ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 2, pp. 162-169. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 2, pp. 194-202.

Molecular Cloning, Characterization, and Expression Studies of Water Buffalo (*Bubalus bubalis*) Somatotropin

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Received August 28, 2006
Revision received September 30, 2006

Abstract—Cloning, high-level expression, and characterization of the somatotropin (ST) gene of an indigenous *Nili-Ravi* breed of water buffalo *Bubalus bubalis* (BbST) are described. Coding, non-coding, and promoter regions of *BbST* were amplified and sequenced. Sequence analysis revealed several silent and two interesting point mutations on comparison with STs of other vertebrate species. One interesting variation in the BbST sequence was the replacement of a conserved glutamine residue by arginine. A plasmid was also constructed for the production of BbST in *Escherichia coli* BL21 (RIPL) CodonPlus, under the control of IPTG-inducible T7-lac promoter. High-level expression could be obtained by synthesizing a codon-optimized *ST* gene and expressing it in the form of inclusion bodies. The inclusion bodies represented over 20% of the *E. coli* cellular proteins. The biologically active conformation of purified BbST was confirmed by its efficient growth promoting activity in Nb2 cell proliferation assay. The expression system and purification strategy employed promise to be a useful approach to produce BbST for further use in structure–function studies and livestock industry.

DOI: 10.1134/S0006297907020058

Key words: somatotropin, nucleotide sequence, T7-lac promoter, overexpression, refolding, Nb2 cell proliferation

The biotechnological industry has considerable interest in products capable of enhancing the performance and efficiency of poultry, aquaculture, and livestock. In this regard, pituitary somatotropin (ST) with its wide-ranging developmental effects on animals has been a prime candidate. In livestock industry, bovine ST (bST) in its recombinant version has successfully been employed to stimulate milk production in dairy cattle [1-3]. Similar stimulation in milk yield is reported for buffaloes, but with varying success. This variability is probably because of the use of bST in buffaloes instead of their own ST. Since, STs from different distinct mammalian species have significant species-specific properties [4], it is anticipated that applications of breed-specific ST can improve the milk producing capabilities of buffalo more effectively than do commercially available bST.

Although the STs of several mammalian and piscine species including, but not limited to, bovine, ovine, caprine, porcine, and human ([5, 6] and references therein) have been cloned and expressed, to our knowledge there has so far been no report of the cloning, sequencing and/or characterization of *Nili-Ravi* water buffalo ST (BbST). Water buffalo (also called bubaline) is a geographical indication of South Asian countries and is abundantly present in Pakistan, India, and Bangladesh. In Pakistan, *Kundi* of Sindh and *Nili*, *Ravi*, and *Nili-Ravi* hybrid breeds of Punjab province are most common and amongst the best dairy breeds of water buffalo.

The present study reports isolation of ST from *Nili-Ravi*, its characterization by nucleotide sequencing, codon optimized high-level expression in *E. coli* using T7-promoter based expression system, and a simple purification scheme, with a potential for up-scaling to obtain ~99% pure recombinant BbST for further applications and analyses.

MATERIALS AND METHODS

Pituitary sample, bacterial strains, plasmid, media, chemicals, and kits. Pituitary glands from freshly slaughtered *Nili-Ravi* water buffalo were collected from a local

Abbreviations: IBs) inclusion bodies; ST) somatotropin; BbST) water buffalo (*Bubalus bubalis*) somatotropin; bST) bovine ST.

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abattoir, and blood samples were obtained from the Animal Nutrition Center, Rakh Dera Chal, Lahore. PCR amplification reactions were performed using GC-RICH PCR amplification system of Roche Applied Sciences (Germany). For RNA isolation, DNA extraction, and plasmid preparation, RNeasy Midi, QIAquick gel extraction, and QIAprep spin miniprep kits (QIAgen Inc., USA), respectively, were used. InsT/A clone PCR product cloning kit was from MBI Fermentas (USA). Restriction endonucleases were purchased from New England Biolabs (USA). T7-promoter based pET22(b) vector used for *BbST* expression was from Novagen (USA). *Escherichia coli* strain DH5α was used for vector propagation and transformation, while *E. coli* BL21 (RIPL) CodonPlus (Stratagene, USA) was used for expression studies. For immunoblot analysis, rabbit anti-bovine growth hormone was obtained from US Biological (USA). Goat anti-rabbit IgG conjugated with alkaline phosphatase was from Bio-Rad (USA). To maintain Nb2 rat lymphoma cell lines (gift from Dr. Imran H. Khan, University of California, USA), Fischer's medium and fetal bovine serum (FBS) were purchased from Quality Biologicals (USA). All other chemicals used were of the highest purity grade commercially available.

DNA extraction and PCR. Genomic DNA was extracted from the blood samples by the proteinase K/phenol extraction method [7]. Based on the published ST sequences (Accession No. J00008, M57764, M27325, D00476), five sets of primers were designed (Table 1) to amplify 459-, 422-, 462-, 639-, and 275-bp fragments of *BbST* coding, non-coding, and promoter regions by PCR. All PCR reactions were performed in a 50 μl reaction mixture using GC-RICH PCR amplification kit with the following PCR conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification (94°C for 1 min, 55-58°C for 45 sec, 72°C for 1 min), and final extension at 72°C for 20 min. PCR amplified product was run on a 0.8% agarose gel. Target DNA bands recovered by gel extraction were ligated into a pTZ57R/T vector, transformed into *E. coli* strain DH5α using InsT/A clone PCR product cloning kit, and sequenced (Bioresource Center, Cornell University, Ithaca-NY, USA).

RT-PCR amplification of BbST cDNA. Total RNA was isolated from pituitary tissue of freshly slaughtered bubaline using QIAgen RNeasy Midi Kit, in accordance with the recommended procedure, and its concentration was determined. Using ST-1/ST-2 primer pair (Table 1), full-length BbST cDNA was reverse transcribed and amplified. The amplicon was ligated into pTZ57R/T vector to generate pWSBS-01 construct, transformed into *E. coli* strain DH5α, and sequenced to ensure correctness of the insert.

Sequence analysis. Sequence alignment, ORF translation, and molecular mass calculation of the predicted protein were carried out using the DNA Star software package, while database searches were performed on the SIB (http://www.expasy.org) server using NCBI BLAST2 software.

Table 1. Oligonucleotides used for PCR amplification and sequencing of the *BbST* gene

Primer	Sequence (5′-3′)
BF1	CCAGTTCACCAGACGACTCAG
BR1	TTGAAGGTGTCAGCAGCCAG
BF2	CCCTGGACTCAGGTGGTG
BR2	GATGGTTTCGGAGAAGCAGA
BF3	GGGACAGAGATACTCCATCCAG
BR3	CCTTCAGCTTCTCATAGACACG
BF4	TCGCATCTCACTGCTCCTTATC
BR4	CAGATGGCTGGCAACTAGAAC
BPF	GCTGGTGGCAGTGGAGA
BPR	AGCCATCATAGCTGGTGAGC
ST-1	ATCCATGGCCTTCCCAGCCATG
ST-2	TAGGATCCGCAACTAGAAGGCA
SS-F1	GCAG*CATATG*GCATTTCCAGCAATGTCCTTG
SS-R1	GAT*AAGCTT*GCAACTAGAAGGCACAGCTGGC
T7-F	TAATACGACTCACTATAGGG
T7-R	GCTAGTTATTGCTCAGCGG

Note: *CATATG* and *AAGCTT* (shown in italics) are restriction sites for *Nde*I and *Hind*III, respectively.

Construction of pET-BbST expression vector. A codon optimized *BbST* was generated by PCR mutagenesis using SS-F1/SS-R1 (Table 1) primers and pWSBS-01 construct as template. Amplified *BbST* was cloned in pET22(b) vector at *Nde*I/*Hind*III sites to create pET-BbST expression plasmid, which was maintained in *E. coli* DH5α with ampicillin as selection pressure. The junction sequences of the recombinant plasmid were verified by nucleotide sequencing on Beckman Coulter CEQ™ 8000 Genetic Analysis System using the T7-F and T7-R primers (Table 1).

Expression and purification of BbST. BbST was expressed by transformation of *E. coli* BL21 (RIPL) CodonPlus with pET-BbST vector. Temperature, induction time, inducer concentration, and other parameters affecting the levels of recombinant protein expression were optimized in test tube cultures. For preparative-scale experiments, 1 liter of bacterial culture was induced with 0.5 mM IPTG at OD_{600} of 0.6. After an incubation time of 4-6 h, cells were harvested by centrifugation at 6000*g* at 4°C for 20 min in a Beckman J25-I centrifuge (JS-10 rotor).

The harvested cells (~4.5 g) were lysed ultrasonically (10 × 30 sec bursts with 1-min interval between pulses) and centrifuged as described above to collect inclusion

bodies (IBs). The IBs were sequentially washed with 0.5% (v/v) Triton X-100, solubilized in 2 M urea solution (pH 12.5), and then refolded as detailed in Patra et al. [8]. Refolded protein was saturated with 0.8 M $(NH_4)_2SO_4$ and purified on an AKTA FPLC system (Amersham Biosciences, Germany) using hydrophobic interaction chromatography (HIC). The FPLC was programmed to equilibrate the RESOURCE-PHE column with equilibration buffer containing 0.8 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl, pH 8.8, followed by sample application. Bound protein was eluted using a decreasing linear gradient of 0.8-0 M $(NH_4)_2SO_4$. During elution, flow rate was maintained at the rate of 2 ml/min and absorption was monitored by an online UV monitor. The eluate was passed through a PD-10 gel filtration column (Amersham Biosciences) and stored at 4°C until biological activity assay.

Biological activity assay. Nb2 rat lymphoma cell lines were maintained in Fischer's medium supplemented with 10% FBS, 10% horse serum (HS), and $5 \cdot 10^{-5}$ M β-mercaptoethanol. The assay was set in 96-well flat bottom culture plates using Fischer's medium with BSA as a negative control. Before the assay, cells were arrested at G_0/G_1 phase (quiescent state) by incubating them in Fischer's medium containing 1% FBS and 10% HS for 24 h. Cellular proliferation was initiated by adding different concentrations (10-50 ng/ml) of recombinant ST and carried out in triplicate runs for 96 h at 37°C under atmospheric pressure with 5% CO_2 [9]. The results were compared with those obtained using a commercial ST preparation.

Protein estimation and immunoblot analysis. Total protein contents of the extracts and purified fractions were determined either by UV absorption or Bradford assay [10] using BSA as standard. Expression levels and purity of BbST were determined by 13% SDS-PAGE [11] and immunoblot analysis. Briefly, the protein fractions resolved by SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane at 50 V for 1 h. The membrane was processed for the detection of ST using rabbit anti-bovine growth hormone and goat anti-rabbit IgG conjugated with alkaline phosphates. The nonspecific sites of the membrane were blocked with 2% gelatin before incubating with anti-bovine growth hormone antisera. The antigen–antibody complexes were detected by developing with 5-bromo-4-choloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) as substrate.

RESULTS AND DISCUSSION

PCR amplification and sequence analysis of *BbST*. PCR amplification of genomic DNA using gene specific primer pairs yielded a single band of expected length (Fig. 1). Since PCR amplification often generates point mutations in the amplicons [12], we performed all reactions at least in triplicate. The amplified products obtained in each case were subjected to sequence analysis both in the forward and reverse directions to resolve discrepancies, if any. The complete nucleotide and deduced amino acid sequences of ST cDNA were deposited to GenBank under the Accession numbers AY940159 and AAX31661, respectively. Like other mammals, the gene coding for *Nili-Ravi* ST was comprised of four introns and five exons and extended over 1.5 kb (Fig. 2). Homology comparison of the sequence with other vertebrate STs showed a very high degree of evolutionary conservation. *Nili-Ravi* ST shared high homologies with *Bos indicus* (99%), *Capra hircus* (99.5%), *Ovis aries* (98.5%), *Giraffa camelopardalis* (98.5%) and relatively lower levels of homologies with dog *Canis familiaris* (91.1%), cat *Felissilvestris catus* (90.5%), rabbit *Oryctolagus cuniculus* (88.5%), African elephant *Loxodonta africana* (90.5%), horse *Equus caballus* (90.1%), camel *Camelus dromedaries* (91.6%), pig *Sus scrofa* (91.1%), and human *Homo sapiens* (67%). This adds support to the idea of an underlying slow rate of molecular evolution in mammalian STs.

While analyzing the sequences, we noticed that the mature ST sequence of *Nili-Ravi* (Accession No. AY940159) differs from bovine STs by two amino acids, i.e. serine (S) in lieu of glycine (G) at position 9, and arginine (R) at position 140 in lieu of glutamine (Q). Variation of G_9 to S_9 has been reported in caprine [13], canine [14], equine [15], cat [16], rabbit [17], elephant

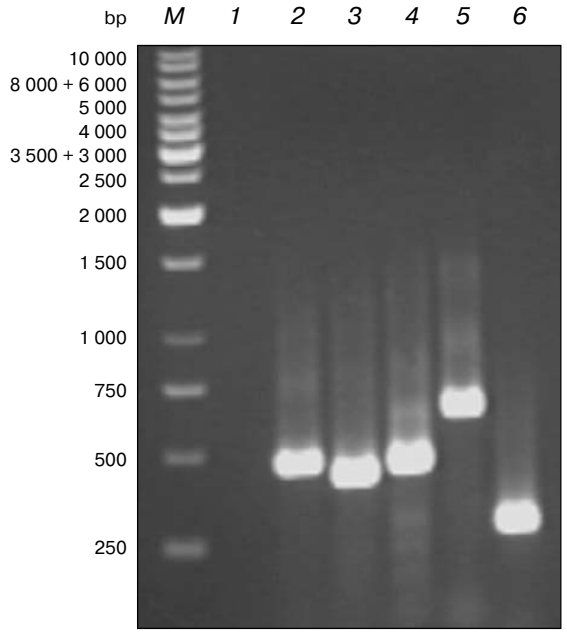

Fig. 1. Analysis of the PCR amplified product resolved on 0.8% agarose gel. Lanes: *M*) 1 kb DNA ladder; *1*) PCR negative control (without template); *2-6*) 459-, 422-, 462-, 639-, and 275-bp PCR amplified products.

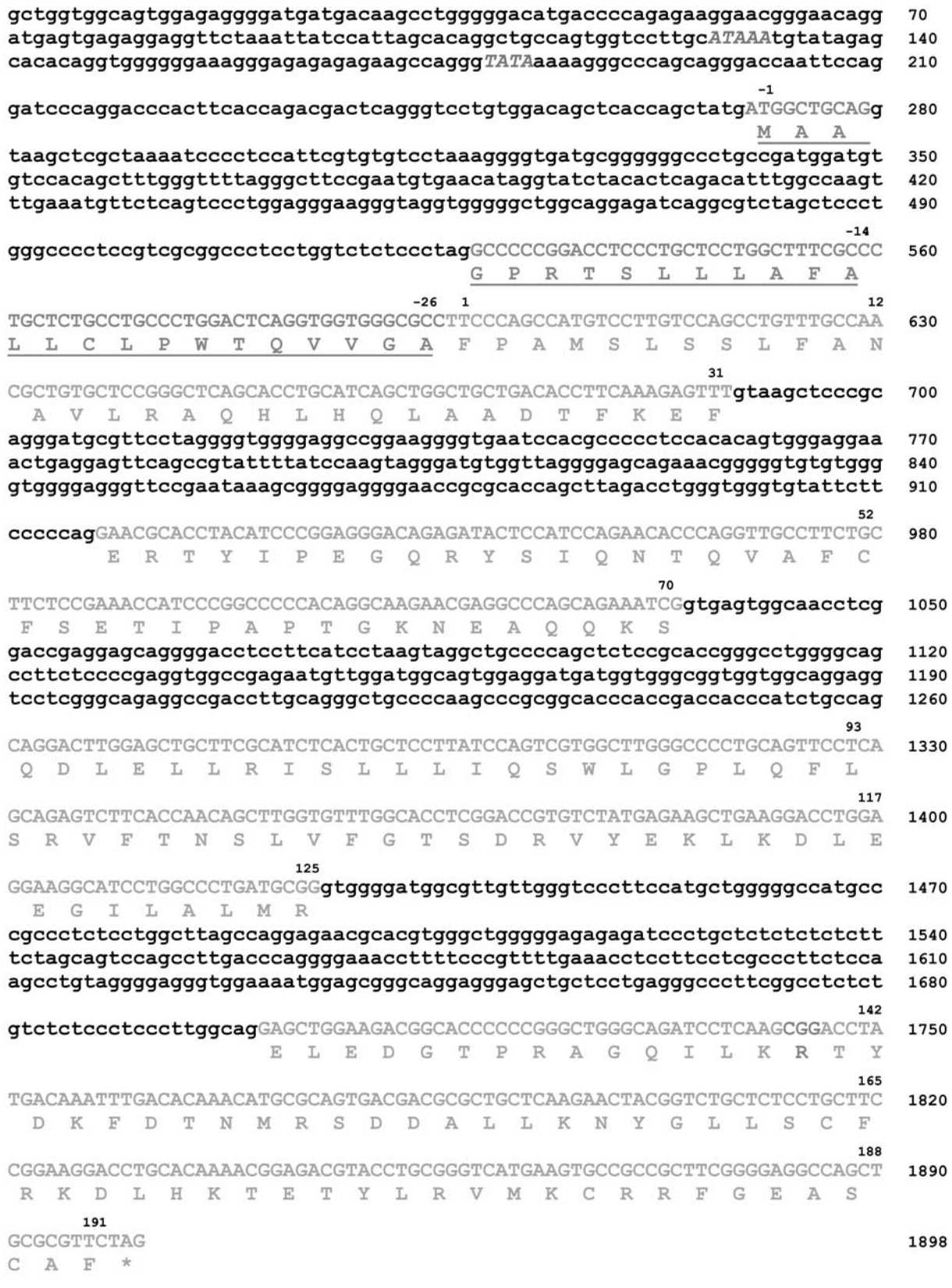

Fig. 2. Nucleotide and deduced amino acid sequence of BbST. Coding and non-coding regions are shown in upper and lower case, respectively. Regulatory elements TATAA and ATAAA are shown in italics. A 26 amino acid long signal peptide is shown in gray and underlined, while the stop codon (TAG) is marked by an asterisk (*). Both DNA strands were sequenced completely, and all discrepancies were resolved. The cDNA sequence has been deposited to GenBank under the Accession No. AY940159.

Fig. 3. Alignment of amino acid sequences of STs from different vertebrate species starting from P*Bb*ST for Pakistani *Bubalus bubalis* (Accession No. AY940159), *Bi*ST for *Bos indicus* (Accession No. Q9TQW9), *Oa*ST for *Ovis aries* (Accession No. P67930), *Ch*ST for *Capra hircus* (Accession No. Q9TU21), *Cf*ST for *Canis familiaris* (Accession No. P33711), *Fc*ST for *Felis silvestris catus* (Accession No. P46404), *Oc*ST for *Oryctolagus cuniculus* (Accession No. P46407), *La*ST for *Loxodonta africana* (Accession No. P20392), *Ec*ST for *Equus caballus* (Accession No. P01245), *Cd*ST for *Camelus dromedaries* (Accession No. Q7YRR6), *Ss*ST for *Sus scrofa* (Accession No. P01248), *Gc*ST for *Giraffa camelopardalis* (Accession No. Q7YQD2), and *Hs*ST for *Homo sapiens* (Accession No. Q61YFO).

[18], and camel [19] STs. The second variation however, is more interesting as the Q_{140} residue is generally conserved in STs of different vertebrate species (Fig. 3). It is pertinent to note that Q_{140} lies between residues 130 to 154, which seems to be the most immunogenic region of STs [4]. In the case of bovine ST, glutamyl (Q_{140}) and asparaginyl (N_{148}) residues have been reported to be susceptible to deamidation (a hydrolytic reaction that is dependent on protein sequence and conformation) and are amongst the post-translational modifications that might play a physiological role [20]. Since both of these residues (Q_{140} and N_{148}) occur most frequently on the surface, deamidation therefore makes the protein entourage more hydrophilic and flexible and may enhance surface accessibility and immunogenicity of this portion of ST [21]. In the present study, variation in BbST sequence (i.e. replacement of Q_{140} by strongly basic, positively charged R_{140}) is likely to affect the post-translational modifications and/or immunogenicity of the ST molecule. It may also be noted that despite the overlapping evolutionary, structural, immunological, and biological properties, STs from different distinct mammalian species have significant species-specific properties [4]. Amino acid variation observed in indigenous BbST sequence, therefore, seems to be breed-specific and may also be associated with enhanced milk production, which bubaline is famous for. A definitive conclusion regarding role in milk production, however, requires further studies with a larger number of animals.

Construction of expression vector. The construction of pET-BbST is shown in Fig. 4. Presence of high GC contents in the 5′-coding region of the gene of interest had been associated with the formation of secondary structure in mRNA. The hairpin loop formed usually makes the ribosome-binding site (RBS) inaccessible to the ribosome, resulting in both decreased or interrupted translation and lower levels of expression [22].

Because of the GC rich (~60%) coding sequence of ST, we modified the sequences in the initial eight codons of BbST mRNA, without changing the native amino acid

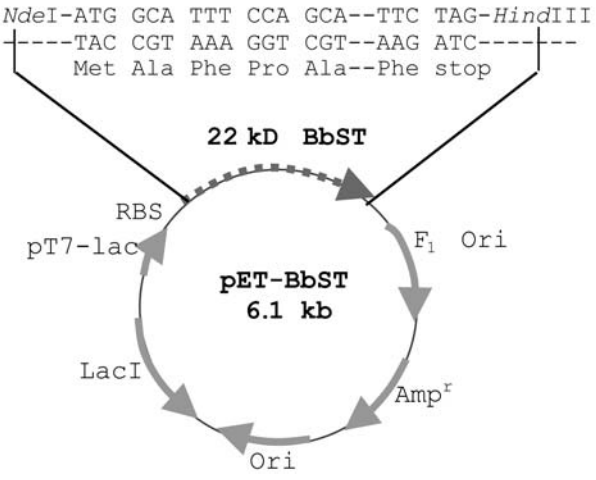

Fig. 4. Construction of recombinant plasmid pET-BbST. A 0.6 kb codon optimized *BbST* was cloned in pET22(b) expression vector downstream of the T7-lac promoter (pT7-lac) to generate pET-BbST construct. RBS, ribosome-binding site; Ori, origin of replication; Amp^R, antibiotic resistance gene.

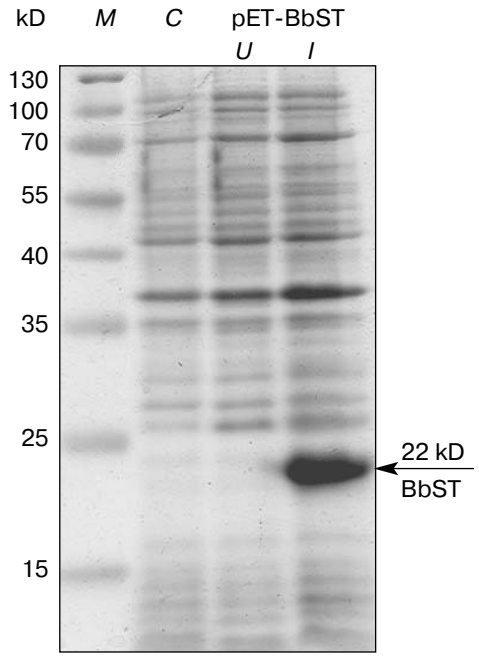

Fig. 5. Expression analysis of BbST in *E. coli*. Equal amounts of total cell proteins were mixed with 2× reduced sample buffer and loaded onto 13% SDS-polyacrylamide mini gel (8.6 × 6.8 cm; thickness 1 mm). *M*, molecular weight markers; *C*, control (*E. coli* transformed with pET22(b) vector); lanes *U* and *I* represent uninduced and induced pET-BbST, respectively.

sequence. The modification ensured higher expression levels of ST by discouraging the formation of possible mRNA secondary structure as reported previously [23]. The resulting pET-BbST was first transformed into *E. coli* DH5α (cloning host) and then BL21 (RIPL) CodonPlus for BbST expression.

BbST expression. Variables like temperature, expression time, inducer concentration, and induction stage (known to influence the yield and reproducibility of expressed protein) were tested *prior to* preparative-scale experiments. Maximal levels of BbST production were observed by inducing *E. coli* harboring pET-BbST with 0.5 mM IPTG for 4-6 h in LB-ampicillin medium at 37°C. A typical induction experiment comparing the polypeptide profiles of uninduced and IPTG-induced *E. coli* cultures is shown in Fig. 5. It is evident that IPTG induction results in the expression of a ~22 kD recombinant protein which was not observed in uninduced or control cells (Fig. 5).

A study of the effect of IPTG concentration (0.25-2.5 mM) showed that beyond 0.5 mM there was no increase in the expression levels. After induction, BbST accumulation increased up to 4 h and stayed constant thereafter. To increase the biomass, the post-induction period could be increased up to 8 h. However, at this stage, the culture reached a saturation point and slightly decreased levels of expressed protein were observed (data not shown). This observation may be a combined effect of increased degradation of expressed protein due to starvation for a required nutrient and entry of *E. coli* into stationary phase. In all subsequent experiments, induction therefore was carried out with 0.5 mM IPTG for 6 h. BbST was expressed in *E. coli* at high levels, but in the form of insoluble IBs. ST was hardly detectable in the soluble fraction when the culture was cultivated at 37°C during IPTG induction. Lowering the culture temperature to 25 or 30°C reduced the growth rate and helped in minimizing the formation of IBs. However, the proportion of soluble protein to total BbST produced was only 5-10%. We therefore opted to grow cultures at 37°C, which yielded BbST in the form of IBs accounting for >20% of the total *E. coli* cellular proteins.

BbST purification and refolding. The IBs were purified from induced cell cultures by low speed centrifugation and solubilized using 2 M urea and alkaline pH. When used in higher concentrations (such as 8 M), urea denatures the proteins completely by disrupting its secondary and tertiary structure. However, mild concentration of urea and alkaline pH, used in present study, helped in efficient solubilization of BbST from IBs without disturbing its native-like secondary structure. The observation is in agreement with previous reports [8, 24] where human and ovine STs were solubilized by employing a similar strategy.

Solubilized protein was refolded by decreasing the pH to 8.8. During refolding, no protein aggregation could be seen when the pH was 8.8 or above; however, lowering the pH below 8.8 resulted in extensive protein aggregation. Using buffer of similar ionic strength and pH, recombinant BbST was further purified on a phenyl-Sepharose column. SDS-PAGE analysis of expressed recombinant protein at different stages of the purification is shown in Fig. 6. It is apparent that the IBs prepared

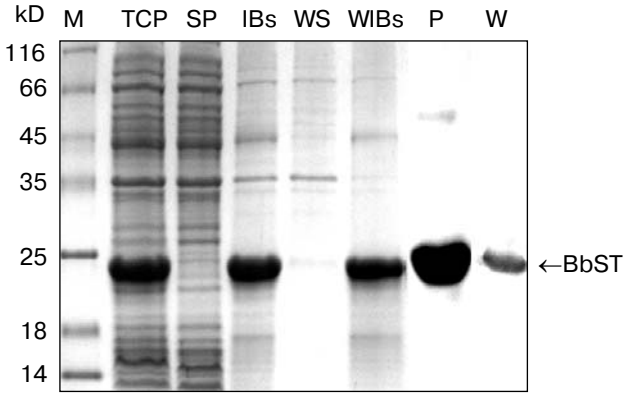

Fig. 6. Analysis of recombinant BbST expression and purification by 13% SDS-PAGE. M) molecular weight markers; TCP) total cell protein; SP) soluble protein; IBs) inclusion bodies; WS) supernatant during washing of IBs; WIBs) Triton X-100 washed inclusion bodies; P) column purified and refolded protein; W) the confirmation of refolded and purified BbST by immunoblot analysis using rabbit anti-bovine growth hormone antibody.

Table 2. Summary of recombinant BbST purification (BbST was isolated from 1 liter culture containing approximately 4.5 g wet weight *E. coli* cells)

Step	Total protein, mg	BbST, mg	Protein recovery, %	Purification, %
Cell lysate	417	84	100	20
Inclusion bodies (IBs)	105	77	91	74
Washed IBs	94	75	89	80
Solubilization	73	62	74	85
HIC purification	52	49	58	95
Gel filtration	47	46	55	99

were considerably pure in comparison to the total cell lysate (Fig. 6). This leads to the conclusion that major purification (~85%) had been achieved at the IB stage itself. Using HIC and gel-filtration chromatography, BbST was purified to 99% homogeneity.

Recovery yield of purified BbST is summarized in Table 2. Final concentration of BbST from 1 liter shake-flask culture was >46 mg and was higher than reported previously for Indian riverine buffalo [25] on the same culture volume scale, using *E. coli* as expression host. The protein also showed efficient growth-promoting activity in Nb2 cell proliferation assays in a concentration-dependent manner indicating that the recombinant BbST produced by *E. coli* has a biologically active conformation (Fig. 7).

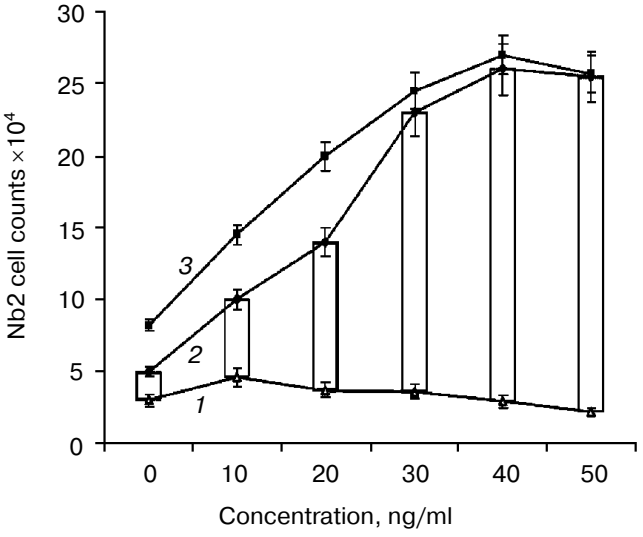

Fig. 7. Assessing biological activity of BbST. Growth promoting effect of different concentrations (10-50 ng/ml) of purified recombinant BbST and a commercial ST preparation on the proliferation of Nb2 rat lymphoma cells was determined by counting cell numbers after 96 h of incubation. Fischer's medium containing BSA was used as negative control. Curves: *1*) BSA (negative control); *2*) purified recombinant BbST; *3*) standard ST.

We conclude that the study contributes new sequencing data for structural–functional and evolutionary studies of vertebrate STs. The T7-promoter based expression system and purification strategy employed are notably simpler and can be used to further enhance the yield of BbST many-fold in a fermenter. This would result in the production of bioactive breed-specific BbST for applications in local livestock industry. The possible immune rejections sometimes seen after repeated usage of heterologous proteins are likely to disappear by using the new ST identical to the endogenous one.

This study is a part of a Higher Education Commission funded project, Government of Pakistan. A major part of this work was carried out in the Department of Molecular Biology and Genetics, Cornell University, Ithaca-NY, USA. We acknowledge the research facilities provided by Cornell University for the conduct of this research project.

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