

Studies on the Specificity of Protaminase

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Received March 21, 1958

INTRODUCTION

In our recent paper (1) evidence was presented that protaminase, described by us (2, 3) years ago, was not identical to any of the known proteolytic enzymes of the pancreas, but represented a new enzyme. The action mechanism of this enzyme appeared to be confined to the hydrolysis of free arginine from the C-terminus of protamines. The present studies describe the extension of this work to the specific action of protaminase and, in addition, evidence is brought forward that carboxypeptidase-B, described by Folk *et al.* (4, 5), is identical with protaminase.

EXPERIMENTAL

The enzyme preparations used in this work were as follows: Protaminase and the "heat-resistant" enzyme were prepared as described previously (1). They were entirely free of tryptic and chymotryptic activities, but contained traces of carboxypeptidase, which were impossible to remove even upon further adsorption with $C\gamma$ -aluminum hydroxide suspension. The preparations were freed of glycerol by dialysis against distilled water in a cellophane tube, and the original volume was restored by directing an air current against it at room temperature. The above treatment did not impair the enzyme activities, and the final solution contained 0.096 mg. protein nitrogen/ml. The twice-crystallized trypsin preparation possessed no carboxypeptidase activity, but about 0.1% chymotrypsin activity. The crystalline chymotrypsin used was free of tryptic and carboxypeptidase activities. The four-time crystallized carboxypeptidase employed was treated with diisopropyl fluorophosphate (DFP) and did not exhibit any tryptic, chymotryptic, and protaminase activities. The enzymic assays were carried out as described previously (1). The enzyme-protein ratio used in all experiments was 1:100 on a dry-weight basis. The α -lactalbumin was crystallized three times and was kindly supplied by Dr. W. G. Gordon of this laboratory. All calculations were based on 15,500 as the molecular weight of α -lactalbumin.

Hydrolysis of α -Lactalbumin by Trypsin and Subsequent Cleavage by Protaminase

Inasmuch as tryptic hydrolysis of a protein results in peptide fragments with C-terminal arginine and lysine (6-10), α -lactalbumin was subjected to hydrolysis

by trypsin. The resultant peptide fragments then were hydrolyzed by protaminase to find out whether the C-terminal basic amino acids were cleaved by this enzyme. For this purpose, α -lactalbumin was hydrolyzed by trypsin in 1:100 enzyme to substrate ratio at 37°. Two-milliliter aliquots, containing 40 mg. protein in 0.05 *M* phosphate buffer solution of pH 8.0, were taken at various time intervals for the determination of the increase in α -amino groups by the Van Slyke method. Simultaneously, the same amount was taken to determine the possible liberation of free amino acids by the method of Moore and Stein (11) using the 15-cm. Dowex 50 column. Preparatory to the above procedure (11), the 2-ml. aliquots were precipitated with 0.5 ml. of 25% trichloroacetic acid (TCA) (final TCA concentration 5%) and centrifuged, and the supernatant was freed of the TCA by extracting three times with ether. After evaporation of ether traces in the water phase by a combination of heat and vacuum, aliquots containing 6 mg. of the original protein were placed on the 15-cm. column for chromatography. The ninhydrin color was developed according to the modified procedure of Moore and Stein (12). The peptide fragments, soluble in 5% TCA, did not interfere with the determination of the amino acids which emerged as a distinct peak from the 15-cm. column, since these fragments appeared as two large sharp fractions, one with a peak at the 6th and the second at the 28th ml. of effluent, and thus did not interfere with the possible determination of tyrosine plus phenylalanine, and tryptophan. After completion of the tryptic digestion of α -lactalbumin, the enzyme was inhibited with DFP (to 10 ml. of digest, 0.1 ml. of 0.05 *M* DFP was added) and protaminase which is unaffected by the inhibitor (1) was added. For this purpose, 2-ml. aliquots of the tryptic digest, containing 40 mg. protein, were incubated with 0.5 ml. protaminase solution for various periods at 37°. The increase in α -amino nitrogen was measured. To determine the subsequent free amino acids liberated by protaminase, the above aliquots were precipitated with 0.5 ml. of 30% TCA and treated in the same manner as described for the tryptic digest.

The results obtained and presented in Fig. 1 show that, as expected from the lysine and arginine content of α -lactalbumin (13) (12 and 1 moles), trypsin cleaved 13 peptide bonds, one of which was due to the liberation of 1 mole lysine. Successive hydrolysis of the tryptic digest by protaminase produced the cleavage of 12 additional peptide bonds after 7 hr. incubation, about 11 of which were due to free lysine (10.6 moles) and the remaining bond due to the liberation of 1 mole arginine. At this point only about 0.3 mole tyrosine + phenylalanine (the 15-cm. column gives only the sum of these two amino acids) was liberated. After 24 hr. hydrolysis by protaminase, the lysine and arginine values remained the same (since all of them were liberated), while the value for tyrosine + phenylalanine increased to about 1.8 moles and, in addition, 0.3 mole of free histidine was released. The moderate concentration of tyrosine + phenylalanine and the histidine traces after 24 hr. incubation are ascribed to slight chymotryptic impurity of trypsin and to the successive action of carboxypeptidase traces present as a contaminant of the protaminase preparation.

Hydrolysis of α -Lactalbumin by Trypsin and Subsequent Cleavage by Carboxypeptidase

To contrast the action of protaminase (Fig. 1) on the tryptic digest of α -lactalbumin with that of carboxypeptidase, the same experiments as described for Fig. 1 were carried out except that four-time crystallized carboxypeptidase was subsequently used instead of protaminase. The results presented in Fig. 2 show that while trypsin acted in the same manner as before, successive action of carboxypeptidase

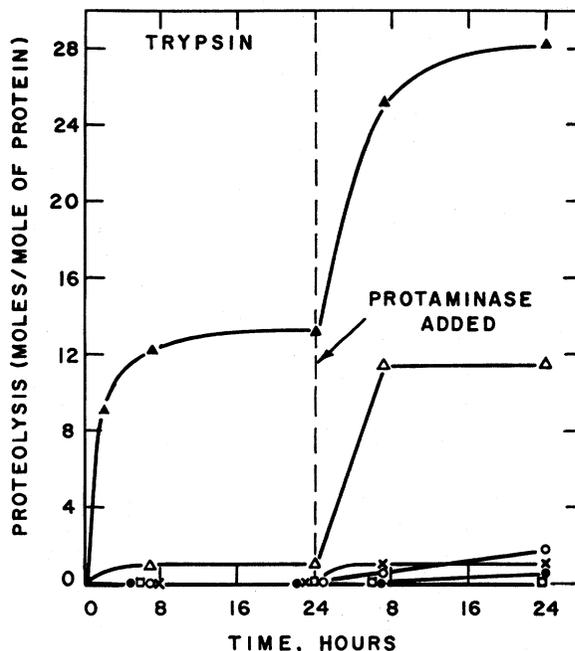


Fig. 1. Hydrolysis of α -lactalbumin by trypsin and subsequent cleavage by protaminase. \blacktriangle — \blacktriangle peptide bond; \circ — \circ tyrosine + phenylalanine; \square — \square tryptophan; \bullet — \bullet histidine; \triangle — \triangle lysine; \times — \times arginine.

produced no hydrolysis after 7 hr. incubation, and only after 24 hr. could we observe the liberation of 1.9 moles tyrosine + phenylalanine, due to the chymotryptic impurity of our trypsin preparation.

Hydrolysis of α -Lactalbumin by Chymotrypsin and Subsequent Action of Protaminase

The experimental details were the same as described in Fig. 1, except that chymotrypsin was used in the place of trypsin. The results obtained and presented in Fig. 3 demonstrate that chymotrypsin hydrolyzed α -lactalbumin to the extent of about 12 peptide bonds per mole of protein, two of which resulted from the liberation of 2 moles tryptophan. Successive action of protaminase on the chymotryptic digest produced a slow increase in peptide bonds cleaved, reaching the value of 2.3 after 24 hr. incubation and due to the liberation of 2.4 moles tyrosine + phenylalanine. The slow moderate increase of these amino acids is very likely attributable to traces of carboxypeptidase in the protaminase preparation.

Hydrolysis of α -Lactalbumin by Chymotrypsin and Subsequent Cleavage by Carboxypeptidase

These experiments were identical with those presented in Fig. 3, except that carboxypeptidase was employed instead of protaminase. The results obtained and pre-

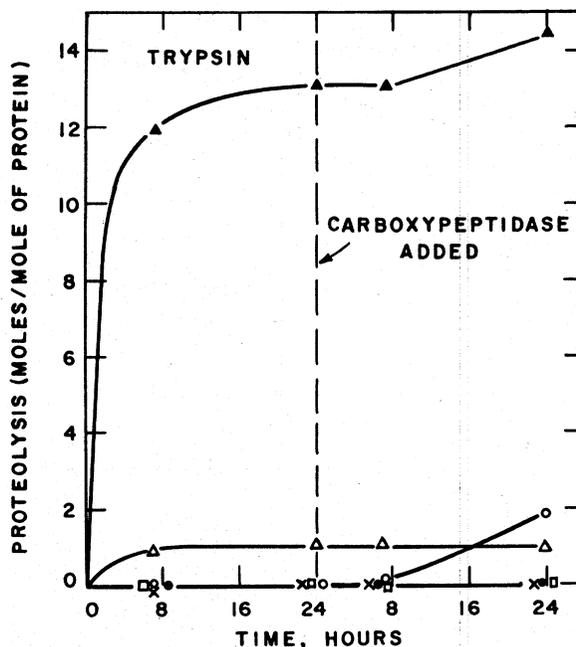


FIG. 2. Hydrolysis of α -lactalbumin by trypsin and subsequent action by carboxypeptidase. \blacktriangle — \blacktriangle peptide bond; \circ — \circ tyrosine + phenylalanine; \square — \square tryptophan; \bullet — \bullet histidine; \triangle — \triangle lysine; \times — \times arginine.

sented in Fig. 4 show that, as in Fig. 3, chymotrypsin hydrolyzed α -lactalbumin to the extent of 12 peptide bonds/mole protein; two of these bonds were due to the liberation of 2 moles tryptophan. The successive action of carboxypeptidase on this digest completely liberated all the tyrosine and phenylalanine present in α -lactalbumin (5 and 4 moles) after 3 hr. incubation and produced 1 mole histidine, with no further change even after 24 hr. incubation. The increase in peptide bonds cleaved due to the successive action of carboxypeptidase amounted to about 19, a value which is about 9 bonds in excess when the 10 due to the liberation of 5 moles tyrosine, 4 moles phenylalanine, and 1 mole histidine are subtracted. This excess is obviously due to the amino acids liberated, which are not determined by the 15-cm. chromatographic column used in our work.

Preparation of *S*-(β -Aminoethyl)cysteine- α -lactalbumin

A few years ago Lindley (14) made the interesting observation that conversion of the cysteine unit within a protein (wool protein) molecule to *S*-(β -aminoethyl)-cysteine resulted in susceptibility of the adjacent peptide bond to trypsin. This susceptibility to trypsin was ascribed to the close similarity of *S*-(β -aminoethyl)-cysteine to lysine. In view of Lindley's (14) observation, it was of interest to convert the cysteine residue of α -lactalbumin to the above derivative and study the action of trypsin and subsequent effect of protaminase on this protein derivative.

To follow this conversion quantitatively, an analytical method for the deter-

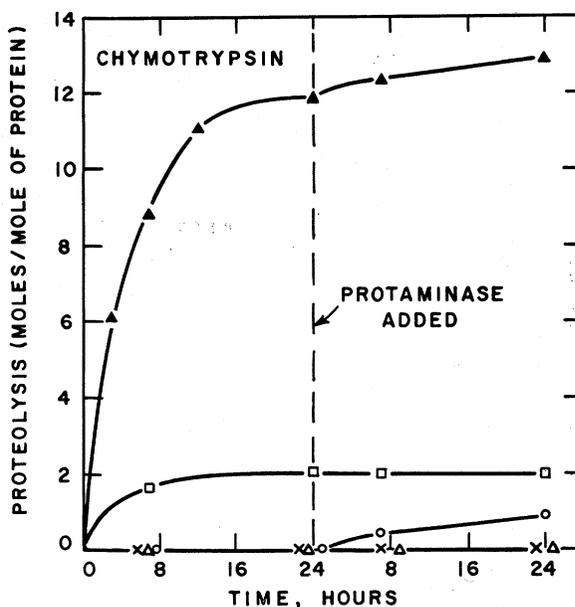


Fig. 3. Hydrolysis of α -lactalbumin by chymotrypsin and subsequent action of protaminase. \blacktriangle — \blacktriangle peptide bond; \circ — \circ tyrosine + phenylalanine; \square — \square tryptophan; \bullet — \bullet histidine; \triangle — \triangle lysine; \times — \times arginine.

mination of *S*-(β -aminoethyl)cysteine had to be worked out. For this purpose, the chromatographic procedure of Moore and Stein (11), using the 15-cm. Dowex 50 column, proved very satisfactory. A reference sample of *S*-(β -aminoethyl)-L-cysteine¹ was placed on the 15-cm. column and, after employing in detail the experimental procedure of Moore and Stein (11), a distinct sharp peak was obtained at the 116th 1-ml. fraction, a position just between the peaks due to lysine and ammonia. The color yield relative to that obtained with leucine was found to be 1.10. The chromatographic recovery of *S*-(β -aminoethyl)cysteine, calculated on this basis was essentially 100%. Analysis of a mixture of lysine and *S*-(β -aminoethyl)cysteine resulted in an excellent separation of these two amino acids, with quantitative recovery. A complication, however, was encountered when these two amino acids were analyzed together with NH_4Cl . The ammonia peak had distinct shoulders on both sides, one of which partially merged with the *S*-(β -aminoethyl)cysteine peak. To avoid this complication, the above experiment was repeated, and to the 1-ml. fractions collected in this region, 0.2 ml. of 2.5 *N* NaOH was added and placed in a drying oven at 110° for 2.5 hr. to drive off the ammonia. After this heating period, the fractions were essentially dry, and to each tube then was added 1 ml. of 6% by volume acetic acid and the ninhydrin color was developed as before (12). Complete recovery of both amino acids was obtained, indicating that the alkali treatment had no destructive effect.

¹ Obtained through the courtesy of Dr. H. Lindley of the Wool Textile Research Laboratory, Parkville, Australia.

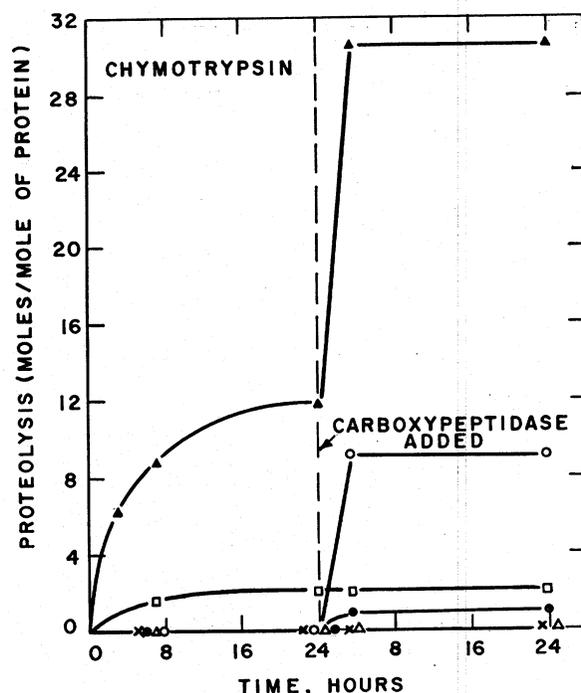


FIG. 4. Hydrolysis of α -lactalbumin by chymotrypsin and subsequent cleavage by carboxypeptidase. \blacktriangle — \blacktriangle peptide bonds; \circ — \circ tyrosine + phenylalanine; \square — \square tryptophan; \bullet — \bullet histidine; \triangle — \triangle lysine; \times — \times arginine.

After establishing the analytical procedure, an attempt was made to convert the eight half-cystine residue present in α -lactalbumin (13) into S -(β -aminoethyl)-cysteine according to the procedure of Lindley (14). Since α -lactalbumin contains eight half cystines, according to the analysis of Gordon and Ziegler (13), theoretically, eight moles S -(β -aminoethyl)cysteine should be formed. Actually, however, only half this value, namely 3.9 moles, was obtained. To reaffirm the position of the S -(β -aminoethyl)cysteine peak, the protein hydrolyzate was spiked with a known amount of reference sample of S -(β -aminoethyl)cysteine and chromatographed as described. The resulting peak was additive, with complete recovery, demonstrating that the new amino acid formed in α -lactalbumin was identical with S -(β -aminoethyl)-cysteine. Owing to the partial substitution of the SH groups of α -lactalbumin with Lindley's procedure (14), it was necessary to decide whether this was produced by an incomplete reaction or a partial reactivity of the SH groups of α -lactalbumin. To resolve these possibilities, the partially substituted α -lactalbumin was subjected again to the same procedure, except that, as advised by Dr. H. Lindley in a personal communication, the substitution was carried out at pH 9.0 and the amine was added in five equal portions over the reaction period. The resulting product, however, did not differ from the first one inasmuch as the S -(β -aminoethyl)cysteine content was again 3.9 moles. It would appear, therefore, that under the experimental conditions employed, half of the SH groups were inert.

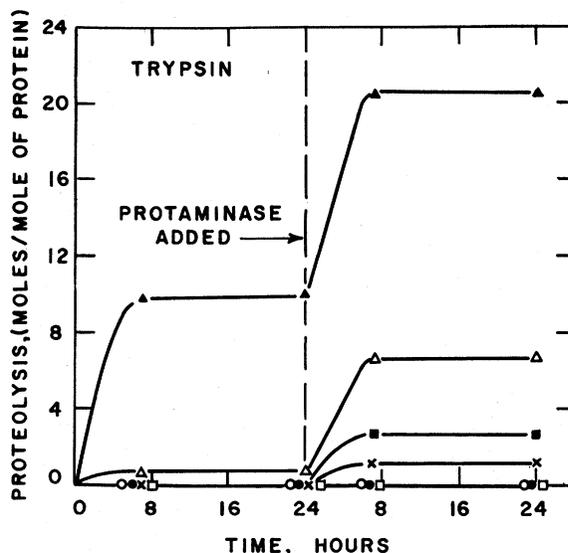


FIG. 5. Hydrolysis of *S*-(β -aminoethyl)cysteine- α -lactalbumin by trypsin and subsequent cleavage by protaminase. \blacktriangle — \blacktriangle peptide bond; \circ — \circ tryptophan + phenylalanine; \square — \square tryptophan; \bullet — \bullet histidine; \triangle — \triangle lysine; \times — \times arginine; \blacksquare — \blacksquare *S*-(β -aminoethyl)cysteine.

*Hydrolysis of S-(β -Aminoethyl)cysteine- α -lactalbumin by
Trypsin and Subsequent Cleavage by Protaminase*

The experimental conditions were the same as described for studies presented in Fig. 1. The substituted protein did not give a clear solution, and an undetermined portion remained in suspension even during the enzymic digestion. The results obtained and presented in Fig. 5 show that trypsin hydrolyzed the substituted protein, yielding only 10 ruptured peptide bonds instead of the expected 17 (due to 12 lysine, 1 arginine, and 4 *S*-(β -aminoethyl)cysteine molecules). Whether incomplete solubility of the substituted protein caused these low hydrolytic values cannot be answered with certainty at present. The tryptic digestion of the substituted α -lactalbumin resulted in the formation of only 0.6 mole free lysine. Addition of protaminase to this tryptic digest produced, after 7 hr. incubation, an additional increase of 10.5 peptide bonds cleaved, which remained constant after 24 hr. incubation. The amino acids liberated during the action of protaminase in this case amounted to an additional formation of 6.1 moles lysine, 2.7 moles *S*-(β -aminoethyl)cysteine, and 1.1 moles arginine. The total number of 10.5 peptide bonds hydrolyzed by protaminase, as measured by the Van Slyke procedure, is in fairly good agreement with the 9.9 moles of free amino acids (6.1 moles lysine + 2.7 moles *S*-(β -aminoethyl)cysteine and 1.1 mole arginine) recovered on the short column. It should be noted, however, that there was a partial release of lysine and *S*-(β -aminoethyl)cysteine (namely, 6.7 moles lysine out of a total of 12, and 2.7 moles *S*-(β -aminoethyl)cysteine out of a total of 4), while there was complete liberation of the 1 mole arginine present in the protein. The incomplete liberation of these amino acids by protaminase, as mentioned before, might be due to the incomplete solubility of this protein derivative.

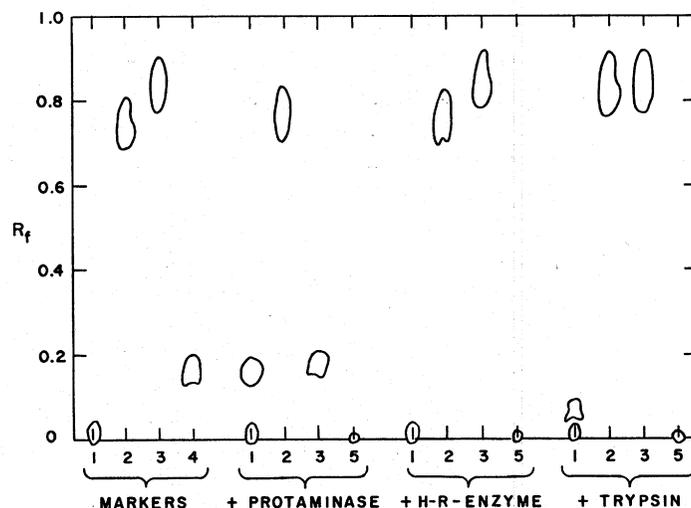


FIG. 6. Action of protaminase, "heat resistant" enzyme, and trypsin on various synthetic substrates. 1 = poly-L-lysine; 2 = hippuryl-L-lysine amide; 3 = hippuryl-L-lysine; 4 = L-lysine; 5 = enzymes.

These findings, nevertheless, sufficiently demonstrate that trypsin can hydrolyze the peptide bonds adjacent to *S*-(β -aminoethyl)cysteine as well as to lysine and arginine, and the subsequent action of protaminase can hydrolyze these C-terminal amino acids to their free form.

The Action of Protaminase on Synthetic Substrates

The action of protaminase was studied on the following substrates: poly-L-lysine² ($n = 38$), hippuryl-L-lysine amide³ and hippuryl-L-lysine.³ To aliquots containing 2 mg., respectively, of hippuryl-L-lysine amide and hippuryl-L-lysine and aliquots containing 5 mg. of poly-L-lysine, the enzyme was added in a 1:100 ratio of enzyme to substrate; the phosphate buffer (of pH 8.0) concentration was 0.05 *M*; the total volume, 1 ml. After 30 min. incubation at 37°, 0.01-ml. aliquots were chromatographed on Whatman No. 1 paper, using the descending technique with *n*-butyl alcohol-acetic acid-water solvent (15). Simultaneously, identical experiments were carried out in which the action of protaminase was compared with that of trypsin and the "heat resistant" enzyme, recently described by us (1). The results obtained and presented in Fig. 6 show that protaminase hydrolyzed poly-L-lysine, liberating free lysine; hippuryl-L-lysine was completely hydrolyzed to free lysine and hippuric acid, while hippuryl-L-lysine amide was not hydrolyzed at all. In contrast, the "heat resistant" enzyme (1) did not hydrolyze any of these substrates; trypsin, in agreement with Katchalski's (16) findings, hydrolyzed poly-L-lysine, but without free lysine liberation, did not cleave hippuryl-L-lysine, and hydrolyzed hippuryl-L-lysine amide to hippuryl-L-lysine and ammonia.

² Kindly supplied by Kremers-Urban Co., Milwaukee, Wisconsin.

³ Kindly supplied by Dr. J. E. Folk of the National Institute of Health, Bethesda, Maryland.

DISCUSSION

The nonidentity of protaminase with any of the known proteolytic enzymes of the pancreas was demonstrated by us recently (1). The enzyme appeared to confine its action to the hydrolysis of free arginine from the C-terminal end of protamines. In the present work we have extended our studies to the specificity of protaminase on the tryptic breakdown products of α -lactalbumin.

It has been repeatedly observed (6-10, 17) that trypsin specifically attacks bonds adjacent to arginine and lysine with the formation of peptide fragments with C-terminal arginine and lysine. In view of these observations, it was of interest to discover whether these tryptic degradation products would provide natural substrates for the action of protaminase. As anticipated, trypsin hydrolyzed α -lactalbumin, resulting in the cleavage of 13 peptide bonds (17). [There are 12 lysine and 1 arginine moles in this protein (13).] Simultaneous chromatographic analysis (11) showed that one of the peptide bonds cleaved was due to the liberation of one mole of free lysine—an occurrence which might be explained by an arginyl-lysine or lysyl-lysine sequence within the α -lactalbumin molecule. Successive action of protaminase on this tryptic digest (Fig. 1) resulted in an additional cleavage of 12 peptide bonds after 7 hr. incubation, while simultaneous chromatographic analysis (11) demonstrated the liberation of 1 mole arginine and 10.6 moles of additional lysine in their free form. The total amount of free basic amino acids liberated would account for the entire concentration of these amino acids in this protein (13). These observations demonstrate that the 12 peptide bonds protaminase cleaved from the tryptic digest of α -lactalbumin were confined entirely to the liberation of all the remaining bound lysine (11 moles) and arginine (1 mole). The appearance of a small amount of free tyrosine plus phenylalanine and histidine after 24 hr. incubation is ascribed to enzymic impurities in both enzyme preparations.

The specificity of protaminase was tested on the chymotryptic digest of α -lactalbumin (Fig. 3). In this case, chymotrypsin should fragment the protein chiefly into peptides with C-terminal tyrosine, phenylalanine, and tryptophan residues, and subsequent action of protaminase should produce no further hydrolysis. This assumption proved correct; however, a slight additional hydrolysis was produced by the carboxypeptidase contamination of our protaminase preparation.

It was of interest to note that 2 moles of free tryptophan were always liberated from α -lactalbumin by chymotrypsin, indicating a possible sequence of tryptophyl-tryptophan, tyrosyl-tryptophan, or phenylalanyl-tryptophan in the α -lactalbumin molecule.

The inertness of carboxypeptidase toward the tryptic digest of α -lactal-

bumin (Fig. 2), in contrast to the reactivity of protaminase (Fig. 1), demonstrates the requirement of C-terminal basic amino acids for the action of protaminase and, at the same time, confirms the known specificity of carboxypeptidase (18).

In contrast to the inertness of protaminase on the chymotryptic digest of α -lactalbumin, the action of carboxypeptidase in this case (Fig. 4) brought about a very rapid release of all the tyrosine and phenylalanine present (5 and 4 moles) and 1 mole histidine. The hydrolysis, produced by carboxypeptidase in excess of these amino acids, was not resolved by the chromatographic column (11) employed and was presumably due to sequential release of amino acid by this enzyme. The above findings are in accord with the existing knowledge on the specificity of chymotrypsin and carboxypeptidase (18). Recently, Lindley (14) observed that the conversion of the cysteine residues to *S*-(β -aminoethyl)cysteine within a protein molecule rendered the adjacent peptide bond susceptible to the action of trypsin. When the above conversion was applied to α -lactalbumin, however, a protein derivative resulted, with half of the total eight half cystines (13) converted into *S*-(β -aminoethyl)cysteine. Tryptic hydrolysis of this protein derivative and subsequent cleavage by protaminase liberated free *S*-(β -aminoethyl)cysteine in addition to lysine and arginine (Fig. 5). These findings would indicate that trypsin, in accordance with Lindley, also cleaves bonds adjacent to *S*-(β -aminoethyl)cysteine, which when present at the C-terminal end of a peptide, is susceptible to the action of protaminase.⁴ The identical behavior of protaminase and carboxypeptidase-B (4, 5) toward such synthetic substrates as hippuryl-L-lysine, hippuryl-L-lysine amide, and poly-L-lysine would indicate the identity of these two enzymes (Fig. 6). The inertness of the "heat resistant" enzyme (1) toward these synthetic substrates, on the other hand, would distinguish this enzyme from both protaminase and trypsin.

On the basis of the above experiments, it is concluded the protaminase is an exo-enzyme, highly specific for the cleavage of C-terminal arginine, lysine, and *S*-(β -aminoethyl)cysteine. It complements the action of trypsin as carboxypeptidase does chymotrypsin.

SUMMARY

The action of protaminase liberates all C-terminal arginine and lysines from the tryptic digest of α -lactalbumin.

Protaminase is inert toward the chymotryptic digest of α -lactalbumin as carboxypeptidase is nonreactive to the tryptic digest of this protein.

⁴ While this paper was in preparation, Tietze, Gladner, and Folk [*Biochim. et Biophys. Acta* **26**, 659 (1957)] made a related observation showing that C-terminal *S*-(β -aminoethyl)cysteine is susceptible to the action of carboxypeptidase-B.

In accordance with the specificity of carboxypeptidase, the addition of this enzyme to a chymotryptic digest of α -lactalbumin effected a rapid liberation of all the C-terminal tyrosine and phenylalanine together with other amino acids.

Protaminase hydrolyzes peptides with C-terminal arginine, lysine, and S-(β -aminoethyl)cysteine, and its action is confined to the liberation of these amino acids.

Protaminase and carboxypeptidase-B are identical enzymes.

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