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# Native chromatographic sample preparation of serum, plasma and cerebrospinal fluid does not comprise a risk for proteolytic biomarker loss

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ABSTRACT

We recently developed a native multidimensional chromatographic method for serum and plasma fractionation for proteomic biomarker search. This method has several advantages:parallelization and automation, high reproducibility and proteome coverage, flexible dynamic range with respect to molecular weight and sample amount, optional enzymatic and immunological analytics additional to mass spectrometry, retaining metabolites, and information on complex formation, modification, and fragmentation of constituents. Nevertheless, native conditions have the probable risk of proteome alteration and biomarker loss by intrinsic proteinases.

Hence, we tried to quantify here intrinsic proteolytic activity in native samples and fractions from serum, plasma and cerebrospinal fluid, as well as the effectiveness of intrinsic anti-proteinases during sample handling and preparation under our fractionation conditions. Therefore, we used several quantitative measures: (1) total proportion of intrinsic protein and peptide fractions, (2) azocasein hydrolysis and (3) mass spectrometric protein coverage and peptide numbers. To 1: In all non-fractionated specimens, neither decrease of protein concentration or molecular weight nor increase of peptide concentration was found after variable clotting or pre-incubation time. To 2: No azocasein hydrolysis was seen in these samples when prepared within a few hours at room temperature. Trypsin, when added in concentrations not higher than 0.85 μg/mL (0.04 μM), even was completely inhibited. Moreover, in native 1-D fractions no proteinase activity could be observed. To 3: Mass spectrometry confirmed that neither protein coverage nor peptide numbers differ significantly in 1-D or 2-D fractions after variable incubation time. These results suggest that intrinsic, native proteinase inhibitors potentially protect the proteomes considered, enabling "top-down" proteomic approaches under native conditions with serum, plasma and cerebrospinal fluid.



## 1. Introduction

Native chromatographic fractionation of intact proteins in "top-down" proteomics could be a valuable approach complementary to denaturing techniques such as 2-DE, and "shotgun" platforms, and also to peptidomics. Recently, we were able to develop a native, multidimensional pre-fractionation method with serum samples as a complementary method to pipelines applying depletion, early denaturation, and "bottom-up" strategies [1]. This method combines size exclusion (SEC, 1-D), followed by anion exchange (AEC, 2-D) and lectin affinity (LAC, 3-D) chromatography. Without exception, all intrinsic components and information are preserved after fractionation, including natural complex formation, fragmentation, and biological activities. Such information is beneficial for comprehensive profiling, for biomarker search, as well as for efficient evaluation. Moreover, the liquid state of all sub-fractions allows parallelization and automation with high reproducibility and recovery.

Blood serum, plasma and cerebrospinal fluid (CSF) are low-invasive, accessible samples favored in routine clinical and biomarker searches. Analyzing these materials is challenging not only with respect to their complexity and the dynamic range

*Abbreviations:* AUC, area under the curve; LMW, low molecular weight; CSF, cerebrospinal fluid; FPA, fibrinopeptide A; SEC, size exclusion chromatography; AEC, anion exchange chromatography.

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of probable biomarker concentrations, but also to their probable degradation activities during sample preparation under native conditions.

In considering the search for biomarkers >15 kDa, it is crucial to monitor the activity of all classes of proteinases, *i.e.* enzymes that cleave within the polypeptide sequence of proteins. These activities could abolish valuable tryptic peptides. Thus, intrinsic, high abundant proteinases, *i.e.* coagulation and complement components, and some tissue-derived enzymes may change proteome composition during fractionation. In considering this risk, inhibitory cocktails are frequently used as a precaution to prevent potential proteolysis in "top-down" proteomics with these materials. However, serum, plasma, and CSF actually contain a high capacity and a broad specificity of natural proteinase inhibitors. Exemplarily, in serum, serine proteinases are inhibited by alpha-1-antitrypsin (20–53 μM) [2], alpha-2-antiplasmin (~1 μM) [3], alpha-2-antithrombin (~2 μM) [4], and alpha-2-macroglobulin (~1 μM) [3]. Furthermore, cysteine proteinases and metalloproteinases are inhibited by kininogen (~2 μM) [5] and TIMP 1/2 (~4 μM) [6], respectively. Indeed, intrinsic proteinase inhibitors impair tryptic digest prior to mass spectrometry [7]. Therefore, retaining these active inhibitors during sample preparation should also protect the proteomes, enabling "top-down" proteomic pipelines with native material. In contrast, specific peptidases known as important and specific modifiers of biologically active oligopeptides are not relevant for this question, as well as studies using artificial peptides.

Typical serum peptide patterns are well known in the low molecular weight (LMW) fraction. They probably stem from inherent natural *in vivo* activities (mainly coagulation and the complement system) [8] which cannot be circumvented and which could be still active during prolonged sample storage [9,10].

So far, studies regarding the influence of subsequent sample preparation conditions have been controversial. The effect of identified peptidase activity varied from virtually none on proteins to the rapid degradation of certain peptides and appearance of distinct new peptide entities. In blood-derived samples, there was no measurable difference in protein composition between serum samples analyzed immediately and after up to 8 h clotting time at room temperature – except for clotting-related components [11,12]. Recently, Zimmerman et al. [13] showed the stability of plasma proteins by using shotgun proteome analysis. Almost no damage on the peptide level could be observed, applying very long sample preparation times and multiple freeze-thaw cycles. In contrast, degradation could be observed when native serum or plasma was spiked with fibrinopeptide A (FPA) [14]. Moreover, peptide-producing activity of added α-thrombin was shown with a LMW fraction of plasma proteins or purified hemopexin [15]. These activities have been used as indicators for distinctive degradation during sample preparation. However, the first analyte, FPA represents a natural metabolite of clotting activity that has known regulatory properties [16,17]. This component is specifically and rapidly cleared from plasma [18] and has been identified as a biomarker in combination with its naturally occurring fragments [19]. The second approach focuses on plasma fractions depleted of components above 30 kDa or even on a purified protein. Thus, both samples are relieved from their natural proteinase inhibitors. Moreover, using amino-terminally truncated peptides as indicators, "exo"-peptidase activities rather than proteinase action are likely revealed. Thus undoubtedly, by monitoring peptides in artificial samples, neither of the approaches could verify proteolytic activities present in common specimens. Similar results have been published for CSF. Significant peptidase activities are likely since profiles of small peptides and amino acids change during pre-analysis [20,21]. Contamination by blood may be one source [21–23], by cells, another [20]; both may be avoided. On the other hand, some proteins are extremely stable when CSF samples are being handled [24]. Thus, proteolysis seems to be dependent on the actual handling conditions. To avoid uncertainty, strictly standardized specimen collection, handling, and prompt analysis [10,25,26] are recommended. The preventative use of proteinase inhibitors is not recommended and has to be examined with respect to the known interference and the side effects of these substances [10,27,28].

Besides sample acquisition, pre-analytical steps comprise long-term storage and delays prior to and during the fractionation process. Although there are many results with special storage conditions that are not in our focus, to our best knowledge there is no information on the actual quantitative impact of intrinsic proteolysis under native fractionation. Therefore, we analyzed here the effectiveness of intrinsic anti-proteinases by quantifying proteolytic products of freshly harvested samples after variable pre-incubation time applying our native multidimensional pre-fractionation method. We analyzed not only the influence of sample pre-treatment but also sample modification during fractionation. Measures used are (1) the percentage of intrinsic peptide fractions of samples as obtained after SEC, (2) disintegration of azocasein as a ubiquitous substrate for all known classes of proteinases by non-fractionated samples both with and without trypsin addition, (3) azocasein hydrolysis by several fractionations of samples from both healthy subjects and patients and (4) protein coverage and peptide hits as quantified after mass spectrometry. All assays were performed under conditions applied to our proteomic pipeline [1] and additionally with prolonged incubation times in order to detect even miniscule effects.

## 2. Materials and methods

### 2.1. Materials

If not stated otherwise, all chemicals used were of analytical grade and purchased from Carl Roth (Karlsruhe, Germany), Fluka (Deisenhofen, Germany), Serva (Heidelberg, Germany), or Merck (Darmstadt, Germany).

### 2.2. Samples

The study was approved by the local Ethical Committee (1940–01/07). The informed consent of all participating subjects was obtained. Blood samples from healthy volunteers and patients exhibiting severe psoriasis were obtained in the morning. Blood was drawn by venipuncture into 9 mL-monovettes® (Sarstedt, Nümbrecht, Germany) using $20G \times 1^1/_2''$ needles. Supernatants were prepared according to standard operation procedures. To obtain serum, blood was collected into serum monovettes (02.1063.001). If not stated otherwise, the clotting time was 90 min at room temperature. Samples were centrifuged at $1500 \times g$ for 10 min at 20 °C, immediately aliquoted and stored at −84 °C. Plasma was obtained by drawing blood into citrate- or EDTA-containing containers (02.1067.001 and 02.1066.001, respectively). To study the time-dependent evolution of peptide fractions, clotted blood and plasma samples were incubated at room temperature up to 10 h. Thereafter, samples were centrifuged, aliquoted and stored at −84 °C until analysis. For the analysis of activated plasma, plasma samples were thawed. Polystyrene granula from serum monovettes and calcium chloride (Sigma–Aldrich, Taufkirchen, Germany, C-3881) were added to a final concentration of 50 mM and incubated at room temperature for up to 10 h. Thereafter, samples were centrifuged at $1500 \times g$ for 10 min at 20 °C, aliquoted and stored at −84 °C. CSF samples were obtained after written informed consent from patients undergoing diagnostic lumbar puncture,

who agreed to give an additional 10 mL CSF for proteomic analyses unrelated to their disease, and subsequently had unremarkable clinical laboratory CSF results. Samples were collected into 4 mL vials (Greiner Bio-One, 115261, Frickenhausen, Germany). The vials were cooled immediately at the bed-side using an ice/NaCl mixture and centrifuged within 15 min (800 × *g* for 5 min). Supernatants were snap-frozen with liquid nitrogen and subsequently stored at −84 °C.

### 2.3. Size-exclusion chromatography (1-D SEC)

SEC, 1-D fractionation, and analysis of the fractions resulting from blood-derived samples were performed as described previously [1]. Briefly, 1 mL of diluted samples was injected (sample content: 0.5–0.9 mL), separated on a HiLoad Superdex™ 200 column (16/60, GE Healthcare, Munich, Germany), and controlled by an Äkta purifier™ system (GE-Healthcare). To fractionate serum and supernatant from activated plasma, 10 mM Tris-HCl (Merck 1.08382.2500) was used as an elution buffer containing 150 mM NaCl (pH 7.4). For citrate and EDTA plasma separation, 1 mM sodium citrate monohydrate or EDTA (Sigma–Aldrich, Taufkirchen, Germany, E-9884) was added to prevent coagulation on the column, respectively. The running temperature was 20 °C. After the void volume of 37 mL, 96 fractions of 1 mL were collected into chilled 96-deepwell micro plates using a flow rate of 1 mL/min. Column calibration was performed using thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), BSA (67 kDa), Chymotrypsin (25 kDa) and RNase (13.7 kDa). The percentage of peptide fractions (<5 kDa), was calculated by Unicorn™ software (GE-Healthcare), using areas under the curves (AUC) at 280 nm.

With CSF, 12.5 μL original sample or concentrated samples (Vivaspin 6, GE Healthcare, MWCO 5 kDa, 28-9322-94) were separated on a Superdex™ 200 GL column (5/150, GE Healthcare) controlled by the Äkta purifier™ system in 10 mM Tris-HCl including 150 mM NaCl (pH 7.4) at 20 °C. After the void volume of 1 mL, 48 fractions of 50 μL were collected into micro plates using a flow rate of 50 μL/min.

### 2.4. Anion exchange chromatography (2-D AEC)

AEC, 2-D fractionation was done as described previously [1] with slight modifications: (i) separation matrix was 100 μL Toyopearl DEAE-650 M (Tosoh Bioscience GmbH, Stuttgart, Germany) instead of 300 μL DEAE cellulose; (ii) consequently, a slightly different elution protocol (see Supplementary Data, Fig. S6) was used, and (iii) two samples were processed simultaneously on one array of 96 micro columns to obtain best reproducibility.

### 2.5. Proteinase assay

The azocasein assay was performed in 384-masterblock deep well plates (Greiner, No. 781270). All pipetting steps into micro plates were performed simultaneously using a CyBi-well™ (CyBio AG, Jena, Germany) with 96 channels. Samples (35 μL unprocessed specimens or fractions therefrom) were mixed with 35 μL 2.5%-azocasein (Sigma–Aldrich, 11610) solution in water and incubated at room temperature up to 19 h. After precipitation with 130 μL TCA (6%, Merck 41443874), 25 μL of the supernatant was neutralized with 75 μL sodium hydroxide (500 mM). Liberated colored LMW fragments were measured at 442 nm using a Spectramax Plus$^{384}$ micro plate reader (Molecular Devices, Sunnyvale, CA, USA). To exclude false positive signals caused by *e.g.* bilirubin-containing protein fractions, a distinct blank value was used for each fraction. For blank values, each fraction was precipitated with TCA immediately after the addition of azocasein.

To determine the sensitivity of azocasein hydrolysis, hyperbola parameters were fitted to data from dilution series of trypsin (Serva 37291) using SigmaPlot 11.0™ (*cf.* Fig. 4).

Additionally, to detect proteolytic activities under 1-D separation, some samples were spiked with azocasein (1 mg/mL) prior to SEC.

The proteinase inhibition effects of serum, plasma, and CSF samples were measured using spiked trypsin. Samples (28 μL) were mixed with 7 μL trypsin (final concentration in sample mixture: 0 (buffer), 50, 100, 200 and 400 mU/mL) and 35 μL 2.5% azocasein solution. A buffer control sample (10 mM Tris/HCl, 150 mM NaCl, pH 7.4) was included to determine the non-inhibited trypsin activity. Additionally, a BSA control sample (40 mg/mL, Serva 11932) was used to determine trypsin inhibition by competition of containing proteins with the azocasein substrate. The azocasein assay was conducted as described above.

### 2.6. Peptide quantification after denaturation

#### 2.6.1. Sample peptides determined by UV measurement

In order to quantify all free peptides and peptides released from their parent molecules, samples were denatured by TCA. 140 μL citrate plasma (non- and ten-fold diluted, with and without 400 mU/mL trypsin) was incubated for 0 h and 16 h at 20 °C. The peptide amount in the supernatant was determined by measuring the absorbance at 280 nm after the addition of 175 μL 6% (w/v) TCA.

#### 2.6.2. Fraction's peptides determined by mass spectrometry

Selected 2-D fraction pairs were analyzed by mass spectrometry. 60 μL of these fractions were digested according to [29]. Alternatively, fractions were filtered through a spin column (Vivaspin 500, Sartorius, MWCO 5 kDa) prior to dialysis and trypsin addition. Liquid chromatographic separation prior to mass spectrometry detection was performed on a Hypersil Gold UHPLC column (1.9 μm, 50 × 1.0 mm) with an Accela 1250 UHPLC system (both Thermo Fisher Scientific, Waltham, MA, USA) using binary gradient elution of the mobile phase, *i.e.* 0.1% formic acid in (A) water and in (B) acetonitrile at a flow rate of 150 μL/min, throughout (0–1 min 5% B, 21 min 30% B, 24 min 40% B, 25–26 min 90% B, 26.1–30 min 5% B). Tandem mass spectrometry (MS/MS) measurements were carried out on a LTQ Orbitrap Discovery (Thermo Fisher Scientific, Waltham, MA, USA) by positive heated electrospray ionization (H-ESI) at a vaporizer temperature of 200 °C. Sheath gas flow (30.0) and auxiliary gas flow (10.0) were used to dry the ionspray (both nitrogen gas flows in arbitrary units). The ionization voltage and the temperature of the ion transfer tube were set to 4.5 kV and to 275 °C, respectively. The MS/MS system operated in data-dependent TOP10 mode using 1 microscan. For this purpose, ions have been monitored in the LTQ ion trap in full scan centroid mode at $m/z$ 350–1700. The ten most intensive ions run through collision-induced dissociation (CID) for further orbitrap high resolution (30,000) analysis (profile data type). Wideband activation was used. The automatic gain control (AGC) target value for the orbitrap mass analyzer in full scan mode was $1.0 \times 10^6$. All measurements were performed with the lock mass option ($m/z$ 445.120025) for internal calibration. The LC–MS/MS operated *via* graphical interface of the Xcalibur software 2.1. Analysis was done with Proteome Discoverer 1.3 and Sequest database search algorithm applying a false discovery rate of 0.01. To identify non-tryptic peptides, samples were processed by omitting enzyme definition. Peptide numbers and protein coverage were used as semi quantitative measure. To detect differences between two samples, analysis with SIEVE 1.3 was done. Results were filtered by the

charge-dependent Xcorr (Xcorr 1.5, 2.0, 2.2 and 2.5 for charge 1, 2, 3, and > 3, respectively) and by a $p$-value of 0.05.

## 3. Results

### 3.1. Peptide fractions in blood-derived samples and CSF

The first step of our multidimensional pre-fractionation approach is size exclusion chromatography (SEC). This chromatography also allows quantification of LMW fractions in protein samples. The separation of serum or plasma showed that about 7% of their proteomes were in the molecular weight range of peptides. As shown in Fig. 1 and Table 1, they typically divided into three fractions with similar percentages. Surprisingly, no augmented peptide concentration was found in patients exhibiting strong chronic inflammation usually accompanied by various proteolytic activities (Table 1). In order to see if proteinase activity does take place during sample preparation, blood products were incubated at room temperature for various periods and subsequently separated by SEC. The overlay in Fig. 1 shows high reproducibility of ratio and molecular weight of peptide and protein fractions, independent of sample preparation time. Neither prolonged clotting time (serum) nor incubation time (plasma) had any impact on the percentage of the peptide fractions after SEC (Fig. 2). Even clotting reactivation time of 10 h at room temperature did not influence native SEC results (data not shown). To insure that proteolytic peptides are released from their mother molecules, SEC conditions were slightly modified. However, neither different NaCl concentrations (25 mM or 500 mM) nor the addition of detergents (0.2% tween 20 or 0.1% deoxycholate) had any impact on peptide fractions. Even TCA denaturation did not reveal additional peptides (data not shown).

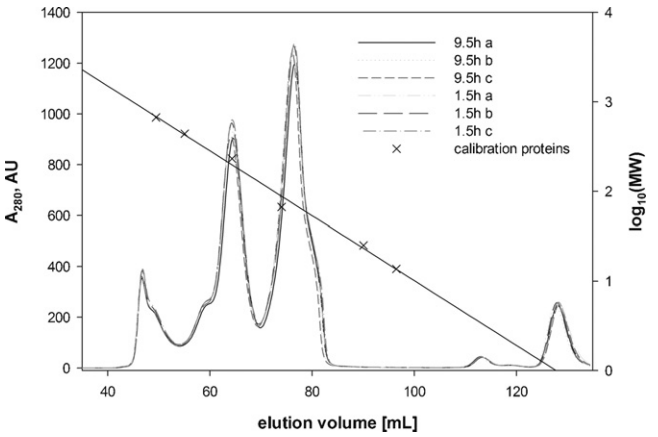


**Fig. 1.** Reproducibility of SEC exemplary for serum. Blood from one healthy volunteer clotted for 1.5 or 9.5 h. 0.68 mL serum was injected onto a HiLoad Superdex$^{TM}$ 200 column and separated in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl at a flow rate of 1 mL/min. Three individual runs were applied (a–c). Five proteins (13.7–660 kDa) were used for calibration.

In contrast to blood, the majority of UV-absorbing material of rapidly processed but non-concentrated CSF was found in three fractions below 5 kDa. The minority consists of intact plasma proteins (*e.g.* Albumin) of high molecular weight (see Supplementary Data Fig. S1). Incubation of CSF samples at room temperature led to a slight shift to small molecular weights only of the LMW content (Fig. 3A). However, the sum of peptide amount remained constant up to 5.5 h and was found only slightly diminished after unusually long pre-incubation at room temperature. Correspondingly, the total amount of protein fractions (>30 kDa) remained constant

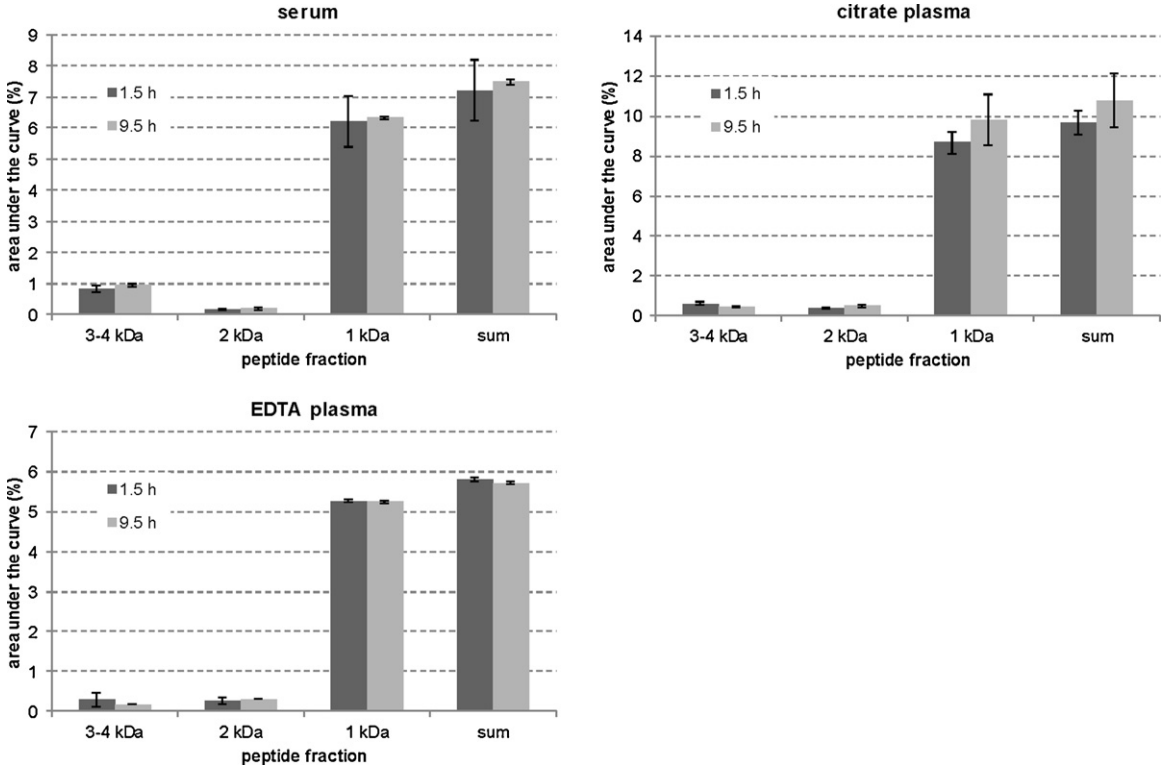


**Fig. 2.** Quantification of peptide fractions by SEC. Blood was drawn from three different healthy volunteers, one for serum, one for EDTA plasma, and one for citrate plasma generation. The samples were incubated at room temperature for 1.5 or 9.5 h and then separated by SEC. The percentage of peptide fractions (<5 kDa), was calculated by Unicorn$^{TM}$ software (GE-Healthcare), using AUC at 280 nm. The data represent mean and SD of technical triplicates. Sum of areas under the curve of all individual peaks was set to 100%.

**Table 1**
Percentage of the three peptide fractions of blood derived samples of various subjects obtained under standard operation procedure.

| Subject type | Sample type | Peptide fraction | | | *n* | *m* |
|---|---|---|---|---|---|---|
| | | Mr ≈4 kDa | Mr ≈2 kDa | Mr ≈1 kDa | | |
| H | Serum | 0.84 ± 0.22 | 0.33 ± 0.23 | 6.37 ± 1.87 | 19 | 6 |
| | EDTA plasma | 0.19 ± 0.10 | 0.29 ± 0.07 | 5.62 ± 1.40 | 5 | 3 |
| | Citrate plasma | 0.59 ± 0.02 | 0.51 ± 0.13 | 6.20 ± 2.49 | 4 | 2 |
| P | Serum | 0.13 | 0.76 | 3.03 | 1 | 1 |
| | EDTA plasma | 0.22 ± 0.19 | 0.44 ± 0.21 | 5.14 ± 1.26 | 4 | 6[a] |

Data represent mean ± SD of the percentage of peptide fractions (AUC, $A_{280}$) after SEC (*cf.* Fig. 2). Number of runs (*n*) includes m subjects. Measurements include single runs and replicates (2–5) of aliquots after storage at −80°.
H, healthy volunteers (laboratory staff members), 3/3 (male/female), 33.7 ± 12.9 years; P, patients suffering from severe psoriasis.
[a] Single samples of 2 patients and 2 pooled samples of 2 patients each, 3/3 (male/female), 59.0 ± 13.3 years, Psoriasis Area and Severity Index >15 [43].

during the whole observation time in three different CSF samples with individually different total protein concentration (Fig. 3B).

Taken together, prolonged sample preparation time did not influence considerably the amount of peptides in different sample types indicating the lack of quantitatively significant proteolysis.

### 3.2. Inhibition of proteinase activity

Besides the direct quantification of peptide fractions in samples, enzymatic assays were performed to investigate the (anti)-proteolytic activity of serum, plasma and CSF. The universal substrate azocasein was applied to a protein sample and supplied with varying amounts of trypsin. Trypsin was used as a model proteinase, since the majority of proteinases in the three body fluids are also serine proteinases. Buffer and BSA were used as controls instead of serum, plasma or CSF. BSA should confirm that proteinase inhibition was not only due to competition of azocasein with the high number of plasma proteins. In case of blood-derived samples, proteinase activity, *i.e.* liberation of LMW fragments from azocasein, was not observed under conditions used for proteomic sample preparation (Fig. 4 and Supplementary Data Fig. S2 (incubation 1.5 h, without trypsin)). The same was true for patient samples (data not shown). Moreover, activity of added trypsin was always strongly inhibited, and to a similar extent by all blood-derived specimens even during 19 h incubation. This inhibition was virtually complete by non-diluted as well as by 10-fold diluted plasma up to 100 mU/mL trypsin and up to 1.5 h. As expected, the inhibitory activity of plasma declined gradually with dilution.

Like blood-derived samples, CSF did not show azocasein hydrolyzing activity during 19 h incubation (Supplementary Data Fig. S3). The same was true with prolonged incubation times up to 72 h which we performed to take the very low protein concentrations in CSF into account (data not shown). CSF also inhibited added trypsin activity. Having a much lower protein concentration, the

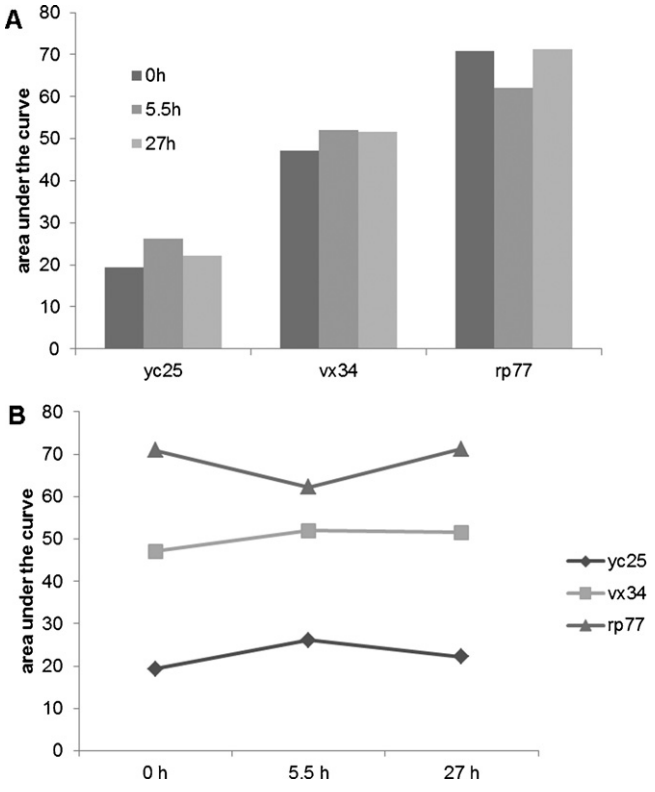


**Fig. 3.** Size of fractions from CSF samples after three pre-incubation times and SEC. CSF samples from three different patients were thawed, stored at room temperature and then analyzed by SEC (see Section 2). (A) Ratio of peptide fractions Data represent mean and SD of the areas of peptide fractions (<5 kDa, see Fig. S1) of three samples. Sum of AUC of all individual peaks of the chromatogram was set to 100%. (B) Sums of absolute areas of the protein fractions (>30 kDa) of the three samples in (A).

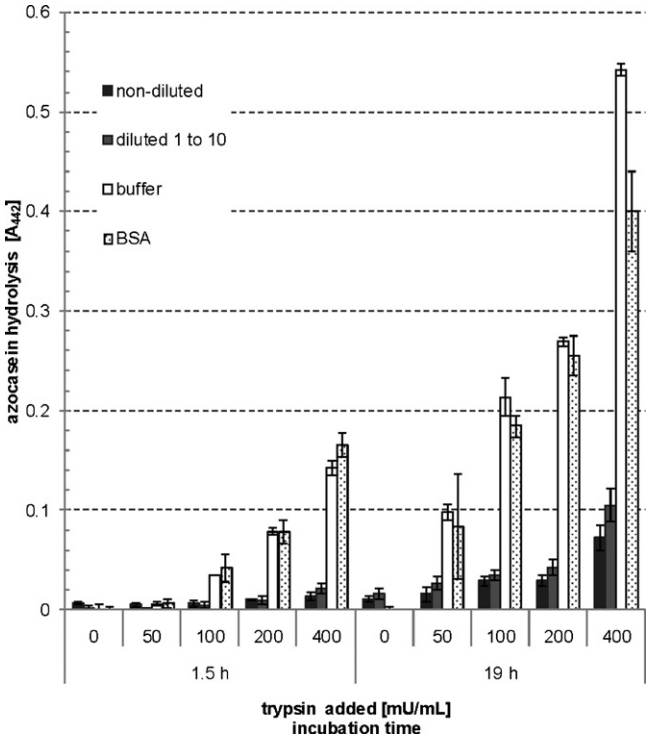


**Fig. 4.** Hydrolysis of azocasein by trypsin and its inhibition by citrate plasma. Azocasein was incubated with increasing concentrations of trypsin for 1.5 and 19 h at 20 °C, Additive: non-diluted and 10-fold diluted plasma; buffer, without any protein, BSA: 40 mg/mL bovine serum albumin. Bars represent mean values ± SD of three experiments. For details, Section 2.

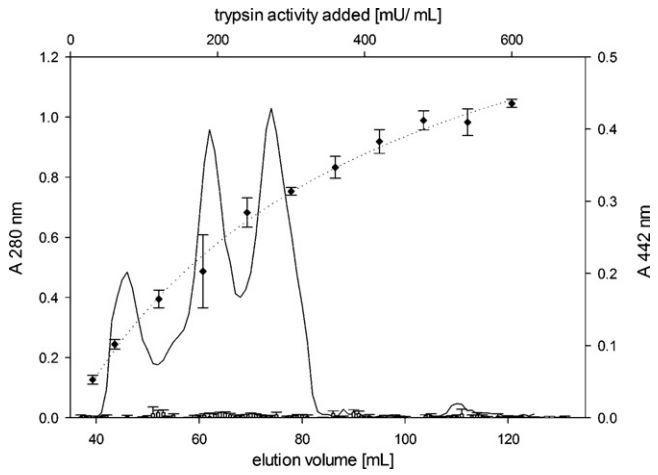


**Fig. 5.** Azocasein hydrolyzing activity of native 1-D fractions of plasma and porcine trypsin. Citrate plasma from a healthy volunteer was fractionated by SEC. The fractions were tested for proteinase activity (see Methods). Left ordinate, solid line: $A_{280}$ of 150 μL aliquots of all fractions determined in micro plates; Right ordinate, bars: LMW products (mean value ± SD of triplicates, $A_{442}$) of 1-D fractions after incubation with azocasein for 19 h at room temperature; right ordinate, dotted line: $A_{442}$, trypsin activity under identical conditions; for details see Section 2.

inhibitory capacity of CSF was predictably lower than serum and plasma (Fig S3).

These results indicate that serum, plasma, and CSF not only lack proteinase activity but even show anti-proteolytic activity under appropriate conditions.

### 3.3. Proteinase activity of SEC fractions

Thus, proteinases in the blood are probably strongly regulated by their corresponding anti-proteinases. However, chromatographic fractionation may separate them from each other. Therefore SEC fractions from serum, plasma and CSF samples were tested for their proteinase activity using azocasein as a substrate. As shown in Fig. 5, within all SEC fractions from healthy citrate plasma, no substantial azocasein hydrolyzing activity could be detected although unusually long incubation times (19 h) have been applied together with high temperature. The same results were obtained with EDTA plasma and serum from healthy volunteers and also for patient samples. SEC fractions of CSF were also tested for their proteinase activity. Due to their low protein concentration, CSF samples were 195-fold concentrated (Vivaspin 6, MWCO 5 kDa) prior to SEC. As shown in Fig. S4 (Supplementary Data), no azocasein hydrolyzing activity could be identified.

In order to test proteinase activity during SEC, azocasein was added to EDTA plasma prior to chromatography. Subsequently, azocasein was recovered completely and showed an identical molecular weight to the control (without plasma). Furthermore, no azo-fragments appeared within LMW peptide fractions (data not shown).

To check proteinase activity in fractions in more depth by mass spectrometry, SEC fractions were incubated for 0 h or 9.5 h at room temperature. To analyze the probable formation of LMW compounds, five randomly selected fractions (Mr ~ 85, 150, 230, 420, 820 kDa) were filtered through a spin column (MWCO 5 kDa) prior to digestion and then processed by omitting enzyme definition. Tryptic digest was still necessary, since the obtained peptides (≤10 kDa) were too large for proper mass spectrometric analysis. To compare fractions after 0 h or 9.5 h incubation, analysis with SIEVE 1.3 was done for each sample pair. Taking all samples together only one peptide of 96 hits increased by factor ≥4. All other peptides were unaffected or even decreased (Supplementary Data, Fig. S5). In accordance to our recent publication [30], we use factor 4 as confident criterion here. Corresponding to the law of error propagation, mass spectrometric results strongly depend on the number and precision of several sample preparation steps. In absence of certain information about these parameters, the use of factor 2 appears not to be sufficient.

Additional analysis with Proteome Discoverer 1.3 showed that the total number of peptides and the sum of the coverage of all fractions did not show significant differences between 0 h and 9.5 h (8 ± 1.5 *vs.* 5.5 ± 3 peptides, 19.4 ± 5.6% *vs.* 17.3 ± 5% coverage, respectively). These data indicate that there are only minuscule intrinsic amounts of LMW peptides and no considerable changes occurred due to longer incubation time of HMW 1-D fractions.

### 3.4. Protein distribution and coverage in AEC fractions

The second step of our native, multidimensional pre-fractionation method is anion exchange (AEC, 2-D). Due to the high dilution rate in these fractions, azocasein assays were not applicable. Consequently, 2-D fractions were analyzed by mass spectrometry after various pretreatments of EDTA plasma. One freshly taken and unprocessed sample was divided into two aliquots and incubated for 1.5 or 9.5 h. Thereafter, both were simultaneously processed with our complete native multidimensional chromatographic method, including SEC and AEC. As shown in Supplementary Data, Fig. S6B, the distribution pattern of protein concentrations in the space of 2064 2-D fractions of EDTA plasma were very similar after 1.5 and 9.5 h pre-incubation. Thus, with only a few exceptions ratios of related fractions were mainly ~1.0 (Fig. S6A). 22 2-D fractions (ratio <0.6 or >1.7, marked in Fig. S6A) were selected from both samples, digested and analyzed by mass spectrometry. Analysis with SIEVE 1.3 of all these samples together revealed 45 proteins. 2 proteins were actually decreased, but they were supported by only 1 peptide and had a very high standard deviation (see Supplementary Data, Fig. S6C). Additionally, as shown in Table 2, neither protein coverage nor the number of peptides decreased in the samples after 9.5 h pre-incubation.

To analyze non-tryptic peptides, selected fractions were further processed by omitting enzyme definition. Due to the very sparse differences, the limited fraction volume, and to the long processing time of the Proteome Discoverer software (up to 40 h per sample), only 5 fractions from each sample were analyzed. Again, neither protein coverage nor the number of peptides decreased in the samples after 9.5 h pre-incubation. Additionally, an increase of the proportion of non-tryptic peptides in the 9.5 h sample was not observed in any of these fractions (data not shown). This is in agreement with no additional peptide formation by proteolysis during SEC.

Additionally, the influence of processing time of SEC fractions on protein distribution and size of peptide fraction was determined. Therefore, SEC fractions were incubated for 0 h or 9.5 h and processed subsequently and simultaneously with AEC. As shown in Supplementary Data, Fig. S7, distribution pattern of protein concentrations is also similar after 0 h and 9.5 h pre-incubation. Six AEC fractions were selected: two with increased, two with decreased, and two with unaffected UV absorbance after 9.5 h pre-incubation. To analyze the LMW content, denatured fractions were filtered through a spin column (MWCO 5 kDa) prior to digestion and then processed by omitting enzyme definition. Tryptic digest was still necessary, since the obtained peptides (≤10 kDa) were too large for proper mass spectrometric analysis. Analysis with Proteome Discoverer 1.3 showed that the total number of peptides was very low (5.6 ± 3.7), since approximately 200–600 peptides per sample were found in non-filtrated samples. Peptide numbers and the sum of the coverage of all fractions together did not show significant differences between 0 h and 9.5 h (31.5 ± 7.5 *vs.* 36.5 ± 8.5

**Table 2**
Selected 22 fractions (see Supplementary Data, Fig. S6A) from each 2-D AEC were digested and analyzed by mass spectrometry. Processing with Proteome Discoverer revealed protein coverage and peptide numbers for each protein. Coverage and peptide numbers of all proteins were summed up for each fraction and the ratio (9.5 h *vs.* 1.5 h) was calculated.

| | 1.5 h | | | | 9.5 h | | | | Ratio | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Coverage | | Peptides | | Coverage | | Peptides | | Coverage | Peptides |
| Fraction | Mean | SD | Mean | SD | Mean | SD | Mean | SD | | |
| 11_16 | 437.2 | 9.2 | 163.0 | 1.0 | 375.1 | 3.6 | 147.5 | 0.5 | 0.86 | 0.90 |
| 11_17 | 327.2 | 5.6 | 105.0 | 0.0 | 534.3 | 28.1 | 207.0 | 4.0 | 1.63 | 1.97 |
| 13_19 | 433.1 | 8.9 | 159.0 | 0.0 | 389.9 | 11.2 | 120.0 | 2.0 | 0.90 | 0.75 |
| 13_26 | 254.4 | 17.8 | 105.5 | 10.5 | 479.8 | 1.3 | 256.0 | 0.0 | 1.89 | 2.43 |
| 13_27 | 763.4 | 28.0 | 389.0 | 24.0 | 774.8 | 1.8 | 419.0 | 8.0 | 1.01 | 1.08 |
| 14_19 | 441.9 | 3.2 | 200.5 | 0.5 | 509.9 | 11.2 | 215.5 | 1.5 | 1.15 | 1.07 |
| 25_31 | 470.6 | 14.5 | 223.5 | 15.5 | 389.5 | 37.7 | 174.0 | 14.0 | 0.83 | 0.78 |
| 25_32 | 326.7 | 51.7 | 133.0 | 21.0 | 390.1 | 56.9 | 165.5 | 31.5 | 1.19 | 1.24 |
| 27_34 | 401.8 | 13.7 | 190.5 | 0.5 | 381.3 | 8.6 | 181.0 | 2.0 | 0.95 | 0.95 |
| 27_35 | 261.0 | 9.7 | 148.0 | 5.0 | 306.2 | 3.7 | 165.0 | 3.0 | 1.17 | 1.11 |
| 35_13 | 556.0 | 11.5 | 195.5 | 2.5 | 577.8 | 17.9 | 199.5 | 7.5 | 1.04 | 1.02 |
| 36_13 | 455.0 | 19.8 | 175.5 | 2.5 | 364.3 | 24.3 | 142.0 | 9.0 | 0.80 | 0.81 |
| 36_18 | 700.3 | 59.6 | 272.5 | 19.5 | 489.1 | 31.9 | 187.0 | 12.0 | 0.70 | 0.69 |
| 36_19 | 394.6 | 6.9 | 152.0 | 3.0 | 462.1 | 29.5 | 199.5 | 3.5 | 1.17 | 1.31 |
| 38_01 | 338.9 | 4.2 | 122.0 | 3.0 | 306.7 | 3.4 | 100.0 | 2.0 | 0.90 | 0.82 |
| 38_02 | 322.4 | 56.5 | 142.0 | 18.0 | 346.3 | 16.9 | 158.5 | 2.5 | 1.07 | 1.12 |
| 40_02 | 274.8 | 5.6 | 131.0 | 7.0 | 298.5 | 21.2 | 132.5 | 10.5 | 1.09 | 1.01 |
| 40_03 | 322.2 | 4.8 | 246.5 | 4.5 | 390.7 | 5.4 | 299.5 | 9.5 | 1.21 | 1.22 |
| 40_04 | 619.2 | 18.3 | 329.5 | 40.5 | 386.5 | 12.6 | 222.5 | 2.5 | 0.62 | 0.68 |
| 40_05 | 408.8 | 15.3 | 169.0 | 5.0 | 366.6 | 13.5 | 191.5 | 26.5 | 0.90 | 1.13 |
| 41_02 | 490.0 | 27.2 | 341.5 | 15.5 | 357.3 | 21.1 | 274.0 | 20.0 | 0.73 | 0.80 |
| 41_03 | 463.7 | 48.0 | 158.5 | 13.5 | 539.1 | 45.8 | 187.0 | 10.0 | 1.16 | 1.18 |

peptides, 131.6 ± 56.6% *vs.* 145.2 ± 29.7% coverage). Additionally, samples were analyzed with SIEVE 1.3 for each sample pair. Taking all samples together, 195 protein hits were found, from which each was supported by no more than a single peptide (data not shown). Neither peptide decreased considerably (≥factor 4) in the 9.5 h sample, and only 2 actually increased (≥factor 4).

Again, it could be shown here that proteolysis does practically not take place during native sample handling and preparation in non-fractionated plasma samples nor in their corresponding 1-D SEC fractions.

## 4. Discussion

Native size exclusion chromatography separates all natural plasma polypeptides according to their native molecular weights. Natural interactions are preserved and 3–4 main groups of high molecular weight protein complexes can be observed above ~ 50 kDa. In addition, unprocessed serum, plasma, and CSF samples contain 3 distinct peptide fractions below a molecular weight of 5 kDa. In serum and plasma, this peptide proportion is small (below 7%) in comparison to the whole protein content. The ratio of these fractions is virtually unaffected by pretreatment at various running conditions of SEC and by denaturation. In unprocessed CSF, the peptide content amounts to 60–80% of the whole UV-absorbing material, in accordance with [31]. This ratio remains constant during various pretreatment times whereas the molecular weights of peptides shift partially to smaller entities. Thus, in blood these peptides seem to be pre-formed *in vivo* and not to be produced by proteinases during sample preparation *in vitro*. In CSF a similar conclusion can be drawn, but some peptidase activity has to be considered during sample preparation affecting LMW constituents in accordance with [20,21]. In line with these results, no azocasein degradation, *i.e.* proteinase activity can be identified in native blood samples and CSF from healthy subjects or from patients that are obtained under conditions of our native multidimensional prefractionation method. Even trypsin-spiked serum, plasma, or CSF samples do not affect azocasein, indicating strong intrinsic antiproteinase activity. A lack of proteinase activity is also shown for the first separation step in our multidimensional chromatographic method. In SEC fractions of blood serum, plasma and CSF, azocasein disintegration is not found at all. In order to maintain these results, estimates on the specificity and sensitivity of azocasein disintegration are crucial. *Specificity*: Since azocasein has been used as a ubiquitous substrate for all known classes of proteinases, *e.g.* [32–36], a broad spectrum of proteinase activities, likely to be present in blood, should be indicated by azocasein hydrolysis. *Sensitivity*: To our best knowledge, there are no published estimates of "normal" proteinase activity in blood since proteinases are produced as inactive zymogens and become active only under defined conditions. When activated, proteinases may form pathways and interact to form the proteinase web. Thus, blood clotting and complement activation show strong interplay [37] producing vast total proteolytic activities of serine proteinases that should be traced by azocasein hydrolysis. The analytical sensitivity of our assay has been determined in parallel to sample measurements at identical conditions using a dilution series of pure porcine trypsin. With freshly prepared trypsin dilutions, the detection limit is 0.3 μg/mL, defined as mean + 3*SD of $A_{442}$ of blanks. This value corresponds to an absorbance of 0.011, to an activity of 15 mU/mL in the assay mixture, and to 30 mU/mL added to the assay (as outlined in the figures). The obtained detection limit is equivalent to about 0.3% of the mean plasma concentration of prothrombin [38] and one thousandth of the trypsin concentration used by us for tryptic digests [1].

Semi quantitative mass spectrometric analysis supports our results to some extent since neither protein coverage nor the number of peptides change significantly (≥factor 4) in 1-D fractions after incubation at room temperature. Azocasein hydrolyzing activity was not analyzed in 2-D fractions with respect to protein concentrations below the detection limit of our assays. However, mass spectrometry analysis of 2-D fractions showed no significant differences of the distribution pattern of protein concentrations. Both protein coverage and the number of peptides remained virtually constant after incubation of samples or 1-D fractions at room temperature. Nevertheless, we believe the mass spectrometric results to be much more uncertain in comparison to spectrophotometric

quantification of concentrations of peptides and azo-compounds. Generally, according to the law of error propagation, mass spectrometric results depend on the precision and number of sample preparation steps and on the linearity and precision of mass spectrometric signals [30]. Since we have no reliable information about these parameters, factor 4 is used as confident criterion tracing an alteration just as in our ongoing biomarker search.

Taken together, we show here by application of several different methods suitable for our native multidimensional chromatographic method that serum, plasma and CSF proteins are more stable than generally assumed. Our results are in accordance with some other studies who showed the stability of serum [11,12,27,39], plasma [13,39] and CSF [40] proteomes without proteinase inhibitors added. Proteinase inhibitors failed to reduce proteome alteration over time [28] or even interfered mass spectrometric results [10,27,28]. The use of proteinase inhibitors is not applicable for our method and apparently not necessary. Probably, intrinsic proteinase inhibitors adequately protect the proteomes, enabling "top-down" proteomic pipelines with native material.

Our results do not necessarily contradict the fragmentation of distinct peptides seen by others under slightly different conditions, as we have only quantified the total amount of peptides and not analyzed probable qualitative changes of the peptide pattern.

Noticeable proteinase activity which overcomes intrinsic inhibition either *in vivo* and/or during sample handling can be considered both to "artificially" alter constituents and to indicate imbalance and dysfunction. Actually, proteinase activity may serve as a valuable biomarker, measured by the "degradome" of parent components [41,42]. Nevertheless, the impact of proteolysis needs to be checked with each specific search method and sample set.

## 5. Conclusions

Native and non-depleted samples of human serum, plasma and CSF as well as 1-D fractions do not exhibit any significant endo-proteolytic activity when handled for an appropriate time.

Since added active trypsin is inhibited by all sample types, endogenous proteinase inhibitors potentially protect the proteomes from major endo-proteolytic activities.

Thus, all three sample types could be used for proteomic studies and biomarker search with our native chromatographic fractionation.

## Conflict of interest statement

The authors declare no financial/commercial conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.02.014.

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