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C-Terminal Sequence of Crystalline Bovine and Human Serum Albumins: Relationship of C-Terminus to Antigenic Determinants of Bovine Serum Albumin

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A study of the rates of release by carboxypeptidase of amino acids from crystalline bovine serum albumin and crystalline human serum albumin revealed that the two proteins have different C-terminal sequences. Within the limits of error of the analytical methods used, the immunochemical studies with carboxypeptidase-treated bovine serum albumin and specific antibody to native bovine serum albumin did not indicate that the structural changes at the C-terminus affected the reaction between antigen and antibody in the quantitative precipitin test.

Introduction

Similar proteins of different species, *i.e.*, serum albumins, differ from each other by the number and probably by the sequence of amino acid residues in their peptide chain.[1-3] For this reason, it has been conjectured that the antigenic determinants may be composed of the side chains of amino acids arranged in a specific pattern.[4] Little information is available on the total number (valence), chemical composition, size or area and relative positions of the determinants or combining sites of native protein antigens.[4-6] The immunochemical reaction between antigen and homologous antibody has shown that the antigen is multivalent.[7] The antigenic determinants of the albumin molecule may reside either at the ends or the longitudinal faces of the protein or both.[4] It is important to differentiate between the ends of the amino acid chains, commonly referred to as N- and C-termini, and the geometric or spacial ends as defined by Pauling and Corey and others in the helical structures.[8-10] The amino acid chain ends may or may not be at the geometric end. These ends again may be different from the antibody-combining sites or ends as defined by Pauling.[11] An example of the possibility that one of the major antigenic determinants could reside in the amino acid chain ends seems to occur in the ovalbumin–plakalbumin transformation.[12] The removal of six amino acid residues from ovalbumin by enzymatic means resulted in a definite change in the immunochemical reaction between the resulting protein, plakalbumin, and a specific antibody to native ovalbumin.[13,14] Since native ovalbumin shows no amino acid ends,[15-17] the removal of the six amino acid residues could have resulted in a major transformation in the structure of the protein molecule. Since attempts to identify the configuration of the antigenic determinants by reagents that affect specific groups occurring in the amino acid side chains have not been too successful,[4,6] an enzymatic approach to the problem was investigated. The removal of C-terminal amino acids from native proteins by carboxypeptidase has recently been extensively investigated.[18-21] The present report describes the use of this enzymatic method to study the relationship between the C-terminal residues in bovine and human serum albumins. Crystalline bovine and human serum albumins were degraded at the carboxyl end by means of carboxypeptidase. The rate of release of amino acids indicated marked differences in the C-terminal sequences of bovine and human serum albumin. A study of the effect of removal of C-terminal residues in bovine and human serum albumin on the quantitative precipitin reaction was then carried out. No change in the quantitative immunochemical reaction was observed after treatment of bovine serum albumin with carboxypeptidase.

Experimental

Enzymatic Treatment of Proteins.—The carboxypeptidase was a six times-crystallized preparation.[22] It was tested for chymotrypsin contamination by treating it with lysozyme for 24 hours. The carboxypeptidase was used in an amount equal to 10% of the weight of the lysozyme. In accordance with the findings of Thompson[23] only leucine was liberated under these conditions whereas the slightest contamination by chymotrypsin caused the release of aromatic amino acids in large quantities. Thus, it appeared that the carboxypeptidase was free from significant contamination by chymotrypsin and, to prevent confusion of the immunochemical study, diisopropylfluorophosphate[24] was not used in the main experiments. However, small batches of both albumins were run with DFP as controls. In no case was any difference noted in the results. Further proof of the freedom of the enzyme from chymotrypsin is the absence of aromatic amino acids in the list of those released from either bovine or human serum albumin.

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The enzymatic reactions were done as follows. The protein[25] was dissolved in *p*H 7.5 0.10 *M* ammonium acetate buffer[26] at a concentration of 20 mg./ml. and then the solution was re-adjusted to *p*H 7.5. A suspension of the enzyme and a small amount of toluene were added and the mixture was agitated gently at room temperature (24°). Aliquots were removed at suitable intervals and were de-proteinized by the addition of 3 volumes of anhydrous ethanol. The supernatants were taken to dryness *in vacuo* and the residues were assayed for amino acids by quantitative paper chromatographic method.[27] At the end of the digestion period, the reaction mixture was taken to *p*H 6.0 and centrifuged to remove carboxypeptidase. The clear supernatant was then dialyzed against cold running tap water (4°) for 18 hours. After re-clarification, the solution was lyophilized.

It was realized that the step of de-proteinizing samples by the addition of 3 volumes of alcohol was subject to the criticism that some of the liberated amino acids might have been adsorbed on the precipitated protein. To test this possibility, one of the 24 hour digests was dialyzed batchwise against limited volumes of distilled water. The dialysates were combined and concentrated to dryness. The resulting solid was assayed for amino acids both before and after complete acid hydrolysis. No difference was noted in the identity of the principal amino acids or in the quantitative ratios between them (Table I).

TABLE I

STEPWISE CARBOXYPEPTIDASE TREATMENT OF BOVINE SERUM ALBUMIN

(Values expressed as mcg. α-amino nitrogen/mg. bovine albumin.)

Amino acid	1st. Step (1% enzyme) EtOH super.	1st. Step (1% enzyme) Hydrolyzed dialysate	2nd Step (2% enzyme)	3rd Step (4% enzyme)	Total α-amino N	Total residues
Alanine	0.23	0.23	0.09	0.07	0.39	2.0
Leucine	.08	.07	.07	.07	.22	1.0
Threonine	.06	.07	.08	.06	.20	1.0
Valine	Trace	Trace	.07	.09	.16	0.8
Serine	...	...	.04	.06	.10	0.5

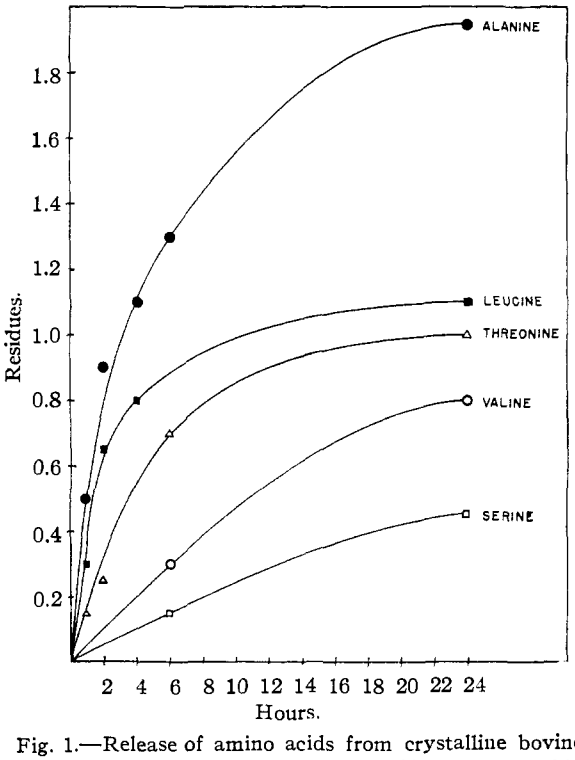

Fig. 1.—Release of amino acids from crystalline bovine serum albumin by carboxypeptidase–2.5% enzyme at 24° with gentle agitation.

Quantitative Precipitin Test.[28,29]—The reaction was carried out in 25-ml. conical Pyrex centrifuge tubes. To varying amounts of antigen, first diluted to 1.0 ml. with 0.15 *M* NaCl, was added successively 1.0 ml. of a specific rabbit anti-bovine albumin serum and 1.0 ml. of a *p*H 7.4, 0.067 *M* phosphate buffer. The tubes were immediately shaken and then incubated in a water-bath at 37° for two hours. After standing for three days in a 1° water-bath, the antigen–antibody precipitates were removed by centrifugation for one hour at 3000 r.p.m. at 1° in an International Refrigerated Centrifuge. They were washed twice by centrifugation with ice-cold 0.15 *M* NaCl. The nitrogen in the precipitates was determined by a biuret method.[30] All reactions were carried out in the zone of the antibody excess.

Results and Discussion

The amino acids liberated in the course of the reaction of carboxypeptidase on bovine serum albumin are plotted in Fig. 1. At the end of 24 hours, two residues of alanine, one of leucine and one of threonine are split off from all of the albumin molecules and an additional valine residue and serine residue are removed from a large fraction of them.

Figure 2 shows the corresponding results obtained with human serum albumin. Here, only one residue, leucine, is split off from all the protein molecules although an alanine, a valine, and a glycine residue are removed from a good proportion of them.

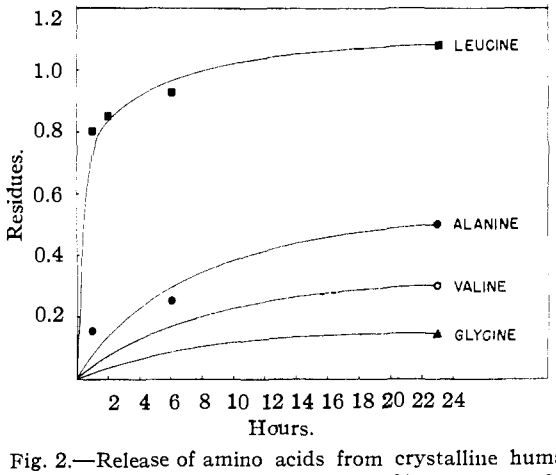

Fig. 2.—Release of amino acids from crystalline human serum albumin by carboxypeptidase–2.5% enzyme at 24° with gentle agitation.

From the data it is obvious that the C-terminal arrangements of the two albumin molecules are different, although the results are not sufficient to fix the exact sequences. This is especially true in the case of the bovine albumin molecule where the

(25) Crystalline human serum albumin, #179, obtained from Dr. W. L. Hughes, Jr., Harvard Medical School. Crystalline bovine serum albumin, Armour #128176.

(26) Ammonium acetate was chosen as a buffer because of its volatility. Salts interfere with the paper chromatography of the amino acids. Despite the low capacity of the ammonium acetate, no change was noted in the *p*H during the enzymatic reactions.

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double alanine residue introduces several possibilities even on the assumption of a single peptide chain. On the other hand, if a single chain be assumed, the data for human albumin seem to fit the C-terminal sequence: . . . Gly.Val.Ala.Leu.

It was clear, therefore, that human and bovine serum albumin possess different C-terminal amino acid sequences. The more easily degraded of the two proteins, bovine serum albumin, was tested in the quantitative precipitin reaction.

In order to ensure maximum degradation of the bovine serum albumin, the carboxypeptidase reaction was done in three steps, using progressively more enzyme at each step and removing the accumulated amino acids by dialysis at each step. Table I summarizes the results. After each step, the amino acid values were determined on an aliquot by the technique described above. In addition, the amino acids were run on the first dialysate after complete acid hydrolysis. This served not only to check the extent of the removal of the amino acids, but also eliminated the possibility that impurities in the enzyme had split off larger fragments. As shown in the table, no evidence was found for such fragments.

The total fragmentation (last column of Table I) was not significantly different from that obtained in the single-step experiment of Fig. 1. This is true despite the fact that the cumulative amount of enzyme was almost three times as much in the three-step experiment as in the single step one. Thus, the accumulated amino acids did not appear to influence the extent of the enzymatic reaction.

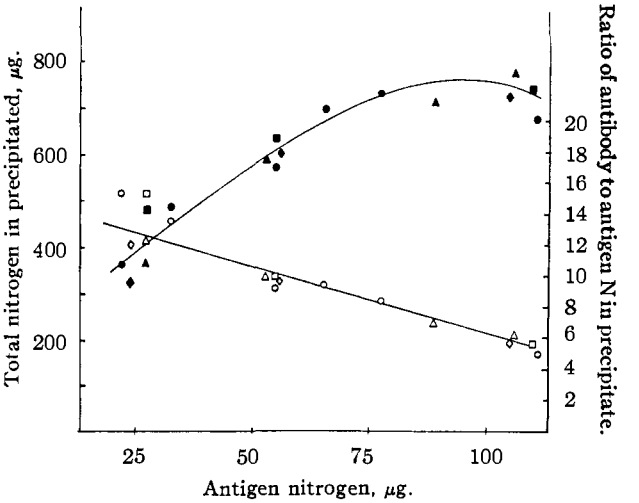

Fig. 3.—Quantitative precipitin reaction, addition of increasing of antigen to 1.0 ml. of rabbit anti-bovine albumin serum (plot of data in Table II): O, bovine serum albumin; △, bovine serum albumin–carboxypeptidase treated (step 1); □, bovine serum albumin–carboxypeptidase treated (step 2); ◇, bovine serum albumin–carboxypeptidase treated (step 3). The closed symbols represent plot of antigen nitrogen against total nitrogen precipitated and the open symbols represent plot of antigen nitrogen against ratio of antibody nitrogen to antigen nitrogen in precipitate.

TABLE II

QUANTITATIVE PRECIPITIN REACTION: ADDITION OF INCREASING AMOUNTS OF ANTIGEN TO 1.0 ML. OF RABBIT ANTI-BOVINE ALBUMIN SERUM

Preparation	Antigen nitrogen, μg.	Total nitrogen precipitated, μg.	Antibody nitrogen by difference, μg.	Ratio of antibody nitrogen to antigen nitrogen in precipitate
1. Bovine serum albumin	22	360	338	15.4
	33	480	447	13.6
	55	565	510	9.3
	66	690	624	9.5
	77	724	647	8.4
	111	670	560	5.0
2. Bovine serum albumin–carboxypeptidase-treated (step 1)	27	360	333	12.3
	53	585	532	10.0
	89	710	621	7.0
	106	770	664	6.3
3. Bovine serum albumin–carboxypeptidase-treated (step 2)	27	425	398	15.3
	55	630	575	10.0
	110	735	625	5.7
4. Bovine serum albumin–carboxypeptidase-treated (step 3)	24	323	299	12.1
	56	605	549	9.8
	105	720	615	5.9

The carboxypeptidase-treated bovine serum albumin, at each step of the degradation, was studied in the quantitative precipitin test in the region of antibody excess. As indicated in Table II and Fig. 3, the treatment of bovine serum albumin with carboxypeptidase did not change the quantitative immunochemical reaction. The ratios between antibody and antigen in the specific precipitates were also the same. Thus, the removal of 5 to 6 residues from the C-terminus of bovine serum albumin does not appear to have affected the quantitative precipitin reaction. Either the method may not be sensitive enough to detect a significant change in the antigenic determinants or the C-terminus does not contain any antigenic determinants. The electrophoretic mobility of three times carboxypeptidase-treated bovine serum albumin was the same as the native albumin at *p*H 8.6 in veronal buffer, ionic strength of 0.1. Equal mixtures of native and three times-carboxypeptidase-treated bovine serum albumin moved as a single component.

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