Short reports

Purification of α_1 -antitrypsin monomer by preparative electrophoresis

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Abstract

Alfa₁-antitrypsin (α_1 AT) was purified by pseudoligand chromatography and preparative electrophoresis from the serum of a patient with α_1 AT deficiency. The combination of the two techniques yielded a high grade batch of α_1 AT monomer and this was successfully used to purify the protein from the serum of PiMIM1, PiMIM2, and PiZZ phenotype subjects. This procedure should facilitate structural studies of α_1 AT variants susceptible to intracellular accumulation.

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Alfa₁-antitrypsin (α_1 AT) is a serum glycoprotein with serine protease inhibitor activity.¹ It is mainly synthesised by the liver where it undergoes specific processing before secretion. In serum α_1 AT is microheterogeneous over a narrow range of pIs. The major phenotypes are labelled according to their isoelectric properties, from M (medium mobility) to Z, the most alkaline species. In general, the electrical charge of PiM species reflects changes in carbohydrate side chains,² but other major differences are due to amino acid substitutions which render α_1 AT less soluble and prone to form globular inclusions within hepatocytes.^{3,4} This was described for both the Z variant, which presents a 342 Glu → Lys substitution,³ and for rare M-like variants, one of which (M Cagliari) is characterised by a deletion of 52 Phe.⁵ Technologies for purifying discrete amounts of α_1 AT warrant structural studies, including the amino acid sequence of α_1 AT pathological variants.

In this paper we describe a two-step method for α_1 AT purification based on pseudoligand chromatography and preparative electrophoresis. This innovative method was successfully used to purify α_1 AT from the serum of a patient with the PiZZ phenotype and severe α_1 AT deficiency.

Methods

Blood samples were obtained from 20 normal volunteers and from a patient with PiZZ homozygosity for α_1 AT, severe serum deficiency, and clinical features of chronic liver disease. Blood was collected after an overnight fast and immediately centrifuged; serum was stored at -20°C for less than one month.

The characterisation of the PiZZ molecule was carried out using the monoclonal antibody against PiZZ that is specific only for this mutant⁶; DNA analysis from the same patient was performed according to a method described before.⁵

After 24 hours of dialysis at 4°C against 0.03M phosphate buffer (pH 7) 20 ml of serum was passed through a 16 × 1.5 cm column of Affi Gel Blue (BioRad, Richmond, California) equilibrated with the same dialysis buffer. The initial 30 ml volumes were discharged while the following 45 ml were recovered and processed further. In these conditions albumin interacts with the Cibacron Blue F3GA dye, immobilised on agarose via a mechanism which is still unclear, while α_1 AT has low affinity for the dye and elutes in the recovered fraction.

Preparative electrophoresis was performed in thick (2 mm) gels of polyacrylamide (18 × 16 cm) without detergents. Gradients of polyacrylamide were cast by mixing two solutions of acrylamide (T10% and 18%), both containing constant 0.9% BIS (C = 2.5%), 40% sucrose, 0.033% ammonium persulphate and 0.033% TEMED in 37 mM TRIS-HCl (pH 8.8). The stacking gel was constant in BIS and in acrylamide concentrations (C = 4%; T = 4%) in 125 mM TRIS-HCl (pH 6.8). Electrode buffer was 25 mM TRIS-glycine (pH 8.3). The sample application point corresponded to the whole length of the upper margin of the gel. The run was continued overnight with an initial 200 volts and constant 60 mA using a Protean 11 apparatus (Bio Rad) equipped with a 1000-500 power supply. After electrophoresis the strips relative to α_1 AT were cut with a razor blade and the protein, after homogenisation, was recovered with electroelution. Gel strips fragmented in 50 mM TRIS-glycine buffer (pH 8.9) were put into tubes (16 mm in diameter) of a vertical electrophoretic chamber (Bio Rad 175 model tube cell), each tube being closed at the bottom with a plug of polyacrylamide gel of the same composition of the stacking gel. Tubes were connected with a dialysis membrane (Amicon PM 10 000 milliwatts cutoff) containing 50 mM TRIS-glycine which was immersed in the same buffer and acted as the trapping site. Electroelution was continued overnight by applying a constant 120 Volts corresponding to the initial 80 mA. The final α_1 AT concentration was determined using the Coomassie G-250 dye binding assay.

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Figure 1 (a) Separation efficiency of preparative electrophoresis in non-denaturing conditions of an effluent from Affi Gel Blue; numbers from 1 to 9 identify the uncharacterised proteins which were separated by preparative electrophoresis. Immunoblotting with polyclonal antibodies was used to characterise the migrating position in the preparative gels of different α_1 AT phenotypes (d-e) and then of purified band 8 before (c) and after chromatography on Affi Gel Blue. All α_1 AT phenotypes migrated into two major bands, one of which (band 8) was easily recovered from the preparative gel.

Discontinuous electrophoresis was performed in gradient sodium dodecyl sulphate-polyacrylamide gels (T=10–20%; C = 2.5%) according to the method of Laemmli.⁷ Gels (0.7 mm) were cast by mixing two polyacrylamide solutions (10%–20%) containing 0.9% BIS, 30% sucrose, 0.033% ammonium persulphate and 0.033% TEMED in 30 mM TRIS-HCl (pH 8.8). The run was continued overnight at an initial 60 Volts (constant 16 mA) using the Bio Rad Protean II apparatus.

For immunolocalisation of α_1 AT, proteins were transblotted to Hybond C-Super (Amersham, Little Chalfont, England) nitrocellulose membranes, using a BioRad dry

transblot cell. Transfer was carried out for three hours with 300 mA in 20 mM TRIS, 190 mM glycine (pH 8.3) plus 10% methanol. Polyclonal antibodies against α_1 AT (Dako, Copenhagen, DK) were used for immunoblotting and colour was developed with goat-anti rabbit IgG linked to alkaline phosphatase in the presence of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. Washing steps were done according to the manufacturer's instructions (Bio Rad).

Fused rocket immunoelectrophoresis was carried out in agarose gels using antibodies against α_1 AT and against total human serum proteins (Dako)

For sequencing, proteins were transblotted electrophoretically to PVDF membranes in a semi-dry apparatus (Hofer, San Francisco, California). Sequences were recorded using a Millipore Pro-Sequences 6625 (Bedford, Massachusetts) and the resulting phenylthiohydantoin amino-acid derivatives were identified using the on-line Waters 600 E PTH analyser (Millipore).

Ultrathin isoelectric focusing was performed in polyacrylamide gels containing carrier ampholytes (Pharmacia, Uppsala, Sweden) in a narrow range of pIs (pI 4.2–4.9). Runs were continued for 12 hours at a constant 1200 volts. Proteins were stained with Coomassie R-250.

Results

The main problem we encountered in purifying α_1 AT was the removal of serum albumin, owing to the striking homology in molecular weight and isoelectric point between the two proteins. This was eventually achieved by devising a two-step procedure in which albumin was first immobilised on Affi Gel Blue due to the strong affinity of this protein with the blue dye. Alpha α_1 AT, which does not bind the resin, was instead recovered and further purified using preparative electrophoresis.

Figure 2 Analytical SDS-PAGE (a–b) and immunoblotting (c–d) of an effluent from Affi Gel Blue and of purified band 8 from preparative electrophoresis. Band 8 from preparative electrophoresis corresponds to the α_1 AT monomer.

The recovery of α_1 AT at this stage was nearly 100%. During preparative electrophoresis without detergents, α_1 AT migrated in three different bands (fig 1, a) one of which (band 8) corresponded to the α_1 AT monomer of the purest grade on the basis of the immunoblot (fig 2), and the NH2 amino terminal sequence. Further analysis of band 8 showed the presence of M1/M2 phenotypes. This technique was then used to purify serum α_1 AT in a case of PiZZ homozygosis. The use of monoclonal anti-Z α_1 AT antibody confirmed the Z identity; DNA analysis from the same patient showed a point mutation at the level of the V exon (GAA-GAG). As already reported for the M species, the analysis of α_1 AT composition by immunoblot showed that band 8 accounted for the major part of the Z variant (fig 2, e), showing that our method is very effective for purifying this variant. The final recovery of α_1 AT was about 50% in both the case of M and Z α_1 AT species.

Discussion

Previous techniques for α_1 AT purification have all been based on serial chromatographic passages or on affinity chromatography.^{8,9} The one presented here is based on preparative electrophoresis after separation of α_1 AT from albumin, which is the most direct contaminant, owing to its strict homology in molecular weight and isoelectric point.

There are at least two major advantages to the newly described method: it allows high grade α_1 AT monomer to be purified, thus excluding other components with a different size (dimeric α_1 AT or fragmentation products); it works perfectly with both normal and

abnormal α_1 AT molecules. The demonstration that PiZZ α_1 AT can be easily purified electrophoretically makes it a method of choice for structural studies of α_1 AT both in vitro and in vivo. It is also simple and highly reproducible.

The highly purified monomers obtained with this method should facilitate physico-chemical analysis (crystallography, circular dichromism, amino acid fingerprinting) of the molecule, thus potentially helping to solve the unknowns on polymerisation and the insolubility of all α_1 AT variants.¹⁰

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