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ISOLATION AND CHARACTERIZATION OF TWO MINOR FRACTIONS OF α_1-ANTITRYPSIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHROMATOFOCUSING

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SUMMARY

α_1-Antitrypsin is a glycoprotein that separates into five electrophoretic fractions, *viz.* M_2, M_4, M_6, M_7 and M_8. Con A-Sepharose separates the protein into three fractions according to the branching degree of the three oligosaccharide chains. The Con A affinity is identical for M_4 and M_7 and for M_6 and M_8. Within each pair the proteins were isolated by rapid chromatofocusing. The M_7 and M_8 have the same carbohydrate structure as the major M_4 and M_6 respectively, but have lost the first five N-terminal amino acids (Glu-Asp-Pro-Glu-Gly) as compared to the majority of the protein.

INTRODUCTION

α_1-Antitrypsin (α_1-protease inhibitor) is a human plasma protein with an unusual microheterogeneity revealed by electrophoresis at pH 5 or by electrofocusing. There are two major and three minor fractions with isoelectric points between 4.4 and 4.7[1]. The molecule consists of a single polypeptide chain with 394 amino acid residues and three carbohydrate chains, giving a molecular weight of 51 000[2]. The carbohydrate side-chains contain a common core sequence: Asn-GlcNAc-GlcNAc-Man-(Man)2 and an outer antenna sequence of three sugars: GlcNAc-Gal-NeuNAc, attached through the GlcNAc to the terminal mannose residues of the core[3]. Variation in the number of antennae (branching degree) from two to three is responsible for the major microheterogeneity (M_2, M_4 and M_6) within each homozygous phenotype[4]. The aim of this study was to isolate and characterize the M_7 and M_8 fractions.

MATERIALS AND METHODS

Purification of α_1-antitrypsin

EDTA-treated blood was collected from a single blood donor. The α_1AT phenotype M_1M_1 was checked by isoelectric focusing[1], and α_1AT was then purified with preserved microheterogeneity by using ammonium sulphate precipitation followed by thiol–disulphide interchange reactions and two other steps of affinity chromatography with Blue Sepharose and AH-Sepharose[5].

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Equipment

Con A-Sepharose 4B and a FPLC® system equipped with a Mono P™ HR 5/10 column and Polybuffer™ were from Pharmacia (Uppsala, Sweden).

Con A-Sepharose chromatography

Purified α_1AT with preserved microheterogeneity (50 mg) was applied to a 90 × 1.6 cm I.D. column of Con A-Sepharose as described in the legend to Fig. 1. Fractions II and III were collected, freeze-dried and subjected to chromatofocusing.

Chromatofocusing on Mono P

Fractions Con A II and III (5 mg of each), respectively, were dissolved in 500 µl of starting buffer: 25 m*M* methylpiperazine and 10 m*M* NaCl (pH 5.4) and dialysed for 30 min at room temperature—immediately before chromatofocusing as described in the legends to Fig. 2 and 3. Each tube in the fraction collector contained 250 µl of 1 *M* glycine (pH 7.0) for an immediate adjustment of pH from 4.4–4.7 to neutrality.

Identification of the microheterogeneous fractions

Collected peaks after chromatofocusing were pooled, concentrated 10-fold on a Minicon B15 filter and subjected to isoelectric focusing in a pH gradient of 3.5–5.0[1].

Con A affinity crossed immunoelectrophoresis

The glycosylation pattern of the isolated α_1AT isoforms from chromatofocusing on Mono P were analysed by Con A affinity crossed immunoelectrophoresis according to Wells *et al.*[6]. In the first dimension there was a short stretch of 1% agarose, then a 1% gel containing 1 mg/ml of Con A. The second-dimension gel contained 50 mmol/l of α-methyl mannoside and 1% rabbit antiserum raised against α_1-antitrypsin.

Amino acid sequence determination

N-terminal sequence determination of 20 nmoles of each of five fractions was performed by automatic Edman degradation in a Beckman 890 C sequencer. The Beckman No 127974 Quadrol program was used and 2 mg of Polybrene was added to the spinning cup. Phenylthiohydantoin derivatives of amino acids were identified by high-performance liquid chromatography[7].

RESULTS

Isoelectric focusing of α_1AT purified by the thiol–disulphide interchange system showed a protein with preserved microheterogeneity. The Con A-Sepharose chromatography (Fig. 1) separated α_1AT into three groups Con A I–III depending on the branching degree of the carbohydrate chains[4]. Rapid chromatofocusing further separated Con A II fraction into M_4 and M_7 with small impurities of M_6, and Con A III into M_6 and M_8 with small impurities of M_4. The pattern from isoelectric focusing of pooled fractions from chromatofocusing are included in the chromatograms (Figs. 2 and 3). The purified M_2, M_4, M_6, M_7 and M_8 from chromatofocusing were further analysed by Con A crossed immunoelectrophoresis. In this system the

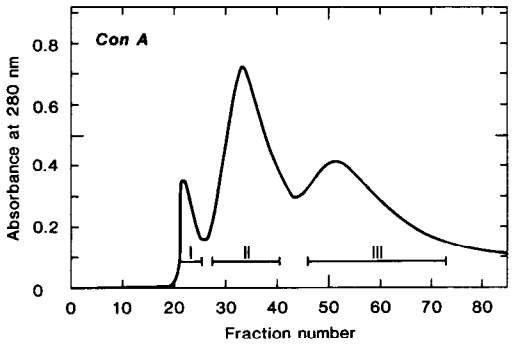
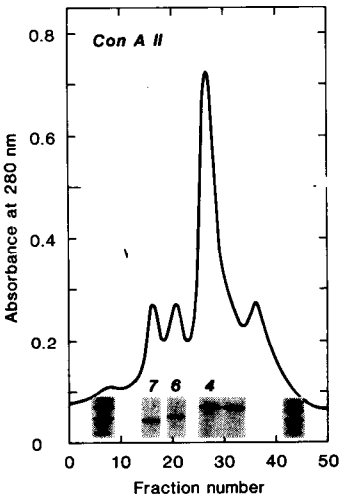


Fig. 1. Affinity chromatography of purified α_1AT on Con A-Sepharose 4 B (90 × 1.6 cm I.D.) Flow-rate, 18 ml/h. Starting buffer A: 50 m*M* Tris–HCl, 50 m*M* NaCl, pH 7.5. Eluting buffer B: Buffer A containing 40 m*M* α-methyl-D-mannoside. A linear gradient was formed by mixing equal volumes (250 ml) of A and B.



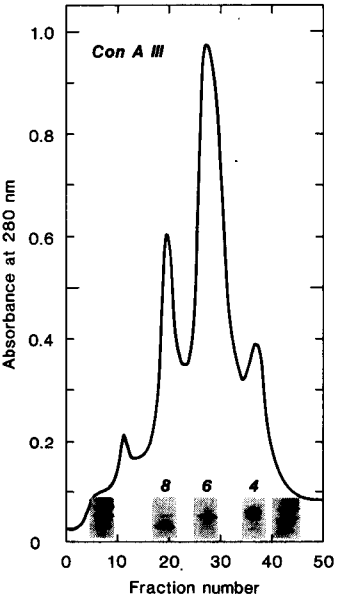


Fig. 2. Chromatofocusing of Con A II. The Mono P column was equilibrated with 25 m*M* methylpiperazine and 10 m*M* NaCl, pH 5.4. A gradient was formed by Polybuffer™ (1:10), 10 m*M* NaCl, pH 4.0. Flow-rate, 1 ml/min. The isoelectric focusing patterns are included.

Fig. 3. Chromatofocusing of Con A III fraction. Conditions as in Fig. 2.

M_2 form had a low affinity, the M_4 and M_7 isoforms an intermediate affinity, and the M_6 and M_8 forms had a very high affinity for Con A.

For further evaluation of charge differences between forms with identical carbohydrate chains, all five purified forms were subjected to N-terminal degradation. The first 25 N-terminal amino acids were identical to those cited in previous publications, except for the minor fractions M_7 and M_8. These forms lacked the first five regular N-terminal amino acids: Glu-Asp-Pro-Gln-Gly and started at position 6 with Asp-Ala-Ala-Glu...

DISCUSSION

Two previously uncharacterized forms of α_1AT, M_7 and M_8 have been purified and analysed. The minor fractions seem to be parallels to thhe major forms M_4 and M_6, respectively (Fig. 4), concerning oligosaccharide chains, but they are missing the first five N-terminal amino acids. Recent results obtained on culture of normal liver biopsy material demonstrate that α_1AT in the liver is produced in three forms. They represent the normal M_2, M_4 and M_6 isoforms on isoelectric focusing of plasma[8].

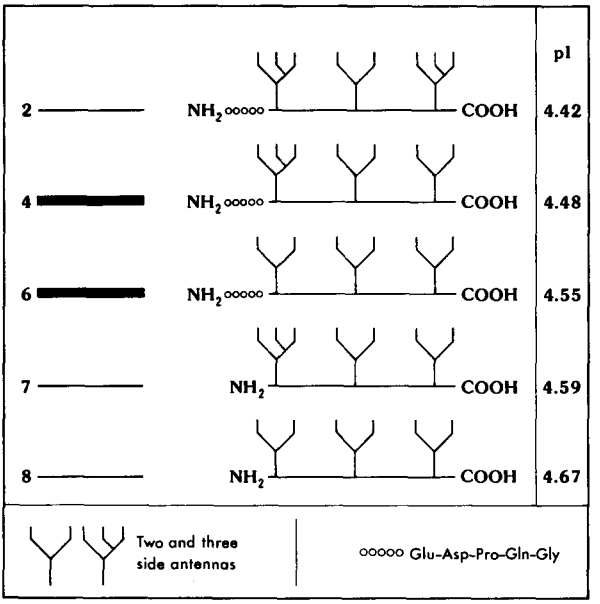


Fig. 4. A summary of the structural differences explaining the microheterogeneity of α_1-antitrypsin. The isoelectric points for the fractions M_2–M_8 are included. The attachments of the oligosacharide chains given for Asn in position 46, 83 and 246 are schematic.

Thus, a cleavage of a peptide bond Gly-Asp in the N-terminal part may be a post-ribosomal phenomenon. A corresponding N-terminal cleavage of the M_2 fraction willl give contamination of M_4, but this will be more difficult to evaluate because of its low concentration. Rate of synthesis and concentration in blood of M_2, M_4 and M_6 fractions are influenced by inflammatory response and by oestrogens[9]. The microheterogeneity of M_2–M_6 has earlier been shown to be predominantly due to variations in the branching degree of the three oligosaccharide chains attached to molecule[4]. Our results show that the pairs M_4–M_7 and M_6–M_8 have the same proportions of triantennary and biantennary oligosacharide chains, but we cannot conclude any difference in attachment to the polypeptide chain. Isoelectric focusing after total neuraminidase digestion of purified α_1AT resulted in one major fraction and two minor ones, one anodal and one cathodal[10]. The anodal one may represent a deamidation and the cathodal the asialo form of M_7 and M_8.

Rapid chromatofocusing is a prerequisite to obtaining a good yield of M_7 and M_8. Earlier systems took 4–10 h and sialic acid disappeared during a long residence at low pH causing an abnormal microheterogeneity. An important technical detail was the need to increase the ion strength during chromatofocusing in order to obtain a reasonable yield of isoforms.

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