

Analysis of Methods to Improve the Solubility of Recombinant Bovine Sex Determining Region Y Protein

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Abstract

Background: Inclusion body formation in *E. coli* is a significant problem in recombinant protein production. The aim of this study was to improve the solubility of recombinant bovine sex determining region Y protein (SRY) in BL21 (DE3) *E. coli* cells.

Methods: In this research two recombinant bovine SRY (rbSRY) sequences were analyzed; these were wild-type SRY (wtbSRY) and codon-optimized SRY (cobSRY). Their expression in various culture conditions was examined; these differences included IPTG concentrations, temperatures, and media stabilizers.

Results: IPTG and temperature significantly affected rbSRY solubility ($P < 0.001$). The optimum IPTG concentration and temperatures for wtbSRY and cobSRY induction were 0.3 mM at 27 and 32 °C, respectively. In addition, arginine and sorbitol concentrations significantly affected rbSRY solubility ($P < 0.01$). Solubility of rbSRY protein was highest from the cobSRY construct in the presence 0.2 M arginine and 0.3 M sorbitol. The highest inclusion body production occurred with high glucose concentrations.

Conclusions: We found that modifications in temperature and IPTG and stabilizer concentrations affected rbSRY solubility.

Keywords: Cobsry, Inclusion Bodies, Recombinant Bovine SRY Protein, Solubility, Wtbsry.

Introduction

Production of recombinant proteins in *E. coli* is a great revolution in biochemistry, biotechnology, and therapeutic drug production (1, 2); however, the use of this organism to express heterologous proteins has some disadvantages; these include diminished recombinant protein folding, inclusion body production, codon usage toxicity of the inserted protein to the host and non-biological activity of the recombinant protein (2-5). To counter these disadvantages, several strategies have been employed; these include decreasing the inducer concentration, reducing induction temperature, and developing the methods to tightly control protein expression using specialized promoters (2, 3, 6).

Another strategy to increase the solubility, protein folding, and stability of recombinant proteins produced in prokaryotes is the use of

stabilizers. These are generally small organic compounds that influence protein folding without creating or breaking covalent bonds (7, 8). Finally, codon adaptation factors can influence recombinant protein solubility in *E. coli* (9).

In most mammals, male sex is initiated by the sex-determining region on the Y chromosome (SRY). SRY is an intron-less gene located on the Y chromosome and containing three domains; the N-terminal, HMG-box, and C-terminal domains (12). The 687 bp bovine SRY gene length encodes the 229 amino acid 27 kDa SRY protein (13).

In this study, the codon adaptation index, temperature, IPTG, and stabilizers were examined for their effect on rbSRY solubility in BL21 (DE3) *E. coli* cells.

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Materials and methods

Materials

Genetically engineered *E. coli* strains, including DH5 α and BL21 (DE3), were gifts from the Royan Institute (Novogene, USA, Madison, Wisconsin). SRY gene-specific primers were purchased from SinaCloneBioScience Co, Iran. Lysozyme (PI-90082), DNase A (EN0525), proteinase K (E00491), RNase A (EN0531), and isopropyl β -D-1-thiogalactopyranoside (IPTG) (BP 1755-1) were purchased from Thermo Scientific, Munich, Germany. Platinum *Pfx* DNA polymerase (11708-013) was obtained from Invitrogen, Carlsbad, California, USA. The plasmid extraction kit (GF2001) and 1 kb marker (MD104) were purchased from VIOGENE Co, New Taipei City, Taiwan. The Luria Bertani (LB) Broth (L3022), agar (05040), arginine (A5006), glycerol (G5516), sorbitol (S1876), glucose (G8270), and NI-NTA His Select affinity chromatography gel (P6611) were from Sigma Aldrich.

Syntheses of Bovine SRY gene, construction of cloning and expression vectors

The 687 bp open reading frame (ORF) of bovine SRY was obtained from the NCBI database (accessibility code NM-001014385.1). Native SRY (wtbSRY) and the optimized codon SRY sequence (cobSRY), encoding the same protein, were selected. Online software from biotechnologies web (<http://genomes.urv.es/CAIcal>) was used to determine the codon adaptation index (CAI) for cobSRY. To produce the SRY-pET32a (+) constructs, wtbSRY and cobSRY were chemically synthesized (GeneRay Co) and cloned into pET32a (+). Competent DH5 α *E. coli* were transformed with the constructs (14) and cultured on Luria Bertani or LB-agar plates (10 g tryptone; 5 g yeast extract; 10 g NaCl, 15 g agar per liter containing 30 μ g/mL of kanamycin) at 37 °C overnight. Colony-PCR and sequencing techniques were used to screen for positive clones. The forward and reverse primer sequences for colony-PCR were 5' ATGTTTCAGAGTATTGAACGA 3' and 5' TCAATATTGAAAATAAGCAC 3', respectively. Materials used to amplify bovine SRY gene were as follows; 5 μ L of 10X *Pfx*

amplification buffer, 1.5 μ L of 10 mM dNTPs mixture, 1 μ L of 50 mM MgSO₄, 2 μ L of primer mix (10 μ M each), template DNA from touched positive clones, 0.5 μ L of Platinum *Pfx* DNA Polymerase, and 39.5 μ L of ddH₂O. Thermal cycling conditions were 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 45 sec, 68 °C for 1 min, and then a final extension for 10 min at 68 °C. The plasmid stability was evaluated as described by Yari et al. (2011) with some modifications(15). BL21 (DE3) *E. coli* cells were transformed (14)with the stable constructs to produce the wtbSRY-pET32a (+)-BL21 (DE3) and cobSRY-pET32a (+)-BL21 (DE3) expression systems. To express rbsRY protein, fresh LB medium supplemented with 50 μ g/mL kanamycin was inoculated with the stable clone and incubated at 37 °C overnight with shaking at 130 rpm. When the absorbance of the culture media at 600 nm reached approximately 0.8, the cultures were added to 1000 mL of LB media (pH 7.4) plus kanamycin until OD 600 reached 0.6-0.8. Then the induction program was performed with 0, 0.3, 0.6, 0.9 and 1.2 mM IPTG, at 27, 32, or 37 °C and the cultures were shaken at 130 rpm for five hours. Protein expression was visualized by SDS-PAGE.

The effects of stabilizers on expression and solubility of rbsRY

Next, stabilizers were analyzed for their effects on expression and solubility using the IPTG concentrations and temperatures that gave the highest protein expression. Glucose at 5, 10, 15, and 20 mM, sorbitol at 0.1, 0.2, 0.3, and 0.4 M, arginine at 0.1, 0.2, 0.3, and 0.4 M, and glycerol at 5, 10, 15, and 20 mM were added to the batch media that containing 0.3 mM IPTG. Each assay was performed in triplicate. After completion of the growth phase, the bacteria were pelleted at 10733 x g at 4 °C for 10 min, and the pellets were stored at -70 °C until further analysis. To analyze protein production during cultivation, the total extracted proteins and soluble or inclusion bodies from BL21 (DE3) *E. coli* were analyzed by SDS-PAGE. Control cultures with no IPTG or stabilizer were analyzed in parallel.

Extraction and purification of rbSRY protein
 SRY proteins from bacteria and soluble/inclusion bodies were phase-separated as described (16) with some modifications. Briefly, cell pellets collected from cultures were washed with phosphate-buffered saline (PBS), pH 7.4 and pelleted at 5,000 g for 12 min at 4 °C. The pellets were lysed in lysis buffer, pH 7.8 (3 mM EDTA.4 Na, 300 mM NaCl, 10 mM imidazole, and 1% Triton X100) containing 30 µg/mL DNase, 800 µg/mL lysozyme, 12 µg/mL RNase and 1 mM protease inhibitor cocktail tablets. The mixtures were incubated at room temperature for 2 h and then sonicated 15 times with 25-sec bursts and 45-sec rest intervals, centrifuged at 37,500 x g for 50 min at 4 °C, and the upper phase, considered as the soluble fraction, was collected. The precipitate in the lower phase, considered as the insoluble phase containing inclusion bodies, was suspended in a solution containing 8 M urea pH 8, 100 mM sodium phosphate (NaH₂PO₄.2H₂O), 100 mM Tris/HCl and 5 mM DTT for 14 h at room temperature and sonicated three times with 45 sec bursts and 90 sec rest intervals. The resultant mixtures were centrifuged at 49,500 x g for 60 min at 4 °C and the supernatant was collected. The soluble and inclusion body phases were applied separately on NI-NTA His Select Affinity columns and rbSRY was purified according to the manufacturer's recommendation. Each fraction from the soluble and inclusion body phases was assayed for protein concentration at 280 nm using a

CECIL spectrophotometer (CE 7250, Cambridge, UK) and analyzed by SDS-PAGE.

Detection of rbSRY

The rbSRY proteins were analyzed by mass spectrometry by isolating a single band from the SDS-PAGE gel. Proteins were sequenced at York University, England.

Statistical analysis

The effects of different temperatures and IPTG and stabilizer concentrations on rbSRY solubility from the wtSRY and cobSRY sequences were analyzed using Graph Pad Prism 6 Demo.

Results

Bovine SRY amino acids and gene sequences

The wtSRY and cobSRY nucleotide and amino acid sequences are shown in Fig. 1. The codon adaptation index (CAI) between wtSRY and cobSRY and *E. coli* were 51 and 85%, respectively. The wtSRY sequence contains 15 rare arginine codons including 6 AGGs, 4 AGAs, and 5 CGAs. The wtSRY sequence contains four rare proline CCC codons. The other rare codons in the wtSRY sequence including four leucine CTA codons and three isoleucine ATA codons. To increase the CDI of bovine SRY with BL21 (DE3) *E. coli*, the rare arginine, proline, leucine, and isoleucine codons were substituted with the most common *E. coli* codons.



Fig. 1. The wtSRY and cobSRY sequences are shown. The wild-type bovine SRY (in black) with a CAI of 51% and codon optimization sequence of cobSRY (in yellow) with a CAI of 85% are shown. The wtSRY and cobSRY amino acid sequences (blue) are identical.

The effects of IPTG and temperature on rbSRY expression and solubility

Different temperatures and IPTG concentrations significantly affected the expression and solubility of the two rbSRY sequences ($P < 0.001$). (Fig. 2 and Table 1). The following cultivation at 37 °C in 1.2 mM IPTG, a considerable amount of total protein was produced; however, most of it was in inclusion bodies. By shifting the cultivation temperature to 27 °C in 0.3 mM IPTG, more soluble rbSRY was produced. In contrast, incubating the cobSRY construct in 0.3 mM IPTG at 32 °C produced more soluble protein than at 37 or 27 °C. It appears that codon optimization improved the amount of soluble recombinant SRY in comparison with inclusion bodies (Figs. 3). As shown in figure 3 the amount of soluble recombinant bovine SRY protein in the presence

of IPTG 0.3 mM for wtSRY (at 27 °C) and cobSRY (at 32 °C) was seen at the highest quantity. In contrast the largest amount of inclusion bodies in this protein for two sequences observed in 1.2 mM IPTG at 37 °C of cultivation. The solubility of the SRY proteins encoded by wtSRY and cobSRY sequences at the different temperatures and IPTG concentrations are shown in Table 1. The solubility of rbSRY encoded by the cobSRY sequence in 0.3 mM IPTG was greater at 32 °C than at 27 or 37 °C, while the solubility of rbSRY encoded by wtSRY in 0.3 mM IPTG was greatest at 27 °C. In general, rbSRY protein solubility was directly correlated with the CAI and inversely correlated with increased temperature and low IPTG concentrations. The apparent molecular weight of the recombinant protein determined by mass spectrometry was 27 kDa.

Table 1. The percentage solubility of the recombinant bovine SRY protein encoded by the two different constructs at different cultivation temperatures and IPTG concentrations.

Seq. IPTG	Percentage of soluble /total rbSRY in LB media at different temperatures and IPTG concentrations											
	LB Media cultivation at 27 °C				LB Media cultivation at 32 °C				LB Media cultivation at 37 °C			
	0.3	0.6	0.9	1.2	0.3	0.6	0.9	1.2	0.3	0.6	0.9	1.2
wtSRY	14	13	11	7	9	7	6	6	9	6	5	5
cobSRY	21	11	17	14	24	18	17	15	12	11	11	12

The different temperatures and IPTG concentrations had the difference significant on the solubility of rbSRY. For wtSRY and cobSRY constructs, the best concentration of IPTG at 27, 32 and, 37 °C was 0.3 mM. the best temperature for induction of recombinant bovine SRY as the soluble form for wtSRY and cobSRY constructs was 27 and 32 °C, respectively.

The effects of stabilizers on the solubility of rbSRY

The effects of various concentrations of stabilizers on the solubility of rbSRY from wtSRY and cobSRY constructs grown in 0.3 mM IPTG at 27 and 32 °C, respectively, were examined. The protein profiles of wtSRY and cobSRY soluble/inclusion body phases on SDS-PAGE in the presence of the different stabilizers are shown in Fig. 4. Different arginine concentrations significantly affected cobSRY solubility, but not wtSRY, while different sorbitol concentrations significantly affected SRY

solubility from both constructs ($P < 0.01$). Different glucose and glycerol concentrations had no significant effects on rbSRY solubility from either construct (Table 2). The greatest amount of soluble protein was achieved with 0.2 M arginine on the cobSRY construct (Fig. 4 and Table 2). At the highest glucose concentration (20 mM) the insolubility of rbSRY from wtSRY and cobSRY sequences was increased (Fig. 5 and Table 2), while at the lower glycerol and glucose concentration (5 mM), rbSRY solubility was relatively improved.

The Solubility Increase of Bovine Recombinant SRY

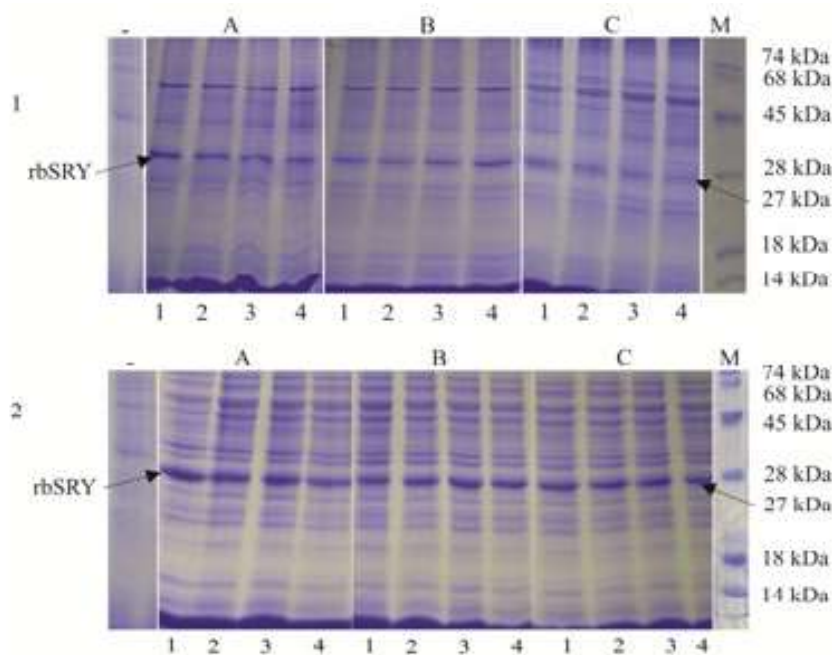


Fig. 2. The expression of rbSRY produced at 27 (A), 32 (B), and 37 (C) °C in 0.3 (bands 1), 0.6 (bands 2), 0.9 (bands 3), and 1.2 mM (bands 4) IPTG from wtbSRY (gel 1) and cobSRY (gel 2) sequences. Band (-) shows a control sample without inducer. As shown in figures the recombinant bovine SRY protein for wtbSRY and cobSRY sequences was expressed in the highest amount incomparable with total protein for BL21 (DE3) *E. coli* host strain. The expressed recombinant SRY protein was shown by Fletcher (approximately 27 kDa). Lane M shows the low molecular weight marker.

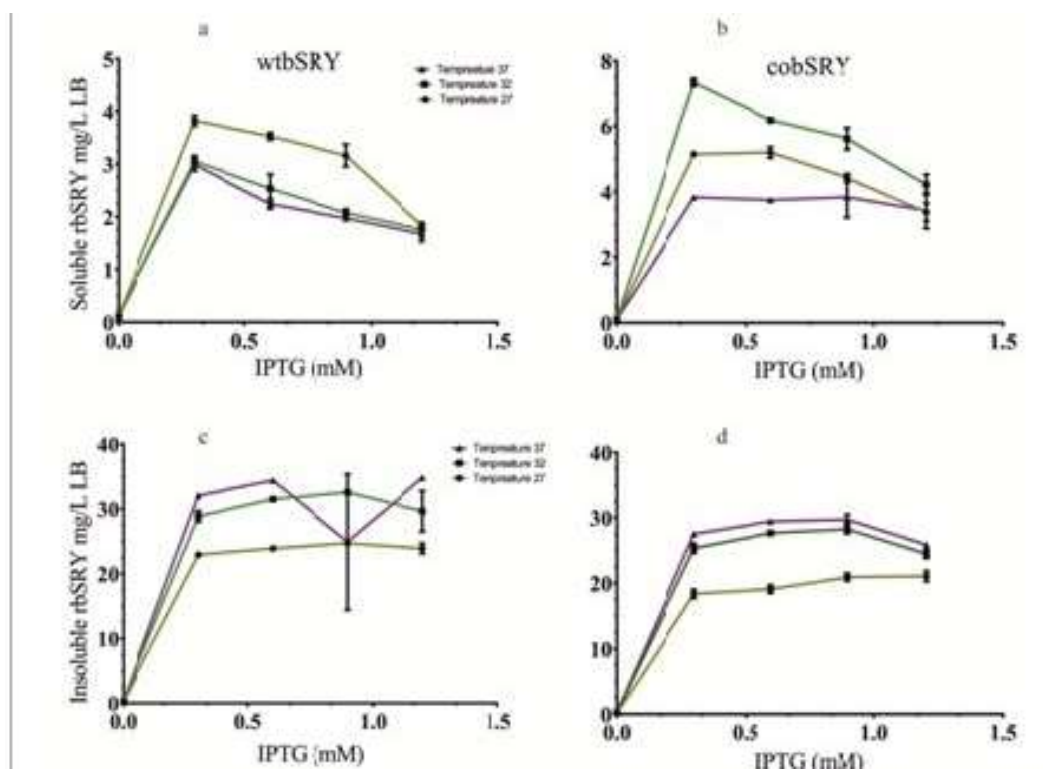


Fig. 3. The effects of different temperatures and IPTG concentrations on amount soluble rbSRY (a and b) or inclusion body production (c and d). *E. coli* BL-21 cells were transformed with wtbSRY and cobSRY sequences and grow at 27, 32, or 37 °C in 0.3, 0.6, 0.9, or 1.2 mM IPTG. Data from this research indicated that the highest amount of rbSRY as soluble and inclusion bodies form was seen in 0.3 mM at 27 or 32 °C. Protein products were analyzed by SDS-PAGE and mass spectrometry. The solubilities of rbSRY in wtbSRY and cobSRY sequences were greatest at 27 and 32 °C, respectively.

Table 2. The percentage soluble recombinant bovine SRY protein for wtSRY and cobSRY constructs that cultivated in the presence of different concentrations of stabilizers.

Culture media/ Seq	LB	LB-Arginine (M)				LB-Sorbitol (M)				LB-Glycerol (mM)				LB-Glucose (mM)			
	0.3	0.1	0.2	0.3	0.4	0.1	0.2	0.3	0.4	5	10	15	20	5	10	15	20
wtSRY	14	14	14	14	14	12	11	15	11	11	12	13	13	13	12	12	11
cobSRY	22	26	34	32	23	21	22	27	23	23	21	21	19	18	16	16	15

The best concentration of stabilizers for achieving the highest percentage of soluble rbSRY protein in two constructs were 0.2 M Arginine, 0.3 M Sorbitol, 5 mM Glycerol and, 5 mM Glucose. For the experiment, the effects of stabilizers the best concentration of IPTG (0.3 mM) that mentioned in the last previous section was used.

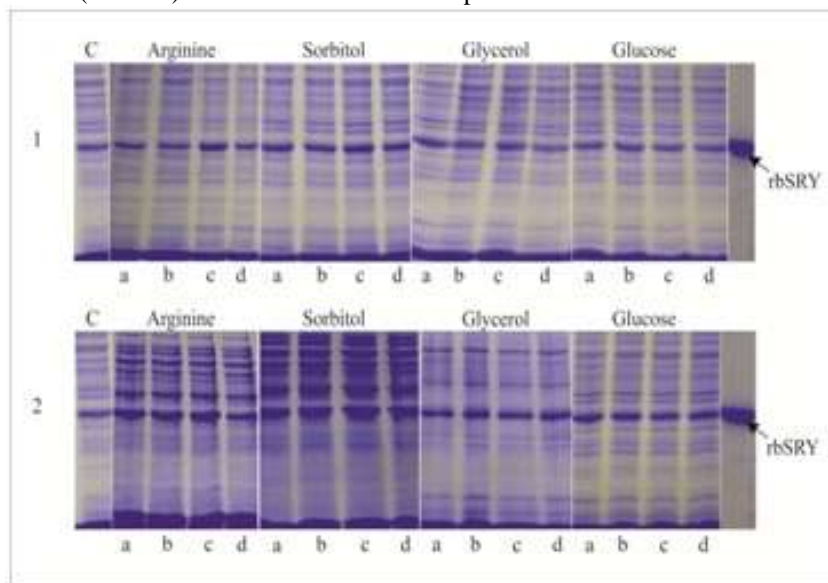


Fig. 4. The expression of recombinant bovine SRY protein as the soluble form in wtSRY (1) and cobSRY (2) constructs that grown in LB media containing 0.1, 0.2, 0.3, or 0.4 M arginine or sorbitol and, 5, 10, 15, or 20 mM glycerol or glucose. As shown in this figure the solubility of rbSRY protein in the presence of 0.2 M arginine and 0.3 M sorbitol for two constructs. The best concentration of glycerol and glucose was 5 mM. The purified recombinant bovine SRY as a marker is shown on the right. a, b, c and, d include the different concentration of stabilizers.

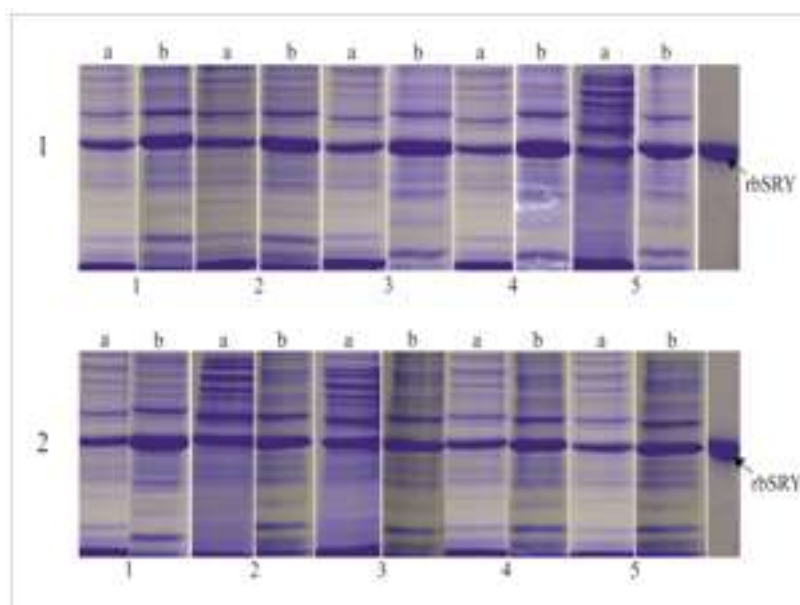


Fig .5. The effects of best concentrations of stabilizers on the solubility (lane a) of and inclusion bodies (lane b) recombinant bovine SRY in wtSRY (1, upper at 27 °C) and cobSRY (2, down at 32 °C) constructs on 12.5% SDS-PAGE. Pairs lane 1 IPTG 0.3 mM without stabilizer, Pairs lane 2 0.2 M of arginine, Pairs lane 3 0.3 M sorbitol, Pairs lane 4 10 mM glycerol and, Pairs lane 5 5 mM glucose is shown. The purified recombinant bovine SRY is shown on the right.

Discussion

In our hands, the solubility of rbSRY in wtSRY and cobSRY clones was highest in 0.3 mM IPTG. As the IPTG concentration was increased, rbSRY solubility decreased. This has previously been seen in other systems (17-19). Rabhi-Essafi et al. (2007) demonstrated that reducing cultivation temperature and IPTG concentration increased the production of soluble recombinant IFN α 2B protein yield up to 70% in *E. coli* BL21 cells (20). In the pET system with the strong T7 promoter and high inducer concentrations, product yields can exceed 50% of the total cytoplasmic proteins; however, this leads to increased misfolding and inclusion body formation in *E. coli* (21). IPTG induces T7 RNA polymerase and protein production in *E. coli*. Low IPTG concentration (0.3 mM) probably the strong affinity of T7 promoter and lac repressor occurred in a suitable form; therefore, rbSRY production in during cultivation also occurred slowly, increasing the solubility of rbSRY. One possible explanation for this increased rbSRY solubility at low IPTG concentration is that its slow production, increased solubility. In contrast at higher IPTG concentrations, the induction program was not efficient, perhaps due to additive toxicity of the inducer. We also found that the expression of rbSRY using wtSRY-pET32a (+)-BL21(DE3) at 27 °C and cobSRY-pET32a at 32 °C resulted in the greatest solubilities. One well-known strategy to limit the aggregation of recombinant proteins in *E. coli* is to reduce the cultivation temperature (17). Generally, at higher temperatures inclusion body formation in is favored in *E. coli* because the higher temperature increases hydrophobic interactions, leading to aggregation (22). Reducing the cultivation temperature also reduces the expression of heat shock proteases (23), increasing recombinant protein stability and solubility. Suddenly decreasing the cultivation temperature inhibits recombinant protein replication and transcription (24). Besides, at 30 °C, the activity and expression of the number of chaperones are increased in *E. coli* (24, 25). Recombinant protein stability and the potential for correct folding at low temperatures are partially explained by these factors (26). Low cultivation temperature also results in reduced protein yields (3), but solubility is

increased. Soleymani et al. (2017), using the pET28a(+)-SRY-BL21 (DE3) expression system, increased rbSRY solubility using 0.5 mM IPTG (0.5mM) at 20 °C (5). When we optimized codon usage in the cobSRY sequence, the optimum cultivation temperature for protein solubility was 32 °C, while the optimum solubility culture temperature for the wtSRY sequence was 27 °C. We propose that differences in codon frequency between the target gene and the expression host may lead to translational stalling, premature translation termination, and amino acid misincorporation. These differences may be overcome by using bacterial strains containing plasmids encoding the rare tRNAs needed to express genes that utilize these rare codons. 0.3 M sorbitol increased rbSRY solubility. Sorbitol inhibits cell growth and recombinant protein aggregation by a mechanism similar to that of other polyhydric alcohols, increasing their solubility (27-29). The low growth rate in sorbitol may provide sufficient time for protein folding, improving recombinant protein solubility.

The role of arginine as an element to prevent recombinant protein aggregation has been widely studied (30). In our study, arginine improved rbSRY solubility. Arginine likely prevents aggregation by binding recombinant proteins, one or more membrane transporters, and proteins that affect the growth rate. In our study, 20 mM arginine increased the cell biomass but rbSRY stability was decreased. The cell biomass was lowest in 5 mM glucose, but rbSRY solubility was greatest at this concentration. At this low glucose concentration, the culture the doubling time was increased; thus, rbSRY folding time and subsequent solubility were likewise increased. Wang et al. (2014) reported that high glucose concentrations in *E. coli* cultures led to acetate accumulation, inhibiting bacterial growth and recombinant protein production (31). As a result, limiting glucose is regarded as a valid strategy for reducing acetate accumulation, subsequently improving cell growth and recombinant protein solubility.

We found that cultures grown in low IPTG concentration at low and moderate temperatures

increased the solubility of rbSRY protein solubility from the wtSRY and cobSRY constructs. Codon optimization of the bovine SRY sequence with *E. coli* increased the solubility of rbSRY from cobSRY. In high IPTG concentrations at 37 °C, inclusion bodies in two constructs were observed in the largest amount from both constructs. The rbSRY protein solubility was increased by adding arginine or sorbitol, but not glycerol or glucose, to

induced batch cultures during protein expression.

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