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A Radioimmunoassay for Ovalbumin

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A sensitive and specific radioimmunoassay for chicken ovalbumin is reported. Egg white ovalbumin was labelled with ^{125}I by the chloramin-T method. The bound and free ovalbumin were separated with a second antibody bound to a solid matrix, or by filtration on a Millipore filter. After linearization by logit-log transformation the standard curve was linear from 1 to 100–200 ng of ovalbumin/ml with separation by the double antibody solid phase technique and from 5 to 200 ng/ml with a filter technique.

Ovalbumin is one of the most extensively studied chicken egg white proteins.[1] During the past decade it has assumed an important role in the study of steroid hormone action and the regulation of gene expression.[2-5] In addition, ovalbumin is acquiring a central role in the study of gene structure and function.[5-9] In most of these studies ovalbumin concentration has been measured using an immunoprecipitation method, where proteins labelled with a radioactive amino acid are precipitated by specific antisera.[10-12] The concentration of a given protein can however usually only be measured proportionally with immunoprecipitation methods. Ovalbumin has also been determined quantitatively using thin layer isoelectric focusing in polyacrylamide gel[13] and recently by a radioimmunoassay.[14]

We are studying the regulation of protein synthesis in the chick oviduct by progesterone and certain non-hormonal treatments.[15-17] The action is observed by assaying concentrations of such specific proteins as avidin and ovalbumin. To determine ovalbumin concentration an assay system had to be developed which is highly sensitive, easily and quickly performed and which measures specifically the given protein. The development and details of a radioimmunoassay for ovalbumin are reported here.

EXPERIMENTAL

Materials. Chemicals were obtained from the sources indicated: avidin, bovine serum albumin (96–99% pure) and ovalbumin from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); chloramin-T, diethylstilboesterol and progesterone from E. Merck (Darmstadt, Germany); merthiolate from BDH Chemicals (Poole, Dorset, U.K.); carrier-free $Na^{125}I$ (3.7 GBq/ml) from Amersham International Limited (Amersham, Bucks., U.K.); Freund's complete adjuvant from Difco (Detroit, MI, U.S.A.); Sephadex G-50 (medium) from Pharmacia Fine Chemicals (Uppsala, Sweden) and sheep anti-(rabbit γ-globulin) immunosorbent from Organon (Oss, Holland). MF-Millipore filters, HAWP 00010 and GSWP 00010, were from the Millipore Co. (Bedford, MA, U.S.A.).

Animals and tissue samples. Immature Leghorn chicks (2–3 days old) were pretreated daily with diethylstilboestrol [0.5 mg/animal] and tissues removed, stored and homogenized as described previously by Kulomaa *et al.*[18] Ovalbumin was measured in the supernatant of the homogenate after centrifugation for 25 min at $2500 \times g$ and +4 °C.

Radioimmunoassay. Antiserum against ovalbumin was prepared in rabbits by subcutaneous injections of an emulsion containing 1 mg of ovalbumin in Freund's complete adjuvant at 2-week intervals for 3–5 months. Serum was separated by centrifugation (15 min, $3000 \times g$, +4 °C) and stored in small fractions at −80 °C.

The iodination of ovalbumin was carried out by the chloramin-T method of Greenwood *et al.*,[19] and ^{125}I-labelled ovalbumin was purified on a Sephadex G-50 column as described in the radioimmunoassay for avidin.[18] The tracer was stored in small fractions at −20 °C.

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All dilutions were made with a sodium phosphate buffer (0.05 M sodium phosphate buffer −0.15 M NaCl−0.01 M EDTA −0.01 %(w/v) methiolate; pH 7.5) containing 0.5% bovine serum albumin. Ovalbumin antiserum diluted $2-5\times10^{-5}$ (100 μl) and the ^{125}I-labelled ovalbumin (50 μl; 10−15 ng) were added to sample or standard (400 μl) and incubated overnight at room temperature. Bound ovalbumin was separated by sheep anti(rabbit γ-globulin) immunosorbent as described previously,[18] or by filtration on a Millipore filter (diameter 13 mm) of pore size of 0.45 or 0.22 μm (HAWP 00010 and GSWP 00010, respectively). The tube and the filter were washed three times with 2 ml of sodium phosphate buffer and the filter dried for 30 min at +60 °C. The radioactivity of the pellet from the double antibody solid phase technique and the filter was counted with a gamma counter (LKB/Wallac, Ultrogamma II). All samples and standards were assayed at least in dublicate.

RESULTS

The ^{125}I-labelled ovalbumin usually contained 20 −25% of the total radioactivity after a 40 s iodination procedure. The specific activity of the product was 10−35 μCi/μg of labelled protein. The tracer could be used for at least 4−5 weeks. A little degradation was found after 3−4 weeks storing at −20 °C, but it could be removed by gel filtration on Sephadex G-50.

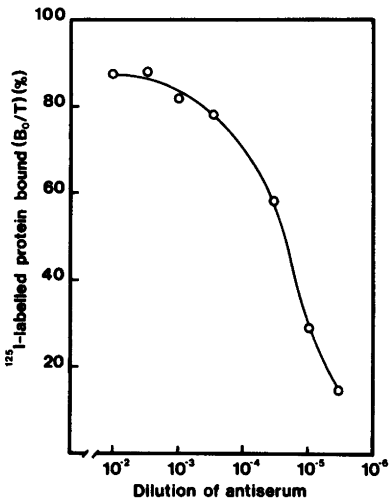

Fig. 1. Titration of ^{125}I-labelled ovalbumin with antiserum. ^{125}I-labelled ovalbumin was mixed with different dilutions of ovalbumin antiserum. After incubation overnight at room temperature 1 ml of a dilution (1:5−1:20) of sheep anti-(rabbit γ-globulin) immunosorbent was added and the tube was incubated for 2 or 20 h depending upon the dilution of immunosorbent. The tube was centrifuged and bound radioactivity in the precipitate counted with a gamma counter.

The titration of the ^{125}I-labelled ovalbumin with antiovalbumin serum (Fig. 1) was carried out in the absence of unlabelled antigen (B_0). About 90% of the iodination product was precipitated with the highest antibody concentration by the double antibody solid phase technique. The antiserum was diluted in the assay ($2-5\times10^{-5}$) such that 40−50% of the tracer was bound. In the background control, *i.e.* in the absence of the antibody against ovalbumin, only 2−3% of the radioactivity was bound in both of the separation techniques.

The rate of equilibrium of immunoreaction was studied with different incubation times before addition of the second antibody solution. The balance was reached in 2 h and it continued constant up to 70 h. The time of the second antibody reaction was chosen according to the dilution of ovalbumin antiserum, this being usually 2 h. The logit-log presentation of the standard curve was linear from 1 to 100−200 ng of ovalbumin/ml with the double antibody solid phase technique (Fig. 2) and from 5 to 200 ng/ml with the filtration technique with both pore sizes of filter.

The supernatant of the oviduct homogenate of oestrogen-treated chick displaced ^{125}I-labelled ovalbumin in the same way as ovalbumin standards (Fig. 3). The cross-reactivity of avidin in the ovalbumin radioimmunoassay was also checked. Avidin did not displace ^{125}I-labelled ovalbumin at concentrations up to 1 μg of avidin/ml.

DISCUSSION

Iodination with chloramin-T as oxidizing agent[19] is the most popular method for labelling proteins with ^{125}I. Mercereau-Puijalon *et al.*[14] labelled ovalbumin according to Bolton and Hunter,[20] where iodination takes place in milder conditions than in the chloramin-T method. Iodination of ovalbumin can also be performed with the latter method, since ovalbumin seemed to be quite resistant to changes in its antigenity.

The dilution of antiovalbumin serum was chosen according to antibody titration, where bound radioactivity was precipitated by a double antibody

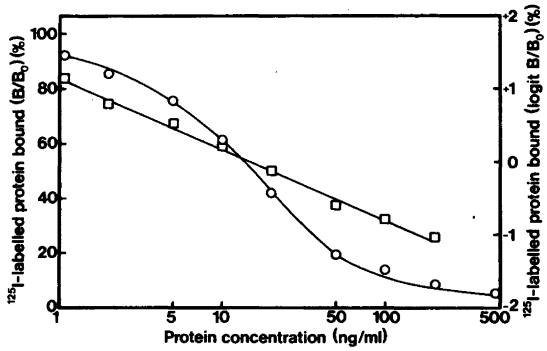

Fig. 2. Ovalbumin standard curve. Standard samples from 1 to 500 ng of ovalbumin/ml were assayed. The ratio of the bound (B) over the maximal (B_0) radioactivity (○), and its logit value (□), were plotted on the function of the ovalbumin concentration on a semi-logarithmic paper. Ovalbumin concentration of the sample was determined from the linear logit-log presentation.

solid phase technique (Fig. 1). The same dilution was also used in the filter technique. Background for both of the separation techniques was below 2% of the total radioactivity with new tracer, but increased to 3% when the tracer got older. Mercereau-Puijalon *et al.*[14] separated antibody-bound radioactivity by the addition of Na_2SO_4 and centrifugation. It is an easy and rapid technique, but the background was quite high, 2–3 times higher than in the present methods. In addition, purification of IgG-fraction from antiserum is not necessary for the present techniques.

The double antibody solid phase technique was used in routine, since it gave a wider standard curve than the filter technique. It was also easier and quicker to perform. One technician can daily assay 30–60 supernatant samples and an ovalbumin standard. The sensitivity of the radioimmunoassay was 1–2 ng of ovalbumin/ml of supernatant with the double antibody solid phase and 5 ng/ml with the filtration technique. Palmiter and Wrenn[11] determined ovalbumin concentration by the immunoprecipitation method in combination with measurement of the total proteins according to Lowry *et al.*[21] The sensitivity of their method was, however, much lower, being 100 ng of ovalbumin/mg of tissue. The radioimmunoassay introduced by Mercereau-Puijalon *et al.*[14] had

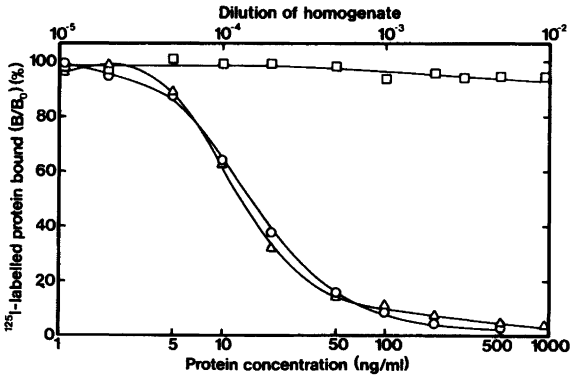

Fig. 3. Specificity of the assay. Ovalbumin (○) and avidin (□) were dissolved and diluted with the phosphate buffer used in the assay. The oviduct of oestrogen-treated chick (5 mg/day for 6–7 days) was homogenized and the supernatant diluted with the radioimmunoassay buffer (△). Samples were assayed as described in the text.

approximately the same sensitivity as reported here. The logit-log transformation of the standard curve, however, makes calculation of ovalbumin concentration easier and more accurate, and the linear part of the curve becomes wider.

The supernatant of the oviduct homogenate of oestrogen-treated chick gave a shape of dilution curve identical to that of ovalbumin standards (Fig. 3). Avidin could not interfere with ovalbumin radioimmunoassay, since its concentration should be at least 200 times higher than the ovalbumin concentration, whereas avidin comprises only approximately 1 % of ovalbumin content in the oviduct of the hormone-stimulated chick. We did not study cross-reactions in grater detail, since a high ovalbumin concentration was found with the radioimmunoassay only in the oviduct of oestrogen-treated chicks.[15] Very low cross-reaction (or ovalbumin concentration) can, however, be seen in some non-oviductal tissues (plasma, intestine). Thus it would be preferable for specificity to be checked thoroughly before the ovalbumin concentration is assayed in these tissues or other samples.

In conclusion, a radioimmunoassay is a valid method of assaying low ovalbumin concentrations in chicken tissues or other protein solutions. It can also be used to detect the bacteria having ovalbumin-containing recombinant plasmids after cloning experiments. There are several techniques for separating free and bound radioactivity after immunoreaction. The double antibody solid phase technique is rapid and easy to perform, background is low and the standard curve fairly wide. Because of its sensitivity and specificity, radioimmunoassay is the most favourable method for the assay of unknown ovalbumin concentrations in biological samples.

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