NOTE

Competitive Enzyme Immunoassay for Bovine Growth Hormone

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Abstract. We developed an enzyme immunoassay (EIA) for bovine GH (bGH) which is based on indirect competitive immunoassay in culture medium from a bovine pituitary cell culture. 40 μl cell culture samples (or bGH standard) and bGH antibody (rabbit anti-bGH) were added to the 96 well microplate coated with secondary antibody (Goat anti-rabbit IgG), and incubated for 24 h at 37 °C. Biotin-label bGH was added and incubated further for 24 h at 37 °C, and biotinylated bGH was linked with streptavidin-peroxidase. Substrates for peroxidase were added to the plate and incubated for 1 h at 4 °C. The enzyme reaction was stopped with 4N H2SO4, and the absorbency at 450 nm was measured with an ELISA Reader. The coefficients of intra-assay and inter-assay variations were 4.13–7.59% and 3.71–8.27%, respectively. The regression equation and correlation coefficients with the radioimmunoassay (RIA) were y(RIA) = 1.9986 x (ETA)− 1.3921 and 0.9701 (n=27), respectively. Collectively, the present assay provides a reliable alternative to RIA and offers the major advantage of eliminating radioactive reagents and counting equipment.

Key words: Enzyme immunoassay (EIA), Radioimmunoassay (RIA), Bovine GH (Endocrine Journal 44:195-198, 1997)

BOVINE GH (bGH) is closely related to the growth, development and production of livestock. A radioimmunoassay (RIA) for the measurement of bGH plays an important role in research on GH in cattle. For the past decade, enzyme immunoassay (EIA) with higher sensitivity and precision has been reported in several pituitary hormones [1-4]. EIA for bGH makes it possible to perform a large number of measurements in a short time at substantially lower cost. In many EIA procedures, an indirect assay based on competition of labeled antigen with an unknown sample for the antibody is the strategy of choice. This parallels the approaches used in double antibody RIA. Since primary antibody is a limiting reagent, we chose a strategy based on indirect competition by using the secondary antibody.

In this paper, we aimed to establish an indirect competitive EIA system for the assay of bGH, especially for the medium derived from primary bovine pituitary cell culture. This method offers advantages in the study of GH secretion in bovine pituitary cell culture.

Materials and Methods

Materials

Rabbit anti-bGH (TE-R-1-1-4) and bGH (USDA-bGH-I-2) for reference and labeling preparation were generously supplied by the USDA Animal Hormone Program. Goat anti-rabbit IgG (H+L)
was bought from Seikagaku Corporation (Japan). Biotin labeling kit for biotinylation of bGH was bought from Boehringer Mannheim Biochemica. Streptavidin peroxidase (SAP) (S5512), urea hydrogen peroxide (U1753) and bovine serum albumin (BSA) (A6793) were obtained from Sigma Chemical Company. Human growth hormone releasing factor (1–29 amide) (hGRF) was obtained from Peptide Institute, Inc. (Japan). All other reagents were purchased from Wako Chemical (Japan).

**Biotinylation of bGH**

Biotinylation of bGH was done with a biotin labeling kit. 500 ng bGH was dissolved in 0.5 ml of NaHCO₃ buffer (0.05 M, pH 9.5). The molar reaction mix was 1:20, i.e. 1 molecule of bGH was reacted with 20 molecules of D-biotinoyl-aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS). Preparation of the column, labeling with biotin-7-NHS and column chromatography were done by kit protocol.

**EIA steps**

Goat anti-rabbit IgG (H+L) was diluted to 50 µg/ml in coating buffer (0.015 M Na₂CO₃ and 0.034 M NaHCO₃: pH 9.6). 100 µl Goat anti-rabbit IgG (H+L) was put into a 96-well microplate (Nunc immuno plate) and coated for 2 h at room temperature. The plate was decanted and blocked with 250 µl blocking buffer (0.04 M Na₂HP₂O₄, 0.145 M NaCl and 0.1% BSA: pH 7.2) for 2 h at room temperature, and then stored at 4 °C. Before using, the plate was washed two times with washing buffer (0.05% Tween 80). 40 µl of culture medium samples (bGH standards) and 100 µl of bGH-antiserum (Rabbit anti-bGH, x 100,000) diluted in assay buffer (0.042 M Na₂HP₂O₄, 0.008 M KH₂PO₄, 0.02 M NaCl, 4.8 mM EDTA 2Na and 0.05% BSA: pH 7.5) were put into the wells. Standards were diluted with GH-free Dulbecco’s Minimal Essential Medium (DMEM). After 24 h incubation at 37 °C, the plate was decanted and 100 µl of biotin-labeled bGH (360 pg) diluted in assay buffer was added, and then incubated for another 24 h at 37 °C. The plate was washed four times with the washing buffer. 100 µl substrate solution (mixed with the same volume of substrate A and B) was added and then incubated for 1 h at room temperature. Substrate A (pH 5.0) consisted of 0.1% urea hydrogen peroxide, 0.1 M Na₂HP₂O₄, 0.05 M citric acid, and substrate B (pH 2.4) contained 0.05% 3,3′,5,5′-tetramethylbenzidine, 4% dimethyl sulfoxide and 0.05 M citric acid. The enzyme reaction was stopped by adding 25 µl of 4N H₂SO₄ to each well. The absorbency at 450 nm was measured directly through the bottom of the plates with an ELISA Reader (International Reagents Corporation, Japan).

**Preparation of culture medium samples from primary bovine pituitary cell culture**

Culture medium samples of various bGH concentrations were prepared from bovine pituitary cells treated with hGRF. Fresh bovine whole pituitaries were obtained from a local abattoir and were divested of encapsulating tissue, the neurohypophysis and pituitary stalk tissues. The anterior tissues were sectioned at about 1 mm³ and incubated with 0.15% (w/v) collagenase/calcium-free HEPES buffer solution for 120 min at 37 °C. After centrifugation at 800 g, the cells were resuspended in the DMEM and counted by trypan blue exclusion test. Cell culture (5 x 10⁵ cells/well) was carried out in DMEM containing 10% fetal bovine serum under 5% CO₂-95% air. After 4 days, the cells were washed two times with DMEM (without fetal bovine serum), and then tested with DMEM containing hGRF (10⁻⁴ M).

**RIA of bGH**

bGH in the culture medium was assayed by RIA as previously reported [5]. The sensitivity was 0.54 ng/ml, and intra- and inter-assay coefficients of variation (CV) were less than 5 and 10%, respectively.

**Results**

**Standard curves for bGH**

Figure 1 shows the typical standard curves for bGH. Reproducible results for competitive binding were obtained in the dynamic range (0.78–200 ng/ml). When using 40 µl culture medium samples,
the sensitivity was 0.2 ng/ml. Samples containing a high concentration of bGH were prepared with bovine primary pituitary cell culture and serially diluted with GH-free culture medium. As shown in Fig. 1, the curves produced by the samples closely paralleled those of the standard, showing that there is practically no problem in assessing bGH released into culture medium when using this EIA.

Assay variation in bGH EIA

The assay variations in the standard immunoassay were examined at 4 different bGH levels in culture medium. The intra-assay and inter-assay coefficients of variation were 4.13–7.59% (n=5) and 3.71–8.27% (n=5), respectively (Table 1).

Correlation coefficients between EIA and RIA

bGH levels of 27 samples were determined by EIA and a double antibody RIA. The regression equation and correlation coefficient were \( y (\text{RIA}) = 1.9986 \times (\text{EIA}) - 1.3921 \) and 0.9701 (n=27), respectively (Fig. 2).

Discussion

We developed a highly sensitive competitive EIA for bGH secreted from bovine primary pituitary cell culture. The dynamic range of the standard curves seemed to be sufficient and reproducible enough to detect the bGH from culture medium. The parallelism of cell culture samples to standard curves indicates that this EIA can measure the high concentration of bGH in diluted samples. Although we used the reference for bovine, this assay system may be used for ovine samples also (data not shown). The present EIA, though it uses the same polyclonal antibody as in RIA, tended to give lower

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**Table 1. Intra- and inter-assay coefficients of variation (CV)**

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
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<tbody>
<tr>
<td>1.88</td>
<td>6.49</td>
<td>8.27</td>
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<tr>
<td>20.62</td>
<td>4.13</td>
<td>5.76</td>
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<tr>
<td>50.32</td>
<td>7.46</td>
<td>3.71</td>
</tr>
<tr>
<td>120.07</td>
<td>7.59</td>
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</tr>
</tbody>
</table>

*Intra and inter-assay coefficients of variation in EIA for GH were determined from 5-replicated measurements of bovine anterior pituitary cell culture media with four different hormone concentrations.
values by approximately 50% than RIA. The cross activity of iodine-labeled bGH with anti-bGH is far higher than that of biotin-labeled bGH. This may be due to the difference of antigen-antibody reaction between RIA and ETA.

Secchi et al. [3] reported a sandwich enzyme-linked immunosorbent assay of bGH in bovine plasma. The sandwich assays require the preparation of a large amount of antibody, but indirect competitive assays with labeled antigens are good alternatives to overcome this limitation. The use of a second antibody instead of a hormone-specific antibody is preferred for coating the well as it reduces assay variations associated with uneven binding of the latter antibody to the wells and further reduces the amount of hormone specific antibody needed in the EIA [4, 6]. Our results showed that the amount of bGH antibody needed is only one tenth that needed for our bGH RIA system [5]. 1 mg of second antibody was sufficient to perform more than 200 assays.

Other advantages include the elimination of problems and additional effort associated with using ¹²⁵I as the assay indicator. The chemical modification step for iodination is eliminated. Further, the biotin-label bGH used in ETA is stable for more than 6 months when stored at -80 °C whereas radioactive iodine has a half life of only 60 days. Secondary antibody reagents can also be stored in ready to use concentrations for at least 2 months at -20 °C. The counting equipment is replaced by a less expensive and less cumbersome absorbency detector. The time required to take readings on the assay plate is 1 min or less. Finally the EIA assay can be completed in approximately 48 h compared to 72 h for RIA. The least detectable amount of bGH was 0.2 ng/ml in the present assay, while it was 0.54–0.82 ng/ml in the previous reported RIA [5]. This new assay is reproducible, sensitive and satisfies the criteria of a valid immunoassay. Nevertheless, bovine plasma samples were not completely parallel to the curve of the bGH standard samples diluted with the culture medium. There may be many unknown factors that interfere with the antigen-antibody reaction in the plasma. Further study is required for the development of the indirect ETA system for plasma samples.

The results demonstrated that ETA for bGH has the same sensitivity, precision and reproducibility as RIA. Owing to the advantages of the method (e.g. elimination of radioactivity, small sample volume and speed), our ETA system will be a practical alternative in bovine pituitary cell culture.

**Acknowledgments**

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**References**


