VIP Fast Proteolysis

DOI: 10.1002/anie.201004152

Efficient Tryptic Proteolysis Accelerated by Laser Radiation for Peptide Mapping in Proteome Analysis**

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Proteomics has become one of the fastest-developing areas of biological research.[1,2] It aims to provide a global perspective of changes in the amount of a protein through the characterization of a large number of proteins.[3] The commonly used strategy for protein identification consists of protein digestion and subsequent peptide-mass measurements based on mass spectrometry (MS). Because the conventional in-solution digestion of proteins is prone to such intrinsic limitations as prolonged digestion time, autolysis, and sample loss, the development of novel methods for highly efficient proteolysis is of high importance for MS-based peptide mapping.

Many efforts to increase tryptic-digestion efficiency by immobilization of the enzyme on various substrates have been reported.[4–17] The benefits of the use of immobilized enzyme molecules for the characterization of proteins include the reusability and stability of the enzyme, the higher efficiency of the digestion of protein analytes, and the lack of enzyme-autolysis products. In a previous study,[18] we successfully developed magnetic carbonaceous microspheres as a new substrate for enzyme immobilization and applied them to fast protein digestion. Digestion was complete within 30 minutes. The efficiency of conventional in-solution proteolysis could also be accelerated by microwave irradiation, ultrasonic waves, and infrared (IR) radiation. Juan et al.[19] demonstrated that the optimum conditions for in-gel microwave-assisted tryptic digestion was treatment at 195 W for 5 minutes. In 2007, Rial-Otero et al.[20] demonstrated that ultrasonic waves could decrease the digestion time of conventional in-solution proteolysis to 1 minute. More recently, Chen and co-workers used IR radiation as an energy source to promote in-solution tryptic proteolysis: digestion was complete within 5 minutes.[21]

Laser radiation has been shown to modulate many biological progresses. It offers great promise for lesion treatment and selectivity of tissue destruction. In particular, lasers emitting a wavelength of approximately 800 nm have attracted increasing attention.[22–26] This near-infrared coherent light can penetrate readily through water with little energy deposition even under high power and prolonged irradiation. When the near-infrared laser beam is applied under certain thresholds, organized tissue will not sustain irreversible damage. Furthermore, if the laser power is high enough, it may accelerate heat accumulation and raise the tissue temperature in the process. Besides photothermal effects, the high-energy near-IR irradiation could excite overtone or harmonic vibrations of the chemical bonds within the organic components of the tissue. These vibrations might lead to more cleavage sites of proteins exposed to trypsin and thus result in easier cleavage of peptide bonds and better digestion efficiency. It should therefore be feasible to employ an 808 nm laser to enhance the efficiency of conventional in-solution proteolysis with little damage to the enzyme.

In this study, protein solutions containing trypsin were allowed to digest directly with the assistance of laser irradiation (808 nm) both in sealed transparent Eppendorf tubes and on the spots of a stainless steel MALDI plate. Laser irradiation (808 nm) was also employed to enhance the efficiency of the in-gel tryptic digestion of proteins separated by SDS-PAGE. The laser-assisted proteolysis technique was coupled with MALDI-TOF MS for protein digestion and peptide identification. High digestion efficiency was observed for both standard proteins and real protein samples. Thus, this novel digestion method has promise for high-throughput protein identification.

A continuous laser was used to irradiate the protein sample perpendicularly (Figure 1). The laser used in our study was a diode infrared laser module (CNI, Changchun, China). It emits a wavelength of 808 nm with a power of 5 W. The laser-beam output was coupled to a fiber-lens system, which led to a spot diameter at the sample of 5 mm. The resulting irradiance was 25.5 $W\,cm^{-2}$. The distance between the fiber lens and the liquid surface was fixed at 4 cm.

Three replicate experiments were carried out for the optimal reaction time (see Figure S1 in the Supporting Information). The average sequence coverage found upon comparison with structures recorded in the Swiss-Prot database was 42% for bovine serum albumin (BSA; Figure 2a), 89% for myoglobin (see Figure S2a in the Supporting Information), and 83% for cytochrome *c* (see Figure S3a in the Supporting Information). For comparison purposes, we also measured the MALDI mass spectra of the digests obtained by conventional in-solution digestion of the proteins for 12 hours (Figure 2b; see also Figures S2b and S3b in the Supporting Information). Detailed identification results are listed in Tables S1, S2, and S3 of the Supporting Information.

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[**] This research was supported by the National Natural Science Foundation of China (Projects: 20875017, 21075022, and 20735005), the Technological Innovation Program of Shanghai (09JC1401100), the National Basic Research Priorities Program (Project: 2007CB914100/3), and the Shanghai Leading Academic Discipline Project (B109).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201004152.

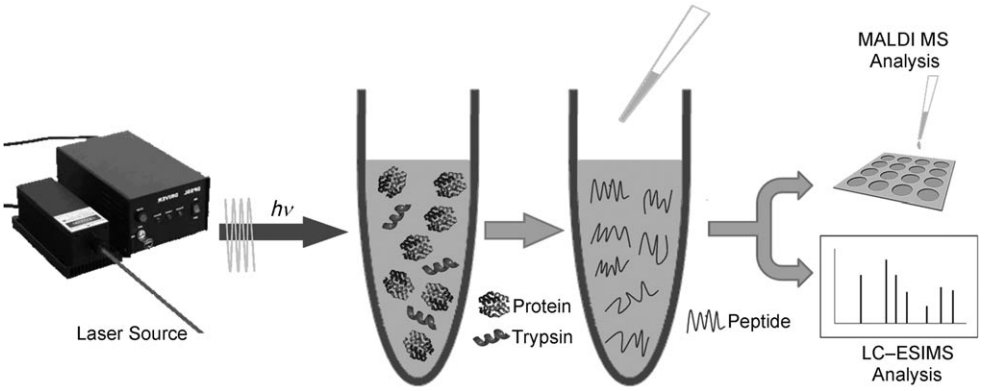

Figure 1. Schematic illustration of laser-assisted in-solution proteolysis.

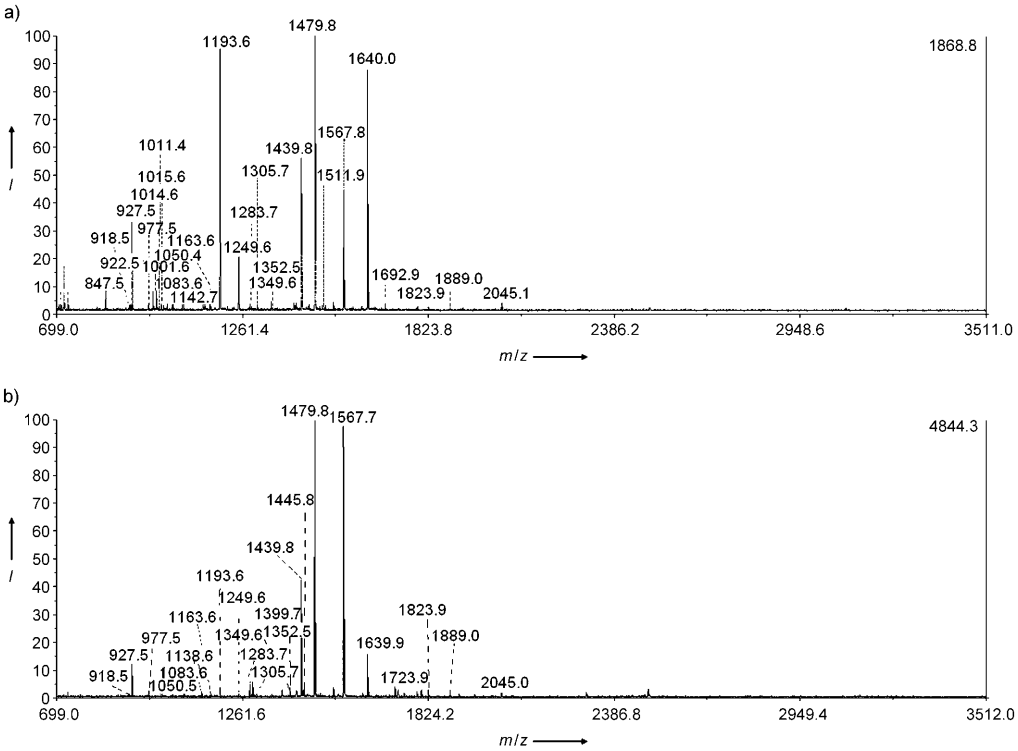

Figure 2. The MALDI mass spectra of digests of BSA obtained by a) laser-assisted in-solution digestion at 37 °C for 45 s (power: 5W) and b) conventional in-solution digestion at 37 °C for 12 h. The peaks of all matched peptides are labeled with mass values. Conditions: 1 μL trypsin (0.1 μg μL^{-1})/20 μL proteins (0.2 μg μL^{-1}). *I* = % intensity.

When laser (808 nm) radiation was used to accelerate in-solution digestion, the number of matched peptides and the sequence coverage increased from 23 to 28 and from 36 to 42% for BSA, from 12 to 14 and from 83 to 89% for myoglobin, and from 10 to 12 and from 81 to 83% for cytochrome *c* (Table 1). The results indicate that the efficiency of in-solution proteolysis was substantially enhanced by laser radiation, whereas the digestion time was significantly reduced from 12 hours for conventional in-solution digestion to less than 1 minute for the present laser-assisted (808 nm) digestion. Given the overlap in the results observed for these two different digestion methods in terms of matched peptide sequences, there can be no significant divergence in digestion selectivity between laser-assisted in-solution digestion and traditional digestion for 12 h (see Figure S4 in the Supporting Information).

To investigate the detection limit of this proposed approach, we conducted the laser-assisted in-solution digestion of cytochrome *c* at concentrations of 100, 50, and 25 ng μL^{-1} (see Figure S5 in the Supporting Information). Proteolysis was still efficient when the concentration of the protein was decreased to 25 ng μL^{-1} (see Figure S5c in the Supporting Information). If the protein concentration was less than 25 ng μL^{-1}, the mass-spectral peaks of the tryptic peptides of cytochrome *c* would be barely detectable. Four, six, and 11 tryptic peptides were matched, with a corresponding amino acid sequence coverage of 36, 56, and 77% for the three protein concentrations of 25, 50, and 100 ng μL^{-1}, respectively (see Table S4 in the Supporting Information). The results indicated that the proposed approach can be used for the fast digestion of proteins at low concentrations.

We next sought to demonstrate the feasibility of laser-assisted on-plate proteolysis. Following the laser-assisted on-plate digestion of BSA and myoglobin, MALDI mass spectra of the digests were obtained (see Figure S6 in the Supporting Information). Three replicate experiments were carried out for the optimal reaction time (see Figure S7 in the Supporting Information), and an average sequence coverage of 35% for BSA and 90% for myoglobin was obtained against the Swiss-Prot database (Table 1).

The intrinsic effects of laser irradiation on tryptic proteolysis are difficult to determine because of the photothermal effects resulting from high-power laser irradiation. To solve this problem, we chilled the reaction mixture in an ice bath to reduce the catalytic activity that arises from thermal

Table 1: Summary of MALDI-TOF MS results for digestion products obtained by different proteolysis methods.

Digestion method	Protein	Digestion time	Sequence coverage [%]	Peptides matched
laser-assisted in-solution	BSA	45 s	42	28
	myoglobin	30 s	89	14
	cytochrome *c*	30 s	83	12
conventional in-solution	BSA	12 h	36	23
	myoglobin	12 h	83	12
	cytochrome *c*	12 h	81	10
laser-assisted in an ice bath	BSA	45 s	26	15
laser-assisted on-plate	BSA	5 s	35	20
	myoglobin	5 s	90	13
laser-assisted in-gel	BSA	45 s	38	24
	HSA	45 s	26	19
conventional in-gel	BSA	16 h	37	25
	HSA	16 h	24	19

heating. MALDI mass spectra were recorded of BSA digested under laser irradiation in an ice bath (see Figure S8 in the Supporting Information). Three replicate experiments were carried out, and an average sequence coverage of 26% for BSA was obtained from the database (Table 1). This result demonstrates that lasers can trigger significant effects on tryptic proteolysis. The observed specific laser effect most likely results from overtones of the vibration, stretching, and bending of C–H, C–C, N–H, C–O, and O–H bonds in protein chains upon irradiation with the near-infrared laser.[27,28]

To further confirm the feasibility of laser irradiation at 808 nm for the analysis of complex protein mixtures, we applied this method to rat-brain extract. After reduction and alkylation, the brain extract was digested by trypsin enzyme for only 1 minute and directly subjected to LC–MS/MS(ESI) (the resulting total-ion chromatogram is shown in Figure S9 of the Supporting Information). After a database search according to the SEQUEST criteria (see the Supporting Information), 758 peptides and 134 proteins were identified with $p < 0.01$ (see Table S5 in the Supporting Information). The MS/MS spectrum corresponding to the ion at m/z 2797.34 in the precursor mass scan at 139.23 minutes (see Figure S10 in the Supporting Information) is shown in Figure 3. Most y ions together with b ions produced from the precursor ion matched together and resulted in the high reliability observed for the peptide sequence SGPFGQIFRPDNFVFGQS-GAGNNWAK. These results clearly show that this novel digestion approach can be used for large-scale proteomic analysis.

Having demonstrated the suitability of laser irradiation for in-solution digestion, we next sought to demonstrate that laser irradiation could enhance the efficiency of in-gel proteolysis. After SDS-PAGE (see Figure S11 in the Supporting Information), the target protein bands were excised, cut into small pieces, destained, dehydrated, and then rehydrated in trypsin solution at 4°C for 30 minutes. Subsequent laser-assisted in-gel digestion involved digestion of the treated gel pieces in transparent Eppendorf tubes with the assistance of an 808 nm laser.

Figure 4a shows the MALDI mass spectrum of the extracted digests of 1 μg of BSA ($1\ \mu g\,\mu L^{-1} \times 1\ \mu L$) from gel pieces obtained by laser-assisted in-gel digestion for 45 s. The identified peptide residues obtained are presented in Table S6 of the Supporting Information. A total of 24 tryptic peptides were found to match to BSA, with a corresponding amino acid sequence coverage of 38%. Conventional in-gel tryptic digestion gave a comparable result but required a reaction time of 16 hours (Figure 4b; see Table S6 in the Supporting Information).

However, over the same irradiation time, the protein-digestion efficacy of laser-assisted in-gel digestion was not as good as that of laser-assisted in-solution digestion. This phenomenon may mostly depend on the specific transmission mechanism of near-IR light: laser light (808 nm) can better penetrate samples with a higher water content, and consequently, photons of the 808 nm laser can excite the vibration of molecules of liquid samples more efficiently. It is therefore reasonable to assume that a longer irradiation time (e.g. 5 min or even longer) would result in higher efficiency of laser-assisted in-gel proteolysis.

The suitability of laser-assisted in-gel proteolysis for the study of complex proteins was demonstrated by digesting HSA separated from human serum by SDS-PAGE. The protein concentration of the sample was $60.0\ mg\,mL^{-1}$ according to the modified Bradford method. The weight percentage of HSA in the total protein content of human serum is in the range of 53.3–70.5%.[29] The darkest protein band on the polyacrylamide gel after SDS-PAGE of diluted human serum ($1\ \mu g\,\mu L^{-1} \times 2\ \mu L$) and Coomassie blue staining (see Figure S11b in the Supporting Information) was excised, digested, extracted, and finally identified by MALDI-TOF MS (see Figure S12a in the Supporting Information). A total of 19 peptides (see Table S7 in the Supporting Information) were found to match to HSA with a sequence coverage of 26%, and the darkest band on the gel in Figure S11b was identified to be Coomassie blue stained HSA. The results were comparable to those obtained by conventional in-gel tryptic digestion, which required a reaction time of 16 hours (see Figure S12b and Table S7 in the Supporting Information).

In summary, we have demonstrated that laser-assisted proteolysis coupled with MALDI-TOF MS is a promising strategy for efficient protein digestion and peptide mapping. This laser-assisted approach accelerated in-solution/in-gel digestion and on-plate proteolysis to the extent that they required only seconds. The efficacy of protein digestion was comparable to that observed with traditional methods. Furthermore, this laser-assisted digestion protocol ensured fast and efficient results even at low protein concentrations of only $25\ ng\,\mu L^{-1}$. The successful digestion of complex samples, such as rat-brain extract, by laser-accelerated proteolysis indicates the strong potential of this straightforward, fast, efficient, and low-cost approach in high-throughput proteome analysis.

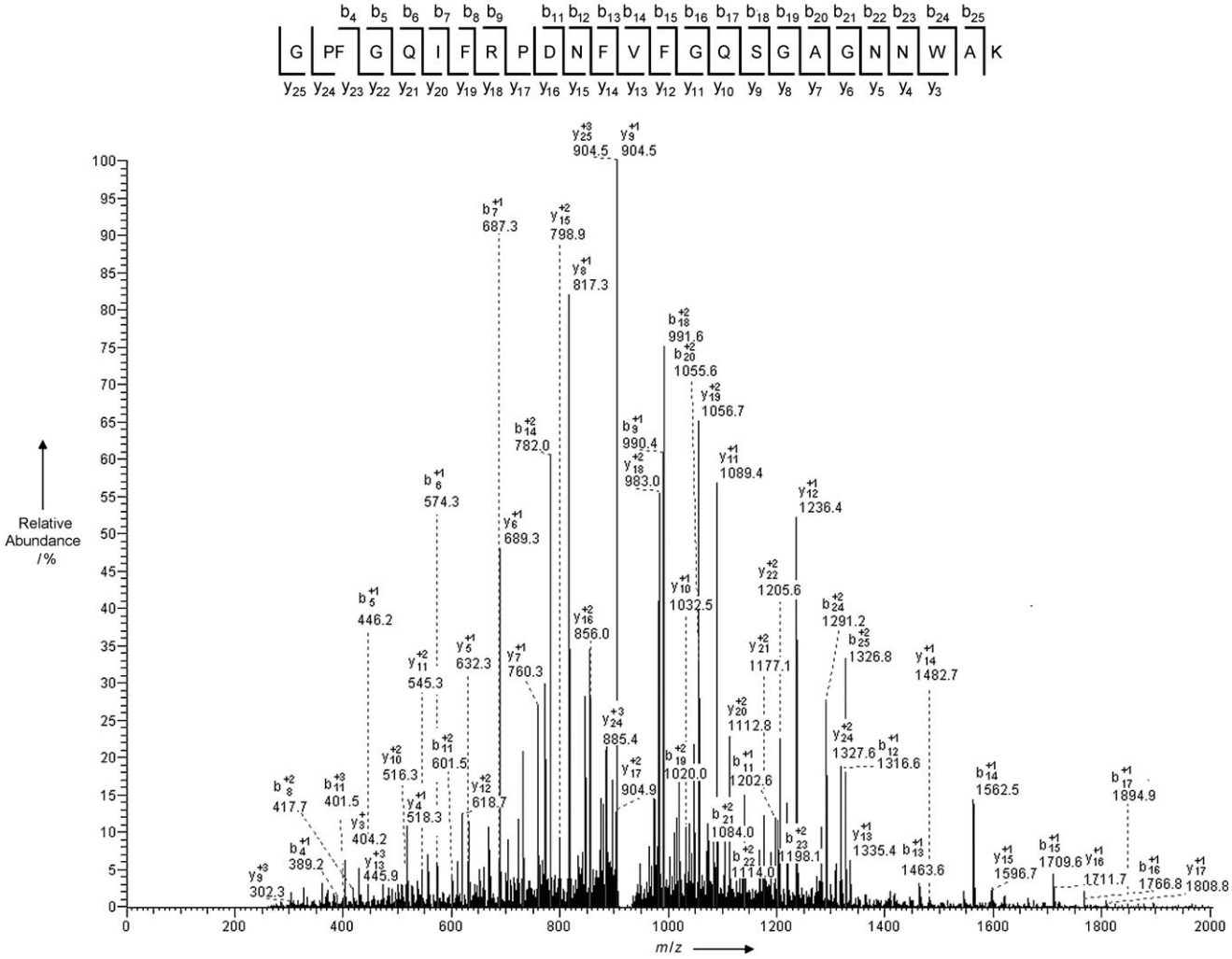

Figure 3. MS/MS spectrum of the peak at 2797.34 *m*/*z* in the precursor mass scan at 139.23 min of rat-brain extract after laser-assisted digestion by trypsin for 1 min (see Figure S10 in the Supporting Information). The sequence SGPFGQIFRPDNFVFGQSGAGNNWAK could be confirmed to a high level of certainty on the basis of b ions and y ions.

Experimental Section

Healthy rat brain was provided by Zhongshan Hospital, Fudan University. The rat-brain tissue was cut into small pieces and cleaned with cold physiological saline solution (0.9% NaCl) to remove blood and other contaminants. The tissue debris were then suspended in the protein-extraction buffer (9.0 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM dithiothreitol, 1.0 mM phenylmethanesulfonyl fluoride). Lysis was performed by homogenization on ice and aided by vortexing for 30 min. The lysate was centrifuged at 18000 *g* for 15 min at 4 °C. The supernatant was collected, fractionated in aliquots, and stored at −80 °C until further analysis. Protein concentration was measured by using the Bradford assay with BSA as a standard (13 $\mu g\,\mu L^{-1}$) for rat-brain tissue.

SDS-PAGE was performed on 1 mm thick 12% 2-amino-2-hydroxymethylpropane-1,3-diol (Tris)–glycine polyacrylamide mini-gels loaded with 1–4 μg of protein. Following electrophoresis, the gel slab was stained with colloidal Coomassie blue for 4 h and then destained in water overnight. Protein gel bands of interest were excised, cut into small cubes (1 mm^3), and then subjected to a repeated washing and dehydration cycle (incubation for 20 min with 50 mM ammonium bicarbonate solution (200 μL) containing 50% (v/v) acetonitrile (ACN), removal of the solution, incubation for 5 min with ACN (100 μL), removal of ACN). Next, the dehydrated gel cubes were rehydrated in 50 mM ammonium bicarbonate solution (20 μL) containing trypsin (25 $ng\,\mu L^{-1}$) and incubated at 4 °C for 30 min.

Laser-assisted in solution/in-gel digestion was performed by the digestion of protein solutions containing trypsin (1:40) or trypsin-treated gel pieces in Eppendorf tubes with the aid of laser radiation (Figure 1). The distance between the fiber lens and the liquid surface was fixed at 4 cm. After laser-assisted in-solution proteolysis, the sample solution was deposited directly on a MALDI plate. After laser-assisted in-gel proteolysis, tryptic peptides were recovered by repeated extraction (3×) with 50% ACN aqueous solution /0.1% trifluoroacetic acid (TFA; v/v, 100 μL). The peptide extracts were lyophilized and dissolved in 50% ACN aqueous solution /0.1% TFA (10 μL) for analysis by mass spectrometry.

Received: July 7, 2010
Published online: September 24, 2010

Keywords: lasers · mass spectrometry · proteolysis · proteomics

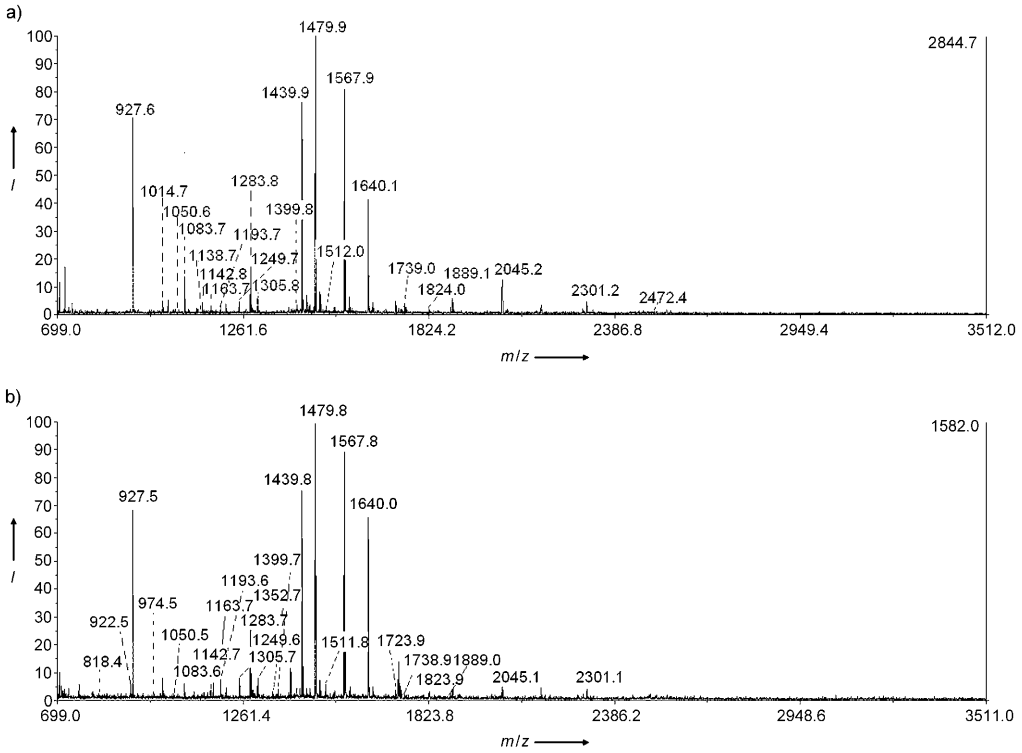

Figure 4. MALDI mass spectra of the extracted digests of 1 μg of BSA bands (1 μg μL^{-1} × 1 μL) from gel pieces obtained by a) laser-assisted in-gel digestion at 37 °C for 45 s and b) conventional in-gel digestion at 37 °C for 16 h. The peaks of all matched peptides are labeled with mass values.

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