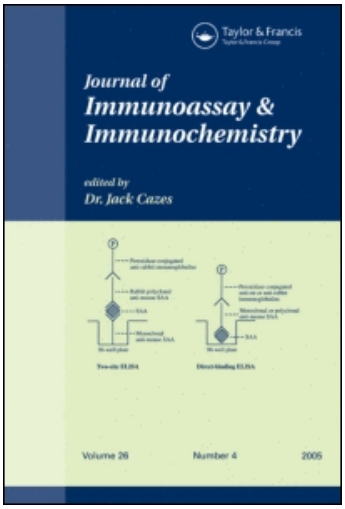

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development and Validation of a Simple Sensitive Enzyme Immunoassay (EIA) for GH Determination in Buffalo Plasma

B. S. Prakash[a]; Mohan Mondal[a]; N. Anandlaxmi[a]
[a] Division of Dairy Cattle Physiology, National Dairy Research Institute, Karnal, Haryana, India

Online publication date: 11 June 2003

To cite this Article Prakash, B. S. , Mondal, Mohan and Anandlaxmi, N.(2003) 'Development and Validation of a Simple Sensitive Enzyme Immunoassay (EIA) for GH Determination in Buffalo Plasma', Journal of Immunoassay and Immunochemistry, 24: 4, 409 — 420
To link to this Article: DOI: 10.1081/IAS-120025778
URL: http://dx.doi.org/10.1081/IAS-120025778

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 24, No. 4, pp. 409–420, 2003

Development and Validation of a Simple Sensitive Enzyme Immunoassay (EIA) for GH Determination in Buffalo Plasma

B. S. Prakash, Mohan Mondal,* and N. Anandlaxmi

Division of Dairy Cattle Physiology, National Dairy Research Institute, Karnal, Haryana, India

ABSTRACT

A simple and highly sensitive enzymeimmunoassay (EIA) for GH determination in buffalo plasma on microtitreplates using biotin-streptavidin amplification system and the second antibody coating was developed. Biotin was coupled to GH and used to bridge between streptavidin-peroxidase and immobilized antiserum in competitive assay. The EIA was carried out directly in 100 μL buffalo plasma. The GH standards ranging from 0.05 ng/well/100 μL to 12.8 ng/well/100 μL were prepared in hormone free plasma collected from an aged (>15 years) senile buffalo. The sensitivity of the EIA procedure was 50 pg/well GH, which corresponded to 0.50 ng/mL

*Correspondence: Mohan Mondal, Division of Dairy Cattle Physiology, National Dairy Research Institute, Karnal, Haryana, 132001, India; E-mail: mohan.mondal@rediffmail.com.

DOI: 10.1081/IAS-120025778 1532-1819 (Print); 1532-4230 (Online)

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

plasma; the 50% relative binding sensitivity was seen at 800 pg/well/100 μL. Plasma volumes for the EIA, viz., 25, 50, and 100 μL did not influence the shape of standard curve, even though a slight drop in the OD_{450} was seen with higher plasma volumes. For the biological validation of the assay, 12 Murrah buffalo calves were used. Six of these were administered synthetic bovine growth hormone-releasing factor (10 μg/100 kg body weight, i.v., and the remaining six animals were administered sterile normal saline and kept as controls. Jugular blood samples were collected at −60, −45, −30, −15, −10, −5, 5, 10, 15, and 30 min and, thereafter, at an interval of 15 min using an indwelling jugular catheter, beginning 1h prior to GRF injection up to 8 h post treatment. In all animals, a peak of GH was recorded within 5 to 20 min of GRF administration, which confirms the biological validation of the EIA. To confirm homogeneity of buffalo GH with bovine GH, a parallelism test was conducted between the buffer standard curve of bovine GH and GH measured from serial dilution of buffalo plasma containing a high level of endogenous growth hormone.

Key Words: Buffalo; EIA; GH; Plasma; Validation; IgG.

INTRODUCTION

Growth hormone (GH) is necessary for most aspects of the postnatal somatic growth.[1,2] The exogenous GH has been found to stimulate a number of physiological processes, including body growth and milk production.[3–6] Exogenous growth hormone has also been reported to improve fertilization of in vitro matured oocytes,[7] as well as to improve the superovulatory response,[8] and to increase the number of follicles in heifers.[9] Estimation of GH in blood plasma of neonatal calves to assess its genetic potential for milk production at an early stage has also been suggested.[10] To facilitate further research into the action of GH in buffaloes, an efficient GH assay is needed. GH measurements in buffalo plasma are currently being carried out by sensitive radioimmunoassay (RIA) procedures using ^{125}I as the label.[11,12] Although these methods are reliable and accurate, they suffer from the problems associated with the use of radioisotopes, which restricts their use to specialized laboratories. Easy availability of ^{125}I for radio iodination is also a serious limitation for conducting GH RIA. The RIA procedure also suffers from the disadvantage of using ^{125}I as the label, which has a short half-life. While EIA procedures have been developed for bovine GH,[13] LH,[14] and FSH,[15] which do not require radioisotopes, and are faster and less

expensive, no EIA has been established for buffalo GH. We, therefore, decided to develop a sensitive and convenient second antibody EIA for GH determination in buffalo plasma using a biotin-streptavidin peroxidase amplification system.

EXPERIMENTAL

Preparation of Biotinyl-GH Conjugate

To 40 μg bovine GH (USDA-bGH-B-1), dissolved in 200 μL of 0.1 M carbonate buffer (50 mM, pH 9.6), 20 μL biotinamidocarproate-*N*-hydroxysuccinimideester (biotin; Sigma, Germany) dimethylsulfoxide (5 mg/mL, Sigma, Germany) was added and the mixture was immediately vortexed and incubated further for 3 h at room temperature under constant agitation. The coupling reaction was then stopped by the addition of 20 μL NH_4Cl (1M) and the reaction mixture was incubated for a further 30 min before addition of 2 mL of a solution of 0.1% bovine serum albumin (BSA) in phosphate buffered saline solution (PBS: 50 mM NaPO4; 0.15 M NaCl, pH 7.4). For the isolation of biotin-GH conjugate, the mixture was dialyzed overnight at 0°C with four changes in PBS. After dialysis, the conjugate was mixed with an equal volume of glycerol to prevent freezing and was preserved at −20°C in 1 mL aliquots.

bGH Antibody

The bovine growth hormone antiserum (Rabbit 3-anti-bGH, Pool 7–12) used in the present investigation was specific for estimation of growth hormone. It was generously gifted by Dr. M. Hennies (Germany) and the details of the specificity of the antiserum have been given by Hennies and Holtz.[13]

Preparation of Affinity Purified Goat IgG Antirabbit IgG

The affinity purified goat IgG antirabbit IgG was developed following the procedure of Anandlaxmi and Prakash.[16] Briefly, about 40 mL plasma from a goat immunized against rabbit IgG containing 20 I.U heparin/mL of blood was vortexed with rabbit IgG agarose and loaded onto a small column. First, nonspecific proteins were eluted with PBS (0.5 M, 0.15 M NaCl, pH 7.2) buffer. Proteins bound specifically

were eluted with 15 mL of 0.1 M glycine–HCl (pH 2.0). All steps were performed at room temperature. The eluted fractions (3 mL each) were collected in vials containing 0.2 mL of 1M Tris-HCl (pH 8.0). The eluted IgG was dialysed overnight against PBS and the protein content determined by measuring the absorbance spectrophotometrically at 260 and 280 nm, and extrapolated from a normograph.

EIA Procedure

First Coating

The first coating was performed by adding 0.63 μg of goat IgG anti-rabbit IgG dissolved in 100 μL of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH9.6) per well of the microtiter plate (Greiner Labotrechnik). The plates were subsequently incubated overnight at 4°C.

Second Coating

For blocking the remaining binding sites, 300 μL of 1% BSA in phosphate buffer was added to all the wells and incubated for 40 to 50 min at room temperature under constant shaking.

Washing

The coated plates were washed twice with 350 μL/well of washing solution (0.05% Tween 20) using an automated microtiter plate washer (Model: EL 50 8 MS, USA).

Assay Protocol

Duplicates of 100 μL of unknown plasma or bovine GH standards (USDA-bGH-B-1) prepared in hormone free plasma ranging from 50 pg/100 μL/well to 12,800 pg/100 μL/well were simultaneously pipetted into respective wells along with 100 μL of GH antibody diluted 1:40,000 in assay buffer (50 mM Na_3PO_4, 0.15 M NaCl, 0.02% thiomersal; pH 7.4) with the aid of a dilutor-dispensor. Plates were incubated overnight at room temperature after 30 min constant agitation. They were then decanted and washed two times with washing solution before addition

of 100 μL of biotinyl-GH conjugate per well diluted 1:3000 in assay buffer. The plates were further incubated for 30 min with constant agitation, decanted, and washed four times. Then, 20 ng streptavidin-peroxidase (Sigma, Germany) in 100 μL of assay buffer was added to all the wells and the plates, now wrapped in aluminium foil, were incubated for a further 30 min under constant agitation. All steps were performed at room temperature.

Substrate Reaction

The plates were washed five times with washing solution and incubated further in the dark for 40 min after addition of 150 μL of substrate solution per well (Substrate buffer: 0.05 M citric acid, 0.11 M Na_2HPO_4, 0.05% ureum peroxide; pH4.0 adjusted with 5N HCl; substrate solution: 17 mL substrate buffer plus 340 μL 3,3′,5,5′-tetramethylbenzidine; 12.5 mg/mL dimethyl sulfoxide; Sigma, Germany). The reaction was stopped by the addition of 50 μL 4N H_2SO_4 and the color was measured at 450 nm with a 12-channel microtiter plate reader (Model: ECIL, Microscan, India).

Biological Validation of the Buffalo Plasma GH EIA

For the biological validation of the assay, 12 Murrah buffalo calves within the age group of 6 to 8 months of age were used. Six of these were administered synthetic bovine growth hormone releasing factor (GRF; at 10 μg/100 Kg body weight, intravenously) containing 1 to 44 amino acids (Product Code # G-0644, Sigma-Aldrich Chemie, Germany), and the remaining six animals were kept as controls and administered sterile saline. Blood samples were collected at −60, −45, −30, −15, −10, and −5 min before administration, and 5, 10, 15, 30 min and thereafter at intervals of 15 min post administration up to 8 h, by using an indwelling jugular catheter. All experimental protocols and animal care met IACUC regulations. Before catheterization, local anesthesia (Xylocan®) is given and, after removal of the catheter, the animal is treated with antibiotic (Oxytetracycline®) for 7 days.

The blood samples were collected in heparinized plastic tubes and immediately kept in ice-box (4°C) and then centrifuged at 3000 rpm for 20 min at 4°C, and the separated plasma was stored at −20°C until assayed for GH.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

RESULTS

Standardization of Enzyme Immunoassay for Buffalo Plasma GH Determination

Titration of Biotinyl-GH Antiserum

A two-dimensional titer determination for the optimum dilution of GH label and the antiserum was carried out. Antibody dilutions ranging from 1:10,000 to 1: 640,000 and the biotinyl-GH dilutions of 1:1000 to 1:32,000 were tested. The antibody titer of 1:40,000 and the biotinyl-GH conjugate titer of 1:3000 were found to be the most suitable and achieved an OD_{450} of around 1.471.

Assay Validation

Assay Interference and Sensitivity

To determine the possible interference of plasma with the assay sensitivity, bovine GH standards in various amounts of plasma (0, 25, 50, and 100 μL) were run in an assay (Fig. 1). There was no difference in the absolute binding sensitivity among 25, 50, and 100 μL plasma volumes.

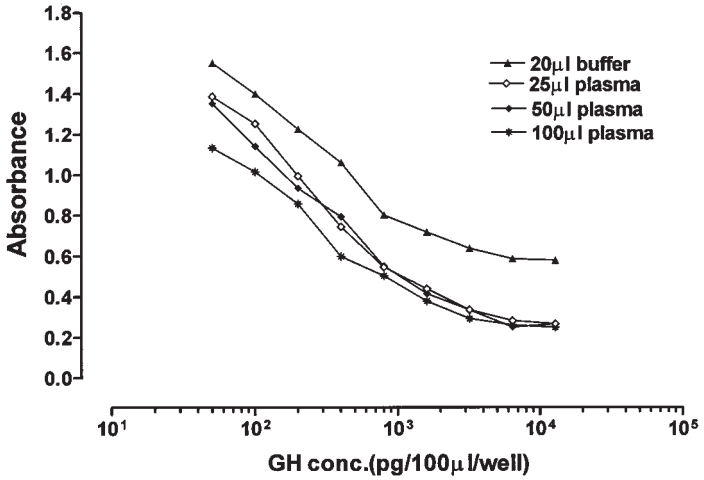

Figure 1. Influence of different volumes of 25, 50, and 100 μL of buffalo plasma on optical density displacement in GH standard curve. Along with different volumes of plasma, the standards were also prepared in assay buffer. Optical density was measured at 450 nm.

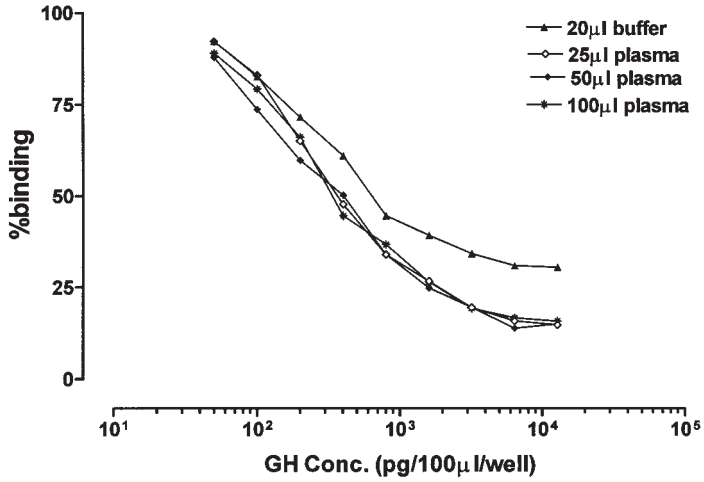

Figure 2. Influence of different volumes 25, 50, and 100 μL of buffalo plasma on percentage binding in GH standard curve. Along with different volumes of plasma, the standards were also prepared in assay buffer. Optical density was measured at 450 nm.

Increasing plasma volumes from 25 to 100 μL did not influence the shape of standard curve; however, a slight drop in the OD_{450} was seen with higher plasma volumes (Figs. 1 and 2). Keeping these aspects in view, standards were subsequently prepared in hormone-free plasma and run along with the unknowns in the assay. The nonspecific binding (OD_{450}) using all these volumes of plasma was recorded to be within the range of 0.091 to 0.139. All the assays were, therefore, conducted taking 100 μL of unknown plasma samples and standards per well just to increase assay sensitivity per mL of plasma in duplicates. The range over which GH could be measured was between 0.5 to 128 ng GH/mL and covered all possible physiological variations. The lowest GH detection limit significantly from zero concentration was 50 pg/100 μL plasma, which corresponded to 0.5 ng/mL plasma. The 50% relative binding (B/B0) sensitivity was 800 pg/20 μL plasma/well, which corresponded to 8.0 ng GH/mL plasma.

Intra- and Inter-Assay Precision

Intra- and inter-assay coefficients of variation were determined using pooled plasma containing 2.0 and 64.0 ng/mL; they were found to be 2.62 and 0.75% and 3.83 and 4.12%, respectively from 85 assays.

Biological Validation

Mean GH concentrations measured in blood samples collected from the six Murrah buffalo calves administered with GRF and another six animals (controls) which were not given any hormonal treatment, but an equal volume of sterile normal saline are presented in Fig. 3. In all the animals administered with GRF, a peak of GH was recorded within 5 to 20 min after GRF administration. The mean GH concentration of six buffalo calves administered with GRF rose sharply to a peak value of 89.013 ± 5.923 ng/mL after 10 min of GRF administration. Following GRF administration, GH concentrations remained at higher levels for 150 min post-treatment and, thereafter, became similar to that of the control group (Fig. 3).

bGH Parallelism with Buffalo Plasma

To assess the homology between bovine standards used and endogenous GH in buffalo plasma, a parallelism test was carried out between standard bGH and serial dilutions of buffalo plasma containing high concentrations of endogenous GH. For this purpose, a buffalo plasma sample containing a high level of endogenous GH was serially diluted (containing 100, 50, 25, 12.5, and 6.25 μL buffalo plasma sample size) and run along with bGH standards (in assay buffer) in an assay. It showed a good parallelism with the bovine standard curve, though a small deviation was found at very low plasma volumes (Fig. 4). This suggests that buffalo GH has a considerable structural homology to bGH. Since buffalo GH is not available, the GH EIA can use bovine specific GH in the assay.

DISCUSSION

Many assay procedures, most of which are RIA, have been developed for estimation of growth hormone in plasma or serum but, to the best of our knowledge, the GH EIA described here is the first report of direct EIA in unextracted buffalo plasma using the second antibody coating technique and GH-biotin-streptavidin system. The use of a second antibody for coating the microtitreplate wells, instead of a hormone-specific antibody, is preferred as it reduces assay variabilities associated with uneven binding of the latter antibody to the wells and further reduces the amount of hormone specific antibody needed in the EIA.[17]

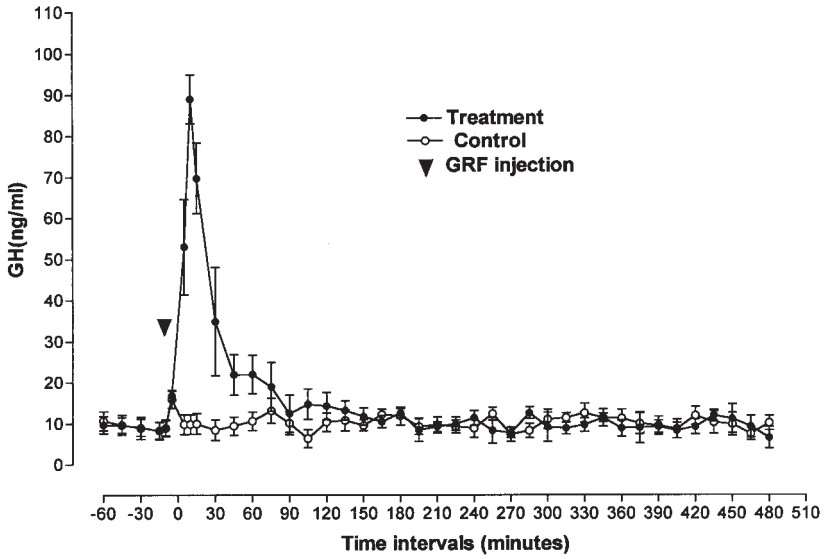

Figure 3. GH (Mean ± SEM) profile in growing Murrah buffaloes prior to and after GRF ($n = 6$) or normal saline treatment (Control; $n = 6$). Synthetic GRF [GRF (1–44)-NH_2] (10 μg/100 Kg body weight) or equal volume of normal saline was administered intravenously and blood samples were collected at −60, −45, −30, −15, −10, and −5 min before administration, and 5, 10, 15, and 30 min, and thereafter, at an interval of 15 min post administration up to 8 h.

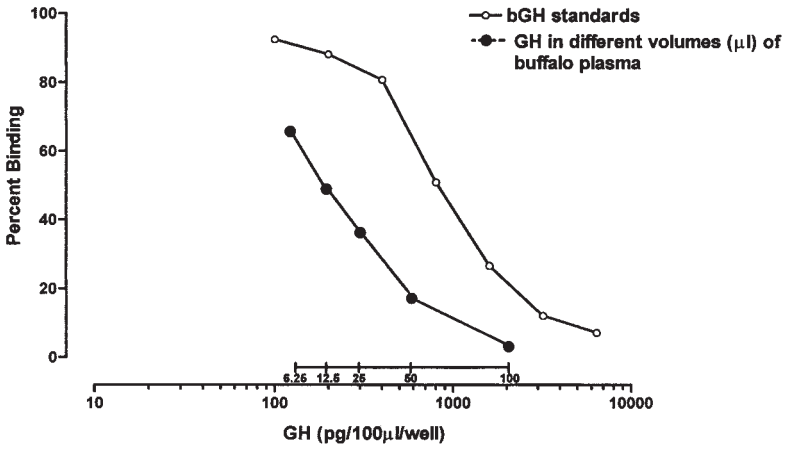

Figure 4. Parallelism for bovine GH standards with serially diluted different volumes of 6.25, 12.5, 25, 50, and 100 μL of buffalo plasma. Standards for bovine GH ranged from 100 pg/100 μL/well to 6400 pg/100 μL/well.

In the direct EIA, less sample volume is desirable to reduce the nonspecific binding and plasma matrix effects and, thus, a high degree of sensitivity.[14] This requires the use of a highly specific antibody, a very efficient amplification system, and optimum ligand antibody dilutions at a suitable incubation temperature. In our GH EIA, there was a slight drop in the optical densities (OD_{450}) with increasing plasma volumes, although the sensitivities per well and the relative binding percentages did not change when 25, 50, and 100 μL plasma were taken along with standards (Figs. 1 and 2). This effect of plasma on optical densities can be compensated by using the same plasma volumes for standards and unknown samples. A high assay sensitivity of 50 pg/well GH (0.5 ng/mL) was obtained, while 100 μL plasma was taken for estimation. This was sufficient for determining the low physiological levels (controls) as well as high GH concentrations after GRF administration in Murrah buffalo calves (Fig. 3).

The good parallelism obtained with bGH standards is ample evidence of a high degree of homology in the structures of bovine and buffalo GH and, hence, the use of bGH in the assays will provide a true reflection of the actual GH profile in buffalo plasma. The GH EIA described here offers a reliable alternative to RIA. Apart from being nonradioactive in nature, the EIA procedure has several other advantages over RIA, namely, (a) it may be more sensitive than RIA procedures described in buffalo plasma; (b) it requires less plasma volumes as compared with the conventional RIA methods; (c) biotinylated GH has a much longer shelf life (several years) as compared to iodinated-GH (60 days); and (d) it is less labour intensive, quick, and relatively simple to perform. Further, since the procedure requires less capital investment in terms of instruments, the GH EIA provides a safe, cost-effective, and less time consuming alternative to the conventional RIA procedures and can, therefore, be adopted in the laboratories of the developing countries where financial constraints limit the adoption of RIA. Highly purified GH preparations from cattle and other species of animals are available, and biotinylation of GH is not difficult as compared to iodination procedures. Biotin and streptavidin peroxidase of good quality are also commercially available at rather lower costs than ^{125}I preparations. In conclusion, the GH EIA procedure described here is an economical and sensitive alternative to RIA.

ABBREVIATIONS

GH	Growth hormone
EIA	Enzymeimmunoassay

PBS	Phosphate buffered saline
BSA	Bovine serum albumin
GRF	Growth hormone-releasing factor

ACKNOWLEDGMENTS

The authors wish to thank Dr. Mark Hennies, Institute of Tieranatomic, Bonn, Germany, for the generous gift of highly specific bGH antibody. The supply of a highly purified reference preparation of bovine GH by USDA Animal Hormone Program, Beltsville, MD, USA is gratefully acknowledged. Financial assistance provided by National Agricultural Technology Project PSR No. 47 for this study is also duly acknowledged.

REFERENCES

1. Nalbandov, A.V. Symposium on growth: endocrine causes of growth and growth stasis. J. Animal Sci. **1963**, *22*, 558–560.
2. Etherton, T.D.; Kensinger, R.S. Endocrine regulation of fetal and postnatal meat animal growth. J. Animal Sci. **1984**, *59*, 511–528.
3. Machlin, L.J. Effect of growth hormone on milk production and feed utilization in dairy cows. J. Dairy Sci. **1973**, *56*, 575–580.
4. Bauman, D.E.; McCutcheon, S.N. In *The Effects of Growth Hormone And Prolactin on Metabolism*, Proceeding of Sixth International Symposium on Ruminant Physiology, Control of digestion and Metabolism in Ruminants; Milligan, L.P., Grovum, L., Dobson, A., Eds.; Prentice-Hall: Englewood Cliffs, NJ, 1986; 436–455.
5. McDowell, G.H.; Hart, I.C.; Kirby, A.C. Local intra-arterial infusion of growth hormone into the mammary glands of sheep and goats: effects on milk yield and composition, plasma hormones and metabolites. Austral. J. Biol. Sci. **1987**, *40*, 181.
6. Burton, J.H.; MacLeod, G.K.; McBride, B.W.; Burton, J.L.; Bateman, K.; McMillan, I.; Eggert, R.G. Overall efficacy of chronically administered recombinant bovine somatotropin to lactating dairy cows. J. Dairy Sci. **1990**, *73*, 2157–2167.
7. Izadyar, F.; Colenbrander, B.; Bevers, M.M. Growth hormone enhances fertilizability of in vitro matured bovine oocytes. Theriogenology **1997**, *47*, 191.

8. Gong, J.G.; Wilmut, I.; Bramley, T.A.; Webb, R. Pretreatment with recombinant bovine somatotropin enhances the superovulatory response to FSH in heifers. Theriogenology **1996**, *45*, 611–622.
9. Tripp, M.W.; Ju, J.C.; Hoagland, T.A.; Riesen, J.W.; Yang, X.; Zinn, S.A. Influence of somatotropin and nutrition on bovine oocyte retrieval and in vitro development. Theriogenology **2000**, *53*, 1581–1590.
10. Woolliams, J.A.; Lovendahl, P. Physiological attributes of male and juvenile cattle differing in genetic merit for milk yield: a review. Lives. Prod. Sci. **1991**, *29*, 1–12.
11. Singh, M.; Ludri, R.S. Plasma growth hormone profile and milk yield response of Murrah buffaloes (Bubalus bubalis) treated with slow release formulation of somidobove. Buffalo J. **1994**, *10*, 81–84.
12. Jailkhani, S.; Madan, M.L. Plasma growth hormone profile in buffalo neonates. Ind. J. Anim. Sci. **1999**, *69*, 217–218.
13. Hennies M.; Holtz W. Enzymeimmunoassay for the determination of bovine growth hormone using avidin-biotin-peroxidase complexes. J. Immunol. Meth. **1993**, *157*, 149–157.
14. Mutayoba, B.H.; Meyer, H.H.D.; Schams, D.; Schallenberger, E. Development of a sensitive enzymeimmunoassay for LH determination in bovine plasma using the streptavidin-biotin technique. Acta Endocrinol. (Copenh.) **1990**, *122*, 227–232.
15. Prakash, B.S.; Wallenhorst, S.; Metten, M.; Holtz, W.; Wuttke, W. Development of a sensitive enzymeimmunoassay (EIA) for FSH determination in bovine plasma. Animal Reprod. Sci. **1999**, *55*, 183–192.
16. Anandlaxmi, N.; Prakash, B.S. Production and purification of goat antirabbit IgG. Ind. J. Dairy Sci. **2001**, *54*, 332–334.
17. Meyer, H.H.D. In *Possibilities to Improve Enzymeimmunoassay* (*EIA*) *Techniques and their Application in Animal Production*, Proceedings of International Symposium on the Use of Nuclear Techniques in Studies of Animal Production and Health in Different Environments, IAEA: Vienna, 1986; 255–262.

Received January 23, 2003
Accepted February 28, 2003
Manuscript 3091