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Isolation and Partial Characterization of Rabbit Plasma α_1-Antitrypsin

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α_1-Antitrypsin was isolated from rabbit plasma by salting out with $(NH_4)_2SO_4$ followed by ion-exchange chromatography either on DEAE-Sephadex or DEAE-cellulose (each at pH 8.8 and 6.5), and affinity chromatography on Sepharose–Cibacron Blue and Sepharose–concanavalin A. The protein thus obtained was homogeneous during crossed immunoelectrophoresis by using an antiserum to whole rabbit plasma, but it migrated as two broad bands when electrophoresed in alkaline polyacrylamide gels. Under optimal loading conditions, two or three subcomponents could be distinguished in each band. The two major forms of rabbit α_1-antitrypsin, designated components F and S, were separated by preparative polyacrylamide-gel electrophoresis, and some of their physicochemical properties were established. Both forms reacted with trypsin at a molar ratio of 1:1. Their elution volumes from a Sephadex G-200 column were identical, corresponding to a mol.wt. of 58000; however, some heterogeneity was observed after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Isoelectric focusing in polyacrylamide gel in a pH 4–6 gradient revealed a multiple-band pattern for each form in the range of pH 4.4–4.9. The two forms of rabbit α_1-antitrypsin possessed the same *N*-terminal amino acid (glutamic acid) and had very similar amino acid and carbohydrate compositions.

A major proteinase inhibitor of mammalian blood plasma present in the α-globulin fraction and known as α_1-antitrypsin has been isolated in a pure state from human blood by a number of investigators (Crawford, 1973; Kress & Laskowski, 1973; Pannell *et al.*, 1974; Glaser *et al.*, 1975; Miller *et al.*, 1976; Saklatvala *et al.*, 1976; Hercz & Barton, 1977). This glycoprotein occurs in multiple genetically determined variants, in some of which the deficiency in antitrypsin activity is responsible for pathological changes. The protein is generally regarded as an acute-phase reactant, e.g. it circulates in increased amounts after injury (cf. Koj, 1974). Work in this laboratory necessitated the isolation of α_1-antitrypsin from rabbit plasma. In the present paper we report purification procedures for this protein from small or large volumes of starting material, together with some of the physicochemical properties of pure rabbit α_1-antitrypsin.

Materials and Methods

Materials

Sepharose 4B, cross-linked Sepharose CL4B, DEAE-Sephadex A-50, Sepharose–concanavalin A and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; heparin, benzoyl-L-arginine ethyl ester (Bz-Arg-OEt), 1-*O*-methyl α-D-glucopyranoside, bovine serum albumin and ovalbumin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; DEAE-cellulose DE-52 was from Whatman, Maidstone, Kent, U.K.; *p*-nitrophenyl guanidinobenzoate was from ICN, Cleveland, OH, U.S.A.; bovine trypsin was from P–L Biochemicals, Milwaukee, WI, U.S.A.; soya-bean trypsin inhibitor (three-times crystallized) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; human immunoglobulin G and goat antiserum to whole rabbit serum were from Miles–Yeda, Kankakee, IL, U.S.A.; $Na^{125}I$ and $Na^{131}I$ were from New England Nuclear, Boston, MA, U.S.A.; Diaflo ultrafiltration membrane UM10 was from Amicon Corp., Lexington, MA, U.S.A.; 5-dimethylaminonaphthalene-1-sulphonyl (dansyl, Dns) chloride was from BDH Chemicals, Poole, Dorset, U.K.; ethylenediamine was from Fisher Scientific Co., Fair Lawn, NJ, U.S.A.; Cibacron Blue 3G-A was a gift from Ciba–Geigy, Toronto, Ont., Canada. Human transferrin was prepared as described by Regoeczi *et al.* (1977) and antiserum to rabbit α_1-antitrypsin was raised in guinea pigs by Dr. J. Gauldie (McMaster University, Hamilton). Antibody production was initiated by injecting a total amount of

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25 μg of antigen in complete Freund's adjuvant at four intramuscular sites. The treatment was repeated without the use of the adjuvant 3 weeks later. A blood sample was taken 1 week later and tested for specificity and titre. The animals were bled at biweekly intervals and received monthly booster injections. Sera exhibiting a high titre were pooled.

All other chemicals were A.R. grade, where available.

Purification of α_1-antitrypsin

The starting material was fresh citrated plasma from adult male New Zealand rabbits (final concentration of citrate 0.38%). When a large volume of plasma was required, blood was pooled from several animals. For comparison, in one case plasma was obtained from a rabbit of the Griffen strain.

The purification procedure was derived from that of Pannell *et al.* (1974) for human α_1-antitrypsin. Two methods were devised, depending on the initial volume of plasma: A for 20–40ml of plasma and B for 100–200ml. In method A, plasma was mixed at room temperature (22°C) with saturated $(NH_4)_2SO_4$ solution to a final saturation of 40%. The precipitate was removed by centrifugation (10000*g* for 10min), the supernatant dialysed exhaustively against 0.15M-NaCl and passed through a Sepharose–heparin column (1cm×5cm) to remove antithrombin III (Miller-Anderson *et al.*, 1974). To the effluent, solid $(NH_4)_2SO_4$ was added to 55% saturation and, after 2h at room temperature, the precipitate removed by centrifugation (10000*g* for 10min). The concentration of $(NH_4)_2SO_4$ was increased to 85% saturation and, after 3h at 4°C, the precipitate was collected by centrifugation for 20min at 20000*g*, dissolved in 10–20ml of 0.05M-Tris/HCl, pH8.8, and dialysed against several changes of 0.05M-Tris/HCl, pH8.8, containing 0.05M-NaCl (buffer *A*). All subsequent procedures were carried out at 4°C. The protein solution was loaded on a column (2cm×30cm) of DEAE-Sephadex A-50 equilibrated with buffer *A*. After washing with 100ml of buffer *A*, a linear gradient [200ml of buffer *A* and 200ml of buffer *B* (0.05M-Tris/HCl, pH8.8, containing 0.2M-NaCl)] was applied. Fractions (5ml) were collected and their A_{280} was determined. By measuring trypsin-inhibitory capacity, the fractions rich in α_1-antitrypsin were located on the ascending slope of the albumin peak. These fractions were pooled, concentrated by ultrafiltration with an Amicon UM10 membrane and passed through a Sepharose–Cibacron Blue column (1cm×10cm), equilibrated with buffer *B*, to remove albumin. The fractions showing the highest A_{280} were pooled, dialysed against 0.005M-sodium phosphate buffer, pH6.5, containing 0.15M-NaCl, and loaded on to a DEAE-Sephadex A-50 column (1cm×15cm) equilibrated with the same buffer. After washing with 50ml of this buffer, a linear gradient was applied from 0.05M- to 0.2M-NaCl in 0.005M-sodium phosphate, pH6.5. The effluent was monitored at A_{280} for protein content and for antitryptic activity, and the most active fractions were pooled, exhaustively dialysed against 0.005M-Tris/HCl, pH8, and either kept at 4°C or frozen at −20°C.

In method B, plasma was passed through a Sephadex–Cibacron Blue column (5cm×50cm) equilibrated with 0.05M-Tris/HCl/0.15M-NaCl, pH8.0. The effluent was subjected to fractional salting-out with $(NH_4)_2SO_4$ and the precipitate obtained between 50 and 80% saturations was dialysed against buffer *A*. All subsequent steps were done at 4°C. The dialysed protein was applied to a DEAE-cellulose column (2cm×40cm) equilibrated with buffer *A*. After washing with 100ml of this buffer, a linear gradient (400ml) was started from 0.05M-NaCl to 0.2M-NaCl, both in 0.05M-Tris/HCl, pH8.8. The fractions showing the highest antitryptic activity were pooled and loaded directly on to a Sepharose–concanavalin A column (2cm×20cm) equilibrated with 0.1M-Tris/HCl, pH7.5, containing 0.5M-NaCl, and $MgCl_2$, $MnCl_2$ and $CaCl_2$, each at 0.001M. The column was washed with 5 column volumes of this buffer and the glycoproteins were eluted by 1-*O*-methyl α-D-glucopyranoside (0.1M) in the equilibration buffer. The eluate was concentrated by pressure dialysis (4°C) against 0.005M-sodium phosphate, pH6.5, containing 0.05M-NaCl and loaded on to a DEAE-cellulose column (1cm×20cm) equilibrated with this buffer. α_1-Antitrypsin was eluted by a linear gradient (300ml) from 0.05M- to 0.2M-NaCl in 0.005M-sodium phosphate, pH6.5 at a concentration of 0.09M-NaCl.

Separation of the two forms of rabbit α_1-antitrypsin was obtained by preparative electrophoresis in a polyacrylamide-gel column (2.8cm×2.8cm; 7.5% acrylamide) with an apparatus described by Pajdak (1973) with 0.005M-Tris containing 0.039M-glycine, pH8.2 (Clarke, 1964), in the anode and cathode chambers. After loading 1.0–1.5ml of α_1-antitrypsin (approx. 9mg/ml), electrophoresis was carried out at a constant current of 20mA (approx. 300V) for approx. 2h at 4°C until the tracking dye (Bromophenol Blue) reached the anode chamber. By subsequently collecting fractions at 2–3min intervals, it was possible to isolate a fast component (designated as α_1-antitrypsin F or component F), the mixture of two components, and a slow component (α_1-antitrypsin S or component S). The proteins were concentrated by pressure dialysis against water (4°C) and freeze-dried.

Assay of antitryptic activity

The concentration of trypsin solution was determined spectrophotometrically at 280nm by using $A^{1\%}_{1cm} = 15.4$ and mol.wt. 23900 (Robinson *et al.*,

1971). The commercial preparation of trypsin contained 70% of active enzyme, as determined by titration with *p*-nitrophenyl guanidinobenzoate (Chase & Shaw, 1967), and appropriate correction was made when calculating antitryptic activity. Inhibition of trypsin was measured with Bz-Arg-OEt as a substrate by a spectrophotometric method (Rick, 1965) after 10min preincubation of enzyme and antitrypsin at room temperature. One unit of antitryptic activity was defined as the amount of preparation able to completely inhibit 1 μg of trypsin. Specific activity of tested preparations was expressed as units of antitryptic activity per mg of protein.

Analytical electrophoretic methods

Analytical electrophoresis in polyacrylamide-gel cylinders was carried out at pH8.2 (Clarke, 1964). Gels were stained with 0.05% Amido Black in 7% (v/v) acetic acid and destained in 7% acetic acid.

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate was carried out as described by Weber *et al.* (1972). Pyronin Y was the tracking dye and the following proteins served as standards for molecular-weight determination: human transferrin (mol.wt. 80000), bovine serum albumin (68000), ovalbumin (43000), trypsin (23900) and soya-bean trypsin inhibitor (22000).

Analytical isoelectric focusing was carried out in cylindrical polyacrylamide gels containing 7.5% (w/v) acrylamide monomer and 2% (w/v) Bio-Lyte carrier ampholyte as described by Wrigley (1971). With a narrow gradient (pH4–6) a mixture of Bio-Lyte 3/10 and Bio-Lyte 4/6 (2:3, v/v) was used. The anode solution was 0.2% H_2SO_4 and cathode solution 0.4% ethylenediamine. Proteins (50–100 μg) in 20% (w/v) sucrose were layered on top of paired gels covered with 10% sucrose solution and then the reservoir was filled with ethylenediamine solution. After 8–10h at 400V (4°C) one gel of each pair was soaked in 10% (w/v) trichloroacetic acid and then stained with 0.05% (w/v) Coomassie Brilliant Blue dissolved in methanol/water/acetic acid (5:5:1, by vol.), and the other was frozen and cut into 5mm sections. The slices were soaked overnight in 1ml of 0.01M-KCl, centrifuged at 1400*g* for 10min, and the pH of the solution was measured with a Radiometer model 26 pH-meter. Finally, radioactivity in the gel slices was determined in a Packard model 5986 multichannel analyser.

Sephadex gel filtration

The molecular weight of α_1-antitrypsin was also estimated on a Sephadex G-200 column (2.2cm × 80cm) equilibrated with 0.5M-NaCl in 0.05M-sodium phosphate buffer, pH7.4, at room temperature. The column was calibrated with samples of Blue Dextran (mol.wt. 2000000), human immunoglobulin G (mol.wt. 160000), bovine serum albumin (mol.wt. 68000) and soya-bean trypsin inhibitor (mol.wt. 22000). The fractions were monitored by measuring their A_{280} or, when trace-labelled markers were used, by radioactivity.

Immunochemical techniques

The purity of rabbit α_1-antitrypsin was assessed by crossed immunoelectrophoresis in 1% agarose on glass plates (8.2cm × 10cm) in Tris/barbital buffer (I 0.05) at pH8.8 as described by Weeke (1973). Electrophoresis in the first dimension (24V/cm for 100min at 4°C) was followed by immunoelectrophoresis (5V/cm for 15h at 4°C) in 1% agarose supplemented with either 1% (v/v) goat antiserum to whole rabbit plasma or 1% (v/v) guinea-pig antiserum to rabbit α_1-antitrypsin. Plates were washed in 0.9% NaCl, dried and stained in 0.25% (w/v) Coomassie Brilliant Blue dissolved in methanol/water/acetic acid (5:5:1, by vol.).

Protein concentration and specific absorption coefficient

Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard; a specific absorption coefficient of 6.6 (1mg/ml) was used to determine albumin concentration in the standard, as suggested by Gordon (1976).

The u.v. spectrum of purified α_1-antitrypsin in 0.005M-Tris/HCl, pH8.0, obtained with a Cary 118C spectrophotometer, showed a maximum at 277–278nm. The readings at 280nm were corrected for non-specific absorption based on extrapolation of the absorption in the 330–350nm region and then used for calculation of the specific absorption coefficient $A^{1\%}_{1cm}$. Unfractionated preparations of rabbit α_1-antitrypsin yielded an $A^{1\%}_{1cm}$ value of 5.1 (±0.18S.E.M.), and isolated F and S components gave the values of 5.2 (±0.22S.E.M.) and 4.8 (±0.23S.E.M.) respectively ($n = 5$). These coefficients were used as an alternative to the Lowry *et al.* (1951) method for measuring protein concentrations of purified α_1-antitrypsin preparations.

Determination of N-terminal amino acid

The *N*-terminal amino acid of α_1-antitrypsin was determined by the dansyl chloride technique of Gray & Hartley (1963). Dns derivatives were separated by two-dimensional t.l.c. on polyamide layers by using 1.5% (v/v) formic acid solution and heptane/butan-1-ol/acetic acid (3:3:1, by vol.; Woods & Wang, 1967). Standard Dns-amino acids were prepared by the method of Boulton & Bush (1964).

Amino acid analysis

Protein samples were hydrolysed in freshly distilled 6M-HCl in sealed tubes at 105°C for 24, 30 and 48h and were analysed with a Beckman 120C analyser. To quantify cysteine and methionine

residues accurately, two samples of protein were oxidized with performic acid as described by Bailey (1967). Glucosamine was measured from a sample after 3h hydrolysis in 3.5M-HCl at 100°C.

The tryptophan content of α_1-antitrypsin was determined by a colorimetric procedure (Opienska-Blauth *et al.*, 1963), and by measuring the molar ratio of tyrosine to tryptophan by a spectrophotometric method (Beaven & Holiday, 1952).

Carbohydrate analysis

Analyses of carbohydrate constituents were undertaken by using the g.l.c. procedure of Zanetta & Vincendon (1974). After methanolysis (1.0M-HCl) of the protein samples for 20h at 80°C, trifluoroacetyl derivatives of the methyl glucosides were prepared. A Varian 2100 gas chromatograph was equipped with a Hewlett–Packard 3380 electronic integrator and a 3% OV-210 (on Chromosorb W, 80–100 mesh) column (length 1.8m; internal diam. 4mm; carrier gas N_2 at 40ml/min). The initial column temperature was 110°C, increasing with a gradient of 2°C/min, the final temperature was 200°C.

In addition, the sialic acid content was measured by a colorimetric method (Aminoff, 1961), after acid hydrolysis of the protein by 0.05M-H_2SO_4 for 1h at 80°C.

Other methods

Heparin was coupled to Sepharose 4B by the CNBr method as described by Miller-Anderson *et al.* (1974), and Cibacron Blue to cross-linked Sepharose 4B as described by Travis *et al.* (1976). Proteins were trace-labelled with ^{131}I or ^{125}I by the ICl method of McFarlane (1958).

Results

Isolation of α_1-antitrypsin

When purified by method A, approx. 25% of the plasma antitryptic activity was recovered in the final fraction (Table 1). The true recovery of α_1-antitrypsin was probably slightly higher considering that plasma contains other proteins, e.g. antithrombin III, that also inhibit the esterolytic activity of trypsin. From the specific activity of the final preparation it can be calculated that α_1-antitrypsin reacts with trypsin at a molar ratio of 1:1, assuming a mol.wt. of 23900 for trypsin and 58000 for rabbit α_1-antitrypsin. However, in some experiments the specific activity of the final product was 10–30% below the expected value, despite the fact that no impurities could be detected by electrophoresis and immunological techniques. A possible explanation is that some of the α_1-antitrypsin molecules became denatured during the purification procedure.

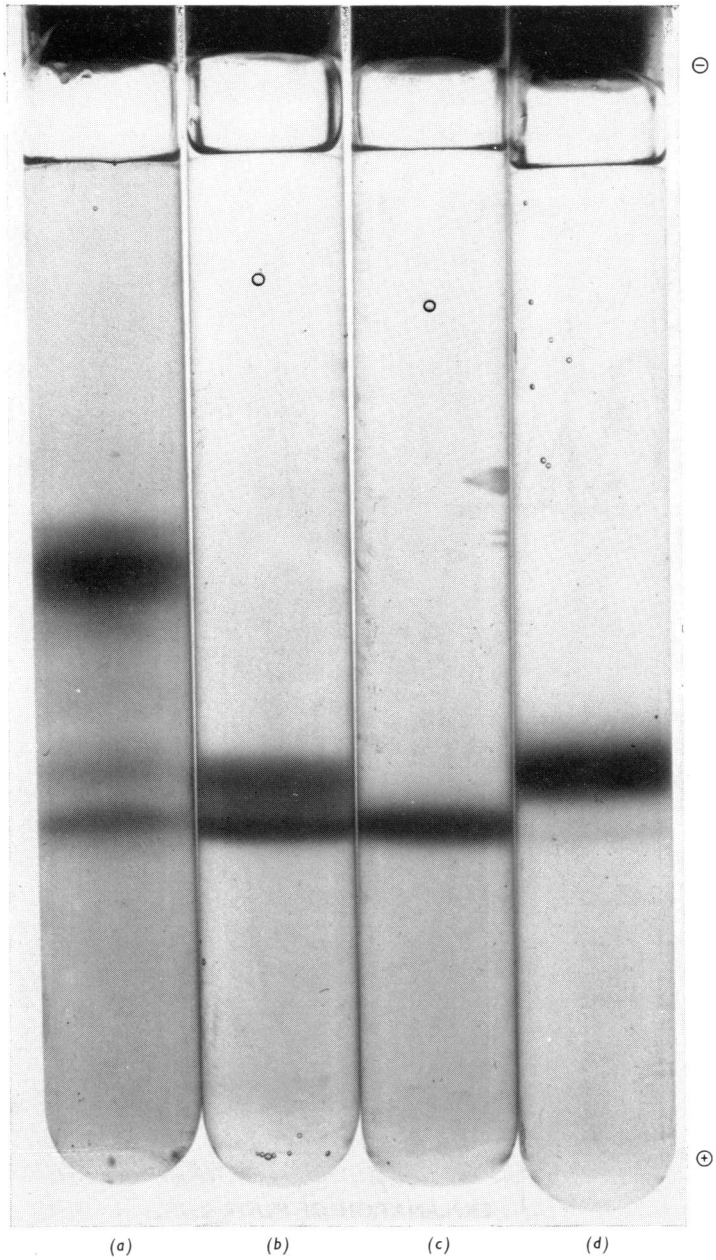
In the purified state, α_1-antitrypsin exhibited a variable tendency to spontaneous deterioration, as manifested by decreases in the specific activity of the protein during storage. Although no systematic studies were undertaken to establish the relationship between the viability and age of α_1-antitrypsin, random re-determinations of the specific activities of stored preparations provided some insight. Thus decreases in the specific activity of preparations kept at pH 7.4–8.0 and 4°C for 4 weeks varied between 0 and 21% of the initial values. From aged preparations of rabbit α_1-antitrypsin, slowly migrating components often appeared on polyacrylamide-gel electrophoresis, and they are thought to be analogous to the protein aggregates described for human α_1-antitrypsin by Glaser *et al.* (1977). Dilute samples (3mg/ml or less) formed aggregates less readily and lost their viability more slowly. 2-Mercaptoethanol (10mM), reported to be essential for the preservation of the activity of human α_1-antitrypsin during and after isolation (Miller *et al.*, 1976), had no comparable effect on rabbit α_1-antitrypsin. Freeze-drying or repeated freezing-thawing caused losses of up to 30% of the antiproteinase activity of rabbit α_1-antitrypsin.

Purified rabbit α_1-antitrypsin always showed a two-band pattern after alkaline polyacrylamide-gel electrophoresis (Plate 1*b*). Both bands reacted with trypsin (Plate 1*a*) and they shared common antigenic determinants as evidenced by the results of crossed

Table 1. *Purification of rabbit α_1-antitrypsin by method A*

Values listed are from an experiment that is representative of the method with respect to purity and yield of the inhibitor.

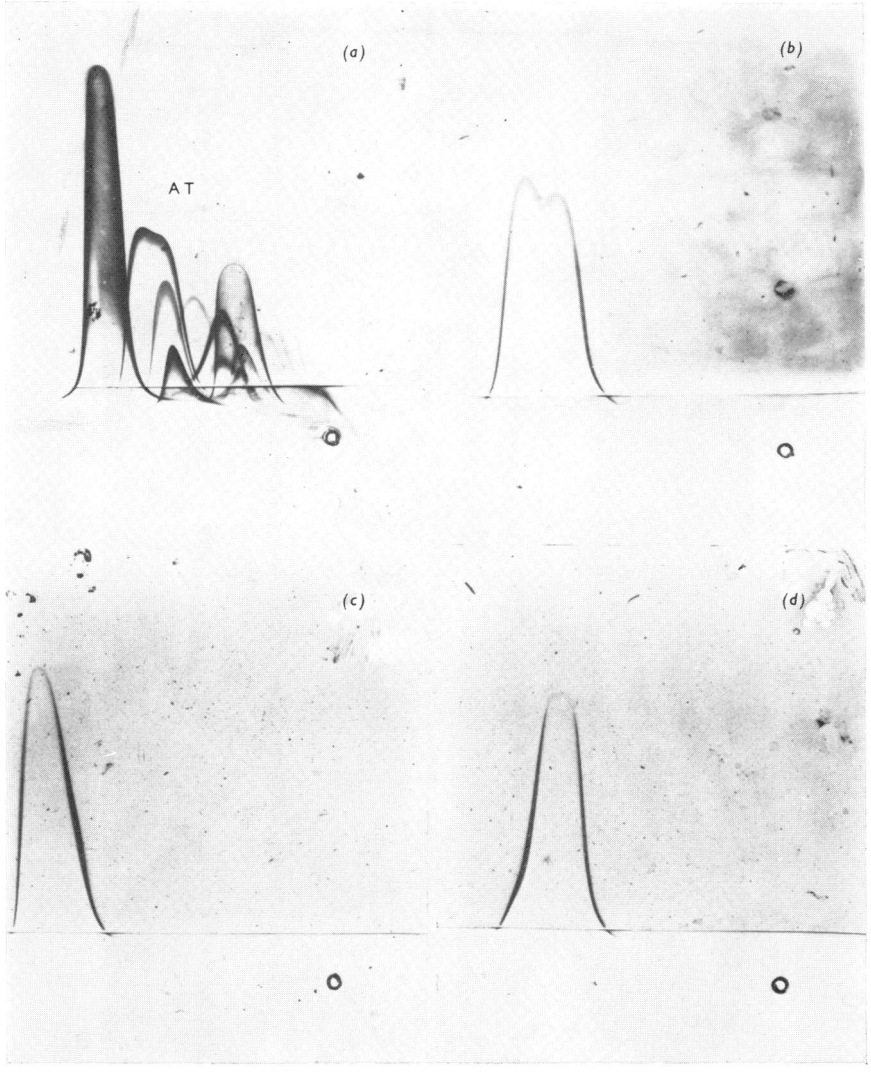
Purification step	Protein content (mg)	Antitryptic activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery of inhibitory activity (%)
1. Plasma (20ml)	1160	11620	10	1	100
2. $(NH_4)_2SO_4$: 40% saturation	577	9232	16	1.6	79
3. Eluate: Sepharose–heparin	478	9082	19	1.9	78
4. $(NH_4)_2SO_4$: 55–85% saturation	364	8735	24	2.4	75
5. Eluate: DEAE-Sephadex, pH8.8	36	5270	146	15	45
6. Eluate: Sepharose–Cibacron Blue	12.3	4711	383	38	40
7. Eluate: DEAE-Sephadex, pH6.5	6.9	2891	419	42	25



EXPLANATION OF PLATE 1

Electrophoresis of rabbit α_1-antitrypsin in polyacrylamide-gel

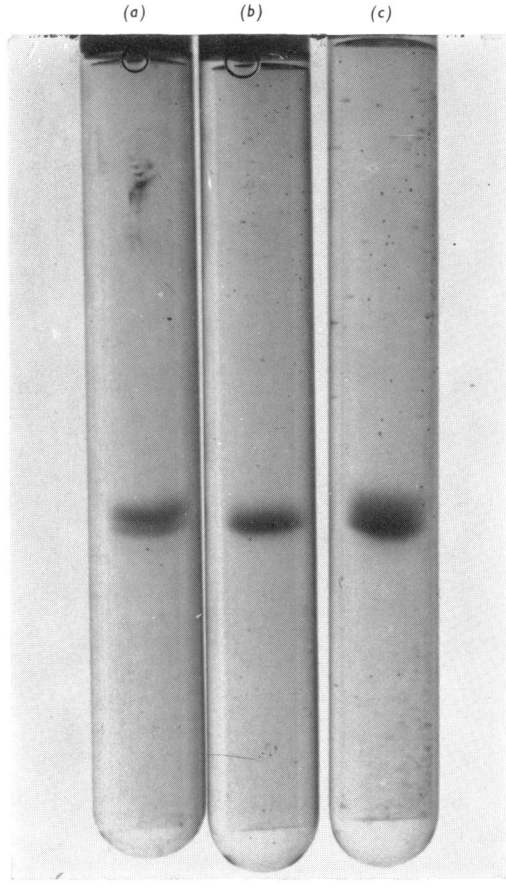
(*a*) α_1-Antitrypsin after incubation with trypsin (5:4 molar ratio) at pH8.0 for 10min; (*b*) fresh α_1-antitrypsin; (*c*) fast component (F) and (*d*) slow component (S) as obtained by preparative polyacrylamide-gel electrophoresis. Each gel was loaded with approx. 40μg of protein and run for 1h at 4mA/gel at pH8.2.



EXPLANATION OF PLATE 2

Two-dimensional crossed immunoelectrophoresis of rabbit α_1-antitrypsin in agarose gel with goat antiserum to whole rabbit plasma

(*a*) Whole rabbit plasma; AT = α_1-antitrypsin; (*b*) purified α_1-antitrypsin; (*c*) fast component (F) and (*d*) slow component (S) as obtained by preparative polyacrylamide-gel electrophoresis. In each case approx. 5μg of α_1-antitrypsin was analysed.



EXPLANATION OF PLATE 3

Electrophoresis of rabbit α_1*-antitrypsin in polyacrylamide gel containing sodium dodecyl sulphate*
Migration was from top to bottom, anode at the bottom. (*a*) Fast component; (*b*) slow component; (*c*) mixture of fast and slow components. Gel loads were 3–6μg of protein.

immunoelectrophoresis (Plate 2). Crossed immunoelectrophoresis with either monospecific antiserum to rabbit α_1-antitrypsin or multivalent antiserum to whole rabbit plasma (Plate 2*a*) showed that the two forms of α_1-antitrypsin were also present in fresh plasma samples from two unrelated strains of rabbits (New Zealand White and Griffen). In individual plasma samples from eight animals, the relative proportions of the F and S proteins appeared to be approximately equal. However, the separation of the two arcs from one another was rather poor, whereby no reliable estimate for individual variations in the F:S ratios could be obtained.

Rabbit α_1-antitrypsin prepared by method B often contained trace impurities seen as more-slowly migrating components on analytical polyacrylamide-gel electrophoresis. These were easily removed by preparative polyacrylamide-gel electrophoresis. The yield of antitryptic activity in the scaled-up method was slightly lower than in method A (approx. 20%). However, the large DEAE-cellulose column, equilibrated with 0.005M-sodium phosphate, pH6.5, together with a shallow NaCl gradient (0.05–0.15M-NaCl), made a partial separation of the two molecular forms of rabbit α_1-antitrypsin possible. Under these conditions, the slowly migrating component was eluted first from the column in an almost pure state, but a complete separation of components F and S was not possible.

Regardless of whether method A or B was used to isolate α_1-antitrypsin, final purification and separation of the two molecular forms was achieved by preparative polyacrylamide-gel electrophoresis. The best results were obtained with loads up to 10–12mg of protein. The F and S components thus obtained were essentially homogeneous on analytical polyacrylamide-gel electrophoresis (Plate 1*c*,*d*) and crossed immunoelectrophoresis (Plate 2*c*,*d*). However, as a rule, the antiproteinase activity of α_1-antitrypsin subjected to preparative electrophoresis was 10–30% below expected values.

Molecular weight of rabbit α_1-antitrypsin

When a mixture of α_1-antitrypsin components F and S was chromatographed on Sephadex G-200, the protein was eluted in a volume corresponding to a mol.wt. of approx. 58000. No separation of components F and S occurred, as concluded from analyses of chromatographic fractions by polyacrylamide-gel electrophoresis. However, some heterogeneity of rabbit α_1-antitrypsin was observed during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. When 10–20μg of F protein was analysed by this method, only one band appeared, which migrated with a relative mobility corresponding to a mol.wt. of approx. 58000–59000. If the load was decreased to 3–4μg, component F migrated as two sharp and closely spaced bands (Plate 3*a*), the difference between their relative mobilities corresponding to less than 4000 daltons. Judged by the relative intensities of the bands to the naked eye, the proportion of the slow and the fast subcomponents in α_1-antitrypsin component F appears to be approx. 4:1. Similar loads of the S protein always yielded a single band with a mobility similar to that of the faster band of component F and corresponding to a mol.wt. of 57000–58000. The resolution of mixed samples varied depending on the relative proportions of the F and S components contained in them: when α_1-antitrypsin was in excess the mixture yielded two closely spaced bands of comparable intensities, whereas in the reverse situation the mixture gave a single band with some trailing (Plate 3). Increased concentrations of sodium dodecyl sulphate (up to 4% in the protein sample), or the presence of 2-mercaptoethanol, had no influence on the mobility or the staining pattern of α_1-antitrypsin subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Isoelectric point of rabbit α_1-antitrypsin

Additional evidence for the heterogeneity of rabbit α_1-antitrypsin was obtained by isoelectric focusing in polyacrylamide gel. Pure but unfractionated α_1-antitrypsin formed two bands in the region of 4.8–5.0 when focused in the pH gradient 3–10. In a more narrow gradient (pH4–6) the apparent isoelectric points were shifted to slightly lower values, and a multiple-band pattern was observed in the region of pH4.4–4.9. The positions of the bands plotted against the pH gradient partly overlapped, component F being localized mainly in the region of pH4.4–4.7 and component S in the region of pH4.6–4.9.

Amino acid and carbohydrate compositions

By the dansyl technique, rabbit α_1-antitrypsin components F and S each gave a single *N*-terminal amino acid, which was tentatively identified as glutamic acid by comparison with standard Dns-amino acids. The overall amino acid compositions of proteins F and S were comparable (Table 2), except for phenylalanine (difference of three residues) and possibly alanine and threonine (difference of two residues each). The compositions shown in Table 2 account for 98.0–100.6% of the material, assuming a molecular weight of 58000 for α_1-antitrypsin. Tryptophan analyses are not included in Table 2 because of a great discrepancy that was observed in the results from colorimetric and spectrophotometric methods. By the colorimetric procedure, component F was found to contain 0.84 residue of tryptophan/molecule and component S 0.62 residue/molecule (mean of two determinations), whereas the tryptophan content calculated from spectrophotometric measurements was in the range of 2–3 residues/molecule with no significant differences between components F

Table 2. *Amino acid and carbohydrate compositions of rabbit α_1-antitrypsin components F and S*

Unless otherwise stated the values are the means (±S.E.M.) of three hydrolysates (24, 30 and 48h). The carbohydrate results from g.l.c. analyses after methanolysis in 1M-HCl were calculated as molar ratios relative to various molar mixtures of the standard monosaccharides that had also undergone methanolysis. These values were fitted relative to the results for sialic acid and glucosamine obtained from colorimetric estimations. The results for sialic acid from two analyses, by the method of Aminoff (1961), after acid hydrolysis, were 116 and 124nmol/mg for components F and S respectively. The glucosamine content of component S determined by amino acid analysis after hydrolysis for 3h at 95°C in 3.6M-HCl was 227nmol/mg.

	Quantity			
	(nmol/mg of protein)		(residues/molecule)	
Component ...	F	S	F	S
Aspartic acid	704± 3.7	684± 4.5	35.2	34.2
Threonine	679±11.7	648±16.5	35.6*	33.7*
Serine	490±16.5	514±19.7	27.5*	29.1*
Glutamic acid	1586±12.3	1577± 8.5	79.3	78.9
Proline	551±27.2	542±18.3	27.5	27.1
Glycine	398±22.3	408±18.0	19.7	20.4
Alanine	777±14.5	814±15.0	38.9	40.7
Half-cystine†	61	57	3.1	2.9
Valine	493±47.5	472±24.6	24.6	23.6
Methionine†	0	0	0	0
Isoleucine	217±18.3	207± 9.3	10.9	10.3
Leucine	997±19.7	968±16.0	49.9	48.4
Tyrosine	130± 6.4	126± 0.9	6.5	6.3
Phenylalanine	416±18.6	480±20.9	20.8	24.0
Lysine	698±22.2	692±21.6	34.9	34.6
Histidine	346± 3.7	334± 1.9	17.3	16.7
Arginine	331± 2.8	350± 2.0	16.5	17.5
N-Acetylglucosamine			10.2	10.6
Galactose			7.3	8.2
Mannose			9.0	9.0
Sialic acid			6.5	6.5

* Extrapolated to zero hydrolysis time.
† Obtained by performic acid oxidation.

and S. We have no explanation to offer for this discrepancy.

Carbohydrates account for 11.4–11.8% of the weight of rabbit α_1-antitrypsin. Components F and S contained comparable quantities of *N*-acetylglucosamine, galactose, mannose and *N*-acetylneuraminic acid (Table 2). Fucose, glucose and *N*-acetylgalactosamine were not detected in either protein.

Discussion

Isolation of immunologically pure rabbit α_1-antitrypsin for the present study was accomplished by two different methods. In the procedure used to process large volumes of starting material, early elimination of serum albumin by affinity chromatography on Sepharose–Cibacron Blue greatly decreased the quantities of ion-exchangers required for the subsequent steps.

The summary of the results of our physicochemical measurements on rabbit α_1-antitrypsin is given in Table 3, together with comparative values for the corresponding human and rhesus-monkey proteins. A broad similarity is apparent among the α_1-proteinase inhibitors from the three species. Nevertheless, the ratio of polar to apolar amino acids for rabbit α_1-antitrypsin strongly deviates from the ratios for the corresponding primate inhibitors. Also, rabbit α_1-antitrypsin is notably devoid of methionine, but perhaps the most prominent difference among these proteins is the resolution of the rabbit inhibitor into two major components at alkaline pH.

The results indicate that α_1-antitrypsin components F and S have different isoelectric points. From the results in Table 2 it seems unlikely that the charge difference reflects uneven distribution of carbohydrates and, indeed, high-voltage electrophoresis of the glycopeptides from rabbit α_1-antitrypsin yielded only one glycopeptide species (M. W. C. Hatton & H. Kaur, unpublished work). Results of the amino acid analyses favour the view that subtle

Table 3. *Comparison of some physicochemical properties of rabbit, human and rhesus-monkey α_1-antitrypsins*
The data for rabbit α_1-antitrypsin are from the present study, those for human α_1-antitrypsin have been compiled from Crawford (1973), Pannell *et al.* (1974) and Saklatvala *et al.* (1976), and those for rhesus-monkey α_1-antitrypsin from Berninger & Mathis (1976). Ratios of polar/apolar amino acids were calculated as described by Hatch (1965).

	Rabbit α_1-antitrypsin		Human α_1-antitrypsin	Rhesus-monkey α_1-antitrypsin
	F	S		
Specific absorption coefficient ($A^{1\%}_{1cm,280}$)	5.2	4.8	4.4–5.3	?
Molecular weight	58000–59000	57000–58000	50000–53000	60000
pI	4.4–4.8	4.6–4.9	4.85–5.10	?
N-Terminal amino acid	Glu	Glu	Glu	?
Ratio: polar/apolar amino acids	1.72	1.70	1.33	1.32
No. of polypeptide chains	1	1	1	1
Carbohydrate content (%)	11.4	11.8	11.5	11.7
Sialic acid (residues/molecule)	6–7	6–7	2–6	9

differences may exist between the fast and the slow proteins (see values for phenylalanine, aspartic acid and arginine in Table 2).

Evidence for further heterogeneity within the F and S proteins was obtained after isoelectric focusing, when each protein split into several bands. Furthermore, small loads of whole α_1-antitrypsin often migrated on electrophoresis in alkaline polyacrylamide gel as a pair of two or three narrow bands, rather than a pair of broad bands.

The results of the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis suggest that α_1-antitrypsin component S is slightly smaller than α_1-antitrypsin component F. The resolution of the F protein into a larger and a smaller subcomponent by this method was unexpected. Since the small subcomponent of F protein was indistinguishable from the S protein by its mobility in sodium dodecyl sulphate/polyacrylamide gel, special care was taken to analyse only such α_1-antitrypsin component F preparations that exhibited no contamination with S protein on alkaline polyacrylamide-gel electrophoresis. There are several possible explanations for the apparent size heterogeneity of the F protein. One possibility is that there are two differently sized but similarly charged F components, in addition to a third major component (component S) of rabbit α_1-antitrypsin. A perhaps simpler assumption is that there are only two major forms (F and S) of the protein as far as size is concerned, and that the small subcomponent that separates from component F in the presence of sodium dodecyl sulphate is actually an S protein that possesses more charged groups at pH 8.2 and consequently migrates with component F in an alkaline gel in the absence of the detergent.

The origin of the heterogeneity of rabbit α_1-antitrypsin is not altogether clear at present. Artifacts generated by chemicals during purification are an unlikely explanation considering that the F and S components are readily demonstrated also in whole rabbit plasma by crossed immunoelectrophoresis. In view of the difference in molecular weights, a precursor–product relationship, similar to the one described by Sherman *et al.* (1969) for fibrinogens of different solubilities, could exist between F and S components. In this case the S protein would arise from F protein through the loss of a small portion of its *C*-terminal end, the *N*-termini being identical. However, unpublished kinetic observations (A. Koj & E. Regoeczi) do not support this explanation. A more plausible assumption is that synthesis of rabbit α_1-antitrypsin, similarly to that of the four main transferrin phenotypes in homozygous cattle (Ashton, 1958), is effected by allelic genes. Alternatively, it is conceivable that variants of the gene responsible for the synthesis of α_1-antitrypsin exist in rabbits and all eight animals, from which individual plasma were examined, were heterozygous. [Genetic variants of the human inhibitor are known: cf. Kueppers & Black (1974).] However, the probability for such a distribution of samples from randomly selected animals is very small ($P = 0.0039$).

The present demonstration of the existence of a complex α_1-antitrypsin system in the rabbit follows the earlier demonstration of the presence of two trypsin-binding macroglobulins in the serum from this species (Got *et al.*, 1965; Berthiller *et al.*, 1968), and thus it serves as a further example for species-related differences among mammalian plasma anti-proteinases.

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