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Isolation and purification of two major serum amyloid A isotypes SAA1 and SAA2 from the acute phase plasma of mice

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Abstract

A new procedure was developed for isolation of two major serum amyloid A (SAA) isotypes SAA1 and SAA2 from acute-phase plasma of mice. The procedure included preparation of high-density lipoproteins (HDLs) and their separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SAA proteins (M_r 12 000) were electroeluted and afterwards purified from SDS by gel permeation chromatography on a Fractogel TSK-40F column in aqueous 50% acetonitrile–0.1% TFA. Finally, the SAA proteins free from SDS were fractionated by high-performance liquid chromatography on a Vydac 214TP54 column (250×4.6 mm I.D., particle size 5 μm), yielding two major fractions with $k=5.2$ and $k=5.5$. The N- and C-terminal sequence analyses and mass spectrometry demonstrated the purity of these two major fractions and their identity with apo SAA1 ($k=5.2$) and apo SAA2 ($k=5.5$). The developed procedure is applicable to small amounts of pooled murine plasma (6–7 ml) and could be readily modified from small to large scale preparations.

Keywords: Serum amyloid A isotypes; High-density-lipoprotein apoproteins

1. Introduction

Serum amyloid A (SAA) is a family of high-density-lipoprotein (HDL) apoproteins found in mammals and duck. Apo SAA are highly homologous proteins (M_r 12 000) representing some of the most dramatic acute-phase reactants. In the mice, the SAA family is made up of four genes (SAA1, SAA2, SAA3 and SAA5) and pseudogene (SAA4) [1–4]. Apo SAA1 and SAA2 are the major acute-phase reactants, whose amount could increase up to 1000-fold during inflammatory response. Apo SAA3 is present as a minor apolipoprotein on acute-phase HDL. Apo SAA5 is constitutively expressed and comprises the major form of SAA proteins in normal HDL.

The normal function of apo SAA proteins and the pathologic processing of these proteins in AA amyloidogenesis are widely studied, but remain as yet not fully elucidated [2,5–10]. It is still unclear why one of two major SAA isoforms, the apo SAA2, is deposited in tissues and processed into insoluble AA protein in the experimentally induced murine amyloidosis [8,9]. Further studies are needed to understand better the structure of SAA proteins and their association with HDL in normal state and

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disease. Although the complete primary structure of SAA isotypes was deduced from the corresponding nucleotide sequences [1,3,11], the direct biochemical studies including the preparative isolation, purification and analysis of murine SAA isotypes are still missing.

Here we present a new technique for isolation and purification of two major murine acute-phase reactants SAA1 and SAA2 and their biochemical examination.

2. Experimental

2.1. Chemicals

Acetonitrile (Bio-Labs., Jerusalem, Israel) was of HPLC grade. Sodium dodecyl sulphate (SDS) and Tween-20 were obtained from BDH (Poole, UK). Lipopolysaccharide *E. coli* (LPS, 4-2630), trifluoroacetic acid (TFA), acrylamide, β-mercaptoethanol, Coomassie Blue, bovine serum albumin (BSA), 4-chloro-1-naphtol, 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) and goat anti-rabbit IgG peroxidase conjugate were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-mouse protein AA antibodies were kindly provided by Prof. K. Miura (Dept. of Pathology, Hamamatsu University, Hamamatsu, Japan). Rainbow coloured protein high-molecular-mass markers (M_r 14 000–220 000) were obtained from Amersham (Little Chalfont, UK).

2.2. Preparation of mouse HDL

Acute inflammation was induced in male Swiss mice ($n=20$) by intraperitoneal (i.p.) injection of 50 μg of LPS (dissolved in 4% normal mouse serum). 24 h after LPS administration the animals were bled from the eye choroid plexus with heparinized Pasteur pipette. Plasma was separated by centrifugation at 4°C with precooled rotor at 1500 *g*. Mouse HDL was obtained from the pooled acute-phase plasma as described elsewhere [12]. Briefly, the plasma density (ρ) was adjusted to 1.063 g/ml with solid KBr and the plasma was centrifuged for 24 h at 252 000 *g* (Ti 60 rotor, Beckman Instruments) at 4°C. The infarnatants containing HDL were harvested, re-adjusted to $\rho=1.21$ g/ml and centrifuged for 48 h at 252 000 *g* (Ti 60 rotor, Beckman Instruments) at 4°C. The pellicles containing HDL were collected, dialyzed against distilled water and lyophilized.

2.3. Western blotting

The specimens of HDL preparation were run on 17% SDS–polyacrylamide gels [13], 1.5 mm thick, using mini Protean II vertical electrophoresis system (Bio-Rad Labs., Richmond, CA, USA). The proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using 25 m*M* Tris, 192 m*M* glycine (pH 8.3) buffer containing 20% methanol (v/v) and 0.1% SDS. Unbound sites were blocked with 1% BSA in TBS (pH 7.5) containing 0.05% Tween-20. Rabbit antibodies to mouse amyloid A protein (40 μg/ml) were used as primary antibodies. Goat anti-rabbit IgG peroxidase conjugate was used as secondary antibody (1:1000). Immunoreactive bands were visualized using 4-chloro-1-naphtol and hydrogen peroxide.

2.4. Isolation of total apo SAA

Isolation of total SAA was performed by SDS-polyacrylamide gel electrophoresis (PAGE) on 17% polyacrylamide gels, 1.5 mm thick, with 5 mm wide sample wells. The aliquots of HDL preparations were dissolved in a sample buffer containing 125 m*M* Tris–HCl (pH 6.8), 6% SDS, 2% β-mercaptoethanol and 0.2 *M* sucrose. The samples, 10 μg/μl, were loaded on the gel, 20 μl/well. The prestained protein markers were used for the localization of SAA proteins, M_r 12 000. The gel slices containing the localized unstained SAA proteins were placed to a Biodialyser sample chamber with the pre-cut MWCO 1000 membranes (Sialomed, Columbia, MD, USA). The electroelution was carried out in a horizontal electrophoretic cell using 25 m*M* Tris–192 m*M* glycine buffer (pH 8.3) containing 0.1% SDS. The content of the sample chamber was centrifuged, and the supernatant containing the electroeluted proteins, buffer salts and SDS was collected.

2.5. SDS removal

The removal of SDS was performed as described earlier [14,15]. The aliquots of the electroeluted proteins (see Section 2.4) were acidified with 10%

TFA (final concentration 0.1% TFA), diluted with 60% acetonitrile–0.1% TFA (1:2) and applied onto a Fractogel TSK HW-40 (F) (Merck, Darmstadt, Germany) column (200×10 mm I.D.). The elution was performed using aqueous 50% acetonitrile–0.1% TFA. The void volume material containing SAA proteins free from SDS and buffer salts was collected and lyophilized.

2.6. Fractionation of SAA proteins by RP-HPLC

The HPLC equipment consisted of Spectra-Physics 8700 solvent delivery system, 8500 dynamic mixer and 8750 organizer, coupled to Jasco Uvidec 100IV spectrophotometer with an 8 μl cassette type cell (10 mm pathway) and a Hewlett-Packard 3390 A integrator. The electroeluted SAA proteins free from SDS (see Section 2.5) were applied on a Vydac 214TP54 (Alltech, Deerfield, IL, USA) column (250×4.6 mm I.D., pore diameter 300 Å, particle size 5 μm) and eluted using a linear gradient from 20 to 80% acetonitrile–0.1% TFA over 30 min. Preparation of sample for injection included the dissolution of SAA proteins (obtained from 4 mg HDL) in 6 *M* urea–0.1% TFA (50–70 μl) and dilution with 20% acetonitrile–0.1% TFA (1:2). The elution of proteins was monitored by UV absorbance at 220 nm. The HPLC peaks were collected and lyophilized.

2.7. N-terminal sequence analysis

The specimens of HDL preparation and the HPLC peaks of the SAA proteins were run on 17% polyacrylamide gels and electrotransferred to the Immobilon-P membranes (Millipore, Bedford, MA, USA) using 10 m*M* CAPS buffer (pH 11)–10% (v/v) methanol at 400 mA for 45 min. The electroblotted proteins were visualized by staining with Coomassie Blue. Bands of interest were excised and sequenced using one of the following protein sequencers, Beckman LF 3000 or ABI 4-76, according to the manufacturer's protocols.

2.8. Cleavage and purification of C-terminal SAA fragments

The HPLC peaks of the SAA proteins were further characterized by taking advantage of the Asp–Pro peptide bond located near the C-terminus of SAA [1]. For the cleavage of this peptide bond, the proteins were dissolved in 50% acetic acid and incubated at 55°C for 96 h [16]. The resulting peptides were resolved using a Beckman System Gold HPLC system on an Upchurch narrow-bore reversed-phase high-performance liquid chromatography (RP-HPLC) column (C_{18}, 300 Å pore-size, 50×2.1 mm I.D.). A linear gradient from 5% to 60% acetonitrile–0.1% TFA was run over 60 min at flow-rate of 200 μl/min. Fractions were monitored at both 225 and 280 nm. The expected peptide should have three tyrosines, while the starting material and large fragments contain both tryptophan and tyrosine. Thus, the peaks of interest should have 280 nm absorbance, and the peaks without 280 nm absorbance would be non specific. The fractions which had 280 nm absorbance were subjected to amino acid sequence and mass spectral analyses.

2.9. Mass spectral analysis

The peptide peaks recovered from the Asp–Pro cleavage were analyzed by matrix-assisted-laser desorption time-of-flight mass spectrometry (MALD–TOF-MS) on a PerSeptive Biosystems Voyager Biospectrometer. A solution of α-cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile–0.1% aqueous TFA) was used as matrix. One μl of peptide recovered from HPLC was spotted with 1 μl of matrix, dried and then analyzed.

2.10. Amino acid analysis

Amino acid analysis was carried out for quantitation of the yield of the purified SAA proteins. Samples were hydrolized in the vapor-phase at 160°C for 1 h using 6 *M* hydrochloric acid and analyzed on a Beckman 6300 amino acid analyzer using System Gold software.

3. Results

The HDL fraction obtained from the acute-phase murine plasma yielded about 0.8 mg dry HDL material/ml plasma. The electrophoretic analysis of the obtained acute-phase plasma HDL revealed two major protein bands with M_r values of about 12 000

and 28 000 (Fig. 1, lane 2). Western blotting showed that protein band with M_r of 12 000 was immunoreactive with the anti amyloid A antibodies, thus corresponding to apo SAA (Fig. 1, lane 3). Protein with M_r about 28 000 was electroblotted on a PVDF membrane for microsequencing which revealed N-termini DEPQSQWDKVKDFAN identical to that of apo A1.

The electroelution of SAA proteins from the gels was followed by SAA purification from SDS and afterwards their separation by HPLC. This procedure was performed repeatedly in three independent experiments, each time using about 4 mg of starting HDL material. The obtained HPLC profiles (1–2 runs/experiment, $n=5$) were highly reproducible and yielded two major fractions, peak IV (mean k value=5.2, C.V.=0.5%) and peak VI (mean k value=5.5, C.V.=0.45%) (Fig. 2). Fractions IV and VI and the minor fractions I, II, III and V were characterized by automated Edman degradation. N-terminal sequence

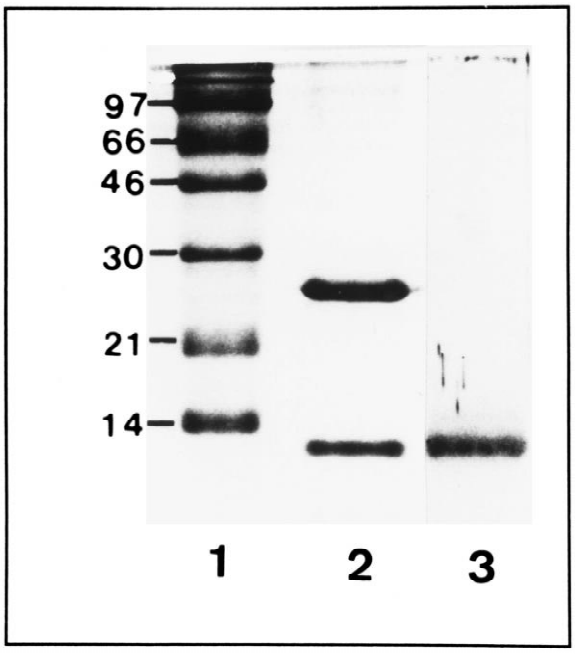

Fig. 1. The SDS-PAGE (2) and Western blotting (3) of the HDL fraction obtained from the acute-phase plasma of mice. (1) Molecular mass markers ($M_r \cdot 10^{-3}$); (2) major proteins of acute-phase plasma HDL: apo A-1 (M_r 28 000), apo SAA (M_r 12 000); (3) Western blot of HDL fraction performed using anti-SAA antibodies.

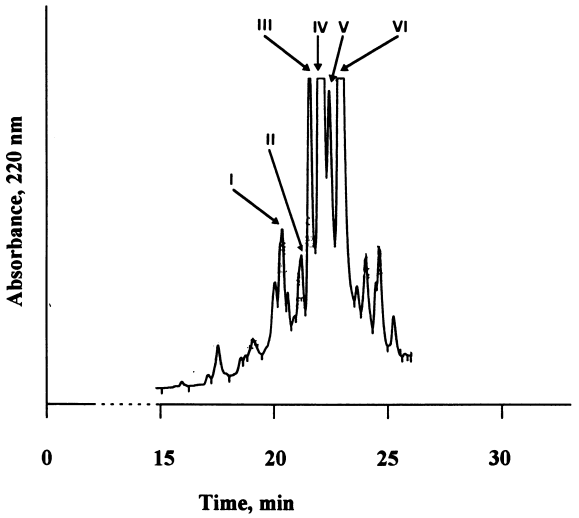

Fig. 2. RP-HPLC of the SAA proteins isolated from the acute-phase plasma of mice. SAA obtained from about 4 mg HDL by preparative SDS-PAGE and purified from SDS was injected into Vydac 214TP54 column and eluted using a linear gradient from 20 to 80% acetonitrile in 0.1% TFA over 30 min as described in Section 2.6. The effluent was monitored by UV absorbance at 220 nm, sensitivity 0.53 a.u.f.s. The elution time of the unretained solute (ammonia solution), $t_0=3.2$, was determined and used for the calculation of the k values.

analysis allowed identification of the components, with major peaks IV and VI representing apo SAA1 and SAA2, respectively (Tables 1 and 2). Small amounts of SAA isotypes were also revealed in the minor fractions: SAA1 was predominant in fractions I–III, SAA2 in fraction V (Table 2).

The C-terminal domains of two major HPLC fractions were cleaved (as described in Section 2.8 above) and purified by narrow-bore RP-HPLC (Fig. 3). The peptides cleaved from fractions IV and VI and eluted at 31.3 min and 31.4 min, respectively, were subjected to amino acid sequence and mass spectral analyses. The amino acid sequences of the C-terminal domains of fractions IV and VI were found to be identical to those of apo SAA1 and SAA2, respectively (Table 1). These findings were confirmed by mass spectral analysis of the C-terminal peptides: peaks at m/z 1585.5 (fraction IV) and 1541.8 (fraction VI) corresponded to the molecular ions for C-termini of apo SAA1 and apo SAA2, respectively (Fig. 4).

The separated SAA1 and SAA2 proteins were

Table 1
N- and C-terminal amino acid sequence analyses of the SAA fractions IV and VI separated by HPLC

	N-terminal sequence	C-terminal sequence
SAA1[a]	GFFSFVHEAFQGAGDMWRAY	PNYYRPPGLPDKY
Fr. IV[b]	GFFSFVHEAFQGAGDMWRAY	PNYYRPPGLPDKY
SAA2[a]	GFFSFIGEAFQGAGDMWRAY	PNYYRPPGLPAKY
Fr. VI[b]	GFFSFIGEAFQGAGDMWRAY	PNYYRPPGLPAKY

[a] Amino acid sequences deduced from the corresponding nucleotide sequences [1,3,11].
[b] Amino acid sequences determined presently by protein sequencing.

quantitated by amino acid analysis. The amounts of fractions IV (SAA1) and VI (SAA2) obtained from 4 mg of the starting HDL material corresponded to about 12 and 8 μg, respectively.

4. Discussion

Presence of different apo SAA isoforms in acute-phase mice plasma was shown in early studies [17–20] which demonstrated two distinct SAA isotypes by using SDS-PAGE and isoelectrofocusing techniques. The latter studies, however, were focused mainly on the examination of the SAA gene family structure and their expression [1–4,11,21–23], by paying less attention to isolation and biochemical analysis of these proteins. This is in contrast to the numerous biochemical studies of human apo SAA isoforms which included application of different chromatographic techniques for isolation and purification of SAA proteins [24–30]. In most cases, separation of human apo SAA isomers was performed by combined use of gel permeation and ion-exchange chromatography [24–27,29], preparative isoelectrofocusing [30] and hydrophobic interaction chromatography [30]. In these studies, however, large amounts of human plasma (100–300 ml) were used, that makes it difficult to apply directly these techniques for analysis of small amounts of plasma obtained from mice.

Here we present a new procedure for isolation of two major serum amyloid A isotypes SAA1 and SAA2 from the acute-phase plasma of mice. The procedure included preparation of HDL fraction, purification of apo SAA by preparative by SDS-PAGE and separation of the SAA isomers by RP-HPLC. Since the presence of SDS interferes with RP-HPLC by decreasing the resolution of proteins and retarding their elution from the column [31], removal of SDS prior to RP-HPLC is essential. We found that SDS, insufficiently removed even after extensive dialysis of proteins, could affect their behavior on RP-HPLC columns and cause difficulties in obtaining reproducible elution profiles (unpublished data). In the present study the SDS removal technique developed by us previously [14,15,32] was

Table 2
N-terminal amino acid sequence analysis of SAA fractions I–VI separated by HPLC

HPLC fraction	N-terminal amino acid sequence	SAA1 and SAA2 content (%)
I	GFFSF**VH**EAFQGAGDMWRAY (SAA1)	99
II	GFFSF**VH**EAFQGAGDMWRAY (SAA1)	75
	GFFSF**IG**EAFQGAGDMWRAY (SAA2)	25
III	GFFSF**VH**EAFQGAGDMWRAY (SAA1)	99
IV	GFFSF**VH**EAFQGAGDMWRAY (SAA1)	90
	GFFSF**IG**EAFQGAGDMWRAY (SAA2)	10
V	GFFSF**VH**EAFQGAGDMWRAY (SAA1)	20
	GFFSF**IG**EAFQGAGDMWRAY (SAA2)	80
VI	GFFSF**IG**EAFQGAGDMWRAY (SAA2)	99

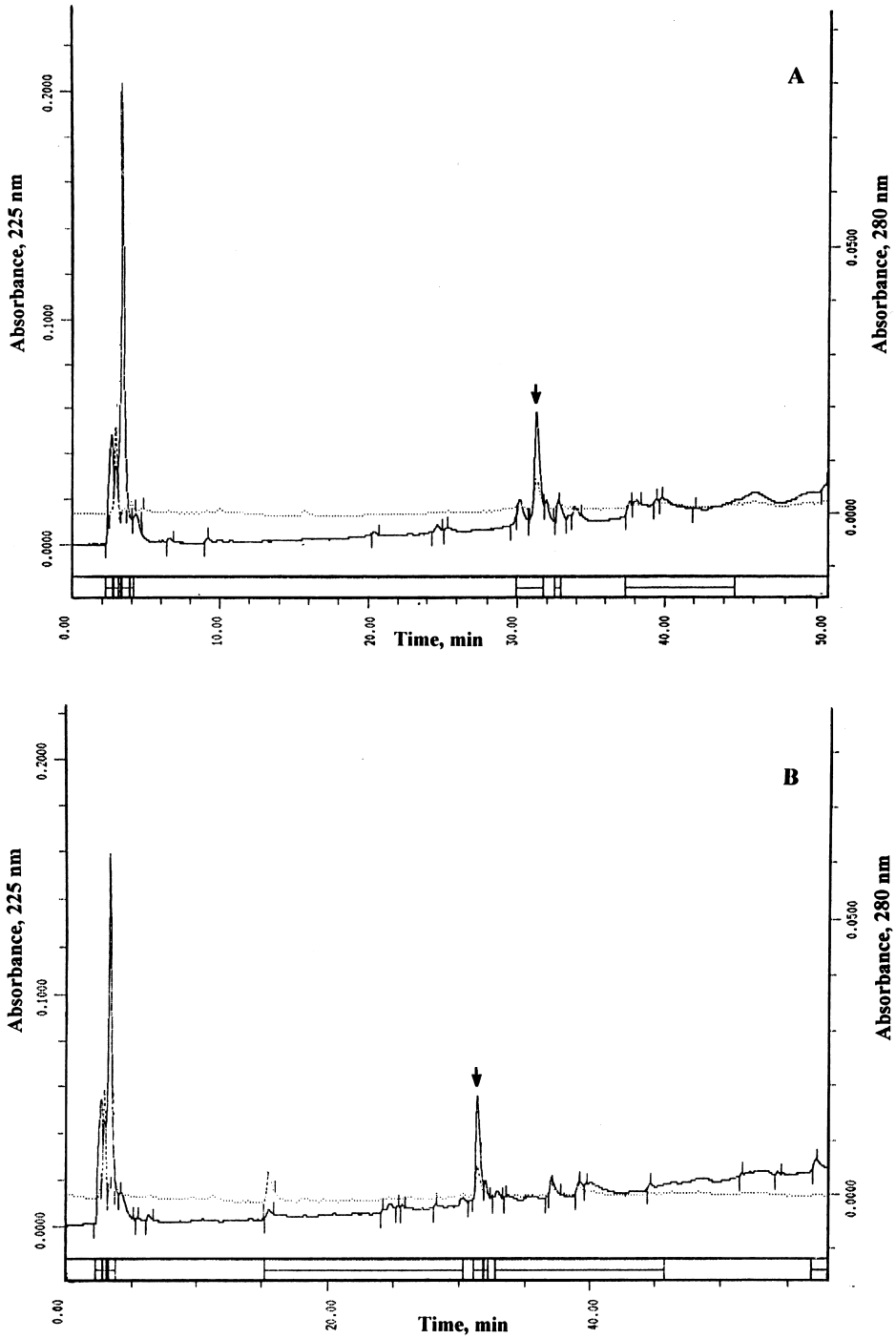

Fig. 3. Narrow-bore RP-HPLC purification of C-terminal peptides of SAA1 (A) and SAA2 (B) obtained by Asp–Pro bond cleavage. The peptides were cleaved and separated on narrow bore RP-HPLC column as described in Section 2.8. A linear gradient from 5% to 60% acetonitrile in 0.1% TFA was run over 60 min at flow-rate of 200 μl/min. The effluent was monitored by UV absorbance at 225 nm (solid line) and 280 nm (dotted line). The peptides eluted at 31.3 min (A) and 31.4 min (B) were subjected for amino acid sequence and mass spectral analyses.

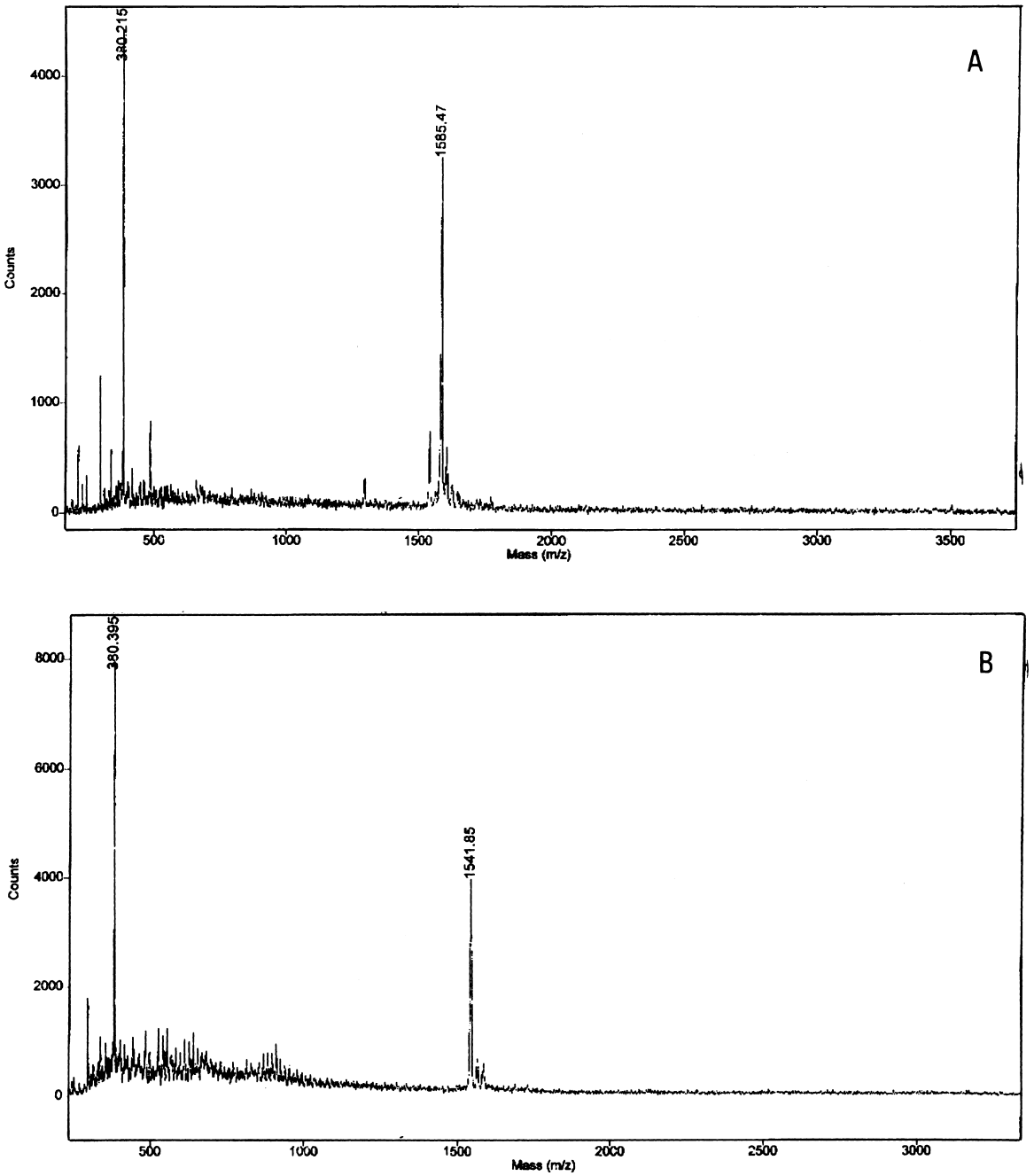

Fig. 4. Mass spectral analysis of C-terminal domains of the SAA proteins: (A) HPLC fractions IV and (B) fraction VI. C-terminal peptides were recovered from the Asp–Pro cleavage as described in Section 2.8. Peaks at m/z 1585.47 (A) and 1541.85 (B) correspond to the molecular ions of C-termini of apo SAA1 and SAA2, respectively.

applied. The proteins free from SDS were recovered in a soluble form, thus avoiding some problems arising from the purification of small amounts of protein by acetone or TCA precipitation techniques [33]. The techniques applied presently allowed one to obtain reproducible HPLC elution profiles in three independently performed SAA isolation experiments. The identity and purity of the SAA1 and SAA2 isotypes separated by RP-HPLC were demonstrated and confirmed by N- and C-terminal amino acid sequence analyses and mass spectrometry. Finally, the developed procedure is applicable to small amounts of pooled murine plasma (6–7 ml) and could be readily adapted to large scale preparations.

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