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Selective improvement of peptides imaging on tissue by supercritical fluid wash of lipids for matrix-assisted laser desorption/ionization mass spectrometry

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Short title: Improvement of peptide imaging by $scCO_2$ wash

ABSTRACT

There is a high analytical demand for improving the detection sensitivity for various peptides in matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS), because exhaustive distribution analyses of various peptides could help to reveal the function of peptides in vivo. To improve the sensitivity of peptide detection, we used supercritical fluid of CO_2 ($scCO_2$) as washing solvent for a pretreatment to remove lipids. We evaluated whether our wash method using $scCO_2$ with an entrainer improved the detection of peptides and suppressed lipid detection in MALDI-IMS. Our analysis revealed that the signal intensities of peptides such as *m/z* 3339.8, 3530.9, 4233.3, 4936.7, and 4963.7 were increased in $scCO_2$-washed samples. The greatest improvement in the signal-to-noise ratio (S/N) was found at *m/z* 4963.7, which was identified as thymosin β4, with the S/N reaching almost 190-fold higher than the control. Additionally, all of the improved signals were associated with the morphological structure. Our method allows us to analyze the distribution of molecules, especially in the region of *m/z* 3000-5200. For these improvements, the polarity difference between $scCO_2$ and the matrix solution used was considered as a key. A wider variety of molecules can be analyzed in the future due to this improvement of the detection sensitivity by optimizing the polarity of $scCO_2$ with various entrainers.

Key words: imaging mass spectrometry, supercritical fluid of CO_2, peptides, pretreatment, matrix-assisted laser desorption/ionization

Introduction

Distribution analyses of peptides are performed using positron emission tomography (PET) by isotope-labeled peptides [1], immunohistochemistry [2], imaging mass spectrometry (IMS) [3], and other techniques. Each of these methods has distinct advantages and disadvantages. The advantage of PET is that it allows non-invasive distribution analyses with pharmacokinetics of an injected peptide [4], and the advantage of immunohistochemistry is that it provides detailed information at sub-cellular spatial resolution [5]. The disadvantage of both PET and immunohistochemistry is the limitation in the number of peptide species that are simultaneously detected. To evaluate the relationships and the functions of peptides associated with morphological structure in the body, comprehensive distribution analyses are needed [3]. IMS is a powerful tool that satisfies the requirement of simultaneous analysis of various molecules without labeling [3]. Matrix-assisted laser desorption/ionization (MALDI)-IMS in particular provides direct information about the localized peptides since MALDI-IMS analyses enables the simultaneous detection of various intact molecules without fragmentation [6]. However, the peptide analysis that MALDI-IMS provides is not satisfactory, due to the low signal intensities of peptides. Improving the sensitivity of peptide detection by MALDI-IMS is thus a major analytical demand.

Several research groups have attempted to improve the detection of peptides by reducing the ion suppression caused by lipids and salts, which are abundant in tissue [7]. For reducing the level of lipids in the tissue, many types of organic solvent [8, 9] have been used in MALDI-IMS analyses. On the other hand, supercritical fluids have been used for the extraction or separation of various lipids in chromatographic analyses [10].

A supercritical fluid can easily infiltrate deeply into tissue and is expected to clear specific molecules completely, since supercritical fluids have low viscosity (e.g. under 8×10^{-5} Pa•s for CO_2) [11] and high diffusivity (e.g. under 10^{-4} cm^2/s for CO_2) [12]. In addition, a supercritical fluid can be coupled with other types of organic solvent (called "entrainer") to effectively remove other molecules such as phospholipids [12]. Among the various types of supercritical fluid, the supercritical fluid of CO_2 ($scCO_2$) is widely used, since it can be used under relatively mild conditions (over 7.38 MPa and 31.1°C). In addition, CO_2 is inexpensive and nontoxic [13]. We hypothesized that the use of $scCO_2$ for removing lipids could maintain the spatial distributions of peptides and enhance their signals.

In this study, we used $scCO_2$ as a wash solvent in a pretreatment for a MALDI-IMS analysis, and we evaluated whether our method could improve the detection of peptide molecules. We used methanol (MeOH) as the entrainer to remove phospholipids more effectively [12].

Materials and Methods

Chemicals

We purchased 1,5-diaminonaphthalene (DAN) from Tokyo Chemical Industry Co. (Tokyo) and obtained 2,5-dihydroxybenzoic acid (DHB), peptide calibration standard, and protein calibration standard I from Bruker Daltonics (Billerica, MA, USA). Cesium iodide (CsI), bradykinin fragment 1–7, and angiotensin II were purchased from Sigma-Aldrich (St. Louis, MO). Liquid chromatography/mass spectrometry-grade ultrapure water, MeOH for the spray in the matrix application, and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemical Industries (Osaka, Japan).

High-performance liquid chromatography (HPLC)-grade MeOH for the $scCO_2$ pretreatment was purchased from Kanto Chemicals (Tokyo). Thymosin β4 was purchased from Tocris Bioscience (Bristol, UK).

Animals

All experiments were conducted according to protocols approved by Animal Care and Use Committee of Hamamatsu University School of Medicine. C57BL/6JJcl Eight-weeks-old female mice were purchased from Japan SLC (Hamamatsu, Japan). Mouse brains were rapidly frozen in powdered dry ice after harvest. The tissue was mounted on a sample holder using O.C.T. compound (Sakura Finetek Japan, Tokyo), and sectioned at 10-μm thickness along the sagittal axis by a cryostat system (CM1950; Leica Biosystems, Wetzlar, Germany) with the temperature of the working area maintained at −16°C. Sections were thaw-mounted on electroconductive glass slides coated with indium-tin-oxide (ITO) (100Ω; Matsunami Glass, Osaka, Japan). Samples were stored at −80°C until use.

Pretreatment

The entire system for $scCO_2$ washing is shown in Supplementary Figure S1. $scCO_2$ fluid was pumped by a PU-1586 Intelligent HPLC Pump (JASCO, Tokyo). The sample glass slides kept in the extractor unit (Nikkaki Bios, Tokyo) were soaked in the $scCO_2$ fluid (flow rate, 10 mL/min) for 1 hr at 40°C under 20 MPa, with MeOH (flow rate, 5 mL/min with SCF-Get pump; JASCO) used as an entrainer. In a previous report, 94.70±8.45% of phospholipids was extracted within 1 hr in a one-dimensional chromatographic extraction with 20% ethanol [14]. We therefore set the extraction time

in the present study to 1 hr, which was sufficient for removal of the phospholipids. The extract in MeOH was collected in glass vials and dried under a vacuum. The dried extract was stored at 4°C.

MALDI-IMS for m/z 1800-6000

One mL of matrix solution (DAN; 10 mg/mL in 70% MeOH, 0.1% TFA) was sprayed manually for each section on the glass slide, using an air-brush with a 0.2-mm nozzle (Procon Boy FWA platinum; Mr. Hobby, Tokyo). Tissue samples were analyzed using a MALDI-Fourier transformation ion cyclotron resonance (FT-ICR)-type IMS instrument, the solariX XR (Bruker Daltonics) with a 355-nm Nd:YAG laser at a 2000 Hz repetition rate. The laser power was set at 40%, and 500 laser shots were irradiated for each measured point. The raster scan pitch was set at 100 μm, and a medium laser diameter (approx. 70 μm) was used.

Measured regions were manually set and data were acquired at *m/z* 1800-6000 in a positive ion mode. External calibration was performed using CsI clusters, the peptide calibration standard, and protein calibration standard I. The following conditions were used for the analysis: plate offset (100 V), deflector plate (200 V), time of flight delay (3 ms), transient length (2.2 s), and resolving power (23615 at *m/z* 4961.6). Averaged mass spectra and ion images were reconstructed using flexImaging 4.0 data analysis software (Bruker Daltonics). The signal-to-noise ratio (S/N) was calculated with the data exported by the flexImaging 4.0 program.

Results

During the experiments, neither slippage nor deformation of the tissue sections on the glass slide was observed. Figure 1 shows the spectra at *m/z* 1800-6000 obtained from mouse brain tissue sections washed by $scCO_2$ with the entrainer (upper, blue) and the control without the wash (lower, red). Some of the signal intensities, such as *m/z* 3339.8, 3530.9, 4233.3, 4936.7, and 4963.7, were increased by the $scCO_2$ wash (Fig. 1a,b; black arrow). The greatest improvement in the S/N was found at *m/z* 4963.7; the ratio was almost 190-fold greater than the control value (Fig. 1c). In contrast, several signals such as those around *m/z* 2292.8, 3127.4, and 3888.0 were diminished by the wash with $scCO_2$ and the entrainer.

The region at *m/z* 700-900, where the signals of phospholipids (especially phosphatidylcholines; PCs) are concentrated [15], showed few signals in the $scCO_2$-washed samples, whereas the corresponding *m/z* region in the control samples showed abundant signals (Suppl. Fig. S2a,b). PCs were consistently detected from the extract of a concentrated solution used in $scCO_2$ washing (Suppl. Fig. S3). These results clearly demonstrated that phospholipids were extracted in the $scCO_2$ from tissue sections by the wash. Supercritical fluid can easily infiltrate deeply into tissue and is expected to clear specific molecules completely since $scCO_2$ have low viscosity (under 8×10^{-5} Pa•s) [11] and high diffusivity (under 10^{-4} cm^2/s) [12].

The distributions of the signals improved by the $scCO_2$ wash are shown in Figure 2. We classified these signals into four groups according to the distributions. The first group was *m/z* 3339.8, 3477.9, 3633.0, and 4248.3, which were distributed on the callosum, anterior commissure, stria medullaris, optic nerve, midbrain, white matter of the cerebellum, and medulla oblongata (Fig. 2a). The second group was signals at *m/z* 3417.9, 3503.9, 3976.2, 4105.2, 4233.3, 4919.7, 4947.7, 4963.7, and 4979.7, which

were similarly distributed on the olfactory bulb, callosum, anterior olfactory nucleus, and hippocampus (Fig. 2b). The third group was signals at *m/z* 3530.9 and 3544.9, which were similarly distributed on the callosum, anterior commissure, midbrain, and white matter of the cerebellum (Fig. 2c). The fourth group has only one signal at *m/z* 4936.7, which was distributed on the callosum, striatum, and anterior commissure (Fig. 2d). Signals found only in control samples, such as *m/z* 2292.8, 3127.4, and 3888.0, were distributed in the cerebral cortex and gray matter of the cerebellum (Suppl. Fig. S4). The region of *m/z* 500-2500, which includes the *m/z* values of several bioactive peptides, was also analyzed (Suppl. Fig. S5a,b), and signals at *m/z* 942.5 and 1834.0 were found to be associated with the morphological structure on $scCO_2$-washed samples (Suppl. Fig. S5c,d).

High-spatial-resolution analysis in the hippocampus revealed that the signal of *m/z* 4963.7 (which was most improved by the $scCO_2$ wash) was distributed at CA1 and the dentate gyrus, which are subregions of the hippocampus (Suppl. Fig. S6). The signal of *m/z* 4963.7 showed almost the same distribution as that reported in a study using MALDI-IMS [16], which assigned the signal as thymosin β4. Consistently, tandem mass spectrometry (MS/MS) using a MALDI-time of flight (TOF)/TOF-type instrument for the signal, showed almost the same pattern of product ions between the thymosin β4 standard and the signal obtained from tissue (Suppl. Fig. S7).

It was reported that an alcohol-based wash also improved the detection of thymosin β4 [16]. We compared the wash of 33% MeOH only and that of $scCO_2$ with an entrainer since 33% MeOH was used as an entrainer in our method. The results showed that the $scCO_2$ wash increased the signal intensity more than the 33% MeOH wash did (Suppl. Fig. S8a,b). In addition, phospholipid signals had remained only in the section

washed with 33% MeOH (Suppl. Fig. S8c–e). As 33% MeOH only was not enough to improve the detection of the signal or the removal of lipids by itself, it is clear that $scCO_2$ is essential for increasing the signal intensity.

For our evaluation of the improvement of signal intensity, we compared washing by $scCO_2$ with entrainer with conventional washing by two types of organic solvent, MeOH and isopropanol [9]. We observed that the $scCO_2$-washed samples showed higher signal intensity of thymosin β4 compared to the samples washed with MeOH or isopropanol (Suppl. Fig. S9a–c). Additionally, the baseline of the signals was decreased in the washes by $scCO_2$ with an entrainer compared to the wash with isopropanol around *m/z* 3000-5000.

Discussion

The intensity of some signals at *m/z* 3000-5200 was increased by the wash with $scCO_2$ (Fig. 1). On the other hand, the intensity of signals at *m/z* 700-900 corresponding to phospholipids decreased on $scCO_2$-washed tissue, and the same signals were detected from extract solution (Suppl. Figs. S2, S3). These results indicate that our method removed phospholipids from tissue sections and reduced the ion suppression by phospholipids, enabling the detection of peptide signals at *m/z* 3000-5200.

All of the improved peaks from the $scCO_2$-washed samples corresponded to the molecular weights of the peptides and were associated with the morphological structure. We classified the peaks into four groups according to the patterns of distribution (Fig. 2, Suppl. Fig. S5). The morphological structures were maintained even in the high-spatial-resolution analysis (Suppl. Fig. S6). These results suggested that the

distribution of detected signals considered to represent peptides was not affected by the $scCO_2$ wash.

Other signals at *m/z* 3000-5200 were specific to the control sample and were not detected in $scCO_2$-washed samples (Suppl. Fig. S4). It appeared that the $scCO_2$ wash removed the signals specific to the controls in addition to phospholipids, which is the opposite of the increase in the peptide signal intensities in the $scCO_2$-washed samples. We therefore propose that our method allows simultaneous distribution analyses of peptides other than phospholipids.

MS/MS analysis for the highest signal at *m/z* 4963.7 on the $scCO_2$-washed samples identified the signal as thymosin β4 (Suppl. Fig. S7), and thus the marked improvement of the signal intensity offers benefits for both MALDI-IMS and MS/MS analyses on tissue. Unfortunately, most of the signals from $scCO_2$-washed samples were not enough for identification by the MS/MS analysis. Among the unidentified signals, we assigned *m/z* 4936.7 as thymosin β10 based on a literature search [16]. We expect that the uses of different entrainers will improve these signal intensities enough to enable an MS/MS analysis to identify the rest of the signals.

The use of 33% MeOH only was not enough to increase the sensitivity of the detection of thymosin β4 or to remove phospholipids signals (Suppl. Fig. S8). This result suggests that $scCO_2$ was crucial for our method to increase signal intensities at *m/z* 3000-5200, and this is because characteristics of $scCO_2$ such as low viscosity and high diffusivity were essential for the removal of phospholipids with the assistance of a low volume of MeOH. The detection of peptide signals including the signal of thymosin β4 was considered to be improved by the removal of the interference from phospholipids.

Our wash method successfully improved both the signal intensity and the S/N of thymosin β4, and better results were observed compared to those obtained by conventional methods using an organic solvent (Suppl. Fig. S9). Our findings indicate that our method can completely remove lipids and chemical noise, which are thought to suppress the ionization of peptides. Our method has the advantage of sensitivity for peptide signal intensities compared to conventional wash methods, whereas the conventional methods have the advantage of being easy and quick.

We consider that the improved detection of thymosin β4 in this study was due to the difference in the polarity of solutions between the $scCO_2$ wash step and the matrix application step (Suppl. Fig. S10). Polarity is usually estimated as the partition coefficient XlogP3 [17], which is an atomic-based prediction of the logarithmic ratio, (concentration in octanol)/(concentration in water). We therefore considered XlogP3 for each step in our method. The first step was the removal of phospholipids from the tissue to the $scCO_2$. We calculated the partition coefficient of $scCO_2$ by using the XLogP3 value of CO_2. We used CO_2 with MeOH to remove phospholipids such as PC (32:0), which was likely to be PC (16:0/16:0). The XlogP3 values were 0.9 for CO_2, −0.5 for MeOH, and 13.5 for PC (16:0/16:0) [18].

The solution used in these experiments consisted of 70% CO_2 and 30% MeOH, and thus the total XlogP3 is calculated as $0.7 \times (0.9) + 0.3 \times (-0.5) = 0.63 + (-0.15) = 0.48$, indicating that the solution is hydrophobic and sufficient to dissolve PC (16:0/16:0). The second step was performed in the matrix application from the tissue to the matrix solution. The XlogP3 of MeOH and water is −0.5, and that of thymosin β4 is −43.5 [18]. The total XlogP3 of the solution containing 70% MeOH and 30% water is calculated as

$0.7 \times (-0.5) + 0.3 \times (-0.5) = -0.35 + (-0.15) = -0.5$, which represents sufficient hydrophilicity for the extraction of thymosin β4. The removal of phospholipids makes it easy to extract peptides in place of lipids, and to that end extracted peptides were well-mixed with matrix in the second step.

As thymosin β4, a major intracellular peptide, is an abundant and highly hydrophilic peptide [19], thymosin β4 was the most improved signal in the present analysis. We consider that the unidentified signals improved in the $scCO_2$ extracted sample were also hydrophilic peptides. In the future, our method could be used to detect various peptides related to diseases, such as Alzheimer's disease [20]: as the XlogP3 for amyloid-beta peptide (Aβ) 40 is estimated as −13.1, and that for Aβ 42 as −12.2, Aβ 40 and Aβ 42 are potential targets for MALDI-IMS using our method.

In conclusion, we investigated a new pretreatment method using $scCO_2$ with an entrainer for distribution analyses by MALDI-IMS. Our results demonstrate the effective removal of phospholipids and the increased detection of peptides at *m/z* 3000-5200, including unidentified molecules. The pretreatment method using $scCO_2$ described herein is thus a powerful technique to enhance distribution analyses of peptides. Further improvements for analyses of other molecular specie could also be achieved by optimizing the polarity of the wash conditions, such as by using different supercritical fluids, and adding various entrainers. We propose that our wash method with a supercritical fluid could be used to reveal the distributions of a variety of molecules in tissue samples.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

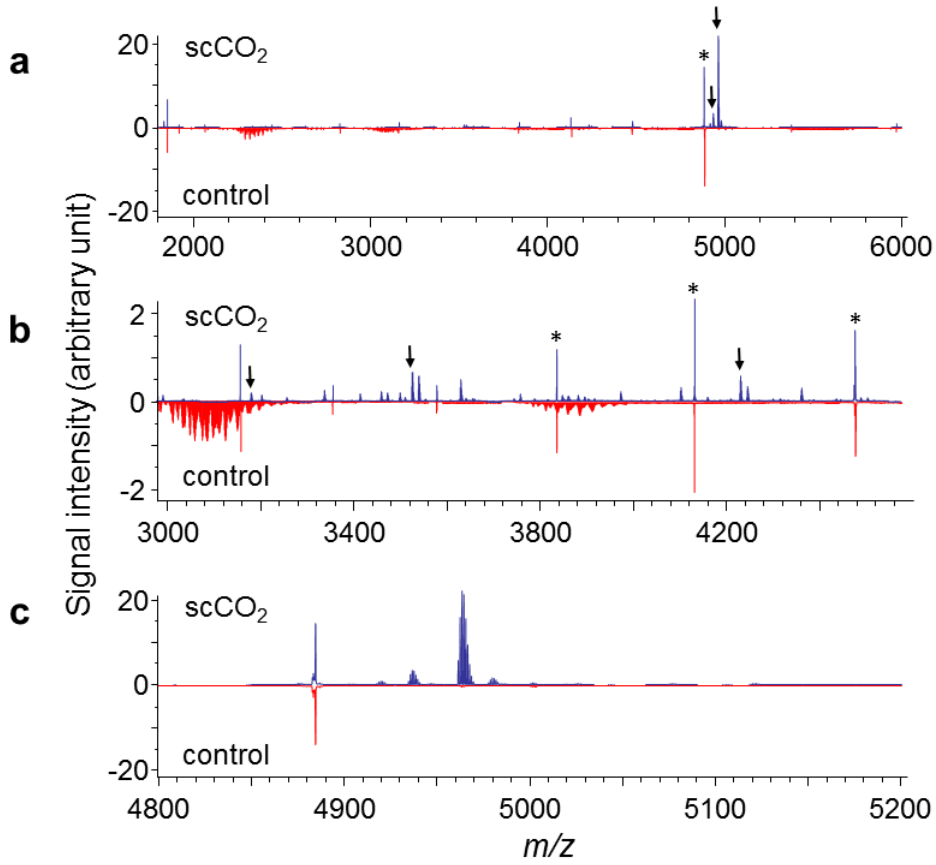

Fig. 1. Spectra obtained from a $scCO_2$-washed tissue (upper, blue) and a control tissue (lower, red) sample. (**a**) Whole spectra of *m/z* 1800-6000. (**b**) Enlarged spectra of *m/z* 3000-4400. (**c**) Enlarged spectra of *m/z* 4800-5200. *an artifact signal.

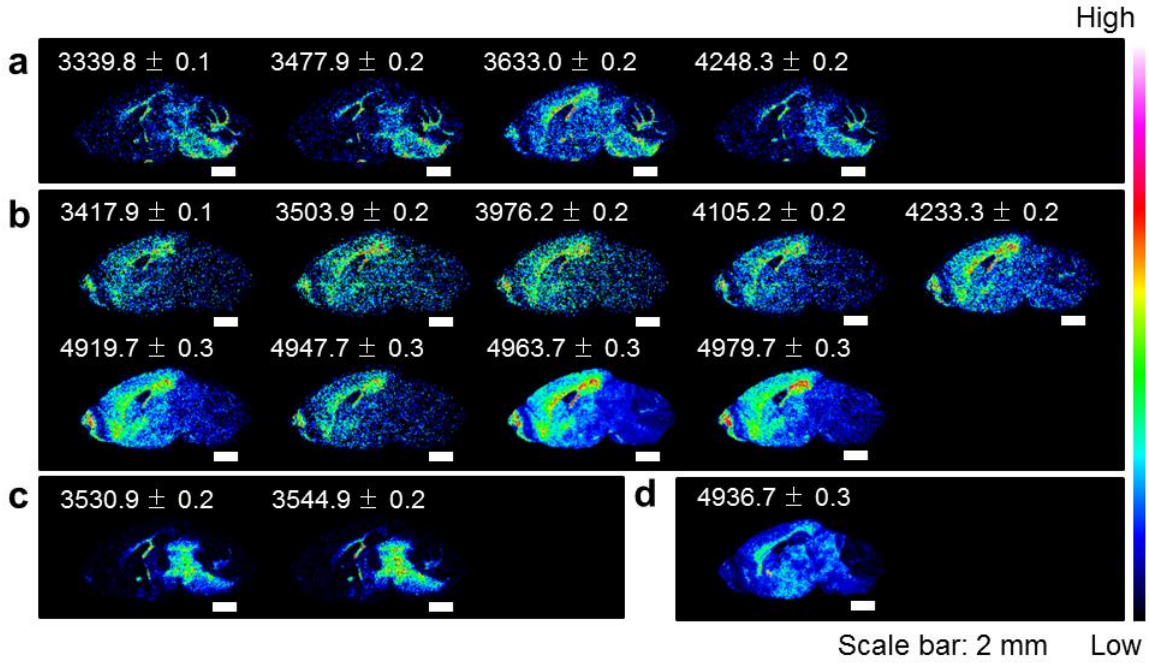

Fig. 2. Ion distribution of the signals specifically detected from $scCO_2$-washed samples. We grouped several peaks according to their distributions. (**a**) Signals distributed on white matter of the cerebellum and medulla oblongata. (**b**) Signals distributed in the hippocampus. (**c**) Signals distributed in the midbrain. (**d**) Signal distributed in the callosum.