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PROCEDURE FOR THE ISOLATION OF A LARGE PEPTIC FRAGMENT OF HUMAN SERUM ALBUMIN

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SUMMARY

For a thorough investigation of the drug-binding behaviour and other physicochemical properties of human serum albumin, one needs large amounts of specific fragments of albumin. Such fragments were obtained by careful proteolysis of the native protein with pepsin at pH 3.70. The fast protein liquid chromatographic technique was used to find the optimum experimental conditions for the separation of the fragments. By means of anion-exchange chromatography, chromatofocusing and gel permeation, it was possible to obtain a large fragment with a relative molecular mass of 46 000. The fragment could be assigned to segment 1–387 and therefore consists of domains 1 and 2 of the albumin structure. A 1-g amount of albumin produced 50 mg of a fragment that was 98% homogeneous.

INTRODUCTION

The binding of drugs to blood proteins can have a marked effect on the pharmacokinetic behaviour of the drugs [1,2]. Albumin* is one of the most important of these drug-binding blood proteins. There are only a limited number of drug-binding sites on the albumin molecule and most drugs bind to the so-called warfarin site (site I) or diazepam site (site II) [3–9]. Both sites I and II are sensitive to a conformational change around physiological pH, the neutral to base or N–B transition [10–15]. At pH 6 albumin exists mainly in the neutral form and at pH 9 the basic form predominates.

In an attempt to elucidate the characteristics of the drug-binding sites and the molecular mechanism of the N–B transition, we are currently investigating the drug-binding behaviour and other physicochemical properties of fragments of albumin.

*The term albumin is used to represent human serum albumin, unless indicated otherwise.

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The primary structure of albumin was elucidated simultaneously by Behrens et al. [16] and Meloun et al. [17]. Albumin is a single peptide chain of 585 amino acids containing 17 disulphide bridges. These disulphide bridges form the basis of the organization of albumin. It is commonly accepted that the primary structure is folded into three domains, each domain being built up of three loops [18].

Because albumin can be regarded as three more or less independent domains linked together by a small peptide, we thought it advisable to start our study with fragments containing intact domains. In the first instance, we focused our attention on fragments with two domains. The albumin structure suggests that careful proteolysis with enzymes might lead to fragments containing one or two domains. The successful isolation of such fragments has already been described for bovine serum albumin [19–23]. However, for reasons not fully explained, similar proteolysis studies with human serum albumin have been less successful [18,24]. One of the problems was apparently the lack of homogeneity of the fragments; the homogeneity generally did not exceed 90%. Moreover, the amounts of fragments isolated were just enough for identification purposes in most instances.

To perform drug-binding and other physicochemical studies, one requires large amounts (up to 50 mg) of homogeneous material. Therefore, we tried to improve the preparation and isolation methods (for tryptic and peptic fragments of human serum albumin) described in earlier papers [24–27]. In the cited work, the albumin fragments were usually isolated using "common" chromatographic procedures, such as DEAE or CM ion-exchange chromatography and gel permeation. In order to improve these chromatographic procedures, we made use of fast protein liquid chromatography (FPLC). The high resolution and the fast elution times associated with this technique made it very suitable for use in such an investigation.

In this paper, a procedure for isolating large amounts of an almost homogeneous peptic fragment of albumin is described.

EXPERIMENTAL

Reagents

Mono Q HR 5/5, Mono P HR 5/20, Superose-12 HR 10/30, Sephacryl S-200, Q-Sepharose Fast Flow, PBE-94 and polybuffer-74 were obtained from Pharmacia (Uppsala, Sweden). Pepsin (from pig stomach mucosa) was purchased from Boehringer (Mannheim, F.R.G.). All other chemicals were of analytical-reagent grade (J.T. Baker, Deventer, The Netherlands; Janssen Chimica, Beerse, Belgium; Sigma, St. Louis, MO, U.S.A.; Merck, Darmstadt, F.R.G.).

Human serum albumin

Albumin was isolated from human plasma according to the method described by Kremer [28]. The albumin concentration was determined as described elsewhere [12].

Fast protein liquid chromatography

We used the analytical set up of the FPLC technique (Pharmacia) to find the optimal experimental conditions for separating the fragments obtained after the

pepsin digestion of albumin. Because this FPLC system has a low loading capacity and therefore a low overall yield of fragments, the results found for the FPLC system were applied to a conventional system with a 100-fold higher loading capacity. The conditions for separating the fragments with the conventional system were the same as those determined for the FPLC system. The procedure for the conventional system will be described.

Pepsin digestion

A 1.0-g amount of albumin was dissolved in 100 ml of 50 m*M* ammonium formate buffer (pH 3.70 ± 0.02) containing 0.15 *M* sodium chloride. To this solution was added a freshly prepared 0.1% (w/v) pepsin solution in water, with an extinction of 1.0 in a 1-cm cell at 278 nm, in order to obtain a 1:1000 (w/w) ratio of pepsin to albumin. The reaction vessel was stirred continuously and kept at 22 ± 2°C. The reaction was stopped after 20 min by increasing the pH to 8.0 with 2 *M* Tris. The reaction vessel was stirred for a further 1 h to ensure that all pepsin was inactivated.

Isolation of a large peptic fragment of albumin

The temperature was kept at 4–8°C during all isolation steps. The solution obtained after the pepsin digestion was concentrated to 10–15 ml by ultrafiltration in an Amicon stirred cell equipped with a PM-10 filter. The concentrated protein solution was diluted 100-fold with 20 m*M* Bis–Tris–HCl pH 6.0, concentrated to 10 ml by ultrafiltration and loaded on a Q-Sepharose Fast Flow column (25 × 2.6 cm I.D.) equilibrated with a 20 m*M* Bis–Tris–HCl buffer (pH 6.0). The applied digest solution was eluted with a 0.1–0.35 *M* sodium chloride gradient in the same buffer; the gradient had a total volume of 1000 ml. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS), used to assay the relative molecular mass (M_r) of the peptides, showed that the third UV absorption peak (Fig. 1A) contained a fragment with $M_r = 46\,000$ (the P46 fragment). The fractions containing this fragment were pooled and concentrated to 6–8 ml by ultrafiltration. In order to remove the salt and to change the molarity of the Bis–Tris–HCl buffer, the concentrated protein solution was diluted in 25 m*M* Bis–Tris–HCl buffer (pH 5.9) and the solution concentrated to 5–10 ml by ultrafiltration. These last two steps were repeated once and the concentrated material obtained was loaded on a PBE-94 column (30 × 1.6 cm I.D.) equilibrated with 25 m*M* Bis–Tris–HCl (pH 5.9). The proteins were eluted with 550 ml of ten-fold diluted polybuffer-74 (pH 4.2). The P46 fragment was found to be eluted from the column when the pH gradient reached a pH of about 4.7. The fractions containing the fragment (Fig. 1B) were pooled, concentrated to 3–4 ml by ultrafiltration and loaded on a Sephacryl S-200 column (80 × 2.6 cm I.D.) equilibrated with 0.1 *M* Tris–HCl buffer (pH 8.0) containing 0.3 *M* sodium chloride. The same buffer was used for the elution. The fractions containing the P46 fragment (Fig. 1C) were pooled and concentrated to ca. 4 ml by ultrafiltration. After deionization, the fragment solution obtained was immediately used for further experiments. Deionization was performed as described elsewhere [12] and the protein content was determined as described by Martree [29].

Amino acid analyses

Amino-terminal residues were determined with an Applied Biosystems Model 470A protein sequencer, equipped on-line with a Model 120A PTH analyzer.

The amino acid composition was determined with a Pharmacia/LKB Alpha-Plus amino acid analyser, using a 20-cm Ultropac-8 cation-exchange column (particle size 8 ± 0.5 μm) and a 0.2 *M* sodium citrate (pH 3.20/4.25) buffer system. The amino acid analyses were performed after hydrolysis of the peptide for 24 h in 6 *M* hydrochloric acid in sealed evacuated ampoules.

RESULTS AND DISCUSSION

Experimental conditions for the digestion of human serum albumin

According to Ledden et al. [24] and Geisow and Beaven [27], the optimum pH for obtaining high yields of large peptic fragments of albumin is 3.70. At this pH and at the optimum pepsin-to-albumin ratio of 1:1000, fifteen fragments can still be identified in the albumin digest.

Our purpose is to study drug-binding and other physicochemical properties of selected fragments of albumin (i.e., fragments containing one or two domains). To obtain reliable results, we wanted to start the digestion with unmodified, fatty acid-free albumin. Therefore, in contrast to the situation described in the literature [24,25,27], we did not block the free thiol group of albumin before the digestion, nor did we perform the digestion in the presence of octanoic acid [24,25].

Isolation and characterization of the P46 fragment

Fig. 1A–C show the results of the isolation procedure used to obtain the P46 fragment. The insets give the elution patterns obtained with the FPLC system. The figures show that the results found for the FPLC columns could well be transferred to the conventional columns.

However, in spite of the FPLC system, the purification of the P46 fragment

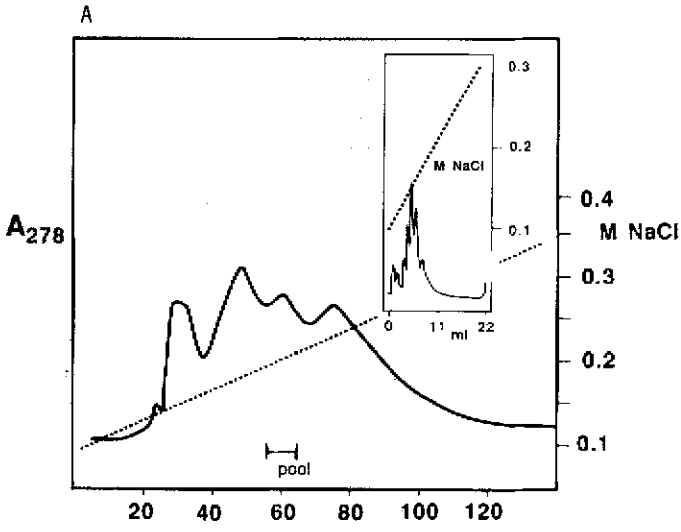

Fig.1.

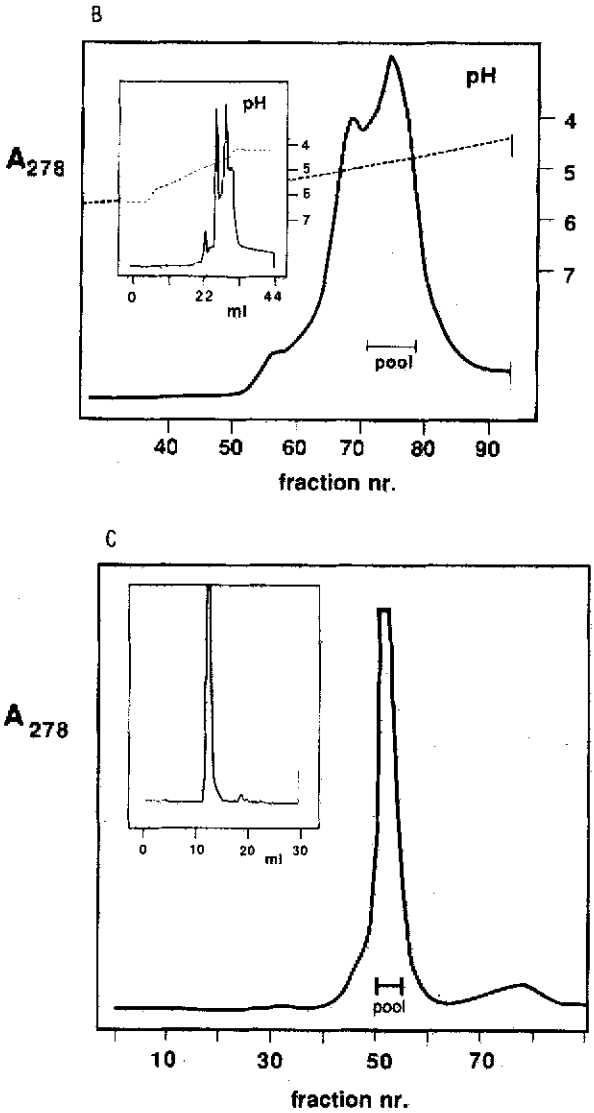

Fig. 1. Isolation of the P46 fragment. The experimental procedures are described in the text. (A) Anion-exchange chromatography on Q-Sepharose Fast Flow; (B) chromatofocusing on PBE-94; (C) gel permeation on Sephacryl S-200. The flow-rates were 50 ml/h and fractions of 5 ml were collected. The insets show the patterns obtained when the FPLC system was used: results for (A) Mono Q HR 5/5; (B) Mono P HR 5/20; (C) Superose-12 HR 10/30. The buffers used were the same as those described for the Q-Sepharose Fast Flow, PBE-94 and Sephacryl S-200 columns.

turned out to be an arduous task. This was not only because we had to isolate one fragment from a pool of fifteen fragments, but also because all fragments emanated from a protein which has rather a proportional distribution of its constitutive amino acids. Therefore, it was desirable to devise an isolation method that could distinguish between two fragments differing by only one amino acid. It appeared that especially chromatofocusing, a method of separating proteins ac-

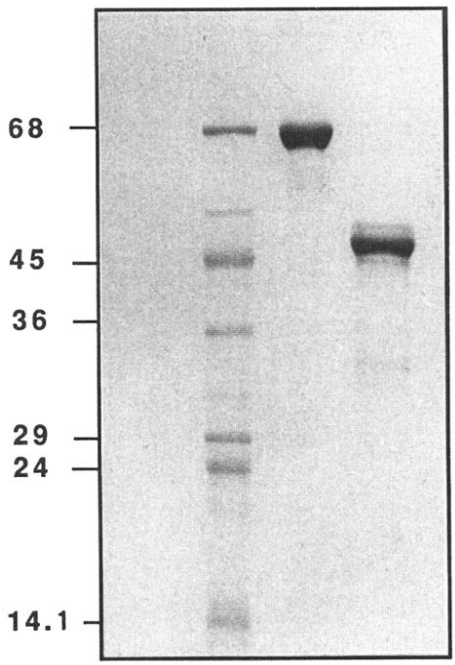

Fig. 2. SDS-PAGE. Polyacrylamide (12.5%) gel electrophoresis was performed in the presence of 0.1% SDS (according to Laemmli [31]). Left lane, low M_r markers: bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), glyceraldehyde-3-phosphate dehyrogenase (M_r 36 000), carbonic anhydrase (M_r 29 000), trypsinogen (M_r 24 000) and lactalbumin (M_r 14 100). Middle lane, 15 μg of the albumin isolated according to Kremer [28]. Right lane, 15 μg of the P46 fragment.

cording to their isoelectric point, was a powerful and appropriate technique. As can be seen in Fig. 2, when the P46 fragment was evaluated by SDS-PAGE, we were able to isolate an almost homogeneous fragment. The homogeneity of the P46 fragment was further verified by determining the amino acid composition and the N-terminal sequence. The amino acid composition of the fragment is listed in Table I. The N-terminal sequence of the fragment, as determined with a gas-phase sequenator, appeared to be Asp–Ala–His–Lys–Ser–Glu–Val. These results resemble those of Ledden and co-workers [24,25] and Geisow and Beaven [27], who described the isolation of a fragment containing segment 1–387 or domains 1 and 2 of the albumin structure. Therefore, it is probable that we also had isolated segment 1–387 or domains 1 and 2 of the albumin structure. However, as described by the above authors, the problem was how to isolate large amounts of fragments that were more than 90% homogeneous. Utilizing the conventional column system as outlined above and starting with 1 g of albumin, we were able to isolate 50 mg of a fragment that was more than 95% homogeneous. Using the FPLC system, we were able to obtain a fragment that was more than 98% homogeneous.

Table I gives not only the experimental amino acid composition of the P46 fragment but also the composition of segment 1–387 according to the sequence deduced from the nucleotide sequence of albumin [30]. When we calculate the

TABLE I

AMINO ACID COMPOSITION OF THE PEPTIC FRAGMENT

Each value represents the mean of two different experiments. The 95% confidence limit of the mean values is ±3% relative. For comparison is shown the amino acid composition of segment 1–387 according to the nucleotide sequence of Dugaiczyk et al. [30].

Amino acid	Peptic fragment	Segment 1–387
Asp	39.4	28
Asn		11
Glu	54.9	41
Gln		11
Leu	43.5	42
Ile	4.9	5
Val	21.4	22
Ala	44.4	45
Gly	7.2	8
Pro	16.2	16
Phe	22.1	22
Tyr	13.5	14
Ser	14.7	15
Thr	13.9	15
Trp*	1	1
½Cys	21.6	23
Met	3.7	4
His	11.5	12
Arg	18.0	17
Lys	35.0	35
Total residues	386.9	387

*Trp could not be determined and was taken as unity.

relative molecular mass of the fragment on the basis of the number of residues found in segment 1–387, we obtain an M_r of 44 080. This value is lower than the M_r of 46 000 obtained by gel permeation and SDS-PAGE (Fig. 2). In this paper we call the peptic fragment the P46 fragment, according to the experimental M_r value of 46 000. If we assume a 95% accuracy in determining the relative molecular mass by gel permeation or SDS-PAGE, then the experimental M_r value of 46 000 agrees well with the theoretical value of 44 080.

Table II lists some physicochemical properties of albumin and the peptic fragment. The sulphydryl content of albumin was 0.70 mol SH per mol albumin and of the fragment 0.68 mol SH per mol fragment. We expected the sulphydryl contents to be almost equal, because the single thiol group of albumin is located in domain 1 (amino acid number 34) and this domain is present in the peptic fragment.

The amount of free fatty acid bound to albumin is almost negligible. We did not expect this amount to change during the purification of the peptic fragment. Therefore, we did not attempt to determine the free fatty acid content of this fragment.

The specific extinction [the extinction of a 1% (w/w) solution measured in a

TABLE II

PHYSICOCHEMICAL PROPERTIES OF ALBUMIN AND THE PEPTIC FRAGMENT

All determinations were performed with deionized protein solutions. Data are expressed as mean ± S.D. (four to seven experiments).

Property	HSA[a]	Peptic fragment
$A_{278}^{1\%}$	5.7 ± 0.1	6.0 ± 0.2
SH content[b]	0.70 ± 0.05	0.68 ± 0.05
Fatty acid content[c]	< 0.01	Not determined
M_r (kDa)	66 500[d]	44 080[e]
Absorption maximum (nm)	277.8 ± 0.2	278.0 ± 0.4

[a]Albumin isolated according to Kremer [28].
[b]Sulphydryl content in mol/mol of protein, determined according to the method of Grassetti and Murray [32].
[c]Free fatty acid content expressed in mol/mol of protein, determined according to the method of Chen [33].
[d]According to the literature [16–18,30].
[e]On the basis of the number of residues found in segment 1–387. The M_r determined by means of gel permeation on the Superose-12 HR 10/30 column and by means of PAGE was 46 000.

1-cm cell at 278 nm] of the peptic fragment is higher than that of albumin. This is as expected, because the relative concentration of aromatic residues in the fragment is higher than that in albumin.

CONCLUSION

The isolation procedure described here (i.e., first determining the optimal isolation conditions by FPLC, then applying these conditions to a conventional system) is an elegant method for obtaining large amounts of an almost homogeneous fragment. This means that, using this procedure, it should also be possible to isolate large amounts of homogeneous fragments containing only one domain of the albumin structure. Because our purpose is to obtain a better insight into the physicochemical properties of albumin by studying fragments of albumin, it might be worth isolating large amounts of fragments with one domain.

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