

The present report is the extension of our previous work (1) on the primary structure of α -lactalbumin. It describes the amino acid sequences of four peptides isolated from the peptic digest of α -lactalbumin.

EXPERIMENTAL

MATERIALS

The α -lactalbumin employed was three-times crystallized according to the method of Gordon and Ziegler (2). Crystalline carboxypeptidase A was from a commercial source. Carboxypeptidase B was prepared from pig pancreas, as described before (3), and leucine aminopeptidase according to the procedure of Hill *et al.* (4). The latter had a C_1 value of 4.5. Crystalline pepsin and trypsin were of commercial origin. Carboxypeptidase A, carboxypeptidase B, and leucine aminopeptidase were treated with diisopropylfluorophosphate prior to use.

METHODS AND RESULTS

DIGESTION OF α -LACTALBUMIN WITH PEPSIN

One gram of α -lactalbumin was dissolved in 20 ml. of water by adjusting the pH to

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

2.0 with 1 *N* HCl. After the addition of 10 mg. of crystalline pepsin, the solution was incubated at 37°C., and (using a micro-pipette) 0.01-ml. aliquots were withdrawn to follow the hydrolysis by the ninhydrin procedure (5). As Fig. 1 indicates, after 7 hours of digestion, the hydrolysis was complete.

CHROMATOGRAPHIC SEPARATION OF THE PEPSIN DIGEST OF α -LACTALBUMIN

For the separation of the peptides, a Dowex 50-X 4 column of 1.8 \times 150 cm. dimension was used. Prior to packing the column, the resin was converted to its Na-form, then to its H-form, and finally equilibrated with a 0.05 *M* pyridine-formic acid buffer of pH 3.1. After packing the column, the same buffer was passed through until the pH of the effluent was 3.1. At this point, 1 g. of α -lactalbumin-pepsin digest (7-hour digestion), contained in 20 ml. (pH 2.0), was placed in its entirety on the column; and a linear ionic gradient, consisting of 1150 ml. of 0.05 *M* pyridine-formic acid buffer of pH 3.1 and 1150 ml. of 0.5 *M* pyridine-acetic acid buffer of pH 5.2 (Gradient I), was passed through the water-jacketed column at a rate of 32 ml. per hour at 35°C. Gradient I was followed by linear ionic Gradient II,

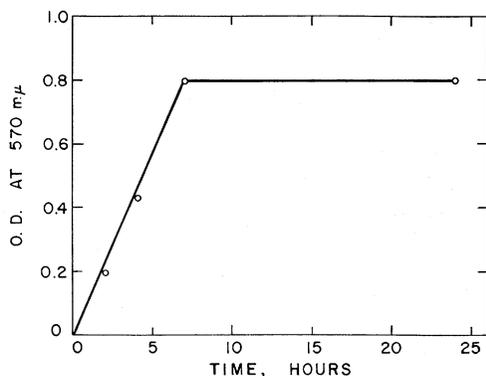


FIG. 1. Hydrolysis of α -lactalbumin with pepsin (100:1).

consisting of 1150 ml. of 0.5 *M* pyridine-acetic acid buffer of pH 5.2 and 1150 ml. of 2.0 *M* pyridine-acetic acid buffer of pH 5.2, and finally by linear ionic Gradient III which was composed of 1150 ml. of 2.0 *M* pyridine-acetic acid buffer of pH 5.2 and 1150 ml. of 4.0 *M* pyridine-acetic acid buffer of pH 5.2. The molarity of the three gradients refers to pyridine concentration. Fractions of 8 ml. were collected every 15 minutes, and 0.2-ml. aliquots were taken for the development of ninhydrin color (5) to follow the emergence of the peptides. The effluent curve obtained in this fashion is shown in Fig. 2.

Gradient I produced numerous unresolved peaks, but between the effluent volume of 992 and 1064 ml., a distinct peak appeared and was designated as "Peptide I."

Gradient II gave rise to seven well-separated peaks, but the yields were extremely small for structural studies.

Gradient III afforded three well-resolved peaks. The first peak emerged between the effluent volume of 208 and 320 ml. and was designated "Peptide II"; the second emerged between the effluent volume of 360 and 472 ml. and was designated "Peptide III"; and the third emerged between 1098 and 1248 ml. of effluent and was designated "Peptide IV".

The effluents containing the above peptides were flash-evaporated and freed of the pyridine salts at 60°C. in high vacuum.

AMINO ACID SEQUENCE OF PEPTIDE I

The homogeneity of this peptide was checked by the use of high voltage paper

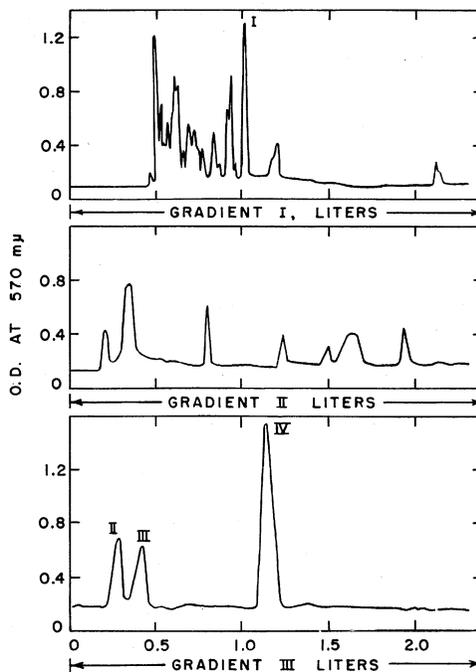
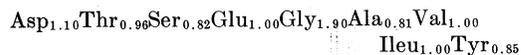


FIG. 2. Elution diagram of the peptic digest of α -lactalbumin on Dowex 50-X 4 column at 35°C. Details in text.

electrophoresis. The peptide was placed on Whatman 3 MM paper on a horizontal, water-cooled plate and operated with the buffers and by the technique of Ingram (6). The samples tested were run at pH 1.9 and 6.4, respectively, at 4000 V. for 1 hour. The papers were then dried and dipped into 0.2% ninhydrin in acetone, dried again, and heated 2 minutes in a 110°C. oven. Preparative electrophoretic runs were made under the same conditions with aliquots containing about 4 mg. of the peptide. After drying the paper, a guide strip was cut from the edge and the bands located by ninhydrin. The section of the paper involved was then cut out and the peptide eluted with 1.0 *M* acetic acid. The eluates were combined and flash-evaporated in vacuum. Peptide I, applied in this manner, migrated as one spot at both pH values, indicative of its purity. The yield obtained was 22%, based on the amino acid composition.

Amino acid composition. The hydrolysis of the peptide was carried out in 6 *N* HCl in a vacuum-sealed tube for 24 hours at 110°C. Using the analytical procedure of Moore *et*

al. (7), the following composition was obtained:

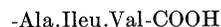


None of the peptides described contained tryptophan if tested by the method of Spies and Chambers (8).

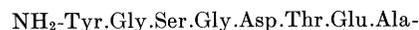
The amide contents of the peptides were determined by measuring the ammonia formed during the acid hydrolysis and subtracting the blank ammonia value obtained when the same hydrolysis procedure was carried out without the peptide. The amide content obtained in this manner was 0.08 equivalents per mole of peptide.

Carboxy-terminal sequence. The carboxy-terminal sequence of Peptide I was established by hydrolysis with carboxypeptidase A at pH 8.0 at 37°C. (ratio of substrate to

enzyme, 100:1). One ml. of aliquots containing 0.5 μ mole of the peptide were withdrawn at various time intervals, and the liberated amino acids were determined on the amino acid analyzer (7). The results obtained and presented in Fig. 3 indicate that the carboxy-terminal sequence of Peptide I is:



Amino acid terminal sequence. To determine its amino terminal sequence, Peptide I was hydrolyzed with leucine aminopeptidase at pH 7.0 (substrate-enzyme ratio, 5:1). One-ml. aliquots containing 0.5 μ mole of the peptides were analyzed for the liberated amino acids, as described for Fig. 3. Our findings, presented in Fig. 4, are in accord with an amino-terminal sequence of:



A qualitative confirmation of a portion of the amino terminal sequence of Peptide I was obtained by the Edman degradation procedure as applied for paper strips by Fraenkel-Conrat *et al.* (9) and Schroeder *et al.* (10). Paper chromatographic analysis of the acetone extracts from the first and second degradations established the amino-terminal amino acid as tyrosine and the penultimate amino acid as glycine. Successive degradation steps resulted in complex chromatograms which made further identifications dubious. On the basis of the experimental evidence presented, the proposed structure of Peptide I is as follows:

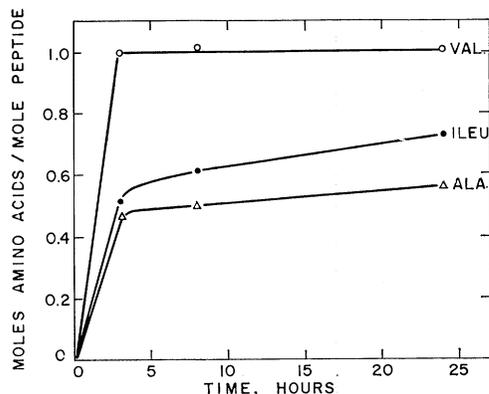
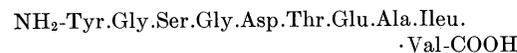


FIG. 3. Hydrolysis of Peptide I with carboxypeptidase A (100:1).

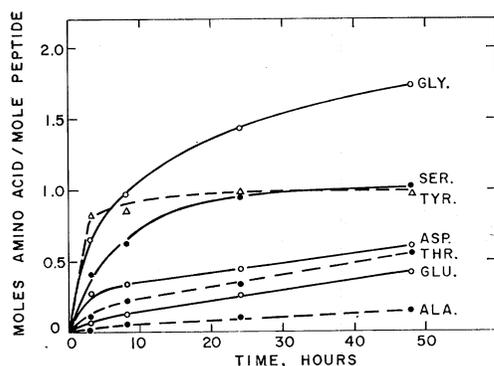
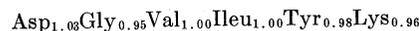


FIG. 4. Hydrolysis of Peptide I with leucine aminopeptidase (5:1).

AMINO ACID SEQUENCE OF PEPTIDE II

Peptide II proved to be heterogeneous when tested by high voltage electrophoresis at pH 6.4 and 1.9. One major component and two minor components were noticeable. The major component was isolated by preparative electrophoretic runs as described for Peptide I at pH 6.4. The yield obtained was 20%.

Amino acid composition of Peptide II. The following composition was obtained:



The amide content of Peptide II was 1.12 equivalents per μ mole of peptide, as deter-

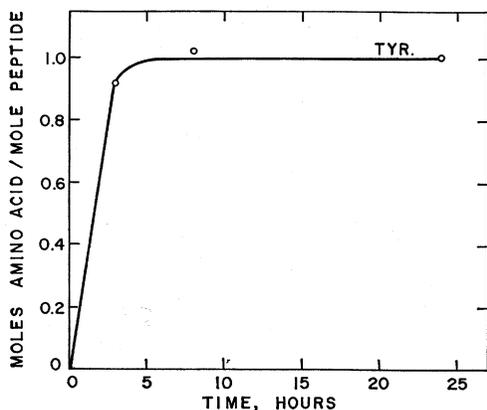


FIG. 5. Hydrolysis of Peptide II with carboxypeptidase A (100:1).

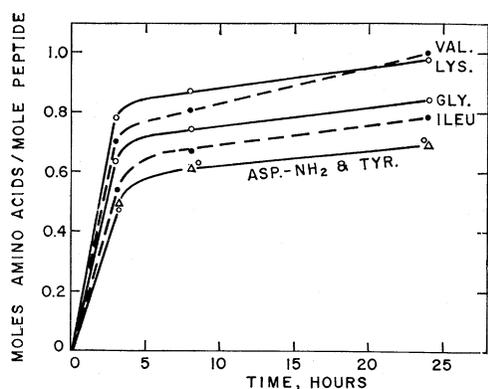


FIG. 6. Hydrolysis of Peptide II with leucine aminopeptidase (5:1).

mined by the procedure used for Peptide I.

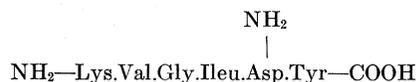
Carboxy-terminal sequence of Peptide II. For the determination of the carboxy-terminal sequence of Peptide II, hydrolysis was carried out with carboxypeptidase A as described for Peptide I. The results obtained and presented in Fig. 5 show only a rapid liberation of stoichiometric amounts of tyrosine. Addition of carboxypeptidase B to the carboxypeptidase A digest of this peptide had no further effect. The results would indicate that the carboxy-terminal amino acid of Peptide II is tyrosine and that the penultimate amino acid is not lysine.

Amino-terminal sequence of Peptide II. The amino-terminal sequence of Peptide II was established by hydrolysis with leucine aminopeptidase, as described before for Peptide I, and is presented in Fig. 6.

Edman degradation, adapted for paper strips (9, 10), was successfully employed up to five steps of degradation, and in agreement with the findings in Fig. 6, supported the following amino terminal sequence:



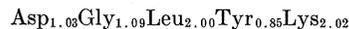
On the basis of all the experimental evidence presented, the structure of Peptide II is as follows:



AMINO ACID SEQUENCE OF PEPTIDE III

High-voltage paper electrophoresis, both at pH 1.9 and 6.4, showed that Peptide III was contaminated with one minor component and was therefore purified on a preparative scale at pH 6.4, as described before.

The amino acid composition of Peptide III, determined as described before, is as follows:



The amide content of Peptide III was 0.06 equivalent per mole of peptide.

Carboxy-terminal sequence of Peptide III. The carboxy-terminal sequence of Peptide III was elucidated by hydrolysis of the

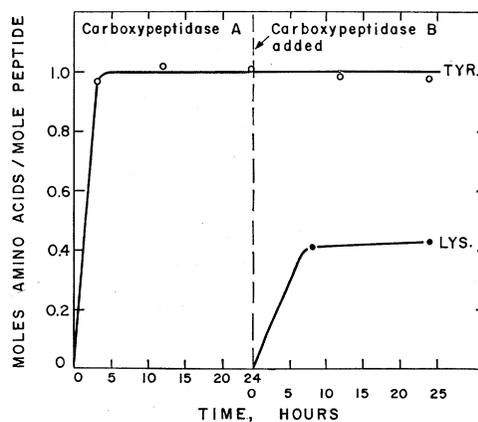


FIG. 7. Hydrolysis of Peptide III with carboxypeptidase A followed by carboxypeptidase B (100:1).

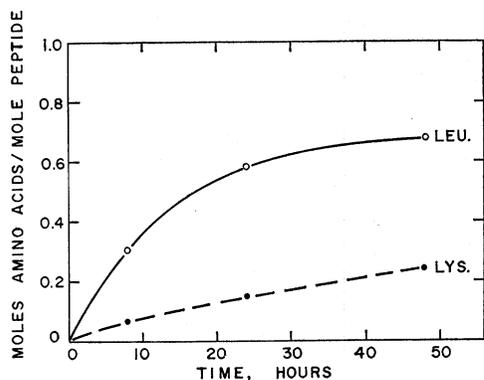
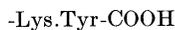


FIG. 8. Hydrolysis of Peptide III with leucine aminopeptidase (5:1).

peptide with carboxypeptidase A and the subsequent action of carboxypeptidase B, as described before (ratio of substrate to enzyme 100:1). Results presented in Fig. 7 indicate that the carboxy-terminal sequence is:

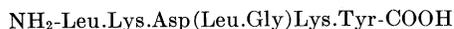


Amino-terminal sequence of Peptide III. Hydrolysis of Peptide III with leucine aminopeptidase was carried out as described before (ratio of substrate to enzyme 5:1), and the results are given in Fig. 8.

Edman degradation of Peptide III as applied previously could be carried through three steps and gave supporting evidence for the amino-terminal sequence:



Our experimental data in the case of Peptide III are insufficient for the elucidation of the complete sequence, but on the basis of the evidence presented, the following sequence is proposed:



AMINO ACID SEQUENCE OF PEPTIDE IV

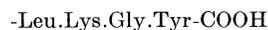
Peptide IV, as isolated from the ion exchange column and tested by high-voltage paper electrophoresis, proved to contain two minor components which were removed by repetition on a preparative scale of the electrophoretic step at pH 6.4. Peptide IV, purified in this manner, was obtained in a 35% yield.

Amino acid analysis of Peptide IV gave the following composition:



The amide content of Peptide IV was found to be 0.05 equivalent per mole of peptide.

Carboxy-terminal sequence of Peptide IV. The carboxy-terminal sequence of Peptide IV was established by hydrolysis with carboxypeptidase A and by the subsequent action of carboxypeptidase B, as described before. The action of the first enzyme, as shown in Fig. 9, resulted in the rapid liberation of tyrosine, followed by glycine. Subsequent action of carboxypeptidase B on the carboxypeptidase A and Peptide IV digest produced an additional liberation of lysine, followed by leucine, the latter being cleaved by the action of carboxypeptidase A already present in the digest. Evidence presented in Fig. 9 supports the following carboxy-terminal sequence for Peptide IV:



Amino-terminal sequence of Peptide IV. The action of leucine aminopeptidase on Peptide IV, carried out as described before and presented in Fig. 10, supports the following amino-terminal sequence for this peptide:



The paper strip modification of the Edman procedure (9, 10), if applied to Peptide IV, gave unsatisfactory results, probably due to the lability of this peptide under the experimental conditions employed.

Tryptic hydrolysis of Peptide IV. 5.4 μ moles of Peptide IV were hydrolyzed at pH 8.0 with 0.06 mg. of trypsin for 16 hours at 37°C.; total volume was 2.5 ml. Aliquots were subjected to high-voltage paper electrophoresis at pH 1.9 and 3000 V. for 90 minutes. As Fig. 11 shows, Peptide IV was hydrolyzed into 3 fragments, and specific tyrosine stain, α -nitroso- β -naphthol, gave a positive reaction only with segment 1, while the Sakaguchi reagent for arginine reacted only with segment 3. Preparative electrophoretic runs were made under the same conditions, and the 3 fragments were isolated as described before.

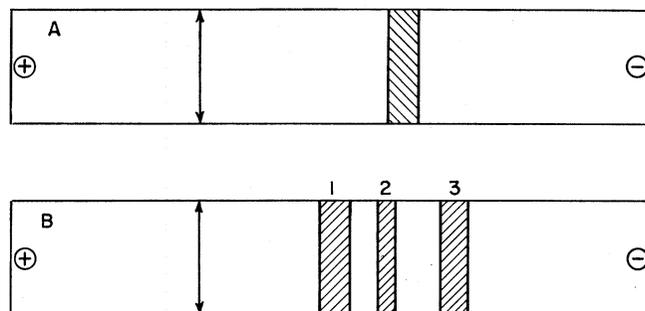


FIG. 11. High voltage electrophoresis diagram of the tryptic digest of Peptide IV. (A) Before hydrolysis; (B) after hydrolysis.

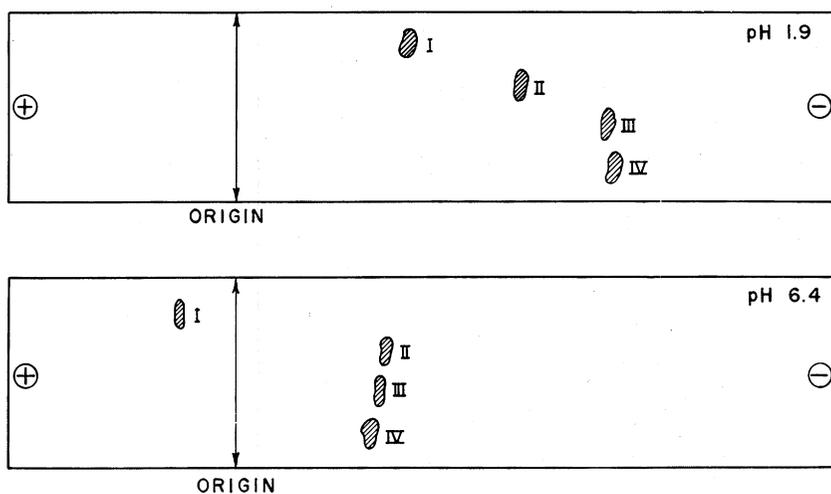


FIG. 12. High voltage electrophoresis diagram of Peptide I, II, III, and IV at pH 1.9 and 6.4.

the position of peptide bonds hydrolyzed by trypsin.

ELECTROPHORETIC BEHAVIOR OF THE PEPTIDES

The migration of the pure peptides upon high voltage paper electrophoresis was tested at pH 1.9 and 6.4 by the technique of Ingram (6) at 4000 V. for 1 hour. The results are presented in Fig. 12. At pH 1.9 (where all the carboxyl groups of the peptides are unionized), Peptide I should carry one, Peptide II two, Peptide III three, and Peptide IV four positive charges on the basis of the established sequences. This is reflected in Fig. 12 for Peptides I, II, and III, inas-

much as the rates of migration toward the negative pole appear to be proportional to the number of positive charges. However, Peptide IV, which should migrate at the fastest rate, behaved like Peptide III. Whether this retardation in rate is due to some binding of the peptide by the paper remains to be established. At pH 6.4, on the basis of the established amino acid sequences, Peptide I should have 2 negative charges, while Peptides II, III, and IV should have 1 positive charge. These deductions appear to be in accord with the results presented in Fig. 12, where Peptides II, III, and IV migrated at about the same rate toward the negative pole, while Peptide I, with 2 negative charges, migrated at a slower rate toward the positive pole.

DISCUSSION

The amino acid sequences of the four peptides isolated from the peptic digest of native α -lactalbumin account for 25% of the amino acids present in this protein molecule. The position of these peptides within the entire peptide chain, however, remains to be established by further investigation.

The proteolytic enzymes used for our structural studies as a whole conformed to the known specificity requirements. One might point, however, to the limited action of carboxypeptidase A on Peptide II (see Fig. 5) which was confined to the cleavage of tyrosine only. Whether this was due to the penultimate asparagine or to some other steric factors remains to be established.

Peptide IV, which contains two lysines (in 2nd and 7th position) and the only arginine residue (located in the 4th position) present in the whole molecule, when subjected to tryptic hydrolysis, was cleaved only at two points—namely, the peptide bond involving the carboxyl group of arginine and the peptide bond involving the carboxyl group of lysine located in 7th position. The resistance of the peptide bond of lysine in the 2nd position to the action of trypsin might be due to the close proximity of the free α -amino group to this linkage.

The absence of amide groups in Peptides I, III, and IV was established by three criteria: (1) lack of ammonia formation during acid hydrolysis; (2) enzymatic hydrolysis of these peptides resulting in the liberation of glutamic acid and aspartic acid

instead of glutamine and asparagine; and (3) high-voltage electrophoresis patterns which were in general agreement with the number of positive and negative groups of the peptides, as postulated in this paper.

Similarly, the occurrence of asparagine in Peptide II was ascertained three ways: (1) by the stoichiometric formation of ammonia during acid hydrolysis; (2) by the asparagine liberation during enzymatic hydrolysis; and (3) by the migration behavior during high-voltage electrophoresis.

Further work is in progress toward the establishment of the primary structure of α -lactalbumin.

REFERENCES

1. WEIL, L., AND SEIBLES, T. S., *Arch. Biochem. Biophys.* **93**, 193 (1961).
2. GORDON, W. G., AND ZIEGLER, J., *Biochem. Prep.* **4**, 16 (1955).
3. WEIL, L., AND TELKA, M., *Arch. Biochem. Biophys.* **71**, 204 (1957).
4. HILL, R. L., SPACKMAN, D. H., BROWN, D. M., AND SMITH, E. L., *Biochem. Prep.* **6**, 35 (1958).
5. MOORE, S., AND STEIN, W. H., *J. Biol. Chem.* **211**, 907 (1954).
6. INGRAM, V. M., *Biochim. Biophys. Acta* **28**, 539 (1958).
7. MOORE, S., SPACKMAN, D. H., AND STEIN, W. H., *Anal. Chem.* **30**, 1185 (1958).
8. SPIES, J. R., AND CHAMBERS, C., *Anal. Chem.* **20**, 30 (1948).
9. FRAENKEL-CONRAT, H., HARRIS, J. I., AND LEVY, A. L., in "Methods of Biochemical Analysis" (D. Glick, ed.) Vol. 2, p. 359 (1955). Interscience Pub. New York.
10. SCHROEDER, W. A., SHELTON, J. R., AND SHELTON, J. B., *Anal. Biochem.* **2**, 87 (1961).