



## Purification, identification and profiling of serum amyloid A proteins from sera of advanced-stage cancer patients

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### ABSTRACT

Surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) is a powerful tool for screening potential biomarkers of various pathological conditions. However, low resolution and mass accuracy of SELDI-TOF-MS remain a major obstacle for determination of biological identities of potential protein biomarkers. We report here a refined workflow that combines ZipTip desalting, acetonitrile precipitation, high-performance liquid chromatography (HPLC) separation and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis for the profiling, purification and identification of the targeted serum proteins found by SELDI-TOF-MS. By using this workflow, we purified ten targeted proteins from the sera of patients with various types of advanced stage (stage III–IV) cancers. These proteins were identified as isoforms of the human serum amyloid protein A (SAA) family with or without truncations at their N-terminals. This was confirmed by Western blot analysis. Different SAA expression patterns were observed by MALDI-TOF-MS profiling. SAA has long been reported as a biomarker for various cancer types such as lung cancer, ovarian cancer, and breast cancer. However, in this study we found increased SAA expression in the sera of advanced-stage cancer patients with different cancer types. Our results suggest that maybe SAA should not be used alone as a biomarker for any specific cancer type.

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### 1. Introduction

Surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) has played an important role in the area of biomarker discovery over the last decade. With the advantages of simple operation, high-throughput capability, relatively high sensitivity, and the capability of profiling small proteins with molecular weight less than 20 kDa, it has been widely used as a screening tool for clinical samples, and led to potential protein biomarker discovery in many cancer types, including lung can-

cer [1], prostate cancer [2], ovarian cancer [3] and pediatric acute lymphoblastic leukemia [4].

However, the inherent low resolution and mass accuracy have prevented SELDI-TOF-MS from application in identification of targeted proteins. As a consequence, many reports were limited to a general description of protein peaks, without further elucidation of protein identities and clinical and pathological validation. Therefore, developing novel approaches for the purification and identification of targeted proteins remain a major challenge in SELDI-TOF-MS-based biomarker discovery.

By use of SELDI-TOF-MS, researchers in Shanxi Cancer Hospital have found a cluster of highly expressed proteins with molecular weight between 11,100 and 11,900 *m/z* in sera of patients with advanced-stage cancers of various origins. The increase in expression of these low-molecular-weight proteins has been shown to be associated with disease progression and a general deterioration of the patient's conditions [5].

In this study, we developed an approach combining ZipTip  $\mu$ -C18 pipette tip desalting, acetonitrile (ACN) precipitation, reverse phase (RP) C18 high-performance liquid chromatography (HPLC) purification and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis, and applied it to the profiling, purification and identification of these reported

**Abbreviations:** ACN, acetonitrile; BSA, bovine serum albumin; CHCA,  $\alpha$ -cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; FA, formic acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IAM, iodoacetamide; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PMF, peptide mass fingerprinting; RP, reverse phase; SA, sinapinic acid; SAA, human serum amyloid protein A; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELDI-TOF-MS, surface-enhanced laser desorption/ionization time of flight mass spectrometry; SPE, solid phase extraction; TBST, Tris-buffered saline Tween-20; TFA, trifluoroacetic acid; WCX, weak cation exchange.

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proteins [5]. Our data demonstrated that these low-molecular-weight proteins are members of the human serum amyloid protein A (SAA) family with or without truncations at their N-terminals.

## 2. Materials and methods

### 2.1. Materials

Studies using human tissues blood were approved by Human Research Ethics Committees of Shanxi Cancer Hospital. Informed consents were obtained from all participating patients and healthy donors. Serum samples were collected according to standard operating procedures as described previously [5], and were stored at  $-80^{\circ}\text{C}$  until use. All samples had been analyzed by SELDI-TOF-MS using weak cation exchange (WCX) protein chips. Representative samples with high expression levels of the targeted protein peaks were randomly chosen for purification and identification of the targeted proteins.

Reverse phase C18 resin (IR-60-40/60-C18) and reverse phase C18 HPLC column (4.6 mm  $\times$  250 mm, 5  $\mu\text{m}$ , 300 Å) were purchased from the Great Eur-Asia Sci & Tech Development Company (Beijing, China); monoclonal antibody for human SAA and the secondary antibody coupled with HRP were purchased from Abcam (Cambridge, MA, USA); prestained protein markers were purchased from Invitrogen (Cadrilsbad, CA, USA); ultrapure water was obtained from a Milli-Q system, the ZipTip $\mu$ -C18 pipette tips and PVDF membrane (Immobilon-P) were purchased from Millipore (Bedford, MA, USA); sequencing grade modified trypsin was the product of Promega (Madison, WI, USA); ACN and formic acid (FA) were from J.T. Baker (Phillipsburg, NJ, USA); Super enhanced chemiluminescence detection reagents were purchased from Applygen Technologies (Beijing, China). All other chemicals used in the experiment, including  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), trifluoroacetic acid (TFA), dithiothreitol (DTT), iodoacetamide (IAM), bovine serum albumin (BSA) and ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) were all purchased from Sigma Aldrich (St. Louis, MO, USA). All chemicals used were ACS or HPLC grade.

### 2.2. Methods

#### 2.2.1. Serum desalting and MALDI-TOF-MS profiling

10  $\mu\text{L}$  serum from each sample was diluted in 60  $\mu\text{L}$  0.1% TFA in water (solvent A) and the serum sample solution was then desalted using ZipTip  $\mu$ -C18 Pipette Tips following the standard protocol provided by Millipore. Proteins were eluted with 10  $\mu\text{L}$  0.1% TFA in 60% ACN, and evaporated to about 2  $\mu\text{L}$  in vacuum. Each sample solution was mixed with matrix (10 mg/mL SA in 60% ACN containing 0.1% TFA) at the ratio of 1:1 and left to crystallize on the MALDI plate at room temperature. MALDI-TOF-MS protein profiling of the serum samples was performed using an AXIMA-CFR<sup>TM</sup> plus MALDI-TOF mass spectrometer (Shimadzu Biotech, Japan) equipped with a pulsed nitrogen laser operated at 337 nm. MALDI mass spectra were acquired in linear mode; detection mass range was set at 5000–15,000  $m/z$  and was optimized at 11,500  $m/z$ ; the parameters (laser power, accumulated shots) were optimized for each sample to get the best spectra. Before detection, the instrument was calibrated using appropriate protein standards.

#### 2.2.2. ACN precipitation and purification of targeted proteins

The workflow chart of the purification and identification of the targeted proteins was shown in Fig. 1. Serum samples were first pretreated by ACN precipitation to get rid of the abundant proteins and reduce the sample complexity. In detail, 100  $\mu\text{L}$  serum sample was thawed at  $4^{\circ}\text{C}$  and diluted in 300  $\mu\text{L}$  water. 600  $\mu\text{L}$  ACN

was added to the sample solution, and then the mixture was incubated in  $4^{\circ}\text{C}$  for 30 min for precipitation. The sample solution was centrifuged at  $10,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , and the supernatant was collected and evaporated to less than 400  $\mu\text{L}$ , reconstituted with solvent A (0.1% TFA in water) and desalted using home-made SPE (solid phase extraction) C18 column. Briefly, the C18 column was equilibrated for sample binding using solvent A; the supernatant was loaded onto the column 3 times for sufficiently binding and washed using 3 column volumes of solvent A. Finally, the proteins were eluted using 2 mL 60% ACN containing 0.1% TFA. The elution solution was evaporated to 50  $\mu\text{L}$  in vacuum and a small aliquot (about 1–3  $\mu\text{L}$ ) was analyzed by MALDI-TOF-MS to trace the targeted proteins.

The purification of the targeted proteins was performed by HPLC (Shimadzu Biotech, Japan) at a flow rate of 0.5 mL/min. The mobile phases used were solvent A (0.1% TFA in water) and solvent B (80% ACN containing 0.1% TFA). After sample loading, the following gradient program was used: 100% solvent A (0 min) – 100% solvent A (15 min) – 20% solvent B (15.1 min) – 40% solvent B (30 min) – 70% solvent B (80 min) – 100% solvent B (80.1 min) – 100% solvent B (90 min). The detection wavelengths were set at 214, 254 and 280 nm. The eluate was collected based on peaks, and evaporated to less than 20  $\mu\text{L}$ . A small amount (about 1  $\mu\text{L}$ ) was analyzed by MALDI-TOF-MS to trace the targeted proteins and measure their molecular weights. Mass accuracy obtained by external calibration in the linear mode for proteins was within 0.05%.

#### 2.2.3. Tryptic digestion and protein identification

Each purified targeted protein sample was alkalinized to pH 8.0 using 100 mM  $\text{NH}_4\text{NCO}_3$ , reduced with 5 mM DTT at  $56^{\circ}\text{C}$  for 1 h and then treated with IAM at a concentration of 10 mM in the dark for 45 min. About 0.08  $\mu\text{g}$  trypsin was added, and the sample solution was incubated at  $37^{\circ}\text{C}$  overnight. Digestion was ended by adding TFA to final concentration of 0.1% in the next morning.

Each digested sample was analyzed using the MALDI-TOF mass spectrometer in reflection mode with CHCA (4 mg/mL in 60% ACN containing 0.1% TFA) as matrix to obtain peptide mass fingerprinting (PMF) spectra. Detection mass range was set at 700–2500  $m/z$  and was optimized at 1500  $m/z$ . Before detection, the instrument was calibrated using appropriate peptide standards.

PMF spectra were processed using Launchpad 2.4 software (Shimadzu Biotech, Japan) and searched online using the Mascot algorithm and the NCBIInr human database. Mass tolerance was set at 0.3 Da calculated using mono isotopic masses. Enzyme cleavage specificity was set to trypsin and no more than one missed cleavage was allowed. Fixed modification was set for cysteine carbamidomethylation with the addition of 57.052 Da. Variable modification was set for methionine oxidation with the addition of 15.999 Da. The results were filtered using the default parameter  $p \leq 0.05$ .

#### 2.2.4. Western blot validation of the identified proteins

Western blot experiments were performed to validate the identification result. For each sample, 10  $\mu\text{L}$  serum was diluted to 30  $\mu\text{L}$  with water, fully mixed with 10  $\mu\text{L}$  4 $\times$  electrophoresis loading buffer by vortex shaking, heated at  $100^{\circ}\text{C}$  for 5 min, and was centrifuged at  $12,000 \times g$  for 5 min after it cooled to room temperature. Equal amount (1.6  $\mu\text{L}$ , equivalent to 0.4  $\mu\text{L}$  original serum) of the supernatant of each sample was loaded on an 18% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) for separation. The protein bands were transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine, 20% methanol, blocked with 2% BSA in TBST (Tris-buffered saline Tween-20) (100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) at  $4^{\circ}\text{C}$  overnight. The proteins were probed with a mouse anti-human SAA

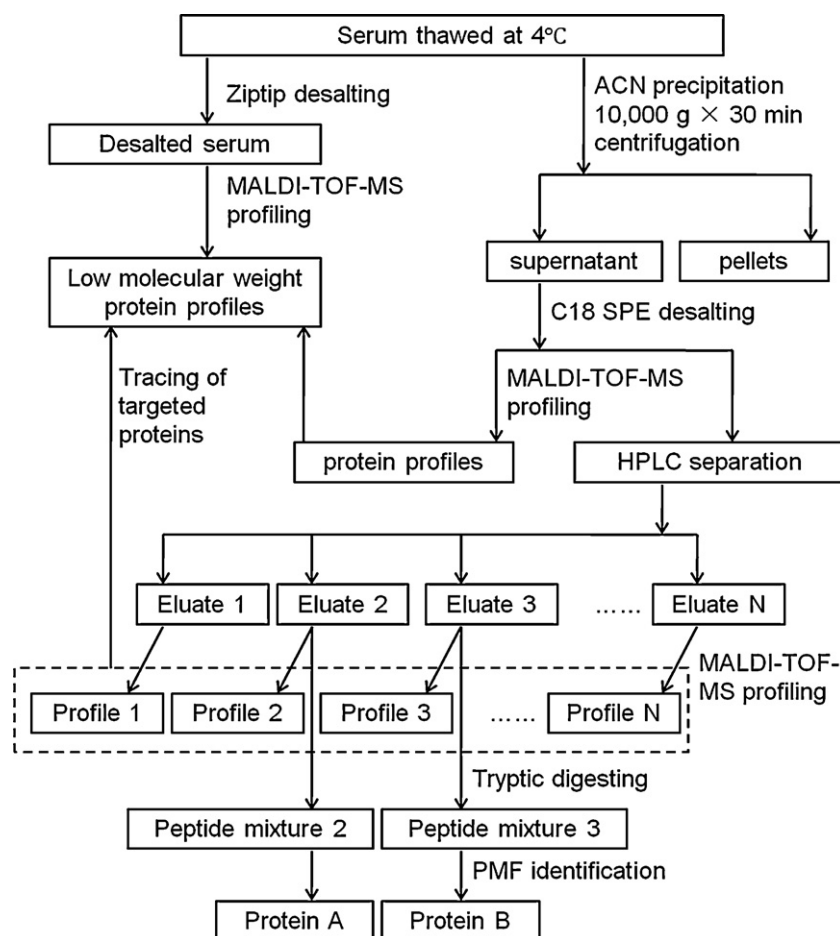


Fig. 1. The workflow of targeted protein purification and identification from human sera.

antibody (0.2  $\mu\text{g}/\text{mL}$ ) at 4 °C for 2 h, washed three times in TBST and incubated with a goat anti-mouse IgG (H + L)(HRP (horseradish peroxidase)) secondary antibody (0.2  $\mu\text{g}/\text{mL}$ ) at room temperature for 1 h. After washing in TBST for three times again, the proteins were detected by enhanced chemiluminescence.

### 3. Results and discussion

#### 3.1. MALDI-TOF-MS profiling of low-molecular-weight proteins in sera

MALDI-TOF-MS was successfully used to obtain the low-molecular-weight protein profiles of human serum (Fig. 2A). Fig. 2B shows that MALDI-TOF-MS spectra (right) are of much higher resolution and mass accuracy than SELDI-TOF-MS spectra (left) in the targeted mass range (11,000–12,000  $m/z$ ). Therefore the targeted proteins could be traced using MALDI-TOF-MS during purification. MALDI-TOF-MS provided a reliable profiling method with high reproducibility. As shown in Fig. 2A, while the targeted proteins were present only in the spectra of advanced-stage cancer patients' sera, other proteins were repeatedly observed in all the spectra.

#### 3.2. Purification and identification of the targeted proteins

ACN precipitation was performed first to get rid of most "large" and high-abundance proteins in serum (Supplementary Fig. 1A). After precipitation, MS analysis of serum low-molecular-weight proteins was greatly improved with enhanced signal intensity and

higher resolution (Supplementary Fig. 1B), which facilitated tracing of the targeted proteins.

RP C18 HPLC separation was carried out next, and the targeted proteins were found to be eluted using 50–55% solvent B (Supplementary Fig. 2). Using our workflow, ten targeted proteins were purified from more than 20 human serum samples of advanced-stage cancer patients (Fig. 3). Their observed  $m/z$  values were: 11,387.1, 11,439.5, 11,472.9, 11,491.3, 11,525.3, 11,583.7, 11,627.3, 11,647.3, 11,655.1 and 11,682.9.

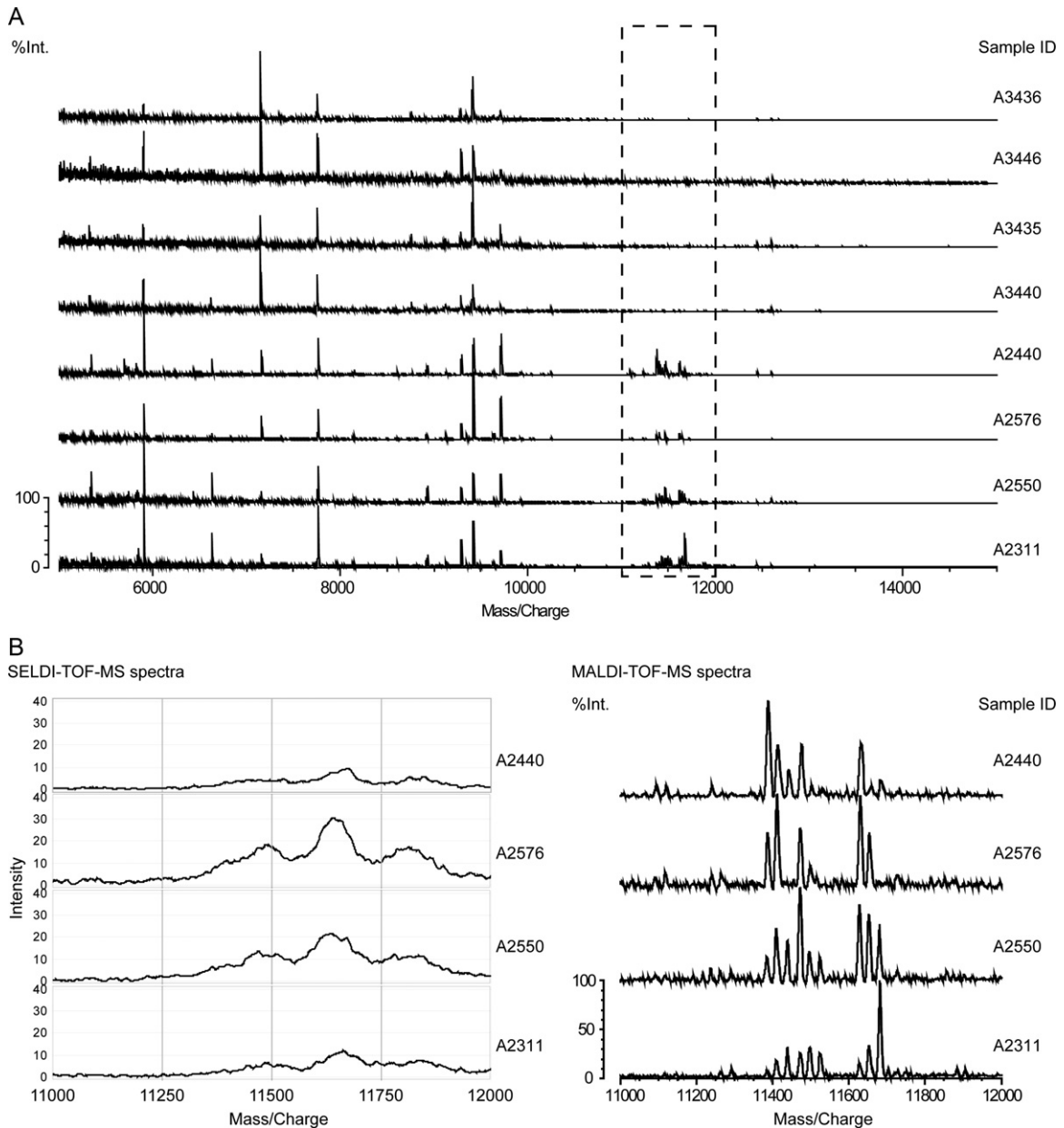
The purified proteins were then digested with trypsin, analyzed by MALDI-TOF-MS and confidently identified as a series of isoforms of human serum amyloid A (SAA) or truncated SAA by online Mascot search engine. With the combination of the accurate molecular weight information and the PMF results, the whole amino acid sequence of each purified proteins was assigned (Table 1). Table 1 indicates that the identified target proteins include three forms of SAA1 $\alpha$ , one of SAA1 $\beta$ , one of SAA1 $\gamma$ , three of SAA2 $\alpha$  and two of SAA2 $\beta$ , respectively. The PMF spectra and database search results are shown in Supplementary Fig. 3.

The heterogeneity of human SAA has been reported earlier [6–8]. Based on the identification results, we examined the protein peaks that were not only purified but were also in the targeted mass range. These protein peaks were found to have similar performance with the identified SAA proteins, and their observed molecular masses were in good accordance with the reported SAA isoforms with or without N-terminal truncations. It is therefore conceivable that these proteins similarly belong to the SAA family. The identified and speculated SAA isoforms and their

**Table 1**  
Identification results of the targeted proteins.

Protein name and length <sup>a</sup>	Theoretical MW (Da)	Observed MW (Da)	Sequence coverage (%)	Mascot score	Identified peptides	Protein sequence
SAA1 $\alpha$ (1–104)	11,682.7	11,682.9	80.77	102	-.RSFFSLGAEAFDGDAR.D. R.SFFSLGAEAFDGDAR.D. R.EANYIGSDKYFHAR.G. K.RGPGGVWAAEISDARE. R.GPGGVWAAEISDARE. R.FFGHGAEDSLADQAANEWGRS. K.DPNHFRPAGLPEKY.-	<u>RSFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHAR</u> <u>GNYDAAKRGPGGVWAAEISDARENIQRFFGHGAEDSLAD</u> <u>QAANEWGRSGKDPNHFRPAGLPEKY.</u>
SAA1 $\alpha$ (2–104)	11,526.5	11,526.6	81.55	104	R.SFFSLGAEAFDGDAR.D. R.DMWAYSMDMR.E. R.AYSMDMR.E. R.EANYIGSDKYFHAR.G. K.RGPGGVWAAEISDARE. R.GPGGVWAAEISDARE. R.FFGHGAEDSLADQAANEWGRS. K.DPNHFRPAGLPEKY.-	<u>SFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHAR</u> <u>GNYDAAKRGPGGVWAAEISDARENIQRFFGHGAEDSLAD</u> <u>QAANEWGRSGKDPNHFRPAGLPEKY.</u>
SAA1 $\alpha$ (3–104)	11,439.4	11,439.5	67.65	72	R.EANYIGSDKYFHAR.G. K.RGPGGVWAAEISDARE. R.GPGGVWAAEISDARE. R.FFGHGAEDSLADQAANEWGRS. K.DPNHFRPAGLPEKY.-	<u>FFSFLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYD</u> <u>AAKRGPGGVWAAEISDARENIQRFFGHGAEDSLADQAANE</u> <u>WGRSGKDPNHFRPAGLPEKY</u>
SAA1 $\beta$ (2–104)	11,584.6	11,583.7	100	145	-.SFFSLGAEAFDGDAR.D. -.SFFSLGAEAFDGDARMWR.A. R.AYSMDMR.E. R.EANYIGSDKYFHAR.G. K.YFHAR.G. K.YFHARGNYDAAK.R. K.RGPGGAWAAEVISDARE. R.GPGGAWAAEVISDARE. R.GPGGAWAAEVISDARENIQR.F. R.FFGHDAEDSLADQAANEWGRS. R.SGKDPNHFRPAGLPEKY.Y. K.DPNHFRPAGLPEKY.-	<u>SFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYDAA</u> <u>KRGPGGAWAAEVISDARENIQRFFGHDAEDSLADQAANEWGRSG</u> <u>KDPNHFRPAGLPEKY.</u>
SAA1 $\gamma$ (1–104).	11,654.7	11,655.1	84.62	90	-.RSFFSLGAEAFDGDAR.D. R.SFFSLGAEAFDGDAR.D. R.AYSMDMR.E. R.EANYIGSDKYFHAR.G. K.RGPGGAWAAEISDARE*. R.FFGHGAEDSLADQAANEWGRS. R.SGKDPNHFRPAGLPEKY.Y. K.DPNHFRPAGLPEKY.-	<u>RSFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYDAAK</u> <u>RGPGGAWAAEISDARENIQRFFGHGAEDSLADQAANEWGRSGKD</u> <u>PNHFRPAGLPEKY.</u>
SAA2 $\alpha$ (1–104)	11,628.7	11,627.3	76.92	84	R.SFFSLGAEAFDGDAR.D. R.AYSMDMR.E. R.AYSMDREANYIGSDK.Y. K.RGPGGAWAAEVISNARE. R.GPGGAWAAEVISNARE. R.LTGHGAEDSLADQAANK.W. R.SGRDPNHFRPAGLPEKY.Y. R.DPNHFRPAGLPEKY.Y. R.DPNHFRPAGLPEKY.-	<u>RSFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYDAAK</u> <u>RGPGGAWAAEVISNARENIQRLTGHGAEDSLADQAANKWGRSGRDP</u> <u>NHFRPAGLPEKY</u>
SAA2 $\alpha$ (3–104)	11,385.4	11,387.1	49.02	64	R.EANYIGSDKYFHAR.G. K.RGPGGAWAAEVISNARE. R.GPGGAWAAEVISNARE. R.LTGHGAEDSLADQAANKWGRS	<u>FFSFLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYDAAK</u> <u>RGPGGAWAAEVISNARENIQRLTGHGAEDSLADQAANKWGRSGRD</u> <u>PNHFRPAGLPEKY</u>
SAA2 $\alpha$ (2–104)	11,472.5	11,472.9	84.47	104	R.SFFSLGAEAFDGDAR.D. R.AYSMDMR.E. R.EANYIGSDKYFHAR.G. K.RGPGGAWAAEVISNARE. R.GPGGAWAAEVISNARE. R.LTGHGAEDSLADQAANK.W. R.LTGHGAEDSLADQAANKWGRS. R.SGRDPNHFRPAGLPEKY.Y. R.DPNHFRPAGLPEKY.-	<u>SFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYDAAK</u> <u>RGPGGAWAAEVISNARENIQRLTGHGAEDSLADQAANKWGRSGR</u> <u>DPNHFRPAGLPEKY</u>
SAA2 $\beta$ (1–104)	11,647.8	11,647.3	78.85	100	-.RSFFSLGAEAFDGDAR.D. R.SFFSLGAEAFDGDAR.D. R.EANYIGSDKYFHAR.G. K.RGPGGAWAAEVISNARE. R.GPGGAWAAEVISNARE. R.LTGHGAEDSLADQAANKWGRS. R.SGRDPNHFRPAGLPEKY.Y. R.DPNHFRPAGLPEKY.-	<u>RSFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYDAAK</u> <u>RGPGGAWAAEVISNARENIQRLTGRGAEDSLADQAANKWGRSGRD</u> <u>PNHFRPAGLPEKY</u>
SAA2 $\beta$ (2–104)	11,491.6	11,491.3	62.14	94	R.SFFSLGAEAFDGDAR.D. R.AYSMDMR.E. R.EANYIGSDKYFHAR.G. K.RGPGGAWAAEVISNARE. R.GPGGAWAAEVISNARE. R.DPNHFRPAGLPEKY.-	<u>SFFSLGAEAFDGDARMWRAYSMDREANYIGSDKYFHARGNYDAAK</u> <u>RGPGGAWAAEVISNARENIQRLTGRGAEDSLADQAANKWGRSGR</u> <u>DPNHFRPAGLPEKY</u>

<sup>a</sup>Protein lengths were calculated for mature proteins without signal sequences. Arginine (R) was considered as the first amino acid of the N-terminal of the protein.



**Fig. 2.** (A) MALDI-TOF-MS profiles of serum low-molecular-weight proteins. The upper four spectra were from healthy individuals, while the lower four were from advanced-stage cancer patients. Targeted mass range (11,000–12,000  $m/z$ ) was marked with a dashed box. (B) Magnified protein profiles of the targeted mass range obtained from SELDI-TOF-MS (left) and MALDI-TOF-MS (right), respectively. Information of serum donors could be found in Supplementary Table 1.

truncated forms detected in this study are listed in Supplementary Table 2, annotated as identified results or speculated results, respectively.

### 3.3. Analysis of SAA profiling patterns in sera from advanced-stage cancer patients

To clarify SAA expression patterns in sera from patients with different types of advanced-stage cancers, we profiled 180 serum samples (data not shown) using the method described in Section 2.2.1. Diverse SAA expression patterns were discovered, with different peak numbers, various peak combinations and distinct signal intensities (Fig. 4). We have made an effort to find out if there is any correlation between the different patterns and the cancer types or the tumor sites, but could not draw any statistically significant

conclusion so far. Maybe the amount of samples was too small for a study of multiple cancer types, or maybe the SAA expressional differences were simply due to molecular heterogeneity from person to person, as showed by Nedelkov et al., in their investigation of the diversity of human proteins [9,10].

### 3.4. Western blot validation of the identified results

Western blot experiments were performed for results validation. The results indicated that sera from terminal cancer patients showed obvious chemiluminescent lanes of SAA, while SAA could not be detected in sera from healthy controls when using the same volume of serum. Furthermore, the SAA protein abundance revealed by western blot was consistent with the MALDI-TOF-MS profiles for the corresponding samples (Fig. 5).

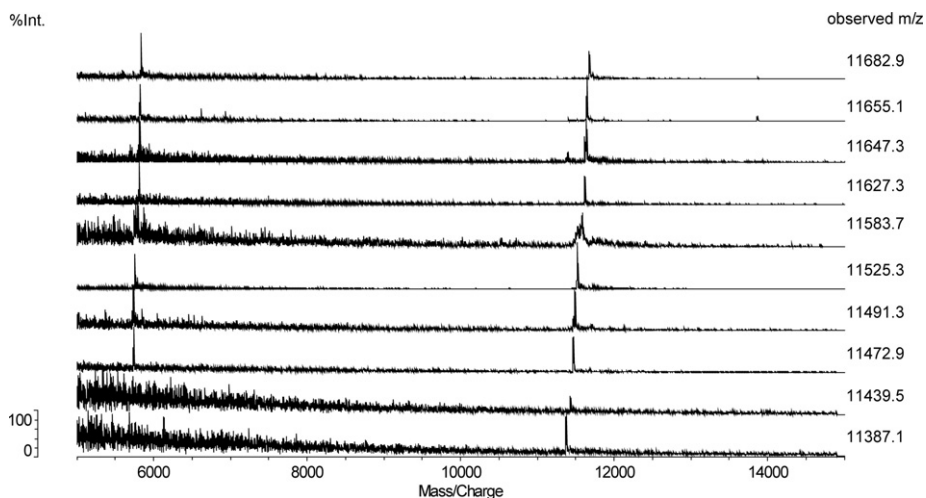


Fig. 3. MALDI-TOF-MS spectra of the ten purified target proteins. Their observed  $m/z$  values were listed on the right.

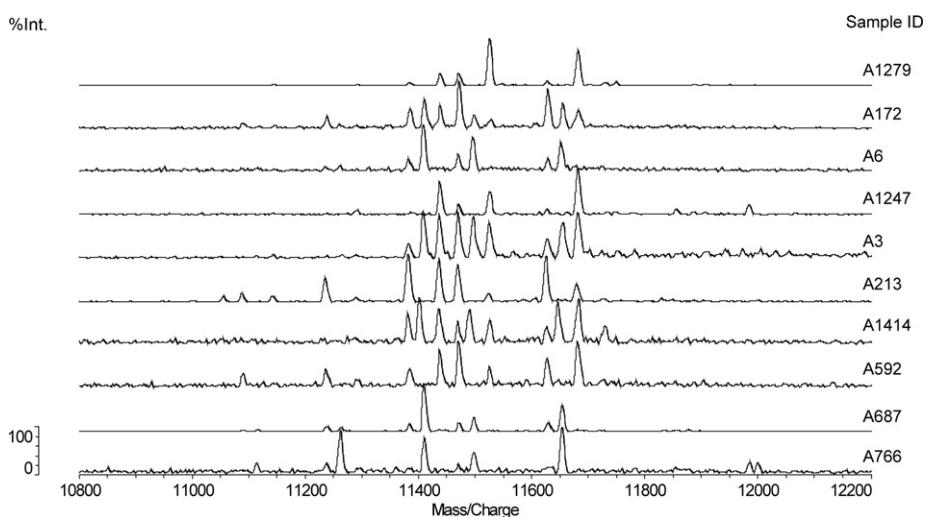


Fig. 4. Examples of SAA expression patterns of advanced-stage cancer patients. Information of serum donors could be found in Supplementary Table 1.

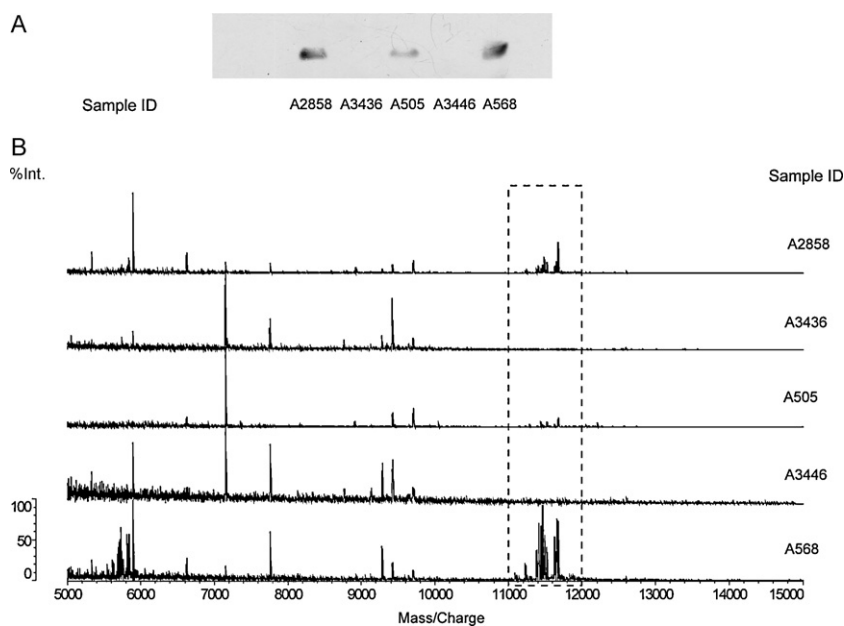


Fig. 5. The result of Western blot experiment (A) and MALDI-TOF-MS profiling (B) of sera from healthy individuals (A3436, A3446) and advanced-stage cancer patients (A2858, A505, A568). The targeted mass range (11,000–12,000) was marked with a dashed box. Information of serum donors could be found in Supplementary Table 1.

#### 4. Conclusion

In this study, we established an efficient workflow for the profiling, purification and identification of low-abundance, low-molecular-weight targeted proteins in human serum. It is a simple, fast and robust method that can be readily adopted to other similar studies.

Using this workflow, we purified and identified ten SAA isoforms with or without truncations from sera of advanced-stage cancer patients. Some of them were purified for the first time. Although detection of human SAA proteins using MALDI-TOF-MS has been previously reported [9,11,12], our study appears to be the first to detect such a large number of SAA isoforms with high mass accuracy and resolution.

The proteins identified in this study (SAA1 $\alpha$ , SAA1 $\beta$ , SAA1 $\gamma$ , SAA2 $\alpha$  and SAA2 $\beta$ ) all belong to the acute phase SAA family. They are major acute-phase reactants and have been suggested to have immune-related functions. They are secreted during the acute phase of inflammation, taking part in the processes including transport of cholesterol, recruitment of immune cells and induction of some enzymes [13,14].

SAA has long been a hotspot in the field of biomarker research. Elevated expression level of SAA has been reported in many types of cancers including: nasopharyngeal cancer [15–17], ovarian cancer [3,11,18], lung cancer [19–22], pancreatic cancer [23–25], prostate cancer [26–28], colorectal cancer [29,30], breast cancer [31,32], gastric cancer [33], pediatric osteosarcoma [34], neuroblastoma [35] and renal cell carcinoma [36,37] and other diseases such as amyloidosis, coronary artery disease, rheumatoid arthritis and severe acute respiratory syndrome [38–42].

This study detected SAA in sera from multiple cancer types for the first time and found high expression levels of SAA in sera of almost all advanced-stage cancer patients. Based on this and the previous studies [5,21,43], it seems that SAA may be a biomarker or an indicator of deteriorating physical conditions found in advanced-stage cancers, but should not be used alone as a biomarker of any specific cancer type.

#### Conflict of interest

The authors have declared no conflict of interest

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.01.002.

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