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Full Length Research Paper

Expression and sequence characterization of growth hormone binding protein of *Nili-Ravi* buffaloes (*Bubalus bubalis*)

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The growth hormone binding protein (GHBP) was isolated from the liver of *Nili-Ravi* buffaloes (*Bubalus bubalis*), reverse transcriptase-polymerase chain reaction (RT-PCR) amplified and sequence characterized. RT-PCR analysis demonstrated high degree sequence identities (97.3 to 99.6%) of *Bb*GHBP cDNA with *Bos taurus, Ovis aries* and *Capra hircus*. An expression plasmid was constructed for the production of *Bb*GHBP in *Escherichia coli* BL21 (RIPL) CodonPlus under the control of T7*lac* promoter. On induction with isopropyl β-D thiogalactopyranoside, the *Bb*GHBP was expressed at levels >30% of the total *E. coli* proteins. The target protein expressed as inclusion bodies was solubilized in denaturing solution and refolded by step/pulsatile dilution method using cysteine and cystine redox potential. Purification to near homogeniety (>98%) was achieved by ion-exchange chromatography with a recovery yield of 64%. Mass spectrometric analysis of the purified *Bb*GHBP showed a single peak of 30,756 Da. A radioprotein assay evaluated the binding affinity of recombinant *Bb*GHBP with iodinated bovine growth hormone (bGH) which demonstrated active conformation of *Bb*GHBP. These results demonstrate high expression and sequence characterization of *Bb*GHBP in *Nili-Ravi* buffaloes and provide the basis for the assessment of *Bb*GHBP in other breeds of buffalo.

Key words: Liver, *Nili-Ravi* buffalo, GHBP, MALDI-TOF mass spectrometry, radioprotein binding assay, refolding.

INTRODUCTION

The growth hormone system is comprised of growth hormone (GH), growth hormone receptor (GHR) and growth hormone binding protein (GHBP) (Edmondson et al., 2003). GH, a 22 kDa polypeptide also known as somatotropin is produced by somatotroph cells of the anterior pituitary gland (Ayuk and Sheppard, 2006). It acts on various organs and systems to stimulate growth, development, cell proliferation and other metabolic activities of the body (Leung and Ho, 2001). Liver is the major site for GH action as it contains transmembrane receptors for GH binding that ultimately stimulates production of insulin like growth factor (IGF), through which most of the GH actions are mediated.

GHR, a transmembrane protein encoded by GHR gene, belongs to a family of cytokine receptors and is mainly expressed in the liver (Bazan, 1990). It is comprised of 634 amino acids, having extracellular, transmembrane and cytoplasmic domains. It has a signal peptide of 1 to 18 amino acids, an extracellular domain (240 amino acids) from 19 to 258 amino acids which corresponds to the serum GHBP (which helps in circulating the GH throughout the body), as GH binds to this domain (Yang et al., 2007).

The GHBP is a transporter protein for the GH and is

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Abbreviations: GHBP, Growth hormone binding protein; **RT-PCR,** reverse transcriptase-polymerase chain reaction; **GH,** growth hormone.

encoded by the same GHR gene, as the extracellular domain of GHR (Leung and Ho, 2001). GH binds with high affinity and specificity to the GHBP as it binds with its receptor (Edens and Talamantes, 1998). In most of the mammals such as humans, monkey's serum GHBP is generated by the proteolytic cleavage of the extracellular domain of the GHR (Baumann et al., 1989) while in rodents it is produced by alternative splicing (Edens et al., 1994). The serum GHBP measurement might be a useful indicator for the hepatic GHR status, as liver is the major source for GHBP mRNA (Tiong and Herington, 1991). It also prolongs the half-life of the GH through competition with the GHR *in vivo* (Amit et al., 2000). Moreover, it acts as a reservoir or buffer for the GH system (Edmondson et al., 2003). However, the importance of GHBP in the GH system is widely increasing for its therapeutic use; for example in case of gigantism or acromegaly in which GH is circulating in an excess amount, GHBP/GHR administration can reduce the circulating levels of GH in humans (Molitch et al., 2006). GHBP can also be useful in the treatment of disorders associated with the GH deficiency (dwarfism or GH deficiency syndrome); when GH is administered in combination with GHBP, it increases the stability and efficiency of GH *in vivo* (Ayuk and Sheppard, 2006).

Bubalus bubalis is widely present in South Asian countries including Pakistan, as it plays leading role in enhancing the economy of the country by producing milk, meat and draught power. It is reported that milk and meat production of livestock can be improved by exogenous administration of GH (Bauman, 1999). Since GHBP prolongs the half-life of GH, it is anticipated that GHBP can increase the activity of GH, hence enhancing the animal productivity such as milk and meat. GHR/GHBP of rabbit (Sakal et al., 2000), human (Postel-Vinay et al., 1991) and mouse (Moffat et al., 1999) have been isolated, cloned and expressed. In contrast, the assessment of GHBP in buffaloes (*B. bubalis*) has not been fully demonstrated in spite of the fact that buffaloes are reared in many parts of the world. Accordingly, the present study was conducted on the liver of *Nili-Ravi* breed in Pakistan to describe in detail sequence characterization, expression profile and refolding of *Bb*GHBP in biologically active conformation.

MATERIALS AND METHODS

Animals, sample collection, chemicals, kits, plasmids and bacterial strains

Liver samples of three *Nili-Ravi* buffaloes (15 years of age, about 300 to 450 kg in body weight) were freshly collected from a local abattoir (Lahore, Pakistan). The liver samples were sliced into 10 x 20 mm pieces and stored at -80°C till further analysis. Chemicals/reagents used in this study were of the highest purity grade commercially available. GF-1 Gel DNA Recovery and GF-1 Plasmid DNA extraction kits used for DNA extraction and plasmid minipreparation were acquired from V*ivanti*s Technologies Sdn. Bhd., Malaysia. DTCS Quick start kit (Beckman Coulter) was used for sequencing of the gene. The vectors used for cloning and expression of *Bb*GHBP include pTZ57R/T (Fermentas Inc. USA) and pET22b(+) (Novagen EMD Biosciences, Germany). *E. coli* host strains used in this study were DH5α and BL21 (RIPL) CodonPlus (Stratagene, USA). Iodinated bovine GH (^{125}I-bGH) used for biological activity assay was a kind gift from NETRIA, UK.

RT-PCR amplification and sequence analysis

The total RNA was isolated from *B. bubalis* liver tissue using standard guanidinium isothiocynate phenol chloroform extraction method (Sambrook and Russell, 2001) and its concentration (µg/ml) was determined at 260 nm. A set of primers [GHBP-F (5´-GTACATATGGATCTCTGGCAGCTGCTG-3´) and GHBP-R (5´-ATGCTCGAGATCTTCTTCACATGCAGATG-3´)], designed on the basis of published nucleotide sequences of Bovidae species GHR/GHBPs (Accession nos. AY608917, NM_176608 and NM_001009323), was used for reverse transcription-based PCR amplification of *Bb*GHBP. Conditions used for PCR were: initial denaturation at 95°C for 2 min followed by 30 cycles of amplification (94°C for 45 s, 57°C for 45 s and 72°C for 1 min) and final extension at 72°C for 20 min. The amplified product was analyzed on 1% agarose gel, purified and T/A cloned in pTZ57R/T vector and subcloned between *Nde*I/*Xho*I sites of pET22b(+) vector. The recombinant plasmid (pET-*Bb*GHBP) obtained was used to transform *E. coli* strains DH5α (cloning host) and subsequently BL-21 (RIPL) CodonPlus for expression. The transformants were selected on LB-ampicillin plates, sequence and correct integration of *Bb*GHBP in the recombinant plasmid was confirmed by sequence analysis on Beckman Coulter CEQ8000^{TM} Genetic Analyzer.

The nucleotide sequence was translated using EMBL-EBI European Bioinformatics Institute (www.ebi.ac.uk) tool and aligned with GHBPs of Bovidae species such as Indian *B. bubalis*, *Bos taurus, Ovis aries* and *Capra hircus* using a multiple sequence alignment program MUSCLE (www.phylogeny.fr). The tertiary structure was predicted by I-TASSER version 1.1 (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and visualized on VMD version 1.9.

Expression analysis

For expression analysis of *Bb*GHBP, *E. coli* BL21 (RIPL) CodonPlus cells transformed with pET-*Bb*GHBP were grown overnight in 10 ml LB medium containing 100 µg/ml ampicillin at 37°C, 150 rpm in an orbital incubator shaker. One percent of this overnight culture was used to inoculate 200 ml fresh LB-ampicillin broth in 2-L baffled flask and grown under same conditions mentioned above.

Protein expression was induced with 0.5 mM IPTG when the culture A_{600} reached 0.5 to 0.7. After 8 h of induction, cells were harvested by centrifugation (6,000 rpm, 20 min, 4°C), resuspended in lysis buffer [50 mM Tris-Cl (pH 8.5), 100 mM NaCl, 5 mM EDTA, 1 mM phenylmethyl sulfonyl flouride (PMSF)] and subjected to sonication (30 pulses with an interval of 1 min). The soluble and insoluble protein fractions were collected by centrifugation at 9,000 rpm, 4°C for 15 min and analyzed by 12% SDS-PAGE (Laemmli, 1970). The inclusion bodies (IBs) containing *Bb*GHBP were washed twice with washing buffer [50 mM Tris-Cl (pH 8.5), 5 mM EDTA, 0.5% Triton-× 100] followed by washing with 50 mM Tris-Cl (pH 8.5) and then solubilized in solubilization buffer [50 mM Tris-Cl (pH 9.5), 10 mM EDTA, 8 M urea, 4 mM dithiothreitol]. The solution was incubated at room temperature for an hour and centrifuged (20,000 rpm, 4°C, 15 min) to obtain clear supernatant for purification and refolding.

Refolding and purification

The expressed *Bb*GHBP, obtained in the form of IBs, was refolded by the removal of denaturant and step dilution. Briefly, 50 mg solubilized protein was added in the refolding buffer [50 mM Tris-Cl (pH 9.5), 1 mM EDTA, 1 mM PMSF, 50 mM glycine, 1 mM cystine and 5 mM cysteine] gradually with gentle stirring at 4°C. DTNB [5, 5′-dithiobis (2-nitrobenzoic acid)] assay or Ellman's test was performed to estimate the free thiols at different stages of refolding process (Ellman, 1959). After 24 h of air oxidation, the solution was centrifuged at 20,000 rpm for 30 min and the clear solution obtained was dialyzed overnight against 20 mM Tris-Cl (pH 8.3) and then purified on Resource-Q column (1.6 x 3.0 cm) by fast protein liquid chromatography (FPLC). Prior to purification, the column was equilibrated with 2 column volumes of the running buffer [20 mM Tris-Cl (pH 8.3)]. The bound protein was eluted with continuous NaCl gradient (0.1 to 1 M) at a flow rate of 1 ml/min and dialyzed against 20 mM Tris-Cl (pH 8.3) to remove salt traces.

Mass spectrometry and protein quantification

Mass spectrometric analysis of the refolded, purified *Bb*GHBP was performed using MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany). *Bb*GHBP (1 µg) after mixing with 3,5 dimethoxy-4-hydroxycinnamic acid was deposited onto the stainless steel target plate of mass spectrometer and analyzed in linear positive mode taking bovine serum albumin (BSA) as standard.

Protein concentration was determined either by Bradford (1976) assay or by absorbance measurements at 280 nm on a UV-visible spectrophotometer (UV-2450, SHIMADZU) taking the absorbency value of 0.7 as the extinction coefficient for a 0.1% (1 mg/ml) solution.

Radioprotein binding assay

The biological activity of *Bb*GHBP was studied by radio protein binding assay set for 4 h at 4°C in duplicates. Different concentrations (200, 400, 800 and 1000 µg) of *Bb*GHBP were made in 20 mM Tris-Cl (pH 8.3) and 100 µl was added in the assay tubes. Iodinated bovine GH (^{125}I-bGH) of 20,000 c.p.m./100 µl and 100 µl assay buffer [20 mM Tris-Cl (pH 8.3), 0.01 M $CaCl_2$, 0.1 % BSA] was added and incubated for 4 h at 4°C. Non-specific binding was calculated by adding assay buffer and iodinated bGH. 1 ml polyethylene glycol (25% w/v) was added to each assay tube and centrifuged at 6,000 rpm for 30 min. Supernatant was discarded, tubes were dried and counts were read on gamma counter (LB2111, BERTHOLD TECHNOLOGIES GmbH & Co. KG) as specific binding or total binding. The percentage specific binding was calculated by the formula [(total binding – nonspecific binding) / total counts added × 100] (Cadman and Wallis, 1981).

RESULTS AND DISCUSSION

RT-PCR amplification and sequence analysis

Total RNA isolated from liver tissue of indigenous *B. bubalis* (*Nili-Ravi*) was RT-PCR amplified using gene-specific primers to obtain 0.77 kb long *Bb*GHBP cDNA. The amplicon was T/A cloned and sequenced using M13 universal primers both in the forward and reverse directions to resolve discrepancies, if any. The nucleotide and deduced amino acid sequence was submitted to the NCBI GenBank Database and has accession no. JN558567.

Comparison of the sequence with corresponding sequences from the selected *Bovidae* species (such as Indian *B. bubalis*, *B. taurus, O. aries* and *C. hircus*) showed high degree of sequence similarities that ranged between 97.3 and 99.6% (Figure 1). Here, of particular interest was the observation that GHBP of indigenous bubaline is more closely related to bovine (*B. taurus*) as compared to Indian bubaline GHBP. Three amino acid variations that is, $Gly_{60}\rightarrow Glu_{60}$, $Thr_{87}\rightarrow Ile_{87}$ and $Arg_{127}\rightarrow Ser_{127}$ were noticed when the sequences of the two *Bb*GHBPs were compared. With bovine, the *Bb*GHBP of the indigenous bubaline showed just a single amino acid difference at position 251 where Ser_{251} has been replaced by Asn_{251} which is the only variation which is common in the two *Bb*GHBPs. Although, the observed variations were apparently not present in the binding region of the GHBP (Allan et al., 1999; de Vos et al., 1992), yet to see the impact of three additional amino acid variations on 3-dimensional conformation of *Nili-Ravi* *Bb*GHBP, we built the 3D-model using I-TASSER program and visualized on VMD (Figure 2). As shown, the amino acids difference in the *Bb*GHBP are in the turns/coil regions and none is in the binding subdomains 1 and 2 (characterized by the presence of seven extended β-sheets in antiparallel fashion) suggesting that the binding properties of GHBPs are less likely to be influenced as a consequence of three additional variations reported in the case of Indian *Bb*GHBP.

Construction of expression plasmid

Approximately 0.77 kb long cDNA of *Bb*GHBP containing *Nde*I and *Xho*I overhangs was cloned in T7*lac* promoter-based pET22b(+) vector between *NdeI/Xho*I sites to generate pET-*Bb*GHBP expression plasmid (Figure 3). The recombinant plasmid was initially maintained in *E. coli* DH5α for vector propagation and then transformed into *E. coli* BL21 (RIPL) CodonPlus for expression studies. Prior to expression analysis, the correct/in-frame integration of insert in the expression plasmid was confirmed by restriction digestion and nucleotide sequencing (data not shown).

Proteins from the induced *E. coli* cells harboring pET-*Bb*GHBP showed a prominent band at position corresponding to ~30 kDa within initial one hour of IPTG induction, when analyzed by SDS-PAGE. The expression levels increased rapidly reaching a maximum of ~30% of the total *E. coli* cellular proteins with 3 h of induction and remained constant thereafter. As expected, a band of similar size and intensity was absent in uninduced cells (Figure 4, Lane 1). After 8 h of induction, a gradual decrease in expression and cell mass was observed most probably due to cell lysis and depletion of nutrients.

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BbGHBP       MDLWQLLLTLAVAGSSDAFSGSEATPAFLVRASQSLQILYPVLETNSSGNPKFTKCRSPE  60
BbGHBP(Ind)  MDLWQLLLTLAVAGSSDAFSGSEATPAFLVRASQSLQILYPVLETNSSGNPKFTKCRSPG
BtGHR/GHBP   MDLWQLLLTLAVAGSSDAFSGSEATPAFLVRASQSLQILYPVLETNSSGNPKFTKCRSPE
OaGHR/GHBP   MDLWQLLLTLAVAGSSDAFSGSEATPAFFVRASQSLQILYPGLETNSSGNLKFTKCRSPE
ChGHR/GHBP   MDLWKLLLTLAVAGSSDAFSGSEATPAFFVRASQSLQILYPGLETYSSGNLKFTKCRSPE

BbGHBP       LETFSCHWTDGANHSLQSPGSVQMFYIRRDIQEWKECPDYVSAGENSCYFNSSYTSVWTP  120
BbGHBP(Ind)  LETFSCHWTDGANHSLQSPGSVQMFYTRRDIQEWKECPDYVSAGENSCYFNSSYTSVWTP
BtGHR/GHBP   LETFSCHWTDGANHSLQSPGSVQMFYIRRDIQEWKECPDYVSAGENSCYFNSSYTSVWTP
OaGHR/GHBP   LETFSCHWTDGANHSLQSPGSVQMFYIRRDIQEWKECPDYVSAGENSCYFNSSYTSVWTP
ChGHR/GHBP   LETFSCHWTDGANHSLQSPGSVQMFYIRRDIQEWKECPDYVSAGENSCYFNSSYTSVWTP

BbGHBP       YCIKLTSNGGIVDHKCFSVEDIVQPDPPVGLNWTLLNISLTEIHADILVKWEPPPNTDVK  180
BbGHBP(Ind)  YCIKLTRNGGIVDHKCFSVEDIVQPDPPVGLNWTLLNISLTEIHADILVKWEPPPNTDVK
BtGHR/GHBP   YCIKLTSNGGIVDHKCFSVEDIVQPDPPVGLNWTLLNISLTEIHADILVKWEPPPNTDVK
OaGHR/GHBP   YCIKLTSNGGIVDHKCFSVEDIVQPDPPVGLNWTLLNISLTEIHADILVKWEPPPNTDVK
ChGHR/GHBP   YCIKLTSNGGIVDHKCFSVEDIVQPDPPVGLNWTLLNISLTEIHADILVKWEPPPNTDVK

BbGHBP       MGWIILEYELHYKELNETQWKMMDPLMVTSVPMYSLRLDKEYEVRVRTRQRNTEKYGKFS  240
BbGHBP(Ind)  MGWIILEYELHYKELNETQWKMMDPLMVTSVPMYSLRLDKEYEVRVRTRQRNTEKYGKFS
BtGHR/GHBP   MGWIILEYELHYKELNETQWKMMDPLMVTSVPMYSLRLDKEYEVRVRTRQRNTEKYGKFS
OaGHR/GHBP   MGWIILEYELHYKELNETQWKMMDPLLVTSVPMYSLRLDKEYEVRVRTRQRNTEKYGKFS
ChGHR/GHBP   MGWIILEYELHYKELNETQWKMMDPLIVTSVPMYSLRLDKEYEVRVRTRQRNTEKYGKFS

BbGHBP       EVLLITFPQMSPSACEED  258
BbGHBP(Ind)  EVLLITFPQMSPSACEED
BtGHR/GHBP   EVLLITFPQMNPSACEED
OaGHR/GHBP   EVLLITFPQMNPSACEED
ChGHR/GHBP   EVLLITFPQMNPSACEED
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Figure 1. Multiple sequence alignment of *Bb*GHBP (accession no. JN558567) with selected members of Bovidae family viz., Indian *B. bubalis* [bubaline, *Bb*GHBP(Ind.), accession no. AY608917], *B. taurus* [bovine, *Bt*GHBP, accession no. NM_176608], *O. aries* [ovine, *Oa*GHBP, accession no. NM_001009323], *C. hircus* [caprine, *Ch*GHBP, accession no. EF559245]. The amino acid residues different amongst the Bovidae species are boxed.

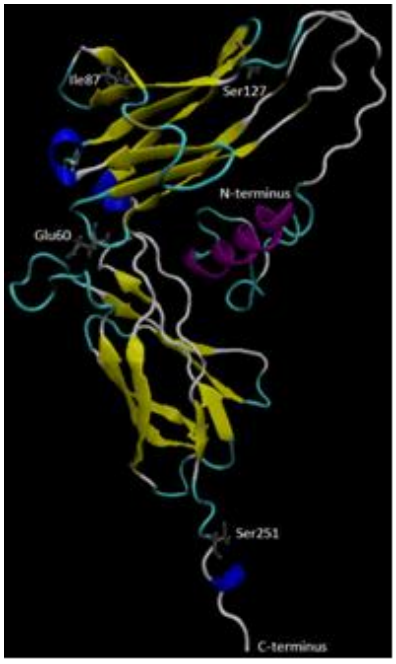

Figure 2. Three-dimension model of *Nili-Ravi* buffalo *Bb*GHBP built using I-TASSER program.

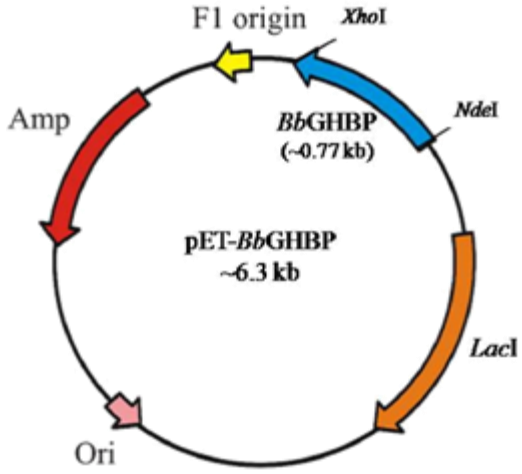

Figure 3. Construction of pET-*Bb*GHBP expression plasmid. ~0.77 kb long *Bb*GHBP cDNA was inserted between *Nde*I/*Xho*I sites of pET22b (+) through restriction digestion and ligase-mediated cloning.

In all subsequent experiments, induction was therefore continued for a maximum of 8 h.

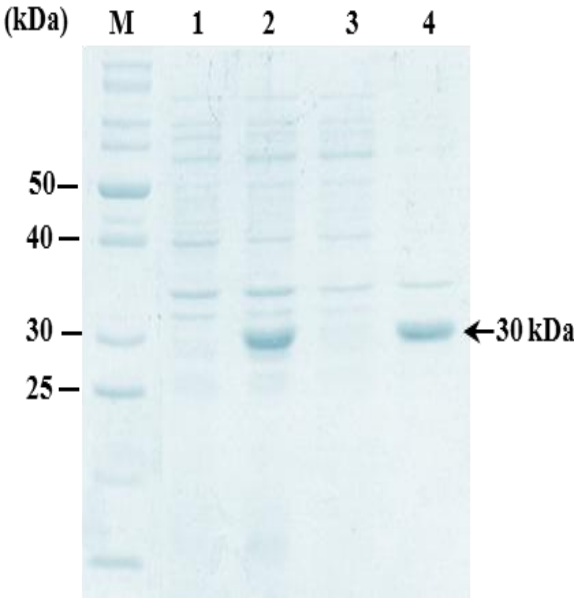

Figure 4. Analysis of induced *E. coli* cell proteins by 12% SDS-PAGE. Lanes: M, protein marker; 1 to 4, uninduced (control), total cell proteins, soluble proteins, and insoluble proteins, respectively.

Further, the analysis of soluble and insoluble protein fractions on SDS-gel revealed that *Bb*GHBP expression is in the form IBs, which constitute over 70% of the total insoluble protein fraction (Figure 4, Lane 4). In prior studies, rat (Baumbach et al., 1989), monkey (Martini et al., 1997) and mouse (Moffat et al., 1999) GHR/GHBP had been expressed in bioactive form using the mammalian expression systems. In the present study, however, *E. coli* based expression system resulted in high-level expression of *Bb*GHBP in the form of IBs as the system lacks refolding machinery. IBs thus obtained were therefore recovered, solubilized and refolded to obtain biologically active recombinant protein.

Refolding and purification

The IBs containing *Bb*GHBP were recovered from the lysed *E. coli* cells by low speed centrifugation and washed twice with Triton-X 100. The purity of *Bb*GHBP attained at this stage was over 75% as judged by densitometric analysis of 12% SDS-gel (Figure 4, Lane 4). Following washing, the native and non-native intra- and inter-chain interactions of IB proteins were disrupted completely using high concentration of urea (8 M) and dithiothreitol. While, for the solubilization of extracellular domain of rabbit GHR, lower molar concentration of urea (4.5 M) was used (Sakal et al., 2000). The refolding was then initiated through pulsatile addition of solubilized protein at a rate of 0.1 ml/min in a refolding sink containing renaturation solution, which was 10 times the volume of solubilized protein, followed by removal of denaturant, which is, urea by dialysis.

Table 1. DTNB assay for the estimation of free thiols. A_{412} measurements showing the free thiol groups present in the refolding sink before and after the addition of solubilized IB proteins at different time intervals.

Sample		A_{412}
Solubilized IB proteins		0.60
Refolding sink	Before the addition of solubilized protein	0.21
	1 h post-addition	0.37
	5 h post-addition	0.21
	12 h post-addition	0.14
	18 h post-addition	0.09
	24 h post-addition	0.06

Too high concentrations of protein during the refolding process often lead to the formation of aggregated species thus resulting in poor refolding yields (Li et al., 2004). In the present study, the final concentration of solubilized protein in the refolding sink was maintained at 0.1 mg/ml to prevent the aggregate formation. Presence of glycine, an aggregation suppressor (Qoronfleh et al., 2007), in the refolding sink further assisted the prevention of misfolded or unfolded protein aggregates. On the other hand, oxidized and reduced cysteine provided appropriate redox potential for the formation of disulfide bonds. During the refolding process, the presence of free thiols in the refolding sink was monitored by DTNB assay (Fuh et al., 1990) and the results are indicated in Table 1. In the beginning, the concentration of free thiols was high as reflected by A_{412} measurements. But with the passage of time, a gradual decrease in free thiols has been demonstrated and after 24 h of air oxidation, the A_{412} dropped down to 0.06 indicating an almost complete oxidation of cysteine residues, which was present at the beginning of the experiment in the reduced state.

Following refolding step, dialysis was performed for buffer exchange and the dialysate was subjected to anion exchange-based Resource-Q column chromatography (Figure 5A). The bound protein could be eluted with 0 to 0.8 M NaCl gradient. Similar strategy was employed for the purification of rabbit extracellular domain of GHR (Sakal et al., 2000). The fraction numbers 128 to 146 containing *Bb*GHBP were pooled and dialyzed against 20 mM Tris-Cl (pH 8.3) to remove salt traces. The percentage purity attained was >98%, whereas the recovery was ~64% (Table 2).

Mass spectrometry and biological activity assessment

The identity of purified and refolded protein was

Table 2. Summary of the percentage recovery of *Bb*GHBP at different stages of purification.

Purification stage	Total protein (mg)[a]	Purity (%)[b]	Recovery (%)
Inclusion bodies	50.0	75	100
Refolding & dialysis	45.8	80	92
FPLC	32.0	98	64

[a] Protein concentration was determined by absorbance measurements at A_{280}. [b] The percentage purity was determined by densitometric analysis of 12% SDS-gel.

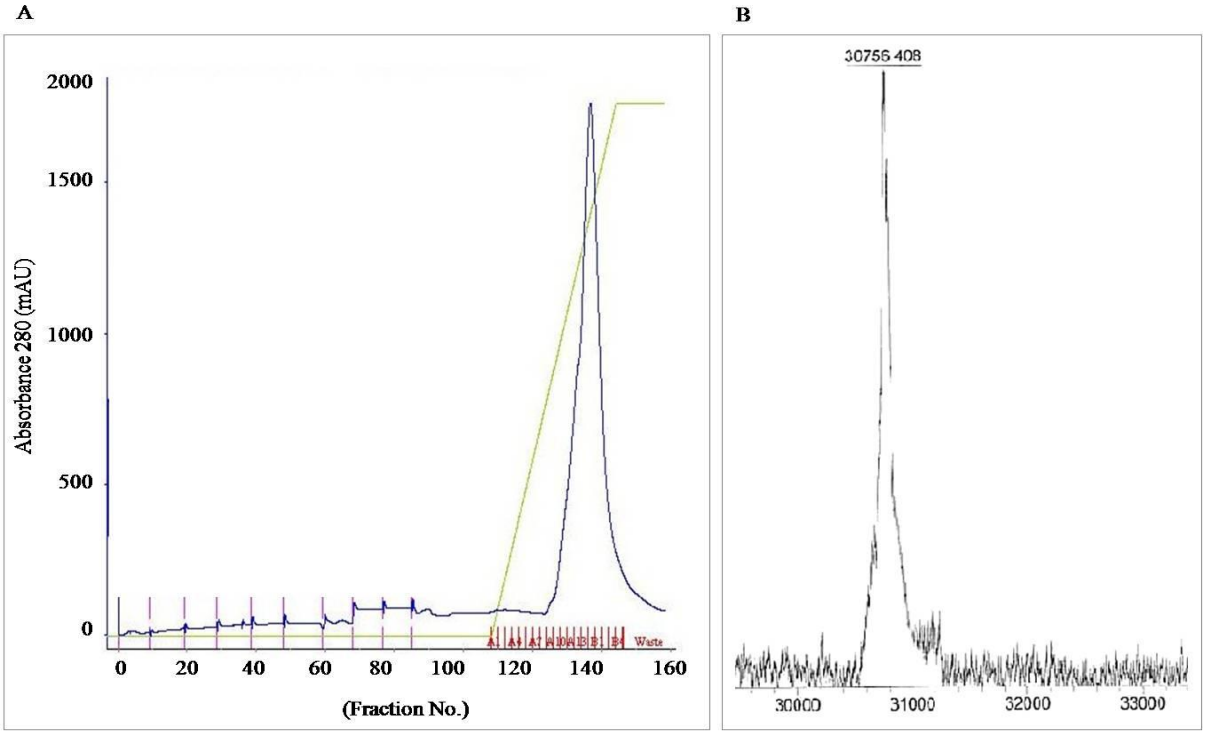

Figure 5. A) Elution profile of *Bb*GHBP fractionated on Resource-Q column. B) MALDI-TOF analysis of purified recombinant *Bb*GHBP.

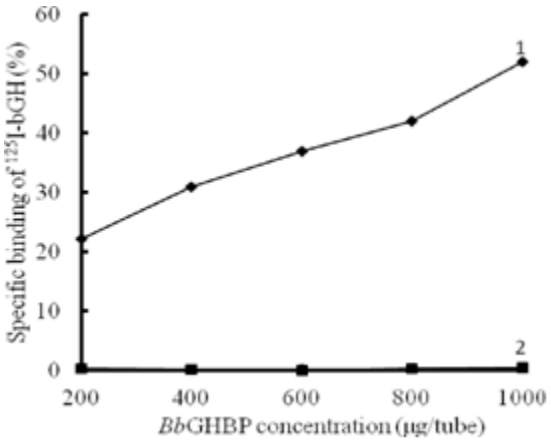

Figure 6. Assessment of biological activity by radioprotein binding assay. Binding of the tracer (^{125}I-bGH, 20,000 c.p.m.) to different *Bb*GHBP concentrations (200, 400, 600, 800 and 1000 µg/tube) is shown. 1) specific binding, 2) non-specific binding.

monitored by MALDI-TOF/TOF mass spectrometry, which showed a single peak of 30,756 Da (Figure 5B). Furthermore, the biological activity of *Bb*GHBP was assessed by its ability to bind with iodinated ^{125}I-bGH in a radioprotein binding assay. As shown in Figure 6, the non-specific binding was negligible, whereas the specific binding of *Bb*GHBP with the ^{125}I-bGH was over 50%. These results confirmed the biologically active conformation of recombinant *Bb*GHBP (Staten et al., 1993).

Conclusion

The current study describes sequence and high-level expression of *Bb*GHBP in the *Nili-Ravi* buffaloes, for the

first time. The findings would certainly be used as reference data for comparison in other breed of buffaloes in Pakistan as well as in other regions of the world. Also, the results would contribute towards developing procedures for large-scale production of *Bb*GHBP for further studies where the use of GH in combination with GHBP would enhance the galactopoetic and somatogenic activities of the livestock more effectively.

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