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Studies on Biotransformation of Lysozyme. IV.[1] Radioimmunoassay of Lysozyme and Its Evaluation

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The radioimmunoassay of hen egg-white lysozyme (hen lysozyme) was investigated and evaluated in order to examine the biotransformation of the enzyme in men. ^{125}I-labeled hen lysozyme was prepared and separation of the free from the bound form of the enzyme was performed by dextran-treated charcoal method in comparison with two-antibody method. From the result, it was idnicated that dextran-treated charcoal method was useful from the viewpoint of precision, reproducibility and simplicity. The stability of hen lysozyme labeled was determined by the degree of radioactivity bound to antibody. The recovery of radioactivity bound to antibody decreased with the time lapse after labeling and it was found that the recovery rate was restored by dialysis. In this radioimmunoassay system, the competitive binding of labeled and unlabeled hen lysozyme to antibody was examined, and 0.3 to 5.0 ng/ml of the enzyme were detectable. It was found that human lysozyme had an influence on the radioimmunoassay system with over 2 μg/ml. But, the influence did not disturb the assay of hen lysozyme in human serum. Additionally, since it was found that the ratio of the bound form to the free increased with human serum, the assay of hen lysozyme in human serum was carried out using a calibration curve of the enzyme added to untreated human serum. Thus, human serum levels after oral administration of hen lysozyme were measured by the radioimmunoassay in comparison with the determination method by lytic activity. This radioimmunoassay system was found to be appropriate for the determination of hen lysozyme in human serum.

Keywords——lysozyme; ^{125}I-labeled hen egg-white lysozyme; radioimmunoassay; stability of labeled lysozyme; immunological resemblance; oral administration; intestinal absorption; man

We have previously reported details on the biotransformation of hen egg-white lysozyme in rats.[1,3] In order to obtain informations on the biotransformation of hen egg-white lysozyme administered to men, it is first necessary to establish an assay method specific for the hen egg-white lysozyme. The assay method commonly used for lysozyme in biological fluid is not able to distinguish exogenous lysozyme from endogenous one, because of determination based on lytic activity of lysozyme. For this purpose, the radioimmunoassay of hen egg-white lysozyme was investigated using radioiodinated tracer. This paper deals with a radioimmunoassay procedure for the quantitative measurement of the hen egg-white lysozyme and application to an assay of human serum levels after oral administration of the enzyme.

Experimental

Materials——Hen egg-white lysozyme-hydrochloride (abbreviated as hen lysozyme) recrystallized five times was used for ^{125}I-labeling. Rat lysozyme was purified from the urine of lysozymuria induced by a nephrotoxic agent as described previously.[1] Human leukemia lysozyme was kindly supplied by Dr. T. Moriuchi (Cancer Institute, Hokkaido University School of Medicine). Na ^{125}I (The Radiochemical Centre, England), bovine serum albumin (Fraction V, Armour Pharm. Co., abbreviated as BSA), goat anti-rabbit IgG serum (Nakarai Chemical Co.) and Micrococcus Lysodeikticus (Whashington Biochemical Co.) were used.

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3) *a*) T. Yuzuriha, K. Katayama, and T. Fujita, *Chem. Pharm. Bull.* (Tokyo), **23**, 1309 (1975); *b*) *Idem, ibid.*, **23**, 1315 (1975); *c*) T. Yuzuriha, K. Katayama, and S. Ohtake, *ibid.*, **25**, 836 (1977).

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Table I. Preparation of Radioactive Hen Lysozyme

1.	$Na^{125}I$ (5 mCi/50 μl)	1.5—2.0 mCi	20 μl
2.	Hen lysozyme in 0.5 M sodium phosphate buffer (pH 7.4)	2—3 μg	50 μl
3.	Chloramine-T in Buffer A	50 μg	20 μl
	↓ Mixing for 20 sec at room temperature.		
4.	Sodium metabisulfite in Buffer A	100 μg	50 μl
5.	Potassium iodide in Buffer A	25 μg	50 μl
6.	Buffer B		0.5 ml
	↓ Determination of total radioactivity, and yield of labeled hen lysozyme.		
7.	Gel filtration by Sephadex G-25 (1.5 × 30 cm column) Elution; Buffer B, fraction; 2 ml/tube.		

Buffer A; 0.05 M sodium phosphate buffer (pH 7.4).
Buffer B; 0.05 M sodium phosphate buffer (pH 7.4) containing 0.2% bovine serum albumin and 0.15 M NaCl.

Preparation of Radioactive Hen Lysozyme——^{125}I-labeled hen lysozyme (abbreviated as ^{125}I-hen lysozyme) was prepared according to the chloramine-T method described by Greenwood *et al.*[4] The preparation procedure is represented in Table I.

The radiochemical yield of purified ^{125}I-hen lysozyme was approximately 23% by paperchromatography as described previously.[3b] After gel filtration by Sephadex G-25, the radioactivity was measured in each fraction and ^{125}I-hen lysozyme was eluted at fraction number 9 to 11 (2 ml/tube). The specific radioactivity was 127.5—182.3 mCi/mg. One tenth ml of the diluted labeled protein solution contained 1×10^4 cpm in 0.05 M sodium phosphate buffer (pH 7.4) comprising 0.2% BSA and 0.15 M NaCl was used for radioimmunoassay of hen lysozyme.

Anti-Hen Lysozyme Serum——Ten mg of hen lysozyme in 1 ml of 0.15 M NaCl was mixed with 1 ml of Freund's incomplete adjuvant (Difco Laboratories) to form a water-in oil emulsion. Two ml of emulsion was injected into the hind footpads and the femoral hypodermis of rabbit weighing about 2 kg. The administration of the emulsion was repeated 3, 5 and 10 weeks after the first immunization. Two weeks later, the serum was obtained from blood of the ear vein. From the sedimentation curve by antigen-antibody reaction of hen lysozyme, the concentration of anti-hen lysozyme IgG in antiserum was 1.19 mg/ml as rabbit IgG (A_{280nm}, $E^{1\%}_{1cm}=13.5$)[5] equivalent. The antibody was found to bind 60% of the diluted ^{125}I-hen lysozyme in absence of unlabeled hen lysozyme.

Separation of the Free from the Bound Form——Antibody-bound labeled lysozyme was separated from the free labeled lysozyme based on the charcoal-dextran method described by Herbert *et al.*[6] Dextran T-70 (pharmacia) treated charcoal ("Norit A, American Norit Co.) was prepared by mixing equal volumes of 10% charcoal suspension and 1% dextran T-70 solution in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.2% BSA and 0.15 M NaCl (abbreviated as Buffer B). One half ml of this solution was used. Hen lysozyme bound to antibody remained in supernatant fluid after centrifugation.

In two-antibody method, 0.1 ml of normal rabbit serum (1: 200 dilution) and 0.1 ml of goat anti-rabbit IgG serum (1: 2 dilution) were used. Following incubation at 4°, hen lysozyme bound to antibody was recovered in the precipitate after centrifugation.

Human Serum Samples——Human serum samples were obtained from four individuals at various time intervals after oral administration of 900 mg of hen lysozyme. The sera had been ascertained to have no anti-hen lysozyme antibody by preliminary experiment.

Determination of Radioactivity and Lysozyme Activity——The counting of ^{125}I was determined in a well-type scintillation counter (Aloka JDC-207). The counting efficiency of radioactivity was 70%. Lytic activity of lysozyme was determined according to the method described previously,[3a] which could detect the amount of lysozyme more than 0.5 μg per ml of serum.

Results

Effect of the Concentration of Antiserum on the Recovery of ^{125}I-Hen Lysozyme by Charcoal-Dextran and Two-Antibody Method

The effect of the concentration of antiserum on the recovery of ^{125}I-hen lysozyme was examined using charcoal-dextran method or two-antibody method. Results are shown in Fig. 1.

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5) M.J. Crumpton and J.M. Wilkinson, *Biochem. J.*, **88**, 228 (1963).
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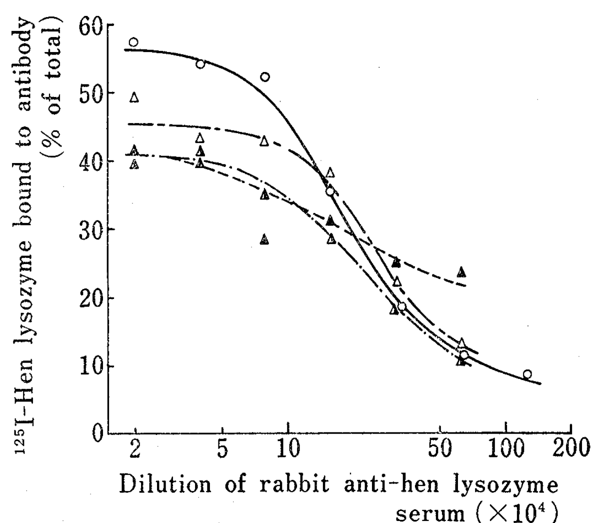

Fig. 1. Effect of the Concentration of Antiserum on the Recovery of ^{125}I-Hen Lysozyme by Charcoal-Dextran and Two-Antibody Methods

Incubation: —○—; 2 hr (37°), —△—; 24 hr (4°), —▲—; 48 hr (4°), —▲—; 72 hr (4°).

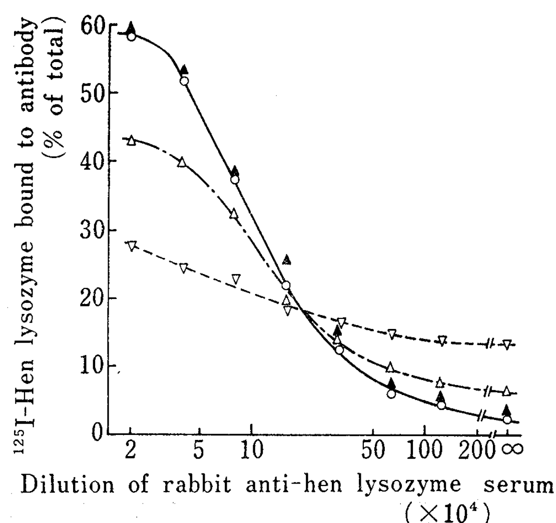

Fig. 2. Stability of ^{125}I-Hen Lysozyme determined by the Degree of Radioactivity Bound to Antibody

Time after labeling: —○—; 5 days, —△—; 35 days, —▽—; 64 days and —▲—; 1 day following dialysis of ^{125}I-hen lysozyme 35 days after labeling.

With antiserum in dilutions of 1: 20000 to 1: 50000, 55 to 58% of radioactivity was recovered in this supernatant by charcoal-dextran method. On the other hand, the recovery of radioactivity in the precipitate by two-antibody method was 40 to 50% with no relation to incubation time. With further dilution of the anti-hen lysozyme serum, there was a diminution in the fraction of ^{125}I-hen lysozyme recovered in the supernatant or in the precipitate. The recovery pattern of ^{125}I-hen lysozyme by charcoal-dextran method was found to be satisfactory as compared with that by two-antibody method. Since charcoal-dextran method was simple, rapid and reliable, this method was used in the separation of labeled antigen bound to antibody from free labeled antigen. Anti-hen lysozyme serum was used at dilution of 1: 80000 or 1: 100000 in this study.

Stability of ^{125}I-Hen Lysozyme

The stability of hen lysozyme after labeling was determined by the degree of radioactivity bound to antibody. To 0.1 ml of ^{125}I-hen lysozyme, each 0.1 ml of various diluted antiserum and Buffer B was added and mixed. After incubation for 2 hr at 37°, ^{125}I-hen lysozyme bound to antibody was separated by charcoal-dextran. The result is shown in Fig. 2.

As indicated in Fig. 2, with dilution at 1: 20000 the recovery of radioactivity bound to antibody decreased with the time lapse after labeling of hen lysozyme. ^{125}I-Hen lysozyme 35 days after labeling was dialized sufficiently against Buffer B, and then the dialized ^{125}I-hen lysozyme was mixed with the various diluted antiserum. The recovery pattern of radioactivity bound to antibody was in good agreement with that of ^{125}I-hen lysozyme 5 days after labeling.

The stability of labeled hen lysozyme was also examined by paperchromatography and dialysis against Buffer B. The radiochemical purity of ^{125}I-hen lysozyme by dialysis was 60 to 70% at one month and 40 to 50% at two months after labeling, respectively. These results were in good agreement with those by paperchromatography. The change of radiochemical purity of ^{125}I-hen lysozyme in freezing storage was identical with those in solution storage (4°) as described above. From these results, ^{125}I-hen lysozyme was used for the radioimmunoassay within at least two weeks after labeling.

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TABLE II. Standard Procedure for the Radioimmunoassay of Hen Lysozyme

1.	^{125}I-Hen lysozyme (10000 cpm/0.1 ml)	0.1 ml
2.	Hen lysozyme standards, or samples	0.1 ml
3.	Anti-hen lysozyme serum diluted 1 : 80000 or 1 : 100000	0.1 ml
	↓ Mixed and incubated for 2 hr at 37°	
4.	Dextran treated charcoal; 1% dextran T-70 : 10% charcoal (1/1, v/v)	0.5 ml
	↓ Centrifugation for 15 min at 3000 rpm (4°)	
5.	Radioactivity of the supernatant (B) was measured (B); ^{125}I-Hen lysozyme and unlabeled hen lysozyme bound competitively to antibody remain in supernatant fluid. (F); Free hen lysozymes are removed by dextran treated charcoal.	

The preparation and dilution of materials were made in Buffer B (see text).

Procedure of Radioimmunoassay

The radioimmunoassay of hen lysozyme was performed according to the scheme shown in Table II.

Most of tests were done in duplicate in 10 ml plastic tubes. The reagents were added in the volume and sequence indicated in Table II. The tubes were incubated for 2 hr in a 37° water bath. After incubation, 0.5 ml of dextran-treated charcoal was added to all tubes and mixed. They were then centrifuged for 15 min at 3000 rpm. After the charcoal formed a solid at the bottom of the tubes, the supernatant fluid was decanted into counting tubes and then counted in a well-type scintillation detector.

Instead of hen lysozyme standard, the known quantity of human or rat lysozyme prepared with Buffer B was used to examine their effects on the radioimmunoassay system. Additionally, hen lysozyme standard was prepared with buffer containing 4% BSA or 8% BSA or human serum instead of Buffer B. The respective standards were also treated according to the scheme in Table II.

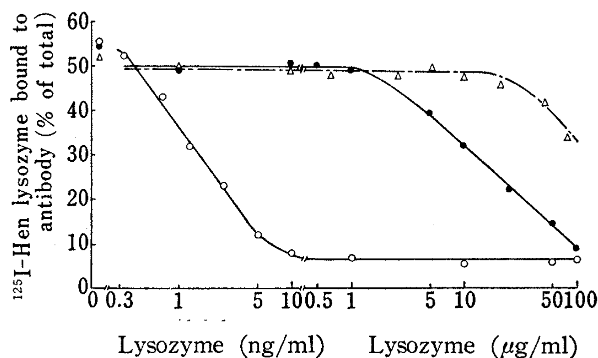

Fig. 3. Effects of Human and Rat Lysozymes on the Radioimmunoassay of Hen Lysozyme

—○—; hen lysozyme, —●—; human lysozyme, —△—; rat lysozyme.

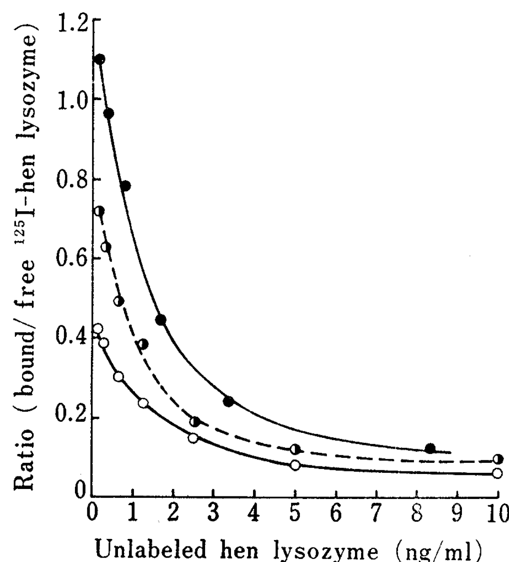

Fig. 4. Effects of Bovine Serum Albumin and Human Serum on the Radioimmunoassay of Hen Lysozyme

—○—; radiodilution curve of hen lysozyme prepared with Buffer B, —◑—; with 4% instead of 0.2% bovine serum albumin in Buffer B, —●—; with human serum.

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Effects of Human and Rat Lysozymes on the Radioimmunoassay of Hen Lysozyme

The cross-reactivity between hen lysozyme and human lysozyme has been suggested. Therefore, the cross-reactivity is examined by measuring the degree of displacement of ^{125}I-hen lysozyme bound to anti-hen lysozyme antibody by human lysozyme. Theoretically, if the two lysozymes bear any immunological resemblance, a decrease in the amount of ^{125}I-hen lysozyme bound to antibody would be expected when the amount of the competing human lysozyme increases.

According to the scheme shown in Table II, the effects of different concentration of unlabeled hen lysozyme were determined. The result is shown in Fig. 3.

The competitive binding of ^{125}I-hen lysozyme and unlabeled hen lysozyme to anti-hen lysozyme antibody were tested. With increasing of unlabeled hen lysozyme, the recovery of labeled hen lysozyme decreased. In this procedure, 0.3—5.0 ng/ml of hen lysozyme were detectable.

As shown in Fig. 3, the effects of human and rat lysozymes on the radioimmunoassay of hen lysozyme were found at the concentration of over 2 μg/ml and 20 μg/ml, respectively. The inhibitory concentrations of lysozymes at which the recovery of ^{125}I-hen lysozyme was reduced to 30% were 1 ng/ml in hen, 10 μg/ml in human and 100 μg/ml in rat. From these results, the cross-reactivity between hen and human lysozyme was found to be 1: 10000 and that between hen and rat lysozyme be 1: 100000.

Effects of BSA and Human Serum on the Radioimmunoassay of Hen Lysozyme

In the scheme shown in Table II, unlabeled hen lysozymes which were prepared with 4% BSA instead of 0.2% BSA in Buffer B and with human serum, were used as standard. Results are shown in Fig. 4.

As shown in Fig. 4, the ratio of antibody-bound ^{125}I-hen lysozyme to free ^{125}I-hen lysozyme (B/F) was plotted against a range 0.078 to 10 ng/ml of unlabeled hen lysozyme. This ratio increased with 4% BSA and more with human serum. The effect of 8% BSA

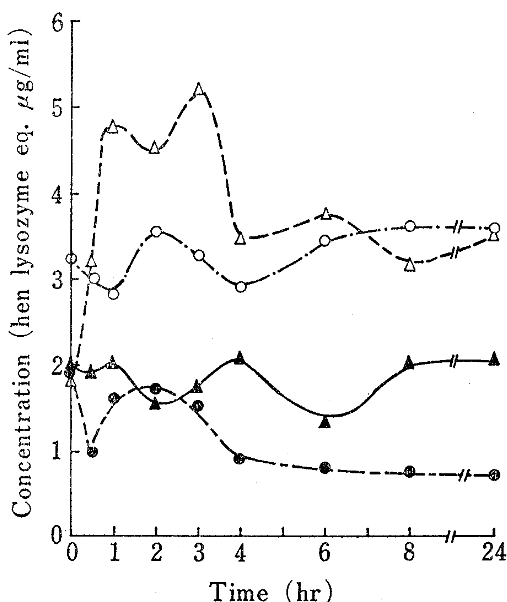

Fig. 5. Human Serum Levels measured by Enzyme Activity following Oral Administration of Hen Lysozyme

The serum levels represent the respective data of 4 experiments.
—○—; male, age 29, weight 62 kg, —●—; male, age 35, weight 54 kg, —△—; male, age 29, weight 61 kg, —▲—; male, age 28, weight 59 kg.

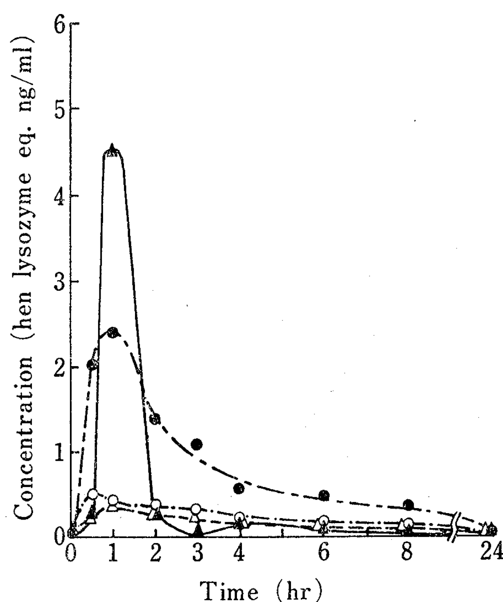

Fig. 6. Human Serum Levels measured by Radioimmunoassay following Oral Administration of Hen Lysozyme

The serum levels represent the respective data of 4 experiments corresponded to the plots in Fig. 5.

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containing unlabeled hen lysozyme was also the same as 4% BSA. Although the ratio (B/F) in human serum differed from that in 4% BSA, the assay range of 0.3 to 5.0 ng/ml of hen lysozyme was the same in both. Therefore, the determination of the serum levels after oral administration of hen lysozyme in men was carried out using the calibration curve by human serum.

Assay of Hen Lysozyme in Human Serum

Human serum levels after oral administration of hen lysozyme were determined by lytic activity for the purpose of comparing with human serum levels determined by the radioimmunoassay. The result on the lytic activity in four men is shown in Fig. 5.

As shown in Fig. 5, it was not found that serum levels determined by lytic activity had a specific increase following oral administration of hen lysozyme.

Subsequently, human serum levels after oral administration of hen lysozyme were measured by the radioimmunoassay according to the scheme of Table II, with the same samples used for the lytic activity. The result is shown in Fig. 6.

As shown in Fig. 6, the serum concentrations attained a peak 30 min to 1 hr after oral administration of hen lysozyme and thereafter decreased. The maximum level of hen lysozyme varied in individuals, ranging from 0.35 to 4.55 ng/ml.

Discussion

In labeling of hen lysozyme with ^{125}I, the suitable quantity of hen lysozyme used as starting material was 3 μg. When 1 or 0.5 μg of hen lysozyme was used, the yield of labeled lysozyme decreased. This was assumed to be due to adsorption of hen lysozyme to glass-tube in labeling reaction. When oxidation time by chloramine-T was 1 min or 5 min, the yield of ^{125}I-hen lysozyme decreased and the antigenicity of hen lysozyme was almost lost. The specific radioactivity of ^{125}I-hen lysozyme prepared according to Table I was 127.5 to 182.3 mCi/mg and that prepared using smaller amount of Na^{125}I was 48 mCi/mg. However, assay ranges of hen lysozyme by the radioimmunoassay were identical in both. This finding indicated that sensitivity of the radioimmunoassay was dependent on the quality of anti-serum used.

From the stability of ^{125}I-hen lysozyme, it was indicated that ^{125}I-hen lysozyme should be used within two weeks after labeling. ^{125}I-Hen lysozyme one month after labeling might be also used at the radioimmunoassay after being dialysed, since the recovery rate of ^{125}I-hen lysozyme was found to be restored by the treatment.

Radioimmunoassay ranges of hen lysozyme were 0.3 to 5.0 ng/ml, and human and rat lysozymes had an influence on the radioimmunoassay system with over 2 μg/ml and 20 μg/ml, respectively.

It was reported that human lysozyme concentration in normal serum was 3 to 10 μg/ml[7] by lytic activity of lysozyme. On the other hand, Canfield *et al.*[8] reported that human lysozyme concentration in normal serum was 0.79±0.35 μg/ml (n=35) by means of the radioimmunoassay of human lysozyme. In our study, the binding between ^{125}I-hen lysozyme and anti-hen lysozyme antibody was not inhibited by human serum obtained before oral administration of hen lysozyme. The fact supports indirectly the result of Canfield *et al.*

It was found that the ratio of the bound form to the free increased at the presence of human serum and high concentration of BSA. The cause remains to be elucidated. However, since it was thought that these effects were avoidable by drawing up a calibration curve using

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the same solution with assay sample, human serum levels after oral administration of hen lysozyme were calculated based on a calibration curve given by human serum containing the known quantity of hen lysozyme.

In conclusion, this radioimmunoassay system was appropriate for the determination of hen lysozyme in human serum. It is thought that the difference of serum levels after oral administration of hen lysozyme between by radioimmunoassay and by lytic activity was dependent on whether or not assay method was specific to hen lysozyme. As to the variation of the maximum levels of hen lysozyme in human serum as seen in Fig. 6, it might be resulted from the degree of degradation of hen lysozyme by pepsin in stomach and that of absorption from intestine.

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