短　報

Analytical assay for β-conglycinin in soybeans using cryogenic milling and the absolute quantification (AQUA) method

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ABSTRACT

ß-Conglycinin, a major protein in soybeans (*Glycine max* (L.) Merr.), improves human lipid metabolism and influences the processing properties of soybeans. Recently, an analytical method based on the absolute quantification (AQUA) strategy for ß-conglycinin was developed by us. In this study, the performance of an assay combining trichloroacetic acid (TCA)/acetone protein extraction and the AQUA method with cryogenic milling in dry ice was evaluated, which is used for measuring pesticide residues in foods to prepare homogeneous comminuted samples. The homogeneity of soybean powder obtained by cryogenic milling and the linearity and precision of the analytical assay were satisfactory; however, its recovery was not high. Guanidinium-mediated denaturation and trypsin digestion of ß-conglycinin in the AQUA method, following the TCA/acetone extraction, were suggested to have a substantially higher negative effect on recovery than the TCA/acetone method. Overall, as this assay is based on AQUA strategy using stable isotope-labelled peptides as internal standards, and its performance except for recovery was satisfactory, it is thought to be available for the analysis of ß-conglycinin in cases where a high trueness of measurement is not needed (e.g. comparative semi-quantification of ß-conglycinin).

Keywords: ß-conglycinin, soybean, *Glycine max* (L.) Merr., liquid chromatography/tandem mass spectrometry (LC/MS/MS), stable isotope-labelled peptide

Introduction

Approximate 70–80% of storage proteins of soybeans (*Glycine max* (L.) Merr.) is constituted by two proteins: glycinin and ß-conglycinin (Singh et al. 2015). Glycinin exists as a hexamer and ß-conglycinin is composed of the α, α′, and ß subunits. ß-Conglycinin improves human lipid metabolism (Nishimura et al. 2016) and influences the processing properties of soybeans. The allergenicity of various kinds of miso manufactured in Japan were evaluated by Western blotting using specific antibodies including anti-ß-conglycinin antibody (Moriyama et al. 2013).

My colleagues and I have recently reported a quantitative method for ß-conglycinin based on the strategy of the absolute quantification, or AQUA, termed by Gerber et al. (2003) using liquid chromatography/tandem mass spectrometry (LC/MS/MS) and three stable isotope-labelled internal standard (SIIS) peptides, LQSGDALR[$^{13}C_6$,$^{15}N_4$], NILEASYDTK[$^{13}C_6$,$^{15}N_2$], and NPIYSNNFGK[$^{13}C_6$,$^{15}N_2$]. Multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer is employed to quantitatively determine tryptic peptides produced from the targeted proteins (Gillette et al. 2013). Comparison between the peak areas of the labelled peptides and the tryptic peptides LQSGDALR, NILEASYDTK, and NPIYSNNFGK produced from ß-conglycinin α, α + α′, and ß subunits, respectively, in the MRM chromatogram (Fig. 1) indicates the content of ß-conglycinin (Ippoushi et al. 2019). Out of the phenol, trichloroacetic acid (TCA)/acetone, and thiourea/urea methods used for protein extraction, the TCA/acetone method has proven to be the most efficient method for ß-conglycinin extraction from raw soybeans (Ippoushi et al. 2020). In this study, the performance of the assay combining TCA/acetone extraction and AQUA-based method with cryogenic milling in dry ice (solid carbon dioxide),

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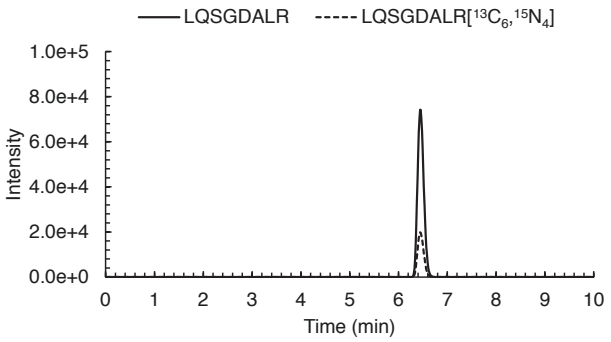

Figure 1. An example of MRM chromatogram of a tryptic digest to analyse LQSGDALR and LQSGDALR[$^{13}C_6$,$^{15}N_4$].

which is used for measuring pesticide residues in foods to prepare homogeneous comminuted samples (AOAC international 2007, Chiba et al. 2015), was evaluated. Cryogenic milling soybeans in dry ice can treat more samples than grinding them in liquid nitrogen using a mortar and pestle. As a result of the evaluation, the performance of this assay was satisfactory except for recovery.

Materials and methods

Materials

Ammonium bicarbonate, dithiothreitol, and TCA were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Guanidinium chloride and ß-mercaptoethanol were purchased from Sigma–Aldrich (MO, USA). Iodoacetamide was purchased from FUJIFILM Wako Pure Chemical Corporation and Sigma–Aldrich. LQSGDALR[$^{13}C_6$,$^{15}N_4$] (molecular weight: 869.0), NILEASYDTK[$^{13}C_6$,$^{15}N_2$] (molecular weight: 1161.3), NPIYSNNFGK[$^{13}C_6$,$^{15}N_2$] (molecular weight: 1161.3), and trypsin (MS grade) were obtained from Thermo Fisher Scientific (MA, USA). Acetonitrile and formic acid (Sigma–Aldrich, LC/MS grade solvent) and water obtained from the Milli-Q water purification system (Merck, Darmstadt, Germany) were used for LC/MS/MS analysis. ß-Conglycinin, whose purity was not less than 85%, was provided by Fuji Oil (Osaka, Japan). The soybean cultivars 'Fukuyutaka' (Ohba et al. 1982) (National Agriculture and Food Research Organization, Ibaraki, Japan) and 'Nanahomare' (Yagasaki et al. 2010) (Nagano Vegetable and Ornamental Crops Experiment Station, Nagano, Japan) were purchased from a local store.

Sample preparation

Twenty grains of 'Fukuyutaka' (6.6 g) and fifty grains of 'Nanahomare' (13.4 g) were stored over overnight at -80 °C, and comminuted with dry ice into a fine powder using a crusher (LAB MILL, Osaka Chemical, Osaka, Japan) (AOAC international 2007, Chiba et al. 2015). After dry ice was sublimed from the powder at -20 °C, the soybean powder (20 mg) was suspended in 0.2 mL of 10% (w/v) TCA in acetone mixed with 2% (v/v) ß-mercaptoethanol; and the suspension was kept at -20 °C overnight. The sample was then centrifuged at 5000g for 30 min at 4 °C, and the supernatant was discarded. Next, cold acetone (0.2 mL) was added to the pellet, centrifuged at 5000g for 10 min at 4 °C, and the supernatant was discarded. Rinsing with acetone was repeated twice further. The pellet was then air-dried.

The extracted proteins were denatured, reduced, and alkylated as reported previously (Ippoushi et al. 2019). The treated sample was diluted with 50 mM ammonium bicarbonate (851 µL). An aliquot (20 µL) of the diluted solution was mixed with 980 µL of 50 mM ammonium bicarbonate. An aliquot (50 µL) of this sample was then mixed with 25 µL of 4 µg/mL trypsin in 50 mM ammonium bicarbonate and 25 µL of 80 fmol/µL LQSGDALR[$^{13}C_6$,$^{15}N_4$], NILEASYDTK[$^{13}C_6$,$^{15}N_2$], and NPIYSNNFGK[$^{13}C_6$,$^{15}N_2$] in 50 mM ammonium bicarbonate, and incubated for 24 h at 37 °C. After incubation, the sample was stored at -20 °C until LC/MS/MS analysis.

LC/MS/MS conditions

The trypsin-digested sample (5 µL) was subjected to LC/MS/MS instrument consisting of an ACQUITY UPLC system connected to an XEVO TQD by way of a Zspray ion source (Waters, MA, USA). The LC and MS/MS conditions were set as described in a previous study (Ippoushi et al. 2019).

ß-Conglycinin content

ß-Conglycinin content (g) was calculated according to the following equation:

ß-Conglycinin (g) = α subunit (mol) × 64,800 + α′ subunit (mol) × 66,900 + ß subunit (mol) × 48,300

The molecular weights of α, α′, and ß subunits reported by Picariello et al. (2013) were used.

Assay evaluation

The homogeneity of soybean powder was determined as follows: twenty grains of 'Fukuyutaka' (6.8 g) were comminuted

with dry ice as described above. Ten fractions (ca. 0.4 g) were acquired from the resulting soybean powder, and each fraction was quantitatively determined in duplicate. Homogeneity was analysed using one-way analysis of variance (Excel, Microsoft, WA, USA).

The linearity of three tryptic peptides, LQSGDALR, NILEASYDTK, and NPIYSNNFGK was evaluated using serial dilutions (0.4–1700, 0.8–1700, and 2.5–310 fmol, respectively, molar quantity injected to LC) of trypsin digest prepared from the 'Fukuyutaka' protein extract. These serial dilutions were obtained as follows: after adding 50 mM ammonium bicarbonate (851 µL) as described in the section **Sample preparation**, an aliquot (40 µL) of the solution was mixed with 160 µL of 50 mM ammonium bicarbonate and 100 µL of 20 µg/mL trypsin in 50 mM ammonium bicarbonate, and then incubated for 24 h at 37 °C. After incubation, two-fold serial dilutions of the tryptic digest were prepared using 50 mM ammonium bicarbonate. The amounts of LQSGDALR, NILEASYDTK, and NPIYSNNFGK in the tryptic digest were determined by LC/MS/MS measurement of the tryptic digest mixed with their SIIS counterparts.

Precision was assessed based on repeatability relative standard deviation (RSDr) and intermediate precision relative standard deviation (RSDint), which were obtained by quantitatively determining the content of ß-conglycinin in soybean 'Fukuyutaka' and 'Nanahomare' in duplicate on five different days. The data were analysed using one-way analysis of variance in Excel.

Recovery was evaluated by spiking ß-conglycinin (0.4 mg) to 'Fukuyutaka' soybean powder (20 mg). The recovery (%) of ß-conglycinin was calculated as follows: the difference between the quantitively determined amount of the spiked sample and that of the non-spiked sample was divided by the spiking amount.

Results and discussion

Homogeneity

In a previous study (Ippoushi et al. 2019), soybean powder was obtained by grinding soybeans in liquid nitrogen using a mortar and pestle; however, this approach is inadequate for treating many samples. Therefore, I adopted a method using pre-freezing at -80 °C and cryogenic milling in the presence of dry ice, which is used for analysing pesticide residues in foods, to obtain homogeneous comminuted samples (AOAC international 2007, Chiba et al. 2015). A homogeneity test was conducted as follows: ten fractions were sampled from the soybean powder prepared by cryogenic milling, and the content of ß-conglycinin in each fraction was quantitatively determined in duplicate (Table 1). As the *F* value (variance ratio) was lower than the *F* critical value, no significant difference was found between the fractions (Santos et al. 2015). Therefore, the soybean powder comminuted with dry ice was considered homogeneous.

Linearity

The linearity of the three tryptic peptides measured by LC/MS/MS, LQSGDALR used for quantifying the ß-conglycinin α subunit, NILEASYDTK for the α + α′ subunits, and NPIYSNNFGK for the ß subunit, is shown in Fig. 2, and the results are summarised in Table 2. The concentrations of these three tryptic peptides generated from ß-conglycinin in 'Fukuyutaka' and 'Nanahomare', a ß-conglycinin-rich cultivar (Yagasaki et al. 2010), used in this study, were above the limits of quantifica-

Table 1. Homogeneity test of soybean powder comminuted with dry ice.

Mean (mg/g weight[a])	40
Between-fraction variance	9.62
Within-fraction variance	20.9
F value (variance ratio)	0.460
F critical value ($\alpha = 0.05$, $\nu_1 = 9$, $\nu_2 = 10$)	3.020

Ten fractions prepared from soybean powder ('Fukuyutaka') were analysed in duplicate. [a], Mean is presented as per weight of soybean powder.

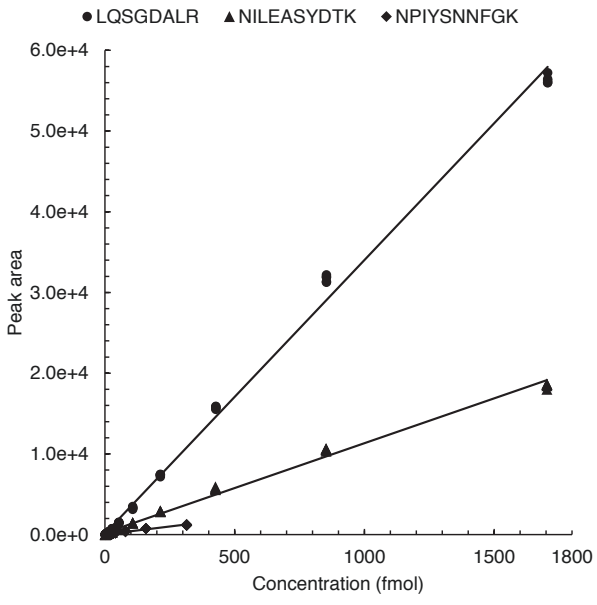

Figure 2. The linearity of LQSGDALR, NILEASYDTK, and NPIYSNNFGK. *n* = 5.

Table 2. Linearity and limits of detection and quantification of LQSGDALR, NILEASYDTK, and NPIYSNNFGK.

Peptide	ß-Conglycinin subunit	Linearity range (fmol)	r^2	Limit of detection (fmol)	Limit of quantification (fmol)
LQSGDALR	α	0.4–1700	0.997	0.1	1
NILEASYDTK	α + α′	0.8–1700	0.994	0.2	1
NPIYSNNFGK	ß	2.5–310	0.975	0.6	5

The limit of detection was established at a signal-to-noise ratio of 3. The limit of quantification was estimated as the concentration detected with a relative standard deviation of 20%.

tion and in the range showing linearity in the detector response of LC/MS/MS (data not shown). In a previous study (Ippoushi et al. 2019), the linearity was assessed using the SIIS peptides in place of their native peptides. Although the linearity of the native peptides was evaluated in this study, the limits of detection and quantification of NPIYSNNFGK, whose counterparts were highest among three SIIS peptides in the previous study, became lower than the previous values.

Precision

Table 3 shows the RSDr and RSDint evaluated by measuring the ß-conglycinin content in 'Fukuyutaka' and 'Nanahomare'. According to the Guidelines for the Validation of Analytical Methods presented by the Ministry of Agriculture, Forestry and Fisheries of Japan (2019), RSDr should be less than or equal to 25% over the whole dynamic range of the analytical method for specific proteins. The RSDr in the analyses of 'Fukuyutaka' and 'Nanahomare' fulfilled this criterion. According to the guidelines, the reproducibility relative standard deviation (RSDR) is required to be less than or equal to 35%. The RSDint in the analyses of 'Fukuyutaka' and 'Nanahomare', but not their RSDR, were below 35% which is the upper limit of RSDR as required in the guidelines.

Recovery

The recovery of ß-conglycinin evaluated using ß-conglycinin-spiked 'Fukuyutaka' was 40% (Table 4). As per the above-mentioned guidelines, the trueness of the analytical method for specific proteins, which is verified using a reference material, is required to be between 70% and 120%. According to the Analytical Method for Foods Containing Allergens, Notification No. 286 of the Consumer Affairs Agency of Japan (2010), the recovery of proteins derived from specific raw materials analysed by enzyme-linked immunosorbent assay (ELISA) is required to be between 50% and 150%. The recovery of the analytical method evaluated in this study did not reach these targets.

Table 3. Mean and repeatability and intermediate precision relative standard deviations in the quantitative determination of ß-conglycinin content in soybeans.

Cultivar	Mean (mg/g weight[a])	RSDr[b] (%)	RSDint[c] (%)
Fukuyutaka	34	6	27
Nanahomare	44	14	22

ß-Conglycinin content in 'Fukuyutaka' and 'Nanahomare' was analysed in duplicate on five different days. [a], Mean is presented as per weight of soybean powder. [b], RSDr, repeatability relative standard deviation. [c], RSDint, intermediate precision relative standard deviation.

Table 4. Recovery of ß-conglycinin spiked into 'Fukuyutaka' powder.

	Mean ± standard deviation
Recovery (%)	40 ± 11

Recovery (%) was calculated based on the purity of spiked ß-conglycinin (85%). $n = 5$.

Soybeans contain 6–7% ß-conglycinin (Urade 2011). As shown in Table 3, the content of ß-conglycinin in 'Fukuyutaka', a standard soybean cultivar, was determined to be 3.4%, which corresponds to 49%–57% of the known value. This is in approximate agreement with the recovery (40%) of the analytical assay. Hill et al. (2017) quantitatively determined ß-conglycinin contained in ground and lyophilised soybean varieties using heavy isotope labelled NILEASYDTK alone based on the AQUA strategy. In their study, the concentrations of ß-conglycinin in many soybean samples were between 30 and 40 mg/g dry tissue weight. The concentration (34 mg/g weight, Table 3) of ß-conglycinin in 'Fukuyutaka' was in this range, even though its powder was not lyophilised. These results indicate that both AQUA-based assays show similar recovery of ß-conglycinin.

AQUA peptides are added relatively late from the trypsin digestion process in the analytical workflow, and thus cannot serve to compensate for the loss of ß-conglycinin during protein extraction and sample treatment before trypsin digestion. Moreover, because the AQUA strategy depends on the complete

formation of target peptides during trypsin digestion (Wohlgemuth et al. 2015), the analytical assay used in this study underestimated the content of ß-conglycinin in case of incomplete formation of tryptic peptides LQSGDALR, NILEASYDTK, and NPIYSNNFGK produced from ß-conglycinin. These factors are thought to result in not reaching the required target value for recovery as per the above-mentioned guidelines.

ß-conglycinin used in this work was estimated to be 0.40 ± 0.05 g/g weight (mean ± standard deviation, n = 3) by the analytical assay without TCA/acetone extraction, which thus results in 47% of the recovery based on 85% ß-conglycinin purity. This value is close to the recovery (40%, Table 4) of ß-conglycinin spiked into 'Fukuyutaka' powder. These results suggest that guanidinium-mediated denaturation and trypsin digestion of ß-conglycinin in the AQUA method, following the TCA/acetone extraction, have a substantially higher negative effect on recovery than the TCA/acetone method (Ippoushi et al. 2020).

Lund et al. (2012) developed an analytical method for the quantification of human chorionic gonadotropin (hCG) using immunoaffinity extraction and the AQUA approach. In this study, the recovery of hCG was approximately 40% from serum and 60% from urine. They also described that the AQUA peptide does not correct any variability due to immunocapture or tryptic digestion, which results in a low recovery of the target.

The Protein Standard Absolute Quantification (PSAQ) standard, an isotope-labelled equivalent of the full-length target protein, is added to the samples at a very earlier stage of the analytical procedure than the AQUA standard. PSAQ is compatible with any type of sample prefractionation, which is shared by the PSAQ standard and target protein. Moreover, the PSAQ strategy can prevent differences in tryptic digestion yields between the standards and analytes. Therefore, the PSAQ strategy is markedly more accurate than the AQUA strategy. The PSAQ technique is thought to improve the recovery of ß-conglycinin obtained using the AQUA-based assay. However, use of the PSAQ method is limited because of its cost and difficulty in preparing protein standards (Brun et al. 2009).

Because the performance of the AQUA-based assay evaluated in this work was satisfactory except for recovery, this assay is thought to be available for the analysis of ß-conglycinin in cases where a high trueness of the measurement is not needed. For example, the assay is expected to contribute to determine the relative abundance of ß-conglycinin across various soybeans, similar to Western blotting, which is used for the comparative semi-quantification of ß-conglycinin.

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Declaration of conflict of interest

The author has no conflict of interest to declare.

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