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PROTEOLYTIC CLEAVAGES IN α_1-ANTITRYPSIN AND MICROHETEROGENEITY

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SUMMARY: Antitrypsin was resolved into two pools by ion-exchange chromatography. Pool 2 contained three anodal isoinhibitors and an N-terminal sequence identical with the one found by others. Pool 1 contained, in addition to the anodal ones two cathodal isoinhibitors as well. The sequencing data of Pool 1 indicate that the cathodal proteins are formed from the anodals by a cleavage of the Gly5-Asp6 bond in the molecule.

INTRODUCTION: In the human serum antitrypsin is present in the form of 6-8 immunologically identifiable isoproteins (1). It contains three Asn-linked complex oligosaccharides, with 6-7 mols of sialyl residues per mol protein. Individual isoproteins, obtained from human (2) and rat serum (3) antitrypsin were found to contain increasing numbers of sialyl residues with increasing acidity of the isoforms consistent with the view that the microheterogeneity of the protein would be due to variations in the sialylations of the isoforms. On the other hand, the microheterogeneity of this protein could not be abolished by desialylation (4,5). Further, in spite of the fact that avian glycoproteins do not contain sialyl residues, chicken antitrypsin is no less microheterogeneous than mammalian antitrypsins (6). In this communication, limited proteolytic cleavages are shown to contribute to the microheterogeneity of human serum antitrypsin.

METHODS: Preliminary purification of antitrypsin from blood bank plasma was carried out by precipitation with ammonium sulfate, followed by thiol-exchange chromatography (7,8). Proteins were sequenced in a Beckman Sequenator, Model 890C, modified with a cold trap and PTH derivatives

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were identified by HPLC on an Altex 5 µm ultrasphere-ODS. Isoelectric focussing in 5% polyacrylamide gel was carried out in an LKB Multiphore instrument. Sialic acid was measured according to the method of Warren (9).

RESULTS AND DISCUSSION: The partially purified protein was subjected to ion-exchange chromatography on a QAE-Sephadex column as illustrated in Figure 1 and the antitrypsin-containing fractions were pooled as indicated by brackets. Pool 1 and Pool 2 were both homogenous and indistinguishable from each other by SDS-PAGE (not shown).

Analytical isoelectric focussing of these proteins is illustrated in Figure 2. Of the 6 antitrypsin bands (indicated by dots) visible in the plasma (sample 4), only three anodal bands are present in appreciable quantity in Pool 2 (sample 3). In contrast, the Pool 1 protein (sample 2)

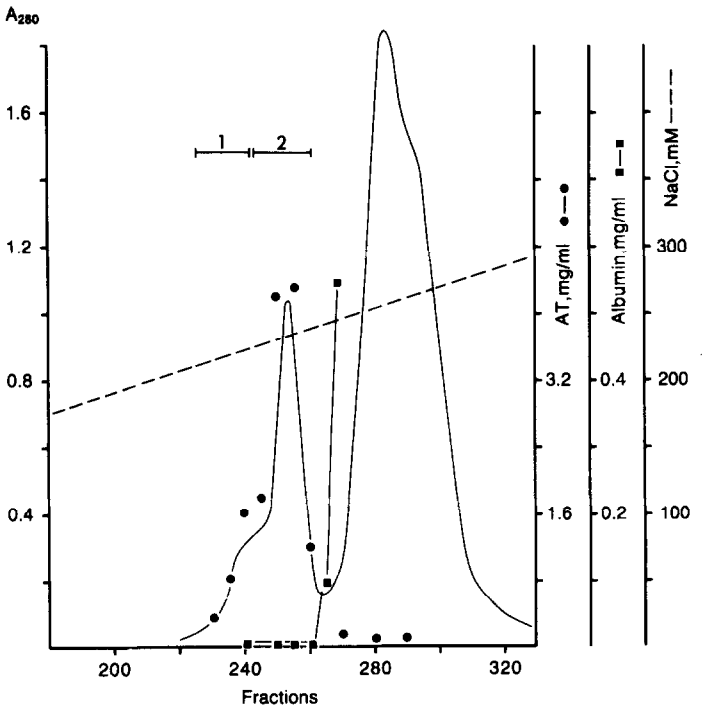

Figure 1: Ion-exchange chromatgraphy of partially purified alpha$_1$-antitrypsin on a column (2 cm x 40 cm) of QAE-Sephadex A-50 (13). Chromatography was carried out with 230 mg of antitrypsin in 230 ml of 50 mM Tris-HCl, pH 8.6, containing 100 mM NaCl and 0.05% Na-azide. After sample application, the column was washed with 560 ml of buffer and the elution was carried out with a linear salt gradient. The flow rate was approximately 40 ml/h and 7 ml fractions were collected. The fractions were pooled as indicated by brackets, dialyzed and then lyophilized. Forty-two mg antitrypsin was recovered from Pool 1 and 143 mg from Pool 2.

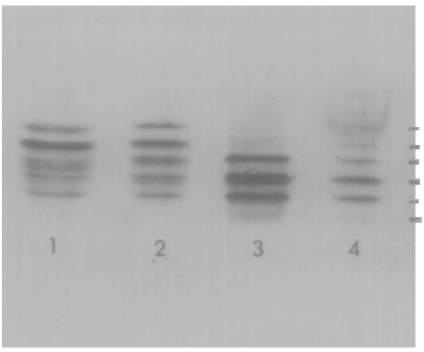

Figure 2: Analytical isolectric focussing (pH 4-6) of proteins in polyacrylamide gel. Sample 1: Pool 2 protein, after 12 weeks of storage at 4°C, 18 µg. Sample 2: Pool 1 from Figure 1, 22 µg (fresh solution). Sample 3: Pool 2 from Figure 1, 22 µg (fresh solution). Sample 4: supernate of human plasma, precipitated by 50% saturation with ammonium sulfate: 4 µl. Cathode is uppermost.

contains two additional cathodal bands, while its content of anodal isoproteins is diminished. Pools 1 and 2 contained 5.7 and 5.8 mols of sialyl residues per mol protein respectively.

The proteins were subjected to five consecutive cycles of sequencing, with results documented in Table 1. With the exception of cycle 4, the Pool 2 protein (column 2) yielded only a single residue in each cycle. The presence of glutamic acid, in addition to glutamine, in cycle 4 is most

Table 1

Cycle	Pool 1	Pool 2
1	a, Glu 2.34 b, Asp 4.96	Glu 4.80
2	a, Asp 4.07 b, Ala 3.64	Asp 5.90
3	a, Pro 1.99 b, Ala 3.05	Pro 2.90
4	Glu 2.30 a,b, Gln 3.05	Glu 1.80 Gln 2.40
5	a, Gly 2.20 b, Lys 3.01	Gly 3.60

Analysis of the amino acid sequences in Pool 1 (14 nmol) and Pool 2 (11 nmol) from Figure 1. The figures in the table are expressed in nmol-s.

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                          5                  10
A:  Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr ...
B:  Glu Asp Pro Gln Gly ...
C:                      Asp Ala Ala Gln Lys ...
D:  Glu Asp Pro Gln Gly ...
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Figure 3: Comparison of the amino-terminal sequences of preparations described in this report with that of alpha$_1$-antitrypsin. A: the amino-terminal sequence of antitrypsin (10). B: sequence a from Pool 1. C: sequence b from Pool 1. D: sequence from Pool 2.

likely due to an oxidative breakdown of the latter during sample handling. Apart from this, the sequence, listed in column 2, is in complete agreement with the known amino-terminal sequence of antitrypsin (10) (compare line D with line A, Fig. 3). In contrast to this, the Pool 1 protein (column 1) yielded two different residues - a and b - in each cycle. Of these, sequence a (line B, Fig. 3) was also identical with the N-terminal sequence of antitrypsin and sequence b, (line C, Fig. 3) with the sequence Asp6...Lys10 in antitrypsin. Since sequence a belongs to the isoproteins in Pool 2 (sample 3, Fig. 2), sequence b has to be assigned to the cathodal isoproteins in Pool 1 (sample 2, Fig. 2).

The absence of the N-terminal pentapeptide from the cathodal isoproteins could be due to either a biosynthetic variation or to a postsynthetic proteolytic cleavage of the bond Gly5-Asp6. The observation in Figure 2, showing that two cathodal bands were formed from anodals during extended storage of Pool 2 protein supports the second alternative (compare sample 1 with sample 3).

These results show that two anodal isoproteins are formed from cathodal proteins by proteolytic action. Since the missing pentapeptide contains two negative residues (Glu1 and Asp2), the cleavage leaves the isoproteins with an increased positive charge, hence increased cathodal mobility (Fig. 2) and diminished binding to the ion-exchange column (Fig. 1).

Recently Lobermann et al described the cleavage of the Thr11-Asp12 bond in antitrypsin by minute amounts of a firmly bound serum protease (11), but they did not attempt to correlate the cleavage with microheterogeneity. Although the cleavage in this paper is different from the former, in all

probability it would be due to the same or similar serum protease. The involvement of bacterial proteases in these events can be ruled out with near certainty, as all purification steps were carried out in the presence of 0.05% sodium azide, and bacterial growth in these samples was not in evidence after more than three months of standing. Further, bacterial proteases are unlikely to produce isoproteins, recognizable in human plasma.

One has to give consideration to the possibility that this cleavage affects not only the polymorphic expression of antitrypsin, but its inhibiting function as well. Finally, cleavages may contribute to the polymorphism of other proteins as well. Indirectly this is suggested by the finding that the microheterogenity of alpha-fetoprotein could not be abolished by desialylation (12).

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