Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 526-533

A RADIOIMMUNOASSAY FOR CHIRONOMID HEMOGLOBIN

Patrick M. Trewitt, Gerald Bergtrom and Ku-Chuan Hsiao

Department of Zoology, University of Wisconsin-Milwaukee, Milwaukee, WI, 53201, U.S.A.

Received August 12, 1982

A double antibody, competitive radioimmunoassay was developed for the quantitation of stage-specific hemoglobin from the insect Chironomus thummi. The radioimmunoassay will detect as little as 150 picograms of Hb 3, a hemoglobin synthesized and secreted into the hemolymph of larvae during the 4th, but not the 3rd instar. The assay also detects cross-reacting hemoglobins purified from 4th instar larvae and in freshly laid eggs. Application of this radioimmunoassay is discussed in light of the cross-reacting Hbs in the insect.

INTRODUCTION

Among the many hemoglobins (Hbs) present in the hemolymph of Chironomus thummi , at least 11 are expressed by different genes, based on the number of non-allelic globin amino acid sequences already reported (1-11). The synthesis and secretion of Hbs by 4th instar fat body tissue was directly demonstrated using radioactive precursors in organ culture (12). While Hbs are first detectable during the second larval instar as a red pigment, at least two Hbs are synthesized specifically in the 4th instar (13) and their coordinate induction appears to be directed by juvenile hormone (14, 15). Furthermore, consistent with the fact that chironomid Hbs are secreted, fat body cells show cytochemical evidence of Hb (pseudoperoxidase activity) in rough endoplasmic reticulum, Golgi and secretory vesicles (16-18). Similar cytochemical studies suggest that Hbs are accumulated from the hemolymph by the yolk granules of developing oocytes in pharate adults (19,20), at which time much of the larval hemoglobin is being degraded (19,21).

Studies of Hb synthesis, secretion or uptake have been limited by the sensitivity of trace isotope incorporation techniques and by uncertainty in the interpretation of pseudoperoxidase cytochemistry. In order to study the early kinetics of induction of stage-specific Hbs, a 4th instar-specific Hb was

0006-291X/82/180526-08$01.00/0

purified and used as an immunogen to raise antibodies in rabbits (22) with the goal of developing a more sensitive, Hb-specific radioimmunoassay (RIA). The ability of the antibodies obtained to cross-react with several C. thummi Hbs (22) led to the RIA characterized in this report and allows us to address reservations, inherent in previously reported ultrastructural studies, about the identification of Hb.

MATERIALS AND METHODS

Proteins, chemicals and immunological reagents. C. thummi Hbs 1-6 were extracted, and each was purified to apparent homogeneity as previously described (22). Bovine lactoperoxidase and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Reagent grade 30% hydrogen peroxide was obtained from Fisher (Itasca, IL); G50 and G75 Sephadex from Sigma (St. Louis, MO); and carrier-free $Na^{125}I$ (17 Ci/mg) from New England Nuclear (Boston, MA).

The rabbit anti-Hb 3 serum used in these experiments has been previously characterized (22). Normal rabbit serum (NRS) was obtained from a non-immunized animal. Goat anti-rabbit serum (GARS), prepared against the globulin fraction of rabbit serum, was purchased from Gibco (Grand Island, NY).

Iodination of C. thummi Hb 3 and fractionation of [^{125}I]Hb 3. Pure Hb 3 was iodinated by the enzymatic method of Thorell and Johansson (23). One mCi (10 ul) of ^{125}I was added to 7.5 ug/10 ul of Hb 3 in iodination buffer (0.1M Na-PO_4, pH 7.5) in a 1 ml vial at room temperature (22°C) under a ventilation hood. The radioisotope was transferred from its shipping vial by microsyringe in order to contain volatile iodine. Bovine lactoperoxidase (1 ug in 20 ul distilled water) and H_2O_2 (0.1 ug in 20 ul distilled water) were added sequentially to initiate the iodination reaction. After 3 minutes of gentle agitation, the reaction was terminated by dilution with 300 ul of iodination buffer. The mixture was transferred to a G50 Sephadex column (10 x 0.8 cm I.D.) along with 300 ul of additional iodination buffer used to rinse the reaction vial. The sample was eluted with iodination buffer containing BSA (0.2%) and collected in 10 drop (0.3 ml) fractions. A 2 ul aliquot of each fraction was monitored in a Packard 400 CGD gamma counter (Downers Grove, IL). Tubes containing the high molecular weight ^{125}I peak were stored at -20°C for up to 3 months.

Before use, freshly iodinated or thawed, stored iodinated [^{125}I]Hb 3 was refractionated by G75 Sephadex chromatography. Between 0.15 and 0.30 ml of the G50 eluent was applied to the G75 column (12 x 0.8 cm I.D.), eluted with RIA buffer (0.1M Na-PO_4, pH 7.5, 0.2% BSA, 0.02% NaN_3), and collected in 20 drop fractions. A 5 ul aliquot of each fraction was assayed in the gamma counter. The radioactive protein peaks obtained were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) according to previously detailed procedures (22). Fractions that contained homogenous undamaged [^{125}I]Hb 3 as determined electrophoretically were pooled, and the concentration of [^{125}I]Hb 3 was adjusted with RIA buffer to 0.15 - 0.5 ng/100 ul. Storage at 4°C for up to 7 days had no adverse effects on experimental assays.

Radioimmunoassay (RIA). The double antibody method of RIA was used to precipitate antibody-bound [^{125}I]Hb 3. All reagents were diluted as required with RIA buffer. In the primary reaction, anti-Hb 3 serum was allowed to react with a fixed amount of [^{125}I]Hb 3, in the absence or presence of unlabelled competitor (C. thummi Hbs or cleared egg mass homogenates). After addition of NRS in order to increase the concentration of rabbit antibodies to a precipitable level, anti-Hb 3 antibodies bound to [^{125}I]Hb 3 were removed from solution by the secondary reaction with GARS.

Optimal levels of primary antiserum (anti-Hb 3), NRS and GARS for the RIA were determined following the guidelines of Hunter (24).

Assays were performed in 10 x 75 mm glass culture tubes. In the first reaction, optimal concentrations of RIA reagents in RIA buffer were added to the tubes in sequence: 100 ul of [^{125}I]Hb 3; 100 ul of competitor (or 100 ul of RIA buffer alone); 100 ul primary antiserum. Following incubation at 37°C for 2 hours, the reaction tubes were allowed to cool to room temperature. In the second reaction, 100 ul each of optimal concentrations of NRS and GARS were added in sequence to the reaction tubes. Following incubation at 4°C for 18-22 hrs, all reaction tubes were centrifuged in a GSA rotor with appropriate adaptors (Dupont-Sorvall, Norwalk, CT) at 5000 xg, 4°C, 10 minutes. Supernatants were decanted and the tubes inverted onto absorbent toweling. Each tube was counted to within 1.5% error or for 5 minutes.

Some unbound [^{125}I]Hb 3 remained in the assay tubes due to trapping during pellet formation and/or adsorption onto the glass during decanting. This non-specific binding (NSB) level was estimated for each assay by including a set of tubes in which 100 ul of RIA buffer was substituted for the primary serum. NSB values (3-7% of input cpm) have been subtracted from the data.

All RIA experiments were performed at least twice, each time with replicate or triplicate tubes. Intra-assay variability was less than 5%. Comparing repeated assays, the averaged values for a given experimental point were within 6% of one another. A calibration competition curve, using unlabelled Hb 3 as competitor, was generated for each experimental RIA.

For RIA of egg Hb, single freshly laid egg masses from laboratory colonies of C. thummi were obtained within 1/2-1 hr of laying, and the number of eggs per egg mass was counted using a dissecting microscope. Each egg mass was homogenized in RIA buffer. After centrifugation at 10,000 xg, 10 min. at room temperature, the supernatants were brought to 1.0 ml with RIA buffer.

RESULTS

Fractionation and characterization of radiolabelled Hb 3. Approximately 25-35% of input ^{125}I was recovered bound to protein following G50 Sephadex fractionation. Since pure Hb 3 was used for iodination, and since iodination of lactoperoxidase is negligible under these reaction conditions (24), the specific activity of iodinated Hb 3 (MW = 16000 daltons) typically ranged from 530-750 mCi/uMole. The calculated molar ratio of ^{125}I/Hb 3 (less than 0.25) indicates that only mono-iodinated reaction products were obtained. Due to concern that the introduction of more than one iodine might interfere with the recognition and binding of Hb 3 by primary antiserum, no attempt was made to further increase the specific activity of the labelled Hb 3.

G75 Sephadex chromatography of the [^{125}I]Hb 3 resolved 3 peaks of ^{125}I activity (Fig. 1a). Protein peaks from a typical refractionation were analyzed by SDS-PAGE. The first peak contained ^{125}I-labelled high molecular weight aggregates, which tend to form during storage of the Hbs, while fractions from the middle peak consisted almost entirely of ^{125}I-labelled Hb 3

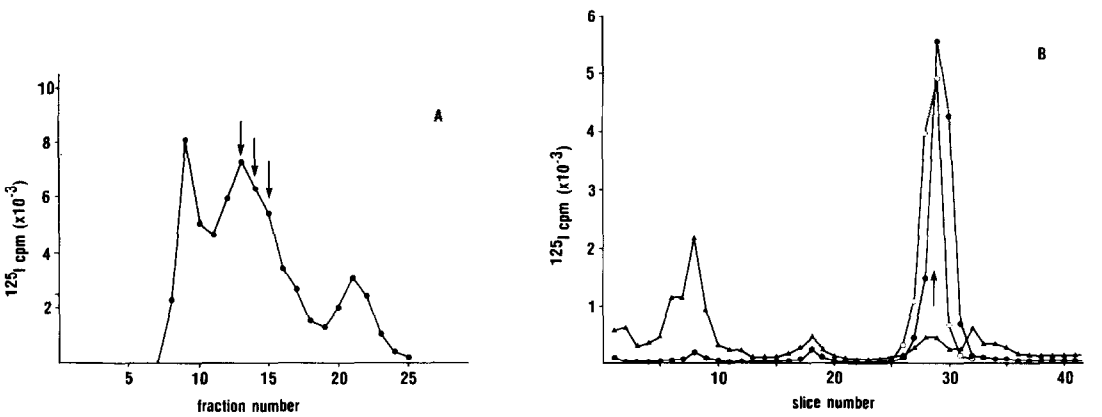

Fig. 1. Separation and analysis of iodinated antigen. A. G75 Sephadex refractionation of an aliquot of [^{125}I]Hb 3. Arrows indicate the [^{125}I]Hb 3 fractions pooled for RIA experiments. B. Denaturing (SDS) polyacrylamide gel electrophoresis of selected G75 Sephadex fractions. Arrow indicates position of unlabelled Hb marker. Fraction 9 (▲); fraction 12 (●); fraction 14 (o).

which comigrated with unlabelled Hb 3 marker (Fig. 1b). The 3rd peak contained non-fixable material, presumably low MW peptides or free ^{125}I generated by nucleolytic processes during storage (data not shown). Those fractions comprising the middle peak, i.e. undamaged [^{125}I]Hb 3, which contained less than 5% overlapping material from adjacent peaks, were pooled for RIA determinations.

Optimization of the precipitation reaction. The percent of input labelled antigen which can be precipitated using a double antibody technique is typically about 70%, even when the primary antibody concentration is in excess compared to labelled antigen (24). However, maximal sensitivity for a competitive RIA is achieved at a primary antiserum titer that will bind about 50% of the label in the absence of a competitor (25). In order to achieve maximum precipitation of radiolabelled antigen without affecting the primary binding reaction, the secondary reagents (NRS and GARS) must be optimized at concentrations high enough to minimize the contribution of primary serum to immunoprecipitation. Therefore, in the absence of competitor and using fixed amounts of [^{125}I]Hb 3 and primary antiserum (diluted 1:1000), NRS and GARS were varied with respect to each other, generating a series of precipitation curves (Fig. 2). Optimized concentrations of NRS (1:500) and GARS (1:14) were

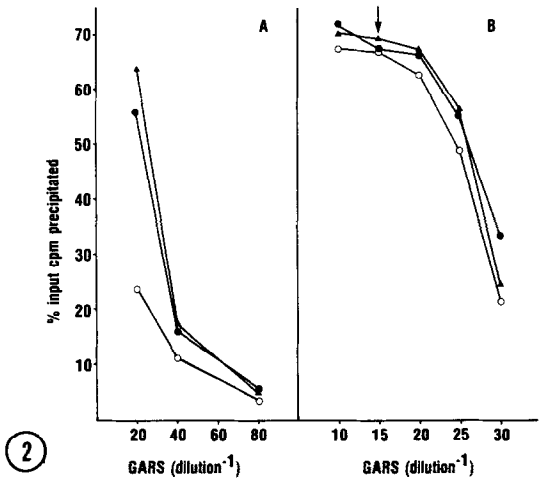

Fig. 2. Optimization of RIA precipitation reaction. [^{125}I]Hb 3 = 0.2 ng/100 ul; anti-Hb 3 serum = 10^{-3} dilution. A. NRS = 1:200 (o); 1:400 (▲); 1:800 (●). B. NRS = 1:400 (o); 1:500 (▲); 1:600 (●). Arrow indicates the balanced ratio of NRS and GARS which conforms to the optimization criteria of Hunter (24).

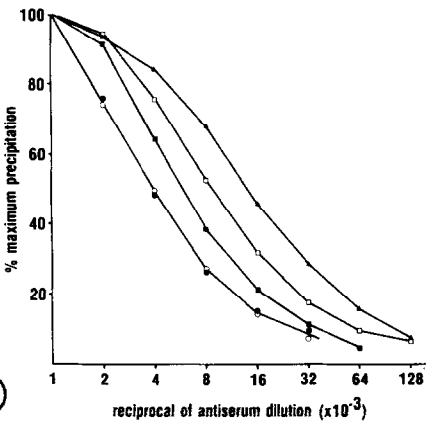

Fig. 3. Reactivity of primary (anti-Hb 3) antiserum for ^{125}I-labelled and unlabelled antigen. NRS = 1:500; GARS = 1:14. [^{125}I]Hb 3 per 100 ul: 0.2 ng (▲); 0.5 ng (□); 1.0 ng (■); 2.0 (●); 0.2 ng + 1.8 ng unlabelled Hb 3 (o).

selected from well within the maximum precipitation plateau to insure reproducibility of the RIA.

Reactivity of antibodies for [^{125}I]Hb 3. The ability of primary antiserum to discriminate between different levels of [^{125}I]Hb 3 and between labelled and unlabelled Hb 3 was determined. The results indicate that maximum precipitation levels for each primary antiserum titration were between 65-71% of input label (set at 100% for each curve in Fig. 3). As input label was decreased, the proportional decrease in primary antiserum titer (at 50% of maximum precipitation) indicates no difference in avidity of primary antiserum for [^{125}I]Hb 3, within the range of 0.2 to 2.0 ng (Fig. 3). In addition, the superimposition of the titration curve for 2 ng of [^{125}I]Hb 3 over that for 0.2 ng of [^{125}I]Hb 3 + 1.8 ng of unlabelled Hb 3 (total = 2 ng), indicates that iodination of Hb 3 to the specific activities reported above does not alter its reactivity with anti-Hb 3 serum.

RIA of C. thummi Hbs. The sensitivity and range of a competitive RIA was tested using optimized concentrations of NRS, GARS and primary antiserum. Competition curves were drawn based on a 100% level (B_o) of [^{125}I]Hb 3

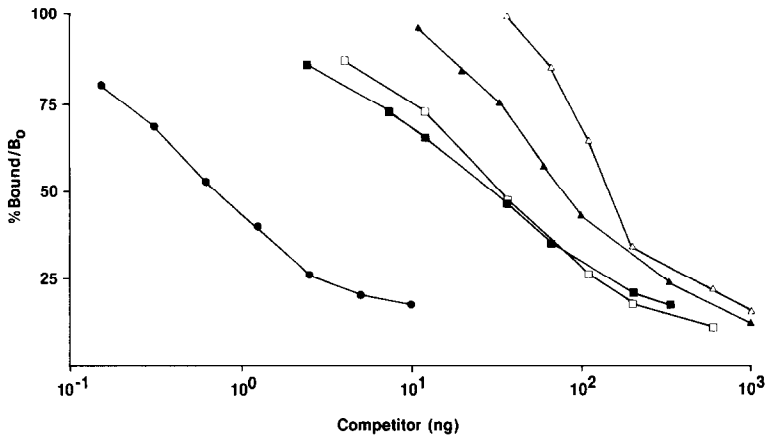

Fig. 4. RIA competition curves for pure C. thummi Hbs. [^{125}I]Hb 3 = 0.15 ng; primary antiserum = 1:32000; NRS = 1:500; GARS = 1:14. Unlabelled competitors: Hb 3 (●); Hb 4 (■); Hb 5 (□); Hb 2 (▲); Hb 6 (△).

precipitated in the absence of competitor. The results (Fig. 4) show that 0.15 to 5.0 ng of Hb 3 are readily assayed with greatest sensitivity at about 0.72 ng. Though several C. thummi Hbs cross-react with antiserum prepared against Hb 3 (22), the RIA is at least 40-160 times more sensitive for Hb 3 based on the concentrations of the different Hbs needed to reduce antibody-bound [^{125}I]Hb 3 to 50% of B_o. Furthermore, the slope of the competition curves for some non-immunogen Hbs differs from the slope obtained with the homologous competitor. This implies that these C. thummi Hbs share some, but not all antigenic determinants of Hb 3, confirming previous observations (22). Finally, one of the C. thummi Hbs (Hb 1), as well as sperm whale myoglobin and human Hb, failed to be detected by the RIA at concentrations at or greater than 10^5 ng/100 ul (data not shown).

Hb in C. thummi eggs. Since all but one of the C. thummi larval Hbs cross-react with anti-Hb 3 serum, the possibility was tested that similar cross-reacting Hbs could be detected immunologically in the eggs of the insect. Our results (Fig. 5) demonstrate that Hbs are indeed present in freshly laid eggs. The slopes of the competition curves are similar, suggesting that the same proportions of different Hbs are present in the eggs of each egg mass. Finally, the apparent biological variability in egg mass Hb content, reflected by a 25% difference between the two curves at 50% B_o, can be

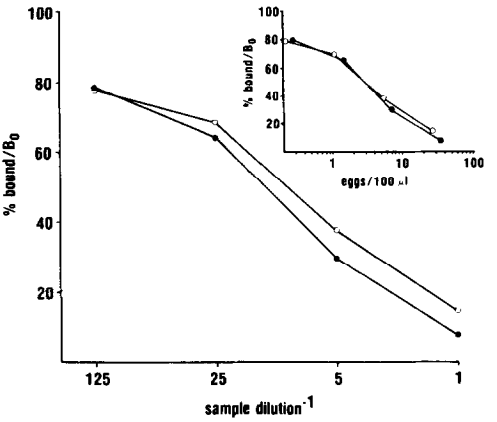

Fig. 5. RIA competition curves for C. thummi egg mass nomogenates. Number of eggs per egg mass: 345 (•); 268 (o). Inset: data adjusted and redrawn to account for the number of eggs in the competitive samples.

virtually eliminated when the competition curves are redrawn to account for the number of eggs in each egg mass (inset, Fig. 5).

DISCUSSION

Due to the cross-reactions of some chironomid hemolymph Hbs with the primary antiserum raised to Hb 3, application of this RIA to the quantitation and kinetics of stage-specific Hb synthesis will require prior separation of Hbs in samples suspected of containing Hb 3. This separation is easily achieved in one step, even in small samples, by ion-exchange chromatography or PAGE of native proteins (12, 22). Moreover, the cross-reactivity of the several Hbs, not unexpected for a family of closely related gene products, will be useful to screen tissues for the presence of Hb. In the present study, this RIA was used to detect Hbs in the freshly laid eggs of C. thummi. The presence of Hbs in eggs could be due to egg-specific synthesis or uptake of larval Hbs from the hemolymph by developing oöcytes. Ultrastructural study of the ovary has shown the accumulation of pseudoperoxidase-active material into yolk granules during development (19,20). The application of the RIA to fractionated pharate adult ovary and to fractionated egg mass homogenates should reveal any differences between Hbs in larval hemolymph and in eggs and, therefore, the extent to which egg Hb is due to accumulation from hemolymph.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the helpful discussions with Dr. John Buntin. This research was supported in part by NSF grant PCM8010915 and in part by a University of Wisconsin-Milwaukee Graduate School Committee award. Additional support was provided by the Center for Great Lakes Studies at the University of Wisconsin-Milwaukee and by a Freshwater Biomedical Center support grant PHSE501985. This is contribution No. 240, Center for Great Lakes Studies, the University of Wisconsin-Milwaukee, Milwaukee, WI 53201.

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