Semicomprehensive and Semiquantitative Determination of Proteins with Mass Probes

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We have designed and synthesized a series of mass-probes, which could be a model of a new tool for the semicomprehensive quantitative assays of proteins using mass spectrometry. In the system with mass-probes, the molecular weights were used as markers. We have synthesized the two probes, KMP-174 and KMP-188, and ovalbumin (OVA) and the hen egg lysozyme (HEL) were evaluated by antibodies labeled with those probes.

Rapid profiling of proteins is essential to meet the widely varying demands of biological detection. We have developed a semicomprehensive and semiquantitative method, which can rapidly measure multiple proteins by a single measurement.

A mass spectrometry (MS) measurement takes only a few seconds per sample, the detection is sensitive, and the obtained spectrum is generally sharp with very little overlap; therefore, it is potentially an ideal tool for multiplex determination. However, the determination of protein using mass spectrometry is performed only in "proteomics" research in combination with 2D gel electrophoresis[1–6] such as the ICAT (isotope code affinity tag)[7,8] method. In ICAT method, target proteins from two samples are digested by an enzyme and the cleaved peptides are bound to isotope/non-isotope labeled probes and introduced to MS. The existence ratio of the protein in two samples is obtained by as the intensity ratio of the isotope/nonisotope peptide. In the method, proteins are digested by an enzyme because whole proteins are detected as fragment ion peaks, which result in a complicated analysis, and also, samples have to be isotope labeled because the efficiency of the ionization is different for each molecule. It is known that the efficiencies of ionization are different depending on its chemical structure, solvent, and pH.[9–11] In our newly developed system, these problems have been resolved because cleavable marker sites containing quaternary amines are sensitively detected by mass spectrometry instead of the whole protein in the method. The derivatization of samples with quaternary amines is known to raise the efficiency of the detection by mass spectrometry by raising the ionization efficiency.[12–14] We have previously proposed adductive mass probes that are easily introduced to carboxylic acids, primary amines, alcohols, carbonyl groups, and their derivatives in both polar and nonpolar solvents.[15] However, they were not available for biological molecules with a number of functional moieties.

The protein measurement scheme using mass probes is shown in Figure 1. Sample proteins are immobilized on a solid phase and many kinds of antibodies, which are labeled with mass probes having different marker site, are added. After washing off unbound antibodies, marker sites are cleaved by UV irradiation and detected by MS. The amounts of the antigens are calculated by the intensity ratios. The molecular structures of KMP-174 and

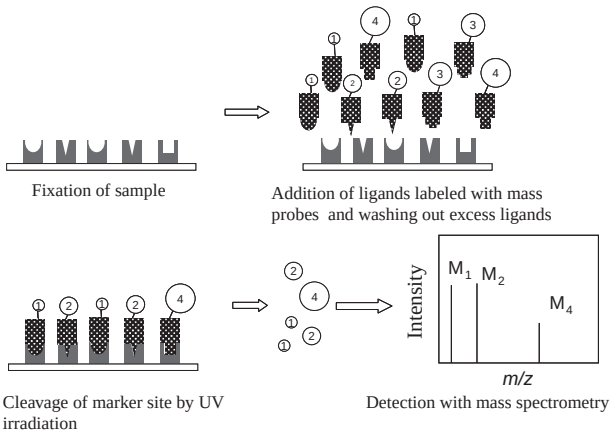

Figure 1. Schematic outline of the analyte-ligand detection using mass probes.

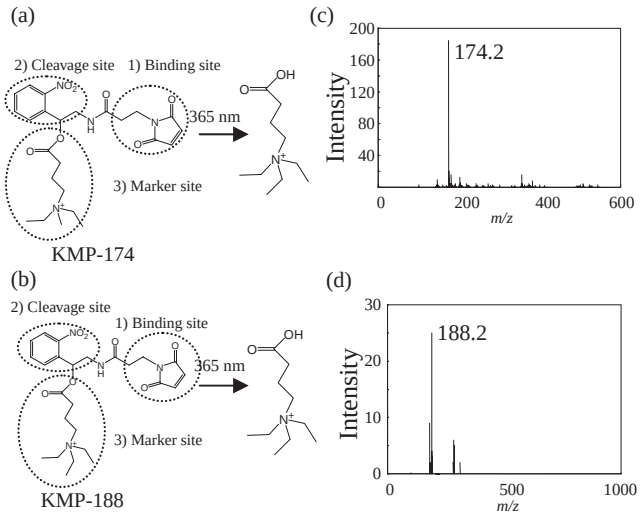

Figure 2. The molecular structure of the cleaved marker site of KMP-174 (a) and KMP-188 (b) and mass spectra of these marker sites (c: KMP-174, d: KMP-188). The exact masses of the expected marker sites of both probes are 174.15 and 188.16, respectively.

KMP-188 are indicated in Figures 2a and 2b, respectively. The mass probes are composed of (1) a binding site which binds to ligand molecules in a specific manner, (2) a cleavage site which can be cleaved by UV irradiation, and (3) a marker site which has various molecular weights (see Figure 2). Each probe synthesized in this study has a maleimide group which binds to a sulfide as the binding site. The maleimide group can be replaced

by other groups, for example, NHS or isothiocyanate groups, which bind to amine groups. These replacements can be easily achieved by the substitution of the appropriate compound in our established synthesis scheme. Nitrobenzyl groups, which are photoreactive,[16,17] and alkyl chains were introduced as the cleavage sites and marker sites, respectively. The attachment of the marker site was made the last step of the synthesis so that the mass probes with various molecular weights were easily produced. The marker site does not necessarily have to be composed of alkyl chains. The spectra of the molecules after cleavage by UV irradiation for 1 min are shown in Figures 2c and 2d. The marker sites of both molecules were detected as single main peaks at the exact molecular weights (174.2 and 188.2). After the probes were confirmed to be cleaved and detected in a quantitative manner with using 10 μM benzyltriethylammonium bromide as the internal standard and inally (data not shown), we measured HEL and OVA using anti-HEL IgG and anti-OVA IgG labeled with KMP-174 and KMP-188, respectively. HEL and OVA were dissolved at 0 to 120 μg/mL in buffers, respectively, and bound to SG (styrene glycidyl) particles by incubation at room temperature for 24 h. The SG particles are particles having styrene as the core with modification of the glycidyl groups on the surface. One milligram of anti-OVA-KMP188 or anti-HEL-KMP-174 was added to the mixture in microtubes and incubated overnight at 4 °C. After washing the particles, 2.0 mL of absolute methanol was added and the marker sites were cleaved by UV irradiation. After centrifugation, the supernatant was collected and benzyltriethylammonium bromide was added as the internal standard at 1.0 μM to each tube. The solutions were subjected to ESI TOF mass spectroscopy and the relative intensities of the marker sites derived from KMP-174 and MP-188 were measured. The results show that both OVA and HEL were detected with high correlation coefficients in the range of 1–100 μg (Figure 3). The difference of the slopes of two antigens was thought to be caused by the different binding property of respective antigen–antibody. The lack of the linearity of the graph under 10 μg may be due to the detection limit of the mass spectrometer used in the experiment. HEL and OVA are food allergens both derived from eggs.[18] Since the unknown intake of food allergens from commercially processed food causes serious allergies by anaphylactic shock, the indication of allergen contamination of processed foods is mandatory in Japan in case when the contamination level of allergens originating from certain foods is above 10 μg/g. The results showed that mass probes may be useful in measurement of those allergens. It is expected that the sensitivity can be much more enhanced by improvement of application and also by minimizing the volume used in the detection with mass spectrometry. If applying MALDI TOF MS or ESI TOF MS by a nanospray, the introduction of only 1 μL of sample is sufficient for each measurement, while we introduced 2.0 mL of dissolved injection sample to compensate for the dead volume in the injection loop.

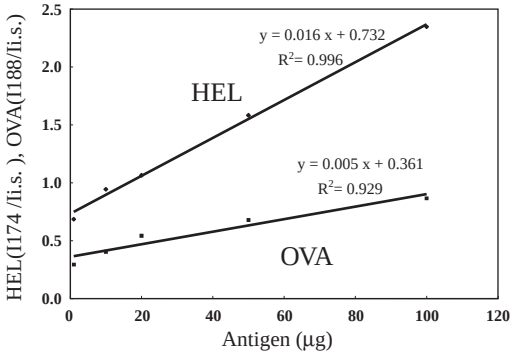

Figure 3. Standard curves of OVA and HEL measured by anti-OVA and anti-HEL antibodies labeled with KMP-174 and KMP-188.

As mentioned in the introduction, we developed adductive mass probes which give charges to small molecules to be effectively detected by mass spectrometry[15] and mass probes for ions which control the charge number and molecular size although they have not yet been published.[19] We call the method of adding a charge to various molecules via probes for detection by mass spectrometry, "MPAI (Mass Probe Aided Ionization) method." Our final goal is to combine our newly developed mass probes for proteins with other probes and realize the total analysis of biomolecules such as proteins, ions, and other small molecules together by the MPAI method.

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