

**BONE MORPHOGENETIC PROTEIN 2 (BMP2): FROM CLONING TO
PURIFICATION, CHARACTERIZATION, STABILITY AND INTERACTION
STUDIES WITH ITS RECEPTOR**

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by

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Submitted

In the fulfilment of the requirement of the degree of Doctor of Philosophy

To the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

JANUARY 2024

Dedicating this thesis to my Parents

CERTIFICATE

This is to certify that the thesis which is entitled **“Bone Morphogenic Protein 2 (BMP2): From Cloning to Purification, Characterization, Stability and Interaction Studies with its Receptor”** being submitted by **“Ms. Nitika Patwa”** to the “Indian Institute of Technology Delhi” for the award of **“Doctor of Philosophy”** is a record of bonafide research work carried out by him. She has worked under my guidance and supervision and has fulfilled all the necessary requirements for the submission of this thesis.

To the best of my knowledge, the results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree.

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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 (T_m) 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 K_d 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.

सारांश

"बोन मॉर्फोजेनिक प्रोटीन 2 (बीएमपी2): क्लोनिंग से पुनःशोधन, विशेषण, स्थिरता और इसके प्रतिसंबंध साथी के साथ अध्ययन" शीर्षक वाले थीसिस में प्रोटीन के कम-लागत पुनःशोधन का प्रस्तुतिकरण किया गया है और प्रोटीन की pH और तापमान स्थिरता के साथ ही प्रोटीन के साथ प्रतिसंबंध अध्ययन किया गया है।

अध्याय 1 (परिचय) में टीजीएफ-बीटा परिवार की प्रोटीनों के बारे में संक्षेपित जानकारी प्रदान की गई है। इस अध्याय में बीएमपी2 के संरचना और कार्यों की विस्तृत जानकारी है। बीएमपी2 की सिधता और सेल प्रसंस्करण के बारे में भी इस अध्याय में चर्चा है, साथ ही बीएमपी2 के थेरेप्यूटिक भूमिका के बारे में भी। इस अध्याय में BMP रिसेप्टर के साथ जुड़ने पर BMP साइनलिंग का प्रारंभ होता है। इस अध्याय में E.coli से प्रोटीन उत्पन्न करने और इनक्लूजन बॉडी सॉल्यूबिलाइजेशन विधियों की चुनौतियों की व्याख्या है। एसिडिक pH पर रिफोल्डिंग की उपयोगिता के साथ सल्फाइड-रिच प्रोटीन की रिफोल्डिंग में विभिन्न यौगिकों की भूमिका को उदाहरणों के साथ बताया गया है। इस अध्याय में मानव प्रोटीन्स की फोल्डिंग पर केमिकल और मॉलेक्युलर चैपरों के प्रभाव की चर्चा भी है। अंत में, इस अध्याय में इस थीसिस के संदर्भ में समस्या की उत्पत्ति पर चर्चा की गई है और वर्तमान अनुसंधान को रूपरेखित किया गया है।

अध्याय 2 (सामग्री और विधियाँ) एक्सपेरिमेंट्स में उपयोग किए गए सभी रासायनिक, एंजाइम, बैक्टीरियल स्ट्रेन्स, प्लाज्मिड्स, और सेल कल्चर्स के बारे में जानकारी प्रदान करता है। इस अध्याय में, क्लोनिंग, प्रोटीन अभिव्यक्ति, और शोधन प्रोटोकॉल्स जैसी सभी विधियों का विस्तृत विवरण दिया गया है। इसके अलावा, अध्यय में अध्ययन में उपयोग होने वाली विभिन्न प्रयोगशील तकनीकों का एक सारांश प्रदान किया गया है।

पॉलिमरेस चेन रिएक्शन और यूवी-विसिबल स्पेक्ट्रोस्कोपी, फ्लोरेसेंस स्पेक्ट्रोस्कोपी, और सर्कुलर डाइक्रोइजम स्पेक्ट्रोस्कोपी जैसी मोलेक्युलर बायोलॉजी तकनीकें वर्णित की गईं। इसमें एफिनिटी क्रोमैटोग्राफी और आयन-एक्सचेंज क्रोमैटोग्राफी जैसी सेपरेशन तकनीकों की भी संक्षेप चर्चा है। इस अध्याय में उनके उपयोग के संदर्भ में आगरोस जेल इलेक्ट्रोफोरेसिस, एसडीएस-पीएज, और नेटिव पेज की आलेखित हैं।

अध्याय 3 (BMP2 का क्लोनिंग और अभिव्यक्ति) इस अध्याय में, हमने बीएमपी2 के पूरे प्रोटीन सीक्वेंस की जाँच की, शुरूवात में प्राइमर डिज़ाइन पर ध्यान केंद्रित करके। हमने विभिन्न तापमानों और प्राइमर अनुपातों पर पॉलिमरेस चेन रिएक्शन किए ताकि बीएमपी2 प्रोटीन के परिप्रेक्ष्य में मैच्योर क्षेत्र को बढ़ावा मिल सके। अगले में, हमने व्यक्तित्व वेक्टर और पीसीआर प्रोडक्ट (बीएमपी2) के मैच्योर क्षेत्र के विघटन और उनके लिगेशन का क्लोन बनाने पर ध्यान केंद्रित किया। हमने आगरोस जेल पर क्लोन्स के डबल डाइजेस्ट के माध्यम से और कोलोनी पीसीआर से उचित क्लोनिंग की पुष्टि की। आखिरकार, बीएमपी2 क्लोन को सेंगर सीक्वेंसिंग द्वारा पुष्टि की गई। प्रोटीन का सफल अभिव्यक्ति ई. कोलाई BL21 DE3 में हुआ, और प्रोटीन की योग्यता और अपचयन के लिए, अभिव्यक्ति को रोजेता पीएलआईएसएस और रोजेता BL21 DE3 में भी जाँचा गया। BMP2 अभिव्यक्ति के लिए तापमान और इंड्यूसर संघटन को अनुकूलित किया गया।

अध्याय 4 (बीएमपी2 और Alk3 की शोध, शोध संग्रहण, और वर्णन): इस अध्याय में, हमने लक्षित किया कि इनक्लूजन बॉडीज (आईबी) से मॉनोमर्स की अलगाव करें और बीएमपी2 की शोध सुनिश्चित करें। हमने उसी समय थायोरेडॉक्सिन-टैग्ड Alk3 रिसेप्टर की शोध की, जो E.coli BL21 DE3 में अभिव्यक्त हुआ था, नाइ-एनटीए क्रोमैटोग्राफी द्वारा शोधित, डेनेचरेट, रिफोल्ड, और थ्रॉम्बिन द्वारा थायोरेडॉक्सिन टैग हटाने के

लिए काटा गया। शोधित A1k3 को ओलिगोमर्स को हटाने और पूर्ण A1k3 प्राप्त करने के लिए एनियन एक्सचेंज कॉलम से और एक चरण में शोधा।

बीएमपी2 आईबी के लिए, हमने दो विधियाँ उपयोग की: यूरिया सॉल्यूबाइलाइजेशन और एसिड सॉल्यूबाइलाइजेशन। यूरिया-सॉल्यूबाइलाइज्ड प्रोटीन्स (बीएमपी2) को एसपी सेफारोज़ क्रोमैटोग्राफी द्वारा शोधा गया था ताकि एक पूर्ण मोनोमर प्राप्त हो सके। हालांकि, यूरिया-सॉल्यूबाइलाइज्ड प्रोटीन्स यूरिया को हटाने पर अधिक संघटन के प्रवृत्ति थी, जिससे प्रोटीन की हानि होती थी। एसिड या यूरिया-सॉल्यूबाइलाइज्ड मोनोमर को विभिन्न यौगिकों का उपयोग करके रिफोल्ड किया गया था ताकि एक डाइसल्फाइड-लिंक्ड बीएमपी2 डाइमर बन सके, जिसे नॉन-रीड्यूसिंग एसडीएस-पेज द्वारा पुष्टि की गई थी। हमने मोनोमर और डाइमर को हेपारिन क्रोमैटोग्राफी द्वारा अलग किया। प्रोटीन की संरचना को सर्कुलर डाइक्रोइसम (सीडी) द्वारा पुष्टि की गई थी।

इसके अलावा, हमने सी2सी12 सेल्स को संस्कारित किया और उन्हें बीएमपी2 के साथ अंशात्मक गतिविधि की आकलन के लिए बूफरियानी के माध्यम से उत्तेजित किया गया था। क्लोन किए गए बीएमपी2 की क्रियात्मक गतिविधि को और भी सुनिश्चित किया गया था एक पुनर्मूढ़न यांत्रा के साथ, जिसमें पोषित A1k3 रिसेप्टर के साथ बाइंडिंग एसे पेज का उपयोग करके।

अध्याय 5 - (बीएमपी2 के पुनर्मूढ़न में मॉलेक्युलर और रासायनिक चेपरों का योगदान): इस अध्याय में, मूल रूप से मॉलेक्युलर और रासायनिक चेपरों के योगदान का अध्ययन करने पर ध्यान केंद्रित था, जो बीएमपी2 को सॉल्यूबल फ्रैक्शन में अभिव्यक्त करने में सहायक होते हैं। चेपरों का अच्छी तरह से प्रोटीन को फोल्ड करने में उनकी भूमिका के लिए प्रसिद्ध है, और जब ये बैक्टीरिया में अभिव्यक्त होते हैं, तो बीएमपी2 का रूपबद्ध और विशेष डाइसल्फाइड-रिच संरचना के कारण इनक्लूजन बॉडीज बनाने की प्रवृत्ति होती है।

बीएमपी2 की सॉल्यूबल फ्रैक्शन में अभिव्यक्ति को बढ़ाने के लिए हमने कई प्रयोग किए, जिनमें BMP2 के साथ मोलेक्युलर चेपरों (GroES-GroEL, TF, और DnaK-DnaJ-GrpE) के सह-अभिव्यक्ति, यौगिक चेपरों (त्रेहलोज़ और आर्जिनाइन) के साथ BMP2 की अभिव्यक्ति, और यौगिक चेपरों के साथ मोलेक्युलर चेपरों के सह-अभिव्यक्ति शामिल है।

हमने देखा कि शफल टी सेल्स ने मोलेक्युलर और रासायनिक चेपरों के साथ अच्छी उपज, न्यूनतम तापमान पर सॉल्यूबल, क्रियाशील बीएमपी2 को अभिव्यक्त किया। यौगिक और मोलेक्युलर चेपरों के सह-प्रभाव ने न्यूनतम तापमान पर प्रोटीन की अधिक उत्पादन की ओर की। सॉल्यूबल, क्रियाशील प्रोटीन को नैटिव पेज के माध्यम से उसके रिसेप्टर Aik3 के साथ बाइंड होने की पुष्टि की गई।

अध्याय 6 (बीएमपी2 की स्थिरता और बाइंडिंग अध्ययन): बीएमपी2 की तापमान स्थिरता का अध्ययन 10 °C से 85 °C तक प्रोटीन की फ्लोरेसेंस स्पेक्ट्रा रेकॉर्ड करके किया गया। ट्राइटोफ़ैन को 295 नैनोमीटर पर उत्तेजित किया गया और प्राप्त डेटा का विश्लेषण किया गया। प्रोटीन का मेल्टिंग टेम्परेचर (T_m) 325 के रूप में पाया गया। इसके अलावा, बीएमपी2 के दूर-यूवी सीडी स्पेक्ट्रा को तापमान के फलस्वरूप रेकॉर्ड किया गया, और डेटा को विश्लेषण के लिए फिट किया गया ताकि प्रोटीन के द्वितीयक संरचना में परिवर्तनों का अध्ययन किया जा सके।

बीएमपी2 की स्थिरता को फिर भी pH के साथ बदलते हुए अध्ययन किया गया। सीडी, फ्लोरेसेंस, और एब्जॉर्बेंस स्पेक्ट्रोस्कोपिक मापनों का उपयोग करके pH के साथ प्रोटीन की द्वितीयक और तृतीयक संरचना में परिवर्तनों को देखने के लिए किया गया। इसका परिणाम साबित करता है कि बीएमपी2 अम्लता pH पर स्थिर है, विशेषकर pH 3 और 4 पर। हालांकि, pH 5 के ऊपर, प्रोटीन गुच्छा बनाने लगा। pH 11 और ऊपर में, कोई दृष्टिगत गुच्छाएं नहीं देखी गईं, लेकिन फ्लोरेसेंस में स्पेक्ट्रा की रेडशिफ्ट से प्रोटीन की संरचना

विकर्षित हो गई थी। सम्पूर्ण रूप से, परिणाम इस सुझाव करते हैं कि बीएमपी2 अम्लता pH पर स्थिर है और 325 K तक विभिन्न तापमानों को सहन कर सकता है। ये फ़िंडिंग्स, बायोटेक्नोलॉजी और चिकित्सा क्षेत्रों सहित विभिन्न अनुप्रयोगों में बीएमपी2 का उपयोग के लिए महत्वपूर्ण हैं।

बीएमपी2-AIk3 समर्थन कम्प्लेक्स के गठन को समझने के लिए कई तकनीकों का उपयोग किया गया। पहली तकनीक नेटिव-पेज विश्लेषण थी, जिससे बीएमपी2 और AIk3 के बीच बाइंडिंग स्टोइकियोमेट्री की निर्धारण की गई। एक बीएमपी2 के लगातार बढ़ते संघटन के साथ, जो BMP2 को AIk3 के साथ टाइट्रेट करके और उलटे अनुप्रयोग करके प्राप्त हुआ, की गई।

उपयोग किया गया अगला तकनीक रोटेशनल एनिसोट्रोपी डिफे है, जिसमें AIk3 को Alex flour 488 डाई से लेबल किया गया और लेबल प्रोटीन के एनिसोट्रोपी कमी का मॉनिटरिंग किया गया। बीएमपी2 को जोड़ने से घूर्णन समय बढ़ता गया, जिससे एक कम्प्लेक्स का गठन सूचित हुआ।

स्थिर-स्थिति फ्लोरेसेंस प्रयोग भी किए गए, जिसमें एक स्थिर मात्रा AIk3 को pH 4 पर बीएमपी2 के बढ़ते संघटन के साथ टाइट्रेट किया गया। यौगिक का स्वाभाविक फ्लोरेसेंस मॉनिटर किया गया, और बीएमपी2 मात्रा में वृद्धि के साथ फ्लोरेसेंस इंटेन्सिटी की चित्र एक यू-आकार की कर्व दिखाई दी। यौगिक सांख्यिकी के रूप में कुल फ्लोरेसेंस को यौगिक मात्रा के साथ फिट करने से एक केडी मूल्य प्राप्त हुआ, जिससे बीएमपी2 और AIk3 के बीच मजबूत संबंध की सूचना मिली।

अध्याय 7 (संक्षेप और भविष्य की दृष्टि): यह अध्याय अध्ययन के मुख्य बिंदुओं को प्रस्तुत करता है। हमारा क्लोन प्रोटीन बीएमपी2 मसूदा और ऑर्थोपेडिक्स जैसे क्षेत्रों में एक चिकित्सात्मक प्रोटीन के रूप में उपयोग किया जा सकता है। मोलेक्युलर चैपरोन्स और केमिकल चैपरोन्स साथ में सॉल्यूबल प्रोटीन उत्पन्न करने में सहायक हो सकते हैं, जो उनकी अनुपस्थिति में समाहित होते हैं, जो शोधन कठिन होता है। इसके अलावा,

प्रोटीनों की अमिलता और थर्मल स्थिरता को उच्च करने के लिए उपयुक्त योजकों को जोड़ने से भी हो सकता है। बीएमपी2 का चिकित्सात्मक प्रोटीन के रूप में उपयोग करने के लिए, विभिन्न विषाणु परीक्षण और प्रोटीनों को शरीर में या चोट के स्थान पर एक उपयुक्त कैरियर का उपयोग करके डिलीवरी की अध्ययन पर और अधिक गहराई से अध्ययन करना महत्वपूर्ण है। ये अध्ययन सीमाओं में बीएमपी2 के चिकित्सात्मक प्रोटीन की सुरक्षा और प्रभाव की निर्धारण में महत्वपूर्ण हैं।

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List of abbreviations

1.	BMP2	Bone morphogenetic protein 2
2.	BMPR1	Bone morphogenetic protein receptor, type I
3.	ALP ASSAY	Alkaline phosphatase assay
4.	ELISA	Enzyme-linked immunoassay
5.	IPTG	β -D-1-thiogalactopyranoside
6.	LB Media	3 Luria Broth Media
7.	PBS	Phosphate-Buffered Saline
8.	PCR	Polymerase Chain Reaction
9.	CD	Circular Dichroism
10.	EDTA	Ethylene Diamine Tetraacetic Acid
11.	DTT	Dithiothreitol
12.	FPLC	Fast protein Liquid Chromatography
13.	CHAPS	3-[(3-Cholamidopropyl) Dimethylaminonio]-1-Propanesulphonate
14.	GF	Growth factors
15.	PC	Proprotein Convertase