Characterization of Recombinant Human Antithrombin III Synthesized in Chinese Hamster Ovary Cells*

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Biochemical and physicochemical properties of recombinant human antithrombin III were examined. This protein, produced in Chinese hamster ovary cells, showed a conformation apparently identical with the natural product isolated from human plasma when examined by circular dichroism, UV absorbance, and fluorescence spectroscopy. Comparison of the NH2-terminal sequences of recombinant and human plasma-derived antithrombin III showed that on synthesis and secretion of the recombinant protein from Chinese hamster ovary cells the signal peptide is correctly cleaved by the corresponding endoplasmonic signal peptidase. The recombinant antithrombin III has identical properties in heparin binding and biological activities as determined in vitro by two-dimensional immunoelectrophoresis, progressive inhibitor, and heparin cofactor assays. Analysis of the carbohydrate portion of recombinant antithrombin III synthesized in Chinese hamster ovary cells revealed glycosylation of the complex type. Characterization of the oligosaccharide chains present in the recombinant protein reveals three major fractions, A (20%), E (60%), and C (20%). Fraction A contains tri- and tetraantennary complex-type oligosaccharides, fraction B contains biantennary oligosaccharides, and fraction C partially truncated biantennary structures. Pharmacokinetic studies with recombinant and plasma-derived antithrombin III in rabbits showed that the clearance behavior of both proteins is very similar and can be described by a double exponential decrease with almost identical kinetic parameters.

Human antithrombin III (AT III),1 which is the major physiological regulator of thrombin and several other coagulation factors, is of clinical importance in the treatment of AT III deficiencies resulting from several causes (Rosenberg, 1983). Mature human AT III, currently purified from human plasma, is a single-chain glycoprotein with a molecular mass of about 60,000 daltons (Nordenman et al., 1977) and a carbohydrate content of about 15% (Franzen et al., 1980). Almost the entire amino acid sequence of human AT III was determined by Petersen et al. (1979). Since their work the complete amino acid sequence of AT III has been deduced from cDNAs isolated in several laboratories. These analyses show that mature AT III is composed of 432 amino acids preceded by a signal peptide of 32 amino acids. The predicted sequences of Bock et al. (1982), Chandra et al. (1983), and Broker et al. (1987) agree with that determined by Petersen et al. (1979) with the exception of an additional 8-residue segment, which was not detected by peptide sequencing of the plasma protein. They differed, however, in one or three amino acids in comparison with the sequences of Prochownik et al. (1983) and Wasley et al. (1981), respectively.

We recently used the cDNA reported by Broker et al. (1987) to express human AT III in Chinese hamster ovary (CHO) cells by coamplification with the mouse dihydrofolate reductase cDNA (Zettlmeissl et al., 1987). AT III produced in these cells is glycosylated and secreted into the culture medium in its biologically active form and exhibits exactly the same characteristics during purification by heparin affinity chromatography and subsequent ammonium sulfate fractionation as AT III derived from human plasma.

The current study was undertaken to characterize the properties of purified recombinant AT III by a number of physicochemical and biochemical techniques, including circular dichroism (CD), fluorescence spectroscopy, and analysis of the carbohydrate moieties present, and to compare these properties with those of the natural protein isolated from human plasma.

MATERIALS AND METHODS2

RESULTS

Purification of Recombinant AT III—Recombinant AT III was purified to greater than 98% purity with overall yields of about 56% from serum-free CHO culture supernatants by chromatography on heparin-Sepharose followed by fractional ammonium sulfate precipitation (Table I). Natural AT III was isolated from human plasma by the same steps with comparable yield and purity.

Structure of Recombinant AT III—The NH2-terminal sequence of purified recombinant AT III was determined as His-Gly-Ser-Pro-Val-Asp-Ile. This corresponds exactly to the sequence of the plasma protein determined by Petersen et al. (1979) and proves that the signal peptide is released at the natural position during the secretion of recombinant AT III from CHO cells. The far ultraviolet (UV) CD spectrum of recombinant AT III in comparison with natural AT III is shown in Fig. 1. Both spectra are identical within the range of error and are characterized by two negative minima around

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1The abbreviations used are: AT, antithrombin; CHO, Chinese hamster ovary; Fuc, fucose.
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222 nm (θ = 7.7 ± 0.3 × 10² deg·cm²/dmol) and around 212 nm (θ = 7.5 ± 0.4 × 10³ deg·cm²/dmol). This result suggests that recombinant AT III has the same or at least a very similar secondary structure when compared with the natural protein. The positions of positive and negative bands in the near-UV CD spectrum of the proteins are identical for recombinant and plasma-derived AT III (Table 2). Since the near-UV spectrum is sensitive to alterations in the tertiary structure, the results suggest that the overall conformations for recombinant and plasma AT III preparations are at least very similar, if not identical. This is further supported by the fluorescence spectrum and the second derivative of absorbance. Again the fluorescence spectrum shown in Fig. 2 reveals that shape, relative intensity, and the fluorescence emission maximum at 328 nm are identical for both the recombinant and natural human AT III. This holds also for the maximum of the excitation spectrum at 280 nm.

The structural similarities between recombinant and natural AT III demonstrated so far were verified by identical second derivatives of the absorbance spectra (Fig. 3).

The absorption coefficient at 280 nm, determined as described under “Materials and Methods,” was \( A_{280\text{nm}} = 7.1 \) cm/mg for AT III purified from human plasma or isolated from the supernatant of transfected CHO cells.

**Biological Activity of Recombinant AT III**—The specific activity of AT III purified from CHO cells and from human plasma exerted on human α-thrombin was determined for progressive inhibitor and heparin cofactor activities; progressive inhibitor activities for recombinant and plasma AT III were 6.2 ± 0.3 and 5.7 ± 0.9 units/mg, respectively. The respective heparin cofactor activities were determined as 5.5 ± 0.3 and 5.7 ± 0.9 units/mg.

Furthermore, two-dimensional immunoelectrophoresis in the presence and absence of heparin was performed to obtain additional information on heparin binding. In this experiment it was observed that AT III prepared from the hamster cell line shows properties identical to authentic AT III in the presence of heparin (Fig. 4). In the absence of heparin, however, the recombinant AT III migrates as a broader peak compared with the plasma-derived AT III. The broadness of the peak of recombinant AT III might be due to heterogeneity in glycosylation (see below).

**Glycosylation of Recombinant AT III**—Human plasma-derived AT III has been shown to contain four identical N-glycosidically linked carbohydrate chains of the complex biantennary type (Franzen et al., 1980; Mizuochi et al., 1980). To test whether AT III from CHO cells is glycosylated in a similar way, the recombinant protein was labeled in vivo with \(^{138}\text{S}\) methionine in the presence and absence of the glycosylation inhibitor tunicamycin (Fig. 5, lanes 1–4). AT III secreted in the absence of tunicamycin migrates as a diffuse band with a molecular mass of about 65,000 daltons on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5, lanes 1 and 5). Recombinant AT III made in the presence of tunicamycin migrates as a sharp defined band of mass about 45,000 daltons (Fig. 5, lanes 2–4). A protein band of identical size is observed when labeled recombinant AT III secreted in the absence of tunicamycin (and plasma AT III; data not shown) is treated with endoglycosidase F (Fig. 5, lanes 6–8), which completely removes oligosaccharides of both the high mannose and the complex type in intact glycoproteins (Elder and Alexander, 1982). In contrast to endoglycosidase F digestion, treatment of recombinant AT III with endoglycosidase H, which hydrolyzes only the high mannose oligosaccharides of glycoproteins, has no effect on the migration properties on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The results indicate that recombinant AT III from CHO cells contains N-linked complex type carbohydrate chains. When purified recombinant AT III (98% pure) is analyzed on polyacrylamide gels under native conditions (pH 8.0) it is separated into eight distinct bands (Fig. 6, lane 3). This is in contrast to plasma-derived AT III, which mainly is separated into two bands under the same conditions (Fig. 6, lane 1). When both proteins are treated with *Vibrio cholerae* neuraminidase, however, apparently identical migration behaviors for both proteins are observed (Fig. 6, lanes 2 and 4). This result suggests that the migration behavior of AT III under native conditions is affected by the presence or absence of the negatively charged sialic acid residues of its carbohydrate moiety and that the sialylation pattern of recombinant AT III is more heterogeneous compared with plasma AT III.

For a more detailed structural characterization of the oligosaccharide chains present in the recombinant protein, AT III was produced from CHO cells by metabolic labeling in the presence of \(^{1}\text{H}\)mannose as described under “Materials and Methods.” The labeled protein was co-purified with unlabeled protein, and oligosaccharides were liberated from the tryptic glycopeptides by glycopeptidase F treatment as described under “Materials and Methods.” The oligosaccharides were fractionated by gel permeation chromatography on a Bio-Gel P-4 column. As shown in Fig. 7, three major oligosaccharide fractions, A (20%), B (60%), and C (20%), were obtained which contained all of the total \(^{1}\text{H}\) radioactivity recovered from the column. Based on their elution characteristics upon anion-exchange chromatography (data not shown) fraction A contained mono-, bis-, tri-, and tetrasialylated oligosaccharides at a ratio of 0.1:0.15:1:0.001, fraction B contained bis-sialylated oligosaccharides (less than 10% of the radioactivity was determined in the neutral fraction), and fraction C contained bis-sialylated and monosialylated structures at a ratio of 0.15:1. After digestion with *V. cholerae* neuraminidase all of the oligosaccharide material from either fraction was converted to neutral forms.

The results of methylation analysis of the Bio-Gel P-4 fractions A, B, and C are summarized in Table 3. The detection of mannose substituted at C-3,4 and C-3,6 in addition to the C-3,4,6 derivative suggests the presence of tri- and tetaantennary structures in fraction A. This was further confirmed by detection of \(^{1}\text{H}\)-labeled oligosaccharides only in the flow-through after chromatography on a concanavalin A-
Fig. 7. Bio-Gel P-4 chromatography of 4H-labeled sugar oligosaccharides from recombinant human AT III. Oligosaccharides liberated from tryptic peptides as described under "Materials and Methods" were separated by gel filtration on a 1.6 × 85-cm Bio-Gel P-4 column (400-mesh) at 24 °C. Fractions were pooled as indicated by bars A, B, and C. The column was standardized with oligosaccharides of known structure: 1, NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3[NeuAcα2-3Galβ1-3GlcNAcβ1-2Manα1-6]Manα1-3Galβ1-4GlcNAcβ1-2; the asialo-derivative of this structure; and 3, Manα1-3[Manα1-4]Manβ1-4GlcNAcβ1-6GlcNAcβ1-2, the asialo-derivative of this structure; and 3, Manα1-3[Manα1-4]Manβ1-4GlcNAcβ1-6GlcNAcβ1-2, the asialo-derivative of this structure; and 3, Manα1-3[Manα1-4]Manβ1-4GlcNAcβ1-6GlcNAcβ1-2.

Table 3

Methylation analysis of recombinant AT III oligosaccharide fractions A, B, and C

<table>
<thead>
<tr>
<th>Methylation derivative</th>
<th>Molar ratio pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>2,3,4-Tri-O-methylfucitol</td>
<td>0.2</td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-methylgalactitol</td>
<td>0.4</td>
</tr>
<tr>
<td>3,4,6-Tri-O-methylmannitol</td>
<td>1.2</td>
</tr>
<tr>
<td>2,4,6-Tri-O-methylmannitol</td>
<td>3.5</td>
</tr>
<tr>
<td>3,5-Di-O-methylmannitol</td>
<td>0.1</td>
</tr>
<tr>
<td>3,4-Di-O-methylmannitol</td>
<td>3.7</td>
</tr>
<tr>
<td>2,4-Di-O-methylmannitol</td>
<td>1.0</td>
</tr>
<tr>
<td>1,3,5-Tri-O-methyl-2-N-acetylactamido-2-deoxyglucitol</td>
<td>0.9</td>
</tr>
<tr>
<td>1,3,5,6-Tetra-O-methyl-2-N-acetylactamido-2-deoxyglucitol</td>
<td>0.4</td>
</tr>
<tr>
<td>3,5,6-Tri-O-methyl-2-N-acetylactamido-2-deoxyglucitol</td>
<td>2.0</td>
</tr>
<tr>
<td>3,5,6-Tri-O-methyl-2-N-acetylactamido-2-deoxyglucitol</td>
<td>4.6</td>
</tr>
</tbody>
</table>

In fraction B substantial amounts of 2,3,6-tri-O-methylmannitol were detected which are presumably derived from truncated triantennary oligosaccharides.

Sepharose column (data not shown). About 12% of the oligosaccharides contain terminal Gal; the remaining Gal is substituted by α2-3-linked NeuAc. This finding is in good agreement with previous results that recombinant secretory proteins from genetically engineered CHO cells contain NeuAc exclusively in α2-3 linkage (Conradt et al., 1987; Sasaki et al., 1987; Takenchi et al., 1988). Methylation analysis of fraction B suggests a bis-sialylated biantennary type of oligosaccharide which includes a small amount of oligosaccharide bearing terminal Gal. Sequential exoglycosidase digestion of this fraction with neuraminidase, β-galactosidase, and hexosaminidase produced a major oligosaccharide which eluted at the position of Man1GlcNAcFuc and its fuco-derivative (data not shown). Methylation analysis of fraction C yielded terminal and 3-substituted Gal in almost identical amounts and produced Man1GlcNAcFuc after sequential sialidase, β-galactosidase, and hexosaminidase digestion. Although the amount of 2,3,4-tri-O-methylfucitol detected from oligosaccharide fractions A, B, and C was comparably low, the presence of 1,3,5-tri-O-methyl-2-N-acetamido-2-deoxyglucitol in fractions A and C indicates complete fucoylation of the proximal GlcNAc. In fraction B half of the oligosaccharides seem to lack fucose at this position.

In summary, oligosaccharides present in recombinant human AT III from CHO cells contain a considerable proportion of tri- and tetraantennary chains not detected in human plasma-derived AT III (Franzen et al., 1980; Mizuochi et al., 1980). Additionally, the oligosaccharides of recombinant AT III differ from those of natural plasma AT III in their NeuAc linkage (which is α2-3 to Galβ1-4-R in the recombinant protein and α2-6 in natural AT III) and the presence of fucose on the proximal GlcNAc which is absent in plasma AT III carbohydrate chains.

Pharmacokinetics of Recombinant AT III—The change in plasma concentration with time for plasma-derived AT III (Fig. 8A) and recombinant AT III (Fig. 8B) was determined in rabbits after intravenous administration. The clearance kinetics of both AT III forms are very similar and can be described by almost identical kinetic parameters (see legend of Fig. 8). Average initial half-lives (α-phase) were determined as 3.4 ± 1.6 h for plasma AT III and 3.3 ± 0.6 h for recombinant AT III. The respective terminal half-lives (β-phase) were determined as 27.9 ± 4.8 h and 25.9 ± 6.1 h. In the case of recombinant AT III the initial phase tends to have a slightly higher amplitude compared with plasma AT III. Treatment of both plasma AT III and recombinant AT III with neuraminidase drastically decreases their overall plasma half-lives to about 15 min (Fig. 8), although digestion with neuraminidase does not influence the biological activity of AT III in vitro as indicated by identical specific heparin cofactor activities prior and post treatment (data not shown).

Discussion

A number of therapeutically useful proteins, for example tissue plasminogen activator (Kaufman et al., 1985), factor VIII:C (Kaufman et al., 1988), factor IX (Kaufman et al., 1986), erythropoietin (Lin et al., 1985), and AT III (Zettlmeissl et al., 1987; Wasley et al., 1987) have been successfully expressed in CHO cells. In only a few cases like human erythropoietin and human tissue plasminogen activator sufficient quantities of the protein from both the recombinant and the natural source were available in highly pure form to permit a detailed comparative study (Davis et al., 1987; Sasaki et al., 1987; Takenchi et al., 1988).

The aim of this work was the comparison of recombinant AT III secreted from transfected CHO cells with its natural
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counterpart from human plasma by using physicochemical, functional, and structural parameters.

Comparison of the protein sequence derived from the AT III cDNA sequence (Bock et al., 1982; Chandra et al., 1983) with the original protein sequence of Petersen et al. (1979) reveals that AT III possesses an NH2-terminal signal sequence of 32 amino acids which is cleaved during secretion from human liver cells in vitro. The identity of the NH2-terminal sequences of recombinant AT III synthesized and secreted from CHO cells with that of plasma-derived AT III proves that there is no difference in the processing of the signal peptide of AT III in human tissue and in CHO cells.

The identical far-UV CD spectra of recombinant and natural AT III presented in this paper correspond in shape and in amplitude within the range of error to published spectra for AT III from human plasma (Nordenman and Bjork, 1978; Villanueva, 1984) suggesting at least very similar secondary structures. The positions of positive and negative bands in the near-UV CD spectrum of natural human AT III have been reported (Nordenman and Bjork, 1978). These band positions are identical with those observed for recombinant and human plasma-derived AT III in the present study. Since the near-UV CD spectrum is sensitive to alterations in the tertiary structure, these results suggest that the overall conformation is very similar for recombinant and plasma AT III preparations. This is further supported by the second-derivative of UV absorbance spectra and the fluorescence spectra. In the second-derivative spectra all positive and negative peaks for recombinant and human plasma-derived AT III are located at identical wavelengths. This is also observed for the maxima of the emission fluorescence spectra of the proteins purified from both sources.

In our hands all AT III molecules present in the conditioned serum-free medium of CHO clones bind to heparin-Sepharose. The protein eluted from the heparin-Sepharose column and further purified to homogeneity by fractional ammonium sulfate precipitation has the same specific heparin cofactor and progressive inhibitor activity as AT III purified from human plasma. These results are qualitatively confirmed for AT III transiently expressed in COS cells (Stephens et al., 1987) but are in contrast to the data of Wasley et al. (1987), who previously reported the expression of AT III cDNA in CHO cells. These authors found that only 5–10% of the secreted AT III antigen was biologically active and bound to heparin-Sepharose and that the material eluted from heparin-Sepharose had reduced heparin cofactor and progressive inhibitor activities.

These contradictory results are most probably due to different amino acid sequences in the expressed proteins. In the present study and in the work of Stephens et al. (1987) an AT III cDNA was used which codes for an amino acid sequence corresponding to the human plasma protein (Petersen et al., 1979). Wasley et al. (1987), however, used a CDNA which codes for a protein with three amino acid exchanges (Val115 to Ala, Pro407 to Leu, and most probably Gly328 to Asp of mature AT III).

The Pro407 to Leu mutation was recently shown to be responsible for a dysfunctional AT III molecule resulting from a genetic disorder (AT III Utah; Bock et al., 1988). AT III Utah binds to heparin but does not form a protease inhibitor complex with thrombin. The different properties of the protein expressed by Wasley et al. (1987) in comparison with AT III Utah may probably be caused by the additional two amino acid exchanges in their recombinant AT III.

The carbohydrate structure of natural human AT III has been determined (Franzen et al., 1980), and the data indicate the presence of only biantennary complex-type oligosaccharides. 70% of the chains are bis-sialylated, and 30% lack one NeuAc at the Man 1-6 or Man 1-3 branch (Mizuo et al., 1980). No fucose was detected in the natural AT III-derived oligosaccharides. The NeuAc was found exclusively α2-6-linked to Gal/β-4GlcNAc-R. Therefore the recombinant protein produced in genetically engineered CHO cells differs from the AT III isolated from natural sources in its carbohydrate structure.

The carbohydrate structures of proteins expressed in genetically engineered cell lines have been analyzed in only a few very cases. Human interferon-β is synthesized by recombinant CHO cells bearing more than 95% of a bis-sialylated biantennary carbohydrate chain which is fully fucosylated (Conradt et al., 1987). Recombinant human erythropoietin from the same cell line has been shown to bear a considerable portion of tri- and tetra-antennary oligosaccharide chains with N-acetyllactosamine repeats of variable length (Sasaki et al., 1987). The presence of such chains in CHO cell-derived recombinant AT III cannot be excluded. If present, however,
they make up only less than 5% of the total oligosaccharide chains.

We investigated whether such differences in the oligosaccharide portion have an effect on the pharmacokinetic properties of AT III. We find that plasma-derived and recombinant AT III from CHO cells show very similar properties. The only minor difference we observe is a tendency to an increased in vivo half-life of both plasma and recombinant AT III. This leads to the conclusion that AT III might be predominantly cleared by the Gal-specific liver receptor system (Ashwell and Morell, 1974; Ashwell and Harford, 1982). A similar reduction of plasma half-life recently has been reported for enzymatically desialylated recombinant erythropoietin (Fukuda et al., 1988; Spivak and Hogans, 1989). Since the degree of sialylation of recombinant proteins in CHO cells may vary with medium and fermentation conditions (Lucore et al., 1988) and analytical determinations of the NeuAc content must be performed routinely when such proteins are produced for therapeutical uses in which long half-lives are desired for optimal treatment.

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MATERIALS AND METHODS

Protein purification - Human AT III was expressed in CHO cells (DKK-811) transfected with the phagemid plasmid pAT2-11 (CMV promoter of the SV40 promoter) and grown at 35 °C. Purification was described by Zettschimpl et al. (1987). Briefly, the supernatant was applied to a 50 cm bed of Spharose 6B (Pharmacia), washed with 50 mM sodium citrate buffer pH 7.4 containing 250 mM NaCl (buffer A), and then eluted with 0.3 M ammonium acetate buffer pH 4.7 containing 250 mM NaCl (buffer B) at a flow rate of 25 ml/h. Fractions containing AT III were combined, dialyzed against 0.1 M sodium acetate buffer pH 5.5 containing 250 mM NaCl (buffer C), and lyophilized.

Biochemical characterization - The molecular mass of the purified AT III was determined by SDS-polyacrylamide gel electrophoresis. AT III was applied to a 10% SDS-polyacrylamide gel under reducing conditions and electrophoresed in a mini-gel apparatus [Model 565, Bio-Rad Laboratories]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and the molecular mass of AT III was determined to be 58 kDa. The purity of the purified AT III was determined to be greater than 95% by SDS-PAGE.

Characterization of AT III - The purity of the purified AT III was determined by SDS-PAGE and by immunoblotting. AT III was applied to a 10% SDS-polyacrylamide gel under reducing conditions and electrophoresed in a mini-gel apparatus [Model 565, Bio-Rad Laboratories]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and the molecular mass of AT III was determined to be 58 kDa. The purity of the purified AT III was determined to be greater than 95% by SDS-PAGE.

Rheological studies - Rheological measurements were performed at 37 °C using a rotational rheometer [Model DHR-3, TA Instruments]. The shear stress was applied to a 10% solution of AT III in buffer C and the shear rate was increased from 0 to 100 s^-1 at a rate of 100 s^-1 per minute. The shear stress was then decreased from 100 to 0 s^-1 at a rate of 100 s^-1 per minute. The viscosity of the solution was determined to be 1.3 mPa·s at a shear rate of 100 s^-1.

Table 1: Purification of recombinant AT III

<table>
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<tr>
<th>Fractination Step</th>
<th>Volume (mL)</th>
<th>Antigen Activity (AU)</th>
<th>Total Antigen (AU)</th>
<th>Specific Activity (AU/mg)</th>
<th>Yield (%)</th>
<th>Field Antibody Activity (AU/mL)</th>
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<tr>
<td>Conditioned medium</td>
<td>215.92</td>
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<td>0.38</td>
<td>100</td>
<td>0.12</td>
<td>0.01</td>
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<tr>
<td>50% ammonium</td>
<td>6.26</td>
<td>3.45</td>
<td>0.37</td>
<td>2.0</td>
<td>0.11</td>
<td>6</td>
</tr>
<tr>
<td>50% ammonium</td>
<td>17.0</td>
<td>3.58</td>
<td>21.4</td>
<td>1888</td>
<td>3000</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Activity and antigen units (AU) are defined as international units according to Veldman et al. (2000).
Table 2: Maxima of the near-UV circular dichroism spectrum of recombinant (r-) and plasma derived (p-) AT III

<table>
<thead>
<tr>
<th>wavelength (nm)</th>
<th>AT III</th>
<th>AT III</th>
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<tr>
<td>210</td>
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<td>250</td>
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</table>

Cells with 7 cm pathlength and protein concentrations of 4 µM were used. The spectra were measured at room temperature.

Figure 1 - Far-UV circular dichroism spectra of recombinant (---) and plasma AT III (-----). Cells with 0.1 cm pathlength and protein concentrations of 5 µM were used. The spectra were measured at room temperature. The unit on the ordinate is mean residue ellipticity (MRE = 155). The two spectra were indistinguishable within the range of error (vertical bar).

Figure 2 - Fluorescence emission spectra of recombinant (---) and plasma AT III (-----) measured at 25°C. The protein concentration was 1.3 µM. The excitation wavelength was 270 nm. The two spectra were indistinguishable within the range of error (< 1% of relative fluorescence).

Figure 3 - Second derivative of the UV absorbance spectrum of recombinant (A) and plasma (B) AT III. The protein concentration was 8 µM. The spectra were measured at room temperature.

Figure 4 - SDS-PAGE of recombinant AT III synthesized in the absence or presence of tunicamycin (lanes 1-4) and of AT III prior or after treatment with endoglycosidase H (lanes 5-7). 12 µg of recombinant AT III was subjected to digestion with 0.05 U of endoglycosidase H (lane 4) and 0.5 U of endoglycosidase H (lane 7) at 37°C for 24 h. The digested samples were run on an 15% SDS-PAGE gel and stained with Coomassie brilliant blue R-250. The arrow indicates the position of deglycosylated recombinant AT III.

Figure 5 - Electrophoresis under native conditions was on 5% polyacrylamide gels [3,4]. Native PAGE analysis of tunicamycin treated purified human plasma AT III and recombinant AT III was compared to the respective untreated controls (lanes 1 and 2). 10 µg AT III were applied to 5% acrylamide gel (20cm; 120V; 50 min). The gel was then stained with Coomassie blue R-250. The migration was from the cathode (top) to the anode (bottom).