Human serum amyloid A protein

Complete amino acid sequence of a new variant

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Serum amyloid A protein (SAA), an acute-phase reactant and apolipoprotein of high-density lipoprotein, is a polymorphic protein with six reported isoforms. These are the products of three genes, i.e., cDNA pA1, cDNA pSAA82 and genomic DNA SAAg9, the last two being allelic variants at a single locus. We have identified an individual with additional novel SAA isoforms on isoelectric-focusing analysis. By using 3-bromo-3-methyl-2-(2'-nitrophenylsulphenyl)-indolenine (BNPS-skatole) cleavage of the protein at tryptophan residues we obtained the complete amino acid sequence of a novel isoform. Additional cleavage by endoproteinase Asp-N allowed verification of the tryptophan residues and complete amino acid sequence of both isoforms. The suitability of this approach to the rapid sequencing of SAA was demonstrated. Sequence analysis and quantification suggest that these isoforms are the result of the first confirmed allelic variation at the SAA1 locus. We designate the protein products of this allele SAA1 β (pI 6.1) and SAA1 β des-Arg (pI 5.6).

INTRODUCTION

Serum amyloid A protein (SAA) is an acute-phase reactant and apolipoprotein of high-density lipoprotein (HDL) [1]. SAA is the precursor of amyloid A protein (protein AA), which aggregates to form the fibrils characteristic of systemic amyloidosis [2]. SAA is a polymorphic protein with six reported isoforms falling into three pairs, pI 6.0/6.4, pI 7.0/7.5 and pI 7.4/8.0 [3,4]. These pairs correspond to cDNA pA1 [5], cDNA pSAA82 [6] and genomic DNA SAAg9 [7]. The second of each isoform pair (i.e. pI 6.4, pI 7.5 and pI 8.0 isoforms) is the primary gene product [4]. Each 104-amino acid-residue primary product is processed to either a 103-residue or a 102-residue isoelectrically indistinguishable polypeptide by the loss of an arginine or an additional serine residue from its *N*-terminus. These secondary products have the lower pI values of 6.0, 7.0 and 7.4 [3]. The segregation characteristics of the pI 7.0/7.5 (SAA2 α [4]) and pI 7.4/8.0 (SAA2 β [4]) isoform pairs, which are the products of the respective genes pSAA82 and SAAg9 [6,7], suggest that these are allelic variants at a single locus distinct from the pI 6.0/6.4 [SAA1 α (4)] isoform pair.

Recent evidence has emerged for the existence of other SAA molecules distinct from those recognized up to now. Sack & Talbot have described another human SAA family member, GSAA1 [8], at the gene level. Computer modelling by us [3] predicts a pI of 7.8 for it, but we have found no evidence for its existence on HDL (results not shown). In addition, cDNA sequence analysis by Steinkasserer *et al.* [9] and amino acid sequence analysis of a peptide derived from protein AA [10] establish that novel SAA genes and gene products exist that have not yet been characterized.

Given this complexity, we consider it important to define the SAA family both at the protein and at the gene level. To this end we have pursued techniques that would allow rapid and complete amino acid sequence analysis of isoelectrically separated SAA

isoforms. A patient (C.F.) was identified with a unique isoelectric SAA isoform distribution identical with that described by Steinmetz *et al.* [11]. As this SAA isoform pattern was theoretically compatible with allelic variation at the SAA1 locus, we purified the variant isoforms and obtained a complete amino acid sequence from peptides generated by chemical cleavage at tryptophan residues. This approach is very applicable to human SAA as the cleavage of the three tryptophan residues in the molecule yields four peptides of ideal sequencing length. We suggest that allelic variation of SAA does occur at the SAA1 locus, albeit rarely, and designate the new protein SAA1 β .

MATERIALS AND METHODS

Preparation of HDL

Blood was obtained with informed consent from patients with rheumatoid arthritis, including patient C.F. HDL was isolated from plasma essentially as described previously [1,12]. Plasma density was adjusted to 1.09 g/ml with solid KBr and centrifuged for 5.3 h at 55000 rev./min in a VTi80 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) at 10 °C. The density of the infranatants, which contained the HDL, was adjusted to 1.21 g/ml with solid KBr and they were re-centrifuged for 9.4 h under the same conditions. The pellicles, containing HDL, were extensively dialysed against 0.15 M-NaCl/0.1 % (w/v) EDTA, pH 7.4.

Electrofocusing

Portions (200 μ g) of HDL were freeze-dried and delipidated with 0.5 ml of chloroform/methanol (2:1, v/v) [13]. The delipidated proteins were resuspended in sample buffer consisting of 7 M-urea, 1 % (w/v) sodium decyl sulphate (Eastman Kodak Co., Rochester, NY, U.S.A.) and 5 % (v/v) 2-mercaptoethanol. Samples were electrofocused on 0.3 mm polyacrylamide gels containing 7 M-urea and an Ampholine gradient consisting of

Abbreviations used: SAA, serum amyloid A protein; HDL, high-density lipoprotein; BNPS-skatole, 3-bromo-3-methyl-2-(2'-nitrophenylsulphenyl)indolenine.

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20% (v/v) Ampholines pH 3–10, 40% (v/v) Ampholines pH 4–6.5 and 40% (v/v) Ampholines pH 7–9 (Pharmacia–LKB Biotechnology, Piscataway, NJ, U.S.A.) [12], which would expand the basic pH range of electrofocusing gels. Alternatively, an Ampholine gradient of 20% (v/v) Ampholines pH 3–10 and 80% (v/v) Ampholines pH 4–6.5 was used to expand the acidic pH range of electrofocusing gels.

Immunochemical analysis

The SAA isoform distributions in patients with active rheumatoid arthritis were investigated by means of immunochemical analysis. Plasma from individual patients (20 μ l) was freeze-dried, delipidated and subjected to isoelectric focusing as described above. Samples on electrofocused gels were pressure-blotted on to 0.2 μ m-pore-size nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.) for 20 h at room temperature [12]. The membrane was wetted with 25 mM-Tris/HCl buffer, pH 8.3, containing 192 mM-glycine and 15% (v/v) methanol. Following pressure-blotting the membrane binding sites were blocked overnight at 4 °C with 5% (w/v) non-fat dry milk in 0.137 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, containing 2% (w/v) BSA. Screening for SAA isoforms was performed with rabbit anti-[human SAA-(95–104)-peptide] antibody, a gift from Professor A. Steinmetz (University of Marburg, Marburg, Germany). An alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody was used as secondary antibody (A8025, lot no. 39F-88961; Sigma Chemical Co., St. Louis, MO, U.S.A.). The chromogenic substrates for alkaline phosphatase, 5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt and Nitro Blue Tetrazolium chloride, were applied according to the manufacturer's instructions (Bethesda Research Laboratories Life Technologies, Bethesda, MD, U.S.A.).

Electroblotting

HDL (6.8 mg) from a female rheumatoid arthritis patient (C.F.) expressing variant pI 6.1 and 5.6 SAA isoforms was electrofocused in 200 μ g portions in an Ampholine gradient of 20% (v/v) Ampholines pH 3–10 and 80% (v/v) Ampholines pH 4–6.5. The pI 6.1 and pI 5.6 bands were excised from Coomassie Blue-stained electrofocused gels and resolved in a second-dimension SDS/5–20% polyacrylamide gel with a 3% polyacrylamide stacking gel [14]. Approximately seven to ten bands were pooled, boiled in SDS sample buffer and loaded into a single well. Subsequently the isoforms were electroblotted [15] for 2.5 h at 200 mA on to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA, U.S.A.) with 25 mM-Tris/HCl buffer, pH 8.3, containing 192 mM-glycine, 10% (v/v) methanol and 0.05% SDS as transfer buffer. Electroblotted protein was identified by staining with Amido Black.

Electroelution

HDL (10 mg) from patient C.F. was electrofocused in 200 μ g portions in an Ampholine gradient in 20% (v/v) Ampholines pH 3–10 and 80% (v/v) Ampholines pH 4–6.5. The desired Coomassie Blue-stained bands were excised and equilibrated in elution buffer (25 mM-Tris/HCl buffer, pH 8.3, containing 192 mM-glycine and 0.01% SDS) before electroelution in a model UEA unidirectional electroelutor (International Biotechnologies, New Haven, CT, U.S.A.) according to the manufacturer's instructions. Electroelution was carried out for 45 min at 125 V and the electroeluted protein was trapped in 7.5 M-ammonium acetate. The pooled eluates were dialysed against 20 mM-Tris/HCl buffer, pH 8.4, containing 1 mM-EDTA and 150 mM-NaCl and the protein was precipitated overnight at 4 °C with a final concentration of 20% (w/v) trichloroacetic acid. Residual trichloroacetic acid was removed by washing with

diethyl ether at 4 °C. The total amount of electroeluted protein was 60 μ g of pI 5.6 isoform and 92 μ g of pI 6.1 isoform.

Fragmentation of protein

Chemical cleavage at tryptophan residues. Electroeluted protein was dissolved in 0.5 ml of 75% (v/v) acetic acid. Solid 3-bromo-3-methyl-2-(2'-nitrophenylsulphenyl)indolenine (BNPS-skatole) (Pierce Chemical Co., Rockford, IL, U.S.A.) was added to a final concentration of approx. 1 mg/ml and the solution was heated at 47 °C, in the dark, for 2 h. To prepare the sample for subsequent reverse-phase h.p.l.c., the BNPS-skatole and most of the acetic acid were removed by two or three extractions with 2–4 vol. of diethyl ether. Approx. 0.2–0.4 vol. of water was added to the cleavage mixture before extraction with diethyl ether, and the yellowish ether phase was removed by aspiration. After the final extraction, residual diethyl ether was removed from the aqueous phase by vacuum centrifugation.

Enzymic cleavage by endoproteinase Asp-N. In order to create overlapping peptides containing the critical tryptophan residues, pI 5.6 and pI 6.1 SAA isoforms electroblotted on to poly(vinylidene difluoride) membranes were digested with 0.04 μ g of endoproteinase Asp-N (Boehringer Mannheim, Indianapolis, IN, U.S.A.) for 20 h at 37 °C essentially according to Bauw *et al.* [16].

Chromatographic separation of protein fragments

All chromatography was done with a Hewlett–Packard (Palo Alto, CA, U.S.A.) 1050 series h.p.l.c. system with an automatic sampler, quaternary pump, variable-wavelength detector and model 3396A recording integrator. The fragments created by cleavage with BNPS-skatole were separated by a Vydac (Hesperia, CA, U.S.A.) 4.6 mm $\times$ 50 mm C_4 reverse-phase column and the peptides created by cleavage by endoproteinase Asp-N were separated by a Vydac 4.6 mm $\times$ 250 mm C_{18} column. A linear gradient of acetonitrile in 0.06% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min was used. The column effluent was monitored at 214 nm and u.v.-absorbing peaks were collected for subsequent amino acid sequence analysis.

Amino acid sequence analysis

Amino acid sequencing of protein fragments was performed with either a model 477A or a model 475A automated protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.) with on-line analysis of amino acid phenylthiohydantoin derivatives. The samples were dried on to precycled Polybrene-coated glass-fibre discs and the standard sequencer cycles employed pulsed-liquid chemistry.

Quantification of total SAA or isoforms

Total SAA or individual isoforms were excised from Coomassie Blue-stained SDS/PAGE gels or electrofocused gels, the dye was extracted with 25% (v/v) pyridine and the absorbance was measured at 605 nm. Proteins were quantified by comparing the colour yield of unknown samples to that of reference proteins of known protein concentration [17].

RESULTS

SAA isoform distribution

We characterized the SAA isoforms in 31 acute-phase rheumatoid arthritis patients by means of immunochemical analysis (results not shown) and, on the basis of our previous assignment of the major isoforms to published gene sequences [3], found that 3% (one out of 31) were heterozygous at the SAA1 locus with SAA1 β present. At the SAA2 locus 26% (eight out of 31) were

Fig. 1. Immunoblot of isoelectrically separated SAA isoforms

HDL (10 µg) from patients with active rheumatoid arthritis was electrofocused utilizing an Ampholine gradient of (a) 20% (v/v) Ampholines pH 3–10, 40% (v/v) Ampholines pH 4–6.5 and 40% (v/v) Ampholines pH 7–9 or (b) 20% (v/v) Ampholines pH 3–10 and 80% (v/v) Ampholines pH 4–6.5. The SAA isoforms were detected with a rabbit anti-[human SAA-(95–104)-peptide] antibody. Lanes 1, electrofocused HDL (10 µg) of a patient who expressed all three SAA isoform pairs, i.e. pI 6.0/6.4 (SAA1α), pI 7.0/7.5 (SAA2α) and pI 7.4/8.0 (SAA2β). Lanes 2, electrofocused HDL (10 µg) of a patient who expressed only two SAA isoform pairs, i.e., pI 6.0/6.4 (SAA1α) and pI 7.0/7.5 (SAA2α). Lanes 3, electrofocused HDL (10 µg) of patient C.F., who expressed novel acidic SAA isoforms with pI values of 5.6 and 6.1.

heterozygous. Whereas 6% (two out of 31) of the patients were homozygous for SAA2β, no patient homozygous for SAA1β has been detected.

The SAA isoform distributions of three patients with rheumatoid arthritis are shown in Fig. 1(a), where the SAA isoforms had been separated in an Ampholine gradient of 20% (v/v) Ampholines pH 3–10, 40% (v/v) Ampholines pH 4–6.5 and 40% (v/v) Ampholines pH 7–9. Patient no. 1 expressed all three SAA isoform pairs, i.e. pI 6.0/6.4 (SAA1α), pI 7.0/7.5 (SAA2α) and pI 7.4/8.0 (SAA2β). This patient was therefore heterozygous at the SAA2 locus. Patient no. 2 expressed only two SAA isoform pairs, i.e. pI 6.0/6.4 (SAA1α) and pI 7.0/7.5 (SAA2α), and was therefore homozygous at the SAA2 locus. Evidence of novel SAA isoforms expressed by patient no. 3 (patient C.F.)

Fig. 2. Chromatographic separation of peptide fragments generated by BNPS-skatole cleavage of SAA1β pI 6.1

The results from cleavage of the pI 5.6 isoform were similar and are not shown. The fragments were separated by a 4.6 mm × 50 mm Vydac C₄ reverse-phase column with gradient elution conditions. The mobile phase was water versus acetonitrile with 0.06% trifluoroacetic acid throughout, flow rate was 1 ml/min, gradient slope was 1.4% acetonitrile/min, and spectrophotometric monitoring of the effluent was at 214 nm. Four peaks are labelled W1–W4, and the column effluent corresponding to these peaks was subjected to amino acid sequence analysis.

could be seen at the acidic region of the electrofocused gel. In order to establish the nature of these novel isoforms, the SAA isoforms of the same three patients were separated in an Ampholine gradient consisting of 20% (v/v) Ampholines pH 3–10 and 80% (v/v) Ampholines pH 4–6.5 (Fig. 1b). Patient C.F. clearly expressed novel SAA isoforms with pI values 5.6 and 6.1 apart from the SAA isoform pairs pI 6.0/6.4 (SAA1α) and pI 7.0/7.5 (SAA2α).

Quantification of total SAA and SAA isoforms

Quantification of the relative contribution of the SAA1 locus to the total SAA (Table 1) confirmed that this locus contributed

Table 1. Quantification of total SAA and SAA isoforms

Total SAA or SAA isoforms were quantified in the HDL isolated from four patients (nos. 1–4) suffering from active rheumatoid arthritis. All four were homozygous at the SAA2 locus and expressed only SAA2α. Subject 4, known as patient C.F., was a female patient expressing the novel SAA isoforms of pI values 6.1 and 5.6. The total SAA content of the various HDLs was calculated after excising the SAA band from Coomassie Blue-stained SDS/PAGE gels and extracting the dye with 25% pyridine as described in the Materials and methods section. In a similar fashion, the SAA isoforms were excised from Coomassie Blue-stained electrofocused gels and the dye extracted with 25% pyridine. The relative contribution of each SAA isoform to the total complement is expressed as a percentage of the total colour yield of all the SAA isoforms. Abbreviation: N.D., not detected.

Patient no.	SAA (% of total HDL apolipoprotein)	Isoform...	Colour yield (% of total)					
			pI 5.6	pI 6.0	pI 6.1	pI 6.4	pI 7.0	pI 7.5
1	7.8		N.D.	38	N.D.	42	11	9
2	9.7		N.D.	39	N.D.	38	15	8
3	10.6		N.D.	29	N.D.	35	19	17
4	8.9		23	10	26	13	16	12

Fig. 3. Chromatographic separation of peptides generated by endoproteinase Asp-N cleavage of SAA1 β des-Arg (pI 5.6)

The results from cleavage of the pI 6.1 isoform were identical and are not shown. The peptides were separated by a 4.6 mm $\times$ 250 mm Vydac C₁₈ reverse-phase column with linear gradient elution conditions of 0.84% acetonitrile/min and other chromatographic conditions as described for Fig. 2. Three peaks in the chromatogram are labelled D1–D3, and the column effluent corresponding to these peaks was subjected to amino acid sequence analysis. Because of their relative peak heights and locations, these three peaks were chosen for further analysis, as it was correctly surmised that they represented tryptophan-containing peptides. Peak D1 was shown to have two peptides, only one of which contained tryptophan.

approx. 70% of the SAA in HDL containing approx. 9% SAA. In contrast, in patient C. F. SAA1 α contributed only 23% of the total SAA, with SAA1 β contributing 49%.

Amino acid sequencing of peptides

After treatment of electroeluted protein with BNPS-skatole for fragmentation at tryptophan residues, the fragment mixture was subjected to reverse-phase h.p.l.c. on a C₄ column. The chromatograms obtained from the pI 5.6 and pI 6.1 isoforms were similar. The chromatogram from the pI 6.1 isoform fragmentation is presented in Fig. 2 and the column effluent fractions subjected to amino acid sequence analysis are marked. In several cases, e.g. peaks W1 and W4, sequencing revealed more than one species in a chromatographic peak. The sequencing yields of each species were different enough (the yield of one species being at least twice that of the other) to allow unambiguous separation of the sequences.

Fig. 3 shows the chromatogram produced by cleavage of the pI 5.6 isoform by endoproteinase Asp-N with sequenced peaks identified. Endoproteinase Asp-N cleavage of the pI 6.1 isoform resulted in a chromatogram indistinguishable from that of the pI 5.6 isoform (results not shown). The amino acid sequences of the novel isoforms are given in Fig. 4 with the contributions from individual peptides found beneath each sequence. The pI 5.6 isoform was shown to be the des-Arg derivation of the pI 6.1 isoform.

The cleavage of proteins at tryptophan residues by BNPS-skatole is an inefficient process [18], and several peptides were generated from the pI 6.1 isoform that represented the partial nature of the cleavage, e.g. a peptide spanning Trp-18 found in

Fig. 4. Amino acid sequences of SAA1 β des-Arg pI 5.6 and SAA1 β pI 6.1

The sequences of the two isoforms were identical with the exception of an N-terminal arginine residue in the pI 6.1 protein that was not present in the pI 5.6 protein. Amino acid numbering begins with position number 2 in the des-Arg form of SAA1 β . Linear sequences of peptides generated by BNPS-skatole cleavage (W1–W4) or endoproteinase Asp-N cleavage (D1–D3) of the parent protein are marked by a line indicating amino acid residues identified.

peak W4 and a peptide spanning Trp-85 found in peak W3. Also sequenced (in peak W1) was a peptide covering residues 92–104 that was generated by the cleavage of an Asp–Pro bond.

Sequencing of tryptophan-containing peptides created by protein cleavage of the pI 5.6 isoform by endoproteinase Asp–N allowed positive identification of tryptophan residues. However, when sequencing residues of BNPS-skatole-generated peptides corresponding to a tryptophan cleavage site, two unusual peaks were noted on the chromatogram of amino acid phenylthiohydantoin derivatives. In every case the taller of the two peaks was eluted immediately before tyrosine phenylthiohydantoin derivative and the shorter of the two peaks was eluted immediately before arginine phenylthiohydantoin derivative, arginine phenylthiohydantoin derivative being positioned immediately before proline phenylthiohydantoin derivative. As these peaks were uniquely associated with identified tryptophan residues in the sequencing of the pI 5.6 isoform, their appearance in the sequencing of BNPS-skatole-generated peptides of the pI 6.1 isoform was interpreted as the location of a tryptophan residue.

DISCUSSION

The amino acid sequences of the two variant isoforms revealed that they adhered to the same pattern described for other SAA gene products [3] in that the pI 5.6 isoform was the des-Arg derivation of the primary protein (pI 6.1). We designate this variant isoform pair SAA1 β and SAA1 β des-Arg. No evidence was seen that an additional N-terminal serine residue was removed [3]. A comparison of the sequence of SAA1 β with the published sequences of SAA2 α , SAA2 β and SAA1 α is given in Fig. 5 [5–7]. Sequence analysis of SAA1 β at residues 52 and 57 corresponded to that of SAA2 α and SAA2 β . At the five remaining residues (60, 68, 69, 84 and 90) where SAA1 α differs from SAA2 α and SAA2 β , SAA1 β was identical with SAA1 α . At position 71 SAA2 β differs from the other SAAs in having an arginine substitution. These sequencing data imply that exon 3 of SAA1 β and the alleles at the SAA2 locus were identical. Possibilities of exon shuffling, gene conversion and mutation of the gene duplication between these alleles need to be considered. In addition, SAA1 β had a unique substitution at position 72 where glycine was replaced by aspartic acid. This acidic substitution was responsible for the isoelectric differentiation between SAA1 α and SAA1 β . Thus SAA1 β differed from SAA1 α at three, from SAA2 α at six and from SAA2 β at seven amino acid residues.

The absence of SAA2 β and concomitant appearance of SAA1 β in the two patients studied made it theoretically possible that SAA1 β could be created by amino acid substitutions in the SAA2 β allele that made the resulting isoforms markedly more acidic. The greater sequence similarity between SAA1 α and SAA1 β makes this hypothesis less likely. Additional evidence that SAA1 β is an allelic variation at the SAA1 locus was presented by the comparative quantification of the various isoforms (Table 1). In all acute-phase individuals SAA1 α was always quantitatively the major isoform even when these individuals were homozygous for either SAA2 α or SAA2 β [12,19]. In individuals that were heterozygous at the SAA2 locus the total concentration of SAA2 α plus SAA2 β was roughly equal to the SAA2 concentration found in their homozygous counterparts at similar SAA concentrations, whether the SAA2 be α or β . At a specific locus each allele thus contributes approx. 50% of the protein produced. In our patient SAA1 α constituted only 23% of the total SAA. When the amounts of SAA1 α and SAA1 β were added together they constituted 72% of the total SAA, a value that approximated the expected contribution of the SAA1 locus at this SAA concentration. It is unclear why SAA1 β should

Fig. 5. Comparison of amino acid sequences of three SAA isoforms with the sequence of SAA1 β

The amino acid sequences of SAA2 α , SAA2 β and SAA1 α were derived from the three published cDNA/genomic DNA SAA sequences pSAA82 [6], SAAg9 [7] and pA1 [5]. Differences are marked with an asterisk and the location of the unique aspartic acid residue found in SAA1 β at position 72 is enclosed in a box. Regions of the protein encoded by exons 2, 3 and 4 are defined below the sequence.

constitute 49 % and SAA1 α only 23 % of the SAA locus products. One possibility that needs to be considered is differential clearance of SAA1 α and SAA1 β .

It was previously reported that an anti-peptide antibody raised against SAA1 α amino acid residues 58–69 does not cross-react with SAA derived from the SAA2 locus [11]. We confirmed that this antibody (gift from Professor A. Steinmetz) stained SAA1 β (results not shown). However, when isoelectric-focusing gradients were employed that allowed resolution of the SAA2 locus products, these proteins also stained in all the rheumatoid arthritis patients studied, precluding its specificity for the SAA1 locus (results not shown).

The novel charged amino acid substitution that we identified at position 72 is very close to the Ser–Leu bond at position 76–77 where SAA is commonly cleaved during amyloidogenesis. Protein AA subspecies can, however, vary in length from 45 to 94 amino acid residues [10]. It is interesting to speculate that the aspartic acid at position 72 might alter the cleavage site, resulting in an AA species of a different length.

The convenient siting of tryptophan residues in all human SAAs and protein AA for internal amino acid sequence analysis has previously been exploited for a form of protein AA [10]. However, no peptide separation was attempted. Cleavage at the three tryptophan residues in SAA would yield four fragments of 18, 35, 32 and 19 amino acid residues. As purification of peptide fragments would be essential for complete amino acid sequence analysis, we chose to separate and purify peptides by reverse-phase h.p.l.c. To overcome the problem of background peaks generated by the cleavage reagent, we extracted the BNPS-skatole reagent from the peptide mixture with diethyl ether, allowing definable reverse-phase chromatography. The distinctive colour difference of the aqueous blue Coomassie Blue-containing phase and the yellow BNPS-skatole-containing ether phase allowed easy monitoring of the extent of reagent extraction.

All fragments were identified, indicating that all of the tryptophan residues were accessible for cleavage. The extra peptide peaks visible in the chromatograms (Fig. 2) probably represented the fragments generated by inefficient cleavage at tryptophan as well as acid hydrolysis of a labile Asp–Pro bond (position 91–92). It was advantageous that cleavage at tryptophan residues by BNPS-skatole was less than 100 % efficient because sequencing through tryptophan residues allowed generation of overlapping data. Thus the complete sequence of a protein might be obtained by a single cleavage with BNPS-skatole.

The techniques for rapid and complete amino acid sequence analysis that we describe in the present paper should be applicable to SAAs from many other species. This would benefit characterization of the diversity of SAA and assist in definitive probe design for gene analysis.

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