

EVALUATION OF SEX AND SPECIES SPECIFICITY OF POLYCLONAL ANTIBODIES AGAINST EGG-YOLK PROTEIN FROM AN ISTIOPHORID FISH

R. C. Simon

U.S. Fish and Wildlife Service, National Fish Health Research Laboratory, Kearneysville, West Virginia, U.S.A.

SUMMARY

Polyclonal antisera were prepared in rabbits using protein purified from sailfish ovaries as the antigen. These antisera were tested for reactivity with males and females of several fish species, and with pure phosvitin from hen eggs. Serum, and red muscle extracts from mature or maturing female fish reacted with the antisera, as did hen phosvitin. Serum or muscle extracts from males and immature females failed to react. Reaction intensity was measured by an enzyme-linked immunosorbent assay using 96-well microplates, spectrophotometer measurements at 410 nm, and goat anti-rabbit IgG linked to alkaline phosphatase. The test described can be applied to fresh or frozen fish filets, eviscerated carcasses, or to living specimens which can be repeatedly tested without harm. Frozen storage of samples at -20°C for 18 months did not diminish their reactivity appreciably.

RESUME

Des antisera polyclonaux ont été préparés sur des lapins en utilisant de la protéine purifiée d'ovaires de voilier comme antigène. Ces antisera ont été testés pour la réactivité avec les mâles et les femelles de plusieurs espèces de poissons, et avec phosvitine pure d'œufs de poule. Le sérum et les extraits de muscle rouge de poissons femelles matures ou en période de maturité ont réagi avec les antisera, de même qu'avec la phosvitine de poule. Le sérum ou les extraits de muscle de mâles et de femelles immatures n'a pas réagi. L'intensité de réaction a été mesurée par un dosage d'enzyme en chaîne immunosorbante en utilisant des microplaques de 96 réceptacles, des mensurations de spectrophotomètre à 410 nm, et IgG chèvre anti-lapin associé à phosphatase alcaline. Le test décrit peut être appliqué à des filets de poissons frais ou congelés, à des carcasses éviscérées ou à des spécimens

vivants qui peuvent être testés à plusieurs reprises sans danger. L'entrepôt des échantillons congelés à -20°C pendant 18 mois n'a pas diminué leur réactivité à un degré appréciable.

RESUMEN

Se prepararon antisera policlonales en conejos utilizando proteína purificada de ovarios de pez vela como antígeno. Estos antisera se probaron para conocer su reactividad con hembras y machos de diversas especies de peces, y con fosvitina pura de huevos de gallinas. El serum y los extractos de músculos rojos de hembras maduras o en proceso de maduración reaccionaron con los antisera, como ocurrió con la fosvitina de gallina. El serum o extractos musculares de machos y hembras inmaduras no presentó reacción. La intensidad de la reacción se midió mediante una muestra de ensayo inmunoabsorbente de enzimas en cadena utilizando microplacas con 96 receptáculos, mediciones de espectrofotómetro a 410 nm y IgG de cabra anti-conejo unido a fosfatasa alcalina. El test que se describe puede aplicarse a filetes de pescado fresco o congelado, a carcassas evisceradas o a especímenes vivos, que pueden ser repetidamente testados sin sufrir daño. El almacenaje de las muestras a -20°C durante 18 meses no disminuyó de forma apreciable su reactividad.

Introduction.

Several commercially valuable fish species are landed at transshipment ports with gonads and other viscera removed. This practice prevents assessment of sex-ratios in the catch, and also prevents estimates of fishing mortality for sexes separately. Still another problem may lie in uncertainty about size at first maturity, which would be necessary information if regulations were intended to protect spawning stocks.

The present study was begun in 1989 with the intent of developing an immune reagent that would enable identification of maturing and fully mature females. An ideal protein to be used as an antigen, would be female-specific. The protein chosen, lipovitellin, is found abundantly in egg yolks of many animals, along with a separate protein, phosvitin, but neither protein is present in males. These proteins are produced by the liver and are attached together when transported by the bloodstream as a large molecule called vitellogenin, which is cleaved into its constituent proteins during yolk deposition in ova.

Materials and Methods

Ovaries were obtained from two maturing sailfish (Istiophorus platypterus), stripped of their exterior membranes and washed with saline. Individual eggs were crushed in distilled water, and the resulting milky precipitate was collected by centrifugation. The precipitate was dissolved in 1.0% NaCl then two volumes of saturated ammonium sulfate were added. The precipitate containing lipovitellin was collected and again

dissolved and precipitated as above, followed by dialysis against four changes (2 liters each) of phosphate buffered saline at 4C. The solution was adjusted to a protein concentration of 60 micrograms per milliliter and 0.5 ml was mixed with 0.5 ml of complete Freund's adjuvant for each rabbit injected. After the initial injection, two boosters were given at 2 and 4 weeks, each consisting of 0.5 ml protein solution and 0.5 ml incomplete Freund's adjuvant. Rabbits were bled two weeks after the last booster injection. Enzyme-linked immunosorbant assays (ELISA) were modified from Johnstone and Thorpe (1982, pp 252-255). First and second antibody incubations were each for 1 hr at 37C. Incubation with alkaline phosphatase substrate solution was for 1 hr at 37C followed by overnight at 4C. Chicken (hen) phosvitin was purchased from the Sigma Chemical Co. (St. Louis, USA). Fish samples (red muscle or serum) representing seven families were obtained from brokers, tournaments, hatcheries, or from fish markets. Non-destructive serum samples were from anesthetized fish and were drawn from caudal vessels without anticoagulants. ELISA tests were replicated 3 or 4 times for each fish. Extracts (for ELISA) from eggs or red muscle were prepared by crushing or grinding in 6-10 volumes of phosphate-buffered saline. Antiserum dilutions in the range of 1:1000 to 1:3000 generally provided the best separation of males and females, as concluded from serial dilution trials on two antisera produced. Dilution of the second (goat) antiserum to 1:1000 was used throughout. As a precaution, rabbit antisera were absorbed with an equal volume of male fish serum, although this step was probably not necessary.

Results

In 136 samples known to be males, one serum tested positive for lipovitellin (suspected but not proven to have been misnumbered) and several serum samples from Atlantic salmon that were known to be fully mature females failed to react. Examination of female serums that did not react showed very heavy hemolysis to be present, while those that reacted were invariably clear. Some serums of female rainbow trout with small ovaries, and known to be one year before spawning condition, produced good reactions while other females of the same age failed to react. In a sample of red muscle from 14 sailfish, 9 of 11 known females reacted positively. The smallest female in the sample (19.1 kg) reacted with the antibody, while the females not reacting were the second and third smallest in the sample (19.8 and 21.1 kg). None of three males in the sample reacted. A sample of 21 small, frozen Sebastes fillets showed no reaction from extracts of red muscle, while large fillets from the same species were variably positive. Hen phosvitin reacted strongly at concentrations of 5 micrograms per microplate well, but one microgram quantities were sometimes not detected. Extracts from eggs of several fish species invariably reacted. Three of 9 red muscle samples, taken from swordfish carcasses of unknown sex, reacted strongly.

Discussion

The substantial affinity of rabbit anti-lipovitellin antibodies for hen phosvitin, and for serum or tissue extracts from females representing several fish species, suggests that these egg-yolk proteins have evolved very slowly. An antibody prepared against

lipovitellin, phosvitin, or vitellogenin from one species is thus likely to be useful for measuring the presence of one or more of these proteins in many (perhaps most) other vertebrates. Such antibodies are useful for detecting the onset of female maturation, and for following its subsequent course. Males are not known to produce egg proteins, except by experimental induction with estradiol, where vitellogenin can account for 90% of total protein synthesis in the liver (Clemens, et al. 1975). Detection of lipovitellin at the much lower levels present in young female fish may be enhanced by radioactive tagging of antibodies--perhaps enabling accurate measurement of sex-ratios at earlier ages than were possible using ELISA. Furthermore, because many variables in ELISA can be adjusted, which might increase sensitivity of the method, the technique should not be abandoned without further attempts at refinement. At the present state of application, ELISA appears to be suitable for answering a variety of questions concerning maturation schedules in female fish.

Literature Cited

- Clemens, M.J., R. Lofthouse, and J.R. Tata 1975. Sequential changes in the protein synthetic activity of male Xenopus laevis liver following induction of egg-yolk proteins by estradiol-17. *Journal of Biological Chemistry* 250 (6): 2213-2218.
- Johnstone, A., and R. Thorpe 1982. *Immunochemistry in practice*. Blackwell Scientific Publications, London: 298 pp.