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Letter to the Editor

## Development of a liquid chromatography-tandem mass spectrometry method for quantification of the egg allergen ovalbumin in rat plasma

Dear Editor,

Monitoring of serum concentrations of food allergens during challenge tests provides important information when diagnosing food allergies.<sup>1</sup> Sandwich ELISA has been used to measure the concentrations of food allergens in foodstuffs<sup>2</sup> and in biological samples.<sup>1</sup> However, the antibodies used in ELISA sometimes show cross-reactivity and/or interference with multiple components in samples, resulting in overestimation and/or underestimation. Thus, the widespread use of ELISA is limited because of poor specificity. Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used for the analysis of food allergens in processed foods.<sup>2,3</sup> These reports suggest that detection of food allergens in processed foods using mass spectrometry (MS) is helpful for quality control. Furthermore, MS has been used for quantification of biological origin peptides<sup>4</sup> and protein-based biopharmaceuticals<sup>5</sup> in biological samples. However, there are no studies that the plasma (or serum) concentrations of food allergens were measured using MS. Compared to the detection of food allergens in processed foods using MS, detection of allergen in plasma is complicated since concentrations of food allergen in plasma are generally much lower than those in processed foods. To detect a target protein at low concentrations in biological samples, it is necessary to remove any abundant impurities from the samples by appropriate methods such as immunocapture methods. We applied immunoprecipitation of egg allergen OVA and nanoLC combined with quadrupole time of flight tandem mass spectrometer to measure the concentration of OVA in rat plasma after oral ingestion.

Figure 1 shows our schematic strategy to determine the concentrations of OVA in plasma. In this study, we used Eksper™ nanoLC 425 (AB sciex) combined with a TripleTOF 5600+ (AB Sciex) as a LC-MS/MS system. Experiments involving animals were approved by the animal committee of Hiroshima University (Approval No. A16-138). All details are shown in Supplementary Methods.

To determine the signature peptide ions for OVA quantification, we first confirmed the correlation between the OVA concentrations in water and peak areas of the product ions generated from tryptic digested OVA. When the peptides from tryptic digested OVA were analyzed using nanoLC-MS/MS and Skyline software, 11 precursor ion peaks were obtained (Supplementary Table 1). Among these precursor ions, five OVA-specific precursor ions exhibited correlations between the OVA concentrations and peak areas of the product ions (Supplementary Table 2). In particular, GGLEPINFQTAADQAR [M+2H]<sup>2+</sup> showed the highest intensity

and reproducibility for measuring the OVA concentration in the range of 50–500 µg/mL. GGLEPINFQTAADQAR peptide has been identified as a marker that can be used to quantify the OVA contents in processed foods.<sup>3</sup> In addition, this peptide contains a partial IgE-binding epitope sequence of OVA (RGGLEPINFQ) for patients with type I hypersensitivity to eggs.<sup>6</sup> Thus, we determined GGLEPINFQTAADQAR as a signature peptide and synthesized its stable isotope-labeled (SIL) peptide. SIL-peptide was used as internal standard in OVA quantification.

Next, we tried to detect OVA in rat plasma and confirmed correlation between the standard OVA concentration in rat plasma and peak area ratios of product ions from OVA to SIL-peptide. To eliminate the quantitative interferences by large amounts of biological proteins, we concentrated OVA from plasma samples by the immunoprecipitation method using polyclonal anti-OVA antibody-coupled magnetic beads. After immunoprecipitation, we could also obtain the signals of GGLEPINFQTAADQAR [M+2H]<sup>2+</sup> (Supplementary Fig. 1). When the peak area ratios of the integrated product ions generated from OVA to those generated from an internal standard were plotted against the OVA concentrations (0–150 ng/mL), a high correlation coefficient was observed ( $r = 0.9956$ , Supplementary Fig. 2). The calculated lower limit of detection (signal to noise ratio = 3) and lower limit of quantitation (signal to noise ratio = 10) of OVA were 6.6 ng/mL and 29.5 ng/mL, respectively.

Finally, we measured OVA concentration in plasma after oral administration of OVA in rats. When OVA (50 mg) was administered orally 30 min after the vehicle (PBS), the plasma concentration of OVA was low (approximately 74 ng/mL). By contrast, after pretreatment with aspirin, the plasma concentration of OVA was approximately 149 ng/mL following ingestion by rats (Table 1). These results agree with our previous finding that aspirin disrupted the intestinal barrier, which was followed by OVA absorption through the paracellular pathway.<sup>7</sup> In this study, 50 mg of OVA was administered to each rat with a body weight of approximately 250 g, which gave an OVA administration rate of 200 mg/kg. Husby *et al.* reported a serum concentration of OVA of 4–178 ng/mL measured by ELISA after 2 mL/kg of raw egg white was administered to children through a gastric tube, which corresponded to a dosage rate of approximately 100 mg of OVA per kilogram.<sup>8</sup> According to Food and Drug Administration guidelines, 100 mg/kg of OVA in human corresponds to 620 mg/kg in rats.<sup>9</sup> Hence, the dosage of OVA (200 mg/kg) in this study is considered to be reasonable.

In conclusion, we developed a new technique to measure the plasma OVA concentrations using LC-MS/MS followed by immunoprecipitation. Our new method enables the measurement of low plasma concentrations of OVA even in clinical challenge tests. This

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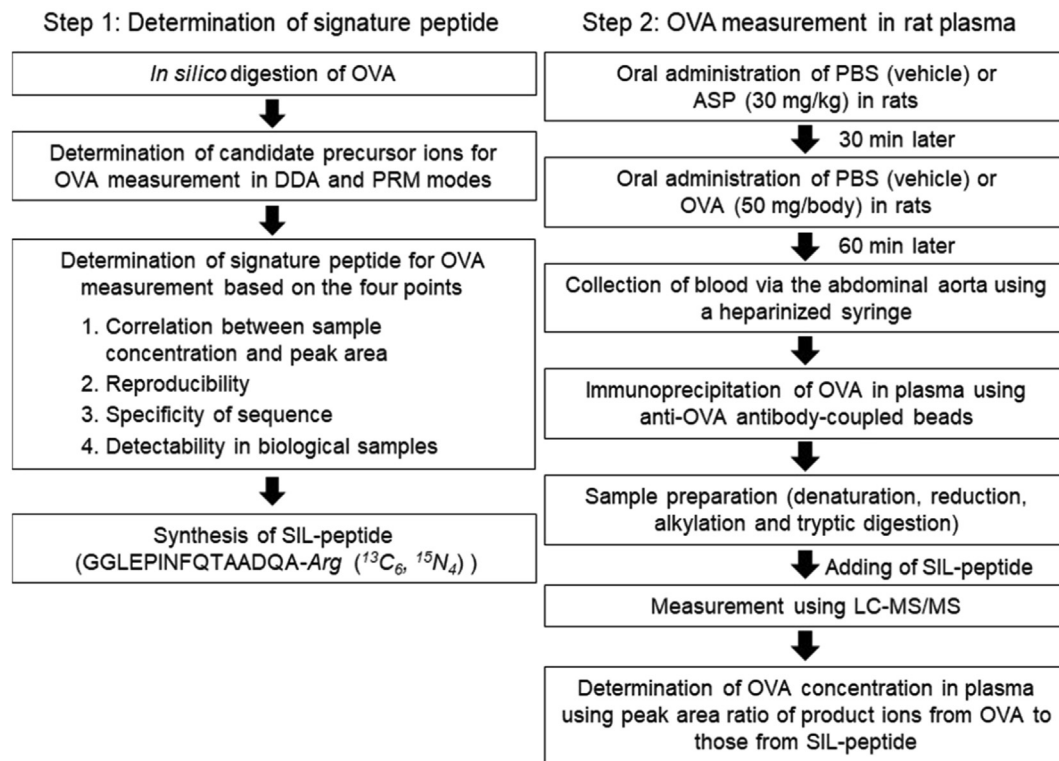


**Fig. 1.** Strategy for measurement of plasma OVA concentration using liquid chromatography tandem mass spectrometry (LC-MS/MS) in this study (schematic). *In silico* digestion and peak area analysis were conducted using Skyline software (Version 3.5, MacCoss Lab Software). Stable isotope-labeled (SIL)- GGLEPINFQTAADQAR peptide were synthesized by Sigma-Aldrich, which has six carbon ( $^{13}\text{C}$ ) and four nitrogen ( $^{15}\text{N}$ ) atoms of the C-terminal arginine substituted with  $^{13}\text{C}$  and  $^{15}\text{N}$ , respectively. ASP, aspirin; DDA, data-dependent acquisition; PRM, parallel reaction monitoring.

**Table 1**  
Effect of aspirin on plasma concentrations of OVA after oral administration in rats.

| Aspirin treatment         | PBS  |      | OVA             |                   |
|---------------------------|------|------|-----------------|-------------------|
|                           | –    | +    | –               | +                 |
| OVA concentration (ng/mL) | n.d. | n.d. | $73.5 \pm 16.3$ | $148.5 \pm 9.2^*$ |

Aspirin (30 mg/kg) was administered orally 30 min before oral administration of OVA (50 mg). Each value is the mean  $\pm$  standard error for three rats.  $^*P < 0.01$ , significantly different from OVA ingestion without aspirin treatment. n.d., not detected.

method could be used to elucidate the relationship between serum allergen concentrations and development of symptoms in food allergy patients after ingestion of an allergen. However, immunoprecipitation is not suitable for measuring large numbers of samples in clinical situations because it is complicated and expensive. Furthermore, it may be difficult to concentrate allergen in plasma from patients with food allergy by immunoprecipitation because immunoglobulins specific to allergen may be increased. A method that is simpler and quicker than immunoprecipitation is required to remove proteins such as albumin and globulins from the plasma samples. Additionally, further studies are also required to confirm the relationship between the plasma concentration of OVA determined by LC-MS/MS and allergic symptoms. The limitation in our assay is method validation. Various validation factors can affect the accuracy quantification by LC-MS/MS, SIL-protein was reported more useful than SIL-peptide to improve method validation.<sup>10</sup> If these problems are resolved, LC-MS/MS may be useful for precise quantification of serum allergen concentrations following ingestion of allergenic foods.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2018.12.005>.

### Conflict of interest

The authors have no conflict of interest to declare.

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## **Supplementary Methods**

### **Animals**

Male Sprague Dawley rats aged 7–8 weeks were obtained from Japan SLC, Inc. (Shizuoka, Japan). The rats were fed a standard laboratory diet and water *ad libitum* for more than 1 week before the experiments. Experiments involving animals were carried out in accordance with the Guide for Animal Experimentation from the Committee of Research Facilities for Laboratory Animal Sciences of Hiroshima University (Approval No. A16-138).

### **Oral administration of OVA in rats**

Absorption of OVA in rats was studied using a modification of our previously reported method [7]. Briefly, 12 rats (body weight 240–260 g) were assigned randomly to four experimental groups, each containing three rats. These rats were fasted overnight and anesthetized with pentobarbital (30 mg/kg, intraperitoneally). The vehicle alone (PBS, pH 7.4) or an aspirin solution (30 mg/kg; Wako Pure Chemical Industries, Kyoto, Japan) was administered orally using a stainless-steel feeding tube. Aspirin was administered to enhance the absorption of OVA into the blood [7]. An OVA solution (100 mg/mL in PBS, Grade V; Sigma-Aldrich, St. Louis, MO) was prepared. The vehicle (PBS) or OVA solution (500  $\mu$ L) was administered orally 30 min after aspirin treatment. Blood was collected via the abdominal aorta using a heparinized syringe 1 h after PBS or OVA administration. After centrifugation, the plasma samples were stored at  $-30^{\circ}\text{C}$  until required for use.

### **Preparation of magnetic beads coupled with anti-OVA antibody**

To purify OVA from plasma by immunoprecipitation, we prepared magnetic beads (Dynabeads<sup>®</sup> M-280 Tosylactivated; Life Technologies, Carlsbad, CA) coupled with anti-OVA antibody according to the manufacturer's instructions with some modifications. Briefly, beads (5 mg) were washed with 0.1 M sodium phosphate buffer (pH 7.4) and resuspended in 165  $\mu$ L of fresh 0.1 M sodium phosphate buffer (pH 7.4). The bead suspension was placed on a magnet (DynaMag<sup>™</sup>-Spin, Life Technologies), and the supernatant was removed. Polyclonal anti-OVA antibody (100  $\mu$ g, GTX21221; GeneTex, Irvine, CA) was added, and the volume was adjusted to 150  $\mu$ L with 0.1 M sodium phosphate buffer (pH 7.4). The bead suspension was mixed with 100  $\mu$ L of 0.1 M sodium phosphate buffer (pH 7.4) containing 3 M ammonium sulfate and incubated at 37°C overnight. To inactivate excess tosyl groups on the surfaces of the beads, the beads were incubated with 500  $\mu$ L of 0.1 M Tris-HCl (pH 8.0) at 37°C overnight. The beads were washed twice with the washing buffer [0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.01% Tween 20]. The supernatant was removed, and the beads were resuspended in 0.01 M sodium phosphate buffer containing 0.15 M NaCl, 0.01% Tween 20, and 0.1% sodium azide. The prepared beads were stored at 4°C until required for use.

### **Immunoprecipitation of OVA**

Plasma (500  $\mu$ L) obtained from rats that ingested OVA was diluted twice with ultrapure water in a tube with low protein adsorption (PROTEOSAVE<sup>™</sup> SS 1.5 mL

Microtube; Sumitomo Bakelite Co., Ltd, Tokyo, Japan). To prepare standard OVA samples, a solution of OVA was spiked with 50% rat plasma containing no OVA at concentrations of 20–150 ng/mL. These samples were incubated with 400  $\mu$ g of antibody-coupled beads at 4°C for 18 h. The beads were washed five times with washing buffer, starting with 500  $\mu$ L and reducing the volume of washing buffer by 100  $\mu$ L at each washing step. Then, 20  $\mu$ L of 20 mM glycine-HCl (pH 2.5) was added, and the solution was incubated for 1 h on ice with occasional stirring to dissociate the OVA from the antibodies. The supernatant containing OVA was immediately neutralized with an appropriate volume of a 100 mM NaHCO<sub>3</sub>-NaOH solution (pH 10) and lyophilized (FRD-50M freeze dryer; IWAKI, Shizuoka, Japan). These samples were stored at –80°C until required for urea denaturation.

### **Sample preparation for MS**

Lyophilized samples were reconstituted with 20  $\mu$ L of ultrapure water. Then, 50  $\mu$ L of 500 mM Tris-HCl (pH 8.0) containing 10 M urea and 2.91  $\mu$ L of 500 mM dithiothreitol (DTT) were added, and the mixture was incubated at 60°C for 1 h. Alkylation was performed by addition of 3.03  $\mu$ L of 1 M iodoacetamide, with incubation at room temperature for 30 min protected from light, and terminated with 1.54  $\mu$ L of 500 mM DTT. After alkylation, the urea concentration in each mixture was diluted to  $\leq$  1 M with 422.5  $\mu$ L of ultrapure water. Then, each mixture was added to 2.0  $\mu$ L of a 1 mg/mL trypsin solution and incubated at 37°C overnight. The tryptic digestion samples were lyophilized and then reconstituted with 100  $\mu$ L of 1% formic acid. The samples were lyophilized again following desalting using MonoSpin™ C18

(GL Sciences, Tokyo, Japan) according to the manufacturer's instruction. These samples were reconstituted with 20  $\mu$ L of 50% ACN containing 5% formic acid and stored at  $-80^{\circ}\text{C}$  until required for MS.

### **Determination of signature peptide ions for OVA quantification**

Signature peptide ions for OVA quantification was determined using an Ekspert<sup>TM</sup> nanoLC 425 (AB Sciex, Framingham, MA) combined with a Q-TOF MS/MS system (TripleTOF 5600+, AB Sciex). The tryptic peptides from OVA were separated by the Ekspert<sup>TM</sup> nanoLC 425 with a reversed phase column (ChromXP C18-CL, 3  $\mu\text{m}$  120  $\text{\AA}$ , 150 mm  $\times$  5  $\mu\text{m}$ ; AB Sciex) and trap column (ChromXP C18-CL, 3  $\mu\text{m}$  120  $\text{\AA}$ , 0.5 mm  $\times$  200  $\mu\text{m}$ ; AB Sciex). Aliquots (1  $\mu\text{L}$ ) of the tryptic peptides were injected using an autosampler (Ekspert<sup>TM</sup> nanoLC 400; AB Sciex). Then, the peptides were eluted with a linear gradient of 2–40% B over 30 min at a flow rate of 300 nL/min. Mobile phase A was water containing 0.1% formic acid and mobile phase B was ACN containing 0.1% formic acid. After elution, the column was washed with 90% B for 5 min and re-equilibrated to the initial conditions with 2% B over 10 min. The eluted peptides were analyzed with the TripleTOF 5600+ system in data-dependent acquisition (DDA) mode or parallel reaction monitoring (PRM) mode. In DDA mode, selected precursor ions and/or the top ten precursor ions with the highest peak intensities were analyzed in second MS dimension (MS2). The analyzed ions are summarized in Supplementary Table 1. MS was performed with positive electrospray ionization and the scan ranges were 400–1250  $m/z$  for the precursor ions and 200–1600  $m/z$  for the product ions. The source parameters were as follows: curtain gas, 20 psi (1 psi = 6894.76 Pa); ion source

gas 1 (nebulizing), 20 psi; ion source gas 2 (desolvating), 0 psi; ion spray voltage floating, 2300 V; and interface heater temperature, 150°C. One cycle was 1.3 s. Peptide identification was performed with ProteinPilot™ Software (Version 4.5, AB Sciex) using the UniProtKB/Swiss-Prot database. To calculate the peak areas, data were analyzed using Skyline software (Version 3.5, MacCoss Lab Software; University of Washington, Seattle, WA). The specificities of selected peptide sequences of OVA were confirmed using BLAST® (National Center for Biotechnology Information, Bethesda, MD) with the UniProtKB/Swiss-Prot database.

### **Determination of the OVA concentration**

The stable isotope-labeled (SIL)-peptide GGLEPINFQTAADQAR (Sigma-Aldrich), which has six carbon ( $^{12}\text{C}$ ) and four nitrogen ( $^{14}\text{N}$ ) atoms of the C-terminal arginine substituted with  $^{13}\text{C}$  and  $^{15}\text{N}$ , respectively, was dissolved in dimethyl sulfoxide at 1 mg/mL. Then, the peptide solution was diluted to 50 ng/mL with 50% ACN containing 5% formic acid. SIL-peptide (2  $\mu\text{L}$ ) was added to each sample following immunoprecipitation and digestion treatments for quantification of OVA as an internal standard. To determine the OVA concentrations in samples, product ions generated from a signature peptide ion (precursor ion) derived from digested OVA and SIL-peptide were monitored using the TripleTOF 5600+ system in PRM mode. To confirm OVA detection, 16 other transitions were monitored simultaneously. The collision energies and approximate retention times of the ions are shown in Supplementary Table 2. Quantitative data were also analyzed using Skyline software. The quantitative values are ratios of the OVA-derived peptide (light ions) peak areas to the internal standard

(heavy ions) peak areas. The lower limit of detection and the lower limit of quantitation were calculated as the concentrations at signal-to-noise ratios of three and ten, respectively, when the peak areas of standard samples without OVA (0 ng/mL) were used as noise.

### **Statistical analyses**

Data are presented as the mean  $\pm$  standard error. Differences in mean values between groups were assessed by the Kruskal–Wallis test with Tukey's test.  $P < 0.05$  was considered statistically significant

**Supplementary Table 1.** List of precursor ions for ovalbumin (OVA) measurement in data-dependent acquisition (DDA) mode.

| <i>m/z</i> | <i>z</i> | sequence                  | position in OVA |
|------------|----------|---------------------------|-----------------|
| 479.29     | 2        | (R) TQINKVVR (F)          | 51–58           |
| 563.28     | 3        | (R) GGLEPINFQTAADQAR (E)  | 127–142         |
| 844.42     | 2        | (R) GGLEPINFQTAADQAR (E)  | 127–142         |
| 620.33     | 3        | (R) ELINSWVESQTNGIIR (N)  | 143–158         |
| 929.99     | 2        | (R) ELINSWVESQTNGIIR (N)  | 143–158         |
| 527.91     | 3        | (K) LTEWTSSNVMEER (K)     | 264–276         |
| 791.36     | 2        | (K) LTEWTSSNVMEER (K)     | 264–276         |
| 546.30     | 2        | (N) LSGISSAESLK (I)       | 312–322         |
| 444.23     | 4        | (K) ISQAVHAAHAEINEAGR (E) | 323–339         |
| 591.97     | 3        | (K) ISQAVHAAHAEINEAGR (E) | 323–339         |
| 887.45     | 2        | (K) ISQAVHAAHAEINEAGR (E) | 323–339         |

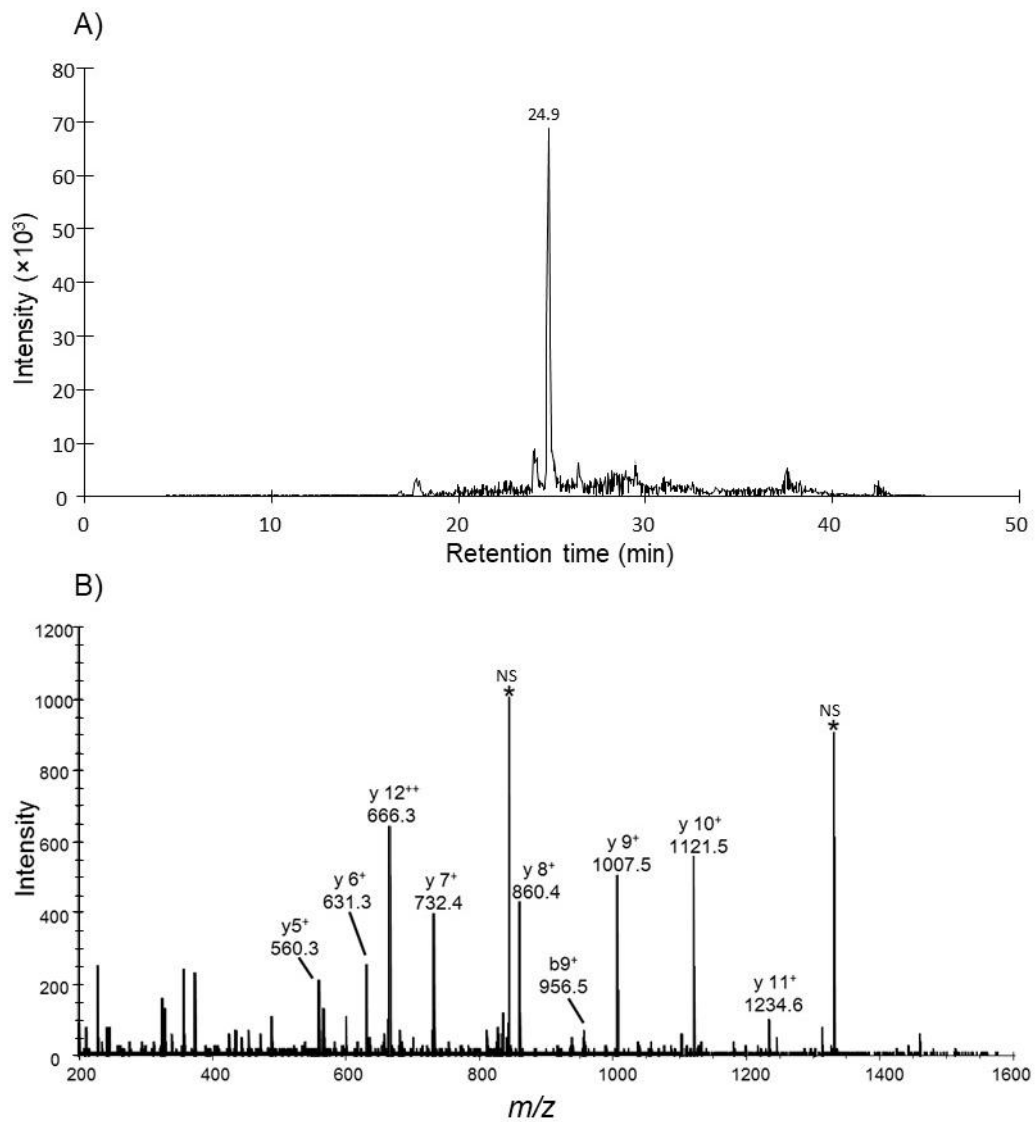
Measurement of OVA in DDA mode was performed to identify candidate ions for parallel reaction monitoring mode. These ions were selected from an OVA identification study using an OVA solution (data not shown). In DDA mode, the listed ions and top ten precursor ions with the highest intensities were further analyzed in second MS dimension (MS<sup>2</sup>).

**Supplementary Table 2.**

List of transitions for OVA measurements in parallel reaction monitoring (PRM) mode.

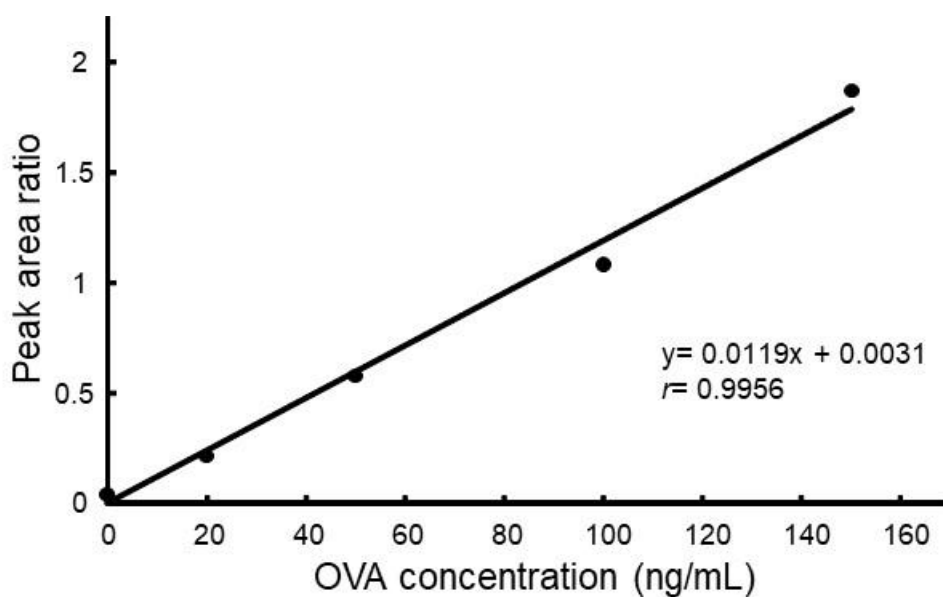
| Precursor sequence ( <i>m/z</i> ) | CE (V) | RT (min) | Transitions ( <i>m/z</i> )   |
|-----------------------------------|--------|----------|------------------------------|
| DILNQITKPNDVYSFSLASR (761.0656)   | 39     | 27       | y8 <sup>+</sup> (930.4860)   |
| [M+3H] <sup>3+</sup>              |        |          | y6 <sup>+</sup> (680.3726)   |
|                                   |        |          | y5 <sup>+</sup> (533.3042)   |
|                                   |        |          | y12 <sup>2+</sup> (678.3331) |
| GGLEPINFQTAADQAR (844.4235)       | 39.3   | 24       | y10 <sup>+</sup> (1121.5334) |
| [M+2H] <sup>2+</sup> , (light)    |        |          | y9 <sup>+</sup> (1007.4905)  |
|                                   |        |          | y8 <sup>+</sup> (860.4221)   |
|                                   |        |          | y12 <sup>2+</sup> (666.3388) |
| GGLEPINFQTAADQAR (849.4277)       | 39.3   | 24       | y10 <sup>+</sup> (1131.5417) |
| [M+2H] <sup>2+</sup> , (heavy)    |        |          | y9 <sup>+</sup> (1017.4988)  |
|                                   |        |          | y8 <sup>+</sup> (870.4303)   |
|                                   |        |          | y12 <sup>2+</sup> (671.3429) |
| LTEWTSSNVMEER (791.3643)          | 37.3   | 22       | y9 <sup>+</sup> (1052.4677)  |
| [M+2H] <sup>2+</sup>              |        |          | y8 <sup>+</sup> (951.4200)   |
|                                   |        |          | y7 <sup>+</sup> (864.3880)   |
|                                   |        |          | y4 <sup>+</sup> (564.2446)   |
| ISQAVHAAHAEINEAGR (591.9712)      | 29.8   | 15       | y16 <sup>2+</sup> (830.9712) |
| [M+3H] <sup>3+</sup>              |        |          | y14 <sup>2+</sup> (723.3658) |
|                                   |        |          | y13 <sup>2+</sup> (687.8473) |
|                                   |        |          | y12 <sup>2+</sup> (638.3131) |
| ISQAVHAAHAEINEAGR (444.2302)      | 29.8   | 15       | y6 <sup>+</sup> (659.3471)   |
| [M+4H] <sup>4+</sup>              |        |          | y5 <sup>+</sup> (546.2630)   |
|                                   |        |          | y4 <sup>+</sup> (432.2201)   |
|                                   |        |          | y14 <sup>2+</sup> (723.3658) |

Measurements of OVA in PRM mode were performed to identify a signature peptide and determine OVA concentrations in plasma from rats after OVA ingestion. The light/heavy peak area ratio of GGGLEPINFQTAADQAR transitions was calculated by Skyline software. This ratio was used to determine OVA concentrations in plasma from rats after OVA ingestion. The other 16 transitions were used as reference ions to confirm the OVA results. CE, collision energy; RT, approximate retention time in Eksper<sup>TM</sup> nanoLC425.



**Supplementary Fig. 1.**

(A) The intensity of total y- and b-ions generated from the signature peptide (GGLEPINFQTAADQAR, 844.4235  $m/z$ ) is shown in the chromatogram. The retention time of total product ions generated from signature peptide was 24.9 min. (B) MS/MS spectra of the product ions generated from signature peptide (844.4235  $m/z$ ,  $[M+2H]^{2+}$ ) at 24.88 min in immunoprecipitated rat plasma. NS, nonspecific peak.



**Supplementary Fig. 2.**

Calibration curve for OVA quantification in plasma. Peak areas were calculated using Skyline software. Peak areas of the four selected y-ions derived from the signature peptide (light) and its SIL-peptide (heavy) were integrated, respectively. To compensate for dispersion of the measurements, light/heavy ratios were plotted against OVA concentrations. The correlation coefficient ( $r$ ) was 0.9956 and the lower limit of quantitation was 29.5 ng/mL. SIL, stable isotope-labeled.