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Quantitative Determination of Recombinant Bovine Somatotropin in Commercial Shrimp Feed Using a Competitive Enzyme-Linked Immunosorbent Assay

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Recombinant bovine somatotropin (rbST), also known as growth hormone, is used to enhance production and development of animals within the agriculture and aquaculture industries. Its use is controversial because of its potential effects on human and animal health. To screen for rbST in shrimp feed, a competitive enzyme-linked immunosorbent assay (ELISA) with an inhibition step was developed. Sample and rbST antibody (rabbit anti-rbST) were incubated at room temperature for 30 min. Subsequently, this competitive reaction was transferred to a microplate coated with rbST, using goat antirabbit IgG linked with horseradish peroxidise as the secondary antibody. Substrates for peroxidise were added, and the absorbance at 410 nm was determined. The applicability of the method was assessed using rbST extracted from "spiked" shrimp feed samples. The assay was reproducible and linear with R^2 values greater than 0.98 over the standard curve range of 20−500 μg/g. The intra- and interday precisions expressed as relative standard deviations were 3.4 and 5.3%, respectively. The mean recovery from 15 spiked feed samples was 105%. This assay will be a valuable tool for quantitative detection of rbST by both governments and commercial companies and can be modified for other types of feed.

KEYWORDS: ELISA; recombinant bovine somatotropin; hormone; rbST; aquaculture

INTRODUCTION

Somatotropin, also known as growth hormone, is a polypeptide with a molecular mass of 22 kDa. It is produced in the anterior pituitary gland of animals (*1*). This peptide acts as a hormone to stimulate somatic growth and development of preadult vertebrates. Recombinant versions of this peptide have been produced, such as bovine somatotropin (rbST) (*2*). The administration of this protein is well-documented in the cattle industry for increasing the efficiency of milk yield (*2*). The application of rbST in dairy cattle was approved in 1994 by the Food and Drug Administration (FDA) for use in the United States where it is now widely used (*2*). In addition, regulatory agencies in other countries (e.g., Brazil and Korea) have approved the use of rbST to increase milk production and to improve the efficiency of feed use (*2*). The European Union, however, has banned the use of recombinant somatotropins due to the potential dangers to milk consumers (*1*, *3*, *4*).

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However, the use of rbST has widespread application, and its use is not limited to agriculture. There have been studies demonstrating that rbST has increased growth rates, time to maturation, and the immune response in many aquaculture species including carp (*Cyprinus carpio*) (*4*), coho salmon (*Oncorhynchus kisutch*) (*5*, *6*), American elver (*Anguille rostrate*) (*3*), channel catfish (*Ictalurus punctatus*) (*7*−*11*), rainbow trout (*Oncorhynchus mykiss*) (*12*−*14*), tilapia (*Tilapia* spp.) (*15*), Korean rockfish (*Sebastes schlegeli*) (*16*), and pacific white shrimp (*Penaeus vannamei*) (personal communication).

The use of this hormone is controversial because of its potential effects on human and animal health; therefore, there is a need to be able to detect the recombinant protein (*1*). Detection requirements can be due to both government legislation to certify levels or the absence of rbST or by industry wanting to conduct quality assurance on their feeds. Currently, there are many published methods of detecting rbST/bST in the biological fluids of animals and injection preparations (*2*, *17*−*19*). However, none of them is employed for the quantitative analysis of rbST in animal feeds. The present study describes the development of a competitive enzyme-linked immunosorbent assay (ELISA) for the quantitative detection of rbST in shrimp feed with the potential as an

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effective technique for use by both commercial companies and regulatory agencies.

MATERIALS AND METHODS

Chemicals. Commercially available rbST was purchased in dry powder formulation from Monsanto (St. Louis, MO). Shrimp feed was purchased in dry powder form from Aquatic Eco-Systems (Apopka, United States). 2,2′-Azino-di-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was obtained from Sigma (St. Louis, MO). ELISA microwell plates were purchased from Nunc (Denmark). All ELISA buffers, including ELISA diluent, ELISA wash buffer, and ELISA blocking buffer, were obtained from TropBio (Townsville, Australia). Primary antibody against bovine growth hormone (rabbit antibovine growth hormone) and the secondary antibody [goat antirabbit IgG (H&L) HRP conjugate] were purchased from Abcam (Cambridge, MA). All chemicals were of analytical grade or better. All water used was Milli-Q water (Millipore Corp., Bedford, MA).

rbST Standard Preparation. A stock solution of rbST was prepared by dissolving 2 g of rbST in 500 mL of 10 mM hydrogen peroxide (pH 9.5). This was diluted with 10 mM hydrogen peroxide (pH 9.5) to a working stock of 0.1 mg/mL and diluted further to various concentrations of rbST between 6.25 and 500 μg/10 mL in 10 mM hydrogen peroxide (pH 9.5). All solutions were freshly prepared on a daily basis.

Extraction. Using a laboratory feed mill, known concentrations of rbST were added to dry shrimp feed prior to cold press extrusion. Two grams of feed was placed into a 50 mL centrifuge tube, and 20 mL of 100 mM NaOH (pH 9.5) was added to each tube. The standards and samples were then mixed for 30 s using a vortex and incubated in a 50 °C water bath for 15 min and then at room temperature for 20 min (the sample was mixed every 5 min during incubation). The standards and samples were then centrifuged at 1000*g* for 10 min. A 1000 μL amount from the middle layer, distinguished from the upper layer, which consisted of fats, of each centrifuge tube was transferred to a microcentrifuge tube and then diluted 2-fold in sodium bicarbonate buffer, pH 9.6, from 1:2 to 1:256.

Competitive ELISA. The assay (**Figure 1**) was a competitive ELISA. Unless otherwise noted, all steps were performed at room temperature, and washing was conducted with ELISA wash buffer. The initial incubation of the assay was carried out in low-binding 96-well U-bottom noncharged microwell plates. First, 100 μL of rabbit antibovine growth hormone IgG (1:5000) was added to each well, followed by 100 μL of either rbST standard or samples. Plates were covered and incubated at room temperature for 30 min. Samples and standards were assayed in triplicate.

For the second incubation, a flat-bottom microplate was coated overnight with 1 μg rbST/well in 10 mM NaOH (pH 9.5). Plates were washed once, blocked with 150 μL of blocking buffer for 1 h, and rewashed once.

A 100 μL volume of each incubate was transferred to the coated and blocked ELISA plate. The plate was covered and incubated for 1 h. After the plate was washed three times, 100 μL of goat antirabbit IgG (H&L) HRP conjugate (1:4000) was added and incubated for 1 h, followed by three washes. ABTS was added and incubated for a further 1 h prior to determining the color formation with an ELISA reader (BioTek, software: Gen5) using a wavelength of 410 nm. Optical densities corrected for absorbance of blank wells (containing no primary antibody) were graphed against logarithm rbST concentration. The standard curve obtained from the dilution with the highest coefficient of determination was used to determine the sample concentration of rbST.

Assay Performance Validation. The characterization of the developed method involved the repeated generation of six-point calibration curves of shrimp feed spiked with different amounts of rbST prior to extraction and analysis by ELISA. The reproducibility of the assay was assessed by comparing the slope of the standard curves of consecutive assays, and a linear working range was determined. Three standards were included on all ELISA plates as an indicator of assay variability. To assay for matrix interference of the shrimp feed, standard curves were prepared in both 10 mM NaOH (pH > 9.5) and shrimp feed.

To demonstrate the applicability of the method, it was used for the determination of rbST in 15 spiked shrimp feed samples. The contents of rbST in these samples were in the range of 0−40 μg/g shrimp feed. The samples were analyzed by an investigator who was blinded to the concentration of rbST in the samples. Recovery was calculated by comparing the analytical results obtained for the extracted spiked feed samples (calculated using a standard curve constructed with standards of rbST in shrimp feed) with the amount of rbST added (representing 100% recovery). The intraday and interday assays were evaluated six times by analyzing shrimp feed spiked with rbST at three concentrations (6.25, 12.5, and 25 μg/g).

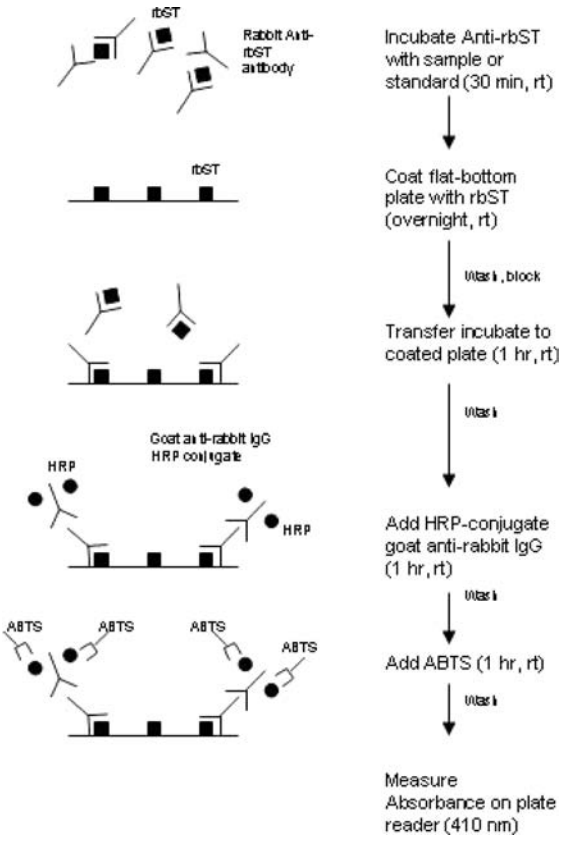

Figure 1. Schematic diagram describing the assay procedure for recombinant bovine somatotrophin (rbST). IgG, immunoglobulin; and HRP, horseradish peroxidise.

RESULTS

Calibration curves of corrected absorbance versus the logarithmic concentration of rbST gave correlation coefficients (R^2) greater than 0.98. A typical reference standard curve constructed using rbST standards extracted from shrimp feed is shown in **Figure 2**. The approximate working linear range for this curve is 20−500 μg/g. No statistical significance difference was observed between the correlation coefficients.

The recovery of rbST from aquaculture feed is shown in **Figure 3**. Although the correlation was high ($r = 0.99$), the assay slightly overestimates samples toward 0 (intercept = 1.05). Recoveries from the spiked shrimp feed samples ($n = 15$) are given in **Table 1**. The mean recovery was 105%, which is within the range of acceptability. The intra- and interday precision studies are shown in **Table 2**. The coefficient of variation (CV) % of intra- and interday variability was less than 10%, which is within the limit of acceptability (±15%).

DISCUSSION

There are numerous reports in the literature employing various analytical techniques to detect rbST in biological fluids (*18*−*21*); however, the present paper is the first report of an ELISA for the detection of rbST in shrimp feed. This paper utilizes a competitive ELISA with an inhibition step to detect rbST. The ELISA can easily be established by any laboratory working in this area,

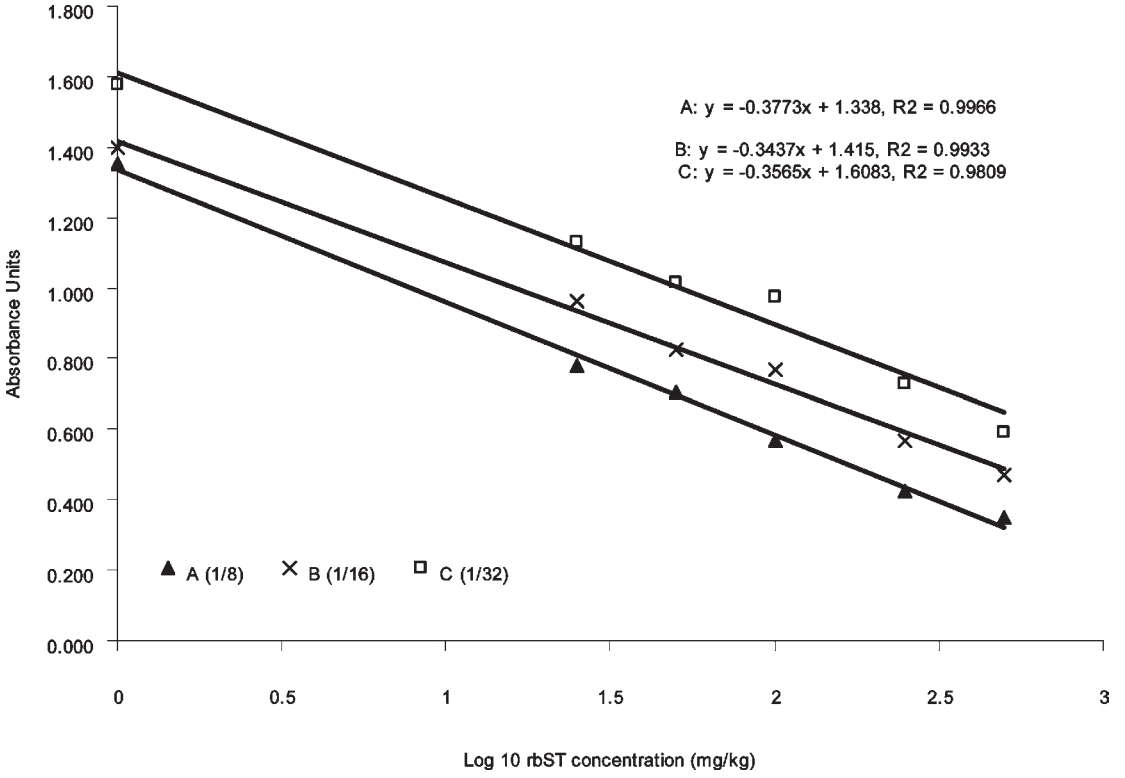

Figure 2. Typical rbST reference standard curve showing the working linear range.

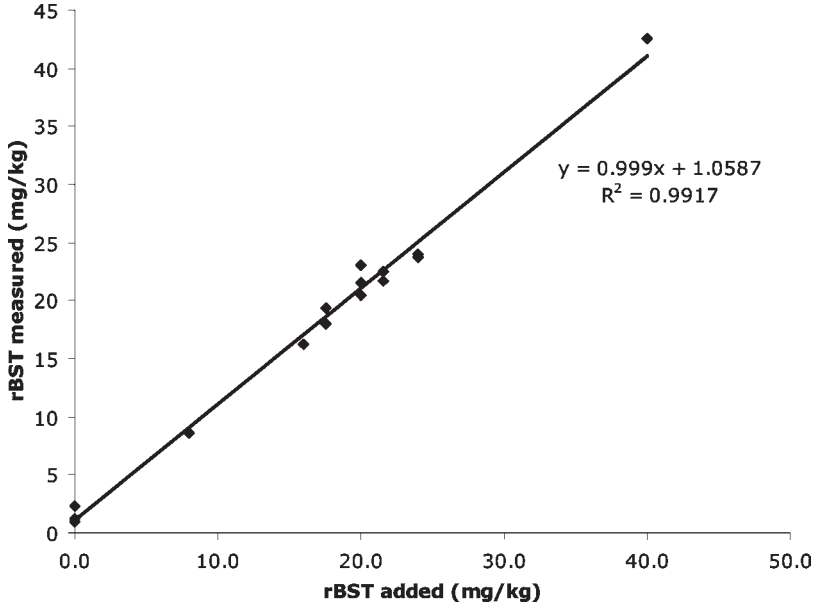

Figure 3. Recovery of increasing amounts of rbST standard added to a constant weight of shrimp feed.

utilizing commercially available antibodies and plates. The assay's working range of 20−500 μg/g is ideal for the concentration range utilized in shrimp feed. The proposed method can be applied successfully for the determination in feed using an ELISA after extraction using 10 mM NaOH (pH > 9.5). The same extraction technique can be employed for the analysis of rbST from other feeds and construction of an in-house standard curve prepared in the feed of interest, to facilitate analysis. The use of standards prepared in the same matrix with samples corrects for the relatively low error from incomplete recovery and possible interference from the matrix via absorbance at 410 nm.

Matrix interference is a major factor influencing assay performance by affecting assay sensitivity or color development or both of these parameters (*22*). Shrimp feeds are composed of soybean meal, fish meal, yeast protein, meat meal, wheat gluten protein, and other fillers dependent on proprietary brands (*23*). Potential matrix interference from the feed ingredients was identified by comparing the absorbance at 410 and logarithmic concentration curves prepared in the shrimp feed with those prepared in 10 mM NaOH (pH > 9.5). There was found to be no significant effect on assay sensitivity and color development. It is anticipated that these results would be similar when using fish feed as it contains similar ingredients (*24*).

Table 1. Recoveries of rbST from Spiked Shrimp Feed

sample	measured rbST concentration (μg/g)	spiked rbST concentration (μg/g)	recovery (%)
A	0.97	0.0	
B	23	20.0	115
C	24	24	100
D	8.56	8.0	107
E	20.5	20.0	102.5
F	1.29	0.0	
G	23.7	24.0	99
H	21.7	21.6	100.4
I	18	17.6	102.
J	42.5	40.0	106
K	2.3	0.0	
L	16.2	16.0	101.2
M	19.3	17.6	119
N	21.5	20.0	108
P	22.5	21.6	104.2
mean			105

Table 2. Intra-assay and Interassay Variability of ELISA for Recombinant Bovine Somatotropin

added rBST (μg/g)	experimental (mean and standard deviation)	coefficient of variation (%)
	intraday precision (n = 6)	
6.25	6.14 ± 0.12	1.9
12.5	13.2 ± 0.6	4.6
25	24.2 ± 0.6	2.5
mean		3
	interday precision (n = 6)	
6.25	6.5 ± 0.2	3.1
12.5	13.8 ± 0.9	6.5
25	28.1 ± 1.8	6.4
mean		5.3

There have been numerous unsuccessful attempts to quantify rbST in aquaculture feed in our laboratory. In the present assay, the recovery of rbST from feed is facilitated by the addition of NaOH at a pH greater than 9.5. The effects of pH on the extraction of rbST indicated that the extraction failed at pH below 9 but could withstand a pH up to 10, without affecting the analytical performance. A similar higher pH buffer was utilized for extraction of rbST proteins from different commercially available injections and improved their extraction efficiency (*2*). The feed has its own buffering capabilities; therefore, solubilization of the protein into the extraction solvent can only be achieved if the pH is greater than 9.5. Depending on the expected concentration of rbST being tested in samples, standards can be optimized to reduce error, and the ELISA can be used to detect samples with a range of 20−500 μg/g of rbST per shrimp feed.

In the current study, the specificity of the assay was not compared to other hormones such as insulin and oxytocin, because these are not presently utilized in the shrimp industry as additives. Given that this ELISA utilizes an initial inhibition step with antibodies designed for rbST, it is likely to be very specific for the recombinant protein; however, some amount of cross-reactivity with other recombinant somatotropin proteins (with similar sequence) is likely.

This ELISA will enable monitoring and control of the hormone as an additive. Feed companies and production facilities can use the ELISA to ensure that the correct concentration of rbST is in the feed. Additionally, organizations can use the ELISA to screen for its use in countries that the substance is controlled. In conclusion, we have developed a rapid extraction technique and sensitive and reliable ELISA, which can be adapted for the routine screening of rbST in animal feeds.

ABBREVIATIONS USED

CV, coefficient of variation; rbST, recombinant bovine somatotrophin; ABTS, 2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonic acid; ELISA, enzyme-linked immunosorbent assay.

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