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# About thiol derivatization and resolution of basic proteins in two-dimensional electrophoresis

The influence of thiol blocking on the resolution of basic proteins by two-dimensional electrophoresis was investigated. Cysteine blocking greatly increased resolution and decreased streaking, especially in the basic region of the gels. Two strategies for cysteine blocking were found to be efficient: classical alkylation with maleimide derivatives and mixed disulfide exchange with an excess of a low molecular weight disulfide. The effect on resolution was significant enough to allow correct resolution of basic proteins with in-gel rehydration on wide gradients (*e.g.* 3–10 and 4–12), but anodic cup-loading was still required for basic gradients (*e.g.* 6–12 or 8–12). These results demonstrate that thiol-related problems are not solely responsible for streaking of basic proteins on two-dimensional gels.

**Keywords:** Basic proteins/Cysteine/Maleimide/Two-dimensional gel electrophoresis PRO 0589

## 1 Introduction

One of the major weaknesses in proteomics using 2-DE lies in the difficulties associated with analysis of basic proteins. Although considerable progress has been made recently [1–4] poor resolution of basic proteins, coupled with extensive horizontal streaking, is still frequently experienced. This has been attributed, at least in part, to cysteine oxidation in the basic reaion. In the classical IPG setup, the gel is rehydrated in a buffer containing a reducing agent such as DTT. Since DTT is a weak acid (pKa close to 9), it migrates out of the basic part of the gel, leaving the proteins without the protection of the reducing agent and thus able to form mixed disulfides (intra or intermolecular). This oxidation is probably promoted by the electrochemical oxidation reactions taking place at the basic, cathodic electrode. In order to prevent these noxious phenomena, a strategy providing continuous influx of DTT from a paper reservoir has been proposed [5]. However, this system is rather cumbersome and difficult to optimize from one sample to another, as it is based on the dynamic influx of reducer during the course of the IPG run. A better strategy, in this case, would be to covalently block the cysteine residues, thereby preventing noxious oxidation reactions such as disulfide formation or cysteine overoxidation. Cysteine blocking can be achieved either irreversibly by alkylation or reversibly by exchange with a large excess of a disulfide. The two strategies have been evaluated previously. Cysteine alkylation with either iodoacetamide or acrylamide has been proposed [6, 7]. Alternatively, cysteine blocking by disulfide exchange with dithiodiethanol (DTDE) [8] has also be proposed and shown to be useful. However, the real efficiency of both approaches has not been thoroughly investigated. We therefore decided to investigate various methods of cysteine blocking in order to determine the role of cysteines in the resolution of the gels and to devise alternative and/or optimized methods for cysteine blocking.

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**Abbreviations: DTDE**, dithiodiethanol; **TBP**, tributylphesphine

## 2 Materials and methods

### 2.1 Sample preparation

In order to enable easy cysteine blocking, a sample must be reduced but contain no thiol. Thus, we devised an alternative buffer for whole cell extraction. The packed cells were first suspended in a minimal volume of 10 mM Tris-HCl, pH 7.5, 250 mM sucrose. The suspension was transferred into an ultracentrifuge tube. Then four suspension volumes of concentrated lysis buffer (8.75 M urea, 2.5 M thiourea, 5% CHAPS, 10 mM Tris carboxyethyl phosphine and 20 mM spermine base) were added. After 30 min at room temperature, the nucleic acids were centrifuged at 200 000 *g* for 30 min. The protein concentration was then estimated with a Bradford-type protein assay (BioRad, Hercules, CA, USA) Carrier ampholytes (0.4% final concentration) were added and the sample was stored at −80°C until use.

* These authors contributed equally to this work

 

Cellular subfractions with a very low content of nucleic acids (*e.g.* mitochondria or microsomes) were also used. They were diluted in 7 M urea, 2 M thiourea, 4% CHAPS, 0.4% ampholytes (3–10 range) and 4 mM tributyl phosphine (prediluted 100 mM in tetramethylurea). All the concentrations given above are final concentrations.

Cysteine blocking was performed using two alternative strategies. In the first strategy, the reduced sample was alkylated with 20 mM alkylating agent at various pHs. Carrier ampholytes of various pH ranges (2 pH units wide) were used as buffers, and the pH was presumed to be at the midpoint of the pH range. To simplify the protocol, the maleimide derivatives were made as 10-fold concentrates in water (maleimide) or dimethyl formamide (maleic hydrazide, *N*-methyl and *N*-ethyl maleimides). Acrylamide was used from a stock water solution, while the other alkylating agents (methylvinylsulfone, methyl methanesulfonate and methyl trifluoromethane sulfonate) were prepared as 200 mM stock solutions in dimethyl formamide (*i.e.* 10 × concentrated). The reaction was left for 6 or 24 h at room temperature. The alkylated extracts were then either loaded in a cup, or mixed with the gel rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.4% ampholytes) [9].

In the second strategy, the reduced sample was either cup-loaded, or mixed directly with the gel rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.4% ampholytes, and 100 mM disulfide). Various disulfides were tested, including DTDE, dithiodiglycerol and dithiodipyridine. Dithiodiglycerol was prepared in the laboratory by oxidation of 2 equivalents of thioglycerol with 1 molar equivalent of hydrogen peroxide.

### 2.2 Gel electrophoresis

Various immobilized pH gradients were tested, including a 3.75–10.5 linear pH gradient interpolated from a previously published 3–10.5 pH gradient [10], a nonlinear 4–12 pH gradient [1] and a linear 6–12 pH gradient [2]. The IPG gels were cast with plateaus at both sides [11]. The samples were applied either by in-gel rehydration or by cup-loading, with the cup positioned at the acidic plateau. The IPG strips were then focused for 50 000 Vh. After focusing, the gels were equilibrated, with or without an iodoacetamide thiol alkylating step [12] and then run on a second dimension 10% T gel. The gels were silver stained with an ammoniacal silver protocol [13] or, when used for subsequent MS analysis, with a fluorescent ruthenium complex [14] or with colloidal Coomassie Blue [15].

### 2.3 MS

#### 2.3.1 In-gel digestion

Rinsing of excised gel slices was performed with the Massprep (Micromass, Manchester, UK) as described previously [14]. Gel pieces were completely dried in a Speed Vac before digestion. The dried gel volume was evaluated and three volumes trypsin (12.5 ng/μL freshly diluted in 25 mM $NH_4HCO_3$; Promega, Madison, USA) were added. The digestion was performed at 35°C overnight. The gel pieces were then centrifuged for 5 min in a Speed Vac and 5 μL of 35% $H_2O$/60% acetonitrile/5% HCOOH was added to extracted peptides. The mixture was sonicated for 5 min and then centrifuged for 5 min. The supernatant was recovered and the procedure was repeated once.

#### 2.3.2 MALDI-TOF MS analysis

Mass measurements were carried out on an ULTRAFLEX™ MALDI TOF/TOF mass spectrometer (Bruker-Daltonik GmbH, Bremen, Germany). The instrument was used at a maximum accelerating potential of 20 kV and was operated in reflector positive mode. Sample preparation was performed with the dried droplet method using a mixture of 0.5 mL of sample and 0.5 mL of matrix solution. The matrix solution was prepared from a saturated solution of α-cyano-4-hydroxycinnamic acid in $H_2O$/50% ACN diluted 3 times. Internal calibration was performed with tryptic peptides resulting from autodigestion of trypsin (monoisotopic masses at $m/z$ = 842.51, $m/z$ = 1045.564, $m/z$ = 2211.105).

#### 2.3.3 MS Data analysis

Monoisotopic peptide masses were assigned and used for databases searches with the search engine MASCOT (Matrix Science, London, UK). All human proteins present in SWISS-PROT were used without any p*I* or $M_r$ restrictions. The peptide mass error was limited to 70 ppm and one possible missed cleavage was accepted.

## 3 Results

### 3.1 Evaluation of published procedures

We first investigated the influence of cysteine blocking by disulfide exchange [8], or by alkylation with acrylamide. In order to be efficient, these options require that the sample is first reduced, and then reacted with a large excess of a low $M_r$ disulfide or acrylamide. As the alkylation process with acrylamide is pH- and time-dependent, we used two

 

different times (6 and 24 h) and three different pHs (6.5, 8 and 9). Typical results are shown in Fig. 1. It can easily be seen that treatment with DTDE sharply increased resolution, while treatment with acrylamide resulted in decreased resolution. In order to obtain more details on the molecular mechanisms at play, and especially to know whether the action of DTDE and acrylamide is on the proteins themselves or on the surrounding medium, we analyzed the separated proteins by peptide mass fingerprinting. Typical results are shown in Fig. 2 and Table 1. They clearly show that irrespective of the alkylation conditions, cysteine alkylation with acrylamide was far from being complete and spurious alkylation on lysine took place, giving rise to artefactual peptides. The proportion of underivatized cysteine at the end of the equilibration period was fairly low (less than 10%) and correlated with the good resolution observed when DTDE was used.

### 3.2 Investigation of other alkylation processes

In order to obviate the spurious alkylation phenomena observed with acrylamide, two approches were explored. The first was to use a more powerful alkylating agent than acrylamide, but at a lower pH to increase the selectivity for thiols over amines. We investigated alkylation with methylvinylsulfone, methyl methanesulfonate and methyl trifluoromethane sulfonate, at pHs ranging from 5 to 6, in the presence of a large excess of amino groups (from carrier ampholytes used as buffers) to decrease the risk of amine alkylation. Typical 2-D gels obtained after this alkylation are shown in Fig. 3. The resolution was terrible, which suggests major problems linked to spurious alkylation on protein amino groups. We thus followed the opposite track, which was to use a less reactive alkylating chemical. Specifically, we focused on the maleimide derivatives. We used the classical *N*-ethyl maleimide as well as maleimide itself, *N*-methyl maleimide and maleic hydrazide. Typical gels are shown in Fig. 4, and clearly demonstrate a good resolution. Here again, peptide mass fingerprinting was carried out to characterize the separated proteins. Typical results are shown in Fig. 5 and Table 2. While it is quite clear that cysteine alkylation with maleimide derivatives is a rather inefficient process (compare with Table 1), at least under our conditions, it appears sufficient to improve the resolution of basic proteins. Furthermore, spurious alkylation on lysine is fairly uncommon (one case detected only), which may explain the increased resolution in comparison to acrylamide-alkylated samples.

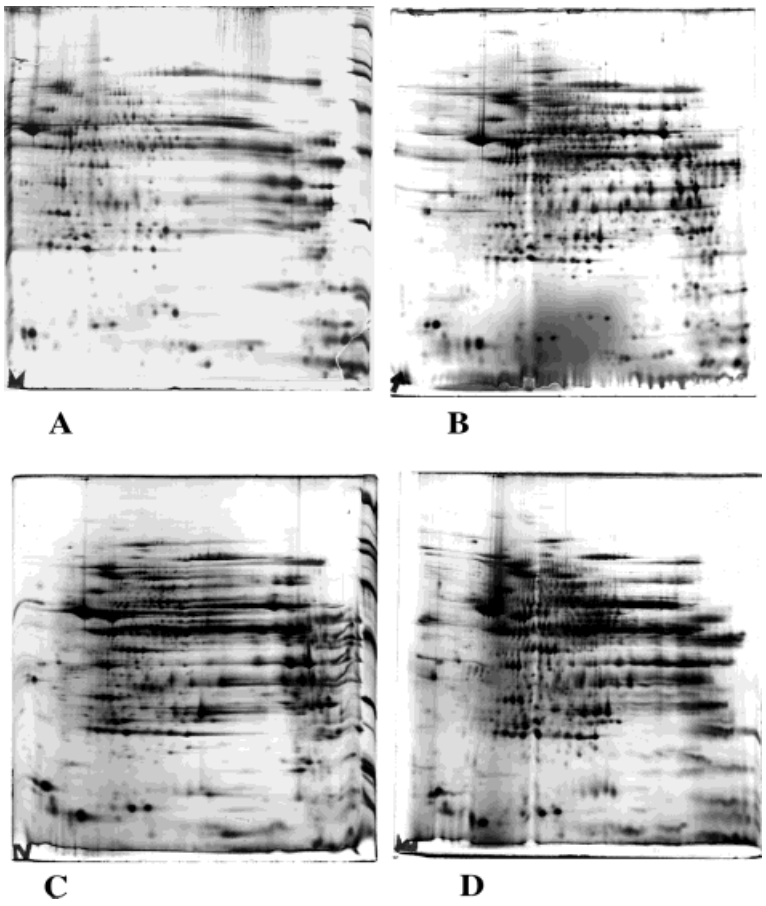


**Figure 1.** Cysteine blocking with acrylamide or DTDE. Bovine mitochondrial proteins were separated by 2-DE. First dimension: linear pH 3.75–10.5 IPG. Equilibration after IPG was performed using the DTT-iodoacetamide two-step method. Second dimension: 10% T gel. Detection was performed with silver staining. A) Proteins were reduced in 50 mM DTT and separated in an IPG gel containing 20 mM DTT. B) Proteins were reduced in 5 mM tributyl-phosphine (TBP) and separated in a gel containing 100 mM DTDE. C) Proteins were reduced with 5 mM TBP and alkylated prior to IPG with 20 mM acylamide for 6 h at pH 6. D) Proteins we reduced and separated as described in c, but alkylation was performed at pH 9.

 

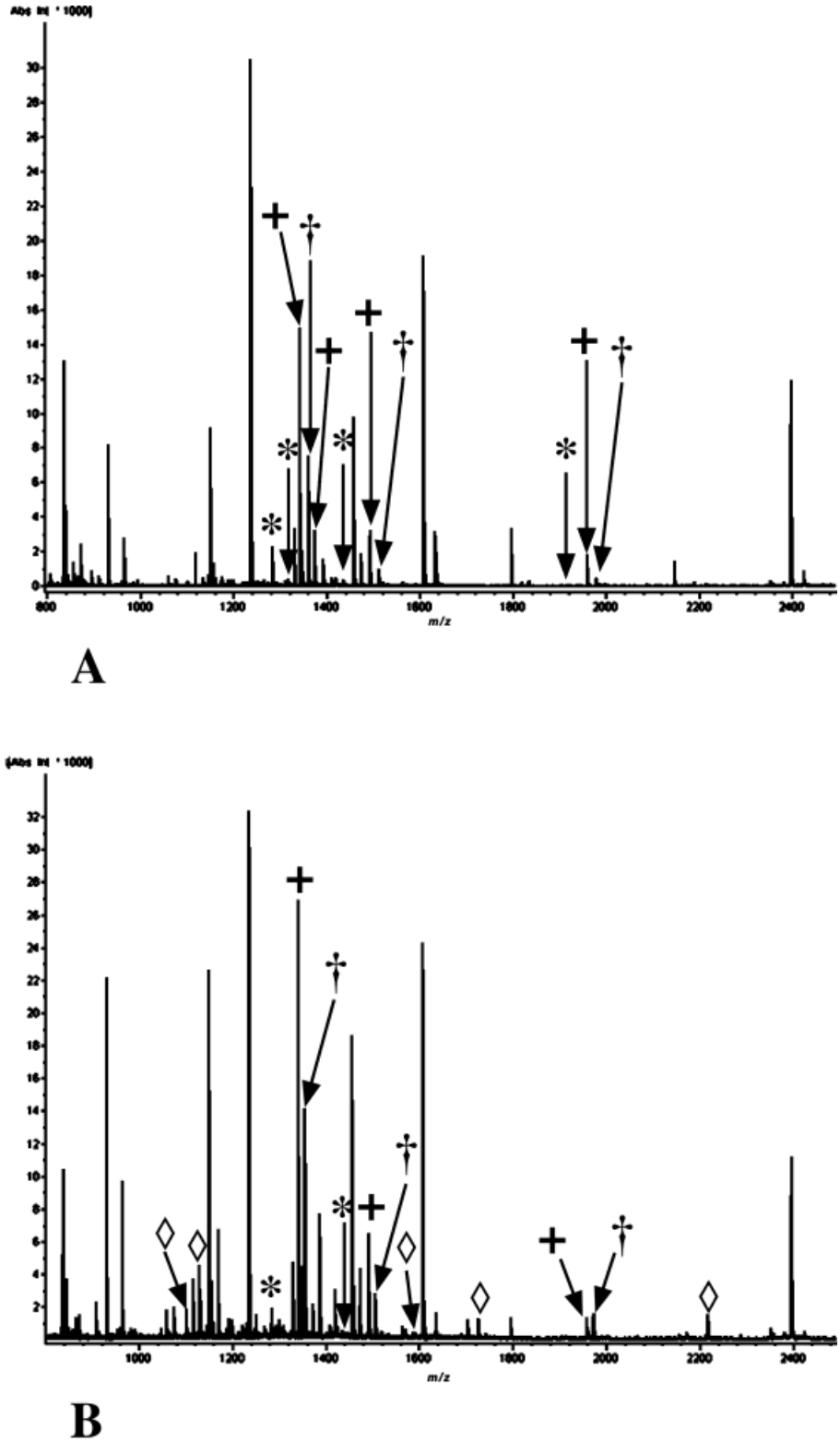


**Figure 2.** MALDI MS spectra of malate dehydrogenase. A) Proteins were alkylated with 100 mM DTDE and equilibrated according to the DTT-iodoacetamide method. The peptides marked with a star correspond to unalkylated cysteine-containing peptides with *m/z* values of 1281.7 (positions 92–104, Cys 93), 1313.7 (positions 204–215, Cys 212), 1432.7 (positions 79–91, Cys 89), and 1898.9 (positions 280–296, Cys 285). The peptides marked with a + correspond to the same peptides with a +57 Da shift, *i.e.* carboxamidomethylated with iodoacetamide. The corresponding *m/z* are 1338.7, 1370.7, 1489.7, and 1955.9. The peptides marked with a † correspond to the cysteine-containing peptides with a 76 Da shift, *i.e.* a mixed disulfide with a mercaptoethanol moiety. The corresponding *m/z* values are 1357.8, 1389.8, 1508.8, and 1975.0. B) Proteins were alkylated with 100 mM acrylamide at pH 9 for 6 h, and equilibrated according to the DTT-iodoacetamide method. The peptides marked with a star correspond to unalkylated cysteine-containing peptides with *m/z* values of 1281.7, 1313.7, 1432.7, and 1898.9. The peptides marked with a + correspond to the same peptides with a +57 Da shift, *i.e.* carboxamidomethylated with iodoacetamide. The corresponding *m/z* are 1338.7, 1370.7, 1489.7, and 1955.9. The peptides marked with a † correspond to the cysteine-containing peptides with a 71 Da shift, *i.e.* alkylated with acrylamide. The corresponding *m/z* values are 1352.7 1384.8, 1503.7, and 1969.9. The peptides marked with an ◇ correpond to spurious alkylation peptides, *i.e.* peptides alkylated on lysine and therefore not digested by trypsin at this position. The corresponding *m/z* values and positions are 1099.6 (298–307), 1127.6 (157–165), 1584.9 (315–328), 1724.9 (240–257), and 2214.2 (158–176).

 

**Table 1.** Cysteine alkylation yield with acrylamide and DTDE

| Alkylation with acrylamide | | | Alkylation with DTDE | | |
|---|---|---|---|---|---|
| +0 Da | +57 Da | +71 Da | +0 Da | +57 Da | +76 Da |
| 0.12 | 0.48 | 0.40 | 0.08 | 0.78[a)] | 0.14 |

The alkylation yield is calculated by integrating the MS peak areas for the unalkylated masses, the +57 Da adduct (alkylated with iodoacetamide), the +71 Da adduct (alkylated with acrylamide) and the +76 Da adduct (alkylated with DTDE). Statistics are carried out on all cysteine-containing peptides for 8 proteins (ATP synthase alpha subunit, isocitrate dehydrogenase (NADP dependent), creatine kinase, malate dehydrogenase, asparate aminotransferase, succinate dehydrogenase, NADH ubiquinone oxidoreductase (B22 and PDSW subunits)).
a) the high yield of iodoacetamide adducts is due to the reversibility of alkylation with DTDE under the equilibration conditions chosen.

**Table 2.** Cysteine alkylation yield with maleimide derivatives

| Alkylation with *N*-methyl maleimide | | | Alkylation with *N*-ethyl maleimide | | |
|---|---|---|---|---|---|
| +0 Da | +57 Da | +111 Da | +0 Da | +57 Da | +125 Da |
| 0.17 | 0.53 | 0.30 | 0.17 | 0.45 | 0.38 |

The alkylation yield is calculated by integrating the MS peak areas for the unalkylated masses, the +57 Da adduct (alkylated with iodoacetamide), the +111 Da adduct (alkylated with *N*-methyl maleimide) and the +125 Da adduct (alkylated with *N*-ethyl malerimide). Statistics are carried out on the cysteine-containing peptides for 8 proteins (ATP synthase alpha subunit, Isocitrate dehydrogenase (NADP dependent), creatine kinase, malate dehydrogenase, aspartate aminotransferase, succinate dehydrogenase, NADH ubiquinone oxidoreductase (B22 and PDSW subunits)).

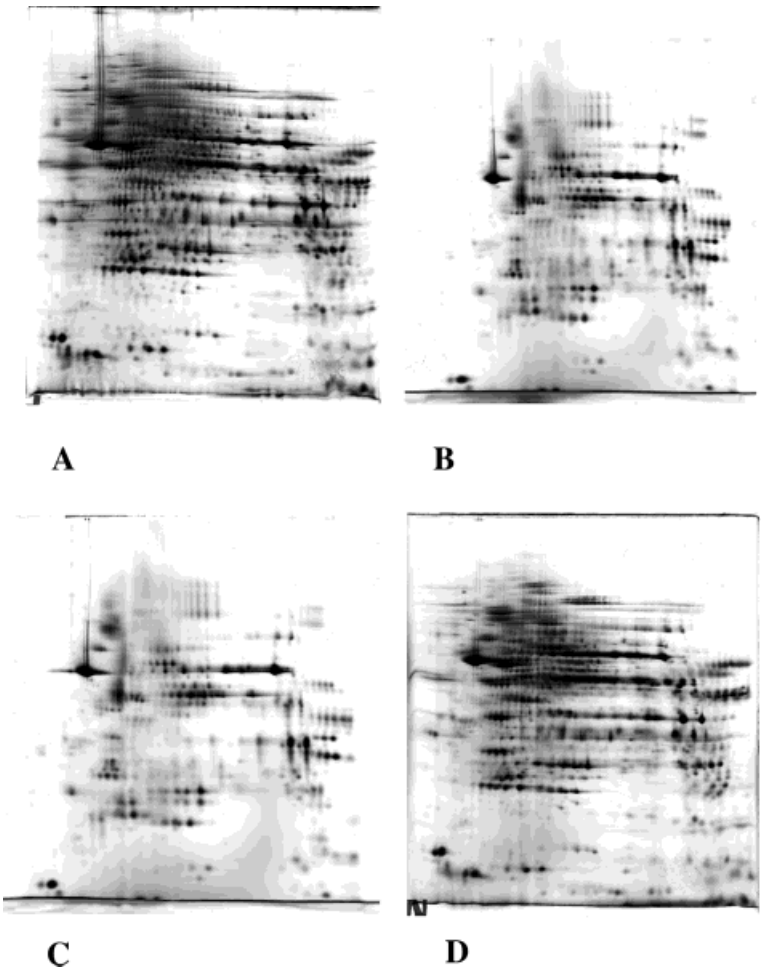


**Figure 3.** Cysteine blocking with highly reactive alkylating agents. Bovine mitochondrial proteins were separated by 2-DE. First dimension: linear pH 3.75–10.5 IPG. Equilibration after IPG was performed using the DTT-iodoacetamide two-step method. Second dimension: 10% T gel. Detection was performed with silver staining. A) Proteins were reduced in 5 mM TBP and separated in a gel containing 100 mM DTDE. B) Proteins were reduced with 5 mM TBP and alkylated prior to IPG with 20 mM methyl methane sulfonate for 3 h at pH 6. C) Proteins were reduced with 5 mM TBP and alkylated prior to IPG with 20 mM methyl triflate for 3 h at pH 6. D) Proteins were reduced with 5 mM TBP and alkylated prior to IPG with 20 mM methyl vinyl sulfone for 3 h at pH 6.

 

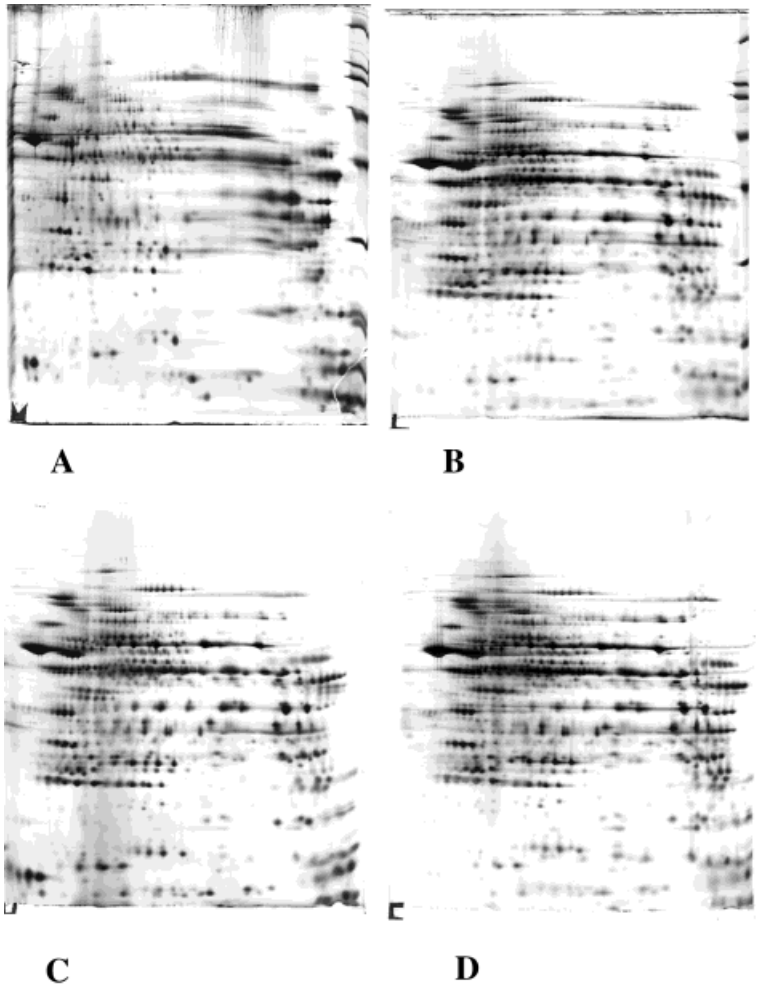


**Figure 4.** Cysteine blocking with maleimide derivatives. Bovine mitochondrial proteins were separated by 2-DE. First dimension: linear pH 3.75–10.5 IPG. Equilibration after IPG was performed using the DTT-iodoacetamide two-step method. Second dimension: 10% T gel. Detection was performed with with silver staining. A) Proteins were reduced in 5 mM TBP separated run in a gel containing 100 mM DTDE. B) Proteins were reduced with 5 mM TBP and alkylated prior to IPG with 20 mM methylmaleimide for 6 h at pH 6. C) Proteins were reduced as described in B, but alkylation was performed with ethyl maleimide: D) Proteins were reduced as described in B, but alkylation was performed with maleic hydrazide

### 3.3 Investigation of the disulfide exchange process

The disulfide exchange process was recently introduced in a short but interesting paper [8]. From the data shown in this paper, the mechanism of action of DTDE is rather unclear. We first sought to establish whether other disulfides would be equally efficient.

The only theoretical constraint on the disulfide used is that it is electrically neutral to prevent spurious transport phenomena at the high disulfide concentration required by this approach. We thus tested three different disulfides: DTDE, dithiodiglycerol and dithiodipyridine. The first two compounds are highly water soluble, the latter being soluble only up to 150 mM. Typical results are shown in Fig. 6. A clear increase in resolution was seen with all three disulfides. As could be anticipated, dithiodipyridine produced a lower resolution in the acidic region, where the pyridine moiety begins to be protonated. Dithiodiglycerol and DTDE were equally effective and had high resolution over the entire pH range. This demonstrated that the positive effect of disulfides do not depend on their structure, within the constraints that they must be neutral and water-soluble.

### 3.4 Pushing the limits of the system

In proteomic studies, analysis of minor proteins often requires that milligram quantities of the starting complex extract are loaded onto the IPG strip. While the alkylation process can be easily scaled up to face this challenge, this is not necessarity the case for the disulfide exchange process, which requires a large excess of low $M_r$ disulfide over the protein thiols. This large excess might not be guaranteed at high protein loads even with 100 mM disulfide, especially if by some unknown mechanism, part of this disulfide is consumed at the basic, reducing electrode. We therefore decided to test higher disulfide concentration (up to 0.5 M, 6% v/v in the gel). At such concen-

 

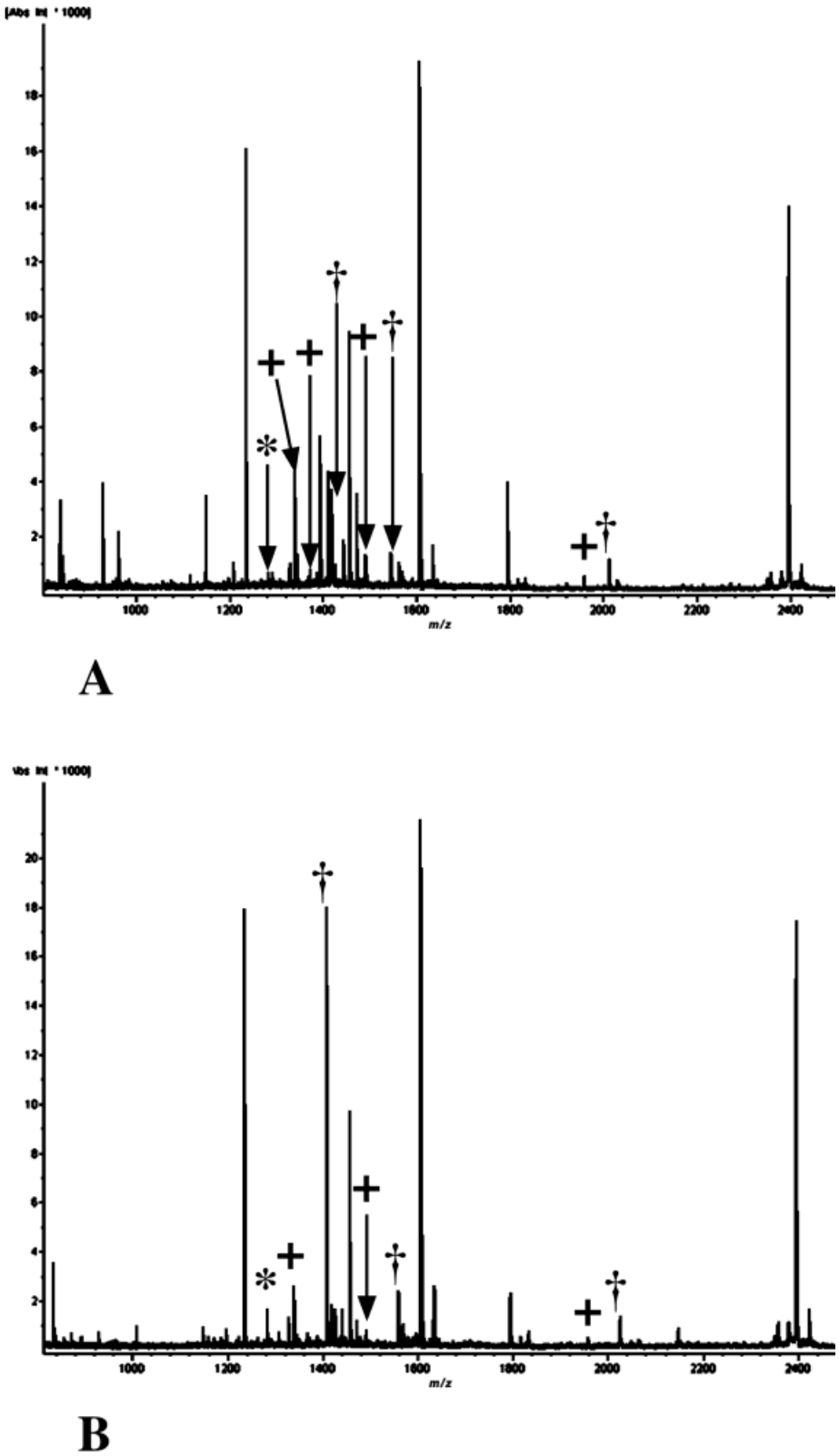


**Figure 5.** MALDI MS spectra of malate dehydrogenase. A) Proteins were alkylated with methyl maleimide and equilibrated according to the DTT-iodoacetamide method. The peptides marked with a star correspond to unalkylated cysteine-containing peptides with *m/z* values of 1281.7 (positions 92–104, Cys 93), 1313.7 (positions 204–215, Cys 212), 1432.7 (positions 79–91, Cys 89), and 1898.9 (positions 280–296, Cys 285). The peptides marked with a + correspond to the same peptides with a +57 Da shift, *i.e.* carboxamidomethylated with iodoacetamide. The corresponding *m/z* are 1338.7, 1370.7, 1489.7, and 1955.9. The peptides marked with a † correspond to the cysteine-containing peptides with a +111 Da shift, *i.e.* alkylated with methyl maleimide. The corresponding *m/z* are 1392.7, 1424.7, 1543.7, and 2009.9. b) Proteins were alkylated with ethyl maleimide and equilibrated according to the DTT-iodoacetamide method. The peptides marked with a star correspond to unalkylated cysteine-containing peptides with *m/z* values of 1281.7 (positions 92–104, Cys 93), 1313.7 (positions 204–215, Cys 212), 1432.7 (positions 79–91, Cys 89), and 1898.9 (positions 280–296, Cys 285). The peptides marked with a + correspond to the same peptides with a +57 Da shift, *i.e.* carboxamidomethylated with iodoacetamide. The corresponding *m/z* are 1338.7, 1370.7, 1489.7, and 1955.9. The peptides marked with a † correspond to the cysteine-containing peptides with a +125 Da shift, *i.e.* alkylated with ethyl maleimide. The corresponding *m/z* values are 1406.8, 1438.8, 1557.8, and 2024.0.

 

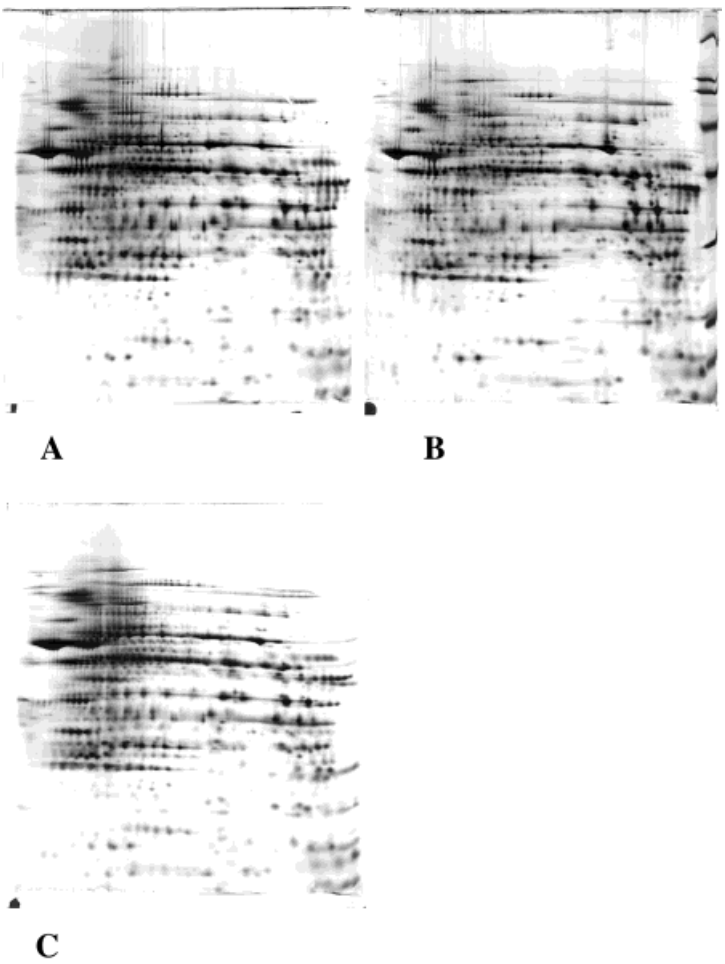


**Figure 6.** Cysteine blocking with organic disulfides. Bovine mitochondrial proteins were separated by 2-DE. First dimension: linear pH 3.75–10.5 IPG. Equilibration after IPG was performed using the DTT-iodoacetamide two-step method. Second dimension: 10% T gel. Detection was performed with silver staining. The proteins were reduced with 5 mM TBP prior to being applied to the IPG strip. A) IPG strip containing 100 mM DTDE. B) IPG strip containing 100 mM dithiodiglycerol. C) IPG strip containing 100 mM dithiodipyridine.

trations, any reduction alkylation process after IEF becomes impractical. Thus, we performed a very simple equilibration in urea-glycerol-SDS buffer. Typical results are shown in Fig. 7, and corresponding mass spectra are shown in Fig. 8. It is easily seen that a correct resolution is achieved at 500 mM DTDE. Furthermore, the cysteine-containing peptides are correctly seen on the MS spectra, but as a mercaptoethanol adduct, *i.e.* with a mass shift of 76 Da. Quantitative analysis of the spectra showed that the derivatization yield was above 85%. No improvement or deterioration was seen when 1 M DTDE was used (data not shown).

Finally, we investigated whether cysteine blocking by this method also increased the resolution in basic pH gradients sufficiently that in-gel rehydration could be used

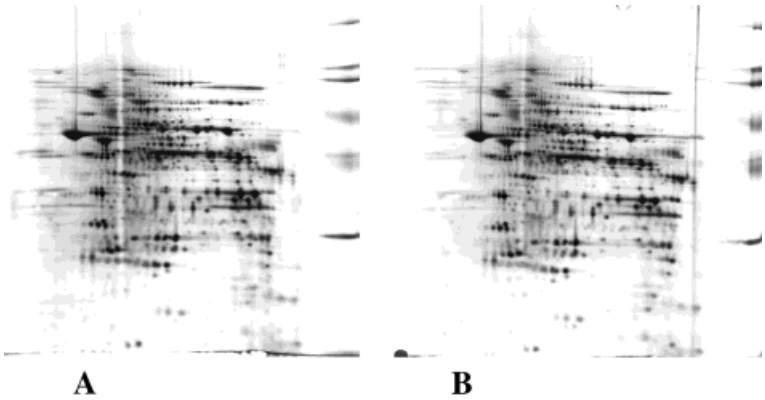


**Figure 7.** Cysteine blocking with high concentrations of DTDE. Bovine mitochondrial proteins (1 mg) were separated by 2-DE. First dimension: linear pH 3.75–10.5 IPG. Second dimension: 10% T gel. Detection was performed with colloidal Commassie Blue. Proteins were reduced with 5 mM TBP prior to application on the IPG strip. A) IPG strip containing 100 mM DTDE. Equilibration after IPG was performed using the DTT-iodoacetamide two-step method. B) IPG strip containing 500 mM DTDE. Equilibration was performed without DTT or iodoacetamide.

 

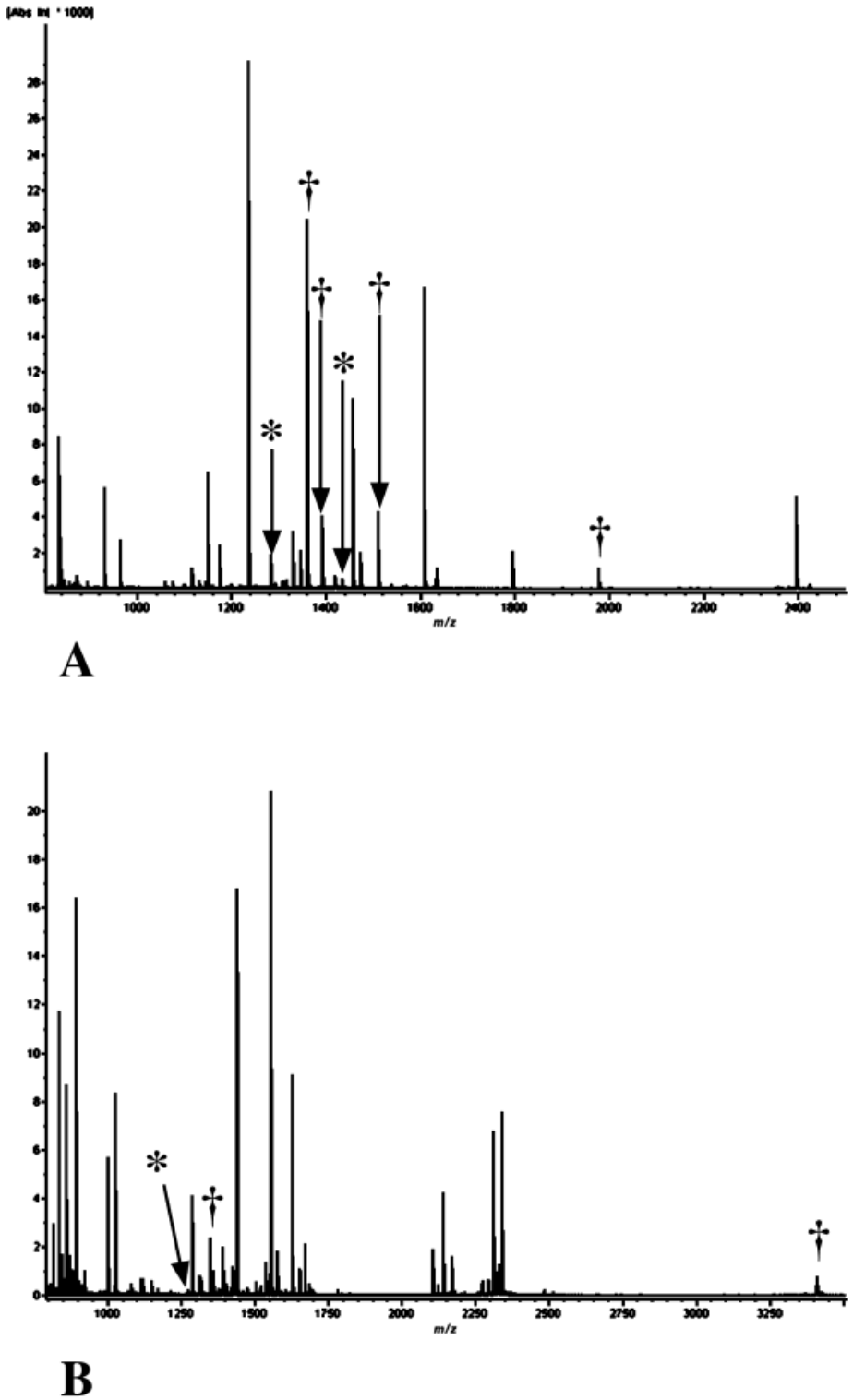


**Figure 8.** MALDI MS spectra of proteins treated with 500 mM DTDE. A single step equilibration without DTT or iodoacetamide was performed. A) Malate dehydrogenase. The peptides marked with a star correspond to unalkylated cysteine-containing peptides with *m/z* of 1281.7 (positions 92–104, Cys 93), 1313.7 (positions 204–215, Cys 212), 1432.7 (positions 79–91, Cys 89), and 1898.9 (positions 280–296, Cys 285). The peptides marked with a † correspond to cysteine-containing peptides with a 76 Da shift, *i.e.* with a mixed disulfide containing a mercaptoethanol moiety. The corresponding *m/z* values are 1357.8, 1389.8, 1508.8, and 1975.0. B) ATP synthase, alpha subunit. The peptides marked with a star correspond to unalkylated cysteine-containing peptides with *m/z* values of 1270.7 (positions 242–252, Cys 244) and 3331.6 (positions 271–301, Cys 294; not detected). The peptides marked with a † correspond to the cysteine-containing peptides with a 76 Da shift, *i.e.* with a mixed disulfide containing a mercaptoethanol moiety. The corresponding *m/z* values are 1346.8 and 3407.7.

 

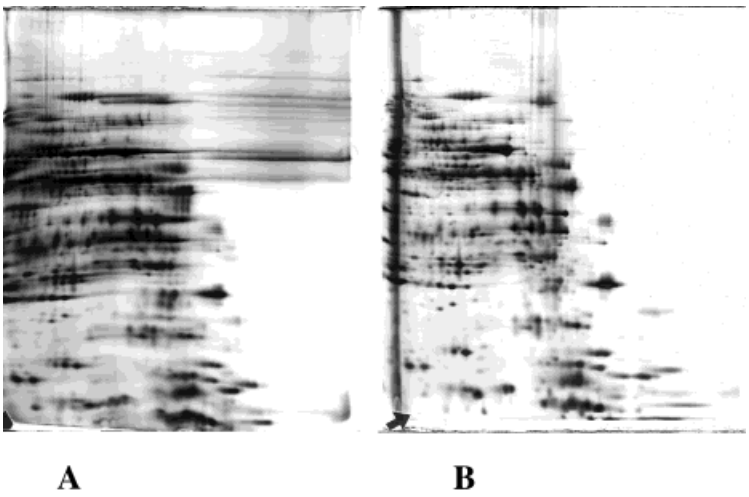


**Figure 9.** Influence of application method for analysis of basic proteins. Bovine mitochondrial proteins (0.1 mg) were separated by 2-DE. First dimension: linear pH 6–12 IPG. The proteins were reduced with 5 mM TBP prior to application on the IPG strip, which contained 0.5 M DTDE. Equilibration was performed without DTT or iodoacetamide. Second dimension: 10% T gel. Detection was performed with silver staining. A) Application by in-gel rehydration. B) Application by cup-loading at the anodic side.

instead of cup-loading (Fig. 9). The results show that this is unfortunately not the case, and that cup-loading is still required for optimal resolution in the basic gradients.

## 4 Discussion

The importance of protein thiols in the resolution problems encountered in the basic range have been reported [5], but no real convenient solution had been described until recently. The DTT process [5] is difficult to control, as is the alkylation process with iodoacetamide or acrylamide [6]. The first user-friendly solution came from a recent paper which introduced thiol-disulfide exchange as a cysteine derivatization process. In order to obtain a better understanding of the molecular processes at play, we performed a combined study by 2-DE in gradients extending into the basic pH range, coupled with peptide mass fingerprinting to characterize the reactions taking place on the proteins.

Our results clearly demonstrate the danger of alkylation procedures performed with acrylamide, which have often been proposed as a convenient alkylation method [6, 7].

Even at moderate pH (6.5), cysteine alkylation was not complete after 6 h, as shown by the presence of underivatized cysteine, while alkylation had already occurred on lysine residues. The same problem was previously demonstrated for iodoacetamide [6], and we show here that it occurs with a reactive alkylating agents. The only alkylating agents which do not give rise to spurious alkylation are those derived from maleimide. However, their alkylation yield is poor under the conditions used for IEF solubilisation buffers (*i.e.* multimolar concentrations of urea and thiourea, as well as the presence of carrier ampholytes to scavenge spurious reactions on lysine and the presence of detergents). Although determination of cysteine derivatization by integration of signals on mass spectra is always questionable, we believed that the solubility and ionization properties were dictated mainly by the peptide backbone and only slightly modulated by the side group on the cysteine chain. This entitled us to perform comparative integration of the mass signals arising from the same peptide but varying in the side group grafted to the cysteine thiol. The only exception to this rule might be the underivatized peptide itself, which may cross-link and give rise to insoluble products. This means in turn that this signal may be underevaluated, thereby leading to an over estimation of the real yield of the derivatization process.

Whatever the real yields are, it is puzzling to see that a substantiable increase in resolution can be reached only with 30% alkylation of the protein thiols, as shown by the maleimide experiments, and that increased blocking of the thiols does not ameliorate the situation. Complete and specific thiol blocking may even improve the situation. However, we believe that phenomena other than simple thiol-linked problems are at play, as demonstrated by previous studies [16].

## 5 Concluding remarks

We suggest the routine use of 0.5 M DTDE in the gel for pH gradients extending above pH 8. This procedure has the additional benefit of simplifying the equilibration procedure (one bath of urea-SDS-glycerol buffer) without compromising the subsequent resolution of the cysteine-con-

 

taining peptides in MS. Simpler treatment of the protein-containing gel plugs prior to digestion is even possible, as no reduction-alkylation is required. However, if special cysteine derivatization must be performed at this stage, the reversibility of the thiol-disulfide exchange still make it possible.

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