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A new ELISA method for the measurement of total α_2-plasmin inhibitor level in human body fluids

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ABSTRACT

The ever-increasing research efforts to develop new antithrombotic therapies have led to the reassessment of the role of alpha-2-plasmin inhibitor (α_2-PI) in pathological conditions. In particular, experimental stroke studies have suggested correlation between increased free α2-PI level and mortality. However there are only a small number of well-characterized and specific assays available for the measurements of free α_2-PI. In plasma α_2-PI undergoes both N- and/or C-terminal cleavages resulting four isoforms with modified susceptibility to FXIII catalyzed cross-linking to fibrin and/or loss of plasmin(ogen) binding.

Present paper describes a new sandwich ELISA method for the determination of free total α_2-PI in plasma and other body fluids. A newly generated biotinylated monoclonal antibody recognizes and captures all the four N- and/or C-terminally modified isoforms of α_2-PI while HRPO-labeled polyclonal anti-α_2-PI antibody detects the captured antigen. Performing the 2-step assay in streptavidin-coated microplate can be completed within three hours. The assay is well reproducible, total (within laboratory) imprecision in the normal, pathological and very low ranges were 7.4%, 9.1% and < 19%, respectively. When examining the plasma samples of 197 healthy volunteers, 100 acute ischemic stroke patients and 102 patients with venous thrombosis, strong correlation was observed between total α2-PI antigen levels and α2-PI activity for each group. Using the assay a reference interval of 45–86 mg/L was established for total α_2-PI mass concentration in the plasma. α_2-PI levels were also measured in cerebrospinal fluid samples of 47 individuals the median value and range was 132 (36–379) μg/L.

In conclusion, our ELISA enables accurate and fast measurement of total free α_2-PI in human body fluids.

1. Introduction

α_2-plasmin inhibitor (α_2-PI; also known as α_2-antiplasmin, α_2-AP) is a single-chain glycoprotein consisting of 491 amino acids produced by the liver with a molecular weight of ~67,000 Da and carbohydrate content of 11–14% (Moroi and Aoki, 1976). Its plasma concentration is about 1 μM. α_2-PI is described as the main physiological inhibitor of plasmin. It regulates fibrinolysis in two ways: by forming an irreversible complex with plasmin and by making fibrin more resistant to local plasmin through covalent cross-linking to fibrin by activated factor XIII (Mullertz and Clemmensen, 1976; Wiman and Collen, 1977; Wiman and Collen, 1979). α_2-PI is a unique member of the serine protease inhibitor *(serpin)* family due to its N- and C-terminal extensions (42 and 55 amino acids, respectively) beyond its core inhibitory domain that gave additional functions to the molecule. Activated factor XIII (FXIIIa) cross-links the N-terminal extension of α_2-PI to the α chain of fibrin (Tamaki and Aoki, 1982) by this way localizes antiplasmin activity to the clot and protect it from plasmin-mediated degradation. The C-terminal extension is important in the initial interaction of α_2-PI with the kringle domains of plasminogen.

α_2-PI circulates in the plasma in different N- and C-terminally truncated forms. The secreted full length Met1-α_2-PI (464 amino acids) is shortened by 12 amino acids (Bangert et al., 1993; Koyama et al., 1994) by the cleavage of *a*ntiplasmin *c*leaving *e*nzyme (APCE) (Lee et al., 2004) resulting in a version with Asn at the N-terminus (Asn1-α_2-PI). The two forms have different susceptibility for cross-linking to fibrin, FXIIIa cross-links Asn1-α_2-PI to fibrin more effectively and faster than Met1-α_2-PI (Sumi et al., 1989; Lee et al., 2004). Only the cross-linked α_2-PI offers efficient protection against fibrinolysis (Sakata and Aoki, 1982). The ratio of Met1-α_2-PI to Asn1-α_2-PI in normal human plasma is reported to be 30:70% (Bangert et al., 1993; Koyama et al., 1994; Lee et al., 2004). A single nucleotide polymorphism in the α_2-PI

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gene resulting in an arginine to tryptophan substitution at position 6 (R6W) strongly effects the N-terminal cleavage rate of α_2-PI in the circulation, because APCE cleaves Met1-α_2-PI(R6) approximately eightfold faster than Met1-α_2-PI(W6) (Christiansen et al., 2007). There are also two C-terminal variants of the α_2-PI molecule, which differ in binding ability to plasminogen (Christensen and Clemmensen, 1978; Wiman, 1980). The plasminogen-binding form is C-terminally intact (PB-α_2-PI), while the non-plasminogen-binding variant (NPB-α_2-PI) is truncated by at least 26 amino acids by an unknown protease. Approximately 35% of the total circulating α_2-PI is C-terminally truncated (Wiman et al., 1982; Kluft et al., 1986; Sasaki et al., 1986; Sumi et al., 1986; Sugiyama et al., 1988).

α_2-PI can be determined in plasma either with enzymatic assays based on the measurement of its plasmin inhibitory activity, or with immunochemical assays to measure antigen concentration using specific antibodies. To measure the concentration of total and differently truncated α_2-PI isoforms, as well as their ratio in the plasma, at the same time, a set of immunoassays needs to be applied.

In the present study we describe a new, highly sensitive immunoassay for the measurement of total circulating α_2-PI in human body fluids using a newly generated monoclonal antibody to capture and a polyclonal tag antibody to detect human α_2-PI. ELISA measurements were performed from 197 normal plasma samples to establish a reference range and from 47 cerebrospinal fluids (CSF) to demonstrate the suitability of the assay for determination of total α_2-PI antigen in other body fluids.

2. Materials and methods

2.1. Materials

Purified human α_2-PI was obtained from Calbiochem (Darmstadt, Germany). Affinity purified goat anti-human α_2-antiplasmin and α_2-antiplasmin deficient plasma were produced by Affinity Biologicals Inc. (Ancaster, ON, Canada). Ready for use 3,3′,5,5′-tetramethylbenzidene (TMB) substrate, bovine serum albumin (BSA) fraction V, horse radish peroxidase (HRPO) type VI, BioStab peroxidase conjugate stabilizer, biotinamido-hexannoic acid hydrazide and streptavidin, immobilized on agarose CL-4B, goat anti-mouse IgG (Fc specific), endoproteinase Asp-N and trypsin (proteomics grade) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Bis(sulfosuccinimidyl)suberate (BS^3) was a product of Pierce (Rockford, IL, USA). Streptavidin coated microplates were the products of Thermo Fisher Scientific (Vantaa, Finland). Normal and pathologic control plasmas were obtained from Siemens Healthcare Diagnostics (Marburg, Germany).

2.2. Samples

Blood samples were obtained from 197 apparently healthy volunteers (115 females/82 males, median age: 32 years, range: 18–69). Healthy subjects were recruited from Eastern Hungary; they completed an extensive questionnaire before blood sampling. Except for moderate hypertension (blood pressure between 145/90 and 165/95 mmHg) in their case history all chronic diseases and any acute illness in the previous 3 weeks were considered as exclusion criteria. The body mass index (BMI) was calculated and smoking habit was recorded. 9 volumes of blood were drawn into a Vacutainer tube (Beckton Dickinson, Plymouth, UK) containing 1 volume of 0.105 mol/L buffered sodium citrate. After centrifugation at 1500 $\times g$ (20 °C, 20 min), plasma was removed and stored at −70 °C until determination of α_2-PI.

Citrated plasma samples were also obtained from patients with thrombotic diseases: 1) 100 patients with acute ischemic stroke (AIS) admitted to the Department of Neurology, University of Debrecen, Hungary. Samples were taken within 4.5 h of their symptom onset, before thrombolytic therapy. 2) 102 patients with venous thromboembolism (VTE) admitted to the Thrombosis Centre of the University of Debrecen. Patients with malignant disease were excluded. Blood samples were taken at least 3 months after the acute event.

CSF samples were obtained from 47 hip or knee replacement surgery patients (32 females/15 males, age: 59 ± 14 years) collected by lumbar puncture at the occasion of spinal anesthesia. Fuchs-Rosenthal chamber was used to count red blood cells (RBCs) in the CSF within 2 h after draining. Samples with > 120 red blood cells/μL were discarded. Samples were centrifuged at 1500 x*g* for 10 min to pellet cells. The fluid fraction was separated and stored at −20 °C until analysis. Total protein concentration was measured by turbidimetry using precipitation by benzethonium-chloride (Roche, Mannheim, Germany).

Ethical approval was obtained from the University of Debrecen Ethics Committee, and all participants or their relatives provided informed consent in accordance to the Helsinki Declaration.

2.3. Measurement of fibrinogen and α_2-PI activity

Fibrinogen levels were measured with a clotting assay according to Clauss. α_2-PI *activity was measured using the Berichrom* α_2-PI *activity assay* on BCS Coagulation System (Siemens Healthineers, Erlangen, Germany).

2.4. Production of monoclonal antibodies against α_2-PI

Balb/c mice (8–12 week old) were immunized subcutaneously with 10 μg purified α_2-PI emulsified in complete Freund's adjuvant. Ten micrograms of antigen adsorbed on aluminium hydroxide gel were administered intraperitoneally as booster immunizations at two occasions in two-week intervals. Four days before cell fusion a final boost of 10 μg antigen was given into the peritoneal cavity. Immune spleen cells were fused with Sp2/o myeloma cells employing a modification (Stahli et al., 1980) of the method described by Köhler and Milstein (Kohler and Milstein, 1975). The hybridoma cell supernatants were tested for reaction with purified α_2-PI in indirect ELISA systems and antibody-producing hybridomas were selected and cloned by limiting dilution technique. Antibodies were purified from cell supernatants using Protein G affinity chromatography. Animal welfare was in accordance with the University of Debrecen's guidelines.

2.5. Labeling of anti-α_2-PI antibodies

The newly generated 10/5A8 monoclonal antibody (Isotype: IgG_1) was selected based on its high affinity as capture antibody and was biotinylated via its sugar moieties (O' Shanessy, 1990). The biotinylated antibody was stored at −20 °C in PBS (pH: 7.2), supplemented with 1% BSA and 120 mg/L methylisothiasolone. Commercial affinity purified goat anti-human α_2-PI was labeled with HRPO (Wilson and Nakane, 1978) and used as detection antibody. It was stored in 200-fold dilution in BioStab conjugate stabilizer at +4 °C.

2.6. ELISA method

The optimal concentration of biotinylated capture antibody, 0.5 μg/mL, was established in preliminary titration experiments using increasing amount (0.1 to 2 μg/mL) of the antibody. At this concentration of the capture antibody the optimal final dilution of the HRPO-labeled tag antibody was 1:4000 (*v*/v).

α_2-PI specific antibodies, plasma or CSF samples and purified α_2-PI were diluted in 0.15 mol/L, pH 7.2 phosphate buffer containing 0.5 M NaCl, 0.05% Tween 20 and 0.5% BSA. Routinely the assay was performed with 1:2000 (v/v) dilution of plasma or 1:10 (v/v) dilution of CSF samples. Highly purified lyophilized α_2-PI reconstituted in ultrapure water and further diluted in 20 mM Tris, 200 mM NaCl, 2% BSA, 120 mg/L methylisothiasolone buffer, pH 7.0 to a final protein concentration of 50 μg/mL was used as calibrator. Aliquots were stored at −70 °C until the preparation of calibration curves.

The assay was performed in streptavidin-coated microplate at room temperature as described below:

70 µL of diluted sample or calibrator and 70 µL of diluted biotinylated 10/5A8 monoclonal antibody were added to a streptavidin-coated microwell. The mixture was incubated for 1 h with gentle shaking. The plate was then washed thrice with 0.15 mol/L phosphate buffer (pH 7.2) containing 0.05% Tween 20 (300 µL/well) before 1-h incubation with 140 µL of diluted HRPO-labeled detecting antibody. After washing the enzyme reaction was visualized with 140 µL of TMB substrate for 30 min and the reaction was terminated by the addition of 50 µL 2 mol/L H_2SO_4. Absorbance was measured at 450 nm in a Labsystems iEMS MF microplate reader (Labsystems Oy, Helsinki, Finland).

2.7. Assay evaluation

The evaluation of precision performance was carried out according to the EP5-A2 guideline of the Clinical and Laboratory Standards Institute (NCCLS, 2004) performing single runs per day with duplicate determinations of α_2-PI in normal, pathological and α_2-PI deficient plasma spiked with 1.98 mg/L of purified α_2-PI for 20 days. The limit of detection (LOD) and limit of quantification (LOQ) were determined as described in the CLSI EP-17-A protocol (NCCLS, 2004b). To establish the LOD the partly nonparametric approach was applied (Linnet and Kondratovich, 2004).

Recovery was determined in normal, pathological and α_2-PI deficient plasma samples spiked with various concentrations of purified α_2-PI. One volume of spiking solution or buffer was added to 9 volumes of plasma samples. α_2-PI levels in the spiked plasmas were measured as described in section 2.5. In order to assess whether our α_2-PI ELISA is able to recognize α_2-PI in complex with plasmin, 5-, 10-, and 20-fold molar excess plasmin was added to plasma of known α_2-PI concentration or to purified α_2-PI. After incubation for 1 h at 37 °C the samples were analyzed in the α_2-PI ELISA.

α_2-PI antigen concentrations determined by our α_2-PI ELISA were compared to α_2-PI activity values measured by a chromogenic α_2-PI activity assay (Berichrom α_2-Antiplasmin assay, Siemens, Munich, Germany). Measurements were carried out on citrated plasma samples obtained from 197 healthy subjects, 100 patients with AIS and 102 patients with VTE.

2.8. Epitope mapping of the 10/5A8 monoclonal antibody

Epitope mapping was performed as previously published (Hager-Braun and Tomer, 2006). Briefly: The primary antibody (goat anti-mouse Fc-specific Ig) was bound to CNBr-Sepharose beads then the secondary antibody (monoclonal anti-human α_2-PI antibody) was added and covalently cross-linked to the primary antibody. 25 µL of washed and drained beads was incubated with 400 µL 1:3 diluted plasma (in PBS) or PBS alone at room temperature for an hour in compact reaction cups (CRC). After washing with 0.1 M Tris/HCl, pH 8.5 buffer, 50 µL 1 µg/mL trypsin or 1 µg/mL Asp-N in 0.1 M Tris/HCl, pH 8.5 were added to the CRC-s and were incubated overnight at 37 °C. The supernatants were transferred to low protein binding cup, the peptide fragments were purified and desalted using ZipTip™ pipette tips with C18 matrix (Millipore, Billerica, MA, USA). The bound peptides were eluted with 1:5 dilution of saturated α-cyano-4-hydroxycinnamic acid (in 50% acetonitrile, 0.3% trifluoroacetic acid), and air-dried on stainless steel MALDI plate. To investigate the peptide fragment that remained attached to the surface bound antibody approximately 1 µL of washed beads were mixed with 1 µL of saturated α-cyano-4-hydroxycinnamic acid in ethanol/water/formic acid (45/45/10 *v*/v/v) on the MALDI target and air-dried. The supernatants and the beads were analyzed by MALDI-TOF mass spectrometry on a Voyager DE STR (Applied Biosystems, Foster City, CA, USA) operated in positive reflectron mode. Amino acid sequence of α_2-PI (Accession number: P08697) was retrieved from the UniProt database (www.uniprot.org) and was subjected to in silico trypsin and Asp-N digestion by the PeptideCutter software (http://web.expasy.org/peptide_cutter). Proteolytic fragments identified by mass spectrometry were compared to the fragments obtained by in silico digestion.

2.9. Statistical methods

All standards and samples were measured in duplicates. Four-parameter logistic regression was used for curve fitting and concentration calculation using Ascent Software version 2.4.2. (Thermo Fisher Scientific, Waltham, MA USA). The distribution of α_2-PI concentration values was investigated by Kolmogorov-Smirnov and Shapiro-Wilk tests. The reference interval was calculated by parametric method. The justification of partitioning the reference values according to gender was tested by the z-test and by comparing standard deviations of the male and female reference groups. Spearman's correlation was calculated to investigate the relationship between two methods. The GraphPad Prism software (GraphPad, La Jolla, California, USA) Version 5.0 was used for statistical analyses. Multiple regression modeling was used to test multivariate relationships of variables with total α2-PI levels using the SPSS software (version 24, SPSS Inc).

3. Results

3.1. Evaluation of total α_2-PI ELISA

The LOD was 0.7 µg/L. As at this level the estimated total error (21.8%) was below 25%, the LOQ was considered equal to the LOD (as described in the EP17-A CLSI protocol). The assay has a wide measuring range between 0.7 µg/L and 100 µg/L (Fig. 1).

Using 1:2000 plasma dilutions this range corresponds to 1.4–200 mg/L α_2-PI antigen concentration (2–300% of normal average, see later). Using 1:10 CSF dilution the measurement range is 7–1000 µg/L. A four parameter polynomial equation resulted in a curve with excellent fitting to the calibration points. The total (within laboratory) imprecision of the assay in the normal and pathological ranges were 7.4% and 9.1% respectively and even at very low concentration range it was 18.9% (Table 1). The mean recovery of added α_2-PI varied between 91.6 and 101.7% with a mean value of 98.3% (Table 2).

Next, measurement of α_2-PI in PAP complex was carried out after incubation of plasma with increasing molar excess of plasmin. Measured total α_2-PI antigen decreased upon addition of plasmin to the plasma, however approximately 10% of α_2-PI was still recognized after adding plasmin in 20-fold molar excess (Fig. 2). Similar results were obtained with purified α_2-PI (data not shown).

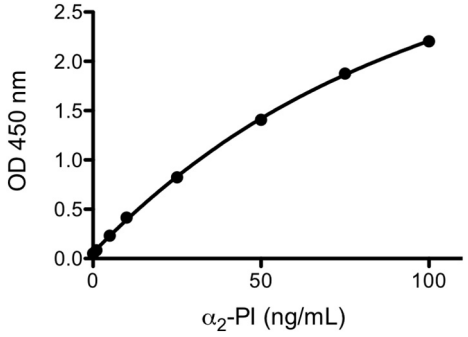

Fig. 1. Calibration curve for the total α_2-plasmin inhibitor (α_2-PI) ELISA. The mathematical formula for fitting the calibration curve is the following $y = 0.06665x^2 + 0.03287x - 0.0001157$ ($r^2 = 0.9996$, Sy.x = 0.01909).

Table 1
Imprecision of total α_2-PI ELISA.

Sample	Mean (mg/L)	Within run CV%	Between day CV%	Within laboratory CV%
Normal	60.77	4.63	5.8	7.4
Pathologic	19.51	5.34	7.3	9.1
Low	1.98	9.09	16.6	18.9

CV, coefficient of variation, Quality control plasmas with normal and decreased assigned α_2-PI activity were obtained from Siemens (Control Plasma N and P), preparation of plasma sample with very low α_2-PI activity is described in the Methods.

Table 2
Analytical recovery of total α_2-PI in the α_2-PI ELISA.

Sample	α_2-PI in the sample (μg/mL)	Added α_2-PI (μg/mL)	Measured α_2-PI (μg/mL)	Recovery %
Normal control	49.42	45.62	95.43	99.2
		8.29	57.68	98.8
Pathologic control	17.81	41.74	58.9	98.4
		7.48	25.41	101.7
α_2-PI deficient plasma	0.035	41.74	38.26	91.6
		7.48	7.50	99.8

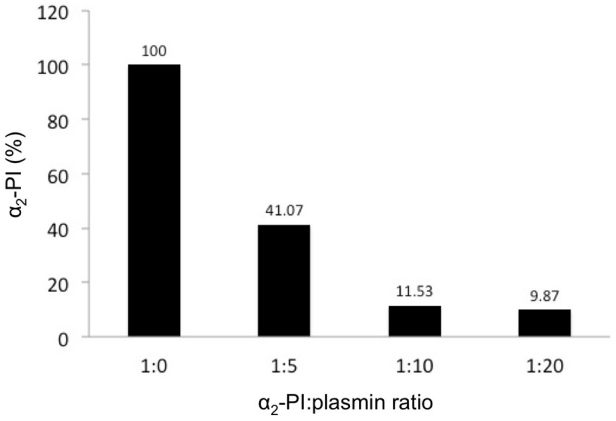

Fig. 2. Effect of complex formation between α_2-plasmin inhibitor (α_2-PI) and plasmin on the measurement of total α_2-PI in plasma. The assay mixture contained 35.4 mg/L α_2-PI with or without added plasmin.

3.2. α_2-PI levels in the plasma of healthy subjects and its association with different variables

Total α_2-PI concentrations in citrated plasma samples from 197 healthy individuals showed Gaussian distribution and the plasma concentration was 66.34 ± 9.19 mg/L (mean ± SD) (min-max: 35.66–90.00 mg/L; interquartile range: 59.95–72.96 mg/L) (Fig. 3). For males and females the concentration of total α_2-PI in the plasma was almost identical 66.45 ± 9.67 mg/L and 66.25 ± 8.88 mg/L (mean ± SD), respectively. Neither the z test nor the ratio of the standard deviations (1.089) justified partitioning the reference interval according to gender. Thus a common reference interval (middle 95 percentile) of 47.96–84.72 mg/L (72–128% of normal average) was established for both sexes.

Fibrinogen concentration and BMI in the healthy group was 3.35 ± 0.65 g/L and 24.3 (21.3–27.9), respectively. The presence of hypertension was 18.3% and the ratio of current smokers was 21.6%. Total α_2-PI antigen levels correlated significantly with body mass index (BMI) ($r = 0.357$, $p < .001$), fibrinogen levels ($r = 0.281$, $p < .001$) and hypertension ($r = 0.222$, $p = .012$), but did not show correlation with age ($r = 0.059$, $p = .410$) and smoking ($r = 0.197$, $p = .333$).

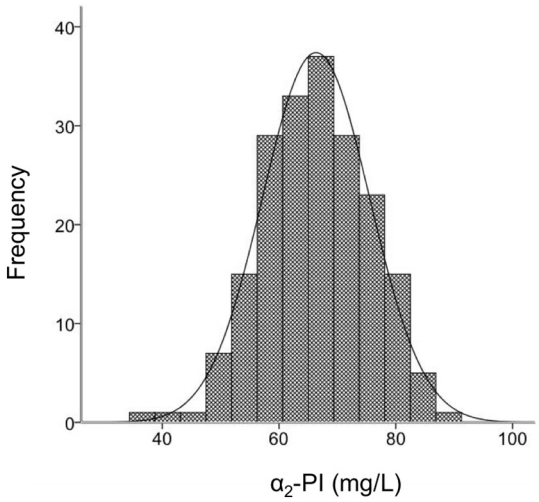

Fig. 3. The distribution of α_2-plasmin inhibitor concentration values measured in plasma of healthy volunteers.

However, multiple linear regression analysis confirmed that only BMI and fibrinogen level showed independent association with total α_2-PI antigen levels.

3.3. Total α_2-PI levels in cerebrospinal fluid

α_2-PI and total protein concentrations were measured in CSF samples of 47 individuals who underwent hip or knee replacement. The distribution of total α_2-PI concentrations was non-Gaussian, the median α_2-PI concentration was 132 μg/L, (min-max: 36–379; interquartile range: 74–160), however total α_2-PI results normalized for protein concentration showed normal distribution (Fig. 4), concentration of total protein and normalized total α_2-PI concentration were 0.304 ± 0.09 g/L and 418.8 ± 172.7 μg α_2-PI/g protein (mean ± SD), respectively.

3.4. Method comparison

To investigate the correlation between total α_2-PI antigen levels measured by our ELISA and α_2-PI activity levels, we analyzed plasma samples of 197 healthy subjects, 100 acute ischemic stroke (AIS) patients and 102 patients with venous thrombosis (VTE). There was a

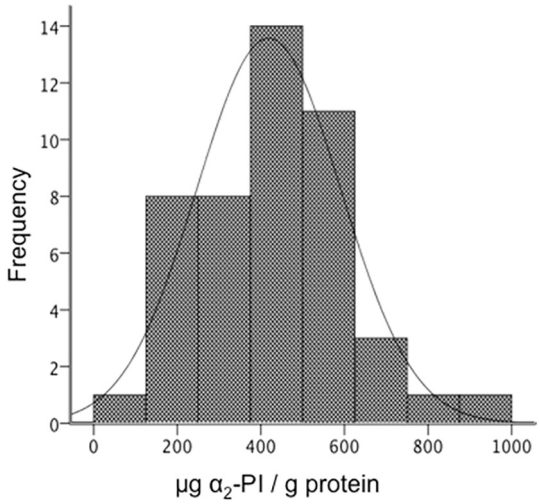

Fig. 4. The distribution of α_2-plasmin inhibitor/total protein values measured in cerebrospinal fluid of healthy volunteers.

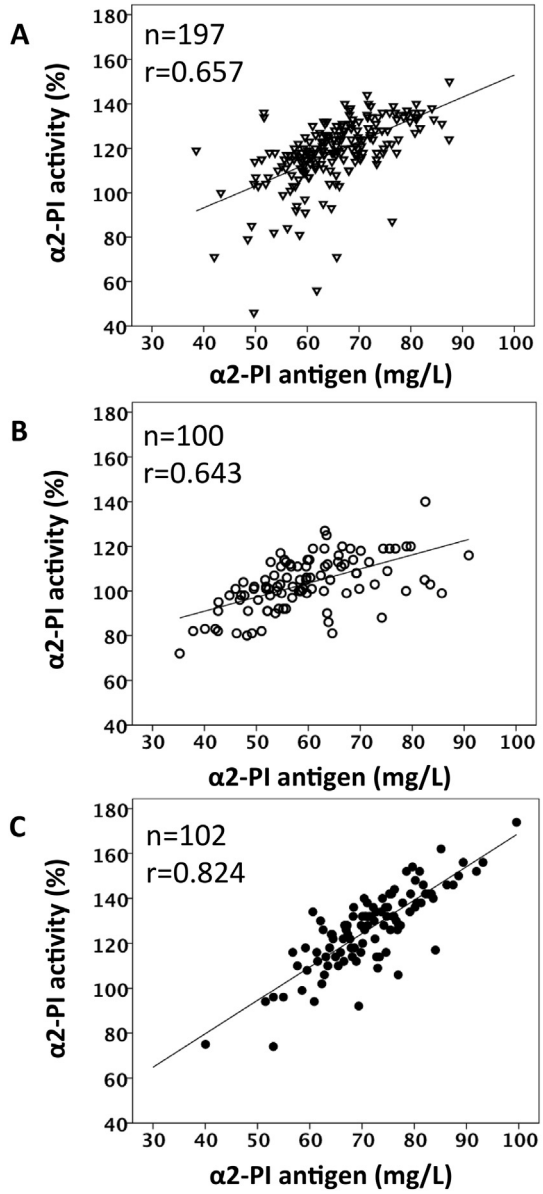

Fig. 5. Correlation of α_2-PI antigen and activity values measured in plasma samples of different groups. A: healthy individuals (n = 197), B: acute ischemic stroke patients on admission (n = 100) and C: venous thromboembolism (n = 102).

strong correlation between total α_2-PI antigen levels and α_2-PI activity for each group (r = 0.657, r = 0.643, r = 0.824, p < .001 for healthy, AIS and VTE group, respectively) (Fig. 5).

3.5. Epitope mapping of the 10/5A8 monoclonal antibody

Immunoblotting experiments indicated that the monoclonal antibody recognize an epitope present in native α_2-PI that was destroyed by denaturation with SDS (data not shown). To prove that the monoclonal antibody used in the ELISA method react equally well with the intact and N- and/or C-terminally truncated isoforms, i.e. measure total α_2PI antigen level, we immunoprecipitated the α_2PI from pooled human plasma with the 10/5A8 monoclonal antibody immobilized on Sepharose 4B beads. After washing, α_2-PI antigen on the beads was digested with trypsin or Asp-N endopeptidase and the proteolytic fragments were identified by MALDI-TOF MS analysis. The identified peptide fragments covered 74% of the full-length protein. Table 3 and

Table 3
The monoclonal capture antibody reacts with all known isoforms of α_2-PI.

Peptide mass (calculated)	Peptide mass (measured)	Position and sequence	α_2-PI isoform identified by the peptide	Enzyme
1953.07	1953.15	7–24 QLTSGPNQEQVSPLTLLK	Met1	Trypsin
1369.76	1369.87	13–24 NQEQVSPLTLLK	Asp1	Trypsin
1833.87	1833.99	449–464 LVPPMEEDYPQFGSPK	C-terminally intact	Trypsin
1749.88	1750.06	422–437 DSPGNKDFLQSLKGFP	C-terminally truncated at position 437	Asp-N
1364.73	1364.66	428–439 DFLQSLKGFPRG	C-terminally intact	Asp-N
1038.49	1038.44	456–464 DYPQFGSPK	C-terminally intact	Asp-N

Mass spectrometric identification of N- and C-terminal peptide fragments obtained after trypsin or Asp-N endopeptidase digestion of α_2-PI immunoprecipitated from pooled normal plasma with the monoclonal antibody 10/5A8.

Fig. 6 show only the peptides identified at the N- and C-terminal part of the molecule. In the case of trypsin digestion the overlapping Q7-K24 and N13-K24 fragments were identified at the N-terminal end, which means that both Met1-α_2PI and Asn1-α_2-PI were captured from the plasma (Fig. 6). Concerning the C-terminal end, both Asp-N and trypsin digested fragments were identified. The overlapping L449-K464 tryptic and D456-K464 Asp-N fragments originate from the non-truncated C-teminal end of the molecule. The D422-P437 fragment obtained by Asp-N digestion could only be identified if α_2-PI was truncated already in the plasma by an unknown proteinase, which can hydrolyze peptide bonds on the C-terminal side of prolyl residues. On the contrary, the fragment D428-G439 originated from the non-truncated form. The results proved that the epitope of the antibody is located in the central part of the protein because it precipitated all of the four isoforms of α_2-PI present in the plasma.

4. Discussion

Some earlier studies have generated mAbs reacting with α_2-PI and characterized their epitope specificity. Monoclonal antibodies JTPI-1 and JTP-2 directed towards the internal region of α_2-PI were used for the detection of free α_2-PI (not in complex with plasmin) in a sandwich type immunoassay (Mimuro et al., 1987). However the assay was not evaluated. In another study affinity-purified polyclonal antibodies reacting with native α_2-PI or with the N-terminal peptide of Met-α_2-PI were used as capture antibodies and a monoclonal antibody specific for the C-terminus of α_2-PI was used as tag antibody to develop ELISA methods for the measurement of C-terminally intact α_2-PI total plasminogen binding α_2-PI and non-truncated α_2-PI (Met-PB-α_2-PI), respectively (Uitte de Willige et al., 2013). The antibody specific to the N-terminal 12-mer peptide however did not respond to the W allele of SNP R6W located in the middle of the peptide, thus the assay can be used only for RR homozygote individuals. In another study affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids Gln14 to Pro30 of the α_2-PI protein was used as capture and HRP-conjugated rabbit anti-human α_2-PI antibody was used as tag antibody to measure total free α_2-PI in the plasma. Pooled normal plasma was used for calibrating the assay and results were expressed in arbitrary units. Total α_2-PI free α_2-PI antigen and activity levels did not show clear correlation (Uitte de Willige et al., 2011).

In our study from mouse monoclonal antibodies raised against α_2-PI an antibody specific to the internal region of the protein was selected. Using this antibody as biotinylated capture antibody and an HRPO-

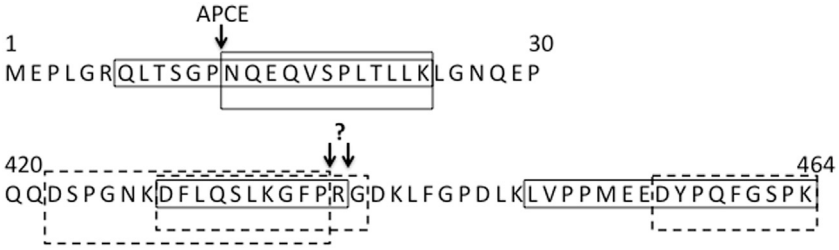

Fig. 6. Peptid fragments identified on the N- and C-terminal end of α_2-PI. APCE, *a*ntiplasmin *c*leaving *e*nzyme;?, unidentified proteolytic enzyme; amino acids encircled with solid line, tryptic fragment; amino acids encircled with dotted line, Asp-N fragment.

labeled polyclonal anti- α_2-PI antibody we developed a new sandwich ELISA for the measurement of total free α_2-PI antigen level in human body fluids. The assay could be performed within three hours. The ELISA was shown to be highly sensitive and reproducibility and recovery tests demonstrated good performance.

A reference interval of 48–85 mg/L (72–128%) for plasma total α_2-PI mass concentration was established. The distribution of total α_2-PI levels measured in 197 healthy individuals was normal and no gender-difference was observed. It is to be noted that the mean plasma value (66.34 mg/L) well agreed with the estimated 1 μM plasma concentration of α_2-PI based on electroimmunoassay measurements (Collen and Wiman, 1978). Total α_2-PI antigen concentration in our healthy control group showed an independent association with BMI and fibrinogen level and was not influenced by age and gender. Positive association between BMI and α_2-PI activity was also published by Meltzer et al. (Meltzer et al., 2010) however they found a negative correlation between α_2-PI activity and age. We observed a similar trend between α_2-PI activity and age, but the association was not statistically significant ($r = -0.096$, $p = .180$).

The assay did not measure PAP complex therefore it allows an accurate determination of the total free plasma α_2-PI concentration in cases when PAP complex levels are markedly increased such as disseminated intravascular coagulation or in recipients of thrombolytic therapy. The total α_2-PI antigen levels measured by our ELISA showed a good correlation with α_2-PI activity levels even though the activity assay is mainly sensitive to the levels of C-terminally intact α_2-PI (Uitte de Willige et al., 2011). Interestingly, the correlation between the total α_2-PI antigen and activity levels was much stronger in the VTE group. This may be simply the result of the wider range of α_2-PI levels in this patient group. Another possible explanation may be that the amount of α_2-PI form that inhibits soluble plasmin is increased in this patient group, however this assumption needs to be confirmed by further studies.

Proteins related to the fibrinolytic system are known to exist also in other body fluids. Determination of α_2-PI in body fluids could be helpful for understanding the physiological significance of the fibrinolytic system. To demonstrate that the assay is suitable for the determination of α_2-PI in other body fluids we determined α_2-PI and total protein concentrations in CSF samples of 47 healthy individuals. α_2-PI was well measurable from 1:10 diluted CSF samples. The calculated median α_2-PI concentration was 132 (range: 36–379) μg/L. When normalized for total protein concentration the results showed normal distribution. We found only one report investigating the α_2-PI antigen concentration in CSF samples with a mean value of 244 ± 66 mg/L (mean ± SD) measured in 8 patients' CSF (Matsuo et al., 1982).

The epitope mapping indicated that the monoclonal antibody captured all four isoforms of α_2-PI from the plasma that means the assay, indeed, measures total α_2-PI. In addition, using Asp-N digestion we identified a peptide fragment (D422-P437) on the C-terminal part of the molecule, which could be found only if the C-terminal truncation of α_2-PI was the results of cleavage between P437 and Arg438 by an unidentified plasma protease. The exact position of C-terminal cleavage is yet to be identified. Experiments in which α_2-PI was digested with trypsin and the plasminogen binding ability of the truncated protein was analyzed supposed that non plasminogen binding α_2-PI is truncated by at least 26 amino acids at its C-terminus. In vitro experiments have identified enzymes that can cleave PB-α_2-PI, such as elastase (Klingemann et al., 1981; Brower and Harpel, 1982; Kolev et al., 1994) and MMP-3 (Lijnen et al., 2001), but the in vivo responsibility of these enzymes was not confirmed. Our results indicate the cleavage of 27 amino acids from the C-terminus in the plasma, however further studies are needed to confirm the result and find the proteolytic enzyme which can hydrolyze peptide bonds on the C-terminal side of prolyl residues and responsible for the cleavage of α_2-PI.

In conclusion we developed a new sandwich ELISA method for the determination of free total α_2-PI in plasma and other body fluids. We generated a biotinylated monoclonal antibody specific to the internal part of the α_2-PI molecule this way it captured all isoforms present in the plasma and HRPO-labeled polyclonal anti-α_2-PI antibody was used to detect the captured antigen. The assay was well reproducible and showed good recovery and strong correlation with the activity measurement. Using the assay a reference interval of 45–86 mg/L was established for total α_2-PI mass concentration in the plasma.

Conflict of interests

None of the authors has a conflict of interest to declare.

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