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Impact of un-polymerized acrylamide monomer residues onto protein identification by MALDI TOF MS

Research Article

Ivan Talian[1], Veronika Kováčová[1], Marián Petrovič[1], Ján Sabo[1]

[1]*Department of Medical and Clinical Biophysics, Pavol Jozef Šafárik University 040 11 Košice, Slovakia*

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Abstract: Polyacrylamide gel electrophoresis is a powerful tool for protein mixture separation frequently used in proteomic studies. This article describes the problems which can arise during the identification process of separated proteins by matrix assisted laser desorption/ionization mass spectrometry. Presence of residual acrylamide monomers in the peptide samples leads to a significant peptide signal suppression. In the case of protein prohibitin, isolated from the MCF 7 breast cancer cell line, the peptide signal suppression was observed in 50% of all detected peptides.

Keywords: *MALDI • Acrylamide • Peptide mass fingerprinting • Gel electrophoresis*

1. Introduction

Polyacrylamide gel electrophoresis (PAGE) is one of the most used separation technique for protein mixture analysis in the proteomic studies [1,2]. The polymerization is rarely quantitative and the polymer usually contains some un-polymerized acrylamide molecules [3,4]. Chiari and co-workers calculated that for 5%T gel (20×20cm×1mm) is the concentration of unreacted free acrylamide about 30-60mM [5-7]. Acrylamide, with respect to its chemical properties, is able to react with various types of chemical compounds. During the electrophoretic separation acrylamide can react with free sulfhydryl groups in proteins and form cysteine-acrylamide adducts [8]. In the proteomic analysis the relevant reactions of acrylamide with primary and secondary amines and eventually with tertiary amines (the last mentioned create buffer solution of electrolyte within PAGE separation) can occur [9]. The elimination of amines adducts formation is especially important in peptide mass fingerprinting (PMF). Acrylamide can covalently bind proteins by reacting with the amino group [10,11]. For a long time there has been a well-known reaction mechanism of carbamides with amines. Nucleophilic addition of ammonia, primary and secondary amines [12-14], and amino acids [15,16] to α,β - unsaturated carbonylic compounds is a well-characterized chemical reaction [17]. Since protein identification after PAGE is mostly performed *via* mass spectrometry, especially by MALDI TOF [18,19], formation of adducts during PAGE is not desirable. Proteins that were modified by these adducts have different mobility in comparison with unmodified proteins during the PAGE. The reason for this phenomenon is the fact that the adduct changes the proteins overall charge and eventually its size. Tertiary amines – used in solutions of electrolytes by PAGE – change their pH value due to the influence of adducts with acrylamide. Proteins, *e.g.* bovine serum albumin and cytochrome c showed a change in electrophoretic mobility in PAGE after the interaction with acrylamide monomer under alkaline conditions [20]. In MALDI MS technique, acrylamide adducts increases the probability of prior ionization of acrylamide instead of peptide ionization (peptide signal suppression) or the measured masses shift to a higher m/z due to these adducts. This leads to problematic protein identification and increases the number of false positive identifications. The most likely reaction of un-polymerized acrylamide is with the cysteine [21]. Sulphilic bond of cysteine is prone to carbamidoethylation. For example Barber and co-workers observed adducts of acrylamide with cysteine

* *E-mail: ivan.talian@upjs.sk*

on the peptides of digested protein (recombinant NSF protein) where the peptide masses were shifted to higher m/z values. The reaction of cysteine was in this case carbamidoethylation (mass difference 71 m/z) despite expected carbamidomethylation (mass difference 57 m/z) [22]. The other amino acids underlying possible carbamidoehtylation are serine, valine and glutamine. Recent results indicate that also glycine is able to create adducts of various types and it can lead to serious complications concerning the evaluation of mass spectra [23].

The article describes problems in protein identification by PMF with MALDI TOF caused by un-polymerized acrylamide residues presented in the digested protein sample. The influence of acrylamide on peptide ionization and following peptide mass fingerprinting is discussed.

2. Experimental procedure

2.1. Materials

Cytochrome c and acetonitrile (ACN) were purchased from Sigma Aldrich (St. Louis, Mo, USA), Tris base, ammonium persulfate, TEMED, 0.5% agarose, Tris-glycine-SDS buffer, 30% acrylamide/bis solution, coomassie brilliant blue R-250 destaining solution were purchased from Bio-Rad (Hercules, CA), ammonium bicarbonate (NH_4HCO_3) was from Applichem (Darmstadt, Germany), trifluoroacetic acid (TFA) was from Acros-Organics (New Jersey, USA), proteomics grade purity trypsin was purchased from Promega (Medison, USA), ultra-pure water with current resistance of more than 18.2 mΩ was used for preparation of the electrophoresis buffers and digestion solutions (Millipore, Bedford, MA, USA).

2.2. Methods

2.2.1. MCF 7 nuclear proteins isolation

The source of cytoplasmic protein was the cell line from breast cancer, from American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM with the addition of FBS, Penicillin and Streptomycin at 37ºC in medium containing 5% CO_2 and 95% humidity. Cells were collected at 80-90% confluence using trypsin and ice-cooled PBS. The cells were washed 3 times with cooled PBS. Pellet was formed by centrifugation at 2500 g and stored at -80ºC. The number of cells in the pellet was approximately 2×10^6. The cell lysis was made according to the instruction manual from the ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear) from Bio-Rad Laboratories (USA).

2.2.2. UV control of acrylamide polymerization reaction

UV absorption spectra were recorded on a double beam Shimadzu UV 1800 spectrometer (Wavelength range 190 to 1100 nm, Spectral bandwidth, Wavelength accuracy 0.1 nm), using a 1cm path length.

2.2.3. Two-dimensional electrophoresis and protein spot analysis

Nuclear proteins were diluted in a chaotropic 2-D rehydration/sample buffer from BioRad kit. 125 µL of diluted proteins (1.23 mg mL^{-1}) were loaded into IPG strips by in-gel passive rehydration strips overnight. Afterwards isoelectric focusing of the proteins was conducted at 20ºC with a current limit of max. 50 µA per strip in linear mode from 250 V to 4000 V until reaching 20000 V hrs in a PROTEAN IEF cell from Bio-Rad (USA). The IPG strips were subsequently transferred to an equilibration buffer (6 mol dm^{-3} urea, 2% SDS, 0.375 mol dm^{-3} Tris HCl, pH=8.8, 20% glycerol, 0.130 mol dm^{-3} DTT), 2 mL per strip, followed with equilibration buffer (6 mol dm^{-3} urea, 2% SDS, 0.375 mol dm^{-3} Tris HCl, pH=8.8, 20% glycerol, 0.135 mol dm^{-3} iodoacetamide). SDS-PAGE was performed on 12.5% SDS-polyacrylamide gel using Mini PROTEAN Tetra Cell apparatus (Bio-Rad Laboratories, USA). Strips were fixed in place with an agarose overlay. Electrophoresis was carried out at 200 V for approximately 50 min until the dye (bromophenol blue) front reached the end of the gel. The running buffer (0.025 mol dm^{-3} Tris, 0.192 mol dm^{-3} glycin, 0.1% SDS) was not cooled. The staining procedure was performed using Coomassie brilliant blue (for 1 hour) and afterwards the gels were washed 3x with proteomic grade water. The stained gels were scanned using Calibrated Densitometer GS – 800 and data were analyzed using the PDQuest software (Bio-Rad Laboratories (USA). Selected protein spots were manually cut.

2.2.4. Protein digestion

The gel pieces were transferred to 500 µL tubes and washed with 200 µL of 50% ACN/25 mM ammonium bicarbonate buffer for gel decoloration. Supernatant was discarded and the gel pieces were washed once with 100 µL 100% ACN. After a few minutes the gel pieces turned white and ACN was removed. The gel pieces were dried in the air. Dried gel pieces were rehydrated in 25 µL of 25 mM ammonium bicarbonate containing 12.5 µg trypsin. After 60 minutes on ice, the non-absorbed trypsin solution was discarded. 20 µL of 25 mM ammonium bicarbonate was added to the

gel pieces to prevent the gel from drying out and the samples were incubated at 37ºC for at last 12 h. To stop the digestion process 5 µL of 5% TFA was added and the samples were shaken out for 15 min. The samples were separated with a spinning process and supernatant was transferred to 500-µL tube. Peptides were extracted twice with 20 µL of 50% ACN/0.1% TFA and afterwards combined again.

2.2.5. MALDI TOF MS

1 µL of samples were mixed with 2 µL of MALDI matrix α-cyano-4-hydroxycinnaminic acid (4 mg mL^{-1} in acetonitrile/methanol/0.1% TFA in water, 84/14/2 v/v/v) and 1 µL was spotted on a MALDI target and allowed to dry. Samples were analysed on a mass spectrometer UltrafleXtreme MALDI TOF/TOF (Bruker Daltonik, Germany). Spectra were acquired in the reflection mode in the range of 700 – 3500 Da. Mass calibration was performed externally with PepCal standard. The data were evaluated using the Mascot algorithm (Matrix Science Ltd., UK). Mass spectra and tandem mass spectra of peptides were compared against NCBIr database. The search parameter used was: taxonomy – *Homo sapiens* (human), global modifications Carbamidomethyl (C), variable modificat ions Oxidation (M), enzyme Trypsin, the number of maximum missed cleavages 1, mass error tolerance 0.1 Da (MS).

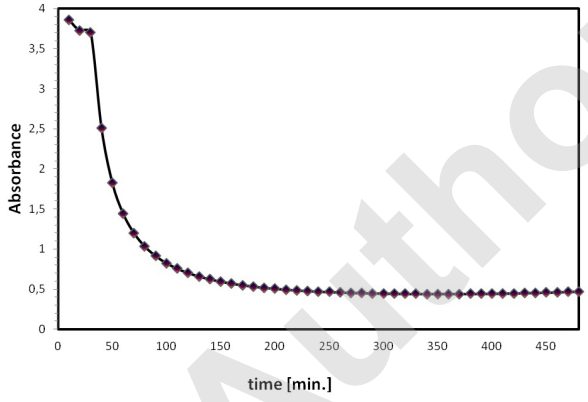

Figure 1. The acrylamide polymerization process controlled by UV absorbance at the wavelength of 260 nm.

3. Results and discussion

The PAGE resolution and reproducibility is strongly influenced by the gel preparation conditions. The polymerization can be easily controlled by UV absorbance due to the fact that the absorbing double bonds are during polymerization eliminated. The reaction was controlled at the wavelength of 260 nm (Fig. 1).

Although the reaction has exponential behavior (the UV absorbance decreases with the increasing reaction time) and the most of the monomers have reacted already after approximately 1 h the monomer residues still presented in the gel strongly influence not only the protein mixture separation, but also their identification. The resolution and the reproducibility of the 2D PAGE is decreasing with the rising concentration of acrylamide monomer residues. As an example, the image of 2D PAGE separation of nuclear proteins isolated from the MCF 7 cell line is shown in Fig. 2.

Fig. 2a shows the separation on the gel prepared with one hour of solidification time and 2b with 24 hours respectively. On the gel 2a we can see the unresolved spots. This leads to the problems connected with the spot cutting and the number of identified proteins decreases. The reproducibility of such prepared gels is also rather poor. For instance the protein HNRF marked 1 or the 60 kDa heat shock protein marked 2 are in the gel 2a unresolved from its variants, showed almost as one unresolved line. Meanwhile on the gel 2b we can see the distinct spots. Except for the resolution and separation problems the protein identification utilizing MALDI TOF is also influenced. The conditions during

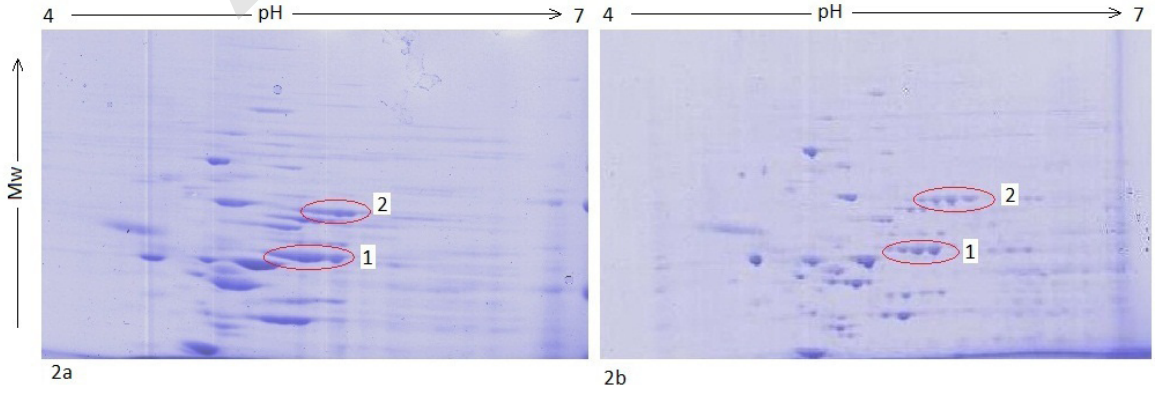

Figure 2. The image of 2D PAGE separation of nuclear proteins isolated from the MCF 7 cell line, (a) gel solidification time of 1 hour and (b) a solidification time of 24 hours.

the ionization process are crucial. As an example, the MALDI identification of cytochrome C (horse) was performed after the gel electrophoresis on the gels prepared with different solidification times. The selected solidification times were 1, 3, 6, 12 and 24 hours. This time scale should ensure minimal un-reacted acrylamide presented in the gel after polymerization. The number of matched peptides and their intensity in the MALDI spectrum were compared. The results are listed in the Table 1.

From Table 1 it is clear that the gel solidification time/concentration of un-reacted acrylamide can considerably improve the identification score of the separated proteins. Due to more matched peptides a better sequence coverage is achieved. Also the MS spectrum acquisition is much easier; less laser power leads to better signal to noise ratio, better mass resolution and the acquired mass spectra are at least in one order of magnitude more intensive. This combination ensures also a more accurate peptide mass determination and so protein identification with minimal false positive matches. The protein prohibitin from the MCF 7 cell was chosen as a model protein for the peptide mass fingerprinting after 2D SDS-PAGE. In Fig. 3 we can see the comparison of prohibition PMF spectrum from the two gels with different solidification time (1 h and 24 h).

Table 1. The number of matched peptides for cytochrome C in PMF MALDI analysis after PAGE on the gels with the different solidification times.

gel solidification time (hours)	number of matched peptides
1	7
3	7
6	8
12	8
24	13

The number of matched peptides was 5 in the 1 h gel and 10 in the 24 h gel. The sequence coverage raised in this case from 16% up to 41.5% and the Mascot score from 68 to 153. Notice that especially heavier peptide masses are missing (mass 1791.7 m/z do not belong to peptide masses of protein prohibition). However it should be mentioned that also few lighter peptides were not detected in the 1 hour gel. The comparison of these MS spectra points out on the ionization problems of proteins related with acrylamide monomer residues from the gel. The possible explanation is the surface-protein interaction between the peptide and the un-polymerized acrylamide monomer during the desorption event. In the extreme case (high concentration of un-reacted acrylamide, in this case the gel solidification time was only 30 minutes), the polymerization of acrylamide induced by the laser pulse can be observed. The free acrylamide monomer residues show polymeric behavior (Fig. 4).

The repeating unit 72 m/z belongs to acrylamide monomer residue $[MH]^+$, 44 m/z belongs to the

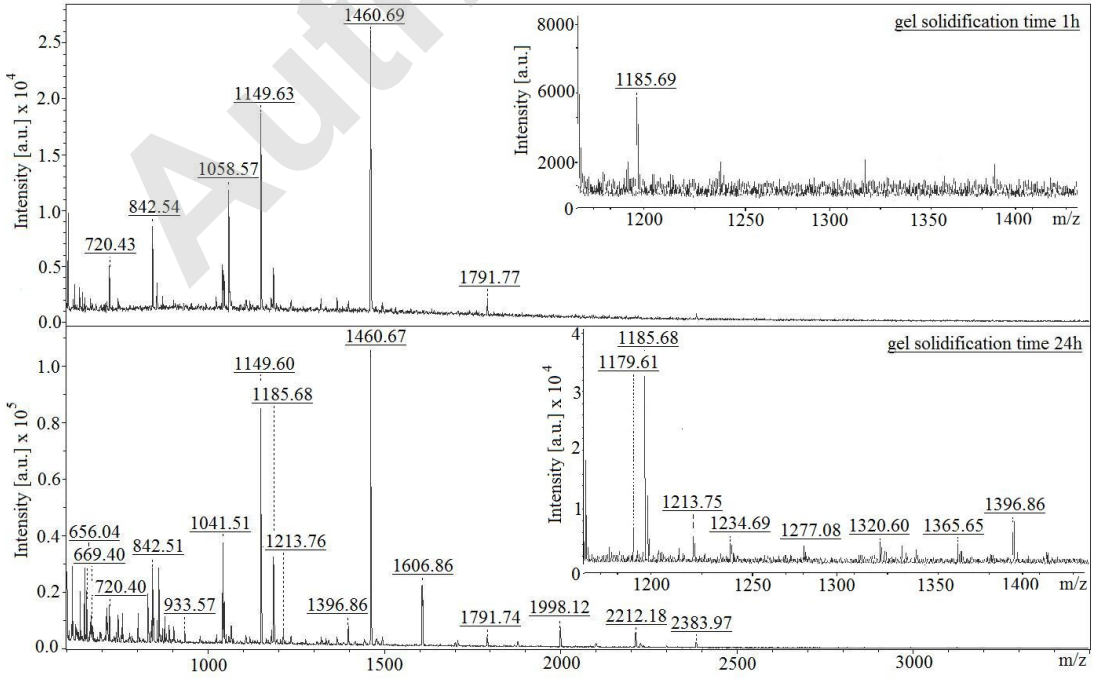

Figure 3. The comparison of prohibition PMF spectrum from the two gels with different solidification time (1 h and 24 h).

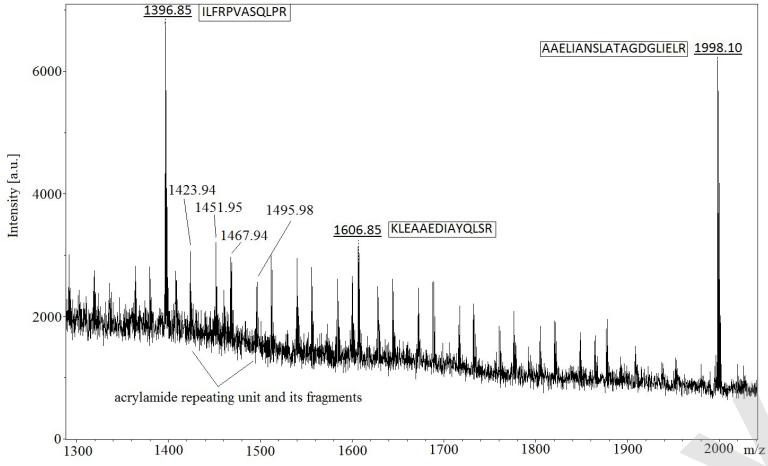

Figure 4. Detail of prohibition PMF spectrum with the repeating acrylamide monomer units and its fragments. Underlined masses are peptides of prohibition origin.

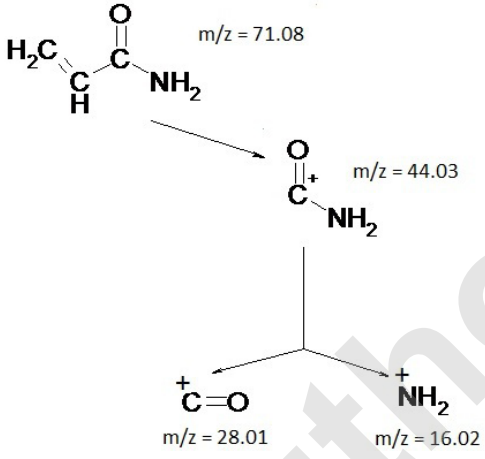

Figure 5. Acrylamide fragmentation scheme.

$[O = C - NH_2]^+$ group and 28 m/z to this group $[C = O]^+$. The scheme of acrylamide fragmentation is shown in the Fig. 5.

In the case where the un-polymerized acrylamide reacts with the protein already during the PAGE the mass spectrum usually contains few peptide masses and the others do not correspond to the peptide masses of this certain protein (spectrum not shown). These peptide masses are shifted to higher masses due to acrylamide reaction with cysteine (carbamidoethylation of cysteine).

4. Conclusions

The presented results clearly show the influence of acrylamide monomer residues from 2D PAGE on the peptide mass fingerprinting of separated proteins. Considerable signal suppression of peptides was observed. In some cases the peptide signal even disappeared especially for the heavier peptides which are usually used in MS/MS identification. As an example the protein prohibition isolated from the MCF 7 cell line was chosen. The number of matched peptides for prohibition rises from 5 (in the one hour solidification time gel) to 10 (in the gel solidification time of 24 hours) and for protein cytochrome c the number of matched peptides rises from 7 to 13. The prohibition sequence coverage was improved from 16% to 41.5% and the intensity of the MS spectrum was also considerably improved. The possible explanation of this behavior is in the protein/peptide – surface/acrylamide interaction during the desorption/ionization in MALDI analysis. The acrylamide polymerization during the laser pulse was also observed and can be seen on the prohibition MS spectrum where repeating acrylamide monomer units are marked. The problems with the un-polymerized acrylamide monomers can be partially overcome by utilizing pipette tips with incorporated packing material such as C_{18}. However, some strongly hydrophobic or hydrophilic peptides can be lost.

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