

Commercial plasma α_1 -antitrypsin (Prolastin®) contains a conformationally inactive, latent component

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Commercial plasma α_1 -antitrypsin (Prolastin®) contains a conformationally inactive, latent component. D.A. Lomas, P.R. Elliott, R.W. Carrell. ©ERS Journals Ltd 1997.

ABSTRACT: Fractionated plasma α_1 -antitrypsin is widely-used as replacement therapy in patients with Z α_1 -antitrypsin deficiency-related emphysema. We have recently shown that purified antitrypsin may be induced to adopt an inactive latent conformation by heating at high temperatures in stabilizing concentrations of sodium citrate. Such a conformation was predicted to be present in commercial preparations of antitrypsin, as these require heating under similar conditions for viral inactivation.

Native antitrypsin was purified from plasma, and commercial antitrypsin (Prolastin®) was obtained from Bayer Corporation. Western blot analysis of transverse urea gradient (TUG) gels showed that commercial antitrypsin migrated as two bands: one with an unfolding profile of native antitrypsin and the second with a profile of latent antitrypsin. A latent fraction, comprising approximately 8% of the total antitrypsin, was separated from the native antitrypsin in Prolastin® by anion exchange chromatography. The specific activity of this latent form against bovine α -chymotrypsin increased from 1 to 2% to 50% over 3 h after refolding from 6 M guanidine hydrochloride.

These data show that commercial antitrypsin contains a latent component. The significance of this conformation *in vivo* is unknown, although Prolastin® has shown few adverse side-effects in prolonged clinical usage.

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Alpha₁-antitrypsin is the most abundant circulating proteinase inhibitor and the archetypal member of the serine proteinase inhibitor or serpin superfamily [1]. The most clinically relevant deficiency variant, the Z mutation (³⁴²Glu→Lys) [2], results in misfolding of the protein [3], and a spontaneous interaction between the reactive centre loop of one molecule and a β -pleated sheet of a second [4–6]. This loop-sheet polymerization accounts for the hepatic intracellular inclusions [4] that predispose, in conjunction with genetic factors [7], to hepatitis, cirrhosis [8], and subsequent hepatocellular carcinoma [9]. The combination of misfolding and polymerization result in plasma levels that are only 10–15% of the normal M antitrypsin. This level of protein is insufficient to protect the lungs against proteolytic attack, and so predisposes the Z antitrypsin homozygote to early onset panlobular basal emphysema [10]. There is no proven therapy for the treatment of congenital Z anti-trypsin deficiency-related emphysema, although many patients receive intravenous replacement therapy based on trials showing biochemical efficacy [11]. This replacement therapy utilizes fractionated concentrates of human plasma antitrypsin, that have been pasteurized by prolonged heating (60°C for 10 h) in the presence of 0.38 M citrate and 37% w/v sucrose.

The molecular structure of antitrypsin, like that of all serpins, is based on a dominant A β -sheet and a mobile reactive centre loop [1]. This loop acts as a pseudosubstrate for the target enzyme neutrophil elastase forming

a 1:1 enzyme-inhibitor complex, which is then cleared from the circulation. The mobility of the loop allows the molecule to adopt a range of conformations (fig. 1), from the inhibitory conformation [12] that is responsible for docking with neutrophil elastase to full insertion of the loop into the A sheet following reactive loop cleavage [13]. The loop may also be stably incorporated into the A β -sheet in the absence of cleavage in the latent conformation, as illustrated in figure 1. We have recently described a protocol for the preparation of latent antitrypsin by heating for prolonged periods of time at high temperatures in the presence of stabilizing sodium citrate [15]. We show here that the latent conformation is present in a commercial antitrypsin preparation (Prolastin®, Bayer Corporation) used for the treatment of Z antitrypsin deficiency-related emphysema.

Methods

Native antitrypsin was purified from plasma [5] and used to derive the inactive latent, loop-sheet polymerized and reactive loop cleaved conformations using protocols detailed previously [15]. All the conformations of antitrypsin were checked by sodium dodecyl sulphate (SDS), nondenaturing and transverse urea gradient polyacrylamide gel electrophoresis (PAGE). Commercial fractionated plasma antitrypsin used for replacement therapy (Prolastin®) was obtained from Bayer Corporation, Berkeley, California, USA.

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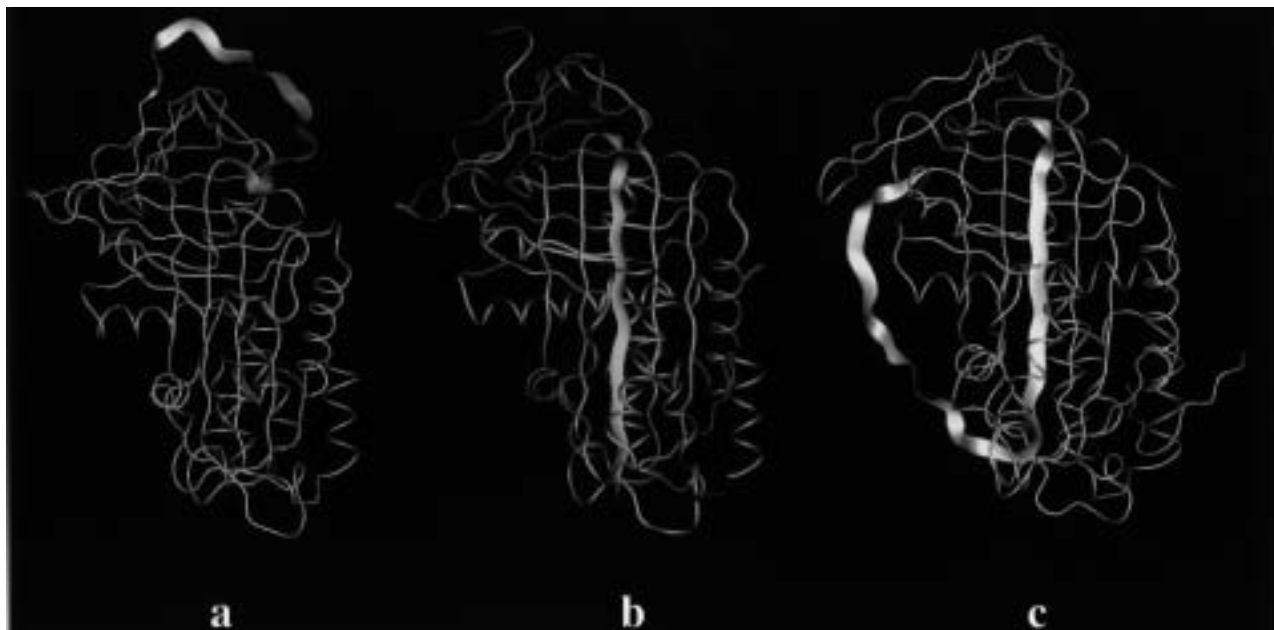


Fig. 1. – Illustration of the reactive loop conformations (bold) in: a) the inhibitory [12]; and b) reactive loop cleaved [13] conformations of antitrypsin. In the inhibitory conformation, the loop is present as a β -pleated strand that is able to dock with and inhibit the proteinase, neutrophil elastase. Following reactive loop cleavage, the molecule is inactive, as the loop is inserted into the A sheet of the protein. c) In the latent conformation of antitrypsin, the reactive loop is fully inserted into the A β -sheet of the molecule in the absence of cleavage [14], as shown here.

Separating latent from native antitrypsin in Prolastin®

The separation of latent from native antitrypsin in commercial Prolastin® was based on previous observations that the latent form is resistant to temperature-induced polymerization [15]. Native antitrypsin (25 mg) was induced to form loop-sheet polymers by heating at approximately $2 \text{ mg}\cdot\text{mL}^{-1}$ in 20 mM Tris pH 8.0, at 60°C for 15 h. The resulting mixture was loaded onto a Q-Sepharose anion exchange column ($22 \times 1.6 \text{ cm}$) at $60 \text{ mL}\cdot\text{h}^{-1}$ in 20 mM Tris pH 8.6 (4°C), washed to baseline and eluted with 0–0.4 M NaCl in 20 mM Tris pH 8.6 (4°C). The latent fraction was characterized as described previously, and refolded with the denaturant guanidine hydrochloride [15] in an attempt to restore inhibitory activity.

Results

Commercial Prolastin® was resuspended in water according to the manufacturers instructions. The antitrypsin concentration was shown to be $32 \text{ mg}\cdot\text{mL}^{-1}$ by immunonephelometry, and the preparation was 66% active as an inhibitor of bovine α -chymotrypsin, in keeping with the findings of others [11]. The protein migrated as two bands on SDS-PAGE (fig. 2, lane 4), the upper of which (arrowed) was identified as antitrypsin by Western blot analysis. In addition, there was a minor band with electrophoretic mobility similar to reactive centre loop cleaved antitrypsin that was not seen on Coomassie stained gels but was apparent when the SDS gel of Prolastin® was probed for antitrypsin by the more sensitive Western blot analysis. Transverse urea gradient gel electrophoresis followed by Western blot analysis for antitrypsin identified a constituent of Prolastin® that was resistant to unfolding in 8 M urea (fig. 4d), as is characteristic both of latent and cleaved antitrypsin [15].

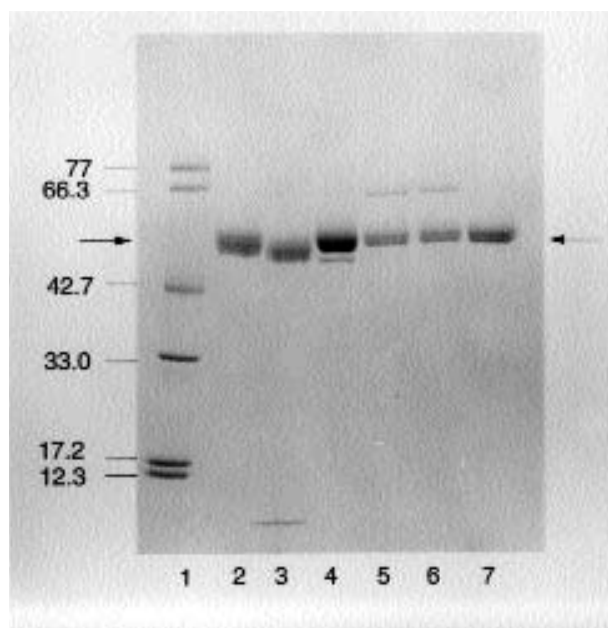


Fig. 2. – SDS-PAGE, 7.5–15% w/v, to show the purification of latent antitrypsin from commercial Prolastin®. Prolastin® ($2 \text{ mg}\cdot\text{mL}^{-1}$) was heated at 60°C for 15 h and the monomers separated from polymers by anion exchange chromatography. The arrows represent the antitrypsin bands detected by Western blot analysis. Lane 1: molecular weight markers (kDa); lane 2: native M antitrypsin control (5 μg); lane 3: M antitrypsin cleaved with *Staphylococcus aureus* V8 proteinase (5 μg); lane 4: Prolastin® starting material (5 μg); lanes 5 and 6: adjacent fractions from the monomeric latent peak (L) shown in figure 3 (5 μg each lane); lane 7: polymeric fraction from the polymeric peak (P) shown in figure 3 (5 μg). SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis.

The urea-resistant fraction could not be separated from the native antitrypsin in Prolastin® on a Q-Sepharose anion exchange column as both fractions eluted at the same sodium chloride concentration. An alternative strategy was

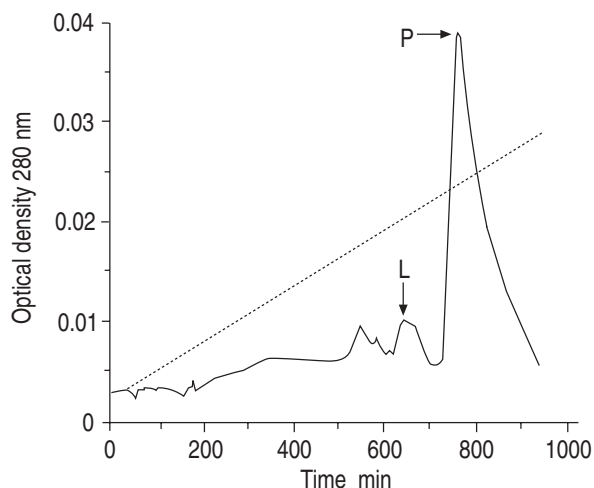


Fig. 3. — Isolation of latent and polymeric antitrypsin from Prolastin® by Q-Sepharose anion exchange chromatography. Twenty five milligrams of Prolastin® was heated at $2 \text{ mg}\cdot\text{mL}^{-1}$ (60°C for 15 h) and loaded onto a Q-Sepharose anion exchange column. Protein was eluted with a 0–0.37 M NaCl gradient (dotted line) in 15 mL fractions. L: latent antitrypsin; P: polymeric fraction.

adopted to isolate the latent or cleaved component. This was based on our previous findings that, upon incubation at 60°C , both latent and reactive loop cleaved antitrypsin were more resistant to heat-induced polymerization than native antitrypsin [15]. Commercial Prolastin® was,

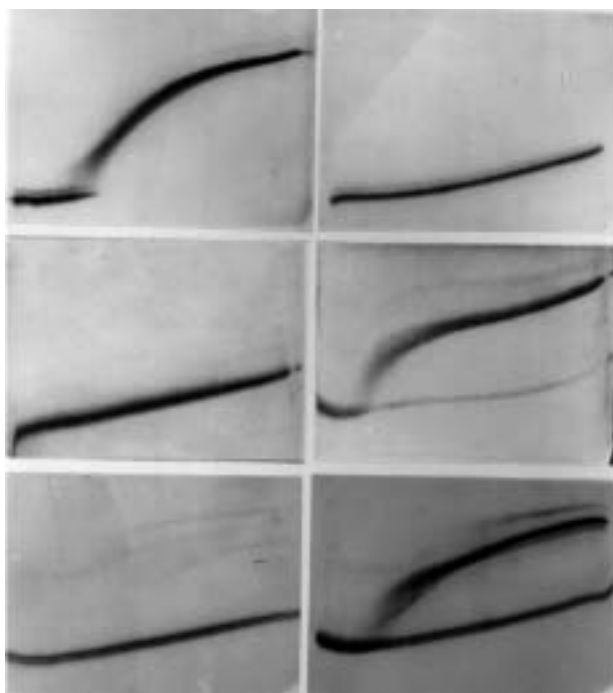


Fig. 4. — Transverse urea gradient gel electrophoresis, 7.5% w/v, of conformations of antitrypsin isolated from Prolastin®. The left of each represents 0 M urea and the right 8 M urea. All gels contain 50 µg protein. As the protein unfolds in urea it is retarded by the acrylamide matrix, producing a characteristic profile. a) native M antitrypsin; b) antitrypsin cleaved at the reactive centre loop with *Staphylococcus aureus* V8 proteinase; c) latent antitrypsin prepared as described previously [15]; d) Western blot of Prolastin® for antitrypsin showing native antitrypsin and an antitrypsin fraction that is resistant to unfolding in 8 M urea; e) latent antitrypsin isolated from the Q-Sepharose anion exchange column shown in figure 3, the major band was confirmed as antitrypsin by Western blot analysis; f) latent antitrypsin isolated from the Q-Sepharose anion exchange column (50 µg) mixed with native antitrypsin (50 µg).

therefore, heated at approximately $2 \text{ mg}\cdot\text{mL}^{-1}$ at 60°C for 15 h to induce the native antitrypsin to form loop-sheet polymers. The unpolymerized latent or cleaved component was then separated from the polymers by Q-Sepharose anion exchange chromatography. The heat-resistant peak, corrected for contaminating impurities, represented approximately 8% of the total antitrypsin in Prolastin®, as determined by area under the curve of the anion exchange profile (fig. 3). Biochemical characterization showed that this fraction contained monomeric, intact antitrypsin that was resistant to unfolding in 8 M urea when assessed by SDS (fig. 2) native (data not shown) and transverse urea gradient (fig. 4) PAGE followed by Western blot analysis.

This heat-resistant fraction of antitrypsin from Prolastin® was 1.6% active as an inhibitor of bovine α -chymotrypsin. The cardinal feature of the latent serpins is the restoration of activity following treatment with denaturants [15–18]. Refolding of the inactive fraction of antitrypsin from Prolastin® resulted in an increase in specific activity against bovine α -chymotrypsin from 1 to 2% to approximately 50% over 3 h (data not shown), with a refolding profile similar to that described previously [15]. The antitrypsin concentration for this assay was determined by immunonephelometry (Department of Clinical Chemistry, Addenbrooke's Hospital, Cambridge) to avoid confounding results from minor contaminants.

Discussion

Alpha₁-antitrypsin replacement therapy is widely-used in some countries for the treatment of patients with congenital deficiency to prevent the development, or slow down the progression, of the associated panlobular emphysema. This is based on evidence of biochemical efficacy rather than randomized controlled clinical trials showing a reduction in lung damage. The common source of replacement antitrypsin is from fractionated human plasma, which is heat-treated to inactivate viral particles, such as hepatitis B and the human immunodeficiency virus. Heat treatment of native antitrypsin induces long chains of inactive loop-sheet polymers [5] and, therefore, stabilizing agents must be added to maintain antitrypsin inhibitory activity during pasteurization. These agents prevent polymer formation but permit the heating process to drive a fraction of antitrypsin to adopt an inactive, monomeric, latent species, with properties similar to those of latent antitrypsin described previously [15]. The stabilizing concentration of citrate is lower than we have used to prepare latent antitrypsin but clearly has the same effect when combined with sucrose.

Latent antitrypsin is stabilised by the insertion of the reactive site loop into the central A β -sheet of the molecule (fig. 1c). Such a conformation is resistant to unfolding both in urea and guanidine hydrochloride, and is inactive as an inhibitor of proteinases, such as neutrophil elastase. As a consequence, this conformation of antitrypsin is unable to protect the lungs against proteolytic damage when administered as part of Prolastin®. The latent conformation comprises only 8% of the total antitrypsin in Prolastin® but provides an important demonstration that the pasteurization process itself may induce novel conformational changes that are inactive as proteinase inhibitors. In total, 34% of antitrypsin in

Prolastin® is inactive as an inhibitor of proteolytic enzymes, and this fraction may contribute to the failure to sustain adequate plasma concentrations described by some workers [19]. New strategies to stabilize antitrypsin during pasteurization would increase the specific activity of Prolastin®, with a consequent reduction in the quantity that has to be administered to provide adequate antiproteinase activity in the lungs.

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