

## Generation of soluble recombinant human acute phase serum amyloid A2 (A-SAA2) protein and its use in development of a A-SAA specific ELISA

Christine C. McCormack<sup>a</sup>, Audrey H. Hobson<sup>a</sup>, Sean Doyle<sup>b</sup>, John Jackson<sup>c</sup>,  
Cormac Kilty<sup>b</sup>, Alexander S. Whitehead<sup>a,\*</sup>

<sup>a</sup> Department of Genetics and Biotechnology Institute, Trinity College, Dublin 2, Ireland

<sup>b</sup> Biotrin International, 93 The Rise, Mount Merrion, Dublin 4, Ireland

<sup>c</sup> Department of Immunology, St James Hospital, Dublin 8, Ireland

Received 3 August 1995; revised 13 May 1996; accepted 12 July 1996

### Abstract

Human acute phase serum amyloid A (the A-SAA2 isoform) was expressed at high levels using the pGEX bacterial expression system. A-SAA2 protein was expressed in *E. coli* NM544 as part of a fusion protein facilitating rapid purification. A-SAA2 was cleaved from the fusion moiety in the presence of a non-ionic detergent (Triton X-100) to release a soluble A-SAA2. Further purification using ion exchange chromatography yielded a pure A-SAA2 (3 mg per litre of culture). Antibodies generated against recombinant A-SAA2 were specific for the acute phase SAAs, A-SAA1 and A-SAA2 and showed no cross-reactivity with the constitutively expressed SAA (C-SAA). These antibodies were used to develop a rapid enzyme-linked immunosorbent assay (ELISA) specific for the measurement of A-SAA in serum.

**Keywords:** Recombinant; Serum amyloid A protein; Acute phase protein; Amyloidosis; ELISA

### 1. Introduction

The mammalian acute phase response is the first line of systemic defence elicited by stimuli such as

Abbreviations: SAA, serum amyloid A protein; A-SAA, acute phase SAA; C-SAA, constitutive SAA; APR, acute phase reactant; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; AA, amyloid A; GST, glutathione *S*-transferase; IPTG, isopropyl  $\beta$ -D-thiogalactosidase; NIBSC, National Institute of Biological Standards and Controls; CRP, C-reactive protein; HRP, horseradish peroxidase.

\* Corresponding author. At: Department of Genetics, Trinity College, Dublin 2, Ireland. Tel.: 353-1-6081289; Fax: 353-1-6083442.

infection, trauma, myocardial infarction, neoplasms, and surgery (Koj, 1985). It is initiated and maintained by a large number of proinflammatory mediators including cytokines, glucocorticosteroids and anaphylatoxins and involves a wide range of complex physiological changes including elevated circulating concentrations of hepatically synthesised acute phase reactants (APRs). In man, this latter class includes the 'major' APRs, serum amyloid A (SAA) and C-reactive protein (CRP) (reviewed by Steel and Whitehead, 1994).

The human SAA gene family is comprised of four known genes that have been localised to the short arm of chromosome 11p15.1 (Sellar et al., 1994).

The *SAA1* and *SAA2* genes specify the two acute phase SAA (A-SAA) proteins A-SAA1 and A-SAA2 respectively which are both 104 amino acid, 12.5 kDa proteins that share 93% amino acid sequence identity (Woo et al., 1987; Betts et al., 1991). A number of allelic forms have been identified by amino acid sequence analysis of A-SAA isolated from plasma. The A-SAA1 protein has three allelic forms whereas A-SAA2 has two (Dwulet et al., 1988; Baba et al., 1993). A third gene *SAA3* which shows 71% nucleotide identity with *SAA1* and *SAA2* is a pseudogene (Kluve-Beckerman et al., 1991). Constitutive SAA (C-SAA) is the third expressed SAA family member (Whitehead et al., 1992) and is the product of the *SAA4* gene (Steel et al., 1993). C-SAA levels characteristically do not increase as a result of inflammation and exist in serum at concentrations between 80–140 mg/l (Yamada et al., 1994a). C-SAA differs from A-SAA with respect to peptide length, being eight amino acids longer, and shares only 55% identity with A-SAAs. Additionally, C-SAA may be post-translationally modified by glycosylation at a single site (Whitehead et al., 1992). In common with the A-SAAs, C-SAA rapidly associates with high density lipoprotein (HDL3) when released into the circulation (Eriksen and Benditt, 1980; De Beer et al., 1995).

Circulating concentrations of A-SAA can increase up to 1000 mg/l within 24–48 h of an acute stimulus (Marhaug, 1983) indicating an important protective role for these proteins; however, no definitive function has been demonstrated for the A-SAA proteins. Recent studies variously suggest that A-SAA has chemoattractant activity (Badolato et al., 1994), may play a role in lipid metabolism and immunosuppression (Aldo-Benson and Benson, 1982; Guyton and Kemp, 1992) and may inhibit the oxidative burst in neutrophils (Linke et al., 1991).

During chronic inflammation A-SAA levels remain significantly elevated reflecting the continued persistence of underlying pathological inflammatory processes that can contribute to long term tissue damage. An occasional consequence of chronic inflammation is reactive secondary amyloidosis (Pepys, 1994), a progressive fatal condition in which amyloid A protein (AA), a cleavage product of A-SAA, is the major component of insoluble fibrous deposits that accumulate in major organs (Husebekk et al.,

1985; Tape et al., 1988; Stone, 1990). The sustained elevation of A-SAA in chronic inflammatory conditions suggests that A-SAA is an important indicator of disease status. However, the measurement of A-SAA concentration has not been used for routine clinical diagnosis and clinical management, due in part to the difficulty in raising specific antisera against human A-SAA (Pepys, 1984). Several methods including radioimmunoassays, single radial immunodiffusion and ELISA based procedures have, however, been reported for the measurement of A-SAA levels (Chambers and Whicher, 1983; Marhaug, 1983; Taktak and Lee, 1991; Sipe et al., 1989; Yamada et al., 1989 and Casl and Grubb, 1993). As A-SAA in serum exists as one of the apolipoproteins associated with HDL3 particles many of these methods require denaturation and delipidation of the serum samples prior to assay.

Biological studies necessary to define clearly the role of A-SAA have been hampered due to problems encountered when working with the protein. Isolation of A-SAA from blood is laborious and results in poor yields of insoluble protein (Godenir et al., 1985; Strachan et al., 1989). Recombinant protein technology offers a means of generating a renewable homogeneous source of A-SAA. We report here the production of recombinant human A-SAA2 in *E. coli* as a GST fusion protein using the pGEX expression system (Smith and Johnson, 1988). Expression of A-SAA2 in this system permits the recovery of soluble recombinant A-SAA2 protein following thrombin cleavage of the fusion protein when used in the presence of a mild non-ionic detergent. Antibodies raised against recombinant A-SAA2 were specific for the A-SAAs and were used to develop a sandwich ELISA to quantify A-SAA levels in serum.

## 2. Materials and methods

### 2.1. Construction of the A-SAA2 protein expression vector

The coding region of A-SAA2 was amplified from the A-SAA2 cDNA clone by the polymerase chain reaction (PCR) with the concomitant introduction of sequence specifying an additional glycine residue and a *Bam*HI restriction site at the 5' end

(oligonucleotide primer sequence 5'-CGGGATCCG-GCGAAGCTTCTTTTCGTTTC-3') and an *EcoRI* site at the 3' end (oligonucleotide primer sequence 5'-CGGAATTCAGTATTTCTCAGGCAGGCC-3'). The PCR product was digested with *Bam*HI and *EcoRI*, gel purified, and ligated into the glutathione S-transferase (GST) fusion protein expression vector pGEX-2T (Pharmacia Fine Chemicals, Milton Keynes, UK). DNA sequence analysis of the resulting pGEX-(A-SAA2) confirmed that it carried the entire unmodified A-SAA2 coding region positioned downstream of the GST coding region with no mutations resulting from the PCR process.

## 2.2. Induction of *E. coli* cultures for high level expression of recombinant A-SAA2 protein

Plasmid pGEX-(A-SAA2) was transformed into the *E. coli* expression strain NM554. Transformants were isolated and grown overnight at 37°C. Overnight cultures were diluted 1/100 in Luria broth containing 100 µg/ml ampicillin (Boehringer Mannheim, East Sussex, UK) and grown to an OD<sub>600</sub> value of 1.0. Expression of recombinant fusion protein was induced in culture with 0.1 mM isopropyl β-D-thiogalactosidase (IPTG; Sigma, Dorset, UK) for 5 h at 37°C. Cultures were centrifuged at 5000 × *g* for 10 min at 4°C. Cell pellets were resuspended in 1/50 of the starting volume in lysis buffer (PBS pH 7.3 (Gibco/BRL, Paisley, UK) containing 0.2 mg/ml lysozyme (Sigma); 5 mM EDTA (BDH, Merck, Dorset, England); 0.1% (v/v) Triton X-100 (BDH); 50 mM benzamidine (Sigma); 0.1 mM PMSF (Sigma) and 0.5 mg/ml iodoacetamide (Sigma)) and incubated for 1 h at room temperature. Lysates were sonicated on ice (3 × 20 second bursts) to obtain complete lysis, centrifuged at 10000 × *g* for 10 min at 4°C and filtered through a 0.45 µM millipore filter to remove particulate material. Clarified sonicates were passed through a Glutathione Sepharose 4B column (Pharmacia) to which the GST-(A-SAA2) fusion protein bound. Contaminating *E. coli* proteins were removed by washing with ten column volumes of PBS (pH 7.3), and the recombinant A-SAA2 protein was directly cleaved from the GST moiety on the glutathione Sepharose 4B column using thrombin (Sigma) (5 U/mg protein bound) in PBS (pH 7.3)

0.1% (v/v) Triton X-100 at room temperature for 6 h. The column eluate containing recombinant A-SAA2 was collected and stored at 4°C. The recombinant A-SAA2 sample was further purified by ion exchange chromatography using a column of high performance Sepharose Q (Pharmacia) equilibrated with 0.1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 10.0) and eluted with 0.1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 10.0), 0.1 M NaCl. Fractions were collected and analysed by SDS-PAGE (Laemmli, 1970; Sambrook et al., 1989) and immunoblotting (Towbin et al., 1979; Sambrook et al., 1989) using anti(A-SAA) antiserum (see below) with peroxidase-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody. The protein content of the fractions was determined using Bicinchoninic Acid solution (Sigma) with crystalline bovine serum albumin (Sigma) as standard. Recombinant A-SAA2 was stored at 4°C in buffer A (20 mM Tris-HCl (pH 8.4), 150 mM NaCl and 0.1% (v/v) Triton X-100).

## 2.3. N-terminal sequencing of recombinant A-SAA2

Recombinant A-SAA2 was electroblotted onto a ProBlott membrane and stained with amido black prior to N-terminal amino acid sequencing on a Biosystems model 473A protein sequencer.

## 2.4. Antibodies to A-SAA2

Rabbits were immunised intramuscularly with recombinant A-SAA2 purified from SDS-PAGE gels (Hager and Burgess, 1980) as follows: day 1, 1 ml of 1 mg/ml recombinant A-SAA2 in Freund's complete adjuvant (Sigma); days 14 and 21, 1 ml of 1 mg/ml recombinant A-SAA2 in Freund's incomplete adjuvant (Sigma). Blood was drawn on day 28. IgG-anti(A-SAA) was isolated by affinity chromatography on immobilised protein A (Pharmacia).

## 2.5. IgG-anti(A-SAA)-horseradish peroxidase (HRP) conjugate

A direct conjugate of IgG-anti(A-SAA)-HRP was produced essentially as described by Duncan et al. (1983). HRP was obtained from Biozyme, UK.

### 2.6. A-SAA enzyme-linked immunosorbent assay (ELISA)

Microtitre maxisorp plates (Nunc, Roskilde, Denmark) were coated with 1  $\mu\text{g}/\text{ml}$  protein A purified IgG-anti(A-SAA) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed twice with PBST (PBS (pH 7.3), 0.05% (v/v) Tween 20) and stabilised using a BSA (Sigma) solution. The microtitre plates were sealed and stored at 4°C until required. Recombinant A-SAA2 protein was used to generate standard curves. The standard curve range was 5–750  $\mu\text{g}/\text{l}$  and was prepared in sample dilution buffer (20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 25% (v/v) propan-2-ol). Serum samples to be analysed were diluted 1/200 in sample dilution buffer and 100  $\mu\text{l}$  of each dilution were added in duplicate to microwells. After incubation at room temperature (20–25°C) for 60 min with uniform shaking, wells were washed four times with 350  $\mu\text{l}$  PBST. Enzyme conjugate, IgG-anti(A-SAA)-HRP, was diluted 1/800 in conjugate dilution buffer (50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 1% (w/v) BSA) and 100  $\mu\text{l}$  aliquots were added to the wells. Microtitre plates were incubated at room temperature (20–25°C) for a further 60 min with uniform shaking. The wells were again washed four times with 350  $\mu\text{l}$  PBST. 100  $\mu\text{l}$  of stabilised tetramethylbenzidine (TMB: Moss, Pasadena, M.D, USA) substrate were added to each well using a multichannel pipette, and plates were incubated at room temperature for 15 min. Colour development was stopped using 100  $\mu\text{l}$  1 N  $\text{H}_2\text{SO}_4$  (BDH) and plates were read immediately at  $\text{OD}_{450}$  nm. The standard curve obtained using the recombinant A-SAA2 was used to calculate the A-SAA concentration in the test samples. In order to examine serum samples with low concentrations of A-SAA the conjugate was titred appropriately.

### 2.7. Serum samples

Normal serum samples were obtained from blood donors aged 18–65 years. Rheumatoid arthritis serum samples were obtained from patients undergoing routine assessment in the Rheumatology Clinic of St James Hospital, Dublin. Samples were stored at –20°C prior to use.

### 2.8. SAA standard

Recombinant A-SAA2 protein concentrations were determined using bicinchoninic acid solution (Sigma) with crystalline bovine serum albumin (Sigma) as standard. The recombinant A-SAA2 was diluted to a concentration of 150 mg/l and used as the A-SAA standard in the ELISA.

### 2.9. Assay validation

The assay was validated by examining the following: linearity of dilution; reproducibility (intra- and interassay variation) and normal range determination. Experiments where recombinant A-SAA2 was added (spiked) into sample dilution buffer and into normal serum were conducted to examine the extent of masking of the A-SAA signal by serum.

## 3. Results and discussion

The A-SAAs constitute a major class of human acute phase reactant whose concentration can increase up to 1000-fold following an acute inflammatory stimulus. No function for A-SAA has yet been definitively defined. While further studies are necessary to clearly define the role of A-SAA, problems obtaining a soluble purified native A-SAA have been encountered: purification of A-SAA protein from large volumes of blood is characterised by poor yields (Godenir et al., 1985), limited solubility (Bausserman et al., 1983) and the heterogeneous nature of the A-SAA recovered. In addition, A-SAA purified from serum may contain trace amounts of other serum components thereby potentially compromising studies of A-SAA function that involve sensitive bioassays. The production of A-SAA by recombinant methods offers a reliable, homogeneous source of large quantities of highly purified A-SAA. We report the production of A-SAA2 using the pGEX bacterial expression system. The A-SAA2 coding region was inserted in frame into the pGEX-2T vector to produce a construct in which A-SAA2 expression was under the control of the IPTG inducible *tac* promoter and GST ribosome binding site. Upon induction a 38.5 kDa GST-(A-SAA2) fusion protein was produced (Fig. 1, lane 4) consti-

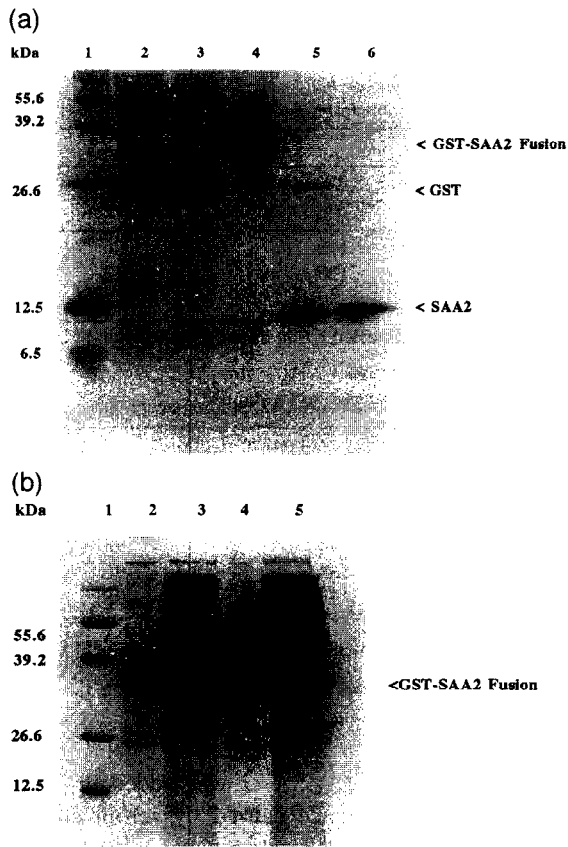


Fig. 1. *a*: expression of the recombinant A-SAA2 protein from pGEX(A-SAA2) analysed by SDS-PAGE. Lane 1: protein molecular weight markers 55.6, 39.2, 26.6, 12.5, 6.5 kDa; lane 2: pGEX(A-SAA2) uninduced; lane 3: pGEX-2T expression vector induced with IPTG; lane 4: pGEX(A-SAA2) induced with IPTG; lane 5: thrombin cleaved mature A-SAA2 product; lane 6: purified recombinant A-SAA2 following ion-exchange chromatography. *b*: solubility of recombinant GST-(A-SAA2) fusion protein. Recombinant GST-(A-SAA2) fusion protein was tested for solubility in the presence and absence of nonionic detergent 0.1% (v/v) Triton X-100. SDS-PAGE analysis: lane 1: protein molecular weight markers 55.6, 39.2, 26.6, 12.5 kDa; lane 2: insoluble fraction after cell lysis without the presence of 0.1% (v/v) Triton X-100; lane 3: soluble fraction without the presence of 0.1% (v/v) Triton X-100; lane 4: insoluble fraction after cell lysis in the presence of 0.1% (v/v) Triton X-100; lane 5: soluble fraction in presence of 0.1% (v/v) Triton X-100.

tuting approximately 5% of the total cellular protein. Solubilisation of GST-(A-SAA2) from lysed *E. coli* cell pellets requires the presence of 0.1% (v/v) Triton X-100 (Fig. 1b). Following capture of the fusion protein on GST Sepharose 4 $\beta$ , the protein

was cleaved by thrombin in the presence of 0.1% (v/v) Triton X-100 to yield a soluble A-SAA2 product of 12.5 kDa (the predicted size for mature A-SAA2) (Fig. 1, lane 5). Further purification of recombinant A-SAA2 was achieved using ion exchange chromatography and the resulting protein could be resolved as a single band on SDS-PAGE (Fig. 1, lane 6). N-terminal amino acid sequence of the 12.5 kDa product was Gly-Ser-Gly-Arg-Ser-Phe-Phe-Ser-(Phe/Asp/Ala)-Leu-Gly-(Glu/Asp)-Ala-Phe-Asp-Gly-Ala-Arg-Asp, confirming its identity as A-SAA2 with an amino terminal Gly-Ser-Gly extension derived from the fusion protein. Approximately 3 mg of recombinant A-SAA2 was obtained per litre of bacterial culture.

Previously reported methods for producing recombinant A-SAA2 require the presence of strong chaotropic agents such as urea and guanidine hydrochloride to achieve maximum soluble protein recovery (Kluve-Beckerman et al., 1993; Yamada et al., 1994b). The use of such harsh denaturing agents renders the task of isolating active protein more difficult (Marino, 1989; Schein, 1989). As no clear biological function has been established for A-SAA it is impossible to determine whether the use of such strong denaturing agents compromises or abolishes A-SAA protein activity. Nonionic detergents are less likely to disrupt protein-protein interactions as these detergents have an uncharged head group and have been shown to be particularly useful for isolating functional proteins (Scopes, 1982).

Recombinant A-SAA2 was used to raise antibodies in rabbits. The resulting antiserum was tested for cross reactivity with other human SAA protein family members and serum components by immunoblot analysis (Fig. 2). It reacted with (i) purified recombinant A-SAA2 (Fig. 2, lane 1); (ii) A-SAA (but no

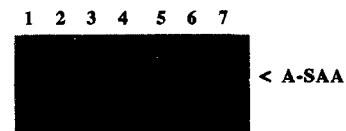


Fig. 2. Specificity of anti(A-SAA2) antibodies. Immunoblot analysis using antiserum raised against recombinant A-SAA2. Lane 1: recombinant A-SAA2; lane 2: serum of an acute phase patient; lane 3: non-acute phase serum; lane 4: NIBSC A-SAA; lane 5: recombinant C-SAA; lane 6: recombinant A-SAA1; lane 7: recombinant A-SAA2 spiked into non-acute phase serum.

other molecular species) present in the serum of a patient with inflammation (Fig. 2, lane 2) and A-SAA obtained from the NIBSC (Fig. 2, lane 4); (iii) recombinant A-SAA2 spiked into non-acute phase serum (Fig. 2, lane 7); and (iv) recombinant A-SAA1 (expressed and purified as for A-SAA2; unpublished) (Fig. 2, lane 6). Antibodies raised against recombinant A-SAA2 generate equivalent signals with both recombinant A-SAA1 and recombinant A-SAA2 in the immunoblot (Fig. 2, compare lanes 1 and 6) indicating that the binding capacity for each isoform is essentially equivalent. In addition, the antibodies raised against recombinant A-SAA2 do not cross react with purified C-SAA (expressed and purified as for A-SAA2, unpublished) (Fig. 2, lane 5) or any component of non-acute phase serum (Fig. 2, lane 3).

There is considerable interest in measuring A-SAA concentrations in serum as sustained high levels of circulating A-SAA may constitute an important aspect in the pathogenesis of some inflammation associated conditions (e.g. secondary amyloidosis) and may be an accurate marker for other pathogenic factors in a number of diseases with an inflammatory component (Husebekk et al., 1986; Mozes et al., 1989; Maury et al., 1988; Muller et al., 1992; Liuzzo et al., 1994). We used the polyclonal antibodies raised against recombinant A-SAA2 to develop a simple and rapid sandwich ELISA for measuring A-SAA levels in serum. Some of the previously reported A-SAA immunoassays used SAA isolated from acute phase serum to generate antibodies (Marhaug, 1983; Sipe et al., 1989; Wilkins et al., 1994). As C-SAA is present in both normal and acute phase serum, antibodies raised against SAA isolated from acute phase serum may exhibit cross-reactivity with C-SAA and pre-absorption of antibodies with normal serum is required to eliminate this cross-reactivity (Wilkins et al., 1994). The anti(A-SAA) antiserum reported here was raised against recombinant A-SAA2, is specific for A-SAA, and requires no additional absorption steps prior to use.

Some previous reports of methods to measure A-SAA in patient serum have identified problems associated with quenching of the A-SAA signal by serum components (Casl and Grubb, 1993). To address this issue, we conducted experiments in which

Table 1

Masking experiments: spiking of recombinant A-SAA2 into non-acute phase serum

(a) Spiking of recombinant A-SAA2 into non-acute phase serum

Dilution buffer		
Recombinant A-SAA2 $\mu\text{g}/\text{l}$ spiked	Recombinant A-SAA2 $\mu\text{g}/\text{l}$ recovered	% recovery
750	190	26
375	120	32
188	40	21

Serial dilutions were carried out in dilution buffer without 25% (v/v) propan-2-ol.

(b) Spiking of recombinant A-SAA2 into non-acute phase serum

Dilution buffer with 25% (v/v) propan-2-ol		
Recombinant A-SAA2 $\mu\text{g}/\text{l}$ spiked	Recombinant A-SAA2 $\mu\text{g}/\text{l}$ recovered	% recovery
750	740	98
375	358	95
188	178	95

Serial dilutions were carried out in dilution buffer with 25% (v/v) propan-2-ol.

purified recombinant A-SAA2 was added to serum (spiked) and interference caused by serum components was quantified. Recombinant A-SAA2 at a known concentration was spiked into (i) buffer A and (ii) non-acute phase serum, and assayed using the sample dilution buffer without 25% (v/v) propan-2-ol. Only 26% recovery of signal was observed following the spiking of recombinant A-SAA2 into non-acute phase serum (Table 1a). The above spiking experiment was repeated using sample dilution buffer containing 25% (v/v) propan-2-ol. In the presence of the organic solvent almost complete recovery of signal was observed following spiking of recombinant A-SAA2 into non-acute phase serum (Table 1b). The propan-2-ol sample dilution buffer most likely achieves signal recovery by disrupting the hydrophobic apolipoprotein complexes thereby facilitating antibody access to otherwise hidden A-SAA epitopes. It has been reported that SAA purified from acute phase serum exhibits a different immunoreactive dilution profile from that of purified SAA reconstituted back into normal serum (Godenir et al., 1985; Yamada et al., 1989). From our studies purified recombinant A-SAA2 in buffer A, A-SAA2

spiked into non-acute phase serum, and native A-SAA in serum from a rheumatoid arthritis patient, all show similar serial dilution profiles when diluted in our sample dilution buffer (Fig. 3). The presence of propan-2-ol in the sample dilution buffer offers a simple, rapid alternative to previous methods (delipidation and denaturation) used to unmask the A-SAA in serum samples (Marhaug, 1983; Chambers and Whicher, 1983; Casl and Grubb, 1993).

In the A-SAA sandwich ELISA serum samples were routinely run at 1/200 dilution. The lower and upper limits of the low range standard curve were 5  $\mu\text{g}/\text{l}$  and 100  $\mu\text{g}/\text{l}$  respectively. The lower and upper limits of the high range standard curve were 50  $\mu\text{g}/\text{l}$  and 750  $\mu\text{g}/\text{l}$ , respectively (Fig. 4), and samples falling above this range were diluted appropriately so that their A-SAA levels fell within the range of the curve. For assay validation the linearity

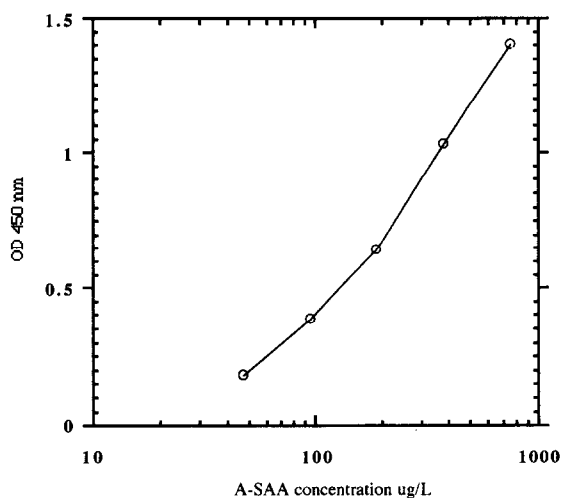


Fig. 4. Standard curve for A-SAA. The standard curve (50–750  $\mu\text{g}/\text{l}$ ) using recombinant A-SAA2 protein.

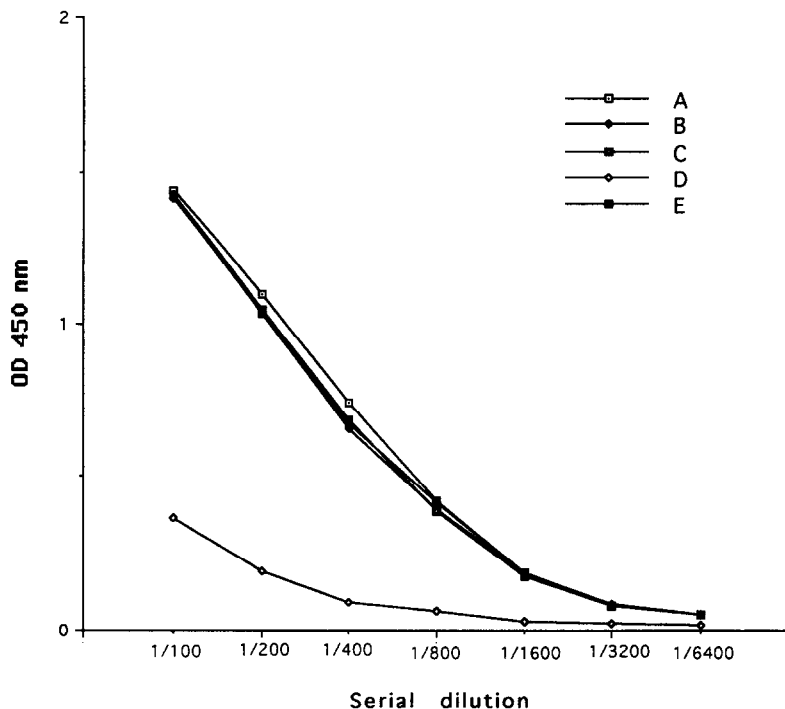


Fig. 3. Serial dilution profile. Native A-SAA (A), recombinant A-SAA2 spiked into normal serum (B) and recombinant A-SAA2 (C) were serially diluted in buffer A using sample dilution buffer containing 25% (v/v) propan-2-ol. Dilution profile for recombinant A-SAA2 spiked into normal serum (D) and recombinant A-SAA2 in buffer A (E) using dilution buffer without 25% (v/v) propan-2-ol.

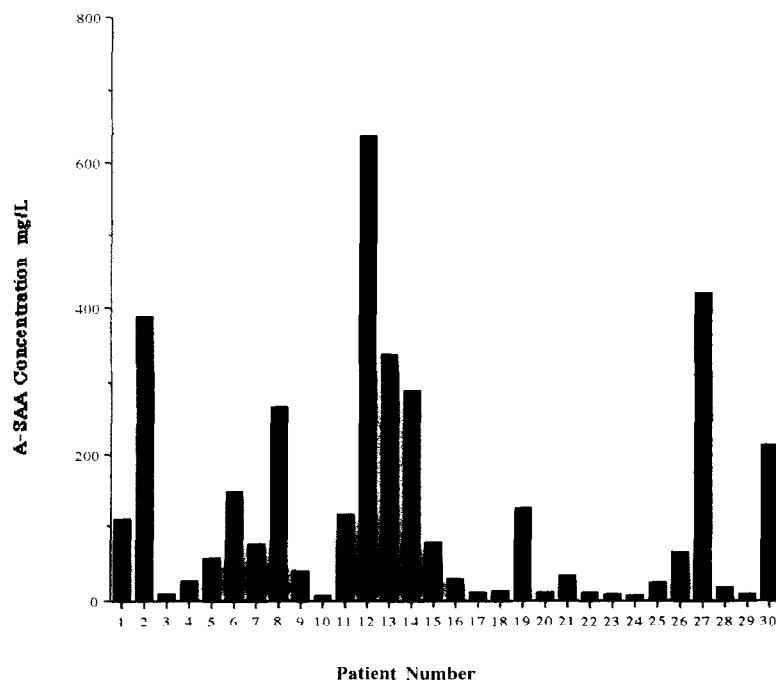


Fig. 5. Measurement of A-SAA concentration in the serum of rheumatoid arthritis patients.

of sample dilution was analysed by carrying out serial dilutions and the resulting data show that assay parallelism is observed in the ELISA. The reproducibility of the ELISA method was analysed by intra-assay and interassay variability. The intra-assay coefficient of variation from 20 replicate assays of three A-SAA serum samples (A-SAA concentrations were 5, 130 and 244 mg/l) were 4.8, 5.0 and 6.7%, respectively. The interassay coefficient of variation in ten replicate assays on the same serum samples were 8.0, 6.2 and 6.0%, respectively.

The normal range for A-SAA was analysed using 50 serum samples from healthy individuals, and determined to be  $0.4 \text{ mg/l} \pm 0.57 \text{ mg/l}$  using the standard equation: mean  $\pm$  2 SD. The A-SAA concentrations in 30 serum samples from rheumatoid arthritis patients undergoing routine assessment in the Rheumatology Department, St James Hospital, Dublin were analysed using the ELISA procedure and 95% of rheumatoid arthritis patients showed an elevated level of A-SAA (Fig. 5).

A-SAA proteins are difficult to isolate, purify and solubilise. The production of A-SAA2 by thrombin

cleavage from a GST-(A-SAA2) fusion protein in conjunction with the use of triton X-100 for solubilisation offers a means of generating large amounts of homogeneous A-SAA. As the recovery of soluble A-SAA2 by this method is possible without the use of harsh denaturants the resulting material may be particularly suited to future studies of A-SAA structure and biological function. Antibodies generated against recombinant A-SAA2 were shown to be specific for A-SAAs and used to develop an ELISA for quantifying A-SAA in patient serum. Monitoring acute phase protein levels is of considerable clinical importance in the assessment of inflammatory disease activity and response to therapy and the ELISA reported here provides a simple, rapid and reproducible method for such monitoring.

#### Acknowledgements

C. McC. holds a Wellcome Trust Prize Studentship (037361/Z/92/Z).



## References

- Aldo-Benson, M.A. and Benson, M.D. (1982) Serum amyloid A suppression of immune response in vitro: evidence for an effect on T cell-macrophage interaction. *J. Immunol.* 128, 2390.
- Baba, S., Takahashi, T., Kasama, T., Fujie, M. and Shirasawa, H. (1993) A novel polymorphism of human serum A protein, SAA1 gamma, is characterized by alanines at both residues 52 and 57. *Arch. Biochem. Biophys.* 303, 361.
- Badolato, R., Wang, J.M., Murphy, W.J., Lloyd, A.R., Michiel, D.F., Bausserman, L.L., Kelvin, D.J. and Oppenheimer, J.J. (1994) Serum amyloid A is a chemoattractant: induction of migration, adhesion and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J. Exp. Med.* 180, 203.
- Bausserman, L.L., Herbert, P.N., Forte, T., Klausner, R.D., McAdam, K.P.W.J., Osborne, J.C. and Rasseneu, M. (1983) Interaction of serum amyloid A proteins with phospholipid. *J. Biol. Chem.* 258, 10681.
- Betts, J.C., Edbrooke, M.R., Thakker, R.V. and Woo, P. (1991) The human acute phase serum amyloid A gene family: structure, evolution and expression in hepatoma cells. *Scand. J. Immunol.* 34, 471.
- Casli, M.T. and Grubb, A. (1993) A rapid enzyme-linked immunosorbent assay for serum amyloid A using sequence-specific antibodies. *Ann. Clin. Biochem.* 30, 278.
- Chambers, R.E. and Whicher, J.T. (1983) Quantitative radial immunodiffusion assay for serum amyloid A protein. *J. Immunol. Methods* 59, 95.
- De Beer, M.C., Yuan, T., Kindy, M.S., Asztalos, B.F., Roheim, P.S. and De Beer, F.C. (1995) Characterization of constitutive human serum amyloid A protein (SAA4) as an apolipoprotein. *J. Lipid. Res.* 35, 526.
- Dwulet, F.E., Wallace, D.K. and Benson, M.D. (1988) Amino acid structures of multiple forms of amyloid related serum protein SAA from a single individual. *Biochemistry* 21, 1677.
- Duncan, R.J.S., Weston, P.D. and Wrigglesworth, R. (1983) A new reagent which may be used to introduce sulfhydryl groups into proteins, and its uses in the preparation of conjugates for immunoassay. *Anal. Biochem.* 132, 68.
- Eriksen, N. and Benditt, E.P. (1980) Isolation and characterisation of the amyloid-related apoprotein (SAA) from human high density lipoprotein. *Proc. Natl. Acad. Sci. USA* 77, 6860.
- Godenir, N.L., Jeenah, M.S., Coetzee, G.A., Van der Westhuyzen, D.R., Strachen, A.F. and De Beer, F.C. (1985) Standardisation of the quantitation of serum amyloid A protein (SAA) in human serum. *J. Immunol. Methods* 83, 217.
- Guyton, J.R. and Kemp, K.F. (1992) Early extracellular and cellular lipid deposits in aorta of cholesterol fed rabbits. *Am. J. Pathol.* 141, 925.
- Hager, D.A. and Burgess, R.R. (1980) Elution of proteins from sodium dodecyl sulphate-polyacrylamide gels, removal of sodium dodecyl sulphate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase and other enzymes. *Anal. Biochem.* 109, 76.
- Husebekk, A., Skogen, G., Husby, G. and Marhaug, G. (1985) Transformation of amyloid precursor SAA to protein AA and incorporation in amyloid fibrils in vivo. *Scand. J. Immunol.* 21, 283.
- Husebekk, A., Permin, H. and Husby, G. (1986) Serum amyloid protein A (SAA): an indicator of inflammation in AIDS and AIDS-related complex (ARC). *Scand. J. Infect. Dis.* 18, 389.
- Kluve-Beckerman, B., Drumm, L. and Benson, M.D. (1991) Non expression of the serum amyloid A three (SAA3) gene. *DNA Cell Biol.* 10, 651.
- Kluve-Beckerman, B., Song, M., Benson, M.D. and Liepnieks, J.J. (1993) Recombinant murine serum amyloid A from baculovirus-infected insect cells: purification and characterization. *Biochim. Biophys. Acta* 1182, 303.
- Koj, A. (1985) In: A.H. Gordon and A. Koj (Eds.), *The Acute Phase Response to Injury and Infection*. Elsevier, Amsterdam, p. 139.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680.
- Linke, R.P., Bock, V., Valet, G. and Rothe, G. (1991) Inhibition of the oxidative burst response of N-formyl peptide stimulated neutrophils by serum amyloid A protein. *Biochem. Biophys. Res. Commun.* 176, 1100.
- Liuzzo, G., Biasucci, L.M., Gallimore, J.R., Grillo, R.L., Rebuzzi, A.G., Pepys, M.B. and Maseri, A. (1994) The prognostic value of C-reactive protein and serum amyloid A protein in severe unstable angina. *New Engl. J. Med.* 331, 417.
- Marhaug, G. (1983) Three assays for the characterisation and quantitation of human serum amyloid A. *Scand. J. Immunol.* 18, 329.
- Marino, M.M. (1989) Expression systems for heterologous protein production. *BioPharmaceutics* 2, 18.
- Maury, C.P.J., Totterman, K.J., Gref, C.G. and Ehnholm, C. (1988) Serum amyloid A protein, apolipoprotein A-1, and apolipoprotein B during the course of acute myocardial infarction. *J. Clin. Pathol.* 41, 1263.
- Muller, T., Schindler, S., Sprenger, H., Steinmetz, A., Hocke, G., Ebel, H., Gerns, D. and Lange, H. (1992) Prospective analysis of 10 different parameters of acute renal allograft rejection. *Transplant. Proc.* 24, 2731.
- Mozes, G., Friedman, N. and Shainkin-Kestenbaum, R. (1989) Serum amyloid A: An extremely sensitive marker of tissue damage in trauma patients and indicator of acute response in various diseases. *J. Trauma* 29, 71.
- Pepys, M.B. (1984) Measurement of serum amyloid A protein concentrations as a test of renal allograft rejection. *Biol. Med. J.* 288, 859.
- Pepys, M.B. (1994) Amyloidosis. In: M.M. Frank, K.F. Austen, H.N. Clannan and E.R. Unanue (Eds.), *Samter's Immunological Diseases*, 5th edn. Little Brown, Boston, MD, p. 637.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) In: *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.
- Sellar, G.C., Jordan, S.A., Bickmore, W.A., Fantes, J.A., Van Heynigen, V. and Whitehead, A.S. (1994) The human serum amyloid A protein (SAA) superfamily gene cluster: Mapping to chromosome 11p15.1 by physical and genetic linkage analysis. *Genomics* 19, 221.

- Schein, C.H. (1989) Production of soluble recombinant proteins in bacteria. *Bio/Technology* 7, 1141.
- Scopes, R.K. (1982) In: *Protein Purification: Principles and Practice*. Springer-Verlag, New York p. 185.
- Sipe, J.D., Gonnerman, W.A., Loose, L.D., Knapschaefer, G., Xie, W.J. and Franzblau, C. (1989) Direct binding enzyme-linked immunosorbent assay (ELISA) for serum amyloid A (SAA). *J. Immunol. Methods* 125, 125.
- Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* 67, 31.
- Steel, D.M. and Whitehead, A.S. (1994) The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol. Today* 15, 81.
- Steel, D.M., Sellar, G.C., Uhlar, C.M., Simon, S., De Beer, F.C. and Whitehead, A.S. (1993) A constitutively expressed serum amyloid A protein gene (C-SAA) is closely linked to, and shares structural similarities with, an acute phase serum amyloid A protein (A-SAA) gene. *Genomics* 16, 447.
- Stone, M.J. (1990) Amyloidosis: a final common pathway for protein deposition in tissues. *Blood* 75, 531.
- Strachan, F.A., Shepard, E.G., Bellstedt, D.U., Coetzee, G.A., Westhuuzyen, D.R. and De Beer, F.C. (1989) Human Serum Amyloid A protein. Behavior in aqueous and urea-containing solutions and antibody production. *Biochem. J.* 263, 365.
- Taktak, Y.S. and Lee, M.A. (1991) A solid phase enzyme immunoassay for serum amyloid A (SAA) protein. *J. Immunol. Methods* 136, 11.
- Tape, C., Tan, R., Nesheim, M. and Kisilevsky, R. (1988) Direct evidence for circulating apoSAA as the precursor of tissue AA amyloid deposits. *Scand. J. Immunol.* 28, 317.
- Towbin, J., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350.
- Whitehead, A.S., De Beer, M.C., Steel, D.M., Rits, M., Lelias, J.M., Lane, W.S. and De Beer, F.C. (1992) Identification of novel members of the serum amyloid A protein (SAA) superfamily as constitutive apolipoproteins of high density lipoprotein. *J. Biol. Chem.* 267, 3862.
- Wilkins, J.W., Gallimore, R., Tennent, G.A., Hawkins, P.N., Limburg, P.C., Van Rijswijk, M.H., Moore, E.G. and Pepys, M.B. (1994) Rapid automated enzyme immunoassay of serum amyloid A. *Clin. Chem.* 40, 1284.
- Woo, P., Sipe, J., Dinarello, C.A. and Colten, H.R. (1987) Structure of a human serum amyloid A gene and modulation of its expression in transfected L cells. *J. Biol. Chem.* 262, 15790.
- Yamada, T., Kazuaki, U., Yakat, M. and Gejyo, F. (1989) Sandwich enzyme immunoassay for serum amyloid A protein (SAA). *Clin. Chim. Acta* 179, 169.
- Yamada, T., Kluge-beckerman, B., Kuster, W.M., Liepnieks J.J. and Benson M.D. (1994a) Measurement of serum amyloid A<sub>4</sub> (SAA<sub>4</sub>): its constitutive presence in serum. *Int. J. Exp. Clin. Invest.* 1, 114.
- Yamada, T., Kluge-beckerman, B., Liepnieks, J.J. and Benson, M.D. (1994b) Fibril formation from recombinant human serum amyloid A. *Biochim. Biophys. Acta.* 1226, 323.