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RESEARCH ARTICLE

Quantitative determination of kokumi compounds, γ-glutamyl peptides, in Korean traditional fermented foods, *ganjang* and *doenjang*, by LC-MS/MS

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Abstract Recent studies have shown that γ-glutamyl peptides (GGPs) are recognized by the calcium-sensing receptor and induce kokumi taste. The contents of GGP have been reported in some fermented foods such as cheese and Japanese soy sauce but not in *ganjang* and *doenjang* which are representative Korean fermented-soybean products. In this study, the qualitative and quantitative analyses of GGPs in several *ganjang* and *doenjang* were carried out by LC-MS/MS using 11 synthetic GGPs as reference compounds. The total GGP contents ranged from 92 to 620 µg/mL for *ganjang* and from 203 to 387 µg/g for *doenjang*, respectively. Interestingly, the levels of GGPs were not related to manufacturing types of traditional and industrial products. These data provide a basis for the taste of *ganjang* and *doenjang* which was expressed abstractly as mouthful and long-lasting taste.

Keywords γ-glutamyl peptide · Kokumi taste · LC-MS/MS (MRM mode) · *Ganjang* · *Doenjang*

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Introduction

Ganjang (Korean soy sauce) and *doenjang* (Korean fermented-soybean paste) are the representative fermented-soybean products in Korea (Shin and Jeong, 2015). They have been used favorably as natural condiments by consumers. *Ganjang* is usually used to impart salty and savory taste to foods. Several ingredients such as amino acids, peptides, saccharides, and organic acids contribute to the taste of *ganjang* (Lee et al., 2002; Shin and Jeong, 2015). *Doenjang* has a unique taste and flavor, which are very different from those of *ganjang* (Lee et al., 2018). The tastes of *ganjang* and *doenjang* are often described very abstractly as '*gusuhada*' in Korean, which means mouthful and long-lasting taste.

The sixth taste called 'kokumi' was first introduced in 1990 (Ueda et al., 1990, 1997). Kokumi taste is described as mouthful, thick, and long-lasting taste (Ueda et al., 1990), which is very similar to the description of the unique taste of *ganjang* and *doenjang*. Kokumi taste is induced by γ-glutamyl peptides (GGPs) and sulfur-containing compounds which are perceived through the calcium-sensing receptor (CaSR) (Maruyama et al., 2012; Ohsu et al., 2010). The presence of GGPs has been confirmed in various foodstuffs including edible legumes, Alliaceae, and fermented foods such as cheeses, soy sauce, fermented fish, and yeast extract (Yang et al., 2019). Especially, GGPs are responsible for the kokumi taste of some kinds of cheese (Hillmann et al., 2016; Roudot-Algaron et al., 1994; Toelstede et al., 2009; Toelstede and Hofmann, 2009). Some GGPs were also identified in Japanese soy sauce, however, only five kinds of GGPs have been reported (Frerot and Chen, 2013; Kuroda et al., 2013).

Ganjang and *doenjang* are expected to contain GGPs given their kokumi taste and the involvement of several microorganisms which can produce GGPs in proteinous substrates. Traditionally, *ganjang* and *doenjang* are made from *meju*, which is a brick of boiled soybeans fermented by the natural inoculation of a diverse group of microorganisms. Traditional *meju* contains different molds depending on the types and the regions. Despite kokumi-like taste of *ganjang* and *doenjang*, to the best of our knowledge, the qualitative and quantitative analyses of their GGPs have not been reported.

In this study, we analyzed the profile of GGPs in *ganjang* and *doenjang* to understand their unique taste which cannot be explained by previously reported taste and flavor ingredients. Therefore, our study will provide the scientific basis for the abstractive description of the taste of *ganjang* and *doenjang*.

Material and methods

Chemicals and *ganjang* and *doenjang* samples

Reference compounds of GGPs (γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, γ-Glu-His, γ-Glu-Phe, γ-Glu-Trp, γ-Glu-Tyr, γ-Glu-Val, γ-Glu-Val-Gly) were purchased from Bachem (Weil am Rhein, Germany). γ-Glutamyl-valyl-glycine was chemically synthesized, as previously reported (Ohsu et al., 2010). The stable isotope of ^{15}N-uniformly labelled L-Arg (Arg-UN) purchased from Sigma Aldrich (Tokyo, Japan) was used as internal standard (ISTD). LC-MS grade acetonitrile (Junsei Chemicals Co., Ltd., Osaka, Japan), ammonium acetate (99%, Wako Pure Chemical Industries Ltd., Osaka, Japan) and water (Fisher Scientific, Hampton, VA, USA) were used to make the mobile phase.

A total of 12 samples for *ganjang* and 9 samples for *doenjang* were purchased from Korean markets in 2018. *Ganjang* samples were grouped into three types: traditional *ganjang*, industrial brewed *ganjang*, and industrial mixed *ganjang* that are made by mixing industrial brewed *ganjang* and acid-digested *ganjang*. *Doenjang* samples were grouped into traditional and industrial *doenjang*.

Ganjang and *doenjang* sample preparation for LC-MS/MS

A *ganjang* sample was directly diluted 50 times with deionized water containing the final concentration of 1 ppm ISTD. The water-soluble extracts (WSE) of a *doenjang* sample were prepared according to a protocol reported recently (Desfontaine et al., 2018). Briefly, a *doenjang* sample (10 g) was mixed with 40 mL of deionized water and homogenized for 5 min using a homogenizer in a Falcon tube. The homogenized sample was centrifuged at 10,062xg for 20 min at 4 °C. The upper layer and the precipitate were removed and the aqueous layer containing the *doenjang* WSE was transferred to a new tube. After re-extraction of the pellet with 40 mL of deionized water, the aqueous layers were combined with the first extract. The combined extracts were centrifuged at 10,062xg for 20 min at 4 °C, and the supernatants were filtered using a Whatman No. 1 filter paper (GE Healthcare, Chalfont St. Giles, UK). The filtered extract was freeze-dried and resuspended in deionized water. The WSE of a *doenjang* sample was stored at − 20 °C until further analysis.

Like a *ganjang* sample, the WSE of a *doenjang* sample were directly diluted 50 times with deionized water including ISTD prior to LC/MS. The diluted samples were filtered through a 0.45 µm syringe filter (25 mm GD/X disposable filter device, Whatman Corporation, Maidstone, UK) to remove the insoluble materials before analysis by LC/MS.

Standard GGPs, *ganjang*, and *doenjang* samples were derivatized using an AccQ Fluor reagent kit (Waters Corporation, Milford, MA, USA) following the protocol provided by Waters. Briefly, each sample (40 µL) was mixed with 60 µL of AQC reagent, 120 µL of borate buffer, 20 µL of 0.1 mg/dL ISTD, and 160 µL of 0.1% formic acid.

Analysis of GGPs by LC-MS/MS

GGPs were analyzed using a LC-MS/MS system at the Western Seoul Center of Korea Basic Science Institute (Seoul, Korea). Separation of GGPs were carried out by reversed-phase high-performance liquid chromatography using an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a Waters BEH Amide column (1.7 µm, 2.1 × 75 mm; Waters, Corporation, Milford, MA, USA). The column temperature was maintained at 30 °C. Mobile phase A (MP A) consisted of aqueous 20 mM ammonium acetate in distilled water (D.W.) and mobile phase B (MP B) consisted of 20 mM ammonium acetate in 90% ACN and 10% D.W. The elution conditions were 0–4 min (95% MP B), 4–10 min (85% MP B), 10–12 min (50% MP B), and 12–15 min (95% MP B). The flow rate was set to 0.30 mL/min. Injection volume was 2 µL.

A 6495 Triple Quad MS system (Agilent Technologies) was used for detection. The Agilent Jet Stream Electrospray ion source was operated in positive mode at 3.5 kV. Peak detection of GGPs and ISTD were conducted using the MRM (multiple reaction monitoring) method. The following GGPs were analyzed using mass transitions given in parentheses: γ-Glu-Val-Gly (m/z 304 → 175), γ-Glu-Glu (m/z 277 → 148), γ-Glu-Met (m/z 279 → 150), γ-Glu-His (m/z 285 → 156), γ-Glu-Gln (m/z 276 → 147), γ-Glu-Gly (m/z 205 → 142), γ-Glu-Leu (m/z 261 → 132), γ-Glu-Val (m/z 247 → 118), γ-Glu-Tyr (m/z 311 → 182), γ-Glu-Trp (m/z 334 → 205), γ-Glu-Phe (m/z 295 → 166), respectively. ISTD (m/z 117 → 116) was monitored under the condition of the MRM transition channel. The collision energy was 15 V for each GGP and ISTD.

Quantitative analysis was performed in triplicate by comparing the peak areas obtained for the corresponding mass traces with those of defined standard solutions of each reference peptide ranging from 0.01 to 10 ppm (6-point calibration).

Results and discussion

Ganjang and *doenjang* samples were analyzed by LC-MS/MS with the MRM transition of each GGP. The identity of GGP peak was confirmed by spike test given that multiple peaks were measured in samples (Supplementary Fig. 1).

Contents of GGPs in *ganjang*

GGPs were present in all analyzed *ganjang* samples. The contents of total GGPs ranged from 76.53 to 445.61 mg/mL in traditional *ganjang* samples, from 84.58 to 620.88 mg/mL in industrial brewed *ganjang* samples, and from 20.20 to 153.31 mg/mL in industrial mixed *ganjang* samples (Table 1). These results indicate that GGPs are commonly contained in *ganjang*. The contents of γ-Glu-Val-Gly, the strongest tripeptide of kokumi taste, were determined to be below 5.38 mg/mL in *ganjang* samples whereas the tripeptide was found from 15 to 61 mg/mL in Japanese soy sauce (Kuroda et al., 2013). On the other hand, Ferrot and Chen (2013) reported that GGPs in the acidic fraction of soy sauce were analyzed to be 70 mg/kg (Frerot and Chen, 2013). These data suggest that the GGP contents vary among soy sauces.

Contents of GGPs in *doenjang*

The contents of GGPs in *doenjang* are summarized in Table 2. GGPs were present in all analyzed *doenjang* samples, and their contents ranged from 1,425.14 to 2,940.76 μg/g in traditional *doenjang* and from 1,425.14 to 14,464.75 μg/g in industrial *doenjang*. These results indicate that GGPs are commonly distributed in various types of *doenjang*. There was no obvious correlation between the contents and manufacturing types of *doenjang*. γ-Glu-Met (1.60-fold), γ-Glu-His (1.55-fold), γ-Glu-Glu (1.51-fold), γ-Glu-Leu (1.17-fold), γ-Glu-Val (1.15-fold), γ-Glu-Gln (1.10-fold) were determined at higher levels in traditional *doenjang* compared to industrial one.

As expected based on kokumi-like taste of *ganjang* and *doenjang*, they contained various GGPs which might impart their kokumi taste (Tables 1 and 2). Regardless of manufacturing types, all analyzed *ganjang* and *doenjang* contained the analyzed eleven GGPs. To the best of our knowledge, only five kinds of GGPs have been reported in other soy sauces (Frerot and Chen, 2013; Kuroda et al., 2013) and GGPs in *doenjang* have not been reported. The previously reported GGPs such as γ-Glu-Glu, γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe, and γ-Glu-Val-Gly in soy sauces were identified except for γ-Glu-Ile in *ganjang*. The fewer number of GGPs identified in other soy sauces may have resulted from limitations in the analysis methods, and other soy sauces may also contain additional GGPs as in *ganjang*. Our study was limited to the GGPs whose reference molecules were available. Therefore, *ganjang* and *doenjang* might contain other GGPs including γ-Glu-Ile which were not analyzed.

The contents of GGPs were higher in *doenjang* than *ganjang* by more than twofold. This may be a result due to differences in their manufacturing processes, in which *ganjang* uses only the liquid part of fermented soybeans in brine while *doenjang* is made of the solid portion of the fermented soybeans. This may explain why the taste of *doenjang* is described as more kokumi-like taste compared to *ganjang* by consumers.

Soybeans, the raw starting materials for *ganjang* and *doenjang* production, contain GGPs such as γ-Glu-Tyr and γ-Glu-Phe at variable levels depending on their origin (Shibata et al., 2018). Therefore, some GGPs may originate from the raw soybeans. However, the variable levels of GGPs amongst *ganjang* produced by the same manufacture suggest that the majority of them are produced during the manufacturing processes. Most GGPs in fermented foods are formed by microbial activity and enzyme present in the raw materials (Yang et al., 2019). Because all enzymes of soybeans are inactivated during boiling process before fermentation, microbial enzymes such as γ-glutamylpeptidase (GGT) and certain L-glutaminase may be responsible for the production of GGPs during fermentation. Several microorganisms are involved in *ganjang* and *doenjang* fermentation. Some microorganisms isolated from *meju* such as *Aspergillus oryzae*, *Aspergillus sojae*, *Penicillium roqueforti* exhibit GGT activity (unpublished data), suggesting that GGP in *ganjang* and *doenjang* samples are produced by enzyme activity of major fermenting microorganisms. *Ganjang* and *doenjang* are produced using traditional *meju* which is fermented by various microorganisms such as molds, yeasts, and bacteria, or using whole soybean *meju* which is usually fermented by a starter mold. Therefore, additional factors to microorganisms seem to be important for the contents of GGPs.

Table 1 Contents of GGPs in selected soy sauce samples (μg/mL)

GGP	Traditional soy sauce				Industrial brewed soy sauce					Industrial mixed soy sauce		
	KM	JM	SM	HM	S701Y	S501Y	SY	MY	CY	SF3B	HB	NB
γ-Glu-Trp	0.09 ± 0.00	1.38 ± 0.00	LOD*	0.55 ± 0.00	0.85 ± 0.00	0.66 ± 0.00	0.76 ± 0.00	0.18 ± 0.00)	LOD*	0.39 ± 0.00	0.66 ± 0.00	LOD*
γ-Glu-Phe	11.24 ± 0.19	13.45 ± 0.79	11.52 ± 0.22	71.10 ± 1.27	81.99 ± 2.48	48.21 ± 0.92	38.76 ± 0.88	10.92 ± 0.20	16.28 ± 0.33	19.97 ± 0.57	12.22 ± 0.06	1.26 ± 0.13
γ-Glu-Leu	9.59 ± 0.08	24.08 ± 0.58	15.78 ± 0.41	78.83 ± 1.73	77.60 ± 2.79	42.49 ± 0.94	34.95 ± 1.07	3.48 ± 0.14	3.34 ± 0.07	17.06 ± 0.65	6.01 ± 0.03	0.10 ± 0.06
γ-Glu-Met	2.60 ± 0.05	3.09 ± 0.15	2.38 ± 0.08	7.26 ± 0.19	37.99 ± 1.39	23.56 ± 0.46	21.69 ± 0.21	5.12 ± 0.14	LOD*	8.15 ± 0.19	3.92 ± 0.05	1.19 ± 0.02
γ-Glu-Tyr	1.70 ± 0.09	2.41 ± 0.18	1.93 ± 0.11	18.74 ± 0.68	11.52 ± 0.42	11.05 ± 0.44	17.00 ± 0.30	17.69 ± 0.32	20.63 ± 0.19	4.17 ± 0.21	10.55 ± 0.00	1.48 ± 0.11
γ-Glu-Val	5.53 ± 0.51	9.80 ± 0.64	13.65 ± 0.28	54.25 ± 1.33	109.13 ± 3.16	68.50 ± 2.12	50.18 ± 0.90	6.15 ± 0.22	3.11 ± 0.17	20.82 ± 0.86	5.44 ± 0.03	1.59 ± 0.06
γ-Glu-Val-Gly	0.64 ± 0.07	LOD*	0.47 ± 0.04	4.37 ± 0.10	5.38 ± 0.21	5.20 ± 0.15	5.34 ± 0.12	1.69 ± 0.02	1.40 ± 0.08	1.08 ± 0.08	1.57 ± 0.02	0.07 ± 0.02
γ-Glu-Gly	7.79 ± 0.21	3.52 ± 0.16	10.29 ± 0.29	5.54 ± 0.02	45.70 ± 1.23	30.24 ± 0.86	29.55 ± 0.84	8.43 ± 0.20	9.37 ± 0.12	12.33 ± 0.61	12.01 ± 0.12	4.18 ± 0.07
γ-Glu-Gln	7.00 ± 0.10	LOD*	LOD*	15.64 ± 0.10	8.84 ± 0.23	10.19 ± 0.18	12.39 ± 0.28	6.68 ± 0.25	7.64 ± 0.18	2.42 ± 0.08	6.82 ± 0.03	1.33 ± 0.02
γ-Glu-His	15.40 ± 0.15	0.69 ± 0.06	5.84 ± 0.26	80.33 ± 1.01	28.23 ± 0.63	20.44 ± 0.42	31.77 ± 0.88	6.082 ± 0.27	6.88 ± 0.42	10.35 ± 0.23	12.90 ± 0.05	1.96 ± 0.11
γ-Glu-Glu	36.071 ± 0.34	18.12 ± 0.65	33.471 ± 0.56	109.01 ± 2.11	213.65 ± 6.90	137.50 ± 2.16	139.66 ± 3.29	18.16 ± 0.66	23.61 ± 2.13	54.58 ± 2.50	31.48 ± 0.4	7.04 ± 0.46
∑ γ-Glu-X	97.64	76.53	95.32	445.61	620.88	397.99	382.05	84.58	92.26	151.32	103.58	20.20

Values represent the mean ± standard deviation

LOD*; Limit of determination

Table 2 Contents of GGPs in selected *doenjang* samples (μg/g)

GGP	Traditional *doenjang*				Industrial *doenjang*				
	KT	MT	PT	ST	SC	HC	CC	KC	IC
γ-Glu-Trp	12.47 ± 1.21	9.82 ± 0.43	16.26 ± 0.09	5.80 ± 0.36	232.63 ± 9.80	8.19 ± 0.73	26.18 ± 0.86	7.578 ± 0.35	9.31 ± 0.23
γ-Glu-Phe	292.28 ± 21.03	559.42 ± 25.45	394.15 ± 18.19	393.72 ± 3.61	1551.04 ± 75.18	648.52 ± 44.32	761.02 ± 31.01	552.84 ± 14.35	822.32 ± 31.60
γ-Glu-Leu	79.30 ± 4.91	72.08 ± 3.49	173.55 ± 7.11	32.94 ± 0.46	1355.98 ± 62.59	11.33 ± 0.71	17.60 ± 0.24	241.81 ± 6.64	10.50 ± 0.30
γ-Glu-Met	65.52 ± 5.14	19.60 ± 0.87	87.11 ± 5.50	23.16 ± 0.34	568.99 ± 31.04	6.19 ± 0.56	10.75 ± 0.50	86.91 ± 2.36	6.43 ± 0.11
γ-Glu-Tyr	310.84 ± 21.42	567.54 ± 17.73	323.55 ± 11.57	366.21 ± 8.21	554.40 ± 19.74	493.48 ± 33.73	583.28 ± 15.15	429.68 ± 3.98	525.97 ± 18.36
γ-Glu-Val	188.21 ± 13.79	58.17 ± 2.18	239.26 ± 13.46	72.18 ± 1.02	1672.23 ± 90.81	16.36 ± 1.65	22.80 ± 0.84	393.98 ± 9.78	12.14 ± 0.49
γ-Glu-Val-Gly	17.73 ± 1.50	14.02 ± 1.00	25.17 ± 1.81	12.64 ± 0.33	52.23 ± 4.16	6.39 ± 0.98	7.298 ± 0.49	49.64 ± 0.70	4.62 ± 0.22
γ-Glu-Gly	259.54 ± 15.30	162.34 ± 4.66	280.21 ± 9.03	151.24 ± 5.04	857.78 ± 51.57	19.08 ± 2.28	25.40 ± 1.41	381.11 ± 6.58	23.60 ± 0.51
γ-Glu-Gln	107.06 ± 5.26	225.26 ± 6.43	123.48 ± 7.47	48.16 ± 1.97	168.76 ± 7.07	100.02 ± 7.98	149.09 ± 4.47	67.82 ± 0.67	103.25 ± 4.87
γ-Glu-His	200.78 ± 15.03	434.02 ± 12.13	422.65 ± 20.89	155.38 ± 4.05	3958.77 ± 166.22	80.37 ± 7.58	127.82 ± 6.00	432.71 ± 5.40	77.83 ± 4.23
γ-Glu-Glu	535.77 ± 36.16	818.49 ± 20.80	782.76 ± 35.61	163.71 ± 5.14	3491.96 ± 53.06	139.44 ± 9.5	286.89 ± 6.17	818.32 ± 14.52	154.34 ± 5.62
∑ γ-Glu-X	2069.49	2940.76	2868.16	1425.14	14,464.75	1529.37	2018.14	3462.39	1750.32

Values represent the mean ± standard deviation

The taste of *ganjang* and *doenjang* has been described very abstractly as "*gusuhada*" in Korean which is very similar to kokumi taste. The main ingredients that contribute to this taste had not been recognized. This research confirmed that *ganjang* and *doenjang* contain various GGPs which could contribute to the unique taste of *ganjang* and *doenjang*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10068-021-00993-x.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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