unstable and could not be detected under the experimental conditions used.

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Registry No. D-Glucose, 50-99-7; L-galactose, 15572-79-9; D-mannose, 3458-28-4; mannose phosphate isomerase, 9023-88-5.

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Purification and Biochemical Characterization of Recombinant α_1-Antitrypsin Variants Expressed in *Escherichia coli*

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ABSTRACT: Site-directed variants of α_1-antitrypsin (α_1AT) expressed in a recombinant strain of *Escherichia coli* have been isolated with an overall process yield of 50% following tangential flow ultrafiltration, anion-exchange, immobilized metal affinity, and hydrophobic interaction chromatography. The primary structure of the purified variants including the integrity of the N- and C-termini has been verified by electrospray mass spectrometry of the intact molecules (44 kDa) for two of the variants (α_1AT Leu-358 and α_1AT Ala-357, Arg-358). Complementary classical peptide mapping and automated amino acid sequencing have verified 75% of the primary sequence of α_1AT Ala-357, Arg-358. Isoelectric focusing in an immobilized pH gradient revealed some microheterogeneity which proved to be reproducible from one purification batch to another. The isolated variants of α_1AT did not show any signs of proteolytic degradation during the purification process and proved to be fully active against their target proteases. The described process also allowed the complete removal of endotoxins from the preparations, opening the possibility to evaluate these novel protease inhibitors for their in vivo efficacy in different animal models of human disease.

α_1-Antitrypsin (α_1AT),[1] one of the major protease inhibitors in human plasma, is present at concentrations of 1.5–3.5 mg/mL (Travis & Salvesen, 1983). Its primary physiological role is the inhibition of neutrophil elastase, with an insufficiency leading to the development of pulmonary emphysema. α_1AT deficiency can be either hereditary or acquired (Laurell & Eriksson, 1963; Eriksson, 1964; Kueppers & Black, 1974; Morse, 1978a,b; Carrell et al., 1982; Gadek & Crystal, 1982; Brantly et al., 1988). Attempts are presently being made to provide a protective shield against excess neutrophil elastase

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[1] Abbreviations: α_1AT, α_1-antitrypsin; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; *E. coli*, *Escherichia coli*; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate (tetrasodium salt); PTH, phenylthiohydantoin; ELISA, enzyme-linked immunosorbent assay; IMAC, immobilized metal affinity chromatography; rtPA, recombinant tissue type plasminogen activator; RID, radial immunodiffusion; AEX, anion-exchange chromatography.

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in the lung by replacing the missing anti-protease with a concentrate of the inhibitor obtained from human plasma (Hubbard & Crystal, 1988; Schmidt et al., 1988; Hubbard et al., 1988).

α_1AT is a member of the serine protease inhibitor (serpin) protein family (Carrell et al., 1987a,b; Huber & Carrell, 1989) having a methionine residue in its P1 position (Met-358) which determines its specificity. However, this residue can be readily oxidized by chemical oxidants or oxygen radicals released from stimulated neutrophils or macrophages, thus decreasing significantly the efficiency of the inhibitor at sites of inflammation or in the lungs of cigarette smokers (Beatty et al., 1980; Rosenberg et al., 1984; Courtney et al., 1985a; Travis et al., 1985; Janoff et al., 1986; Hubbard et al., 1987; Padrines et al., 1989). Approaches have been made to overcome this problem by using recombinant DNA technology and site-directed mutagenesis. Variants of α_1AT which retain their inhibitory efficacy against neutrophil elastase under oxidative conditions have been constructed containing either Leu or Val in position 358 (Jallat et al., 1986). However, only the Leu-358 variant also retains its inhibitory activity against cathepsin G, the second neutrophil protease which is inhibited by the natural molecule.

In an attempt to further widen the scope of α_1AT variants with therapeutic potential, nonconservative amino acid changes have been made in its P1 position. This has led to efficient inhibitors of either α-thrombin (α_1AT Arg-358) (Schapira et al., 1986; Scott et al., 1986; George et al., 1989) or plasma kallikrein (α_1AT Ala-357, Arg-358) (Schapira et al., 1987). Both of these α_1AT variants can be envisaged as therapeutic agents in cases where excess thrombin or plasma kallikrein activation occurs such as in sepsis or septic shock (Colman, 1984, 1989a,b).

In order to evaluate these novel inhibitors in appropriate animal models, sufficient amounts of highly purified, active, and endotoxin-free material have to be produced. In this paper, an efficient isolation procedure which is applicable to all of the above-mentioned α_1AT variants will be described. The variants are expressed intracellularly in *Escherichia coli* (*E. coli*), purified by anion-exchange and hydrophobic interaction chromatography on a silica-based anion exchanger (Alpert & Regnier, 1979; Kennedy et al., 1986) in combination with metal chelate chromatography (Porath et al., 1975; Kurecki et al., 1979). The isolated variants are active against their target proteases, homogeneous according to different analytical criteria, and free of endotoxins.

Materials and Methods

Fermentation. α_1AT variants were expressed in a recombinant *E. coli* strain (TGE 7213) under the control of the leftward promoter of phage λ using a temperature-sensitive repressor and a synthetic ribosome binding site (Courtney et al., 1985a; Tessier et al., 1986). Cultures of 15 L were grown at 30 °C and pH 7.0 under selective conditions (Degryse, 1987) in a 20-L fermenter (LSL Biolafitte, Saint-Germain en Laye, France). At approximately 4 g/L cell dry weight (10 optical density units at 600 nm), expression was induced by changing the temperature of the culture to 42 °C for 6 h. After reaching the final biomass of 15 g/L cell dry weight, cells were harvested by centrifugation at 5000*g* for 20 min (Sorvall RC3B rotor, Du Pont, Wilmington, DE).

Purification. Soluble α_1AT variants were released from the cells by two passages through a high-pressure homogenizer (Model 15M 8TA, Gaulin Corp., Everett, MA) at 550 bar in 300 mM NaCl, 20 mM sodium phosphate, pH 6.8, and 5 mM EDTA. Cell debris was removed by centrifugation at 10000*g* (Sorvall, GSA rotor) for 1 h.

The supernatant was subsequently diafiltered against 20 mM sodium phosphate, pH 6.8, using a tangential flow filtration system with a 10-kDa cutoff membrane and 0.9 m^2 surface area (Amicon, Danvers, MA) until the conductivity reached 2.5 ± 0.2 mS. This concentrate (approximately 2.5 L) was loaded on a 2-L column (inner diameter, 10 cm) packed with a silica-based anion-exchange matrix (PAE-300, 50 μm particle diameter, 30-nm pore diameter; Amicon) equilibrated in 20 mM sodium phosphate, pH 6.8. Elution was performed with a gradient from 0 to 0.25 M NaCl in 20 mM sodium phosphate at pH 6.8 with a flow rate of 50 mL/min and a total gradient volume of 10 L. α_1AT variants were recovered between 9- and 20-mS conductivity as detected by radial immunodiffusion against a polyclonal antiserum to human plasma-derived α_1AT (Behring, Marburg, FRG).

The pool from the anion-exchange step was subsequently adjusted to 50 mM Tris-HCl and a final pH of 8–8.5 (conductivity, approximately 15 mS) by adding the appropriate volume of 2 M Tris-HCl, pH 8.5. This material was directly applied to the next chromatographic column (inner diameter, 4 cm) filled with 200 mL of chelating Sepharose Fast-Flow (Pharmacia, Uppsala, Sweden) previously charged with Zn^{2+} ions according to the manufacturer's recommendations. Elution was performed at pH 8.5 with a gradient from 0 to 0.1 M glycine in 150 mM NaCl and 50 mM Tris-HCl at 7 mL/min (gradient volume, 1.5 L). α_1AT variants were collected according to the elution profile at 280 nm and pooled based on the results of analytical PAGE in the presence of SDS (Laemmli, 1970).

The α_1AT-containing fractions were pooled and adjusted to a concentration of 2 M ammonium sulfate by adding the corresponding volume of a saturated solution for the following hydrophobic interaction chromatography. Insoluble proteins were removed by centrifugation at 8000*g* for 30 min (Sorvall, GSA rotor). The supernatant was loaded onto a 200-mL column (inner diameter, 4 cm) packed with PAE-300 equilibrated in 2 M ammonium sulfate, 50 mM Tris-HCl, and 10 mM EDTA at pH 8. Elution was performed with a descending gradient from 2 to 1 M ammonium sulfate in 50 mM Tris-HCl, pH 8, and 10 mM EDTA at a flow rate of 14 mL/min (gradient volume, 1.2 L of 2 M and 2.4 L of 1 M ammonium sulfate). Fractions containing α_1AT were pooled based on the chromatographic profile at 280 nm.

Final formulation of the different α_1AT variants was done by diafiltration against 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% (w/v) poly(ethylene glycol) (MW 6000), and 5% (w/v) sucrose to reach a final concentration of 5 mg/mL using a membrane with a cutoff of 10 kDa (Amicon). This preparation was sterile-filtered at 0.2 μm (Nalge, Rochester, NY), aliquoted into sterile tubes, and lyophilized (Model 12, Virtis, NY).

Regeneration of Chromatographic Matrices. PAE-300 used in the initial purification step was regenerated by washing with 5 column volumes of 0.5 and 1 M NaCl in 20 mM sodium phosphate, pH 6.8. After removal of the salt with water, strongly bound material was removed with 5 column volumes of 0.1 M phosphoric acid in 20% (v/v) aqueous 2-propanol. The stationary phase was finally washed with water and reequilibrated with 20 mM sodium phosphate, pH 6.8, for further use. Otherwise, the stationary phase was stored in 20% (v/v) aqueous 2-propanol to prevent microbial growth. PAE-300 used in the final purification step was regenerated as described above but omitting the washes with NaCl.

Chelating Sepharose was regenerated by washing with 5 column volumes of 50 mM EDTA in 0.5 M NaCl and 50 mM

Tris-HCl, pH 7, followed by a passage of 5 column volumes of water to remove the EDTA. Five column volumes of 0.1 M NaOH were subsequently used to remove strongly bound material. The column was finally washed with water to remove the NaOH and reequilibrated according to the manufacturer's recommendations. For storage, the stationary phase was left in 20% (v/v) aqueous 2-propanol.

The tangential flow ultrafiltration membrane was regenerated with 50 mM NaOH and stored in aqueous formaldehyde according to the manufacturer's recommendations.

Peptide Mapping. (*a*) *Digestion with Trypsin.* Fifty micrograms of α_1AT Ala-357, Arg-358 was heated in 10 mM Tris-HCl, pH 8.5, and 150 mM NaCl (50 μL) containing 2 M urea for 5 min at 95 °C to inactivate the inhibitor. After the solution was cooled to room temperature, 5 μg (10 μL) of bovine trypsin (Boehringer Mannheim, Mannheim, FRG) was added and the mixture incubated at 37 °C for 4 h with agitation. The digestion was stopped by the addition of 1% TFA (Pierce, Rockford, IL) to obtain a final concentration of 0.1% TFA.

(*b*) *Digestion with Endoproteinase Glu-C.* α_1AT Ala-357, Arg-358 was dialyzed against 0.1 M sodium formate, pH 4, and 0.1 M urea at 4 °C. Seventy-five micrograms (100 μL) of the dialyzed variant was subsequently heated to 95 °C for 5 min and incubated with 7.5 μg (50 μL) of endoproteinase Glu-C from V8 *Staphylococcus aureus* (Boehringer Mannheim). Digestion was performed at 37 °C for 17 h with agitation. The reaction was stopped by adding 1% TFA to a final concentration of 0.1%.

(*c*) *Reversed-Phase HPLC.* Peptides were separated by reversed-phase HPLC on a HP-1090 chromatograph connected to a diode array detector and a microcomputer (Hewlett-Packard, Waldbronn, FRG) using a C_{18} column (25 × 0.46 cm, 5-μm particle diameter, 30-nm pore diameter; Vydac, The Separations Group, Hesperia, CA). A flow rate of 1 mL/min and a gradient of acetonitrile from 0 to 60% in 0.1% aqueous TFA were employed with a gradient steepness of 1%/min. Eluting peptides were detected by their absorbance at 205 nm.

Amino Acid Sequencing. Amino acid sequence analysis of the purified variants and the separated peptides was performed by automated Edman degradation using a 477A microsequencer equipped with an on-line PTH-amino acid HPLC analyzer and a microcomputer (Applied Biosystems, Foster City, CA) after sample preparation by reversed-phase HPLC.

Isoelectric Focusing. Isoelectric focusing was done using an Immobiline system in the range of pH 5–6 as described by the manufacturer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Briefly, the gel was prerun at 5000 V (15 mA) for 60 min; 10–20 μg of purified α_1AT Ala-357, Arg-358 was subsequently deposited and focused at 500 V (5 mA) overnight. Proteins were subsequently fixed in the gel with trichloroacetic acid (11.5% w/v) and sulfosalicylic acid (3.5% w/v) and stained with Coomassie Brilliant Blue R-250.

Electrospray Mass Spectrometry. Samples were prepared by reversed-phase HPLC using an acetonitrile gradient in 0.1% TFA as described below. The eluting material was directly collected into 1-mL Eppendorf vials; 2–10 μL of the collected sample containing between 30 and 100 pmol/μL was subsequently introduced into the ion source of a VG Biotech Bio Q mass spectrometer (VG Biotech, Altrincham, U.K.) at a flow rate of 2 μL/min. The quadrupole mass analyzer was operated with a mass range of 4000, and the mass spectrometer was scanned from m/z = 700 to m/z = 1500 in 20 s at unit resolution. Multiple scans were accumulated, and the molecular mass was subsequently calculated from a series of multiply charged ions as previously described (Covey et al., 1988; Mann et al., 1989; Van Dorsselaer et al., 1990).

Analytical HPLC. All analyses of purified α_1AT variants were performed by using an HPLC system equipped with a variable-wavelength detector connected to a microcomputer (Gilson, Villiers-le-Bel, France).

Reversed-phase HPLC was performed on a Vydac C_4 column (25 × 0.46 cm, 5-μm particle diameter, 30-nm pore diameter; The Separations Group) at a flow rate of 1 mL/min and a gradient from 0 to 80% acetonitrile in 0.1% aqueous TFA in 60 min.

Anion-exchange HPLC was performed on a PL-SAX column (10 × 0.46 cm, 8-μm particle diameter, 100-nm pore diameter; Polymer Laboratories, Church Stretton, U.K.) at a flow rate of 1 mL/min and a gradient from 0 to 0.5 M NaCl in 20 mM sodium phosphate, pH 7.5, in 45 min.

Gel filtration HPLC was performed on two coupled TSK-G 2000 SW columns (60 × 0.75 cm each, 10-μm particle diameter; Pharmacia LKB Biotechnology) at a flow rate of 0.5 mL/min in 10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl.

Polyacrylamide Gel Electrophoresis (PAGE). Intermediate pools of partially purified α_1AT variants and the final purified material were analyzed by PAGE in the presence of SDS under reducing conditions as previously described (Laemmli, 1970) using 10% (w/v) acrylamide. Staining of protein bands in the gels was done with Coomassie Brilliant Blue R-250.

Total Protein Determination. The total protein content in the intermediate pools was determined by the method of Bradford (1976) using bovine serum albumin as the standard protein according to the manufacturer's description (Biorad, Richmond, CA). The total protein content in the final pool was determined by using purified α_1AT as a standard.

Endotoxin Determination. Endotoxins in preparations of the final purified α_1AT variants and throughout the purification process were determined by using the limulus lysate method in combination with a chromogenic substrate as described by the manufacturer (Kabi Diagnostica, Mölndal, Sweden). Absorbance readings at 405 nm were performed in a 96-well ELISA plate (Nunc, Denmark, obtained through Poly Labo, Strasbourg, France) on an automated ELISA reader equipped with a microcomputer (Molecular Devices, Palo Alto, CA). All protein samples were heated to 75 °C for 5 min and centrifuged prior to performing the assay to avoid inhibitory effects of some of the α_1AT variants on the test.

Quantitation of α_1AT Activity. Bovine pancreatic trypsin (TPCK-treated) and human α-thrombin (Sigma, St. Louis, MO) were quantified by active-site titration as previously described (Chase & Shaw, 1970). The average of five independent determinations was taken.

Quantitation of active α_1AT Leu-358 was performed by titrating against bovine pancreatic trypsin (1 μg/mL, 42 nM) at room temperature in 150 μL of 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.1% (v/v) Brij 35. A preincubation time of 120 min at room temperature was employed for complete complex formation. The stability of bovine pancreatic trypsin throughout this reaction period was assured by measuring the enzymatic hydrolysis rate of a chromogenic substrate (Chromozym TH, tosyl-Gly-Pro-Arg-4-nitroanilide acetate; Boehringer Mannheim) in the absence of the inhibitor. α_1AT Leu-358 was added in aliquots of 1.3 pmol to a final concentration of 42 nM. After 120 min at room temperature, the reaction mixture was added to 850 μL of 0.1 M Tris-HCl, pH

8.5, 0.15 M KCl, and 0.1% (w/v) PEG-6000 containing 50 μM Chromozym TH and the residual proteolytic activity measured at 410 nm for a period of 5 min at 37 °C. The quantity of active α_1AT Leu-358 was determined from the *x*-axis intercept when plotting residual proteolytic activity against the amount of α_1AT Leu-358 added assuming a 1:1 enzyme–inhibitor complex.

The thus quantitated α_1AT Leu-358 variant was subsequently used to quantitate human leukocyte elastase (Elastin Products, Owensville, MO) by titration using a preincubation time of 5 min at room temperature in 100 μL of 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.1% (v/v) Brij 35. α_1AT Leu-358 was added to this mixture in aliquots of 0.64 pmol, and the residual proteolytic activity was determined by adding the incubation mixture to 800 μL of 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.1% (v/v) Brij 35 containing 2 mM methoxy-L-alanyl-L-alanyl-L-prolyl-L-valine-*p*-nitroanilide (Calbiochem, La Jolla, CA) Residual proteolytic activity was measured at 30 °C for 5 min at 410 nm. The amount of active leukocyte elastase was determined from the amount of α_1AT Leu-358 needed for complete inhibition based on 1:1 complex formation.

Active α_1AT Arg-358 as well as α_1AT Ala-357, Arg-358 was quantified after purification by inhibition of active-site-titrated human α-thrombin using a preincubation time of 30 min at room temperature in 40 μL of 0.1 M Tris-HCl, pH 8.5, 0.15 M NaCl, and 1% (w/v) PEG-6000 at a thrombin concentration of 125 nM. α_1AT variants were added in aliquots of 0.8 pmol, and the residual proteolytic activity was measured by adding the incubation mixture to 960 μL of incubation buffer containing 0.2 mM Chromozym TH. Residual proteolytic activity was determined at 37 °C over a time period of 5 min. The amount of active α_1AT was determined from the *x*-axis intercept when the residual proteolytic activity was plotted against the quantity of the individual α_1AT variants added assuming a 1:1 enzyme–inhibitor complex.

Determination of Second-Order Association Rate Constants. The velocity of inhibition of human leukocyte elastase by α_1AT Leu-358 was determined by incubating equimolar amounts of the inhibitor and the protease (43 nM) at room temperature in 100 μL of 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.1% (w/v) PEG-6000. At defined time intervals between 0 and 2 min, 20 μL of the incubation mixture was added to 800 μL of the incubation buffer containing 2 mM chromogenic substrate (see above) thermostated at 30 °C and the residual proteolytic activity measured for 5 min at 410 nm.

The velocity of inhibition of human α-thrombin by α_1AT Arg-358 was determined by incubating equimolar amounts of human α-thrombin (105 nM) at room temperature in 250 μL of 0.1 M Tris-HCl, pH 8.5, 0.15 M NaCl, and 0.1% (w/v) PEG-6000 with α_1AT Arg-358. Aliquots of 40 μL were withdrawn each 30 s for up to 2.5 min and added to 960 μL of incubation buffer containing 0.2 mM Chromozym TH thermostated at 37 °C. Residual proteolytic activity was measured at 410 nm for 5 min.

The inhibition of human plasma kallikrein by α_1AT Ala-357, Arg-358 was determined by incubating a 5-fold excess of the inhibitor with the protease. Lyophilized human plasma kallikrein (Kabi Vitrum, Stockhom, Sweden) was reconstituted with water to 0.12 unit/mL in 50 mM Tris-HCl, pH 7.9, and 0.2 M NaCl as described by the manufacturer. Kallikrein was quantitated by measuring the hydrolysis rate of the chromogenic substrate S-2302 (H-D-Pro-Phe-Arg-*p*-nitroanilide·2HCl) (Kabi Vitrum) at 37 °C in 50 mM Tris-HCl, pH 7.9, and 0.2 M NaCl using a substrate concentration of 1.7 mM and 0.012 unit/mL kallikrein. The absorbance change at 410 nm was measured for 5 min, and the concentration of kallikrein was calculated from the relation: 0.061 absorbance unit/min = 1 nM (M. Schapira, personal communication).

Inhibition of plasma kallikrein by α_1AT Ala-357, Arg-358 was subsequently determined at room temperature under pseudo-first-order conditions in 675 μL of 50 mM Tris-HCl, pH 7.9, and 0.2 M NaCl containing 12.4 nM kallikrein and 62 nM inhibitor. Aliquots of 103 μL were withdrawn each minute up to 5 min, and residual proteolytic activity was measured at 37 °C for 5 min at 410 nm in a final volume of 1 mL of 50 mM Tris-HCl, pH 7.9, and 0.2 M NaCl containing 1.7 mM chromogenic substrate S-2302.

Results

Site-directed variants of α_1AT were expressed as intracellular proteins in *E. coli* driven by temperature induction of the leftward promoter of phage λ and a specially designed 3′ noncoding region as previously described (Courtney et al., 1984, 1985a; Jallat et al., 1986; Straus et al., 1985; Tessier et al., 1986). The five N-terminal amino acids (Glu-Asp-Pro-Gln-Gly) have been deleted to increase the level of expression (truncated variants) (Courtney et al., 1985b). The following vectors were employed: pTG 2952 (α_1AT Leu-358), pTG 7914 (α_1AT Arg-358), and pTG 2953 (α_1AT Ala-357, Arg-358). Truncated versions of the inhibitors showed identical inhibitory kinetics when compared to the full-length molecules. Previously reported results demonstrated that a truncated form of α_1AT missing its five N-terminal amino acids could be found in normal human plasma (Jeppsson et al., 1985). All variants appeared to be nonglycosylated as deduced from their apparent molecular weight of 44K on PAGE in the presence of SDS in comparison to α_1AT isolated from human plasma (52K) which contains three N-linked carbohydrate moieties (Travis & Salvesen, 1983). This was expected, since the proteins were expressed in *E. coli*.

Purification of the Variants. The described purification procedure was based on three chromatographic separation steps employing anion-exchange, immobilized metal affinity (IMAC), and hydrophobic interaction chromatography. The procedure was designed to facilitate further scale-up. Thus, it was assured that partially purified α_1AT variants obtained after one chromatographic separation step could be directly applied to the following matrix with only minor adjustments in either salt concentration or pH value. This opened the possibility for rapid processing with a significantly reduced risk of proteolytic degradation during purification. The process necessitated the use of only two different stationary phases, since the anion-exchange support employed in the first chromatographic step could also be used in the hydrophobic interaction mode upon changing the mobile phase conditions (Kennedy et al., 1986).

α_1AT variants containing either Leu or Arg residues in position 358 as well as the variant with Ala-357, Arg-358 have been purified by the process outlined in Table I with an overall yield of approximately 50%, giving about 300 mg of homogeneous inhibitor from a fermentation volume of 15 L at a biomass of 15 g/L cell dry weight. Recovery was at least 85% after each purification step based on quantitation by radial immunodiffusion (RID) against a polyclonal antiserum to human plasma-derived α_1AT.

After the cells were harvested by centrifugation and cell breakage in a high-pressure homogenizer, α_1AT variants were recovered in the supernatant of the following centrifugation step at about 0.8% of the total soluble cell protein. The initial ultrafiltration step at a cutoff of 10 kDa removed low mo-

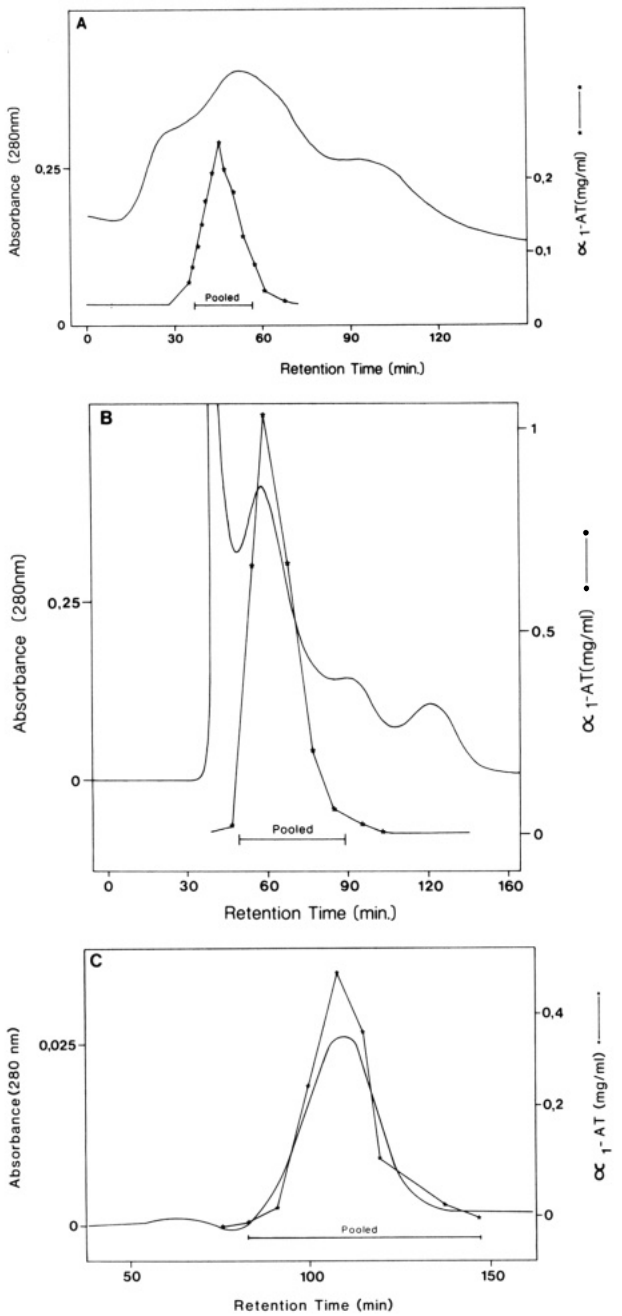

FIGURE 1: Detection of total protein by absorbance at 280 nm (—). Detection of α_1AT by radial immunodiffusion (*—*). Fractions were pooled as indicated. (A) Anion-exchange chromatography of α_1AT Ala-357, Arg-358 after cell breakage, centrifugation, and ultrafiltration at a cutoff of 10 kDa (see Table I) on a silica-based stationary phase (PAE-300, 50-μm particle diameter, 30-nm pore diameter) using a gradient from 0 to 0.25 M NaCl in 20 mM sodium phosphate, pH 6.8, at 50 mL/min. (B) Immobilized metal affinity chromatography (IMAC) of α_1AT Ala-357, Arg-358 obtained after anion-exchange chromatography on chelating Sepharose Fast Flow containing Zn^{2+} ions bound to iminodiacetic acid groups using a gradient of 0–0.1 M glycine in 50 mM Tris-HCl, pH 8.5, and 150 mM NaCl at 7 mL/min. (C) Hydrophobic interaction chromatography of α_1AT Ala-357, Arg-358 obtained after IMAC on PAE-300 using a gradient from 2 to 1 M ammonium sulfate in 50 mM Tris-HCl, pH 8, and 10 mM EDTA at 14 mL/min.

lecular weight contaminants and reduced the ionic strength to a conductivity of 2.5 mS, which was compatible with the following anion-exchange chromatographic purification step. The still slightly cloudy solution was directly applied to the anion-exchange stationary phase which served to remove the majority of proteins. This was a prerequisite for the efficacy

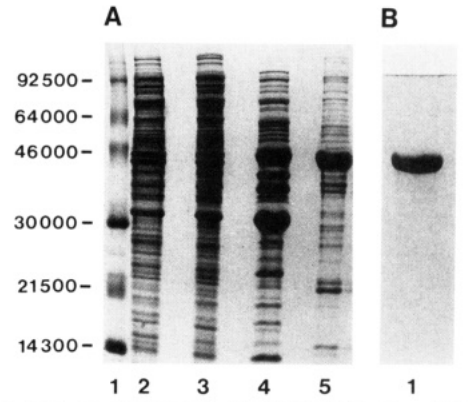

FIGURE 2: Polyacrylamide gel electrophoresis of α_1AT Ala-357, Arg-358 under denaturing and reducing conditions. (A) Lane 1, molecular weight markers. Lane 2, starting material after cell breakage and centrifugation. Lane 3, retentate after ultrafiltration at a cutoff of 10 kDa. Lane 4, eluted pool after anion-exchange chromatography (see Figure 1A). Lane 5, eluted pool after immobilized metal affinity chromatography (see Figure 1B). (B) Lane 1, α_1AT Ala-357, Arg-358 after purification and formulation.

of the following IMAC with immobilized Zn^{2+} ions. It also removed traces of EDTA which was added during cell breakage and would seriously impair the performance of the IMAC. α_1AT variants eluted in a well-defined region between conductivity values of 9 and 20 mS and were collected solely based upon the measured conductivity. A trace of the eluted proteins as followed by their absorbance at 280 nm showed that the variants eluted early during the employed sodium chloride gradient, well before most of the other adsorbed proteins (Figure 1A). It is important to note that the silica-based stationary phase could be regenerated with 0.1 M phosphoric acid in 20% (v/v) aqueous 2-propanol for repeated use even when this crude starting material was loaded.

The following IMAC was highly complementary to the initial anion-exchange purification step, since the major part of contaminating proteins did not adsorb to the stationary phase loaded with Zn^{2+} ions (Figure 2A, lanes 4 and 5). Thus, IMAC proved to be the purification step with the highest purification factor (Table I). All α_1AT variants were tightly bound to the immobilized Zn^{2+} ions, indicating strong metal ion binding sites. This property of the inhibitor has already been used in isolation procedures of the natural molecule from human plasma (Kurecki et al., 1979) and of its nonglycosylated recombinant counterpart from yeast (Hoylaerts et al., 1987), indicating that glycosylation was not necessary for binding to the immobilized Zn^{2+} ions. Highest recoveries of active variants were obtained by using a gradient of increasing glycine concentration (Figure 1B) which proved to be more effective than the previously employed descending pH gradients which resulted in low recoveries presumably due to some loss of inhibitory activity. An attempt to use this chromatographic method as the first purification step failed due to low recoveries and to a significantly reduced binding capacity of the stationary phase for the α_1AT variants.

After having employed the ionic and metal ion binding properties of the variants for their isolation, final purity was obtained by using the anion-exchange support in the hydrophobic interaction mode. This alternative has been described for other proteins which adsorb to mildly hydrophobic anion-exchange supports in the presence of elevated concentrations of antichaotropic anions such as sulfate (Kennedy et al., 1986). Elution was subsequently performed with a gradient of decreasing ammonium sulfate concentration, giving α_1AT variants of high purity as determined by PAGE under reducing

Table I: Purification Chart of α_1-Antitrypsin Ala-357, Arg-358 (AAT)

purification step	AAT (mg)[a]	protein (mg)[b]	purification factor	yield (%)	endotoxin (units/mg)[c]
starting material	533	77694	1	100	1.2×10^7
retentate 10 kDa	508	32098	2.3	95	1.9×10^5
pool AEX	465	10810	6.3	87	1.6×10^4
pool IMAC	348	590	86	65	<0.22
final pool	294		146	55	<0.22

[a] Quantitated by RID using an antiserum raised against human plasma-derived α_1AT (Behring, Marburg, FRG). [b] Coomassie Blue R-250 binding assay using bovine serum albumin as a standard (Bio-Rad, Richmond, CA; Bradford, 1976). [c] 1 endotoxin unit corresponds to 83 pg.

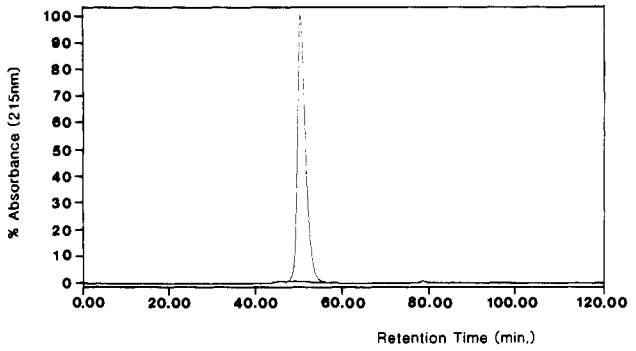

FIGURE 3: Gel filtration HPLC analysis of purified α_1AT Ala-357, Arg-358 on tandem TSK-G 2000 SW columns (60 × 0.75 cm each, 10-μm particle diameter) in 10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl at 0.5 mL/min.

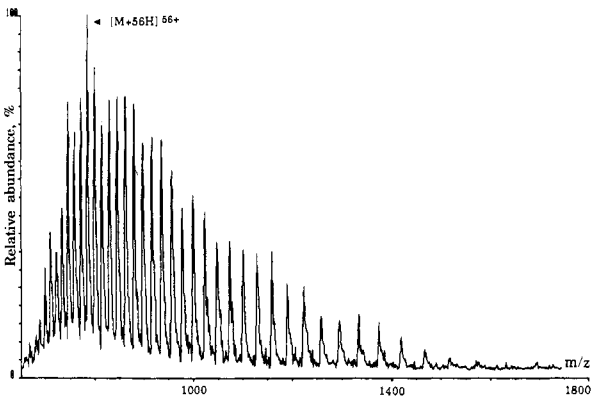

FIGURE 4: Electrospray mass spectrometry of purified α_1AT Ala-357, Arg-358 after sample preparation by reversed-phase HPLC; 2–10 μL of the sample containing approximately 30 pmol/μL was introduced into the ion source at 2 μL/min. The quadrupole mass analyzer was operated at a mass range of 4000, and the spectrum was scanned from m/z = 700 to 1500 in 20 s with unit resolution. Four measurements of different purification lots gave the following mass values: 43 932 ± 10 Da; 43 933 ± 7 Da; 43 934 ± 9 Da; and 43 939 ± 10 Da, giving an average molecular mass of 43 935 Da (expected mass, 43 928 Da). $[M+56H]^{56+}$, molecular ion containing 56 positively charged hydrogen ions.

conditions in the presence of SDS (Figures 1C and 2B).

Since α_1AT variants have pronounced affinities to proteases of either the trypsin or the chymotrypsin type, it was pivotal to have a rapid and efficient way of processing once the cells had been broken. With the described procedure, no indication of proteolytic degradation during purification was observed by PAGE even when the gel was overloaded (Figure 2B).

Endotoxin removal is one of the critical parameters when purifying recombinant proteins from *E. coli* that are destined for therapeutic use. Table I gives an overview of the efficacy of the individual purification steps in reducing the amount of endotoxin relative to the amount of the protein of interest. Although each individual purification step reduced the amount of endotoxin by approximately 1 order of magnitude, it was the IMAC purification step which dropped the level by approximately 5 orders of magnitude to undetectable amounts.

Analytical Characterization. The isolated variants have been characterized by a number of different analytical methods to verify their primary structure, assess their purity, determine their inhibitory activities, and assure the absence of endotoxins. Analytical HPLC on reversed-phase and anion-exchange stationary phases did not reveal any contaminants within the detection limit of approximately 1%. Gel filtration HPLC showed the absence of aggregates in the final preparations (Figure 3). This was especially important, since both plasma-derived α_1AT and recombinant α_1AT have been reported to form aggregates (Coan et al., 1985; Coan, 1988; Yu et al., 1988).

Automated sequence analysis showed that the variants contained an additional methionine residue at the N-terminus. This result was confirmed by determination of the molecular mass of purified α_1AT Ala-357, Arg-358 by electrospray mass spectrometry (Figure 4). Four mass determinations were made on four different purified lots of the variant, and the average was taken. The measured molecular mass of 43 935 ± 10 Da was in good agreement with the expected value of 43 928 Da based upon the amino acid sequence missing the five N-terminal amino acids but including an additional methionine residue. The mass difference of 7 Da lies within the accuracy of the employed method.

The precision of the mass measurement excluded the possibility of C-terminal degradation of the isolated variant, since a missing C-terminal lysine residue (128.2 Da) would have been easily detected. The integrity of the C-terminus was independently verified by tryptic mapping followed by sequence analysis of the isolated C-terminal peptide (Val^{383}-Val-Asn-Pro-Thr-Gln-Lys^{389}). To assure that the inhibitory site in the different variants corresponded to the expected sequence, a digestion with endoproteinase Glu-C from V8 *Staphylococcus aureus* was performed, giving a peptide which covered the inhibitory site. Automated sequence analysis of this peptide confirmed the correct sequence. In combination, digestion of the purified variants with trypsin and endoproteinase Glu-C followed by peptide mapping using reversed-phase HPLC and amino acid sequencing allowed the determination of 75% of the entire protein sequence which corresponded to the one predicted from the sequence of the complementary DNA used for expression.

Analysis of the purified α_1AT Ala-357, Arg-358 variant by isoelectric focusing in an immobilized pH gradient (Righetti, 1989) between pH 5 and 6 revealed some microheterogeneity (Figure 5). When the final material obtained from multiple purification processes was analyzed, it appeared that the microheterogeneity was found in all purified batches, thus emphasizing the reproducibility of the isolation process (Figure 5). The calculated isoelectric point for α_1AT Ala-357, Arg-358 based on the amino acid sequence was 5.72 (DNASTAR, Madison, WI), which was slightly higher than the observed values (5.43–5.58, Figure 5). This might indicate partial deamidation of susceptible asparagine or glutamine residues. The structural basis for this microheterogeneity is presently being investigated.

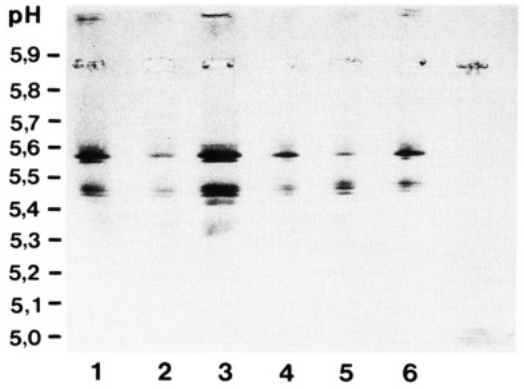

FIGURE 5: Isoelectric focusing of purified α_1AT Ala-357, Arg-358 obtained from different purification processes in an immobilized gradient from pH 5 to 6. Lanes 1–6, different purification lots. β-Lactoglobulin A (p*I* = 5.2) and bovine carbonic anhydrase B (p*I* = 5.85) were used as isoelectric point markers (not shown). The gels were run at 500 V (5 mA) overnight and subsequently stained with Coomassie Brilliant Blue R-250.

Inhibitory activity of the purified α_1AT variants was determined by incubating each variant with its major target protease in a purified system assuming the following reaction mechanism: P + I → PI, where P represents the target protease, I the corresponding inhibitor, and PI the irreversibly formed protease–inhibitor complex (Beatty et al., 1980; Wiman & Collen, 1978; Kitz & Wilson, 1962). The PI complex is considered to remain stable throughout the time of the measurement.

For α_1AT Leu-358 and α_1AT Arg-358, inhibition of purified neutrophil elastase and α-thrombin, respectively, was measured under second-order rate conditions using equimolar amounts of the inhibitor and the protease. When the reciprocal of the residual proteolytic activity was plotted against the incubation time with the inhibitor, a straight line was obtained from which the time ($t_{1/2}$) needed to reduce the proteolytic activity to 50% of the initial value could be deduced. The second-order association rate constants, k_{assoc}, were subsequently calculated as 3×10^6 M^{-1} s^{-1} in the case of α_1AT Leu-358 with neutrophil elastase and as 3.6×10^5 M^{-1} s^{-1} in the case of α_1AT Arg-358 and α-thrombin by using the following relation: $k_{assoc} = 1/t_{1/2}$[inhibitor], with [inhibitor] representing the molar concentration of inhibitor in the test. The results were in good agreement with previously published values (Jallat et al., 1986; Schapira et al., 1986; Scott et al., 1986).

The α_1AT Ala-357, Arg-358 variant was incubated with plasma kallikrein using a 5-fold excess of the inhibitor. Since under these conditions the reaction proceeded according to pseudo-first-order kinetics, it was possible to obtain a pseudo-first-order rate constant, k', from the relation $k' = (\ln 2)/t_{1/2}$. $t_{1/2}$ was obtained by plotting the logarithm of residual proteolytic activity against the incubation time with the inhibitor and graphically determining the time after which 50% inhibition had occurred. A straight line indicated that pseudo-first-order conditions were fulfilled. The second-order association rate constant k_{assoc} was subsequently calculated according to the following relation: $k_{assoc} = k'/$[inhibitor]. This gave a k_{assoc} of 1.8×10^5 M^{-1} s^{-1} for the inhibition of plasma kallikrein by α_1AT Ala-357, Arg-358, which is slightly lower than the previously published value (Schapira et al., 1987). This discrepancy might be due to the different buffer and pH conditions during the assay.

Discussion

Procedures have been developed over the last 16 years for isolating α_1AT from human plasma (Pannell et al., 1974; Kurecki et al., 1979; Coan et al., 1985; Coan, 1988; Glaser et al., 1982; Laurell et al., 1983) and more recently from recombinant yeast strains (Travis et al., 1985; Hoylaerts et al., 1987). While procedures using human plasma or fraction IV-1 of the Cohn process as starting materials have resulted in recoveries of approximately 50% for the natural inhibitor, recoveries from recombinant yeast strains have tended to be lower (approximately 25%). It has also been reported that final α_1AT preparations isolated from recombinant yeast strains proved to be unstable in the absence of stabilizers such as citrate (George et al., 1989). In addition, it was necessary to use a number of protease inhibitors during the processing of α_1AT from the intracellular fraction of recombinant yeast strains, which not only increase the production cost but also have to be absent in the final preparation due to their toxicity. On the other hand, an isolation procedure which used human plasma as starting material necessitated a heating step to assure that no pathogenic viruses were present in the final preparation. In the case of α_1AT, this was done by treating the pool after the final chromatographic purification step at 60 °C for 10 h in the presence of stabilizers which led to some aggregate formation and necessitated the subsequent removal of the stabilizers (Coan, 1988).

The described process avoided most of these problems when purifying α_1AT variants from the intracellular fraction of a recombinant *E. coli* strain. Process yields of 50% have been achieved which are comparable to the reported recoveries for processes using human plasma. In addition, only EDTA was used as a protease inhibitor which is easily removed by ultrafiltration at the end of the process. α_1AT variants prepared in this way prove to be stable for several months at room temperature in the lyophilized state.

The procedure has the potential for further scale-up, since it allows rapid processing avoiding lengthy treatments between the individual purification steps. It relies on only two chromatographic stationary phases which should also facilitate its use on a pilot or even industrial scale. Thorough regeneration of the individual chromatographic media permits their repeated use without loss in either capacity or chromatographic performance. The removal of endotoxins during the purification process is of prime importance in view of the therapeutic applications that are envisaged for the inhibitors.

The analytical characterization of recombinant proteins that are destined for therapeutical use is a crucial part of the production process. In the present work, α_1AT variants were characterized by peptide mapping in combination with amino acid sequencing to verify the major part of their primary structure including the N- and C-termini as well as the sequence covering the inhibitory site. Since complete peptide mapping of larger proteins represents a considerable task, alternative techniques are being investigated to obtain a rapid and reliable analysis of the primary structure of proteins. In this respect, mass spectrometry is starting to play a more and more important role, as it allows the rapid analysis of the primary structure of recombinant proteins by determining their molecular mass (Van Dorsselaer et al., 1989, 1990). In the case of α_1AT expressed intracellularly in a recombinant yeast strain and isolated therefrom, fast atom bombardment mass spectrometry in combination with specific cleavage reactions allowed the identification of an additional N-terminal acetylmethionine residue (Greer et al., 1988). The recently developed technique of electrospray mass spectrometry allows the analysis of proteins up to at least a molecular mass of 50 kDa with a precision that is sufficient to obtain reliable data on the integrity of both N- and C-termini. This technique

allowed verification of the primary structure of α_1AT Ala-357, Arg-358 (measured mass, 43 935 Da; expected mass, 43 928 Da) as well as that of α_1AT Leu-358 (measured mass, 43 914 Da; expected mass, 43 911 Da) (Van Dorsselaer et al., 1990). With further developments in the areas of sample preparation, resolution, and sensitivity, this method is likely to become a major tool for protein analysis.

High-resolution isoelectric focusing in an immobilized pH gradient revealed some microheterogeneity with respect to the isoelectric point of the purified α_1AT Ala-357, Arg-358 variant. This is a phenomenon which was previously observed in the case of other complex proteins such as recombinant tissue-type plasminogen activtor (rtPA) (Anicetti et al., 1989). While part of the charge heterogeneity in rtPA could be attributed to the glycosylation pattern, there was still some microheterogeneity left after enzymatic deglycosylation which resulted most likely from the protein core. Since recombinant α_1AT Ala-357, Arg-358 expressed in *E. coli* is not glycosylated, the observed charge heterogeneity is a result of modifications within the amino acid sequence of the protein. Deamidation of asparagine or glutamine residues could be one explanation, since the observed isoelectric points were all slightly lower than the theoretical value that was calculated from the amino acid sequence of the protein (5.43–5.58 observed versus 5.72 calculated). As asparagine residues followed by glycine were reported to be particularly susceptible to deamidation, Asn-116 and Asn-314 are two potential sites that have to be considered (Geiger & Clarke, 1987; Kossiakoff, 1988). However, calculation of isoelectric points for proteins of this size tends to be slightly erroneous, since effects of the local environment on the isoelectric points of the charged amino acids within the three-dimensional structure of the protein are not accounted for. The structural basis for the observed microheterogeneity is therefore still unclear and merits further investigation.

Since serine proteases and their corresponding inhibitors play crucial roles in controlling numerous physiological mechanisms of vital importance, it is of considerable interest to develop novel inhibitors as therapeutics for diseases where proteolytic activities in the organism are no longer correctly controlled. One area of interest is inflammatory disorders where a massive activation of neutrophils occurs at the site of inflammation. The thus released neutrophil proteases, elastase and cathepsin G, are able to degrade connective tissue which may lead to the formation of edema and irreversible lesions (Janoff, 1985; Peterson, 1989). Since neutrophils also release reactive oxygen radicals upon stimulation and since natural α_1AT contains an oxidation-sensitive methionine residue in its inhibitory site, it is of importance to see whether an oxidation-resistant variant of α_1AT would be a more efficient therapeutic agent than the natural inhibitor. As α_1AT Leu-358 shows almost identical inhibitory kinetics against neutrophil elastase and cathepsin G when compared to the natural inhibitor, it is conceivable that it will be a more effective agent in the case of severe inflammatory disorders.

Numerous publications have outlined the importance of both the kallikrein–kinin-forming system and also the different pathways leading to the formation of thrombin for complications encountered during clinical sepsis and septic shock (Colman, 1984, 1989a,b; Emerson et al., 1987; Taylor et al., 1987, 1988; Redens et al., 1988). In addition, results have been presented showing that pretreatment of septic pigs with the α_1AT Arg-358 variant had a marked beneficial effect on their hemodynamic condition (Colman et al., 1988). It is thus conceivable that inhibition of both plasma kallikrein and α-thrombin by either the α_1AT Ala-357, Arg-358 or the α_1AT Arg-358 variant could be of major importance in the treatment of sepsis and septic shock. The high mortality that is currently observed for this clinical complication makes the development of effective treatments an urgent necessity. The described purification procedure should allow the evaluation of the therapeutic potential of these novel serine protease inhibitors in clinically relevant animal models.

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Registry No. α_1AT, 9041-92-3; elastase, 9004-06-2; thrombin, 9002-04-4; kallikrein, 9001-01-8.

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