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RESEARCH ARTICLE

AL-hajaj M. A. Maarich*
Othman R. Monther **

CLONING AND EXPRESSION OF BOVINE SOMATOTROPIN GROWTH HORMONE GENE FROM LOCAL COWS BREEDS OF BASRAH CITY, IRAQ

ABSTRACT:

The present study, have undertaken the cloning of bovine growth hormone or bovine somatotropin gene from local cows breed in Basrah city, and optimizing the conditions for its high-level expression in *Escherichia coli*. To achieve this goal the cDNA for bovine growth hormone was inserted in to the *Pst*1 site of pBR322 via the poly dC: poly dG joining technique to produce the recombinant vector. The cloning vector was then transformed into *E. coli* HB101. The efficiency of transformation also determined to be 2×10^7 cfu/µg. The fermentation strategy for high cells density growth of *E. coli* harboring the bGH gene was carried out and the highest cells density level was determined to be 1.540 at OD_{600} after 8 hs of cells growing. Following solubilization and refolding, bovine growth hormone was purified by using ion exchange chromatography and the result was a single visible band with 22KDa on 12% SDS-Polyachrylamid gel which represents the purified bGH.

KEY WORDS:

Bovine Growth hormone, Recombinant bovine somatotropin, milk, cow reproduction.

CORRESPONDENCE:

Mohamad A. Maarich AL-hajaj *

*Dept. of Biology, College of Science, Basrah University, Iraq

Email:

Rasha Monther Othman **

**Dept. of Microbiology, College of Veterinary Medicine, Basrah University,Iraq

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INTRODUCTION:

One of the first potential biotechnology products for animal production is bovine somatotropin (bST). Bovine somatotropin biotechnology research has involved scientists and is supported from universities, as well as private industry (Bauman, 1992). In contrast to steroids, bST is a protein hormone composed of 190-191 amino acids with a molecular mass of 22 KDa naturally secreted from the anterior mammalians pituitary gland. The amino acids sequence of somatotropin is known for many species, including cattle (Wallis, 1975; Kostyo and Reagan, 1976). The first step in all cloning project was hunt for a suitable gene. A variety of growth hormone (GH) genes and cDNAs have been cloned, including those derived from cow (Gordon *et al*., 1983) and sheep (Orian *et al*., 1988). The full length of bST gene was reported to be 2856 bp, which is arranged as five exons interrupted by four introns with 5` and 3` untranslated regions (Gordon *et al*., 1983).

Bacterial expression systems are the preferred choice for the production of many prokaryotic and eukaryotic proteins. The reasons for this lie in the cost-effectiveness of bacteria, their well-characterized genetics, and the availability of many different bacterial expression systems. Among the hosts available for the recombinant expression, *Escherichia coli* is in an exceptional position. It is especially valued because of its rapid growth rate, capacity for continuous fermentation, low media costs and achievable high expression levels (Yin *et al*., 2007).

Most expression systems for *E. coli* are plasmid-based. This ensures, in most cases, a relatively easy cloning of the recombinant gene into the host cell and facilitates many copies of the gene in a single cell. On the other hand, the recombinant gene expression from plasmids demands high plasmid stability that is often achieved by the use of antibiotic resistance gene markers (Yin *et al*., 2007).

MATERIAL AND METHODS:

Bacterial strain and plasmid:

Plasmid pBR322 (Amp ^{s}, Tet^{s}) was used as cloning vector and the *E. coli* HB101 competent cells of genotype (F–, *thi*-1, *hsd*S20 (rB–, mB–), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (str^{r}), *xyl*-5, *mtl*-1) was used for transformation and expression, both plasmid and bacterial strain was purchased from Promega®

Total RNA Purification:

Total RNA was purified from fresh blood sample of the cow according to the protocol of the QIAamp® RNA Blood Mini Kit (Qiagen), and its concentration was determined according to Sambrook *et al*. (1989).

Reverse transcription and cDNA amplification:

RT-PCR was carried out for the amplified bovine growth hormone cDNA on the previously extracted RNA sample using (QIAGEN One Step RT-PCR Kit) and specific set of primers which have the following sequences , forward primer 5`- ATG ATG GCT GCA GGC CCC -3` and reverse primer 5`- GGC AAA CAA CAG ATG GCT -3 (Bioneer , Korea). The PCR conditions were 94ºC for 1.5 min., followed by 30 cycles of denaturing at 94Cº/1min and annealing at 52.3°C/1 min. and extension at 72Cº/2 min. The final extension cycle was at 72Cº for 10 min. The PCR product was electrophoresed in 2% agarose gel and successfully amplified PCR band was purified by using SV Gel and PCR Clean- up system kit (Promega, USA).

Cloning Project:

The cloning technique for the production of bovine growth hormone was carried out according to Buell (1987), de Boer *et al*. (1993), and Miller *et al*. (2006) with simple modification, using the dC/dG Homopolymer tailing technique. The bovine somatotropin cDNA was prepared and tailed with dCTPs for further ligation with a vector using Terminal Transferase Enzyme which was used according to manufacturer's instructions (Promega®). On the other hand, the expression plasmid pBR322 was linearized by restriction cleavage using the *Pst*I endonuclease restriction enzyme and then tailed with dGTPs using Terminal Transferase Enzyme procedure. The annealing reaction was then prepared according to the protocol of Powell and Gannon (2001) with simple modification: In a reaction tube, 5 µl of 1 × annealing buffer (500 mM Tris-HCl, 10 mM EDTA, 1 M NaCl) was mixed with 10 µl of dC tailing bGH cDNA and 10 µl of dG tailing PBR322 at the ligation molar ratio 1:1. The reaction was heated to 70°C for 30 min in a water bath then heat was stopped and the sample reaction was allowed to cool in the water bath to room temperature over a period of 12 h.

The tailing and ligation reaction product were electrophoresed in 2% agarose and detected under the UV transilluminator Fro transformation step, one µl of the recombinant DNA (annealed mixture) was directly added to 100 µl of *E. coli* HB101competent cells according to the protocol provided along with the genetic modified competent *E. coli* HB101 (Promega®), then each transformation reaction was added to 900µl of cold 4°C LB broth medium, and incubated for 60 minutes at 37°C with shaking (approximately 225rpm). For each transformation reaction, the cells were diluted 1:10; 1:100 and 1:1000. Then 100µl of each diluted and undiluted cells were plated on LB plates containing 100µg/ml tetracycline antibiotic. To confirm the results of the transformation step, random choose of single positive transformed colonies that growing on tetracycline LB medium were spread on LB agar supported with Ampicillin (final concentration 100µg/ml). On the other hand, the plates with single colonies (10 - 100) were selected for determining the efficiency of transformation, which was carried out according to method of Hanahan (1985).

Bovine ST Expression in *E coli* HB 101:

The protein expression and production in bacteria was carried out according to Aviv *et al.* (2001). The fermentation media were prepared and bacteria grown at 37°C., with shaking at 250 rpm. Fermentation was continued up to 14 h for each flask and cells density level and growth were monitored at an interval of two hours, till the OD600 was reached to 0.5 - 1.5 A°. The bacterial cells at this point were harvested and prepared for refolding and purification.

Refolding and Purification of Produced bST:

Cells were grown, harvested and broken, and broken cells, pellet fractions were obtained which has been carried out according to Wingfield *et al*. (1987). Then 20 µl of purified bGH were analysis by SDS-PAGE according to method of Laemmli (1970), and the rest of the purified products were concentrated using freeze dryer Labconco for the gel chromatography steps based on anion exchangers DEAE-Sepharose which was carried out according to Wingfield *et al*. (1987). BGH did not bind to the column resin and was located in the 'flow-through' fractions then the fractions contents were subjected to 12% SDS-PAGE. The peak profile with 22 KDa was filtered through a millipore filter (0.22 µm) and the protein concentration of each purified steps (protein concentration of inclusion bodies, protein

concentration of refolded protein and protein concentration after ion–exchange purification) were estimated according to Lowry *et al.* (1951) method.

RESULTS AND DISCUSSION:

Bovine Total RNA Purification:

The total RNA was isolated from whole bovine blood and its concentration was determined (Table 1). On the other hand, the agarose gel electrophoresis was carried out to check the quality and purity of the extracted RNA. The bands of 28S rRNA and 18S rRNA were observed on the gel as shown in figure 1. The agarose gel electrophoresis showed clearly that RNA does not undergo any degradation during extraction.

Table 1. Absorptions and concentrations of purified bovine total RNA

Whole blood (500 µl)		
Sample NO.	Abs at 260nm.	Concentration of total RNA(µg/ml)
1	0.910	36.4
2	0.563	22.52
3	0.941	37.64
4	0.706	28.24
5	0.968	38.72
6	0.667	26.68
7	0.858	34.32

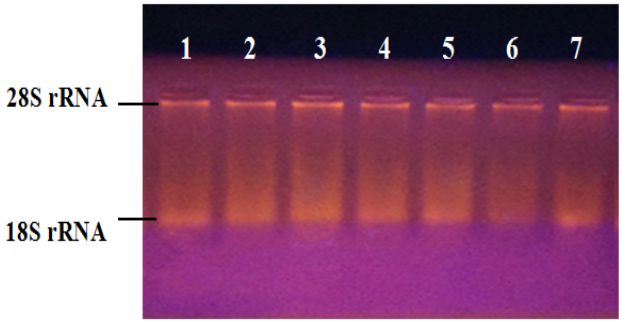

Fig. 1. 0.7% agarose gel electrophoresis of bovine total RNA bands show the discrete 28S &18S ribosomal RNAs; All Lane = Positive Samples (presence of RNA).

RT-PCR Products of bST cDNA:

The bovine somatotropin growth hormone cDNA was prepared from total RNA by RT-PCR using specific primer as detailed in the materials and methods. The best amplification of the bST growth hormone cDNA was observed at 52.3°C annealing temperature. Under these optimal conditions, the expected fragment of 677 bp was successfully amplified as shown in figure 2. These results are in accordance with those reported by Khalid (2008).

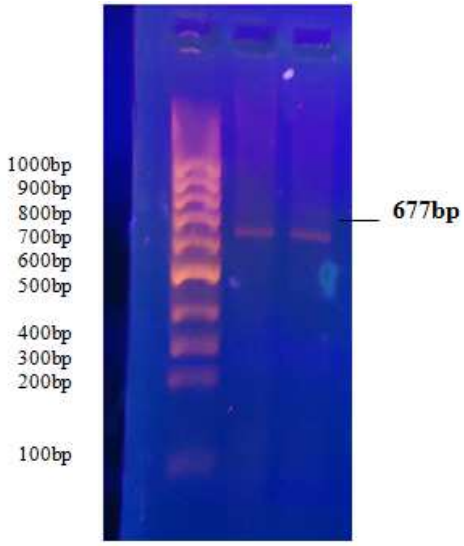

Fig. 2. RT-PCR products of Bovine Somatotropin GH1 cDNA

Lane 1: 100 bp DNA Ladder.

Lanes 2 and 3: Bovine GH1 cDNA

Addition of Homopolymer dC Tracts to the 3'-Ends of bST Double-Stranded

The strategy of producing a cloning vector using dC/ dG technique is demonstrated in figure 3. The double stranded cDNA RT-PCR products were prepared for cloning by using the terminal deoxynucleotidyl transferase enzyme (TDT).

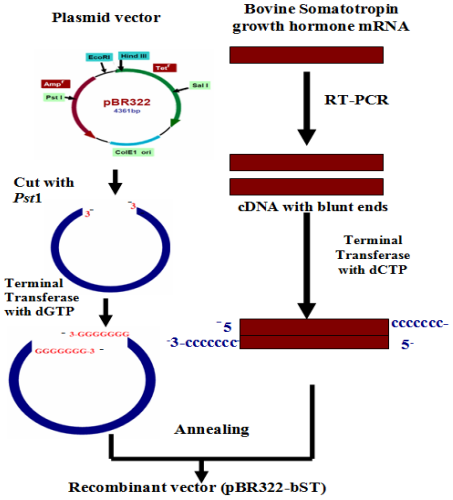

Fig. 3. Schematic Outline of dC/dG Cloning Technique.

This enzyme has the property of adding the same type of nucleotides to the blunt ends of the duplex cDNA in the presence of Co2+ ions instead of Mg2+ ions, which allows the enzyme to accept double-stranded DNA as a primer instead of a single-stranded DNA (Roychoudhury *et al.*, 1976; Brutlag *et al.*, 1977). The gel electrophoresis method was applied routinely to determine the dC tail length of cDNA molecules. Since the optimal conditions were established for the enzyme to produce tails of approximately 20 residues in each 3--OH end of ds cDNA in 10 minutes (Deng and Wu, 1981; Powell and Gannon,

2001). So, the expected fragment of bST cDNA 677bp in addition to ~ 40 bp dC tail were ~717bp as demonstrated in figure 4.

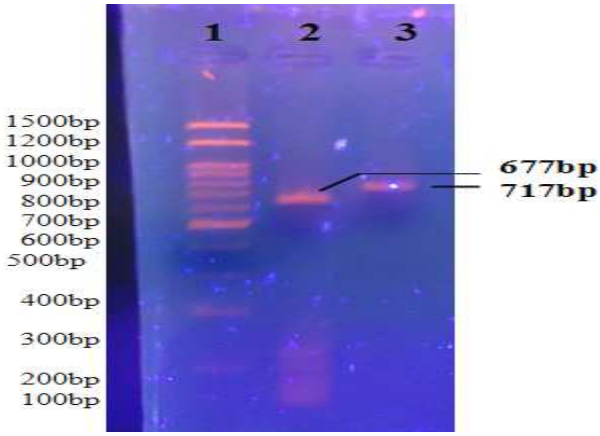

Fig. 4. Bovine Somatotropin GH1 cDNA /dC Tailing Ligation.

Lane 1: 100 bp DNA Ladder.

Lane 2: Bovine GH1 cDNA Ligated to /dC Tailing Ligation >700 bp.

The Pst1 restricted vector was then homopolymer tailed with dGTP, and consequently ligated to the bST cDNA fragments previously tailed with the complementary dCTP. Moreover, the results of the ligation steps were documented by the agarose gel electrophoresis and displayed in figure 5. In this figure, the pBR322 with a length of 4361bp was linearized by restriction cleavage using the endonuclease PstI (Lane 2). Then tailed with dGTP under the conditions described in the Materials and Methods (Lane 3) and consequently to construct a hybrid plasmid. The ds cDNA/dC tailing was annealed with pBR322/ dG tailing .In this case, the expected fragment band was ~ 5118bp as observed clearly in lane 4. These results indicated that the ligation steps were successful and this finding agreed with other studies conducted on cloning the bST gene using the dC/dG tailing technique such as Keshet *et al*. (1981), Kim et al. (1987), Buell (1987), de Boer *et al*. (1993), Aviv *et al*. (2001), and Miller *et al*. (2006). In all these studies we can obviously observe that inserting bST cDNA into the Pst 1 cleavage site of pBR322 by using the oligo dG / oligo dC tailing was promising and this procedure has many advantages than other methods (Sutcliffe, 1978; Vila-Komaroff *et al*., 1978; Owens and Cresst, 1980; Deng and Wu, 1981; Otsuka, 1981; Protter *et al*., 1982; Bolivar *et al*., 1992; Powell and Gannon, 2001).

Figure 6 shows the attempt to draw the possible experiment designed to determine a hybrid plasmid containing a 717bp segment of bST with the orientation of inserted cloned. Since only 573 base pairs are required to encode bST (Miller *et al*., 1980).

Therefore, we conclude that all the cDNA sequences required for synthesis of the bST growth hormone, as well as most, if not all, of the non-translated 3' region of the mRNA are included in the clone. In this figure we use the promoter of β- lactamase gene (*bla*) of pBR322 as bST growth hormone sequences promoter for further expression of the hormone. Since the *Pst*1 cleavage site is located at 3607 bp in the penicillinase gene of the plasmid pBR322 (Ambler and Scott, 1978; Sutcliffe, 1978). In this case, the location map of the region encoding the first amino acid of the bST is 740bp upstream from the *Pst*1 site (Keshet *et al*., 1981).

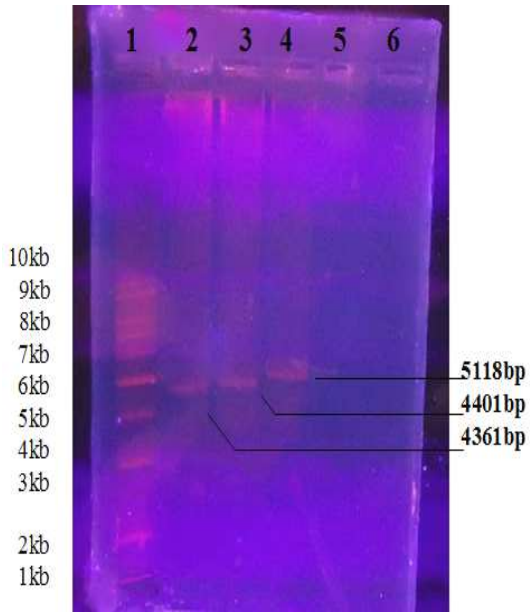

Fig. 5. The dC /cDNA and dG/ Plasmid Annealing Reaction.

Lane 1: 1 kb DNA Ladder.

Lane 2: 4361bp Plasmid pBR322.

Lane 3: Plasmid pBR322 dG/Tailing.

Lane 4: dC /cDNA and dG/ Plasmid.

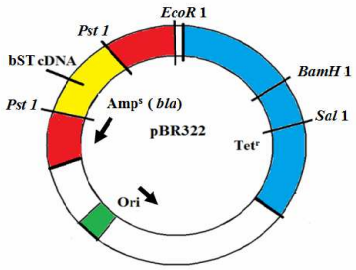

Fig. 6. The constructed designed of pbr322 carrying bST growth hormone cDNA sequences fused to the β-lactamase gene (Amp) and the orientation of insertion. The expression operon of β- lactamase gene of pBR322 is used for expression the bST cDNA

Transformation of *E. coli* Competent Cells:

The transformed *E. coli* cells were demonstrated by their culturing on the LB agar selectable medium supported with 100µg/ml tetracycline, the results were observed and given in figure 7 A. In this figure many single colonies were obtained and selected as transformed colonies. In addition, figure 7 B shows the sensitivity of transformed *E. coli* cells subcultures on LB agar supported with

100µg/ml ampicillin. The growth of bacteria in LB / Tet^r – Amp^s plates revealed that cloned genes with the promoter were transformed well into *E. coli* competent cells. Beside, the use of LB medium containing antibiotics is popular for screening clone genes (Shinichi *et al*., 2004). The results of the cloning of pBR322 with bST and transformation of signal sequence in *E. coli* HB101 were also successfully reported by other researchers (Keshet *et al*., 1981; Kim *et al*., 1987).

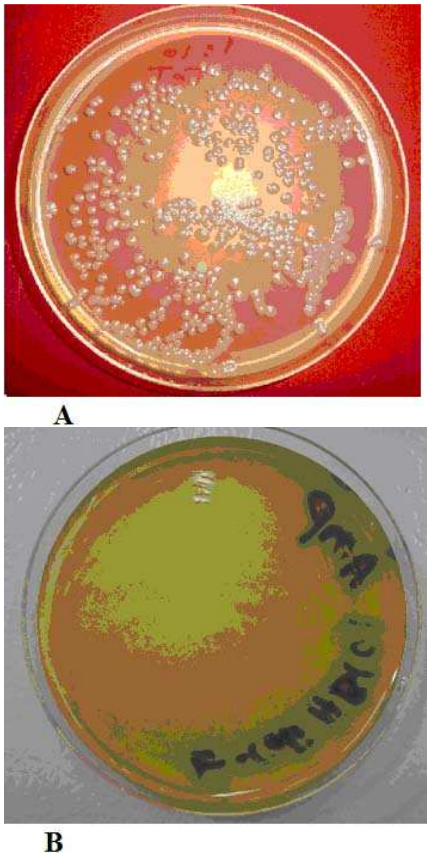

Fig. 7. Transformed *E. coli* HB101 competent cells on 1:10 LB selectable medium. (A) Transformed *E. coli* HB101 competent cells resistant to tetracycline. (B) Transformed *E. coli* HB101 competent cells sensitive to ampicillin.

On the other hand, figure 8 displays the result of 1:1000 dilution of transformed *E. coli* HB101 which was used to determine the efficiency of transformation. In this figure only 20 single colonies were counted and transformation efficiency was found to be sufficiently high (2×10^7 cfu/ µg pBR322 DNA) on LB/tet^r plates at 100µg/ml concentration. These findings are in agreement with the findings of Cohen *et al*. (1972), Inoue *et al*. (1990), and Sambrook and Russell (2001) who observed that the transformation efficiency was enhanced when using SOC medium. In these studies, the transformation efficiency reached 8.27×10^7 cfu/ µg DNA and 1.04×10^8 cfu/µg DNA when the bacteria were grown in SOC medium, but only at 3.40×10^7 cfu/µg DNA and 3.33×10^7 cfu/µg DNA in the LB medium, respectively. On the other hand and according to the manufacture (Promega), *E. coli* HB101 competent cells have normally high transformation efficiency greater than 10^8 cfu/µg. Since the optimal condition was established to obtain high transformation efficiency. The possible explanations of results could be attributed to the factors that affected the efficiency of transformation (Tu *et al*., 2005).

These factors are including the size and form of the transformed plasmid according to Hanahan (1983), the transformation efficiency declines linearly with increasing plasmid size. Another important consideration is the type of medium and the composition used for culturing recombinant strain. In the present study, we choose the LB broth medium. This medium is easy to make, has a fast growth rate of most *E. coli* strains, and can support *E. coli* growth OD_{600} two to three under a normal shaking incubation condition (250 rpm). In contrast , some researchers reported that the replacement of LB medium by SOB and SOC media significantly increased the transformation efficiency of a recombinant plasmid (Luria *et al* ., 1960; Bertani, 2004; Nikaido, 2009).

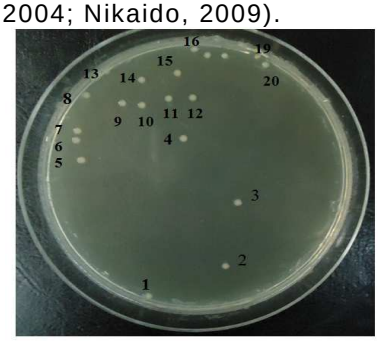

Fig. 8. Single colony transformed E. coli HB101 competent cells on 1:1000 LB selectable medium supported with tetracycline for determining the efficiency of transformation.

Bovine Somatotropin Expression in *E coli* HB 101:

Figure 9 shows the time profile of *E. coli* cells density in fermentation media. The data indicate that under the specific fermentation conditions used in this study, including 2% glucose to support the growth of *E. coli*, the curve growth level was almost the same, but the cell growth was highest at 8 h incubation and the maximum density level at OD_{600} was 1.540. After this point, the density level clearly descended. According to the cultivation results, the *E.coli* HB101 harboring recombinant plasmid grew well in the fermentation media supported with 2% glucose. These results are in accordance with the studies reported by Aviv *et al*. (2001), Gosset (2005), and Tan *et al*. (2007).

The other important finding in this study is the possibility of using the tetracycline as an indicator. The ability of *E. coli* HB101 to grow well in the media supported with a low concentration of tetracycline or even in 100 µg /ml as an indicator has no effect on the growth of *E. coli* (Skerra, 1994; Lutz and Bujard, 1997).

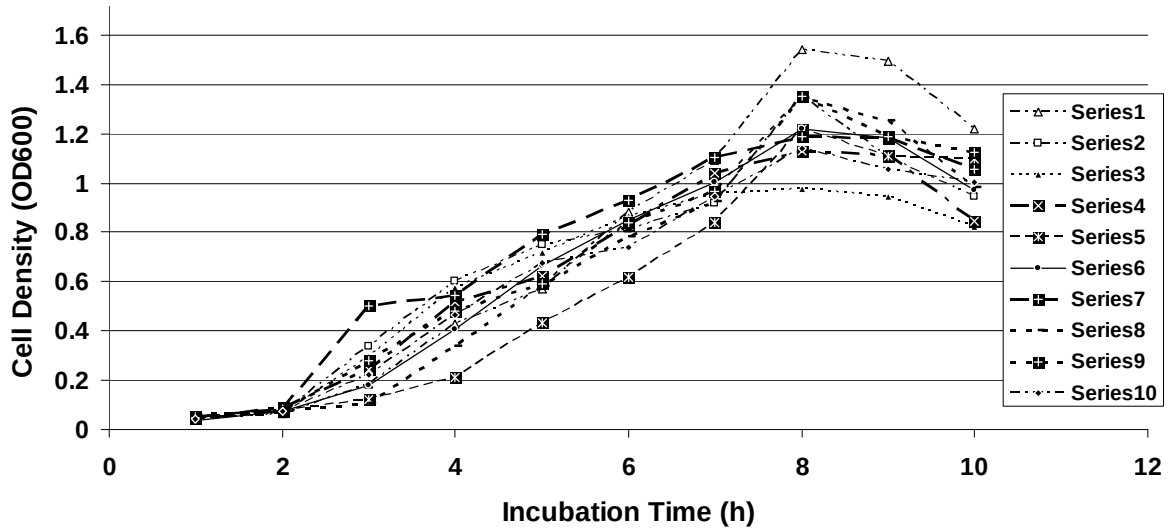

Fig. 9. Time profiles of E. coli HB101 cells harboring cloning vector pBR322, containing bST cDNA inoculated in to LB medium supplemented with 2% glucose and 100µg /ml tetracycline, cultured at 37°C with shaking at 250 rpm. The cells density OD600 (Y-Axis) for each flask culture was measured every hour (X-Axis).

Refolding and Purification of Produced bovine Somatotropin:

Figure 10 represents the array of proteins present in the broken cell suspensions. The lanes from two to six of the SDS/polyachrylamide gel show the 22KDa-stained band that was identified as a refolded bST. It was obvious from these findings that molecular mass of the reduced and active form of bST was ~22 kDa. While, the unreduced bST form had a lower molecular mass than the reduced form so it migrates faster and these results are in accordance with the results of Langley *et al*. (1987). These findings are common observations for disulfide containing proteins (Schoemaker *et al*., 1985).

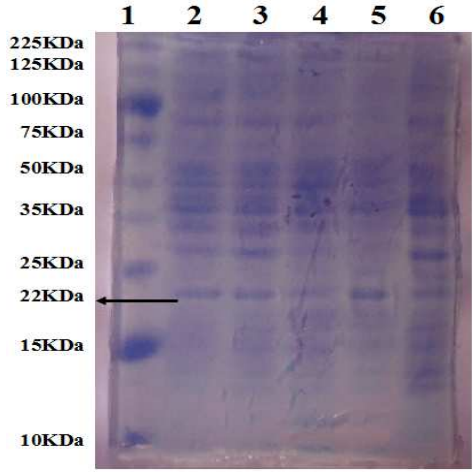

Fig. 10. SDS-PAGE analysis of bST Expression in *Escherichia coli* HB101 inclusion bodies induction.

Lane 1: Protein Marker.

Lanes 2 to 6: Total Cell Protein

~ 22 KDa of bST Expression in *E. coli*.

The high-level expression of eukaryotic proteins in *Escherichia coli* often results in accumulating them as biologically inactive and insoluble aggregates known as inclusion bodies. The first step to purify the bST from the inclusion bodies is solubilizing the contents of the inclusion bodies. To achieve this goal, we first lysis the bacterial cells using sonication and enzymatic digestion of cells and the suspension is then homogenized twice to reduce viscosity, this critical step is reported by Palmer and Wingfield (2000).

Then the cell solubilization was completed by exposure of inclusion bodies to a combination of strong detergents including urea, GdmCl and DTT. The use of these chaotropic media has been reported by a number of researchers (Schein, 1989; Eisenmesser *et al*., 2000; Palmer and Wingfield, 2000). Tsumoto *et al*. (2003), Fischer *et al*. (1993), Rudolph *et al*. (1997), Clark (1998), and Lilie *et al.* (1998) explained the effect of the solubilization step in protein recovery. In these studies, the recombinant proteins were solubilized from the inclusion body by using high concentrations of chaotropic reagents which resulted in the loss of the secondary structure leading to the random coil formation of the protein structure and exposure of the hydrophobic surface (Dill and Shortle, 1991).

In order to purify the bST growth hormone from the refolded system, ion exchange column chromatography is the most widely used and efficient method for purifying proteins, this method has always been the first option for protein purification as folded and unfolded forms of the same protein do not have the same overall charge (Scheich *et al*., 2004) .The IEC column is also useful for the removal of contaminations, nucleic acids and product-related impurities. The elution profile of the mixture and the entire fractions on the analytical column are shown in figure 11.

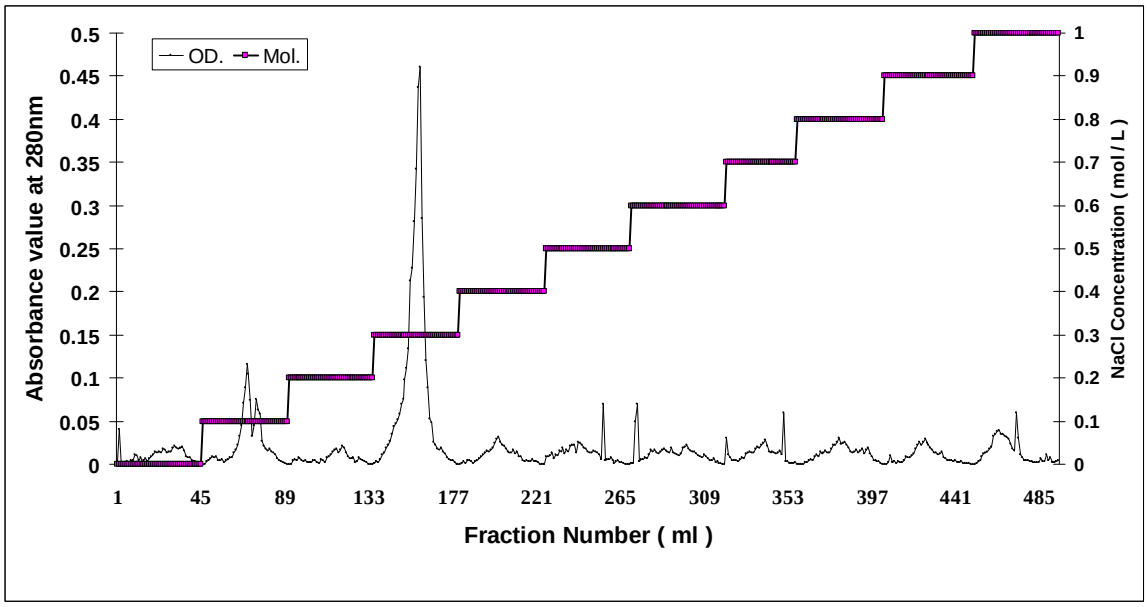

Fig. 11. Final purification of bST on anion exchange chromatography using DEAE-sepharose column. The peaks represent the elution profile of Ion-exchange chromatography conditions: DEAE sepharose with Linear Salt Gradient (0.0-1M NaCl).

In this figure, the refolded bST was eluted by a linear salt gradient solution which started with 0.0 M NaCl until it reached to 1M NaCl. Each peak was then analyzed by 12% SDS/polyachrylamide gel electrophoresis to check and determine the molecular weight of the purified protein. It was observed that the target peak of bST was present within the 0.3 M and this peak represented the eluted ~ 22KDa bST which is demonstrated in figure 12.

Our results indicated that the refolded bST was successfully purified from the extracellular proteins using the ion–exchange (DEAE-Sepharose) column. The constructions of an affinity chromatography system as a current purification step in the process of purification of bST growth hormone have several advantages. This system is easy to build and use; it has a low cost and the possibility of purifying in a gentle process a large volume of samples solution, which is often much greater than the volume of the column itself. On the other hand, these results agree with other results reported by Wingfield *et al.* (1987) and Sami *et al*. (2008).

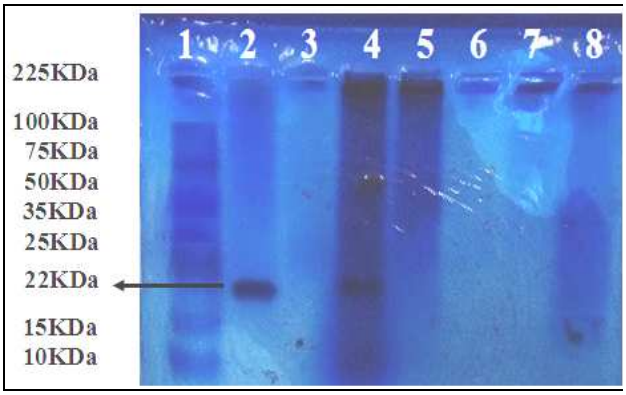

Fig. 12. The purity of the bST after IEC gel filtration, analyzed by 12% SDS-polyachrylamide gel electrophoresis

Lane 1: Protein Marker.

Lane 2 and 4: Purified Refolded bST growth hormone with 22KDa.

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تنسيل وتعبير جين السوماتوتروبين من الابقار المحلية في البصرة

محمد عبدالمحسن معارج الحجاج*، رشا منذر عثمان**

*كلية العلوم، جامعة البصرة، جمهورية العراق

**كلية الطب البيطري، جامعة البصرة، جمهورية العراق

تم في هذه الدراسة تنسيل ألجين المختص بهرمون النمو ألبقري (السوماتوتروبين ألبقري) من سلالات الأبقار المحلية لمدينه البصرة وتهيئه الظروف المثلى لتعبيره في بكتريا *Escherchia coli*. أخذين بنظر الاعتبار أيضا استخدام طرق قليله التكلفة لعمليه إنتاج هرمون كامل البنية وتنقيته للحصول على هرمون فعال بيولوجيا، للوصول إلى هذا الهدف تم عزل واستخلاص الحامض النووي الرايبي RNA من دم الأبقار لاستخدامه كقالب لبناء وتضخيم cDNA الخاص بهرمون النمو ألبقري بواسطة زوج خاص من بادئاته و باستخدام تفاعل البلمرة المتسلسل/ الناسخ العكسي -RT PCR. إن الناتج من التضخيم والمتمثل 677 زوج قاعدي تم إقحامه بالموقع الخاص بأنزيم القطع *Pst*1 في بلازميد pBR322 عبر استخدام تقنيه poly dC /dG للربط وإنتاج ناقل منسل مع ألجين المحدد. تم تحويل الهجين المنسل إلى خلايا *Escherichia coli* HB101 المهندسة وراثيا بعمليه التحول البكتيري، وان الخلايا المتحولة تم انتقائها على أساس مقاومتها للمضاد الحيوي التتراسيكلين وحساسيتها للبنسلين. تم احتساب قابليه التحول البكتيري للخلايا البكتيرية المنسله وكانت 2×10^7cfu/µg. كما استخدمت عمليه التخمر للبكتريا المتحولة بجين السوماتوتروبين ألبقري للحصول على أعلى مستوى لنمو الخلايا وذلك باستخدام طريقه الاستزراع في وسط LB في دورق زجاجي والمدعوم ب 2% من سكر الكلوكوز وحضن الدورق بالحاضنه الهزازه عند 37 درجه مئوية . و بلغت أعلى نسبه امتصاصية لنمو الخلايا بطيف امتصاصي OD_{600} لتكون 1.540 بعد 8 ساعات من نمو الخلايا. وبما أن ناتج تعبير جينات حقيقية النواة في الخلايا البكتيرية يكون بشكل تجمعات تعرف بالمكتنفات الخلوية, لذلك فان الظروف المثلى تم الأخذ بها لاستخلاص و أذابه وتنقيه هرمون النمو ألبقري من المكتنفات الخلوية وكان الناتج بروتين كامل البنية والذي تم الكشف عنه باستخدام 12% من هلام متعدد الاكريلاميد . تبع هذه الخطوة تنقيه الهرمون بمتبادل ايوني سالب باستخدام عمود DEAE-Sepharose وكانت النتائج الحصول على حزمه مفرده واضحة بوزن جزيئي 22 كيلو دالتون باستخدام 12% من هلام متعدد الاكريلاميد والتي تمثل هرمون النمو السوماتوتروبين ألبقري المنقى.

المحكمون:

أ.د. سعاد محمد أبو السعود	قسم النبات، علوم طنطا
أ.د. عبد الفتاح بدر	قسم النبات، علوم حلوان