Determination and quantification of the \textit{kokumi} peptide, γ-glutamyl-valyl-glycine, in commercial soy sauces

Motonaka Kuroda\textsuperscript{a,∗}, Yumiko Kato\textsuperscript{b}, Junko Yamazaki\textsuperscript{b}, Yuko Kai\textsuperscript{b}, Toshimi Mizukoshi\textsuperscript{b}, Hiroshi Miyano\textsuperscript{b}, Yuzuru Eto\textsuperscript{b}

\textsuperscript{a} Institute of Food Research & Technologies, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210-8681, Japan
\textsuperscript{b} Institute for Innovation, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210-8681, Japan

\textbf{Abstract}

Recent studies have demonstrated that \textit{kokumi} substances, such as glutathione, are perceived through the calcium-sensing receptor (CaSR), and screening by CaSR assay and sensory evaluation has shown that γ-glutamyl-valyl-glycine (γ-Glu-Val-Gly) is a potent \textit{kokumi} peptide. In this study, the contents of γ-Glu-Val-Gly in six commercial brands of dark-coloured soy sauces, two brands of light-coloured soy sauce, and one brand of white soy sauce, were investigated by high performance liquid chromatography–tandem mass spectrometry (LC/MS/MS), followed by derivatization with 6-aminoquinoyl-N-hydroxy succinimidyl-carbamate (AQC). The analyses indicated that γ-Glu-Val-Gly was present in all investigated soy sauces at concentrations ranging from 0.15 to 0.61 mg/dl, demonstrating that it is widely distributed in soy sauces.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Recent developments in molecular biology have demonstrated that the five basic tastes, sweet, salty, sour, bitter, and umami, are recognized by specific receptors and transduction pathways. However, some foods are known to have flavours that cannot be explained by the five basic tastes alone, such as thickness, complexity, and mouthfulness. Ueda, Sakaguchi, Hirayama, Miyajima, and Kimizuka (1990) previously investigated the flavouring effects of a water extract of garlic that enhances continuance and mouthfulness. They postulated that substances, such as glutathione, are perceived through the calcium-sensing receptor (CaSR) in humans (Maruyama, Yasuda, Kuroda, & Eto, 2012; Ohsu et al., 2010). These studies confirmed that glutathione can activate human CaSR, as well as 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. Furthermore, these compounds were shown to possess the characteristics of \textit{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 they have no tastes themselves at the concentrations tested (Dunkel, Koster, & Hofmann, 2007; Toelstedt, Dunkel, & Hofmann, 2009; Toelstedt & Hofmann, 2009; Ueda et al., 1997). The CaSR activity of these γ-glutamyl-peptides has also been shown to be positively correlated with the sensory activity of \textit{kokumi} substances, suggesting that \textit{kokumi} substances are perceived through the CaSR in humans. Among these, γ-Glu-Val-Gly has been reported to be a potent \textit{kokumi} peptide, and the sensory activity of \textit{kokumi} substances was found to be 12.8-fold greater than that of glutathione (Ohsu et al., 2010). Although it is possible that γ-Glu-Val-Gly is present, several studies have been conducted to determine the contents of this peptide in foods. Nevertheless, a recent investigation has revealed the presence of γ-Glu-Val-Gly in fishery food products, such as raw scallops and processed scallop products (Kuroda et al., 2012), as well as in various commercial fish sauces (Kuroda et al., 2012). Despite these findings, there have been no studies conducted to investigate the presence of this peptide in other foodstuffs.

In this series of our study, the distribution of γ-Glu-Val-Gly in various foods was investigated. Previous studies had revealed that fishery products such as scallops and fermented fish sauces contained γ-Glu-Val-Glyz, with fermented fish sauces containing...
especially high contents of γ-Glu-Val-Gly. However, no reports have indicated the existence of γ-Glu-Val-Gly in vegetable foods. Since fermented fish sauces contained γ-Glu-Val-Gly, fermented vegetable sauces, such as soy sauces, were assumed to contain this peptide. In the present study, to clarify the presence of γ-Glu-Val-Gly in vegetable foods, the presence and quantity of γ-Glu-Val-Gly in various soy sauces was investigated.

2. Materials and methods

2.1. Chemicals

γ-Glutamyl-valyl-glycine was chemically synthesized, as previously reported (Ohsu et al., 2010). The stable isotope of 15N-uniformly labelled 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).

2.2. Soy sauce samples

All soy sauce samples were purchased in Japanese markets in 2008. Six brands of dark-coloured soy sauce, two brands of light-coloured soy sauce, and one brand of white soy sauce were purchased and used for the investigation. Dark-coloured soy sauce (koikuchi-shoyu in Japanese) is produced using almost equal amounts of soybean and wheat as raw materials, and is characterized by a strong aroma and a deep reddish brown colour. Light-coloured soy sauce (usukuchi-shoyu) is also produced using almost equal amounts of soybean and wheat as raw materials, and is characterized by a very high ratio of wheat to soybean and has a very light yellow colour because it is fermented under conditions that prevent colour development (Yokotsuka, 1961, 1986). As shown in Table 1, soy sauce samples were divided using a Milli-Q system (Millipore, Bellerica, MA).

2.3. Sample preparation prior to derivatization

All soy 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 7500g and 4 °C for 15 min. The resulting filtrates were diluted 50 times with deionized water and stored at −20 °C prior to the derivatization step.

2.4. Derivatization procedure

LC/MS/MS was determined using pre-column derivatization with AQC reagent (AccQ Fluor reagent kit) and LC/MS/MS analysis. The AQC solution in acetonitrile was prepared according to the manufacturer’s protocol. The derivatization procedure was carried out as follows: a 10 μl aliquot of each soy sauce sample was mixed with a 20 μl internal standard solution containing 0.089 mg/dl of Arg-UN. Next, 10 μl of γ-Glu-Val-Gly solution (for spiked samples) or deionized water (for unspiked samples) were added and vortexed, after which 10 μl aliquots of the mixture and 10 μl of AQC solution were added to 30 μl of borate buffer included in the kit. The resulting 50 μl solutions were subsequently put into 1.5 ml microtubes, vortexed and heated at 55 °C for 10 min on a block-heater. After cooling to room temperature, the reaction mixture was added to 100 μl of 0.1% aqueous formic acid.

2.5. Apparatus

Analysis of γ-Glu-Val-Gly was performed using a LC/MS/MS system after derivatization. An Agilent 1200 series HPLC system (Agilent Technologies), equipped with a binary pump, a degasser, an auto-sampler, and a column compartment, was used for the separation. An AB SCIEX 3200 QTrap LC/MS/MS system (AB SCIEX) was used for detection. The turbo ion spray interface was operated in positive mode at 5500 V and 650 °C. Peak detection was conducted using the MRM (multiple reaction monitoring) method with a dwell time of 170 ms. The parameters of CUR, GSI, GS2, CAD, EP, DP and CXP were set to 15, 80, 8, 6, 41 and 4.

Table 1

<table>
<thead>
<tr>
<th>Type of soy sauce</th>
<th>Class</th>
<th>Grade</th>
<th>Contents (g/dl)</th>
<th>Dry matter of extract^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark-coloured soy sauce</td>
<td>Standard</td>
<td>Ordinary</td>
<td>&gt;1.20</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Super</td>
<td>&gt;1.35</td>
<td>&gt;14</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Ultrasuper</td>
<td>&gt;1.50</td>
<td>&gt;16</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>Ordinary</td>
<td>&gt;1.65</td>
<td>&gt;16</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>Super</td>
<td>&gt;1.80</td>
<td>&gt;16</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>Ultrasuper</td>
<td>&gt;1.80</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Light-coloured soy sauce</td>
<td>Standard</td>
<td>Ordinary</td>
<td>&gt;0.95</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Super</td>
<td>&gt;1.05</td>
<td>&gt;12</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Ultrasuper</td>
<td>&gt;1.15</td>
<td>&gt;14</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>Ordinary</td>
<td>&gt;1.15</td>
<td>&gt;15.4</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>Super</td>
<td>&gt;1.15</td>
<td>&gt;16.8</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>Ultrasuper</td>
<td>&gt;1.15</td>
<td>&gt;16.8</td>
</tr>
<tr>
<td>Light-coloured soy sauce</td>
<td>Standard</td>
<td>Ordinary</td>
<td>&lt;0.90</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Super</td>
<td>&lt;0.90</td>
<td>&gt;13</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Ultrasuper</td>
<td>&lt;0.80</td>
<td>&gt;16</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Ultrasuper</td>
<td>&lt;0.80</td>
<td>&gt;17.6</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Ultrasuper</td>
<td>&lt;0.80</td>
<td>&gt;19.2</td>
</tr>
</tbody>
</table>

^a Established as Japanese Agricultural Standard (JAS).

^b Dry matter except for NaCl.
respective. Operations were controlled using the Analyst software (version 1.4.2).

2.6. Separation of derivatized γ-Glu-Val-Gly

Separation of derivatized γ-Glu-Val-Gly was carried out by reverse-phase high-performance liquid chromatography using a CAPCELL PAK C18 MG II column (2.0 mm ID × 100 mm, 3 μm; Shimadzu) while maintaining the column temperature at 40 °C. Mobile phase A (MP A) consisted of aqueous 25 mM formic acid (pH 6.0, adjusted by an aqueous ammonium solution), while mobile phase B (MP B) consisted of water/acetonitrile (40/60). The 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 20 μl aliquot of each derivatized sample was injected for analysis.

2.7. Detection of AQC-derivatized γ-Glu-Val-Gly

Peak detection of derivatized γ-Glu-Val-Gly was conducted using the MRM method. Six MRM transition channels were monitored, and 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). Internal standard (of Arg-UN) was monitored under the condition of the MRM transition channel at 349.0/171.1 (Q1/Q3) and the collision energy at 30 V.

2.8. Quantification of AQC-derivatized γ-Glu-Val-Gly

The concentration of AQC-derivatized γ-Glu-Val-Gly in the samples was determined, based on a single-point standard addition approach at the most sensitive channel (474.2/171.2; Q1/Q3). The internal standard of Arg-UN was monitored in the MRM transition channel at 349.0/171.1 (Q1/Q3) with collision energy at 30 V. The equation used was X = S × hX/(I0 − hX), where X is the calculated γ-Glu-Val-Gly concentration in the sample, S is the spiked standard concentration, and I0 and hX are the relative peak intensities against the internal standard peak (Arg-UN) for the spiked and unspiked samples, respectively.

2.9. Analysis of the general components

 Moisture levels were analyzed by measuring the change in weight after drying for 5 h. Since the main nitrogen-containing compounds in soy sauces are amino acids and peptides (Yokotsuka, 1986), 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 (Alcock, 1946); while the crude fat content was analyzed by the Soxhlet extraction method, using diethyl ether as the solvent (Firth, Ross, & Thonney, 1985). Ash levels were determined from the weight after heating at 550 °C for 16 h. The sodium content was determined through atomic absorption spectrochemical analysis (Sanui & Pace, 1968), using a Spectro AA240FS spectrometer (Varian Technologies Japan Ltd., Tokyo, Japan).

2.10. Statistical analysis

The data describing the γ-Glu-Val-Gly contents in the soy sauces were distributed normally; therefore, Pearson’s 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 Kolmogorov–Smirnov test, using Excel Statistics 2008 (SSRI Co., Ltd., Tokyo, Japan). The contents of γ-Glu-Val-Gly and general components in each type of soy sauce were compared by Mann–Whitney’s U-test, using the StatView version 5.0 statistical package (SAS Institute, Inc., Cary, NC).

3. Results and discussion

3.1. Contents of general components in soy sauce samples

Table 2 shows the general components of various soy sauces. The results indicated that, except for moisture and salt, the major component of the commercial soy sauces is crude protein. Among the three types tested, the crude protein content was highest in the dark coloured soy sauce, while it was lowest in the white soy sauce. The crude protein content in the dark-coloured soy sauces was significantly higher than that in light-coloured soy sauces (p < 0.001). In addition, the NaCl content in light-coloured soy sauces was significantly higher than that in dark-coloured soy sauces (p < 0.05). These results are consistent with those of previous studies (Kagawa, 2001; Yokotsuka, 1986).

3.2. Identification of γ-Glu-Val-Gly in soy sauce samples

Identification of γ-Glu-Val-Gly was achieved using a LC/MS/MS method. Fig. 1A(a) and B(a) show the mass chromatograms of the derivatized standard γ-Glu-Val-Gly and the white soy sauce sample monitored at the most sensitive MRM transition channel of 474.2/171.2. The fragment ion of 171.2 was assigned as the AQC reporter moiety. The complex chromatogram pattern in Fig. 1B(a) indicates that it is difficult to confirm the existence of γ-Glu-Val-Gly in the white soy sauce, based on the retention time and single MRM transition information alone. Although some other peaks were not assigned, it was assumed that they were derived from other peptides or compounds with a specific pair of precursors and fragment ions identical to those of γ-Glu-Val-Gly.

Table 2

The characteristics and the contents of general components in various commercial soy sauces.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Manufacturer</th>
<th>Grade</th>
<th>Raw materials</th>
<th>Moisture</th>
<th>Protein</th>
<th>Crude fat</th>
<th>Ash</th>
<th>Sodium</th>
<th>NaCla</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark-coloured soy sauce A</td>
<td>P</td>
<td>Super</td>
<td>Soybean, wheat, salt</td>
<td>67.9</td>
<td>8.2</td>
<td>&lt;0.1</td>
<td>15.2</td>
<td>5.36</td>
<td>14.1</td>
</tr>
<tr>
<td>Dark-coloured soy sauce B</td>
<td>Q</td>
<td>Super</td>
<td>Soybean, wheat, salt</td>
<td>67.6</td>
<td>8.1</td>
<td>&lt;0.1</td>
<td>14.6</td>
<td>5.13</td>
<td>13.5</td>
</tr>
<tr>
<td>Dark-coloured soy sauce C</td>
<td>Q</td>
<td>Ordinary</td>
<td>Defatted soybean, wheat, salt, soybean, ethanol</td>
<td>69.1</td>
<td>7.6</td>
<td>&lt;0.1</td>
<td>14.5</td>
<td>5.11</td>
<td>13.6</td>
</tr>
<tr>
<td>Dark-coloured soy sauce D</td>
<td>R</td>
<td>Ordinary</td>
<td>Defatted soybean, wheat, salt, soybean, ethanol</td>
<td>68.6</td>
<td>7.6</td>
<td>&lt;0.1</td>
<td>15.3</td>
<td>5.48</td>
<td>14.3</td>
</tr>
<tr>
<td>Dark-coloured soy sauce E</td>
<td>Q</td>
<td>Ultrasuper</td>
<td>Defatted soybean, wheat, salt, soybean</td>
<td>65.4</td>
<td>9.2</td>
<td>&lt;0.1</td>
<td>14.6</td>
<td>5.17</td>
<td>13.4</td>
</tr>
<tr>
<td>Light-coloured soy sauce A</td>
<td>T</td>
<td>Ordinary</td>
<td>Salt, soybean, wheat, ethanol</td>
<td>68.6</td>
<td>6.1</td>
<td>&lt;0.1</td>
<td>17.2</td>
<td>6.32</td>
<td>16.3</td>
</tr>
<tr>
<td>Light-coloured soy sauce B</td>
<td>P</td>
<td>Ordinary</td>
<td>Soybean, wheat, rice, salt, ethanol</td>
<td>70.7</td>
<td>5.8</td>
<td>&lt;0.1</td>
<td>16.6</td>
<td>6.09</td>
<td>15.8</td>
</tr>
<tr>
<td>White soy sauce</td>
<td>T</td>
<td>Ordinary</td>
<td>Wheat, soybean, salt, ethanol</td>
<td>66.9</td>
<td>3.3</td>
<td>&lt;0.1</td>
<td>15.5</td>
<td>5.73</td>
<td>15.1</td>
</tr>
</tbody>
</table>

a NaCl content was calculated from the sodium content.
of the MS/MS fragment pattern between an authentic and unknown compound is commonly used for structural determination of unknown peaks but, in this case, the intensity of the putative derivatized \(\gamma\)-Glu-Val-Gly peak was too low to obtain fragment information. In a previous study, we employed multiple MRM transition channels monitoring, to confirm the presence of \(\gamma\)-Glu-Val-Gly in scallop samples (Kuroda et al., 2012) and fish sauce samples (Kuroda et al., 2012). This approach was effectively applied to the present study as described below.

Fig. 1A(b–f) and B(b–f) show the mass chromatograms monitored at MRM transition channels of 474.2/145.3, 474.2/300.3, 474.2/229.4, 474.2/304.0 and 474.2/72.1. It is assumed that the fragments of 145.3, 300.3, 229.4 and 304.0 are derived from aminoquinoline, aminoquinoline-\(\gamma\)-Glu, \(\gamma\)-Glu-Val and \(\gamma\)-Glu-Val-Gly, respectively. The fragment structure of 72.1 is unclear. The relative peak intensity was 1.00/0.38/0.11/0.06/0.27/0.21 for the white sauce sample (Fig. 1B), while that of the standard \(\gamma\)-Glu-Val-Gly was 1.00/0.38/0.11/0.06/0.27/0.20 (Fig. 1A). These findings demonstrate that the white sauce sample contained \(\gamma\)-Glu-Val-Gly. The results from other soy sauce samples are summarized in Fig. 2. The x-axis of the figure represents the six MRM transition channels mentioned above, while the y-axis indicates the relative intensity standardized by the intensity at the 474.2/171.2 channel. The large open circle shows the results from the derivatized standard \(\gamma\)-Glu-Val-Gly. Good agreement was observed among plots, indicating that these soy sauce samples contained \(\gamma\)-Glu-Val-Gly.

3.3. Reproducibility, linearity and recovery during quantification of \(\gamma\)-Glu-Val-Gly in soy sauce samples

The reproducibility of the peak area was determined by performing nine injections of a 0.03 mg/dl \(\gamma\)-Glu-Val-Gly solution after derivatization, and the %RSD was found to be 3.6%. The linear dynamic range for the system was confirmed to be 0.003–1.5 mg/dl based on a standard calibration plot of the \(\gamma\)-Glu-Val-Gly concentration and the peak area ratio of \(\gamma\)-Glu-Val-Gly to Arg-UN \((r^2 > 0.999)\). To overcome the matrix effect, determination of \(\gamma\)-Glu-Val-Gly in the soy sauce samples was conducted by a
single-point standard addition approach. The equation \( X = S \times l_0x \) was used, where \( X \) is the \( \gamma \)-Glu-Val-Gly content in the sample, \( S \) is the spiked standard concentration, and \( l_0 \) and \( x \) are the relative peak intensities against the internal standard for the spiked and unspiked samples, respectively. The recovery rates were between 91% and 114%.

3.4. Contents of \( \gamma \)-Glu-Val-Gly in soy sauce samples

The contents of \( \gamma \)-Glu-Val-Gly in various types of soy sauce are given in Table 3. \( \gamma \)-Glu-Val-Gly was present in all samples of soy sauce. The contents of dark-coloured soy sauce ranged from 0.31 to 0.61 mg/dl, while those in light-coloured soy sauce ranged from 0.34 to 0.37 mg/dl. The content of the white soy sauce sample was 0.15 mg/dl. These results indicate that \( \gamma \)-Glu-Val-Gly is widely distributed in commercial Japanese soy sauces.

The results indicated that \( \gamma \)-Glu-Val-Gly was most abundant in dark-coloured soy sauce, followed by light-coloured soy sauce, and then white soy sauce. Although there was no significant difference, \( \gamma \)-Glu-Val-Gly contents in dark-coloured soy sauce tended to be higher than those in light-coloured soy sauce (\( p < 0.1 \)). Among the dark-coloured soy sauces, the \( \gamma \)-Glu-Val-Gly content in the ultrasuper grade was highest (0.61 mg/dl), followed by the super grade (average = 0.47 mg/dl), and the ordinary grade (average = 0.37 mg/dl). Dark-coloured soy sauce \( (\text{kokkuchi-shoyu} \text{ in Japanese}) \) is produced using almost equal amounts of soybean and wheat as raw materials, and is characterized by a strong aroma and a deep reddish brown colour. Light coloured soy sauce \( (\text{usukuchi-shoyu}) \) is also produced using almost equal amounts of soybean and wheat as raw materials, but has a lighter, red-brownish colour and milder aroma than has dark-coloured soy sauce. The properties of light coloured soy sauce are developed by applying different fermentation conditions and treatments. White soy sauce \( (\text{shiroshoyu}) \) is produced using a very high ratio of wheat to soybean and has a very light yellow colour because it is fermented under conditions that prevent colour development \( (\text{Yokotsuka, 1961; Yokotsuka, 1986}) \). The differences in the \( \gamma \)-Glu-Val-Gly contents likely reflect differences in the raw material and fermentation process. To clarify the reason for the variations in the contents of \( \gamma \)-Glu-Val-Gly, 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 \( \gamma \)-Glu-Val-Gly contents \( (r = 0.860, p < 0.01) \) (Fig. 3). These findings suggest that the high level of \( \gamma \)-Glu-Val-Gly was partly caused by the crude protein contents of soy sauces. The \( \gamma \)-Glu-Val-Gly contents of different types of soy sauce were compared. Dark coloured soy sauce A and light coloured soy sauce B were produced by the same manufacturer, P, and light coloured soy sauce A and white soy sauce were produced by manufacturer T. Since the \( \gamma \)-Glu-Val-Gly contents in the different types of soy sauce produced by the same manufacturer were different, one reason for the variation of \( \gamma \)-Glu-Val-Gly contents is likely the difference in the fermentation process used in the production of the various types of soy sauces. In addition, the results shown in Tables 2 and 3 revealed that relatively low amounts of \( \gamma \)-Glu-Val-Gly were present in the soy sauce produced from raw materials containing ethanol. Ethanol is usually added to soy sauce after the fermentation process to suppress deterioration by microbes. Ethanol is also often added to soy sauces with low total nitrogen contents to compensate for their high water activity; therefore, ethanol in raw materials is not a cause of the low contents of \( \gamma \)-Glu-Val-Gly. Although a detailed investigation should be performed to clarify the reasons for the variations in \( \gamma \)-Glu-Val-Gly contents, the results of this study and the above observations suggest that the major factors responsible for the variation in \( \gamma \)-Glu-Val-Gly contents are differences in the fermentation process that lead to differences in the total nitrogen (crude protein) contents.

It has been reported that the dipeptide, Val-Gly, can serve as a substrate of \( \gamma \)-glutamyltransferase (GGT) \( (\text{Suzuki & Yamada, 2007}) \). Therefore, it was assumed that \( \gamma \)-Glu-Val-Gly was biosynthesized via GGT. During the production of soy sauces, it is assumed that GGT from koji fungi, such as \text{Aspergillus oryzae}, yeast, such as \text{Saccharomyces} species and lactic acid bacteria, which have been reported to exist in soy sauces, are involved in the biosynthesis of \( \gamma \)-Glu-Val-Gly. Since the action of proteases has been shown to occur during the fermentation of soy sauces, Val-Gly is considered to be produced by the degradation of soybean protein and wheat protein via proteases. A database search of the sequence of proteins contained in the seeds of soybean and wheat revealed that the Val-Gly sequence was present in soybean \( \beta \)-conglycinin \( (\text{Tierney et al., 1987; Sebastiani et al., 1990}) \), soybean basic 7S globulin \( (\text{Kaga-awa & Hirano, 1989}) \), wheat \( \alpha \)-gliadin \( (\text{Garcia-Maroto, Marana, Garcia-Olmedo, & Carbonero, 1990}) \), and wheat low molecular weight glutenin \( (\text{Colot, Bertels, Thompson, & Flavell, 1989}) \). Therefore, it is possible that Val-Gly was liberated from the above proteins via protease activity and then converted to \( \gamma \)-Glu-Val-Gly via GGT. However, detailed studies are needed to clarify the biosynthesis of \( \gamma \)-Glu-Val-Gly during the production of soy sauces.

In the present study, the \text{kokumi} peptide, \( \gamma \)-Glu-Val-Gly, was identified and quantified in various soy sauces. This is the first report to confirm the existence of \( \gamma \)-Glu-Val-Gly in vegetable foods. The contribution of these peptides to the taste of soy sauces is

![Fig. 3. Correlation of the crude protein contents and the contents of \( \gamma \)-Glu-Val-Gly in commercial Japanese soy sauces.](image-url)
currently being investigated in our laboratory, as is the distribution of \( \gamma \)-Glu-Val-Gly in various foods.

In conclusion, the kokumi peptide, \( \gamma \)-Glu-Val-Gly, was widely distributed among various Japanese soy sauces.

Acknowledgements

We sincerely thank Dr. Kiyoshi Miwa, Dr. Tohru Kouda, and Mr. Hiroaki Takino of Ajinomoto Co., Inc., for encouragement and continued support of this work. We are grateful to Ms. Yuko Iida, Dr. Hiroaki Takino of Ajinomoto Co., Inc., for encouragement and continuous support of this work. We are grateful to Ms. Yuko Iida, Dr. Hiroaki Takino of Ajinomoto Co., Inc., for encouragement and continuous support of this work.

References


