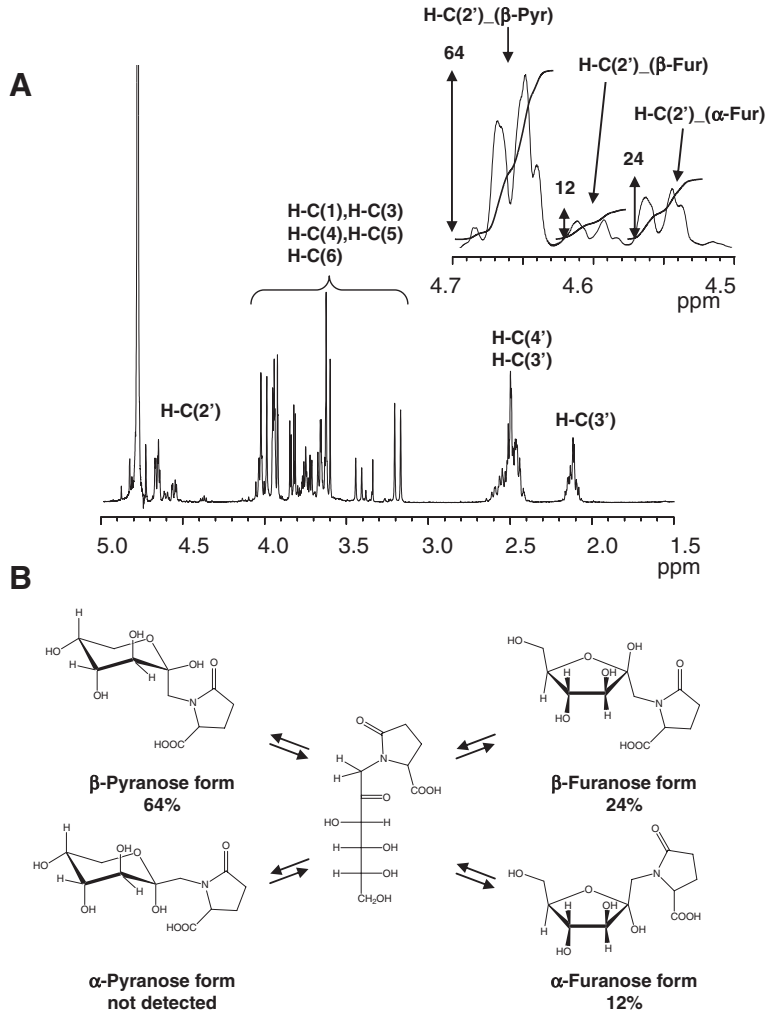


**Fig. 5.** $^{1}$H NMR Spectrum (400 MHz, $D_2O$) (A) and Cyclic Structures of *N*-(1-Deoxy-D-fructos-1-yl)pyroglutamic Acid (Fru-pGlu) (B).

**Table 5.** LC-TOFMS Spectra of Amadori Compounds and Pyroglutamylpeptides Isolated from the Soy Sauce

| Compound | Exact mass $m/z$ | | Ions |
|---|---|---|---|
| | measured | calculated | |
| Fru-pGlu | 292.1028 | 292.1032 | $[M + H]^+$ |
| | 274.0913 | 274.0927 | $[M + H–H_2O]^+$ |
| | 256.0826 | 256.0821 | $[M + H–2H_2O]^+$ |
| | 238.0708 | 238.0715 | $[M + H–3H_2O]^+$ |
| pGlu-Gly | 187.0712 | 187.0719 | $[M + H]^+$ |
| pGlu-Gln | 258.1097 | 258.1090 | $[M + H]^+$ |
| Fru-Val | 280.1397 | 280.1396 | $[M + H]^+$ |
| | 262.1274 | 262.1291 | $[M + H–H_2O]^+$ |
| | 244.1182 | 244.1185 | $[M + H–2H_2O]^+$ |
| | 234.1372 | 234.1341 | $[M + H–H_2O–CO]^+$ |
| Fru-Met | 312.1123 | 312.1117 | $[M + H]^+$ |
| | 294.0998 | 294.1011 | $[M + H–H_2O]^+$ |
| | 276.0907 | 276.0906 | $[M + H–2H_2O]^+$ |

C(5), C(1), and C(1′), appeared at 182.8 ppm, 176.0 ppm, and 173.5 ppm in $^{13}C\{^{1}H\}$ NMR and HMBC experiments. C(2) and C(2′) appeared at 57.3 ppm and 41.4 ppm as parts of the carbamoyl and amide moieties respectively. The signals of the methylene carbons C(4) and C(3) were determined to be 29.6 ppm and 25.5 ppm respectively. By LC-TOFMS analysis, the exact mass ion $m/z$ 187.0712 (calculated 187.0719) was determined as the pseudomolecular ion (Table 5). All spectroscopic data were confirmed by comparison with the commercially available chemical.

Because fraction B-II-13 was detected as a single peak at 214 nm on HPLC, identification was achieved without any purification step. 1D/2D-NMR and LC-TOFMS analysis led to the identification of pGlu-Gln in fraction B-II-13 (Fig. 1). $^{1}$H NMR showed resonance signals at 4.44 ppm and 4.40 ppm for the H-C(2′) and H-C(2) protons with double doublet coupling as parts of the carbamoyl and amide moieties respectively. The chemical shifts and the assignment of protons H-C(3), H-C(4), H-C(3′), and H-C(4′) were achieved in homonuclear (COSY) and heteronuclear (HMQC, HMBC) correlation experiments. Carbonyl carbons, C(5), C(5′), C(1), and C(1′), appeared at 182.7 ppm, 178.4 ppm, 175.5 ppm, and 175.2 ppm by $^{13}C\{^{1}H\}$ NMR and HMBC experiment. C(2) and C(2′) appeared at 57.2 ppm and 52.7 ppm as parts of the carbamoyl and amide moieties respectively. The signals of methylene carbons C(4′), C(4), C(3′), C(3) were determined at 31.6 ppm, 29.6 ppm, 26.6 ppm, and 25.5 ppm respectively. By LC-TOFMS analysis, the exact mass ion $m/z$ 258.1097 (calculated 258.1090) was determined as the pseudomolecular ion (Table 5). All spectroscopic data were confirmed by comparison with the commercially available chemical. pGlu-Gln has been identified in the enzymatic hydrolysate of wheat gluten by FAB-MS/MS

**Table 6.** Taste Qualities, Concentrations, Taste Thresholds, and Dose-over-Threshold (DoT) Factors of Amadori Compounds and Pyroglutamyl-peptides in the Soy Sauce

| Compound | Taste quality | Concentration[abc] (mmol/L) | One-fifth concentration[abc] (mmol/L) | Taste threshold (mmol/L) | DoT factor[d] |
|---|---|---|---|---|---|
| Fru-pGlu | astringent | 1.6 | 0.3 | 2.6 | 0.1 |
| Fru-Val | bitter | 1.5 | 0.3 | 1.8 | 0.2 |
| Fru-Met | astringent | 1.0 | 0.2 | 1.6 | 0.1 |
| pGlu-Gln | sweet | 1.6 | 0.3 | 1.9 | 0.2 |
| pGlu-Gly | sweet | 0.2 | 0.05 | 2.2 | >0.1 |

[a]Data are mean values of triplicate measurements.
[b]The fraction B was used for quantification.
[c]Data were calculated as the concentration in the soy sauce based on the yield of fraction B, as described in Table 2.
[d]The DoT factor was calculated as the ratio at one-fifth concentration in the soy sauce and the taste threshold concentration of the compound.

measurement and by amino acid analysis of its hydrolysate by pyroglutamate aminopeptidase.[18)]

Because many compounds were still present in fraction B-II-19, further separation was done by RP-HPLC using a C-30 column. 1D/2D-NMR and LC-TOFMS measurements led to the identification of Fru-Val and Fru-Met as the major and second major components at 214 nm in fraction B-II-19 (Fig. 1). The $^{13}C\{^{1}H\}$ NMR spectrum of Fru-Val was assigned by comparing the spectroscopic data with a reported reference.[12)] The $^{1}H$ NMR resonance signals at 3.47–4.18 ppm were attributed to the D-fructose protons of H-C(3), H-C(4), H-C(5), H-C(6), and H-C(2′). Chemical shifts and the assignment of protons H-C(1), H-C(3′), H-C(4′), and H-C(5′) were achieved in homonuclear (COSY) and heteronuclear (HMQC, HMBC) correlation experiments. By LC-TOFMS analysis, the pseudomolecular ion $m/z$ 280.1390 (calculated 280.1396) was determined as a major peak as well as minor peaks of the product ions 262.1274 (calculated 262.1091), 244.1182 (calculated 244.1185), and 234.1372 (calculated 234.1341) from the loss of one or two molecules of water and one molecule of water and a carbonyl group respectively (Table 5).

The $^{13}C\{^{1}H\}$ NMR spectrum of Fru-Met was assigned by comparing the spectroscopic data with a reported reference.[12)] The $^{1}H$ NMR resonance signals at 3.63–4.20 ppm were attributed to the D-fructose protons of H-C(3), H-C(4), H-C(5), H-C(6), and H-C(2′). The chemical shifts and the assignment of protons H-C(1), H-C(3′), H-C(4′), and H-C(5′) were achieved in homonuclear (COSY) and heteronuclear (HMQC, HMBC) correlation experiments. By LC-TOFMS analysis, the pseudomolecular ion $m/z$ 312.1123 (calculated 312.1117) was detected as a major peak as well as minor peaks of the product ions 294.0998 (calculated 294.1011), and 276.0907 (calculated 276.0906) from the loss of one or two molecules of water respectively (Table 5). All spectroscopic data were confirmed by comparison with the synthesized compounds. Fru-Val has been isolated from soy sauce and identified by IR measurement,[19)] and Fru-Met has been identified in the deamidated wheat gluten hydrolysate by HILIC-ESI-MS measurement.[20)] These amadori compounds were also detected in fraction B-II-18.

Because fraction B-II-11 contained many further compounds, specification of the key compounds is currently in progress.

*Quantitative analysis and sensory studies of the umami-enhancing compounds*

To investigate the umami-enhancing effect on the soy sauce of the identified amadori compounds and pyroglutamyl peptides, quantification of concentrations in the soy sauce by LC-TOFMS and determination of taste-threshold concentrations by triangle test was performed, followed by sensory evaluation of the taste qualities of the individual compounds above the threshold concentration and calculation of the dose-over-threshold (DoT) factor (Table 6).

Fru-pGlu, Fru-Val, and Fru-Met were present at 1.6 mmol/L, 1.5 mmol/L, and 1.0 mmol/L in the soy sauce respectively. Fru-pGlu and Fru-Met caused an astringent oral sensation above threshold concentrations of 2.6 mmol/L and 1.6 mmol/L, whereas Fru-Val tasted bitter above a threshold concentration of 1.8 mmol/L. pGlu-Gln and pGlu-Gly were present at 1.6 mmol/L and 0.2 mmol/L in the soy sauce, and they tasted sweet above threshold concentrations of 1.9 mmol/L and 2.2 mmol/L respectively.

According to the DoT factor calculated as the ratio at one-fifth concentration in the soy sauce and the taste threshold concentrations of the individual compounds, no compound contributed directly to the taste of the soy sauce, because they were at sub-threshold concentrations in the soy sauce.

*Taste reconstitution experiment*

To confirm the contributions of the identified amadori compounds and pyroglutamylpeptides to the umami taste of the soy sauce, a reconstituted soy sauce (rSS) solution mixed in accordance with quantitative analyses of the compounds at one-fifth concentration in the soy sauce listed in Table 3 was evaluated by the sensory panel. The rSS solution was then compared to the soy sauce and the revised reconstituted soy sauce (rrSS) solution which was added to the identified amadori compounds and pyroglutamylpeptides listed in Table 6 besides the rSS solution. As shown in Fig. 6, the umami tastes of the rSS solution, the rrSS solution, and the soy sauce were evaluated with intensities of 2.8, 3.2, and 3.4 on the 5-point scale, respectively. Although there were few differences in umami intensity, there were significant differences between the rSS solution and the soy sauce as well as the rrSS solution. These results indicate that the identified compounds play important roles in the umami taste of the soy sauce. Whereas previous reports

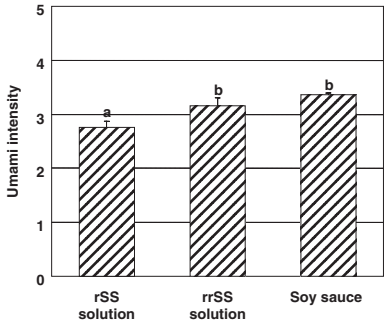


**Fig. 6.** Umami Taste Intensity of the Soy Sauce, the Reconstituted Soy Sauce (rSS) Solution, and the Revised Reconstituted Soy Sauce (rrSS) Solution.

have suggested that the low molecular weight fraction of a wheat-gluten-digested *koji* mold, which contained high amounts of pyroglutamylpeptides, enhanced the umami taste of MSG and soy sauce,[21,22] and some pyroglutamylpeptides have been identified tentatively as umami compounds in an enzymatic hydrolysate of wheat gluten,[23] a contribution to the umami taste of the specified pyroglutamylpeptides has not been reported. The taste characteristics and the taste interactions to the umami taste of the amadori compounds have not been reported.

To clarify the umami-enhancing effects of the individual identified compounds on the soy sauce, omission experiments on the respective compounds from the rrSS solution and addition experiments of the respective compounds from the rSS solution were performed at one-fifth concentration in the soy sauce. There were no significant differences among them (data not shown). On the other hand, fraction B-II-13, which contained only pGlu-Gln, enhanced the umami taste of a 6.0 mM MSG solution with the addition of one-fifth concentration in the soy sauce (Fig. 4). These results might due to differences in the concentrations of the added umami-contributing compounds. The omission experiments and the addition experiments were performed at high concentrations of umami compounds, such as glutamic acid (20.8 mmol/L) and aspartic acid (6.2 mmol/L), and umami-enhancing compounds such as sodium (544.6 mmol/L) (Table 3), whereas the screening experiment on HILIC fractions was performed at a 6.0 mM MSG solution. Considering these experiments, there appeared to be an optimum ratio between an umami compound and an umami-enhancing compound for an umami-enhancing effect as well as for the relationship between MSG and disodium 5′-inosinate, as reported by Yamaguchi.[24] Further investigation is needed to clarify this.

In conclusion, application of sensory-guided fractionation of the soy sauce using preparative chromatographic separation revealed that the umami taste of the soy sauce was due to the known tastants listed in Table 3 as well as to pyroglutamylpeptides and amadori compounds. Although present at sub-threshold concentrations, pyroglutamylpeptides and amadori compounds were identified for the first time, and all of them enhanced the umami taste of the soy sauce.

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