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Control of the cultivation process of antithrombin III and its characterization by capillary electrophoresis

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Abstract

The production by baby hamster kidney cells of recombinant antithrombin III (r-AT III), the main inhibitor of thrombin, factor Xa and other proteases of the clotting cascade, was monitored by capillary isotachophoresis using mixtures of continuous spacers. The results were compared with those obtained by capillary zone electrophoresis (CZE). The downstream process, which incorporated anion-exchange and heparin affinity chromatography, was monitored by CZE under acidic conditions and voltage ramping. The purified product was characterized by its isoelectric point and molecular mass. Isoelectric points of the three major and three minor isoforms of AT III were evaluated by capillary isoelectric focusing using a pH range of 4–6 and various mobilization procedures. The molecular mass of AT III was investigated by capillary gel electrophoresis (CGE), applying removable dextran gels. Both parameters could be determined within 30 min using only one coated capillary. The results showed an excellent correspondence with those achieved with conventional slab gels. The affinity complex between AT III and thrombin could also be detected by CGE and the heparin dependence of the affinity reaction could be investigated.

1. Introduction

Antithrombin III (AT III) is one of several pharmaceutically relevant proteins that are produced biotechnologically. Analysis of such proteins produced by recombinant mammalian cells requires the use of the most powerful tools of analytical protein chemistry to ferret out any contaminants [1]. A detailed knowledge of the protein of interest in all phases of the production by the recombinant organisms and its downstream processing to the purified product is essential for the whole process. Process development, production monitoring, quality control and regulatory submissions calling for the rapid and reliable analysis of the product intended as a medical drug are the most important aspects [2]. Biosynthetic proteins for pharmaceutical applications have to be highly purified and the main analytical methods to confirm the purity and the protein characterization rely on high-performance liquid chromatography (HPLC) [3], electrophoresis and immunological techniques [4,5].

The recent advances in capillary electrophoresis (CE) offer an instrumental technique capable of yielding remarkable information in a variety of applications, especially in the analysis of peptides and proteins. CE combines the quantification and handling benefits of HPLC with the separating power of conventional electrophoretic techniques [6,7]. With its simple

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automated instrumentation and analysis times up to 20 min, CE is an obvious choice as a potential on-line analysis technique for monitoring the bioproduction of pharmaceutical proteins and peptides. Further, CE offers the advantage of reducing the sample preparation to simple centrifugation, instead of filtration employed for column protection in HPLC. The variation of the analytical conditions such as buffers, additives or gels in CE putatively yields a wide range of information about the sample. With regard to this, CE offers an advantage over HPLC, as it is easier to change methods in CE than in HPLC.

So far, only a few groups have reported the application of CE for monitoring the cultivation, the downstream process and the characterization of recombinant pharmaceutical proteins. Using CE, the protease savinase was determined in culture fluids by Vinther et al. [8], while the purification of hirudin was controlled at different stages of the purification process by Paulus and Grassmann [9]. Recombinant human growth hormone (hGH) was characterized by Frenz et al. [10], Banke et al. [11] analysed cultures of *Aspergillus* and Hurni and Miller [12] used CE for the monitoring of a recombinant hepatitis B vaccine expressed in *Saccharomyces cerevisiae*. The humanized monoclonal antibody anti-TAC was characterized [2] and the effect of the temperature of recombinant interleucin-1α was investigated by Guzman et al. [13]. The human recombinant tissue plasminogen activator (rt-PA) was characterized and its glycoforms were determined according to their isoelectric points by Yim [14]. Recombinant bovine somatotropin (r-bSt) was characterized by capillary gel electrophoresis (CGE) by Tsuji [15]. Further, the purification of r-DNA proteins, such as monoclonal antibodies against the gp-41 of the AIDS virus and of human recombinant superoxide dismutase, was monitored by Wenisch et al. [16] using capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF).

Human antithrombin III (h-AT III) is a well characterized, therapeutically important single-chain glycoprotein [17,18], which inhibits serine proteases such as factor IXa, Xa and XIa and thrombin [19], of which the last is its principal physiological target. Heparin was found to enhance the rate of inactivation of thrombin by AT III [20]. Based on the data already available on human AT III, the capabilities of CE for the analysis of recombinant antithrombin III (r-AT III) were evaluated in this work. Capillary isotachophoresis (cITP) and CZE were applied for monitoring the cultivation and the downstream process. The isoelectric points and the molecular mass of the final purified product were determined by cIEF and CGE. Finally, the affinity of the purified r-AT III to thrombin and the heparin dependence of the complex formation kinetics were investigated.

2. Experimental

2.1. Instrumentation

CE was performed on a Beckmann P/ACE 2210 instrument. For data collection, data analysis and system control the System Gold software from Beckman (Palo Alto, USA) on a PS2 computer (IBM) was applied. Detection was by UV absorbance (200, 254 and 280 nm). Capillaries were supplied from CS-Chromatographie Service (Langerwehe, Germany). If necessary the capillaries were coated with 6% linear polyacrylamide (PAA) according to Hjertén and Kiesling-Johansson [21]. In some specially indicated experiments, the dextran coating proposed by Hjertén and Kubo [22] was used instead. The liquid chromatography was preformed on a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala Sweden), using a Protein G column (HiTrap, Pharmacia, Sweden). Slab gel electrophoresis and isoelectric focusing were performed in a Sartophor System from Sartorius (Göttingen, Germany).

2.2. Materials

AT III was produced and excreted into the culture supernatant by baby hamster kidney (BHK) cells. Culture supernatants were kindly donated by members of the Cell Culture Technology Group, Institut für Technische Chemie,

University of Hannover, Hannover, Germany. Unless indicated otherwise, the culture media contained 10% foetal calf serum (FCS). Precise cultivation conditions are given elsewhere [23]. The cultivation samples were stored at −80°C after cell harvest. Water was purified with a Milli-Q system from Millipore (Bedford, MA, USA). All buffer chemicals, methanol, ethanol and Triton X-100 were obtained from Fluka. Dextran (M_r $2 \cdot 10^6$), hydroxypropylmethylcellulose (HPMC), ammediol, tris(hydroxymethyl)aminomethane (Tris), 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylethylenediamine (TEMED) and all protein standards, thrombin and heparin, were purchased from Sigma (St. Louis, MO, USA). All substances were of analytical-reagent grade and used without further purification. Ampholine 4/6 and Pharmalytes 3/10, 2.5/5 and 4/6.5 were obtained from Pharmacia (Freiburg, Germany), precast gels from Serva (Heidelberg, Germany) and the silver stain kit from Bio-Rad (Munich, Germany).

2.3. Methods

Cultivation analysis

For cITP 500 μl of the cultivation supernatant were treated with cold methanol (1:5, v/v), stored at −80°C for 30 min. and centrifuged at 15 000 *g*. The supernatant was discarded and the pellet was resuspended in the sample buffer (Pharmalyte 3/10, 2.5/5, 4/6.5 and leading electrolyte in the ratio 1:2:2:15, v/v). The leading electrolyte consisted of 10 m*M* HCl–ammediol and 0.1% HPMC. The terminating electrolyte was 10 m*M* β-alanine–$Ba(OH)_2$ (pH 9.0). The sample was injected by pressure for 5 s. The dimensions of the PAA-coated capillary were 27 cm length (20 cm to detector) × 50 μm I.D. The applied voltage was 20 kV at 25°C. UV detection was performed at 254 nm.

For CZE the pellet was resuspended in equilibration buffer for protein G affinity chromatography (25 m*M* phosphate, pH 5.5) and loaded on a protein G column. The breakthrough, containing all proteins except the immunoglobulins, was collected and analysed by CZE in an uncoated capillary [27 cm length (20 cm to the detector) ×50 μm I.D.]. Electrophoresis was performed using 50 m*M* phosphate buffer (pH 2.0)–0.1% HPMC. The sample was injected by applying pressure for 5 s. The applied voltage was 10 kV at 20°C. The components were detected at 200 nm.

Downstream process analysis

The cultivation supernatant was dialysed for 10 min (membrane cut-off M_r 12 000) and further purified using ion-exchange and heparin affinity membrane adsorber (MA) chromatography as described previously [24]. After each step of the purification, 500 μl of the product containing fraction were removed. The sample was analysed by CZE under conditions identical with those described above except for the application of a voltage ramp (5 to 15 kV in 10 min).

Characterization

For CGE the sample was dissolved 1:1 (v/v) in 50 m*M* Tris–CHES buffer (pH 8.6)–1% SDS and heated at 90°C for 5 min. The standard proteins pepsin (porcine stomach mucosa; M_r 34 700), ovalbumin (M_r 45 000), bovine serum albumin (M_r 69 000) and phosphorylase b (rabbit muscle; subunit M_r 97 400) were dissolved in the same buffer containing an additional 5% of 2-mercaptoethanol. The sample was injected by electromigration at 10 kV for 10 s. The electrophoresis buffer contained 0.1% SDS and 100 m*M* Tris and was titrated to pH 8.6 with 100 m*M* CHES, also containing 0.1 % SDS. The PAA (or dextran)-coated capillary [27 cm (7 cm to detector) × 100 μm I.D.] was filled with gel containing 1 g of dextran (M_r $2 \cdot 10^6$) in 10 ml of electrophoresis buffer. The degassed gel solution was injected into the capillary by pressure before each run and subsequently removed. Detection was by UV absorbance measurement at 200 nm.

The complex formation of r-AT III and thrombin was investigated by CGE under identical conditions. A 10-μl volume of r-AT III (1 mg/ml in 50 m*M* Tris–HCl, pH 7.5) was mixed with 10 μl of thrombin (250 μg/ml in 50 m*M* Tris–HCl, pH 7.5). After 10 s, 5 min and 10 min of

incubation, the samples were mixed with 10 μl of the 2-mercaptoethanol-free sample buffer and analysed. The influence of heparin on the affinity reaction was investigated under conditions identical with those above, with the exception that the r-AT III–thrombin mixture also contained 150 μg of solid heparin.

For cIEF the sample was desalted by cold methanol precipitation and centrifugation as described above. The sample pellet was resuspended in a buffer containing 0.01% HPMC, 0.1% TEMED, 0.001% Triton X-100, 2% Ampholine 4/6, 0.5% Pharmalytes 3/10, 0.5% Pharmalytes 2.5/5 and 0.5% Pharmalytes 4/6.5 in deionized water. The standard proteins trypsin inhibitor from soybean (p*I* 4.6), β-lactoglobulin A from bovine milk (p*I* 5.1) and carbonic anhydrase II from bovine erythrocytes (p*I* 5.4 and 5.9) were treated in the same way. The anolyte consisted of 10 m*M* phosphoric acid and the catholyte of 20 m*M* sodium hydroxide. The dextran-coated capillary had the dimensions 27 cm length (20 cm to the detector) × 50 μm I.D. A focusing voltage of 12 kV was applied for 2 min. Hydrodynamic mobilization was achieved by pressure afterwards, while a voltage of 8 kV was still applied. UV absorption was measured at 254 nm.

SDS–polyacrylamide gel electrophoresis (PAGE) in slab gels was performed according to Walker, using precast gels of 7% PAA (Serva) [25]. Isoelectric focusing was also performed according to Walker, applying precast gels for the pH focusing range 4–6 (Serva). Silver staining was used to reveal the proteins.

3. Results and discussion

Recombinant antithrombin III is produced by BHK cells which are cultivated in a culture media containing approximately 10% FCS and additives such as sugars and amino acids. Therefore, the determination of r-AT III in the culture supernatant and the monitoring of the cultivation process by CE faces some serious problems. First, the UV detection is limited to wavelengths of 254 or 280 nm, as the additives would interfere with any protein detection at lower wavelengths. The application of such high wavelengths has an adverse effect on the detection limit of the proteins. Further, AT III belongs to the so-called serpin (serpin protease inhibitor) superfamily [26], and therefore shows close physical and chemical relationships to other serpins, such as α_1-antitrypsin. The isoelectric points of AT III (p*I* 4.9–5.3) and its molecular mass (M_r 58 000) [27] are close to those of bovine serum albumin (p*I* 4.9, M_r 69 000) and transferrin (p*I* ≈ 5.8, $M_r \approx 75\,000$), two proteins which make up to 60% of the FCS proteins. Results obtained by common protein analysis such as size- or charge-dependent electrophoresis are therefore difficult to interpret. As the r-AT III concentration in the culture supernatant usually ranges between 5 and 50 μg/ml [20], a preconcentration step is necessary for the determination of r-AT III by CE.

3.1. Cultivation analysis

Our attempts to overcome these problems included a quantitative protein precipitation to combine a preconcentration of the proteins with the removal of the low-mass components, which would interfere with UV detection at lower wavelengths. In the following analysis of the culture supernatant by cITP, r-AT III could be identified in one distinct peak (9.2 min) and two neighbouring peaks, but only by peak spiking (Fig. 1a). Moreover, the sensitivity of the cITP method was low, as detection has to be done at 254 nm because the UV absorption of the spacers became a problem at lower wavelengths. Therefore, all proteins of interest had to be preconcentrated to at least 1 mg/ml. Further, exact r-AT III determination was not possible by cITP, as two of the three peaks ascribed to r-AT III appeared to be contaminated by other proteins. In another approach, the concentrated sample was in addition chromatographed on a protein G column for removal of the immunoglobulins. The breakthrough was analysed by CZE under acidic conditions (Fig. 1b). Since the resolving power of CZE is lower than that of cITP, r-AT

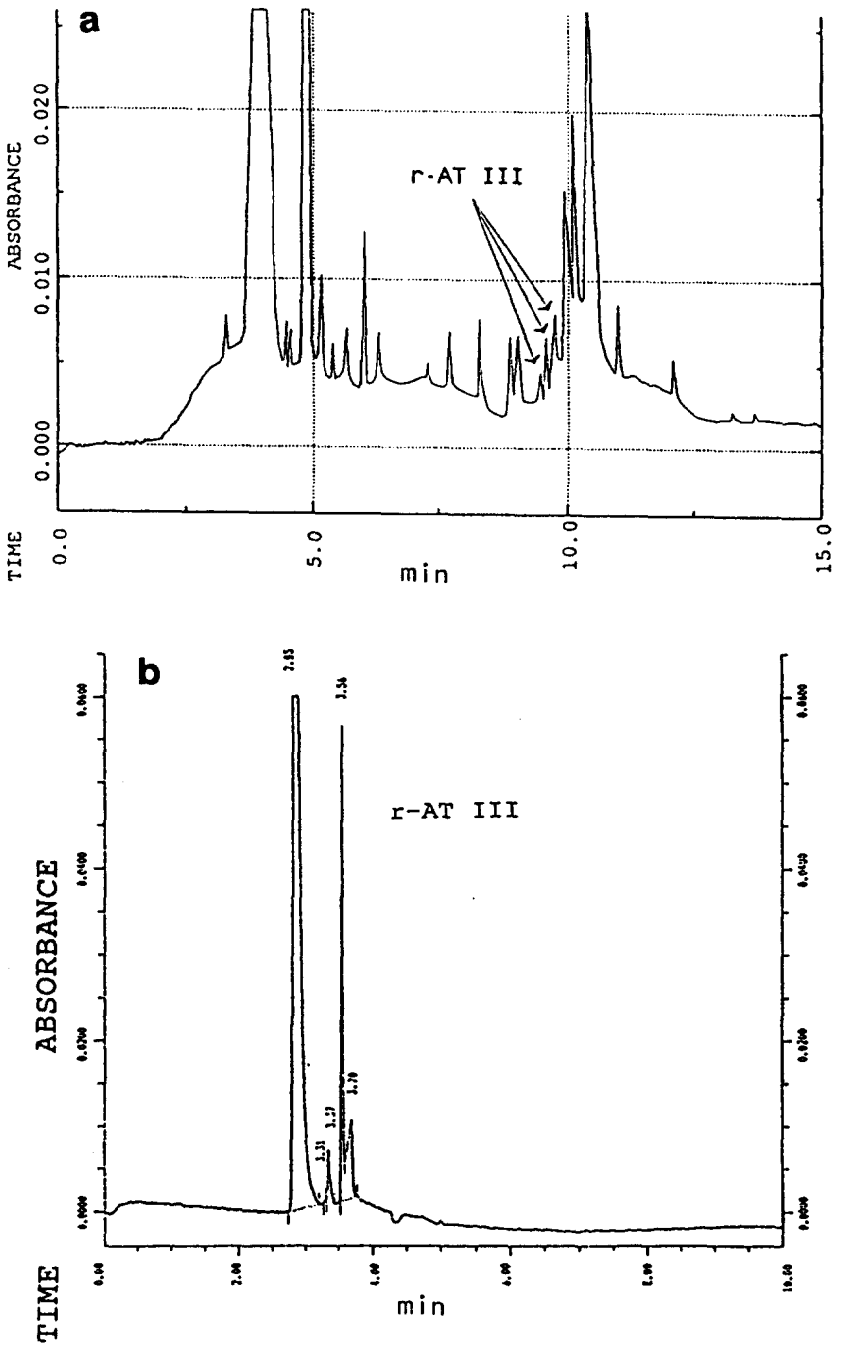


Fig. 1. (a) Determination of r-AT III in cultivation supernatant by cITP. (b) Determination of r-AT III in cultivation supernatant by CZE after removal of immunoglobulins by protein G affinity chromatography.

III could easily be determined in a single peak. The peak was well separated from BSA and transferrin. The detection limit was found to be ca. 50 μg/ml r-AT III, as CZE allows detection at 200 nm, which increases the sensitivity. Relative standard deviations of less than 1% were calculated for the CZE analysis. The additional step in sample preparation took no longer than 5 min, resulting in a total analysis time of 60 min from sample collection to data analysis, which was regarded as acceptable for the intended purpose, as changes in the cultivation considered here occur in hours rather than minutes.

3.2. Downstream process analysis

CZE was also used for monitoring the down-stream process, which called for an anion-exchange and a heparin affinity chromatographic separation. The electropherograms in Fig. 2 show the analysis of the r-AT III-containing fractions after each step. Compared with the cultivation supernatant (Fig. 2a), r-AT III was already enriched by the ion-exchange step and several other proteins of the FCS, such as most γ-globulins, were removed (Fig. 2b). The main remaining contaminants were BSA and transferrin [24]. After the following purification step utilizing heparin affinity MA, electrophoretically pure r-AT III was obtained (Fig. 2c). As the down-stream processing of large batches of r-AT III-containing supernatants takes several hours, the analysis time achieved in CZE can be regarded as acceptable, especially as no preconcentration of the sample is necessary.

3.3. Characterization

The purified product was characterized according to its isoelectric points and molecular mass and the results were compared with those determined by slab gels of the product itself and also those already published for h-AT III isolated from human serum. The determination of the isoelectric point was done by cIEF and the determination of the molecular mass by CGE. One of the more difficult aspects of cIEF is the correlation of the pI values. One option is to calculate directly the respective values from the currents characterizing the peaks during salt mobilization [28], but this method cannot be used in the case of pressure mobilization. If this is intended, as here, standard proteins have to be used instead to establish a pI vs. migration time calibration graph, albeit with the acknowledged disadvantage that variations between the standard runs and samples would bias the results. Desalting of the sample is crucial for the correctness of the determined isoelectric point. Further, the coating of the capillary has to be stable even at extreme pH values for at least ten subsequent runs to provide sufficient data.

We examined two different types of coating for cIEF: PAA and dextran. PAA started to hydrolyse after the fourteenth run, resulting in unreproducible results through an increase in electroendosmotic flow (EOF). The dextran coating was found to be stable for at least 50 runs, showing results comparable to those achieved with the PAA-coated capillaries. Following the suggestion of Zhu et al. [29], we added Triton X-100 to the sample buffer to prevent protein precipitation and TEMED in order to prevent the focusing of sample components behind the detector. The applied spacer mixture was found to constitute a good compromise between resolving power and pH gradient stability. A single ampholyte mixture for the focusing range pH 4–6 gave the highest resolving power, but was unsuited to building a stable pH gradient in the capillary. The cIEF of the standard proteins [Fig. 3a(I)] showed a linear relationship between the isoelectric points of the standard proteins and their average retention times (t_R) [Fig. 3a(II)]. Relative standard deviations were between 2 and 4%.

Based on this calibration graph, the isoelectric points of the r-AT III fractions were determined. We found a pattern of six fractions focused in the pH range 4.7–5.2 [Fig. 3b(I)], with three major peaks corresponding to pI values of 4.7, 4.75 and 4.85 and three poorly resolved minor peaks corresponding to pI values of 5.0, 5.1, and 5.3. The poor resolution of the minor peaks might be due to microheterogeneities of the recombinant AT III or to an inferior resolving power of the

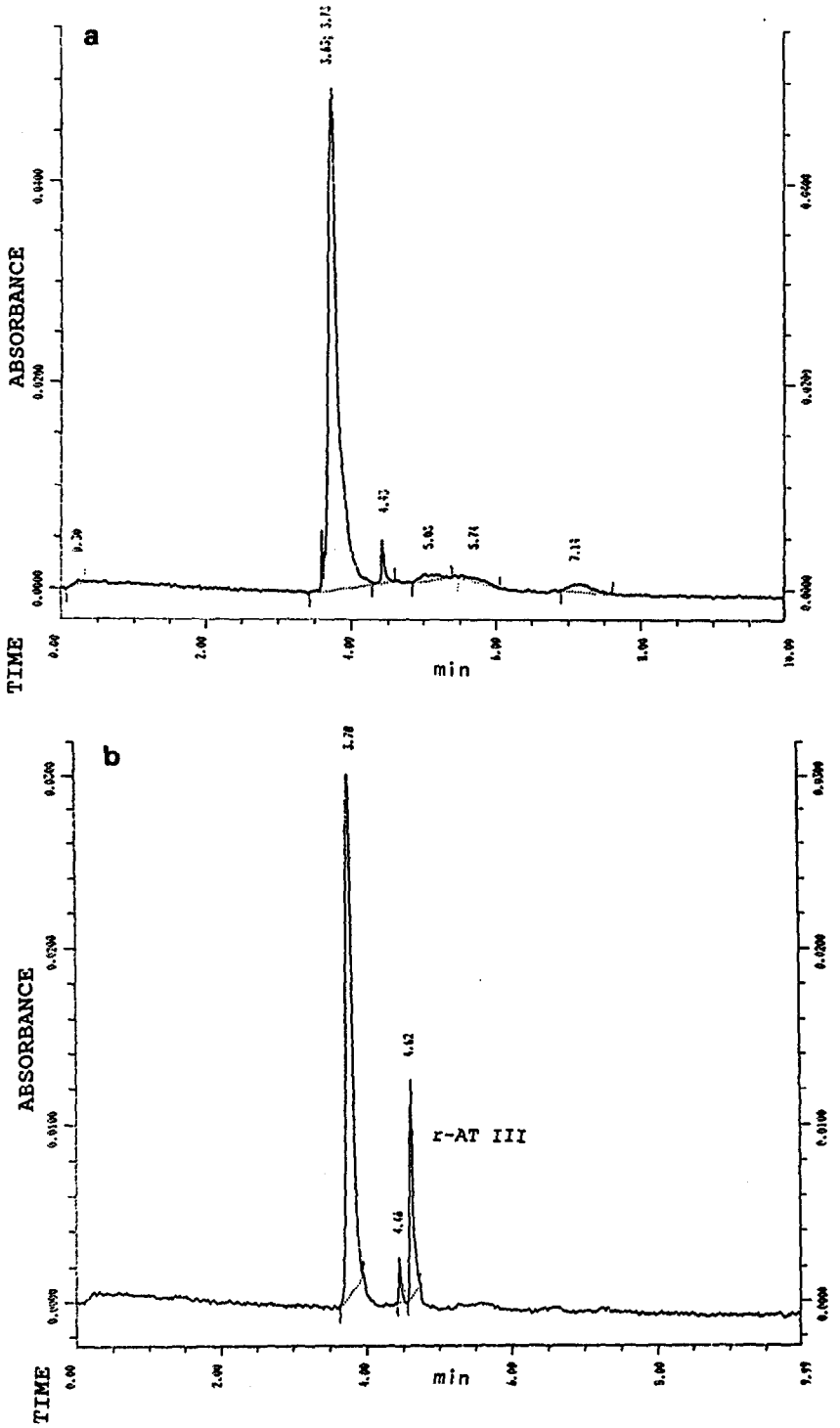


Fig. 2. *(Continued on p. 390)*

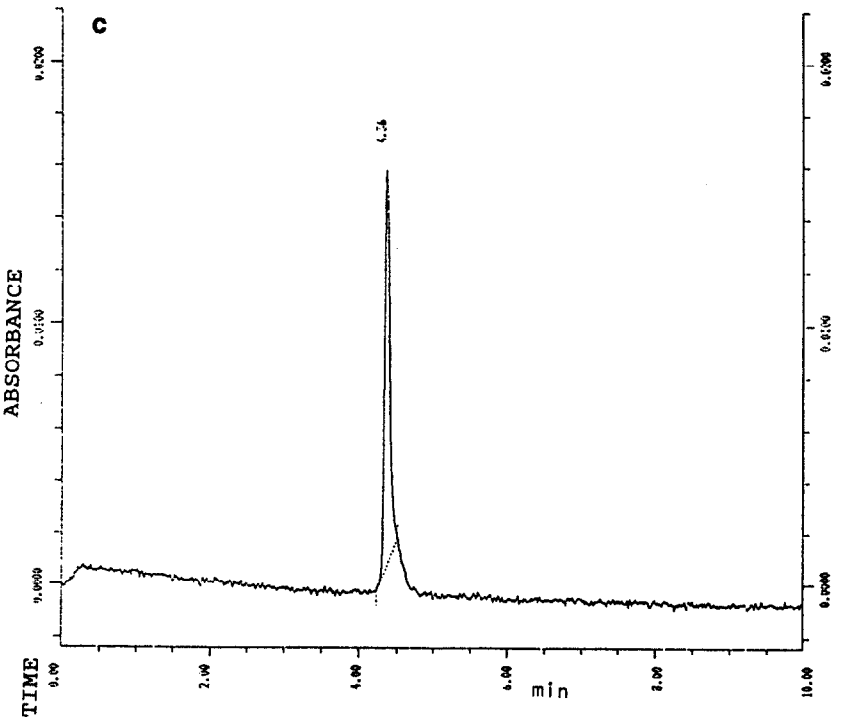


Fig. 2. Monitoring of downstream process of r-AT III by CZE. (a) Cultivation supernatant; (b) r-AT III-containing fraction from the ion-exchange step; (c) r-AT III-containing fraction from the heparin affinity chromatographic step.

chosen ampholytes in that particular pH range. The cIEF results were comparable to those achieved with conventional IEF using PAA slab gels in the focusing range pH 4–6 [Fig. 3b(II)]. Further, the determined isoelectric points fit those already published for human serum AT III, based on IEF in PAA slab gels [30]. For human serum AT III, three major bands were found to have p*I* values of 4.75, 4.8 and 4.85 and three minor bands with p*I* values of 5.0, 5.05, 5.2, in addition to a doublet at p*I* 4.7. Except for the doublet at p*I* 4.7, all of these bands were also seen here, by both cIEF and IEF. The missing doublet might simply be covered by the major peak at pH 4.7, but it is also possible that this fraction is indeed under-represented in the r-AT III owing to faulty glycosylation of the protein by the producing organisms.

The molecular mass was determined by CGE, using external standards as suggested by Lausch et al. [31] [Fig. 4a(I)]. No differences could be found between PAA- and dextran-coated capillaries. The standard proteins showed an excellent correlation between the M_r and the retention time [Fig. 4a(II)]. Relative standard deviations of approximately 2% were found, partly because the gel in the capillary was replaced after each run. Microheterogeneities of the polymer solution can never be excluded, even though they should by reduced here, as the same gel stock solution was used for each run. In this case, for example, the protein standards and the r-AT III sample would be analysed under slightly different conditions. Nevertheless, with a relative standard deviation of 2%, the results are within an acceptable range of error. Taking the calibration into account, the purified r-AT III was found to have M_r 59 000 (Fig. 4b). This result is close to that published for human AT III, i.e. 58 000, a value supported by protein and cDNA sequencing [32]. The difference of 1000 may be due to variations of the protein itself, but is also within the limits of the standard deviation. Using an automated system and a single coated capillary, the isoelectric point and the molecular mass of a purified protein can be determined

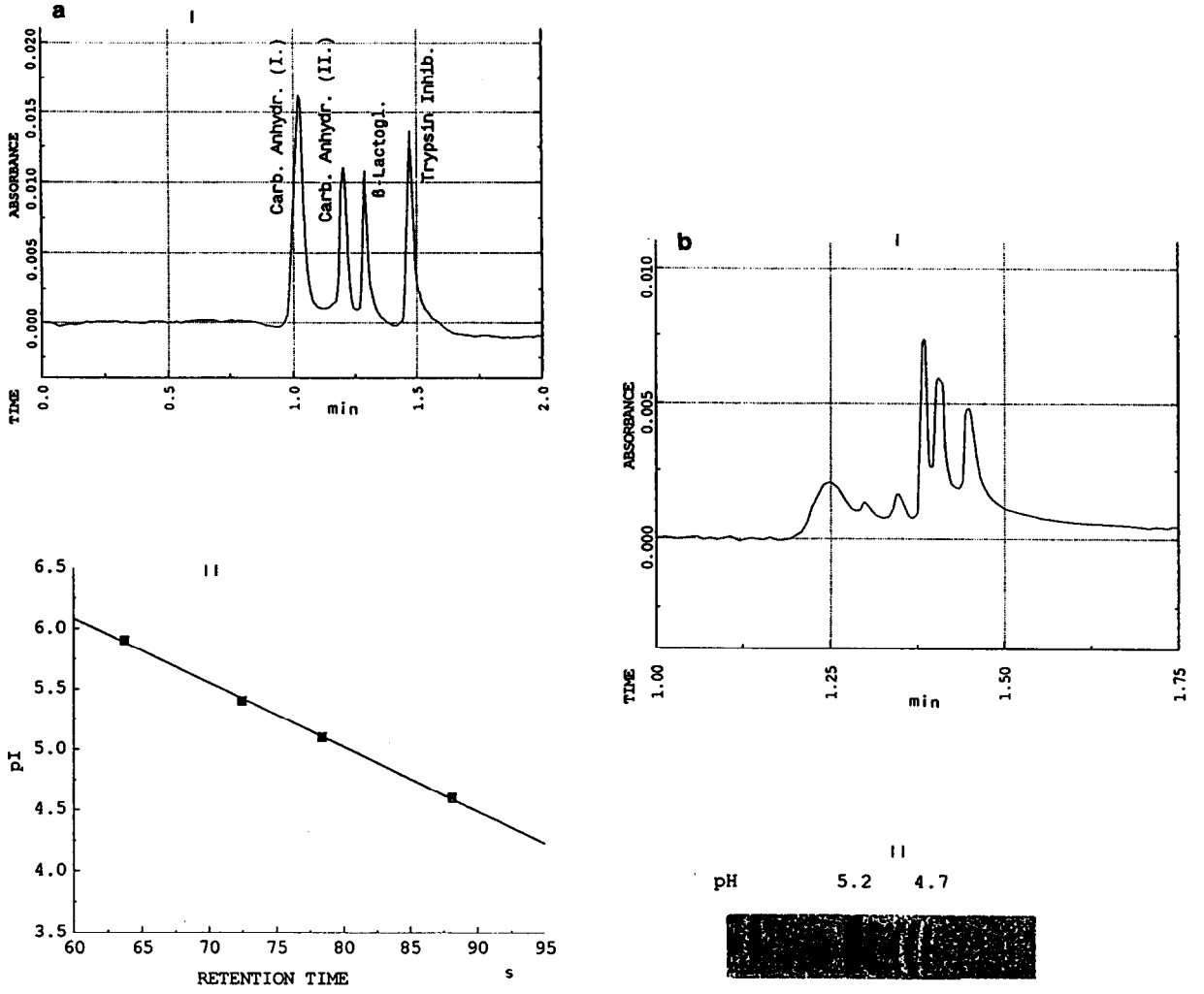
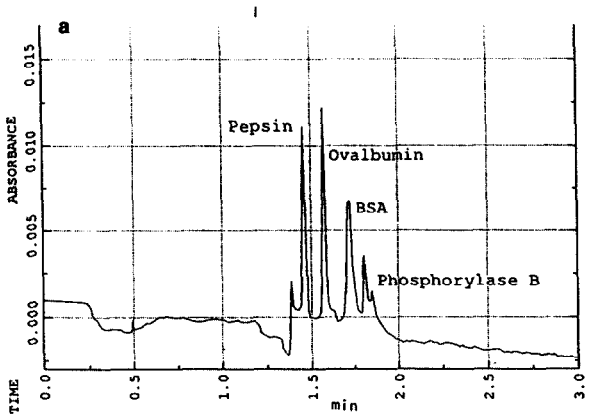


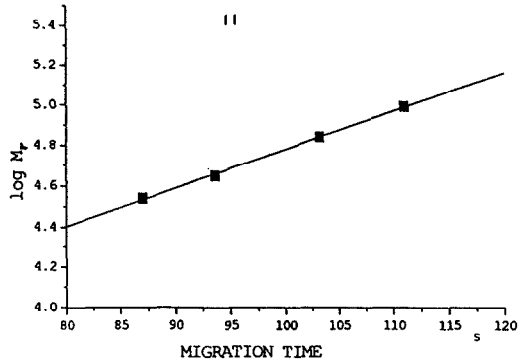
Fig. 3. Characterization of the isoelectric points of r-AT III by cIEF. (a) Standard proteins by cIEF (I) with calibration graph (II); (b) r-AT III determined by cIEF (I) and IEF in slab gel (II).

within 30 min, proving that CE is an adequate technique in protein characterization.

3.4. Antithrombin–thrombin complex formation

The complex formation between r-AT III and thrombin was investigated by CGE using conditions identical with those described above. After an extra-capillary incubation of 10 s, the two components were detected as individual peaks corresponding to M_r 32 000 (thrombin) and 59 000 (r-AT-III) (Fig. 5a). After an incubation time of 5 min, however, a third peak appeared, which corresponded to a molecular mass of 92 000, while the thrombin peak nearly vanished (Fig. 5b). This new peak represents the r-AT III–thrombin complex, when the incubation time was prolonged up to 10 min, no further complex formation could be detected (Fig. 5c). However, when heparin was added to the incubation mixture, the complex appeared even after 10 s in amounts comparable to those found after incubation for 5 min in the absence of heparin (Fig. 6a), demonstrating that the rate of complex





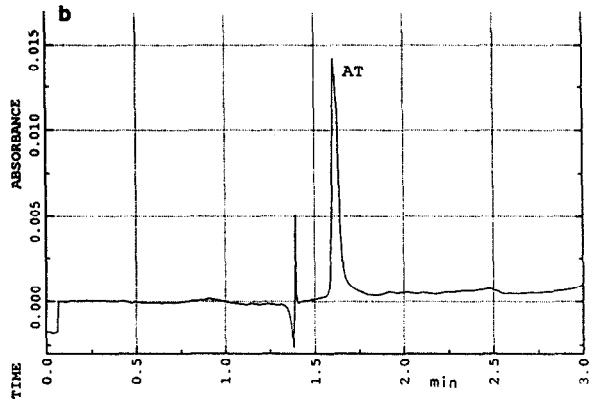
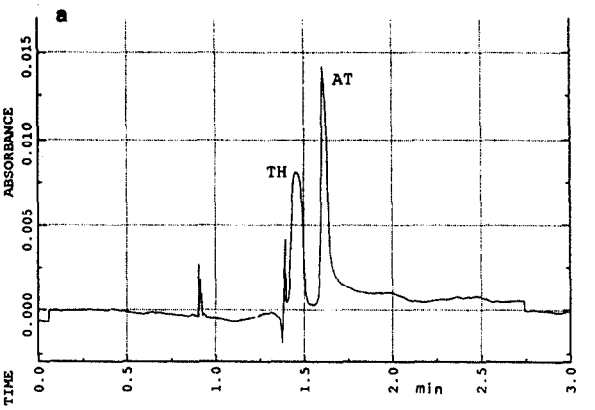
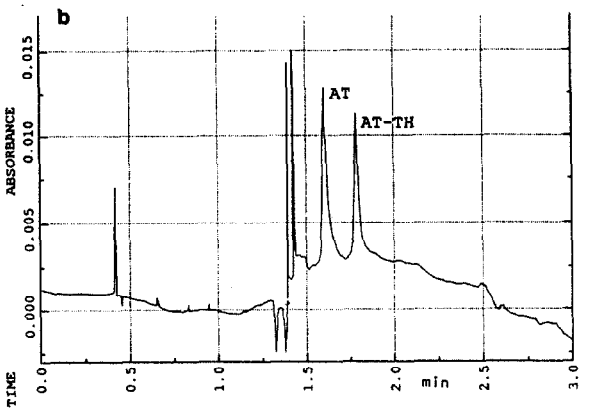


Fig. 4. Characterization of the M_r of r-AT III by CGE. (a) Standard proteins (I) with calibration graph (II); (b) r-AT III.





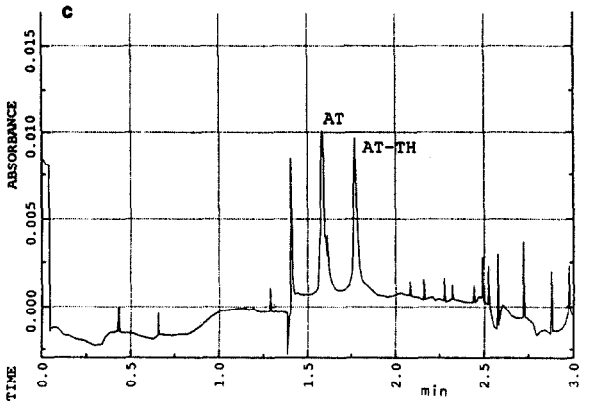


Fig. 5. Complex formation between r-AT III and thrombin. AT = Antithrombin III; TH = thrombin; AT–TH = complex. (a) r-AT III and thrombin after incubation for 10 s; (b) after 5 min; (c) after 10 min.

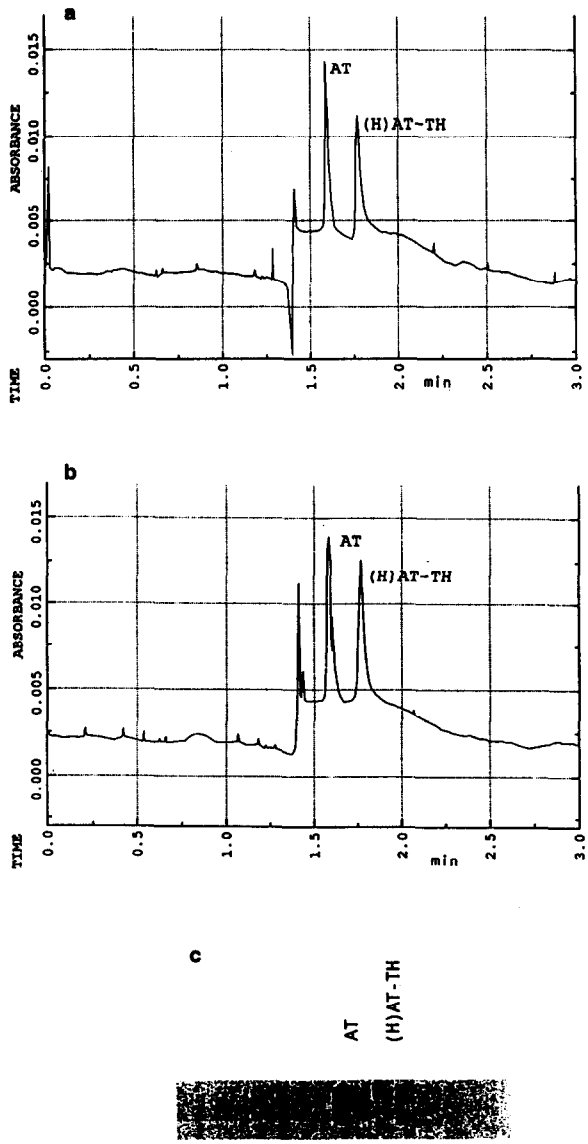


Fig. 6. Complex formation between r-AT III and thrombin in the presence of heparin. AT = Antithrombin III; TH = thrombin; (H)AT—TH = complex. (a) r-AT III, thrombin and heparin after incubation for 10 s; (b) after 5 min; (c) same sample as (b), but SDS-PAGE in slab gel.

formation in enhanced by catalytic amounts of the polysaccharide heparin. When the incubation time in the presence of heparin was increased to 5 min, no further complex formation could be observed (Fig. 6b); apparently the complex formation had reached equilibrium after 10 s. The results obtained with CGE were comparable to those achieved with the PAA slab gel (Fig. 6c) and also to published data [33]. Heparin enhances the enzyme–inhibitor reaction without affecting the stochiometry of the reaction if the heparin is at least 18 monosaccharide units long [32]. Thus CGE proved to be an adequate method even for the evaluation of biological affinity reactions.

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