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An Enzyme-Linked Immunosorbent Assay for Sampling Blood Plasma Insulin Concentrations in Largemouth Bass, *Micropterus salmoides*

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Abstract: Insulin assays could be a valuable tool in assessing carbohydrate utilization in fish. Therefore, we conducted procedures to validate a non-radioisotopic Enzyme-Linked Immunosorbent Assay (ELISA) for detection of plasma insulin concentrations in Largemouth Bass (LMB), *Micropterus salmoides*. Wild caught LMB were subjected to oral glucose stimulation or fasted for 2 d. Farm-raised, pellet-trained bass were fed a commercial diet containing approximately 25% soluble carbohydrates. All three groups of fish were then bled and plasma was used for validation of the assay. A solid phase two-site enzyme immunoassay in which two monoclonal antibodies are bound to separate antigens on the insulin molecule was conducted using the Food and Drug Administrations Center for Veterinary Medicine validation of analytical procedures methodology. The results from this assay validation study indicate that an ELISA insulin kit could be used for experimental detection of blood plasma insulin concentrations in LMB. The linearity, precision and reproducibility of the data were acceptable. However, the accuracy is unproven as no "gold standard" for LMB insulin exists. This ELISA method would be most useful for the detection of insulin, general increases or decreases in insulin (insulin response) and general comparative trends in insulin concentration in LMB and possibly other fish.

Key words: ELISA, insulin, bass, enzyme-linked immunosorbent assay, validation

INTRODUCTION

Largemouth bass (LMB), *Micropterus salmoides*, is one of the most popular freshwater sport fish in the United States (U.S.) and is often used to control forage species and reduce stunting in small impoundments[1]. Niche foodfish markets in ethnic communities also exist on the eastern and western coasts of the U.S.[2]. Due to the popularity of LMB, commercial production has intensified and investigation into dietary requirements and feeds has increased[3-8] .

One of the key issues of diet development for LMB is the amount of carbohydrate that can be included in the diet as an energy source. Plant ingredients contain starch, the primary source of soluble carbohydrates in practical diets[9]. Higher inclusion of starch-containing plant ingredients would reduce diet cost and increase the environmental sustainability of feeds for LMB and other carnivorous fishes. Unfortunately, dietary carbohydrate levels of 27% in LMB feeds resulted in excessive liver glycogen accumulation and hepatocellular necrosis[2]. Natural diets of LMB are very low in carbohydrate and it is possible that dietary levels in excess of 27% cannot be used. However, there have been few studies of the ability of LMB to utilize carbohydrates as an energy source.

One of the best physiological determinants of metabolism, utilization and storage of carbohydrates is insulin secretion. Insulin is an anabolic polypeptide hormone secreted by the β-cells of the fish pancreas. Glucose stimulates insulin secretion in fish as in mammals, although insulin secretion can also be triggered by increased levels of arginine in fish[10]. Fish appear to be glucose intolerant compared to mammals and fish tissues may have less ability to take up glucose in response to insulin[11]. Glucose utilization and storage can take three routes; utilization for energy by skeletal muscle tissue, storage by the adipose tissues, or storage as glycogen by the liver. All three of these pathways are facilitated by insulin secretion. At this time, knowledge of the role insulin plays in muscle glucose metabolism in fish is limited[10]. The lack of insulin assay and detection tools has limited our understanding of carbohydrate metabolism in fishes.

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In mammals, adipose and liver tissues are important target tissues for insulin[10]. Fish are poikilothermic and lack the subcutaneous layer of fat needed by mammals for insulation. Consequently, fish typically have lesser amounts of adipose tissue available for glucose storage as triglycerides compared to mammals. As a result, the liver is the primary storage site for glucose as glycogen in fish. Insulin suppressed hepatic gluconeogenisis in rainbow trout, *Onchorhynkis mykiss*,[12] and encouraged glycogen formation *in vitro* in the presence of glucose in catfish[13], while decreased insulin concentrations produced hyperglycemia in salmonids[14].

Insulin assays could be valuable tools to assess carbohydrate utilization in fish. However, the current standard method for assaying fish insulin is the radioimmunoassay (RIA). 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[15]. Development of non-radioisotopic assays that are safe and effective would eliminate most of these problems. Therefore, we performed a partial validation of a non-radioisotopic enzyme-linked immunosorbent assay (ELISA) for detection of plasma insulin concentrations in LMB.

This ELISA assay uses two monoclonal antibodies that are bound to separate antigens on the insulin molecule. The endpoint is read spectrophotometrically and has no hazardous by-products. We chose an insulin ELISA kit that should be capable of detecting fish insulin because the primary sequence of amino acids, which will be bound in the assay, is highly conserved among vertebrates[16]. The mature insulin molecule in fish and other vertebrates is 51 to 58 amino acids in length with A and B chains and a molecular weight of 5,600 Da[10]. Validation of an ELISA assay for insulin will facilitate studies of insulin production in relation to carbohydrate intake and subsequent utilization in LMB. This information is needed to assess the potential for inclusion of different types and amounts of carbohydrate in practical diets for LMB.

MATERIALS AND METHODS

Fish: Two groups of LMB were used for this experiment. The first group consisted of wild fish captured by electro-fishing from the Arkansas River near Pine Bluff Arkansas. The LMB (N = 9) ranged from 812 to 1,210 g and mean (±SE) weight was 1,026.3±39.9 g. The fish were held for 2 d without feed in three 190 L tanks operated as flow through systems with well water and an air stone for supplemental aeration. These fish were used for glucose induced insulin response because their natural diet (mostly fish) would preclude any compensatory insulin response related to a history of eating commercial diets containing large amounts of carbohydrate.

The second group of LMB (N = 30) were obtained from F and L Anderson Farms, a commercial sport fish farm in Lonoke, Arkansas. These fish were ranged from 162.3 to 238.4 g with a mean weight of 212.6±13.9 g and were trained to accept commercial pelleted diets (Silver Cup® salmon slow-sinking diet (45% protein, 25% fat, <27% carbohydrate). They were held in two outdoor 2.74 m diameter, 4,500 L pools and fed once daily to slight excess of satiation with Silver Cup® (Nelson and Sons Inc., Murray, Utah) salmon diet. The Silver Cup diet is relatively low in carbohydrate content (approximately 25%) due to the high protein and extremely high fat content compared to other bass diets, but it contains more carbohydrates than the fish would ingest in a natural environment. These fish were used to measure insulin responses in fish fed commercial diets.

Glucose stimulation treatment: After the 2 d acclimation/fasting period, five wild LMB were tranquilized using 100 mg L^{-1} MS-222 (tricaine methane sulfonate) and given 10 mL kg^{-1} body weight of a 1 kg L^{-1} glucose solution by intubation directly into the stomach via a 10 mL syringe. The glucose solution was a prepared stock solution with a ratio of 1 g glucose : 1 mL distilled water. Six hundred mL of the solution was mixed, heated to facilitate glucose dissolution and cooled to room temperature prior to use. All fish recovered from anesthesia in less than 4 min. Thirty min later and the fish were again anesthetized and 1.5 to 2 mL blood was colelcted from the caudal artery/vein into a heparinized syringe (21 gauge, 38 mm needle). The blood was placed in 15 mL centrifuge tubes and centrifuged at 5,000 rpm for 7 min to separate the plasma. The plasma was removed and placed in 1.5 mL cryotubes for storage. Part of the blood plasma from each of the five LMB that received glucose stimulation was pooled to make the "standard". This standard was assayed repeatedly, used to make serial dilutions and spiked with insulin standards for the validation of the ELISA insulin assay. The plasma was stored at -20°C until the insulin assays were conducted.

Fasting treatment: Four other wild fish were anesthetized with 100 mg L^{-1} MS-222 and bled as described previously after 2 d of fasting. Plasma collection and storage was conducted as in the previous treatment. These fish were considered to be the baseline insulin response and were used to compare insulin response after glucose stimulation to response after consumption of a commercial diet.

Commercial diet treatment: Farm raised LMB (pellet trained) were fed the commercial diet to satiation once daily for 30 d prior to the experiment. They fed aggressively on the commercial diet when offered. The day of the experiment, fish were fed pellets to satiation. Fish were allowed a 30 min digestion/response period at the conclusion of active feeding (≈13-15 min after initial feeding). At the conclusion of the 30 min digestion/response period, five fish were randomly netted, anesthetized with 100 mg L^{-1} MS-222 and bled as above. Plasma collection and storage was conducted as in previous treatments.

Insulin assay procedure: A commercial 96 well insulin ELISA assay (Mercodia AB, Uppsala, Sweden) was used for quantitative determination of plasma or serum insulin concentrations. It is a solid phase two-site enzyme immunoassay in which two monoclonal antibodies are bound to separate antigens on the insulin molecule. During the incubation reaction, insulin in the plasma sample will react with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound in the titration well. Detection limits as established by Mercodia AB for the assay are = 0.10 μg L^{-1} to 345 μg L^{-1} .

Assay reagents and standard solutions were prepared as per the kit instructions the morning of the assay. The previously collected plasma samples were set out to thaw as the assay reagents, well plate and unknown samples must be used at room temperature for valid assay results. All calibrators (standards), controls and unknowns were run in duplicate. Twenty-five μL 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 unknown was added to two wells for each sample. All cryotubes that contained the unknowns were mixed with a vortex mixer just prior to addition to the well in order to help insure uniformity of plasma. Finally 25 μL of each control was added to two wells for each control. Fifty μL of enzyme conjugate was then added to each well using an 8-channel pipette to bind insulin molecules. The well plate was then incubated on a shaker plate (700 cycles per min, orbital movement) for 2 h at room temperature (26°C). After the incubation period, the well plate was washed with a wash-buffer solution and aspirated 5 times with a twelve-channel manifold automatic plate washer to remove unbound enzyme labeled antibodies. After the final wash the plate was inverted and tapped on absorbent paper to remove any remaining solution. Two hundred μL of substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well using an 8-channel pipette to detect the bound conjugate. The plate was then 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 an 8-channel pipette to create a colorimetric endpoint that was read spectrophotometrically at 450 nm on a (Spectramax 340PC plate reader using SOFTmax Pro 4.3.1 LS software, MolecularDevices, Wokingham, United Kingdom). Back calculations from controls, standard curves, unknown insulin concentrations and percent coefficient of variation (% CV) were generated automatically by the SOFTmax software.

Insulin assay validation: The Pooled Glucose Plasma Sample (PGS) from the glucose stimulation test was used for the validation. All other samples were assayed in duplicate to evaluate blood plasma insulin concentrations of various treatments. Validation procedures were followed as in the Validation of Analytical Procedures: Methodology[17].

Accuracy is a measure of exactness of an analytical method and is typically tested against an accepted proven method. No current insulin assay has been validated for LMB so accuracy was partially tested by measurement of PGS samples spiked with additional known amounts of insulin. Accuracy of the assay was tested by comparing the 25 μL PGS sample with 25 μL PGS samples spiked with 25 μL of 0.62, 1.5 and 7.2 ng mL^{-1} insulin or an additional 25 μL of PGS. Precision was tested by repeated assays of PGS and by serial dilutions of 0, 25, 50 and 75% made by adding distilled water to PGS. The PGS was assayed in duplicate four times throughout the assay to test precision by calculation of coefficient of variation. A percent coefficient of variation of = 20.0 or less was set as the acceptable limit for the intra-assay % CV, while = 20.0 was also set as the acceptable limit for the inter-assay % CV[18]. For the intra-assay % CV, anything over 20.0 was rerun as to confirm the variation in precision, or to determine if human error in pipeting and irregularities in the plasma samples accounted for the variation. The original values, rather than the rerun values, were used in all analysis of results.

Linearity and parallelism were tested using serial dilutions of PGS and pooled plasma from fasted (FP) LMB. Duplicates were run of 0, 25, 50 and 75% dilutions made by adding distilled water to PGS and FP. Range was not tested as linearity did not approach the extremes of the specified range (0.5 to 35.0 ng mL^{-1}) for the assay. Inter-assay and intra-assay coefficients of variation were used to confirm precision and to quantify reproducibility of results within and between assays by repeating the procedures with another Mercodia AB insulin kit.

Statistical analysis: Regression of calibrator values for the standard curve and linearity dilutions and the 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 0.984. Mean (± SE) PGS insulin concentrations for duplicates run four times separately were 7.91±0.04 ng mL^{-1} with an intra-assay % CV of 10.80. This insulin concentration is the standard value of PGS used for most of the validation procedures. Insulin concentrations from fish in other treatments are found in Table 1. Linearity Fig. 1 and parallelism Fig. 2 of serial dilutions of 0, 25, 50 and 75% of PGS and FP were good with a PGS R^2 fit of 0.995 and a FP R^2 fit of 0.998.

Accuracy of the assay was low as 25 μL PGS samples spiked with 0.62, 1.5 and 7.2 ng mL^{-1} insulin calibrators yielded assay results of 7.79±0.04, 8.30±0.09 and 12.93±0.07 ng mL^{-1} insulin respectively (expected values: 8.53, 9.41 and 15.11 ng mL^{-1}). This represents spiked recoveries of 91.3, 88.2 and 85.6% respectively, which are considered acceptable to low (Personal communication, Kelly Fecteau, Assistant Professor and Assistant Director, Clinical Endocrinology Service, Department of Comparative Medicine, University of Tennessee, Knoxville, Tennessee). Accuracy of samples spiked with an additional 25 μL of PGS was better with assay results of 16.21 ng mL^{-1} insulin (expected value: 15.82 ng mL^{-1}).

Table 1: Blood plasma insulin concentrations of largemouth bass for three treatments from an experimental insulin enzyme-linked immunosorbent assay. Blood was taken from largemouth bass 30 min after oral glucose stimulation, fasting for 2 d, or 30 min after being fed a commercial salmon diet.

Treatment	Fish	Insulin (ng mL^{-1})	% CV
Glucose stimulation (30 min)	1	2.1	15.8
Glucose stimulation (30 min)	2	9.3	18.4
Glucose stimulation (30 min)	3	21.3	10.6
Glucose stimulation (30 min)	4	8.0	11.64
Glucose stimulation (30 min)	5	1.4	9.87
Glucose stimulation (30 min)	pooled	7.9	10.8
Fasted (2 d)	1	1.1	5.49
Fasted (2 d)	2	1.4	13.9
Fasted (2 d)	3	0.7	19.5
Fasted (2 d)	4	2.1	18.9
Fasted (2 d)	pooled	1.6	15.7
Commercial diet (30 Min)	1	0.5	14.2
Commercial diet (30 Min)	2	1.9	8.5
Commercial diet (30 Min)	3	1.9	10.4
Commercial diet (30 Min)	4	1.7	12.3
Commercial diet (30 Min)	5	1.5	6.6

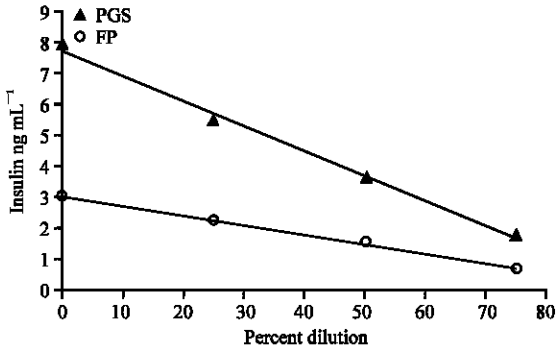

Fig. 1: Linearity test of serial dilutions of pooled plasma from five orally glucose stimulated (PGS) and four fasted (FP) largemouth bass. The linearity test was a portion of a validation effort for an enzyme-linked immunosorbent assay for determining blood plasma insulin responses in fish

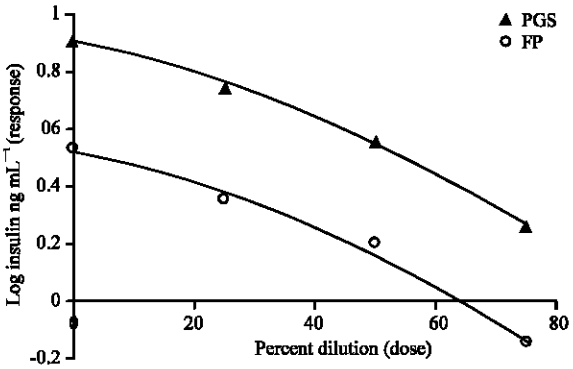

Fig. 2: Parallelism test of serial dilutions of pooled plasma from five orally glucose stimulated (PGS) and four fasted (FP) largemouth bass. The linearity test was a portion of a validation effort for an enzyme-linked immunosorbent assay for determining blood plasma insulin responses in fish

This produces a recovery of 102.5%, which is acceptable for accuracy (Personal communication, Kelly Fecteau, Assistant Professor and Assistant Director, Clinical Endocrinology Service, Department of Comparative Medicine, University of Tennessee, Knoxville, Tennessee).

Assay results for the precision test dilutions were 7.88, 5.49, 3.60 and 1.83 ng mL^{-1} insulin respectively (expected values: 7.91, 5.93, 3.96 and 1.98 ng mL^{-1}). This produced recoveries of 99.6, 92.5, 91.0 and 92.1%, respectively. Linear regression for precision of serial PGS dilutions of 0, 25, 50 and 75% were acceptable with an R^2 fit of 0.995. Additional precision tests by repeating the

PGS analysis four times in duplicate produced mean results of 7.85, 7.90, 8.02 and 7.88 ng mL^{-1} insulin (SE±0.04) with a % CV of 3.0. Other measures of the precision and reproducibility are inter- and intra-assay % CV. Intra-assay % CV for unknowns in the first assay kit ranged from 5.49 to 23.9%. with the mean (±SE) being 15.36±1.51%. Only one sample that yielded a % CV of 23.9 was rerun, but continued to yield a high % CV. Intra-assay % CV for the second assay kit ranged from 2.4 to 19.2% with the mean being 9.46±0.93%. The inter-assay % CV for the two assays was 15.46%.

Specificity defines the ability of the assay to measure the compound of interest and exclusion of other relevant compounds. Specificity tests for determination of impurities were not conducted, although Mercodia AB (Mercodia 2006) reports crossreactivities of <0.05% for C-peptide, <0.02% for IGF-I, <0.02% for IGF-II and <14% for proinsulin. Detection limits were not tested as the range detection limits determined by the manufacturer, = 0.10 to 345 μg L^{-1}, far exceed the range of LMB insulin concentrations that were tested.

The results from this assay validation study indicate that the Mercodia AB insulin ELISA kit could be used for experimental detection of blood plasma insulin concentrations in LMB. We consider the linearity, precision and reproducibility to be acceptable. However, the accuracy is unproven and the types of studies that this assay could be applied to must be considered relative to the specific objectives of each study. We recommend this assay for detection of insulin, general increases or decreases in insulin (insulin response) and detection of general comparative trends of insulin concentrations in nutrition studies with LMB and possibly other fish. The ELISA assay would not be suitable for an insulin endocrinology/physiological study that requires more accurate individual values to precisely identify mechanisms of physiological functions.

This study also demonstrates that insulin response in LMB is highly variable among individuals, although some trends in insulin concentration due to treatment were apparent. Several LMB that received oral glucose stimulation exhibited higher blood plasma insulin concentrations(mean 6.69±4.1, range 0.14 to 22.3 ng mL^{-1} insulin), while the fasted (mean 1.3±0.3, range 0.72 to 2.07 ng mL^{-1} insulin) and pellet-fed fish (mean 1.5±0.3, range 0.50 to 1.90 ng mL^{-1} insulin) appeared to be quite similar in insulin concentrations. More data is needed on insulin response in LMB and time-response curves in relation to feeding need to be developed. With increased understanding of the physiological basis of the insulin response of LMB, economical commercial diets suitable for production of this species can be developed. This non-radioactive insulin ELISA kit could facilitate nutrition research on carnivorous fish at institutions that lack radiation licenses or access to insulin RIA kits.

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