

Study of Urinary Proteomes in Patients with Nephrotic Syndrome

(urinary proteomes / nephrotic syndrome / two-dimensional electrophoresis)

L. VOJTOVÁ¹, T. ZIMA¹, V. TESAŘ², M. KAZDEROVÁ²

¹Institute of Clinical Biochemistry and Laboratory Diagnostics, ²Department of Nephrology, 1st Faculty of Medicine, Charles University, and General Teaching Hospital, Prague, Czech Republic

Abstract. Heavy proteinuria may be caused by either increased glomerular basement membrane permeability or membrane or podocyte structural damage, and also by impairment of secretion-reabsorption tubular processes. The precise composition of modified or degraded urine proteins in proteinuria is not known. However, a possible toxic effect of proteins on tubular cells and disease progression is assumed. In this study, 15 patients with nephrotic proteinuria and other diagnoses (systemic lupus erythematoses with renal involvement (lupus nephritis) and AAV) were analysed by the 2D electrophoresis method. We have studied sample stability during storage, the albumin separation effect on sample analyses using ammonium sulphate, and the effect of proteases on the protein spectrum. In the first step, the proteins were divided by the isoelectric focusing method using polyacrylamide strips (pH 3–10 linear). The second step involved two-dimensional SDS electrophoresis performed in 12% polyacrylamide gel, which separated proteins according to their molecular weight. The proteins were visualized by the silver method. The gels were evaluated by Phoretix 2D expression software 2005. We found out that samples are stable for more than 6 months provided that they are frozen to –80°C. The separation of albumin caused higher lucidity of the urinary proteomes. Without adding protease inhibitors we could detect proteolysis with increased quantity of proteins manifested in the area of about 10 kDa and decreased quantity of proteins detectable in the area with molecular weights about 50 kDa.

Introduction

Proteinuria is a well-recognized negative prognostic factor for patients with kidney disease. The possible presence of proteases among various urinary proteins

should be stressed, as they may play an important role in degradation and changing structure and features of the filtered proteins. Ultimately the reabsorption by tubular cells may be affected. Common laboratory methods do not allow detection of changes in these urinary protein molecules. However, these changes of proteins may contribute to renal damage and its progression. Proteomic analysis (2D, LC-MS/MS, HPLC-MS/MS) provides many advantages compared with conventional methods such as Western blot analysis, because a large complement of expected and unexpected proteins can be examined simultaneously. Western blot analysis is limited by the relatively small number of proteins that can be studied in a single experiment and the availability of a specific antibody to the targeted protein is also necessary. However, HPLC coupled to tandem MS (HPLC-MS/MS) requires complicated instrumentation and MS analyses are required for all experiments. Construction of a urinary proteome map on 2D gel may therefore be a useful technique, because the recent methodology of 2D PAGE provides a consistent protein spot pattern (Thongboonkerd, 2004).

The presented data are focused on the study of urinary protein changes in glomerular renal diseases (predominantly with the nephrotic syndrome) using modern analytical techniques such as isoelectric focusing and two-dimensional (2D) electrophoresis in polyacrylamide gels. A similar technique of protein determination by 2D electrophoresis is being studied in other laboratories, which are focused on different types of diseases such as tubular renal disease (Fanconi syndrome) or various carcinomas (Cutillas et al., 2004; Pieper et al., 2004; Rossing et al., 2005).

Protein analysis using 2D electrophoresis gives a very clear picture about proteins contained in the sample. Earlier studies have shown that this technique is comfortable for protein analysis from animal sources (Gygi et al., 2000; Washburn et al., 2001; Pyo et al., 2004). We have focused on detailed urinary protein analysis (technique optimization, sample preparation, and protein stability) in patients with glomerulopathies accompanied mostly with nephrotic syndrome. Next, we studied the technique of albumin separation from the sample and the protease effect on protein changes in urine samples.

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Corresponding author: Lucie Vojtová, Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University and General Teaching Hospital, Karlovo náměstí 32, 120 00 Prague 2, Czech Republic. Phone: +(420) 224 966 627; e-mail: lucie.vojtova@vfn.cz

The main aim of this study was optimization of the two-dimensional (2D) technique in order to obtain a clear spectrum of proteins in the urine of patients with heavy proteinuria and identification of noticeable protein changes in urine in the presence of proteases.

Material and Methods

The following chemicals and instruments were used in the study:

- 2-DE ampholytes pH 3–10L (Amersham Biosciences, Uppsala, Sweden);
- BenchMark™ Protein Ladder (Invitrogen, Glasgow, Great Britain);
- Protease inhibitor cocktail (Sigma, Saint Louis, MO);
- IPG Strip pH 3–10L (Amersham Biosciences);
- electrophoresis Multiphor II (Amersham Biosciences);
- SDS-PAGE vertical electrophoresis (Scie-Plas, Warwickshire, Great Britain);
- mini vertical electrophoresis (Hofer, San Francisco, CA);
- Power Supply EPS 3501 (Amersham Biosciences,);
- spectrophotometer Modular (Roche, Basel, Switzerland);
- dialysis membrane Visking, 10 kDa (Roth, Karlsruhe, Germany).

Solutions

The following solution were used:

- acetate buffer (50 mM acetate; 0.3 M NaCl; 6 mM EDTA; pH = 5.3)
- Ranford's assay solution (0.1 mM Coomassie brilliant blue G250; 5% (95% ethanol); 10% (85% H₃PO₄))
- Rehydration stock solution (Amersham Biosciences) – 2D electrophoresis
- Equilibration buffer solution (Amersham Biosciences) – 2D electrophoresis
- 30% acrylamide mixture (29% acrylamide; 1% N, N'-methylenebisacrylamide)
- TRIS-glycin solution for SDS electrophoresis (10x) (290 mM TRIS; 2.5 M glycin; 1% SDS)

Preparation of samples

Urinary samples from 15 patients with nephrotic proteinuria and other diagnoses (systemic lupus erythematoses with renal involvement (lupus nephritis) and ANCA-associated vasculitis (AAV)) were analysed. Their proteinuria varied between 0.97 and 10 g per day.

Thirty-ml samples from 24-h collection of urine were used for the analyses. Total protein concentration was determined using pyrogallol red (Modular). Urinary samples from patients with proteinuria less than 1 g were concentrated using Amicon Ultra (Millipore, Billerica, MA) (10 kDa, 5 min, 3000 x g, 4°C) (Joo et al., 2003). Samples were analysed immediately or sample aliquots were stored at –80 °C.

Stability of samples

Samples that were not analysed immediately were stored at –80 °C. Their stability was validated by repeated analyses after 30 and 180 days, respectively. Again, we used the centrifugation method described above where the quantity of proteins in the samples was lower than 1 g/24 h.

To test the stability of proteins, we studied five different samples of patients with the nephrotic syndrome. Protein spectra of samples analysed immediately after collection, 30 and 180 days after storage at –80 °C, were compared using software Phoretix 2D expression 2005.

Separation of albumin

Albumin was separated from samples containing albumin concentrations higher than 2.9 g/ 24 h using ammonium sulphate (Harlow and Lane, 1999). The amount of ammonium sulphate was added to the given volume of each of the five samples studied, so as to get 50% saturated solution. The samples were incubated and mixed at 4°C for 16 h. Exactly 50% saturation of the sample by ammonium sulphate causes protein agglutination and only albumin remains diluted in the solution. The mixture was next centrifuged (Amicon Ultra 10 kDa, 10 min, 10 000 x g, 4°C). The pellet containing all urine proteins except albumin was diluted in distilled water and transferred to the dialysis membrane (molecular mass cutoff at 10 kDa). The following dialysis against 1.5 M Tris buffer, pH = 7.5, removed the ammonium sulphate, which might disturb the subsequent detection of proteins. The protein concentration after removing albumin was determined by Bradford's assay. The samples prepared by this method were analysed immediately.

Effect of proteases

The presence of proteases was studied in samples from five patients with primary glomerulopathy (three of them with nephrotic syndrome) after 48-h incubation at 37 °C.

Three aliquots from each patient were prepared in this part of the study. Two identical 5-ml aliquots from each urine sample were separated immediately after collection. Forty µl of a cocktail containing protease inhibitors (serine, cysteine, aminopeptidase) and sodium azide were added to one aliquot of each sample. The second aliquot contained only sodium azide. These two aliquots were incubated 48 h at 37°C.

Another (third) aliquot obtained from each sample was frozen at –80°C immediately after the collection without adding any other substances.

All aliquots were analysed together at the same time.

Determination of protein concentration

Bradford assay

Bradford's spectrophotometric method (Bradford, 1976) was used to determine protein concentration and bovine albumin was used for calibration. The samples for analysis were prepared by mixing 200 µl of the sam-

ple with 2.5 ml of the Bradford reagent. The next step was 10 min incubation at room temperature (25°C) and absorption was monitored at 595 nm against a standard.

Method using pyrogallol red

We used the method described by Watanabe et al. (1986). After the collection of urine (1057 ml/24 h) the sample was diluted 10-fold using a solution containing 60 µl/l pyrogallol red, 40 µmol/l sodium molybdate and 50 nmol/l succinate, pH 2.3. Absorption was measured after 10-min incubation of the samples at 37°C using the spectrophotometric instrument Modular (Roche).

2D electrophoresis

The proteins were divided by the isoelectric focusing method (IEF) using 7-cm polyacrylamide strips with immobilized linear gradient pH 3–10 (IPG strip pH 3–10L, Amersham Biosciences). The total amount of protein placed on each polyacrylamide strip was 20 µg. Each strip was rehydrated overnight with a rehydration solution. The isoelectric focusing was performed in three steps to reach 3500 V. After completion of the IEF, the proteins on the strip were equilibrated with an equilibration buffer or the strip was frozen at –80°C for later use. The IPG strip was then transferred onto 12% polyacrylamide gel and the second dimensional separation

was performed in SE260 Mini-Vertical Electrophoresis Unit (Amersham Biosciences). The proteins were then visualized by the Silver Method (Silver Bullit Kit, Amresco, St. Louis, MO).

Gel comparison

The software Phoretix 2D expression 2005 was used for protein analysis. The patient's urine sample that was analysed immediately after collection was used as a reference gel. This reference gel was then used to compare proteins between investigated gels with or without added protease inhibitors. The protein content (in %) was calculated using the following formula:

% protein: [total protein in the sample (with or without inhibitors)/total protein in the sample analysed immediately after collection] × 100

Results

Stability of samples

The nephrotic proteinuria samples were investigated. According to the definition of nephrotic syndrome, the protein content of urine in proteinuria is higher than 1 g.

It was therefore not necessary to concentrate urine samples before 2D analysis when silver detection is used for protein visualization. Two different urine samples from patients with the nephrotic syndrome analysed on the first and 30th day after collection of samples are shown in Fig. 1. The study of the protein spectrum using software Phoretix 2D expression 2005 before and after 30 days of storage at –80 °C did not show protein changes in the sample. Even after 6-month storage of the sample, its reanalysis produced identical results (Fig. 1).

Separation of albumin

In some samples, albumin deteriorated the lucidity of the urinary proteomes. Therefore, albumin was removed from the samples by a method based on different protein solubility at a given concentration of salts. Two protein maps of patients' samples with proteinuria are shown in Fig. 2. The separation of albumin from sample (A) and (B) resulted in higher lucidity of the urinary proteomes and higher protein resolution in an area with molecular weight > 30 kDa. The lower amount of albumin in the sample is visible at a 1D electrophoreogram of three fractions, namely proteins before extraction of the albumin, supernatant after centrifugation, and urine proteins after separation of the albumin (see Fig. 3).

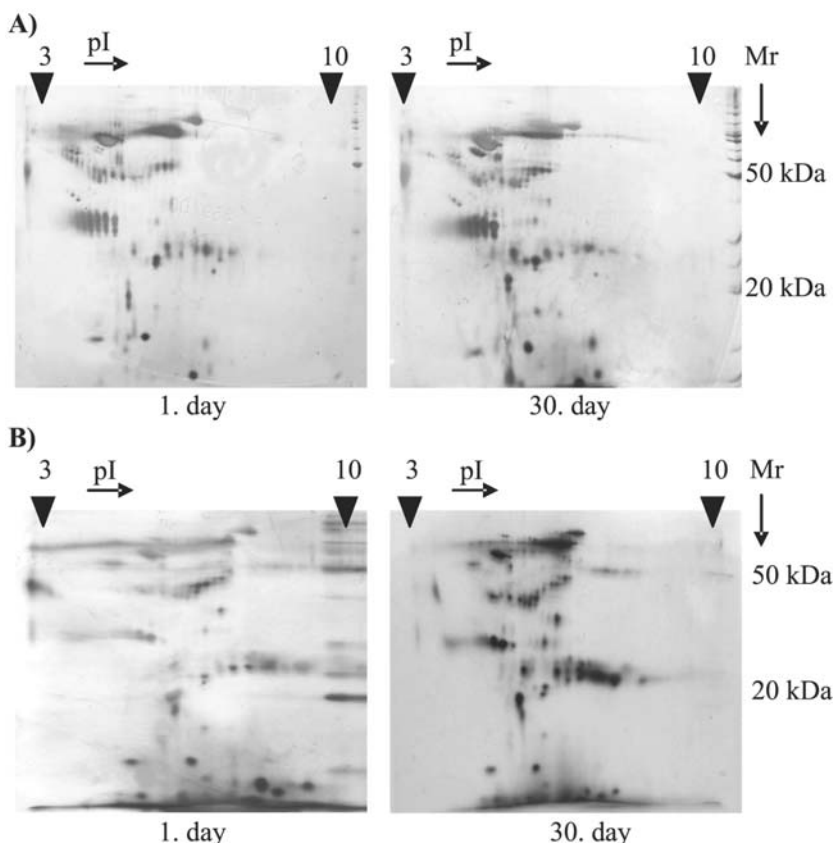


Fig. 1. Comparison of 2D electrophoreograms analysed in different intervals after sample collection. The urine samples were analysed immediately after collection using 7-cm polyacrylamide strips pH 3–10L by the IEF method, where 20 µg of total protein amount were loaded on each strip. The proteins were next separated by SDS-PAGE in 12% polyacrylamide gel and detected by silver.

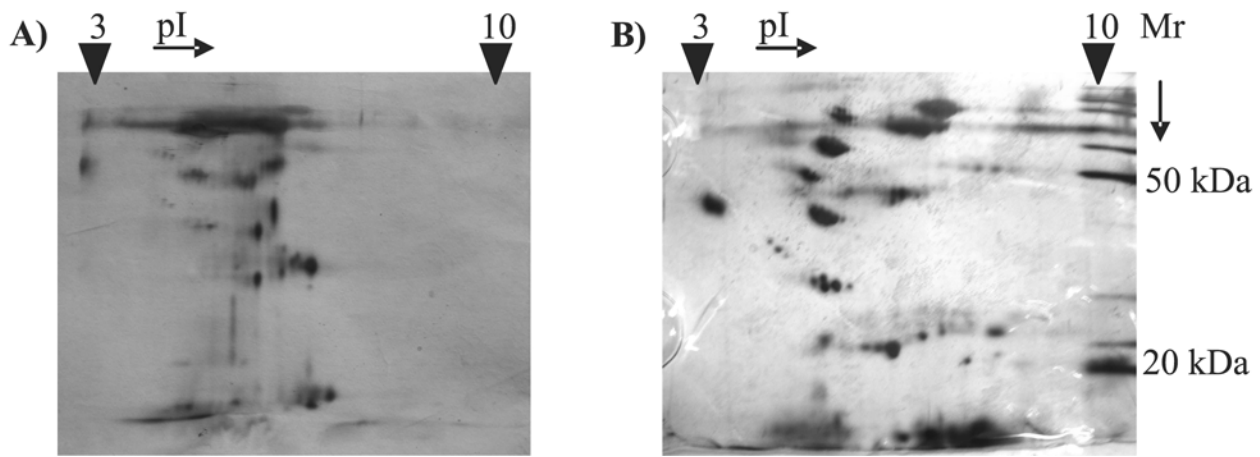


Fig. 2. Comparison of protein map before and after removal of albumin. The urine samples were analysed immediately after collection using 7 cm polyacrylamide strips pH 3–10L by the IEF method, where 20 μ g of total protein amount were loaded on each strip. The proteins were next separated by SDS-PAGE in 12% polyacrylamide gel and detected by silver. The protein spectrum of a patient with nephrotic syndrome before (A) and after (B) removal of albumin.

Effect of proteases

The quantity of enzymes (including proteases) in the urine in kidney disease increases due to damaged plasma filtration and may unfavourably affect protein analysis results. Figure 4 shows four different urine samples from patients with the nephrotic syndrome. The first three electrophoreograms (A, B, C) are focused on the total urinary protein spectrum comparison and the last one (D) is focused on the area of molecular weight higher than 30 kDa.

The study of protein spectra (urine samples from five different patients with primary glomerular disease) using software Phoretix 2D expression 2005 has con-

firmed important protein changes observable at two areas of molecular weight, i.e. range 50–100 kDa and 10–20 kDa (Fig. 4 – diagram marked in black). We could detect proteolysis in samples without adding any protease inhibitors, as manifested by the increased amounts of small proteins (about 10 kDa). On the other hand, a decrease of proteins was observable in the area with molecular weights about 50 kDa. Therefore, we focused on quantitative changes and found strong elevation and drop of the amount of the proteins in these molecular weight groups. The amount of proteins with the lowest molecular weight about 10 kDa in samples without addition of the inhibitors increased more than twice (Table 1 and Fig. 5).

Table 1. Average percentage changes of selected proteins observed using Phoretix 2D expression software version 2005. Samples were collected from five different patients.

Mol. w.	Protein	Sample with inhibitors (average %) ^a	Sample without inhibitors (average %) ^b	Probable mol. w.	Probable pI
>50 kDa	1	↓ 8 ± 1	↓ 13 ± 1	89	7
	2	↑ 23 ± 2	↓ 36 ± 2	64	5
	3	↑ 24 ± 2	↑ 114 ± 5	45	6.2
	4	↑ 6 ± 2	↑ 17 ± 1	48	5.1
>30 kDa	5	↑ 8 ± 1	↑ 28 ± 2	39	6.6
	6	↓ 10 ± 2	↓ 48 ± 3	33	6.3
	7	↑ 5 ± 1	↑ 15 ± 2	30	7
	8	↓ 4 ± 1	↓ 7 ± 1	32	5.5
	9	↑ 9 ± 2	↑ 38 ± 3	33	5.2
	10	↓ 13 ± 1	↓ 30 ± 2	27	5.6
10 kDa	11	↓ 11 ± 1	↑ 32 ± 2	15	5.9
	12	↑ 50 ± 2	↑ 256 ± 16	10	7

^a) N = 5: 100 – [(amount of protein in the sample with inhibitors / amount of protein in the sample analysed immediately after sample collection) × 100]

^b) N = 5: 100 – [(amount of protein in the sample without inhibitors / amount of protein in the sample analysed immediately after sample collection) × 100]



Fig. 3. SDS-PAGE in 12% polyacrylamide gel detected by silver: 1. standard; 2. urine proteins before albumin extraction; 3. supernatant containing albumin after centrifugation; 4. urine proteins after albumin extraction.

On the other hand, the proteins in the area of about 30–50 kDa increased and decreased with lesser contrast in both experiments (with or without inhibitors).

We found similar spectra of proteins in samples analysed immediately after the collection and analysed after incubation with protease inhibitors. This indicates that the effect of proteases can be bypassed either by immediate analysis or by proteolysis inhibition. On the other hand, proteases in the urine may induce marked changes in its protein spectra, namely increase of small molecules (proteins of about 10 kDa) and decrease of molecules with 50–100 kDa (Table 1, Figs. 4, 5).

We found out that samples are stable for more than 6 months provided that they are frozen to -80°C . The separation of albumin caused higher lucidity of the urinary proteomes. Without adding protease inhibitors we could detect proteolysis, with increased quantity of proteins manifested in the area of about 10 kDa and decreased quantity of proteins detectable in the area with molecular weights about 50 kDa.

Discussion

Increasing numbers of scientific laboratories specialize in the study of urine proteins using 2D electrophoresis (Nejedlý et al., 1986; Engliš, 1995; Rossing

et al., 2005) in order to get superior spectra for subsequent MALDI-TOF MS analysis and protein identification. The comparison of protein spectra among certain renal diseases, but also patients' protein maps before and after treatment, can lead to detection of biomarkers typical for a specific disease. As mentioned in the Introduction, insufficient kidney function may lead to a higher amount of proteases in the urine. These proteases may affect the true content and composition of proteins in the urine during analysis. The main aim of our study was the urine sample preparation and 2D technique optimization in order to reduce protein loss or change before analysis and to verify the effect of the proteases on patients' protein maps.

In most cases, the samples were treated immediately after collection to reduce protein loss due to other factors. Other samples were frozen at -80°C for later analysis. The sample stability during storage was confirmed by urine sample reanalysis 30 days after the first analysis. Protein spectrum analysis confirmed that freezing and subsequent storage did not affect the presence of proteins in urine samples. Earlier study of protein detection in urine using 2D electrophoresis called attention to the presence of interfering substances, which can negatively affect analysis using isoelectric focusing (Oh et al., 2004). We applied limited amounts of protein to the polyacrylamide strip and analysis was performed by silver detection to avoid these problems without using sample dialysis and subsequent lyophilization (Oh et al., 2004).

Albumin is the main protein in the serum, and therefore also in the urine. Its molecular weight is about 67 kDa. Besides this form, the patient's urine also contains its various fragments. As shown in Fig. 2, separation of albumin caused higher lucidity of the urinary proteomes and higher protein resolution in the area with molecular weight > 30 kDa. This fact was confirmed by 1D SDS-PAGE electrophoresis, where only albumin was contained in the supernatant fraction after centrifugation (Fig. 3). The ammonium sulphate protein precipitation is suitable for analysis of samples with lower protein content. In this case, the optimal concentration of this salt is 75% (Thonboonkerd et al., 2006). It is possible to separate the albumin from a sample using Affi-Gel Blue agarose kit from the BioRad Company (Oh et al., 2004), where the lucidity of the protein spectra was observed to be similar with our results. Another study (Ahmed et al., 2003) documented similar changes when albumin was isolated from serum samples. These methods do not eliminate those albumin fragments that might have a toxic effect on tubular cells. It was established (Jain et al., 2005) that albumin is not a specific indicator for diagnosis of renal damage. Albumin is common in urine in both healthy and diseased individuals (its amount in healthy subjects is very low). The removal of albumin from the samples does not hinder detection of potential markers for a given disease.

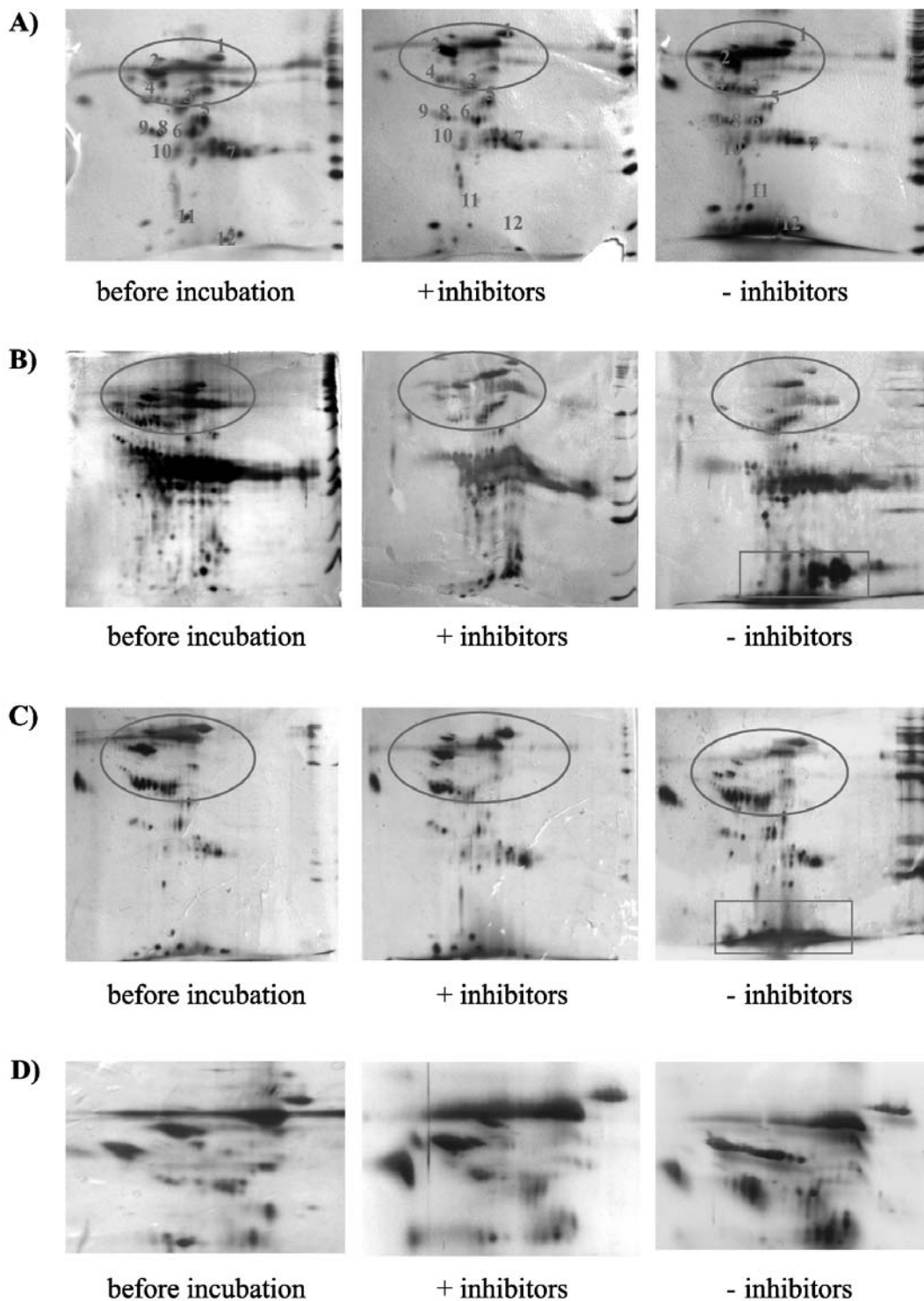


Fig. 4. Changes in the protein map in accordance to the presence of protease inhibitors in the samples of four different patients (A, B, C, D) with nephrotic syndrome. D) detailed comparison of protein map changed in the area of molecular weights higher than 30 kDa. Samples were analysed after incubation using the IEF method on polyacrylamide strips, where 20 μ g of total protein amount were loaded on each strip. The proteins were next separated by SDS-PAGE in 12% polyacrylamide gel and detected by silver.

The occurrence of proteases and their protein degradation effects were confirmed by a study at physiological temperature 37°C for 48 h. When a cocktail of protease inhibitors was added to the samples, there were only slight changes of protein spectra compared

with samples analysed immediately after collection. On the other hand, a decrease in the quantity of a protein with molecular weight about 55 kDa (protein 2) was observed in all samples studied without addition of the inhibitors, which might be explained by the effect of

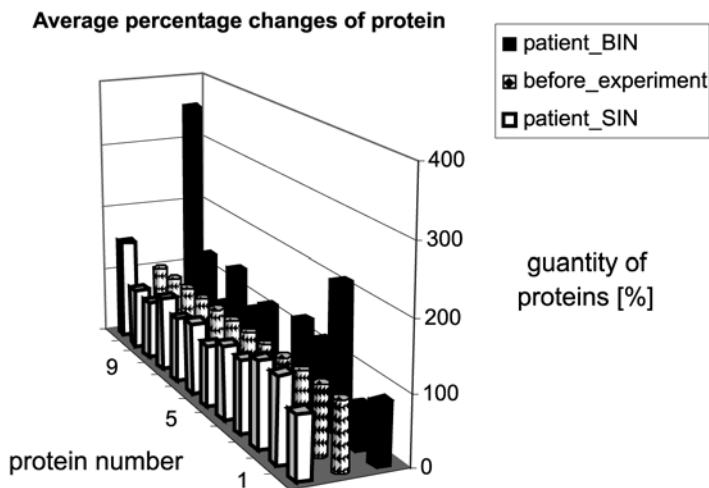


Fig. 5. Graphical demonstration of average changes in the protein content of samples collected from five different patients before 37°C incubation, after incubation with addition of inhibitors (SIN), and after incubation without addition of inhibitors (BIN).

proteases. On the contrary, the quantity of the same protein in samples with added inhibitors was increased. This could be explained by the thermal effect on proteins with higher molecular weights (e.g. albumin) due to the incubation at 37°C for 48 h. The temperature effect is not as noticeable as protein cleavage by the enzyme. Therefore, there is no such strong decomposition of proteins into smaller fragments. Their increased quantity is detectable only in the protein marked 2 compared to samples without inhibitors. This fact is especially seen in Fig. 4D. The incubation of samples without addition of the inhibitors caused more extensive protein changes in the area with molecular weight about 50 kDa. Increased proteolysis was visible in proteins with molecular weight lower than 20 kDa, especially in patient B, whose sample contained an unusually large quantity of proteins in the area with molecular weight about 30 kDa. Consequently, we can assume that degradation products occurred mainly in the area of about 10 kDa. This study implies that proteases have probably some effect on protein degradation in tubular processes and may be necessary for protein reabsorption by tubular cells. It is assumed that proteolytic activity may also occur at room temperature; however, it probably proceeds more slowly. It is therefore advisable to analyse the samples immediately or freeze them at -80°C, because the sample stability established in this way is at least one month. Finally, we should also mention the possibility of increased protease concentration in urine in patients with acute pancreatitis or cancer diseases, as confirmed by the studies of Sáez et al. (2005) or Botchkina et al. (2005), where concentrations of these enzymes were identified as diagnostic markers of the disease.

Conclusion

We optimized the 2D electrophoresis technique for samples of patients with the nephrotic syndrome and other primary glomerulopathies. We found that albumin separation by ammonium sulphate should be applied

when the albumin concentration in a sample is higher than 2.9 g/24 h. We assume that proteases probably play an important role in protein degradation in tubular processes. We focused on the groups of patients with renal disease with different types of heavy proteinuria. The study of urinary protein composition might contribute to the search for relatively specific markers of individual renal diseases and better explore their activity and severity than conventional proteinuria determination.

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