PN 1240

Preliminary contribution to the study of the disulfide bonds in hen's egg-white lysozyme

Two years ago we have indicated a provisional formula of hen's egg-white lysozyme[1] and in 1961 we established the primary structure of this enzyme[2]. Since this date we have studied the location of the –S–S– bonds. Lysozyme contains four cystine residues per molecule[3]. Our present aim was to isolate after an enzymic digestion the four cystine-containing peptides, to submit them to a performic acid oxidation, and to determine which of the half-cystine residues were joined in the native enzyme. Some preliminary experiments made by paper electrophoresis and paper chromatography were unsuccessful. We have then chosen the method described by SPACKMAN, STEIN AND MOORE[4] in their study of the tertiary structure of ribonuclease.

Hen's egg-white lysozyme was obtained from Armour. Native lysozyme (1 g) was dissolved in 60 ml of 0.05 N sodium citrate buffer (pH 1.9) and 18 mg of pepsin were added. After 24 h at 28°, the solution was adjusted to pH 6.75 with NaOH and 10 mg of chymotrypsin were added. The solution remained again 24 h at 28°. To stop the enzymic reaction the solution was brought to pH 2.5 with 1 N HCl. Aliquots were withdrawn for ninhydrin analysis at the beginning of the hydrolysis, before the addition of pepsin and of chymotrypsin, and at the end of the hydrolysis in order to determine the extent of the enzymic reaction.

The entire hydrolysate was chromatographed on a 140 × 2.4 cm column of Dowex-50 X2 with 0.2 N sodium citrate buffer (pH 3.1). Gradient elution was carried out as indicated in Fig. 1. A stepwise elution has been employed with the alkaline buffers. The procedure employed for the analysis of cystine-containing peptides is a modification of the method described by KASSELL AND BRAND[5].

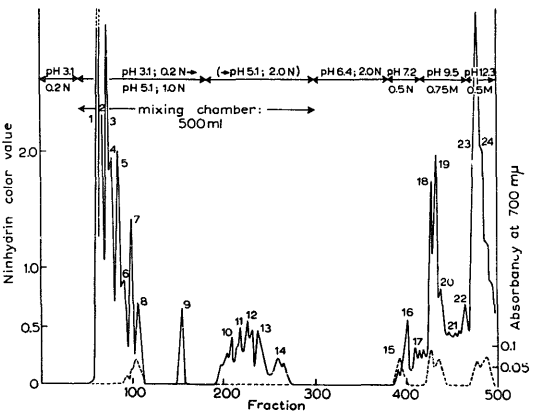


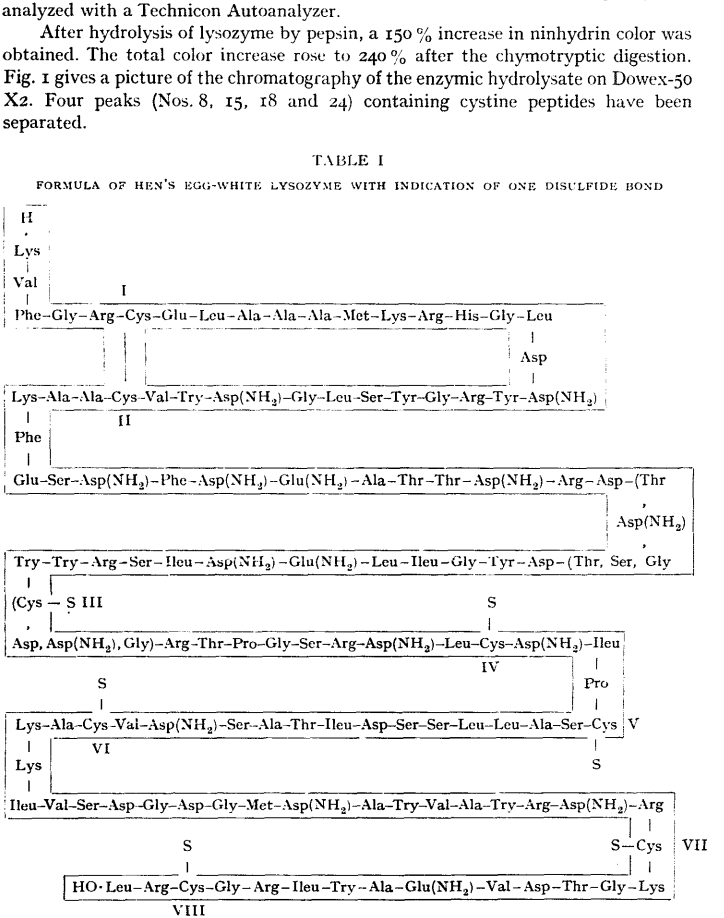
Fig. 1. Chromatography of an enzymic hydrolysate of lysozyme on Dowex-50 X2 (140 × 2.4 cm). ——, ninhydrin color value (after alkaline hydrolysis) expressed as mM leucine equivalent; – – – –, phosphotungstate reaction ($A_{700\,m\mu}$) for –S–S– bonds. 10-ml fractions were collected.

The fractions containing the cystine peptides were pooled, desalted, oxidized with performic acid according to HIRS[6], and individually rechromatographed on a Dowex-50 X2 column (20 × 1 cm) equilibrated with a 0.2 N sodium citrate buffer (pH 3.1). Gradient elution was begun after 0.5 l by allowing a 2 N sodium acetate buffer at pH 5 to flow into the buffer at pH 3.1 in a mixing chamber with a capacity of 250 ml. The cysteic acid peptides were desalted, submitted to different tests of purity and analyzed with a Technicon Autoanalyzer.

After hydrolysis of lysozyme by pepsin, a 150 % increase in ninhydrin color was obtained. The total color increase rose to 240 % after the chymotryptic digestion. Fig. 1 gives a picture of the chromatography of the enzymic hydrolysate on Dowex-50 X2. Four peaks (Nos. 8, 15, 18 and 24) containing cystine peptides have been separated.

TABLE I

FORMULA OF HEN'S EGG-WHITE LYSOZYME WITH INDICATION OF ONE DISULFIDE BOND



Peak 18 has been submitted to a performic acid oxidation and only two cysteic acid peptides have been characterized after rechromatography. Peak 18-A contained Ala_1, Val_1, $CySO_3H_1$ and was obtained with a yield of 8%. Peak 18-B contained Gly_1, Glu_1, Leu_1, Arg_1, $CySO_3H_1$ and was obtained with a yield of 10%. It can be noted here that the yields of these cysteic acid peptides were of the same order as those obtained by SPACKMAN, STEIN AND MOORE[4] in their experiments with ribonuclease. From these amino acid compositions and the sequence previously published[2], we can conclude that the original lysozyme molecule contains a disulfide bond joining half-cystine residues I and II.

The purification of the cysteic acid peptides from Peaks 8, 15 and 24 was very difficult, especially from the two latter. It is, however, worth mentioning that in these peaks only traces of the peptides mentioned above have been detected. The difficulties due probably to some basic or very aromatic peptides enjoined us to use another procedure (chromatography of the enzymic hydrolysate on Dowex-1 X2 columns with very basic volatile buffers). But we can already indicate that we found again the disulfide bond joining half-cystine residues I and II.

The results reported in the present communication allow us to complete our formula of hen's egg-white lysozyme as indicated in Table I. This formula differs from that previously indicated only by the suppression of some brackets obtained thanks to a study of the partial hydrolysate of the corresponding tryptic units and by a slight difference in the relative disposition of half-cystine residues IV and VI; this latter result has been obtained after a new study of the performic acid-oxidized tryptic unit containing half-cystine residues IV, V and VI[7]. After a chymotrypsic digestion, it was found that the C-terminal sequence contains only one half-cystine residue and that the two others are located as indicated in the formula. It is hoped that the details of the present investigations will be published in this same review.

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Laboratory of Biological Chemistry, Faculty of Science, Paris (France)

JACQUELINE JOLLÈS
JUAN JAUREGUI-ADELL
PIERRE JOLLÈS

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