

On the Structure of α -Lactalbumin

I. Degradation Studies with Carboxypeptidase A and Carboxypeptidase B

α -Lactalbumin is a single chain molecule with an N-terminal glutamic acid and a C-terminal leucine.

The sequence of six amino acids at the C-terminal end of the molecule is:

Ileu. Val. Tyr. Thr. Lys. Leu. COOH.

α -Lactalbumin deprived of its C-terminal leucine could be crystallized. Tryptic digestion of α -lactalbumin resulted, in addition to the peptide fragments, in the liberation of free lysine and leucine in stoichiometric amounts. Cleavage of the disulfide bridges of α -lactalbumin did not change the molecular weight of the protein.

INTRODUCTION

In 1939 Sørensen and Sørensen (1) prepared from the albumin fraction of whey crystalline protein which they designated "crystalline insoluble substance." Subsequent works of Gordon *et al.* (2, 3) have further characterized this protein and named it α -lactalbumin. The present paper reports our progress in the investigation of the primary structure of this protein.

EXPERIMENTAL

MATERIALS

Throughout the experiments three-times crystallized α -lactalbumin was employed. For the preparation of this protein the method of Gordon and Ziegler (4), as well as that of Aschaffenburg and Drewry (5), was used with the same results. For the cleavage of disulfide bonds of α -lactalbumin, the protein was converted to its S-sulfo derivative by the procedure described recently by us (6) which was based on Swan's observation (7).

Crystalline trypsin with low chymotrypsin contamination (0.3%) was obtained through the courtesy of Dr. M. A. Mitz, Armour and Company, Chicago, Illinois.

Crystalline carboxypeptidase A² was from a commercial source; it contained no carboxypeptidase B², and it was treated with diisopropyl fluorophosphate (DFP) prior to use. Carboxypeptidase B treated with DFP was prepared as described by us previously (8). It contained traces of carboxypeptidase A and was completely free of trypsin and chymotrypsin.

METHODS AND RESULTS

N-TERMINAL AMINO ACID ANALYSIS OF α -LACTALBUMIN WITH DINITROFLUOROBENZENE (DNFB)

For the conversion of α -lactalbumin into its dinitrophenyl derivative, a modified procedure of Sanger (9) has been used, which permitted the reaction to proceed without precipitation of the protein (10). Two grams of α -lactalbumin and 2 g. NaHCO₃ were dissolved in 24 ml. water (the pH of the reaction mixture was adjusted to 7.0); and after slow addition of 16 ml. isopropyl alcohol, 40 ml. of a 40% solution of isopropyl alcohol containing 1 g. DNFB was added. The clear solution was left at room temperature for 2

² Carboxypeptidase A is equivalent to carboxypeptidase [Anson, M. L., *J. Gen. Physiol.* **20**, 663 (1937)]. Carboxypeptidase B is equivalent to proaminase [see Refs. (8, 17, 20-25) of this paper].

¹ Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

hr., extracted four times with ether, and the protein solution dialyzed against distilled water at 4°. The dialyzed protein solution was then precipitated at pH 4.0, dialyzed again, and finally freeze-dried. Fifty-milligram portions of DNP- α -lactalbumin were hydrolyzed at 105° with 6 N HCl in sealed tubes for 16 and 24 hr. Following the procedure of Levy (11), only DNP-glutamic acid could be demonstrated in the ether-soluble fraction, while the water-soluble fraction contained only ϵ -DNP-lysine. These findings are in complete agreement with the observations of Yasunobu and Wilcox (12). To calculate the yield of DNP-glutamic acid formed during the acid hydrolysis of the DNP-protein, the extent of destruction of the DNP-glutamic acid, due to this procedure, had to be determined. With pure DNP-glutamic acid subjected to acid hydrolysis, the loss incurred was 30%, while the hydrolysis of this derivative in the presence of the DNP-protein resulted in a 55–65% destruction. Calculation based on the latter value gave an average figure of 0.85 molecule of N-terminal glutamic acid per molecule of α -lactalbumin (of molecular weight 15,500).

The identity of DNP-glutamic acid isolated by paper chromatography (11) were verified by regeneration to the original amino acid by the procedure of Mills (13) by hydrolyzing it with saturated Ba(OH)₂ for 1 hr. at 100°. The barium was precipitated with CO₂, and the supernatant was acidified, extracted with ether and, after concentration, subjected to paper chroma-

tography in descending fashion using butanol-acetic acid-water solvent system (4:1:5). Only glutamic acid could be identified.

DETERMINATION OF THE C-TERMINAL AMINO ACID OF α -LACTALBUMIN

α -Lactalbumin was dissolved in water adjusting the pH to 8.0 and was hydrolyzed with carboxypeptidase A in a ratio of 20 at 37°. Four-milliliter aliquots containing mg. protein (4 μ moles) were withdrawn at various time intervals and were precipitated with 1 ml. of 30% trichloroacetic acid (TCA). After centrifugation, aliquots of the supernatants corresponding to μ moles of the original protein were analyzed for the liberated free amino acids using improved chromatographic procedure of Moore, Spackman, and Stein (14).

Aliquots were also withdrawn to determine the number of peptide bonds cleaved by carboxypeptidase A using ninhydrin procedure (15).

The results obtained and presented in Table 1 demonstrate that the action of carboxypeptidase A on α -lactalbumin is confined solely to the splitting of C-terminal leucine in agreement with the previous observations of Davie (16) and with the findings of Yasunobu and Wilcox (12) who obtained the same result by hydrazinolysis. The observed cleavage of one peptide bond accord with the liberation of 1 molecule of leucine.

CRYSTALLIZATION OF α -LACTALBUMIN DEPRIVED OF THE C-TERMINAL LEUCINE

Because of the ease with which α -lactalbumin was deprived of its C-terminal leucine by the action of carboxypeptidase A it was of interest to find out whether residual protein still retained its ability to crystallize. For this purpose, 2 g. of crystallized α -lactalbumin was dissolved in water by adjusting the pH to 7.5 with 1 N NaOH and made to volume of 100 ml. After addition of 10 mg. carboxypeptidase A it was incubated for 4 hr. at 37°. A sample was taken to determine the extent of hydrolysis by the ninhydrin procedure (15) showing cleavage of only one peptide bond. A

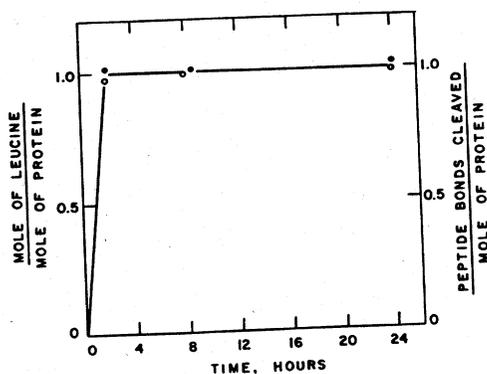


FIG. 1. Action of carboxypeptidase A on α -lactalbumin. ○—○ leucine; ●—● peptide bonds.

incubation period, the digest was subjected to the crystallization procedure of Gordon and Ziegler (4), which resulted in an oily emulsion with some crystalline material. Upon standing at room temperature for several weeks, this was transformed into well formed, needle-like crystalline protein.

HYDROLYSIS OF S-SULFO- α -LACTALBUMIN WITH CARBOXYPEPTIDASE A AND SUBSEQUENT HYDROLYSIS WITH CARBOXYPEPTIDASE B AND CARBOXYPEPTIDASE A

S-Sulfo- α -lactalbumin (1.162 g.) was dissolved in water by adjusting the pH to 8.0, and, after the addition of 5 mg. of DFP-treated carboxypeptidase A, the volume was made up to 48 ml. and incubated under toluene at 37°. At various time intervals 4-ml. aliquots (6 μ moles, the molecular weight of the S-sulfoprotein being 16,140) were withdrawn and precipitated with 1 ml. of 30% TCA. After 24 hr. of incubation with carboxypeptidase A, 10 mg. of dry carboxypeptidase B preparation was added to the digest, and samples were withdrawn at various time intervals as above. The TCA-precipitated digest was centrifuged, and aliquots of the supernatant corresponding to from 3 to 1 μ mole of the original protein (depending on the amount of amino acids liberated) were analyzed for free amino acids by the procedure of Moore, Spackman, and Stein (14). In the earlier phase of our work, the supernatant was extracted with ether to remove the TCA; however, it was found subsequently that this step was not required inasmuch as the positions of the amino acid peaks were not affected by the presence of TCA.

During the course of the chromatographic analysis of the amino acids, it became apparent very soon that due to the enzymic release of glutamine and asparagine with other amino acids from S-sulfo- α -lactalbumin, as well as from the native protein, the chromatographic separation of threonine and serine on the 150-cm. column was rendered impossible. These two amino acids, which as a rule emerge as distinct peaks in volumes of effluent of 137 and 150 ml. respectively, appeared as one peak with a net shoulder. This was due, as shown

below, to the emergence of glutamine and asparagine in this region. To resolve this composite peak into its components and to determine the total glutamine and asparagine content of the enzymic digest, aliquots of the TCA-filtrates were hydrolyzed in 4 *N* HCl for 3 hr. at 110°. This latter step served to convert glutamine and asparagine into their respective acids and at the same time to hydrolyze any pyrrolidonecarboxylic acid formed for glutamine into glutamic acid. The hydrolyzate then was reduced to dryness in a flash evaporator at 40°, redissolved in 2 ml. water, and analyzed for aspartic acid, threonine, serine, and glutamic acid on the 150-cm. column (14). The difference observed in aspartic and glutamic acid values before and after acid hydrolysis was considered to be due to asparagine and glutamine respectively.

Our findings on the enzymic degradation of S-sulfo- α -lactalbumin by carboxypeptidase A and subsequent action of carboxypeptidase B are presented in Fig. 2. The action of carboxypeptidase A as observed before (see Fig. 1) was confined to the liberation of the C-terminal leucine alone. The subsequent addition of carboxypeptidase B brought about the release of lysine and, due to the additional action of carboxypeptidase A present in the digest, the sequential release of threonine, tyrosine, valine, isoleucine, alanine, histidine, and other amino acids. For the sake of clarity, Fig. 2 represents our findings only with those amino acids which were released in stoichiometric (or greater) quantities. The amino acids which were liberated in lower concentrations are presented in Fig. 3 which should be considered, therefore, as an extension of Fig. 2. Inclusion of phenylalanine in Fig. 3 will be dealt with under *Discussion*. The leucine curve is repeated in Fig. 3 to emphasize the slow liberation of the second leucine residue.

HYDROLYSIS OF α -LACTALBUMIN WITH CARBOXYPEPTIDASE A AND SUBSEQUENT CLEAVAGE WITH CARBOXYPEPTIDASE B AND CARBOXYPEPTIDASE A

In order to extend our findings on S-sulfo- α -lactalbumin presented in Figs. 2 and 3, and at the same time to study the influence of disulfide bridges within the protein mole-

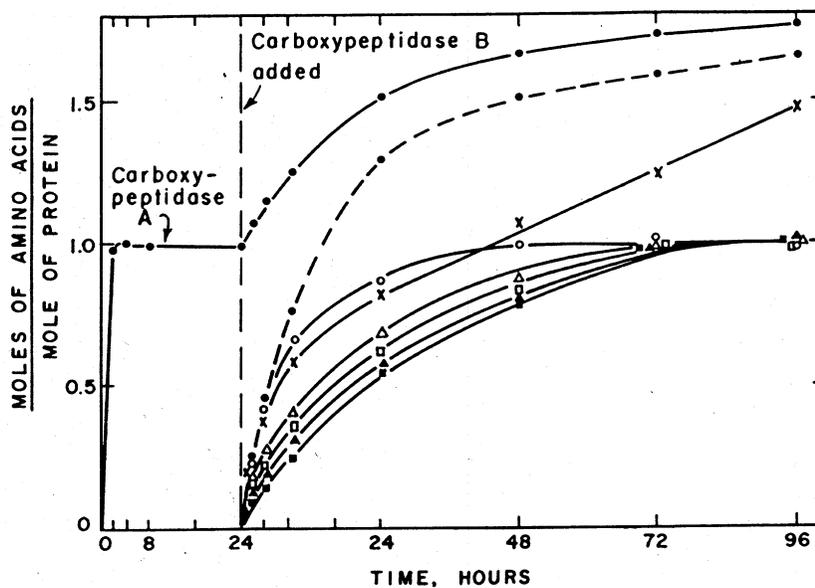


FIG. 2. Liberation of various amino acids from S-sulfo- α -lactalbumin by carboxypeptidase A and by the subsequent action of carboxypeptidase B and carboxypeptidase A. ●—● leucine; ●---● lysine; ○—○ threonine; ×—× tyrosine; Δ — Δ valine; □—□ isoleucine; \blacktriangle — \blacktriangle alanine; ■—■ histidine.

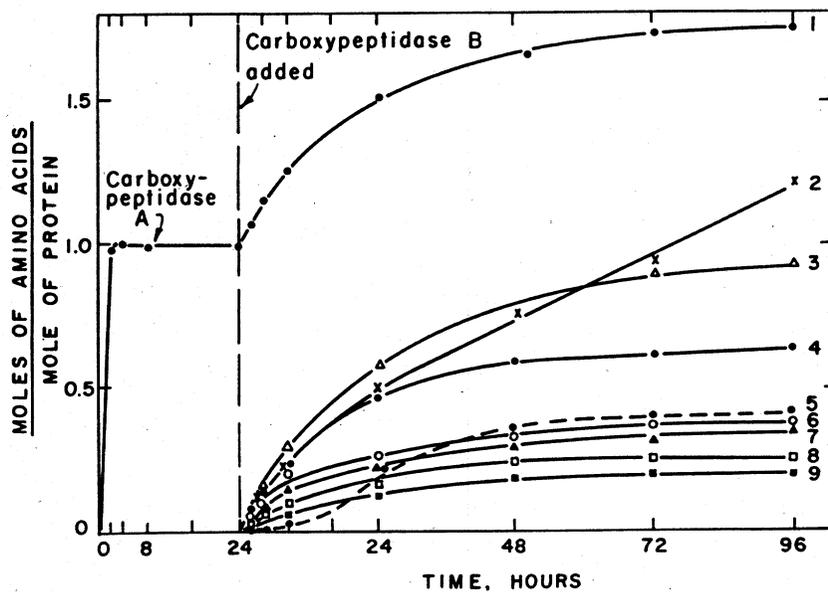


FIG. 3. Liberation of various amino acids from S-sulfo- α -lactalbumin by carboxypeptidase A and by the subsequent action of carboxypeptidase B and carboxypeptidase A. 1 = leucine; 2 = phenylalanine; 3 = glutamine; 4 = glutamic acid; 5 = tryptophan; 6 = glycine; 7 = serine; 8 = aspartic acid; 9 = asparagine.

TABLE I

LIBERATION OF VARIOUS AMINO ACIDS FROM α -LACTALBUMIN BY CARBOXYPEPTIDASE A AND BY THE SUBSEQUENT ACTION OF CARBOXYPEPTIDASE B AND CARBOXYPEPTIDASE A

Time, hr.	Action of carboxypeptidase				Subsequent action of carboxypeptidase B and carboxypeptidase A					
	Moles of amino acids liberated/mole protein									
	2	4	8	24	4	8	24	48	72	96
Leucine	1.02	1.00	1.02	0.98	0.06 ^a	0.14 ^a	0.17 ^a	0.25 ^a	0.34 ^a	0.45 ^a
Lysine	0	0	0	0	0.27	0.41	0.73	1.00	1.16	1.32
Threonine	0	0	0	0	0.25	0.39	0.68	0.88	1.02	1.03
Tyrosine	0	0	0	0	0.22	0.36	0.50	0.75	0.98	1.16
Valine	0	0	0	0	0.15	0.31	0.41	0.54	0.61	0.68
Isoleucine	0	0	0	0	0.13	0.28	0.36	0.46	0.55	0.60
Alanine	0	0	0	0	0.10	0.23	0.29	0.42	0.48	0.53
Histidine	0	0	0	0	0.08	0.17	0.26	0.33	0.45	0.50
Glutamine	0	0	0	0	—	0.20	0.29	0.36	0.39	0.46
Glutamic acid	0	0	0	0	0.06	0.15	0.23	0.28	0.36	0.46
Phenylalanine	0	0	0	0	0.06	0.12	0.29	0.49	0.57	0.71
Glycine	0	0	0	0	0.03	0.11	0.15	0.19	0.25	0.30
Serine	0	0	0	0	0.03	0.11	0.14	0.18	0.21	0.24
Aspartic acid	0	0	0	0	0.00	0.04	0.07	0.13	0.14	0.16
Asparagine	0	0	0	0	0.00	0.04	0.05	0.09	0.12	0.13

^a In addition to the one molecule of leucine liberated by carboxypeptidase A alone.

cule on the enzymic hydrolysis, identical experiments (as presented in Figs. 2 and 3) were carried out with native α -lactalbumin. The results obtained and presented in Table I confirm our findings with S-sulfo- α -lactalbumin. While the liberation of the C-terminal leucine by carboxypeptidase A proceeded with equal rapidity, the successive release of the other amino acids by the action of carboxypeptidase B and carboxypeptidase A progressed at a much slower rate as compared with the S-sulfoprotein. No liberation of tryptophan could be observed for the native protein.

LIBERATION OF FREE AMINO ACIDS DURING THE TRYPTIC DIGESTION OF α -LACTALBUMIN

α -Lactalbumin was hydrolyzed with trypsin (100:1) at pH 8.0 at 37°, and the possible formation of free amino acids was determined as described for experiments presented in Fig. 1. The results obtained and presented in Fig. 4 demonstrate the liberation of one mole of free lysine and one mole of free leucine.

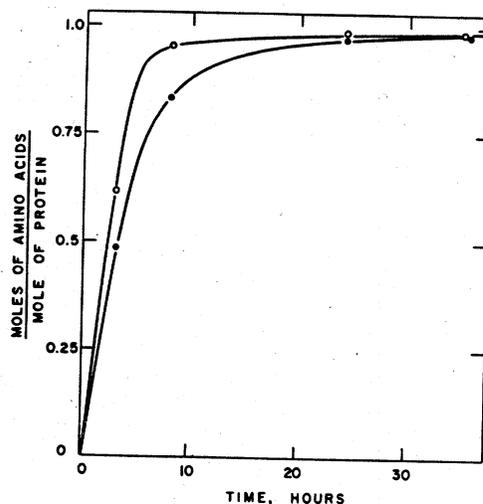


Fig. 4. Liberation of free amino acids during the tryptic digestion of α -lactalbumin. \circ — \circ lysine; \bullet — \bullet leucine.

DISCUSSION

The present report is the first of a series dealing with the primary structure of α -lactalbumin. As prerequisite for this type of

study, consideration was given to the homogeneity of this protein. The works of Zittle (18) and of Klostergaard and Pasternak (19) indicated that under certain conditions the physicochemical properties of this protein gave an appearance of heterogeneity. This was ascribed by these authors to the binding of ions, which might lead to more than one molecular form. Similarly, M. J. Kronman (personal communication) has observed a certain degree of heterogeneity of this protein using ion-exchange techniques. Although the precise meaning of the above observations is not well defined, previous findings (12, 16) and our present work on the terminal amino acids of this protein would indicate that the differences might be only of a minor nature.

In accordance with the single chain structure of α -lactalbumin, only one N-terminal glutamic acid could be demonstrated by Sanger's procedure (9) and one C-terminal leucine using carboxypeptidase A as a hydrolytic agent (Fig. 1). Both of these observations are in accord with similar findings of Yasunobu and Wilcox (12) and of Davie (16).

The single chain structure of α -lactalbumin was confirmed also by sedimentation studies of M. J. Kronman of this laboratory, who showed that the molecular weight of this protein was not altered after cleavage of the disulfide bonds by the sulfide method (6, 7).

α -Lactalbumin deprived of the C-terminal leucine by the action of carboxypeptidase A still retained its ability to crystallize in well-formed needles. Inasmuch as the action of carboxypeptidase A on α -lactalbumin was confined only to the cleavage of one mole of C-terminal leucine, it was of interest to test whether the penultimate amino acid which could act as a barrier for this enzyme might be arginine or lysine. For this purpose, carboxypeptidase B (protaminase), described by us many years ago (20, 21) and reinvestigated recently by various workers (22, 8, 17, 23-25), has been used as a tool. Since the activity of carboxypeptidase B is confined to the cleavage of C-terminal arginine and lysine, the action of this enzyme was tested on both native and

S-sulfoprotein after the C-terminal leucine had been liberated by carboxypeptidase A. Results presented (Fig. 2 and Table I) demonstrate that the penultimate amino acid is lysine. Because of the presence of carboxypeptidase A in the protein digest, used as the first step in the hydrolysis, a sequential release of other amino acids also took place. The release of lysine did not stop at the level of one mole per mole of protein, indicating that this amino acid recurs further down the protein chain. The next amino acid liberated in stoichiometric amount was threonine followed by tyrosine which again, like lysine, was released above the one mole quantity. The approximate position of the second mole of lysine and tyrosine based on the total amounts released after extended incubation (Fig. 2 and Table I) would be as given in Fig. 5. The sequence of amino acids following tyrosine which were liberated stoichiometrically was valine, isoleucine, alanine, and histidine. Other amino acids which were released in less than one mole quantities (see Fig. 3, Table I) are listed in the order of their appearance: glutamine, leucine, glutamic acid, phenylalanine,³ glycine, serine, aspartic acid, asparagine, and tryptophan. The enzymic liberation of glutamine was slightly less than the stoichiometric amount. Therefore, glutamine should be placed in the position following histidine. The fact, however, that the rate of enzymic release of glutamine at the early stages of hydrolysis was very close to that of alanine and histidine, and the possible inherent error in glutamine determination, renders the sequence of these three amino acids doubtful.

Our results on the partial primary structure of α -lactalbumin presented in Fig. 5 are based on the sequential appearance of amino acids at the early stages of hydrolysis, taking into account only those amino

³This amino acid which at the early stages of the hydrolysis lagged behind the release of glutamine and leucine (not the C-terminal), after 48 hr. incubation (Fig. 3) superseded these amino acids, indicating the recurrence of phenylalanine along the chain. Similar behavior has been observed with tryptophan, the last amino acid released.

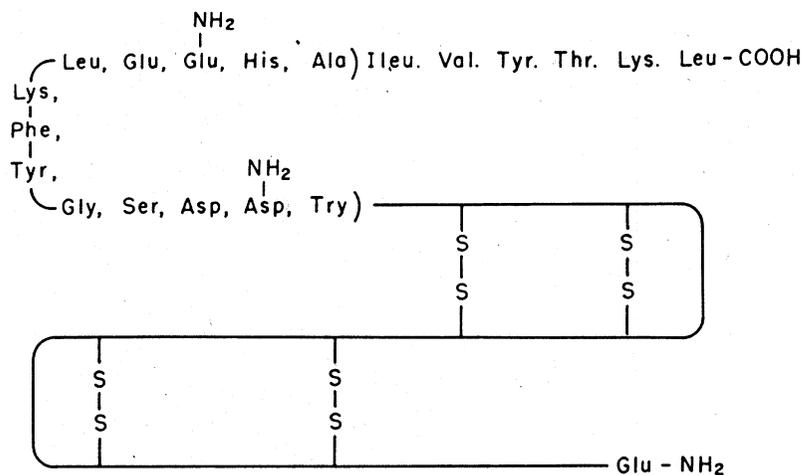


Fig. 5. Schematic partial primary structure of α -lactalbumin.

acids which were liberated in stoichiometric amounts (or above this value). Amino acids released stoichiometrically, but whose position is doubtful for reasons discussed above, and amino acids released in less than one mole per mole of protein are listed in parentheses. The order of amino acids within the parentheses does not reflect their sequence but only their presence in this portion of the protein chain.

The release of the C-terminal leucine by carboxypeptidase was equally rapid whether the native or the S-sulfoprotein was hydrolyzed (Fig. 1 and Table I). The subsequent liberation of the other amino acids was, however, considerably faster for the S-sulfo than the native protein, presumably due to the cleavage of disulfide bonds and the loosening of the secondary structure of the protein. The fact that the enzymic digest of the native and the S-sulfoprotein contained no cystine or S-sulfocysteine would indicate the absence of a disulfide bond in this portion of the protein chain.

The formation of a stoichiometric amount of lysine during the tryptic digestion of α -lactalbumin (Fig. 4) might be due to a lysyl-lysyl or to an arginyl-lysyl sequence within the protein chain. The simultaneous liberation of one mole of leucine per mole of protein can be only ascribed to the tryptic cleavage of the lysyl-leucine bond at the C-terminal end of the protein.

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