

The Carboxyl Terminal Amino Acids of β -Lactoglobulins A and B

EDWIN B. KALAN AND RAE GREENBERG

From the Eastern Regional Research Laboratory,¹ Philadelphia, Pennsylvania

The release of amino acids from the carboxyl terminal end of β -lactoglobulins A and B has been studied with the use of carboxypeptidases A and B. The action of carboxypeptidase A catalyzes the hydrolysis of two moles of isoleucine and about one mole of histidine per mole of native protein at pH 8.5. The S-sulfo derivative releases two moles of isoleucine and two moles of histidine under the same conditions. When the native proteins were hydrolyzed with a combination of carboxypeptidases A and B, a wide spectrum of amino acids was found with leucine appearing as the amino acid following histidine.

It appears that the two phenotypes, β -lactoglobulin A and β -lactoglobulin B, do not differ in the sequence of amino acids in the carboxyl-terminal portion of the molecules. In addition, they do not seem to differ in the manner they are hydrolyzed by the carboxypeptidase preparations.

INTRODUCTION

Previous studies of the carboxyl-terminal amino acids of β -lactoglobulin were carried out by several investigators who employed β -lactoglobulin preparations crystallized from pooled milks. It is known from the work of Polis *et al.* (1) and Aschaffenburg and Drewry (2, 3) that these preparations were mixtures of two proteins, designated as β -lactoglobulin A and β -lactoglobulin B. The differences between these proteins are known to be genetically determined.

Fraenkel-Conrat (4, 5) and Niu and Fraenkel-Conrat (6) found, by chemical methods, two carboxyl-terminal isoleucine residues and two amino-terminal leucine residues per mole of protein (about 36,000 mol. wt.), indicating two subunits in the molecule. Townend *et al.* (7) have shown by physical-chemical means that β -lactoglobulin can be dissociated into two units of $\sim 18,000$ molecular weight.

Neurath *et al.* (8), using carboxypeptidase A, have confirmed Fraenkel-Conrat's findings and, in addition, have shown that

histidine is the penultimate amino acid. More recently, Davie *et al.* (9) have investigated the kinetics of the release of isoleucine and histidine from mixed β -lactoglobulin by the action of carboxypeptidase A. The results of these latter studies made it of some interest to reinvestigate the problem of the carboxyl-terminal amino acids of the individual β -lactoglobulins obtained from genetically typed cows.

This paper reports the results of the action of carboxypeptidases A and B on β -lactoglobulins A and B and their S-sulfo derivatives. The results confirm the findings of Davie *et al.* (9) that the differences between the two proteins are due to a change or changes in the amino acid sequence at some position in the peptide chain other than the carboxyl-terminal portion. The results also indicate that the two proteins are attacked at approximately the same rate by carboxypeptidase A and by a mixture of carboxypeptidases A and B.

EXPERIMENTAL

MATERIALS

β -Lactoglobulins A and B were prepared according to the method of Aschaffenburg and

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

Drewry (10) from milks of genetically typed cows.² The proteins were recrystallized three times, lyophilized, and stored at a constant humidity of 50%.

The S-sulfo β -lactoglobulins A and B were prepared from the native materials by a modification (11) of the method of Swan (12). The sulfur content for the A derivative was 2.58% and for the B 2.41%.³

Carboxypeptidase A⁴ was obtained from Worthington Biochemical Corporation⁵ as a water suspension of three-times recrystallized material. It was treated with diisopropyl fluorophosphate (DFP) prior to use. Carboxypeptidase B (13-15), also known as protaminase (16), was prepared according to the method of Weil and Telka (17) and subsequently treated with DFP.

METHODS

ACTION OF CARBOXYPEPTIDASE A ON NATIVE AND S-SULFO- β -LACTOGLOBULINS A AND B

For the determination of the carboxyl-terminal amino acids, the following hydrolysis procedure was employed. β -Lactoglobulin A and β -lactoglobulin B [mol. wt. 37,300 (11)] were dissolved in water with the aid of 0.1 N NaOH. The reaction was run in an unbuffered system at 37°C., pH 8.5, with a weight ratio of enzyme to protein of 1:50. Four-milliliter aliquots containing 144 mg. protein were withdrawn at specific time intervals from 2 to 96 hr. The aliquots were precipitated with 0.5 ml. of 45% trichloroacetic acid (TCA), centrifuged in the cold, and the supernatants were decanted and frozen until analyzed. The amino acids released were determined by the improved ion-exchange chromatographic procedure of Moore, Spackman, and Stein (18), using 1-ml. portions (0.85 μ mole) of the aliquots for analysis.

The S-sulfo proteins [mol. wt. 38,100 (11)] were treated in exactly the same manner as the native material except that the protein concentration of the aliquots was 0.74 μ mole/ml.

² We wish to thank Dr. C. Kiddy, Dairy Cattle Research Branch, U. S. Department of Agriculture, Beltsville, Maryland, for making these milks available.

³ We are indebted to Mrs. R. Kelly of these laboratories for the sulfur determinations.

⁴ Carboxypeptidase A is equivalent to carboxypeptidase (21).

⁵ It is not implied that the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

HYDROLYSIS OF β -LACTOGLOBULINS A AND B WITH A COMBINATION OF CARBOXYPEPTIDASES A AND B

The same procedure was followed here as in the above experiments. Carboxypeptidases A and B were added at zero time, and the hydrolysis carried out with an enzyme-substrate ratio of 1:50 for both enzymes and both proteins. Aliquots were removed at the same time intervals and treated in like fashion. The final protein concentration of the aliquots was 0.85 μ mole/ml.

RESULTS

The results of the hydrolysis of β -lactoglobulins A and B catalyzed by carboxypeptidase A are shown in Figs. 1 and 2. Both proteins released two moles of isoleucine within 24 hr. but only slightly more than one mole of histidine per mole of protein, even after 96 hr. Davie *et al.* (9) found similar results for mixed β -lactoglobulin when the reaction was carried out at pH 7.6 with an enzyme to substrate ratio of 1:100 (weight basis) for 400 min. Several other amino acids found in relatively small amounts (less than 0.1 mole per mole of protein) are not shown in the figures. It can be seen that there are no significant differences between the proteins in the rate of release of isoleucine and histidine. This is also true for the other experiments described.

The S-sulfo derivatives (Figs. 3 and 4) released the same amino acids as the native proteins but at a faster rate. In this case two equivalents of histidine as well as isoleucine were liberated in about 4 hr., with no increase up to 96 hr. This result is similar to that found by Davie *et al.* (9) for mixed β -lactoglobulin when the reaction was carried out as mentioned above except that the pH was 9.2, a pH at which β -lactoglobulin is known to denature (19).

When the combination of enzymes was used to catalyze the hydrolysis of the native materials, a wide spectrum of amino acids was released as shown in Figs. 5 and 6. Two equivalents of isoleucine and histidine were again liberated in about 4 hr., but, after a slight lag, isoleucine increased to over three equivalents indicating its recurrence farther down the peptide chain. The leucine values also exceeded two equiv-

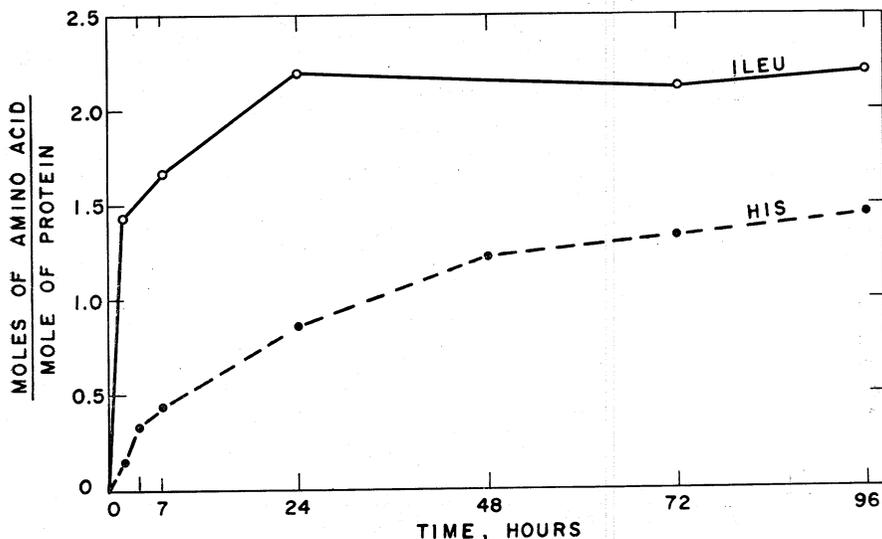


FIG. 1. Hydrolysis of β -lactoglobulin A by carboxypeptidase A at pH 8.5, 37°C., with enzyme-substrate ratio of 1:50.

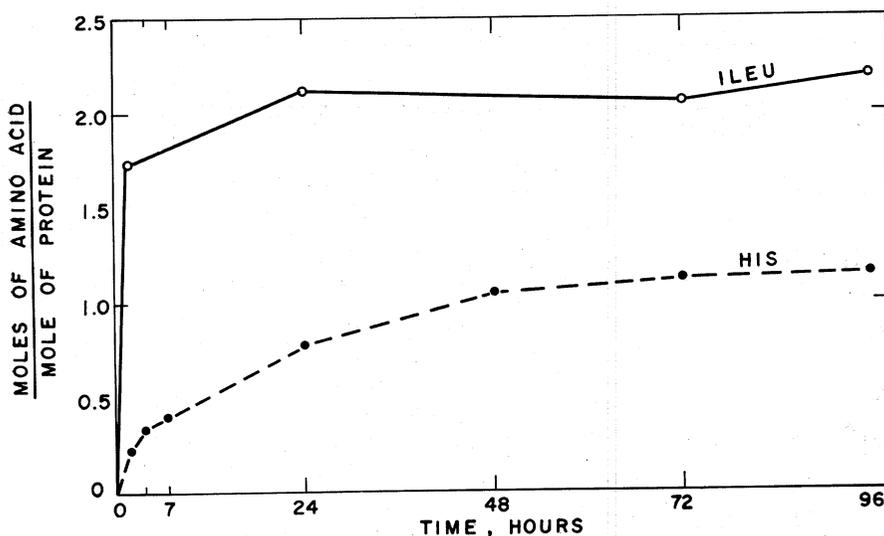


FIG. 2. Hydrolysis of β -lactoglobulin B by carboxypeptidase A at pH 8.5, 37°C., with enzyme-substrate ratio of 1:50.

alents, and this amino acid probably occurs at least twice in the carboxyl-terminal region of the chain. All other amino acids were present in lesser amounts except for the threonine-serine-glutamine combination which will be treated in the *Discussion*.

DISCUSSION

Similar results have been obtained for both phenotypes by hydrolysis with the

carboxypeptidase preparations. Thus it is evident from the data that the differences in the amino acid sequence of β -lactoglobulin A and β -lactoglobulin B do not occur in the region close to the carboxyl end of the molecules.

At pH 8.5, the rates of release of isoleucine and histidine from the two proteins are essentially the same, with only about one mole of histidine released in contrast to

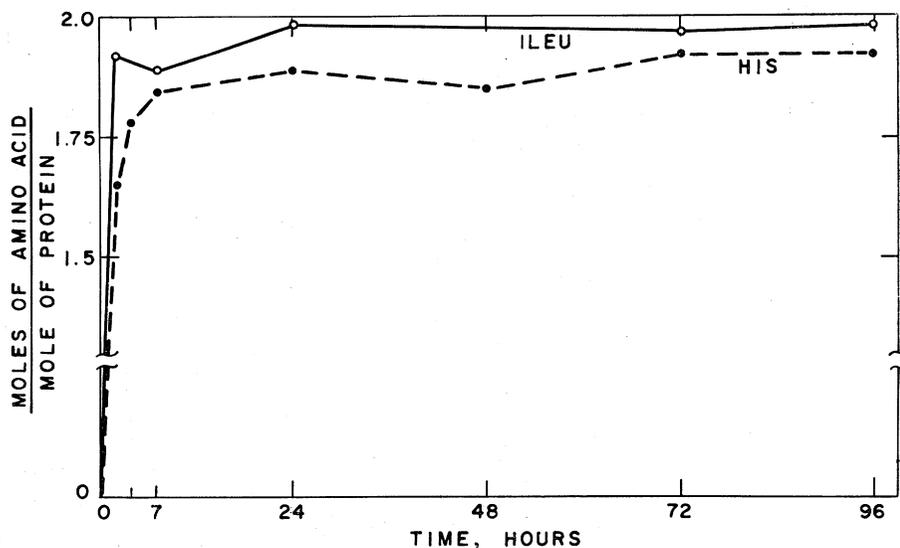


FIG. 3. Hydrolysis of S-sulfo- β -lactoglobulin A by carboxypeptidase A at pH 8.5, 37°C., with enzyme-substrate ratio of 1:50.

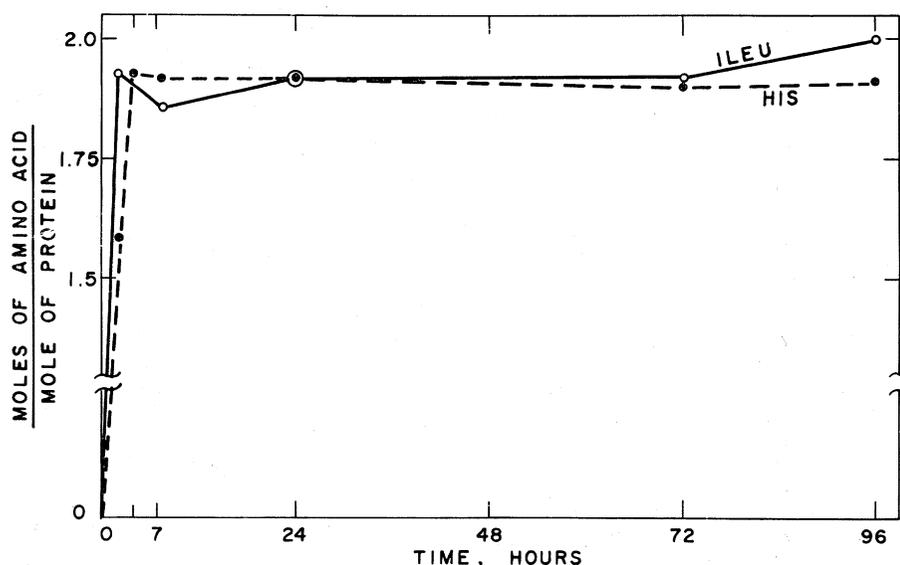


FIG. 4. Hydrolysis of S-sulfo- β -lactoglobulin B by carboxypeptidase A at pH 8.5, 37°C., with enzyme-substrate ratio of 1:50.

two moles of isoleucine per mole of protein. However, two moles of histidine are released from mixed β -lactoglobulin at pH 9.2 (9) and two moles from the S-sulfo compounds at pH 8.5. This would suggest that the slow release of histidine can be attributed to secondary and tertiary structure phenomena which inhibit the release

of the second mole of histidine. In addition, the hybridization experiments of Townend *et al.* (20), the amino acid composition of the two proteins⁶ and the fingerprinting studies of proteolytic digests of the two

⁶ W. G. Gordon, J. J. Basch, and E. B. Kalan, private communication. K. A. Piez *et al.*, private communication.

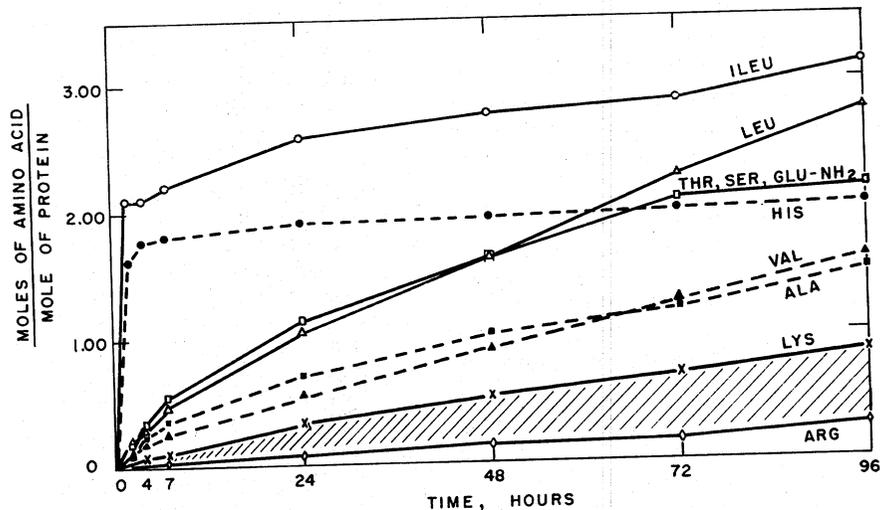


FIG. 5. Hydrolysis of β -lactoglobulin A by carboxypeptidases A and B at pH 8.5, 37°C., with enzyme-substrate ratios of 1:50 for both enzymes. The amino acids found in the shaded area are glutamic acid, tyrosine, phenylalanine, aspartic acid, glycine, methionine, and tryptophan. The threonine, serine, glutamine line represents the sum of these three components (mol. wt. S-sulfo- β -lactoglobulin 38,100).

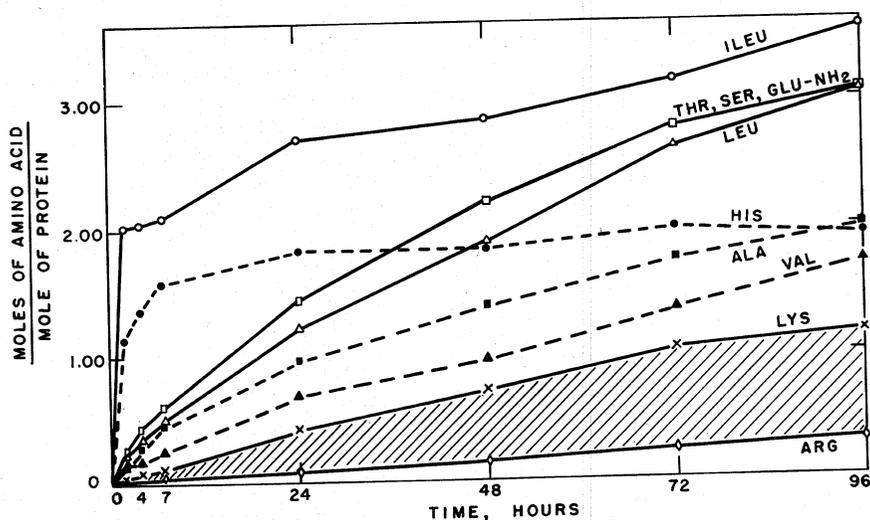


FIG. 6. Hydrolysis of β -lactoglobulin B by carboxypeptidases A and B at pH 8.5, 37°C., with enzyme-substrate ratios of 1:50 for both enzymes. The amino acids found in the shaded area are the same as those in Fig. 5.

phenotypes,⁷ suggest that the two subunits of each of the β -lactoglobulins are identical. If the two subunits are the same, then it is unlikely that each chain would be hydrolyzed at a different rate to account for one mole of histidine released at pH 7.6 (9) or

⁷R. Townend *et al.*, private communication.
E. W. Davie *et al.*, private communication.

at pH 8.5 in the present study. It would seem more likely that both chains are hydrolyzed slowly at these conditions and more rapidly after denaturation at pH 9.2 (9) or when disulfide bonds are broken by derivative formation as with the S-sulfo compounds. The present results do not, however, unequivocally rule out two differ-

ing subunits within a molecule of 36,000 mol. wt. If one chain has a sequence of X.His.Ileu and the second a sequence of Y.His.Ileu, the present kinetic analysis is not able to reveal this subtlety. The possible identity of the amino acid adjacent to histidine is discussed below.

Since only isoleucine and histidine are liberated with carboxypeptidase A, one would assume that a basic amino acid occupies the third position. Carboxypeptidase B should then release an arginine or lysine residue following the histidine. However, when the combination of enzymes is used, leucine appears as the amino acid following histidine. It is apparent that the carboxypeptidase B preparation has in some way affected the rate of release of isoleucine and histidine (compare Figs. 1 and 2 with Figs. 5 and 6). These amino acids appear now at about the same time as in the experiment with the sulfo compounds plus carboxypeptidase A (Figs. 3 and 4). Because of the uncertainty of the enzymic purity of the carboxypeptidase B (proaminase) preparation, the position of the amino acids following histidine is still not unequivocally defined. This aspect is being investigated further. The sequence is further complicated because of the presence of glutamine. This appears exactly coincident with the threonine and serine peaks on chromatographic separation. Since the three amino acids appear as two overlapping peaks, none can be individually calculated. Since the value shown in Figs. 5 and 6 is the sum of the thr-ser-glu-NH₂ peaks, no one of the components can exceed leucine, although the total is of the same magnitude. Alanine and valine appear fairly close to the carboxyl end. The second isoleucine is found in this region also. The remaining amino acids shown are present in less than one mole per mole amounts and little can be said about their relative sequence. More definitive information concerning the

amino acid sequence at the carboxyl end of these two molecules is being sought from peptides obtained from proteolytic digests of the two proteins.

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