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Original Article

Novel expression system for the Large scale Production of Recombinant Human B-type natriuretic peptide (BNP) for therapeutic purpose

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Abstract

Human brain natriuretic peptide (hBNP) is a 32 amino acid peptide, which has been used in the treatment for congestive heart failure. hBNP is expressed as a novel fusion protein which comprises of the N-terminal portion of growth hormone, to overcome proteolysis during its expression in host cells. The GH-hBNP fusion protein was expressed in the form of inclusion bodies with an expression of around 25%.The fermentation process in large scale was optimized to yield 10g dry cell mass per liter of culture. rhBNP was purified by solubilization of inclusion bodies, cleavage and subsequent purification of peptide by reverse phase chromatography. The final purity of the rhBNP was more than 99% and the yield of rhBNP peptide was over 200 mg per liter of culture. The purified rhBNP was confirmed by mass spectrometry, N-terminal amino-acid sequence and by its biological activity.
Keywords: cGMPassay; Peptide purification; Protein expression; Recombinant human BNP; Reversed phase chromatography

Introduction

Brain natriuretic peptide (BNP) is the second member of the natriuretic peptide family originally isolated from the porcine brain (Sudoh *et al.,* 1988). Endogenous hBNP peptide is synthesized in vivo as a 108 amino acid prohormone termed proBNP that is proteolytically cleaved to release the 32-amino acid C-terminal BNP peptide (Goetze *et al.,* 2004; Seilhamer *et al.,* 1989). B-type natriuretic peptide exerts its biological activities via a membrane-bound guanylyl cyclase receptor termed natriuretic peptide receptor A (NPRA) (Kuhn, 2004). Binding of BNP to this receptor on endothelial cells and vascular smooth muscle cells results in the cellular synthesis of cyclic guanosine monophosphate (cGMP), which mediates the vascular effects of this peptide (Holtwick *et al.,* 2002; Sabrane *et al.,* 2005).

BNP is synthesized in the ventricular myocardium, where its levels increase in patients with congestive heart failure (Wei *et al.,* 1993). Systemic infusion of a recombinant human brain natriuretic peptide in patients with congestive heart failure results in beneficial hemodynamic actions, including arterial and venous dilatation (Abraham *et al.,*1998; Holmes *et al.,* 1993; Marcus *et al.,*1996; Mills *et al.,* 1999).

While brain natriuretic peptide offers certain clinical advantages over other drugs that are used to treat congestive heart failure patients, the relatively high cost of production of peptide drugs compared to non-peptide drugs could present an impediment to its acceptance in clinical practice. Consequently there is a need to provide a highly efficient means of producing brain natriuretic peptide in order to minimize its cost. Several approaches have been attempted to express hBNP (Okamoto *et al.,*1997; Sun *et al.,* 2005; Sun *et al.,* 2004). However, none of the published methods for rhBNP yielded quantities that are required for therapeutic purpose.

With an aim to produce high yield rhBNP in the present study, we attempted to develop a method to improve the process yield by using a novel small fusion construct consisting of N-terminal portion of human growth hormone (1-62 AA) with Factor Xa protease encoding cleavage site in between the target rhBNP gene. This approach was not only aimed at increasing the yield, but also to avoid expensive affinity chromatography procedures. This 7kDa GH-BNP fusion protein was expressed as inclusion bodies. After solubilization of these inclusion bodies, the fusion protein was cleaved with the Factor Xa. The human BNP peptide was purified by RP-HPLC with greater than 99% purity. This process yielded around 200mg of pure rhBNP/liter culture.

Materials and Methods

Materials

Plasmid (pET21a) and E. coli BL21 (DE3) host cells were obtained from Novagen (Darmstadt, Germany). Source-30™ reversed phase chromatography matrix was purchased from GE Healthcare (Uppsala, Sweden). Vydac C18 analytical column was purchased from Grace chromatography products (USA). The Oligonucleotide encoding BNP gene and PCR primers were synthesized by IDT technologies (USA). Dynamill was purchased from miltech (Swizerland). The AKTApurifier - High performance purification chromatography system for column purification was from GE Health care (Uppsala, Sweden). The Human aortic endothelial cells were obtained from Lonza Walkersville Inc, (USA). The rhBNP (1-32) standard Natrecor was purchased from Scios (Fremont, California). SDS detection and estimation kit was purchased from G-Biosciences (St Louis, Mo, USA). Factor Xa was prepared in-house by isolation from bovine plasma and activation by a protease from Russell's viper venom (Radcliffe and Barton, 1972).

Cloning and expression

Gene synthesis and Polymerase chain Reaction (PCR) Amplification

The hBNP gene assembly of synthetic gene was performed from component oligo nucleotides. The hBNP nucleotide sequence (GeneBank Accession No BC025785) was designed based on the E.coli rare codon preference. Factor Xa site upstream to BNP encoding sequence was generated by use of oligonucleotide primers. The following primers were used in PCR reaction.

P1 (5'- CC GGA TCC ATT GAG GGT CGC AGC CCG AAA ATG- 3'), P2 (5'-GTT CAG GGC TCT GGC TGC TTC GGC CGT AAA ATG GAC CGT ATC AGC -3'), P3 (5'-TCC TCC AGC GGC CTG GGC TGC AAA GTT CTG CGT CGT CAC TAA TAG- 3'), P4(5'-CCT GAA CCA TTT TCG- 3'), P5 (5'-TGG AGG AGC TGA TAC-3'), and P6(5'- CGG AAT TCC TAT TAG TGA CGA CGC AG- 3'). Equal volumes of ~1mg/ml were mixed together and diluted with water to a final concentration of ~1ng/µl for each oligonucleotide. The final concentration 0.2ng/ µl for each oligonucleotide was used along with 20mM Tris-HCl(pH8.8),10mM for KCl, 10mM$(NH_4)_2$ SO_4 , 6mM $MgSO_4$, 0.1%(V/V) triton –X100, 0.1mg/ml bovine serum albumin,0.2mM each dNTP and 2.5 U Pfu polymerase. The PCR protocol for gene assembly began with one 94^{o}C 5min denaturation step which is followed by 25 cycles of 94^{o}C denaturation for 30s, 56^{o}C annealing for 60s, and 72^{o}c extension for 60s, Final extension of 72^{o}c for 10min . hBNP gene was amplified by using 1µl of the mixture resulting from the gene assembly as the template and the outer most oligonucleotides P1 and P6 as primers. The hBNP gene with Factor Xa cleavage sequence was PCR amplified using gene specific primers. The forward primer 5'-CGGATCCATTGAGGGTCGCAGCCCGAAA ATG-3' contains BamH1 and the reverse primer 5'- CGGAATTCCTATTAGTGACGACGCAG-3' contains EcoR1 site with Stop codon. The PCR amplification cycles were as follows: Initial 94^o C 5 min; 25 cycles of 94 ^{o} C for 30sec, 56 ^{o}C for 1min, 72 ^{o} C for 1min, final extention of 72 ^{o} C for 10min with a final hold at 4 ^{o} C.

Construction of pGFB-1 and expression of hBNP (1-32) fusion protein

The full length growth hormone gene was used as a template to amplify 186 bps growth hormone using gene specific primers. The primers used for amplification contains Nde1 site in forward direction and BamH1 in reverse primer.

Forward primer F': 5'-CGCATATGTTCCCAACTATTCCACTGAGT-3' and Reverse primer R':5'-CGGGATCCAGGGGTCGGGATACTTTCAGA CAAACTCAA-3'. The PCR amplification cycles were as follows: Initial 94 ^{o}C for 5 min; 30 cycles of 94 ^{o}C for 30 sec, 55^{o}C for 1min, and a 72^{o}C for 1min and a final extension of 72 ^{o}C for 10min with a final hold at 4^{o}C cycle. The Plasmid pET21a and PCR product was restriction digested with Nde1 and BamH1 endonucleases and ligated by using T4 DNA ligase to yield plasmid pGH. The modified plasmid (pGH) containing 7kDa growth hormone encoding tag under the control of T7 promoter was used to clone hBNP gene. The hBNP PCR product and plasmid pGH were digested individually with restriction endonucleases BamH1 and EcoR1. The digested samples were ran on agarose gel. The agarose gel electrophoresis purified PCR fragment and plasmid pGH were ligated with T4DNA ligase to yield the expression plasmid pGFB-1. The expression construct with hBNP gene was sequenced by Sanger's Dideoxy method and Escherichia coli strain BL21 (DE3) was transformed with recombinant plasmid containing the hBNP gene.

Fermentation and harvesting

The seed culture was prepared in LB Media (Sambrook *et al.,* 2001) from the Glycerol Stock and incubated for 7-8 hours (O.D of 1.7to2.3) at 35ºC on rotary shaker. Fermentation was carried out in a Biostat C fermentor (B. BRAUN Germany) in fed-batch mode. Modified LB medium was used for fermentation. The temperature of the fermentation cultures was controlled at 35ºC for a period of 5 hours (O.D of 13to16) and induced with 0.5 mM IPTG and harvested after 3 hours. The pH was controlled and adjusted to 7.0- 7.2 with 50% Ammonia solution. The rate of agitation was 300 to 700 rpm, and the dissolved oxygen (PO_2) was maintained above 40%-50% saturation. Foam formation was suppressed by the addition of sterilized 10% silicon based antifoam. The samples for expression analysis were collected and whole cell lysates were analyzed by SDS PAGE. The culture was centrifuged at 6600g for 10 min, the supernatant was removed and the biomass (525g) was determined by weighing the wet pellet. The cells were frozen immediately at -70ºC until further analysis was performed.

Purification and characterization of the recombinant hBNP

Cell lysis, isolation of inclusion bodies (IB) and solubilization of IB

The cell pellet was thawed on ice, suspended in 787.5ml of buffer A (50mM Tris and 5mM EDTA pH 8.0) Cell disruption was done in dynamill (miltech Switzerland) with 0.5mm of Glass beads up to 80% of the total chamber volume. Cell lysate was passed at a flow rate of 100ml/min which takes about 10 mins for each cycle and the process was optimized to 3 cycles. The crude IB preparation was initially washed with Buffer A, then with 0.1%deoxycholate (sodium salt) in buffer A and finally with buffer A plus 2 M urea. Traces of deoxycholate or urea were removed by a final wash with the buffer B (20mM Tris pH 7.5). IB pellet was collected by centrifugation at 12,000 g for 20 min at 4 ^{o}C. The isolated inclusion body was suspended in 570ml solublization buffer (20mM Tris pH 7.5, 100mM Nacl, 0.3% Sodium dodecyl sulfate (SDS)) and dissolved by stirring overnight at 4°C on a magnetic stirrer. The solution was centrifuged at 12,000g at 4 °C for 10 min. The protein concentration was measured according to the method of Lowery *et al*., (1951). The supernatant was then diluted with 20mM Tris PH 7.5, to adjust 0.3% SDS to less than 0.1% of final concentration.

Cleavage of GH-hBNP with Factor Xa

The solubilized fusion protein was kept for cleavage at final concentration of 3-5mg/ml with Factor Xa at 1:100 dilutions for 3hr at 25°C to release hBNP.

Purification of rhBNP by reverse phase chromatography (RPC)

hBNP, after cleavage was purified with reverse phase HPLC on a preparative column. SOURCE 30RPC column (30mm x 400mm; 240ml) was equilibrated with solvent A (0.1%TFA) at a flow rate of 20ml/min until a stable base line was attained. After sample injection, elution was performed with solvent B Acetonitrile at a linear gradient of 10%- 25% at flow rate of 20ml/min. peptide elution was monitored at 214nm. Fractions containing rhBNP were collected, dialyzed with 20mM Citric acid buffer and lyophilized.

SDS PAGE and analytical reverse phase HPLC (RP-HPLC) analysis

Electrophoresis of the expressed samples, purified fusion protein and the pure rhBNP were carried out on SDS–PAGE, stained with coomassie brilliant blue (CBB) G-250. The purity of rhBNP product was evaluated using reverse phase HPLC on a vydac C18 analytical column (4.6 x 150mm). The retention time was compared with that of hBNP standard during a linear gradient of acetonitrile containing 0.1%TFA from 0 to100% over a 55min at a flow of 1ml/min.

Mass spectrometry MALDI- MS and N-terminal anino acid analysis

MALDI- MS and N-terminal amino acid sequence were analyzed at proteomics core facility of CCMB, HYDERABAD. The Mass spectrometry was performed on a Voyager MALDI MS mass spectrometer and the peptide to be sequenced was subjected to Edmen degradation using a (procise cLc, Applied Biosystem) protein/Peptide sequencer.

Biological activity assay of rhBNP

Human aortic endothelial cells at passages 3 through 5 were grown in EGM-2 MV complete medium containing 5% fetal bovine serum (FBS). Cells were changed to serum-free medium and pre-incubated with 0.1 mmol/l 3-isobutyl-1-methylxanthine for 1 h, then treated with varying concentrations of human BNP ranging from 0.1 nmol/l to 1,000 nmol/l for 10 min. Cells were lysed with 0.1 mol/l HCl at room temperature for 20 min. The levels of cGMP in the supernatant were measured using a cGMP enzyme immunoassay kit from Assay Designs (Ann Arbor, Michigan).

Results

Cloning of expression construct of hBNP(1-32)

The (Figure 1) shows hBNP gene and expression vector construction. In the first PCR cycle, the primers 1, 2 and 3 code for the sense strand of hBNP along with Factor Xa cleavage sequence were annealed with complementary primers 4,5 and 6 at optimum annealing Tm temperature. The Factor Xa-hBNP gene amplification was performed from the annealed product using gene specific primers P1 and P6. In second PCR, 186 base pair fragment of growth hormone encoding gene was amplified, digested with Nde1and BamH1 and ligated with digested pET21a (+) vector. The construct was named as pGH. The amplified Factor Xa- hBNP was inserted down stream to the 7kDa GH encoding sequence. The sequencing results of cloned construct revealed that the construct was intact without any manipulation of Factor Xa linker and hBNP genes.

Fermentation, expression of GH–BNP fusion protein and harvesting

hBNP expressing *E.coli* cells were grown in a fed batch fermentation process to produce large quantities of GH-BNP fusion protein. The cultured cells at an OD of 13-16 were induced with 0.5mM IPTG and grown for another 3h, and batch was terminated at an OD of 27-30. Large scale fermentation batch was standardized to get a wet cell mass of about 35g wet cell mass per liter culture. The SDS-PAGE densitometry analysis indicated GHBNP fusion protein as11kDa band with expression level of about 25% of total proteins (Figure 2A). A maximum of 1.4g/L (Table-1) of GH-hBNP fusion protein was expressed as inclusion bodies.

Table- 1: Purification of recombinant BNP per l of E.coli culture.

Step No.	Purification Step	Total proteins (mg)	Fusionprotein[a] / rhBNP[c]	Purity of fusion protein/ rhBNP	Yield[b]
1	whole cell lysate	5733	1400 mg[a]	25%	100%
2	Cell lysis	3600	1260 mg[a]	35%	90%
3	Solubilization of IB	1133.3	850 mg[a]	75%	60%
4	Fusion protein cleavage with Factor Xa	1132.9	235 mg[c]	--	54%
5	RP chromatography and dialysis	1130	200 mg[c]	99%	46%

[a] The amount of fusion protein was determined by quantifying in each gel lane by densitometry.
[b] The purification yield is calculated based on the amount of fusion protein.
[c] The peptide amount of rhBNP.

Cell lysis, Isolation of inclusion bodies (IB) and solubilization of IB

GH-hBNP fusion protein expressed as inclusion bodies (IB) were isolated by means of cell disruption by dynamill. The crude IB isolated in this step were up to 35% pure with a fusion protein concentration around 1-1.2g/L (Table 1). The cell lysis step was efficient and there was a minimal loss of fusion protein in the supernatant (Figure 2A). Extensive washing of IB with 2M urea and 0.5% deoxycholate resulted in 60% pure IB. Washing's with chaotrope and detergents containing buffers helped in removing other *E.coli* host protein. These IB were solubilized in 0.3% SDS, 50mM tris-HCl pH 8.0. The solubilization step was efficient and helped in improving the purity of fusion protein to 75% by leaving other host proteins unsolublized in the pellet. Next, the supernatant was diluted three times to adjust the concentration of SDS to 0.1% and fusion protein to 3-5 mg/ml for Factor Xa cleavage.

Cleavage of GH-hBNP with Factor Xa

The fusion protein (GH-Factor Xa-hBNP) at concentration 3-5 mg/ml was optimum for cleavage with Factor Xa at 25^{o}C. The bovine Factor Xa (serine protease) specifically recognizes and cleaves at the c-terminal of linker site and release the hBNP without any N-terminal modification. The digestion showed greater than 90% cleavage resulting into 7kD GH and 3.4kD hBNP peptide when analyzed by SDS-PAGE (Figure 2A).

Reverse phase chromatography purification of rhBNP

Two major Peaks were eluted with linear acetonitrile gradient. The peptide fractions eluting between 11to18% were proved to be >99% pure by analytical RP-HPLC. The pure peptide was dialyzed against 20 mM citric acid buffer with a pH 4.2. The final yield obtained by this method was approximately 200 mg of pure rhBNP /liter cell culture (Table1).

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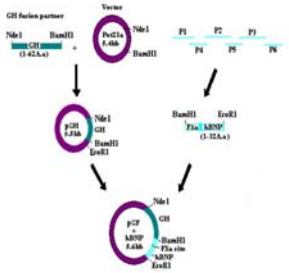


Fig.1: The Construct design of pGFB-1, Pet21a plasmid and PCR amplified 62 amino acids growth harmone gene was digested with Nde I and BamH I, and ligated. The hBNP gene with Factor Xa site sequence was prepared with component oligonucleotide assembly and PCR cycles. The GH tag construct (pGH) and the synthetic hBNP was digested with BamHI and EcoRI, and ligated. The constructed plasmid pGFB-1 from the transformants of E.coli DH5α was confirmed by DNA sequencing.

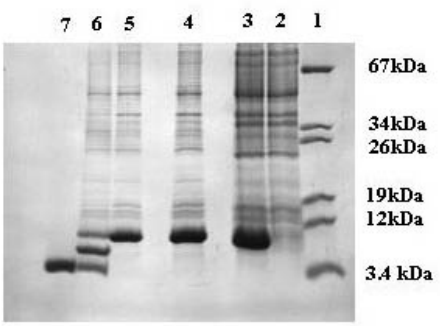
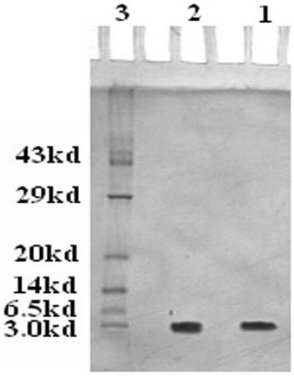


Fig.2a: The purity of the hBNP fusion protein and rhBNP at different process stages was observed by SDS-PAGE gel (A) Lane 1, molecular weight marker; lane 2, 15µg of un-induced total cell lysate; lane 3, 15 µg of induced total cell lysate; lane 4, 10 µg 0.3% SDS solubilized GH-hBNP fusion protein from inclusion body; lane 5, 5µg un-cleaved GH-hBNP fusion protein; lane 6, 5µg cleaved fusion protein; lane 7, 3µg RPC purified pure rhBNP fraction.(B)The purified peptide was ran on SDS-PAGE. Both the standard peptide and rhBNPH(1-32) migrated in the same manner and were > 99% pure.Lane 1, 2.0 µg hBNP standard; lane 2, 3.0 µg pure rhBNP.

Characterization of rhBNP

The purity and identity was confirmed by SDS-PAGE and analytical RP-HPLC over C18 vydac column along with the standard BNP. The analyzed SDS-PAGE pattern showed only single band corresponding ~ 3.4kDa with the standard BNP (Figure 2B). The retention time of both hBNP standard as well as purified rhBNP was similar with purity greater than 99.0% (Figure 3). Finally MALDI-TOF analysis demonstrated a mass of 3467.5 Da. The molecular weight of rhBNP that was determined by mass spectrometry is consistent with the theoretical value of human BNP (Shimizu *et al*., 2002). The N-terminal sequence of purified rhBNP was NH_2-SPKMVQGSGC which was similar to published sequence (Kambayashi *et al*., 1990).



2b:The purified peptide was ran on SDS-PAGE. Both the standard peptide and rhBNPH(1-32) migrated in the same manner and were > 99% pure.Lane 1, 2.0 µg hBNP standard; lane 2, 3.0 µg pure rhBNP.

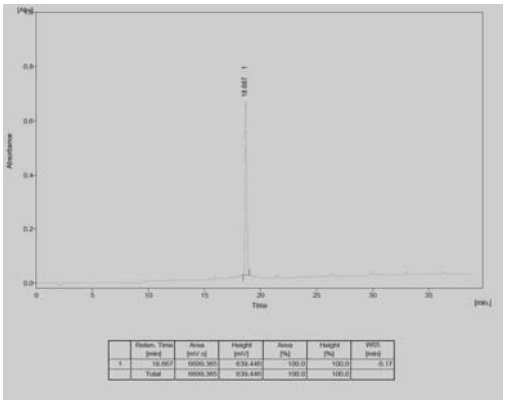


Fig. 3: Analysis of rhBNP on C18 reverse phase HPLC. rhBNP purified by preparative source 30 reverse phase HPLC was analyzed by loading the sample onto a C18 RP-HPLC column and eluting with a linear gradient of acetonotrile. The peptide was eluted at18.6min retension time similar to standard. The purity of the peptide was estimated to be > 99%.

Determination of Biological activity of purified rhBNP

It is suggested that the central ring structure of BNP formed by a disulfide bridge between two cysteine residues is necessary for binding to specific receptors. Hydrolytic disruption of the bridge leads to a loss of

biological activity. The graph (Figure 4) shows the biological activity at different concentrations of rhBNP in comparison to the standard which was estimated as increased levels of cGMP. The calculated EC50 value 35 ± 2.3nmol/l was similar to the standard peptide. The biological activity of purified rhBNP indicated that it has the same potency as the standard.

Fig. 4:

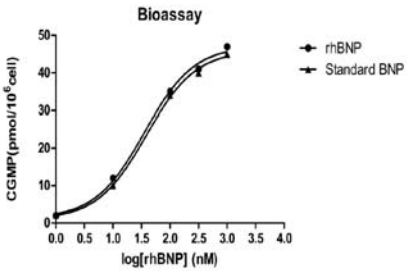


Fig. 4: Comparison of rhBNP with standard BNP on stimulation of intracellular cGMP in Human aortic endothelial cells (HAEC). Human aortic endothelial cells were grown in EGM-2 MV complete medium containing 5% fetal bovine serum (FBS). After confluence, cells were split and cultured in 6-well plates for the experiment. Cells were treated with varying concentrations of human BNP ranging from 0.1 nmol/l to 1,000 nmol/l for 10 min. Cells were lysed with 0.1 mol/l HCl at room temperature for 20 min. The lysates were measured for levels of cGMP in the supernatant using a cGMP enzyme immunoassay kit. The rhBNP stimulated intracellular accumulation of cGMP production as the concentration of rhBNP was increased. The calculated EC50 value 35 ± 2.3nmol/l was similar to the standard peptide.

Discussion

The development of cost-effective systems for the production of smaller peptides using recombinant DNA technology is of great interest due to the increasing use of peptides as pharmaceutical agents. E.coli is the most widely used host for expression of heterologus proteins because it is a well characterized microbial system that offers many advantages for easy manipulation (Hanning *et al.,* 1998). However, expression of smaller polypeptides in E.coli is inefficient mainly due to the proteolytic degradation of expressed products (Gottesman *et al.,* 1989).The problems encountered in over expression of smaller peptides have been successfully overcome by the use of fusion protein expression strategies (Nilsson *et al.,* 1997; LaVallie and McCoy,1995). The fusion partner, with affinity system helps in purifying fusion proteins but many of the fusion protein tags used to express peptides are high molecular weight proteins. Hence the ratio of fusion partner is more, which in turn leads to lower yields of purified peptide. The selection of fusion partner plays a major role as it directly relates to expression and final yield of the target peptide. Several attempts have been made to produce rhBNP but all these methods used longer fusion partner strategies (Sun *et al.,* 2005; Sun *et al.,* 2004). In one attempt chitin binding domain (CBD), was used as a fusion partner to produce intact rhBNP but the final yield was 2-3mg/liter culture. Another fusion expression strategy resulted in an effective expression of hBNP under the control of the T7 promoter in E.coli using thioredoxin as a fusion partner was reported. This method has reported a yield of 4mg/liter culture with 95% pure rhBNP. The end product obtained by adopting the methods cited in the available literature was not directly suitable as an active pharmaceutical ingredient (API) for formulation. The drawbacks with the reported methods are low yield and less purity. Hence, the process and final product is not applicable for industrial scale and therapeutic application.

This study was focused on using a novel small fusion partner to express hBNP in *E. coli* and there by developed a simplified downstream process to recover high yield, biologically active rhBNP for therapeutic application. The hBNP was prepared in large scale from *E. coli* BL21 (DE3) transformed with a plasmid construct (pGFB-1) that consists of N-terminal growth hormone (GH) encoding sequence fused to hBNP encoding sequence. There are several advantages with this approach. First, the use of *E. coli* codon usage and T7 RNA polymerase promoter system, BL21 (DE3)/PGFB-1 could produce high level of expression which accounted for 25% of total proteins. Second, by linkage to hydrophobic region of GH, the fusion protein was in insoluble aggregates. The protein production is in the form of inclusion bodies that has a number of merits, such as high levels of enriched protein production and protection of desired protein from proteolytic degradation. The use of a smaller partner, reduces the amount of energy required to obtain a certain number of molecules, diminishes steric hindrance and simplifies downstream applications(Smith and Johnson,1988).Using a commercially available vector Pet21a(+), hBNP was fused to truncated GH and it provided a favorable intracellular accumulation of

insoluble inclusion bodies. The problems encountered with IB solubilization and refolding of the fusion protein for Factor Xa cleavage was successfully overcome by using SDS as solubilizing agent (Kim and Lee, 2000). SDS is known to interact preferentially with hydrophobic regions of proteins to solubilize protein aggregates (Hermann,1993). Hence, SDS was chosen as the denaturant for solubilization. Post solubilization, the purity of fusion protein was increased to 75% where majority of high molecular host proteins were lost in the pellet after solubilization. The solubilized fusion protein was directly cleaved with Factor Xa without any further refolding process. In our process we displayed a very high degree (90%) of Factor Xa cleavage. To obtain high purity, most previous procedure for purification of rhBNP involved RP-HPLC which involves highest resolution sensitivity. We used SOURCE 30 reverse phase chromatography for large scale preparation which is the critical step in the procedure. The intact rhBNP was eluted at early gradient due to highly hydrophilic nature of the hBNP. The Factor Xa and 7kDa tag along with uncleaved fusion protein were eluted later as there is much variation in the hydrophobic plot.

Conclusion

In this study, by using a novel small fusion insoluble protein strategy we demonstrated an approach to prepare highly pure, biologically active rhBNP at high yield of over 200mg/l. The possible critical keys were provided with simple approaches. In our scheme, solublization and cleavage was achieved efficiently by opting for SDS solublization and thereby developing a simple and cost effective downstream process to purify a therapeutically important peptide.

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