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An Enzyme-Linked Immunosorbent Assay Is Not Effective for Sampling Blood Plasma Insulin Concentrations in Red Pacu, *Piaractus brachypomus* and Black Pacu, *Colossoma macropomum*

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Abstract: Culture of Red Pacu (RP), *Piaractus brachypomus* and Black Pacu (BP), *Colossoma macropomum* is increasing due to increasing demand from human populations and declining supply caused by depletion of wild fish so practical diet formulations need to be developed for pacu. Insulin assays are a valuable tool in assessing carbohydrate utilization in fish for diet development. Therefore, we conducted procedures to validate an Enzyme-Linked Immunosorbent Assay (ELISA) for detection of plasma insulin concentrations in RP and BP. Red and black pacu were fed a commercial catfish diet containing approximately 40% soluble carbohydrates (32% protein, 6% fat). Both species were then bled and plasma was used for validation of the assay. An ELISA was conducted using the Food and Drug Administration's Center for Veterinary Medicine validation of analytical procedures methodology. The results from this assay validation study indicate that an ELISA insulin kit was not suitable for experimental detection of blood plasma insulin concentrations in RP and BP. Almost no insulin (0.34 to 0.48 ng mL^{-1} for red pacu; 0.40 to 0.67 ng mL^{-1} for black pacu) was detected in unknown blood plasma samples from the fish. This indicated that the mammalian insulin antibodies are more derived or that the molecular structure of the insulin variants produced by pacu are not capable of being bound by the antibodies in the ELISA assay. The accuracy (mean recovery of spiked samples was 56.0% for RP and 68.6% for BP), linearity ($R^2 = 0.0011$ for RP and $R^2 = 0.1822$ for BP), precision (mean recovery of serial dilutions was 212.8% for RP and 209.2% for BP) and reproducibility of the data were poor.

Key words: ELISA, insulin, pacu, immunosorbent assay, sampling blood

INTRODUCTION

Red pacu, *Piaractus brachypomus* and black pacu, *Colossoma macropomum* are South American fishes from the Amazon River basin. Culture of these species is increasing due to demand for fish products by an expanding population base and depletion of wild freshwater fish in the region (Fernandes and Lochmann, 2004). Pacu are often considered to be frugivores, but are really omnivores consuming primarily leaves, fruits, tiny fish and small crustaceans (Silva, 1985). Pacu's ability to utilize large quantities of plant material and associated carbohydrates may be facilitated by a greater insulin response compared to carnivorous fish. The development of assay tools to quantify insulin concentrations in pacu may help to develop practical diets for this species.

The current standard method for assaying fish insulin is the Radioimmunoassay (RIA) (Andoh, 2006). Homologous RIA techniques are currently available only for salmon and catfish. Other problems with RIA include potential health hazards and cost of handling the radioisotopes, as well as the short stability time of the competitive antibodies (Andoh, 2006). However, Sink and Lochmann (2006) validated a non-radioisotopic Enzyme-Linked Immunosorbent Assay (ELISA) that was capable of detecting insulin in largemouth bass, *Micropterus salmoides*. This assay may be capable of detecting insulin in RP and BP. Development of non-radioisotopic insulin assays for pacu would eliminate all of the safety and stability problems of RIA. Therefore, validation procedures for RP and BP were conducted for the ELISA insulin assay used by Sink and Lochmann (2006). This ELISA assay has no hazardous by-products and uses monoclonal antibodies bound to separate antigens on the insulin molecule to produce a colorimetric endpoint. The insulin ELISA kit may be capable of detecting pacu insulin because the primary sequence of amino acids, which will be bound in the assay, is highly conservative among vertebrates (Conlon, 2000).

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MATERIALS AND METHODS

Red pacu (mean, 92±12.3 SE g) and black pacu (295±23.8) were used for this experiment. Each species was held in a 3,785-L tank with flow-through reservoir water and fed a commercial 32% protein floating catfish feed. The fish were fed to excess and 30 min post feeding, 7 RP and 4 BP were tranquilized using 100 mg L^{-1} MS-222 (tricaine methane sulfonate) and 1.5 to 2 mL blood was collected from the caudal artery/vein into a heparinized syringe (21 gauge, 38 mm needle). Additionally, 3 RP were bled at time 0, 30, 60, 90, 120, 180, 240 and 300 min post-feeding to develop insulin time response curves.

The blood was centrifuged at 5,000 rpm for 7 min. The blood plasma was removed, pooled for each species to make the "standards" and placed in 15-mL cryotubes for storage. The plasma standards were assayed repeatedly, used to make serial dilutions and spiked with standardized insulin solutions for the validation of the ELISA insulin assay. The plasma was stored at -20°C until the insulin assays were conducted.

A commercial 96 well insulin ELISA assay (Mercodia AB, Uppsala, Sweden) was used for quantitative determination of plasma insulin concentrations (Sink and Lochmann, 2006). Detection limits as established by Mercodia AB for the assay are ≤0.10 to 345 μg L^{-1}. Assay reagents and standard solutions were prepared as per kit instructions the morning of the assay. The plasma samples, reagents and well plate were brought to room temperature. The cryotubes containing the unknowns were mixed with a vortex mixer prior to addition to the well. All calibrators (standards) controls and unknowns were run in duplicate. Twenty-five microliter of each of the calibrators (0.0, 0.21, 0.62, 1.5, 3.9 and 7.2 μg L^{-1}) were added to 12 wells. Then 25 μL of each pacu plasma unknown was added to two wells for each sample.

Fifty microliter of enzyme conjugate was added to each well using a 12-channel pipette. The well plate was then incubated on a shaker plate (700 cycles per min, orbital movement) for 2 h at room temperature (26°C). The well plate was washed with a buffer solution and aspirated 5 times with a twelve-channel manifold automatic plate washer to remove unbound enzyme labeled antibodies. The plate was then inverted and tapped on absorbent paper to remove any remaining solution. Two-hundred microliter of substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well using a 12-channel pipette to detect the bound conjugate and the plate was incubated for 15 min at room temperature. Then 50 μL of stop solution (0.5 M H_2SO_4) was added to each well with a 12-channel pipette to create a colorimetric endpoint that was read spectrophotometrically at 450 nm on a Spectramax 340 PC plate reader using softmax Pro 4.3.1 LS software (Molecular Devices, Wokingham, United Kingdom). Back calculations from controls, standard curves, unknown insulin concentrations and percent Coefficient of Variation (%CV) were calculated automatically using a linear standard curve by the softmax software.

The pooled plasma sample from the RP and BP were used for the validation. The RP timed samples were assayed in duplicate to an insulin time response curve. Validation procedures were followed as in the Validation of Analytical Procedures: Methodology (FDA. CVM, 1999). No current insulin assay has been validated for pacu so accuracy was partially tested by measurement of pooled samples spiked with additional known amounts of insulin and evaluated against results for largemouth bass (Sink and Lochmann, 2006). Precision was tested by repeated assays of pooled plasma and by serial dilutions of 0, 25, 50 and 75% made by adding distilled water to pooled plasma. The pooled plasma was assayed twelve times on the same plate throughout the assay to test precision by calculation of the coefficient of variation. A percent coefficient of variation (% CV) of = 20.0 or less was set as the acceptable limit for both the intra-assay and interassay %CV (Pifat, 2006). Linearity and parallelism were tested using serial dilutions of pooled plasma. Duplicates were run of 0, 25, 50 and 75% dilutions made by adding distilled water to pooled plasma.

Regression of calibrator values for the standard curve and linearity dilutions and the percent coefficient of variation for the unknown samples were calculated automatically by the SOFTmax Pro software. The percent coefficient of variation of unknown samples was also done automatically when the plates were read. All other regression, recovery and inter-assay % CVs were calculated using SPSS 11.0 (2000) statistical software.

RESULTS AND DISCUSSION

The standard curve, generated by the calibrators, was linear with a R^2 fit of 0.993. Mean (±SE) insulin concentrations for duplicates run 12 times were 0.391±0.01 ng mL^{-1} for RP and 0.450±0.02 ng mL^{-1} for BP. Linearity and parallelism of serial dilutions of 0, 25, 50 and 75% of RP and BP were poor with a RP R^2 fit of 0.0011 and a BP R^2 fit of 0.1822. Linear regression of the log of insulin concentrations for the two pooled samples are not parallel and cross during the range of concentrations detectible by the assay. Assay results for the precision test dilutions were 0.395, 0.428, 0.358 and 0.417 ng mL^{-1} insulin (expected: 0.391, 0.293, 0.196 and 0.098 ng mL^{-1}) for RP

and 0.452, 0.433, 0.494 and 0.458 ng mL^{-1} insulin (expected: 0.450, 0.338, 0.225 and 0.113 ng mL^{-1}) for BP. This produced recoveries of 101.0, 146.1, 182.7 and 421.5%, respectively for RP and 100.4, 111.6, 219.5 and 405.3%, respectively, for BP. Additional precision tests by repeating the pooled plasma analysis twelve times produced an intra-assay %CV of 10.9 for RP and 15.9 for BP. The inter-assay %CV was not conducted due to poor validation results from other procedures.

Accuracy of the assay was poor as 25 μL plasma samples spiked with 0.21, 1.5 and 3.9 ng mL^{-1} insulin yielded assay results of 0.455, 0.798 and 2.438 ng mL^{-1} insulin for RP (expected: 0.660, 1.891 and 4.291 ng mL^{-1}) and 0.445, 1.080 and 3.321 for BP (expected: 0.601, 1.950 and 4.350 ng mL^{-1}). This represents spiked recoveries of 68.9, 42.2 and 56.8% for RP and 74.0, 55.4 and 76.3 % for BP (90-110% recovery was previously set as the acceptance limit for dilutions and spiked samples). The time response curve was not plotted due to uniformly low insulin readings throughout the duration of the sampling periods.

The results from this assay validation study indicate that the Mercodia AB insulin ELISA kit should be not used for experimental detection of blood plasma insulin concentrations in red or black pacu. We consider the accuracy, linearity, precision and reproducibility to be poor. The ELISA assay would not be suitable for nutritional, endocrinological, or physiological studies that require accurate insulin values to precisely identify mechanisms of functions. The validity appears doubtful and we cannot state with certainty that the assay was truly detecting insulin in the pacu plasma samples. This is possibly due to a different amino acid sequence in the pacu insulin molecule at the binding site sequence used by the antibody for binding in this assay. A likely explanation is that although the insulin molecule is highly conserved among vertebrates, the mammalian antibodies used by the kit are more derived and are not capable of binding pacu insulin.

The range of insulin given by Thorpe and Ince (1976) for 7 fish species was 0.26±0.15 ng mL^{-1} for pike to 6.35±0.81 ng mL^{-1} for cod. While all the values acquired during this validation fall within this range, the many failed parameters of this assay disallow us to state that these values are correct for pacu. Although non-radioactive insulin ELISA kits could facilitate nutrition research in pacu at institutions that lack radiation licenses or access to insulin RIA kits, kits that are more suitable for insulin detection in pacu must first be developed.

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