

Application of MALDI-TOF-Mass Spectrometry to Proteome Analysis Using Stain-Free Gel Electrophoresis

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Abstract The combination of MALDI-TOF-mass spectrometry with gel electrophoretic separation using protein visualization by staining procedures involving such as Coomassie Brilliant Blue has been established as a widely used approach in proteomics. Although this approach has been shown to present high detection sensitivity, drawbacks and limitations frequently arise from the significant background in the mass spectrometric analysis. In this chapter we describe an approach for the application of MALDI-MS to the mass spectrometric identification of proteins from one-dimensional (1D) and two-dimensional (2D) gel electrophoretic separation, using stain-free detection and visualization based on native protein fluorescence. Using the native fluorescence of aromatic protein amino acids with UV transmission at 343 nm as a fast gel imaging system, unstained protein spots are localized and, upon excision from gels, can be proteolytically digested and analyzed by MALDI-MS. Following the initial development and testing with

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standard proteins, applications of the stain-free gel electrophoretic detection approach to mass spectrometric identification of biological proteins from 2D-gel separations clearly show the feasibility and efficiency of this combination, as illustrated by a proteomics study of porcine skeleton muscle proteins. Major advantages of the stain-free gel detection approach with MALDI-MS analysis are (1) rapid analysis of proteins from 1D- and 2D-gel separation without destaining required prior to proteolytic digestion, (2) the low detection limits of proteins attained, and (3) low background in the MALDI-MS analysis.

Keywords Gel electrophoresis · MALDI-TOF-mass spectrometry · Native fluorescence · Protein identification · Skeleton muscle proteomics

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Abbreviations

1D	One-dimensional gel electrophoresis
2D	Two-dimensional gel electrophoresis
MALDI-TOF	Matrix assisted laser desorption/ionization–time-of-flight
MS	Mass spectrometry
PMF	Peptide mass fingerprinting
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

1 Introduction

Matrix assisted laser desorption/ionization–mass spectrometry (MALDI-MS), introduced by Karas and Hillenkamp in 1988 [1, 2], is now widely used in proteomics studies. Initially developed for the ionization of large polypeptides

and proteins [3], applications of MALDI-MS have significantly broadened and incorporated glycoproteins, oligonucleotides, carbohydrates, and small biomolecules [4]. MALDI is referred to as a “soft” ionization technique, because it causes minimal or no fragmentation and allows the molecular ions of analytes to be identified, even in complex mixtures of biopolymers. The ionization–desorption principle of MALDI-MS is based on the co-crystallization of analytes with an organic, light-absorbing matrix (e.g., α -cyano-4-hydroxy cinnamic acid or sinapinic acid) which, when activated by a laser, ionizes the analyte as it enters the gas phase. The ions once formed are accelerated in an electric field and separated according to their mass-to-charge ratio (m/z) in the mass spectrometer analyzer. Typically, MALDI is coupled with time-of-flight (TOF) analyzers that determine the mass of intact biopolymers.

The most common lasers used in MALDI-MS are ultraviolet (UV) lasers. Most of the commercially available MALDI mass spectrometers are equipped with nitrogen lasers ($\lambda = 337$ nm) which are used as the standard device, although Nd:YAG lasers ($\lambda = 266$ or 355 nm) are also employed. MALDI-MS can also use infrared (IR) lasers such as Er:YAG lasers ($\lambda = 2.94$ μ m) or CO₂ lasers ($\lambda = 10.6$ μ m), and thus can be employed in applications to proteome analysis [5, 6].

MALDI-TOF-MS is a well established method in peptide and protein analysis because of its robust, simple operation and high sensitivity, and the coupling of MALDI-TOF as well as high resolution analyzers, such as FTICR with gel electrophoretic separation has enabled successful protein identifications in recent years [7–14]. The sequence of steps in a typical proteomics experiment is schematically outlined in Fig. 1: (1) first, proteins of interest from a biological mixture are separated by one-dimensional (1D) or two-dimensional (2D) gel electrophoresis; (2) following the gel electrophoretic separation, proteins are visualized using a staining procedure; (3) the protein bands (spots) of interest are excised from the gel and digested by a protease of high specificity (e.g., trypsin); (4) the resulting mixture of proteolytic peptides is analyzed by MALDI-MS yielding a peptide mass map; and (5) identification of proteins is obtained by searching for the best match between the experimentally determined masses of the peptide map and peptide masses calculated from theoretical cleavage of proteins in an appropriate sequence database [15].

In order to visualize proteins separated by gel electrophoresis a number of techniques have been developed in recent years. Most mass spectrometric proteomics studies employ staining procedures with such as Coomassie Brilliant Blue or silver salts, but fluorescent dyes of high detection sensitivity have also been used (Flamingo, SYPRO[®] Ruby) [16–18]. Although several of these approaches provide high sensitivity and are easy to use, major problems are frequently encountered with the compatibility of staining procedures with the mass spectrometric analysis [19]. A more recently explored alternative to omit the use of dyes in the visualization procedure has been the development of methods based on the fluorescent properties of proteins [20, 21]. During fluorescence labeling studies of glycoproteins, Zhao and co-workers observed a fluorescent signal for non-glycosylated proteins such as hen eggwhite lysozyme, which was attributed to

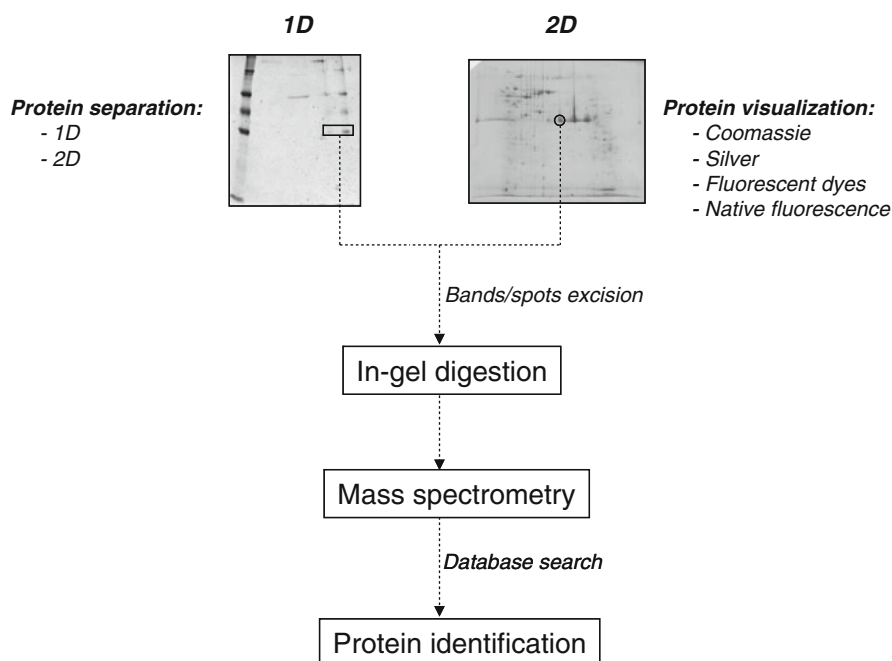


Fig. 1 Scheme of the steps involved in a proteomics experiment. After gel separation (1D or 2D) proteins are visualized by staining methods such as Coomassie, silver, and fluorescent dyes, or by “stain-free” native fluorescence. Protein spots are excised from gels, digested with trypsin, and digestion mixtures are analyzed by MALDI-MS

intrinsic (native) protein fluorescence [22]. A first detection method for unstained proteins based on UV fluorescence was developed by Roegerer et al. who used laser excitation with 280 nm UV light and demonstrated protein visualization in both 1D- and 2D-gel separations with detection limits in the low nanogram range (1–5 ng) [23]. Recently, a commercial gel-analyzer based on native fluorescence has been developed (LaVision-BioTec; Bielefeld, Germany) and employed in the present study.

In this work we have developed and applied native fluorescence detection of proteins in stain-free one- and two-dimensional gel electrophoretic separations as a sensitive and efficient approach for mass spectrometric identifications in proteome analysis. In initial testing experiments 1D-gels of model proteins were analyzed to investigate (1) the relation between fluorescence intensity observed and the relative amounts of aromatic amino acids in proteins, (2) detection sensitivity of the native fluorescence in comparison with Coomassie and silver staining sensitivities, and (3) the applicability of native fluorescence detection to mass spectrometric protein identification. In a second part, the stain-free gel bioanalyzer was successfully employed in applications to porcine skeleton muscle proteomics, providing

identifications of proteins at high detection sensitivity, without the need for staining and destaining isolated protein bands.

2 Methods

2.1 Protein Separation by Gel Electrophoresis

Model proteins used for evaluation in the stain-free gel bioanalyzer were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 1-mm gels using the standard Laemmli method with a Mini-PROTEAN[®]3 cell gel system (Bio-Rad, München, Germany). Myoglobin, ubiquitin, bovine serum albumin (BSA), carbonic anhydrase, lysozyme, and α -casein were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Pepsin was from Fluka Chemie GmbH (Buchs, Switzerland), and human γ -globulin was from Merck4Biosciences (Darmstadt, Germany).

Porcine skeleton muscle samples for 2D-gel separations were prepared as previously described [24] (the samples were isolated from *Longissimus dorsi* muscle and were provided by the Department of Animal Breeding, University of Bonn, Germany). Samples of 800 μ g total protein were applied for 12 h on 17-cm IPG strips (pH 3–10) using a passive in-gel rehydration method. Isoelectric focusing (IEF) was carried out using a Multiphor horizontal electrophoresis system (Amersham Biosciences, München, Germany). For the second separation step, the Bio-Rad Protean-II-xi vertical electrophoresis system was used, and 12.5% SDS-PAGE gels of 1.5 mm thickness were prepared. Electrophoresis was performed in two steps: (1) 25 mA/gel for approximately 30 min, and (2) 40 mA/gel until the dye front reached the anodic end of the gels. All buffers and solutions used for 2D-gel electrophoresis have been described elsewhere [7].

2.2 Gel Bioanalyzer for Protein Detection and Visualization

Proteins separated by 1D- or 2D-gel electrophoresis were visualized with the gel bioanalyzer (LaVision-Biotec, Bielefeld, Germany; <http://www.lavisionbiotec.com/en/microscopy-products/gelreader/>). The experimental setup of the gel bioanalyzer is based on a UV excitation source and a detection system within the UV range. The UV excitation light was generated by a 300-W xenon lamp (265–680 nm). The irradiation area was set to 1 cm² at 35 mW/cm² and imaged by three lenses onto a photomultiplier detector. A UV bandpass filter (280–400 nm) is incorporated to block the excitation light from the detection system. From four filter positions (one for UV excitation, three for visible fluorescence), the UV filter transmitting light at $\lambda = 343 \pm 65/2$ nm was employed. The large reading area

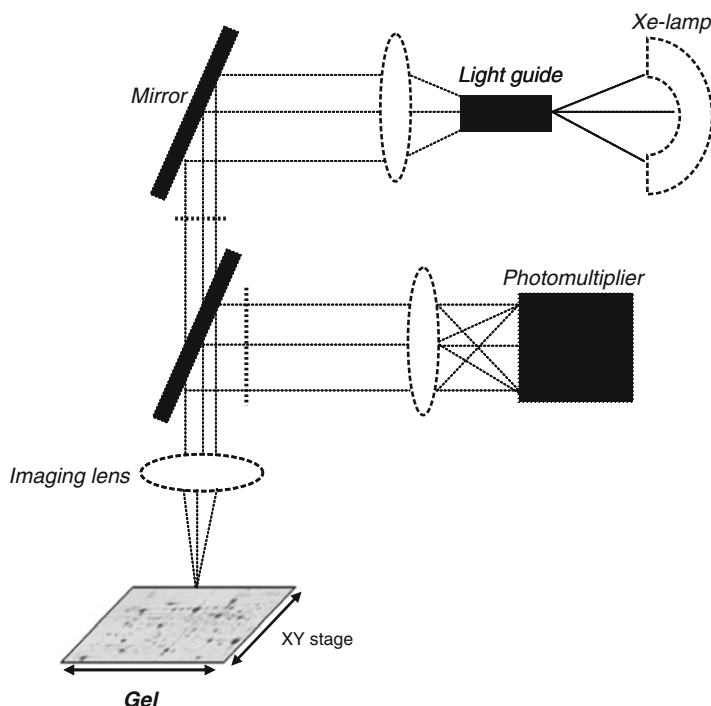


Fig. 2 Scheme of the gel bioanalyzer (LaVision-Biotec, Bielefeld, Germany), modified after <http://www.lavisionbiotec.com/en/microscopy-products/gelreader/>

($30 \times 35 \text{ cm}^2$) provided scanning of both 1D- and 2D-gels. The instrument has a removable gel tray and is equipped to read unstained as well as stained protein gels (Fig. 2). In the present study only scanning of unstained gels was performed. High precision polycarbonate tools for localization and isolation of protein spots were prepared in our Laboratory [7]. Following fixation in position on the gel tray, localization and excision of gel spots was carried out by moving the gel tray, with positioning and scanning of the gel controlled by the LaVision-Biotec scanning software. Using polycarbonate tools, different small holes were made in the scanned gel in order to isolate the protein bands (four fine holes were necessary for the localization of each protein band), and upon gel ejection from the gel bioanalyzer bands were excised with a scalpel.

2.3 In-Gel Proteolytic Digestion

Following detection, visualization, and localization, spots were manually excised from the gels and subjected to in-gel trypsin digestion according to Mortz et al. [25].

No destaining steps were required, since no visible staining procedure was used. The complete protocol has been previously described [7]. The resulting supernatant and elution fractions were combined and lyophilized to dryness for mass spectrometric investigation.

2.4 MALDI-TOF-Mass Spectrometry

MALDI-TOF-MS was carried out with a Waters VG-Micromass TOFSpec-2DE mass spectrometer (Waters Micromass, Manchester, UK) equipped with a nitrogen UV laser (337 nm), channel plate detector, and MASSLynx 4.0 data system for spectra acquisition and instrument control. A saturated solution of α -cyano-4-hydroxy-cinnamic acid (HCCA) in acetonitrile/0.1% trifluoroacetic acid in water (2:1 v/v) was used as the matrix. Aliquots of 0.8 μ L of the sample solution and saturated matrix solution were mixed on the stainless steel MALDI target and allowed to dry. Acquisition of spectra was carried out at an acceleration voltage of 20 kV.

2.5 Database Search

Digestion mixtures determined by MALDI-MS were directly used for a database search employing the MASCOT peptide mass fingerprinting (PMF) search engine (<http://www.matrixscience.com>), employing search and acceptance criteria for protein identification as follows: 0.5–1.2 Da mass error tolerance; two missed cleavage sites permitted; methionine oxidation as variable modification; carbamidomethyl (cysteine) as fixed modification. The database employed was NCBI nr 20060712 (3,783,042 sequence entries, 1,304,471,729 residues), a compilation of several databases including SWISS-PROT, PIR, PRF, PDB, and GenBank CDS translations.

3 Results and Discussion

3.1 Evaluation of Stain-Free Native Fluorescence for Protein Detection and Visualization

Conventional staining procedures used to visualize proteins within gel electrophoretic separations present a number of problems, such as high background and compatibility problems with mass spectrometry procedures (e.g., solvents) [19], high costs of fluorescent dyes, and extensive analysis times required for staining

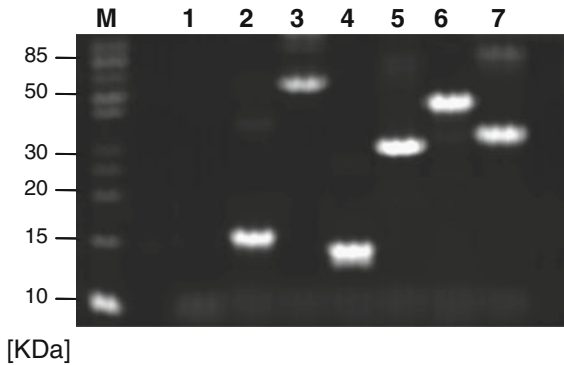


Fig. 3 Native fluorescence visualization and detection for a 15% SDS-PAGE separation. Fluorescence intensity depends on the amount of aromatic amino acids in proteins (see Table 1 for aromatic amino acid and tryptophan amounts, given in pmol). *M* – molecular weight marker (Fermentas; 10–200 kDa; 5 μ L); (1) ubiquitin (5 μ g); (2) myoglobin (5 μ g); (3) bovine serum albumin (BSA; 5 μ g); (4) lysozyme (5 μ g); (5) carbonic anhydrase (5 μ g); (6) pepsin (5 μ g); (7) α -casein (5 μ g)

Table 1 Tryptophan and aromatic amino acid amounts (pmol) in seven different proteins. Proteins were separated in 15% SDS-PAGE gels (see Fig. 3). Fluorescence intensity values exhibited by these proteins and protein amounts (pmol) applied on each band are also listed

Lane	Protein	MW (Da)	Protein amount/ band (pmol)	Amount of aromatic aa/ band (pmol)	Amount of tryptophan/ band (pmol)	Fluorescence intensity (no. of counts)
1	Ubiquitin	8,565	583.8	22.8	0	2,816
2	Myoglobin	17,070	292.9	20.8	3.8	16,820
3	BSA	69,239	72.2	6.4	0.4	10,381
4	Lysozyme	14,309	349.4	32.5	16.4	19,028
5	Carbonic anhydrase	29,100	171.8	17.2	4.6	21,086
6	Pepsin	41,300	121.1	12.4	3.3	19,132
7	α -Casein	26,019	192.2	20	1.7	14,081

aa Amino acids

and destaining of gels [25]. The native fluorescence of a protein is a composite of the fluorescence from individual aromatic residues. Most of the native fluorescence emission of a protein is due to tryptophan residues, with minor contribution from tyrosine and phenylalanine residues. In order to characterize the native fluorescence contributions, several model proteins with different contents of aromatic amino acids (percentage of Trp) were separated by gel electrophoresis using 15% SDS-PAGE, and gels were scanned with the gel-bioanalyzer (Fig. 3, Table 1). From each protein 5 μ g were applied on the gel. In the gel shown in Fig. 3, ubiquitin (lane 1) which has only three aromatic amino acids (22.8 pmol) and no tryptophan gave only a weak fluorescence signal (see Table 1). When comparing fluorescence intensities for carbonic anhydrase (lane 5) and α -casein (lane 7), both having similar molecular weights and similar contents of aromatic amino acids (carbonic

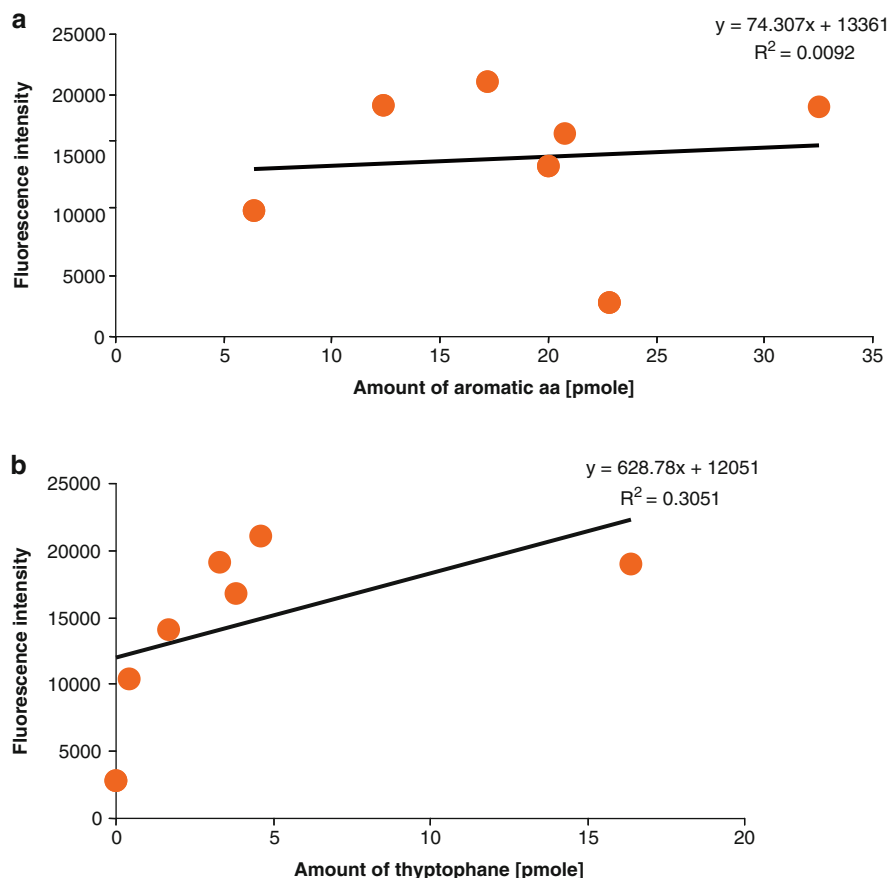


Fig. 4 (a) Linear regression for the investigation of the contribution of aromatic amino acid amounts (pmol) to the fluorescence signal intensity. (b) Linear regression for the investigation of the contribution of tryptophan amounts (pmol) to the fluorescence signal intensity. Fluorescence intensity values, aromatic amino acid amounts, and tryptophan amounts (pmol) correspond to the proteins separated in the gel presented in Fig. 3 (see Table 1)

anhydrase – 17.2 pmol; α -casein – 20 pmol) but different contributions of tryptophan to the aromatic protein content (4.6 pmol for carbonic anhydrase which represents 26.7% tryptophan contribution to the total aromatic protein amount; 1.7 pmol, 8.5%, respectively, for α -casein), a higher fluorescence intensity signal was observed for carbonic anhydrase (see Table 1 for fluorescence intensity values). Another comparison was made between myoglobin (lane 2) and lysozyme (lane 4). Lysozyme with 50.5% tryptophan contribution to the fluorescence signal (32.5 pmol aromatic amino acids and 16.4 pmol tryptophan) showed a higher fluorescence than myoglobin (18.3% tryptophan contribution to the fluorescence signal; 20.8 pmol aromatic amino acids and 3.8 pmol tryptophan) (Fig. 3,

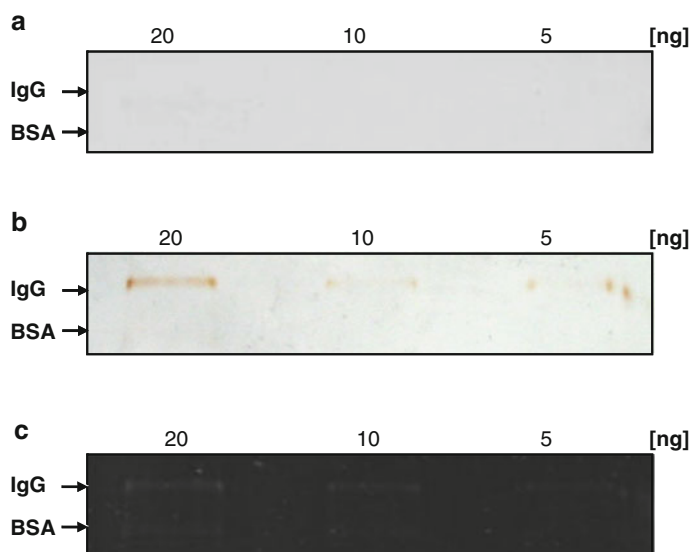


Fig. 5 Sensitivity of stain-free fluorescence detection and visualization in comparison with Coomassie and silver visualizations. Protein samples, IgG (150 kDa heavy and light chain dimer) and BSA (67 kDa) were separated in 3 lanes at 20–5 ng. Gel areas presented are zoomed regions from 12% SDS-PAGE separations. (a) Coomassie stained gel; (b) silver stained gel; (c) native fluorescence gel

Table 1). These results clearly illustrate the dependence of fluorescence intensity on the amounts of tryptophan and other aromatic amino acid residues in proteins.

Furthermore, linear regression was used in order to find the relationship between the amount of aromatic amino acids and tryptophan (given in pmol) towards the detection signal intensity of the proteins separated in the gel from Fig. 3 (Fig. 4a, b).

A further step in the evaluation of the stain-free native fluorescence detection method was to test its sensitivity. Sensitivity tests were performed with 1D-gel separations of mixtures of two model proteins, immunoglobulin-G and BSA, which were scanned with the gel bioanalyzer (Fig. 5c) at concentrations of 20–5 ng/band and compared with gels prepared at identical conditions but visualized using standard staining procedures (Coomassie – Fig. 5a and silver – Fig. 5b). These results showed comparable sensitivities for the UV fluorescence detection and silver staining, with detection limits of approximately 1–5 ng [7]. The detection limit in the low nanogram range is in good agreement with sensitivity data reported by Roegenier et al. [23].

In another set of experiments it was shown that the fluorescence intensity of the protein bands in 1D-gels increases linearly as the protein amount, the amount of aromatic amino acids, and the mass of tryptophan increases, as shown in Fig. 6a–d.

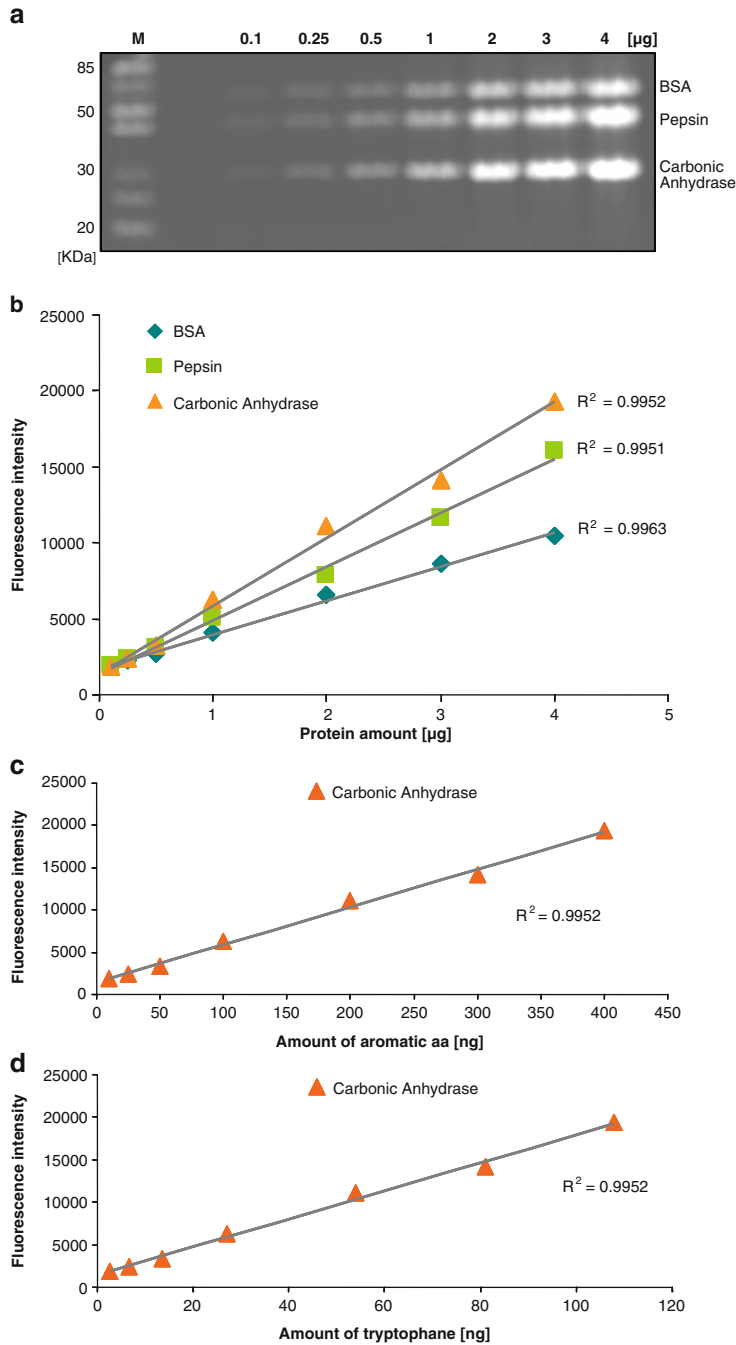


Fig. 6 (a) 15% SDS-PAGE separation of a protein mixture (bovine serum albumin – BSA; pepsin; carbonic anhydrase) at different concentrations (0.1–4 µg). Proteins were visualized by native fluorescence using the gel bioanalyzer instrument. Fluorescence intensity increases linearly with protein concentration (b), with the amount of aromatic amino acids (c), and with the content of tryptophan in a protein band (d). *M* – molecular weight marker (Fermentas; 5 µL)

Fifteen percent SDS-PAGE was used to separate a protein mixture (BSA; pepsin; carbonic anhydrase) at different concentrations (0.1–4 μg) (Fig. 6a). Proteins were visualized by native fluorescence using the gel bioanalyzer instrument. All the proteins in the gel were detectable at 0.1 μg . UV-fluorescence detection offers a linear dynamic range from 0.1 to 4 μg with a correlation coefficient of 0.99 (Fig. 6b–d).

3.2 Application of Stain-Free Native Fluorescence Detection to MALDI-TOF-MS Identification of 1D-Gel Separated Proteins

Following development and optimization, the stain-free detection method in gel electrophoresis was subjected to mass spectrometric identifications of 1D-gel separated proteins. The identification of horse heart myoglobin (5 $\mu\text{g}/\text{gel}$) from an unstained gel (see Fig. 3, lane 2) was successfully achieved. After gel bioanalyzer scanning the protein band was excised, subjected to in-gel digestion with trypsin, and the digestion mixture analyzed by MALDI-TOF-MS. The resulting masses were used for a database search with the MASCOT PMF search engine, and provided unequivocal identification of horse heart myoglobin with 18 identified peptides (data not shown). Since no destaining step was required for the in-gel digestion, high sensitivity and considerably lower sample preparation time were needed compared to conventional Coomassie staining. Identifications were obtained with significantly lower protein amounts used for 1D-gel separation, with a score of 86 (64% sequence coverage) and 320 ng protein band (Fig. 7a). For comparison reasons the same amount of myoglobin (320 ng) but from a gel stained with Coomassie was used and led to protein identification with a score of 117% and 82% sequence coverage (Fig. 7b). In summary, these model studies suggested that gel separation of proteins with native fluorescence detection represents an efficient and sensitive approach for MALDI-MS identification in proteomics.

3.3 Application of Stain-Free Native Fluorescence to Mass Spectrometric Proteome Analysis of Porcine Muscle Tissue

In subsequent proteomics studies, the stain-free detection approach was successfully applied to protein identifications from 2D gels by MALDI-TOF-MS (Fig. 8). Examples of proteome analyses of porcine muscle skeleton proteins isolated post-mortem using the stain-free gel bioanalyzer are summarized in Figs. 9, 10, and 11. The rate and extent of post-mortem metabolic processes of skeleton muscle proteins have recently found increasing interest, and it is generally believed that structural

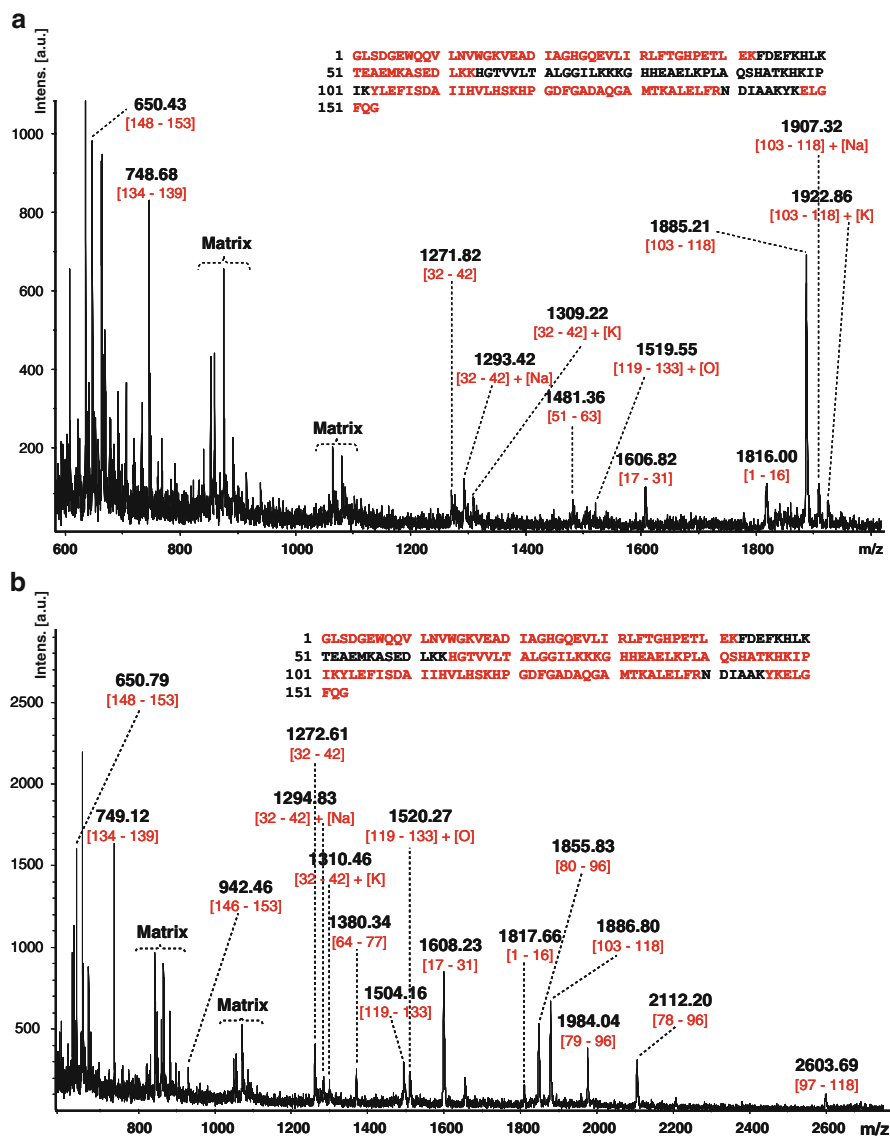


Fig. 7 MALDI-TOF-mass spectrometric identification of horse heart myoglobin (320 ng) from a stain-free gel (a) and from a Coomassie stained gel (b)

changes such as degradation and oxidation forms post-mortem may be indicative as biomarkers and affect meat properties [26]. Thus, tenderization processes have been associated with calpains and calpain inhibitors, calpastatins, that potentially influence proteolytic changes, and with proteins involved in carbonylation that may be potential oxidation biomarkers [27–29].

Fig. 8 The 2D-gel of a post-mortem porcine muscle sample (12.5% SDS-PAGE; 800 µg total protein per gel) was visualized by native fluorescence. Spots 1–6 were excised from the gel, digested with trypsin, and used for protein identification

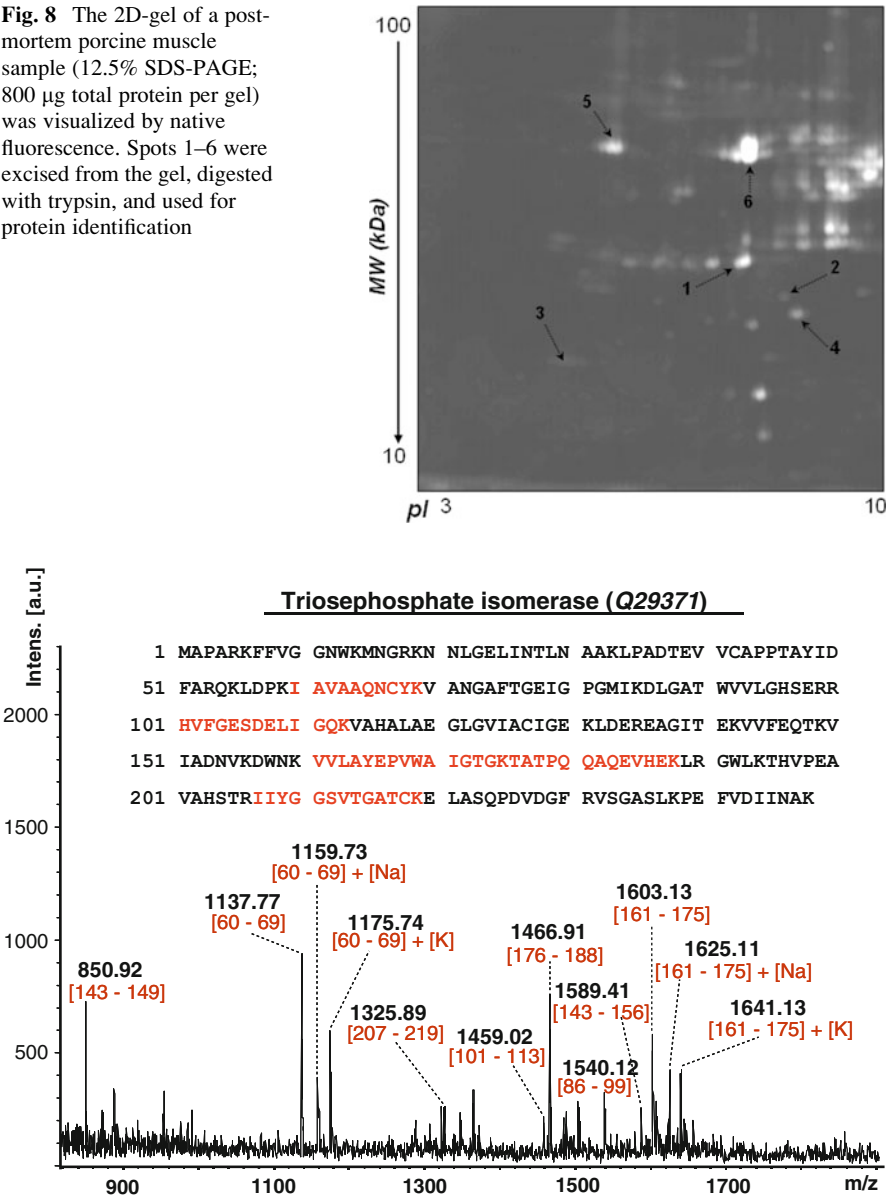


Fig. 9 MALDI-TOF-mass spectrum of the digestion mixture of spot number 1 (see Fig. 8). Labeled peaks correspond to the identified peptides from porcine skeletal triosephosphate isomerase (identified peptides are shown in red in the amino acid sequence of the protein)

A total amount of 800 µg was used for the 2D-gel electrophoretic separation of porcine muscle proteins (see Fig. 8). The gel was scanned with the gel bioanalyzer and proteins to be analyzed by MALDI-TOF-MS were excised using high-precision

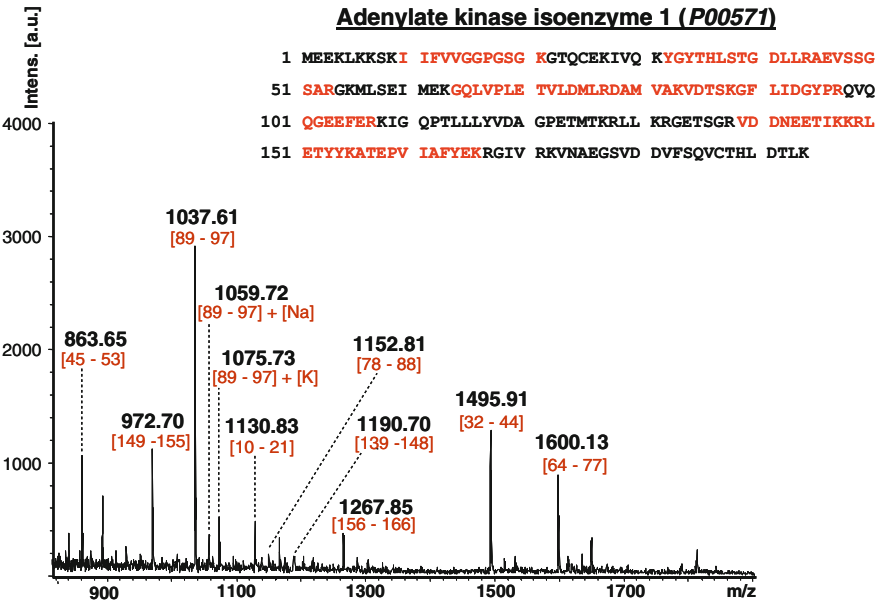


Fig. 10 MALDI-TOF-mass spectrometric identification of porcine adenylate kinase isoenzyme 1. Following gel reader visualization, spot 2 (from Fig. 8) was excised, in-gel digested with trypsin and analyzed by MALDI-MS. Labeled peaks denote the identified peptides (identified peptides are also shown in the amino acid sequence of the protein)

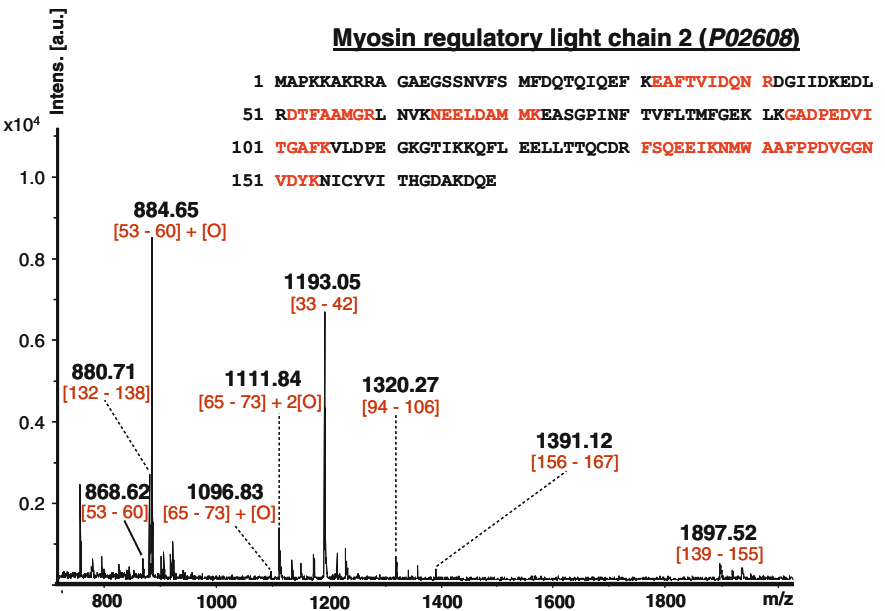


Fig. 11 Mass spectrometric identification of spot 3 (from Fig. 8) as porcine myosin regulatory light chain 2 (with labels for the identified peptides). Identified peptides are denoted in the amino acid sequence

Table 2 Protein identifications in proteome application to post-mortem porcine muscle sample. After native fluorescence visualization and localization, spots 1–6 were excised from 2D-gel (see Fig. 8), in-gel digested with trypsin, and measured by MALDI-TOF-mass spectrometry. Upon database search, proteins were successfully identified

Spot no. ^a	Protein	Score	No. of identified peptides	Sequence coverage (%)	Accession no. ^b
1	Triosephosphate isomerase	78	12	28	Q29371
2	Adenylate kinase isoenzyme 1	98	9	49	P00571
3	Myosin regulatory light chain 2	76	7	44	P02608
4	Alpha-crystallin	70	6	29	P02470
5	Skeletal alpha actin	92	13	70	P68137
6	Creatine kinase M chain	78	20	90	Q5XLD3

^aSpot numbers correspond to the 2D-gel shown in Fig. 8

^bAccession numbers are from SWISS-PROT or TrEMBL database

spot-picking tools [7]. Following tryptic digestion of isolated gel spots, the MALDI-MS analysis provided unequivocal identifications of several proteins, as summarized in Table 2. Figure 9 shows the identification of triosephosphate isomerase from spot 1 (see Fig. 8). Nine peptides (labeled in Fig. 10) provided unambiguous identification of adenylate kinase isoenzyme 1 (spot 2 in Fig. 8), while myosin regulatory light chain 2 was identified from spot 3 based on seven peptides (Figs. 8 and 11). From the proteins identified, alpha-actin (spot 5), creatine kinase M (spot 6), and myosin regulatory light chain 2 (spot 3) showed modifications by oxidation (Table 2, Fig. 11) [24].

4 Concluding Remarks

In this study we show stain-free detection and visualization of proteins in gels using native protein fluorescence as an efficient and sensitive approach for MALDI-mass spectrometric proteome analysis. The stain-free gel bioanalyzer enabled the detection and MALDI-MS identification of proteins from gel spots at detection limits in the low nanogram range, comparable to silver staining. Moreover, this approach does not require any post-electrophoretic manipulation by destaining, thus enabling direct MALDI-MS analysis with reduced background and time needed for sample preparation. The use of fluorescence detection with two-dimensional gel electrophoresis should be feasible for the development of automated, high-throughput technologies in proteome analysis. Thus, the stain-free fluorescence visualization approach should prove useful as both a complement and an alternative to staining techniques for mass spectrometric proteome analysis.

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