Full Length Research Paper

Binding properties of beetal recombinant caprine growth hormone to Bovidae liver microsomal membranes

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The aim of the study was to illustrate the radio-receptor assay of beetal recombinant caprine growth hormone (rcGH). Tracer (^{125}I-rcGH) was prepared by iodinating beetal rcGH with iodine-125 and its biological activity was analyzed by rabbit anti-rcGH antibodies. Liver microsomal membranes of the Bovidae species (caprine, ovine and bovine) were prepared to study the binding properties of beetal rcGH. The tracer binding was dependent on receptor protein concentration, tracer counts, temperature, time of incubation and assay pH. The maximum percentage specific binding of the tracer to the microsomal membrane was 45% in 32 h at 4°C. In cross-reactive study, human GH competed and displaced the ^{125}I-rcGH by binding effectively to the cGH receptor. Scatchard analysis of the rcGH binding suggested a single class of binding site to the caprine microsomal membrane with an affinity of $337.1 \pm 82.94 \times 10^9$ M^{-1} and capacity of 57.61 ± 4.23 fmol mg^{-1} while rcGH binding affinity and capacity to the ovine and bovine receptor protein showed $365.4 \pm 66.82 \times 10^9$ M^{-1}, 57.69 ± 3.23 (fmol mg^{-1}) and $392.6 \pm 56.25 \times 10^9$ M^{-1}, 56.8 ± 2.56 (fmol mg^{-1}) respectively. This study provides data for beetal rcGH interaction with microsomal membrane that shall be beneficial to study hormone receptor interactions of other Bovidae species.

Key words: Beetal recombinant caprine growth hormone, Bovidae, iodine-125, microsomal membrane, receptor protein.

INTRODUCTION

Growth hormone (GH) a 22 kDa polypeptide is produced by somatotroph cells of the anterior pituitary gland and its mode of action is widespread (Edmondson et al., 2003). It is accepted that receptors for the GH are widely occurring on the cell membrane and crude membrane preparations of various tissues (Posner et al., 1974). But the most specific binding site for the GH is the liver, where it acts and ultimately produces insulin-like growth

factors (IGF-I and II), which targets other tissues of the body (adipose tissues, bones, muscles) thus leading to cell proliferation and increases the protein, lipid and carbohydrate metabolism (Leung and Ho, 2001).

The initial step in studying the mechanism of signal transduction of a hormone (Piwien-Pilipuk et al., 2002) and its receptor protein (Alves dos Santos et al., 2001) is to explore the hormone-receptor characteristics. Several works have been done on the characterization of human GH (Herington et al., 1976; Cadman and Wallis, 1981), Bovidae GHs (Wingfield et al., 1987; Sami et al., 2008) and on GH receptors (Waters and Friesen, 1979; Jiang et al., 1999; Connor et al., 2002). Bovidae, a family of artiodactyla contains 140 species (Grubb, 1993) and has 10 known subfamilies (Allard et al., 1992; Grubb, 2005). Out of these subfamilies, two are extensively documented that is, bovinae (for example, cattle, bison, and buffalos) and caprinae (for example, sheep, goat) (Maj and Zwierzchowski, 2006). One of the breed of caprinae is beetal; which is widely present in South Asian countries including Pakistan. It has a great potential of milk production and may play the leading role in enhancing the economy of the country by producing milk and meat. It is reported that milk and meat production of the livestock can be improved by exogenous administration of the GH (Bauman, 1999). By the use of genetic engineering, recombinant growth hormone (rGH) has shown its importance in cattle to increase their milk and meat production (Machin, 1973). In the family Bovidae, the percentage homology of caprine GH with the mammalian GHs is high that is, ovine (100%), bovine and *Bubalus bubalis* (99%), porcine (92%), rat (87%) and human (66%).

The current study shares the possible of understanding the receptor binding characteristics of recombinant beetal caprine GH. These findings would eventually contribute the valuable information regarding the Bovidae GH system and ultimately lead to explore and comprehend the hormone-receptor interaction.

MATERIALS AND METHODS

Liver samples of Bovidae species (bovine, caprine and ovine) were freshly collected from a local abattoir (Lahore, Pakistan). The liver samples were sliced into 10 × 20 mm pieces, immediately frozen in liquid nitrogen and stored at -80°C till further analysis. Chemicals / reagents used in this study were of the highest purity grade commercially available from Sigma-Aldrich. Free ^{125}I-sodium iodide-37MBq was purchased from Amersham, GE Healthcare (1mCi, carrier free). Recombinant beetal cGH was produced in the laboratory (School of Biological Sciences, University of the Punjab, Lahore, Pakistan) and human GH (hGH) used for the cross reactive study was a gift from NETRIA, UK. Rabbit anti-rcGH antibodies

were raised against beetal rcGH in a local animal house.

Iodination of rcGH

Iodination of rcGH was done by chloramine-T method (Edwards, 1999). For the purification of the iodinated rcGH or tracer (^{125}I-rcGH), 0.05 M phosphate buffer pH 7.4 (1% BSA and 1% potassium iodide) was used as a carrier (Herington et al., 1976; Edwards, 1999). Gel exclusion chromatography was done to remove the unchanged I$^-$ from the conjugated rcGH (^{125}I-rcGH). Chromatographic column (30 × 0.9 cm) was packed with Sephacryl S300 and equilibrated with elution (0.05 M phosphate buffer pH 7.4 containing 0.01% NaN$_3$) at a flow rate of 6 ml h^{-1}. Tracer was loaded on the column and 1 ml fractions were collected at flow rate of 3 ml h^{-1} adjusted by peristaltic pump (Amersham, GE Healthcare, Pump-P1). Radioactivity of each fraction was checked by gamma counter (LB2111, Berthold Technologies GmbH & Co. KG) and chromatogram was plotted between the number of fractions and counts per 30 s.

Analysis of the tracer (^{125}I-rcGH)

The binding of the ^{125}I-rcGH was analyzed by using rabbit anti-rcGH antibodies. Total number of counts of ^{125}I-rcGH (200 c.p.m. µl^{-1}) was made in an assay buffer (0.05 M phosphate buffer, pH 7.4). Rabbit anti-rcGH antibodies were diluted (1: 100, 1: 1K & 1: 10K) and 100 µl of each antibody dilution, 200 µl assay buffer and 100 µl tracer (200 c.p.m. µl^{-1}) were added in the tubes (duplicates), and incubated for 4 h at room temperature. Reaction was stopped by adding 1 ml of ice cold assay buffer, followed by 1 ml of 25% (w/v) PEG 6000.

Suspension was mixed and centrifuged at 3,000×*g* at 4°C for 30 min. Supernatant was decanted and radioactivity of the pellet as total binding (TB) of the tracer was checked by gamma counter. Non-specific binding (NSB) or control was determined by adding 200 µl of assay buffer and 100 µl tracer. Specific binding was calculated by subtracting non-specific binding (NSB) from total binding (TB) (Cadman and Wallis, 1981). 1% BSA (20 µgml^{-1}) was added in the purified tracer and aliquots were stored at -20°C.

Preparation of microsomal membrane (receptor protein)

The crude microsomal membranes were prepared from the livers of slaughtered Bovidae species (caprine, ovine and bovine) according to the method described by Rad cliff (Rad cliff et al., 2003). Approximately 2.0 g of liver pieces (10 × 20 mm) were homogenized in ice cold 0.025 M Tris-HCl buffer pH 7.8 containing 0.3 M sucrose, 0.01M EDTA, 0.01M EGTA (ethylene glyco-bis [β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid) and 0.001 M PMSF (phenylmethyl sulfonyl fluoride) by using Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 11,000 x *g*, 4°C for 20 min to remove the tissue fragments. The supernatant was transferred to clean tube and volume was adjusted to 30 ml and was further centrifuged at 100,000 x *g*, 4°C for 120 min. The supernatant was discarded and pellet was homogenized in 0.025 M Tris-HCl buffer pH 7.8 containing 0.01 M calcium chloride. Aliquots were made and

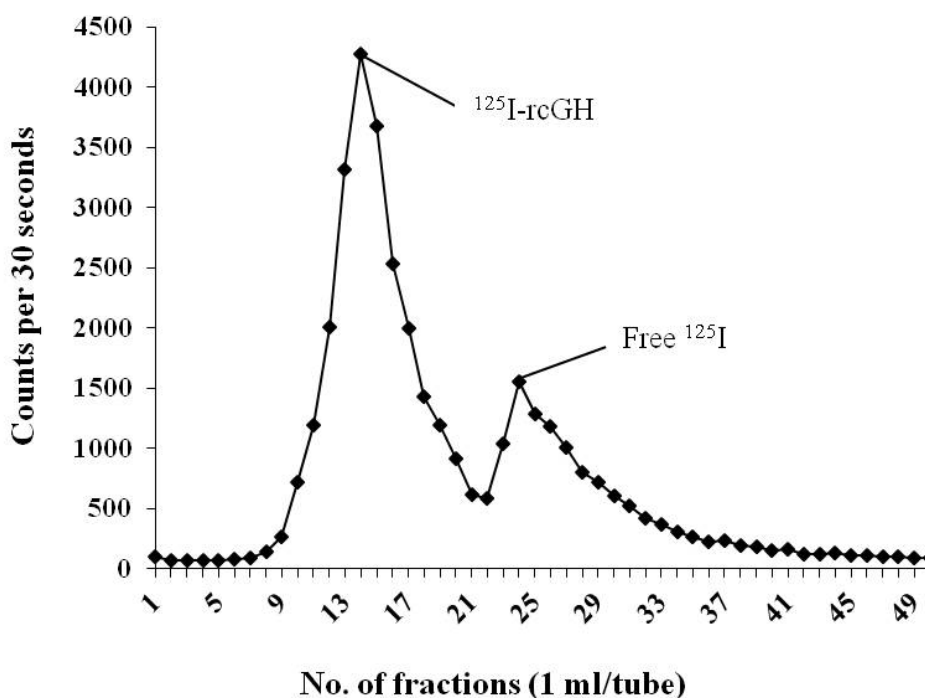*Corresponding author. E-mail: roquyya@yahoo.com, ruqayya.gul@imbb.uol.edu.pk. Tel: 0092 423004177639.

Figure 1. Chromatographic profile of the tracer (^{125}I-rcGH). Purification of the tracer by gel exclusion chromatography (Sephacryl S-300 packed column 30 × 0.9 cm). Two peaks were obtained; big and small peak showing the purified tracer and free ^{125}I, respectively.

stored at -80°C up till analysis. The protein estimation of the microsomal membranes (also known as membrane bound receptors) was measured by Bradford method (Bradford, 1976).

Factors affecting the binding of the tracer to receptor protein

The radio-receptor assay of the rcGH was set in triplicates and the method was based on previously described protocol by Cadman and Wallis, (1981). Factors affecting the binding of the tracer to receptor protein such as effect of receptor protein concentrations, tracer counts, temperature, time of incubation, assay pH and binding to different Bovidae receptor proteins (caprine, ovine and bovine) were studied. Cross reactivity of the tracer was investigated in the presence of unlabeled rcGH and human GH. Scatchard analysis of rcGH binding to Bovidae receptors was analyzed by Prism statistical program provided by Graph Pad I (Manson, 1990).

RESULTS AND DISCUSSION

Preparation of ^{125}I-rcGH

Preparation of the tracer is a primary step for the characterization of beetal rcGH by radio-receptor assay. There are number of methods by which iodination of a protein is done (Edwards, 1999). In the current study, rcGH was iodinated by chloramine-T method (Herington et al., 1976); previously GH has been iodinated by iodogen method (Cadman and Wallis, 1981) and by

lactoperoxidase method (Tsushima and Friesen, 1973). Gel exclusion chromatography was carried out to remove the free iodine (I⁻) from the labeled rcGH (^{125}I-rcGH). Total 50 fractions were collected and radioactivity of each fraction was checked by gamma counter. Chromatographic profile is shown in Figure 1. Two peaks were obtained; big and small peak represented the purified ^{125}I-rcGH and free ^{125}I, respectively. From total of 50 fractions, fraction no. 11-19 were pooled and their binding activity was analyzed by using different dilutions of rabbit anti-rcGH antibodies. The maximum binding was obtained at 1: 100 dilution of the antibody, however, the binding was decreased with the increase in the dilution of the antibody (Table 1). This gave the idea that the tracer was in working condition or rcGH was not damaged during the iodination process (Edwards, 1999).

Preparation of microsomal membrane (receptor protein)

For the receptor assay, another important step is the preparation of the microsomal membrane or receptor protein. These membranes play significant role in learning protein-protein and lipid-protein interactions and understanding their functional properties for membrane bound enzymes. It is commonly accepted that polypeptide hormone receptors are associated with the cell or

Table 1. Analysis of the tracer (^{125}I-rcGH): total binding (TB), non-specific binding (NSB) and specific binding of the tracer at different rabbit anti-rcGH antibody dilutions.

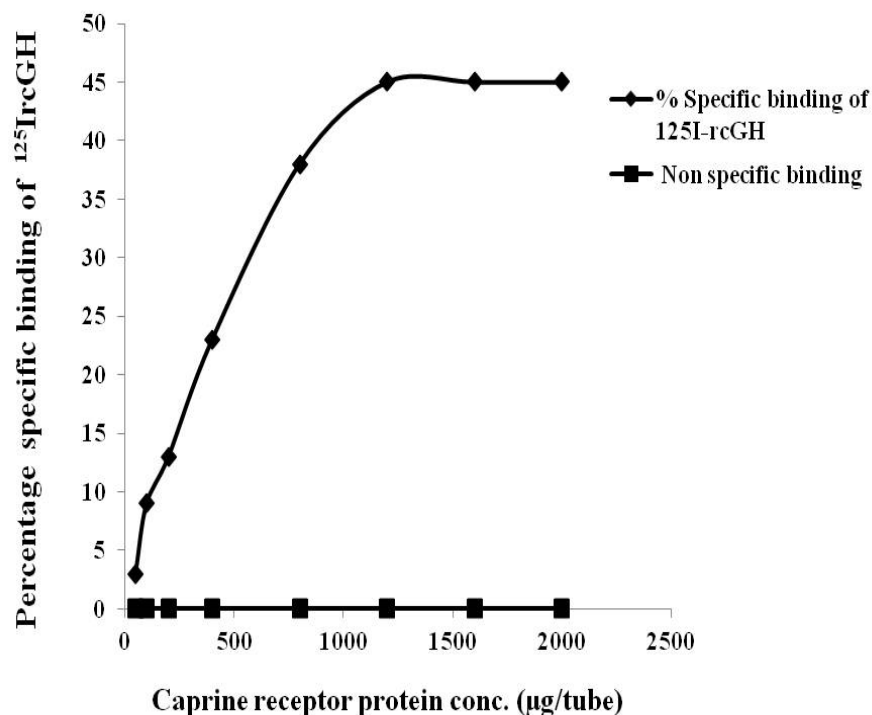| Antibody dilution	TB	NSB	Specific binding = TB - NSB
1: 100	13,904	180	13724
1: 1K	7712	183	7529
1: 10K	3320	180	3140

Figure 2. Effect of binding of the tracer (^{125}I-rcGH) to caprine receptor protein. Tracer (50,000 c.p.m.) and different receptor protein concentrations used per assay tube and maximum 45% specific binding ◆ of the tracer is shown at 1200 µg of the receptor protein and non-specific binding ■ is shown at 0% at all protein concentrations.

plasma membranes of various tissues like liver, lungs, mammary glands etc. (Labbe et al., 1992), however liver microsomal membrane fraction exhibit highest binding affinity for the GH (Herington et al., 1976). Therefore, crude microsomal membranes were prepared from fresh liver tissues of the Bovidae species (caprine, ovine and bovine) to investigate the rcGH biological activity that is, whether the recombinantly produced caprine GH is functionally active.

Effect of different factors on ^{125}I-rcGH binding

Effect of receptor protein concentration

To study the effect of binding of ^{125}I-rcGH to the caprine

receptor protein concentrations (50, 100, 200, 400, 800, 1200, 1600 and 2000 µg), assay was set. The receptor proteins were incubated with 50,000 c.p.m. of ^{125}I-rcGH for 24 h at 4°C and then centrifuged to separate the bound and unbound ^{125}I-rcGH.

The maximum binding of ^{125}I-rcGH occurred at the concentration of 1200 µg receptor protein, however, further increase or decrease in protein concentration had no significant effect on the specific binding of the ^{125}I-rcGH to the caprine receptor protein as shown in Figure 2. Since maximal binding was observed at 1200 µg, this receptor protein concentration was therefore selected to perform subsequent experiments. Contrary to this, specific binding of the ^{125}I-rbGH (iodinated recombinant bovine GH) was shown to be 48% with the crude receptor membrane (Haro et al., 1984).
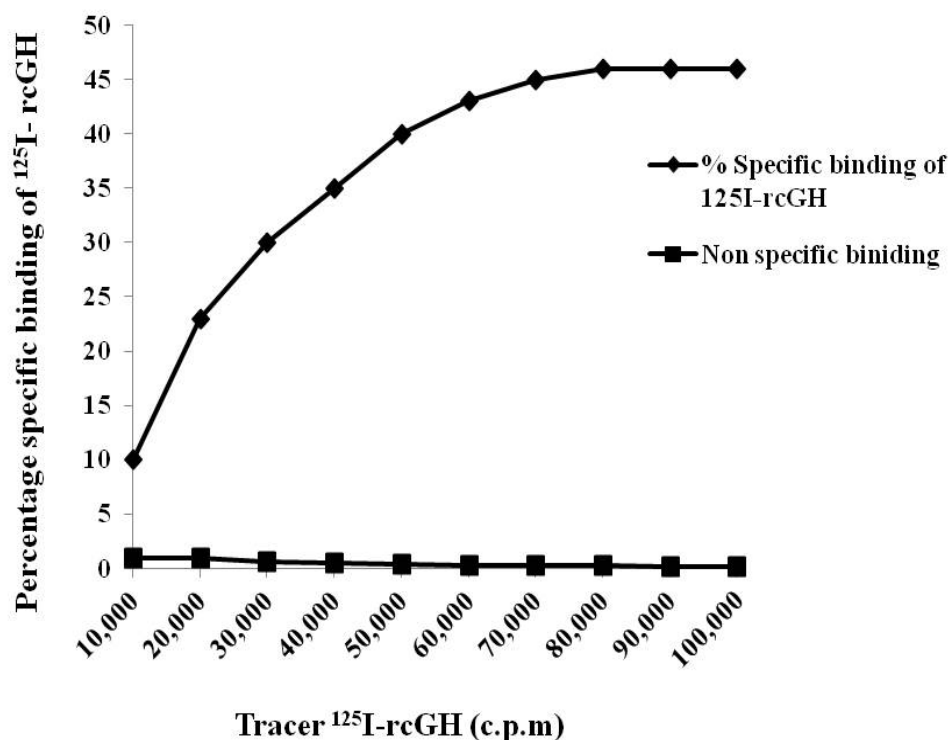
Figure 3. Effect of binding of different tracer (^{125}I-rcGH) counts. Maximum percentage specific binding (♦) is shown at 70,000 c.p.m. Non-specific binding (■) is shown at 0% at all tracer counts.

Effect of tracer counts

The effect of different tracer counts (10,000-100,000 c.p.m.) on ^{125}I-rcGH binding was analyzed for 24 h at 4°C with selected caprine receptor protein concentration that is, 1200 µg. Maximum 45% specific binding was observed at 70,000 c.p.m. and saturation of the tracer binding was seen above 80,000 c.p.m. as shown in Figure 3. Hence, tracer 70,000 c.p.m. was selected for subsequent experiments. Although, non-specific binding was negligible in all the ^{125}I-rcGH counts.

Effect of temperature

Effect of binding of the tracer was studied at two different temperatures that is, 4 and 37°C for 24 h (Figure 4). At 37°C, the tracer binding was fast at the beginning of the assay as compared to 4°C. Although, the specific binding was too low that is, 5%, this could be due to the degradation of the receptor protein or tracer after 24 h of incubation. These results are in agreement with the previous work done in which ^{125}I-human GH was observed to be degraded when incubated with rat microsomal membrane (Posner et al., 1974; Herington et

al., 1976). Whereas, at 4°C, the percentage specific binding showed to be 45% in contrast to 37°C. This might be the reason of non-degradation of the tracer and the receptor protein or tracer remained bound to the receptor protein which ultimately led to the prolonged equilibrium time of hormone-receptor complex and thus %age binding of the hormone with its receptor was increased (Posner et al., 1974; Haro et al., 1984). Thus, 4°C was selected as an appropriate temperature for further assay. While, non-specific binding at both the temperatures was found to be insignificant. The effect of temperature was in consensus with the findings of Kelly et al. (1973) who observed that hGH had a very long equilibration time at 4°C due to non-degradation.

Effect of assay pH

The influence of assay buffer pH on ^{125}I-rcGH binding to receptor protein was studied over the pH range 6-9 (Figure 5). Tracer (70,000 c.p.m.) and caprine receptor protein (1200 µg) were used in the assay as optimized from the earlier experiments. Maximal specific binding (45%) of the tracer was attained at pH 7.0, however, below and above pH 7.0 binding of the tracer to receptor
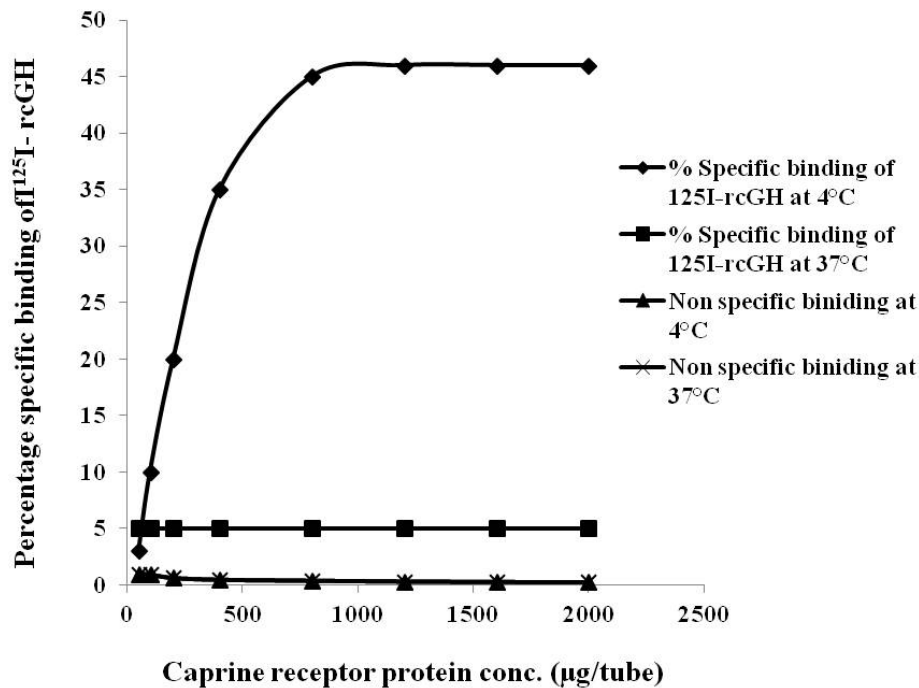
Figure 4. Effect of temperature. Tracer (70,000 c.p.m.) and different caprine receptor protein concentrations (μg per assay tube) used. Maximum percentage specific binding was seen at 4°C (◆) while at 37°C (■) percentage specific binding was low. Non-specific binding at 4°C (▲) and 37°C (×) was negligible.
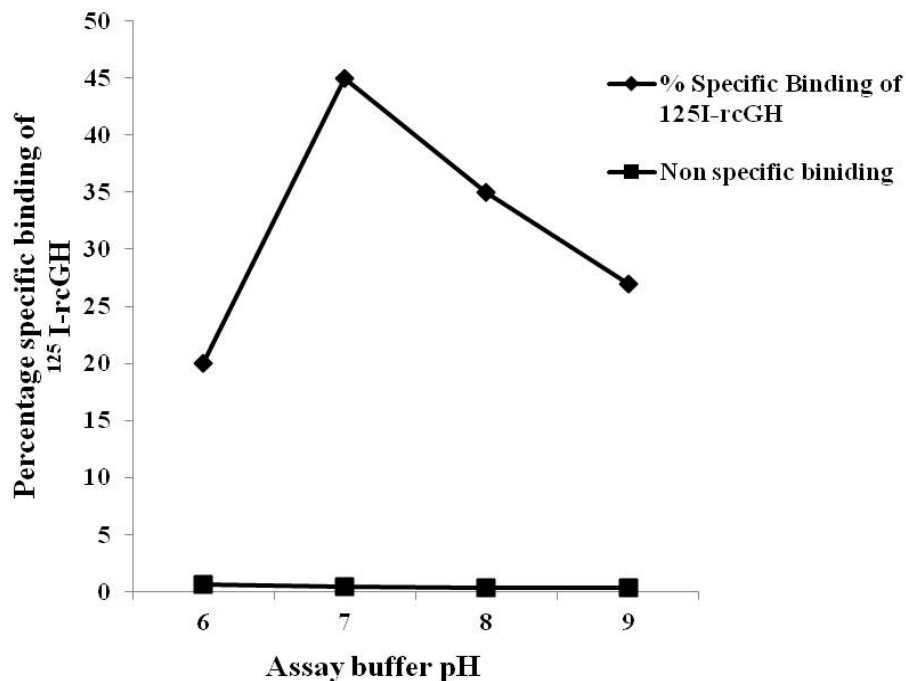

Figure 5. Effect of pH. Tracer (70,000 c.p.m.) and receptor protein concentration (1200 μg) per assay tube used. Maximum of 45% age specific binding (◆) was seen at pH 7. Non-specific binding (■) was shown to be insignificant.
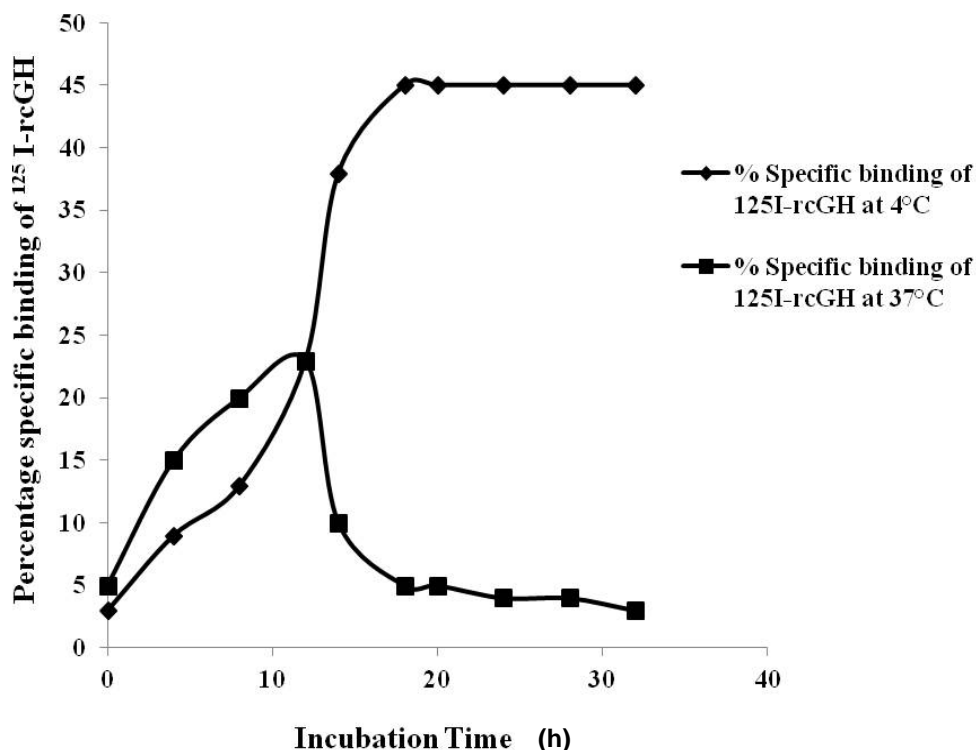
Figure 6. Effect of time. Tracer (70,000 c.p.m.) and receptor protein concentration (1200 μg) per assay tube used. Incubation was given at different durations (0-32 h) at 4 and 37°C respectively. Maximum percentage specific binding (◆) was seen at 45% at 18h incubation for 4°C. At 37°C, maximum %age specific binding (■) reached 23% at 12 h incubation time.

protein was significantly lost. The results reveal that pH 7 was favorable for binding of the tracer to its receptor protein. The optimum pH for binding of Bovidae GH to receptor protein was reported as 7.8 (Haro et al., 1984).

Effect of incubation time

The binding of the ^{125}I-rcGH to the caprine receptor protein was analyzed at different incubation time at two respective temperatures that is, 4 and 37°C under the optimized conditions of incubation time (32 h), receptor protein (1200 μg) and tracer counts (70,000 c.p.m.). After every 4 h, specific and non-specific binding of the ^{125}I-rcGH was checked by taking the counts on gamma counter (Figure 6). Maximum specific binding i.e. 45% was observed at 4°C. The binding of the ^{125}I-rcGH showed to be slow at the start of the assay, however it reached at its maximum levels after 18 h of incubation. After 18 h binding of the ^{125}I-rcGH remained steady, up till 32 h. While at 37°C binding of the hormone to its receptor was fast and the %age binding reached 23% at 12h of incubation. However, after 12 h binding drastically dropped to 5%. This could be due to the degradation of

the hormone and its receptor protein at 37°C, as degradation of the protein is seen to be extensive at 37°C (Herington et al., 1976).

Binding of the ^{125}I-rcGH to different Bovidae receptor proteins

The effect of binding of the ^{125}I-rcGH to different microsomal membranes of Bovidae species (caprine, ovine and bovine) was studied. Different concentrations of Bovidae receptor proteins (50, 100, 200, 400, 800, 1200, 1600 and 2000 μg) were incubated with 70,000 c.p.m. of tracer for 24 h (Figure 7). It was observed that all the microsomal membranes of the Bovidae species showed 45% specific binding. Thus, showing all the receptor proteins of the Bovidae species are similar in nature. The result was in the agreement with the statement that Bovidae family has well conserved GH receptor nucleotide and amino acid sequences. However, Maj and Zwierzchowski (2006) have reported 99 and 97% amino acid sequence homology of the caprine growth hormone receptor (GHR) with ovine and bovine species, respectively. While, non-specific binding was
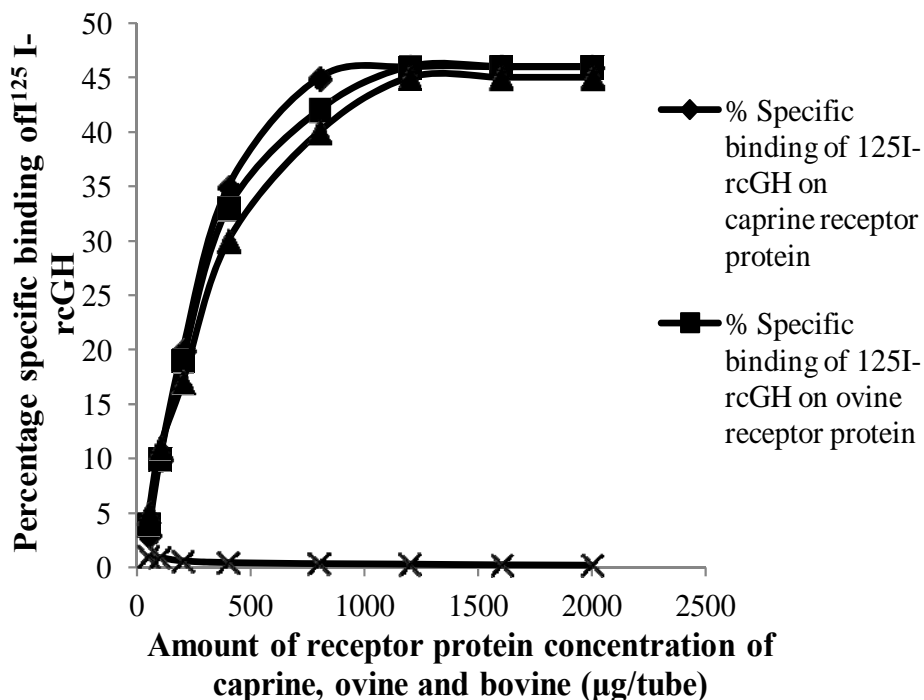
Figure 7. Binding of the tracer (^{125}I-rcGH) to different microsomal membranes of Bovidae species (caprine, ovine and bovine). Tracer 70,000 c.p.m. per assay tube was used, incubation given for 24 h at 4°C. Maximum percentage specific binding in all the Bovidae species, caprine (♦), ovine (■) and bovine (▲) seen to be 45 %. Non-specific binding (×) showed to be negligible.

observed to be negligible in all the Bovidae species.

Cross reactive study of the ^{125}I-rcGH

The cross reactive study was done by using two unlabeled GHs that is caprine and human, and competitive assay was performed in the presence of ^{125}I-rcGH (Figure 8). Different concentrations of unlabeled caprine and human GH (0, 0.1, 1, 10, 100 and 1000 μg) were used in the assay and results showed that 1000 μg concentration of both unlabeled hormones disrupted the binding of the ^{125}I-rcGH at a maximum level. These results were in consensus with that of Tech et al. (1988). The cross reactive results explained that human GH binds with high affinity to the Bovidae microsomal membrane or with non-primates GHR and similar results have also been reported by Souza et al. (1995). Whereas, non-specific binding was found to be insignificant in both cases of unlabeled rcGH and hGH.

Scatchard analysis of rcGH binding

The Scatchard analysis of rcGH binding to Bovidae

receptors indicated single class of binding site. The Scatchard plot was analyzed to calculate the dissociation constant (K_d). As K_d is defined as the ratio of unbound and bound molecules at equilibrium that is,

$$K_d = \frac{[A][B]}{[AB]}$$

Thus small K_d value shows high affinity interaction while large K_d value indicates low affinity interaction. The Scatchard analysis of rcGH binding to Bovidae receptor protein that is, caprine, ovine and bovine is shown in Figure 9. The analysis showed that rcGH has 57.61 ± 4.32 fmol mg^{-1} binding capacity and 337.1 ± 82.94 × 10^9 M^{-1} affinity for caprine receptor protein. Ovine receptor protein also exhibit high binding capacity that is, 57.69 ± 3.23 fmol mg^{-1} and slightly low affinity (365.4 ± 66.82 × 10^9 M^{-1}) for rcGH as compared to caprine receptor protein. However, with bovine receptor protein, both the binding capacity (56.8 ± 2.56 fmol mg^{-1}) as well as affinity (392.6 ± 56.25 × 10^9 M^{-1}) of rcGH were found to be the least when compared with other two receptor proteins as shown in Table 2. The possible reason of differences in binding affinity of rcGH with the caprine, ovine and bovine
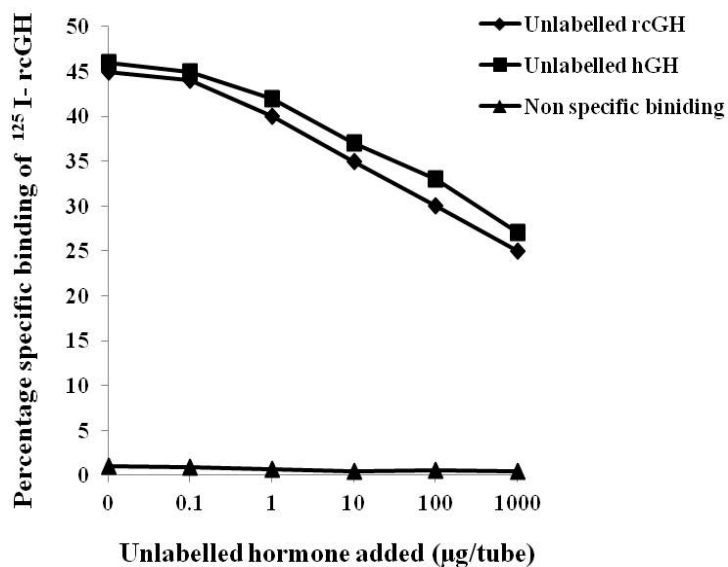
Figure 8. Cross reactive study of the tracer (^{125}I-rcGH) in the presence of unlabeled rcGH and unlabeled hGH. Tracer (70,000 c.p.m.) and 1200 μg receptor protein per assay tube was used in the presence of unlabeled rcGH and hGH. Incubation given for 24 h at 4°C and displacement of the tracer observed in the presence of unlabeled rcGH (♦) and hGH (■). Non-specific binding (▲) showed results to be insignificant.
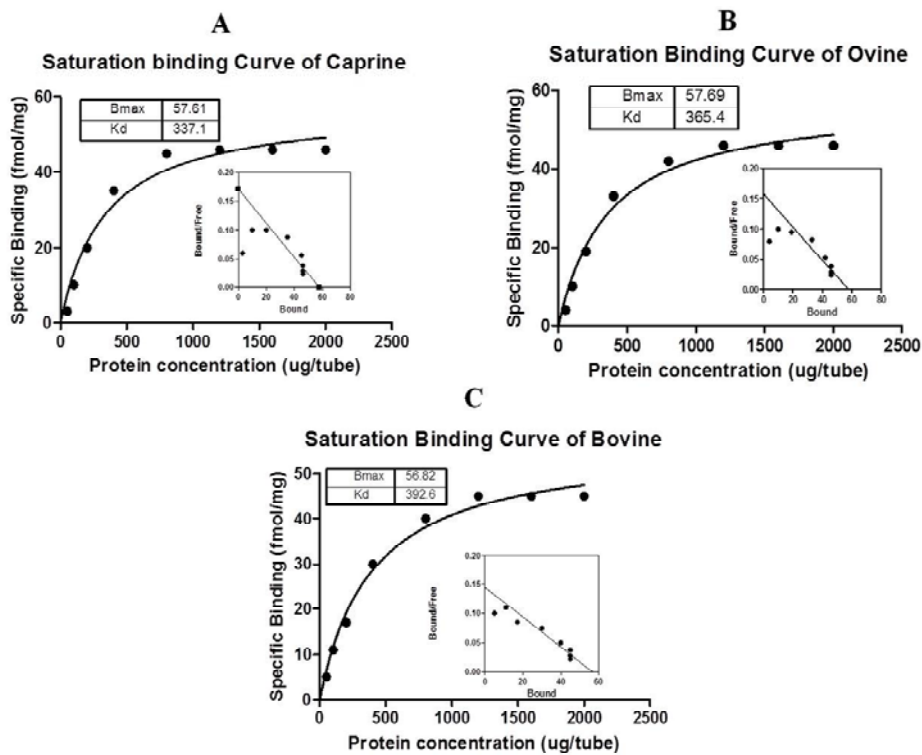

Figure 9. Scatchard analysis of rcGH binding to Bovidae receptors. **A.** rcGH binding to caprine microsomal membrane. **B,** rcGH binding to ovine microsomal membrane. **C.** rcGH binding to bovine microsomal membrane.

Table 2. Saturation binding data of rcGH with the Bovidae receptor proteins that is, caprine, ovine and bovine.

Saturation binding data		Caprine receptor protein	Ovine receptor protein	Bovine receptor protein
B_{max}	Mean	57.61	57.69	56.82
	Standard Error	4.23	3.23	2.56
	Confidence Interval (95%)	47.26-67.95	49.77-65.61	50.55-63.09
K_d	Mean	337.1	365.4	392.6
	Standard Error	82.94	66.82	56.25
	Confidence Interval (95%)	134.1-540	201.9-528	255-530

receptor protein could be due to the amino acid variations found in amino acid sequence alignment of GHR among the Bovidae species (Gul et al., 2012).

Conclusion

Our results conclude that the specific binding of the radiolabeled rcGH to the caprine liver microsomal membrane is dependent on various factors like receptor protein concentration, tracer counts, temperature, time of incubation and assay pH. Moreover, the binding of the ^{125}I-rcGH to the Bovidae (caprine, ovine and bovine,) receptor proteins was 45%. These findings would add to study further the hormone-receptor interaction of the Bovidae growth hormones.

Conflict of Interest

The author(s) have not declared any conflict of interest.

ACKNOWLEDGEMENT

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