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Review

Enrichment of serum amyloid proteins by hydrophobic interaction chromatography combined with two-dimensional electrophoresis with immobilised pH gradients

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Abstract

Serum amyloid A protein was subjected to one-step octyl-Sepharose extraction in three different dimensions. Elution was performed partly without UV recording, and with urea or guanidine-based buffers. The eluent was applied directly to denaturing two-dimensional electrophoresis with immobilised pH gradient, or octyl-Sepharose extracted fractions were pooled and lyophilised before application. Proteins were characterised by N-terminal analysis or mass spectrometry. In most of the species that were studied, previously undescribed serum amyloid proteins were detected. Compared to conventional strategies, the presented techniques are more rational and yield more comprehensive information. The presented data also provide a basis for novel perspectives regarding certain inflammatory conditions.
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Keywords: Reviews; Hydrophobic interaction chromatography; pH gradients; Isoelectric focusing; Two-dimensional electrophoresis; Proteins

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1. Introduction

The paper is based on selected data from a doctoral thesis on serum amyloid A protein (SAA), which forms the foundation of the presented methodology [1–8].

1.1. Amyloidosis and amyloid A protein

Amyloidosis is a group of diseases characterised by proteinaceous tissue deposit (amyloid) [9]. AA amyloid, of which the main protein component is AA, is a complication of certain chronic inflammatory diseases, and can infiltrate and destroy vital organs [9]. Inflammation is the local response of the host to tissue injury. AA is smaller than serum amyloid A protein (SAA), and has been found in a variety of amyloid laden tissues, but not in serum. Because it shares N-terminal sequence homology with SAA, it is believed to be derived from acute phase SAA by proteolytic cleavage [10].

1.2. Serum amyloid A protein

The SAA proteins comprise a superfamily of acute phase and constitutively expressed proteins, containing 102 (man) and 122 (mouse) amino acids [11]. The acute phase response is the systemic response of the host to tissue injury. Human SAA was first detected by Levin et al. in 1973, because of its crossreactivity with (AA) amyloid fibril preparations [12]. Human SAA is encoded by the two allelic genes SAA1 and SAA2. The presence of two SAA2 (α/β) and four SAA1 phenotypes ($\alpha/\beta/\gamma/Asp_{72}$) have been demonstrated, either with or without an N-terminal arginine residue [13–17]. The existence of a fifth (SAA δ) pair(s) of human SAA protein has also been suggested. The aforementioned greek letters symbolise amino acids differences in positions of heterogeneity of the SAA sequence. Asp_{72} refer to Asp in position 72. Human and mouse acute phase SAA differ by an equal number of heterogeneous positions, which are by far most clustered in human SAA [9]. Mouse SAAs are designated according to coding genes, breed and isoelectric point value [17,18]. Known post-translational modifications (PTMs) of SAA are the removal of the N-terminal Arg (human SAA), blocked N-termini (various species) and the presumed cleavage of SAA to amyloid A protein (AA) [19,20]. In general, PTMs are known to confer physicochemical and biological activities on proteins [21].

The SAA sequences from different species have been found to be remarkably conserved, and SAA serum levels increase dramatically during the acute phase response. The protein is found in the serum of most species. It has also been found in a variety of cell-types and extra-hepatic tissues in health and disease. SAA is thus believed to possess an important biological function, which has been associated with amyloidogenesis, atherogenesis, lipid-transport, pro- and anti-inflammatory activities [8]. Atherogenesis refers to the process that creates atheromas. These are the plaques that are found in the vessel-wall in cardiovascular disease.

1.3. SAA and protein technology

Isolation of SAA from serum has traditionally been accomplished by ultracentrifugation and delipidation, and/or in combination with various other large scale, multi-step chromatographic methods, requiring dialysis and lyophilisation [22–28]. Electrophoresis and Western blot have been based on one (or two) dimensional sodium dodecylsulphate–polyacrylamide gel (SDS–PAGE) electrophoresis and carrier ampholyte isoelectric focusing, with dye or immunodetection utilizing poly- or monoclonal AA or SAA antibodies [17,29,30]. The technique carrier ampholyte isoelectric focusing is explained in Section 1.5.

Characterisation of SAA has been performed

mainly by amino acid composition and N-terminal analysis. Prior to the latter, and because these proteins are frequently blocked, internal sequence fragments have been prepared by subjecting chromatographic [22,31] and electrophoretic fractions [29] to chemical and enzymatic cleavage, additional chromatographic steps (including RP-HPLC) and several micro-manipulative purification steps [32].

1.4. Hydrophobic interaction chromatography and SAA

Hydrophobic interaction chromatography (HIC) columns act by providing hydrophobic moieties that bind to hydrophobic areas on the protein surface. Proteins are eluted by reducing the polarity of the ionic strength of the eluent, hereby decreasing the hydrophobic forces in the protein–resin assembly. The HIC resin consists of a hydrocarbon ligand (butyl, octyl, phenyl or alkyl), that is coupled to a crosslinked agarose matrix (Sepharose or Superose) [33].

The foundation of HIC was laid down by Tiselius in 1948, who suggested that proteins that precipitated at high concentrations of neutral salts (salting out), often are adsorbed quite strongly in salt solutions of lower concentrations than is required for their precipitation. Since then, several theories regarding HIC have been proposed, including aspects such as thermodynamic stability, surface tension and van der Waals attraction forces [33].

As the N-terminal SAA sequence contains a cluster of hydrophobic and nonpolar side-chains [34], the protein is particularly suitable for HIC, and for octyl-Sepharose extraction (OSE) in particular [26]. Compared to the lipoprotein purification approach, which is outlined in the beginning of Section 1.3, the yield of SAA from OSE is at least three times higher [26]. Conventional SAA OSE methods, however, have utilised sequential, large-scale chromatography [26,35,36].

1.5. Two-dimensional electrophoresis with immobilised pH gradients

Two-dimensional (2D) electrophoresis separates proteins according to their mass (first dimension) and charge (second dimension). 2D immobilised pH gradients electrophoresis (2D-IPG) is currently the most powerful tool to resolve complex protein mixtures. The technique was first described by Smithies and Poulik in 1956 [37], and was improved in 1975 by O'Farell [38] and by Hochstrasser in 1988 [39].

Related to advances in gel casting and staining, blotting membranes and protein sequencing techniques [40–45], the use of 2D-IPG expanded in the end of the 1980s and in the beginning of the 1990s [46].

In the first dimension, proteins are separated according to their electrical charge, by isoelectric focusing (IEF). IEF means that proteins migrate in a pH gradient, until they have no net charge. The corresponding pH value is the isoelectric point, or the p*I*, of the protein [21]. The p*I* of a protein depends on its net-charge, and thus on its amino acid composition and PTMs. Traditional and modern IEF differ as to whether the substances creating the pH gradient are present in the sample, or if they are immobilised within the material to which the sample is applied. These substances are called carrier ampholytes (CA) and immobilines, respectively. The corresponding IEF techniques are called CA-IEF and IPG-IEF. The disadvantages of CA-IEF, namely low loading capacity, salt-intolerance, electroendosmosis, limited resolution and poor reproducibility, have been largely abolished with the introduction of immobilines [40]. Electroendosmosis refers to an imbalance of ionic migration across the electrophoretic surface that results in attraction of basic components and water towards the cathode [40,47,48]. In the second dimension, proteins are separated according to their molecular mass by SDS–PAGE. 2D-IPG fractions are detected as spots.

Combined with advances in computer-technology and DNA sequencing, 2D-IPG has greatly enhanced identification and characterisation of proteins [45,49,50]. Because of the high loading and purity that can be achieved with 2D-IPG, 2D spots can be subjected more or less directly to N-terminal sequence analysis. If the proteins are blocked, chemical cleavage can be performed in situ (meaning when present in the membranes). Also from small amounts of material, proteins in 2D spots can be identified by mass spectrometry (MS). The technique can also be used to identify PTMs [45,49–52].

Because of its high resolution and reproducibility, 2D-IPG also makes it possible to identify proteins merely by matching 2D patterns, and to perform quantitative studies. This is referred to as comparative 2D analysis. It is performed with the aid of computerised image analysis, special software programs and federated 2D databases [53–55].

The third application area of 2D-IPG is proteomics, which is the protein equivalent of genomics. The term proteome refers to the proteins expressed by the genome of an organism, cell or tissue [49]. Unlike genomics, proteomics combines analytical methods with bioinformatics. Recent trends regarding biomedical applications in proteome research have been summarised elsewhere [54,56]. A useful website regarding the above issues is Ref. [57].

2. Experimental

2.1. OSE

2.1.1. Method A

Column dimensions were 40×1.6 cm. The resin consisted of octyl-Sepharose CL4B (Pharmacia), which had been equilibrated with phosphate-buffered saline (PBS). The settled resin-height was 30 cm. A 50-ml volume of pooled undiluted plasma or serum was applied to the column. The resin was subsequently washed with PBS, until the absorbance of the effluent was below 0.1 (UV monitoring λ 280 nm). Elution was performed automatically, with 100% 4 *M* Gua·HCl–30% ethylene glycol–10 m*M* NaOH throughout. Fractions within the first major peak with absorbance above 0.1 were pooled, dialysed and lyophilised. The method was used for the N-terminal studies of human SAA [4].

2.1.2. Methods B and C

Method B: Column dimensions were 10×1 cm. The resin consisted of octyl-Sepharose CL4B, which had been equilibrated with PBS. The settled bed-height was 4 cm. A 2-ml volume of plasma or serum was applied to the column. Elution was performed with 100% 4 *M* Gua·HCl–30% ethylene glycol–10 m*M* NaOH. The eluent was dialysed and lyophilised before application to 2D-IPG [1]. Further details are given in the end of this section.

Method C: A microcolumn was constructed from a 500-μl Eppendorf tube, whose bottom part had been excised and whose lid-centre was replaced by a nylon membrane. The resin consisted of octyl-Sepharose CL4B, which had been equilibrated with PBS. Elution was performed with 9 *M* urea throughout. A 250-μl volume of plasma or serum was applied to the microcolumn. The eluent was frozen and/or applied directly to 2D-IPG after centrifugation [2,6].

For both method B and C, elution was performed manually, without UV monitoring, until no more liquid could be obtained from the resin. In practice, the relation between the resin height (in cm) and sample volumes (in ml) was 1:2, and between the sample, equilibration and elution volumes, was 1:6:3. The methods were used for the studies of mink (method C) [3,4], and mouse SAA (method B) [2,6].

2.2. 2D-IPG

Before application in the first dimension, lyophilisate was dissolved in 8 or 9 *M* Urea, 0.5% Triton, 2% β-mercaptoethanol and 2% Pharmalyte pH range 3–10. The eluent that had been obtained by OSE method C (9 *M* urea), however, was largely applied in an unmodified state to 2D-IPG. The reason for this is discussed further in the end of this section. Amounts applied in the first dimension were 25 (silver staining) and 240 μl eluent (blotting), and 0.5 (Coomassie staining) and 6.6 mg lyophilisate (blotting). In the second dimension, Excel Gels (SDS) 8–18% (Pharmacia), were used. 2-D gels containing OSE eluent and lyophilisate samples were subjected to silver and Coomassie staining, respectively. The 2D-IPG protocol from Pharmacia was used for all the above procedures [1–4,6].

In the initial experiments with the OSE method C, it was observed that the presence of Triton in the rehydration and sample buffer resulted in a yellow discolouration of the gels [2]. Secondly, the presence of CA resulted in black colour complexes in the basic end of the IEF strip [2]. Thirdly, and because they both produced UV-absorbance artifacts, their presence distorted the interpretation of some of the N-terminal [2] and MS studies [6]. Fourthly, their presence made no difference with regards to the

resolution of mouse SAA. Thus, for OSE method C, both CA and Triton may be omitted from the above buffers [6,8].

It is well known that Triton can cause discolouration, and that CA can interact with metallic ions in the dye. Both are also known to produce UV absorbance [47,58]. Prior to the present study, however, their effects had been examined mostly in complex protein mixtures, on large and membrane proteins [47,59–64]. Consistent with the presented observations, one of these studies proved that the resolution of small proteins was unaffected by the presence of CA [61].

2.3. Semidry blotting

Semidry blotting was performed in the Nova Blot electrophoretic transfer Unit (Pharmacia), using poly(vinylidine difluoride) (PVDF) membranes from Millipore, Coomassie [2] or amido black stain [5]. The procedures were performed according to the protocols of the manufacturers.

2.4. Protein sequence and MS studies

Electroblotted 2D spots present in the 14 kDa region, which were not present in control gels, were excised from the PDVF membranes, and subjected to protein sequence studies. N-terminal amino acid sequence studies were performed in a 477A Sequencer from Applied Biosystems. The pyrrolidine ring of the amino terminal pyrrolidine carboxylic acid was opened by methanolysis, and cyanogen bromide (CNBr) cleavage was performed directly on excised PVDF spots. Peptides were analysed directly in the sequencer. Sequence searches were performed with the FASTA and TagIdent programs [1–4,6]. The human material was also subjected to 2-(2-nitrophenyl-sulphenyl)-3-methyl-3-bromoindolenine (BNPS) skatole cleavage [4], and to liquid chromatography–electrospray ionisation tandem mass spectrometry [5].

In the presented sequence studies, N-terminal analysis of material from three spots, which later proved to contain SAA, revealed co-elution of amino acids [3,4,6]. In order to exclude that this was not caused by nonoptimal separation, also the areas between two of these spots (mouse spot 2 and 3) were subjected to N-terminal analysis [6].

3. Results

3.1. 2D-IPG

In the acute phase plasma from all species that were analysed (fox, goat, man, mink, mouse, rabbit), three to five major spots, in the pH range 3–9, and with a molecular mass corresponding to 14 000, were identified. Selected 2D gels are shown in Fig. 1. These spots were not present in the control samples. In general, the same 2-D pattern was seen both in acute phase samples from individual specimens, and in pooled samples from several specimens.

In the presented 2-D gels, a variety of other proteins could also be seen. As judged by the electrophoretic behaviour in the second dimension, however, their molecular mass was not in accordance with that of SAA. Secondly, they were also found in control samples. Thus, it was considered highly unlikely that these spots represented SAA. Consequently, they were not subjected to protein sequence studies.

3.2. Protein sequence studies

Except for the material from mouse spot 5, N-terminal, CNBr (all species) and BNSP skatole cleavage studies (human material) revealed varying amounts of partial SAA sequence in all spots of interest [2–4,6]. Selected data, with focus upon known heterogeneous positions of the SAA sequence, are shown in Table 1. In mouse spots 2 and 3, the yield of Ile and Val in position 6 and 7 was 150:30, and 80:16 pmol, respectively. This corresponds to a relative ratio of 5 (Ile):1 (Val) in both spots. In the area between the mouse SAA spot 2 and 3, N-terminal studies revealed no amino acids. In mouse spot 5, no SAA sequence could be identified, due to either insufficient material, or to N-terminal blockage (a well-known phenomenon for SAA) [6]. In human spots 3 and 4, N-terminal studies revealed no amino acids in any of the heterogeneous positions of the SAA sequence. The MS studies, however,

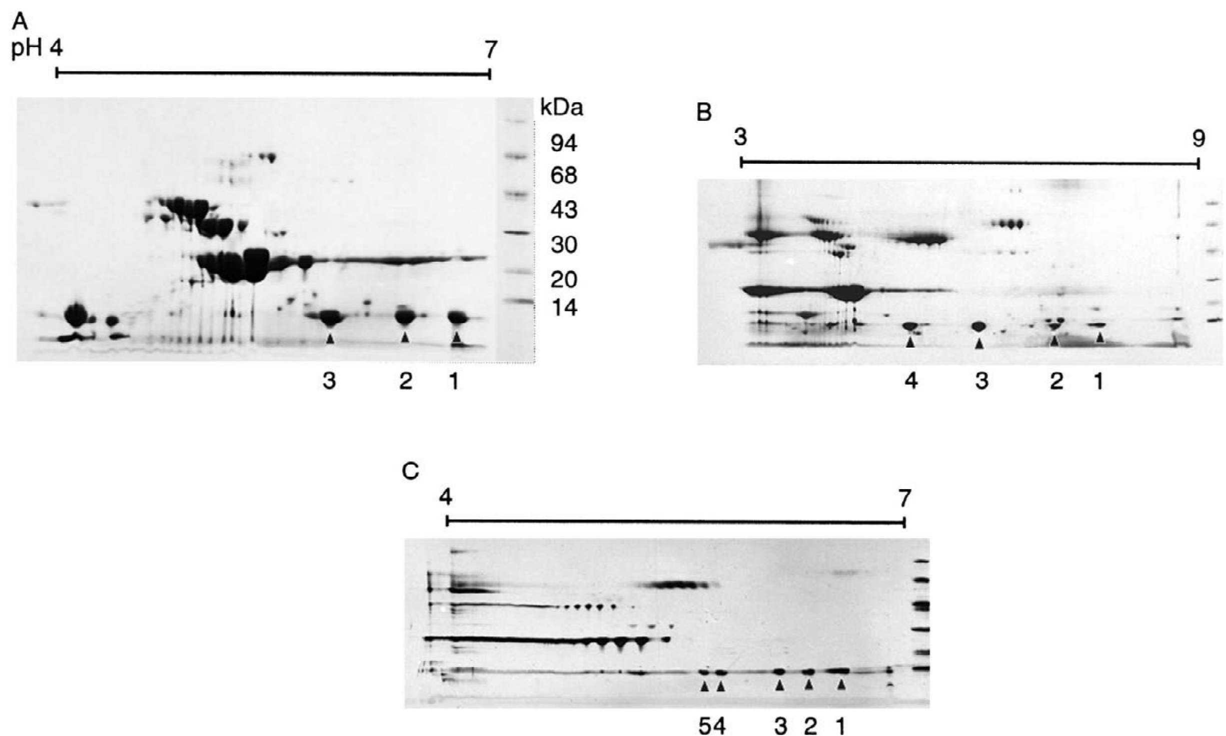

Fig. 1. Computerised images of original photographs of selected 2D-IPG gels. The gels contain Coomassie (A,B) and silver stained (C), acute phase, plasma proteins from mink (A), man (B) and mouse (C). 2D-IPG samples were lyophilisate (A, B) and eluent (C), which had been extracted by OSE method B (A), A (B) and C (C). Gels used were Immobiline dry strips 11 (A) and 18 cm (B, C), with linear (A,C) and nonlinear (B) pH gradients, and ExcelGels SDS 8–18 (A–C). Numbered arrows indicate SAA IEF variants, as identified by N-terminal analysis (A, C) and mass-spectrometry (C). The spots contained (A) Mink SAA1-like proteins (spots 1–3). Spot 3 also contained an SAA2-like protein. (B) Human SAA2a-Arg (spot 1), SAA2a-desArg (spot 2), SAA1a-Arg (spot 3) and SAA1a-desArg. The two most basic spots, located in the 14 kDa and 20 kDa region, are nonglycosylated and glycosylated forms of constitutive human SAA, respectively (5). The unlabelled, minor spots in the 14 kDa region have been characterised in the latter report. (C) Mouse SAA1 (spot 1) and SAA2 (spot 2)-like proteins, an $SAA_{SJL/L}$ p*I* 5.9-like protein (spot 3), and an SAA2 or $SAA_{SJL/L}$ p*I* 5.9-like protein (spot 4). Spot 5 most likely represents a novel SAA protein (6). Spots 2 and 3 also contained minor amounts of SAA1-like proteins. In (A–C), the identity of the unlabelled proteins can partly be deduced from comparison with the web-based 2D map of human plasma [57]. Accordingly, the complex of 2D spots located with between 20 kDa and 30 kDa, most likely represents mink, human and mouse apoAI. OSE, octyl-Sepharose extraction, 2D-IPG=two-dimensional electrophoresis with immobilised pH gradients.

revealed extensive stretches of various SAA peptides, allowing a more comprehensive identification of the individual SAA phenotypes [5].

In summary, the presented sequence data were regarded to be in accordance with mink SAA1 (mink spots 1–3) and SAA2 (mink spot 3), and mouse SAA1 (mouse spots 1-3) and SAA2 or $SAA_{SJL/L}$ p*I* 5.9 (mouse spots 2–4) [3,6]. The human SAA spots proved to be Arg and des-Arg variants of SAA1α and SAA2α [5]. A summary of the results is shown in Fig. 1.

4. Discussion

Previously, two acute phase mink and rabbit SAA IEF variants have been identified by immunological detection. In mouse, four such variants have been found. In the present study, altogether three (mink and rabbit), and most probably five (mouse) variants were partly characterised [3,4,6].

N-Terminal studies of mink (spot 3) and mouse (spots 2 and 3) 2D fractions, which proved to contain SAA, yielded co-elution of amino acids. One might

Table 1
Selected amino acid sequence analysis of isolated SAA IEF variants. Only heterogeneous positions of the SAA sequence are shown. Asterisk symbolises reference sequences [3,6]

Position	6	7	11	24	27	30	31	76	101
Mink SAA1*				Ile	Lys				
Mink SAA2*				Arg	Asn				
Mink spot 1				Ile	Lys				
Mink spot 2				Ile	Lys				
Mink spot 3				Ile	Lys				
					Asn				
BALB/c SAA1*	Val	His	Glu		Asn	Asn	Ser	Ile	Asp
BALB/c SAA2*	Ile	Gly	Glu		Gly	Asp	Gly	Met	Ala
$SAA_{SJL/L}$ pI 5.9*	Ile	Gly	Glu		Gly	Asp	Gly	Met	Asp
$SAA_{CE/J}$ pI 6.15*			Leu						
Mouse spot 1	Val	His	Glu		Asn	Asn	Ser		
Mouse spot 2	Ile	Gly	Glu		Gly	Asp	Gly		
	Val	His			Asn	Asn			
Mouse spot 3	Ile	Gly	Glu						
	Val	His							
Mouse spot 4						Gly		Met	

argue that this could be due incomplete separation and/or to co-migration of other proteins than SAA. The phenomenon occurred, however, only in a few positions of the SAA sequence, which are known to be heterogeneous [3,4,6]. Considering the co-elution found in positions 6 and 7 in the material from mouse spots 2 and 3, the relative ratio between the amounts of Ile and Val was identical in both spots [6]. N-Terminal analysis revealed no amino acids in the areas between these spots [6]. Consequently, incomplete separation could be excluded. A sequence search on the presence of Val and His in positions 6 and 7, revealed no other proteins than SAA [6]. Thus, the co-eluting amino acids were believed to be derived from SAA exclusively.

In conclusion, mink spots 1 and 2 contained SAA1-like proteins exclusively. Mink spot 3 contained a mixture of SAA1 and SAA2-like proteins. Mouse spot 1 contained an SAA1-like protein exclusively. Mouse spot 2 was contained a mixture of SAA2 and SAA1-like proteins. Mouse spot 3 contained a mixture of $SAA_{SJL/L}$ pI 5.9 and SAA1-like proteins. The above conclusions are based on correlation between the presented and previous SAA sequence data, and with SAA cDNA clones. The distinction between the mouse SAA2 (spot 2) and SAA pI 5.9-like (spot 3) proteins, however, is based on differences in pI values. The proteins differ only by one amino acid substitution, namely Ala (SAA2) and Asp (SAA p$I_{SJL/L}$ 5.9) in position 101. Consequently, SAA $_{SJL/L}$ pI 5.9 focus even more acidic than SAA2. With regards to mouse spot 4, however, not sufficient material was obtained to say whether it represents an SAA2 or an $SAA_{SJL/L}$ pI 5.9-like protein [6]. Mouse spot 5 probably also represents SAA, as it was not present in the control sample, and its electrophoretic properties were in accordance with SAA.

The major mouse SAA proteins found in spots 1 and 2, were regarded to represent products of the SAA1 and SAA genes [6]. With regard to the major mouse SAA proteins found protein in spot 3, or to the minor mouse SAA proteins in spots 2 and 3, no conclusions can be drawn as to whether these were products of the SAA1 or SAA2 genes, or of yet undefined genes. They may well also be post-transitionally modified. In mink, the amino acid heterogeneity that was demonstrated in spot 3, is in accordance with the cDNA sequences of SAA1 and SAA2 [4]. A very similar conclusion to this could also be made regarding the co-eluting amino acids that were found in one of the rabbit spots [4].

In conclusion, previously undescribed SAA proteins were identified in all species that were investigated, except for in man. The reason why these had not been detected previously was attributed to the

limited resolution of CA IEF relative to that if IPG IEF. Secondly, the extensive prefractionation utilised in the conventional strategies might have resulted in protein loss. In particular, ultracentrifugation and/or multiple chromatographic steps may well have resulted in loss of SAA [42,65].

Compared to conventional purification, the presented methodology is fast, minimise protein loss, require small amounts of material and yield superior resolution of the SAA proteins, including simple 2D patterns. Because the eluate can be applied directly in the first dimension, OSE method C is most optimal with regards to protein conservation.

The presented methodology can probably also be adapted to alternative samples and dimensions, and to alternative 2D enrichment [66] and blotting procedures [67]. It can probably also be adjusted to other types of HIC and affinity chromatography, and to ''in-gel'' rehydration [68]. It also provides a basis for elucidation upon each of the individual SAA proteins and their biological function in amyloidosis and cardiovascular disease, conditions with substantial mortality and morbidity worldwide. Accordingly, the presented data might have novel pathophysiological and therapeutical implications.

Similar to those proposed for 2D electrophoresis [20] and for proteomics in general [56,57], the methodology might also be useful for other biomedical applications. In particular, and due to its small dimensions, the OSE method C can facilitate the study of hydrophobic proteins in situations where only small blood volumes are available. This is particularly relevant for mice, which are frequently used in research. For SAA, the difficulty in obtaining substantial volumes of blood from mice has in fact been one of the arguments for making recombinant preparations of this protein [69,70]. These do, however, have limitations compared to natural preparations [21,51].

In conclusion, the presented methodology introduces a novel concept for the enrichment of SAA proteins, and highlights its usefulness as a supplement to genomic studies. By its application, more efficient enrichment, and more comprehensive information about SAA and a variety of other proteins might be obtained. Thus, the presented data might ultimately have important implications regarding the understanding and therapy of amyloidogensis, cardiovascular disease, and of many other chronic inflammatory conditions.

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