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Short communication

Resolution of fibronectin and other uncharacterized proteins by two-dimensional polyacrylamide electrophoresis with thiourea

Luca Musante, Giovanni Candiano*, Gian Marco Ghiggeri

Nephrology Section, G. Gaslini Children Hospital, Genoa, Italy

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Abstract

Several proteins, which are recognized components of serum, are not resolved by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) under standard conditions. One major example is fibronectin, which is detected in fairly high concentration (milligram range) by immunoassays, while undetectable in 2D-PAGE gels. Following several experiments with a combination of zwitterionic and chaotropic substances we obtained a good resolution of the protein in gels containing 0.5 *M* thiourea plus 8 *M* urea. By this technique, fibronectin was, for the first time, found to be microheterogeneous between p*I* values of 5.3 and 5.6 . Besides fibronectin we detected three other families of uncharacterized proteins with M_r of 130 000, 110 000 and 34 000 respectively, whose identity and function are currently under investigation. © 1998 Elsevier Science B.V.

Keywords: Fibronectin; Proteins

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the method of choice for resolving single proteins from a mixture, with a wide application in the field of isotype analysis and purification [1].

Historically, 2D-PAGE was based on a first separation of proteins according to their p*I* values utilizing a steady-state gradient of pH values obtained with carrier ampholytes [2], which are however unstable over prolonged separation, produce artefacts due to an interaction with proteins and require very low amounts of sample application. Most of these drawbacks have been overcome by the introduction of 2D-PAGE in immobilized gradients (IPG) which allows better reproducibility, good stability and maximal loading capacity [3]. However, IPG still present a drawback, with major loss of some high-molecular-mass proteins due to reasons which are so far not well understood [4]. One example is fibronectin (M_r 240 000) which is practically absent in 2D-PAGE with IPG in current run conditions, much effort being required for improving protein solubility at its own p*I* value. This protein is detectable by immunoassays in serum at relatively high concentrations (in the milligram range) and tends to increase during selective pathological conditions such as asthma, gestosis and chronic inflammatory process [5].

At the end of a detailed evaluation of several combinations of detergents with chaotropic, zwitterionic and other substances we obtained satisfactory results with a combination of urea and thiourea

*Corresponding author.

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which allowed, an evaluation of fibronectin isoforms in plasma and the detection of three families of uncharacterized proteins which are currently under investigation.

2. Experimental

2.1. Chemicals

Carrier ampholytes, Resolyte, were purchased by BDH (Poole, UK); IPG strips were from Pharmacia (Uppsala, Sweden). All chemicals for electrophoresis, of the purest grade, were obtained from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

Plasma/serum sample preparation was described by Hochstrasser et al. [6]. Briefly, 5 μl of plasma/serum were mixed with 10 μl of a denaturing and reducing solution (sodium dodecyl sulphate (SDS) 1 g, dithioerythritol (DTE) 0.302 g, water to 10 ml), and heated at 95°C for 5 min. After a short cooling, various combinations of DTE, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), (6–8 *M*) urea, (0.5–3 *M*) thiourea and other alkyl derivatives were added. Best results (see below) were obtained with 8 *M* urea, 0.5 *M* thiourea, 4% CHAPS, 65 m*M* DTE in 40 m*M* Tris. For routine analysis, 65 μl of the final diluted sample (corresponding to 0.65 μl of serum/plasma) were loaded onto the first dimension.

The amount of fibronectin in serum was evaluated, prior to electrophoresis, by nephelometric assay utilizing polyclonal anti-fibronectin antibodies (Boehring, Mannheim, Germany). The usual concentration of fibronectin loaded in 0.65 μl of serum was about 100–150 ng.

2.3. Rehydration of IPG gel strips

Hydration of Immobiline dry strips in nonlinear pH 3–10 was performed according to Bjellqviest [7], using the Pharmacia cassette for reswelling of dried Immobiline gels, following the manufacturers instructions. The following solutions were utilized: 8 *M* urea, 0.5 *M* thiourea, 2% (w/v) (CHAPS); 0.6% (w/v) carrier ampholytes Resolyte with a wide-range mixture (60% pH 4–8, 40% pH 3.5–10) 13 m*M* DTE and trace of Bromophenol Blue. Reswelling of IPG strips was carried out overnight for 15–20 h at 4°C.

2.4. Immobilized pH gradients (first dimension)

Isoelectric focusing using immobilized pH gradients (IPGs) was performed as described by Görg et al. [8], and later modified by Bjellqviest et al. [7]. All samples were routinely loaded onto the cathodic end of the rehydrated IPG strips, and covered with low-viscosity paraffin oil. Isoelectric focusing was performed at 10°C. The voltage was progressively increased from 300 to 3000 V during the first 3 h, followed by 5000 V for a total of 100 kV/h.

Before the second-dimensional run, IPG strips were equilibrated within the strip tray for 15 min with a solution of 100 ml of 0.05 *M* Tris–HC1 buffer (pH 6.8) containing 6 *M* urea, 30% (w/v) glycerol, 2% (w/v) SDS and 2.6% (w/v) DTE. SH groups were subsequently blocked by equilibration (10 min) with the same solution containing iodoacetamide (3.5% w/v) instead of DTE and trace of Bromophenol Blue.

2.5. SDS-PAGE (second dimension)

The vertical dimension was performed on 180×160×1.5 mm slabs of polyacrylamide gradient gels (T 8–16%) using piperazine diacrylamide (PDA) as cross-linking agent. The electrophoretic equipment included a Protean II Multi-Cell vertical chamber and a power supply 3000×1 (Bio-Rad, Hercules, CA, USA). SDS was not incorporated into the gels, and stacking gels were not employed. IPG strips were cut to size with second-dimensional gels, and sealed onto slab gels with the upper buffer solution containing 0.5% agarose. The gels were run at 45 mA/gel constant current and maintained at a temperature between 8 and 12°C.

2.6. Staining and western blot

Staining of proteins was performed with the methyl-trichloroacetate negative staining [9] followed by silver staining [10]. Transblot of proteins

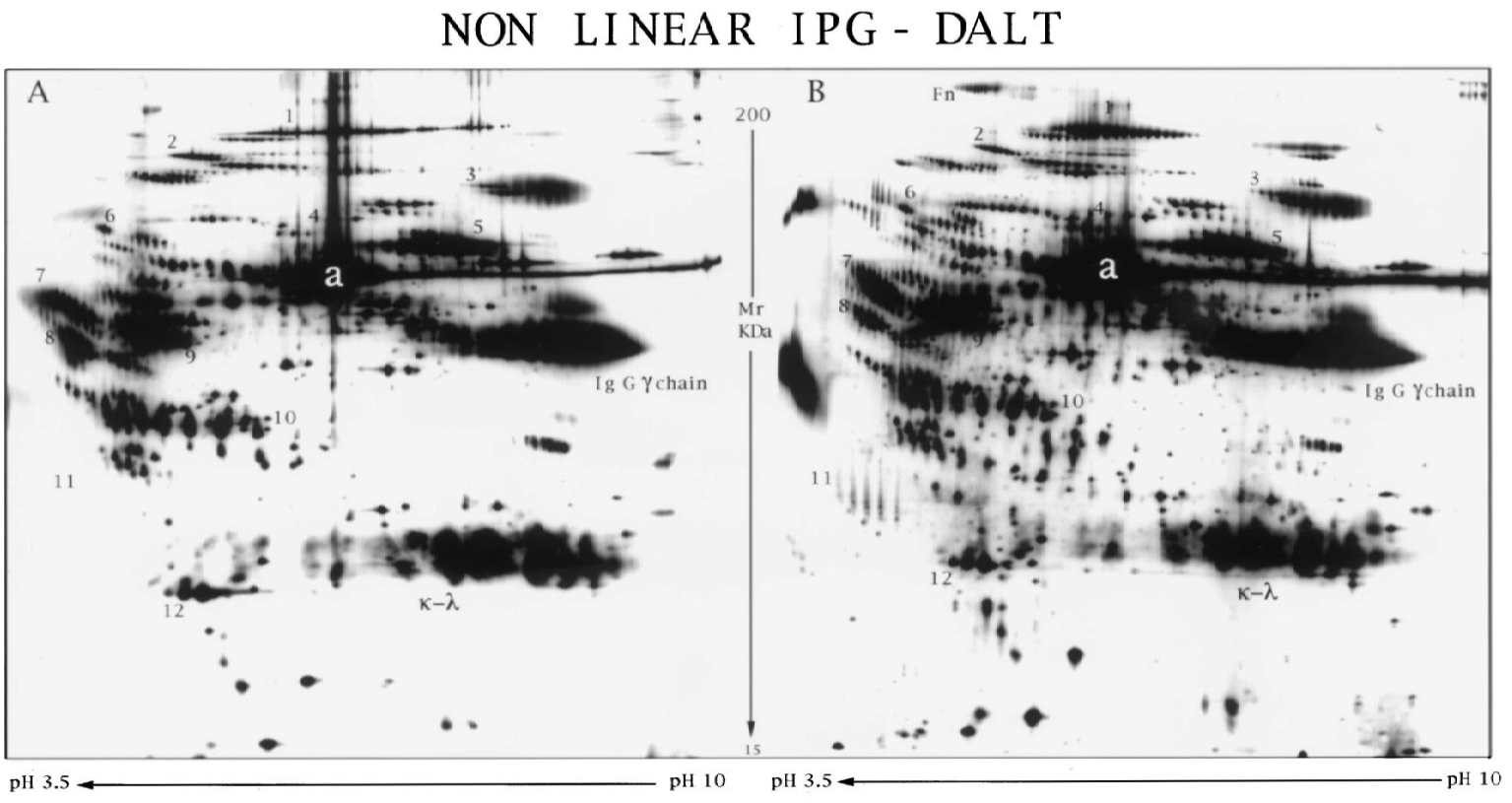

Fig. 1. 2-PAGE of serum proteins from a normal patient; (A) classical condition of electrophoresis; (B) current conditions utilizing 0.5 *M* thiourea–8 *M* urea. Abbreviations: a=albumin, Fn=fibronectin; κ–λ=Ig light chain; (1) α_2-macroglobulin; (2) ceruloplasmin; (3) Glu and Lys-plasminogen; (4) IgM heavy chain; (5) transferrin; (6) C 1s; (7) α_1-antichymotrypsin; (8) α_2-HS- glycoprotein; (9) α_1-antitrypsin; (10) haptoglobulin β-chain; (11) Apo-D; (12) Apo A-1.

to Hybond nitrocellulose membrane (Amersham, Little Chalfont, UK) was done in a Nova Blot semidry system (Pharmacia Biotech) utilizing a continuous buffer system: 48 m*M* Tris, 39 m*M* glycine, 0.035% SDS, 20% methanol. The transfer of proteins was achieved at 1.25 mA/cm^2 for 3.5 h.

Immunostaining was performed with polyclonal rabbit antihuman fibronectin antibodies (Bohering) and phosphatase development with the secondary antibody being goat-anti rabbit linked to alkaline phosphatase (Bio-Rad).

3. Results and discussion

A few high-molecular-mass plasma/serum proteins are not resolved by 2D-PAGE with IPGs under current run conditions [11]. The loss of resolution is an extremely selective phenomenon involving some components such as fibronectin which is a high-molecular-mass protein (240 000) detectable in fairly high concentration (milligram range) in serum by immunoossays while other proteins, such as α2-macroglobulin, with a comparable molecular mass, are readily resolved. Since the putative reason for the loss of fibronectin in 2D-PAGE is poor solubility and/or interaction with IPGs at its isoionic point we first tried to ameliorate the solubility of the protein and to inhibit any interaction with the gel. Having performed several experiments with different detergents and/or combination of detergents with zwitterionic and chaotropic substances we obtained best results with a combination of urea and thiourea. As shown in Figs. 1B and 2A fibronectin was resolved by 2D-PAGE in gels containing 8 *M* urea and 0.5 *M* thiourea while it was not detectable in traditional conditions (Fig. 1A, Fig. 2B). The effect of thiourea was strictly dose-dependent since at higher amounts we found loss of resolution at acid pH values (<4.5). This effect involved, in fact, several acid proteins such as α2-HS-glycoprotein, α_1-antichymotrypsin and orosomucoid (data not shown). At the final conditions of urea and thiourea utilized, we could handle gels with very sensitive staining such as the negative one and/or classical silver stains and also were able to blot proteins to nitrocellulose for immunostaining. We have no clear explanations for the ameliorative effect of thiourea, however, a few possibilities can be mentioned. The first is that thiourea can inhibit better than urea any hydrophobic interaction of proteins with themselves and/or with matrix. A second possibility is that thiourea acts synergically with DTE to maintain proteins in their reduced state. Finally, a possible interaction of thiourea with proteins cannot be excluded, although it seems rather improbable due to

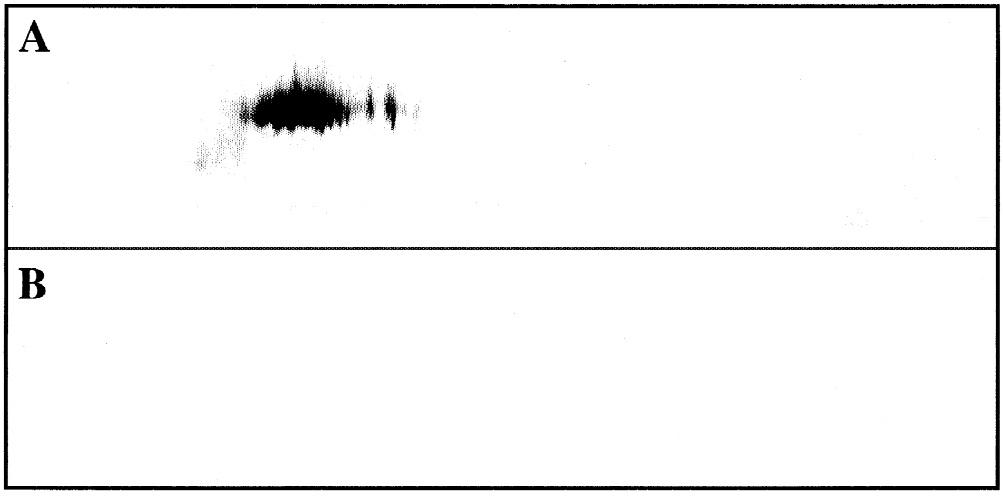

Fig. 2. Western blot and immunodetection of fibronectin with polyclonal anti-human fibronectin antibodies: (A) current conditions of electrophoresis in 0.5 *M* thiourea–8 *M* urea; (B) traditional conditions.

the extreme reproducibility of p*I* in 2D-PAGE protein. In fact, together with fibronectin, thiourea allows the detection of only three other families of proteins with molecular masses of 130 000, 110 000 and 34 000 (Fig. 3B and D) respectively which are not resolved in absence of thiourea and whose identity and functions are currently under investigation. Our observations are in full agreement with Rabilloud et al. [12] who recently published a detailed analysis of factors which improve resolution of membrane and nuclear proteins in IPG gels and clearly demonstrated a marked improvement with a combination of 5 *M* urea and 2 *M* thiourea. Our data extend therefore their original observation [12] to high-molecular-mass proteins of plasma/serum, with the distinguishing characteristics of the lower concentration of thiourea.

Based on our data on fibronectin analysis by 2D-PAGE in thiourea, this protein appears microhetergeneous over a range of p*I* values between 5.3 and 5.6.

The concept the fibronectin is microheterogeneous is not new, although the most up-to-date reports indicate different molecular mass subunits generated by alternative splicing and only two isoforms in respect to charge. Our observations indicate a wider microheterogeneity likely due to glycosylation, phosphorization and sulfation [5].

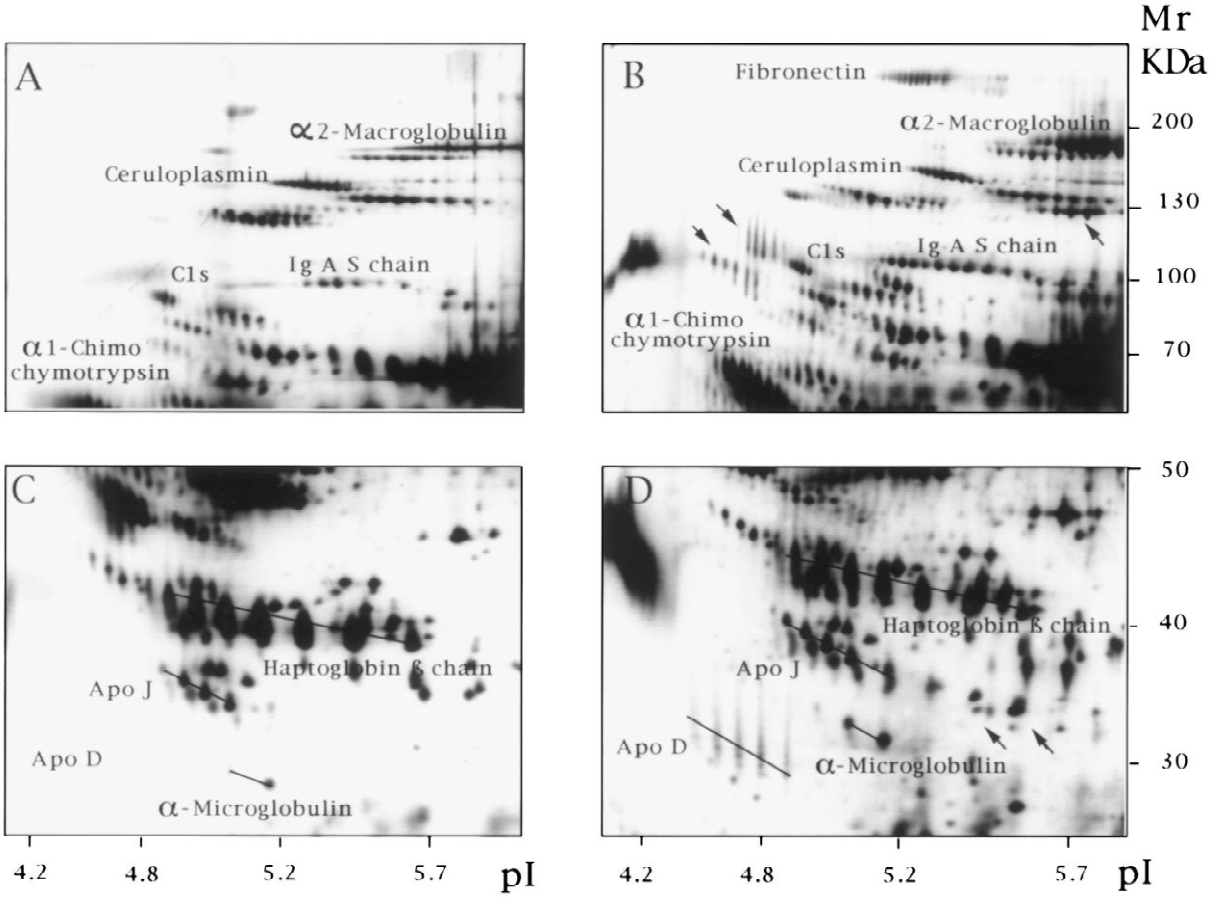

Fig. 3. Enlarged area from Fig. 1 showing high- and low-molecular-mass proteins in traditional conditions of electrophoresis (A, C) and in the presence of 0.5 *M* thiourea (B, D). Arrows indicate proteins resolved in the presence of thiourea.

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