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Measurement of Thymosin α_1 by Disassociation MicroELISA

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A disassociation microELISA was devised for the estimation of thymosin α_1, a chemically characterized thymic polypeptide isolated from bovine thymosin fraction 5. Antiserum to synthetic thymosin α_1 was raised in rabbits. Thymosin α_1 in liquid phase competed with a solid-phase-bound thymosin α_1 for this highly specific antibody. The method is specific, sensitive, reproducible and capable of detecting as little as 100 pg/ml of thymosin α_1.

Key words: *thymosin α_1 — disassociation ELISA*

Introduction

Thymosin α_1 is an acidic peptide with a MW of 3108 that has been isolated from bovine thymosin fraction 5. Purified thymosin α_1, prepared from calf thymus tissue, and chemically synthesized thymosin α_1 prepared by solution synthesis methods (Wang, 1978), are potent inducers of T cells (Low et al., 1979) and can influence immunoregulatory T cell function. Antibodies to the synthetic thymosin α_1 have been used for a radioimmunoassay (RIA) to measure serum levels of thymosin α_1 (McClure et al., 1982) which will permit careful monitoring of patients receiving this thymic preparation.

The RIA is an expensive and tedious procedure made all the more complicated by the necessity to substitute a tyrosine for a serine residue in the polypeptide for the purpose of radioiodination. Enzyme-linked immunosorbent assays (ELISA) are sensitive, rapid and simple for the detection of a variety of antibodies (Rissing et al., 1980; Flynn et al., 1981; Mutchnick and Keren, 1981) and antigens (Engvall et al., 1971; Miedema et al., 1972; Stiffler-Rosenberg and Fey, 1978). This report describes the development of a novel disassociation microELISA which can detect as little as 100 pg/ml of thymosin α_1. The method employed is unique in that a solid-phase-bound antigen (thymosin α_1) competes with an identical liquid-phase antigen for a specific antibody.

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Materials and Methods

Synthetic N-Ac-thymosin α_1 was generously provided by Hoffmann-LaRoche, Nutley, NJ. Antiserum to synthetic thymosin α_1 was prepared as previously described (McClure et al., 1982) and used in the non-absorbed form. Goat anti-rabbit IgG (GARG) was obtained by immunizing a goat with 6.6 mg of Fc piece from rabbit IgG in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Serum was collected weekly and tested for GARG activity by gel diffusion. Specific GARG antibodies were absorbed onto rabbit IgG insolubilized by glutaraldehyde (Avrameas and Ternynck, 1969). After washing this IgG sorbent with saline, the GARG antibodies were eluted with 0.1 M glycine–HCl buffer, pH 2.8. Na_2CO_3 was added to adjust the pH to 8.0 at a final concentration of 200 μg/ml of GARG.

Wells of a U-bottom microtiter Immulon plate (Dynatech Laboratories, Alexandria, VA) were coated with 0.5 μg/ml of thymosin α_1 (except where stated) in 50 mmol/l carbonate buffer, pH 9.6 (coating buffer), at 4°C for 18 h. The plates were covered with Parafilm to prevent evaporation. Immediately before testing the antigen solution was discarded and the plates washed 4 times (5 min, with agitation, per wash) with PTA: phosphate-buffered isotonic saline containing per liter, 50 μl of Tween 20 (Fisher Scientific Co., Pittsburgh, PA) and 20 mg of sodium azide. Rabbit antiserum to thymosin α_1 was diluted at 1×10^{-3} (or as stated) in PTA and 0.2 ml added to each of the antigen-coated wells and to uncoated wells as a control for nonspecific adsorption. The plates were incubated at room temperature for 18 h (or as stated) on a horizontal rotary shaker and then washed 4 times with PTA.

A stock solution of thymosin α_1 in phosphate-buffered saline was aliquoted into plastic tubes, stored at −20°C and thawed only once. The stock solution was diluted with PTA to 6 concentrations suitable for the working range of the assay (100–10,000 pg/ml) Liquid-phase thymosin α_1 in varying concentrations was added (0.2 ml) to the wells. The plates were incubated at 4°C for 48 h on a horizontal rotary shaker, and then washed 4 times with PTA. GARG, 200 ng/ml (or as stated) was added (0.2 ml) to the wells and allowed to react for 8 h at room temperature on the shaker. After washing the plates 4 times with PTA, a 30 μg/ml concentration of alkaline phosphatase conjugated to *Staphylococcus aureus* protein A (AP-SA; Zymed Laboratories, Burlingame, CA) was added (0.2 ml) to the wells and allowed to react for 18 h at room temperature with agitation. Following 4 washes with PTA, 200 μl of the substrate solution were added which contained 1 mg of disodium *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) per ml of 50 mmol/l carbonate buffer (pH 9.8) and 1 mmol of magnesium chloride per liter.

The reaction was permitted to proceed at room temperature until the absorbance at 400 nm was approximately 1.0 (the enzyme substrate reaction is linear to this level (Engvall and Perlmann, 1971)). Optical density (OD) readings were obtained at 10, 20, 25, 50 and 100 min using a Titertek Multiskan automated spectrometer (Flow Laboratories, McLean, VA). All tests were done in quadruplicate and the results extrapolated to 100 min (Engvall and Perlmann, 1971). The absorbances of the uncoated wells at 400 nm were subtracted from those of the coated wells containing the same solutions.

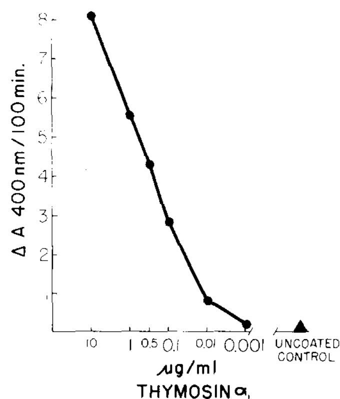
Results

Optimal thymosin α_1 coating of wells

We determined the optimal concentration of thymosin α_1 to coat the wells by incubating various concentrations of thymosin α_1 in coating buffer for 18 h at 4°C (Fig. 1). The wells were tested with a 1×10^{-3} dilution of antisera to thymosin α_1. Although 10 μg/ml of thymosin α_1 yielded the strongest sensitization, we used a concentration of 0.5 μg/ml for the subsequent experiments in order to conserve reagents and because of minimal variation in OD readings within and between assays.

Kinetics of primary antibody binding

Three dilutions of antisera to thymosin α_1 were used (10^{-2}, 10^{-3}, 10^{-4}) to investigate the rate at which thymosin α_1 antibodies were bound to the thymosin α_1 coating the wells and to determine whether the kinetics of primary antibody binding was related to antibody concentration. Antisera were allowed to react in the wells for varying intervals (Fig. 2). The wells were then washed, GARG was added and allowed to react for 4 h. After washing, AP-SA was added for 18 h and was followed by the substrate reaction. As shown in Fig. 2, the primary antibody was completely bound after 4 h of incubation with little change in reactively at 8 h and 24 h. Little or no reactivity was noted in coated wells reacted with pooled sera (10^{-2} dilution) obtained from non-immunized rabbits (OD < 0.090 at 24 h).



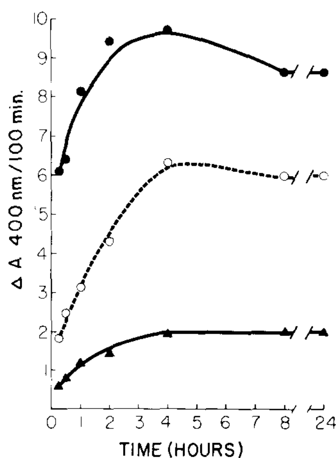


Fig. 1. Determination of optimal thymosin α_1 concentration for coating wells. Plates were incubated at 4°C for 18 h with the indicated concentrations of thymosin α_1, then incubated with rabbit antisera to thymosin α_1 (10^{-3} dilution). GARG was used at a concentration of 200 ng/ml and AP-SA at 30 μg/ml.

Fig. 2. Kinetics of the binding of primary antibodies to wells coated with 0.5 μg/ml thymosin α_1. Rabbit antisera to thymosin α_1 were diluted to 10^{-2} (●), 10^{-3} (○), and 10^{-4} (▲), incubated for the interval indicated and then reacted with GARG (200 ng/ml) and AP-SA (30 μg/ml).

Kinetics of secondary antibody (GARG) binding

Wells were coated with 0.5 μ/ml of thymosin α_1. The rate of binding for 2 concentrations of GARG (200 ng/ml and 20 ng/ml) was determined using a 10^{-3} dilution of primary antibody. The primary antibody was allowed to react for 18 h followed by reaction with GARG for various intervals. The reaction was complete at 8 h (Fig. 3) with little change observed when the reaction was extended to 24 h.

Conjugate reaction

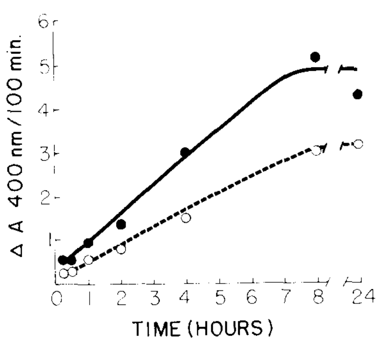
With the optimal conditions described above, we used 2 concentrations of AP-SA (30 μg/ml, 3 μg/ml) to react in the wells for various intervals. The conjugate reaction was completed by 8 h with little change observed when the reaction was extended to 24 h (Fig. 4). A concentration of 30 μg/ml was used for the remaining experiments.

Disassociation reaction

Wells were coated with 0.5 μg/ml of thymosin α_1. Antisera to thymosin α_1 at a dilution of 1×10^{-3} were added and allowed to react for 18 h. The wells were washed and varying concentrations of liquid-phase thymosin α_1 in PTA were added to the wells and allowed to react for 48 h. After washing, an aliquot of 200 ng/ml GARG and of 30 μg/ml AP-SA were added in stepwise fashion as described above. As shown in Table I, thymosin α_1 antibody activity decreased as the concentration of liquid thymosin α_1 increased.

Reproducibility

Using the optimum conditions described above, we assayed 10 replicate samples



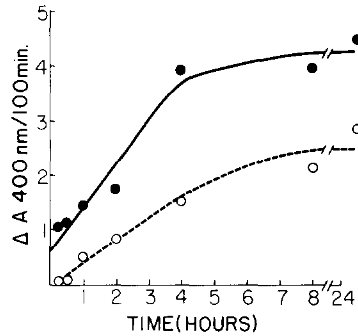


Fig. 3. Kinetics of the binding of secondary antibody (GARG). Wells were coated with 0.5 μg/ml thymosin α_1, reacted with antisera to thymosin α_1 (10^{-3}) for 18 h. This was followed by reaction with GARG at 200 ng/ml (●) or 20 ng/ml (○) for the interval indicated, then reacted with AP-SA (30 μg/ml).

Fig. 4. Conjugate reaction. Wells were coated with 0.5 μg/ml thymosin α_1, reacted with antisera to thymosin α_1 for 4 h. This was followed by reaction with GARG (200 ng/ml) for 18 h. Two dilutions of AP-SA were added at 30 μg/ml (●) and 3 μg/ml (○) for the interval indicated.

TABLE I

DISASSOCIATION REACTION WITH THYMOSIN α_1

[Thymosin α_1] [a]	ELISA results					
	Expt. 1		Expt. 2		Expt. 3	
	OD [b]	Δ [c]	OD	Δ	OD	Δ
0	5.277	0	5.402	0	6.331	0
100 pg/ml	4.505	−0.772	4.561	−0.841	5.621	−0.710
500 pg/ml	4.307	−0.970	3.500	−1.902	5.183	−1.148
1 ng/ml	4.092	−1.185	2.752	−2.650	4.820	−1.511
2.5 ng/ml	3.460	−1.817	2.333	−3.069	4.349	−1.982
5 ng/ml	3.188	−2.089	1.950	−3.452	4.012	−2.319
10 ng/ml	2.808	−2.469	1.306	−4.096	3.769	−2.562

[a] PTA containing increasing concentrations of liquid-phase thymosin α_1 was allowed to react in wells for 48 h.

[b] Absorbance at 400 nm after reaction for 100 min.

[c] The difference (Δ) in OD readings from wells not containing thymosin α_1 in the liquid-phase.

of 3 concentrations of liquid-phase thymosin α_1 to determine the percentage coefficient of variance (% CV) in OD readings within a single assay as well as between assays accomplished over a period of 2 months. As shown in Table II the % CV ranged from 6.3% to 12.7% within a single assay and from 7.9% to 13.2% between assays.

Linear regression analysis was undertaken to correlate the absorbances of the quadruplicate samples from each of the 3 successive experiments, as shown in Table I, with the $\log_{10}$ of the liquid-phase thymosin α_1 concentrations. As shown in Fig. 5, in the range 0–5 ng/ml thymosin α_1 the slope was −0.9319, y intercept 5.445

TABLE II

REPRODUCIBILITY OF DISASSOCIATION REACTION FOR THYMOSIN α_1

[Thymosin α_1] [a]	Within-assay variation			Between-assay variation		
	n	Mean ± S.E.M. [b]	% CV [c]	n	Mean ± S.E.M.	% CV
0	10	5.743 ± 0.121	6.3	5	5.634 ± 0.198	7.9
100 pg/ml	10	5.212 ± 0.203	12.3	5	5.001 ± 0.240	10.7
1 ng/ml	10	4.924 ± 0.116	7.5	5	4.275 ± 0.252	13.2
5 ng/ml	10	4.332 ± 0.183	12.7	5	3.743 ± 0.197	11.8

[a] PTA containing increasing concentrations of liquid-phase thymosin α_1 was allowed to react in wells for 48 h.

[b] Absorbance at 400 nm after reaction for 100 min.

[c] % CV, percentage coefficient of variance.

and $r = 0.6225$. When the analysis was extended to 10 ng/ml thymosin α_1 the slope changed to -1.6635, the y intercept to 9.29 and r to -0.6474.

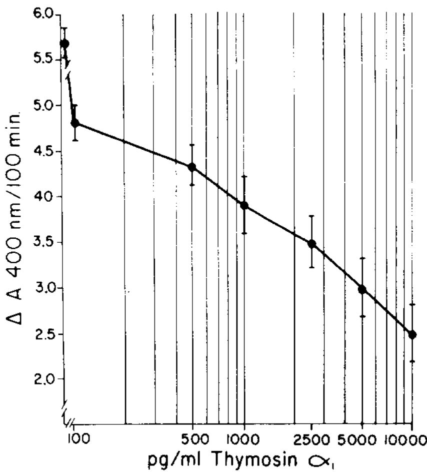


Fig. 5. Mean absorbance ±S.E.M. of each quadruplicate sample from 3 experiments for the indicated concentrations of liquid-phase thymosin α_1.

Antigen specificity of ELISA

We used glucagon (lot no. 258-234B-167-1; Eli Lilly and Co.) and porcine insulin (lot no. 615-07J-256; Eli Lilly and Co.) in the disassociation reaction to ensure that ELISA reactions represented specific responses of liquid-phase thymosin α_1 to the solid-phase-bound antibody. As shown in Table III, thymosin α_1 antibody activity

TABLE III

ANTIGENIC SPECIFICITY OF ELISA

Liquid-phase concentration [a]	ELISA results (Δ) [b]		
	Thymosin α_1	Insulin	Glucagon
100 pg/ml	−0.493	+1.880	+1.778
250 pg/ml	−1.316	+1.996	−0.362
500 pg/ml	−1.615	+0.298	+1.041
1 ng/ml	−1.880	−0.392	−0.654
5 ng/ml	−2.537	+0.077	+1.874
10 ng/ml	−2.612	+0.946	−0.218

[a] Liquid-phase thymosin α_1, insulin or glucagon were reacted in wells containing antibodies to thymosin α_1 attached to solid-phase-bound thymosin α_1.

[b] The difference (Δ) in OD readings from wells not containing thymosin α_1, insulin or glucagon in the liquid-phase. −, decrease in OD readings; +, increase in OD readings.

decreased as the liquid-phase thymosin α_1 concentration increased. In contrast, no activity was lost when liquid-phase glucagon or insulin were added to the wells. It is unclear why OD readings were increased in wells containing insulin or glucagon, but this pattern of response has been observed in other ELISA systems (Flynn et al., 1981; Mutchnick and Keren, 1981).

Discussion

The disassociation microELISA method is unique in that a liquid-phase antigen competes with an identical solid-phase-bound antigen for a highly specific antibody. The 48 h period of incubation in the disassociation reaction was required to attain an equilibrium, and the subsequent substrate reaction was inversely related to the concentration of the liquid-phase thymosin α_1.

The disassociation microELISA is sensitive to changes in the liquid-phase thymosin α_1 concentration in the range 20–2000 pg in the well. In the present study we examined various parameters affecting the ELISA system. Binding of the primary (anti-thymosin α_1) and secondary (GARG) antibodies was essentially complete by 4 h and 8 h respectively. This is similar to results described in previous ELISA systems (Engvall et al., 1971; Carlsson et al., 1976; Zollinger et al., 1976; Butler et al., 1978; Yardley et al., 1978).

The specificity of the antiserum to thymosin α_1 has been shown previously, revealing a lack of significant cross-reactivity with pure preparations of serum proteins, protein hormones and other putative thymic hormones (McClure et al., 1982). Neither insulin nor glucagon affected the solid-phase-bound antibody activity in the disassociation reaction (Table III).

The ELISA method outlined offers an inexpressive means for monitoring thymosin α_1 in a liquid-phase. The advantage over the current RIA method is the absence of the need to introduce a tyrosine residue into the thymosin α_1 moiety. The disassociation microELISA could be particularly useful for quantitating other antigens using highly specific antibodies, including hybridoma-produced monoclonal antibodies. We are currently evaluating the applicability of the disassociation microELISA for measurement of serum thymosin α_1.

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