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Analysis of the bovine plasma proteome by matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry

Ann-Kristin Henning [a,b], Martin H. Groschup [b], Thomas C. Mettenleiter [a], Axel Karger [a,*]

[a] Institute of Molecular Biology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany
[b] Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

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ABSTRACT

In this study, the bovine plasma proteome was analysed using a three step protocol: (1) plasma was treated with a combinatorial peptide ligand library (CPLL) to assimilate the differences in concentrations of different proteins in raw plasma; (2) CPLL-treated material was fractionated by three standard electrophoretic separation techniques, and (3) samples were analysed by nano-liquid chromatography (nLC) matrix-assisted laser desorption/ionisation (MALDI) time-of-flight tandem (TOF/TOF) mass spectrometry. The efficiencies of three fractionation protocols for plasma proteome analysis were compared.

After size fractionation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), resolution of proteins was better and yields of identified proteins were higher than after charge-based fractionation by preparative gel-free isoelectric focussing. For proteins with isoelectric points >6 and molecular weights $\geqslant$63 kDa, the best results were obtained with a 'shotgun' approach, in which the CPLL-treated plasma was digested and the peptides, rather than the proteins, were fractionated by gel-free isoelectric focussing. However, the three fractionation techniques were largely complementary, since only about one-third of the proteome was identified by each approach.

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Introduction

The major obstacle to the proteomic analysis of plasma or serum is the wide range in concentrations of different proteins, which may differ by more than 10 orders of magnitude (Anderson and Anderson, 2002). The 10 most abundant human plasma proteins constitute ~90% of the total plasma protein content (Tirumalai et al., 2003), making the analysis of less abundant proteins (among them potentially valuable biomarkers) very difficult. Strategies to solve this problem are based either on the depletion of highly abundant proteins or on the 'equalisation' of protein concentrations by affinity solid phase extraction using complex combinatorial peptide ligand libraries (CPLLs) (Thulasiraman et al., 2005).

The physicochemical basis of protein equalisation with CPLLs is that, under idealised saturating conditions, the amount of CPLL-bound proteins is not determined by the protein concentrations in the sample, but rather by the number of available binding sites for a certain protein in the library. Thus, extraction with a CPLL offering a wide range of affinities with similar binding capacities will assimilate the concentrations of a complex protein mixture to a certain degree.

In all depletion strategies, there is the risk of concomitant removal of non-targeted proteins. This has been observed for the depletion of the most abundant human plasma protein, serum albumin, using Cibacron blue (CB)-based affinity (Travis and Pannell, 1973; Di Girolamo and Righetti, 2011) and polyvalent immunodepletion (Yadav et al., 2011) matrices. Specific immunodepletion matrices are not available for most veterinary species.

Two-dimensional electrophoretic reference maps are available for cattle (Wait et al., 2002; D'Ambrosio et al., 2005; Skrzypczak et al., 2011; Alonso-Fauste et al., 2012; Marco-Ramell et al., 2012) and pigs (Miller et al., 2009; Sun et al., 2011), but the number of plasma proteins that have been identified by this method is relatively low. Application of state-of-the-art proteomic platforms has resulted in a substantial increase in the number of proteins identified in porcine plasma (Tu et al., 2011).

The aim of the present study was to establish a three step workflow for in-depth proteome analysis of bovine plasma that avoids the use of species-specific immunological reagents. In step 1, the dynamic range of protein concentrations was reduced through albumin depletion by CB affinity extraction or equalisation by CPLL-treatment (Marco-Ramell and Bassols, 2010). In step 2, the material was fractionated using one of three standard protocols: (1) proteins were fractionated on the basis of size by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then digested; (2) proteins were fractionated on the basis of charge

* Corresponding author. Tel.: +49 3835171251.
E-mail address: axel.karger@fli.bund.de (A. Karger).

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by gel-free protein isoelectric focussing (IEF) and then digested, and (3) in a 'shotgun' approach, the entire complement of proteins resulting from step 1 was digested and the resulting peptides (rather than the proteins) were fractionated by gel-free IEF (Chenau et al., 2008; Arrey et al., 2010). In step 3, protein/peptide fractions were analysed by nano-liquid chromatography (nLC) matrix-assisted laser desorption/ionisation (MALDI) time-of-flight tandem (TOF/TOF) MS.

All three workflows were designed to result in 12 fractions to eliminate any bias from the number of MS measurements following fractionation. The performance of the fractionation protocols was compared on the basis of the yields of proteins that were identified. The physicochemical properties and the gene ontology annotations of the identified proteins were analysed. The most efficient fractionation approach, gel-free peptide focussing, was repeated using two different proteolytic enzymes for in-depth analysis. Finally, a list of proteins identified in bovine plasma was compiled from all experiments in this study.

Materials and methods

Animals, sample collection and preparation

For proteome analysis, ethylene diamine tetraacetic acid (EDTA)-treated blood samples were collected from two clinically healthy 12-year-old female German black pied cows which had been tested negative for a broad panel of viral and bacterial agents and parasites, including bovine leukaemia virus, parainfluenza virus type 3, ovine herpesvirus type 2, bovine viral diarrhoea virus, *Mycobacterium avium* subspecies *paratuberculosis* and *Coxiella burnettii*. Blood was centrifuged at 1700 *g* for 30 min at 10 °C and pooled to obtain plasma.

Serum samples originated from the same two cows, or from three additional female German black pied cattle. Porcine sera were collected from clinically healthy 8-month-old or 12-month-old female crossbreed fattening hybrid pigs. Cow and pig blood samples were collected in the course of animal trials approved with reference numbers FLI 05/13 7221.3-2.1-011/13 and FLI 010/06 LALLF M-V/TSD/7221.3-1.2-061/09, respectively, by the German authorities. Human samples were collected from two female and two male healthy adult Caucasian volunteers in the course of routine health checks with informed consent. Blood samples were allowed to clot for 4 h at room temperature and centrifuged at 1700 *g* for 30 min at 10 °C. The supernatant was held at 4 °C overnight and again centrifuged under the same conditions. Samples were stored in aliquots at −80 °C.

Combinatorial peptide ligand library treatment and elution conditions

Equalisation by CPLL treatment (ProteoMiner; BioRad) was performed according to the manufacturer's recommendations. Aliquots with 0.1 mL settled bead volume were transferred to spin columns, washed three times with 1.0 mL wash buffer (0.1% SDS in phosphate buffered saline) and centrifuged at 1000 *g* for 2 min at room temperature to recover the beads. Plasma samples (0.9 mL containing 65–75 mg protein) were mixed with 0.1 mL 10× wash buffer. The sample was mixed with the beads and agitated for 2 h at 20 °C. Protein-loaded beads were recovered by centrifugation at 1000 *g* for 2 min and washed twice with wash buffer and once with purified water (Millipore).

The performance of the CPLL depends not only on the binding characteristics of the library, but also on the efficient elution of the bound proteins (Bandow, 2010; Bellei et al., 2011). Boiling in 4% SDS and 25 mM DTT (Candiano et al., 2009) is the most efficient eluent, but requires the removal of the detergent for downstream applications such as IEF that are not compatible with SDS. Among the alternative elution conditions that were compatible with all three fractionation techniques, the best results were obtained by incubation with 100 µL 70% formic acid (V/V) for 30 min at 20 °C. The eluate was recovered by centrifugation at 1000 *g* for 2 min at 20 °C, dialysed against purified water at 4 °C for 24 h (SnakeSkin Dialysis Tubing, 7 kDa molecular weight cut-off; Thermo Scientific) and dried by vacuum centrifugation (Univapo 150H; Uniequip). Samples were stored at −20 °C and are referred to as 'equalised plasma proteins'.

The proteins that were eluted with boiling SDS solution but not with formic acid (see Appendix A: Supplementary Fig. 1E) amounted to <1% of the formic acid eluate and were analysed by nLC-MALDI-TOF/TOF MS. All 33 proteins identified in this batch were also identified in the formic acid eluate, so that the small loss of protein was without consequence for the following analysis and the formic acid elution protocol was preferred in this study. Comparison of the protein profiles before and after CPLL-treatment (see Appendix A: Supplementary Fig. 1C and D) confirmed the desired assimilation of protein concentrations.

Treatment of serum with CB-Sepharose

Serum samples were diluted with water to 500 µg protein in 360 µL, and were supplemented with 40 µL of a 100 mM citrate/potassium phosphate buffer solution at pH 6, 7 or 7.8, which was prepared by mixing 100 mM citric acid and 100 mM potassium phosphate at ratios appropriate to yield the desired pH. After incubation with a settled bed volume of 100 µL Blue Sepharose 6 Fast Flow beads for 1 h at 20 °C with mild agitation, the beads were sedimented by centrifugation at 2000 *g* for 2 min and the supernatants were transferred to new tubes. Bound proteins were recovered after three washes with 1.0 mL incubation buffer by boiling with 100 µL 4% SDS and 25 mM DTT (Di Girolamo and Righetti, 2011).

Albumin depletion by extraction with CB-Sepharose was tested with human, bovine and porcine samples under various conditions, including variation of the pH (see Appendix A: Supplementary Fig. 2), salinity and the polarity of the buffer (data not shown). Albumin from all three species bound to CB-Sepharose; however, under all conditions tested, the specificity of binding was poor with bovine and porcine sera compared to human sera. This finding was confirmed by densitometric analysis of Coomassie-stained gels of the CB-Sepharose extracts of three more independent samples from each species (see Appendix A: Supplementary Fig. 3).

The protein composition of raw, CB-treated and equalised plasma was compared by nLC-MALDI-TOF/TOF MS. The number of identified proteins in the equalised sample was substantially higher than in raw or CB-Sepharose treated plasma (see Appendix A: Supplementary Fig. 4), indicating that equalisation, but not depletion by CB affinity extraction, is appropriate to prepare bovine plasma for proteome analysis. Thus, equalisation was chosen to prepare samples for the following fractionation step.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Samples were heated to 95 °C for 2 min in a reducing SDS–PAGE loading buffer and separated by electrophoresis in a Mini Protean II apparatus (BioRad) using 7.5–15% polyacrylamide gradient gels at 200 V for 45 min. Proteins were visualised by colloidal Coomassie Brilliant Blue (Neuhoff et al., 1988) or silver (Blum et al., 1987) staining. Densitometry of Coomassie-stained gels was performed with AIDA software version 4.19 (Raytest).

Gel-free isoelectric focussing of plasma proteins

Equalised plasma proteins (500 µg) were fractionated in a 3100 OFFGEL Fractionator (Agilent Technologies) equipped with a 12-well chamber following the manufacturer's protocol (Fractionator Kit User Guide; Agilent Technologies). Immobiline DryStrips (13 cm, pH 3–10; GE Healthcare) were rehydrated with 40 µL OFFGEL solution (8.4 M urea, 2.4 M thiourea, 80 mM DTT, 12% glycerol and 1% Bio-Lyte 3/10 ampholytes; BioRad). The dried samples were solved in 1.8 mL OFFGEL solution, distributed over the 12 chambers in 0.15 mL aliquots and focussed at 20 °C with a maximum voltage of 4500 V, current of 50 µA and power of 200 mW per strip. After exposure to 20 kV h, the 12 fractions were recovered, dialysed as for CPLL-treated proteins and freeze-dried. The samples were stored at −20 °C.

Gel-free isoelectric focussing of peptides

Peptides from digests of 100 µg equalised plasma were fractionated with a 3100 OFFGEL Fractionator (Agilent) using the manufacturer's protocol with minor modifications. Samples were diluted in 1.8 mL of a solution containing 20% methanol and 1% ampholytes (Bio-Lyte 3/10; BioRad) and equally distributed over the 12 chambers. An additional 100 µL of 1% ampholyte solution were added to chambers 1 and 12, and 50 µL to chambers 2 and 11, to prevent these from running dry (Arrey et al., 2010). Electrophoretic conditions were as described for protein IEF.

Mass spectrometry

Protein digestion – Proteins in solution were digested with porcine sequencing grade modified trypsin (catalogue number V5111; Promega) in 5 mM Tris(hydroxymethyl)-aminomethane (Tris; pH 8.0) supplemented with 1 mM $CaCl_2$, or with endoproteinase Glu-C (V8 protease from *Staphylococcus aureus*, catalogue number V165A; Promega) in 100 mM NH_4HCO_3 buffer pH 7.8, respectively, for 16 h at 37 °C using a substrate:enzyme ratio of 50:1. In-gel digestion was performed according to standard protocols (Jahn et al., 2006) with slight modifications. The Coomassie-stained gel was cut into 12 slices (see Appendix A: Supplementary Fig. 5A) and each slice was cut into four pieces. The gel fragments were destained and dehydrated with acetonitrile (ACN), dried, rehydrated with 15 µL trypsin buffer (25 ng/µL trypsin in 5 mM Tris pH 8.0 and 5 mM $CaCl_2$) and digested for 16 h at 37 °C. Peptides were extracted with 0.5% trifluoroacetic acid (TFA) and dried by vacuum centrifugation.

Protein identification – Digested samples were analysed on a platform consisting of an nLC system (EASY-nLC II; Bruker) connected to a Proteineer fcII sample spotting robot (Bruker), and an UltrafleXtreme MALDI-TOF/TOF instrument (Bruker). Peptide samples equivalent to 2 µg protein were diluted in 0.5% TFA

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