Published in final edited form as:

Proteomics. 2011 January ; 11(1): 106–113. doi:10.1002/pmic.201000310.

Examining serum amyloid P component microheterogeneity using capillary isoelectric focusing and MALDI-MS

Noah G. Weiss¹, Jason W. Jarvis², Randall W. Nelson², and Mark A. Hayes¹

¹Department of Chemistry and Biochemistry, Arizona State University

²Biodesign Institute, Arizona State University

Abstract

Serum amyloid P component (SAP) is a glycoprotein of interest due to its presence in amyloid plaque formations. As with most glycoproteins, SAP can possibly vary greatly in its isoforms, which can be an important factor towards understanding the role of SAP. Interestingly, previous characterizations suggest varying degrees of microheterogeneity, some of which are in conflict. In this work, we provide new information to clarify SAP's microheterogeneity profile using capillary isoelectric focusing (cIEF) to carefully analyze pooled samples and by studying individual samples across populations with mass spectrometric immunoassay. With respect to cIEF, a single pI band was observed suggesting that human SAP does not have extensive heterogeneity concluded from gel IEF experiments in the past. Additionally, this is supported by a population study which revealed an overwhelming degree of uniformity. Overall, this work corroborates the idea that SAP is relatively consistent across the population and with respect to microheterogeneity.

Keywords

Isoelectric focusing; Isoforms; Mass spectrometry; Serum amyloid P

1 Introduction

Human serum amyloid P component (SAP) is a member of the pentraxin protein family and is well known for its calcium dependent binding properties and presence in amyloid plaques [1]. It is a decameric/pentameric glycoprotein composed of monomers (M.W. ~ 25 kDa) each having a single complex biantennary N-linked glycosylation [2]. Interestingly, there are several conflicting findings regarding the source and number of existing SAP isoforms [2–6]. Two predominant themes are found in the literature regarding SAP microheterogeneity. One theme, based on mass spectrometry, suggests that SAP is relatively homogeneous and observed glycan variance is primarily a result of different numbers of sialic acid residues [2,6]. Both studies observed varying degrees of sialylation (three forms corresponding to disialo, monosialo, and asialo), but different conclusions were drawn in terms of the endogenous nature of these species. The second theme suggests that SAP has much broader heterogeneity as determined by gel IEF or 2D gel electrophoresis (2DE) visualized by protein staining [3–5]. In each of these gel separation studies a different number of isoforms were identified (4 to 8) with differing isoelectric points (4.7 to 6.1, Table 1). Even more

Correspondence: Professor Mark Hayes, Department of Chemistry and Biochemistry, PO Box 871604, Tempe, AZ 85287-1604, 480-965-2265 (phone), 480-965-2747 (fax), mhayes@asu.edu.

Conflict of Interest

We declare that there is no financial, commercial, or other conflict of interest in carrying out or publishing this research.

incongruous, each of these studies concluded uneven sialylation was not the cause of the multiple bands.

In terms of the second theme, there exists a large amount of experimental and statistical uncertainty. It is difficult to discern quantitative and analytically significant information from these specific gel-based separations for several reasons. In a general sense, the interpretation of the patterns and the intensities of these gels were limited to the broad estimation error associated with manual qualitative assessment. This, along with potential experimental difficulties and a lack of further characterization, may account for the significant differences in data and interpretation. In fact, one study even suggested the additional bands observed in gel IEF characterization to be the result of experimental error associated with impurities or the staining process [7].

No matter the limitations of previous works, pI separations of SAP have frequently revealed multiple bands suggesting mass spectrometric studies may not have completely identified all isoforms (e.g., unresolved peaks). Therefore, it would be insightful to separate SAP using a more controlled IEF method, characterize separated fractions with mass spectrometry, provide sufficient sampling to apply statistical significance, and examine both pooled and individual samples. Such a comprehensive, quantitative analysis will provide new information to either support existing theories or elucidate novel isoforms.

Capillary IEF (cIEF) was chosen for this study as a useful technique to examine SAP microheterogeneity since it is less reliant on manual manipulation (gels require manual preparation, loading, staining, and visualization), known to have comparable resolution to carrier ampholyte gels, and still compatible with mass spectrometry coupling [8]. As a very brief background for IEF, in general, a uniform electric field and pH gradient are applied to the separation length which is most often a gel or capillary. This causes amphoteric species and proteins to change charge states while migrating electrokinetically due to acid/base reactions. Positively charged proteins migrate towards the cathode or higher pH region until becoming neutral and vice versa for negatively charged proteins. The pH where the protein reaches a net neutral state and ceases to electromigrate is defined as the isoelectric point. A more comprehensive comparison of gel and capillary separations can be found elsewhere [9].

After steady state focusing is achieved in cIEF, the capillary contents are typically mobilized allowing for detection of concentrated bands by absorbance or fluorescence [8]. These detection modes have limited applicability since absorbance detection requires high protein concentrations (LOD ~10 μ M) and can suffer from ampholyte/background interference while fluorescence detection often requires the use of linked fluorophores. Additionally, they make it difficult to interpret complex electropherograms, lacking alternative information aside from pI.

A less developed but more universal approach is to interface cIEF with MS [10]. This offers improved detection limits (LOD ~10 fmol or ~10 nM for 1 μ L capillary volume) relative to absorbance detection while providing orthogonal separation by molecular weight, similar to traditional 2DE. Additionally, the accurate and precise determination of molecular weight allows for improved characterization. ESI and MALDI are the two most compatible ionization modes for MS analysis of proteins. With respect to cIEF coupling, ESI receives more attention probably owing to its online coupling ability [11]. On the other hand, cIEF-MALDI-MS has only been demonstrated a few times and its full potential has yet to be realized [12–18]. MALDI-MS uses offline coupling providing semi-permanent sample storage for multiple analyses and can generate simpler spectra. Technically, this increases analysis time and if not performed properly can result in additional band broadening.

In the current work, we present a quantitative examination of SAP microheterogeneity with pooled and individual samples. This work clarifies the microheterogeneity profile of SAP isolated from a pooled serum sample by direct MALDI-MS and with cIEF-MALDI-MS. Only sialic acid variants are observed and SAP is found to be highly homogenous. Additionally, SAP preparations isolated from several hundred individuals were analyzed using mass spectrometric immunoassay (MSIA). Included in this population study were samples from individuals having type-2 diabetes, myocardial infarction, congestive heart failure, and cancer. No significant population heterogeneity was observed amongst healthy and disease state individuals, which supports the uniform microheterogeneity model concluded from the pooled sample.

2 Materials and methods

2.1 Chemicals and materials

Acetonitrile (ACN), ammonium hydroxide, phosphoric acid, sinapinic acid, sodium chloride, sodium phosphate, and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pharmalyte brand carrier ampholyte with a pH range of 4–6.5 was obtained from Amersham Biosciences (Amersham, UK). Fluorescent pI markers 5.1 & 4.0 were obtained from Fluka (Buchs, Switzerland). Neuraminidase and sodium citrate buffer were obtained from New England Biolabs (Ipswich, MA, USA). Polyimide coated fused silica capillaries (75 μ m inner diameter) with an internal coating for electro-osmotic flow suppression were obtained from Microsolv (Long Branch, NJ, USA). Stock serum amyloid p component purified from pooled human plasma using a proprietary modification to a published method was obtained from Calbiochem/EMD Biosciences (La Jolla, CA, USA) [19]. Slide-a-Lyzer mini dialysis units were obtained from Pierce (Rockford, IL, USA). Individual plasma samples were obtained from ProMedDX (Norton, MA, USA) under Institutional Review Board approval. Affinity pipette tips for mass spectrometric immunoassay (MSIA) were obtained from Intrinsic Bioprobes (Tempe, AZ, USA). Anti-SAP polyclonal antibody was obtained from DakoCytomation (Carpinteria, CA, USA). Deionized water was generated using a Nanopure UV ultra pure water system from Barnstead/Thermolyne (Dubuque, IA, USA).

2.2 Mass spectrometric immunoassay

SAP was analyzed from 374 individual plasma samples to carry out a population based study. In addition to healthy individuals, the cohort including the following disease states: type-2 diabetes (T2D), congestive heart failure (CHF), history of myocardial infarction (hMI), and cancer. The sample distribution is as follows: 133 healthy, 29 healthy (serum), 87 T2D, 25 CHF with T2D, 25 CHF and hMI, 29 with CHF, 17 CHF with hMI and T2D, and 29 cancer (9 prostate, 9 breast, and 11 colon cancer).

SAP was isolated from these plasma samples using MSIA tips derivatized with anti-SAP as previously described [20]. The tips were removed from storage in buffer (0.01 M HEPES, 0.15 M sodium chloride) at 4 °C and loaded onto a Beckman Multimeck 96 robotic workstation (Fullerton, CA, USA) capable of processing 96 tips in parallel. Samples for affinity capture were prepared by mixing 67 μ L of plasma and 33 μ L of a detergent/buffer solution (4.5 % Tween 20, 0.15 M octyl- β -glucopyranoside, 1.5 M ammonium acetate, 0.67 M sodium phosphate, and 1 M sodium chloride). Each MSIA tip was washed with 30 μ L of the sample 1000 repetitions: where one repetition equals one aspirate and dispense cycle. After immuno-precipitation was complete, the tips were rinsed (5 repetitions of 150 μ L) with the following solutions in the order listed: PBS (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2), HBS-P (0.01 M HEPES, 0.15 M NaCl, 0.05% v/v Surfactant P20, pH 7.4), water, 0.1 M Tris-HCl (pH 4.6). The tips were rinsed a final time with water (10

repetitions of 150 μL) before they were dried by blotting with a towel. Captured SAP was eluted from the tips by aspirating 4 μL of matrix solution (saturated sinapinic acid in 0.5% TFA/ACN (3/2, v/v)) and dispensing it onto a seeded MALDI plate. The spotted protein preparations were quickly crystallized under vacuum for MALDI-MS analysis. The thin layer of matrix or seeded MALDI plate was prepared by gently rubbing 100 μL of a matrix solution (sinapinic acid in isopropanol/acetonitrile/water (9/2/1 v/v)) across the plate with a towel until dry.

For ESI analysis, MSIA tips were used to extract SAP from plasma and rinsed as described above along with HBS, distilled water, 2 M ammonium acetate/acetonitrile (3:1 v/v), and distilled water. Captured SAP was manually eluted from the tips by aspirating 5.5 μL of a mixture of 100% formic acid/acetonitrile/distilled water (9/5/1 v/v/v), mixing for 20–30 seconds, and dispensing into a 96-conical well polypropylene autosampler tray. An additional 5.5 μL of distilled water was aspirated into the pipette tip which was used to dilute the eluted sample.

2.3 cIEF

Capillary isoelectric focusing was performed on a lab-built instrument using a 40 cm $\times$ 75 μm id internally coated capillary for electroosmotic flow suppression. For all cIEF runs 10 mM phosphoric acid and 20 mM ammonium hydroxide were used as the anolyte and catholyte reservoirs, respectively. The instrument was configured to allow for online absorbance detection and fraction collection for offline MALDI-MS detection. Absorbance was monitored at 220 nm in capillary flow cell using a deuterium light source (Mikropack DH2000), fiber optic cables, and spectrometer (Ocean Optics USB 4000). A sheath flow arrangement, as described previously, allowed for fraction collection without disrupting either the focusing voltage or pH gradient [18]. Briefly, the capillary was threaded through an 18 gauge stainless steel tube (1 mm protrusion) where the ground contact was made. At the time of mobilization, catholyte solution was pumped at a steady rate through the steel tube in order to create catholyte droplets at the capillary tip.

Sample solutions prepared with volumes as low as 5 μL contained 100 ng/ μL SAP, 2% (w/v) pharmalyte (pH 4–6.5), and 20 ng/ μL pI markers 5.1 & 4.0. The sample mixture was pressure injected through the entire capillary volume (1.8 μL) using 10 psi nitrogen. Then the anodic end of the capillary was threaded into the anolyte vial using Upchurch fittings to create an airtight junction and the cathodic end was submerged into the catholyte vial. Focusing was conducted with an applied voltage of 12 kV for 10 min using a Spellman (Hauppauge, NY, USA) high-voltage power supply and the current decreased from 30 to 5 μA . Subsequently, the catholyte vial was removed and a syringe pump was used for pressure mobilization of capillary contents at 0.15 $\mu\text{L}/\text{min}$ and delivery of catholyte sheath flow at 5.25 $\mu\text{L}/\text{min}$. This generated droplets at the capillary tip (5.4 $\mu\text{L}/\text{min}$) where the sheath and capillary flows combined. A MALDI target plate was lightly contacted to droplets in 10–30 second intervals in order to capture them in discrete fractions (0.9–2.7 μL) for MALDI-MS analysis. This led to fractions differing in pI by approximately 0.1 pH units. The matrix solution (saturated sinapinic acid in 0.5% TFA/ACN (2/1, v/v)) was added immediately after collecting fractions on a MALDI plate, since adding it to the sheath flow can cause resolution loss during the spotting process [17].

2.4 Desialylation of SAP

A reaction mixture of stock SAP (250 ng/ μL), neuraminidase (1.25 units/mL), and pH 6 sodium citrate buffer (50 mM) were incubated at 37°C for 1 hour to remove terminal sialic acid residues. Afterwards the sample was dialyzed against 1 L of deionized water at room temperature for 1 hour to remove buffer components found to be problematic for cIEF.

Successful desialylation was confirmed by directly characterizing on MALDI-MS. In one experiment, desialylated SAP was combined with stock SAP in order to enrich the asialo variant to create a mixed sample for cIEF-MS.

2.5 MALDI and ESI Mass Spectrometry

For the MSIA population study, MALDI-TOF MS analysis was performed using a Bruker (Billerica, MA, USA) Ultraflex MALDI-TOF instrument operating in the positive ion, delayed-extraction, and linear mode with the following parameters: ion source 1 at 25.00 kV, ion source 2 at 23.10 kV, lens at 9.00 kV, 90 ns delayed extraction, and deflection signal suppression up to m/z 8000. A 96 spot gold MALDI target was used to collect the eluted proteins. Ten thousand laser-shots were accumulated for each mass spectrum. The spectra were externally calibrated with a mixture of proteins supplied by Bruker (Cat. No. 206355 and 207234), baseline subtracted, and smoothed using the Gauss algorithm (width 1 m/z , cycles 1) within Flex Analysis software.

For ESI analysis, 8 μL of sample was loaded for pre-concentration/solvent exchange before eluting into a Bruker MicroTOF-Q mass spectrometer following the parameters described previously [21]. For data analysis, approximately 1 min of recorded spectra were averaged across the chromatographic peak maximum followed by spectral deconvolution within 1000 Da to all identified peaks using Bruker data analysis v3.4 software.

For analyzing the cIEF fractions, a 96 spot ground steel MALDI target was used for all experiments. Immediately after collecting each cIEF fraction, 2 μL of matrix solution was added to allow for dried droplet deposition. Protein standards without cIEF separation (SAP and desialylated SAP, 500 ng each) were spotted directly onto the target plate and 2 μL of matrix solution was immediately added. Droplets on the MALDI target plate were allowed to air dry at room temperature and pressure. All MALDI-MS spectra were generated on either a Bruker Microflex or Autoflex MALDI-TOF mass spectrometer generated by accumulating 500 shots. The instrument was operated in positive ion mode with a 20 kV extraction voltage and 300 ns delay time. The spectra were calibrated externally using equine myoglobin, baseline subtracted, and smoothed using the Gauss algorithm (width 1 m/z , cycles 1) within Flex Analysis software.

3 Results & discussion

3.1 SAP heterogeneity assessed via MALDI-MS for pooled sample

The heterogeneity of pooled SAP was analyzed by directly placing samples on a MALDI target plate and subjecting them to mass spectrometric analysis (Fig. 1a). The primary signal was at 25,460 m/z which is in good agreement with the theoretical molecular weight of the SAP monomer with two sialic acid residues (disialo) [22]. A peak at 25,170 m/z was observed and assigned to the loss of a single sialic acid residue (-290 Da). Additional peaks were observed (25,670 and 25,380 m/z) with a mass shift of $+220$ Da and are consistent with matrix adducts of sinapinic acid (224 g/mol) of the disialo and monosialo species. To prepare for and be consistent with experiments noted below, relatively high laser power for desorption and ionization was used, resulting in a small additional peak consistent with the asialo form at 24,880 m/z . This is caused by the sialic acid groups being somewhat labile in response to the ablation process, and is considered to be an artifact of the ionization process. In ESI-MS analysis, only the disialo and monosialo signals were observed confirming that the asialo species is virtually non-existent in native SAP preparations and is likely an artifact in MALDI-MS (Fig. 1b). Furthermore, the asialo signal dominates the overall spectra when using much higher laser powers (data not shown).

Supporting this interpretation of the mass spectra is data collected from pooled SAP samples treated with neuraminidase (which selectively and quantitatively removes sialic acid), where signals at 25,460 m/z, assigned to the disialo, and 25,170 m/z, assigned to monosialo, are minimized and the signal consistent with asialo (24,880 m/z) is significantly enhanced (Fig. 1c). Additional peaks are consistent with matrix adducts. These quantitative results are significantly different than the much broader heterogeneity observed in the gel IEF analyses [3–5]. One possible explanation for the difference between these findings and previous results is that direct mass spectrometric analysis may not completely resolve all isoforms and another separation mode is warranted.

3.2 Pooled SAP characterization via cIEF-MALDI-MS

According to gel IEF studies of pooled and individual SAP, multiple bands were interpreted to be isoforms that differ in pI by at least 0.02 and as much as 1.0 pH units which can not be explained in light of mass spectrometry analysis (Table 1) [3–5]. The use of modern capillary-based isoelectric focusing allows for resolution of Δ pH units as small as 0.05 and is compatible with MALDI-MS with minimal separation efficiency loss [17,23]. In support of effective cIEF and MALDI-MS coupling, we previously demonstrated baseline resolution of variants β -lactoglobulin A and B (Δ pI 0.3), although differing by only two amino acids [18].

Only a single band along the pI dimension resulted from quantitative cIEF-MALDI-MS of pooled SAP, in both native and denaturing conditions (8 M urea), in distinct contrast to some gel IEF results (Fig. 2a–b) [3–5]. The single band had a pI 4.2 ± 0.1 (n=4) for the native form and 5.0 ± 0.1 (n=4) for the denatured one. In all cases, the sialic acid variants were found at the same pI and no other mass spectral signals were found in any other pI zones. Higher laser powers were used to ensure optimal detection limits for potentially dilute isoforms, and thus, a weak signal for the asialo protein was observed apparently from fragmentation. The pI shift in the presence of urea is likely due to SAP denaturing and thus disassociating into monomers. The pI was determined by calibrating the pH gradient with the use of pI markers 5.1 and 4 which were detected by online absorbance detection at 220 nm (Fig. 3) [24].

For pooled SAP without urea, the experimental pI agrees with the pI value of 4.1 as determined by chromatofocusing [25] but differs from the pI value of 5.6–6.2 observed in gel IEF [5]. In the later case, it is possible that SAP was denatured in the sample preparation or in the acrylamide gel during focusing since this pI value agrees with pI of denatured SAP in other gel IEF experiments [3–5]. For denatured pooled SAP in presence of 8 M urea, the pI is significantly lower than the average pI of 5.8 (± 0.3) from several gel IEF experiments [3–5,7,26], and can likely be attributed to the contrasting conditions of the medium, exposure of gel to atmosphere, or difference in temperature. The pooled SAP focused close to the pI markers, suggesting possible interaction between the protein and markers. However, experiments with and without markers produced identical results.

The single band observed in all cIEF experiments is in precise agreement with the uneven sialylation peaks identified in direct MS. Sialic acid residues cause a subtle change in pI, on the order of 0.1 pH units for a different protein [27]. SAP sialo-forms are not expected to be resolved by pI since the fraction interval of 0.1 pH unit encompasses the expected pI difference. Additionally, the situation is further complicated by artifacts from the ionization process causing some disialo species to be detected as mono or asialo ones (and mono detected as asialo). The subtle pI difference is further supported in an experiment where the asialo species is enriched using neuraminidase and not completely resolved from disialo (Fig. 2c). It is clear, however, that the species are partially resolved since the asialo species provides a unique signal for the pH 5.1 fraction. Nonetheless, the lack of pI resolution

amongst SAP sialo-forms was not concerning since they are easily separated by mass in the spectra. Overall, these findings support the simple model that uneven sialylation is the main source of isoforms previously suggested using MS [2,6].

These cIEF MALDI-MS results do not support the level of heterogeneity interpreted from previous gel IEF studies [3–5] and a number of experimental details can account for these discrepancies. Individual bands are not resolved from one another in Kubak et al., but data were interpreted as identifying three isoforms within 0.15 pH units (a fourth was observed in 2DE) [3]. Similarly, Serensen et al. reports significant problems with the profile and width of the pI markers suggesting considerable run-to-run variance, yet identifies six isoforms within just 0.22 pH units from only two data sets [4]. Nybo et al. details that the protein markers (carbonic anhydrase and ovalbumin) create multiple bands in the presence of urea, yet claim as many as seven SAP isoforms within 1 pH unit without discussing the obvious experimental problems [5]. Not surprisingly, there is considerable disagreement amongst these results and with other gel IEF studies which did not observe SAP isoforms [7,26]. Although different, the pooled SAP data shown here using cIEF-MALDI-MS is more reliable than the aforementioned gel IEF studies since it is quantitative and relied on minimal manual manipulation.

3.3 Individual SAP characterization via MALDI-MS

Within the pooled samples, an individual's heterogeneous SAP variant may be diluted such that it is no longer detectable. If there is a large variation among individuals, then the provenance of the samples in the previous studies could provide an explanation for the varied results. To determine the extent of heterogeneity across the population, SAP preparations isolated from 374 different individuals were analyzed using mass spectrometric immunoassay [6]. The MALDI-MS instrument was operated at a lower laser power to maximize peak resolution. With the exception of one, identical spectra were obtained for all individuals, regardless of health state (Fig. 4). The disialo and monosialo signals were consistent with direct and cIEF separated pooled SAP samples. The one individual which is an exception showed an additional peak at 25,780 m/z (+ 320 Da relative to disialo). Currently, it is unclear what this peak represents and we are working on further characterization. The spectral uniformity was confirmed quantitatively as shown by the average relative peak areas and standard deviations across the population: disialo (25,460 m/z) $89 \pm 1\%$, monosialo (25,170 m/z) $3.8 \pm 0.8\%$, disialo matrix adduct (25,680 m/z) $4.0 \pm 0.2\%$, and monosialo matrix adduct (25,385 m/z) $3.3 \pm 0.5\%$. The process of matrix adduct formation is poorly understood [28], and thus it is unclear why the relative matrix adduct signals are not proportional to their parent ions. Overall, these results suggest that there is no significant population heterogeneity or any protein modifications specific to the disease states examined (unpublished results). Thus, it is unlikely that previous conflicting findings of SAP isoforms are due to differences in sampling.

4 Concluding remarks

Through this work, a comprehensive examination of SAP microheterogeneity is undertaken using pooled and individual preparations in order to clarify the inconsistent findings across SAP literature. Only sialic acid variation was observed when analyzing pooled SAP with direct MALDI-MS and cIEF-MALDI-MS. Here SAP isoforms disialo (25,460 Da) and monosialo (25,170 Da) were identified and found to have similar pIs of 4.2 ± 0.1 and 5.0 ± 0.1 in its native and urea denatured states, respectively. However, the relative amounts of these two species may be biased depending on the extent of sialic acid loss resulting from the MALDI fragmentation process. Considering the quantitative and instrumental advantages of the methods used, this data provides new information in support of the previous theory suggesting uneven sialylation is the primary microheterogeneity source.

Thus, we conclude SAP has no isoforms, frequent amongst the population, which were previously thought to exist but were uncharacterized. The SAP population study supports this conclusion since 373 of 374 individual SAP preparations, across an array of disease states, generated essentially identical mass spectral data.

Abbreviations

CHF	congestive heart failure
hMI	history of myocardial infarction
MSIA	mass spectrometric immunoassay
SAP	serum amyloid p component
T2D	type-2 diabetes

Acknowledgments

This work was supported in part with NIH grants 2R01EB004761-06 and R21EB010191-01A1.

References

1. Pepys MB, Booth DR, Hutchinson WL, Gallimore JR, et al. Amyloid P Component. A critical review. *Amyloid*. 1997; 4:274–295.
2. Pepys MB, Rademacher TW, Amatayakul-Chantler S, Williams P, et al. Human serum amyloid p component is an invariant constituent of amyloid deposits and has a uniquely homogeneous glycostructure. *Proc. Natl. Acad. Sci. USA*. 1994; 91:5602–5606. [PubMed: 8202534]
3. Kubak BM, Gewurz H, Potempa LA. Identification of multiple forms of the P component of amyloid isolated from human serum. *Int. Arch. Allergy Appl. Immunol*. 1988; 87:194–203. [PubMed: 3192306]
4. Serensen IJ, Andersen O, Nielsen EH, Svehaug SE. Multiple isoforms of the human pentraxin serum amyloid P component. *Int Arch Allergy Immunol*. 1995; 106:25–31. [PubMed: 7812162]
5. Nybo M, Hackler R, Kold B, Nielsen EH, et al. Isoforms of murine and human serum amyloid P component. *Scand. J. Immunol*. 1998; 48:350–356. [PubMed: 9790304]
6. Kiernan UA, Nedelkov D, Tubbs KA, Niederkofer EE, Nelson RW. Proteomic characterization of novel serum amyloid P component variants from human plasma and urine. *Proteomics*. 2004; 4:1825–1829. [PubMed: 15174148]
7. Hawkins PN, Tennent GA, Woo P, Pepys MB. Studies in vivo and in vitro of serum amyloid P component in normals and in a patient with AA amyloidosis. *Clin. exp. Immunol*. 1991; 84:308–316. [PubMed: 1673879]
8. Rodriguez-Diaz R, Wehr T, Zhu M. Capillary isoelectric focusing. *Electrophoresis*. 1997; 18:2134–2144. [PubMed: 9456028]
9. Hille JM, Freed AL, Watzig H. Possibilities to improve automation, speed and precision of proteome analysis: A comparison of two-dimensional electrophoresis and alternatives. *Electrophoresis*. 2001; 22:4035–4052. [PubMed: 11824628]
10. Silvertand LHH, Torano JS, van Bennekom WP, de Jong GJ. Recent developments in capillary isoelectric focusing. *J. Chromatogr. A*. 2008; 1204:157–170. [PubMed: 18565533]
11. Wehr T. Coupling capillary isoelectric focusing with mass spectrometry. *LCGC North America*. 2004; 22:998–1004.
12. Foret F, Muller O, Thorne J, Gotzinger W, Karger BL. Analysis of protein fractions by micropreparative capillary isoelectric focusing and matrix-assisted laser desorption time-of-flight mass spectrometry. *J. Chromatogr. A*. 1995; 716:157–166. [PubMed: 8574384]
13. Chartogne A, Gaspari M, Jespersen S, Buscher B, et al. On-target fraction collection for the off-line coupling of capillary isoelectric focusing with matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom*. 2002; 16:201–207. [PubMed: 11803541]

14. Crowley TA, Hayes MA. Analysis of human blood serum using the off-line coupling of capillary isoelectric focusing to matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Proteomics*. 2005; 5:3798–3804. [PubMed: 16127730]
15. Yu W, Li Y, Deng C, Zhang X. Comprehensive two-dimensional separation in coupling of reversed-phase chromatography with capillary isoelectric focusing followed by MALDI-MS identification using on-target digestion for intact protein analysis. *Electrophoresis*. 2006; 27:2100–2110. [PubMed: 16736452]
16. Lechner M, Seifner A, Rizzi AM. Capillary isoelectric focusing hyphenated to single- and multistage matrix-assisted laser desorption/ionization-mass spectrometry using automated sheath-flow assisted sample deposition. *Electrophoresis*. 2008; 29:1974–1984. [PubMed: 18425755]
17. Silvertand LHH, Torano JS, de Jong GJ, van Bennekom WP. Development and characterization of cIEF-MALDI-TOF MS for protein analysis. *Electrophoresis*. 2009; 30:1828–1835. [PubMed: 19391148]
18. Weiss NG, Zwick NL, Hayes MA. Capillary isoelectric focusing coupled offline to matrix assisted laser desorption/ionization mass spectrometry. *J. Chromatogr. A*. 2010; 1217:179–182. [PubMed: 19945710]
19. Debeer FC, Pepys MB. Isolation of human C-reactive protein and serum amyloid P component. *J. Immunol. Methods*. 1982; 50:17–31. [PubMed: 7086148]
20. Niederkofler EE, Tubbs KA, Kiernan UA, Nedelkov D, Nelson RW. Novel mass spectrometric immunoassays for the rapid structural characterization of plasma apolipoproteins. *J. Lipid Res*. 2003; 44:630–639. [PubMed: 12562854]
21. Borges CR, Jarvis JW, Oran PE, Rogers SP, Nelson RW. Population studies of intact vitamin D binding protein by affinity capture ESI-TOF-MS. *J. Biomol. Tech*. 2008; 19:167–176.
22. Prelli F, Pras M, Frangione B. The primary structure of human tissue amyloid P component from a patient with primary idiopathic amyloidosis. *J. Biol. Chem*. 1985; 260:12895–12898. [PubMed: 4055725]
23. Minarik M, Groiss F, Gas B, Blaas D, Kenndler E. Dispersion effects accompanying pressurized zone mobilisation in capillary isoelectric focusing of proteins. *J. Chromatogr. A*. 1996; 738:123–128.
24. Righetti PG. Determination of the isoelectric point of proteins by capillary isoelectric focusing. *J. Chromatogr. A*. 2004; 1037:491–499. [PubMed: 15214685]
25. Ohkubo I, Sahashi W, Namikawa C, Tsukada K, et al. A procedure for large scale purification of human plasma amyloid P component. *Clin. Chim. Acta*. 1986; 157:95–102. [PubMed: 3719995]
26. Urbanyi Z, Medzihradsky D. Rapid method to isolate serum amyloid p component from human plasma. Characterization of the isolated protein. *J. Chromatogr*. 1992; 578:130–133. [PubMed: 1400779]
27. Yang L, Tang Q, Harrata AK, Lee CS. Capillary isoelectric focusing-electrospray ionization mass spectrometry for transferrin glycoforms analysis. *Anal. Biochem*. 1996; 243:140–149. [PubMed: 8954536]
28. Loboda AV, Chernushevich IV. Investigation of the mechanism of matrix adduct formation in MALDI at elevated pressure. *Int. J. Mass Spectrom*. 2005; 240:101–105.

Figure 1.

Representative mass spectra of (a) SAP from MALDI-MS, (b) SAP from ESI-MS with y-axis zoomed to show details, and (c) MALDI-MS of SAP after 1 hour incubation with neuraminidase at 37°C. For native SAP, the peaks at 25,460, 25,170, and 24,880 m/z represent the disialo, monosialo, and asialo SAP monomers, respectively. Additional peaks in (a) and (c) represent matrix adducts with a shift of 210–220 Da corresponding to sinapinic acid (224 g/mol).

Figure 2. MALDI mass spectra of fractions collected from the cIEF separation using pooled samples (a) SAP, (b) SAP with 8 M urea, and (c) a mixture of SAP & desialylated SAP with 8 M urea. Desialylated SAP generated by incubating SAP with neuraminidase at 37 °C for 1 hour. No spectral signals were observed in any other fractions not shown.

Figure 3. Representative absorbance profile (220 nm) of cIEF run illustrating detection of pI markers (5.1 & 4.0) and steady decay of background absorbance due to ampholyte mixture. The dashed line (10 min) indicates the time at which mobilization is initiated.

Figure 4.

Representative MALDI mass spectra of SAP preparations from 20 individuals overlaid. The primary peaks are at 25,460 m/z (disialo) and 25,170 m/z (monosialo), and the other peaks represent matrix adducts (25,680 and 25,385 m/z). These spectra are representative of 373 out of 374 samples tested from individuals as supported by the relative peak areas and standard deviations shown above each signal.

Table 1

Literature pI values and number of observed isoforms for human SAP

Reference	[Urea]	pI	# of Isoforms	Technique, Sample, Purification Method
[25]	0 M	4.1	N/A	Chromatofocusing, Pool, Sepharose 4B
[5]	0 M	5.6–6.2	N/A	Gel IEF, Individual, Sepharose 4B
[7]	8 M	6	1	Gel IEF, Both, PE Sepharose
[26]	6 M	5.7–5.8	1	Gel IEF, Pool, Sepharose 6B
[3]	9.6 M	5.95–6.08	4	Gel IEF, Pool, Agarose
[4]	8 M	5.65–5.87	6	Gel IEF, Individual, Sepharose 4B
[5]	8 M	4.7–5.7	6 to 8	Gel IEF, Individual, Sepharose 4B