IMMUNOLOGICAL AND RECEPTOR BINDING CHARACTERISTICS OF BOVIDAE GROWTH HORMONES

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By

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In The Name Of Allah, The Most Beneficent, The Most Merciful

"They said: Glory is to You, we have no knowledge except what You have taught us. Verily, it is You, the All-Knower, the All-Wise"

(Al-Quran 2:32)

Dedicated to

my beloved "Mama jan"

(May her soul rest in peace)

for her strong support, eternal warm prayers, infinite love & encouraging attitude all throughout my life

CERTIFICATE

This is to certify that the research work described in this thesis is the original work of Roquyya Gul and has been carried out under my supervision. I have personally gone through all the data/results/materials reported in the manuscript and certify their correctness/authenticity. I further certify that the material included in this thesis have not been used in part or full in a manuscript already submitted or in the process of submission in partial/complete fulfillment of the award of any other degree from any other institution. I also certify that the thesis has been prepared under my supervision according to prescribed format and I endorse its evaluation for the award of Ph.D. degree through the official procedures of the University.

(Prof. Dr. M. Waheed Akhtar)
Research Supervisor

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"Give thanks to Allah, and whoever gives thanks, he gives thanks for (the good of) his ownself. And whoever is unthankful, then verily, Allah is All-Rich (free of all needs), Worthy of All-Praise"

(Al-Quran 31:12)

All Praise to the Most Merciful and Beneficent Allah SWT, Who have helped, gave me courage and ability to complete this task. Without the Graces of Allah SWT it would not have been accomplished.

There is a saying of the Prophet Muhammad (May peace and blessings of Allah SWT be upon him)

"Whosoever is not thankful to the people ---- such is not thankful to Allah"

(Abu-Dawood)

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"My Lord! Inspire me and bestow upon me the power and ability that I may be grateful for Your Favors which You have bestowed on me and on my parents, and that I may do righteous good deeds that will please You and admit me by Your Mercy among Your righteous slaves" amen.

(Al-Quran 27:19)

Roquyya Gul

SUMMARY

Growth hormone (GH), growth hormone receptor (GHR) and growth hormone binding protein (GHBP) are the essential components of the growth hormone system. The recombinant biotechnology has enhanced the importance of GH in livestock especially in Bovidae family by increasing their milk and meat production and eventually increasing the economy of the country. Thus for this purpose recombinant Bovidae GHs are being studied in the laboratory. One of the important aspects of this study was to analyze their immunological, biological and receptor binding characteristics. Current study was therefore undertaken to investigate the immunological and receptor binding characteristics of Bovidae GHs.

For immunological analysis, competitive enzyme immunoassay (EIA) of recombinant caprine (goat) growth hormone (rcGH) was optimized. Polyclonal antibodies against rcGH (rabbit anti rcGH Ab) were raised in two rabbits of local breed. Antisera were collected and by coating on to the microtitre plate at different dilutions, bleeds were titrated. Conjugate was prepared by labeling the enzyme, horseradish peroxidase (HRP) with the rcGH. Titration of the conjugate was done to optimize the optimum conjugate dilution. Standard points i.e. 0, 25, 50, 100, 200 and 400 ng/ml of rcGH was made. The standard points were used to optimize the conditions of the competitive EIA of rcGH like antibody coating time, coating buffer, pH of coating buffer, assay incubation time and substrate, etc. A linear standard curve was plotted between the concentration of standard points and absorbance (450/630 nm). From the plotted standard curve it was deduced that the absorbance of the sample was inversely proportional to the concentration of antigen in the sample.

Data interpretation was done by observing the optical density (OD) or absorbance corresponding to the concentration of standard points on the plotted standard curve. Cross

reactive study among Bovidae species was performed by using recombinant ovine GH in the competitive EIA of rcGH. Recombinant ovine GH bound with the same affinity to the rabbit anti-rcGH antibodies as rcGH. The specificity of the competitive EIA of rcGH was analyzed by using human insulin and it was validated that the assay was specific for the GH quantification. The sensitivity of the assay was checked by determining the lowest range of rcGH.

Another feature which is necessary in the production of recombinant protein is to analyze its biological activity. In the current study the biological analysis of the recombinant caprine GH was investigated by optimizing the receptor binding assay (RBA) or radio receptor assay (RRA). The tracer was prepared by iodinating the rcGH with ^{125}I. The binding activity of the tracer was checked by using rabbit anti-rcGH antibody. Different Bovidae species like caprine, bovine and ovine liver microsomal membranes (receptor proteins) were prepared to analyze the binding of the iodinated rcGH (^{125}I-rcGH). Factors affecting the ^{125}I-rcGH were investigated and percentages of specific and non-specific binding were calculated. The binding of ^{125}I-rcGH was observed to be dependent on various parameters like receptor protein concentration, tracer counts, pH, temperature and time. The maximum percentage specific binding of ^{125}I-rcGH with the different Bovidae microsomal membranes (receptor protein) was found to be same. While, non-specific binding was found to be negligible. Cross reactive study was performed by using unlabeled caprine and human GH of various concentrations on the caprine microsomal membrane. Both the unlabeled GHs displaced the ^{125}I-rcGH. Thus it was concluded that human GH binds with high affinity to non-primates.

To investigate the growth hormone receptor binding characteristics, *Bubalus bubalis* GHR (*Bb*GHR) and its extracellular domain (identical to growth hormone binding protein, GHBP) genes were isolated, cloned and expressed in *Escherichia coli*.

Furthermore, the extracellular domain (GHBP) of the *Bb*GHR gene was refolded, purified and biological activity was investigated. Total RNA was isolated from liver tissue of *Bubalus bubalis* specie and RT-PCR was performed to obtain cDNA. The amplified genes of full length and extracellular domain of *Bb*GHR were cloned in pTZ57R/T cloning vector by T/A cloning technique. The transformants were sequenced and deduced nucleotide and amino acid sequences were compared with the other Bovidae species. High level of similarity was shown among them. The cysteine residues were highly conserved among all the Bovidae species. Phylogenetic tree was generated on the basis of multiple aligned amino acid sequences of different animals. It was observed that the species of Bovidae family are highly interlinked as compared to other animals. The secondary and tertiary structures of the full length and extracellular domain (GHBP) of *Bb*GHR were constructed and their sequence was consistent with the known secondary and tertiary structures of the human GHR. The structures showed that the *Bb*GHR gene is a transmembrane protein. And the extracellular domain is comprised of extended β sheets, transmembrane region is helical while cytoplasmic domain is coiled in nature.

For the expression of full length and extracellular domain (GHBP) of *Bb*GHR genes, pET expression system (pET22b) was used in *E. coli* host. The genes were expressed by using IPTG and lactose as inducers. Approximately 40 % and 30 % full length and extracellular domain (GHBP) of total cell protein was expressed respectively. Both the proteins i.e. full length and extracellular domain (GHBP) were produced in the form of inactive aggregates of inclusion bodies. The inclusion bodies of the extracellular domain (GHBP) were solubilized by using 8 M urea for refolding process. Refolding was performed by applying pulsatile dilution method. The refolded protein (GHBP) was dialyzed to remove the contaminants and proceeded to purification step. Purification was done on FPLC by using Resource-Q column. Mass spectrometry was performed on

MALDI-TOF/TOF of the refolded and purified extracellular domain (GHBP) of *Bb*GHR. A single peak was obtained. The biological activity of the protein (GHBP) was checked on HeLa cell lines in the presence and absence of Bovidae GH. The results showed that, GH in the combination of extracellular domain (GHBP) could increase the half-life of the hormone, thus increasing the proliferation of the cells. Further, GHBP activity was also analyzed by using iodinated bovine GH (^{125}I-bGH) in the radio protein assay. The results confirmed the binding of extracellular domain with the iodinated bovine GH.

In conclusion, the current study laid down the foundation of simple, sensitive and specific competitive enzyme immunoassay of rcGH. It was also concluded that the recombinant Bovidae GHs that are being produced in our laboratory are biologically active. Furthermore, the characterization of *Bubalus bubalis* GHR showed that the extracellular domain (GHBP) of the receptor is a vital portion for the GH binding. Moreover in the combination of extracellular domain (GHBP) and GH, the availability of the GH to cell proliferation is increased.

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