Isolation and Characterization of α -1-Antitrypsin from Rhesus-Monkey Serum

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A simple, relatively gentle, procedure for isolation of rhesus-monkey α -1-antitrypsin from serum is described. The method consists of chromatographic separation of the fraction precipitated by 50–75 % satd. $(\text{NH}_4)_2\text{SO}_4$ from pooled monkey serum on DEAE-cellulose followed by affinity chromatography on Sepharose-bound concanavalin A. Approx. 30% of the trypsin-inhibitory activity present in the original serum was recovered when α -1-antitrypsin was reconstituted with physiological saline (0.85% NaCl). Pure α -1-antitrypsin exhibited a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, with an estimated mol.wt. of 60000, and four bands in acid/starch-gel electrophoresis. The acid/starch-gel-electrophoretic pattern and mobility of isolated material were identical with those of the α -1-antitrypsin bands in the original serum sample. The most rapidly migrating bands resembled the pattern and mobility for the normal human phenotype PiM in 28 monkeys. A starch strip from the acid/starch-gel-electrophoresis as the origin for antigen-antibody electrophoresis was used to examine α -1-antitrypsin for microheterogeneity; no evidence for microheterogeneity was observed in samples from 18 monkeys. In addition, isolated α -1-antitrypsin exhibited a single arc when subjected to immunoelectrophoresis. Amino acid and carbohydrate compositions of isolated monkey α -1-antitrypsin were similar to those of human α -1-antitrypsin.

Proteinase inhibitors in man appear to be essential for maintenance of tissue integrity during the tumultuous events associated with inflammation (Kueppers & Black, 1974). One proteinase inhibitor, α -1-antitrypsin, appears to be especially important. This glycoprotein is the major α -globulin, and accounts for 90% of the anti-proteinase activity in serum (Kueppers & Black, 1974). Experimental procedures necessary to evaluate the role of α -1-antitrypsin *in vivo* during the inflammatory process and in genetic deficiency states preclude the use of human subjects and hence required development of an animal model. Evidence has been obtained that the sub-human primate, *Macaca mulatta*, will fulfil this role (Mathis & Berninger, 1974). An inherent requirement for definitive studies was isolation, purification and characterization of serum α -1-antitrypsin from this primate.

A preliminary account of the present work has already appeared (Berninger *et al.*, 1974).

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Materials and Methods

Serum samples

Healthy rhesus monkeys (3.0–4.2 kg), environmentally conditioned‡ for 6 months to 2 years, were bled at periodic intervals. Blood was allowed to clot for 2–3 h at room temperature (21°C), and separated serum was stored at –70°C. For α -1-antitrypsin-isolation studies, sera from three monkeys were pooled before storage. Individual serum samples obtained from the authors, one known to be of the proteinase inhibitor type M (PiM) phenotype, were treated and stored in an identical manner.

Purification of rhesus-monkey α -1-antitrypsin

Techniques described by Crawford (1973) and Liener *et al.* (1973) were modified to isolate rhesus-monkey α -1-antitrypsin from the serum pool. All

‡ The term 'environmentally conditioned' signifies that the animals were maintained under stable physical conditions (temperature, humidity and light), dietary conditions (time and quantity of feeding as well as type of diet), treatment for bacterial and parasitic diseases, and acclimation to handling procedures required for experimental studies.

procedures were conducted in a cold-room maintained at 4°C. A volume of saturated ultra-pure $(\text{NH}_4)_2\text{SO}_4$ (special enzymatic grade; Schwarz/Mann, Orangeburg, NY, U.S.A.), adjusted to pH 7.4 with NH_3 or H_2SO_4 , was added dropwise to an equal volume of serum, and the mixture was stirred for approx. 1 h with a magnetic stirrer at a rate slow enough to avoid foaming. After centrifugation at 10000g for 10 min (8000 rev./min; Sorvall GSA rotor), the supernatant was adjusted to 75% saturation by addition of solid $(\text{NH}_4)_2\text{SO}_4$ (17.6 g/100 ml of supernatant). This mixture was stirred slowly for 1.5 h and centrifuged as described above. The volume of supernatant was measured carefully, and the precipitate was dissolved in 10 ml of 0.05 M-sodium phosphate, pH 7.6, containing 1 mM-2-mercaptoethanol (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), per 100 ml of supernatant. The 2-mercaptoethanol was added to column buffers to stabilize any half-cysteine residues (Crawford, 1973) which may be present in the α -1-antitrypsin molecule. This solution was dialysed against 10 vol. of the same buffer with at least two changes in 36 h. (The dialysis tubing was cleaned before use by boiling in water.) The non-diffusible material was used immediately or stored at -70°C.

A portion (8 ml, containing about 0.4 g of protein) of the non-diffusible material was chromatographed on a column (2.5 cm $\times$ 32 cm) of DEAE-cellulose (Whatman, DE-52 microgranular; Curtin Matheson, Silver Spring, MD, U.S.A.) equilibrated with 0.05 M-sodium phosphate buffer, pH 7.6, with 1 mM-2-mercaptoethanol. Elution was accomplished by one of two methods. If a Dial-A-Grad (ISCO) was set for 24 h with a starting flow rate of 55 ml/h, a linear gradient from 0 to 500 mM-NaCl (Biological Grade, Fisher Scientific Co., Silver Spring, MD, U.S.A.) was generated in the column buffer. Fractions (11 ml) were collected while the E_{280} of the effluent was recorded. The α -1-antitrypsin fractions were located by radial immunodiffusion and trypsin-inhibitory capacity. Alternatively, when the equal-reservoir method with an in-line mono-staltic pump (Buchler Instruments, Fort Lee, NJ, U.S.A.) was used, 1000 ml of 0.025 M-sodium phosphate, pH 7.6, containing 1 mM-2-mercaptoethanol (N.B. the non-diffusible material must be equilibrated with this buffer) was used to generate a linear gradient with 250 mM-NaCl in 1000 ml of the same buffer.

A pool of active fractions obtained by either technique was applied to a column (1.5 cm $\times$ 13 cm) of Sepharose-bound concanavalin A (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and eluted at 35 ml/h with 0.05 M-sodium phosphate buffer, pH 7.6, containing 1 mM-2-mercaptoethanol. After the E_{280} of the eluate returned to a baseline value, α -1-antitrypsin was eluted from the column with 0.1 M- α -methyl D-glucoside (Nutritional Biochemicals Corp.,

Cleveland, OH, U.S.A.) dissolved in the same buffer. Antigenic activity and trypsin-inhibitory activity were verified. The column was regenerated by washing with the phosphate/2-mercaptoethanol buffer and re-used several times.

Generally, active α -1-antitrypsin fractions were slightly contaminated with albumin. These pooled fractions were dialysed against the phosphate/2-mercaptoethanol buffer to remove the glucoside, and the non-diffusible material was subjected to a second affinity chromatography. Fractions containing α -1-antitrypsin were pooled, and dialysed against water to remove sugar and buffer salts. For freeze-drying, containers with frozen non-diffusible material were packed in ice, and drying was performed at 0°C.

Amino acid analysis

Samples of isolated α -1-antitrypsin were dried over P_2O_5 in a vacuum desiccator at room temperature for 18 h. Hydrolysis of 1–2 mg was accomplished in sealed, evacuated tubes with 6 M-HCl for 24, 48 or 72 h at 110°C. Care was taken to exclude air by evacuating and back-flushing sample tubes with N_2 several times before sealing. The half-cysteine residues were stabilized by 2-mercaptoethanol, which is present in all buffers used in isolation procedures. Hydrolysed samples were analysed on a Technicon TFM amino acid analyser (Eveleigh *et al.*, 1967) with a lithium citrate buffer system (Benson *et al.*, 1967).

Carbohydrate analysis

The carbohydrate portion of α -1-antitrypsin was analysed by g.l.c. by a modification of the procedure described by Chambers & Clamp (1971). All reagents and standards were stored in a desiccator over P_2O_5 . Methanol (Lipopure; Applied Science Laboratories, State College, PA, U.S.A.) was dried in the usual manner by refluxing 500 ml for 1.0 h with 2.5 g of magnesium turnings and 0.1 g of iodine. After distillation into a clean container, 45 ml batches of dry methanol were stored in glass-stoppered flasks over P_2O_5 . To prepare methanolic 1.0 M-HCl, 45 ml of dry methanol was cooled in an ice bath and stirred while 3.5 ml of acetyl chloride (Applied Science Laboratories) was added dropwise from an addition funnel. The mixture was then allowed to warm to room temperature. Each accurately weighed reference standard (D-fucose, D-mannose, α -D-galactose, α -L-glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, from Applied Science Laboratories, and N-acetylneuraminic acid, from Aldrich Chemical Co., Cedar Knolls, NJ, U.S.A.) was prepared at a concentration of 1 mM in 10 ml of methanol. Carefully measured amounts (50–200 μ l) of each monosaccharide solution and a mixture containing 100 μ l of each standard solution were transferred to 2 ml reaction vials (Supelco, Bellforte, PA, U.S.A.) and

dried in a vacuum desiccator over P_2O_5 . Isolated α -1-antitrypsin was dried over P_2O_5 at room temperature for 18h, and 1–2mg samples were accurately weighed in clean, dry 2ml reaction vials. Every vial was processed at the same time with the same reagents. First 1 ml of methanolic 1.0M-HCl was added;

where the area of monosaccharide is a summation of the isomer peaks for each monosaccharide. Rearrangement of the R.F. formula (Clamp *et al.*, 1972) allows μ mol of monosaccharide to be calculated from a g.l.c. analysis of α -1-antitrypsin. Further calculation yields:

$$\frac{\mu\text{mol of monosaccharide}}{\mu\text{mol of } \alpha\text{-1-antitrypsin}} = \frac{\mu\text{mol of monosaccharide}}{\text{mg of } \alpha\text{-1-antitrypsin}} \times \frac{\text{mg}}{10^3 \mu\text{g}} \times \frac{60000 \mu\text{g}}{\mu\text{mol of } \alpha\text{-1-antitrypsin}}$$

dry N_2 was bubbled through the solution for 30s, and the vial was sealed with a Teflon-lined cap. The methyl glycoside was formed by heating at 70–75°C for 24h. D-Mannitol (Applied Science Laboratories) (1mm in methanol), serving as an internal standard, was added in 100 μ l to the cooled vial, and after the solution was neutralized to pH7.0 with solid Ag_2CO_3 (Fisher Scientific Co.), 200 μ l of acetic anhydride (Certified, A.C.S.; Fisher Scientific Co.) was added and mixed thoroughly. The vial was sealed with a Teflon-lined cap, left for 12h at room temperature and centrifuged in a table-top centrifuge at 5000rev./min for 10min. The precipitate was washed with 3 $\times$ 300 μ l of methanol; the washings were combined in a vial with the first supernatant and dried in a vacuum desiccator over P_2O_5 . A 100 μ l volume of trimethylsilylating reagent (Sil-Prep; Applied Science Laboratories) containing pyridine, hexamethyldisilazane and trimethylchlorosilane (9:3:1, by vol.) was added to a vial to yield the trimethylsilyl ether. The vial was heated at 45°C for 1h, allowed to cool to room temperature, centrifuged at 5000rev./min and 2–5 μ l of supernatant was used for chromatographic analyses.

A Packard 7400 (Packard Instrument Co., Downers Grove, IL, U.S.A.) gas chromatograph equipped with flame-ionization detector and a 3m (10ft) $\times$ 2mm (internal diam.) glass column packed with 3% OV-1 (100/120 mesh on Gas-Chrom Q; Applied Science Laboratories) was used in conjunction with a Hewlett Packard 3380A electronic integrator for the analyses. The injection port was maintained at 200°C and the detector at 240°C. The initial column temperature was 120°C, with a programming rate of 0.5°C/min to 190°C, with a 15min hold at this temperature. The flow rates were set as follows: carrier gas N_2 , 15ml/min [165kPa (24lb/in²) back pressure]; O_2 , 400ml/min; H_2 , 40ml/min.

The response factor (R.F.) of each monosaccharide relative to mannitol was determined at several molar ratios by using the following formula:

$$R.F. = \frac{\text{area of monosaccharide peak(s)}/\text{area of mannitol peak}}{\mu\text{mol of monosaccharide}/\mu\text{mol of mannitol}}$$

Blanks (empty vials) which did not contain monosaccharides or mannitol were subjected to the above procedure.

Antisera

Antiserum to rhesus-monkey α -1-antitrypsin was produced in rabbits. For this, 1 vol. of a 0.25% (w/v) solution of isolated rhesus-monkey α -1-antitrypsin was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI, U.S.A.); a portion (1ml) of this mixture was inoculated intramuscularly into each hip of two rabbits. Then 10 days later, a booster dose (2.5mg of α -1-antitrypsin/1ml of sterile water) was administered subcutaneously. Animals were not given food 12h before bleeding on 17, 24, 31 and 38 days after the initial intramuscular injection. Counter-immuno-electrophoresis was used to detect anti-(monkey α -1-antitrypsin); 17-day sera generally exhibited the highest titre. Lipoproteins were removed from pooled antisera by the procedure of Harboe & Ingild (1973). Commercial sources were used to obtain rabbit anti-monkey serum (Microbiological Associates, Bethesda, MD, U.S.A.), goat anti-monkey serum (Cappel Laboratory, Downingtown, PA, U.S.A.) that lacked antibody for the albumin component, and rabbit anti-(human α -1-antitrypsin) serum (Behring Diagnostics, Somerville, NJ, U.S.A.).

Trypsin-inhibitory capacity

The method described by Ericksson (1965) was used to measure trypsin inhibition in sera, column fractions and isolated preparations of α -1-antitrypsin. Three-times-crystallized salt-free bovine trypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) was standardized with salt-free soya-bean trypsin inhibitor (Worthington Biochemical Corp.); benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Nutritional Biochemicals Corp.) was the substrate. Values were expressed as mg of trypsin inhibited/ml of sample.

Immunochemical and electrophoretic techniques

Radial immunodiffusion. Radial-immunodiffusion plates (Melay, Springfield, VA, U.S.A.) for determining human α -1-antitrypsin (Mancini *et al.*, 1965) were used to locate monkey α -1-antitrypsin in column fractions.

A 5 μ l sample of each column fraction was applied to a well; immediately after diffusion (approx. 5 min) a second 5 μ l volume was added. For samples of isolated α -1-antitrypsin (2.35 mg/ml) and for monkey serum, a single 5 μ l volume was used. For calibration, dilutions of monkey serum or of isolated monkey α -1-antitrypsin and human α -1-antitrypsin standards were titrated at the same time on a radial-immunodiffusion plate.

Immunoelectrophoresis. Isolated α -1-antitrypsin was examined by immunoelectrophoresis (Scheidegger, 1955) in 1% agarose (Electrophoretic Grade; Nutritional Biochemicals Corp.) with Tris/barbital/sodium barbital buffer, pH 8.8 ($I = 0.05$ mol/litre). One vial (18 g) of the Gelman high-resolution buffer (Gelman Instrument Co., Ann Arbor, MI, U.S.A.) dissolved in 1200 ml of water was used as reservoir buffer. A 1% agarose solution was prepared in a 1:2 (v/v) dilution of reservoir buffer in sterile water by heating at 100°C for 5 min; 14 ml was pipetted on to a heated glass slide (8.2 cm $\times$ 10.0 cm) precoated with 0.1% agarose. Samples (5 μ l) of monkey serum with the Bromophenol Blue tracking dye or of 2 mg of α -1-antitrypsin/ml in 0.85% NaCl were added to wells (2.5 mm diam.) located 6.25 mm from the serum trough (2.5 mm wide). Electrophoresis was performed at 8 V (d.c.)/cm (measured in the gel with a volt/ohmmeter) for 90 min in a cold-room maintained at 4°C. Then 50 μ l of anti-monkey serum was added to the trough and allowed to diffuse into the gel for 24 h at room temperature in a humid chamber. Gels washed with 0.85% NaCl followed by water were dried at 21°C and stained for protein with Amido Black 10B (see under 'Protein stains').

Crossed immunoelectrophoresis. Antigen-antibody crossed electrophoresis was performed in 1% agarose gel supplemented with 1% (v/v) rabbit anti-(monkey α -1-antitrypsin) or 0.18% (v/v) rabbit anti-(human α -1-antitrypsin) at pH 8.6 with barbital buffer ($I = 0.05$ mol/litre), as described by Laurell (1965). After acid/starch-gel electrophoresis of a 40 μ l sample of serum (1:6 dilution in 0.85% NaCl) or of isolated α -1-antitrypsin (2 mg/ml), a starch strip (80 mm $\times$ 2.5 mm $\times$ 1.5 mm) was inserted into 1% agarose/antiserum gel (8.2 cm $\times$ 10.0 cm plate). Electrophoresis was performed at 4 V (d.c.)/cm for 8 h at 4°C; plates were left for 4–8 h before washing, drying and staining for protein.

Counter-immunoelectrophoresis. Counter-immunoelectrophoresis (Gocke & Howe, 1970) was performed in 1% agarose (electrophoretic grade; Nutritional Biochemical Corp.) with barbital buffer,

pH 8.2 (6.88 g of barbital and 15.14 g of sodium barbital in 2 litres), $I = 0.05$ mol/litre, at 3 V (d.c.)/cm (measured in the gel) for 40 min at room temperature. The wells were 3 mm in diameter and 5 mm apart. Samples (20 μ l) of diluted antibody or undiluted antigen were used to fill the wells. After plates were developed overnight at 4°C in a humid chamber, precipitin arcs were visible without staining.

Acid/starch-gel electrophoresis. Monkey α -1-antitrypsin was compared with human α -1-antitrypsin by electrophoresis [400 V (d.c.)] on a 13% (w/v) gel (22 cm $\times$ 12 cm $\times$ 0.4 cm) of hydrolysed starch (Connaught lot 302-1; Fisher Scientific Co.) in the discontinuous acid-buffer system described by Fagerhol (1968). Samples (40 μ l) of serum or isolated α -1-antitrypsin (2 mg/ml) were applied to the gel and processed in a cold-room at 4°C until the buffer front was 10 cm from the origin; a piece of wet paper towel was placed over the clear plastic Saran Wrap covering the gel to aid in cooling. Gels were either stained for protein or sliced and used as origin material for antigen-antibody crossed electrophoresis.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The purity and estimated molecular weight of rhesus-monkey α -1-antitrypsin were assessed by the method of Weber & Osborn (1969). Samples (50–100 μ l) of eluate fractions from the concanavalin A column or of isolated α -1-antitrypsin (10–20 μ l of a 2 mg/ml solution in 0.85% NaCl diluted with gel buffer to 50 μ l) were applied to columns (0.7 cm $\times$ 8.0 cm) of 0.1% sodium dodecyl sulphate (99% pure); Pierce Chemical Co., Rockford, IL, U.S.A.)/polyacrylamide (10%; Bio-Rad Laboratories, Richmond, CA, U.S.A.) gel at pH 7.2. When electrophoresis (current of 8 mA/gel for approx. 4 h) was terminated, a piece of copper wire was inserted in the gel to denote the terminal position of the tracking dye (Bromophenol Blue) and the gel columns were stained with Coomassie Brilliant Blue (Bio-Rad Laboratories). Reference standards used for estimation of mol.wt. were: phosphorylase (93 000; Worthington Biochemical Corp.), transferrin (90 000; Sigma Chemical Co., St. Louis, MO, U.S.A.), bovine serum albumin (68 000; Schwartz/Mann), egg albumin (43 000; Worthington Biochemical Corp.) and chymotrypsinogen (25 700; Sigma Chemical Co.). A set of standards was included for each electrophoretic determination with α -1-antitrypsin. When logarithms of molecular weights of standards were plotted against electrophoretic mobility, the resultant straight line was used to estimate the molecular weight of isolated α -1-antitrypsin.

Protein stains

Amido Black 10B (Eastman Kodak Co., Rochester, NY, U.S.A.) (0.1%, w/v) and Coomassie Brilliant Blue R250 (Bio-Rad Laboratories) (0.25%, w/v) were prepared by dissolving the appropriate

amount of stain in 500ml of methanol, 500ml of de-ionized water, 100ml of acetic acid. Clearing solution was made in the same manner without the addition of stain. Staining with Amido Black or Coomassie Brilliant Blue showed the same patterns.

Results

Isolation and purification of α -1-antitrypsin

The first step in isolation of α -1-antitrypsin from pooled monkey serum consisted of recovery of serum components precipitated by 50–75%-satd. $(\text{NH}_4)_2\text{SO}_4$. In this and all subsequent steps it should be noted that any deviation from the described procedures resulted in a more contaminated product; processing at 4°C for the time indicated was critical to the results described. In addition to α -1-antitrypsin, the fraction precipitated by 50–75%-satd. $(\text{NH}_4)_2\text{SO}_4$ contained other serum components in-

cluding serum albumin. Partial separation of α -1-antitrypsin from albumin was performed by chromatography of the non-diffusible material from the 50–75%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate on DEAE-cellulose. Elution was accomplished by a linear gradient of NaCl generated by either of two methods: (1) ISCO Dial-A-Grad for 24h or (2) equal-volume reservoirs. Both methods resulted in good separation of α -1-antitrypsin from serum components other than albumin; however, the Dial-A-Grad technique was preferable because, as shown in Fig. 1, α -1-antitrypsin activity was located in Fractions 22–26 and eluted before the major portion of albumin. With the equal-reservoir method, α -1-antitrypsin was located in the leading edge of the albumin peak.

The pooled active fractions from either procedure were further purified by direct application to a concanavalin A-Sepharose column. Elution of serum contaminants was accomplished with 0.05M-sodium

Fig. 1. Purification of monkey α -1-antitrypsin on DEAE-cellulose

The column (2.5cm $\times$ 32cm) was equilibrated with 0.05M-sodium phosphate buffer, pH 7.6, containing 1 mM-2-mercapto-ethanol. The sample (8ml) of non-diffusible material described in the Materials and Methods section was applied to the column. Elution was accomplished with a Dial-A-Grad (ISCO) apparatus set for 24h to generate a linear gradient from 0 to 500mM-NaCl in the same buffer, with a flow rate of 55 ml/h. Active fractions (22–26) were located by trypsin-inhibitory-capacity ($\circ$) and radial-immunodiffusion ($\bullet$) techniques and pooled. The theoretical salt gradient (started in fraction 2, total volume of gradient 1320ml) is shown. No protein was eluted from the column after fraction 50. The fraction size was 11ml.

Fig. 2. Isolation of monkey α -1-antitrypsin on concanavalin A-Sepharose

Pooled active fractions from the DEAE-cellulose column were chromatographed on a concanavalin A-Sepharose column (1.5 cm $\times$ 13 cm) equilibrated with 0.05 M-phosphate buffer, pH 7.6, containing 1 mM-2-mercaptoethanol. After E_{280} (—) returned to baseline value, 0.1 M- α -methyl D-glucoside in the phosphate buffer (indicated by arrow) was used to elute α -1-antitrypsin from the column. The fraction size was 3 ml. Active fractions (59–69) were located by trypsin-inhibitory capacity (○) and radial immunodiffusion (●). These fractions were dialysed against the phosphate buffer to remove sugar, and rechromatographed on the concanavalin A-Sepharose column to remove trace amounts of albumin.

Table 1. Recovery of trypsin-inhibitory activity and quantity of α -1-antitrypsin from 8.4 ml of pooled monkey serum

'Total activity' is expressed as mg of trypsin inhibited [trypsin-inhibitory capacity (mg/ml) $\times$ volume (ml)]; total quantity is expressed as mg of α -1-antitrypsin. For further details, see under 'Isolation and purification of α -1-antitrypsin' in the Results section.

Fraction	Protein (mg dry wt.)	Trypsin-inhibitory capacity		Radial immunodiffusion	
		Total activity (mg)	Recovery (% of starting material)	Total quantity (mg)	Recovery (% of starting material)
Serum pool	660†	7.28	100	20.5*	100
Precipitated by 50–75%-satd. (NH ₄) ₂ SO ₄	440	6.24	86		
Second concanavalin freeze- dried eluate	6.64	1.69	23	6.6	32

* Based on calibration curve for isolated monkey α -1-antitrypsin.

† Value by Lowry method (Lowry *et al.*, 1951) was equivalent to dry weight for 8.4 ml of dialysed serum pool.

phosphate buffer before elution of α -1-antitrypsin with 0.1 M- α -methyl D-glucoside (Fig. 2). Active fractions (59–69) were dialysed against phosphate buffer and rechromatographed on the concanavalin A-Sepharose column to remove traces of albumin. Active fractions were pooled, dialysed against distilled water and freeze-dried at 0°C. Complete loss of trypsin-inhibitory activity occurred if freeze-dried preparations warmed to room temperature.

Estimates for total recovery of trypsin-inhibitory activity were calculated directly for trypsin-inhibitory

capacity values; before processing, the serum pool was estimated to be capable of inhibiting 7.28 mg of trypsin, whereas the non-diffusible material would inhibit 6.24 mg of trypsin (86% recovery) and the chromatographed, freeze-dried preparation would inhibit 1.69 mg of trypsin (28% recovery) (Table 1). The value for quantity of α -1-antitrypsin in the serum pool was derived from a calibration curve for isolated monkey α -1-antitrypsin in radial-immunodiffusion plates containing antiserum to human α -1-antitrypsin. Because of the incomplete serological

cross-reactivity between monkey and human α -1-antitrypsins (Kueppers, 1968), the calculation based on the calibration curve for human standards resulted in abnormally high values for monkey α -1-antitrypsin, e.g. the radial-immunodiffusion reaction for a 2.35mg/ml solution of isolated monkey α -1-antitrypsins indicated a concentration of 3.8mg/ml. Similarly, reactions of individual monkey sera indicated concentrations of 4–6mg/ml with human standards or, with reference to the isolated monkey α -1-antitrypsin, 2.5–3.7mg/ml. The serum pool cited in Table 1 contained 2.56mg/ml or 20.5mg in the 8.4ml serum pool. The freeze-dried fraction from concanavalin A–Sephadex chromatography weighed 6.64mg, representing 32% recovery.

When larger volumes of pooled serum were processed, approx. 30% of the original inhibitory activity was recovered. Similar results were obtained with serum from a single monkey. Overall, specific activity of isolated α -1-antitrypsin varied from 0.26 to 0.40mg of bovine trypsin inhibited/mg of isolated material. This represents 65–100% of the predicted specific activity of 0.40, if it is assumed that inhibition of bovine trypsin (24000mol.wt.) by α -1-antitrypsin (60000mol.wt.) is an irreversible bimolecular reaction.

Electrophoretic mobility

Migration patterns and mobilities for monkey α -1-antitrypsin on acid/starch-gel electrophoresis were identical before and after isolation from serum (Plate 1). Monkey α -1-antitrypsin exhibited two dark-staining bands and two light-staining bands; the two most rapidly migrating bands stained faintly and migrated at the same rate as the two dark-staining bands for human α -1-antitrypsin phenotype PiM. The absence of dark-staining material at the origin for isolated α -1-antitrypsin suggested the absence, or a minimum quantity, of other serum components. A survey of 28 (two females, 26 males) monkey sera revealed that identical acid/starch-gel electrophoretic patterns were obtained in each case. Procedures using DEAE-Sephadex chromatography for isolation of α -1-antitrypsin were unsatisfactory because the patterns for eluates as compared with the pattern for serum indicated that the α -1-antitrypsin components had altered migration patterns and slower mobility.

Purity of isolated monkey α -1-antitrypsin was assessed by immunoelectrophoresis (Plate 2). The immunoelectrophoretic behaviour of isolated monkey α -1-antitrypsin as compared with that of whole serum indicated that isolated preparations were free of all serum contaminants, including albumin. Con-

Table 2. *Amino acid analysis of isolated rhesus-monkey α -1-antitrypsin*

Mean values are calculated from triplicate analyses of 1.08mg (24h) and 0.84mg (48h) and single analysis of 0.72mg (72h). Tryptophan was not determined.

Component	Content		
	(mmol/mmol of α -1-antitrypsin)		Integral residues/ molecule of α -1-antitrypsin
	Mean value	Range	
Asp	42.0	40.7–43.3	42
Thr	25.5, 23.8, 22.9*		27†
Ser	19.9, 16.3, 15.3*		22†
Glu	42.5	41.3–44.4	43
Pro	20.3	19.8–20.9	20
Gly	24.5	23.9–25.0	24
Ala	28.7	27.5–29.4	29
‡-Cys	2.3	2.15–2.50‡	2
Val	27.2	26.7–27.5	27
Met	7.32, 6.67, 5.33*		8†
Ile	17.3	16.8–17.8	17
Leu	42.9	41.8–43.6	43
Tyr	9.6	9.0–10.1	10
Phe	21.5	20.8–21.8	21
Lys	35.1	34.5–35.9	35
His	15.7	15.0–16.4	16
Arg	10.9	10.8–11.1	11

* 24, 48 and 72h values.

† Value corrected by extrapolation to zero time.

‡ 48h values not included in mean.

Fig. 3. G.I.c. analysis of isolated rhesus-monkey α -1-antitrypsin

The g.l.c. conditions and analytical procedures are described in the Materials and Methods section. The chart speed was 1 cm/min. Peak identification was verified by co-injection of monosaccharide standards with α -1-antitrypsin. Peaks were identified as follows: mannose 1, 3; galactose 2, 4, 5; glucose 6, 7; mannitol 8; *N*-acetylglucosamine 9, 10, 11, 12; *N*-acetylneuraminic acid 13, 14.

Table 3. G.I.c. analysis of carbohydrate content of isolated rhesus-monkey α -1-antitrypsin

Mean values were calculated from nine analyses, three each of 1.41, 1.82 and 2.01 mg samples.

Component	Content		Integral residues/molecule of α -1-antitrypsin
	(μ mol of monosaccharide/ μ mol of α -1-antitrypsin)		
	Mean value	Range	
Fuc	None		—
Man	5.27	5.26–5.28	5
Gal	6.03	6.02–6.03	6
Glc	1.24	1.20–1.26	1
GlcNAc	9.38	9.32–9.46	9
GalNAc	None		—
NeuNAc	8.72	8.65–8.80	9

firmation that the reaction involved α -1-antitrypsin, and not albumin, was obtained when the same procedure was performed by using goat antiserum that lacked antibody for monkey albumin.

Isolated monkey α -1-antitrypsin was also subjected to electrophoresis in sodium dodecyl sulphate/polyacrylamide gel to assess purity and to estimate the approximate molecular weight (Plate 3). There was a single protein band, and the pattern for α -1-antitrypsin immediately after affinity chromatography was identical with the pattern for reconstituted, freeze-dried α -1-antitrypsin. The mol.wt. of isolated rhesus monkey α -1-antitrypsin was determined to be 60000 by comparison with the reference standards.

The microheterogeneity of isolated α -1-antitrypsin from monkey serum was examined by crossed immunoelectrophoresis in a 1% agarose gel containing 1% rabbit anti-(isolated monkey α -1-antitrypsin) (Plate 4). The four obvious peaks correspond to nos.

2, 3, 4 and 5 of the six detected by this technique; the same pattern was observed in sera from 18 monkeys (2 females, 16 males). Electrophoretic behaviour of α -1-antitrypsin from human serum is shown for comparison purposes in Plate 5. Six of the eight peaks are readily observed: numbers 2, 4, 6, 7 and 8. Peak 3 of monkey α -1-antitrypsin (indicated by the black dot in Plate 4) exhibited the same mobility as peak 6 (indicated by the black dot in Plate 5) of human α -1-antitrypsin of phenotype PiM.

Amino acid content

Amino acid analysis of isolated monkey α -1-antitrypsin is presented in Table 2. Recovery of amino acids in 24, 48 and 72h hydrolysates was 86.0, 84.6 and 81.3% respectively of the wet weight after correction for loss of threonine, serine, and methionine with time and for carbohydrate content, recovery was calculated to be 97.7%. Insufficient sample was available to perform tryptophan determinations.

Carbohydrate analysis

The g.l.c. analysis for monosaccharides in isolated α -1-antitrypsin is shown in Fig. 3, with the peaks identified by retention time. The trace is similar to those published for reference standards except that two peaks for *N*-acetylneuraminic acid (13 and 14 in Fig. 3) were identified. The area ratio for the two peaks in isolated α -1-antitrypsin was the same as that for the *N*-acetylneuraminic acid standard. The second peak is probably the trimethylsilyl ether of free *N*-acetylneuraminic acid, since subjecting this material to the trimethylsilyl reagent (but not to methanolic HCl) yielded one peak with the same retention time. This peak may result from decomposition in the injection port or from incomplete hydrolysis. Addition of mannitol to α -1-antitrypsin before hydrolysis with methanolic HCl resulted in several unidentifiable peaks which overlapped with other peaks in the trace, whereas addition after hydrolysis, but before neutralization, yielded one mannitol peak. Therefore

EXPLANATION OF PLATE I

Mobility of monkey α -1-antitrypsin and human α -1-antitrypsin on acid/starch-gel electrophoresis

The anode is on the right. Gel 1 represents 40 μ l of a 2 mg/ml solution of isolated monkey α -1-antitrypsin; gel 2, 40 μ l of pooled monkey serum; and gel 3, 40 μ l of human serum α -1-antitrypsin phenotype PiM. The experimental conditions are described in the Materials and Methods section and gels were stained with Amido Black 10B.

EXPLANATION OF PLATE 2

Immunoelectrophoresis of rhesus-monkey serum and isolated monkey α -1-antitrypsin

The anode is on the right. The top well contained 5 μ l of monkey serum and Bromophenol Blue, and the bottom well contained 5 μ l of a 2 mg/ml solution of isolated α -1-antitrypsin. Experimental details are given in the Materials and Methods section; the gel was stained with Amido Black 10B.

EXPLANATION OF PLATE 3

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of monkey α -1-antitrypsin in eluate from concanavalin A-Sepharose before and after freeze-drying

The direction of migration is downward towards the anode. Gel 1, electrophoresis of 100 μ l of active eluate from the concanavalin A-Sepharose column; gel 2, electrophoresis of 20 μ l of a 2 mg/ml solution of freeze-dried α -1-antitrypsin. A piece of wire (near the anode) marks the position of the Bromophenol Blue. Gels were stained with Coomassie Brilliant Blue R-250. Experimental details are described in the Materials and Methods section.

EXPLANATION OF PLATE 4

Antigen-antibody crossed immunoelectrophoresis of isolated monkey α -1-antitrypsin

The first direction of electrophoresis was in acid/starch gel; the gel strip was applied at the origin, with the anode on the left. The second direction of electrophoresis was in 1% agarose with 1% rabbit anti-(monkey α -1-antitrypsin) serum, with the anode at the top. The gel was stained with Coomassie Brilliant Blue R-250. The black dot indicates peak 3 of monkey α -1-antitrypsin, which has the same mobility in starch as peak 6 for human α -1-antitrypsin phenotype PiM. Experimental details are described in the Materials and Methods section.

EXPLANATION OF PLATE 5

Antigen-antibody crossed immunoelectrophoresis of human serum α -1-antitrypsin

The first direction of electrophoresis was in acid/starch gel, by using 7 μ l of human phenotype PiM serum; gel strip (80 mm $\times$ 4.0 mm $\times$ 2.0 mm) was applied at the origin with anode on the left. The second direction of electrophoresis was in 1% agarose with 0.18% rabbit anti-(human α -1-antitrypsin) serum with anode at the top. The gel was stained with Coomassie Brilliant Blue R-250. The black dot denotes peak 6 of phenotype PiM. Experimental details are described in the Materials and Methods section.

mannitol was added to standards and α -1-antitrypsin after hydrolysis but before neutralization.

The carbohydrate analysis of rhesus-monkey α -1-antitrypsin as determined by g.l.c. is shown in Table 3. Fucose and *N*-acetylgalactosamine were not present, even in trace amounts. Since an electronic integrator was used for the analysis, it was possible to inject large amounts of sample into the gas chromatograph and avoid the limitations otherwise imposed by the paper tracing. Thus detection of exceptionally small amounts of monosaccharide was possible. The carbohydrate content of rhesus-monkey α -1-antitrypsin was calculated to be 11.7% of the weight.

The trimethylsilyl reagent alone or mixed with the contents of blanks yielded only a solvent peak. Therefore all peaks in the g.l.c.-analysis tracings were attributed to monosaccharides in either standards or in α -1-antitrypsin preparations. Peak identification was verified by co-injection of monosaccharide standards with α -1-antitrypsin.

Discussion

A variety of procedures have been reported for isolation of human α -1-antitrypsin from plasma (Bellet & Magnan de Bornier, 1975; Horng & Gan, 1974; Pannell *et al.*, 1974; Johnson *et al.*, 1974; Kress & Laskowski, 1974) or serum (Chan *et al.*, 1973; Travis & Pannell, 1973; Murthy & Hercz, 1973; Myerowitz *et al.*, 1972); procedures described by Crawford (1973) and Liener *et al.* (1973) were modified and adapted for isolation of rhesus-monkey serum α -1-antitrypsin. The isolated material exhibited electrophoretic behaviour identical with that exhibited by whole-serum α -1-antitrypsin as determined by acid/starch-gel electrophoresis, crossed immunoelectrophoresis and immunoelectrophoresis. The absence of detectable contaminants with each of these techniques and with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicates that isolated preparations had a high degree of purity.

To our knowledge, isolation and purification of rhesus monkey α -1-antitrypsin has not been previously accomplished. However, the literature abounds with data for isolated human α -1-antitrypsin from plasma or serum. The estimated mol.wt. of 60000 for isolated monkey α -1-antitrypsin is similar to published values of 45000–65000 (Bundy & Mehl, 1959; Heck & Kaplan, 1974) for human α -1-antitrypsin; our mol.wt. estimate is probably 1000–2000 higher than the actual value, because glycoproteins with a 10% carbohydrate content are known to exhibit anomalously high molecular weights (Segrest *et al.*, 1971; Segrest & Jackson, 1972); however, no correction was applied to the molecular weight for calculations. Further confirmation of purity resides in the observation that antisera produced against reconstituted freeze-dried α -1-anti-

trypsin reacted only with a single α -globulin component in whole monkey serum.

Despite the relatively mild conditions, i.e. use of phosphate buffer and approximately neutral pH, considerable loss in trypsin-inhibitory activity occurred during the purification procedures. Although 86% of the original inhibitory capacity was recovered after $(\text{NH}_4)_2\text{SO}_4$ precipitation, only 23% was present in the freeze-dried final product. It is doubtful that the relatively mild chromatographic procedures were responsible for this loss, but the observation that warming the freeze-dried product to room temperature resulted in complete inactivation suggests that freeze-drying may be deleterious, even when the environmental temperature is maintained at 0°C. Nevertheless, our values for recovery of inhibitory activity are similar to those reported for purified human α -1-antitrypsin (Liener *et al.*, 1973; Murthy & Hercz, 1973; Pannell *et al.*, 1974) and electrophoretic and serological activity were unaltered.

The specific activity, 0.26–0.40 mg of bovine trypsin inhibited/mg of α -1-antitrypsin, is in agreement with a 1:1 molar combining ratio for trypsin with isolated α -1-antitrypsin. Reports (Johnson *et al.*, 1974; Travis *et al.*, 1974) have shown a molar combining ratio of 2:1 for trypsin with isolated human α -1-antitrypsin. This discrepancy cannot be resolved until the number of combining sites for isolated monkey α -1-antitrypsin with bovine trypsin has been determined. However, the difference between molar combining ratios for monkey and human α -1-antitrypsin could be related to: (1) species differences, (2) isolation of α -1-antitrypsin from monkey serum rather than plasma, and/or (3) the avoidance of acidic conditions during isolation of monkey α -1-antitrypsin that might have uncovered or activated a second binding site.

The amino acid and carbohydrate composition of rhesus-monkey α -1-antitrypsin are similar to those of human α -1-antitrypsin. However, several subtle differences in amino acid content do exist. For example, glutamic acid residues were somewhat less, whereas tyrosine and arginine residues were somewhat more than those reported for human α -1-antitrypsin (Chan & Rees, 1975).

The carbohydrate moiety of α -1-antitrypsin was determined to be 11.7% (w/w), a value similar to that reported for isolated human α -1-antitrypsin (Chan & Rees, 1975; Chan *et al.*, 1973; Horng & Gan, 1974; Pannell *et al.*, 1974; Crawford, 1973; Kress & Laskowski, 1974; Travis *et al.*, 1974). The finding of nine *N*-acetylneuraminic acid residues is similar to the value reported by Chan & Rees (1975) and Kress & Laskowski (1973) for human PiM phenotype α -1-antitrypsin. In contrast with reported carbohydrate analyses for human α -1-antitrypsin, one glucose residue was detected. It is unlikely that this residue arises from external contamination.

At present no explanation can be given for the

presence of extra peaks introduced by mannitol. However, similar results have been reported in analyses of plant galactolipids (Jamieson & Reid, 1974). Since preparations of human α -1-antitrypsin have not been analysed for carbohydrate content by this method (g.l.c.), it is not known at this time what complications might arise.

Since monkey α -1-antitrypsin bands migrate more slowly than those for normal human PiM phenotype in acid/starch-gel electrophoresis, one might predict that the monkey α -1-antitrypsin has fewer sialic acid residues. This is apparently not the case. Differences in migration could be due to variations in amino acid content or perhaps to sequences of amino acid or carbohydrate components. Since the composition and structure of the bands obtained with acid/starch-gel electrophoresis for humans and monkeys are unknown, no conclusions about differences in migration can be drawn.

It was reasoned that the rhesus monkey might exhibit a system of genetically inherited variants similar to, but perhaps simpler than, that observed for humans (Fagerhol, 1972). Of the 28 monkeys examined by acid/starch-gel electrophoresis and the 18 also tested with antigen-antibody crossed electrophoresis, no evidence for genetic heterogeneity was detected. However, the number of monkeys tested is quite small; the rhesus monkey could very well possess a system of genetically inherited Pi types.

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