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Any use in proteomics for low-tech approaches? Detecting fibrinogen chains of different animal species in two-dimensional electrophoresis patterns

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ABSTRACT

We characterized the two-dimensional electrophoretic patterns of fibrinogen chains α, β, and γ from the plasma of six animal species – *Bos taurus*, *Canis familiaris*, *Equus caballus*, *Felis catus*, *Gallus domesticus* and *Sus scrofa*. Comparing the spots resolved from serum and plasma samples, or exploiting the cross-reactivity of animal fibrinogen with an antiserum raised against the human protein could detect only some of the fibrinogen chains. Conversely, the analysis of the precipitate obtained by heating plasma for some minutes at 56 °C was adequate for the recognition of all fibrinogen chains in all samples. Physicochemical properties of the homologous proteins were found to extensively vary across species, with complete separation among the mapping areas for α, β and γ chains and maximal heterogeneity among β chains.

1. Introduction

Fibrinogen [1] is a 340 kDa plasma glycoprotein made up by three pairs of polypeptide chains – α, β, and γ – linked by disulfide bonds and stabilized by calcium ions. A distinct gene encodes for each of the chains; both genetic [2] and epigenetic [2,3] factors contribute to protein heterogeneity. Besides the human protein, sequence data for 51 mammalian fibrinogens are present in UniProt database (at http://www.uniprot.org/). The overall structure is of a fiber, 45 nm in length, with globular domains (outer = D, central = E) connected by α-helical coiled-coil rods [4,5].

The primary role of fibrinogen is in hemostasis [6,7]. Fibrinogen engagement with the αIIbβ3 integrin induces platelet adhesion and aggregation thereby promoting blood coagulation. Fibrin is formed from fibrinogen after thrombin cleavage of fibrinopeptides A and B from the central region. Association of half-staggered oligomers (via D:E interaction) forms double-stranded fibrils that assemble into a network through lateral interaction and branching. γ Chain pairs are covalently cross-linked by plasma transglutaminase (factor XIIIa) via glutamine–lysine isopeptide bonds that further strengthen the fibrin clot. Degradation of the insoluble fibrin clot is mediated by plasmin, which cleaves fibrin to form soluble fragments such as the D-dimer [8].

Inherited disorders of fibrinogen [9–11] affect either the quantity (afibrinogenemia and hypofibrinogenemia) or the quality (dysfibrinogenemia) of the circulating fibrinogen or both (hypodysfibrinogenemia). Most often, subjects with congenital fibrinogen disorders suffer from a bleeding diathesis but paradoxically may undergo severe thrombotic episodes. In afibrinogenemic women, all pregnancies result in spontaneous miscarriage [12].

In physiological conditions, fibrinogen concentration ranges from 2 to 4 mg/mL, but secretion by liver increases severalfold upon stimulation by interleukin-1 [13]. As one of the main positive acute phase reactants [14], fibrinogen has been extensively used as a marker to assess the presence and persistence of inflammation. It is currently seen also as a mediator of inflammatory diseases, as extravasation within tissues features a danger signal while fibrinogen interacts with different cellular receptors (on neutrophils, monocytes and macrophages/microglia) through its multiple binding motifs [15].

In veterinary clinical medicine, some of the topics recently investigated about fibrinogen in farm animals (cattle, horses, pigs, dogs, excluding for the latter two the use as models of human dis-

Abbreviations: 1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; *C*%, relative bisacrylamide concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CPK, creatine phosphokinase; DTT, dithiothreitol; FFP, fresh-frozen plasma; HRPO, horseradish peroxidase; IgG, immunoglobulin G; IPG, immobilized pH gradient; M_r, molecular weight; p*I*, isoelectric point; SDS, sodium dodecyl sulfate; *T*%, total acrylamide–bisacrylamide monomer concentration.

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ease; source: PubMed database; period: 2004–09) include: changes related to estrous cycle, pregnancy and parturition [16–19], liver [20] and heart disease [21–23], elective and non-elective surgery [24,25], poisoning/envenomation [26–30], parasite infestation [31–33], viral infection [34,35]. The issue most often on focus is however, by far, bacterial infection – especially in respiratory diseases [34,36–41] – and such of its short- or long-term sequelae as septic shock with coagulopathy [42–45], laminitis [46], or osteomyelitis [47].

In addition to fibrinogen, the panel of commercial coagulation tests addresses activated partial thromboplastin time, prothrombin time, thrombin time as well as antithrombin and D-dimer levels. Protein masses are evaluated through latex agglutination and immunoturbidimetric assays. One classical way to measure fibrinogen content of plasma is to compare protein concentration of serum (obtained by heating plasma to 56 °C for 3 min [48–50]) to the original plasma sample: the difference corresponds to fibrinogen. Various aspects of thrombus formation may be assessed by thromboelastography [51,52].

Except for an old report comparing human, cow, pig and sheep fibrinogen [53], no extensive investigation on animal fibrinogen has been reported. Such a survey is the aim of the present report. A seemingly straightforward approach – comparing 2-DE patterns of serum and plasma samples – failed to clearly identify all three fibrinogen chains, even from the same individual. Therefore, we sought other methods to detect the subunits in plasma samples from different species (dog, cat, cow, horse, pig and chicken), including immunoblotting with antibodies against the human homologue. We found enrichment by precipitation a straightforward yet effective procedure able to demonstrate all fibrinogen chains in all samples.

2. Materials and methods

2.1. Samples

The species investigated were *Bos taurus*, *Canis familiaris*, *Equus caballus*, *Felis catus*, *Gallus domesticus* and *Sus scrofa* (common names: cow, dog, horse, cat, chicken and pig). Serum and plasma samples were taken from healthy animals, preferably from the same individual, from routine health checks of animals brought to or held at the University of Veterinary Medicine Vienna (Vienna, Austria).

2.2. Electrophoretic techniques

For two-dimensional electrophoresis (2-DE) an IPG-DALT system was used (isoelectric focusing in immobilized pH gradients followed by electrophoresis in the presence of sodium dodecyl sulfate) with home-made gels for both dimensions [54]. IPGs were run in 11 cm long non-linear gradients in the range of pH 4–10, SDS-PAGE on 140 mm × 140 mm × 1.5 mm gels (separation gel: T = 10–15%, C = 2.7%, stacking gel: T = 5%, C = 2.7%) in a Hoefer SE 600 vertical electrophoresis chamber (HSI, San Francisco, USA) with subsequent silver staining [55]. CPK carbamylation train from the Carbamylyte Calibration Kit (Pharmacia Biotech, Uppsala, Sweden) and low molecular weight marker (GE Healthcare Life Sciences, Munich, Germany) enabled pI and M_r determination, using the computed pI values for the CPK spots [56].

Semi-dry blotting was performed in a Semi-Phor TE 70 (Hoefer) onto a nitrocellulose membrane (GE Healthcare), using the transfer buffer of Bjerrum [57] and applying 0.8 mA/cm^2 for 1.5 h. Blots were processed according to Garfin [58] using the substrate reaction of Young [59], as already described in [60]. The antibodies utilized were an anti-human fibrinogen antiserum (Sigma, St. Louis, USA) and an anti-goat IgG HRPO conjugate (Accurate Chemicals, Westbury, USA).

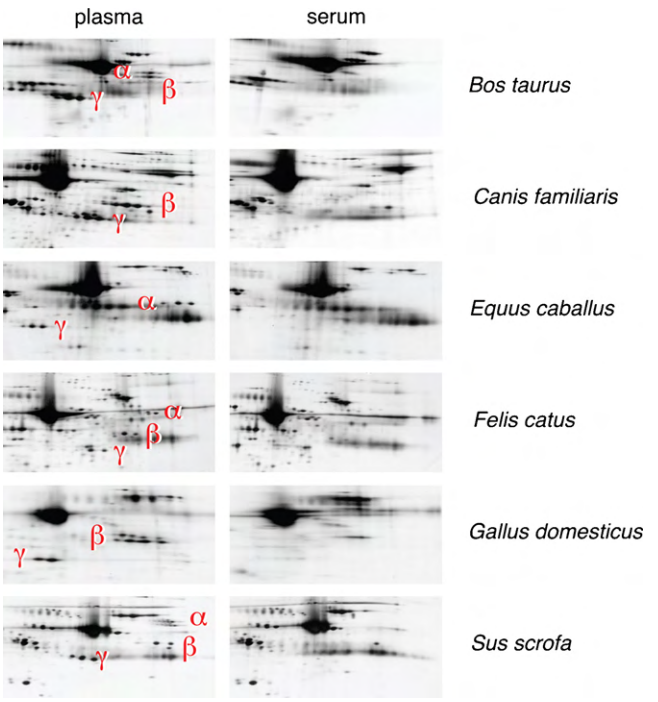

Fig. 1. Comparison between 2-DE patterns of plasma (left panels) and serum (right panels) for various animal species (legend at the right). The applied protein amounts correspond to 0.5 μl serum/plasma. The images are cropped to cover the area between pH 4.8 and 7.2 and between 40 and 90 kDa. The inferred mapping positions of fibrinogen chains are marked.

2.3. Precipitation

Plasma samples were incubated at 56 °C in an Eppendorf Thermomixer 5436 (Eppendorf, Hamburg, Germany) for 3–10 min and then centrifuged at 16,000 × g (Eppendorf Centrifuge 5415C). The precipitate formed was washed with PBS twice and the dry pellet was dissolved in about twice its volume of Laemmli sample buffer containing 2% DTT [61], first by shaking at room temperature and then by heating to 95 °C for 5 min. For 2-DE, samples were appropriately diluted with IPG sample solution (containing urea/CHAPS/DTT/ampholytes).

3. Results

The main difference in protein composition between plasma and serum consists in the presence of fibrinogen only in the former. Accordingly, as a first approach to the characterization of fibrinogen chains in various animal species, serum and plasma samples were run in 2-DE and compared. As we have already pointed out in our previous reports [54,60,62–64], the composition of serum/plasma varies extensively among animal species. Some proteins are specific to just one or few species, some are always present but at exceedingly different levels, and all differ as for pI, M_r and extent of glycosylation. This overall variability was confirmed by the patterns in Fig. 1, which also suggested a large inter-species variability in the physicochemical parameters of the tentatively identified fibrinogen chains. Indeed, in all gel pairs differences were noticed in the 45–80 kDa range, but hardly ever all three fibrinogen chains could be confidently located (Fig. 1).

As there do not exist many immunochemicals for animal proteins, taking advantage of cross-reactivities of anti-human anti-

Table 1
Molecular masses and p*I*s of fibrinogen chains of different species.

Species	α Chain		β Chain		γ Chain	
	p*I*	M_r (kDa)	p*I*	M_r (kDa)	p*I*	M_r (kDa)
Bos taurus	6.15–6.62	61.8–64.2	6.27–7.45	58.9–59.5	4.94–5.28	50.8–52.7
Canis familiaris	6.46–7.09	68.5–69.7	5.84–6.25	55.4–58.0	5.48–5.65	51.5–53.3
Equus caballus	6.65–6.85	73.5–76.2	6.27–7.02	56.5–58.0	4.95–5.12	52.5–52.6
Felis catus	6.12–6.40	64.6–66.9	5.82–6.02	55.3–56.0	5.46–5.67	49.0–49.7
Gallus domesticus	6.28–6.44	79.4–80.6	5.73–6.20	54.8–57.2	4.96–5.15	48.4–48.8
Sus scrofa	6.25–6.49	68.2–71.3	6.01–6.54	58.4–58.8	5.24–5.61	51.1–52.3

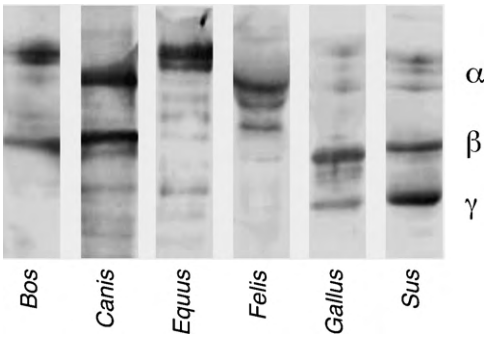

Fig. 2. Immunostain for fibrinogen chains on plasma from various animal species (legend at the bottom, with genus names) using an anti-human protein antiserum, after blotting from a 1-DE gel. The applied protein amounts correspond to 0.5 μl plasma. The inferred migration positions of fibrinogen chains are marked.

bodies is a common practice. Therefore, a commercial antibody against human fibrinogen was tested on a 1-DE (SDS-PAGE) blot. But, as shown in Fig. 2, the antiserum reacted only with some of the chains, depending on the species. For some species (cow, horse and pig), there was also a high degree of unspecific staining (seemingly, albumin and IgG).

Heating of plasma samples, as described in Section 2.3, to concentrate and largely purify fibrinogen (and derived molecular forms) resulted in different amounts of precipitate, depending on the species. The pellet obtained after centrifugation was large with cow and pig samples, hardly visible with cat and chicken. Prolonging the heating to up to 10 min did not markedly improve the yield. After several washes with saline, the pellets were dissolved in SDS-PAGE sample buffer (containing DTT) and boiled for 5 min at 95 °C; some were not completely dissolved, but the supernatant contained enough protein for qualitative analysis. As seen in Fig. 3, using these enriched samples all individual fibrinogen chains could be discerned for all of the species even when fibrinogen had not been quantitatively precipitated from plasma. Abundant precipitates contained as contaminants residual serum proteins (especially albumin and IgG), but their spots did not overlap with fibrinogen patterns.

Table 1 lists p*I* and M_r evaluated for the various fibrinogen chains.

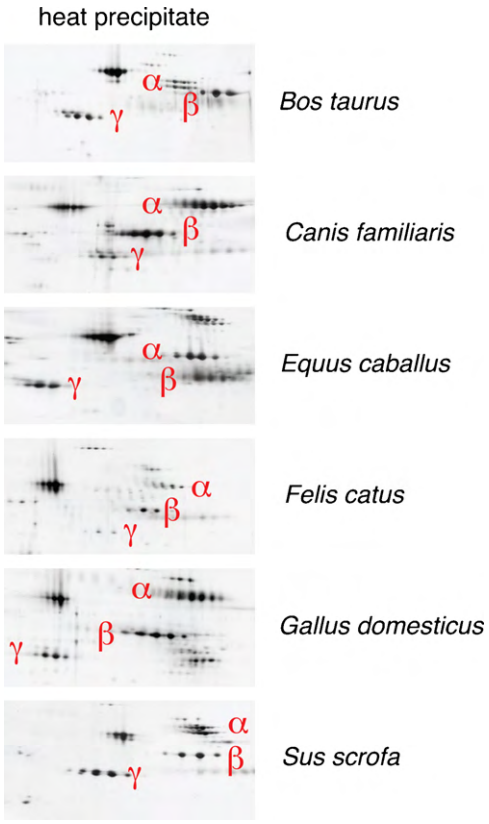

Fig. 3. 2-DE patterns of plasma heat precipitates for various animal species (legend at the right), obtained in correspondence to Fig. 1. The images are cropped to cover the area between pH 4.8 and 7.2 and between 40 and 90 kDa. The mapping positions of fibrinogen chains are marked.

4. Discussion

Among the species we have investigated, UniProt (at http://www.uniprot.org/) records complete sequence of all fibrinogen chains for chicken and cow, but only of short fragments for the others (except cat α chain with a stretch of 463 aminoacids, and pig γ chain with no structural data). Lack of data prevents matching of 2-DE patterns with computed p*I* and M_r.

Table 2
Computed and experimental physicochemical parameters of the three human fibrinogen chains.

Chain	p*I*		M_r (kDa)		Microheterogeneity (number of spots)
	Computed	Experimental	Computed	Experimental	
α	Isoform 1, 5.79 Isoform 2, 8.23	6.66–7.64	Isoform 1, 91.3 Isoform 2, 69.8	62.7–66.8	19
β	7.95	6.11–6.55	50.8	54.6–55.9	4
γ	Isoform A, 5.70 Isoform B, 5.24	5.03–5.65	Isoform A, 49.5 Isoform B, 48.5	44.6–51.2	13

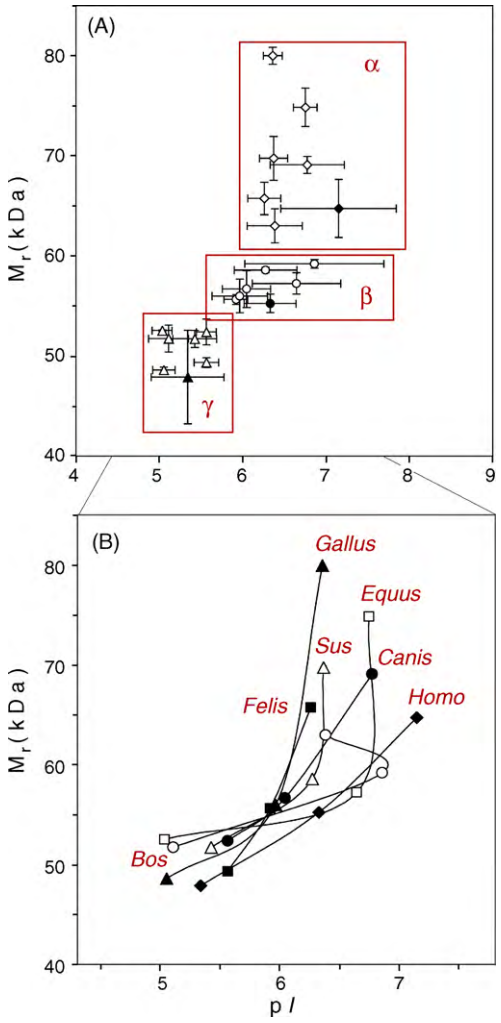

Fig. 4. (A) p*I*–M_r distribution of fibrinogen chain isoforms for various animal species (including humans, solid symbols). α chains are marked with diamonds, β chains with rounds, γ chains with triangles; cumulative data for each of the chains are highlighted by boxing. (B) p*I*–M_r distribution of fibrinogen chains for various animal species; only median values for each of the chains are plotted; lines connect data for each species. Legend (with genus names): *Homo*, solid diamonds; *Bos*, empty rounds; *Canis*, solid rounds; *Equus*, empty squares; *Felis*, solid squares; *Gallus*, empty triangles; *Sus*, solid triangles.

Even when sequence data are available, however, such matching is not straightforward, as exemplified for the human protein. Table 2 compares expected and measured physicochemical parameters of the three human fibrinogen chains – the former computed with the UniProt tools, the latter reported in SWISS-2DPAGE database (at http://www.expasy.ch/ch2d/). All chains display extensive microheterogeneity as brought about by composite post-translational processing.

This observation implies that specific strategies are required for a clear-cut identification of the fibrinogen chains. Of the three we have tried, the subtractive approach comparing serum to plasma pattern and the cross-immunoreactivity test after immunoblotting are informative only for some of the chains in some of the cases. On the contrary, the purification obtained when causing fibrinogen precipitation by heating is adequate for an easy identification in 2-DE of all the chains in all the species, even though yields vary and contamination may be substantial. In no case fibrinogen chains overlap with the spots of the contaminating proteins. The procedure is quick and undemanding and may be performed on small plasma aliquots.

Other seemingly simple procedures for sample enrichment have been reported, but neither is as effective as heating.

Indeed, fibrinogen tends to precipitate upon freezing and thawing of plasma [65]. This point has received attention for its possible effects on the reliability of analytical procedures on clinical samples [66] and on the therapeutic efficacy of fresh-frozen plasma (FFP) preparations [67]. Quality of FFP appears to depend both on freezing [68] and on thawing [69,70] conditions. Slowly thawing FFP at 1–6 °C produces cryoprecipitated antihemophilic factor, or cryoprecipitate for short. Fibrinogen is present in cryoprecipitate together with factor VIII, von Willebrand factor (vWF) and factor XIII; its level is affected by temperature during plasma processing [71]. However, cold precipitation of fibrinogen seems hard to achieve under reproducible conditions with animal plasma. In fact, with fraction-of-a-milliliter-volume samples as collected from laboratory and sometimes from farm animals, handling and processing at controlled temperature throughout is definitely less effective than with samples of human origin drawn in sizeable amount either for clinical testing or for industrial processing.

Fibrinogen also precipitates in 25% saturated ammonium sulfate [65]. However, several other proteins co-precipitate, and fibrinogen chains represent a much lower percentage of the total than with the heating procedure (data not shown). Moreover, a dialysis step is required to achieve ion levels compatible with the subsequent focusing step.

As we pointed out when reporting reference maps of animal sera (cat [60], rat [72], cow [73], mouse [64] and pig [54]), physicochemical properties of homologous proteins may extensively vary across species. This is the case also for fibrinogen chains. In addition to the list of individual data in Table 1, p*I*–M_r distribution for all fibrinogen samples is summarized in Fig. 4. Panel A focuses on the three chain types, panel B on individual animal species; data for the human protein are also included. While a general trend is observed for the relationships among the properties of the three chain types in each species, no rule actually applies. Complete separation is observed among the mapping areas for α, β and γ chains; microheterogeneity varies extensively, being maximal for β chains.

Among the reasons behind mapping heterogeneity, it has been reported that γ chains tend to dimerize [65]. In our 2-DEs of the fibrinogen fractions, these dimers are seen as spot trains with same p*I*, but higher M_r than the parent molecules.

5. Conclusions

With our previous studies on animal body fluids we concentrated on serum and by necessity overlooked proteins connected with blood clotting. For the most abundant of such proteins, fibrinogen, the present survey evaluates/provides the main physicochemical data. These may be a useful reference for all future investigations on plasma using electrophoretic techniques, both in the 1- and in the 2-DE format. For the former, 2-DE data will help recognize with which proteins the bands of fibrinogen chains do overlap, and suggest which precautions to take in band quantification. The collection of species-specific 2-DE references we have produced may allow detecting alterations in the structure of animal fibrinogen resulting either from changes in amino acid sequence or by incorrect post-translational processing. As in human patients, such changes might then be studied as the cause of coagulation disorders or as possible markers of degenerative disease.

A most peculiar aspect of the present investigation is in the identification of protein components through low-tech procedures relying on biochemical properties of a class of proteins (solubility as a function of temperature) instead of on structural data (such as those gathered through MS experiments) or on biological interactions (such as tested through immunoblotting). Since both

comprehensive genomic databases and specialized immunological reagents are seldom available for farm animals and pets as well as for vegetables and herbs, standard identification approaches on such samples have to rely on similarities with more extensively characterized species. We have ourselves exploited the immunological cross-reactivity with human homologues to sharpen the identification of rat serum proteins [62,72,74]. We have also presented and extensively discussed a hierarchical approach to protein identification using MS/MS data as the query which allowed the identification of the majority of bovine serum proteins visible on silver stained gels, even though more than 50% of them lacked specifically bovine database entries at the time the analysis was carried out [73]. While less and less frequently made use of, fractional precipitation methods are not only simple, fast and inexpensive but also robust and portable – which makes them a valuable alternative approach for across species comparisons.

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