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A new rapid and comprehensive peptidome analysis by one-step direct transfer technology for 1-D electrophoresis/MALDI mass spectrometry

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ABSTRACT

We have developed a new target plate for matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). This target plate enables direct electric transfer of analytes from the 1-dimensional gel electrophoresis (1-DE) gel to the target plate in one step. Incorporated with a one-step direct transfer technique, this novel 1-DE/MALDI-MS (1-DE/MS) system eliminates staining, extracting, loading, and many other time-consuming intermediate processes, thereby greatly reducing analysis time while providing high throughput proteome analysis. Furthermore, in peptidome analysis, during the 1-DE step this system separates or removes the high molecular weight plasma proteins in blood and the various low molecular weight substances in tissue extracts, which interfere with mass spectrometry. This system can therefore be used for peptide profiling of any biological sample without special pretreatment. In view of these advantages, the 1-DE/MS system will greatly improve the usefulness of current peptidomic modalities in the discovery and validation of biomarker molecules in various body fluids and tissue extracts, permitting early detection, diagnosis, and treatment of diseases.

Increasingly, researchers are focusing their efforts on the discovery of new biomarkers in human DNA, RNA, proteins, peptides, and metabolites, in order to advance the field of evidence-based health care and permit early detection, diagnosis, and treatment of diseases. The discovery of new, specific, and reliable biomarkers holds the key to future medical treatment. Researchers are exploring a wide variety of biomarkers; increasing evidence indicates that the low molecular weight circulatory peptides in blood [1–3], cerebrospinal fluid [4,5], urine [6,7], and saliva [8] are particularly rich sources of information that may assist in the early detection of diseases and assessment of therapeutic efficacy and safety. In the field of biotechnology, electrophoresis is necessary for purification of proteins, and diverse types of electrophoresis have been developed. In particular, among the various physicochemical properties of proteins, 2-dimensional gel electrophoresis (2-DE) [9,10] uses isoelectric points and molecular weights to separate them. This is the most efficient technique for separating and purifying proteins in a complex biological sample. Furthermore, the development of MALDI-MS [11,12] enables analysis and identification of unstable substances and high molecular weight molecules, i.e., proteins that have not been determined so far by mass spectrometry with conventional ionization. A 2-DE/MS system combining 2-DE and MALDI-MS is therefore considered one of the major techniques for proteome analysis, aiming at comprehensive protein analysis [13]. However, in the 2-DE/MS system it was intrinsically difficult for mass spectrometry to directly analyze the proteins in gel separated by electrophoresis. Cohen and Chait [14] employed MALDI-MS to analyze proteins separated by electrophoresis. For the analysis, they extracted proteins with formic acid/propanol after staining in gel, then mixed the proteins with a matrix. Other research groups transferred proteins onto nitrocellulose [15] or PVDF membranes [16,17] after electrophoresis, and stained them for mass spectrometry. However, these methods had the drawback that proteins in gel or on membrane could not be quantitatively collected. In addition, not only the 2-DE/MS system, but all other proteome analysis techniques as well, have a common drawback that must be remedied immediately: the removal of plasma proteins, such as albumin, from blood samples poses the problem of non-detection of the proteins and peptides adsorbed to the proteins that have been removed [18,19]. To resolve these problems of the 2-DE/MS system and realize high throughput, comprehensive peptidome analysis, we sought to establish a new 1-DE/MS system combining 1-DE and MALDI-MS. To create this system, we devised a new target plate for MALDI-MS, comprising polymer-based material that traps proteins and peptides, and a metal plate. Consequently, after electrophoresis all peptides could be transferred to a target plate in one step, and analyzed directly using MALDI-MS. The novel 1-DE/MS system that we propose can be expected to support the discovery of many new biomarkers that have not been detected by conventional peptidome analysis, due to its technical limitations.

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Materials and methods

A new target plate. To date, many techniques and devices have been investigated for improving the application of samples onto the MALDI-MS target plate [20–23]. To improve the transfer to and retention of peptides on the MALDI-MS target plate for 1-DE/MS, we have investigated the properties of the two components of the target plate, i.e., the protein-adsorbing polymer materials coating the surface of the plate and the plate itself, and have devised a method for binding them. We finally managed to coat the surface of the conductive metal plate with a polyvinylidene difluoride (PVDF) film and realized the above concept [24]. Fig. 1 shows details of target plate structure, sample area size, measuring steps, and measuring spots.

Peptides. Five peptides: substance P (Mw = 1347.6; RPKPQQFFGLM-NH_2), neurotensin (Mw = 1672.9; pyroELYEN KPRRPYIL), adrenocorticotropic hormone (ACTH), 1–24 (Mw = 2933.4; SYSMEHFRWGKPVGKKRRPVKVYP), vasoactive intestinal peptide (VIP) (Mw = 3325.8; HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2), and calcitonin (Mw = 3417.8; CGNLSTCMLGTYTQ DFNKFHTFPQTAIGVGAP-NH_2) were purchased from Peptide Institute Inc. (Osaka, Japan). Normal human serum (Catalog number: S1-100ML) was purchased from Chemicon International Inc. (CA, USA).

Electrophoresis and electro transferring. The five peptides were dissolved in 0.1% trifluoroacetic acid (TFA) and diluted twice with the same solution or normal human serum. Each peptide mixture (3 μl) was mixed with 3 μl of NuPAGE® LDS Sample Buffer (4×) (Invitrogen Corp., Carlsbad, CA, USA) and incubated at 70 °C for 10 min. The sample mixture (6 μl) was then applied to a NuPAGE® Novex 4–12% Bis–Tris Gel (Invitrogen). SDS–PAGE was carried out using NuPAGE® MES SDS Running Buffer (20×) (Invitrogen) at a constant voltage of 200 V for 13 min in an XCell *SureLock*™ Mini-Cell (Invitrogen) powered by a powerStation 1000VC (Atto Corp., Tokyo, Japan). After electrophoresis, the slab gel was sliced into 10 gel strips. The gel strips were cut off above the BPB line located at the electrophoresis point. The strips (length: approximately 3.5 cm) were then placed in pairs on the target plate, in a tail-to-tail manner. A pair of target plates was sandwiched between blotting pads and inserted into the XCell II™ Blot Module (Invitrogen), which was filled with BLOTCHIP buffer (Protosera Inc., Tokyo, Japan) [24]. Following electroblotting at 90 mA for 120 min, α-cyano-4-hydroxy cinnamic acid (CHCA) matrix solution (Bruker Daltonics Inc. MA, USA) was automatically applied to the target plate by a robot (Protosera).

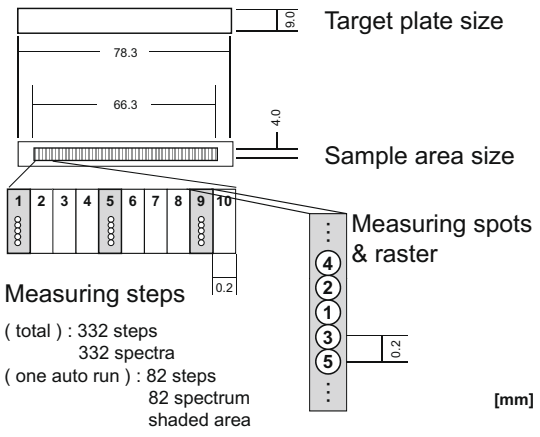

Fig. 1. Schematic of new target plate. A target plate comprising 332 measuring steps at 200 μm intervals was laser-irradiated every fourth step, i.e., 82 steps in total. Each measuring step comprised 20 measuring spots at 200 μm intervals; each of five spots in the central area were irradiated 100 times with the laser as per the order shown in "Measuring spots & raster". A single MS spectrum was acquired from 500 accumulated scans, following these procedures.

Mass spectrometry. Mass spectra were acquired on an ultra-flex II MALDI-TOF/TOF (Bruker Daltonics) interfaced with flexAnalysis Version 2.4 software (Bruker Daltonics) under the following conditions: laser intensity 28–35, detector voltage 1685 V, suppression 500 with fuzzy mode, and molecular mass range of 1000–30,000. The MS instrument was externally mass calibrated with Peptide Calibration Standard II (Bruker Daltonics). The target plate comprised 332 measuring steps at 200 μm intervals. In this study, the specimen was actually irradiated with the laser every fourth step, i.e., 82 steps in total. Each measuring step comprised 20 measuring spots at 200 μm intervals, with each of five spots in the central area irradiated 100 times with the laser, as per the order shown in Fig. 1. Following these procedures, a single MS spectrum was acquired from 500 accumulated scans. Since two gel strips were placed on one target plate, 41 single MS spectra were obtained per gel (Fig. 2A-2 and B-2). Total peak intensities at the same *m/z* value of 41 single MS spectra were combined to generate one integrated MS spectrum (Fig. 2A-3 and B-3), using flexAnalysis Version 2.4 in the molecular mass range of 1000–20,000. All measurements were carried out four times. Significant peak intensity differences between the two groups were analyzed by *t*-test.

Results and discussion

A new 1-DE/MS system

After mixing five peptides (1: substance P, 2: neurotensin, 3: ACTH, 4: VIP, 5: calcitonin) with serum, SDS–PAGE was performed and the gel was stained with Coomassie blue (Fig. 2A-1). In the other part of the experiment, after electrophoresis an unstained gel was electrically transferred to a new target plate and the plate was analyzed by MALDI-MS with irradiation to 41 measuring steps on the surface of the plate, resulting in 41 single MS spectra (Fig. 2A-2). Since peptide peaks with the same *m/z* were detected in several single MS spectra, the intensities of these peaks were summed to estimate the total peak intensity of each peptide (Fig. 2A-3). In this 1-DE/MS system, peptides were first separated from the major plasma proteins by electrophoresis; therefore, in peptidome analysis of blood samples, this system no longer requires pretreatment to remove plasma proteins such as albumin. Consequently, peptides adsorbed to these proteins are expected to be detectable [18]. On the other hand, tissue extracts containing highly concentrated salts, denaturants, and surfactants can be analyzed without such pretreatments as fractionating, desalting, and concentrating, which cause peptide loss and denaturation [25]. Peptides, short chains of amino acids, stain substantially less than proteins and cannot be detected by the usual staining methods. This system, however, no longer requires peptide staining; i.e., using MS it can directly detect peptides on a target plate and determine them comprehensively.

The results for single peptide samples are shown in Fig. 2B-1, -2, and -3. Comparison of the results of peptide analysis in the presence and absence of serum proteins shows that in SDS–PAGE electrophoresis, the migration distance of peptides coexisting with serum proteins is likely to be shorter, suggesting some interaction with serum proteins. In addition, peptides (numbered 1, 3, 4, and 5 in Fig. 2A-3) other than neurotensin (numbered 2 in Fig. 2A-3) coexisting with serum proteins showed two molecular forms, i.e., unchanged and oxidized; conversely, such peptides were completely converted into oxidized forms under serum proteins-free condition (numbered 1, 3, 4, and 5 in Fig. 2B-3). It is suggested that peptides containing methionine were oxidized during

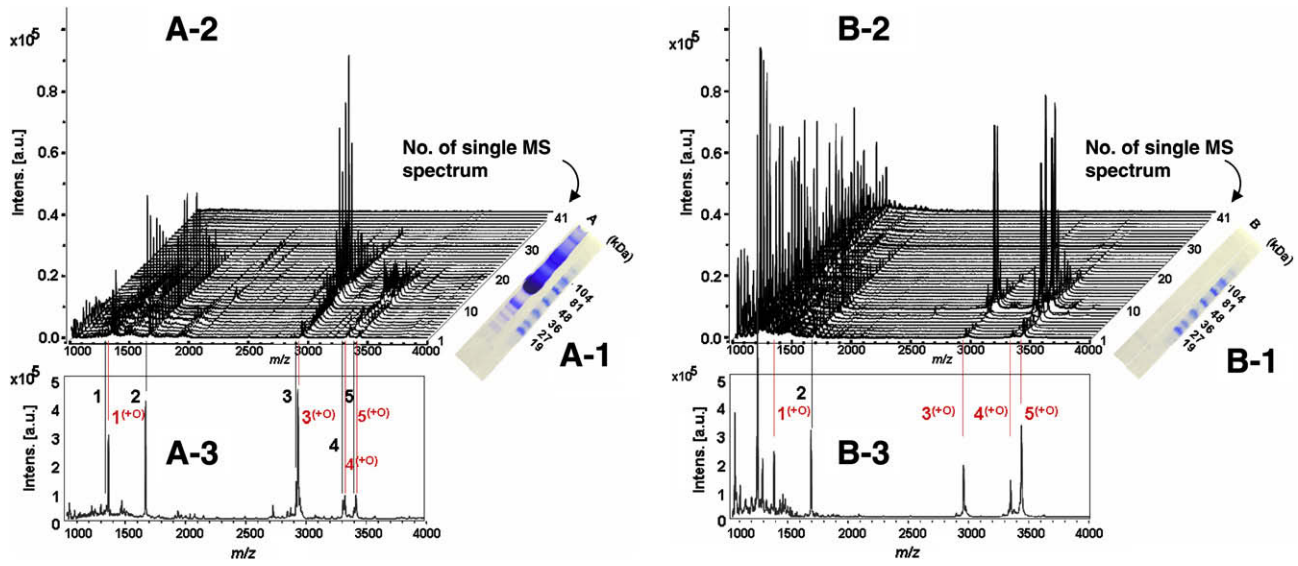

Fig. 2. Integration of MS spectrum. Five peptides (1: Substance P, 2: Neurotensin, 3: ACTH, 4: VIP, 5: Calcitonin) mixed in serum were migrated by SDS–PAGE and stained as shown in A-1. In the other part of the experiment, after electric transfer of an unstained gel, the peptides on the target plate were analyzed by MALDI-MS, yielding a total of 41 single MS spectra (A-2). Total peak intensities at the same m/z value of 41 single MS spectra were combined to generate one integrated MS spectrum (A-3). The results for peptides alone are shown in B-1, -2, and -3.

electrophoresis, electric transfer or mass spectrometry, while the coexisting serum proteins helped attenuate this oxidation.

Reproducibility

The reproducibility of peak intensity was examined for 10 random peaks (*m/z*: 2011, 2046, 2741, 2820, 4164, 4586, 4829, 4993, 9156, and 11686) in normal serum obtained by 1-DE/MS system. Similar peak shapes were detected on all 10 peptides (data not shown). Regarding variance in this analysis, the mean coefficient of variation (CV) was 18% and the maximum CV was 22% (Table 1). On the basis of reproducibility results, this system is considered applicable to peptide determination and differential analysis between two groups, so is expected to be applicable to the peptidome analysis of various biological samples.

Dose dependency

Five peptides were added to normal serum at concentrations of 0.2, 1, 5, and 10 pmol/assay and the dose dependency for each peptide was examined. The experiment was performed 5 times; the results are shown in Fig. 3A and B. For all peptides, good correlation was found between dose and intensity in the range of 0–1 pmol/assay (Fig. 3B). Conversely, for neurotensin, substance P and calcitonin, no dose dependency was found in the range of 5 pmol/assay or higher. We examined the detection limit when these exogenous peptides were added to the serum, and supposed it was under 0.2 pmol/assay (the lowest concentration used in this study) in all peptides.

Differential profiling analysis

Fig. 4 shows the profile of the serum including five peptides at a concentration of 0.2 pmol/assay, superimposed on that of peptide-free serum at the *m/z* range of 1000–3500. All peaks corresponding to the five peptides were detected with a significant difference. Therefore if there is any difference between two peptidomes, they can be expected to be distinguishable via a set of peptides detected by the 1-DE/MS system. Moreover, the system may enable detection of peptides in vivo or ex vivo, and provide detailed information of the peptide profiles of experimental materials.

Table 1
Reproducibility of peak intensities measured by 1-DE/MS system.

Peak no.	*m/z*	Intensity							
		Assay no.					Average	SD	CV (%)
		1	2	3	4	5			
1	2011	98,782	79,101	115,000	114,880	143,657	110,284	23,779	22
2	2046	60,409	53,489	77,922	85,400	73,603	70,164	13,013	19
3	2741	231,960	253,441	254,068	353,197	226,281	263,789	51,515	20
4	2820	208,085	248,328	236,218	284,073	196,484	234,638	34,621	15
5	4164	364,685	370,131	268,490	381,584	266,836	330,345	57,548	17
6	4586	206,474	276,337	229,289	302,483	253,878	253,692	37,810	15
7	4829	113,673	122,638	92,706	103,883	70,013	100,583	20,404	20
8	4993	223,382	162,599	232,011	187,515	278,160	216,733	44,258	20
9	9156	299,336	413,547	346,567	458,785	373,807	378,408	61,202	16
10	11686	87,649	112,064	87,250	69,487	91,037	89,497	15,170	17

Peak intensity reproducibility was examined for 10 random peaks in normal human serum, as measured by the 1-DE/MS system. The average, SD, and CV values are shown.

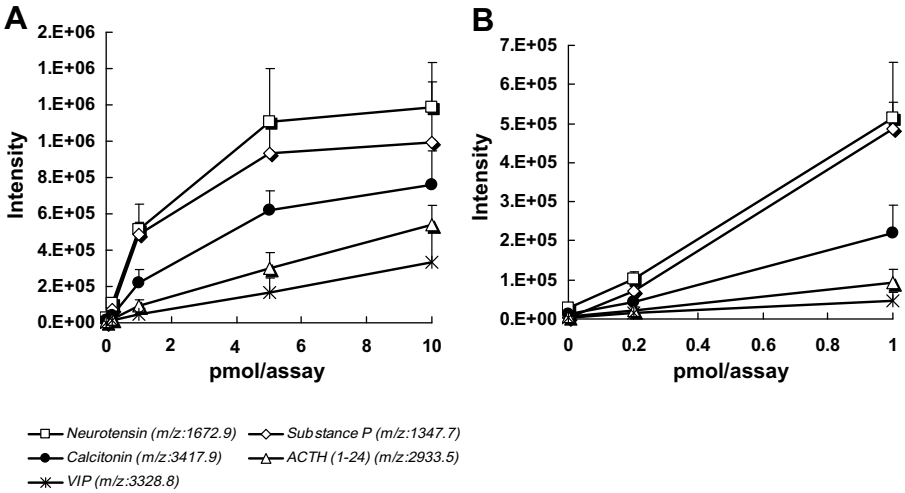

Fig. 3. Correlation diagram between dose and peak intensity. Dose-dependence curves obtained using the new 1-DE/MS system are shown. Each sample was measured 5 times; average and SD are shown. The peptide intensities ranging from 0 to 10 pmol/assay and 0 to 1 pmol/assay, are shown in A and B, respectively. All peptides were detected at 0.2 pmol/assay or higher.

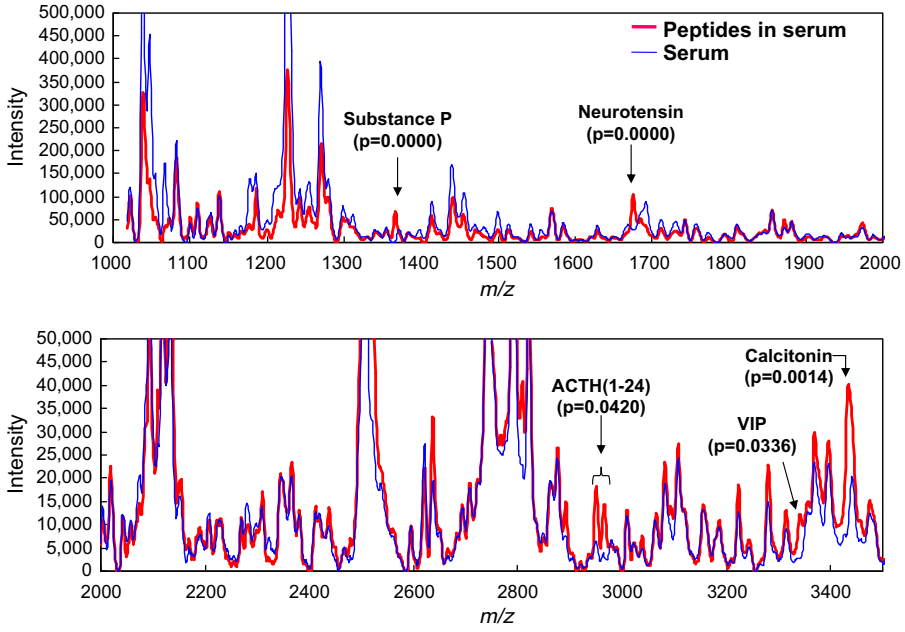

Fig. 4. Differential profiling of exogenous peptides. The profile of the serum including five peptides at a concentration of 0.2 pmol/assay is here superimposed on that of peptide-free serum at the m/z range of 1000–3500. All peaks corresponding to the five peptides were detected with a significant difference.

High throughput analysis of a large number of samples

The main operating processes of this system are electrophoresis, electric transfer and mass spectrometry. The entire process is completed within 4 hours, i.e., electrophoresis (13 min), electric transfer (2 h), and mass spectrometry (20 min), plus associated operations such as gel cutout and matrix application. After installing three target plates in an MTP Cip Adapter (Bruker Daltonics), each target plate was automatically analyzed by MS attached with a Zymark Twister™ microplate handler (Bruker Daltonics), an optional device for robotic target plate supply, leading to high throughput peptidome analysis of 80 samples/day at maximum. This system provides high-speed peptidome analysis owing to the one-step direct transfer of all peptides from gel to target plate. The staining, extracting, and loading steps, essential in conventional methods, are no longer required, greatly reducing total overall processing time for peptidome analysis.

The serum peptidome consists of low molecular weight proteins and peptides that are shed into the local circulatory system from the microenvironment of diseased tissues, and that might be a potential source of diagnostic biomarkers [1]. Our newly established 1-DE/MS platform introduces a new generation of performance that permits rapid and effective peptidome profiling, enabling the discovery in various body fluids and tissue extracts of biomarker molecules for use in the early detection, diagnosis, and treatment of diseases.

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