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Convenient high-resolution isoelectric focusing (IEF) method for the separation of alpha$_1$-proteinase inhibitor (A1PI) isoforms in A1PI concentrates

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Abstract

Currently, high-resolution separation of A1PI is done in highly specialized laboratories using gels made in-house. This paper presents a high-resolution method for the separation of A1PI concentrates and human plasma using commercially available gels. Hybrid IEF was performed with carrier ampholytes and the gels were stained with Coomassie Brilliant Blue G-250. In addition, a sensitive immunoblotting procedure is described. The IEF method allowed the reproducible and convenient determination of the IEF pattern of A1PI in concentrates including resolution of glycan-dependent isoforms and isoproteins with secondary modifications such a C-terminal Lys-truncation. Furthermore, a shift in the IEF pattern of A1PI occurring upon reduction could be detected. Finally, in combination with a sample pretreatment step, the method proved able to monitor complex A1PI isoform patterns in samples with low A1PI concentrations as present for example in bronchoalveolar lavage solutions.

Keywords: Alpha$_1$-antitrypsin; Isoelectric focusing; Isoform pattern; Alpha$_1$-antitrypsin concentrates

1. Introduction

Early work on the microheterogeneity of alpha$_1$-proteinase inhibitor (A1PI) described a combination of paper electrophoresis, agar-gel electrophoresis, and immunoelectrophoresis to analyze isoforms [1]. More recent work used starch gel electrophoresis, which led to the introduction of the multiallelic protease inhibitor (Pi) system [2,3]. These techniques were replaced by isoelectric focusing (IEF) in polyacrylamide gels [4–6], which results in a characteristic pattern of the normal M-type A1PI. This consists of the two main bands M4 and M6, and three minor bands, M2, M7 and M8 that all contribute to the microheterogeneity. The development of immobilized pH gradients (IPG) made it possible to separate A1PI variants that differ only slightly in their isoelectric points (pI), often caused only by deletion or exchange of a single charged amino acid [7–11]. These high-resolution IEF methods can usually only be established in few highly specialized laboratories and require laborious preparation of the gels.

Human A1PI [12] is the major serine protease inhibitor in human plasma. Its most important physiological function is the inhibition of neutrophil elastase, a potent serine protease able to destroy most forms of connective tissues [13]. The effect is seen in subjects with deficiency or functional impairment of this inhibitor who develop emphysema upon destruction of lung tissue [14,15]. For the substitution therapy, three plasma-derived A1PI concentrates – Prolastin [16], Aralast [17] and Zemaira – are available which differ in purity, concentration and content of active, inactive and polymerized A1PI. As recently shown [18] A1PI from each preparation differs in molecular terms including deamidation, cysteinylation and elimination of C-terminal lysine and, in fact, none of the three is identical to native A1PI from human plasma. Because these molecular alterations do not affect

Abbreviations: A1PI, alpha$_1$-proteinase inhibitor; DTE, dithioerythritol; IEF, isoelectric focusing; IPG, immobilized pH gradient; Pi, proteinase inhibitor; pI, isolectric point

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the active center of A1PI, the preparations do not differ in their inhibitory activity against elastase.

In human plasma, the mature 52 kDa highly polymorphic protein with at least 49 gene allelic variations [19] is found as a single 394-amino acid polypeptide chain with three complex type N-glycans. Variations in the N-glycan branching [20] and N-terminal truncation [21] lead to the microheterogeneity observed by isoforms within a given homozygotous phenotype of A1PI.

Here, a convenient high-resolution hybrid IEF method is described that has been developed for analysis of A1PI concentrates used to treat hereditary emphysema. This method uses commercially available IPGs and can be used in any good laboratory. The method offers the possibility of detecting the A1PI isoform pattern even in solutions with very low levels of A1PI. In addition, the method proved suitable for preparing samples for further detailed protein and N-glycan analysis of the separated bands with mass spectroscopy [6].

2. Materials and methods

2.1. A1PI concentrates and other A1PI samples

The following batches of the plasma-derived A1PI concentrates Aralast, Prolastin and Zemaira were used: Aralast batches nos. LH02031A, LH03002A and LH04039A (Alpha Therapeutics, now Baxter); Prolastin batches nos. 26N0361 and PR4HA43A (Bayer, now Talecris); Zemaira batch no. Y400207 (Aventis Behring, now ZLB Behring). The lyophilized preparations were reconstituted according to the manufacturers' instructions. Aliquots were stored frozen at −20 °C until analysis. A lyophilized reference plasma pool (lot no. 1R92) and several single plasma specimens were obtained frozen from Baxter BioScience, the A1PI phenotype controls MM, MZ and SZ were from Canterbury Scientific (Christchurch, New Zealand).

2.2. IEF method

For the optimal IEF method IPG Immobiline gels (pI 4.2–4.9) were re-hydrated for 60 min in 20% glycerol [22] and 1% Pharmalyte 4.2–4.9 (all from GE Healthcare Bio-Sciences, Uppsala, Sweden). The test samples were diluted with distilled water to 0.5 mg A1PI/ml and dithioerythritol (DTE) as reductant at a final concentration of 5 mM. IEF was run on Multiphor II (GE Healthcare Bio-Sciences) at +2 °C. As anodic and as cathodic solutions, 0.2 M H_3PO_4 and 0.2 M NaOH were used, respectively. After pre-focusing the gel for 30 min (3000 V, 150 mA, 5 W), 20 μl samples were applied close to the cathode. Focusing was achieved in a multiphase-step gradient: 15 min, 500 V (150 mA, 5 W); 15 min, 1000 V (150 mA, 5 W); 30 min, 2000 V (150 mA, 10 W); 180 min, 3000 V (150 mA, 15 W). Typically, a total of 11,000 Vh was reached. After fixation with a solution of 11.5% trichloroacetic acid and 3.45% sulfosalicylic acid for 60 min at room temperature, the staining with Coomassie Brilliant Blue G-250 (Bio-Rad, Vienna, Austria) was done as described [23]. Briefly, 160 ml 10% ammonium sulfate in 2% H_3PO_4 were mixed with 40 ml methanol. One-hundred and sixty microliters of a 5% Coomassie Blue G (Serva, Vienna, Austria) solution were then added and the gel was stained overnight under constant shaking at room temperature.

The following conditions were tested to optimize the method: (a) re-swelling in 20 mM DTE used also as anolyte and catholyte, no pre-focusing; (b) re-swelling in 2.5% Pharmalyte 4.2–4.9, 20% glycerol; as anode and cathode solution 40 mM glutamic acid and 0.1 M NaOH, respectively, pre-focusing; (c) 2.5% Pharmalyte 4.2–4.9, distilled water as anode and cathode solution, no pre-focusing. The running conditions were as described above.

2.3. Western blotting and A1PI immunostaining

After the IEF the gel was equilibrated for 10 min with the blotting buffer (25 mM Tris(hydroxymethyl)-aminomethane, 192 mM glycin; 20% methanol), cut from the support and overlaid with a 0.2-μ nitrocellulose membrane (Bio-Rad). Electroblotting at 15 °C and 40 V lasted 16 h. The membrane was incubated for 30 min with phosphate-buffered saline-milk-Tween solution (2 mg/ml skimmed milk, Maresi, Vienna, Austria; 0.05% Tween 20, Bio-Rad). This buffer was also used for diluting the primary antibody (rabbit anti-human alpha$_1$-antitrypsin, A-012; DakoCytomation, Glostrup, Denmark) and the detection antibody (goat anti-rabbit IgG-peroxidase, Bio-Rad). Both incubations were done at room temperature for 60 min. The staining kit Opti 4 CN (Bio-Rad) was used. Samples with low A1PI concentrations or with salt concentrations incompatible with IEF were concentrated and/or desalted as follows: 1 ml sample was mixed with 20 μl rabbit serum (Rockland, Philadelphia, Pennsylvania, USA) and 1 ml 50% (w/w) polyethylenglycol 6000 solution (VWR, Vienna, Austria). The mixes were kept at +4 °C overnight. The precipitate was recovered by centrifuging at 4000 × g at +4 °C, and, dissolved in 200 μl distilled water, used for IEF.

3. Results

The method was developed with samples of A1PI concentrates but also allowed the analysis of A1PI in plasma milieu and even in bronchoalveolar lavage fluids without loosing any resolution power. Fig. 1a shows the gel re-hydrated in 20 mM DTE which was used also as electrode solution. All A1PI isoforms separated to a high-resolution especially visible in the range of the isoform M4. The Aralast concentrate displayed a second more intense band, which focused more cathodically than the M4 band in plasma and Prolastin or Zemaira. In addition, a band was observed in Aralast that focused in the acidic M2 region and was not found in such intensity in the other concentrates. The A1PI protein appeared to migrate on the surface of the gel only, resulting in faintly stained bands. Adding carrier ampholytes did result in higher resolution, but increasing the separation time (data not shown) did not (Fig. 1b–d). The amount of carrier ampholytes added however appeared not to be important because there was no difference when 2.5 or 1% were added to the rehydration cocktail. Consequently, the electrode solutions influenced the separation. Using just water resulted in