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Note

Purification of human serum amyloid A by anion-exchange fast protein liquid chromatography

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The plasma concentration of serum amyloid A (SAA), a plasma apolipoprotein mainly associated with high-density lipoproteins (HDL), is increased by up to 1000-fold as a response to certain acute phase reactions. This enormous increase can be used in the diagnosis of acute allograft rejection [1–3]. SAA, which consists of 104 amino acids, is also believed to be the precursor of the 76-amino-acid amyloid A (AA) protein found in tissue deposits of patients with secondary amyloidosis [4,5]. SAA exists in plasma in several isoforms [6–9]. Two of them, named SAA_1 and SAA_2 by Benditt and Eriksen [10], represent the prominent forms. SAA_1 and SAA_2 differ in their aminoterminal arginine residue, which is missing in SAA_2, giving rise to a p*I* value of 0.5 lower than that of SAA_1.

We have developed a rapid method to isolate both SAA_1 and SAA_2 isoforms from human plasma by a combination of ultracentrifugation and fast protein liquid chromatography (FPLC), using a Mono Q HR 10/10 preparative anion-exchange column.

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EXPERIMENTAL

HDL_3 ($d = 1.125$–1.21 g/ml) was isolated from pooled SAA-containing human plasma by ultracentrifugation according to standard techniques [11], and dialysed extensively against 5 m*M* EDTA buffer (pH 7.4). HDL_3 was then incubated with 6 *M* guanidine hydrochloride for 3 h at 37°C as described for apolipoproteins by Nichols et al. [12]. Thereby the main apolipoprotein of HDL, apo AI, dissociates from the HDL complexes, as well as parts of apo SAA. Recentrifugation at a density of 1.21 g/ml then separated the AI-depleted HDL complexes from the lipid-free fraction containing the dissociated apolipoproteins AI and SAA.

The lipid-free bottom fraction and the HDL_3 fraction depleted of apo AI and SAA were then extensively dialysed against 5 m*M* NH_4HCO_3 and lyophilized. Apoproteins from the bottom fraction dissolved readily in 10 m*M* Tris buffer (pH 8.2) containing 7 *M* urea, whereas the HDL_3 fraction depleted in apo AI and SAA required delipidation by five successive extractions with diethyl ether–ethanol (3:1, v/v) at −20°C (10 ml/mg of protein) prior to further processing. The solution was dissolved in starting buffer (10 m*M* Tris pH 8.2, 7 *M* urea) and then passed through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.) prior to application to the column.

Isolation of apo SAA by anion-exchange FPLC

The FPLC system (Pharmacia, Uppsala, Sweden) was used to purify apo SAA from HDL apolipoproteins. The system employed two P-500 high-pressure pumps and an LCC-500 liquid chromatography controller to form the gradient. The elution was controlled by a single-path UV monitor. A chart recorder with two channels was used to monitor the UV absorbance, the programmed gradient and the collected fractions (FRAC-100 fraction collector, Pharmacia).

All buffers were freshly prepared with deionized urea solution filtered through 0.2-μm membrane filters (Nalge, New York, NY, U.S.A.) and degassed before use. The Mono Q HR 10/10 (10 cm × 1 cm I.D.) column was equilibrated with 10 m*M* Tris–HCl buffer (pH 8.2) containing 7 *M* urea at a flow-rate of 2.0 ml/min. The column was operated at room temperature. A 60–80 mg HDL_1 sample of apoproteins was applied per separation. The column was then eluted for 140 min at the same flow-rate with a segmented gradient of sodium chloride from 0 to 30 mmol for 8 min and from 30 to 65 mmol for 60 min (0 to 3%, 3 to 6.5% buffer B: 10 m*M* Tris–HCl, 7 *M* urea, 1 *M* sodium chloride). The sodium chloride concentration was then increased to 1 *M* to remove the remaining proteins, and the column was reequilibrated with 45 ml of starting buffer. Fractions were analysed for SAA by laser nephelometry [1], and those containing SAA were pooled, dialysed extensively against 5 m*M* NH_4HCO_3 and lyophilized.

Characterization of proteins

Protein concentrations were determined according to Bradford (reagents provided by Bio-Rad, Richmond, CA, U.S.A.) [13]. Analytical isoelectric focusing (IEF) of apolipoproteins in polyacrylamide gels was performed as described by Menzel et al. [14] with slight modifications. The overall concentration of carrier ampholites was maintained, but the composition was changed to one part pH 3–5 (Serva, Heidelberg, F.R.G.), one part pH 3.5–5, two parts pH 5–7 and two parts pH 7–9 (v/v) (Pharmacia, Bromma, Sweden) covering the pH range from 3 to 9. After IEF the proteins were either stained with Coomassie Brillant Blue or electrophoretically blotted onto nitrocellulose sheets and proved immunochemically with specific antibodies to SAA or other apolipoproteins. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 10–22.5% linear polyacrylamide gradient gel as outlined before [15]. Amino acid analysis of SAA was carried out in a Beckman 6300 amino acid analyser after 72 h of acid hydrolysis. The average amount for determination of one amino acid was 532 pmol.

RESULTS

As SAA is mainly associated with HDL we used the isolation of HDL_3 by ultracentrifugation (density fraction 1.125–1.21 g/ml) as a first purification step. This separated SAA from the bulk of plasma proteins. A second centrifugation at the same density was used to further dissociate serum albumin, which was still associated with HDL_3 after the first isolation step. After incubation of HDL_3 with 6 *M* guanidine hydrochloride a proportion of the SAA stayed with the HDL fraction, whereas dissociated SAA could be found together with apo AI in the lipid-free bottom fraction after another centrifugation. Depending on the amount of SAA associated with HDL, 20–40% was found dissociated with apo AI in the bottom fraction. Apolipoproteins C, D and E did not significantly dissociate and also eluted at much higher sodium chloride concentrations than SAA, thus not interfering with SAA isolation. The lyophilized proteins of the bottom fraction were redissolved in a 10 m*M* Tris–HCl buffer with 7 *M* urea and separated by anion-exchange chromatography.

As shown in Fig. 1, chromatography on a Mono Q HR 10/10 column completely separated SAA from apo AI, the main protein of the bottom fraction. Simultaneously SAA_1 was also separated from SAA_2 when a very shallow gradient was chosen between 30 and 65 mmol of sodium chloride. Thereby SAA_1 eluted at 35 mmol and SAA_2 at 40 mmol of sodium chloride. The separation was complete, as demonstrated by IEF (see Fig. 2). Both SAA isoforms purified by FPLC showed the same apparent molecular mass of 12 000 dalton as plasma SAA upon SDS-PAGE (data not shown).

The identities of the isolated proteins with SAA were further established by

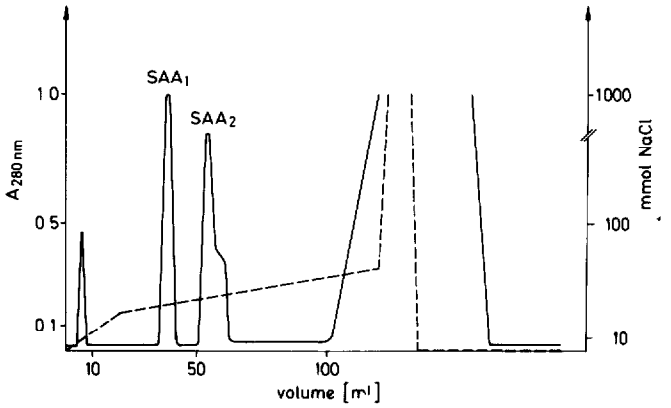

Fig. 1. Elution profile of apo SAA on the Mono Q HR 10/10 column after injection of 70 mg of HDL-dissociated proteins. Flow-rate, 2.0 ml/min; starting buffer, 10 m*M* Tris-HCl (pH 8.2) and 7 *M* urea. The gradient was developed with the same buffer containing 1 *M* sodium chloride.

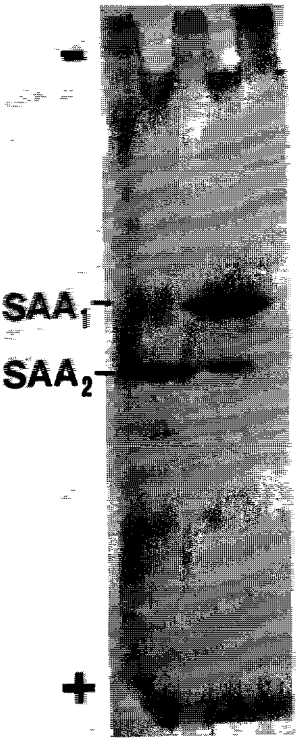

Fig. 2. Isoelectric focusing of purified apo SAA in a pH gradient of 3 to 9. The main isoforms SAA_1 and SAA_2 were obtained after the purification step on the Mono Q HR 10/10 column. The corresponding minor more acidic bands of each major protein probably represent carbamylation products.

TABLE I

AMINO ACID COMPOSITION OF APO SAA ISOLATED BY FPLC

Amino acid	Composition expected[a] (%)	Composition found (%)
Asp + Asn	14	13.47
Thr	0	1.47
Ser	7	7.76
Glu + Gln	9	12 30
Pro	4	7.17
Gly	12	13.40
Ala	16	16.15
Cys	0	0
Val	1	1.58
Met	2	2.02
Ile	3	3.08
Leu	3	5.04
Tyr	5	5.29
Phe	8	8.00
His	3	3.03
Lys	4	5.89
Arg	10	9.89
Trp	3	N.D.[b]

[a]Values from ref. 17.
[b]N.D. = not determined.

immunoblotting after IEF or SDS-PAGE using anti-human SAA from sheep [1]. In addition, amino acid analysis of the purified apo SAA was compatible with the composition derived from protein and DNA sequencing work [7,16–18]. The results are displayed in Table I. Minor differences between the expected and found compositions may be due to minor impurities or fragments of SAA. Other apoproteins were not detected in the preparation.

DISCUSSION

As SAA behaves like an apolipoprotein and is associated in plasma with HDL [10,19,20] ultracentrifugation is commonly used as a first isolation step for this protein. Eriksen and Benditt [21] further separated HDL apoproteins by ion-exchange chromatography on DEAE-Sephadex using a 0.015 *M* Tris buffer (pH 8.2) and 8 *M* urea with a gradient from 0.015 to 0.1 *M* Tris. Bausserman et al. [6] fractionated HDL apoproteins by gel permeation on a Sephacryl S-200 column in a 6 *M* guanidine hydrochloride buffer, and recently Raynes and McAdam [22] described a method to isolate SAA by a combination of gel permeation and DEAE-cellulose chromatography from apoproteins extracted from serum by hydrophobic interaction chromatography.

The present study establishes the use of FPLC for the rapid high-resolution purification of SAA_1 and SAA_2. The bottom fraction containing apo AI and also dissociated SAA was preferred as a source to isolate SAA over the apo AI-depleted HDL_3. Even when SAA was present in only small amounts compared with apo AI in the bottom fraction, its two main isoforms could be completely separated from apo AI. With several runs large amounts of apo SAA can be obtained in a short time. When the purification of SAA from the apo AI-depleted HDL fraction was attempted no complete separation of SAA_2 was achieved from apo AII, which is the major constituent of this fraction. Furthermore, it was necessary to apply a very shallow gradient to obtain complete separation of SAA_1 and SAA_2 and to isolate them free of apo AI.

Several groups working in different fields such as lipid metabolism, immunology and pathology share an interest in SAA. Its increase in plasma can obviously be used as a marker for allograft rejection [1,2]. SAA is the putative precursor of tissue AA in secondary amyloidosis [5], and it has been shown to influence HDL–cholesterol metabolism [23,24]. To facilitate future studies, the technique described here makes it possible to obtain large amounts of SAA to be used in the investigation of problems associated with this unusual acute phase reactant.

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