Estimation of Flexible and Rigid Residues of Disulfide-Bridged and Phosphorylated Proteins Using Matrix-Assisted Laser Desorption/ Ionization in-Source Decay Mass Spectrometry

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Supporting Information

ABSTRACT: Flexible and rigid residues in disulfide-bridged and phosphorylated proteins have been estimated by using MALDI in-source decay mass spectrometry (ISD MS). The MALDI-ISD spectra of bovine α-lactoalbumin, β-lactoglobulin A, and β-casein predict that the backbone amide of Xxx-Asp/Asn/Cys/Ser/pSer and Gly-Xxx residues has higher hydrogen accessibility than other residues, while Xxx-Ile/Val residues have less accessibility. The higher hydrogen-accessible and lower accessible residues as measured by MALDI-ISD are consistent with the flexible and rigid residues determined by X-ray, nuclear magnetic resonance, and fluorescence decay methods. The disulfide bridges and phosphate groups do not prevent the estimation of flexible or rigid residues, whereas some other disulfide bridges inhibit the identification because of decreased sensitivity of ISD fragment ions. The estimation of flexible and rigid residues by means of the matrix-hydrogen accessibility can be explained by exposure or lack thereof to the hydrogen-accessible sites of intact proteins. It is proposed that MALDI-ISD is a powerful tool for identifying flexible and rigid residues of posttranslational modified proteins without the conformation information of the protein data bank.

INTRODUCTION

The study of flexibility of protein molecules is a long standing subject in protein science of great importance, owing to its influence on protein–protein, protein–nucleic acid, and protein–drug interactions. The flexibility concerns with the redox and posttranslational modifications, while such a property is represented by practically important concept of intrinsically disordered proteins. The flexibility nature of the motion of the backbone and sidechains and the interaction properties of proteins can be estimated using X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and fluorescence decay. A number of measures of flexibility are based on these methods. These include the B-factor as a measure of flexibility of amino acid residues, which is defined by the sidechain mobility on the basis of X-ray studies. Another measure, the turn preference factor is also based on X-ray studies and is useful for estimating flexibility of residues. The B-factor and the turn preference factor predict that Asp, Asn, Gly, Pro, Lys, Glu, Gln, and Ser residues and Asp, Asn, Gly, Pro, Cys, and Ser residues are more flexible than other residues, respectively. The protection factor based on the hydrogen/deuterium exchange reaction in NMR suggests that residues that interact most with environmental water molecules are Asp, Asn, Gly, Lys, Thr, Ile, and Met. Measurements using fluorescence decay predict that Asp, Asn, Gly, Ser, and Ala residues are more flexible than others. However, the identification of flexibility of residues in proteins can be time consuming and relies on structural characterization. The development of quick and straightforward techniques to achieve the identification of flexible and rigid residues is strongly desired by protein scientists.

We have reported that a reaction of in-source decay (ISD) coupled with matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is usable for predicting the turn preference or flexible residues of intact proteins. MALDI-ISD is a unique method for rapidly identifying intact posttranslational modified proteins without the need for enzymatic digestion. The ISD experiments result in specific cleavage at the N–Cα bond of the backbone of peptides and proteins when hydrogen-donating matrices such as 2,5-dihydroxybenzoic acid (2,5-DHB) and 5-amino-1-naphthol (5,1-ANL) are used. These matrices generate abundant hydrogen radicals from the anilinic amino group (Ph-NH₂) and the phenolic hydroxyl group (Ph-OH) under ultraviolet (UV) MALDI conditions. The MALDI-ISD experiments result in discontinuous intense peaks corresponding to c-ions originating from cleavage at the N–Cα bond of the Xxx-Asp, Xxx-Asn, Xxx-Cys, and Gly-Xxx residues, which are compatible with the identification of flexible residues by X-ray, NMR, and fluorescence decay methods.

The hydrogen radicals generated under MALDI conditions attach to the backbone carbonyl oxygens to form c-ions (Scheme 1C) and can reduce the disulfide bridge (S–S) to form sulfhydryl groups (–SH HS–) in protein molecules.

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while the presence of a disulfide bridge in a small cyclic peptide does not result in the formation of c-ions. It is of interest to ask whether the resulting hydrogen radicals are able to simultaneously cleave both the backbone N−Cα bond and disulfide bridge (S−S). It is also of interest to examine the influence of disulfide bridges and phosphate groups on the N−Cα bond cleavage in the formation of c-ions and to find discontinuous and lower intensity c-ion peaks. This would thereby identify both residues with higher matrix-hydrogen accessibility and residues with lower accessibility in intact proteins and measure the influence of disulfide bridges. It is expected, therefore, that effective and non-effective cleavage of
the backbone N–Cα bond and the disulfide bridge is a function of whether the backbone carbonyl oxygens and disulfide bridges are exposed or hidden from the matrix molecules.

Here, we estimate flexible and rigid residues of posttranslational modified proteins such as bovine α-lactoalbumin (α-LA, Mr 14220), bovine β-lactoglobulin A (β-LGA, Mr 18363), and bovine β-casein (β-CN, Mr 23983) using the MALDI-ISD. α-LA contains four disulfide bridges (Cys6-Cys120, Cys28-Cys111, Cys61-Cys77, and Cys73-Cys91), β-LGA contains one sulfhydryl (Cys121) and two disulfide bridges (Cys66-Cys160 and Cys106-Cys119), and β-CN has five phosphorylated serine residues (pSer15, pSer17, pSer18, pSer19, and pSer35).

**RESULTS AND DISCUSSION**

MALDI-ISD spectra of bovine α-LA, β-LGA, and β-CN obtained with the 5,1-ANL matrix are shown in Figures 1−3, respectively. All the spectra showed analyte ions such as [M + H]+ (or [M − H]−) and [M + 2H]2+ (or [M − 2H]2−). The resulting fragment c-ions observed in the ISD spectra originate from the cleavage at the backbone N–Cα bond (Scheme 1).

The series of c-ions observed give definite sequence information, because the difference in m/z values Δ (m/z) between adjacent c-ions is equal to the mass of amino acid residues.

The c-ions observed in the MALDI-ISD mass spectrum of α-LA can be divided into three regions in terms of peak intensity, namely high-intensity ions c10 to c27, medium-intensity ions c28 to c60, and low-intensity ions c61 and above (Figure 1). The peak intensity boundaries at c27/c28 and c60/c61 ions correspond to the presence of disulfide bridges Cys66-Cys160 and Cys106-Cys119, respectively. This indicates that the presence of disulfide bridges decreases the intensity of c-ions, because the S–S bridges inhibit the separation of c-ions from covalent S–S-bridged analyte ions [M + H]+. The c-ions in the MALDI-ISD mass spectrum of β-LGA can be divided into two regions, namely high-intensity ions c10 to c65 and low-intensity ions c66 and above (Figure 2). The drastic drop in the c-ion intensity boundary at c65/c66 suggests that simultaneous cleavage of both the disulfide bridge Cys66-Cys160 and the N–Cα bond is rare, while the N-terminal side
backbone chain from Leu1 to Glu56 can easily form c-ions because of the absence of disulfide bridges. The MALDI-ISD mass spectrum of β-CN was obtained in negative-ion mode owing to the electronegative nature of phosphate groups (Figure 3). The spectrum shows a lot of c-ions reflecting both information of the sequence and the sites of phosphorylated-serine residues (pS14, pS17, pS18, pS19, and pS35) (Figure S3).

Higher Matrix-Hydrogen Accessible Residues Giving Discontinuous Intense c-Ions.

The peak intensity of c-ions observed in the MALDI-ISD spectra obtained with hydrogen-donating matrix is mainly governed by two factors, namely, (1) ionization efficiency (protonation/deprotonation) concerned with the presence of basic and acidic residues and (2) hydrogen-accessibility of the backbone carbonyl oxygens.22,23 It should be noted that specific and prompt cleavage at the N–Cα bond of the peptide backbone to form c-fragments takes place independently of the ionization, while the c-ions are produced on several ns time scales in the MALDI ion source.18,19,22,23

The discontinuous intense c-ion peaks of β-LGA were observed with cleavage at the N–Cα bond of the Leu10-Asp11, Gly17-Thr18, Tyr20-Ser21, Ser27-Asp28, Ser36-Ala37, Val41-Tyr42, Gly52-Asp53, Glu62-Asn63, Glu65-Cys66, Leu87-Asn90, Leu95-Asp96, Thr97-Asp98 residues (Figure S3). The spectrum also showed two peaks corresponding to carboxyl (C)-terminal side w13 and w33 ions originating from cleavage at both the backbone N–Cα and sidechain Cβ–Cγ bonds of the Leu109-Cys110 and Met89-Cys90 residues (Scheme 1D), respectively. The observed intense c- and w-ions are shown in Scheme 2 and summarized in Table 1. The discontinuous intense c-ion peaks of β-casein were obtained with cleavage at the N–Cα bond of the Leu6-Asn7, Gly10-Glu11, Glu14-pSer15, Leu16-pSer17, pSer17-pSer18, pSer18-pSer19, Gln62-Asn63, Gly16-Asp47, Glu89-Asn90, Ile49-His50, Phe52-Ala53, Gln56-Ser59 residues (Figure S3).

Higher Matrix-Hydrogen Accessible Residues Giving Discontinuous Intense c-Ions.

The peak intensity of c-ions observed in the MALDI-ISD spectra obtained with hydrogen-donating matrix is mainly governed by two factors, namely, (1) ionization efficiency (protonation/deprotonation) concerned with the presence of basic and acidic residues and (2) hydrogen-accessibility of the backbone carbonyl oxygens.22,23 It should be noted that specific and prompt cleavage at the N–Cα bond of the peptide backbone to form c-fragments takes place independently of the ionization, while the c-ions are produced on several ns time scales in the MALDI ion source.18,19,22,23

The discontinuous intense c-ion peaks in the ISD spectrum of α-LA were observed with the cleavage at the N–Cα bond of the Arg10-Glu11, Lys13-Asp14, Gly17-Tyr18, Ser22-Leu23, Val27-Cys28, Tyr36-Asp37, Gln43-Asn44, Asn44-Asn45, Asn45-Asp46, Ile55-Asn56, Asn56-Asn57, Thr60-Cys61, and Gln65-Asn66 residues (Figure S1). The spectrum also showed two peaks corresponding to carboxyl (C)-terminal side w13 and w33 ions originating from cleavage at both the backbone N–Cα and sidechain Cβ–Cγ bonds of the Leu109-Cys110 and Met89-Cys90 residues (Scheme 1D), respectively. The observed intense c- and w-ions are shown in Scheme 2 and summarized in Table 1. The discontinuous intense c-ion peaks of β-LGA were observed with cleavage at the N–Cα bond of the Leu10-Asp11, Gly17-Thr18, Tyr20-Ser21, Ser27-Asp28, Ser36-Ala37, Val41-Tyr42, Gly52-Asp53, Glu62-Asn63, Glu65-Cys66, Leu87-Asn90, Leu95-Asp96, and Thr97-Asp98 residues (Figure S2). The w- and z-ions were also observed in the spectrum. The resulting fragment ions are shown in Scheme 3 and summarized in Table 1. With regard to the specific formation of w-ions (Scheme 1D), it is proposed that the Cys residue or the sulfhydryl group (–SH) has a high susceptibility to the hydrogen access and the loss of an SH radical. Negative-ion MALDI-ISD experiments with β-CN resulted in sequence reflecting c-ions without the loss of phosphate groups. Relatively intense c-ion peaks were observed resulting from the cleavage at the N–Cα bonds of the Leu6-Asn7, Gly10-Glu11, Glu14-pSer15, Leu16-pSer17, pSer17-pSer18, pSer18-pSer19, Gln62-Asn63, Gly16-Asp47, Glu89-Asn90, Ile49-His50, Phe52-Ala53, Gln56-Ser59 residues (Figure S3).

The results obtained indicate that phosphate groups of the pSer residues do not inhibit the access of hydrogens to backbone carbonyl oxygens in the formation of c-ions. The intense c-ion peaks of

Table 1. Residues with Increased Matrix-Hydrogen Accessibility in MALDI-ISD Spectra of α-Lactoalbumin, β-Lactoglobulin A and β-Casein

| protein       | more hydrogen-accessible residues                                                                 |
|---------------|--------------------------------------------------------------------------------------------------|
| α-lactalbumin | c-ion: Arg10-Glu11, Lys13-Asp14, Gly17-Tyr18, Ser22-Leu23, Val27-Cys28, Tyr36-Asp37, Gln43-Asn44, Asn44-Asn45, Asn45-Asp46, Ile55-Asn56, Asn56-Asn57, Thr60-Cys61, and Gln65-Asn66 |
|               | w-ion: Leu109-Cys110, Met89-Cys90                                                                 |
| β-lactoglobulin A | c-ion: Leu10-Asp11, Gly17-Thr18, Tyr20-Ser21, Ser27-Asp28, Ser36-Ala37, Val41-Tyr42, Gly52-Asp53, Glu62-Asn63, Asn63-Asp64, Glu65-Cys66, Leu87-Asn90, Leu95-Asp96, Thr97-Asp98 |
|               | z-ion: Phe131-Asn152, Phe136-Asp137, Asp129-Asp130, Val128-Asp129                                |
| β-casein      | c-ion: Leu6-Asn7, Gly10-Glu11, Glu14-pSer15, Leu16-pSer17, pSer17-pSer18, pSer18-pSer19, Glu21-Ser22, Lys28-Lys29, Glu34-pSer35, Glu42-Asp43, Gln46-Asp47, Glu89-Asn90, Ile49-His50, Phe52-Ala53, Gln56-Ser59 |
|               | y-ion: Phe175-Leu176                                                                              |

Scheme 3. Preferential Accessible Residues of Matrix-Hydrogen in Bovine β-Lactoglobulin A; (A) Preferential Matrix-Hydrogen Accessible Residues, Disulfide Bridges, and Sulfhydryl Groups, (B) Resulting c- and w-Ions and (C) z-Ions Observed in the MALDI-ISD Mass Spectrum of Bovine β-Lactoglobulin A Obtained with Hydrogen-Donating Reductive Matrix 5,1-ANL.
β-CN are shown in Scheme 4 and summarized in Table 1. In order to investigate the influence of phosphorylation of serine residues, furthermore, a model peptide of bovine β-casein having four phosphate groups (RELEELVPGEIVpSpSpSpS-pSEESITpSK) was used for estimating the flexible amino acids. The MALDI-ISD spectra showed relatively intense peaks corresponding to the c-ions originating from cleavage at the N−Cα bond of the Gly10-Glu, Leu16-pSer, Glu21-Ser, and Ile26-Asn residues, while cleavage at the Glu11-Ile, Ile12-Val, pSer19-Glu, Ser22-Ile, and Arg25-Ile residues gave lower intense c-ions (ISD data not shown). The detailed analysis of this model peptide is described in the later section (Figure S4).

From the results obtained above, the MALDI-ISD experiments of posttranslationally modified proteins predict that Xxx-Asp, Xxx-Asn, Gly-Xxx, Xxx-Ser, and Xxx-Cys are more hydrogen-accessible than other residues. Indeed, it is of interest that the matrix-hydrogen accessible residues identified by MALDI-ISD (Asp, Asn, Gly, Ser, and Cys) are consistent with the flexible residues predicted by B-factor (Asp, Asn, Gly, Pro, Lys, Glu, Gln, and Ser),8,9 the turn preference factor (Asp, Asn, Gly, Pro, Cys, and Ser),6,7 the protection factor (Asp, Asn, Gly, Lys, Thr, Ile, and Met),8,9 and the fluorescence decay factor (Asp, Asn, Gly, Ser, and Ala),10 although identification of some residues depends on the method used.

In order to estimate quantitatively the more and less accessibility of hydrogens to the carbonyl oxygens of amino acid residues of the analyte proteins α-LA, β-LGA, and β-CN, the intensity ratio R(Cn) for n-th c-ion can be defined by the ratio of the intensity of n-th c-ion Int(Cn) to the average intensity of adjacent side c-ion peaks as follows.

\[
R(C_n) = \frac{\text{Int}(C_n)}{\frac{\text{Int}(C_{n-1}) + \text{Int}(C_{n+1})}{2}}
\]

The intensity ratios were calculated from the intensity of c-ions observed in the enlarged MALDI-ISD mass spectra of α-LA, β-LGA, and β-CN (Figures S1–S3), and the cleavage residues and corresponding intensity ratios are summarized in Table S1. The intensity ratio for each amino acid residue averaged with all combinations in the three different proteins indicates that Xxx-Cys/Asp/Asn/Ser and Gly-Xxx residues give the values over 1.0 and Xxx-Val/Ile residues give lower than 1.0, while Xxx-Glu/Ala residues give approximately 1.0, as shown in Figure 4 and Table 2. The intensity ratios estimated from positive- and negative-ion MALD-ISD spectra of the model peptide of β-casein also gave the same tendency with the values for proteins used here (Figure S4). The statistical t-test was performed for Xxx-Asp/Asn/Cys/Ser(pSer)/Ala/Glu, Gly, Ala, Val, and Ile residues estimated from the ISD data of α-LA, β-LGA, and β-CN, as shown in Figures S1–S3.

| amino acid residue | average | p-value | t-value |
|--------------------|---------|---------|---------|
| total average      | 1.06    |         |         |
| Xxx-Asp            | 1.79    | 0.00067 | 3.473   |
| Xxx-Asn            | 1.51    | 0.045   | 2.021   |
| Xxx-Cys            | 5.33    | 0.104   | 1.637   |
| Xxx-Ser/pSer       | 1.25    | 0.105   | 1.626   |
| Gly-Xxx            | 1.37    | 0.211   | 1.256   |
| Xxx-Ala            | 1.04    | 0.893   | 0.135   |
| Xxx-Glu            | 0.93    | 0.311   | 1.016   |
| Xxx-Val            | 0.56    | 0.000061| 4.684   |
| Xxx-Ile            | 0.53    | 0.000189| 4.413   |

Figure 4. Intensity ratio R(Cn) for Cys, Asp, Asn, Ser, Gly, Glu, Ala, Val, and Ile residues estimated from the ISD data of α-LA, β-LGA, and β-CN, as shown in Figures S1–S3.

Table 2. Average Intensity Ratio, Probability (p), and t-Value Estimated by the Statistical t-Test for Asp, Asn, Cys, Ser/pSer, Gly, Ala, Glu, Val, and Ile Residues from the Intensity Ratios of c-Ions Observed in MALDI-ISD Mass Spectra of α-LA, β-LGA, and β-CN
Gly-Xxx, and Xxx-Val/Ile residues, while the data for Xxx-Cys, Lys-Lys, Xxx-Pro, Arg-Xxx, and Phe52-Ala were deleted from a population showed in Table S1 to avoid unnatural deviation. The average intensity ratio, probability (p), and t-value for each residue are summarized in Table 2.

**Lower Matrix-Hydrogen Accessible Residues Giving Discontinuous Lower Intensity c-Ions.** In contrast to the more hydrogen accessible residues described above, the MALDI-ISD spectra also showed discontinuous lower intensity c-ions. The lower intensity c-ions, as observed in Figure 1, for α-LA were c20, c26, c28, c40, c41, c54, c58, and c59 originating from cleavage at the N-Cα bond of the Gly-Val, Trp-Val, Cys-Thr, Ala-Ile, Ile-Val, Gln-Ile, Lys-Ile, and Ile-Trp residues, respectively. The lower intensity c-ions in β-LGA (Figure 2) were c11, c14, c28, c42, and c55 originating from the N-Cα bond cleavage of the Asp-Ile, Lys-Val, Asp-Ile, Tyr-Ile, and Glu-Ile residues, respectively. The ISD spectrum of β-CN also showed lower intensity peaks corresponding to c7, c11, c12, c19, c22, c29, c45, c48, and c51 originating from cleavage of the Asn-Val, Glu-Ile, Ile-Val, pSer-Glu, Ser-Ile, Lys-Ile, Leu-Gln, Lys-Ile, and Pro-Phe residues, respectively. The results obtained above indicate that Xxx-Val and Xxx-Ile are the residues found with high frequency in the lower intensity ions. Thus, the backbone carbonyl oxygens of these residues have decreased hydrogen accessibility. Other residues such as Xxx-Thr/Trp/Phe also seem to show less access to matrix. The identification of Val and Ile residues is in accordance with the rigid residues identified by means of the B-factor, the turn preference factor, the fluorescence decay, and the inhibition of chemical ligation due to steric hindrance.

**Mechanistic Implications of Matrix-Hydrogen Accessibility of Residues.** The matrix-hydrogen accessibility of backbone amides and disulfide bridges is an important factor in the preferential formation of c-ions and sulfhydryl groups. The presence of intramolecular hydrogen-bonded α-helix and β-sheet structures and bulky sidechains inhibits the access of hydrogens to the backbone carbonyl oxygens. In contrast, it is important to recognize that the residues and disulfide bridges exposed at the surface of peptide and protein molecules have increased the likelihood of accepting hydrogen radicals and thus to take part in intermolecular hydrogen bonding (Scheme 1A) and form transient hydrogen-excess radical peptides and proteins (Scheme 1B). The measurement of matrix-hydrogen accessibility of residues by MALDI-ISD indicates that backbone amides of Xxx-Asp/Asn/Cys/Ser and Gly-Xxx residues exposed at the surface of intact proteins are able to interact with matrix active-hydrogens, while those of Xxx-Ile/Val residues are hidden from the active-hydrogens. Such mechanistic implications of the Asp/Asn/Cys/Ser/Gly and Ile/Val residues are compatible with the identification of the flexible and rigid residues obtained from the B-factor, the turn preference factor, the protection factor, and the fluorescence decay factor. Furthermore, the disulfide bridges at Cys6-Cys120 and Cys28-Cys110 of α-LA easily interact with matrix active-hydrogens to form sulfhydryl groups (Scheme 2), while Cys60-Cys78 and Cys73-Cys90 of α-LA and Cys66-Cys160 and Cys106-Cys119 of β-LGA are relatively hidden from the active-hydrogens (Schemes 2 and 3). It is also of interest from the standpoint of protein conformation that the backbone amide carbonyl oxygens of the pSer residues of β-CN preferentially interact with the matrix hydrogens to form intense c-ions, given that there is no conformation information in the protein data bank for β-CN. This indicates that phosphate groups of β-CN do not inhibit the access of matrix hydrogens.

**CONCLUSIONS**

The flexible and rigid residues of posttranslational modified proteins bovine α-lactoalbumin (α-LA), β-lactoglobulin A (β-LGA), and β-casein (β-CN) were estimated by using MALDI-ISD. Discontinuous intense or lower intensity fragment c-ions observed in MALDI-ISD spectra of α-LA, β-LGA, and β-CN predict that backbone amides of the Xxx-Asp/Asn/Cys/Ser and Gly-Xxx residues have higher accessibility to matrix-hydrogens than other residues, while those of the Xxx-Ile/Val residues have lower accessibility. Indeed, the higher matrix-hydrogen accessible Asp, Asn, Cys, Ser, and Gly residues and lower accessible Ile and Val residues estimated by MALDI-ISD are consistent with the flexible and rigid residues, respectively, determined by X-ray, NMR, and fluorescence decay methods.

The disulfide bridges at Cys66-Cys120 and Cys28-Cys110 of α-LA are easily reduced by matrix-hydrogens and do not inhibit the formation of c-ions, while the disulfide bridges at Cys60-Cys78 and Cys73-Cys90 of α-LA and at Cys66-Cys160 and Cys106-Cys119 of β-LGA inhibit the formation of c-ions. This indicates that these disulfide bridges are hidden from the matrix-hydrogens. The phosphate groups of β-CN do not inhibit cleavage at the N-Cα bond, and the c-ions originating from cleavage at the N-Cα bond of the Xxx-Ser/pSer residues show more intense peaks than other residues, suggesting that backbone carbonyl oxygens of the Xxx-Ser/pSer residues of β-CN are exposed to the matrix-hydrogens. Thus, the MALDI-ISD is a powerful tool for predicting flexible and rigid residues of posttranslational modified proteins, owing to the fast and easy capabilities and small sample amount requirements compared to conventional X-ray, NMR, and fluorescence decay methods.

**EXPERIMENTAL DETAILS**

**Chemicals.** MALDI matrix 5-amino-1-naphthol (5,1-ANL) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile was purchased from Wako Pure Chemicals (Osaka, Japan). Water used in all experiments was purified using a MilliQ water purification system from Millipore (Billerica, MA, USA). Bovine α-lactoalbumin, bovine β-lactoglobulin A, and bovine β-casein were purchased from Sigma-Aldrich (Saint Louis, MO, USA). A model peptide of bovine β-casein was supplied from the Peptide Institute (Mino, Osaka, Japan). All reagents were used without further purification.

**Sample Preparation.** Each protein for the ISD experiment combined with MALDI-TOF MS was dissolved in 200 μL of water at a concentration of 100 μM in a 600 μL microtube. The matrix 5,1-ANL (3 mg) was dissolved in a solvent of 150 μL of water/acetonitrile (3:7, v/v). The matrix and analyte solutions were prepared without any additives such as trifluoroacetic acid. A sample solution was prepared by mixing a 10 μL volume of analyte solution with a 10 μL volume of matrix solution by using a shaker just before MALDI-ISD experiments. A 1.0 μL volume of the sample solution was deposited onto a stainless-steel target for the MALDI plate made of stemless steel with a 10 μL micropipette, and the solvents were removed by allowing evaporation in air at room temperature.
Mass Spectrometry. MALDI-ISD spectra were acquired on a time-of-flight mass spectrometer AXIMA-CFR (Shimadzu, Kyoto, Japan) equipped with a nitrogen laser (337 nm wavelength) operating at a pulse rate of 10 Hz. The pulse width of the laser was 4 ns. The laser spot size on the target substrate was ca. 100 μm in diameter. The ions generated by MALDI were accelerated using 20 kV with delayed extraction. The analyzer was operated in a linear mode, and the ions were detected using a secondary electron multiplier. A total of 500 shots were accumulated for each mass spectrum acquisition. The reproducibility of all mass spectra was confirmed by the peak intensity patterns for several runs using the raster function installed on the AXIMA-CFR mass spectrometer. The reproducibility of MALDI-ISD patterns of the analyte proteins with 5,1-ANL matrix was confirmed with different experimentalists and on different days.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02814.

Enlarged MALDI-ISD mass spectra of bovine α-lactoalbinmin, β-lactoglobulin A, and β-casein used for the statistical t-test of the intensity ratios of c-ions, the intensity ratios of c-ions observed in positive- and negative-ion MALDI-ISD mass spectra of a bovine β-casein model peptide, and the list of all residues and intensity ratios of c-ions observed in the enlarged MALDI-ISD mass spectra (PDF)

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The author declares no competing financial interest.

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