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PREPARATION OF RADIOACTIVE EGG WHITE LYSOZYME *IN VIVO*

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A white leghorn hen was injected with tryptophan-3-C¹⁴. The labelled lysozyme was prepared by the DEAE-cellulose chromatography from the radioactive egg white obtained during 24-48 hours after the injection.

It was elucidated that the tryptophan-3-C¹⁴ was incorporated into only tryptophan residues of lysozyme molecule.

INTRODUCTION

It has been expected to obtain the radioactive lysozyme for an investigation of the behaviour of lysozyme in living cells. In 1962, Mandeles and Ducay (1) isolated chromatographically the radioactive conalbumin, ovalbumin and lysozyme from egg white after injection of lysine-1-C¹⁴ and glutamic acid-1-C¹⁴ into a laying hen. We have also prepared the radioactive lysozyme labelled with tryptophan-3 C¹⁴ *in vivo*.

EXPERIMENTAL METHODS

A white leghorn hen was injected intravenously with 1.0 ml of DL-tryptophan-3-C¹⁴ aqueous solution (0.1 mc; specific radioactivity: 52 mCi/mmole; Daiichi Pure Chemicals, Tokyo. Lot No. 210-10). The eggs were collected subsequently, the whites separated from the yolks. The radioactivities and lysozyme contents of the lyophilized egg whites obtained during first 16 days after injection of tryptophan-3-C¹⁴ were determined.

The white was dissolved in 30 ml of 0.02 M glycine and followed by centrifugation (10,000 rpm, 10 min) for removal of insoluble materials. The supernatant was charged on a column (5×50 cm) of DEAE-cellulose, equilibrated with 0.02 M glycine. The elution was stepwise carried out by 0.02 M glycine and a mixture of 0.02 M glycine, 0.1 M KH₂PO₄, 0.1 M NaCl and 0.03 N HCl by the method of Mandeles (2). The radioactive lysozyme fractions were pooled, and followed by

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dialysis in a Visking tube (32/36 inches) against distilled water. The salt-free lysozyme was again dissolved in 10 ml of 0.02 M glycine after lyophilization, and then it was rechromatographed on a column (5×40 cm) of DEAE-cellulose by the elution method as described above. The radioactive lysozyme fractions were pooled, dialysed against distilled water, and then lyophilized. Some preparations were subjected to gel filtration on a column (5×100 cm) of Bio Gel P-2 (50-100 mesh) with 1% acetic acid after DEAE-cellulose chromatography.

Lysozyme content was assayed by lytic activity against *Micrococcus lysodeikticus* (3). The fresh suspension (3.0 ml) which contained 0.3 mg of lyophilized cells per ml in 1/15 M phosphate buffer, pH 6.2, was incubated with 1.0 ml of 0.3 M NaCl-1/15 M phosphate buffer, pH 6.2, for 3 minutes at 35°C. The sample (0.1 ml) was added to 4.0 ml of the cell suspension, and followed by incubation for 5 minutes at 35°C. A decrease of turbidity of the mixture at 540 m μ was measured. Lysozyme content was estimated by reference to the standard hen egg white lysozyme (6X recrystallized, Seikagaku Kogyo Co., Tokyo). Radioactivity was determined for the sample (0.05 ml) in 10 ml of a scintillator (toluene-methanol, 7/3 by volume, 0.4% PPO and 0.02% POPOP) by a liquid scintillation spectrometer, Packard Model 3003. The purified lysozyme was dissolved in water, and followed similarly by the determination of lytic activity and radioactivity.

RESULTS AND DISCUSSION

The analytical results of radioactivities and lysozyme contents of egg whites are shown in Fig. 1. The weight of lyophilized egg white was approximately 4 g and the lysozyme content was about 40 mg per g of lyophilized egg white. It can be seen that the radioactivity is chiefly found in the egg white during 24 to 48 hours after injection of tryptophan-3-C¹⁴. The white (e.g. 4.2 g: 4.85×10^6 cpm; 151.1 mg of lysozyme; from the egg of hen D on the second day as shown in Fig. 1) was treated. Fig. 2 shows the elution diagrams of radioactive lysozyme on DEAE-cellulose column chromatography. 30.5 mg (total counts: 0.34×10^6 cpm) of radioactive hen egg white lysozyme was obtained from this white. The mean recovery of lysozyme and radioactivity in some preparations were 20-40% and 5-10% for the original egg white, respectively.

The lytic activity of radioactive lysozyme was nearly equal to that of the standard one. It moved as a single zone of protein when subjected to Disc electrophoresis at pH 4.0 and 8.6 (4). The amino acid composition of radioactive lysozyme was identical with that of the standard one. Six tryptophan residues were found to react with 2-hydroxy-5-nitrobenzyl bromide (5,6) after denaturation with 5 M guanidine hydrochloride.

With the object of confirming that only tryptophan residues of lysozyme molecule were labelled, the radioactive lysozyme was enzymatically digested with papain which was prepared by the method of Kimmel and Smith (7) and leucine amino peptidase (Worthington Biochemicals Co., LAPDEP 61c) according to the

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Fig. 1 Radioactivity and lysozyme content of egg white obtained after injection of tryptophan-3- C^{14} . Tryptophan-3- C^{14} aqueous solution (total counts; 1.31×10^8 cpm) was injected into hen A (—) two times and into hen D (.....) one time, respectively. Symbols of —●— and ...○... present lysozyme content of hen A and D egg white. Symbols of —▲— and ...△... present radioactivity of hen A and D egg white.

Fig. 2. Chromatography of radioactive lysozyme on DEAE-cellulose. The first chromatography is left, and the second one is right. Symbols of —○—, —●— and —△— present optical density at 280 mμ, lytic activity and radioactivity.

method of Hill and Schmidt (8). The digest was analysed by a amino acid analyser (Hitachi Model 034) which effluent run through a flow cell (2.0 ml) with anthracene as a scintillator in a liquid scintillation spectrometer before it mixed with ninhydrin solution. The elution pattern of basic amino acids and the result of amino acid analysis are shown in Fig. 3. The radioactive peak, in the order of elution, represents tryptophan. It may be evidence for the incorporation of

Fig. 3. Analysis of radioactive tryptophan in lysozyme. A column of Aminex A5 (0.9×15 cm) maintained at 55°C was used for analysis of enzymic digest of radioactive lysozyme (0.66 mg) with papain and leucine amino peptidase. Flow rate was 60 ml per hour. The yield was 64% for original lysozyme. Amino acid composition of radioactive lysozyme observed from acid hydrolysates (6N HCl) at 110°C for 24 hours; Lys: 6.0, His: 0.6, Arg: 10.2, Asp: 20.7, Thr: 6.3 (not corr.), Ser: 10.0 (not corr.), Glu: 4.9, Pro: 1.9, Gly: 12.1, Ala: 12.0 (as standard), Half-Cys: 2.1, Val: 5.6, Met: 1.9, Ileu: 5.8, Leu: 8.1, Tyr: 2.8, Phe: 2.9, Trp: 6.1 (from this figure).

tryptophan-3-C¹⁴ into only tryptophan residues of lysozyme molecule.

It is possible to use the radioactive lysozyme for the determination of distribution and absorption of lysozyme in vivo. Also, the radioactive lysozyme is probably effective as a standard protein for the C-terminal analysis with tritium labelling method (9).

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