ABSTRACT

The thesis entitled **"Bone Morphogenic Protein 2 (BMP2): From Cloning to Purification, Characterization, Stability and Interaction studies with its Receptor"** presents cost-effective purification of protein and also explains the pH and temperature stability of protein along with interaction study.

Chapter 1 (Introduction) provides brief information about the TGF-beta family of proteins. Detailed information about BMP2, such as its structure and functions, is covered in this chapter. The synthesis and cell processing of BMPs are also discussed along with the therapeutic role of BMP2. The initiation of BMP signaling upon binding with its receptors is highlighted in this chapter. The challenges of producing proteins from *E.coli* and inclusion bodies solubilization methods are explained. The role of various molecules in refolding of sulfide-rich proteins is accentuated with examples. The effects of chemical and molecular chaperones upon the folding of human proteins in *E.coli* are described in this chapter. The chapter also explains the thermodynamic aspect of protein stability. Finally, the chapter discusses the origin of the problem in the context of this thesis and outlines the present research**.**

Chapter 2 (Materials and methods) provides information about all the chemicals, enzymes, bacterial strains, plasmids, and cell cultures used in the experiments. The chapter describes in detail all the methods used to perform experiments, such as cloning, protein expression, and purification protocols. Furthermore, an overview of the experimental techniques used in the study is provided. Molecular biology techniques such as polymerase chain reaction and spectroscopic techniques like

UV-visible spectroscopy, fluorescence spectroscopy, and circular dichroism spectroscopy are described. Separation techniques such as affinity chromatography and ion-exchange chromatography are also briefly discussed. Electrophoresis techniques, including agarose gel electrophoresis, SDS-PAGE, and Native Page, are outlined in the context of their use in this study.

Chapter 3 (Cloning and Expression of BMP2) In this chapter, we examined the complete protein sequence of BMP2, focusing initially on primer design. We performed polymerase chain reactions at different temperatures and primer ratios to amplify the mature region of the BMP2 protein. Next, we focused on the cleavage of the expression vector and mature region of the PCR product (BMP2) and their ligation to form a clone. We confirmed proper cloning by double digestion of clones on agarose gel and by colony PCR. Finally, the BMP2 clone was confirmed by Sanger sequencing. Successful expression of proteins was achieved in E. coli BL21 DE3, and for better yield and solubility of proteins, expression was also checked in Rosetta pLysS and Rosetta BL21 DE3. Temperature and inducer concentration were optimized for BMP2 expression.

Chapter 4 (Purification, Refolding, and Characterization of BMP2 and Alk3) In this chapter, we focused on the isolation of monomers from inclusion bodies (IBs) and the purification of BMP2. We also discussed the purification of the Thioredoxin-tagged Alk3 receptor, which was expressed in *E.coli* BL21 DE3, purified by Ni-NTA chromatography, denatured, refolded, and cleaved by thrombin to remove the thioredoxin tag. The purified Alk3 was further purified by anion exchange column to remove oligomers and obtain pure Alk3.

For BMP2 IBs, we used two methods: urea solubilization and acid solubilization. Urea-solubilized proteins (BMP2) were purified by SP Sepharose chromatography to obtain a pure monomer. However, urea-solubilized proteins were more prone to aggregation upon removal of urea,

resulting in protein loss. Acid or urea-solubilized monomer was further refolded using various compounds to form a disulfide-linked BMP2 dimer, which was confirmed by non-reducing SDS-PAGE. We separated the monomer and dimer by heparin chromatography. The protein's structure was confirmed by circular dichroism (CD).

Also, we have cultured C2C12 cells and incubated them with BMP2 to assess the protein's functional activity by measuring the alkaline phosphatase activity of BMP2. The cloned BMP2's functional activity was further confirmed through a binding assay with purified Alk3 receptor using Native PAGE.

Chapter 5- (Role of molecular and chemical chaperones in assisting refolding of BMP2 in *E.coli*): In this chapter, the focus was on studying the role of molecular and chemical chaperones in the soluble expression of BMP2. Chaperones are well-known for their role in assisting protein folding, and BMP2, when expressed in bacteria, tends to form inclusion bodies due to its complex disulfide-rich structure. To increase the expression of BMP2 in the soluble fraction, we carried out several experiments, including co-expression of molecular chaperones (GroES-GroEL, TF, and DnaK-DnaJ-GrpE) with BMP2 at both 18°C and 37°C, expression of BMP2 in the presence of chemical chaperones (Trehalose and Arginine) at both temperatures, and co-expression of molecular chaperones with BMP2 in the presence of chemical chaperones at both temperatures.

We observed that the Shuffle T cells were successful in expressing soluble, functionally active BMP2 in the presence of molecular and chemical chaperones at low temperatures. The combined effect of chemical and molecular chaperones favored a better yield of protein in the best-suited chaperone system (GroESGroEL) at low temperatures. The soluble, functionally active protein was confirmed by its binding with its receptor Alk3 through Native PAGE.

Our experiments showed that BMP2 expression from inclusion bodies to a soluble, active form is possible with the help of molecular and chemical chaperones working synergistically in bacterial cells capable of folding disulfide-rich proteins at low temperatures, which offers an easy and time-saving method of protein production at low temperature.

Chapter 6 (Stability and Binding Study of BMP2)

The thermal stability of BMP2 was studied by recording the fluorescence spectra of the protein in the temperature range of 10 °C to 85 °C. Tryptophan was excited at 295 nm, and the data obtained were analyzed. The melting temperature (������) of the protein was found to be 325 K. In addition to this, Far-UV CD spectra of BMP2 were recorded as a function of temperature, and the data were fitted to analyze the changes in the secondary structure of the protein.The stability of BMP2 was also studied with respect to changes in pH. CD, fluorescence, and absorbance spectroscopic measurements were carried out to observe changes in the secondary and tertiary structure of the protein with pH. It was observed that BMP2 is stable at acidic pH, specifically at pH 3 and 4. However, above pH 5, the protein started to aggregate. At pH 11 and above, no visible aggregates were seen, but the structure of the protein was disrupted as observed from the redshift of spectra in fluorescence. Overall, the results suggest that BMP2 is stable at acidic pH and can tolerate a wide range of temperatures up to 325 K. These findings are important for the use of BMP2 in various applications, including biotechnology and medical fields.

Several techniques were employed to understand the formation of the BMP2-Alk3 complex. The first technique was Native-PAGE analysis, which enabled the determination of the binding

stoichiometry between BMP2 and Alk3. The stoichiometry ratio of one BMP2 to two Alk3(1:2) was established by titrating increasing concentrations of BMP2 with Alk3 and vice versa.

The next technique used was rotational anisotropy decay, which involved labeling Alk3 with Alex flour 488 dye and monitoring the anisotropy decay of the labeled protein. The rotational time increased with the addition of BMP2, indicating the formation of a complex.

Steady-state fluorescence experiments were also carried out, where a fixed amount of Alk3 was titrated with increasing concentrations of BMP2 at pH 4. The intrinsic fluorescence of the complex was monitored, and a plot of fluorescence intensity versus increasing BMP2 concentration showed a U-shaped curve. The global fitting of total fluorescence as a function of ligand concentration yielded a Kd value of 59.8, indicating a strong association between BMP2 and Alk3.

Chapter 7 (Summary and future perspectives) This chapter presents the highlights of the study. Our cloned protein BMP2 can be used as a therapeutic protein in fields like dentistry and orthopedics. Molecular chaperones and chemical chaperones together can help produce soluble proteins that form inclusion bodies in their absence, which are difficult to purify. Furthermore, the pH and thermal stability of proteins can be enhanced by adding suitable additives. To use BMP2 as a therapeutic protein, further toxicity assays and studies on the delivery of proteins into the body or at the site of injury using a suitable carrier can be conducted. These studies are crucial in determining the safety and efficacy of BMP2 as a therapeutic protein in clinical settings.