Research Paper

Application of MALDI-TOF MS analysis for rapid discrimination of free immunoglobulin light chains, kappa and lambda isotypes in patients with monoclonal immunoglobulin gammopathy

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Abstract Among immunoglobulin free light chains (FLCs), kappa and lambda isotypes exist mainly in monomeric and dimeric forms. Under pathological conditions, the levels of total FLCs as well as the ratios between mono- and dimeric FLCs significantly change in samples from patients with monoclonal immunoglobulin gammopathy (MMG). The MALDI-TOFMS technique, which would be correspond to the electrophoresis of proteins under a high vacuum, can be used to easily and rapidly detect any target proteins in body fluids without any specific antibodies. We compared the effectiveness of MALDI-TOF MS and immunoelectrophoresis techniques to detect FLCs in MMG patients. Our preparations were only treated with desalts and concentration using the SpinFilter 3K™ and Affi-Gel™ Blue resin without any antibodies. The ratios of the relative ion intensities between [ca 45 kDa] and [ca 23 kDa] by MALDI-TOF MS were 0.054+/−0.05 (n=26) of the κ-type, and those of the λ-type were 0.572+/−0.45 (n=12), respectively. The ratios of [ca 45 kDa]/[ca 23 kDa] were consisted with those of dimers and monomers in patients' samples by immunoelectrophoresis techniques. Conclusion: The ratios of the relative ion intensity at m/z ca 23 kDa and ca 45 kDa of FLCs in patients' urine may facilitate the rapid discrimination between BJP κ- and λ-types in MMG.

Key words: Bence−Jones protein, MALDI-TOF MS, monoclonal immunoglobulin gammopathy, monomer−dimmer ratios, rapid discrimination

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Received: April 14, 2021. Accepted: May 27, 2021.
Epub June 18, 2021.
DOI: 10.24508/mms.2021.06.007

Introduction

In multiple myeloma (MM) and other monoclonal immunoglobulinemia, monoclonal plasma cells proliferate and produce either κ- or λ-type light chains. There are increases in total amounts of the κ- and/or λ-type, and the ratios between κ- and λ-types are abnormal. In addition, since about 40% more light chains are produced than heavy chains, any the light chain that cannot bind to a heavy chain is called a free light chain (FLC) and released extracellu-

larly[1–3].

Immunoglobulin FLCs, κ- and λ-types, exist mainly in monomeric, dimeric, and multimeric forms[4,5]. Under pathological conditions with an excessive production of plasma cells, the levels of FLCs and ratios between mono- and dimeric FLCs significantly change. Especially, very high levels of FLCs were frequently reported in the urine of patients with monoclonal immunoglobulin gammopathy (MMG), such as multiple myeloma (MM)[6–8]. Various laboratory markers are used for the diagnosis and monitoring of patients with MM. Among protein components, monoclonal peaks and/or BJPs are identifiable in virtually all patients with the disease[9–12]. A major pitfall in the analysis of monoclonal immunoglobulin light chains in a MM patient's urine is the unreliability of recognizing FLCs. Additionally, the conventional tests used for detecting Bence-Jones protein are falsely negative in approximately one-half of patients with MM. Because of the high urinary concentration of FLCs, spot urine has been used for the detection of FLCs in patients with suspected MM. Moreover, the amount of the light chain excreted in the urine often correlates with disease progression. Generally, immunoassay techniques, such as immunoelectrophoresis, immunofixation electrophoresis, and latex coagulating nephelometry, are routinely used for the screening of FLCs in body fluids[13–16]. The immunoassay techniques essentially need specific FLC antibodies to detect FLCs in body fluids, but false results frequently might arise from the titer and specificity of the antibody itself as well as some disadvantages of low reproduction, limits of detection, and expert skills to detect FLCs[17–19].

On the other hand, the MALDI-TOFMS technique, so-called electrophoresis under a high vacuum, can be used to easily and rapidly detect target proteins, such as FLCs, with a wide range of molecular weights in body fluids with or without specific antibodies[20–23]. During the past three decades, our group has applied the immunoprecipitation technique to rapidly separate some disease-specific target molecules in patients' sera, such as a carbohydrate-deficient transferrin, mutant transthyretin and superoxide dismutase-1 in patients' sera with neurodegenerative diseases prior to MS analysis[24–26]. This immunoprecipitation is more suitable to rapidly and simply purify target proteins in body fluids than affinity chromatography. However, in order to more easily apply this MS technique to routine laboratory tests, it might be very important to simply prepare target molecules from body fluids prior to MS analysis. For these reasons, many kinds of spin column devices filled with group-specific resins may be more suitable to rapidly separate the target proteins in body fluids, such as urine, plasma, and cerebral spinal fluid, without specific antibodies against the targets.

In this study, we examined a total of 62 patients with monoclonal immunoglobulin gammopathies, such as a MM, including suspected cases to identify proteins profiles by MALDI-TOF MS. For the simple and rapid preparation of patients' urine, we applied SpinFilter 3K™ or followed with Affi-Gel™ Blue resin. The ratios of the ion intensities between [ca 45 kDa] and [ca 23 kDa] may facilitate discrimination of κ- and/or λ-types of MMG.

Materials and Methods

Spot urines were collected from a total of 62 patients with suspected of confirmed MMG after receiving of informed consent at Osaka Medical College Hospital. This study has been approved by the Ethics Commission and the number is the Clinical division - 969(2884). We selected 38 urine samples from those definitively diagnosed with MMG by immunofixation and then analyzed them by MALDI-TOFMS. Affi-Gel™ Blue resin was purchased from Bio-Rad (Germany) and SpinFilter 3K™ was from Merck (Germany).

The standards of κ (P016) and λ (P017) chains of Bence-John Protein (BJP) were purchased from Nordic-MUbio (Netherlands), and Sinapinic acid (P code: 101006397) for the ionization reagent was from Fluka (CA, USA). The MALDI-TOF mass spectrometer was used at Autoflex speed (Bruker Daltonics KK, Germany) and in a linear mode at a mass range from 10,000 to 100,000.

SDS-polyacrylamide gel, MultiGel II™, and a running buffer were purchased from Cosmo Bio (Tokyo, Japan). Monoclonal specific antibodies against κ (sc-52338) and λ (sc-52339) types of BJP were from Santa Cruz Biotechnology Inc. (CA, USA). Western blot (WB) of separated proteins in the gels was performed with iBlots 2 Dry Blotting System™ (Thermo Fisher Scientific Inc. CA, USA) according to the manufacturer's manual. The other reagents were from Nacalai Tesque (Kyoto, Japan). The quantification analysis module of the Fusion FX7™ software (ver. 17.01) was performed to calculate the ratio between [ca 45 kDa] and [ca 23 kDa] of the Western blot (WB) analysis.

The patients' urine used to diagnose MM was desalted and concentrated with SpinFilter 3K™ prior to MALDI-TOF MS analysis. After this pretreatment, the analyte

was diluted with distilled water 10 to 1000 times. For urine showing severe proteinuria, we pretreated it with Affi-Gel™ Blue resin to remove albumin. The 50–500-times diluted analyte was subjected to MALDI-TOF MS analysis. The diluted analytes of MM patients and saturated sinapinic acid solution with 0.5% trifluoroacetic acid-50% acetonitrile (w/w %: final concentration) were equivalently added, and then 1 μL of the mixture was dropped on the MALDI-target, dried at room temperature, and then applied to MALDI-TOF MS analysis in the linear mode. We checked the *m/z* calibration with the protein I and II calibrator (Bruker Daltonics KK., Tokyo) before daily MS analysis.

Results and Discussion

In the present study, rapid and simple detection of FLCs in spot urines derived from patients with monoclonal immunoglobulin gammopathies was performed by MALDI-TOF MS analysis, immunofixation (routine method), and SDS-polyacrylamide gel electrophoresis/WB/enhanced chemiluminescence (ECL) detection and the methods were compared the ratios of the relative ion intensities between [ca 45 kDa] and [ca 23 kDa] of FLCs in MM patients by MALDI-TOFMS could be used to rapidly discriminate between the κ- and λ-types of MM without any specific antibodies against FLCs.

Fig. 1. shows four typical cases of BJP κ- and λ-types in definite MM patients' urines following immunofixation to compare MALDI-TOF MS analysis and gel electrophoresis under non-reduction/WB/ECL detection. The protein profiles of MALDI mass spectra were predominantly ions at *m/z* 23297 and 24034, shown in two typical cases of the BJP κ-type, and an ion at *m/z* 48224, shown in one case (Fig. 1a and 1b). On the other hand, the two typical cases of the BJP λ-type clearly showed ions at *m/z* 22431 and 22823, with ions at *m/z* 44920 and 45731, respectively (Fig. 1c and 1d). The ratios of the ion intensities between [ca 45 kDa] and [ca 23 kDa] by the MALDI-TOF MS method coincided with those of FLCs in MM patients' urine by gel electrophoresis under non-reduction/WB/ECL detection.

In Table 1a and 1b, the profiles of MALDI mass spectra

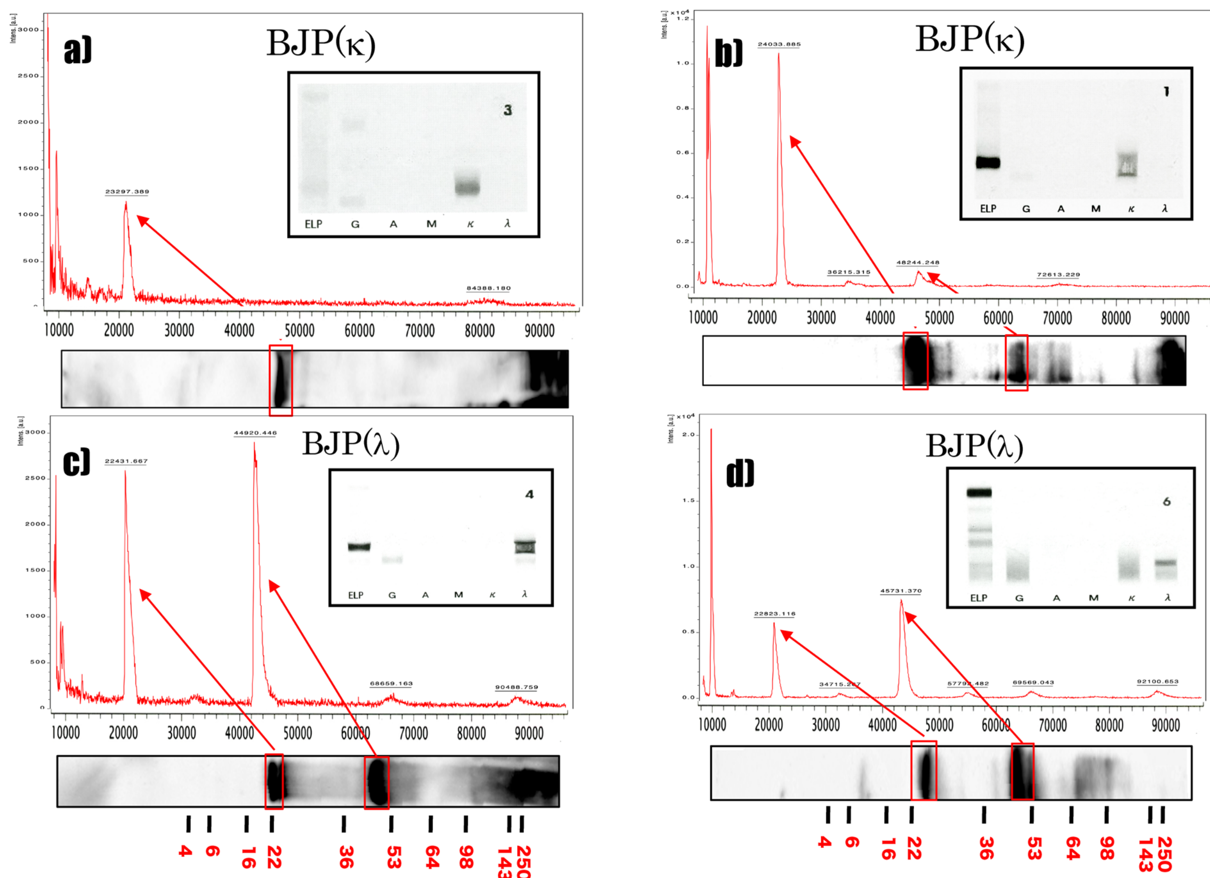

Fig. 1. The data show FLC profiles in the urine of patients' with monoclonal immunoglobulin gammopathy by MALDI-TOF MS as well as immunofixation and Western blotting. a) and b) indicate the BJP κ-types, and c) and d) are BJP λ-types. The inset shows data on immunofixation.

Table 1a. The profiles of MALDI mass spectra of commercially purified BJP κ- and λ-types

	κ-type					λ-type			
	[ca 23 kDa]	[ca 45 kDa]	[ca 72 kDa]	[ca 95 kDa]		[ca 23 kDa]	[ca 45 kDa]	[ca 72 kDa]	[ca 95 kDa]
Mean	23621.3	47165.9	71272.2	94520.3	Mean	22515.4	45134.2	67696.0	90381.5
SD	47.7	71.3	684.9	252.0	SD	29.7	64.0	52.3	113.3
CV (%)	0.2	0.15	0.96	0.03	CV (%)	0.13	0.14	0.08	0.12

Table 1b. The profiles of MALDI mass spectra of BJP κ- and λ-types derived from patients with definite MMs

	κ-type					λ-type			
	[ca 23 kDa]	[ca 45 kDa]	[ca 72 kDa]	[ca 95 kDa]		[ca 23 kDa]	[ca 45 kDa]	[ca 72 kDa]	[ca 95 kDa]
Mean	24151.9	47698.0	72480.1	95271.2	Mean	22803.0	45667.9	67620.6	91643.0
SD	803.9	1491.7	2271.3	1147.9	SD	118.7	582.2	1243.5	1640.5
CV (%)	3.33	3.13	3.13	1.20	CV (%)	0.52	1.27	1.84	1.79

of the commercially purified κ- and λ-types (1a), and those of the corresponding FLCs of MM patients definitely diagnosed with immunofixation (1b) are listed. The MALDI mass profiles (n=10) of the commercially purified κ-type were *m/z* 23621.3+/−47.7 Da, 47165.9+/−71.3 Da, 71272.2+/−684.9 Da and 94520.3+/−252.0 Da, respectively. Those of the commercially purified λ-type were at *m/z* 22515.4+/−29.7 Da, 45134.2+/−64.0 Da, 67696.0+/−52.3 Da, and 90381.5+/−113.3 Da. On the other hand, Table Ib shows that the MALDI mass spectra of 18 cases of the κ-type in MM patients' urine were *m/z* 24151.9+/−803.9 Da, 47698.0+/−1491.7 Da, 72480.1+/−2271.3 Da, and 95271.2+/−1147.9 Da, and those of 12 cases of the λ-type were *m/z* 22803.0+/−118.7 Da, *m/z* 45667.9+/−582.2 Da, *m/z* 67620.6+/−1243.5 Da, and 91643.0+/−1640.5 Da. All of the series of the *m/z* of κ-types were clearly separated and greater than those of the λ-types, the same as clinical samples as well as commercially purified standards. However, there will be a possibility of erroneous identification between the two types using only monomer or divalent ions of FLCs due to their heterogeneities and/or coexistence of low molecular proteins and so we applied to discriminate the two isotypes of BJP with the ratios between dimer and monomer ions.

As shown in Fig. 2, the ratios of ion intensities between [ca 45 kDa] and [ca 23 kDa] in 38 cases of definite MM patients' urine were calculated from the relative ion intensities at *m/z* [ca 45 kDa] and [ca 23 kDa]. The mean +/−standard deviations of the BJP κ-type (n=26) were 0.054+/−0.053 and those of the λ-type (n=12) were 0.572+/−0.448. We also revealed that those of the purified λ-type of BJP from MM patients were 1.112+/−0.616 at the λ-type (n=10). The p-values less than 0.001 were considered significant between the BJP κ-type versus BJP λ-type and those of the κ-type versus purified λ-type was less than 0.0001, but the p-value was 0.06 between the BJP λ-type versus purified λ-type. As

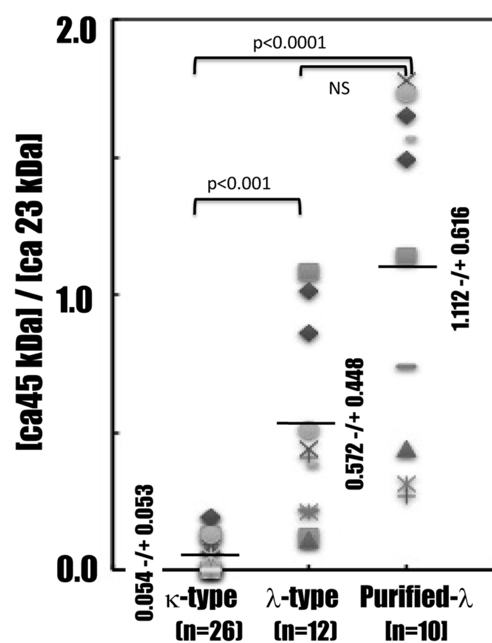

Fig. 2. The ratios of [ca 45 kDa]/[ca 23 kDa] in MM patients' urine were calculated from ion intensities of [ca 45 kDa] and [ca 23 kDa].
The data of [ca 45kDa]/[ca 23 kDa] were 0.054+/−0.06 at the κ-type (n=26) and 0.628+/−0.43 at the λ-type (n=12), and also examined the purified BJP λ-type were and 1.160+/−0.62 at l type (n=10). The p-values less than 0.001 were considered significant between the BJP κ-type versus BJP λ-type, and the κ-type versus purified λ-type, but the p-value was 0.06, not significat, between the BJP λ-type versus purified λ-type.

Table 2. The frequency distributions of the ratios between [ca 45 kDa] and [ca 23 kDa]

The 38 cases with definitive MMG

[ca 45 kDa]/[ca 23 kDa]	<0.02	0.02–0.04	0.04–0.06	0.06–0.08	0.08–0.1	0.1–0.2	0.2–0.4	0.4–0.6	0.6–1.0	1.0–2.0	>2.0
BJP-kappa type	*13	2	1	1	6	3	0	0	0	0	0
BJP-Lambda type	0	0	0	0	0	2	2	3	2	3	0

*The numbers included the 7 cases which [ca 45 kDa] did not detect at MALDI mass spectra.

The 23 cases with suspicious MMG

[ca 45 kDa]/[ca 23 kDa]	<0.02	0.02–0.04	0.04–0.06	0.06–0.08	0.08–0.1	0.1–0.2	0.2–0.4	0.4–0.6	0.6–1.0	1.0–2.0	>2.0
	**12	1	2	0	2	3	2	0	1	0	0

**The numbers included the 8 cases which [ca 45 kDa] did not detect at MALDI mass spectra.

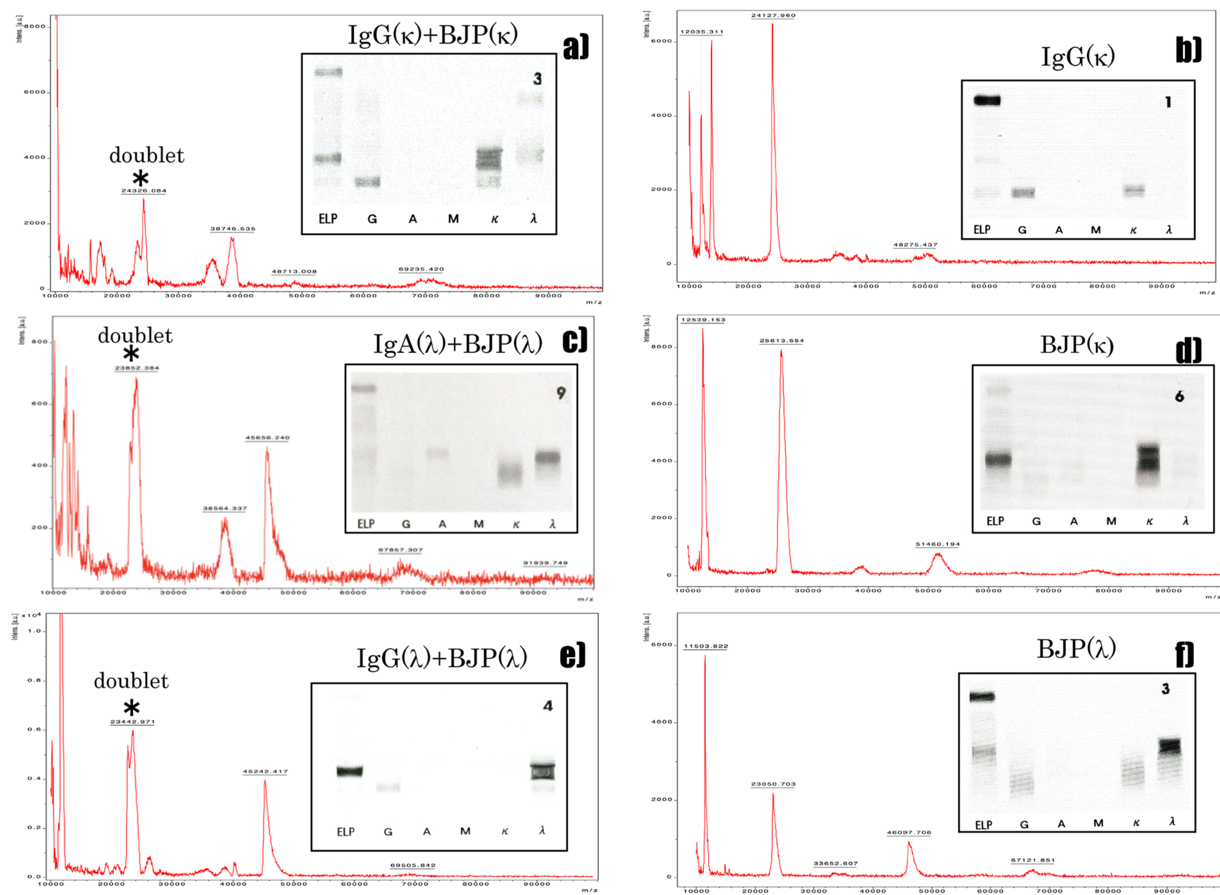

Fig. 3. The MALDI mass patterns of BJP κ- and λ-types coexisting light chains derived from intact immunoglobulin in urine of definitely diagnosed MM patients often detected doublet peaks at *m/z* [ca 23 kDa] as shown in Fig. 3a, 3c, and 3e. On the other hand, the patterns of only BJP κ- or λ-types always showed single and sharp ion peaks, as presented in Fig. 3b, 3d, and 3f.

shown in Table 2, the cut-off values of the ratios of [ca 45 kDa]/[ca 23 kDa] for the discrimination between BJP κ- and λ-types would be estimated as from 0.1 to 0.2. Additionally, the ratios of [ca 45 kDa]/[ca 23 kDa] in 23 cases of suspicious MMG patients' urine were also calculated. The cut-offs in the suspicious MM would be also as from 0.08 to 0.1. From these frequency distributions, we defined that the cut-offs, 0.1, could be clearly discriminated between BJP κ- and λ-types. Using the ratios of [ca 45 kDa]/[ca 23 kDa], rapid and clear discrimination between the BJP κ- and λ-types of MM patients with only 1 μL of spot urine was possible.

T. Nakano et al. [6] and B.M. Bailey et al. [7] reported that the ratio of BJP κ- and λ-types in urines derived from

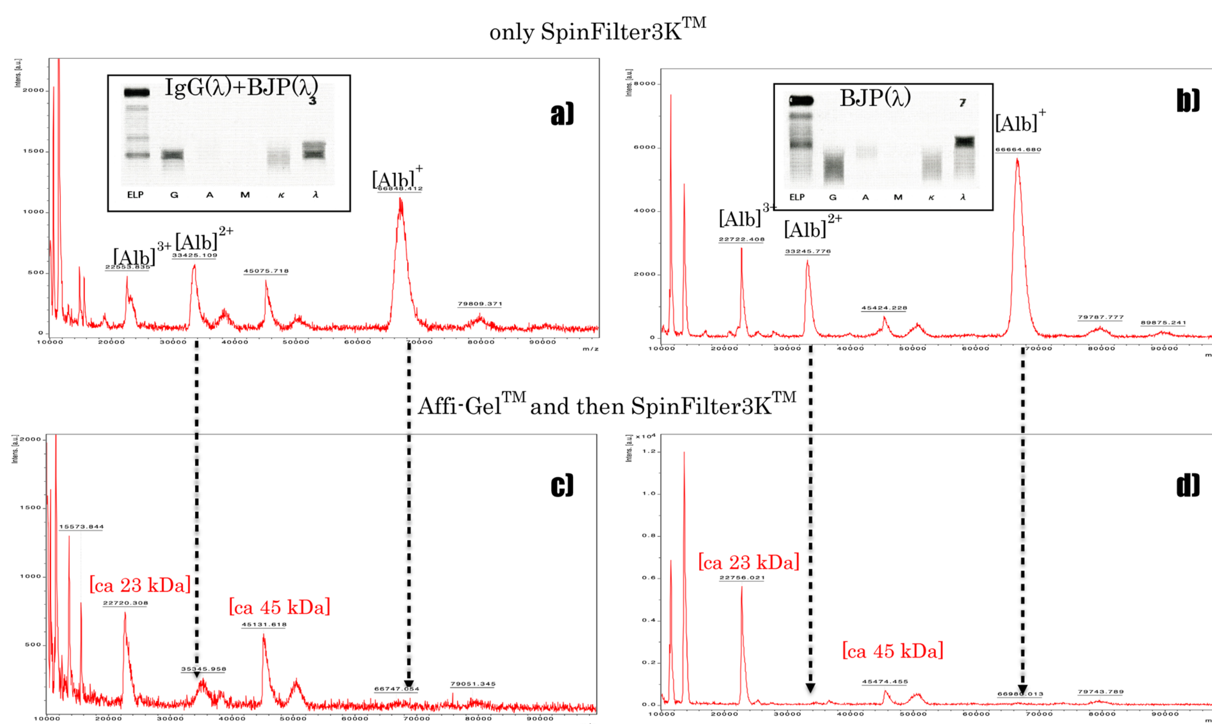

Fig. 4. The typical MALDI-TOF mass spectra of two severe proteinuria after treatment with only SpinFilter3K (Fig. 4a and 4b) and additional treatment with Affi-Gel™ (Fig. 4c and 4d).

Insets show the data on immunofixation.

MMG patients by immunoelectrophoresis and/or immunofixation for the diagnosis of Bence-Jones proteinuria. Their protocols essentially need two devices, one is an electrophoresis to separate the target proteins, and the other is an immunodetection of the target protein with specific antibody. Our protocol using MALDI-TOF MS technique don't need any antibodies to target proteins in the clinical samples.

Fig. 3a, 3c and 3e show typical MALDI mass patterns of BJP κ- and λ-types with the same coexistence of light chains derived from intact immunoglobulin G and A in urine of definitely diagnosed MM patients. We often could observe doublet peaks at *m/z* ca 23 kDa, which overlapped with the ion for the κ- or λ-types. The origin of the doublet ion peaks indicated that one would be BJP itself and the other would be the light chain derived from an intact immunoglobulin. EM. Bailey et al.[7] presented that the light chain "ladder" pattern reflected the heterogeneity of normal light chains. The authors indicated that the ladder pattern probably reflects the limited heterogeneity of normal light chains and so the presence of the ladder pattern in urine might interfere the detection of low levels of BJP sharing the electrophoretic mobility of one of the normal light chain bands. For this reason, the MS analysis has an advantage than the electrophoretic technique. On the other hand, the patterns of only BJP κ- or λ-types showed single and sharp ion peaks, as presented in Fig. 3b, 3d, and 3f. The half width of the [ca 23 kDa] mass spectra would be a quite different between the cases of BJP alone and those of the coexistence of intact immunoglobulin, as shown in Fig. 3. DR. Barnidge et al.[21], MC. Kohlhagen et al.[22], and P. Milani et al.[23] also used immunoaffinity purification targeting the FLCs prior to the MALDI-TOF MS analysis. Indeed, the FLCs in patient's urine could be sufficiently concentrated but the MALDI mass spectra were still interfered by the excessive of albumin. Our fundamental preparations are only desalting and filtration using SpinFilter 3K™ without any purification with specific antibody.

Fig. 4. shows the MLDI-mass spectra of urinary protein profiles with severe proteinuria with and without Affi-Gel™ Blue resin. Before preparation with the resin, multivalent ions of albumin, especially a trivalent ion at *m/z* ca 22.5 kDa, overlapped the ions of the κ- and λ-types. After the preparation, the MALDI mass spectra of albumin completely disappeared and profiles of BJP-λ could be clarified, as shown in Fig. 4c and 4d. In the present study, if the sample revealed severe proteinuria, Affi-Gel™ Blue resin would be used to remove excessive albumin in the sample,

and then followed by SpinFilter 3K™. D.R. Barnidge et al.[21] also applied MALDI-TOF MS analysis using Melon Gel™ and C4 Zip Tips for the enrichment of immunoglobulins in MM patients' sera. They indicated that their clean-up and enrichment preparations still interfered with the detection of the ion of BJP at *m/z* ca 23 kDa, which overlapped with trivalent ions of albumin in clinical samples. Therefore, Affi-Gel™ Blue resin could be effectively removed excessive albumin from patients' samples prior to MS analysis.

In conclusion, the ratios of relative ion intensities between [ca 45 kDa] and [ca 23 kDa] in MM patients' urine and sera determined by the MALDI-TOF MS analysis may facilitate the differential identification of the BJP κ- and λ-types, and also could be used to calculate the molar ratios of the κ- and λ-types in patients' sera. Moreover, the detections of the doublet peak at [ca 23 kDa] in the FLC profiles from MM patients' urine often demonstrated coexistence with light chains from intact immunoglobulin, just as the BJP itself. In order to apply a new technology in clinical laboratory test, it is a very important to be a simply sample preparation and a short turn around time (TAT). As to the simply preparation of urine, it is a very important to remove the salts in urine. If the centrifugal technique (AmiconTM Ultra 3K) could exchange into a pressurization technique, such as an ExtraheraTM LV-200 device (Biotage Japan KK, Tokyo), it might be able to automate urine preparation. Moreover, the TAT of this MALDI-TOF MS method would be within one hour including the sample preparation, and that of the immunoelectrophoresis would be within two days. So, our MS technique coupled with a rapid sample preparation might be used as a routine clinical laboratory test instead of the immunoelectrophoresis assay of FLCs in clinical samples.

Acknowledgements

We would like to thank Professor Emeritus Akira Shimizu for providing many purified FLCs of MM patients for MALDI-TOF MS and immunoelectrophoresis in the present study. This work was partially supported by a 2019–2021 Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 19K07928).

Conflict of Interest

The authors declare no conflicts of interest.

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