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Full Length Research Paper

Optimized conditions for high-level solubilization and purification of recombinant camel growth hormone in *Escherichia coli*

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In this report, we describe the cloning, over-expression, efficient solubilization, purification and **evaluation of bioactivity of camel growth hormone (cGH). The total cellular RNA was extracted from pituitary glands of freshly slaughtered animals and cDNA of cGH was synthesized by a pair of sequence specific primers with a product of 576 base pairs (bps). Amplicons was cloned into T/A cloning vector and positive clones were subjected to sequencing. After sequencing, cDNA was cloned in the prokaryotic expression vector system pET23b$^+$. Conditions for cGH expression were optimized by varying the concentration of isopropyl-L-thio-β D-galactopyronoside (IPTG) and induction time. It was observed that 100 µM concentration of IPTG and 3 h post-induction produced the highest amount of cGH. Expressed GH was sequestered as inclusion bodies (IBs), and was therefore, solubilized using denaturant (urea) and detergents (SDS, CTAB, Tritin X-100, Tween-20). The best solubilization was obtained with 8.5 mM SDS in 100 mM Tris buffer at pH 8.5. The solubilized cGH was purified by gel filtration chromatography using Sephadex G-50 column. The purified protein was refolded by dialysis, analyzed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by Western blot. Further biological activity of purified product was confirmed by efficient growth of rat Nb2 lymphoma cells. This study provided the method for the efficient solubilization of cGH (r-cGH) with comparable bioactivity with commercially available bovine growth hormone (bGH) and could be further used for solubilization of other proteins expressed in prokaryotic system.**

Key words: Recombinant growth hormone, somatotropin, cloning, expression, inclusion bodies, solubilization, purification, bioactivity.

INTRODUCTION

Among several other animals, camel (*Camelus dromederius*) is unique in its physiology and services to humanity. It provides milk, meat and is also used for transportation in the Middle East and several other parts of the world. The animal has ability to thrive in harsh environment of tropical and drought prone regions and is capable of fulfilling milk needs of these regions. It is also a source of earning for many low to middle income families of several countries (Schwartz, 1992). It has been shown that camel milk has some unique qualities when compared with milk from other mammals (Qureshi, 1986). Camel milk is superior in its protein, minerals and vitamins contents and of importance, it has higher stability when compared with other mammalian's milk (Yaqoob and Nawaz, 2007) which is one of the desirable characteristics for the camel rearing regions. Camel milk has also been reported to exhibit medicinal values against a number of diseases like dropsy, jaundice, diabetes and conditions affecting the lung and spleen (Knoess et al., 1986; Mal et al., 2001; Agrawal et al., 2003; Guakhar and Bernard, 2004). Thus, camel has potential of fulfilling the dietary and medicinal needs of human.

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Lactating animals growth hormones (GH) have been exploited through biotechnology to increase milk and meat yield and molecular information regarding animals' GH are crucial for further improving this technology. The GH in addition to its direct role in normal growth and development also regulates several complex physiological processes like metabolism, reproduction and cell proliferation (Kostyo and Isaksson, 1977; Hull and Harvey, 2001). Due to different biological roles and commercial applications especially in livestock, several mammalian GHs have been cloned and expressed in various expression systems (Yamano et al., 1988; Anathy et al., 2001; Mukhopadhyay and Sahni, 2002). As far as cGH is concerned, it has been previously purified and partially characterized using pituitary extract of the camel. In this study, it was found that there is only one form of cGH with no glycosylation (Martinat et al. (1990). Maniou et al. (2004) cloned and sequenced the cGH and reported the molecular evolution of pituitary GH in cetartiodactyla.

It is reported that over expression of GH in prokaryotic system results in formation of insoluble aggregates, that is, IBs (Ouyang et al., 2003). In order to recover the bioactive protein from IBs, four steps are required: cell lysis and isolation, solubilization, refolding and purification (Vallejo and Rinas, 2004). Solubilization and refolding are very critical steps for obtaining high yield of bioactive protein. Therefore, a wide range of buffers with and without denaturants/detergents have been used for solubilization of recombinant protein expressed as IBs (Patra et al., 2000). The solubilized proteins were then purified by gel filtration (Ayson et al., 2000), ion exchange (Funkenstein, 2005) and affinity chromatography (Khalid et al., 2008).

We report here, the results of using SDS as an alternative to urea and guanidine HCl for solubilization and recovery of r-cGH produced as inclusion bodies in prokaryotic system with comparable bioactivity with commercially available bGH.

MATERIALS AND METHODS

Construction of cGH expression plasmid

Pituitary of camel was collected from freshly slaughtered animal and was carried under freeze conditions to the laboratory and stored at -70°C before use. RNA was isolated from pituitary by using the method described by Chomezynski and Sacci (1987) and RNA was stored at -20°C till use. A pair of sequence specific primers (Forward 5` CATATGTTCCCAGCCATGCCTCTGTCC3` and Reverse 5`GCAACTAGAAGG CACAGCTG 3`) was designed based on known cGH sequence (AJ575419) with a restriction site for NdeI at the 5'end of forward primer. Bacterial expression system was used to express cGH cDNA and hence 5' primer was designed in a way not to include signal peptide sequence. Complementary DNA (cDNA) was synthesized by using RevrtAid TM First Strand Synthesis Kit (Fermentas, EU) along with three different primers; random hexamer, oligo dT and sequence specific. A typical polymerase chain reaction (PCR) was run for amplification of cGH gene. The thermal profile for amplification of cGH cDNA was, pre PCR denaturation at 94°C followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 90 s with final extension at 72°C for 20 min.

Putative amplified product was first ligated in a T/A cloning vector followed by its transformation into *E. coli* strain DH5α by the method provided with Ins T/A cloneTM PCR product cloning kit (Fermentas, EU). The cloning was confirmed with restriction digestion of T/A vector with NdeI and SalI. The positive clones were selected and sequenced on Beckman Coulter CEQ TM system by using M13 and sequence specific primers along with provided protocol. DNA sequence of three different clones were aligned by using EBI Clustal W. Open reading frame (ORF) was translated into mature peptide and homology of cGH mature peptide was compared with other mammals by using Justbio Translater and EBI Clustal W, respectively.

Recombinant T/A clone were digested with *NdeI* and *HindIII* to isolate cGH cDNA. The digested product was run on 1% agarose gel and purified with Perfectprep® Gel Cleanup Kit (Eppendorf, Germany). The purified product was then directionally sub cloned into *NdeI* and *HindIII* site of pET23b$^+$ vector which was further transformed into BL21(DE3) cells. The sub cloning of CGH cDNA was confirmed by digestion with *NdeI and HindIII* and confirmed clones were stored at -70°C.

Over expression of cGH

BL21 (DE23) cells harboring pET23b$^+$ vector were streaked on Lauria Bertani (LB) agar containing 50 µg/ml ampicillin and allowed to grow over night at 37°C. A single colony from LB plate was inoculated into 3 ml of LB broth containing 50 µg/ml ampicillin and grown overnight at 37°C with shaking incubator at 250 rpm. About 1 ml of the earlier mentioned culture was added into 100 ml of fresh LB broth containing ampicillin and further grown at 37°C. Once an OD 600 nm was ~ 0.5, representing the logarithmic phase, different concentrations of isopropyl beta-D-1-thiogalactopyranoside (IPTG) was added to test optimal over-expression. Then, inoculum was grown for various time intervals at 37°C under shaking at 250 rpm. The uninduced culture was also grown along with induced culture in each experiment. The cells were harvested by centrifugation and the pellet was resuspended in 1 ml of cell lysis buffer containing 10 mM phosphate buffer saline at pH 7.4 and disrupted by sonicating discontinuously for 2 min to check the cellular localization of expressed protein (Wang et al., 2005).

Solubilization of cGH from inclusion bodies

After cell disruption, the suspension was centrifuged at 10000 g for 20 min at 4°C. The pellet containing insoluble r-cGH IBs was washed three times with 1 ml of 50 mM Tris-HCl at pH 8 containing 10 mM EDTA, 100 mM NaCl and Triton X-100. Finally, IBs were washed with two times 1 ml distilled water to remove contaminating salt and detergent. Purified IBs containing r-cGH was then resuspended in 1 ml of 100 mM Tris buffer (pH 8.0 or 12.5), in the presence of 0, 2, 4, 6 and 8 M urea, and 100 mM Tris buffer at pH 8.0 with 8.5 mM sodium dodecyl sulphate (SDS), 6 mM cetyltrimethyl ammonium bromide (CTAB), 0.5 mM Triton X-100, and 0.06 mM Tween-20, separately. The suspensions were solubilized for 4 h with stirring at room temperature. The solubilized IBs were centrifuged at 12000 g for 30 min. The supernatants and pellets obtained after solubilization using different denaturant and detergents were loaded on 12% SDS-PAGE to check solubilization. The supernatant showing maximum solubilization of r-cGH IBs were used for further purification.

Purification of r-cGH

After optimizing over expression and solubilization, the IBs from 1 L of culture were solubilized in 10 ml of 0.1 M Tris buffer, pH 8.0 containing 8.5 mM SDS. The solubilized IB was concentrated by using Eppendorf concentrator 5301 (USA) and then quantified with BCA assay (Smith et al., 1985). Finally, 1.5 ml of solubilized protein having 8 mg/ml protein was applied on gel filtration column equilibrated and eluted with the same buffer in which IBs solubilization was done. The 2 ml fractions were collected at the rate of 0.5 ml/min and the absorbance of individual fractions were measured at 280 nm. The fractions having proteins were pooled, concentrated and loaded onto Sephadex G-100 column (1.5 x 75 cm) for further purification. The fractions which showed a single r-cGH protein band on SDS-PAGE were pooled together, dialyzed against 20 mM Tris buffer, pH 8 and lyophilized.

SDS–PAGE and Western blotting

The amount of protein expressed, and recovered from IBs and during the purification steps were analyzed by 12% SDS-PAGE (Laemmli, 1970) and r-cGH was confirmed by immunoblot analysis. The protein resolved on 12% SDS PAGE was electrophoretically transferred to a nitrocellulose membrane at 90 V for 1 h. After blocking with BSA, the nitrocellulose membrane was processed using rabbit monoclonal antibody raised against GH and goat anti rabbit antibody conjugated to alkaline phosphatase. The antigen-antibody complexes were detected by developing with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrate.

Biological activity of r-cGH

Nb2 cells obtained from the School of Biological Sciences, Punjab University, Lahore, Pakistan were used for the study. Prior to assay, cell growth was arrested by incubating cells in RPMI 1640 medium with L- glutamine and without sodium bicarbonate supplemented with 1% fetal bovine serum, 10% horse serum and 1% penicillin streptomycin solution (PSS) for 24 h. Different concentrations (0, 10, 20, 30, 40 and 50 ng/ml) of bovine serum albumin, commercially available Boostin (bovine somatotropin; bST, Life Sciences, Korea) and purified r-cGH were added separately into culture medium and then incubated for 72 h in the humidified CO_2 incubator at 37°C. All proteins and controls were done in triplicate and three separate experiments were carried out. The cells proliferation was observed by counting the cells in a haemo-cytometer.

RESULTS AND DISCUSSION

Construction of cGH expression plasmid

The amplification of cGH CDS was done with sequence specific primers and a single band of 576 bp was obtained as expected. The result is consistent with the results of Khalid et al. (2007) in which 576 bp product was obtained from buffalo growth hormone cDNA with sequence specific primers. The amplicons were ligated in T/A cloning vector pTZ57R/T and transformed into DH5α for further propagation. Cloning was confirmed by restriction digestion of recombinant plasmid harvested from transformed cells with *NdeI* and HindIII. A fragment of 576 bp was reproduced upon digestion which confirmed the cloning of cGH CDS.

Positive clones were used for sequencing and sequenced obtained showed 100% homology with previously reported GH sequence of Arabian camel (AJ575419). Similarly, multiple sequence alignments of mature cGH showed high homology with other mammalian GHs like *Bubalus bubalis* (91%), *Bos indicus* (90.5%), *Capra hircus* (90%) and *Ovis aries* (90%). These data further reaffirm the previous concept that GHs are highly conserved among various mammalian species (Nicoll et al., 1986; Chene et al., 1989). In earlier studies, prokaryotic system was used for over-expression of various rGH (Hodgson, 1993; Baneyx, 1999). Therefore, cGH cDNA was directionally sub cloned into pET23b+ expression vector under the control of strong T7 promoter and recombinant plasmid was then transformed into *Escherichia coli* strain BL21(DE3) (Studier and Moffatt, 1986).

Expression of cGH

In optimization strategy, various concentrations of IPTG were used with times of induction to obtain optimal conditions for the production of cGH and maximal amount of expressed protein was obtained at 3 h induction time with 100 µM IPTG concentrations. Expressed protein from induced culture was analyzed by 12% SDS-PAGE along with uninduced culture and the results are shown in Figure 1. Induced cells produced a distinct band corresponding to protein of approximately 21 kDa which constitutes a major part of total cellular proteins. It was observed that molecular weight of r-cGH is almost similar to that of cGH obtained from pituitary glands (Martinat et al., 1990).

The r-cGH was expressed in 100 ml culture as IBs with small amounts in soluble fraction. It was observed earlier that proteins over expressed in prokaryotic system are unable to fold into proper conformation and hence tend to be sequestered in IBs (Clark, 1998). Similarly, production of growth hormone in the form of insoluble aggregates was already reported (Sami et al., 2008; Singh et al., 2009).

Solubilization of cGH inclusion bodies

IBs from 100 ml of culture were washed to remove contaminants and the purified IBs were used for subsequent solubilization which is a critical step towards obtaining maximal amount of desired protein. Different denaturants and detergents such as guanidine HCl, urea, SDS, CTAB, cetyltrimethylammonium chloride (CTAC), 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), N-lauryl sarcosine (NLS), Tween-20,

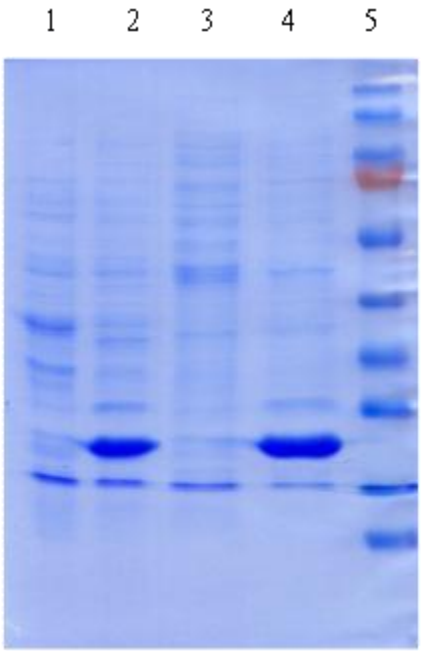

Figure 1. 12% SDS-PAGE analysis of recombinant cGH expressed in BL21 (DE3) cells harboring cGH CDS-pET23b+ vector. Lane 1: lysate of uninduced cells (U), lane 2: lysate of induced cells (I), lane 3 and 4: supernatant and inclusion bodies after washing (S and IBs), lane 5: protein marker (Fermantas, Cat no. Sm0431).

and Triton X-100 were used for the solubilization of animal GH and other recombinant proteins from inclusion bodies (Mitvaki and King, 1989; Sengupta et al., 2008). We used urea as a denaturant from 2 to 8 M concentration, SDS as an anionic detergent, CTAB as a cationic detergent, and Tween-20 and Triton X-100 as a nonionic detergents to determine the best possible solubilizing agent for r-cGH. These reagents have been used at their miceller concentrations (CMC) which has been widely reported for solublizing the IB protein (Arakawa et al., 1994; Burgess, 1996; Stockel et al., 1997). The extent of solubilization was determined by running the solubilized samples on SDS-PAGE. Based on electrophoretic profile of solubilization through denaturant, that is, urea at different concentrations (2, 4, 6 and 8 M) and pH (8 and 12.5), it was observed that 2 and 4 M urea at pH 8 were not effective for solubilization, while 6 and 8 M urea showed little solubilization (Figure 2A). However, maximum solubilization was observed when Tris buffer of pH 12.5 was used with the earlier mentioned concentration of urea (Figure 2B). Our results are in accordance with the previous study in which maximum solubilization of hGH IBs were achieved in 100 mM Tris buffer at pH 12.5 containing 2 M urea (Patra et al., 2000). Among denaturants used, r-cGH protein present in inclusion bodies was found to be highly soluble in SDS, partially soluble in CTAB and Tween-20 and insoluble in Triton X-100 (Figure 3).

In general, denaturants, that is, urea and guanidine HCl are used for solubilization of IBs. But the major draw backs to the use of these denaturants include low recovery, high cost and irreversible modifications to the protein structure (Marston, 1986; Schein, 1989). Solubilization by high concentration of chaotropic reagents results in the loss of secondary structure, leading to the formation of protein aggregates due to the interaction among denatured protein molecules (Dill and Shortle, 1991). It is expected that the overall yield of bioactive protein from the inclusion body would be higher if the existing secondary structure of the protein is protected during solubilization (Khan et al., 1998). It has been shown that the proteins solubilized in detergents retain secondary structure which may yield active protein and eliminate the refolding step (Crivelli et al., 1991). It was found in our studies that solubility achieved in 6 and 8 M urea in 100 mM Tris buffer at pH 12.5 was comparable to that of 8.5 mM SDS in 100 mM Tris buffer at pH 8. Therefore, SDS anionic detergent was preferred over 6 and 8 M urea and used for further experiments.

Purification and refolding of solubilized r-cGH

Recombinant cGH was fractionated by gel filtration chromatography on Sephadex G-50 and further purified by using Sephadex G-100 column. The peak fractions were concentrated and loaded on SDS-PAGE for determining the efficiency of purification. Heterogeneous protein patterns along with a band corresponding to cGH were found after purification from Sephadex G-50 column, whereas a single band of purified r-cGH was obtained from Sephadex G-100 column (Figure 4A). In another study, purification of equine GH was done by using Sephadex G-100 gel filtration chromatography twice (Conde et al., 1973).

After purification, r-cGH protein was dialyzed in 20 mM Tris buffer pH 8 for renaturation with slight difference from the previously published method in which bGH was renatured by dialysis in 10 mM Tris HCl buffer pH 9.1 (Choi et al., 1998). In another study, renaturation was achieved by decreasing concentration gradient dialysis (Wang et al., 2005). Recovery yield of purified r-cGH is summarized in Table 1. Western blot analysis confirmed the presence of r-cGH with expected size of 21 kDa in protein sample solubilized by SDS and purified by gel filtration. The results are presented in Figure 4B.

Biological activity of r-cGH

Biological activity of cGH was then checked by its growth promoting activity on Nb2 rat lymphoma cell lines in the

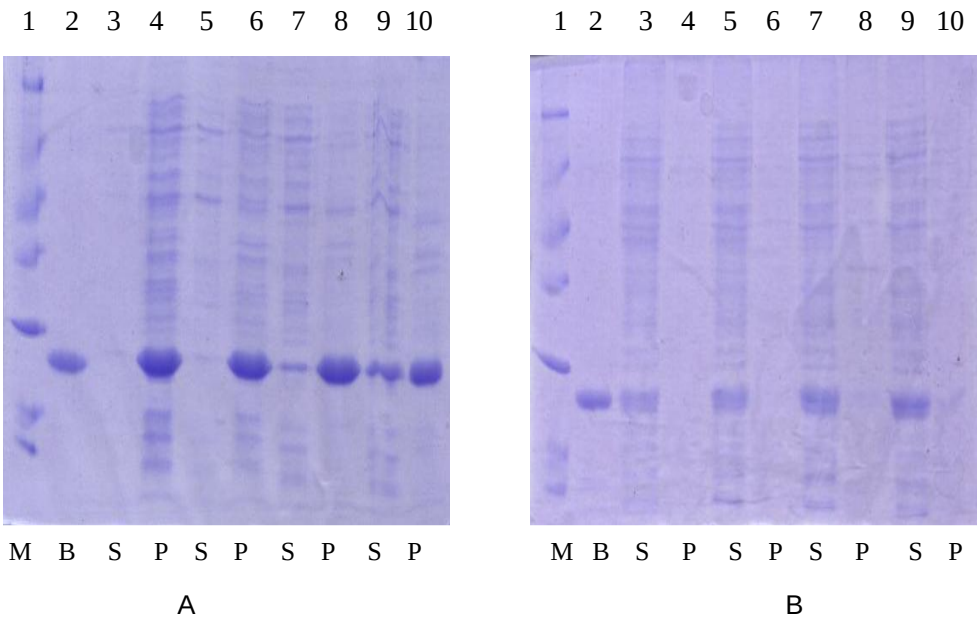

Figure 2. 12% SDS-PAGE showing the solubility of r-cGH IBs in different concentrations of urea in 0.1 M Tris buffer, pH 8 (A) and 12.5 (B). Lane 1, Protein marker (Fermantas, Cat no. Sm0431), lane 2, commercially available bST Boostin (B); Lanes 3 and 4, 2 M urea; lanes 5 and 6, 4 M urea; lane 7 and 8, 6 M urea; lanes 9 and 10; 8 M urea (M, S and P are representing marker, supernatant and pellet, respectively).

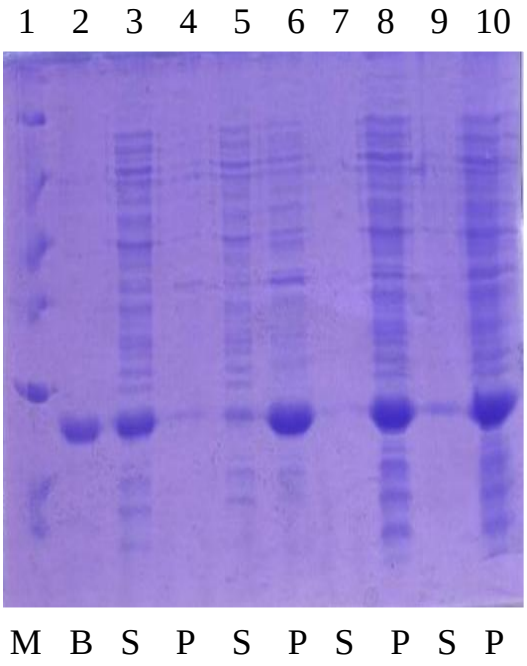

Figure 3. 12% SDS-PAGE showing the solubility of r-cGH IBs in different detergents. Lane 1, Protein marker (Fermantas, Cat no. Sm0431); Lane 2, commercially available bST (Boostin); Lanes 3 and 4: 8.5 mM SDS; lanes 5 and 6, 6 mM CTAB; lanes 7 and 8, 0.5 mM Triton X- 00; lanes 9 and 10, 0.06 mM Tween-20 (M, S and P are representing marker, supernatant an pellet, respectively).

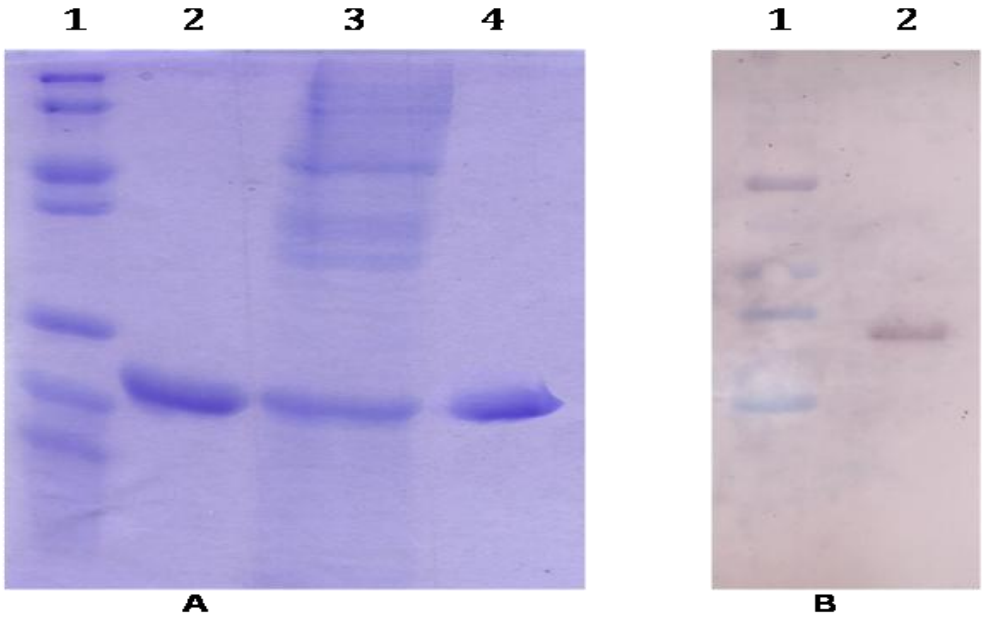

Figure 4. 12% SDS-PAGE and Western blot analysis of purified r-cGH. (A) Lane 1, Protein marker (Fermantas, Cat no. Sm0431); Lane 2, commercially available bST (Boostin); Lane 3, peak I fractions of purification using Sephadex G-50 column (1.5 x 70); Lane 4, peak II fractions of purification using Sephadex G-100 column (1.5 x 70). (B) Lane 1, Prestained protein marker; Lane 2, purified r-cGH by immunoblot analysis using rabbit anti-GH antibody.

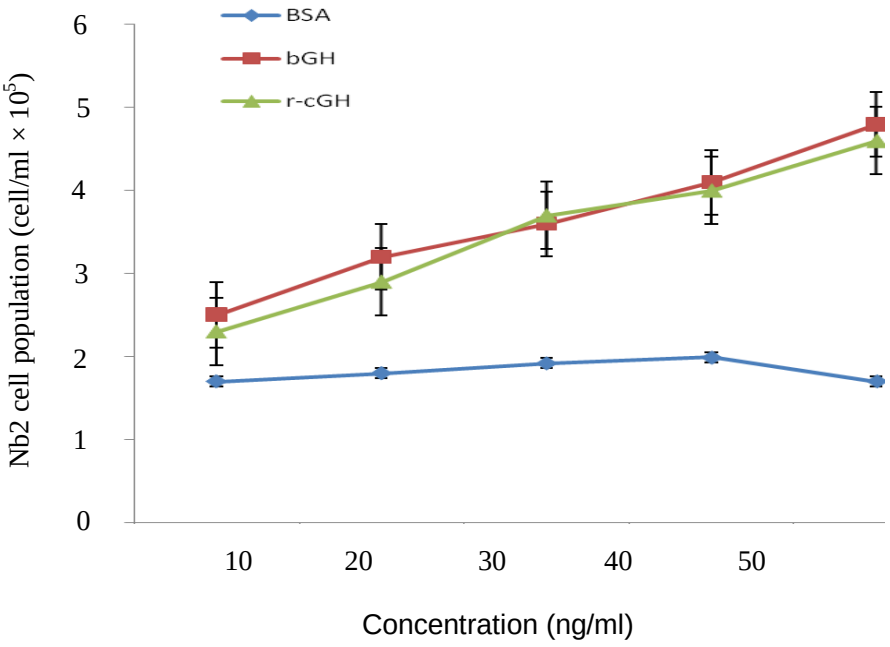

Figure 5. Three independent experiments were run and this is representative experimental results of effect of different concentrations of additives on the growth of Nb2 rat lymphoma cells.

RPMI medium. Effect of additives (BSA, bST and r-cGH) on cell proliferation was observed by counting the cells after 72 h using haemocytometer. The result given in Figure 5 shows a comparable promoting activity of r-cGH on Nb2 lymphoma cells with commercially available bGH, whereas BSA did not show any growth promoting effect.

Using one factor, ANOVA showed a significant difference ($p \leq 0.003$) in the cell number of Nb2 lymphoma cells between the control (BSA) and hormone treated cells (commercially available bGH and r-cGH) which is in line with reported data (Ouyang et al., 2003; Patra et al., 2000; Khan et al., 2009).

It is therefore concluded that r-cGH protein expressed as IBs in *E. coli* can be obtained efficiently in a biologically active conformation by using 8.5 mM SDS in 100 mM Tris-HCl buffer at pH 8.0. This study provide an efficient solubilization procedure for the recovery of bioactive r-cGH from the inclusion bodies which could be further applied for other therapeutic proteins expressed as IBs in prokaryotic system.

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REFRENCES

Agrawal RP, Swami SC, Beniwal R, Kochar DK, Sahani MS, Tuteja FC, Ghouri SK (2003). Effect of camel milk on glycemic control, lipid profile and diabetes quality of life in type-1 diabetes: A randomized prospective controlled cross over study. Ind. J. Ani. Sci., 73: 1105-1110.

Anathy V, Venugopal T, Koteeswaran R, Pandian T, Mathavan S (2001). Cloning, sequencing and expression of cDNA encoding growth hormone from Indian catfish (*Heteropneustes fossilis*). J. Biosci., 26: 315-324.

Arakawa T, Philo J, Kenney WC (1994). Structure and solubility of interleukin-2 in sodium dodecyl sulfate. Int. J. Pept. Protein Res., 43: 583-587.

Ayson FG, Jesus EGT, Amemiya Y, Moriyama S, Hirano T, Kawauchi H (2000). Isolation, cDNA cloning and growth promoting activity of rabbitfish (*Siganus guttatus*) growth hormone. Gen. Comp. Endocrinol. 117: 251-259.

Baneyx F (1999). Recombinant protein expression in *Escherichia coli*. Curr. Opin .Biotechnol. 10: 411-421.

Burgess, RR (1996). Purification of overproduced *Escherichia coli* RNA Polymerase σ factors by solubilizing inclusion bodies and refolding from Sarkosyl. Methods Enzymol. pp. 273: 145-149.

Chene N, Martal J, Llosa PDL, Charrier J (1989). Growth hormones. II. Structure-function relationships. Reprod. Nutr. Dev., 29: 1-25.

Choi JW, Kim S, Lee SY (1998). Purification and characterization of recombinant bovine growth hormone produced in Escherichia coli. Biotechnol. Lett. 20: 269-273.

Clark ED (1998). Refolding of recombinant proteins. Curr. Opin. Biotechnol. 9: 157-163.

Conde RD, Paladinjo AC, Santome JA, Dellacha JM (1973). Isolation, Purification and Characterization of equine growth hormone. Eur. J. Biochem., 32: 563-568.

Crivelli E, Cardamone M, Puri NK (1991). A single step method for the solubilization and refolding of recombinant protein from *E. coli* inclusion bodies. Aust. J. Biotechnol., 5: 78-80.

Dill KA, Shortle D (1991). Denature states of protein. Ann. Rev. Biochem., 60: p. 795.

Funkenstein B, Dyman A, Lapidot Z, Jesus-Ayson EGD, Gertler A, Ayston FG. (2005). Expression and purification of biologically active recombinant rabbitfish (*Siganus guttatus*) growth hormone. J. Aquacult. 250: 504-515.

Guakhar K, Bernard F (2004). A better knowledge of milk quality parameters: A preliminary step for improving the camel milk market opportunity in a transition economy-the case of Kazakhstan. Saving the Camel and Peoples' Livelihoods Building a Multi stockholder Platform for the Conservation of the Camel in Rajasthan, International conference, Sadri, Rajasthan, India. pp. 28-36.

Hodgson J (1993). Expression systems, a user's guide. Bio. Tech., 11: 887-893.

Hull KL, Harvey S (2001). Growth hormone: roles in female reproduction. J. Endocrinol. 168: 1-23.

Khalid S, Mehmood SF, Shahzad MI, Bashir Q, Saba SZ, Mustansar Z, Fatima A, Khanum A (2008). Cloning, overexpression and modeling studies of somatotropin cDNA of local buffalo breed-Nili ravi. J. Chem. Soc. Pak., 30: 712-725.

Khan MA, Sadaf S, Sajjad M, Akhtar MW (2009). Production enhancement and refolding of caprine growth hormone expressed in Escherichia coli. Protein Exp. Purif., 68: 85-89.

Khan RH, Rao KBCA, Eshwari ANS, Toey SM, Panda AK (1998). Solubilization of recombinant ovine growth hormone with retention of native-like secondary structure and its Refolding from the inclusion bodies of Escherichia coli. Biotechnol. Prog., 14: 722-728.

Knoess KH, Makhudum AJ, Rafiq M, Hafeez M (1986). Milk production potential of the dromedary, with special reference to the province of Punjab, Pakistan. World Anim. Rev., 57: 11-21.

Kostyo JL, Isaksson O (1977). Growth hormone and the regulation of somatic growth. Int. Rev. Physiol., 13: 255- 274.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 15: 680-685.

Mal G, Sena DS, Jain VK, Sahani MS (2001). Therapeutic utility of camel milk as nutritional supplement in chronic pulmonary tuberculosis. Livest. Int., pp. 4-8.

Maniou Z, Wallis OC, Wallis M (2004). Episodic molecular evolution of pituitary growth hormone in cetartiodactyla. J. Mol. Evol., 58: 743-753.

Maniou Z, Wallis OC, Wallis M, (2004). Episodic molecular evolution of pituitary growth hormone in cetartiodactyla. J. Mol. Evol., 58: 743-753.

Marston FO (1986). The purification of eukaryotic polypeptides synthesized in *Escherichia coli.* Biochem. J., 240: 1-12.

Martinat N, Anouassi A, Huet JC, Pernollet JC, Segard V, Combarnous Y (1990). Purification and partial characterization of growth hormone from the dromedary (*Camelus dromedarius*). Domest. Anim. Endocrinol. 7: 527-536.

Mitvaki A, King J (1989). Protein folding intermediates and inclusion body formation. Biotechnology, 7: 690-697.

Mukhopadhyay UK, Sahni G (2002). Cloning, characterization, and expression studies in *E. coli* of growth hormone cDNA from Indian Zebu cattle, Reverine buffalo, and Beetal goat. J. Anim. Biotechnol., 13: 179-193.

Nicoll CS, Mayer GL, Russell SM (1986). Structural features of prolactins and growth hormones that can be related to their biological properties. Endocr. Rev., 7: 169-203.

Ouyang J, Wang J, Deng R, Long Q, Wang X (2003). High-level expression, purification and characterization of porcine somatotropin in *Pichia pastoris*. Protein Exp. Purif., 32: 28-34.

Patra AK, Mukhopadhyay R, Mukhija R, Krishnan A, Garg LC, Pandal AK (2000). Optimization of inclusion body solubilization and renaturation of recombinant human growth Hormone from *Escherichia coli.* Prot. Exp. Purif., 18: 182-192.

Qureshi MH (1986). The camel. A paper presented at a seminar on the camel, Kuwait. FAO, Rome. pp. 1-35.

Sami AJ, Wallis OC, Wallis M (2008). Production, purification and characterization of two recombinant DNA-derived N-terminal ovine growth hormone variants: oGH3 and oGH5. Afr. J. Biotechnol., 7: 1859-1864.

Schein CH (1989). Production of soluble recombinant proteins in bacteria. Biotechnology, 7: 1141-1149.

Schwartz HJ (1992). Productive performance and productivity of dromedaries (*Camelus dromedarius*). Anim. Res. Dev., 35: 86-98.

Sengupta P, Meena K, Muukherjee R, Jain SK, Maithal K (2008). Optimized conditions for high-level expression and purification of recombinant human interleukin-2 in *E. coli*. Indian J. Biochem Biophys., 45: 91-97.

Singh SM, Sharma A Panda AK (2009). High throughput purification of recombinant human growth hormone using radial flow chromatography. Protein Exp. Purif., 68: 54-59

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem., 150: 76-85.

Stockel J, Doring K, Malotka J, Jahnig F, Dornmair K (1997). Pathway of detergent-mediated and peptide ligand-mediated refolding of heterodimeric class II major histocompatibility complex (MHC) molecules. Eur. J. Biochem., 248: 684-691.

Studier FW, Moffatt BA (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol., 189: 113-130.

Vallejo LF, Rinas U (2004). Strategy for recovery of active protein through refolding of bacterial inclusion body proteins. Microb. Cell .Fact. 3: 2-12.

Wang BL, Xu Y, Wu CQ, Xu YM, Wang HH (2005). Cloning, expression, and refolding of a secretory protein ESAT-6 of *Mycobacterium tuberculosis*. Protein Exp. Purif., 39: 184-188.

Yamano Y, Oyabayashi K, Okuno M, Yato M, Kioka N, Manabe E, Hashi H, Sakai H, Komano T, Utsumi K (1988). Cloning and sequencing of cDNA that encodes goat growth hormone. FEBS. Lett., 228: 301- 304.

Yaqoob M, Nawaz H (2007). Potential of Pakistani camel for dairy and other uses. Anim. Sci. J., 78: 467-475.