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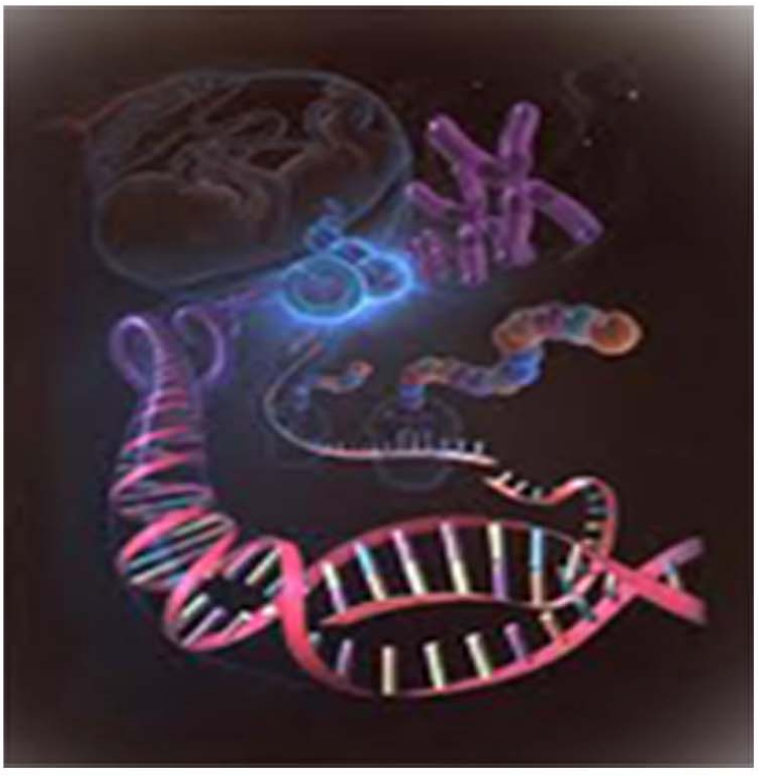

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Research Paper

PROCEDURE FOR ESTABLISHING DOWNSTREAM PROCESS OF RECOMBINANT PLATELET DERIVED GROWTH FACTOR EXPRESSED IN *E. COLI*

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The clone expressing recombinant platelet derived growth factor was constructed and expressed in *E. coli* using standard cloning procedure. The upstream process was developed in shake flask level at 5 L capacity and down stream process was tried using preparative HPLC with different mobile phases and by displacement chromatography. The final process selected was using preparative HPLC with mobile phase composition was 20:35:80 (Acetonitrile: Water: 0.01% Trifluroacetic acid). The purified fractions of PDGF were pooled and freeze dried and the final purity was more than 99% by RP-HPLC.

Keywords: Platelet Derived Growth Factor, Purification, Chromatography, HPLC

INTRODUCTION

The Platelet Derived Growth Factor (PDGF) is a homodimers with five isoforms that are of A-, B-, C-, D-polypeptide chains, i.e., PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD and a heterodimer-AB (Heldin *et al*., 2002). The A- and B- chains are synthesized as inactive precursors but are cleaved during secretion from the producer cell and thus present extracellularly in active forms. In contrast, the C- and D-chains are secreted as inactive forms containing N-terminal CUB domains, which have to be removed before these isoforms can bind to receptors (Li and Eriksson, 2003). PDG-CC and -DD has been shown that they are activated by tissue plasminogen activator and urokinase plasminogen activator (Fredriksson *et al.,* 2004).

PDGF stimulates the proliferation, survival and motility of connective tissue cells and certain other cell types (Heldin and Westermark, 1999). PDGF isoforms have important roles during the embryonal development, particularly to promote the development, particularly to promote the development of various mesenchymal cell types in different organs (Betsholtz, 2004). In the adult, PDGF stimulates normal wound healing (Robson *et al.*, 1992) and regulates the Interstitial Fluid Pressure (IFP) of tissues (Rodt *et al.,* 1996).

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PDGF isoforms are often expressed by epithelial cells in various organs during the embryonal development and PDGF receptors on neighboring mesenchymal cells, suggesting paracrine roles for the PDGF isoforms in the development of different types of mesenchymal cell types in different organs. Detailed insights into the physiological roles of PDGF isoforms have come from the knockout of genes for PDGF isoforms and receptors in mice (Betsholtz, 2004).

PDGF is major mitogens for cells of mesenchymal origin, such as fibroblasts and smooth muscle cells. PDGF is widely expressed in normal and transformed cells and is produced by monocytes and macrophages, vascular endothelial and smooth muscle cells (Antoniades, 1991, Heldin, 1992).

Angiogenesis is critical to embryogenesis, wound repair, diabetic retinopathy, tumor growth, and other conditions. The formation of new vessels requires an increase in proliferation of endothelial cells, the expression of proteolytic enzymes by endothelial cells, the migration of endothelial cell towards an angiogenic stimulus, and the deposition and breakdown of extracellular matrix. The coordinated interplay of these processes leads ultimately to tubular morphogenesis, and, in the presence of pericytes, to capillary formation (Risau, 1990). Based on the multiple applications, Platelet Derived Growth Factor (PDGF-BB) which is a 24.3 kDa disulfide linked homodimer of two B chains with 218 total amino acids. This work has been initiated to development the fermentation and purification process development.

MATERIALS AND METHODS

All the equipments and /instruments required for manufacturing or analysis are procured from reputed manufacturers. All the chemicals used were of analytical/reagent grade obtained from Merck or Qualigens. All the biochemicals, enzymes and other required were obtained from Sigma/Bio-Rad/Invitrogen/New England Biolabs/ Novagen. Primers were procured from Bioserve Biotechnologies India Pvt. Ltd.

Clone Construction and Expression of rhPDGF-BB

Full length cDNA clone containing ORF of PDGF-BB was procured from RZPD, Germany. The PDGF-BB gene was amplified from cDNA by using specific primers

The amplified PDGF-BB gene and pET-23a vector (Novagen) were digested with NdeI and XhoI (New England Biolabs). The digested and gel purified PDGF-BB gene was cloned into digested pET-23a vector. pET23a-PDGF-BB clone was confirmed by colony PCR, restriction digestion and DNA sequencing. pET23a-PDGF-BB plasmid DNA was transformed into *E. coli* strain, BL-21(DE3). The expression of PDGF-BB was checked, shake flask and upstream process was established

Downstream Process

The partially purified protein obtained from cell lysis, inclusion bodies followed by solubilization and refolding was further purified using reverse phase chromatography connected with C18 column and the mobile phase composition was 20:35:80 (Acetonitrile:Water: 0.01% Trifluroacetic acid) at a flow rate of 10 mL per minute, the signal was monitored at 280 nm. The column was properly washed and conditioned for good base line with the same mobile phase.

The refolded sample was concentrated using 10 kDa cutoff membrane to get protein

concentration of about 1.5 ~2.0 mg/mL. 10 mL of sample was injected manually through injection port and the eluted peak of PDGF was collected. In the same manner the entire batch sample was purified and the fractions were pooled. The pooled sample was concentrated and lyophilized using dry ice and by applying high pressure vacuum using vacuum pump in order to remove acetonitrile and to concentrate the sample.

An alternative method "Displacement Chromatography" (Charless Little), was also developed for the purification of recombinant Platelet derived Growth Factor which works on the principle that a molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities.

Sample collected, pooled from refolding step and concentrated sample as describe above was subjected to displacement chromatography. The mobile phase used is mobile phase A (0.1% trifluroacetic acid in water) and Mobile Phase B (0.1% trifluroacetic acid in 90% acetonitrile and 10% water) are used for equilibration of the column and sample loading onto the column. After completion of injection, same mobile phase was passed through the column for 2 min. Then a gradient flow was applied using mobile phase C (0.1% trifluroacetic acid in water + methyltrialkyl (C8-C10) ammonium chloride and mobile Phase B (0.1% trifluroacetic acid in 90% acetonitrile and 10% water + methyltrialkyl (C8-C10) ammonium chloride with a flow rate of 1 mL/min, the absorbance was monitored at 214 nm, column temperature was kept at ambient, auto sampler at 4 °C and the percent purity of the sample is calculated by area normalization mode. PGDF fraction was collected and analyzed for purity and other tests. Column was regenerated with high salt concentration (1 M sodium chloride) re-equilibrated with mobile phase A and B. The collected fraction was checked for purity of the sample by RP-HPLC.

ANALYTICAL METHODS

SDS PAGE

PDGF in process samples and the final purified samples were subjected to SDS PAGE analysis to determine the purity of the sample on 12% gels, stained with coomassie stain.

Western Blotting

The SDS-PAGE samples were electrophoretically transferred to 0.2 µm PVDF membrane and western blotting was performed using primary and secondary antibodies developed with substrate against PDGF-BB.

RP-HPLC

Purity of PDGF was tested by using RP-HPLC which is carried out by using a C18 column on Shimadzu chromatography system with UV detector at 220 nm, mobile phase A is 0.1% TFA in water and mobile phase B is 0.1% TFA in 900 mL ACN and 100 mL water and the flow is 1.0 mL/min and run time is 45 min.

SEC-HPLC

SEC-HPLC was used to check the dimers and aggregates formed during the entire process. TSKgel G2000SWXL was used UV detector at 220 nm, run time is 60 min, and mobile phase is sodium phosphate buffer.

Protein Quantification

Protein estimation was done by Bradford's method using Coomassie brilliant G-250.

Isoelectric Point

The composition of the IEF gel used for determination of pI value of the sample is 1.5 g of acrylamide and bisacrylamide (29:1), 2.2 g urea, ampholyte pH range 3-5 (0.1 mL), ampholyte pH range 3-19 (0.04 mL) 0.07 µL of 10% APS, 0.08 µL of TEMED. 1 M sodium hydroxide is used as cathode buffer and 1 M phosphoric acid as anode buffer. Sample is loaded onto the gel and applied constant voltage of 100 for 1 h followed by 200 V for 1 h and 500 V for 2 h. After completion of run the gel is placed in fixing solution followed by staining and destaining process.

Host Cell Derived Proteins

HCPs contamination is checked by ELISA method provided by kit manufactured by alpha diagnostic international, USA. Acceptable limit of HCPs are less than 100 ppm in final purified sample.

HcDNA

Host cell contamination was tested by PCR method. Standard *E. coli* DNA are diluted to 10, 1.0, 0.1 and 0.01 ng, all the standards and the samples were used for amplification. After completion of PCR run, 10 µL of the PCR product was loaded on a 1% agarose gel with DNA ladder and the gel is visualized using gel documentation system. Acceptable limit of residual DNA contamination is less than 10 ng.

In vitro Potency (ELISA)

ELISA method was used as per the procedure provided by kit and the standard dilutions of 400 pg to 1 pg was prepared to get a linear curve and the samples were diluted that the absorbance of the sample fall in between the lower standard and higher standard value.

100 µl of each standard and samples were added into appropriate wells and incubated for 2.5 h at room temperature with gentle shaking. Solution in the wells was discarded and washed 4 times with 300 µl of wash solution. 100 µl of 1x prepared biotinylated antibody was added to each well and incubated for 1 h at room temperature with gentle shaking and discarded the solution and washed the wells. 100 µl of Streptavidin solution was added to each well. Incubated for 45 min at room temperature with gentle shaking. The solution was discarded and washed the wells. 100 µl of TMB substrate reagent was added to each well and incubated for 30 min at room temperature in the dark with gentle shaking. The reaction was stopped by adding 50 µl of Stop Solution and the absorbance was measured at 450 nm immediately.

Bacterial Endotoxin Test

Gel clot method is simple and well used method in which LAL reagent is added to the sample and incubated at 37°C for 1 h in water bath or dry heating block. Presence of endotoxins is indicated by formation of gel clot and vice versa.

RESULTS AND DISCUSSION

The PGDF-BB gene was cloned into pET vector and transformed into BL21 (DE3) cells. The transformed clone was verified by restriction mapping, gene sequence and conformed by expression check, colony PCR and found that the gene was successfully cloned, transformed into *E. coli*. The protein expressed was with molecular weight of about 24 kDa. Upstream process was developed using different steps like cell lysis, centrifugation and isolation of inclusion bodies, solubilization of inclusion bodies and refolding of the protein.

Preparative HPLC

Semi-preparative HPLC system was used by

using C18 column and we had tried with two different buffer compositions, one is mobile A containing 0.1% TFA in acetonitrile, mobile phase B contains 0.1% TFA in water, flow rate was 10 mL /min, gradient flow was used. The PDGF was eluted at 45% gradient. The fractions collected were pooled and analyzed for its purity by RP-HPLC and SDS-PAGE, the purity of >99% was achieved, but these fractions has high concentration TFA and acetonitrile. For clinical application purified protein has to be free of solvents. Different methods were used to remove TFA and acetonitrile and final method was selected by using rotavapor.

The total HPLC fractions were pooled (~260 mL) and collected into 500 mL round bottom flask and connected to rotavapor and applied vacuum. The water bath in which the RB flask was kept was maintained at 10 ^{o}C by addition ice at regular intervals. The volume of sample was reduced to about 50 mL during this step, indicates most of the solvent was evaporated. Traces of acetonitrile were tested by gas chromatography and trifluoroacetic acid was tested by HPLC. We had manually freeze dried the sample by applying high vacuum, during the entire process of freeze drying the sample was maintained at –20 ~ –30 ^{o}C for 3-4 h, the resultant sample was in solid form, which was tested for RP-HPLC and SDS-PAGE to check any loss in purity during evaporation and freeze drying process and we found there was no objectable loss of purity.

An alternate method was developed using different buffer system was mobile phase A (10 mM phosphate buffer) and mobile phase B (100% acetonitrile), flow rate was 10 mL/min, gradient flow was used. In-order to improve the stability of PDGF, the sample was freeze dried as in above step, sample was tested for purity after this step by RP-HPLC and SDS-PAGE and purity was found to be good. However the purification process was optimized using the first mobile phase composition as the separation and peak shape of PDGF peak was sharp which enables to collect the peak from a particular height and absorbance.

Another method was also tried to purify the protein was one of the three modes of chromatography techniques called displacement chromatography whereas the other two modes are elution and frontal mode. The major advantages of displacement chromatography we found was its flexibility, it can be used in product capture step, in between any of the other purification steps, polishing step during late stage, concentrate and purify the protein present in small quantities, simple to operate, easy to scale up, high yields and large amount of protein can be separated by using an analytical column.

Purification of Platelet derived Growth factor was successful by displacement chromatography. Initial purity was about 78% and other impurities which are very closely eluting impurities in other chromatographic steps and after completion of displacement chromatography the percent purity was greater than 99% with recovery of 72.4%. The recovery obtained through displacement chromatography is far better than any other chromatography purifications. However based on the experience gained during the development of purification process, it was concluded that the initial process was selected, i.e., preparative HPLC using mobile A containing 0.1% TFA in acetonitrile, mobile phase B contains 0.1% TFA in water.

Characterization of PDGF Protein

Protein Estimation by Bradford's Method

The protein content of the purified sample was estimated by Bradfords method and the concentration was found to be about 0.5 mg/mL.

Immunoblotting

Identification of PDGF-BB was done by Immunoblotting technique using reducing SDS gel as mentioned in methods. Following the electrophoresis run, the gel was rinsed with transfer buffer to remove electrophoresis buffer salts. Protein from the gel was transferred from gel to PVDF membrane by Amersham semi dry western blot transfer unit at 25 V for 1 h. The transferred PVDF membrane placed in blocking buffer and washed with TBST buffer. Primary antibodies were added and incubated for 2 h, followed by secondary antibody incubated for 2 h. The membrane was finally washed with TBST buffer and was developed using TMB system. The results after development highlighted the bands specific to PDGF-BB and the protein is confirmed as the migration, band size and shape of purified PDGF-BB band was same with that of standard band (as shown in Figure 1).

SDS-PAGE

Purity and the molecular weight of the product were determined by using SDS-PAGE by applying a constant voltage of 130 V for 60 min. After completion of run, gel was stained with coomassie blue and destained with destaining solution. The gel shows a single band of the test solution and the reference solution match in the position and intensity which indicates the purified sample exactly matches with the standard solution.

The gel showed a single band of the test solution and the reference solution matching in the position and intensity indicating that the purified sample is PDGF-BB.

RP-HPLC

The purity of PDGF-BB sample after the final purification step was determined by RP-HPLC using Inertsil C18 column, 250 mm x 4.6 mm, 5 μm Shimadzu chromatography system with UV

Figure 1: Sample Details: Lane 1: PDGF-BB Standard and Lane 2: Purified PDGF Sample

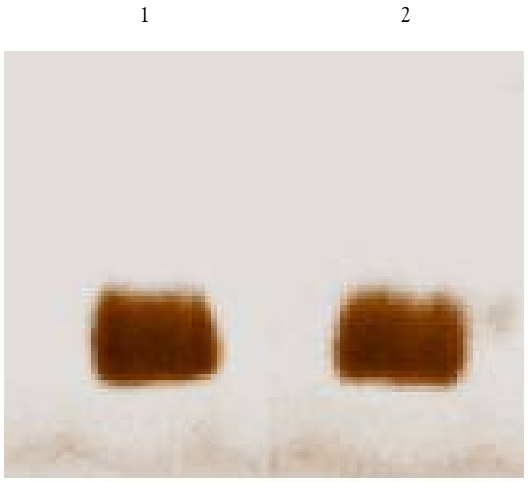
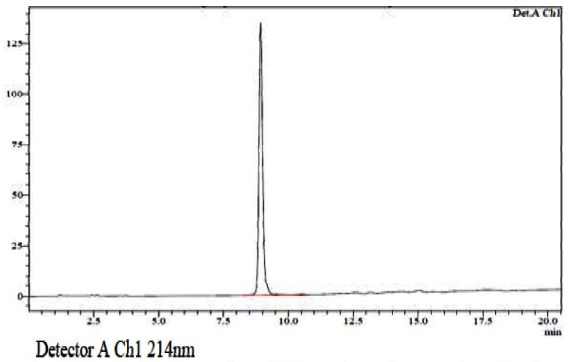


Figure 2: The RP-HPLC Chromatogram of Purified PDGF-BB



Detector A Ch1 214nm

Peak	Ret. Time	RRT	Area	Area %
1	8.930	1.000	1345437	99.557
2	9.618	1.077	2542	0.188
3	10.496	1.175	3449	0.255
Total			1351428	100.000

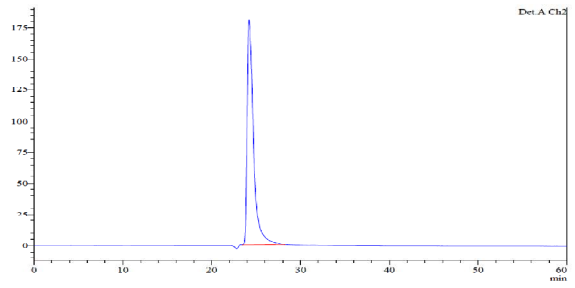
detector and gradient of mobile phase A is 0.1% trifloroacetic acid in water and mobile phase B is 0.1% trifloroacetic acid in 900 mL acetonitrile and 100 mL of water. Injection volume is 100 µL. Analysis was carried at 220 nm and the run time is 60 min. The % purity of PDGF-BB was after semi preparative HPLC step purity followed by freeze drying step was greater than 99% as shown in Figure 2.

Size Exclusion Chromatography

TSKgel G2000SWXL was used UV detector at 220 nm, run time is 60 min, and mobile phase is sodium phosphate buffer pH 7.0. A single peak with purity greater than 99% was observed which indicates that there are no dimers or other related substances (Figure 3).

Formation of dimers and aggregates is a common phenomena for which occurs in some unfavorable condition for all proteins and peptides products, which are identified and quantified by using size exclusion chromatography.

Figure 3: SEC-HPLC Chromatogram of Purified PDGF-BB



Detector A Ch1 220nm

Peak	Ret. Time	Area	Area %
1	24.204	106967815	100.000
Total		106967815	100.000

Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) is an electrophoretic technique by which amphoteric compound are fractionated according to their pI values along a continuous pH gradient. The process of IEF in Carrier Ampholytes (CA) and in Immobilized pH Gradients (IPG) provides an additional force which counteracts diffusion of CA and so maximizes the ratio of separative to dissipative transports; this substantially increases the resolution of the fractionation method. Isoelectric point is very important in any purification process. IEF gel was prepared by mixing 1.5 g of acrylamide and bisacrylamide (29:1), 2.2 g urea, ampholyte pH range 3-5 (0.1 mL), ampholyte pH range 3-19 (0.04 mL) 0.07 mL of 10% APS, 0.08 µL of TEMED. Cathode buffer used is 1 M sodium hydroxide and anode buffer is 1 M phosphoric acid. Sample is loaded onto the gel and applied constant voltage of 100 for 1 h followed by 200 V for 1 h and 500 V for 2 h. After completion of run the gel is placed in fixing solution followed by staining and destaining process. The observed pI value is 9.8.

Host Cell Derived Proteins

HCPs are contaminants generated during fermentation and cell lysis step and may cause immunogenic reactions if present in larger amount. Acceptable limit of HCPs are less than 100 ppm (100 ng) in final purified sample. HCPs contamination is checked by ELISA method by using kit manufactured by alpha diagnostic international, USA. The HCP content is 80 ng which is within the limit of 100 ng as per the WHO guidelines for host cell contamination in purified sample.

Host Cell DNA Contamination

Host cell contamination was tested by PCR method. Host (*E. coli* BL21 (DE3) cells were grown to OD of 2.0 at 600 nm, culture was centrifuged and from the pellet , DNA was isolated by standard procedure as given by kit

manufacturer (Macherey-Nagel). DNA was quantified by measuring absorbance at 260 and 280 nm. Pure DNA has an A260/A280 ratio of 1.8~1.9, lower ratios indicate protein contamination and higher indicates RNA contamination. An A260 is equal to 1.0 indicates a [DNA] =50 μg/ μL. Standard DNA are diluted to 10, 1, 0.1 and 0.01 ng , all the standards and the samples were used for amplification. After completion of PCR run, 10 μL of the PCR product was loaded on a 1% agarose gel with DNA marker and the gel is visualized using gel documentation system. From the results the host cell contamination is less than 10 ng which is less than the acceptable limit of residual DNA contamination of less than 10 ng. The effect of any inhibition of PCR reaction was tested by spiking purified sample with 10 ng of purified *E. coli* DNA. From the results it is concluded that there is no interference in PCR reaction.

Bacterial Endotoxin Test

Endotoxins are fever causing agents which are generated from cell wall disruption of *E. coli* which are detected by gel clot. Gel clot method is simple and well used method which LAL reagent is added to the sample and incubated at 37 °C for 1 h in water bath or dry heat block. Presence of endotoxins is indicated by formation of gel clot and vice versa. The endotoxin limit is less than 20 EU/mg of protein.

In Vitro Potency

In vitro activity of EGF was tested by ELISA. A monoclonal antibody specific for EGF has been precoated onto a microplate. Standards and samples are pipetted into the wells and any EGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme linked polyclonal antibody specific for PDGF is added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a 1:1 ratio mix of hydrogen peroxide and TMB substrate solution is added to the wells and color develops in proportion to the amount of PDGF bound in the initial step. The color development is stopped by adding 2 N sulfuric acid and the intensity of the color is measured within 30 min at 450 nm. The PDGF content is 0.58 mg/mL.

CONCLUSION

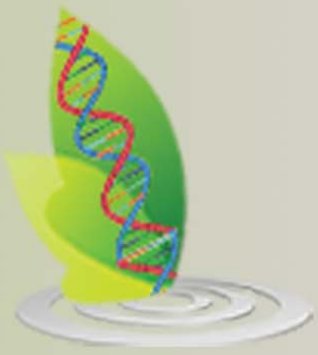
Recombinant PDGF producing clone was developed using established cloning and transformation techniques in *E. coli* cells. The shake flask, fermentation and purification process was optimized for the production and purification of PDGF. The protein was characterized for its identity, purity, safety and *in vitro* activity. The purified protein was found to be greater than 99% pure. From the published data it was known that PDGF plays a major role in various applications like diabetic wound healing, angiogenesis, etc., further formulation studies has to be performed for development of novel formulations.

REFERENCES

1. Antoniades H N (1991), "PDGF: a multifunctional growth factor", *Baillieres Clinical Endocrinology and Metabolism,* Vol. 5, pp. 595-613.
2. Betsholtz C (2004), "Insight into the physiological functions of PDGF through genetic studies in mice", *Cytokine growth Factor Rev.,* Vol. 15, pp. 215-228.
3. Charless Little (2008), "Displacement chromatography comes of age",

Chromatography techniques, pp. 18-23.

4. Fredriksson L, Li H, Fieber C, Li X and Eriksson U (2004), "Tissue plasminogen activator is a potent activator of PDGF-CC", *EMBO J*., Vol. 3, pp. 3793-3802.
5. Guofeng Zhao and Yan Sun (2007), "Displacement chromatography of proteins on hydrophobic charge induction adsorbent column", *Journal of Chromatography* A, Vol. 1165, pp. 109-115.
6. Heldin C H and Wastermark B (1999), "Mechanism of action and in vivo role of platelet derived growth factor", *Physiol. Rev*., Vol. 79, pp. 1283-1316.
7. Heldin C H H, Eriksson U and Ostman A (2002), "New members of platelet-derived growth factor family of mitogens", *Arch. Biochem. Biophys*, Vol. 398, pp. 284-290.
8. Li X and Eriksson U (2003), "Novel PDGF family members: PDGF-C and PDGF-D", *Cytokine Growth Factor Rev.*, Vol. 14, pp. 91-98.
9. Risan W (1992), "Angiogenic growth factors", *Prog. Growth Factor Res*., pp. 71-79.
10. Robson M C, Phillips L G, Thomason, Robson L E and Piece G F (1992), "Platelet derived growth factor BB for the treatment of chronic pressure ulcers", *Lancet,* Vol. 339, pp. 23-25.
11. Rodt S A, Ahlen K, Berg A, Rubin K and Reed R K (1996), "A novel physiological function for platelet-derived growth factor-bb in rat dermis", *J. Physiol.,* Vol. 495 (Pt.1), pp. 193-200.



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