**Bovine Sex Determining Region Y: Cloning, Optimized Expression, and Purification**

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**ABSTRACT**

Sex determining region Y gene (SRY) is located on Y chromosome and encodes a protein with 229 amino acids. In this study, ORF region of SRY with a length of 690 bp was synthesized using PCR and ligated to pET28a (+), then transformed in E.coli DH5α. E.coli BL21 (DE3) strain was chosen to express recombinant bovine SRY protein. A set of optimization steps was taken including different concentrations of IPTG, glucose, and temperatures at differed incubation times after the induction. Results showed that temperature points and different concentrations of IPTG and glucose had a significant effect (p < 0.01) on total protein and recombinant bovine SRY. After purification, various temperatures and concentrations of IPTG showed meaningful effects (p < 0.01) on the solubility of expressed recombinant SRY. Highest soluble rSRY protein amount was achieved where 0.5 mM IPTG and 0.5% glucose was used at 20°C during induction. In the absence of glucose, the highest amount of soluble recombinant SRY levels were achieved at the concentrations of 0.8 mM of IPTG at 28°C, 20°C, and 1.5 mM IPTG at 37°C during induction for 16, 24, and 8 hours, respectively. Regarding the results obtained in this study, it could be stated that by decreasing temperature and inducer concentration, soluble bovine SRY protein expression increases.

**KEYWORDS**

Cloning; expression; purification; recombinant bovine SRY protein; sex determining region Y chromosome

**Introduction**

Sex chromosomes in female and male individuals are mainly responsible for sex determination in various species of mammalian. Sex chromosomes differ widely from autosomal ones in structure, genetic content, and expression levels (1–3). These chromosomes have gained lots of attention among scientists due to a great diversity in size and related sex determining factors (2); unique properties such as being single allelic and inactivation through embryonic development (3, 4); unequal expression of X chromosome in 2 genders (5); X chromosome conservation regarding gene type and order (6, 7); and, last but not least, a vast range of Y chromosome variation (7, 8). In male mammals, the most significant role of Y chromosome is defined as male sex determination and other functions this chromosome takes responsibility for falls into lower levels of importance. Sex Determining Region Y chromosome (SRY) gene has been verified to be behind sex determination and testis development (9). In a classic approach, there is a two-step path to sex determination in mammals, which includes a genetic step (from chromosomes to gonad formation) and a hormone regulatory step (from gonads to phenotyping). The genetic step through which testis differentiation occurs is controlled by Testis Determining Factor (TDF) (10, 11). SRY gene is an intron less gene that is transcribed to produce a protein with 229 amino acid residues (12) belonging to SOX9 transcription factor family. SOX9 family, which by the way is found in all mammals, is composed of 20 members in human, mouse, cow, sheep, monkey, and horse. This family, including the most important one, SRY, is in charge of many different functions in embryonic and many organs development particularly in switching sex determination process (13). Like all other SOX family members, SRY protein possesses a High Mobility Group (HMG) box domain, functioning as a DNA binding region, making it possible for DNA to bend 60-85° in minor groove at an (A/T)ACAA(T/A) motif that helps with double stranded DNA bending (14). HMG box domain has been proved to be the only domain necessary for SRY to begin testis development and male phenotype occurrence (15–17) that contain 79 amino acids...
acid residues (18). In most species, Zhao and Koopman (19) indicated that HMG box domain is located between two sequences of N terminal domain (NTD) (with an average length of about 30-60 amino acids) and C terminal domain (CTD) (with an average length of about 70-100 amino acids). SRY protein possesses two Nuclear Localization Signal (NLS) and target sites for acetylation, and phosphorylation. NLS domain is located at both 3’ and 5’ ends of the HMG box and is mostly preserved in mammals (20). NLS at 3’ and 5’ ends of the HMG box bind to calmodulin and importin, making it possible for the signal transduction from cytoplasm to nucleus and consequent post-translational modifications including phosphorylation and acetylation necessary for SRY to mature (18, 21, 22). Thus far, no extraction and purification of recombinant Bovine SRY protein has been reported. Therefore, the objective of the present study was to amplify, clone, and sequence bovine SRY gene, then to express and produce rBSRY protein in E.coli in an optimized condition and finally purify the product.

Material and methods

Genetically engineered strains of Escherichia coli including DH5α and BL21 (DE3) and also pET28a(+) plasmid were all kind gifts from Royan Institute (Novagene, USA, Madison, Wisconsin). SRY gene specific primers were purchased from SinaClon BioScience Co, Iran. Restriction enzyme Bam HI (Cat. Num. ER0052), EcoRI (Cat. Num. ER0272), lysozyme (Cat. Num. PI-90082), DNase A (Cat. Num. EN0525), proteinase K (Cat. Num. E00491), RNase A (Cat. Num. EN0531), and Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) (Cat. Num. BP 1755-1) were all purchased from Thermo Scientific, Munich, Germany. Platinum pfx DNA polymerase enzyme (Cat. Num. 11708-013) was obtained from Invitrogen, Carlsbad, California, USA. Plasmid extraction kit (Cat. Num. GF2001), 1 kb markers (Cat. Num. MDI04) and standard DNA were purchased from VIOGENE Co, New Taipei City, Taiwan. Simple Safe, as a replacement for ethidium bromide (Cat. Num. E4600-01) and T4 DNA ligase (Cat. Num. E1060-01), were obtained from EURex Co. Frankfurt, Germany. Affinity chromatography media was purchased from Sigma Aldrich. A kit for extracting gene from PCR product and gel (Cat. Num. 11732668001) was provided from Roche, Mannheim, Germany.

DNA extraction, bovine SRY gene synthesis, and cloning

The ORF fragment of 690 bp from bovine SRY gene was selected from NCBI database (accessibility code NM-001014385.1). For DNA extracting and SRY gene amplification, 4 bulls were selected from a farm dairy cattle station at Ravansar, Kermanshah, West of Iran. A 10 mL blood sample was collected from each bull tail vein in tubes containing 0.5 mL 0.5 mM EDTA. Samples were transferred in an ice bucket transferred to Medical Biology Research Center (MBRC) of Kermanshah University of Medical Sciences. A modified salting out method was used for extraction of genomic DNA (23). To amplify bovine SRY gene by PCR (Eppendorf Germany), forward and reverse primer sequences were (5’ to 3’) GGATCCATGTTCAGATTTGAACGA and (5’ to 3’) GAATTCTCAATATTGAAAATACGCAC that contained restriction recognition sites for BamHI and EcoRI enzymes (underlined), respectively. Materials used in a PCR reaction tube to amplify bovine SRY gene were as follows: 5 µL 10 X Pfx Amplification Buffer, 1.5 µL 10 mM dNTPs mixture, 1 µL 50 mM MgSO4, 2 µL Primer mix (10 µM each), 0.5 µL Template DNA (350 ng/µL), 0.5 µL Platinum pfx DNA Polymerase, and 39.5 µL ddH2O. Thermal cycling conditions were 94°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, 58°C for 45 seconds, 68°C for 1 minute, and then a 10 minutes final extension at 68°C. PCR products were then examined by electrophoresis in a 1% (w/v) agarose gel using a 1 kb DNA marker. The PCR products were purified using gel extraction kit as recommended by manufacture, and digested by BamHI and EcoRI restriction enzymes. The expression pET28a (+) vector was also digested by these enzymes according to manufacture kit, and restriction enzymes were deactivated with PCR products/DNA extraction kit using standard protocol. Ligation of PCR digested products and pET28a (+) vector was achieved in a ratio of 3:1 (expression vector: SRY gene) through exposure to T4 DNA Ligase and final ligated sequence was transferred into a susceptible DH5α E.coli (24). The transformed DH5α - pET28a(+) – SRY were consequently cultured on LB agar plate containing 30 µg/mL kanamycin as an antibiotic resistance gene at 37°C overnight. Following step was to run a colony-PCR on colonies grown on the plate. 7 out of 10 colonies were determined + in this test. Plasmid stability was investigated by sub culturing 5 of 7 positive colonies on LB agar plate containing 50 µg/mL kanamycin as an antibiotic resistance gene at 37°C overnight. Following steps were taken to confirm the accuracy of cloning. Colony-PCR and in the case of SRY gene isolation, restriction enzymes BamHI and EcoRI were used. Sequencing nucleotides and amino acids in bovine SRY gene cloned in pET28a(+) and rSRY protein
was carried out by GeneRay Co. Taiwan and York University England, respectively.

**Bovine SRY protein expression in BL21 (DE3)**

According to the protocol of Green and Sambrook (24), the cloned construct of pET28a(+) - Bovine SRY in E. coli BL21 (DE3) was transformed to express the recombinant SRY protein. Transformed colonies were later cultured on an LB agar plate containing 50 µg/mL kanamycin. Colony-PCR was carried out on 10 colonies to confirm the transformation. Positive clones were cultured in 20 mL of LB culture media containing 50 µg/mL kanamycin at 37°C in a shaker incubator overnight at 130 rpm. When the absorbance at 600 nm was reached 0.8, the clones were cryopreserved with a ratio of 1:9 in DMSO and immediately stored at –70°C.

For expression and induction of recombinant bovine SRY in BL21 E.coli (DE3), 15 mL of the overnight-cultivated culture (37°C) was added to 1000 mL of LB media supplemented with 50 µg/mL kanamycin and incubated in a shaker incubator (130 rpm) until OD reached to approximately 0.6 in 600 nm. Then, induction was carried out with various concentrations of IPTG (0, 0.25, 0.5, 0.8, 1, 1.5, and 2 mM) as inducer for expression of recombinant protein at different temperatures (16, 25, 28, 33, and 37°C) in variety of time intervals (2, 6, 8, 12, 16, 24, and 30 hours) after induction. Here, different levels of glucose (0, 0.5, 1, 1.5, and 2%) were also added to the media. The number of replication for each batch in this research was three times. When growth phase finished, bacteria was precipitated at 5000 g for 12 minutes at 4°C and the pellets were stored at –70°C overnight.

**Separating of soluble/insoluble and purification of recombinant SRY protein**

A single protocol was used to both purify SRY protein from bacteria and soluble/insoluble phase separation as follows. Cell pellets collected from 1-L induced culture and 50-mL control culture were washed with phosphate buffered saline (PBS), pH 7.4, and then washed cells were again precipitated at 5000 g for 12 minutes at 4°C and right after were resolved in lyses buffer (EDTA. 4Na 3 mM, 300 mM NaCl, 10 mM Imidazole, and 1% Triton x 100) containing 30 µg/mL DNase, 800 µg/mL Lysozyme, 12 µg/mL RNase, and 1 mM PMSF. The mixtures were incubated at room temperature for 2 hours and then sonicated by 15 times 60 seconds bursts with 90 seconds intervals rest in between, centrifuged afterward at 37500 g for 50 minutes at 4°C. Supernatant, considered the soluble phase, was collected and further used for applying onto a NI-NTA affinity chromatography. The precipitate, on the other hand, considered as the insoluble phase, was resolved in a solution containing 8 M urea, 100 mM sodium phosphate (NaH2PO4,2H2O), 100 mM Tris/Hcl, and 5 mM DTT for 14 hours at room temperature and sonicated by 60-second bursts, and 2-minute intervals. The resulting mixture was centrifuged at 49500 g for 60 minutes at 4°C and the supernatant was collected for purification of the recombinant protein using NI-NTA affinity chromatography. After extracting the recombinant protein from bacteria, the fractions of insoluble and soluble phases were applied separately onto NI-NTA His Select Affinity column and the recombinant SRY was purified according to standard protocol. Each fraction collected at washing and elution steps were analyzed for protein content using a Thermo Scientific 2000 nanodrop.

**SDS-PAGE**

In order to investigate the protein expression pattern in soluble/insoluble phases during culture, extracted proteins were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To do so, 40 µL of each sample with certain concentrations (according to absorbance at 280 nm) were taken at different times and temperatures and mixed with 10 µL sample buffer (SDS 10% w/v, beta-mercaptoethanol 10 mM, Glycerol 20% v/v, Tris/Hcl, pH 6.8), and boiled at 100°C for 5 minutes and then subjected to electrophoresis in a 12.5% separating Polyacrylamide gel.

**Statistical analysis**

In order to investigate the effects of different temperatures, various glucose and IPTG concentrations, and different incubation times on the mixture of total protein and recombinant bovine SRY produced by BL21 (DE3) and purified recombinant bovine SRY protein in the forms of soluble and insoluble, variance analysis table was formulated using SAS Version 9 (26).

**Results**

**Synthesis, cloning, and sequencing of bovine SRY nucleotides and amino acids**

Bovine SRY gene was successfully amplified by PCR (Fig. 1A), cloned, and sequenced. Bovine SRY-pET28a(+) - DH5α construct was developed favorably.
The results of Colony-PCR (Fig. 1B) and restriction enzyme digestion are shown in Fig. 1C. Sequencing results for bovine SRY gene are illustrated in Fig. 2 and as it is clearly observed, no mutation has occurred in the gene sequence. The sequences of amino acids of bovine SRY are also illustrated in Fig. 2. Regarding Fig. 2, NTD, HMG box, and CTD domains contained 53, 69, and 107 amino acids, respectively. The results of mass analysis (York University, England) showed that rBSRY has the largest homology with other species such as Bos Gaurus Frontalis, Bos Javanicus, Bos Mutus Grunniens, Bos Taurus, and Bison Bonasus. These results also suggested that recombinant bovine SRY has 26940 Da molecular mass.

Figure 1. (A) PCR products for bovine SRY gene and 1000 bp marker. As it is evident in the picture, no band is seen in the control sample (C). Purified samples of SRY gene 1, 2, 3, and 4 are gel purified and observed as single bands. (B) Gel electrophoresis (1%) of Colony-PCR product of 690 bp SRY gene, cloned in expression plasmid of pET28a(+) with a 1 kb marker (M), positive clones (1, 2), and negative clone (3). (C) Gel electrophoresis (1%) of products from digesting of pET28a(+) + Bovine SRY by BamHI and EcoR1 (1), restriction enzyme digestion of constructs pET28a(+) + bovine SRY with BamHI (2) and 1 kb marker (M).

Figure 2. Sequence of nucleotides and amino acids in recombinant bovine SRY protein. Sites for EcoRI and BamHI restriction enzymes are shown in pink. The sequences of amino acids for NTD, HMG box, and CTD domains also are shown.
Table 1. Homology between SRY recombinant protein/gene with corresponding ones in other mammalian.

| Homologies       | Human | Mouse | Guar | Buffalo | Sheep | Goat | Deer | Maral | Pig |
|------------------|-------|-------|------|---------|-------|------|------|-------|-----|
| Nucleic acid homologies | 71%   | 43%   | 99%  | 97%     | 94%   | 93%  | 92%  | 91%   | 53% |
| Amino acid homologies  | 52%   | 23.4% | 99%  | 94%     | 88%   | 85%  | 84%  | 83%   | 61% |

Recombinant bovine SRY protein sequence and also the cloned bovine SRY gene sequence had the highest homology with Guar and the lowest with mice.

The homology of nucleotides and amino acid sequences in bovine SRY is shown in Table 1. Based on the data from Table 1, the sequence of amino acids in recombinant bovine SRY protein and also the sequence of the cloned bovine SRY gene nucleotides showed the highest homology with guar and the lowest with mouse. In the recombinant bovine SRY N-TD, HMG box, and C-TD regions consisted of 53, 69, and 107 amino acids, respectively.

**Studying recombinant SRY protein expression profile and statistical analysis**

By applying different optimization factors as mentioned in the Materials and Methods section, it was determined that IPTG concentrations of 0.5 mM, 0.8 mM, and 1.5 mM at temperatures of 20, 28, and 37°C at the presence of 0-1.5% glucose after 8, 16, and 24 hours of incubation after induction yielded the highest total protein compared with other conditions. Glucose and IPTG concentrations had meaningful effects (p < 0.01) on the total protein expression in E.coli BL21 (DE3) and bovine SRY at temperatures of 20, 28, and 37°C (data not shown). In Table 2, the total protein and rSRY protein average amounts in the lyses of bacterial cells are shown. The lowest ratio belonged to no glucose/inducer present in the mixture (Table 2). Regarding variance analysis (ANOVA), it could be concluded that different IPTG concentrations and temperatures affected the solubility of recombinant SRY protein with a meaningful difference (P < 0.01, data not shown). In contrast, the effects of different glucose concentration exerted no significant effects on the solubility of recombinant SRY protein. The greatest and lowest total of mixed protein and rSRY protein average amounts in the lyses was in IPTG 1.5 mM, Glu 1.5% (328.80 mg/L ± 0.21) at 8 hours after induction in 37°C, and IPTG 0.5 mM, Glu 0% (272.57 mg/L ± 0.58) in 20°C at 24 hours after induction, respectively (Table 2). Also, the average amounts of mixed protein and rSRY protein in control groups was shown in Table 2. In order to cooperate averages in each temperature point we used Duncan’s test. In Table 3 summarizes the mean amount of expressed recombinant protein in both soluble and insoluble phase corresponding to each factor being changed. The highest amount of soluble protein (2.143 ± 0.01 mg/L LB) reached where IPTG and glucose concentrations were 0.5 mM and 0.5% at 20°C (24 hours incubation after induction), respectively. Purified insoluble protein had the highest yield in an increasing order when IPTG of 1.5 mM and glucose of 1.5% were used at 20°C (24 hours), 28°C (16 hours), and 37°C (8 hours) after induction. At 37°C, 28°C, and 20°C the highest amounts of soluble protein were 1.236 mg/L ± 0.029 (IPTG 0.5 mM, Glu 0.5%), 2.18 mg/L ± 0.12 (IPTG 0.5 mM, Glu 1%), and 2.143 mg/L ± 0.014 (IPTG 0.5 mM, Glu 1%), respectively (Table 3). In contrast, the greatest amounts of

Table 2. Mean protein value of the mixture of total protein and SRY (mg ±SE/L culture) extracted from E.coli BL21 (DE3) after lyses.

| Culture | Glucose (%) | IPTG mM | 20°C (24 hr) | 28°C (16 hr) | 37°C (8 hr) |
|---------|-------------|---------|--------------|--------------|-------------|
| 1       | 0           | 0       | 269.14±0.58  | 271.79±0.27  | 301.98±0.07 |
| 2       | 0           | 0.5     | 272.57±0.58  | 279.72±0.85  | 310.18±0.46 |
| 3       | 0           | 0.8     | 273.48±0.75  | 279.81±1.4   | 312.03±0.33 |
| 4       | 0           | 1.5     | 275.54±0.61  | 281.53±0.19  | 315.67±0.66 |
| 5       | 0.5         | 0       | 280.04±1.46  | 271.54±0.32  | 302.23±0.34 |
| 6       | 0.5         | 0.5     | 274.67±0.59  | 283.09±0.53  | 316.91±0.32 |
| 7       | 0.5         | 0.8     | 277.96±0.45  | 285.53±0.32  | 325.67±0.34 |
| 8       | 0.5         | 1.5     | 280.98±0.27  | 289.93±0.40  | 326.68±0.37 |
| 9       | 1           | 0       | 272.21±0.62  | 271.35±0.27  | 301.82±0.35 |
| 10      | 1           | 0.5     | 276.02±0.25  | 284.49±0.34  | 320.44±0.19 |
| 11      | 1           | 0.8     | 280.46±0.72  | 287.78±0.52  | 319.16±0.09 |
| 12      | 1           | 1.5     | 283.81±0.28  | 292.39±0.19  | 326.01±0.28 |
| 13      | 1.5         | 0       | 272.7±0.31   | 273.81±0.35  | 304.02±0.51 |
| 14      | 1.5         | 0.5     | 277.02±0.55  | 286.30±0.71  | 321.22±0.64 |
| 15      | 1.5         | 1.5     | 283.34±0.11  | 291.13±0.36  | 323.62±0.24 |
| 16      | 1.5         | 1.5     | 284.13±0.47  | 292.71±0.33  | 328.80±0.21 |

The mean of mixed total and recombinant SRY protein (±standard error, SE) after lysing bacteria cells using lysis buffer (isolating soluble proteins) and 8 M urea (isolating insoluble proteins), in different time periods, temperature points, IPTG concentrations, and glucose levels. a) Different temperatures of 20, 28, and 37°C after 24, 16, and 8 hours incubation after induction, respectively. In Duncan test the averages containing the same letters do not have a significant effect at each temperature point.
The amount of recombinant SRY protein (mg ± SE / L culture) after purification using Ni-NTA affinity chromatography in the form of soluble and insoluble (Inclusion Bodies) on the scale of mg/L of liquid LB media.

| Subject | Glu. (%) | IPTG mM | 20°C | 28°C | 37°C |
|---------|----------|---------|------|------|------|
| 1       | 0        | 0       | 0.01 ± 0.08g | 0.19± 0.01 | 0.13± 0.01 |
| 2       | 0        | 0.5     | 1.80± 0.03 | 1.42± 0.02 | 1.16± 0.06 |
| 3       | 0        | 0.8     | 1.87± 0.04 | 2.07± 0.03 | 1.04± 0.05 |
| 4       | 0        | 1.5     | 1.14± 0.05 | 1.09± 0.04 | 1.18± 0.03 |
| 5       | 0.5      | 0       | 0.09± 0.01 | 0.12± 0.02 | 0.09± 0.02 |
| 6       | 0.5      | 0.5     | 2.14± 0.01 | 1.84± 0.05 | 1.24± 0.03 |
| 7       | 0.5      | 0.8     | 1.26± 0.03 | 1.67± 0.04 | 1.11± 0.04 |
| 8       | 0.5      | 1.5     | 1.27± 0.04 | 0.96± 0.04 | 0.85± 0.03 |
| 9       | 1        | 0.9     | 0.09± 0.01 | 0.13± 0.01 | 0.10± 0.02 |
| 10      | 1       | 0.5     | 1.88± 0.03 | 2.18± 0.12 | 1.17± 0.06 |
| 11      | 1       | 0.8     | 1.14± 0.05 | 1.88± 0.03 | 1.60± 0.05 |
| 12      | 1       | 1.5     | 0.96± 0.05 | 1.04± 0.05 | 0.92± 0.03 |
| 13      | 1.5     | 0       | 0.08± 0.01 | 0.11± 0.01 | 0.09± 0.01 |
| 14      | 1.5     | 0.5     | 1.52± 0.03 | 1.86± 0.05 | 0.97± 0.02 |
| 15      | 1.5     | 0.8     | 1.45± 0.02 | 1.54± 0.14 | 1.13± 0.04 |
| 16      | 1.5     | 1.5     | 0.87± 0.02 | 1.21± 0.05 | 0.87± 0.03 |

The amount of recombinant SRY protein (± standard error, SE) produced in the form of soluble and insoluble (Inclusion Bodies) purified on a Ni-NTA affinity chromatography. a) The effect of different temperatures of 20, 28 and 37°C after 24, 16 and 8 h incubation after induction, respectively. In the Duncan test the averages contain same letters have not significant effect.

Inclusion bodies were attained in concentrations of IPTG 1.5 mM, Glu 1.5% at 37°C (15.11 mg/L ± 0.06), 28°C (12.02 mg/L ± 0.07), and 20°C (6.98 mg/L ± 0.23), respectively (Table 3). When glucose was eliminated from media, with 1.5 mM IPTG, the highest amount of insoluble protein was produced at 37°C (Table 3). In Fig. 3, protein expression profile of both total protein and NI-NTA His Select gel affinity purified protein is shown. Recombinant bovine SRY protein molecular weight was estimated approximately 30 KDa.

Discussion

SRY protein, as a member of transcription factors of SOX9 family, is found in all animal species (13). SRY gene was first cloned by Daneau et al. (27) and determined to have 911, 687, and 202bp in its 5‘-UTR, ORF, and 3’-UTR, respectively, coding a protein of 299 amino acid residues. In the present study, a 690 fragment of SRY gene was successfully cloned using PCR and transferred to the expression vector of pET28a (+). An engineered strain of E.coli, DH5α, was used to clone the gene and finally the cloned gene was sequenced. Sequencing results revealed that the cloned SRY gene (http://molbiol-tools.ca/Homology.htm) had the highest homology with gua (99%) and the least with mouse (43%). In order to produce the recombinant bovine SRY protein, the construct of SRY-pET28a (+) was transferred into an engineered strain of E.coli, BL21 (DE3). Purification step was carried out using a Ni++- linked affinity chromatography. The amino acid sequencing results confirmed the highest homology of
99% with guar and the lowest of 23.4% with mouse. Using Polymerase Chain Reaction (PCR) and Southern Blot techniques to study buffalo sexed fetus, Fu et al. (28) showed that SRY gene is only present in male ones and not in female fetuses. The gene coding regions were highly similar to those in deer, water buffalo and Holstein Friesian bull. Cheng et al. (29) found SRY gene sequence to be genetically changed only slightly especially within coding and 3’domains. Studying on bovine SRY gene (30) indicated that SRY mRNA was less in sperm compared to testicles. Western blot data that they gathered confirmed bovine SRY with molecular weight mass of 27 kDa. They also suggested SRY gene to be located at the sperm head, which encompasses Y chromosome (30). In 1996, Daneau et al. (31) carried out a cloning set of experiments on SRY and reported two fragments of 1664 and 106 bp for genomic DNA and cDNA respectively. Regarding pig SRY, ORF is composed of 624 bp coding a protein with 208 amino acids. In this protein, 3 domains of HMG box, N terminal, and C terminal consist of 79, 59, and 70 amino acids, respectively. From a structural perspective, bovine SRY is highly similar to that in human, pig, sheep, goat, and other mammals, having 3 domains of N terminal, HMG box, and C terminal. This gene greatly differs between bovine and mouse regarding N terminal in mouse, which has 2 amino acids and a glutamine rich (Q-rich) sequence in CTD. The NTD domain in mammalian consists of 30-60 amino acids (32). In the present study, this gene was affirmed to have 53 amino acids in bovine and an N terminal domain similar to that of human, pig, sheep, goat, and mouse with respective percentages of 39, 47, 84, and 69. HMG box in this gene was found to possess 69 amino acids and had the highest similarities with goat and the lowest with mouse. SRY protein CTD on the other side, had 107 amino acids similar to that of human, pig, sheep, and goat with similarity percentages of 28, 32, 86, and 82, respectively. These data suggested that HMG box has undergone the least changes and diversions during time and is preserved up to 75-80% in mammalian. The molecular mass of recombinant bovine SRY protein on SDS-PAGE was approximately 30 kDa. With regard to the sequence of amino acids such as Arg, His, and Lys in SRY protein, the molecular mass of this protein on SDS-PAGE gel was nearly 30 kDa.

E. coli possesses many desired criteria when it comes to expressing recombinant proteins including high rate of replication and recombinant protein production, being cost beneficial, easy to work with and available. However, prokaryotic protein expression system is not all good for eukaryotic protein expressing since the recombinant protein produced is often insoluble (Inclusion Bodies). Adjusting some culturing influential factors may help with overcoming this challenge. By altering temperature, IPTG concentration, and adding osmolites and molecular chaperones, it would be possible to increase the solubility of the recombinant protein expressed in the bacteria. Osmolites increase osmotic pressure by accumulation in cytoplasm and thus provide the produced recombinant proteins with stability and better folding in vivo and in vitro, preventing accumulation whilst they are being expressed with a high rate (33). Here, different levels of glucose, as an Osmolites, had no significant effect on the solubility of recombinant bovine SRY, while the insoluble form (IBs) of SRY at 37°C (15.11 ± 0.06 mg/L LB), 28°C (12.02 ± 0.07 mg/L LB), and 20°C (6.98 ± 0.23 mg/L LB) increased in amount. These results showed that glucose led to increased growth in E. coli cells as well as high expression levels of recombinant bovine SRY protein. Then, rSRY had not enough time to be folded in; therefore, the major fraction of recombinant protein was produced in the form of inclusion bodies. Replication and induction of bacteria cells under osmotic stress at the presence of salt, various sugars, sorbitol, ethanol, glycine betaine, and decreasing the replication temperature after induction led to improved recombinant protein solubility and less accumulation in the form of inclusion bodies in the cytoplasm (34-37). Decreasing the temperature between 28°C and 30°C also increased the solubility in E. coli (38), which was consistent with the results presented in this study. Our results indicated that decreasing temperature from 37 to 20°C increased the rate of “soluble” fraction of the recombinant protein SRY expression (Table 3). By reducing temperature, the growth rate of bacteria cells decreased while the solubility of recombinant bovine SRY increased as a result of a slow rSRY production rate in the media and sufficient time for folding. On the other hand, it has been stated by Prasad et al. (39) that by decreasing the temperature after induction of recombinant GFP protein expression, the soluble (active) amount of this protein decreased significantly, which they resolved by introducing different osmolites into media, changing the inducer concentration and induction time. Rabhi-Essafi et al. (34) successfully increased the rate of soluble IFNα 2B protein yield in E. coli BL21 up to 70% by decreasing both temperature and inducer concentration. Shuo-shuo et al. (40) demonstrated a fall in production of soluble recombinant protein of cold-active lipase Lip-948 to 1.7% where osmolites were wiped out of the media and an increase up to 19.8% where osmolites were of the elements of the mixture. A meaningful difference was observed when
Voulgaridou et al. (41) decreased the temperature from 37 to 25°C during the production of recombinant BMP fused with ALDH3A protein leading to increase the solubility of the protein from 2.5 to 3.5%. They observed no such effect by decreasing the temperature from 25 to 18°C. Using chaperones and osmolites, and under various temperature conditions, Paul and Chaudhuri (42) improved the solubility of recombinant Maltodextrin Glycoside (MalZ) while different temperature points did not make any meaningful improvement in the MalZ expression rate. In the present study, decreasing IPTG concentration from 1.5 mM to 0.5 mM and decreasing the temperature after induction from 37 to 20°C boosted the solubility of recombinant SRY protein synthesized. By adding 1.5% glucose to the media already induced with 1.5 mM IPTG, replication accelerated but the amount of soluble protein expressed at temperature points of 20, 28, and 37°C decreased indicating the effect of high levels of glucose and inducer on the activity of T7 promoter in pET28a(+), leading to accumulation of the recombinant protein, as high inducer concentration leaves the protein with insufficient time for folding. At low temperatures and inducer concentrations, the rate of solubility improved but approximately 85% of recombinant bovine SRY protein was produced in an insoluble (IBs) form. Therefore, we suggest that the effects of other osmolites and chaperones such as Sorbitol, Glycerol, Arginine, Proline, and Glycine would also be tested on improving the rSRY protein solubility.

Conclusion

Herein, bovine SRY gene was successfully cloned in an expression vector and after going through with various optimizing processes, the recombinant SRY protein was produced and purified. In order to express this protein in BL21 (DE3) with an optimized rate and increased soluble to insoluble (Inclusion Bodies) ratio, different possible factors were tested during cultivating the bacteria including temperature, IPTG (inducer), and glucose (as an osmolite) concentration, induction, and incubation time. At high temperatures and high concentration of inducer, the insoluble fraction of produced protein was raised while keeping the temperature at a mediocre point and decreasing the inducer concentration that caused the soluble fraction to rise. By decreasing the temperature to the lowest possible point, the solubility of the protein owned the highest fraction. Also, results of this research shown that by decreasing concentration of inducer, the amount of Inclusion bodies was decreased, while the amounts of soluble or active form or rBSRY was increased.

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