

IMMUNOCHEMISTRY OF SERUM ALBUMIN—II

ISOLATION AND CHARACTERIZATION OF A FRAGMENT FROM THE FIRST THIRD OF BOVINE SERUM ALBUMIN CARRYING ALMOST ALL THE ANTIGENIC REACTIVITY OF THE PROTEIN*

M. Z. ATASSI,† A. F. S. A. HABEEB and CHING-LI LEE†

Department of Chemistry, Wayne State University, Detroit, Michigan 48202; and Division of Clinical Immunology and Rheumatology, Department of Medicine, Microbiology and Biochemistry, University of Alabama Medical School, Birmingham, AL 35233, U.S.A.

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Abstract—Previously we had introduced a novel approach for obtaining fragments from tight (i.e. disulfide-containing, proteolytically inaccessible) proteins. Reversible masking of the amino groups by citraconic anhydride induces in these proteins conformational changes which renders them readily accessible to tryptic cleavage at the arginine peptide bonds. After unmasking of the amino groups, cleavage at the lysyl residues can be achieved, if desired, thus yielding the tryptic peptides with intact disulfide bonds. In the present work, bovine serum albumin (BSA) was subjected by this procedure to cleavage at the arginine residues (giving the Arg-peptides) or at the arginine as well as the lysine residues (i.e. giving the (Arg, Lys)-peptides). The Arg-peptides inhibited 83% the reaction of BSA with its antisera and 97% its reaction with the IgG fraction of the antisera. The (Arg, Lys)-peptides had no (0%) inhibitory activity. A homogeneous fragment was isolated from the Arg-peptides by gel filtration on Sephadex G-100. Its purity was established by disc electrophoresis and its mol. wt was 25,000 by gel filtration on a calibrated Sephadex column and 23,000 by SDS-electrophoresis. From its amino acid composition and N-terminal sequence determination of the first 14 residues, it was possible to assign the location of this fragment to sequence 11-193 (less Arg 144) of BSA. Its mol. wt, calculated from its sequence, was 20,947. This fragment had an inhibitory activity of 80% towards the precipitin reaction of BSA with its antisera and 88% towards the reaction of BSA with the IgG fraction of the antisera. An immunoabsorbent of the peptide held 84-89% of the total anti-BSA antibody. By fluorescence conjugation and co-elution with antibody in gel filtration, two moles of antibody were bound per mole of peptide. Intactness of the disulfide bonds was essential for maintenance of the inhibitory activity of the peptide. Its activity was completely destroyed on rupture of the disulfide bonds by performic acid oxidation or by reduction followed by carboxymethylation. Also, cleavage at the lysyl peptide bonds, without rupture of the disulfides, destroyed the inhibitory activity of the peptide, due to scission of the antigenic reactive sites. Since the peptide comprised less than a third of the BSA molecule but accounted for almost all the BSA antigenic reactivity, it was concluded that native BSA carried repeating identical antigenic reactive sites.

INTRODUCTION

The antigenic structures of disulfide-containing proteins have been difficult to investigate because of their unsusceptibility to enzymatic or chemical cleavage procedures. For delineation of antigenic reactive sites of a protein antigen an important approach relies on studying the immunochemistry of a variety of overlapping peptides from the parent molecule (Atassi, 1972; 1975). Recently a novel approach was introduced from our laboratories (Atassi *et al.*, 1973) for complete hydrolysis disulfide-containing proteins with trypsin. Masking of the amino groups by citraconylation effected conformational changes in the protein

(Habeb & Atassi, 1970) that rendered it completely accessible to tryptic hydrolysis at the arginyl peptide bonds. After unmasking of the amino groups, tryptic hydrolysis at the lysyl peptide bonds may be carried out if desired (Atassi *et al.*, 1973). This approach has been applied to lysozyme yielding fragments which accounted for almost all (90%) of the reaction of lysozyme with its antibodies (Atassi *et al.*, 1973). Before this approach was introduced, only limited success had been obtained with disulfide-containing proteins by employing proteolytic enzymes with a low pH optimum, of course at the expense of specificity, such as pepsin (Canfield & Liu, 1965; Fujio *et al.*, 1968; Maron *et al.*, 1971) or cathepsin (Lapresle & Webb, 1960). Alternatively, the unfolded derivatives with ruptured disulfides were studied (Brown *et al.*, 1959; Brown, 1962; Gerwing & Thompson, 1968; Young & Leung, 1970). These derivatives are immunochemically entirely unrelated to the native protein and, therefore, are not useful to correlate antigenic reactive sites with three-dimensional structure.

In the preceding communication of this series (Habeb *et al.*, 1974), we reported the procedure for

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† Present address: Department of Immunology, Mayo Graduate School of Medicine, Rochester, MN 55901, U.S.A.

cleavage of bovine serum albumin (BSA*) at arginine peptide bonds, yielding peptides which accounted for all (97%) of the antigenic reactivity of native BSA. The present work describes the isolation, purification and characterization of the major fragment in the Arg-peptides of BSA, together with its immunochemical reaction with antisera to BSA.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA) (3 × crystallized) was obtained from Nutritional Biochemical Corp. Trinitrobenzenesulfonic acid and citraconic anhydride were obtained from Eastman Organic Chemicals and 5,5'-dithiobis (nitrobenzoic acid) from Aldrich Chemical Co. Trypsin-TPCK (trypsin which had been treated with tosyl-phenylalanine chloromethyl ketone to eliminate chymotryptic activity, twice crystallized) was obtained from Worthington Biochemical Corp.

Reaction of bovine serum albumin with citraconic anhydride

A 2% solution of BSA in 0.1 M sodium phosphate buffer pH 8 was reacted with citraconic anhydride (2 molar excess of anhydride per free amino group) for 30 min at room temperature with constant stirring. After reaction, the solution was dialyzed exhaustively against water pre-adjusted to pH 8 with NH_4OH and freeze-dried.

Tryptic cleavage of CT-BSA at arginine residues

A solution of CT-BSA (1 g) in water (100 ml) was adjusted to pH 8.40 on the pH-stat. Hydrolysis with trypsin was carried out at 39°C employing trypsin/albumin ratio of 1:100 (by weight). pH was maintained at 8.4 on the pH-stat by the addition of 0.5 M NaOH. Tryptic hydrolysis was for 2 hr. After digestion, soybean trypsin inhibitor, (14 mg; i.e. 1.4-fold excess by weight relative to trypsin) was added to abolish the action of trypsin. Inhibition of tryptic activity was confirmed by absence of action on *p*-tosyl-L-arginine methyl ester hydrochloride. The solution was then adjusted to pH 4 with 1 N HCl and kept at 39°C for 3 hr to deblock the amino groups. On lowering the pH the material comes out of solution. However, the suspension was completely solubilized when the pH was adjusted to 7.0. The hydrolysate was then freeze-dried to give the Arg-peptides.

Tryptic digestion of CT-BSA at arginine and lysine residues

After digestion of CT-BSA with trypsin as described above, the solution was brought to pH 4 and allowed to stir for 3 hr at 39°C to deblock the amino groups. The pH was readjusted to pH 8.4, trypsin was added and digestion was continued for an additional 2 hr, after which the solution was freeze dried to give the (Arg, Lys)-peptides.

Fractionation of the Arg-peptides

Two columns (2.5 × 90 cm each) of Sephadex G-100, pre-equilibrated with 0.01 M NH_4HCO_3 , were connected in series. A solution of Arg-peptides (0.4 g) in 0.01 M NH_4HCO_3 (10 ml) was applied onto the columns and the latter were eluted with 0.01 M NH_4HCO_3 . The eluate was monitored by reading absorbance at 280 nm. Fractions belonging to one peak were pooled and freeze dried.

* Abbreviations: BSA, bovine serum albumin; CT-BSA, citraconylated bovine serum albumin; Arg-peptides, peptides obtained by tryptic cleavage of CT-BSA at arginine peptide bonds followed (after inhibition of tryptic action) by deblocking; (Arg, Lys)-peptides, peptides obtained by tryptic cleavage of CT-BSA at arginine bonds followed, after deblocking, by cleavage at lysine peptide bonds as well; S11-193, an Arg-peptide corresponding to sequence 11-193 of BSA with points of scission at the carbonyl ends of Arg 143 and Arg 144 (i.e. Arg 144 is removed); SDS, sodium dodecyl sulfate.

Determination of mol. wt

(a) *Calibrated Sephadex G100 column.* A column (1.5 × 120 cm) of Sephadex G100 in borate buffer was calibrated with proteins of known mol. wts (BSA, ovalbumin, soybean trypsin inhibitor and ribonuclease) and the elution volume was plotted against log mol. wt. A sample of peptide (5 mg in 1 ml) was applied on the column and eluted with the same buffer. The mol. wt of peptide was derived from its peak elution volume in reference to the calibration curve with the standard proteins.

(b) *Disc electrophoresis in SDS-sodium phosphate.* The mol. wt of peptide was determined from its mobility (Maizel, 1966) by using a plot of mobility vs log mol. wt of proteins of known mol. wt (BSA, lysozyme, ribonuclease soybean trypsin inhibitor and ovalbumin).

Preparation of IgG fraction of anti-BSA

Anti-BSA antiserum was dialyzed exhaustively against 0.0175 M sodium phosphate buffer pH 7 and then applied on a DEAE-cellulose column pre-equilibrated with 0.0175 M sodium phosphate pH 6.2. The unretarded peak constituted the IgG fraction of anti-BSA antiserum and the recovery of antibody activity in this fraction was 96% of the total reactivity of the whole antiserum.

Preparation of immunoabsorbents

Two immunoabsorbents were prepared, one with BSA and the second with fragment S11-193. Sepharose 4B was activated by an acetonitrile solution of cyanogen bromide in 1 M sodium carbonate (March *et al.*, 1974). To a solution of BSA (50 mg) or fragment S11-193 (44 mg) in 0.2 M NaHCO_3 (5 ml) was added activated Sepharose (3.3 g) and the suspension mixed by gentle rotation at 4°C for 18 hr. The amount of protein conjugated was determined from the difference in concentration before and after reaction with the activated Sepharose. After reaction, the Sepharose conjugate was washed with sodium acetate, urea and NaHCO_3 (March *et al.*, 1974) and suspended in water to a total volume of 8 ml. The BSA immunoabsorbent contained 3 mg BSA/ml suspension and that of the peptide contained 2 mg S11-193/ml suspension.

Determination of binding of antihovine serum albumin antibody by the immunoabsorbents

A solution of IgG fraction of anti-BSA was diluted with saline to contain 1.1 mg of specific antibody per ml (determined by quantitative precipitin reaction) and was applied to immunoabsorbent of S11-193 (2 ml suspension) packed in a glass column (1 × 10 cm). The column was washed with 2 ml vol of sodium phosphate-saline buffer pH 7.3. The eluate was collected and the amount of antibody absorbed was determined from extinction at 280 nm. The unabsorbed material was applied to a second column of S11-193 immunoabsorbent and the procedure of applying the effluent unabsorbed material was repeated on a third, fourth and fifth column. The absorbed antibody was eluted with 5 M guanidine hydrochloride from the five columns and the amount of antibody recovered was calculated from the volume and absorbance of the solution.

Reaction of the peptide with fluorescein isothiocyanate

To peptide S11-193 (40 mg) in 0.15 M NaCl (4.5 ml) was added sodium bicarbonate-carbonate buffer (0.5 ml) at pH 9. The solution was reacted with fluorescein isothiocyanate (2.5 mg) at 4°C for 18 hr (Holborow & Johnson, 1967) after which it was applied to a column (1.5 × 20 cm) of Sephadex G-25 which was eluted with 0.01 M NH_4HCO_3 . The first component comprised the fluorescein conjugate of the peptide. The ratio of the fluorescein concentration to the peptide in the conjugate was determined from absorbance at 492, 320 nm (Holborow & Johnson, 1967) and the concentration and mol. wt of the peptide. The concentration of the fluorescein conjugate was determined by the

Folin-phenol reagent (Lowry *et al.*, 1951) using a calibration curve of fragment S11-193.

Binding of fluorescein-peptide conjugate to antbovine serum albumin

Aliquots (0.5 ml) of the IgG fraction of anti-BSA (of known amount of specific antibody) were mixed with varying amounts of the fluorescein derivative of peptide S11-193. The mixture was incubated at 40°C for 1 hr, then at 4°C overnight. It was then applied to a column (1.5 × 86 cm) of Sephadex G-100 in borate buffer (Habeb, 1966b) and eluted with the same buffer. Fractions (3.3 ml) were read at 280 nm. Also, their fluorescence intensity was determined on an Aminco-Bowman Spectrophotofluorometer using an excitation wavelength of 435 nm and an emission wavelength of 520 nm. The amount of fluorescein-peptide bound to the antibody was calculated from the total fluorescence in the IgG peak. Controls were run in which the IgG fraction of anti-BSA was mixed with lysozyme-fluorescein conjugate and also nonspecific human IgG was mixed with fluorescein-peptide conjugate.

Analytical methods

Disc electrophoresis was performed in 7.5% gel using Tris glycine buffer pH 8.9 (0.043 M Tris and 0.046 M glycine) as described previously (Habeb & Atassi, 1969), or in SDS-sodium phosphate gels (Maizel, 1966). The extent of modification of amino groups was determined by the trinitrobenzenesulfonic acid method (Habeb, 1966a). The susceptibility of the disulfide bonds to reduction with 2-mercaptoethanol in CT-BSA in comparison to native BSA was used to evaluate conformational changes (Atassi & Habeb, 1969; Habeb, 1966a) using 5,5'-dithiobis (2-nitrobenzoic acid) (Ellman, 1959). Peptide maps of tryptic digests of BSA were done by applying 1-2 mg samples to Whatman No. 3 MM paper and subjecting it to descending chromatography in butanol-acetic acid-water system (4:1:5 by volume) for 22 hr followed by electrophoresis in pyridine acetate pH 3.6 for 60 min at 3200 V. Performic acid oxidation of BSA-peptides was performed by reacting 5 mg with 1 ml performic acid for 4 hr at 4°C (Hirs, 1967) after which performic acid was removed by dilution with water and evaporation in vacuum over sodium hydroxide.

Amino acid analysis of protein and peptide hydrolysates were done in a Bio-Cal BC-200 amino acid analyzer. Acid hydrolysis was in constant boiling HCl (double-distilled) at 110°C for 22, 48 and 72 hr in nitrogen-flushed evacuated sealed tubes. For tryptophan determination, hydrolysis was in 3 N *p*-toluenesulfonic acid (Liu & Chang, 1971) in nitrogen-flushed evacuated sealed tubes at 110°C for 22 or 48 hr. Cystine was determined as cysteic acid from four replicate acid hydrolysates of performic-acid oxidized samples. Sequence analyses were done on Beckman 890C sequenator in 1 M Quadrol standard program No. 042772. The PTH-amino acids were identified by gas chromatography on Hewlett Packard 7610A GC with 4' 2 mm i.d. columns of DC 560 using the system of Pisano *et al.* (1972), as well as by thin layer chromatography.

Immunochemical methods

Antisera to BSA were raised in three rabbits (Habeb, 1967) and mixed. The serum was freeze dried and was reconstituted in water to contain 1.7 mg anti-BSA/ml. Quantitative precipitin and inhibition experiments were carried out as previously described in detail (Atassi & Saplin, 1968). All immunochemical reactions were carried out in 0.01 M phosphate buffer at pH 7.0 containing 0.15 M NaCl.

RESULTS

Citraconylation of BSA and removal of the masking groups

Reaction of BSA with citraconic anhydride afforded a derivative (CT-BSA) in which 97% of the free amino

groups had been acylated as determined by the trinitrobenzenesulfonic acid method. Disc electrophoresis in Tris-glycine buffer or in SDS-phosphate buffer (Fig. 4) revealed the presence of higher mol. wt polymers in the BSA samples. The mobility of CT-BSA towards the cathode was slightly lower than that of native BSA both in the presence and absence of SDS. The decrease in mobility of CT-BSA was either due to charge effect or to increase in the asymmetry of the molecule or both. The presence of conformational changes in CT-BSA was confirmed by increase in accessibility of its disulfide bonds to reduction. In the absence of denaturing agents, reduction of BSA with 2-mercaptoethanol for 1 hr resulted in the reduction of one disulfide bond. In the case of CT-BSA, eleven disulfide bonds were reduced.

Unmasking of the amino groups yielded an albumin preparation in which 99-100% of the amino groups were free. The preparation was identical to native BSA in antigenic reactivity and had only one reducible disulfide bond.

Tryptic hydrolysis of CT-BSA at the arginine or at both the arginine and lysine peptide bonds

Tryptic hydrolysis of CT-BSA at the arginyl peptide bonds, followed by removal of the masking groups, yielded a preparation which was cleaved only at a few peptide bonds. Peptide mapping at this stage revealed that the bulk of the material moved as a streak near the origin (Fig. 1). Smaller peptides were also present and gave about 14-16 discrete spots. After performic acid oxidation of the tryptic hydrolysate, peptide mapping revealed that streaking had decreased considerably (Fig. 1) and new spots appeared as a result of the oxidation of the disulfides. However, the streaking did not disappear entirely, suggesting the presence of large mol. wt fragments. Disc electrophoresis of the Arg-peptides in SDS (Fig. 4) showed the presence of at least three large fragments.

Cleavage at the arginyl as well as the lysyl peptide bonds gave a large number of peptide fragments as revealed by peptide mapping (Fig. 2). Considerable streaking was also present. After performic-acid oxidation, peptide mapping revealed, as expected, the presence of a large number of new spots and the disappearance of others as a result of rupturing the disulfide bonds (Fig. 2). These maps showed no streaking indicating the absence of large mol. wt fragments.

Immunochemical behavior of the Arg-peptides and the (Arg,Lys)-peptides

The reactivity of the Arg-peptides and the (Arg,Lys)-peptides with antisera to BSA was examined over a wide range of concentrations. Neither the Arg-peptides nor the (Arg,Lys)-peptides gave an immune precipitate with BSA-antisera. However, the Arg-peptides inhibited to the extent of 83% the reaction of BSA with its antisera and completely (97%) its reaction with the IgG fraction of the antiserum. On the other hand, the (Arg,Lys)-peptides possessed no inhibitory activity, even when several hundred molar excess, relative to BSA, was employed. It was, therefore, decided to fractionate the Arg-peptides. Clearly, the (Arg,Lys)-peptides were not useful for immunochemical studies and accordingly no further work was conducted on these preparations.

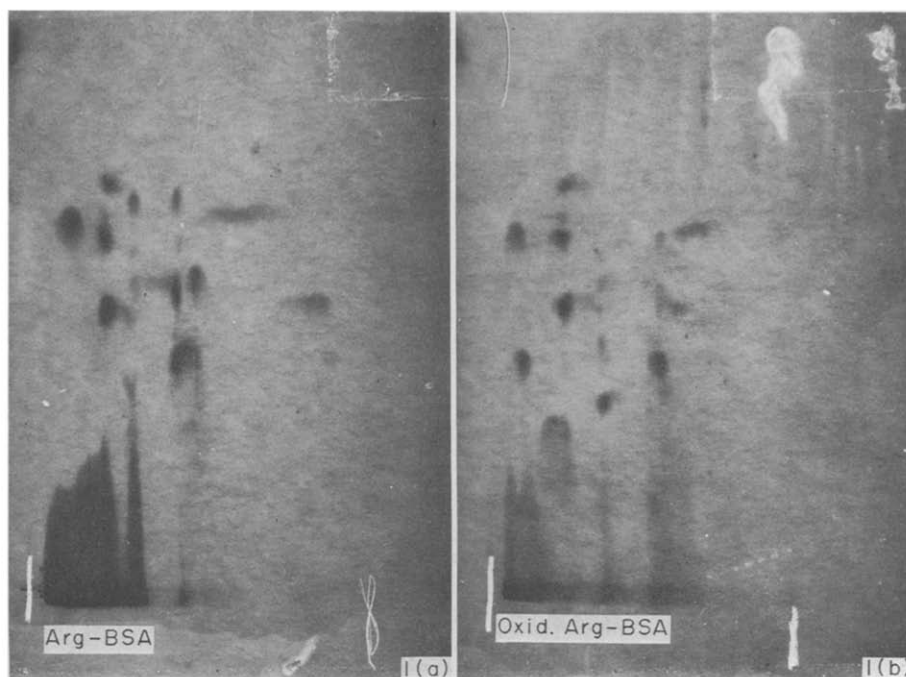


Fig. 1. Peptide maps of (a) Arg-peptides, and (b) performic-acid oxidized Arg-peptides.



Fig. 2. Peptide maps of (a) (Arg,Lys)-peptides, and (b) performic-acid oxidized (Arg,Lys)-peptides.

Fractionation of the Arg-peptides

Gel filtration of the Arg-peptides on Sephadex G100 gave three major components (Fig. 3). Tubes belonging to each fraction were combined, freeze-dried, redissolved and subjected to re-filtration on the same column. Each component was subjected to at least three re-filtration experiments until a single symmetrical peak was obtained. The last emerging fraction (in Fig. 3) comprised small peptide fragments as

revealed by peptide mapping. The first emerging peak (in Fig. 3) corresponded to a high mol. wt fragment. The mol. wt of this fragment was 35,000 in SDS-electrophoresis and 43,000 in calibrated Sephadex G100 columns. The major product was the component eluting in tubes 90–106 (Fig. 3). After several re-filtration experiments, this component was obtained in a pure form as shown by disc electrophoresis (Fig. 4). The mol. wt of this fragment was 23,000 by SDS-electro-

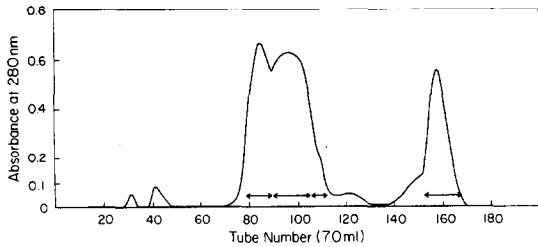


Fig. 3. Gel filtration elution pattern of the Arg-peptides on Sephadex G100. Tubes were pooled as indicated. For details see text.

phoresis and 25,000 by gel filtration on calibrated Sephadex columns. In view of its higher yield and its purity, this fragment was subsequently characterized and its immunochemistry studied. Finally a very small amount of a lower mol. wt (about 15,000 by SDS-electrophoresis) fragment was obtained (Fig. 3, tubes 107–117).

Characterization of the fragment S11–193

Amino acid composition of the fragment was carefully determined. The results summarized in Table 1 were derived from 12 replicate HCl hydrolyses (four each at 22, 48 and 72 hr). Also, four replicate HCl hydrolyses were carried out on performic-acid oxidized fragment for the determination of cystine (as cysteic acid) and an additional six replicate hydrolyses with *p*-toluenesulfonic acid (three each at 22 and 48 hr) for the determination of tryptophan. The exhaustive determination of the composition was carried out because when this work was being done, the primary structure of BSA was not known. However, recently Brown (1975) reported the complete amino acid

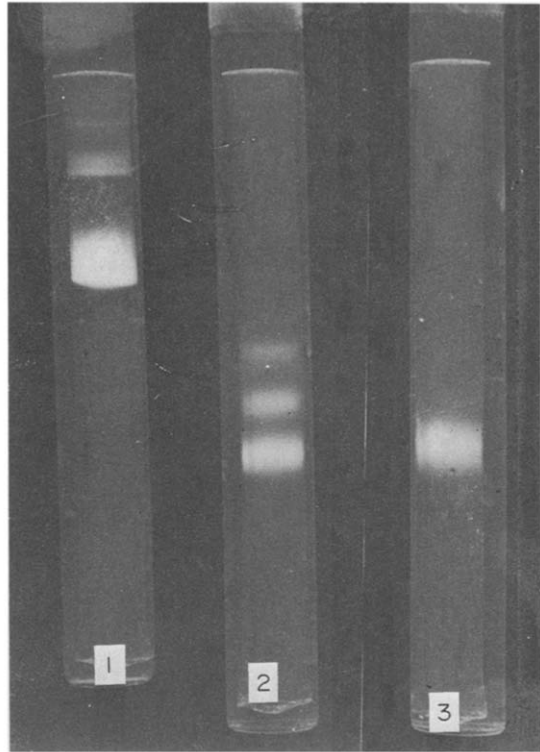


Fig. 4. Disc electrophoresis patterns in SDS-gels of (1) BSA, (2) Arg-peptides and (3) fragment S11–193.

sequence of BSA. Composition of the fragment (Table 1) showed it to correspond with excellent agreement only to sequence 11–193 of BSA. However, the values of tyrosine, phenylalanine and arginine were lower

Table 1. Amino acid composition of the fragment from arginine cleavage of BSA^a

Amino acid	Calcd. for 11–193 (less Arg 144)	Found
Asp	18	18.06
Thr	7	7.15
Ser	8	7.60
Glu	27	26.47
Pro	9	8.85
Gly	8	7.66
Ala	13	13.28
Cys($\frac{1}{2}$)	11	10.92
Val	7	6.68
Met	2	1.91
Ile	3	3.05
Leu	20	19.98
Tyr	8	6.28
Phe	11	9.88
Trp	1	0.99
Lys	18	18.20
His	6	5.86
Arg	5	4.97
Total No. Residues	182	
Mol. Wt.	20,947	
% N	16.11	

^aCompositions are expressed in mole amino acid per mole of peptide. Values were obtained from 22 analyses (see text). The calculated values were based on the sequence of BSA recently determined by Brown (1975). Cystine (1/2) was determined as cysteic acid and threonine and serine have been extrapolated to zero hydrolysis time. For details, see text.

FOUND

Cyclization No.:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sequence:	PHE	—	ASP	LEU	GLY	GLU	GLU	—	PHE	—	—	LEU	VAL	LEU
	—	PRO	TYR	PHE	TYR	ALA	PRO	—	LEU	—	—	ALA	—	—

EXPECTED

Sequence	11	24
11-24:	PHE-LYS-ASP-LEU-GLY-GLU-GLU-HIS-PHE-LYS-GLY-LEU-VAL-LEU	
Sequence	145	158
145-158:	HIS-PRO-TYR-PHE-TYR-ALA-PRO-GLU-LEU-LEU-TYR-ALA-ASN-LYS	

than expected for this sequence by two, one and one residue, respectively. To confirm the identity of the peptide, partial sequence determination of the N-terminal region was carried out. The results are summarized above. The fragment possessed two N-termini and the first 7-9 positions in sequence determination agreed very well with sequences 11-19, and 145-153 of BSA. Carboxypeptidase A had no effect on the peptide. Action of carboxypeptidase B released arginine in the hydrolysates. In view of the established purity of the fragment, its identity must therefore correspond to sequence 11-193 with points of scission between Arg 143-Arg 144-His 145. The disulfide bonds keep the nicked parts of the loop together (Fig. 5). The release of Arg 144 then makes the experimental value of arginine correct. The discrepancies in the phenylalanine and tyrosine values cannot be explained, especially that of tyrosine. The only possibility is that minor errors in the preliminary sequence of BSA may exist. The mol. wt of the fragment calculated from its composition was 20,947 which is in good agreement with the values derived in the foregoing section.

Immunochemistry of the fragment S11-193

For the purpose of quick screening, a mixture of three rabbit antisera to BSA was used. At this level of broad delineation antisera variability should not be expected (Perlstein & Atassi, 1974) and differences will appear only at the stage of fine delineation to ultimate boundaries (Koketsu & Atassi, 1973 and 1974a) and when the investigation approaches that stage antisera should not be mixed (Atassi, 1975).

Peptide S11-193 failed to give an immune precipitate with antisera to BSA when tested over a wide concentration range. However, peptide S11-193

effected substantial inhibition of the precipitin reaction of native BSA with its homologous antisera. The inhibitory activity of the peptide was 80% towards the reaction of BSA with its antiserum (Fig. 6) and 88% towards its reaction with the IgG fraction of the antiserum. In each case, the molar excess of peptide, relative to BSA, required to achieve half the maximum inhibition was 9. Performic acid oxidation of the peptide S11-193 or its reduction by mercaptoethanol followed by carboxymethylation gave preparations that had no (0%) inhibitory activity.

Removal of antibody by peptide immunoabsorbent

Table 2 shows the amount of specific anti-BSA antibodies bound to immunoabsorbents. Sepharose-BSA absorbed out quantitatively all the antibodies in the IgG fraction. However, only 75% was eluted with 0.17 M glycine pH 2.3 while the remainder was eluted with 3 M guanidine. With Sepharose-peptide, the first column bound about 56% and elution required 5 M guanidine. Residual antibody to fragment S11-193 was removed by subsequent passage of effluent fraction through a series of peptide-immunoabsorbent columns. Antibody removed by immunoabsorbent approached a plateau, after five columns, corresponding to a total of 84%. Total bound antibody that could be eluted from the five columns corresponded to 88.6% of antibodies applied in the IgG fraction.

Binding of antibodies to BSA with fluorescent peptide

The fluorescein conjugate was found to contain fluorescein/peptide ratio of 1 and that 1 mg of the fluorescein derivative had a fluorescence intensity of 57,000. Human IgG did not bind any fluorescein derivative but when the IgG fraction of anti-bovine serum

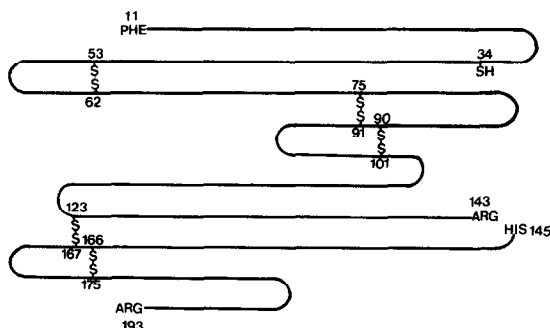


Fig. 5. A line diagram showing the position of the disulfide bonds in fragment S11-193 which enable the pieces to remain covalently attached after scission at Arg 144 and 145 and removal of the latter. For the complete primary structure of BSA see Brown (1975). The diagram shown is not intended to show the actual shape of the fragment.

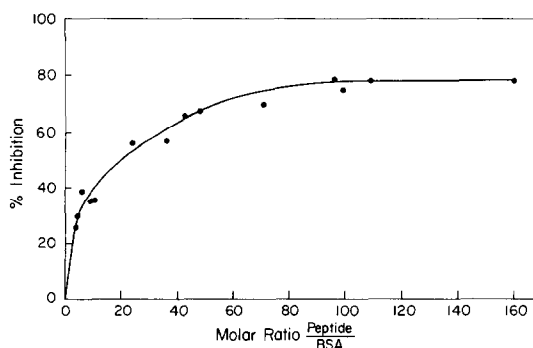


Fig. 6. Inhibition of the precipitin reaction of native BSA with its homologous antiserum by fragment S11-193. Note that inhibition with the IgG fraction of the antiserum was higher (88%). The IgG fraction accounted for 96% of the total antibody activity of the antiserum (see text).

Table 2. Absorption of anti-BSA antibodies by immunoabsorbent carrying BSA or fragment S11-193

Protein or peptide in immunoabsorbent	No. of column	Amounts of antibody applied and reacted			
		Amount Ab applied (mg)	Amount Ab unabsorbed (mg)	Amount Ab bound (mg)	Amount Ab eluted (mg)
BSA	1	1.1	0 ^a	1.1	0.83 ^b 0.36 ^c
S11-193 (per cent)	1	1.1	0.48 ^a (43.6)	0.62 (56.4)	0.53 ^d (48.2)
S11-193	1	1.05	0.48	0.57	0.59 ^d
	2	0.48	0.39	0.086	0.093 ^d
	3	0.39	0.32	0.067	0.121 ^d
	4	0.32	0.21	0.11	0.065 ^d
	5	0.21	0.16	0.05	0.064 ^d
Total recovery (mg)				0.88	0.93
Per cent recovered				83.9	88.6

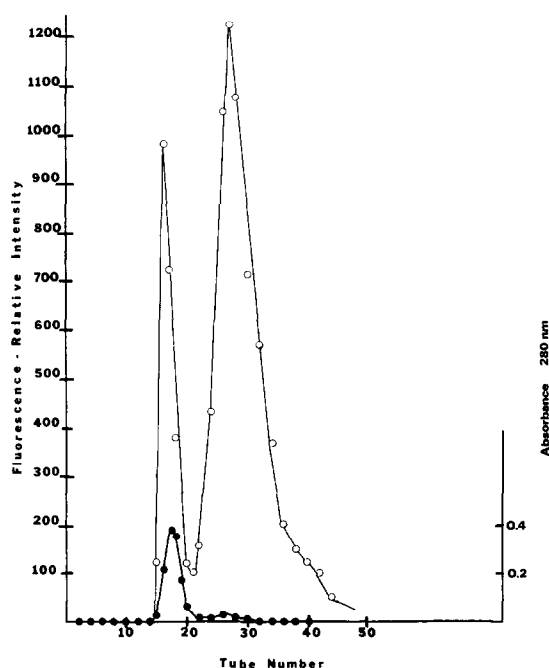
^aBy quantitative precipitin reaction^bEluted with 0.17 M glycine-HCl buffer pH 2.3^cEluted with 3 M guanidine pH 8.5^dEluted with 5 M guanidine pH 8.5

Fig. 7. Elution pattern of a mixture of IgG fraction of anti-BSA antibody and fluorescein derivative of fragment S11-193 on Sephadex G100. (●) absorbance at 280 nm; (○) fluorescence at 520 nm. The first peak corresponds to antibody fraction and the second corresponds to fluorescein-fragment S11-193. Eluent fractions were 3.3 ml per tube.

albumin was mixed with the fluorescein derivative of S11-193, fluorescence eluted with the antibody peak (Fig. 7). Table 3 shows the amount of fluorescein derivative bound to different amounts of anti BSA antibody together with antibody/peptide molar ratio. At various concentrations of peptide and antibody but in the region of peptide excess, it is shown that 2 moles of antibody combine with one mole of peptide.

DISCUSSION

A relatively large molar excess (about 100) of the peptide, relative to BSA, was required to achieve maximum inhibition (Fig. 6). In fact this represents quite an efficient peptide. The high binding efficiency can be seen from the low molar excess required to achieve 50% of the maximum inhibition. The molar excess required of even intact antigenic reactive regions to achieve half the maximum inhibition can be several times higher (e.g. see Koketsu & Atassi, 1973; 1974a, 1974b; Atassi & Pai, 1975). The large molar excess required of the present peptide to achieve full maximum inhibition is also comparatively low. Previously, we had shown that the large excess of an inhibitory peptide was necessary because of conformational factors (Atassi & Singhal, 1970a; 1970b; Singhal & Atassi, 1970; 1971). The immune response to native protein antigens is directed against their native three-dimensional structure (Atassi & Thomas, 1969). A fragment of a protein will undergo some un-

Table 3. Binding of fluorescein derivative of fragment S11-193 to anti-bovine serum albumin antibody^a

Anti-BSA antibody mg	μ mole	Fluorescein-peptide applied (mg)	Fluorescence in Ab peak	Peptide bound		Ab/peptide molar ratio
				mg	μ mole	
1.5	0.00938	1.0	6468	0.1135	0.00454	2.07
1.5	0.00938	1.2	6435	0.1135	0.00452	2.08
2.07	0.01294	0.19	8372	0.147	0.0059	2.19
2.07	0.01294	0.57	8913	0.156	0.00624	2.07
2.07	0.01294	0.76	9033	0.1585	0.00634	2.04
0.207	0.00129	0.19	810	0.0142	0.000568	2.27
0.207	0.00129	0.57	832	0.0146	0.000584	2.21
0.517	0.00323	0.57	2280	0.04	0.0016	2.01

^aBinding studies were carried out by gel filtration on Sephadex G100. For details see text.

folding, the degree of which will depend on its size and the ability of its various segments to form long-range interactions (Singhal & Atassi, 1970; Atassi & Singhal, 1970). For proper binding with antibody, an antigenic reactive site must achieve its native mode of folding (Atassi, 1967; Atassi & Saplin, 1968; Atassi, 1970; Habeeb & Atassi, 1971). The probability of finding such a mode of folding will improve with increase in concentration of the peptide (Atassi & Saplin, 1968). The high efficiency of the present peptide can be attributed to linkage of its various segments by disulfide bonds, thus preventing its complete unfolding. This is analogous to very efficient binding peptides obtained from lysozyme with intact disulfide bonds (Atassi *et al.*, 1973; Lee & Atassi, 1975).

Loss of inhibitory activity of the peptide upon rupture of the disulfides reflects the importance of the disulfide bonds in bringing the two parts of a reactive site in the conformational proximity required for binding with antibody. Also, loss of activity upon further cleavage of fragment S11-193 at the lysyl peptide bonds was most likely the result of scission of the antigenic reactive site on the peptide. It has been shown (Atassi, 1972) that on scission of an antigenic reactive region the resulting fragments will lose most or all of their binding ability. Any retention of binding ability by a portion of a reactive region will depend on the location of cleavage, the size of the fragment, and on the extra non-reactive residues attached to the segment which will be actively engaged in binding with antibody (Atassi, 1972). Fragmentation will also reduce the ability of the resultant parts to achieve a favorable mode of folding for appropriate fit onto the antibody combining site (Atassi & Saplin, 1968).

Pure fragments from human serum albumin, which were isolated from prolonged chymotryptic hydrolysates, gave partial immune precipitation and also, inhibited only partially the immune precipitation of human serum albumin (Porter, 1957; Press & Porter, 1962). Furthermore, the recovery was extremely low (between 0.038%–0.3% of the starting material). A fragment from human serum albumin was isolated (Webb & Lapresle, 1964; Lapresle & Webb, 1964; 1965) from its cathepsin digests, which had a mol. wt of 11,000 and possessed only partial (15–40%) inhibitory activity towards the homologous reaction. The yields of fragment S11-193 reported here were at least 100 times higher than those obtained by the aforementioned workers. Also, its high inhibitory activity was quite remarkable, virtually accounting for almost all (88%) of the antigenic reactivity of the native protein.

It is significant that the fragment did not give an immune precipitate with antisera to the native protein. Our results, which show two antibody molecules are bound per peptide, do not resolve whether the peptide has two antigenic sites or four antigenic sites. With the present antisera, six moles of antibody were bound per mole of BSA in the precipitin reaction, by extrapolation to zero antigen concentration. Accordingly, the existence of two antigenic sites in S11-193, which is approximately a third of the BSA molecule, is more likely. Furthermore, it is perhaps very difficult, for steric reasons, for a single antibody molecule to bind with two sites on the same peptide

antigen. Therefore, if the binding sites of the antibody are filled by two different peptide molecules, then the presence of four antigenic sites on the peptide would tend to favor more the formation of an immune precipitate, which is not observed.

The high inhibitory activity of fragment S11-193 was in good agreement with the results of immunoabsorbent studies where 84–89% of anti-BSA antibodies were removed by the immunoabsorbent. The immunoabsorbent results, unlike inhibition studies will not be obscured by the presence of soluble antigen-antibody complexes. It is relevant to point out that a single immunoabsorbent experiment was not capable of removing all the antibody directed against the fragment. A plateau of antibody removal was achieved in five immunoabsorbent experiments. This was most likely caused by the decreased efficiency of the peptide molecules as a result of modification by the covalent cross-linking onto the Sepharose.

The fact that one fragment, comprising less than a third of the albumin molecule can account for almost the entire antigenic reactivity was unexpected and is highly significant. We can rationalize it best by suggesting that BSA carries repeating identical antigenic reactive sites. This is now under investigation in our laboratories and the results will be reported later in detail.

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