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Molecular cloning, sequence characterization and expression analysis of somatotropin gene of an indigenous chicken breed (*Aseel*)

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Abstract: Somatotropin (ST), an essential polypeptide required for normal growth and development of vertebrates, is commercially important for a variety of applications. Despite the high degree of evolutionary conservation amongst the avian STs, significant variations in sequence at nucleotide and/or amino acid levels have been reported. The present study describes sequence and expression of ST gene of an indigenous chicken (*Gallus gallus*) breed 'Aseel' (GgST-A), one of the oldest known cock-fighting bird, in *Escherichia coli*. Total RNA extracted from the pituitary glands of Aseel was used as template for RT-PCR based amplification of ~600 bp ST cDNA. The amplicon was cloned in pTZ57R/T vector and subjected to sequence analysis. Four sequence variations at the nucleotide level were observed, when the GgST-A sequence was compared with the ST sequence of *G. gallus domesticus* (Desi breed). Amongst these, one point mutation led to F→Y amino acid change at position 184. The GgST-A cDNA was also subcloned in pET-22b (+) vector and its expression was analyzed in *E. coli* strain BL21 (DE3) CodonPlus. High-level expression of GgST-A corresponding to >30 % of the total *E. coli* cell proteins was achieved. Recombinant protein expression was, however, in the form of inclusion bodies (IBs), which were solubilized using mild concentration of urea (2M) under alkaline conditions (pH 12.5) and refolded by dilution and step-dialysis method. In conclusion, the study contributes new sequencing data and highlights the need for protein structural analysis for better insight of the functional and application aspects of GgST-A.

Keywords: Aseel chicken, point mutation, sequence homology, somatotropin, T7*lac* promoter
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INTRODUCTION

Chicken (*Gallus gallus*), a very common domesticated fowl with an average stock of around 20 billion, has been described to be the most populous bird of the world. Based on the physical traits like size, plumage color, comb type, number of toes, amount of feathering, place of origin, etc., a great number of chicken breeds have been reported previously. In Pakistan, "Aseel" is amongst the most common and famous chicken breeds after Desi chicken (i.e., *G. gallus domesticus*, found in almost all villages). This heavily muscled, compact breed with an upright stature is predominantly present in Sindh and Southern part of Punjab and is known for its cock-fighting characters. Sindhi, Mianwali, Jawa and Lasani are some of the world's famous and rare breeds of Aseel in Pakistan.

Somatotropin (ST), MW 22 kDa, is a naturally occurring, non-glycosylated protein, synthesized and secreted from the pituitary glands of vertebrate species. It promotes general protein synthesis and plays important role in number of physiological events including growth, development, metabolism and regulation of hormones. Several research studies have shown that ST is involved in linear growth and milk production in vertebrate system and is commercially important in the areas of medicine, animal husbandry, fish farming and poultry[1,2]. Although the role of ST in regulating the growth of chicken is bit complex and not completely understood but the fact that it exerts positive impact on chicken growth is established by several research groups and so is its importance in poultry industry[3-6]. Several different variant forms of chicken ST ranging from 3-110 kDa have been described, however, only the 22 kDa form is known to exhibit radioreceptor activity[6]. Since STs from different distinct vertebrate species exhibit species-specific properties, sequence characterization seems necessary both from the application view point and for the elucidation of structural-functional relationship.

We, in our lab, have previously sequenced the STs of indigenous bubaline[7], ovine and caprine[8] species and have observed some sequence variations specific to the local/indigenous breeds. The present study aims at cloning, sequence characterization and expression analysis of Aseel ST (GgST-A) in an attempt to contribute new sequence data and expression information of a commercially important protein from indigenous *G. gallus* species. This, to our knowledge, is the first report that describes ST sequence of Aseel, one of oldest documented chicken breeds in the world and parent stock of modern/broiler chicken industry breed, Cornish.

MATERIALS AND METHODS

Chemicals, media and bacterial strains

The chemicals used in this study were of the highest purity grade, commercially available. Trizol reagent used for RNA extraction was acquired from Invitrogen (USA) whereas Gene JET™ Gel

extraction, plasmid minipreparation and InsT/Aclone™ PCR product cloning kits were from MBI Fermentas. *E. coli* strains DH5α and BL21 (DE3) CodonPlus (Novagen, Inc. USA) were used in this study as cloning and expression hosts, respectively. The cells were routinely grown in Lauria Bertani (LB) or M9NG medium[9] supplemented with 100 µg/ml ampicillin. Media were sterilized by either autoclaving (120°C, 15 psi for 20-25 minutes) or by filtration through 0.45 µm filters.

RT-PCR amplification and sequence analysis

Total RNA was isolated from the pituitary glands of two freshly slaughtered Aseel chickens using Trizol reagent (Invitrogen, USA) in accordance with the recommended procedure. Based on the published GgST sequence (GenBank Accession No. NM_204359.2), a set of forward and reverse primers was designed (Table 1), which was subsequently used for RT-PCR based amplification of full-length GgST-A cDNA.

Table 1: Oligonucleotides used for PCR amplification and sequencing of GgST-A.

Oligo name	***Sequence (5′→ 3′)***
GgST-F	GCA**CATATG**ACCTTCCCTGCCATG
GgST-R	GTC**AAGCT**TTCAGATGGTC
T7-F	TAATACGACTCACTATAGGG
T7-R	GCTAGTTATTGCTCAGCGG

The sequences CATATG and AAGCTT (shown in bold) are the sites for *Nde*I and *Hind*III restriction enzymes, respectively.

Using extracted total RNA as template, reverse transcription reaction was performed at 42°C for 60 minutes in the presence of GgST-R primer and MMuLV reverse transcriptase. For amplification, a 50 µl reaction containing cDNA template, forward and reverse primers pair, dNTPs mix and *Taq* DNA polymerase, was set up with following PCR conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification (94°C for 45 sec, 65°C for 45 sec, 72°C for 1 min), and final extension at 72°C for 20 min. The amplified product was analyzed on 1 % agarose gel and the target DNA band recovered by gel extraction, ligated into a pTZ57R/T vector to generate pTZ-GgST-A plasmid, transformed into *E. coli* strain DH5α and sequence analyzed using Beckman Coulter CEQ™ 8000 Genetic Analysis System (SBS, PU). BLAST search, ORF translation, sequence analysis and multiple alignments were all performed on the SIB server (http://www.expasy.org) using ExPASy tools.

Construction of pET-GgST-A expression plasmid

The gene encoding mature GgST-A, obtained from pTZ-GgST-A construct following digestion with *Nde*I and *Hind*III restriction enzymes, was subcloned in similarly digested pET-22b(+) plasmid to generate pET-GgST-A expression plasmid. Restriction enzyme digestion and ligation steps with T4 DNA ligase were essentially performed according to the recommendations of enzyme suppliers and standard protocols[10]. The recombinant plasmid was maintained in *E. coli* DH5α with ampicillin as selection pressure. The transformants were screened for the presence of insert by colony PCR and its sequence/in-frame integration into the plasmid was confirmed by sequence analysis (Beckman Coulter CEQ™ 8000 Genetic Analyzer) using T7 promoter and terminator primers (Table 1).

Expression of GgST-A in E. coli

The expression of *GgST-A* in *E. coli* was approached by transformation of *E. coli* BL21 (DE3) CodonPlus with pET-GgST-A expression plasmid followed by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose. For small-scale expression studies, single colony of positive transformant was inoculated in 10 ml LB-ampicillin (100 µg/ml) medium and allowed to grow in an orbital incubator shaker (37°C, 150 rpm) for overnight. Next day, 3.3 ml of overnight culture was added to 100 ml LB ampicillin medium and the cells were grown at 37°C with shaking until the OD_{600} reached 0.5-0.6, the stage at which the cells were induced with 0.5 mM. Cells were allowed to grow for 10-12 hours post-induction and 1 ml fractions were collected at every 2 hour intervals for the analysis of GgST-A expression by 13 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

For preparative scale experiments, *E. coli* transformants were grown in 1 liter M9NG auto-inducing medium supplemented with 100 µg/ml ampicillin and 10 mM lactose. After an incubation time of 12 hours, the cells were harvested by centrifugation (6000 x *g*, 4°C, 20 min. in a Beckman J25-I centrifuge), resuspended in lysis buffer [50mM Tris-Cl (pH 9.5), 100 mM NaCl, 5 mM EDTA, 1 mM freshly prepared PMSF], lysed ultrasonically (10 × 30 sec bursts with 1 min. interval between two pulses) and centrifuged, as described above, to collect the inclusion bodies (IBs).

Solubilization and refolding of GgST-A

Prior to solubilization, the IBs containing the GgST-A, were sequentially washed with washing buffer I [50 mM Tris-Cl (pH 9.5), 5 mM EDTA (pH 8.5), 0.5% (v/v) Triton X-100], washing buffer II [50 mM Tris-Cl (pH 9.5), 5 mM EDTA (pH 8.5)] and washing buffer III [50 mM Tris-Cl (pH 9.5)]. In all

washing steps, the supernatant was analyzed for the loss of IBs either spectrophotometrically or by 13 % SDS-PAGE. Solubility of IBs was then tested in buffers of different pH and ionic strength both in the presence and absence of denaturing urea (Table 2). Briefly, 1 mg IBs were resuspended in 1 ml of each of the solution, kept at room temperature (25° C) for 2 hours with gentle agitation and then centrifuged (14,000 rpm, 25°C, 10 minutes). Protein concentration in the clarified supernatant was thereafter determined by absorbance measurements at A_{280}.

For GgST-A refolding, 100 mg IBs solubilized in 50 ml of Sol. J, were dialyzed against the 20 fold volume of refolding solution I [10 mM Tris-Cl (pH 9.5), 10 % glycerol, 0.5 mM EDTA, 1 mM PMSF, 5 mM cysteine and 1 mM cysteine] at 4°C. Step-dialysis was then performed to sequentially remove the cysteine/cystine, EDTA/PMSF and glycerol. Dialyzed sample was concentrated through Amicon filters (MWCO: 10kDa; Millipore, USA), lyophilized and stored at 4ºC, until use for further analysis.

RESULTS AND DISCUSSION

Amplification and sequence analysis of GgST-A

The gene encoding GgST-A cDNA was amplified by RT-PCR methodology using the total RNA, extracted from the pituitary gland of Aseel chicken, as template. The desired band of ~0.6 kb could be amplified best at an annealing temperature of 65ºC (data not shown). The amplicon, when cloned in pTZ57R/T vector was referred as pTZ-GgST-A and was subjected to sequence analysis using M13 universal primers sets. To ensure correctness of sequence, the sequencing reactions were performed both in the forward and reverse direction. Furthermore, amplification reactions of GgST-A cDNA were performed in triplicates and the products of each amplification reaction was sequence analyzed, with- and without cloning, in pTZ-57R/T vector, to resolve sequence discrepancies, if any. The final nucleotide and deduced amino acid sequence of GgST-A was deposited to GenBank under the Accession Nos. HQ586894 and ADR74113, respectively. The theoretical mean molecular weight and pI of GgST-A, as calculated using ExPASY server, was 22191.4 D and 7.61, respectively.

We have previously worked out the ST sequence of Desi chicken i.e., GgST-D (unpublished data). It shares the same amino acid sequence as reported for *G. gallus domesticus* (GenBank Accession No. NM_204359.2); at the nucleotide level, five variations were observed in the two sequences but they were all silent. However, in the present study, a comparison of GgST-A nucleotide sequence with GgST-D and the GgST-*domesticus* revealed altogether nine mutations (Fig. 1). Amongst these, one was point mutation, which led to an F→Y amino acid change at position 184 (Fig. 2a). Interestingly, this variation exists in a region which seems to be highly conserved amongst the avian STs. Since the physicochemical properties of the individual amino acids are important determinants of protein structure as well as its interactions with other protein counterparts, a change of F_{184} to Y_{184} in GgST-A is likely to influence the structural and functional/binding properties of the protein in comparison with GgST-D. It is pertinent to note that both phenylalanine (F) and tyrosine (Y) have aromatic side chains but the former is neutral while the later is polar with their respective hydropathy indices of 2.8 and -1.3. Substitution of a uncharged/neutral amino acid with polar residue therefore may conform the ST structure exposing the buried region to the exterior aqueous environment.

Using the X-ray crystallographyically determined structure of human ST as template (pdb: 1hwg.1; shared ~58 % sequence identity with the query sequence), we tried to build the three-dimensional models of GgST-A. SWISS-MODEL (swissmodel.expasy.org/) and I-TASSER (zhanglab.ccmb.med.umich.edu/I-tasser) modelling programs were used for this purpose. GgST-A exhibited the 3-dimensional structure which is typical of the members of cytokines superfamily i.e., dominated by four alpha-helices arranged in up-up and down-down topology (Fig. 3). However, from the predicted model we could not delineate the impact/influence of F_{184}→Y_{184} amino acid substitution on structural and functional aspects of GgST-A. Subtle variations in the structure due to

Figure 1: The nucleotide sequence alignment of *GgST-A* (Aseel; GenBank Accession No. HQ586894) with reported sequence of *Gallus gallus domesticus* (Accession No. NM_204359.2) and GgST-D (Desi; unreported sequence) using Clustal omega alignment program. The conserved sequences are indicated with an asterisk (*) and highlighted area shows the differences in the alignment.

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CLUSTAL O(1.2.1) multiple sequence alignment                    (2A)

GgST-A      -TFPAMPLSNLFANAVLRAQHLHLLAAETYKEFERTYIPEDQRYTNKNSQAAFCYSETIP 59
GgST-D      -TFPAMPLSNLFANAVLRAQHLHLLAAETYKEFERTYIPEDQRYTNKNSQAAFCYSETIP 59
ScST        -TFPAMPLSNLFANAVLRAQHLHLLAAETYKEFERTYIPEEQRHANKNSQSAFCYSETIP 59
ApST        --FPAMPLSNLFANAVLRAQHLHLLAAETYKEFERSYIPEDQRHTNKNSQ-AFCYSETIP 57
AaST        ATFPAMPLSNLFANAVLRAQHLHLLAAETYKEFERSYIPEDQRHTNKNSQ-AFCYSETIP 59
MgST        -TFPTMPLSNLFTNAVLRAQHLHLLAAETYKEFERTYIPEDQRYTNKNSQAAFCYSETIP 59
CcST        -TFPAMPLSNLFANAVLRAQHLHLLAAETYKEFERTYIPEDQRYTNKNSQAAFCYSETIP 59
              **:*******:**********************:****:**::***** *********

GgST-A      APTGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDRVFEKLKDLEEG 119
GgST-D      APTGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDRVFEKLKDLEEG 119
ScST        APTGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDRVYEKLKDLEEG 119
ApST        APTGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDRVFEKLKDLEEG 117
AaST        APTGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDRVFEKLKDLEEG 119
MgST        APTGKDDAQQKSDMELLRFSLVLIQSWLTPMQYLSKVFTNNLVFGTSDRVFEKLKDLEEG 119
CcST        APTGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDRVFEKLKDLEEG 119
            ******************************:******************:*********

GgST-A      IQALMRELEDRSPRGPQLLRPTYDKFDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVM 179
GgST-D      IQALMRELEDRSPRGPQLLRPTYDKFDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVM 179
ScST        IQALMRELEDRSSRGPPLLRSTYDKFDIHLRNEEALLKNYGLPSCFKKDLHKVETYLKVM 179
ApST        IQALMRELEDRSPRGPQLLKPTYDKFDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVM 177
AaST        IQALMRELEDRSPRGPQILKPTYDKFDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVM 179
MgST        IQALMRELEDRSPRGPQLLRPTYDRFDIHLRSEDALLKNYGLLSCFKKDLHKVETYLKVM 179
CcST        IQALMREVEDRSPRGPQLLRPTYDKFDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVM 179
            *******:**** *** :*: ***:******.*:******** *****************

GgST-A      KCRRYGESNCTI 191
GgST-D      KCRRFGESNCTI 191
ScST        KCRRFGESNCTI 191
ApST        KCRRFGESNCTI 189
AaST        KCRRFGESNCTI 191
MgST        KCRRFGESNCNI 191
CcST        KCRRFGESNCTI 191
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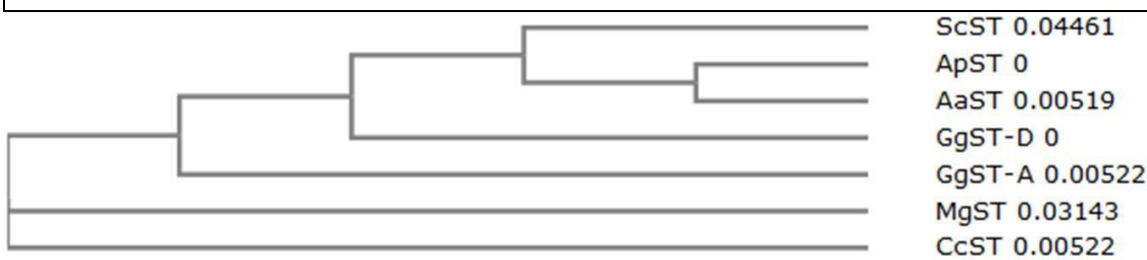

Figure 2A: Amino acid sequence alignment of GgST-A with avian STs i.e., ST of *Gallus gallus domesticus* [Desi chicken; GenBank Accession No. NP_989690.1), *Anser anser* (Fowl; Accession No. AAN37412.1), *Struthio camelus* (Ostrich; Accession No. BAA82959.1), *Coturnix coturnix* (Quail; Accession No. ACJ73931.1), *M. Gallapav*o (Tukey; Accession No. AAA49628.1), *A. platyrhynchos* (Duck; Accession No. CAA30113.1) by using Clustal omega multiple alignment program. The conserved sequences are indicated with an asterisk (*) and highlighted area shows the differences in the alignment. **2B**) Avian STs phylogenetic tree constructed by Neighbor-Joining method using the PHYLIP software. Each name at the terminus represents the species from which protein was originated.

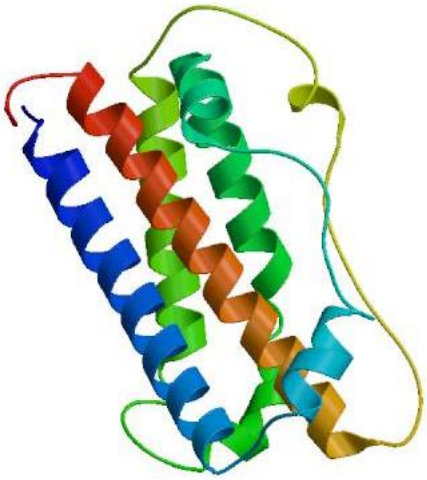

Figure 3: Three-dimensional model of GgST-A predicted using human ST structure, available at Protein Data Bank [pdb: 1hwg.1], as template. The structure shows four alpha-helices in up-up and down-down topology, a structure typically exhibited by the members of cytokine superfamily.

substitution in the predicted models can only be predicted through crystallographic studies of the protein. Since *G. gallus* is included in the class Aves, we further compared the *Gg*ST-A sequence with ST sequences of other members of the class Aves, ailable at GenBank. These include water fowl (*Anser anser*), ostrich (*Struthio camelus*), Turkey (*M. gallapavo*), quail (*Coturnix coturnix*) and duck (*Anas platyrhynchos*). The number of disulfide bonds and the position of cysteine residues (i.e., position 53, 163, 181 and 189) were found highly conserved amongst all the species (Figure 2a).

GgST-A shared maximum 99.4 % sequence identity with GgST-D followed by 98.9 % with CcST, 97.8 % with ApST, 97.3 % with AaST, 96.3 % with MgST and the least 94.2 % with ScST. Based on the amino acid sequence alignment of the aforesaid species, a phylogenetic tree was also constructed using the PHYLIP program (Fig. 2b). While the GgST-A appears to have least identity with ScST, it shares the same distant clad as ScST along with GgST-D, ApST and AaST reflecting as if descended from common ancestors. This adds support to the idea of an underlying slow rate of molecular evolution in avian STs.

Expression analysis of GgST-A

The GgST-A cDNA was cloned in pET-22b (+) expression plasmid between the *Nde*I and *Hind*III restriction sites (Figure 4a) under the control of T7*lac* promoter. In-frame cloning of GgST-A cDNA was confirmed by restriction digestion, colony PCR and nucleotide sequencing. Restriction digestion of pET-GgST-A with *Nde*I and *Hind*III yielded two prominent bands of 5.4 and 0.6 kb long DNA fragments, suggesting the GgST-A was cloned in correct orientation (Fig. 4b). For expression, the positive transformants were induced either with 0.5 mM IPTG or 10 mM lactose. A prominent band of ~22 kDa, contributing >40 % of the *E. coli* total cell protein (TCP), could be seen when analyzed by 13 % SDS-PAGE (Fig. 5). A band of similar size and intensity was absent in control or uninduced cells. The concentration of inducers used was in accordance with some previous studies[7,8,9,11] describing 0.5-1.0 mM IPTG or 10-20 mM lactose for achieving optimal levels of recombinant proteins expression, in *E. coli*.

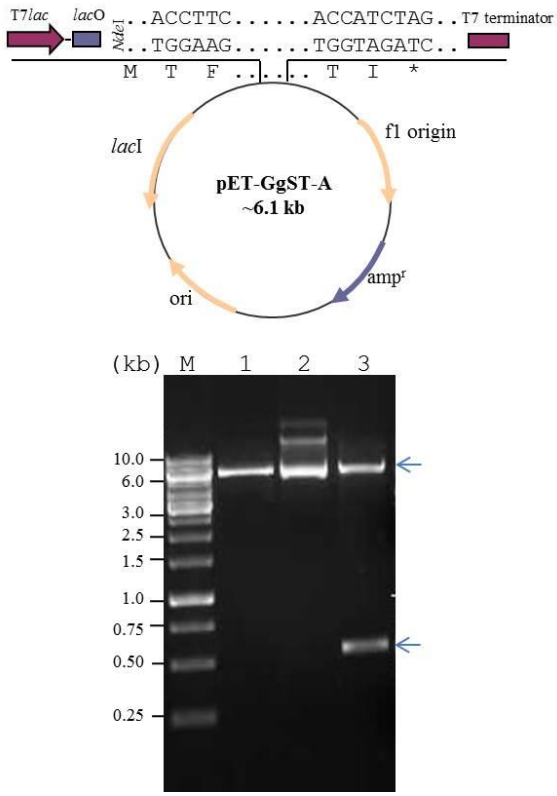

Figure 4: The upper panel shows the map of pET-GgST-A recombinant plasmid used in the study. The cDNA encoding for GgST-A was inserted between *Nde*I/*Hind*III restriction sites and expressed under the regulation of T7-lac promoter. ori (origin of replication); amp^R (ampicillin resistance gene); rbs (ribosome binding site). Lower panel refers to the confirmation of GgST-A in-frame cloning in pET-22b(+) plasmid by restriction digestion. M, DNA size marker; Lanes 1 to 3 respectively represent the linearized, uncut and digested pET-GgST-A, resolved by 1 % agarose gel electrophoresis. Arrows indicate the ~5.5 kb vector and ~0.6 kb insert.

Production of recombinant GgST-A in auto-inducing medium

Lactose-based auto-inducing strategy, described by Studier (2005)[12], Sadaf et al., 2007b[9] and several others, was employed to increase the overall yield of recombinant GgST-A, in shake flask cultures.

Lactose, the auto-inducer, was used as a cheaper alternative to IPTG. Auto-induction is due to the inducer (lactose) which is added right at the beginning of inoculation and the rate of induction is regulated by glucose levels in the medium. Lactose starts induction and production of protein automatically and is advantageous as compared to IPTG induction as culture growth is not required to be monitored before induction. With M9NG auto-inducing medium[9], used in the present study, a high density cell growth corresponding to OD_{600} ~12 (wet cell mass 14.4 g/l) could be attained, which was significantly higher as compared to that observed with IPTG induction (OD_{600} ~8).

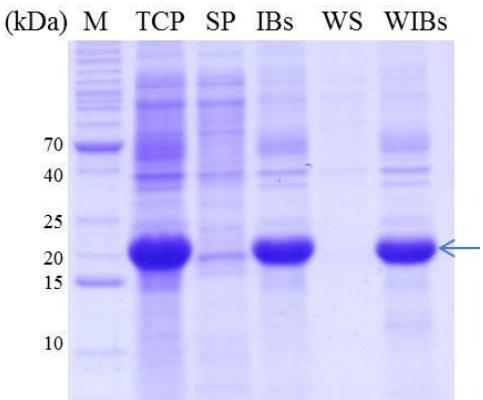

Figure 5: SDS-PAGE analysis of GgST-A during different stages of isolation and washing/purification. Equal amounts of total protein samples mixed with 2xreducing sample buffer were loaded onto 13% SDS polyacrylamide gel (8.6x6.8cm; thickness 0.75mm), run at 150V for 1.5 hours and either stained with Staining solution having R-250. M, Marker; U, uninduced; TCP, total cell protein; SP, soluble protein fraction; IBs, inclusion bodies; WS, washing supernatant; WIBs, washed inclusion bodies. Arrow indicates the position of 22 kDa recombinant GgST-A.

Solubilization and refolding

The expression of GgST-A was examined in both soluble and insoluble fractions, obtained after sonication of *E. coli* cells. When analyzed on 13 % SDS-gel, the recombinant GgST-A was found associated with the pellet fraction in an insoluble form while the supernatant representing the soluble fraction had no band of similar size and intensity (Fig. 5). High-level expression of eukaryotic proteins in bacterial system often leads to their aggregation in the form of insoluble inclusion bodies or IBs[13]. While the IBs are generally regarded as biologically inactive and hence require solubilization and refolding steps for restoration of functional activity, they are often advantageous from the purification view point. In the present study, Triton X-100 washed IBs were found be almost 85% pure, not requiring complex purification schemes prior to functional analysis.

Table 2: Solutions used to solubilize GgST-A IBs.

Solubilization Solutions	*Compositions*
A	100 mM Tris-Cl, pH 8.5
B	100 mM Tris-Cl, 2 M urea, pH 8.5
C	100 mM Tris-Cl, 4 M urea, pH 8.5
D	100 mM Tris-Cl, 8 M urea, pH 8.5
E	100 mM Tris-Cl, pH 10.5
F	100 mM Tris-Cl, 2 M urea, pH 10.5
G	100 mM Tris-Cl, 4 M urea, pH 10.5
H	100 mM Tris-Cl, 8 M urea, pH 10.5
I	100 mM Tris-Cl, pH 12.5
J	100 mM Tris-Cl, 2 M urea, pH 12.5
K	100 mM Tris-Cl, 4 M urea, pH 12.5
L	100 mM Tris-Cl, 8 M urea, pH 12.5

Accumulating evidence suggests that protein molecules inside IBs aggregates have native-like secondary structures and the restoration of this native-like structure by using mild solubilization conditions may ensure enhanced recovery of bioactive protein as compared to the solubilization conditions involving higher concentrations of denaturant[9,14,15]. The degree of IBs solubility in solutions containing different concentrations of urea are shown in Fig. 6. The maximum solubility of recombinant GgST-A was achieved in solubilizing solution D, and J to L (Table 2). In Tris-Cl, pH 8.5, the solubility was quite low (~30 %) but enhanced significantly both by increasing the pH (10.5 or 12.5) and the molar concentration of urea (2-8 M). At pH 12.5, increase in molar concentration of urea (0-8 M) had a slight influence on the solubility of IBs. Maximum solubility was observed in Solution 'D' having higher concentration of denaturant urea (i.e. 8 M); however, Solution 'J' may be preferred as a mild concentration of urea (i.e., 2M only) is likely to have a no influence on native-like secondary structure of IBs. Therefore, in all subsequent experiments, GgST-A IBs were solubilized in 2M urea (pH 12.5) to preserve the structure. Dilution of the solubilized protein directly into the renaturation buffer is the most commonly used method for small scale refolding of recombinant protein. Chelating agent like EDTA used in the renaturaion solutions prevent metal-catalyzed air oxidation of cysteines while 5% sucrose or 10% glycerol stabilize the refolding intermediates by preventing the hydrophobic interactions between some partially folded protein molecules[16-18].

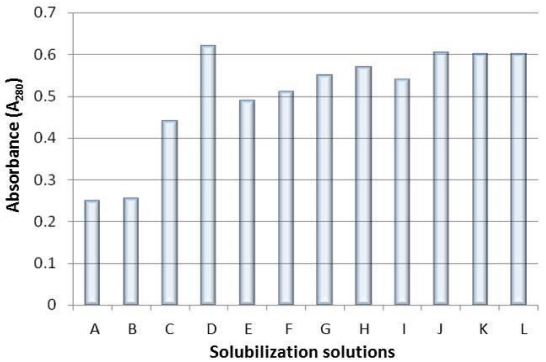

Figure 6: Solubility of IBs in solutions of different pH and ionic strength. A fixed amount of IBs equivalent to 1mg/ml was used for solubility analysis.

In the present study, the refolding was accomplished by slow removal of the denaturant (2M urea) and step-wise drop in pH from 12.5 to 9.5, using dialysis method in the presence of refolding additives. No protein aggregates were observed when the pH was gradually dropped down to 9.5; however, lowering the pH below 8.5 resulted in slight protein aggregation. Aggregation of solubilized mixture results in poor recovery of refolded protein and thus avoided. These are the optimized conditions for expression and refolding of GgST-A in shake-flask cultures. The recombinant protein may further be used for structural characterization and bioactivity assessments. The bioactive GgST-A is likely to serve as a valuable source for poultry and related food industry due to its potential applications in improving chicken growth.

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