

Short communication

Solubilization of Proteins from Human Lymph Node Tissue and Two-Dimensional Gel Storage

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In the present study, we compared six different solubilization buffers and optimized two-dimensional electrophoresis (2-DE) conditions for human lymph node proteins. In addition, we developed a simple protocol for 2-D gel storage. Efficient solubilization was obtained with lysis buffers containing (a) 8 M urea, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 40 mM Tris base, 65 mM DTT(dithiothreitol) and 0.2% carrier ampholytes; (b) 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10 (N-decyl-N, N-dimethyl-3-ammonio-1-propanesulfonate), 40 mM Tris base, 65 mM DTT and 0.2% carrier ampholytes or (c) 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT and 0.2% carrier ampholytes. The optimal protocol for isoelectric focusing (IEF) was accumulated voltage of 16,500 Vh and 0.6% DTT in the rehydration solution. In the experiments conducted for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), best results were obtained with a doubled concentration (50 mM Tris, 384 mM glycine, 0.2% SDS) of the SDS electrophoresis buffer in the cathodic reservoir as compared to the concentration in the anodic reservoir (25 mM Tris, 192 mM glycine, 0.1% SDS). Among the five protocols tested for gel storing,

success was attained when the gels were stored in plastic bags with 50% glycerol. This is the first report describing the successful solubilization and 2D-electrophoresis of proteins from human lymph node tissue and a 2-D gel storage protocol for easy gel handling before mass spectrometry (MS) analysis.

Keywords: Gel storage, Human lymph node tissue, Protein solubilization, Two-dimensional gel electrophoresis

Introduction

The analytical potential of 2-DE is dependent on good sample preparation, in order to obtain reproducibility, good resolution and a great spot number of proteomic maps. In many cases, the proteins of the sample need to be solubilized, disaggregated, denatured and reduced (Shaw and Riederer, 2003). For this purpose, mixtures of chaotropic compounds, detergents or surfactants, reducing agents and carrier ampholytes are employed (Molloy, 2000; Garfin, 2003).

The role of chaotropes, such as urea and thiourea, is to disrupt hydrogen bonding, leading to protein unfolding and denaturation. Surfactants such as CHAPS, SB 3-10, ASB-14 (amidossulfobetaine-14) and SDS act synergistically with chaotropes. Reducing agents, such as DTT and TBP (tributyl phosphine), are used to break intramolecular and intermolecular

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disulfide bonds. Carrier ampholytes enhance protein solubility by minimizing protein aggregation due to charge-charge interactions (Herbert, 1999; Molloy, 2000; Berkelman and Stenstedt, 2002; Shaw and Riederer, 2003).

Due to variable protein expression from one tissue to another, conditions of protein solubilization that are optimal for one particular tissue type may not hold for others. In addition, several 2-DE steps must be optimized for each tissue in order to obtain good results. In the present study, a comparison was made of six previously described solubilization methods for obtaining proteomic maps of human lymph node tissue. We also describe a simple 2-D gel storage protocol for easy gel handling prior to MS analysis.

Materials and Methods

Thirty-one lymph nodes were collected from head and neck squamous cell carcinoma patients at the Cancer Hospital "Arnaldo Vieira de Carvalho", São Paulo, Brazil. Tissue samples were obtained immediately after the removal of the surgical specimen, snap-frozen and stored in liquid nitrogen. The Ethics Committee approved the research, and written informed consent was obtained from all patients.

One lymph node sample was cut into six pieces of about 5 mm³. 500 µL of one out of six different lysis buffers were added to each piece (Table 1). The specimens were disrupted by sonication 12 times at intervals of 10 s at 10°C and vortexed for 2 min. The lysates were centrifuged at 10,000 rpm for 3 min at 4°C. The supernatants were transferred to other tubes, the insoluble pellets were washed with 200 µL lysis buffers and the second supernatants were collected. The protein concentration of the supernatants was determined by the Bradford method (Bradford, 1976). The protein samples were stored at -70°C.

2-DE was performed using IPGphor and SE 600 Ruby (GE Healthcare). For IEF, 500 µg protein were diluted with rehydration

solution (8 M urea, 2% CHAPS, 0.6% DTT, 0.5% IPG buffer, bromophenol blue trace) to a total volume of 250 µL. IPG strips (pH 3-10 L, 13 cm) were rehydrated in this solution for 12 h under mineral oil. IEF was performed at 20°C, with the following parameters: 500 V (1 h), 1,000 V (1 h), 8,000 V (2 h or 3 : 30 h or 5 h), 500 V (0 h or 1 h), until 16,500 Vh, 26,500 Vh or 41,500 Vh were attained. The current was limited to 50 µA/strip. After IEF, the IPG strip was stored at -70°C until analysis by SDS-PAGE.

The individual strips were incubated, at room temperature, in the equilibrium solution A (2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, bromophenol blue trace and 1% DTT), followed by solution B (solution A except that DTT was replaced by 2.5% iodoacetamide), for 15 min each. When the proteins were solubilized using solutions containing TBP, the strips were only incubated in solution C (solution A except that DTT was replaced by 5 mM TBP) for 30 min. The IPG strips were washed in SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), placed on top of 12.5% SDS-PAGE and sealed in place with sealing solution (0.5% low-melting agarose in SDS electrophoresis buffer). The electrophoresis conditions were 15 mA/gel for 30 min, followed by 30 mA/gel for 5 h at room temperature.

Proteins were detected by Coomassie Blue staining. Briefly, gels were incubated overnight in fixing solution (50% ethanol, 10% acetic acid), followed by a destaining solution (50% ethanol, 5% acetic acid) for 3 min, and incubated for 90 min with 0.05% Coomassie Brilliant Blue R-250 solution (0.125 g Coomassie Brilliant Blue R-250, 40% methanol, 10% acetic acid). Subsequently, the gels were washed four times with destaining solution, for 15, 45, 120 and 120 min, respectively, and incubated in preserving solution (5% acetic acid) for approximately 72 h.

For gel storing, five protocols were tested.

- Protocol 1. The gel was washed twice in solution D (30% ethanol) for 30 min, followed by solution E (30% ethanol, 3.5% glycerol) for 60 min.
- Protocol 2. The gel was incubated in 4.3% glycerol for 5 min.
- Protocol 3. The gel was washed four times in water for 4 h.
- Protocol 4. The gel was incubated in 8.7% glycerol for 60 min.

Table 1. Lysis buffer composition. Composition of six lysis buffers tested for protein solubilization efficiency

	Buffer 1	Buffer 2	Buffer 3	Buffer 4	Buffer 5	Buffer 6
Chaotropes	8 M Urea	5 M Urea 2 M Thiourea	5 M Urea 2 M Thiourea	7 M Urea 2 M Thiourea	5 M Urea 2 M Thiourea	7 M Urea 2 M Thiourea
Detergents	4% CHAPS	2% CHAPS 2% SB 3-10	2% CHAPS 2% SB 3-10	4% CHAPS	2% CHAPS 1% SB 3-10 1% ASB -14	2% CHAPS 0.5% ASB -14
Salts	40 mM Tris base	40 mM Tris base	40 mM Tris base			
Reducing agents	65 mM DTT	65 mM DTT	2 mM TBP	65 mM DTT	2 mM TBP	65 mM DTT
Carrier ampholytes	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)
References	(Herbert, 1999; Molloy <i>et al.</i> , 1998)	(Rabilloud <i>et al.</i> , 1997)	(Rabilloud <i>et al.</i> , 1997; Molloy <i>et al.</i> , 1998; Tachibana <i>et al.</i> , 2003)	(Molloy, 2000; Berkelman and Stenstedt, 2002; Rabilloud <i>et al.</i> , 1997; Görg <i>et al.</i> , 2000)	(Garfin, 2003)	(Castellanos-Serra and Paz-Lago, 2002)

• Protocol 5. The gel was incubated in 10% methanol for 48 h. The gels were placed between two cellophane sheets PT (CooperCell) previously embedded in solution E (protocol 1), in water (protocol 2), in 8.7% glycerol (protocol 3 and 4) or stored in a clear plastic bag with 2 ml of 50% glycerol solution applied over both surfaces of the gel. The bag was sealed.

No gel drying equipment was used. Stained and stored gels were scanned with an ImageScanner (GE-Healthcare), and spot detection was manually performed with the Melanie 3.0 software (GeneBio).

One protein spot from 2-DE gel was selected, excised, digested with trypsin and submitted to MALDI-TOF-TOF (Matrix Assisted Laser Desorption - Time of Flight - Time of Flight) 4700 Proteomics Analyser (Applied Biosystems) operated in positive ion reflectron mode to identify the peptides. Such spot was manually cut out from the gel in a clean-air cabinet, to prevent contamination. The protein spot was placed into 0.5 mL tube previously washed with 50% methanol and deionized water. The gel pieces were destained in 250 μ L of 50% acetonitrile (ACN)/50 mM ammonium bicarbonate under constant agitation to complete colourlessness. The gel pieces were then dehydrated with 200 μ L of ACN for 15 min; acetonitrile was discarded and the gel pieces dried in Speed Vac for 30 min. For rehydration and digestion, each gel piece was rehydrated with 20 μ L of a trypsin solution (0.4 μ g modified trypsin in 50 mM acetic acid and 50 mM ammonium bicarbonate). After 30 min incubation at room temperature, a volume of 50 μ L of 50 mM ammonium bicarbonate or the sufficient amount to cover the gel pieces was added, and the sample was incubated for 24 h at 37°C in a water bath, for enzymatic cleavage. Peptides were extracted with 50 μ L 1% trifluoroacetic acid/TFA (first extraction: overnight) and 50 μ L 1% TFA/50% ACN (second extraction: 2 h). The resulting supernatants were mixed and concentrated in a vacuum centrifuge to 10 μ L.

About 1 μ L of this solution was eluted in 1 μ L matrix solution (10 mg/mL α -cyano-4-hydroxycinnamic acid, 0.1% TFA in 50% ACN). Then, 0.5 μ L of the mixture was spotted on a sample plate and introduced into the mass spectrometer after drying. The instrument was calibrated externally using 4700 standard kit (Applied Biosystems).

Proteins were identified by MASCOT MS/MS Ions Search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS). The search parameters were set up as follows: MSDB (Mass Spectrometry Protein Sequence Database); taxonomy *Homo sapiens*; 1 missed cleavage; carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modification, respectively; peptide mass and MS/MS tolerance of 1 and 0.8 Da, respectively; the peptide ion MH^+ and monoisotopic masses.

In the present study, all chemicals used were of highest quality (Merck, Calbiochem, GE Healthcare, Sigma and Bio-Rad).

Results and Discussion

Initial extraction and solubilization is a key factor for proteomic analysis. In the present study, we tested six buffers for solubilization of protein from human lymph node samples, which differed in one or more components, including chaotropes, detergents and reducing agents (Table 1).

Extraction buffer 1 is a standard solution for protein solubilization. About 285 spots were visualized in the gel using this solution (Fig. 1A). Buffers 2 and 3 have similar compositions, except for the reducing agent, and about 281 and 113 spots were visualized in the gels, respectively (Fig. 1B, C). In buffer 4, Tris base was not used for protein solubilization. The results showed 283 spots (Fig. 1D). In lysis buffers 5 and 6, ASB-14 was used as detergent and the gels exhibited poor resolution and streaking (Figs. 1E, F). In conclusion, buffers 1, 2 and 4 were the best solutions for protein solubilization of human lymph node tissue with regard to gel quality. Also, these solutions resulted in high protein concentration (9.8 μ g/ μ L, 8.0 μ g/ μ L and 8.9 μ g/ μ L, respectively), higher than buffer 3 (6.6 μ g/ μ L), but much higher than buffers 5 and 6 (0.3 μ g/ μ L and 1.2 μ g/ μ L). Sharp differences are seen comparing Fig. 1C with 1A, 1B and 1D and reflect the results of the Bradford assay. Although this assay is sensitive to various components of lysis buffers, the reagents of buffers 1-6 probably had no effect on protein quantification, and the gel quality showed to be consequence of the buffer efficiency. Thirty other human lymph node samples were also submitted to buffer 4 and all gels showed excellent quality.

In the buffers tested, urea varied from 5 to 8 M, CHAPS from 2 to 4%, SB 3-10 from 1 to 2%, and ASB-14 from 0.5 to 1%. The carrier ampholytes were used at a low concentration (0.2%), in order to avoid extended running times, because they contribute to the initial conductivity of the sample solution (Garfin, 2003). Tris base was added to three buffers (1, 2 and 3). This compound is used when basic conditions are required for full solubilization or to minimize proteolysis (Rabilloud, 1996). However, addition of ionic compounds in buffers for protein solubilization can result in first-dimension disturbances. Therefore, salts must be removed after the solubilization step or maintained at as low a concentration (lower than 10 mM) in the rehydration solution and IEF (Berkelman and Stenstedt, 2002; Shaw and Riederer, 2003). In our experiments, Tris base was added to the buffers at 40 mM, but the final salt concentration during rehydration was maintained at approximately 10 mM.

Thiourea was introduced in combination with urea, to increase the solubility of proteins, mainly of membrane proteins. The use of this component inhibits the adsorption of protein to the gel matrix, when IEF is conducted in IPG. This efficient chaotrope is poorly soluble in water and requires high concentrations of urea for solubility (optimal condition is 2 M thiourea in 5-7 M urea). This reagent improved the solubility of proteins as compared to urea alone, as already stated by other authors (Rabilloud *et al.*, 1997; Pasquali *et al.*, 1997; Musante *et al.*, 1998; Rabilloud, 1998; Giavalisco *et al.*, 2003; Méchin *et al.*, 2003; Taylor and Pfeiffer, 2003).

We also examined three zwitterionic detergents (CHAPS, SB 3-10, ASB-14). The sulfobetaine CHAPS is the most commonly used detergent for 2-DE, from 2% to 4% in high concentrations of urea. Sulfobetaines with long linear tails (i.e., SB 3-10, ASB-14) have been shown to possess a greater

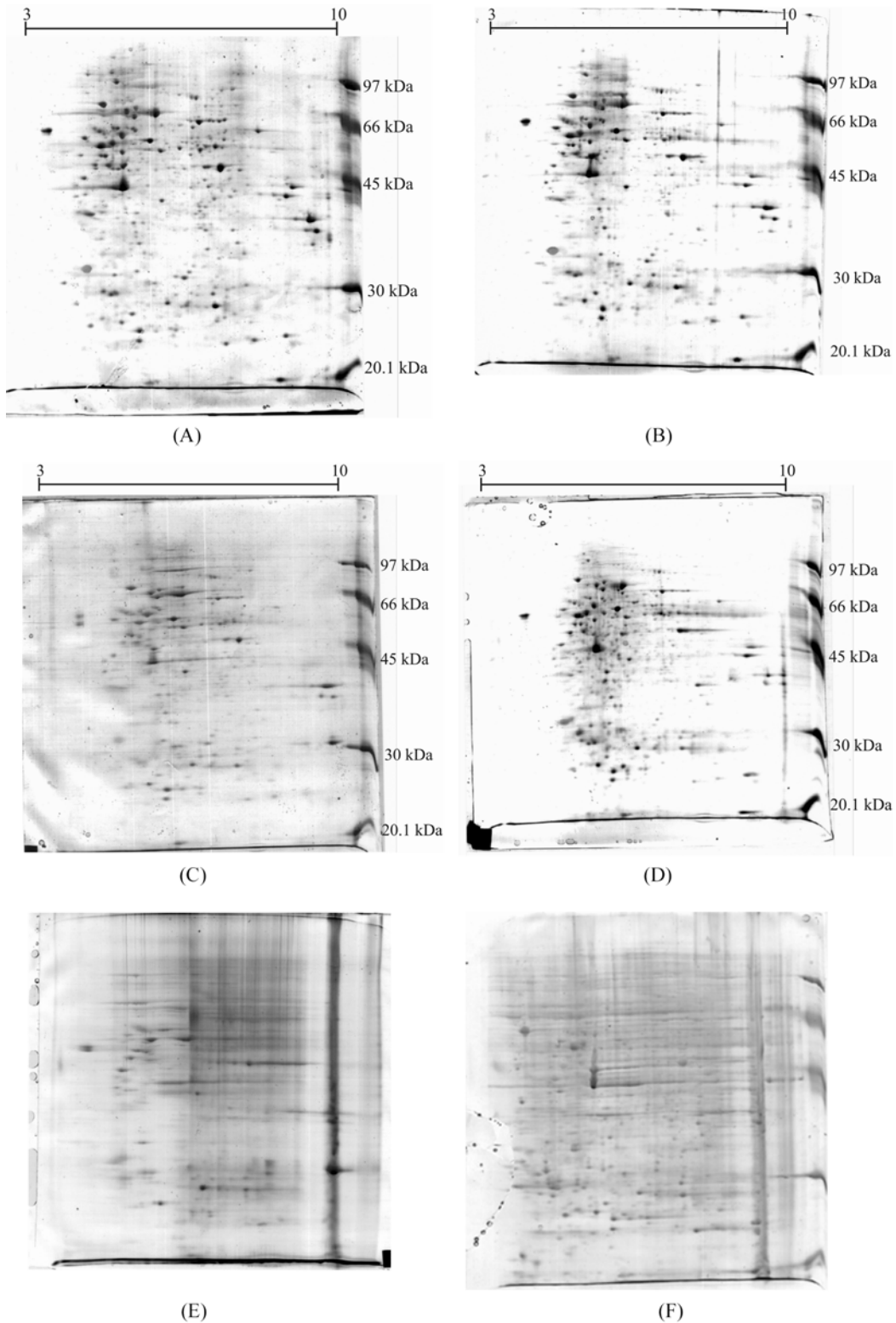


Fig. 1. Comparison of solubilization conditions. Lysis and solubilization from human lymph node tissue proteins were performed using six different buffers (1-6): (A) Buffer 1 (285 spots); (B) Buffer 2 (281 spots); (C) Buffer 3 (113 spots); (D) Buffer 4 (283 spots); (E) Buffer 5 and (F) Buffer 6. Composition of buffers as in Table 1. Proteins were separated on a 13 cm pH 3-10 IPG, 12.5% SDS-PAGE and stained with Coomassie Blue.

ability to solubilize membrane proteins. However, SB 3-10 has poor solubility in high concentrations of urea (Herbert, 1999; Görg and Weiss, 1999). In contrast, ASB-14 is compatible with 9 M urea, but results in large horizontal streaking towards the basic end of the strip. This streaking appears to be an interaction between ASB-14 and the cathode rather than purely an issue with basic proteins, as the effect was independent of the pH range of the IPG strip (pH 3-10 or pH 4-7) (Stanley *et al.*, 2003). Our gels also showed increased streaking in all pH ranges when ASB-14 was used in the extraction solution (Fig. 1E, 1F). Studies using three different detergents (CHAPS, ASB-14 or NP-40) in the solubilization solution also showed more streaking in the second dimension with ASB-14 than with CHAPS (Carboni *et al.*, 2002). The ionic detergent SDS is very effective for protein solubilization, however it is incompatible with IEF (Zuobi-Hasona *et al.*, 2005). For this reason, it was not selected for our optimization experiments.

As for reducing agents, TBP and DTT are commonly used in extraction solutions. When the proteins are solubilized using reagents containing free thiol such as DTT, IPG equilibration requires two steps: reduction (by DTT) and alkylation (by iodoacetamide). DTT has been the standard reducing agent for 2-DE for many years (Molloy, 2000; Görg *et al.*, 2000) and is effective for reducing protein disulfide bonds prior to SDS-PAGE, while iodoacetamide eliminates artifacts of disulfide formation during electrophoresis, for less streaking and better resolution. However, DTT is charged, especially at alkaline pH, and thus migrates out of the pH gradient during IEF, which results in loss of solubility for some proteins. In contrast, TBP lacks a free thiol group, making the second equilibration of the IPG strips unnecessary. In addition, TBP is neutral and does not migrate during IEF, thus, the reducing conditions are maintained over the entire focusing process. On the other hand, TBP has a low solubility, is unstable, volatile and toxic (Rabilloud, 1996; Herbert *et al.*, 1998; Molloy *et al.*, 1998; Berkelman and Stenstedt, 2002). In the present study, the buffers 2 and 3, used for protein solubilization, had similar compositions, except for the reducing agent (DTT in buffer 2 and TBP in buffer 3). Nevertheless, the numbers of spots visualized were very different (281 vs 113 spots), probably due to the reducing agent. Therefore, DTT showed significant improvements in the resolution of proteins by 2-DE. Similar results were reported for myelin proteins (Taylor and Pfeiffer, 2003).

The solubilization of proteins was performed in the absence of protease inhibitors, but using 2 M thiourea, and the sample was processed at 4 to 10°C. According to literature, proteolysis can be inhibited by preparing the sample at such a low temperature, in the presence of Tris base, 2 M thiourea and in strong denaturants such as 8 M urea (Rabilloud, 1996; Carboni *et al.*, 2002; Castellanos-Serra and Paz-Lago, 2002; Berkelman and Stenstedt, 2002).

IEF and SDS-PAGE were also developed using duplicate extracts from buffers 1, 2 and 4. Extracts from other buffers

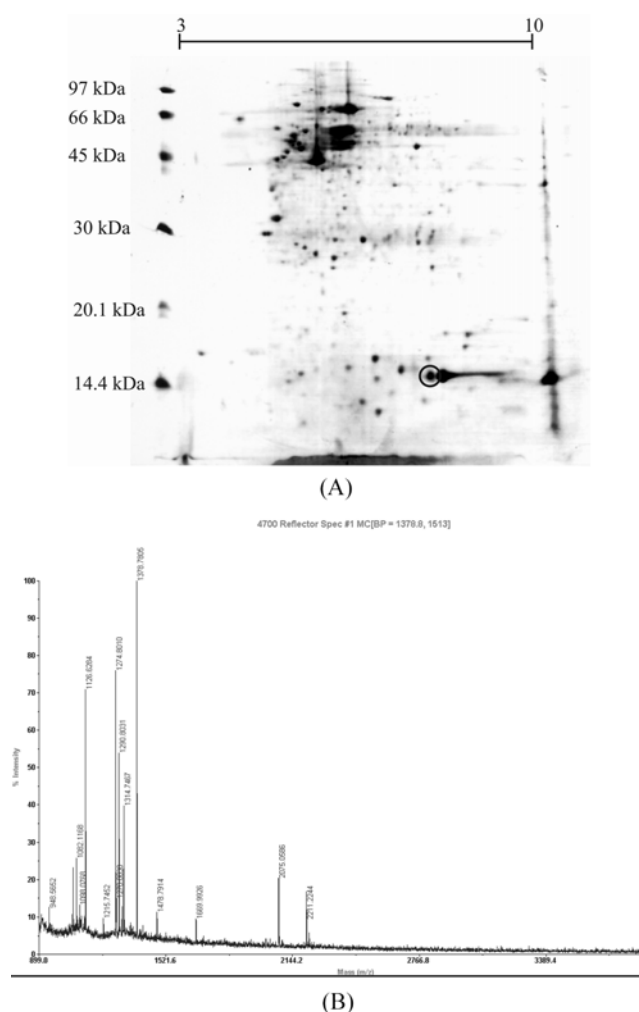


Fig. 2. A one-year-old archived SDS-PAGE and mass spectrum of tryptic peptides derived from hemoglobin. (A) A one-year-old archived SDS-PAGE of a sample from a patient with head and neck squamous cell carcinoma. Highlighted in circle is the spot excised from the gel. (B) Mass spectrum corresponding to a spot with apparent pI and MW of 7.1 and 14.5 kDa, respectively.

were sufficient to perform only one experiment.

In the lysis buffer tests, IEF was carried out with total focusing for 16,500 Vh. After lysis buffer optimization, other IEF conditions were tested in 30 samples (16,500 to 41,500 Vh). The focusing settings are known to be critical for protein separation. In particular, total voltage and slow sample entry have a pronounced effect on the spot pattern quality (Görg *et al.*, 2000). The most remarkable results were the decreasing in horizontal streaking and the well-rounded spots in 16,500 Vh focused gels and by using double concentration of DTT (0.6%) in the rehydration solution (Hoving *et al.*, 2002). DTT is negatively charged at alkaline pH, and migrates towards the anode, causing depletion of DTT at the cathode. In this region the formation of new disulfide bridges could occur, due to oxidation of sulphhydryl groups.

Experiments conducted in 30 samples for the SDS-PAGE

step tested the SDS electrophoresis buffer at a doubled concentration (50 mM Tris-base, 384 mM glycine, 0.2% SDS) in the cathodic reservoir as compared to the anodic reservoir. This condition resulted in decreased protein smearing in the gel, which is usually caused by buffer depletion during electrophoresis (data not shown). The most important component of the typical second-dimension buffer system must be SDS, which binds protein at a relatively constant ratio, thereby allowing for the size-based separation imparted by the sieving matrix. It was observed that buffer depletion during electrophoresis causes dissociation of the SDS from the protein and, consequently, elongated protein patterns or smearing (Werner, 2003).

Five protocols for gel storing were tested and success was attained when the gels were stored in plastic bags with 50% glycerol solution (Protocol 5). This procedure permitted the easy handling of the gel, without the risk of breakage and without harming the image and MS analysis. The gels were submitted to spot excision and MS analysis up to one year after SDS-PAGE. Figure 2A shows a one-year-old archived SDS-PAGE of a sample from a patient with head and neck squamous cell carcinoma. The MS spectrum corresponding to a spot with apparent pI and MW of 7.1 and 14.5 kDa, respectively, is presented in Figure 2B. This protein spot was identified as hemoglobin. The remaining protocols resulted in cracking gels. Preservation of gels for a long time after electrophoresis is frequently desirable, mainly if their transport to another laboratory is necessary. Several methods for drying polyacrylamide gels have been described. These protocols have been routinely applied in thin gels (0.8 mm). However, cracking gels were observed when these methods were performed in thick 2-D gels (1.5 mm).

Progress in sample preparation methodology for 2-DE has focused on improvements in sample buffer constituents to achieve better representation of the proteome. However, it must be taken into account that a variety of factors influence the sample preparation steps, some of which have been reported in this manuscript. We highlighted that this is the first report describing the successful solubilization and 2-DE of protein from human lymph node tissue and a simple protocol for long time preservation of 2D gels.

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