*Pak. J. Biochem. Mol. Biol. 2011; 44(4): 148-152*

# Developing an improved competitive enzyme immunoassay for Beetal caprine growth hormone

*Roquyya Gul[1], Farkhanda Ghafoor[2], Saima Sadaf [3] and Muhammed Waheed Akhtar[1]**
*[1]School of Biological Sciences, University of the Punjab, Lahore, Pakistan,*
*[2]NHRC, Sheikh Zayed Medical Complex, Lahore, Pakistan,*
*[3]Institute of Biochemistry and Biotechnology, Lahore, Pakistan*

**Abstract:** The study aimed to develop a simple and sensitive competitive enzyme immunoassay for growth hormone (GH) in Beetal caprine serum. Antisera (rabbit anti-rcGH) were raised in two rabbits of local breed. Periodate method was used for labeling horseradish peroxidase (HRP) with the purified recombinant caprine growth hormone (rcGH). 96-well microtitre plates were coated with primary antibody (polyclonal rabbit anti-rcGH) for 24 hr at 4°C. Buffer standards ranging from 0-400 ng/ml were prepared with rcGH to be used in each assay batch. Assay parameters like pH, temperature, reaction time and working dilutions of antibody & conjugate were optimized. The assay showed insignificant cross-reactivity with the human insulin. The developed assay is reliable, quick and simple to perform.

**Keywords**: competitive enzyme immunoassay, rabbit anti-rcGH antibody, horseradish peroxidase, Beetal caprine growth hormone.
**Received:** June 15, 2011 **Accepted:** October 21, 2011
***Author for Correspondence**: mwapu@brain.net.pk

## INTRODUCTION

Growth hormone (GH), also known as somatotropin, is a 22 kDa polypeptide produced by somatotroph cells of the anterior pituitary gland. Secretion from the pituitary gland is in a pulsatile manner following which it acts on various organs and perform diverse functions that are associated with complex physiological processes including growth, development, cell proliferation, metabolism and/or protein synthesis[1]. Liver microsomal membrane contains receptors for GH (GHR) and is the major site for GH-mediated actions.

GH is normally produced throughout the life-cycle, although apparently in higher amounts during the pre-adult period. Its deficiency in children is one of the several causes of short-stature, which may be cured by artificially manipulating their plasma GH-levels[2]. Studies have shown that exogenous administration of GH, whether natural or recombinant, enhances the milk and meat production of farm animals like cattle, buffalo, sheep, goat etc. The GH sequences of these mammalian species share high degree of sequence similarity and also show the same physiological activity. Particularly, in family Bovidae, ovine (sheep) and caprine (goat) GHs (oGH and cGH, respectively) differ from bovine (cattle) and bubaline (buffalo) GHs (bGH and BbGH) by only one or two amino acids. The percentage identity of cGH with oGH is 100 %, with bGH and BbGH its ~99 %, with porcine 92 %, and with human only 66 %[3].

The determination of serum GH level in Bovidae species is helpful in identifying the growth performance and it also provides the basal GH level during exogenous GH administration in the animals. Generally, there are number of methods by which small to large molecules (hormones, receptors, cells, viruses, pesticides, etc.) are being analyzed quantitatively or functionally. One of the widely applied sensitive and specific techniques is enzyme immunoassay (EIA) / enzyme linked immunosorbent assay (ELISA). To date there are number of GH EIAs developed for human, porcine, channel catfish, rat[4-7], and for Bovidae species like bovine[8-10], ovine[11], buffalo[12], yak[13,14] and *Bos* frontalis[15]. But to our knowledge this was the first competitive EIA to be optimized for the quantification of exogenous recombinant cGH using nonradioactive labels like enzyme[16].

## MATERIALS AND METHODS

### *Chemicals and reagents*

The chemicals used in the assay were of analytical grade and were acquired from Sigma or Merck. The Beetal caprine and *Lohi* ovine GHs were recombinantly produced in the laboratory. Human insulin (rDNA origin, Humulin R, 100/mL) was acquired from a local pharmacy (FAZAL DIN, Lahore, Pakistan) while rabbits were purchased from Tollinton market Lahore, Pakistan.

### *Conjugation of rcGH with enzyme*

Recombinant Beetal cGH was labeled with horseradish peroxidase (HRP) by periodate method[17]. The conjugate was purified by gel filtration chromatography, fractions were collected and absorbance of each fraction was checked at $A_{280}$ for protein and $A_{403}$ for HRP by using UV/visible spectrophotometer (CECIL 230)[17]. Enzymatic activity of each fraction (10µl) was checked on uncoated microtitre plates (NUNC Maxisorp, Denmark) with 200µl of the HRP substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) and incubated for 20 minutes. While, functional activity of each fraction (100µl) was checked on polyclonal rabbit anti-rcGH

Ab (B5; 1:100) coated plate and incubated for 2 hrs at RT. Washing was done three times with washing buffer (200µl/well) to remove the unbound conjugate. 100 µl of enzyme substrate was added and incubated for 20 minutes. Reaction was stopped with 100 µl of stop solution (1M $H_2SO_4$) and absorbance was read (450/630 nm) on ELISA/EIA reader (HUMAREADER$^{plus}$, Germany). Chromatogram was plotted for the enzymatic and functional activities read at 450/630nm. To find the Reinheitszahl (Rz) value (measure of haemin content of the enzyme) of the purified conjugate, ratio of absorbance at 280 and 403nm was calculated and 100 µl aliquots were made and stored at -20°C.

***Production of rabbit anti-rcGH polyclonal antibodies***

Polyclonal anti-rcGH antibodies (Ab) were raised in rabbits of local breed, Lahore. Recombinant Beetal cGH (1000µg) was dissolved in 5ml of 0.9 % Normal saline solution, aliquots (200µg/ml) were made and stored at -20°C. Freund's complete and incomplete adjuvants (Sigma) were used for primary and booster injections, respectively[17]. 100 µg of rcGH was administered through subcutaneous application at one month interval to the rabbits. Blood was drawn after every four weeks interval from the marginal vein of the ear of each rabbit. First bleed (B0) was drawn before the administration of the first injection. Blood was drawn seven times from each rabbit and was centrifuged (Hettich Mikro 200R zentrifugen) at 6,000rpm for 10 minutes to separate the serum, which was stored at -20°C.

***Competitive rcGH EIA***

Anti-rcGH Ab were coated on the microtitre plates and by the addition of conjugate (HRP-rcGH) and an unknown sample, competition occurred for the limited binding sites of the Ab. Addition of substrate (TMB) produced a signal on binding of a conjugate to the Ab, which was inversely proportional to the concentration of the antigen (GH) in the sample. Standard concentrations of rcGH, 0-400 ng/ml were made in an assay buffer (PBS 0.05M; pH 7.4) to plot the standard curve.

***Coating of polyclonal antibodies on solid phase***

Different buffers were used for antibody coating [50mM $Na_2CO_3$ pH 9.5[18], 10mM PBS pH 7.0, 20 mM Tris-Cl pH 8.5 & Convol-5 (10mM Borate buffer pH 8.0)][17]. Among these Convol-5 pH 8.0 was found appropriate for the coating of polyclonal rabbit anti-rcGH Ab. Antiserum was diluted in Convol-5 [at 1: 100, 1: 1000 (K), 1: 10K, 1: 100K, 1: 150K & 1: 200K]. 200µl/well of each dilution was added and incubated for 2 & 4 hr at RT and for ON at 4°C. After incubation, plates were washed three times with washing buffer (PBS pH 7.4; 0.05M+0.01% Triton X-100) and were ready to use for the assays. Overnight incubation at 4°C for Ab coating was found to be optimum. For the stabilization of Ab coated plates different stabilizers (1 % ByCoA, 1 % ByCo A+2 % Mannitol, 1 % BSA, 2% Mannitol and 2 % Milk powder) were used (200µl/well) and among them 1 % ByCo A+2 % Mannitol showed increase shelf life of the anti-rcGH Ab microtitre plates. Plates were incubated for 30 minutes (RT), aspirated, freeze-dried (CHRIST, ALPHA 1-4LD) for 24 hrs, sealed in plastic bags and stored at -20°C until further assay.

***Optimization of the assay***

Assay incubation was done for two durations i.e. 2 and 4 hrs respectively to optimize the reaction time and tested with two types of substrate; one component and two component substrate. One component substrate was commercially purchased (Neogen's Enhanced K-Blue), while two component substrate was made of 2.5ml stock A (Sodium acetate citrate buffer) and 250µl of stock B (100mg of TMB dissolved in 1 ml of DMSO) and volume was made up to 25ml by distilled $H_2O$. The assay was stopped by using stop solution; 1 M $H_2SO_4$ (54.5ml $H_2SO_4$+945.5 ml $H_2O$).

## RESULTS AND DISSCUSSION

***Preparation of GH conjugate (HRP-rcGH)***

In the present study, conjugate (HRP-rcGH) was made by labeling the recombinant Beetal cGH with HRP by periodate method[19]. The prepared conjugate was purified to remove the free HRP from the labeled enzyme by gel filtration chromatography. Total 96 fractions were collected and their absorbance was measured at 280 & 403 nm (data not shown). The enzymatic (enzyme-substrate complex) and functional (antigen-antibody complex) activity of each fraction was checked. Fractions, 28-33 were pooled that showed both enzymatic and functional activity (Figure 1). Reinheitszahl (Rz) of the purified conjugate calculated was 1.4 (Reference $R_Z$ value = 0.5-3.0). While in the previous studies conjugates were made by labeling the GH with the biotin linked streptavidin or avidin peroxidase[9-11,13,15].

***Production of antibody***

Polyclonal rabbit anti-rcGH Ab[10] were raised and bleeds (B0, B1, B2, B3, B4, B5, B6, B7) were collected. The concentration and affinity of the Ab in the bleeds was measured by EIA. All bleeds of rabbit were coated at different dilutions on the microtitre plate[11] and assay was performed for 2 hrs. Graph was plotted between the dilutions of the harvested bleeds of the rabbit and the absorbance (450/630nm), shown in Figure 2. Maximum binding affinity of the raised

anti-rcGH Ab with the conjugate was seen at 1: 100K dilutions of bleeds B3-B7. Thus, bleeds 3-7 were harvested and 1: 100K dilutions of the antisera were selected for Ab coating. However, in previous studies rabbit anti-bGH antibodies were diluted at 1/250K[10].

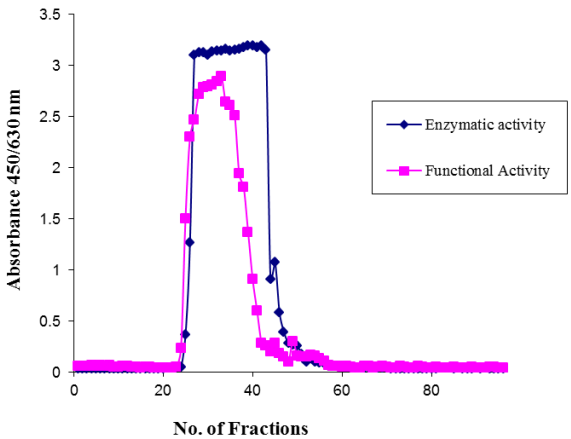


**Figure 1:** Enzymatic & functional activity of 96 fractions. Absorbance read at 450/630nm by ELISA reader. Fractions 28-33 pooled that showed both enzymatic and functional activity.

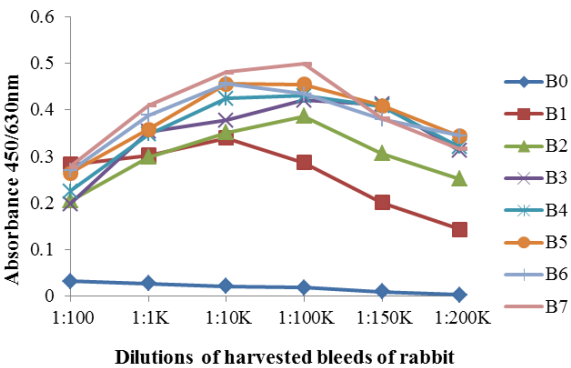


**Figure 2:** Selection and titration of harvested bleeds of rabbit. Absorbance read at 450/630nm, maximum binding affinity of the antibody seen at 1: 100K dilution from bleed B3-B7.

***Optimization of assay parameters***

The reaction time between an antigen and Ab is depended on the temperature or concentration of the reagents that are used. By increasing the temperature, assay time can be decreased[20]. On this basis at 37°C assay was set at two different incubation times (2 & 4 hrs) using 1: 1K, 1: 5K & 1: 10K dilutions of the conjugate and 0 and 400 ng/ml standards to check the maximum displacement. The result showed maximum binding at 1: 1K dilution of the conjugate at 4 hrs incubation time (Table 1). Although, in prior studies for ovine GH EIA 48 hrs assay incubation[11] and for determining the yak GH, assay incubation was selected for ON period[13]. However, our optimized assay was able to generate productive results in less than 5 hrs.

The highest standard concentration was selected 400 ng/ml to plot the standard curve between the concentration of standards and absorbance (450/630 nm) as shown in Figure 3. The normal GH level in the blood of cattle is 41±19ng/100ml[21] but by administrating exogenous GH in *Bos* frontalis, level of GH increased ~ 400ng/ml[22].

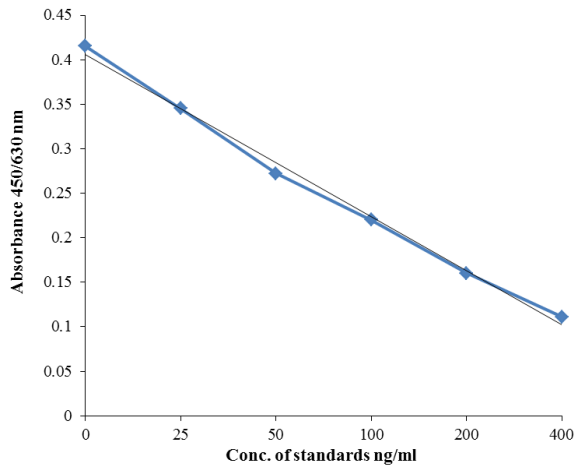


**Figure 3:** Standard curve of recombinant Beetal caprine growth hormone (rcGH) competitive EIA.

**Table 1:** Optimization of assay time, addition of standards and conjugate, incubation time given for 2 and 4 hrs. Maximum absorbance is shown bold in 4 hrs incubation and at 1: 1K dilution of the conjugate.

| Std. points of rcGH | 2 hrs incubation after adding Std. & conjugate | | | 4 hrs incubation after adding Std. & conjugate | | |
|---|---|---|---|---|---|---|
| | Conjugate dilution | | | Conjugate dilution | | |
| | 1: 1K | 1: 5K | 1: 10K | 1: 1K | 1: 5K | 1: 10K |
| Blank | 0.004 | 0.006 | 0.003 | 0.003 | 0.004 | 0.002 |
| | 0.005 | 0.002 | 0.005 | 0.005 | 0.003 | 0.005 |
| 0ng/ml | 0.278 | 0.151 | 0.096 | 0.427 | 0.193 | 0.131 |
| | 0.242 | 0.153 | 0.095 | 0.444 | 0.209 | 0.124 |
| 400ng/ml | 0.067 | 0.049 | 0.033 | 0.176 | 0.052 | 0.04 |
| | 0.060 | 0.044 | 0.036 | 0.158 | 0.052 | 0.037 |

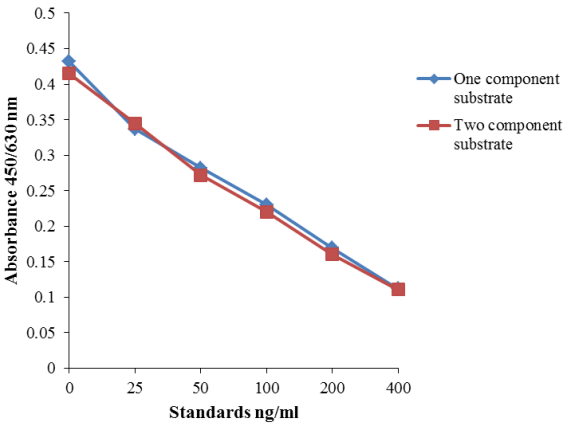


**Figure 4:** Standard curve comparison between commercially available and two component substrates.

Factors depending upon the color development in an assay are composition of the buffer, buffer pH, stability of the enzyme and its substrate, reaction temperature and time[23]. On the basis of these parameters, TMB was prepared and compared with that available commercially and showed encouraging results as of commercially available substrate (Figure 4). The substrate was cost effective for the assay and was in agreement with the previously developed EIA for the ovine GH[11].

***Data Interpretation***

For data interpretation, average absorbance (450/630nm) of duplicate standards and unknown samples were calculated. The concentration of the unknown sample was detected corresponding to the mean OD value on the standard curve.

***Cross-reactivity and specificity of the assay***

The cross-reactivity and specificity of the assay was checked by using human insulin and recombinant *Lohi* ovine GH (roGH) in an assay (Figure 5). The roGH showed substantial competition with the conjugate at different concentrations. Thus, this result was in consensus with the previous study that GHs of the Bovidae species are structurally similar and have almost identical amino acids[24]. While human insulin didn't show any significant binding with the anti-rcGH Ab and also did not compete with the rcGH (Figure 5). Hence, the assay was specific for the quantification analysis of GH and not for any other hormone like insulin[25].

***Final optimized rcGH competitive EIA***

The assay protocol was finalized as; 100µl of standard points (0, 25, 50, 100, 200, 400ng/ml) and unknown sample and 100 µl of conjugate (1:1K dilution) added to rabbit anti-rcGH Ab (1:100K dilution) coated and stabilized microtitre plates.

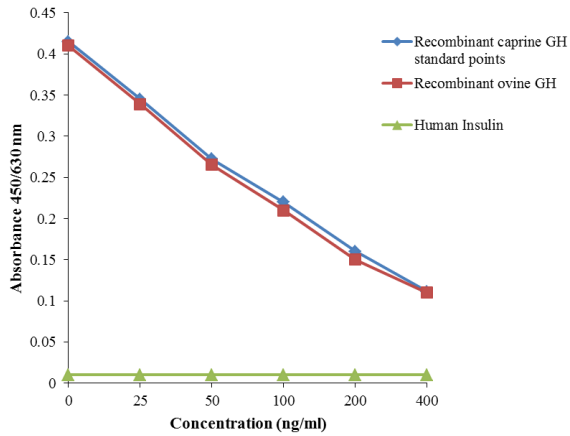


**Figure 5:** Cross-reactive study between the conjugate (HRP-rcGH) and the recombinant ovine GH (roGH) & human insulin. Different concentrations of roGH showed significant competition with the conjugate but human insulin showed negligible binding.

Incubation was done for 4 hrs (RT) and plates were washed three times with washing buffer (PBS 0.05 M pH 7.4+0.01% Triton-X100). 100µl of two component substrate was added in each well and incubated for 20 minutes (RT). 100µl of stop solution (1 M $H_2SO_4$) added to stop the reaction and absorbance read at 450/630nm on ELISA reader. The optimized rcGH competitive EIA was simple, quick, reliable and cost effective.

## ACKNOWLEDGEMENTS

This work was supported by a grant from Higher Education Commission and British Council (HEC-BC), Pakistan.

## REFERENCES

1. Hull KL and Harvey S. Growth hormone: A reproductive endocrine-paracrine regulator? *Rev. Reprod.*, 2000; 5: 175-182.
2. Molitch ME, Clemmons DR, Malozowski S, Merriam GR, Shalet SM, Vance ML and Stephens P. A. Evaluation and treatment of adult growth hormone deficiency: an endocrine society clinical practice guideline. *J Clin. Endocrinol. Met*., 2006; 91: 1621-34.
3. Sadaf S, Khan MA, Wilson DB and Akhtar MW. Molecular cloning, characterization and expression studies of water buffalo (*Bubalus bubalis*) somatotropin. Biochemistry (Moscow), 2007; 72: 162-169.
4. Serpek B, Elsaesser F and Meyer HHD. Development of an enzyme immunoassay for the determination of porcine growth hormone in plasma. *Analytica Chimica Acta*., 1993; 275: 183-187.
5. Drennon K, Moriyama S, Kawauchi H, Small B, Silverstein J, Parhar I and Shepherd B. Development of an enzyme linked immunosorbent assay for the measurement of plasma GH levels in channel catfish (Ictalurus punctatus): assessment of environmental salinity and GH secretogogues on plasma GH levels. *Gen. Comp. Endocrinol.*, 2003; 133: 314-22.

6. Farrington MA and Hymer WC. An enzyme immunoassay for rat growth hormone: applications to the study of growth hormone variants. *Life Sci.*, 1987; 40: 2479-88.
7. Ezan E, Laplante E, Bluet-Pajot MT, Mounier F, Maman S, Grouselle D, Grognet JM and Kordon C, An enzyme immunoassay for rat growth hormone validation and application to the determination of plasma levels and in vitro release. *J. Immunoassay*, 1997; 18: 335-36.
8. Secchi C, Biondi PA, Berrini A, Simonic T and Ronchi S. A biotin-avidin sandwich enzyme linked immunosorbent assay of growth hormone in bovine plasma. *J. Immunol. Methods*, 1988; 110: 123-128.
9. Roh SG, Matsunaga N, Miyamoto A, Hidaka S and Hidari, H. Competitive enzyme immunoassay for bovine growth hormone. *Endocr J*., 1997; 44: 195-8.
10. Hennies M and Holtz W. Enzyme immunoassay for the determination of bovine growth hormone using avidin-biotin-peroxidase complex. *J. Immunol. Methods*, 1993; 157: 149-153.
11. Serpek B and Haliloglu S. Development of an enzyme immunoassay for the determination of ovine growth hormone in plasma. *Turk. J. Vet. Anim. Sci*., 2000; 24: 163-168.
12. Prakash BS, Mondal M and Anandlaxmi N. Development and validation of a simple sensitive enzyme immunoassay (EIA) for GH determination in Buffalo plasma. *J. Immunoassay and Immunochem.*, 2003; 24: 409-420.
13. Prakash BS, Sarkar M, Mishra DP, Nandankar UA. and Bhattacharya M. Development and validation of a simple, sensitive and second antibody format enzyme immunoassay for growth hormone determination in yak plasma. Proceedings of the International Congress on yak, Chengdu, Sichvan, P. R., China, 2004.
14. Sarkar M, Nandankar UA, Duttaborah BK, Das S, Bhattacharya M and Prakash BS. Plasma growth hormone concentrations in female yak (Poephagus grunniens L.) of different ages: Relations with age and body weight. *Livestock Sci.*, 2008; 115: 313-318.
15. Mondal M, Rajkhowa C and Prakash BS. Standardization and validation of a simple, sensitive, second antibody format enzyme immunoassay for growth hormone determination in mithun (*Bos frontalis*) plasma. *Zoo Biology*, 2005; 24: 483-493.
16. Lequin RM. Enzyme immunoassay (EIA)/Enzyme linked immunosorbent assay (ELISA). *Clin. Chem.*, 2005; 51: 2415-2418.
17. Edwards R, Immunodiagnostics; A practical approach Series, Oxford University Press.
18. Catt KJ and Tregear GW. Solid-phase radioimmunoassay in antibody-coated tubes. *Science*, 1967; 158: 1570–1572.
19. Wisdom GB. Conjugation of antibodies to horseradish peroxidase. *Methods Mol. Biol.*, 2005; 295: 127-130.
20. Dev VG and Lasley, J. F. Growth hormone level in the blood of dwarf and normal Herford cattle. *J. Anim. Sci.*, 1969; 29: 384-348.
21. Mondal M, Rajkhowa C and Prakash BS. Exogenous GH-releasing hormone increases GH and LH secretion in growing mithuns (*Bos frontalis*). *Gen. Comp. Endocrinol*., 2006; 149: 197-204.
22. Crowther JR, ELISA Theory and Practice, Methods in Molecular Biology, 42: Humana Press.
23. Lovendahl P, Adamson J, Lund R and Lind P. Technical note: Time-resolved immunofluorometric assay for growth hormone in ruminants. *J. Anim. Sci*., 2003; 81: 1294-1299.
24. Wilkin T, Casey C, Tuck A and Englefield P. An enzyme linked immunosorbent assay (ELISA) for the measurement of serum antibodies to growth hormone. The Wessex Growth study. *J. Immunol. Methods*, 1989; 122: 123-127.