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Analytical Methods

Determination of γ-glutamyl-valyl-glycine in raw scallop and processed scallop products using high pressure liquid chromatography–tandem mass spectrometry

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ABSTRACT

The determination of the *kokumi* peptide, γ-glutamyl-valyl-glycine (γ-Glu-Val-Gly) in raw scallop and processed scallop products was carried out using high pressure liquid chromatography–tandem mass spectrometry (LC/MS/MS). The detection of γ-Glu-Val-Gly was achieved using a multiple reaction monitoring (MRM) method. The optimised condition enabled the precise determination of γ-Glu-Val-Gly. Raw scallop contained 0.08 μg/g γ-Glu-Val-Gly, and the γ-Glu-Val-Gly levels in processed scallop products, such as dried-scallop and scallop extract, were measured to be 0.64 and 0.77 μg/g, respectively. This is the first report to confirm the existence of γ-Glu-Val-Gly in foodstuff.

1. Introduction

The flavour of food is determined by both taste and aroma. Sweet, salty, sour, bitter and umami comprise the five basic tastes, and each is recognised by specific receptors and transduction pathways. In addition, various foods have a *kokumi*-imparting effect. The term "*kokumi*" is used when a flavour cannot be categorised into any of five basic tastes. It was reported that *kokumi* is characterised by four properties, namely thickness, continuity, mouthfulness and harmony of taste (Kuroda, Yamanaka, & Miyamura, 2004). Previously, several studies have aimed to identify *kokumi*-imparting compounds. Ueda et al. studied the *kokumi*-inducing effect of garlic and attempted to isolate the responsible compound (Ueda, Sakaguchi, Hirayama, Miyajima, & Kimizuka, 1990). As a result, sulphur-containing amino acids and peptides were characterised as the *kokumi*-inducing constituents of garlic. γ-Glutamyl peptides such as glutathione (γ-Glu-Cys-Gly) and γ-glutamyl-S-allyl-L-cysteine were identified. Ueda et al. further investigated the *kokumi*-inducing constituents of onion, using the same methodology and identified glutathione and γ-glutamyl-S-(1-propenyl)-L-cysteine sulfoxide (Ueda, Tsubuku, & Miyajima, 1994). These compounds only exhibit a slight flavour in water, but when they are added to an umami solution or various other types of food, they substantially enhance the thickness, continuity and mouthfulness of the food to which they have been added (Ueda, Yonemitsu, Tsubuku, Sakaguchi, & Miyajima, 1997).

Recently, it has been proposed that the *kokumi* of several γ-glutamyl-peptides are perceived through a calcium-sensing receptor (CaSR) in humans (Ohsu et al., 2010). The authors have confirmed that glutathione and several other γ-glutamyl-peptides, such as γ-Glu-Ala, γ-Glu-Val, γ-Glu-Cys, γ-Glu-Abu(aminobutylyl)-Gly (ophthalmic acid) and γ-Glu-Val-Gly, can activate a CaSR and impart the *kokumi*. The CaSR activity of these γ-glutamyl-peptides positively correlates with the intensity of the *kokumi*, as measured by sensory evaluation. This suggests that the *kokumi* is perceived through a CaSR in humans. Of these *kokumi* peptides, γ-Glu-Val-Gly was reported to have the highest intensity of *kokumi*, 12.8-fold of that for glutathione (Ohsu et al., 2010). Although it is possible that γ-Glu-Val-Gly may contribute to *kokumi* in various foods, the presence of this peptide in foodstuff is yet to be reported.

In our study, the distribution of γ-Glu-Val-Gly in various foods was investigated. In a preliminary study that employed LC/MS/MS to detect γ-Glu-Val-Gly in various foods, both raw scallop and scallop extract contained γ-Glu-Val-Gly at trace levels. For the selective detection of the peptide at low concentrations, an LC/MS/MS method was developed and applied for the determination of γ-Glu-Val-Gly in raw scallop and processed scallop products such as dried scallop and scallop extract.

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2. Materials and methods

2.1. Chemicals

γ-Glu-Val-Gly was chemically synthesized in Ajinomoto Co. Inc. The stable isotope of ^{15}N-uniformly labelled-Arg (Arg UN) was purchased from Isotec (Tokyo, Japan). The AccQ Fluor reagent kit was supplied by 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 as the mobile phase and in the derivatization procedure. Deionized water was prepared by a Milli-Q system (Millipore, Bellerica, MA).

2.2. Raw scallop and processed scallop products

Raw scallop (*Patinopecten yessoensis*) ligament (adductor muscle), fished at Hokkaido, Japan, was obtained from a market in Japan. The weight of one piece was approximately 20 g. The best-before date for the raw scallop was 2 days after the processing date. The raw scallop was stored at 5 °C and analysed before the best-before date. Dried-scallop was purchased from the Fisheries Co-operative Association of Sarufutsu village (Hokkaido, Japan). Dried-scallop was produced as follows. Unshelled raw scallop was steamed, and the obtained ligament was boiled in an approximately 8% salt solution at 100 °C for 10 min. After boiling, the ligaments were dried for 50 min at the range of temperature from 100 to 150 °C, cooled in air, and then dried through sun-drying for approximately 40 days. Scallop extract was supplied from Yaidzu Suisan Chemistry Inc. (Shizuoka, Japan). The scallop extract was produced by concentrating under vacuum. After the concentration, the salt was added, mixed and sterilized at 100 °C for 30 min. The dried-scallop and scallop extract were stored at 5 °C prior to the analysis.

2.3. Apparatus

The analysis of γ-Glu-Val-Gly was conducted using a LC/MS/MS system. An Agilent 1100 series HPLC system (Agilent Technologies) was used for the separation of γ-Glu-Val-Gly, and the system was equipped with a binary pump, a degasser, an auto-sampler, and a column compartment. An API 4000 LC/MS/MS system (AB SCIEX) or an AB SCIEX 3200 QTrap LC/MS/MS system (AB SCIEX) was used for detection.

2.4. Preparation of aqueous extracts

The raw scallop was cut into small pieces with stainless steel scissors and then mixed. A sample of scallop (11.19 g) was homogenised in 50 mL of cooled water using a homogenizer (ED-8, Nippon Seiki co. Ltd., Japan) at an operating speed of 9000 rpm for 3 min. The resulting suspension was thoroughly transferred into a 100 mL beaker, using a further 50 mL of water for rinsing. The overall resulting solution was then stirred for 20 min on a magnetic stirrer. A 50 mL aliquot was centrifuged at 5000*g* for 15 min at 4 °C. The resulting supernatant was filtered through a 0.45 μm membrane filter (GD/X Syringe Filters, Whatman), to remove any insoluble matter. The filtrate solutions were further treated using an Amicon Ultra Centrifugal Filter Device (regenerated Cellulose 10,000 MWCO, MILLIPORE, USA) at 7500*g* for 15 min at 4 °C. The samples were stored at −20 °C before use.

A dried scallop adductor muscle, used for making dashi, Japanese soup stock, was ground finely and mixed. A 3.19 g sample was incubated and agitated in 90 mL of hot water at 70 °C for 1 h. After cooling to room temperature, the solution was diluted to 100 mL, and filtered through a 0.45 μm membrane filter (Whatman, GD/X Syringe Filters, 25 mm). A portion of the filtrate was further filtered using a MILLIPORE Amicon Ultra Centrifugal Filter Device (regenerated Cellulose 10,000 MWCO) at 7500*g* for 15 min at 4 °C. The sample was stored at −20 °C before use.

In the case of scallop extract, the sample was diluted with 50-fold water and filtered using a MILLIPORE 10,000 NMWL Ultrafree-MC Filter Unit, at 10,600*g* for 20 min at 4 °C. The sample was then stored at −20 °C prior to the derivatization procedure.

2.5. Derivatization procedure

The concentrations of γ-Glu-Val-Glwy in the scallop samples were analysed using LC/MS/MS after derivatization with 6-aminoquinoyl-N-hydroxysuccinimidyl- carbamate (AQC). A 10 μL aliquot of each appropriately diluted sample was mixed with a 20 μL internal standard solution, containing 0.89 ppm Arg-UN, and 10 μL of a 0.3 ppm γ-Glu-Val-Gly solution (for spiked samples) or deionized water (for unspiked samples). The 10 μL aliquots mentioned above were mixed with 30 μL of borate buffer (AccQ Fluor TM reagent kit) and 10 μL of an AQC reagent (AccQ Fluor TM reagent kit) solution in acetonitrile (prepared as recommended by the supplier). The final solutions were placed in a 1.5 mL microtube, mixed using a vortex mixer and heated at 55 °C 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 analysed by LC/MS/MS.

2.6. LC/MS/MS analysis of γ-Glu-Val-Gly

The separation conditions for γ-Glu-Val-Gly are described as follows. 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. The mobile phase A consisted of aqueous 25 mM formic acid (pH 6.0, adjusted using an aqueous ammonium solution), and the mobile phase B was water/acetonitrile (40/60). The gradient elution conditions were 0 min (15% B), 12 min (25% B), 12.1–14 min (100% B), and 14.1–20 min (15% B). The flow rate was maintained at 0.25 mL/min throughout the analysis. The injection volume was 2 μL.

For mass spectrometric detection, triple quadrupole mass spectrometers were used. The turbo ion spray interface was operated in the positive mode, at 5500 V and 650 °C. Detection was performed via the MRM (Multiple Reaction Monitoring) method, with a dwell time of 170 ms. The MS parameters, CUR, GSI, GS2, CAD, DP, EP and CXP, were set to 15, 80, 80, 8, 41, 6 and 4, respectively. Dwell times were set to 170 ms and 150 ms for the AB ACIEX 3200 OTrap LC/MS/MS system and the API 4000 LC/MS/MS system, respectively. Operations were controlled using Analyst software (version 1.4.2).

The detection of γ-Glu-Val-Gly was achieved using a mass spectrometer with the MRM method. Six MRM chromatograms for γ-Glu-Val-Gly, and one for the internal standard, Arg-UN, were measured simultaneously in a single run. The MRM transition channels (Q1/Q3) and the collision energy (CE) for γ-Glu-Val-Gly were set to 474.2/171.2 (51 V), 474.2/145.3 (30 V), 474.2/300.3 (30 V), 74.2/229.4 (20 V), 474.2/304.0 (20 V) and 474.2/72.1 (50 V), respectively. The parameters for Arg-UN detection were 349.0/171.1 (30 V).

2.7. Analysis of general components

Moisture levels were analysed by measuring the change in the weight after drying at 105 °C for 5 h. Protein content was calculated by multiplying the total nitrogen contents by 6.25. The total nitrogen content was determined via the micro-Kjeldahl method. Crude fat content was analysed by the Soxhlet extraction method using diethylether as the solvent. Ash levels were determined from