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Determination and Quantification of γ-Glutamyl-valyl-glycine in Commercial Fish Sauces

Motonaka Kuroda,*[,†] Yumiko Kato,[‡] Junko Yamazaki,[‡] Yuko Kai,[‡] Toshimi Mizukoshi,[‡] Hiroshi Miyano,[‡] and Yuzuru Eto[‡]

[†]Institute of Food Research & Technologies, and [‡]Institute for Innovation, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210-8681, Japan

ABSTRACT: It was recently reported that *kokumi* substances such as glutathione are perceived through the calcium-sensing receptor (CaSR). In addition, screening by the CaSR assay and sensory evaluation revealed that γ-glutamyl-valyl-glycine (γ-Glu-Val-Gly) was a potent *kokumi* peptide. In this study, the quantities of γ-Glu-Val-Gly in various commercial fish sauces originating from Vietnam (*Nuoc Mum*), Thailand (*Nampra*), China (*Yu-lu*), Korea, Japan (*Shottsuru* and *Ikanago-shoyu*), and Italy (*Garum*) were investigated using a LC/MS/MS method followed by derivatization with 6-aminoquinoyl-*N*-hydroxysuccinimidyl-carbamate (AQC). The analyses revealed γ-Glu-Val-Gly at concentrations ranging from 0.04 to 1.26 mg/dL, indicating that γ-Glu-Val-Gly is widely distributed among various commercial fish sauces.

KEYWORDS: *fish sauce, γ-glutamyl-valyl-glycine, LC/MS/MS*

INTRODUCTION

Recent studies have indicated that *kokumi* substances such as glutathione are perceived through the calcium-sensing receptor (CaSR) in humans.[1,2] These studies have confirmed that glutathione can activate human CaSR and that several γ-glutamyl-peptides including γ-Glu-Ala, γ-Glu-Val, γ-Glu-Cys, γ-Glu-α-aminobutyryl-Gly (ophthalmic acid), and γ-Glu-Val-Gly can also activate the CaSR and possess the characteristics of *kokumi* substances, which modify the five basic tastes, especially sweet, salty, and umami, when they are added to basic taste solutions or food, even though these substances themselves have no taste itself at the concentrations tested.[3−7] The CaSR activity of these γ-glutamyl-peptides has also been shown to be positively correlated with the sensory activity of *kokumi* substances determined by sensory evaluation, suggesting that *kokumi* substances are perceived through the CaSR in humans. Among these *kokumi* peptides, γ-Glu-Val-Gly has been reported to be a potent *kokumi* peptide, and the sensory activity as the *kokumi* substance was 12.8-fold greater than that of glutathione.[1] Although it is possible that γ-Glu-Val-Gly is present in foods, few studies have been conducted to determine the contents of this peptide in foods. However, a recent investigation of edible shellfish revealed the presence of γ-Glu-Val-Gly in raw scallops, dried scallops, and scallop extracts at concentrations of 0.08 μg/g, 0.64 μg/g, and 0.77 μg/g, respectively.[8] Despite these findings, there have been no studies of the existence of this peptide in other foodstuffs.

In this series of our study, the distribution of γ-Glu-Val-Gly in various foods was investigated. Since the contents of γ-Glu-Val-Gly in foods were very low, a new method for the determination and quantification of this peptide using LC/MS/MS followed by derivatization with the 6-aminoquinoyl-*N*-hydroxysuccinimidyl-carbamate (AQC) reagent was developed. In the present study, the presence and quantity of γ-Glu-Val-Gly in various fish sauces was investigated.

MATERIALS AND METHODS

Chemicals. γ-Glutamyl-valyl-glycine was chemically synthesized as previously described.[1] The stable isotopes of ^{15}N-uniformly labeled L-Arg (Arg-UN) was purchased from Isotec (Tokyo, Japan). An AccQ Fluor reagent kit was acquired from Waters (Milford, MA). HPLC grade acetonitrile (Junsei Chemicals Co., Ltd., Osaka, Japan) and formic acid (99%, Wako Pure Chemical Industries Ltd., Osaka, Japan) were used for the mobile phase. Deionized water was prepared using a Milli-Q system (Millipore, Bellerica, MA).

Fish Sauce Samples. Four brands of Vietnamese fish sauce (*Nuoc Mum*) and Thai fish sauce (*Nampra*), one brand of Filipino fish sauce (*Patis*), two brands of Chinese fish sauce, three brands of Korean fish sauce, and five brands of Japanese fish sauce were purchased. In addition, one brand of Italian fish sauce (*Garum*) was purchased.

Sample Preparation Prior to Derivatization. All of the fish sauce samples were filtered through a 0.45-μm syringe filter (25-mm GD/X disposable filter device, Whatman Corporation) to remove the insoluble materials. The filtrates were then further treated using an Amicon Ultra Centrifugal Filter Device (regenerated Cellulose 10,000 MWCO, Millipore, USA) at 7,500*g* and 4 °C for 15 min. The samples were subsequently diluted 50 times with deionized water and stored at −20 °C until the derivatization procedure.

Derivatization Procedure. LC/MS/MS analysis was carried out after derivatization using the AQC reagent (AccQ Fluor reagent kit). The AQC solution was prepared by dissolving AQC powder in dry acetonitrile according to the protocol recommended by the supplier. The derivatization procedure was then conducted as follows. A 10-μL aliquot of each fish sauce sample was mixed with a 20-μL internal standard solution containing 0.089 mg/dL Arg-UN, after which 10 μL of γ-Glu-Val-Gly solution (for spiked samples) or deionized water (for unspiked samples) was added. Next, 10-μL aliquots of the mixture and 10 μL of AQC solution were added to 30 μL of borate buffer (AccQ Fluor TM reagent kit). The 50-μL final solutions were then placed in 1.5-mL microtubes, mixed using a vortex mixer, and heated at 55 °C

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Table 1. Characteristics and the Contents of General Components in Vaious Commercial Fish Sauces

samples	country of origin	raw materials	contents of general components (g/l00g)					
			moisture	crude protein	crude fat	ash	sodium	NaCl[a]
Nampra A	Thailand	anchovy, salt	61.2	10.5	<0.1	22.1	8.22	20.9
Nampra B	Thailand	anchovy, salt, sugar	63.4	10.4	<0.1	21.3	7.64	19.4
Nampra C	Thailand	anchovy, salt	62.8	13.5	<0.1	22.9	8.44	21.5
Nampra D	Thailand	anchovy, salt, sugar	71.9	8.0	<0.1	17.5	6.51	16.6
Nampra E	Thailand	anchovy, salt, sugar	61.7	10.0	<0.1	22.3	8.11	20.7
Nuoc Mum A	Vietnam	anchovy, salt	59.7	18.6	<0.1	21.1	7.73	19.7
Nuoc Mum B	Vietnam	anchovy, salt	59.6	18.3	<0.1	21.4	7.89	20.1
Nuoc Mum C	Vietnam	anchovy, salt	59.4	18.4	<0.1	21.4	7.98	20.3
Nuoc Mum D	Vietnam	anchovy, salt	59.3	18.2	<0.1	21.8	7.92	20.2
Nuoc Mum E	Vietnam	anchovy, salt	59.7	17.9	<0.1	21.8	8.02	20.4
Patis	Philippines	mackerel, salt	69.1	0.9	<0.1	24.8	9.80	25.0
Yu-lu A	China	fish, salt	67.7	8.6	<0.1	23.5	8.58	21.8
Yu-lu B	China	fish, salt	68.5	7.1	<0.1	24.4	9.24	23.5
Myoruchi extract	Korea	sardine, salt	69.4	6.3	<0.1	23.4	8.51	21.7
Kanari extract	Korea	sand lance, salt	68.8	7.5	<0.1	23.2	8.54	21.7
Shottsuru A	Japan	sand fish, salt	67.5	9.0	<0.1	22.9	8.56	21.8
Shottsuru B	Japan	sand fish, salt	67.6	8.6	<0.1	23.0	8.61	21.9
Yoshiru	Japan	sardine, salt	66.3	9.2	<0.1	23.1	8.60	21.9
Ikanago-shoyu	Japan	sand lance, salt	63.5	13.0	<0.1	22.0	8.15	20.8
Garum	Italy	anchovy, salt	66.1	9.1	<0.1	23.4	8.73	22.2

[a]NaCl content was calculated from the sodium content.

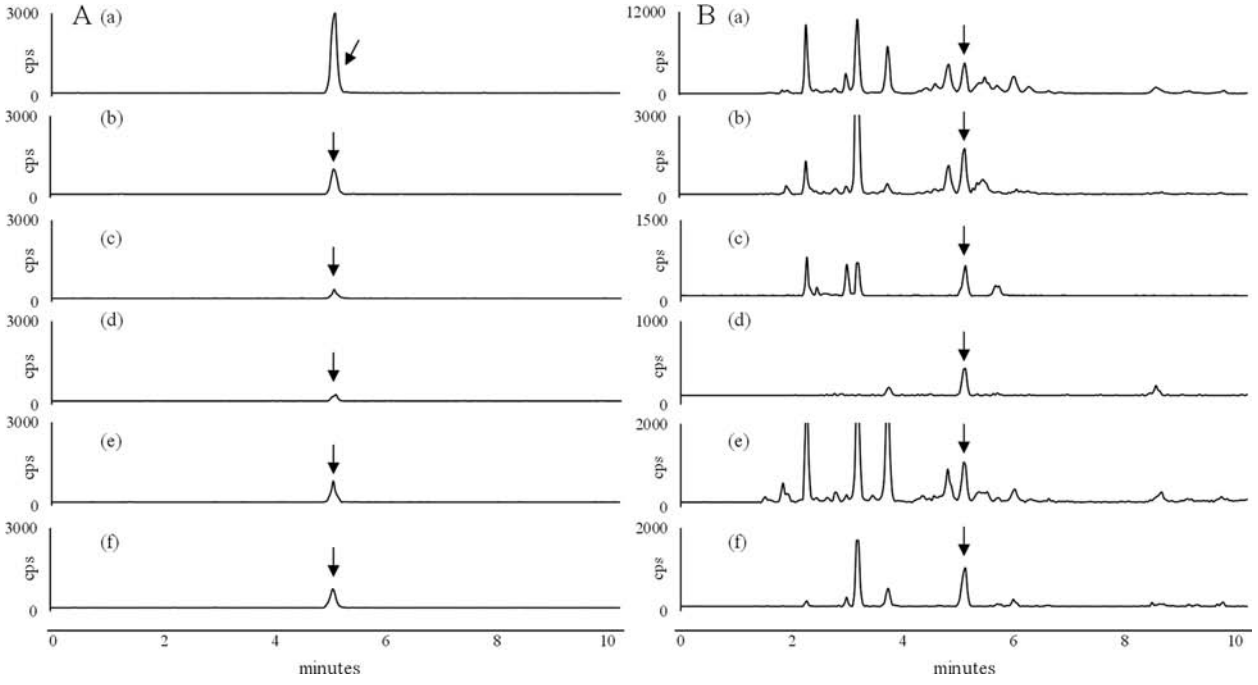

Figure 1. Mass chromatograms of standard 4 μM γ-Glu-Val-Gly (A) and Nuoc Num A (B). The X and Y axes represent the retention time (min) and peak intensity (cps). The MRM transition channels were 474.2/171.2 (a), 474.2/145.3 (b), 474.2/300.3 (c), 474.2/229.4 (d), 474.2/304.0 (d), and 474.2/72.1 (f). Arrows highlight the peaks corresponding to γ-Glu-Val-Gly.

for 10 min on a block-heater. After cooling to ambient temperature, the reaction mixture was added to 100 μL of 0.1% aqueous formic acid and analyzed by LC/MS/MS.

Apparatus. The analysis of γ-Glu-Val-Gly was conducted using an LC/MS/MS system. An Agilent 1200 series HPLC system (Agilent Technologies) was used for the separation. The system was equipped with a binary pump, a degasser, an autosampler, and a column compartment. An AB SCIEX 3200 QTrap LC/MS/MS system (AB SCIEX) was used for identification. The turbo ion spray interface was operated in positive mode at 5500 V and 650 °C. Peak detection was performed using the MRM (Multiple Reaction Monitoring) method with a dwell time of 170 ms. The MS parameters of CUR, GSI, GS2, CAD, DP, EP, and CXP were set to 15, 80, 80, 8, 41, 6, and 4, respectively. Operations were controlled using the Analyst software (version 1.4.2).

Separation of γ-Glu-Val-Gly. Separation of γ-Glu-Val-Gly was conducted using reversed-phase high-performance liquid chromatography. To accomplish this, a CAPCELL PAK C18 MG II column (2.0 mm ID × 100 mm, 3 μm; Shiseido) was used, and the column temperature was maintained at 40 °C. Mobile phase A (MP A)

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consisted of aqueous 25-mM formic acid (pH 6.0, adjusted using an aqueous ammonium solution), while mobile phase B (MP B) consisted of water/acetonitrile (40/60). The gradient elution conditions were 0 min (15% MP B), 12 min (25% MP B), 12.1−14 min (100% MP B), and 14.1−20 min (15% MP B), and the flow rate was maintained at 0.25 mL/min throughout the analysis. A total of 20 μL of each sample was injected for analysis.

Detection of γ-Glu-Val-Gly. Peak detection of γ-Glu-Val-Gly was performed using the MRM (Multiple Reaction Monitoring) method. For γ-Glu-Val-Gly detection, six MRM transition channels were monitored. The precursor/product ions (Q1/Q3) and the collision energies (CE(V)) were 474.2/171.2 (51 V), 474.2/145.3 (30 V), 474.2/300.3 (30 V), 474.2/229.4 (20 V), 474.2/304.0 (20 V), and 474.2/72.1 (50 V), while those of Arg-UN (internal standard) were 417.4/171.1 (51 V), 349.0/171.1 (30 V), and 296.0/171.1 (30 V), respectively.

Analysis of General Components. Moisture levels were analyzed by measuring the change in weight after drying for 5 h. Although fish sauces have been reported to contain amino acids, peptides, proteins, nucleotides nucleosides, and other nitrogen compounds, the main nitrogen-containing components in fish sauces are amino acids and peptides.[9−18] Therefore, the crude protein content was calculated by multiplying the total nitrogen content by 6.25. The total nitrogen content was determined by the micro-Kjeldahl method, while the crude fat content was analyzed by the Soxhlet extraction method using diethyl ether as the solvent. Ash levels were determined from the weight after heating at for 16 h. The sodium content was determined through atomic absorption spectrochemical analysis using a Spectro AA240FS spectrometer (Varian Technologies Japan Ltd., Tokyo, Japan).

Statistical Analysis. The data describing the γ-Glu-Val-Gly contents in the fish sauces were not normally distributed; therefore, Spearman's rank correlation test was performed to investigate the correlation. The correlation analysis was performed using the StatView, version 5.0, statistical package (SAS Institute, Inc., Cary, NC). The normality of data was analyzed by the Kolmogovov−Smirnov test using Excel Statistics 2008 (SSRI Co., Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Contents of General Components in Fish Sauce Samples. Table 1 shows the general components of various fish sauces. The results indicated that, except for moisture and salt, the major component of the commercial fish sauces is crude protein. Vietnamese fish sauces (*Nuoc Mum*) had the highest crude protein levels among the commercial fish sauces investigated in this study. These findings were consistent with previously reported results.[9−18]

Identification of γ-Glu-Val-Gly in Fish Sauce Samples. Identification of γ-Glu-Val-Gly was conducted since this is the first study to investigate its presence in fish sauce products used in Asian countries and Italy. Figure 1B−a shows typical mass chromatograms of *Nuoc Mum* A monitored at the most sensitive MRM transition channel (474.2/171.2). The retention time of the observed peak at 5.04 min was identical to that of standard γ-Glu-Val-Gly. However, the complex peak pattern makes it difficult to confirm its existence. Although the other peaks were not further analyzed, they may correspond to other peptides with a specific pair of precursor (474) and fragment (171 from the AQC reagent moiety) ions, or compounds with close precursor or fragment ions detected owing to the low resolution of the mass window. Overall, this complex peak pattern requires more structural information for confirmation.

The strategy most commonly adopted for structural identification of unknown compounds is comparison of its fragment pattern and the relative intensity of its fragment ions against those of expected compounds during MS/MS analysis. However, in the present study, the low intensity of the provisional γ-Glu-Val-Gly peak was not sufficient to yield a fragmentation spectrum. We previously demonstrated that monitoring multiple MRM transition channels provided alternative MS/MS fragment information regarding γ-Glu-Val-Gly (23); therefore, we implemented the same strategy in this study. Figures 1b−f show the chromatograms monitored at the five MRM transition channels 474.2/145.3, 474.2/300.3, 474.2/229.4, 474.2/304.0, and 474.2/72.1. The relative peak intensity at 5.04 min in mass chromatograms a−f was 1.00/0.39/0.11/0.07/0.24/0.23, while that of standard γ-Glu-Val-Gly was 1.00/0.38/0.12/0.08/0.26/0.24. This good agreement indicates that the *Nuoc Mum* A product contained γ-Glu-Val-Gly. The results from other samples are summarized in Figure 2. The horizontal

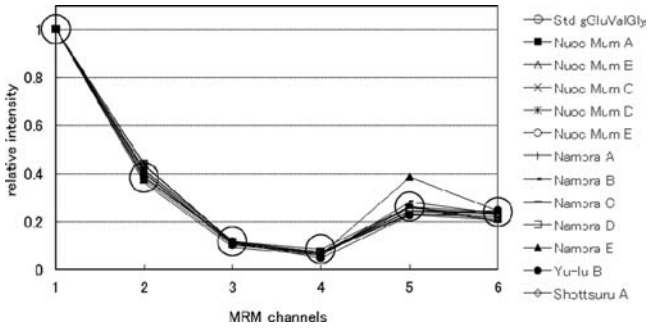

Figure 2. Relative peak intensities normalized by the peak intensities at MRM transition channel 1. The pairs of precursor and fragment ions at each MRM transition channel (1 to 6) are 474.2/171.2, 474.2/145.3, 474.2/300.3, 474.2/229.4, 474.2/304.0 and 474.2/72.1, respectively. The average values based on triplicate analyses are plotted.

axis represents the six MRM transition channels, while the vertical axis indicates the intensity standardized at the 474.2/171.2 channel. The center of the large open circle represents the values obtained upon analysis of standard γ-Glu-Val-Gly analysis. Good agreement of the plots was observed between standard γ-Glu-Val-Gly and other fish sauce samples, indicating that they contained γ-Glu-Val-Gly. One inconsistent plot from *Nampra* E at the 474.2/229.4 channel (closed triangle) was caused by insufficient separation of the γ-Glu-Val-Gly peak from the other samples.

Linearity and Recovery in the Quantification of γ-Glu-Val-Gly in Fish Sauce Samples. The concentration of γ-Glu-Val-Gly in the samples was determined based on the internal standard calibration method at the most sensitive 474.2/171.2 (Q1/Q3) channel. The linearity of the peak-area ratio (γ-Glu-Val-Gly/Arg-UN) to the γ-Glu-Val-Gly concentration was verified at concentrations ranging from 0.003 to 1.5 mg/dL. The squared correlation coefficient (r^2) was greater than 0.999. To consider and overcome the matrix effect that sometimes affects analysis, the concentrations of γ-Glu-Val-Gly in the *Nampra*, *Nuoc Mum*, *Yu-lu* B, and *Shottsuru* A samples were calculated by a single-point standard addition approach. To accomplish this, the equation $X = S \times Ix/(Is - Ix)$ was used, where X is the γ-Glu-Val-Gly concentration of the sample, S is the spiked standard concentration, and Is and Ix are the relative peak intensities against the internal standard (Arg-UN) for the spiked and unspiked samples, respectively (see Derivatization Procedure). The recovery rates for *Nampra* A-E, *Nuoc Mum* A-

E, *Yu-lu* B, and *Shottsuru* A were 114, 105, 103, 113, 131, 85, 91, 97, 108, 102, 96, and 86%, respectively.

Contents of γ-Glu-Val-Gly in Fish Sauce Samples. The contents of γ-Glu-Val-Gly in various types of fish sauce are given in Table 2. γ-Glu-Val-Gly was contained in all samples of

Table 2. Contents of γ-Glu-Val-Gly in Vaious Commercial Fish Sauces

samples	country of origin	contents of γ-Glu-Val-Gly (mg/dL)
Nampra A	Thailand	0.27
Nampra B	Thailand	0.12
Nampra C	Thailand	0.31
Nampra D	Thailand	0.20
Nampra E	Thailand	0.23
Nuoc Mum A	Vietnam	1.20
Nuoc Mum B	Vietnam	1.26
Nuoc Mum C	Vietnam	1.23
Nuoc Mum D	Vietnam	1.06
Nuoc Mum E	Vietnam	1.04
Patis	Philippines	<LOQ[a]
Yu-lu A	China	<LOQ
Yu-lu B	China	0.11
Myoruchi extract	Korea	<LOQ
Kanari extract	Korea	<LOQ
Shottsuru A	Japan	0.28
Shottsuru B	Japan	<LOQ
Yoshiru	Japan	<LOQ
Ikanago-shoyu	Japan	0.05
Garum	Italy	0.04

[a]LOQ: limit of quantification.

Vietnamese fish sauce (*Nuoc Mum*) and Thai fish sauce (*Nampra*). The contents in Vietnamese fish sauce ranged from 1.04 mg/dL to 1.26 mg/dL, while those in Thai fish sauce ranged from 0.12 to 0.31 mg/dL. Among the two Chinese fish sauce samples, one contained γ-Glu-Val-Gly at 0.11 mg/dL. In addition, two of the four Japanese fish sauce samples contained γ-Glu-Val-Gly at 0.28 and 0.05 mg/dL. Furthermore, Italian fish sauce (*Garum*) contained γ-Glu-Val-Gly at 0.04 mg/dL. This peptide was not detected in Korean or Filipino (*Patis*) fish sauces. Although several types of fish sauce did not contain γ-Glu-Val-Gly, the overall results suggest that γ-Glu-Val-Gly is widely distributed among fish sauces.

The results indicated that γ-Glu-Val-Gly was abundant in Vietnamese fish sauces (*Nuoc Mum*) and Thai fish sauces (*Nampra*). Conversely, the levels of this peptide were lower in sauces from the Philippines, China, Korea, Japan, and Italy. The differences in the contents of these peptides likely reflect differences in fish species and fermentation conditions, including the microbial flora. Indeed, fish sauces are produced from more than 100 species of fish, including anchovy, sardine, mackerel, and sand lance. Since many of the fish sauces tested in the present study (11 out of 20) are made from anchovies, the γ-Glu-Val-Gly contents in fish sauces made from anchovies were compared to investigate the effects of raw fish species. The results revealed that the γ-Glu-Val-Gly contents varied from 0.04 to 1.26 mg/dL and that there was no clear correlation between the γ-Glu-Val-Gly contents and raw fish species, indicating that the fermentation conditions contribute to the variation of γ-Glu-Val-Gly levels more than the fish species. To clarify the reasons for the variations in γ-Glu-Val-Gly levels, the correlation between the levels of these peptides and those of various general components was analyzed. Among the general components tested, the crude protein contents were found to be significantly positively correlated with γ-Glu-Val-Gly (correlation coefficient $\rho = 0.842$, $p < 0.001$) (Figure 3). This finding suggests that the high level of γ-Glu-Val-Gly was partly caused by the protein contents in the raw material of fish sauces.

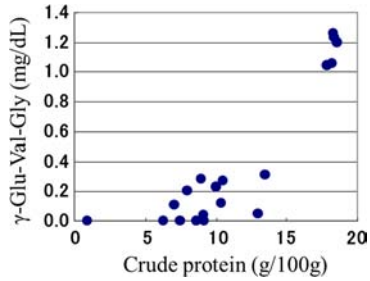

Figure 3. Correlation between the crude protein contents and the contents of γ-Glu-Val-Gly in commercial fish sauces.

It has been reported that the dipeptide Val-Gly can serve as a substrate of γ-glutamyltransferase (GGT);[19] therefore, it was assumed that γ-Glu-Val-Gly was biosynthesized via GGT. During the production of fish sauce, it was assumed that GGT from fish and bacteria such as *Bacillus* species, *Pseudomonas* species, *Halobacterium* species, *Vibrionaceae* species, and *Corynebacterium* species, which have been reported to exist in fish sauces, were involved in the biosynthesis of γ-Glu-Val-Gly.[18,20] Since the action of proteases has been shown to occur during the fermentation of fish sauces, it is possible that Val-Gly was produced by the degradation of fish protein via proteases. A database search of the sequence of fish muscular proteins revealed that the Val-Gly sequence was present in myosin,[21−28] actin,[29−32] parvalbumin,[33−36] creatine kinase,[37] collagen,[38] and elastin[39] from fish. Indeed, the Val-Gly sequences at position 6−7 of the actin-binding site-I in the myosin heavy chain,[21−26] position 342−343 of the light meromyosin domain in the myosin heavy chain,[21−26] position 45−46 of α-actin,[28−32] and position 34−35 of parvalbumin[33−36] are well conserved among various species of fish. Although the existence of the Val-Gly sequence in raw fish used to make fish sauces has not been reported to date, it is possible that Val-Gly was liberated from the above protein via protease activity and then converted to γ-Glu-Val-Gly via GGT. However, detailed studies are needed to clarify the mechanism of γ-Glu-Val-Gly biosynthesis during the production of fish sauces. For example, to clarify the biosynthetic mechanism of γ-Glu-Val-Gly, the Val-Gly contents and GGT activity can be measured during the fermentation of fish sauces. Accordingly, the biosynthetic mechanism of γ-Glu-Val-Gly is currently being investigated in our laboratory.

In the present study, the *kokumi* peptide, γ-Glu-Val-Gly, was identified and quantified in various fish sauces and found to be widely distributed. The contribution of these peptides to the taste of fish sauces is currently being investigated in our laboratory, as is the distribution of γ-Glu-Val-Gly among other foods.

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-44-223-4157. Fax: +81-44-246-6241. E-mail: motonaka_kuroda@ajinomoto.com.

Notes

The authors declare no competing financial interest.

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