Suppression of Matrix-related Ions Using Cyclodextrin in MALDI Mass Spectrometry

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An effective technique for the suppression of matrix-related ions in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry have been developed. Using typical organic matrices such as THAP (2,4,6-trihydroxyacetophenone) and CHCA (α -cyano-4-hydroxycinnamic acid) in a cyclodextrin cavity, we successfully measured the mass peaks of only protonated matrix ions and significantly suppressed their intensities and fragmentation. In addition, it became possible to analyze the mass peak of the analyte molecules (substance P and adenosine) without any interference from the matrix. We believe that this technique could be a powerful tool for MALDI mass spectrometry, particularly for low-molecular-weight compounds.

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Introduction

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has been applied in recent studies of macromolecules, including DNA, proteins and peptides.^{1,2} As one of the soft ionization methods, MALDI is suitable for studying macromolecules because it most often permits the observation of protonated molecules $[M+H]^+$ with little or no fragmentation during desorption/ionization. This feature enables us to predict the molecular structure of the protonated molecules $[M+H]^+$ only from the mass number observed in the spectrum. Furthermore, MALDI offers other features, such as tolerance for contamination, simple spectra since the observed ions are mostly singly charged, and simple instrumentation, to name a few. Although MALDI is considered to be an attractive method for the analysis of macromolecules, it has a drawback: the fragmentation of matrices. When the matrix-analyte system is photoexcited, matrix molecules firstly absorb photons and then transfer some of their internal energy to analyte molecules.^{3,4} However, matrix molecules that do not participate in the excitation energy transfer process often fragment. They produce many matrix-related fragment peaks in area less than approximately 500 Da because the molecular weights of the matrices are mostly less than 500 Da.

MALDI measurements of low-molecular-weight analytes have faced many challenges.⁵⁻⁸ For example, a fine powder of metal or metal oxide was used as an alternative to organic matrix.⁹ Porous silicon¹⁰ and nano-structured plate¹¹ were used as sample support. Niito *et al.* revealed that irradiation of infrared photons with a free electron laser prevented the fragmentation of matrix molecules.¹² There were many attempts to improve the ion yields of protonated molecules $[M+H]^+$ by using sugars,¹³ ammonium salts,^{14,15} proteins¹⁶ with conventional MALDI matrices such as CHCA (α -cyano-4-hydroxycinnamic acid) or

2,5-DHB (2,5-dihydroxybenzoic acid). Phosphoric acid¹⁷ and serine¹⁸ were also used as co-matrix in order to increase the ion yields as well as for the reduction of alkali metal ion adducts. Recently, Zhang *et al.* used β -cyclodextrin (β CD) to separate the mass number of analyte from matrix-related ions region.¹⁹ They put an analyte molecule (RDX; hexahydro 1,3,5-trinitro-1,3,5-triazine) into β CD, and then they carried out MALDI mass measurements of β CD + RDX species in the presence of matrix molecule (SA; sinapinic acid). They clearly separated the mass number of analyte molecule from matrix-related fragment region. However, even with their method, matrix molecules still undergo the fragmentation because this is mere laser desorption/ionization for the matrix molecules, although Zhang *et al.* did not show the region less than 500 Da. In this report, we used the cyclodextrin as host molecules for the matrix. We report that cyclodextrin prohibit fragmentation of a guest molecule. Cyclodextrin-supported matrix enabled us to measure the mass peaks of only protonated matrix ions and to significantly suppress their intensities and fragmentation. In addition, by using this "cyclodextrin-supported matrix" as the new matrix for MALDI mass measurements, it became possible to analyze the mass peak of the analyte molecule without any interference from the matrix.

Experimental

Typical crystalline organic matrices, THAP (2,4,6-trihydroxyacetophenone, 168 Da) and CHCA (α -cyano-4-hydroxycinnamic acid, 189 Da), were used as the matrix for laser desorption/ionization (LDI) and MALDI mass measurements. Matrices (Wako Chemical) were recrystallized from methanol three times. α -Cyclodextrin (α CD, Sigma) (Fig. 1(a)) was dissolved in distilled water to a concentration of 10 mM. THAP (CHCA) was dissolved in a mixture of acetonitrile and water (7:3 in volume) containing trifluoroacetic acid (TFA, 0.1% in volume), and a solution of ~75 mM was obtained. The 120 μ L of α CD and 20 μ L of THAP (CHCA) solutions were pipetted into a vial and the mixture was sonicated

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Fig. 1 Chemical structure of (a) α -cyclodextrin (α CD), (b) substance P (SubP), (c) adenosine, and (d) *N*-acetyl-chitobiose (biase).

with increasing temperature to form a complex between the cyclodextrin and the matrix molecule at a molar ratio of 1:1 (α CD:THAP (CHCA)). The interaction between α CD and the matrix was confirmed by UV-vis absorption spectroscopy. For THAP difference around 320 nm was observed in the absorption spectra by the presence of α CD, whereas the blue shift of absorption maximum and a new peak around 280 nm was observed for CHCA. Observed UV-vis absorption spectra with changing the concentrations of THAP or CHCA revealed that the 1:1 complex was formed between α CD and MALDI matrices, and the equilibrium constants were evaluated to be $\sim 1380 \text{ M}^{-1}$ for THAP and $\sim 930 \text{ M}^{-1}$ for CHCA. Although UV-vis absorption spectra do not give solid information about the molecular structure of the complex between α CD and THAP (CHCA), we define these complexes as the “cyclodextrin-supported matrix” hereafter. The analytes substance P (SubP, Fig. 1(b)), adenosine (Fig. 1(c)), and *N*-acetyl-chitobiose (biase, Fig. 1(d)) were dissolved in a mixture of acetonitrile and water (7:3 in volume) containing TFA (0.1% in volume), and solutions of ~ 75 , 24, and 37 mM were obtained, respectively. Then, 0.5 μL each of cyclodextrin-supported matrix and analyte solution was pipetted onto a stainless-steel plate, left in air for a few minutes to evaporate the solvent, and then analyzed by MALDI mass spectrometry.

MALDI mass measurements were performed with a laboratory-made mass spectrometer. Briefly, a mixture of cyclodextrin-supported matrix and analyte on a stainless-steel plate was put inside a vacuum chamber set at $5 \times 10^{-5} \text{ Pa}$. Although THAP and CHCA are commonly used with 337 nm N_2 laser, the fourth harmonics generation of fundamental laser output from an Nd:YAG laser (266 nm, 10 Hz, 5 ns) was used as excitation light in this work because of the limitation of our apparatus. Typical pulse energy of excitation light for MALDI mass measurements was 10 – 15 μJ . Molecular and other fragment ions were accelerated with an electric field of 2.3 – 4.0 kV, and spectra were acquired with a linear TOF tube and an MCP detector.

Results and Discussion

Figure 2(a) shows the result of laser desorption/ionization (LDI) mass measurement of α -cyclodextrin (α CD; 973 Da). Since α CD has almost no absorption coefficient in the UV-vis region, no mass peak was observed on photoexcitation at 266 nm. Therefore, it is clear that α CD does not interfere with the mass spectrum under this experimental condition. Figure 2(b) shows

Fig. 2 (a) Laser desorption/ionization (LDI) mass measurement of α CD. LDI mass spectra of (b) THAP alone and (c) THAP with α CD. In (c), the mass peak of only the protonated species of the protonated molecules $[\text{M} + \text{H}]^+$ was observed.

the LDI mass spectrum of a typical MALDI matrix, THAP (2,4,6-trihydroxyacetophenone; 168 Da). In this mass region, we could identify many THAP-related peaks, including fragmentation and alkali metal ion adducts of THAP monomer and dimer. Fragmentation of the THAP molecule indicates that soft ionization cannot be realized only by LDI. However, the THAP with α CD significantly suppressed decomposition of the THAP molecule. In the LDI mass spectrum of α CD(THAP) (Fig. 2(c)), we could only recognize a very tiny fragment peak (OH loss, 153 Da) of the protonated molecules $[\text{M} + \text{H}]^+$. It is known that CD stabilizes the chemical properties of guest molecule because of reaction selectivity due to its structure. Therefore, it is possible to consider that the decomposition of THAP was significantly suppressed by the presence of the CD. Furthermore, only the protonated molecules $[\text{M} + \text{H}]^+$ was observed, and mass peaks due to alkali metal ion adducts disappeared. It is surmised that only one THAP can be interacted with one α CD, effectively mono-dispersing THAP molecules, and α CD also prevented adduct formation with alkali metal ions. However, one may consider that adding suitable agents may reduce the thermal load on the THAP molecules under the same pulse energy, and thus the reduced fragmentation was observed. If so, peak intensity would decrease generally keeping the ratio of protonated and alkali metal ion adducts same. In the obtained spectra, the peaks from alkali metal ion adducts as well as the dimer were decreased, indicating the advantage of the CD. We also noted that reduced fragmentation could be also observed up to $\sim 50 \mu\text{J}$, although the fragmentation of THAP was observed in the higher energy region.

The results revealed that α CD can facilitate the soft ionization of a guest molecule and simplify the mass spectrum. Therefore, using the cyclodextrin-supported THAP for MALDI mass

measurements, it can be possible to analyze the mass peaks of an analyte without any interference from the matrix. Figure 3 shows the results of MALDI mass measurements of a model peptide (substance P). It should be noted that THAP is known to be a suitable matrix for oligonucleotides, however we used a peptide as a first example. It should be also mentioned that when 266 nm is used aromatic groups peptide absorb the excitation light, resulting somehow strong ion peaks of peptides.²⁰ In Fig. 3(a), the result obtained from conventional MALDI mass measurement (without α CD) is depicted for comparison. Using THAP as the matrix, the mass peak of the protonated molecules $[M+H]^+$ of substance P (SubP) was observed. In the inset showing the region from 1300 to 1450 Da, mass peaks of not only the protonated ion but also alkali metal ion adducts were observed. Furthermore, we also found many peaks in the mass region less than approximately 500 Da. Many matrix-related peaks as well as fragments appear in this mass region, making it impossible to identify mass peaks related to the analyte if the analyte gives fragments in this region. To overcome this drawback, the cyclodextrin-supported matrix was used for MALDI mass measurements. Figure 3(b) shows the MALDI mass spectrum of SubP using the α CD(THAP). It is clear that the number of mass peaks was significantly reduced in the mass region of the matrix and the protonated molecules $[M+H]^+$. As we can see in the inset, only the protonated ion of SubP was observed. Therefore, it was revealed that the cyclodextrin-supported matrix can simplify the mass spectrum of not only the matrix but also the analyte. Here, we used ~75 mM solution of SubP and the analyte/matrix mixing ratio of ~1:1. Therefore, one can consider that the matrix suppression effect which is common in the conventional MALDI measurement was observed in Fig. 3 rather than the effect by the α CD. We recognize that such a matrix suppression effect might be also observed in the spectra. In such a case, however, all of the matrix-related peaks should decrease equally. In the obtained spectra, the protonated matrix was clearly observed despite the peaks from the alkali metal ion adducts and dimer were completely disappeared. We consider that the observed spectra are not only due to the matrix suppression effect arising from the analyte/matrix mixing ratio but mostly due to the efficiency of the cyclodextrin-supported matrix. It was also confirmed that these effects in MALDI mass spectra could not be achieved by adding α -glucose. Figure 3(c) shows the MALDI mass spectrum of SubP using co-matrix of THAP and glucose with a molar ratio of 1:6 since α CD is composed of 6 glucoses. It was observed from Fig. 3(c) that THAP molecules fragmented as in the case of conventional MALDI although peaks from alkali metal ion adducts fairly disappeared. Therefore, it can be concluded that host-guest interaction between α CD and MALDI matrix is the key factor and α CD efficiently suppress the fragmentation of matrix molecules. This method is also available to other matrix molecules. We also carried out the MALDI mass measurements for SubP with using α CD-supported CHCA matrix (α CD(CHCA)). In Fig. 3(d), we could see only the protonated ion of SubP and no other matrix-related peaks. We consider that not only α CD but also the reaction between matrix and the analyte²¹ led to efficient suppression of CHCA-derived ions.

The above results indicate that cyclodextrin-supported matrix could be a powerful tool for MALDI mass spectrometry, particularly for low-molecular-weight compounds. In the conventional MALDI, matrix molecules may fragment into give many matrix-related fragment peaks in area less than approximately 500 Da. Therefore, it is difficult to analyze the low-molecular-weight compound and its fragments. For this

Fig. 3 (a) MALDI mass spectrum of SubP/THAP. (b) MALDI mass spectrum of SubP/ α CD(THAP). Only the protonated molecules $[M+H]^+$ and matrix molecules were observed. (c) MALDI mass spectrum of SubP using THAP and α -glucose co-matrix (1:6). (d) MALDI mass spectrum of SubP/ α CD(CHCA). Matrix-related peaks completely disappeared.

purpose, we also carried out MALDI mass measurements on one of the nucleosides, adenosine (267 Da) using cyclodextrin-supported matrix (α CD(THAP)). We chose adenosine as a demonstration of this method to the low-molecular-weight compound. Figure 4(a) shows the MALDI mass spectrum in the area less than 500 Da. Equal to the above results in Figs. 2 and 3, THAP-related peaks including fragmentation and alkali metal ion adducts of THAP monomer and dimer disappeared. As for the adenosine, the peaks from alkali metal ion adducts were also disappeared. Furthermore, even the fragment peak (adenine, 136 Da) of adenosine molecule could be recognized without any interference from the matrix. This result clearly revealed that the advantage of cyclodextrin-supported matrix and this method can be a powerful tool especially for low-molecular-weight compounds. Finally, we also carried out MALDI mass measurements for the other low-molecular-weight compound, biose (*N*-acetyl-chitobiose; 424 Da). In the conventional MALDI, biose gave Na^+ adduct peak preferentially. However in this method, we could not observe any biose-related peaks as shown in Fig. 4(b). Cyclodextrin-supported matrix gives only

Fig. 4 (a) MALDI mass spectrum of adenosine/ α CD(THAP). The fragment peak (adenine, 136 Da) of adenosine molecule was observed without any interference from the matrix. (b) MALDI mass spectrum of biose/ α CD(THAP).

protonated peak of the protonated molecules $[M+H]^+$. Therefore, it is considered that this method is not suitable for the compounds that give alkali metal ion adducts preferentially. It may be possible to understand that α CD also works as the sodium and potassium sponge to prevent alkali metal ion adduct formation. However, we consider that this argument should be carefully confirmed by further experiments.

Conclusion

In summary, we have presented a novel technique for MALDI mass spectrometry. By using the cyclodextrin-supported matrix as a new MALDI matrix, we could suppress mass peaks of matrix-related ions as well as alkali metal ion adducts. We believe that this technique could be a powerful tool for MALDI mass spectrometry, particularly for low-molecular-weight compounds.

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