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## RELATIONSHIP BETWEEN THE CONFORMATION OF BOVINE SERUM ALBUMIN AND ITS DIGESTION BY PEPSIN

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### SUMMARY

1. The digestion of bovine serum albumin by pepsin in the pH region of the Normal-Fast transition was studied by means of electrophoresis on polyacrylamide gels. The results suggest a close relationship between the conformation of the albumin molecule and its digestion by pepsin.

2. At pH values just above 3.6 ten well-defined fragments were found. These features might support a model of albumin in which there are only a few, possibly three sites, available for hydrolysis by pepsin.

3. At pH values below 3.6 the more complex electrophoretic patterns suggest the existence of more sites, probably as a consequence of unfolding of the albumin molecule.

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### INTRODUCTION

The behaviour of albumin at low pH has been the subject of many investigations<sup>1-6</sup>. A model which accounts for nearly all conformational transitions observed around pH 4 has been proposed by FOSTER<sup>7</sup>. This model is an extension of the model of HARRINGTON *et al.*<sup>3</sup> which was designed to account for the Normal-Fast transition, *i.e.* the transition from the normal electrophoretic mobility to a faster one upon lowering the pH. In this model it is suggested that the molecule consists of four compact units linked by short randomly coiled polypeptide chains. Optical rotatory<sup>6</sup> and viscosity<sup>2</sup> data reveal that three stages can be distinguished in the behaviour of albumin at low pH. In the pH range 4.5 to 3.9 at least two cooperative steps are observed<sup>5</sup>. Between pH 3.9 and 3.6 a plateau, *i.e.* very little change in various physical parameters, is found. Below pH 3.6 a further change in optical rotation and viscosity appeared to occur<sup>1,2,5,6</sup>. According to the model proposed by FOSTER<sup>7</sup> these observations can be interpreted as an increase of the distance between the units from pH 4.5 to 3.9, a more or less stable situation between pH 3.9 and 3.6 and a partial unfolding of the units below pH 3.6. WEBER AND YOUNG<sup>8</sup> pointed out that at a pH where there is some distance between the units, a protease should preferentially act

on the linking polypeptide chains, thus liberating a small number of fragments. They digested albumin with pepsin at pH 3.0 and isolated two larger fragments. Later the isolation of several fragments obtained after short peptic digestion at pH 2.45 (see ref. 9) and pH 3.0 (see refs. 10, 11), was reported. The amino acid sequence of an amino terminal peptide fragment has recently been determined<sup>10,12</sup>. It is, however, clear that upon lowering the pH an increasing number of sites becomes available for hydrolysis by a protease and therefore more fragments can be expected since a partial unfolding of the compact units occurs below pH 3.6. For this reason the influence of the pH on the proteolytic hydrolysis of albumin should be carefully considered. We have therefore made a detailed study of the digestion of albumin by pepsin between pH 2.5 and 4.5.

#### MATERIALS AND METHODS

##### *Bovine serum albumin*

Bovine serum albumin (Nutritional Biochemicals Corp., 4 × crystallised, lot No. 4710) was deionized over a mixed bed ion exchange column (Amberlite IRA 400 and IR 120) giving an isoionic solution with a pH of about 5.2.

The SH group, present in about 60% of the bovine serum albumin molecules was blocked with iodoacetamide (6 moles/mole bovine serum albumin) at pH 7.4. After standing for one hour at room temperature the excess of reagent was removed by dialysis at 5°. Titration with *p*-chloromercuribenzoate at pH 4.6 revealed that no reactive SH groups were present after this treatment.

Dimer-free albumin was obtained by gel filtration on Sephadex G-150 in 0.1 M NaCl at 5°. After dialysis the dimer-free albumin was concentrated by ultra filtration in a Diaflo Model 50 using a UM 10 filter.

The protein concentration was determined spectrophotometrically, using  $E_{1\text{cm}}^{1\%} = 6.67$  at 279 nm.

##### *Peptic digestion*

Pepsin (porcine stomach mucosa, 3 × crystallised, lot No. 800 537) was obtained from Calbiochem (Switzerland). Only freshly prepared pepsin solutions were used. They were diluted to an absorbance of 0.5 at 279 nm in a 1-cm cuvette and stored (for a short time) at 5°.

The digestion was carried out at  $25^\circ \pm 0.1^\circ$  using 10 ml of a 5% solution of albumin and 0.5 ml of the pepsin solution (pepsin/albumin = 1:3000, w/w). The pH was brought to the desired value (between 2.5 and 4.5) and kept constant during digestion by carefully adding 0.2 M HCl. At regular time intervals 0.5-ml portions were taken from the mixture and, after addition of 0.1 ml 1 M  $\text{K}_2\text{HPO}_4$  placed in ice to stop the hydrolysis. The samples were stored at  $-20^\circ$ . No difference in electrophoretic pattern was found for samples taken before and after freezing.

##### *Polyacrylamide gel electrophoresis*

Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Fluka (Switzerland). Gel dimensions were 5 mm × 60 mm. The protein samples, eight at a time, were diluted with a saccharose solution to a protein concentration of 1%. With a Hamilton microsyringe about 5  $\mu\text{l}$  were carefully layered onto the gel. The gels were

stained in a solution containing 6 g amidoblack and 70 ml acetic acid per l, and then washed in 7% (v/v) acetic acid. Densitometer scans were made with a Kipp lin/log densitometer Model DD2 (Delft, Holland). In most cases electrophoresis was performed at pH 7.3 (see ref. 13) or at pH 3.0 in the presence of 6 M urea. At pH 3.0 an electrophoretic system as described by SCHOENMAKERS *et al.*<sup>14</sup> was used. In both cases the gel concentration was 7.5%. No sample gels were used.

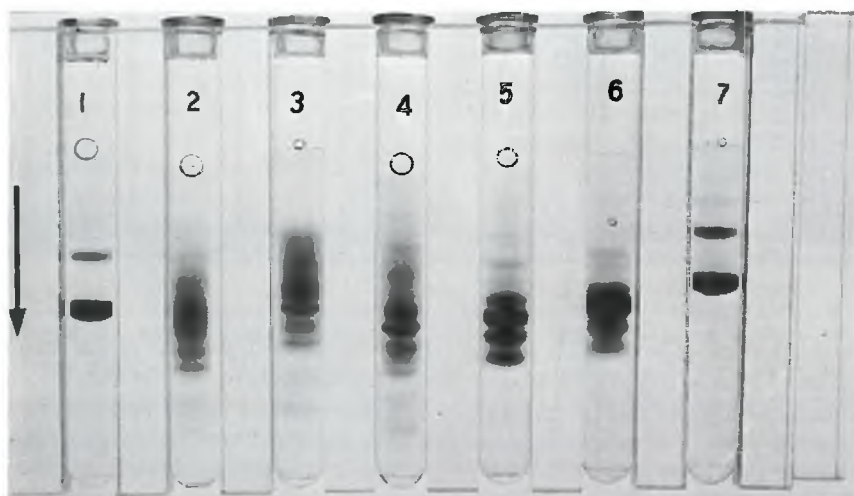


Fig. 1. Electrophoresis at pH 7.3. The arrow indicates the direction of migration. The time of incubation with pepsin was 16 min. 1, albumin, not incubated; 2, pH 2.5; 3, pH 3.0; 4, pH 3.3; 5, pH 3.6; 6, pH 3.9; 7, pH 4.5.

## RESULTS AND DISCUSSION

Fig. 1 shows the electrophoretic patterns of hydrolysates of albumin obtained after peptic digestion at pH values varying from 2.5 to 4.5. The electrophoresis was performed at pH 7.3. Below pH 3.6 rather complex patterns were obtained, but at pH 3.6 and pH 3.9 the pattern is simpler and consists of about five components. At pH 4.5 no hydrolysis occurs, not even after digestion times up to 105 min (not shown). The slowest migrating component in Gels 1 and 7 is the dimer which is always present in commercial preparations of albumin. For all other experiments dimer-free albumin was therefore used. Since the enzymatic activity of pepsin is pH dependent, a comparison of digests obtained after the same time of hydrolysis but at different pH values becomes rather difficult. The time at which the same number of peptide bonds has been broken would be more suitable for this purpose. Experimentally, however, this is difficult to realize. For this reason, during each incubation samples were taken at time intervals varying from 1 to 105 min after the start of the hydrolysis. By doing this at various pH values information about the peptic digestion could be obtained. Representative results are shown in Figs. 1 and 2. In Fig. 2 densitometer scans of some of the gels are given. For each incubation the situation is given after 6 and 16 min digestion. The patterns at pH values below 3.6 are complex

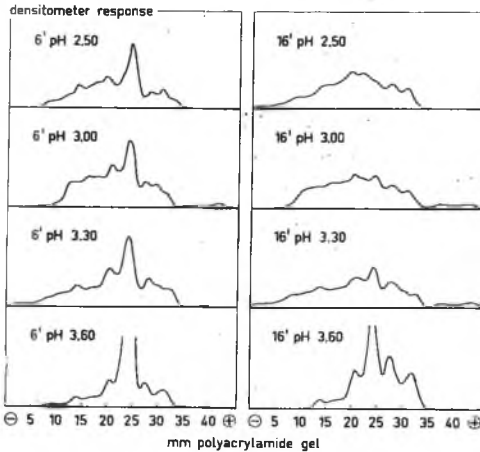


Fig. 2. Densitometer scans of the polyacrylamide gels. Electrophoresis was performed at pH 7.3. At each pH the results (obtained with dimer-free albumin) are given after incubation for 6 min and 16 min.

even after a short time of hydrolysis (6 min). The well-defined pattern in the case of incubation at pH 3.6 is still found after prolonged hydrolysis up to 105 min.

These findings support the proposal that, as a consequence of the unfolding of the molecule below pH 3.6, more sites become available for hydrolysis by the protease than between pH 3.6 and 3.9.

The number of components observed gives some information about the number of sites of hydrolysis. When for example there are two or three sites available for pepsin, six or ten fragments, respectively, can be expected. When electrophoresis is performed at pH 8.9, at pH 7.3 in the presence of sodium dodecyl sulphate or at pH 3.0, no fundamental difference in the patterns is observed, except that there appear to be six components instead of the five found at pH 7.3 in the absence of sodium

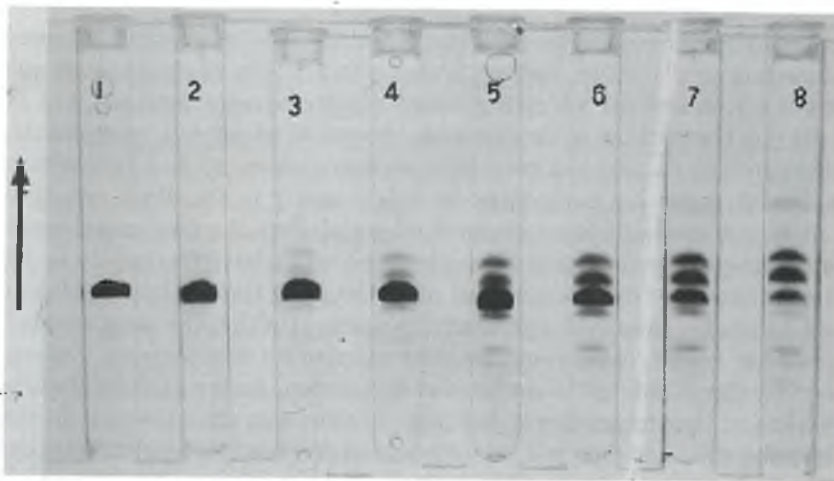


Fig. 3. Electrophoresis at pH 7.3. The arrow indicates the direction of migration. Incubation of dimer-free albumin with pepsin at pH 3.7. 1, 0 min; 2, 2 min; 3, 4 min; 4, 6 min; 5, 10 min; 6, 20 min; 7, 40 min; 8, 105 min.



Fig. 4. Electrophoresis at pH 3.0 in 6 M urea. The arrow indicates the direction of migration. Incubation of dimer-free albumin with pepsin at pH 3.7. The same samples are used as in Fig. 3. 1, 0 min; 2, 2 min; 3, 4 min; 4, 6 min; 5, 10 min; 6, 20 min; 7, 40 min; 8, 105 min.

dodecyl sulphate. However, at pH 3.0 (fragments highly charged) in 6 M urea (to prevent hydrogen and hydrophobic bonding between the fragments) ten components were found. This picture remained unchanged at urea concentrations varying from 2 to 7 M. In Figs. 3 and 4 are shown the results obtained with the same samples using two different electrophoretic systems. The existence of only five or six bands in the absence of urea is difficult to explain. Presumably aggregation of the peptide fragments is possible for only a few combinations.

Supposing that the hydrolysis of the "elementary" fragments of albumin is much slower than the hydrolysis at the linkages between these fragments, only four fragments will continuously increase in quantity after prolonged digestion, assuming that there are three linkages which can be hydrolysed by pepsin. Densitometer studies show the tendency of Fragments 4, 5, 9 and 10 to survive (Fig. 4). While other components first accumulate and then diminish, only these fragments show an increase up to an incubation time of 105 min. These observations support the model proposed by FOSTER<sup>7</sup>.

It is remarkable that pepsin, to which a broad specificity is normally ascribed can, under special circumstances, acquire a high specificity which seems to arise from the conformation of the substrate itself. In this respect it should be mentioned that the use of pepsin at pH values removed from its pH optimum for controlled hydrolysis of proteins has already been suggested by SCHLAMOWITZ and co-workers<sup>15,16</sup> who also found high-molecular-weight intermediates after peptic digestion of albumin at pH 3.5.

From the experiments described, it is clear that the peptic digestion of albumin follows the Normal-Fast transition and although the enzyme is active up to pH 5.5 (see refs. 16-18), the hydrolysis of the Normal form of albumin is so slow that under our experimental conditions no hydrolysis is observed at pH values above 4.4.

The isolation and investigation of the ten fragments obtained after peptic

hydrolysis is now in progress. A paper concerning the isolation and some properties of Fragments 8, 9 and 10 is in preparation.

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