OPEN ACCESS

GAVIN PUBLISHERS

International Journal of Chromatography and Separation Techniques

Sivakumar T, et al. Int J Chromatogr Sep Tech: IJCST-115.

Research Article

DOI: 10.29011/2577-218X. 000015

Purification of Authentic Human Basic Fibroblast Growth Factor Expressed in Both the Cytoplasm and Culture Medium of *Escherichia coli*

T. Sivakumar, Cheuk Yin Nelson Lai, C.C. Lam, Xiuhua Hu, Hao Wang, K.L. Ng, Wan-Keung R. Wong*

Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China.

***Corresponding author:** Wan-Keung R. Wong, Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China. Tel: +85223587299; Fax: +85223581552; Email: bcwkrw@ust.hk. Tel: +85223587299; Fax: +85223581552; Email: bcwkrw@ust.hk

Citation: Sivakumar T, Lai CYN, Lam CC, Hu X, Wang H, et al. (2018) Purification of Authentic Human Basic Fibroblast Growth Factor Expressed in Both the Cytoplasm and Culture Medium of *Escherichia coli*. Int J Chromatogr Sep Tech: IJCST-115. DOI: 10.29011/2577-218X. 000015

Received Date: 05 April, 2018; **Accepted Date:** 21 April, 2018; **Published Date:** 30 April, 2018

Abstract

Through intein-mediated and fed-batch fermentative expression, we have recently reported high-level production of ~600 mg l^{-1} of authentic human basic fibroblast growth factor (abFGF) in *Escherichia coli*. In this communication, we report the development of purification protocols for efficient retrieval of purified abFGF from both culture supernatant and lysate fractions of JM101 [pWK311ROmpAd] cells. Extracellular abFGF in the culture supernatant was efficiently purified using a 3-step approach which included salt precipitation, heparin affinity chromatography, followed by dialysis for desalting. On the other hand, the major portion of abFGF (~85%) present in the cell lysate was purified using a separate 3-step approach involving cation exchange chromatography, heparin affinity chromatography and gel filtration. The cation exchange chromatographic step worked effectively to result in a 24-fold increase in the specific activity of the lysate, thereby greatly facilitating the achievement of homogeneously pure abFGF by subsequent heparin affinity chromatography. Using the two purification protocols described, good recoveries of 70% and 61% of abFGF activity were obtained from the supernatant and cell lysate samples, respectively. The simplicity and high reproducibility of the methods support that expression and purification of abFGF from JM101 [pWK311ROmpAd] presents an attractive approach to cost-effectively produce quality abFGF for various applications.

Keywords: bFGF; CM Sephadex Cation Exchange Chromatography; Heparin Affigel Chromatography; Intein; Salt Precipitation; Size-Exclusion Chromatography

Introduction

Basic fibroblast growth factor has been shown to possess a wide spectrum of medical applications, including wound and bone healing [1,2], burn vascular grafting [2], lens and limb regeneration [3], and treatment for myocardial infarction [4]. Moreover, findings of other beneficial effects of bFGF on angiogenesis [5], stem cell signalling [6], neuron extension and survival [7] support the potential use of bFGF in treating neurodegenerative diseases like Alzheimer's and Parkinson's disease in the future [8]. Although bFGF has been isolated from various biological sources including placenta [9], bovine brain and pituitaries [10-12], chick embryo brain, retina and vitreous [13], carpus luteum [14], and rat brain microglia [15], the small quantities of bFGF available from these natural specimens inflicted high costs on the extraction and purification of the target protein. Recombinant DNA technology has offered a promising solution for cost-effective production of heterologous useful/valuable proteins [16]. Expression of bFGF has been undertaken using various host systems including yeast, *E. coli* and even rice endosperm [17-21]. However, due to various difficulties including expression as fusion products, formation of protein aggregates and imprecisely processed variants, bFGF derivatives or isoforms have been commonly obtained as final products [17,21,22].

Our laboratory has been involved in engineering various expression systems using both *E. coli* and *B. subtilis* as hosts to produce valuable proteins [16,23-30]. In the 1990s, our group pioneered the development of excretory *E. coli* systems, which had been employed for large-scale production of a medically important

skin growth protein, human epidermal growth factor (hEGF) cost effectively [16,27,29,31,32]. The extracellular approach facilitated not only the implementation of a facile purification process, but also the attainment of authentic and highly potent hEGF as the final product. The purified hEGF has been demonstrated to be effective in treating various skin problems including diabetic foot ulcers [33], bedsores [34], Stevens-Johnson syndrome [35], scalds [36] and surgical wounds [37]. Recently, we have developed an effective approach to enable the production of both hEGF and bFGF in different compartments of *E. coli* [25]. The findings showed that both hEGF and bFGF, for the first time, were not only co-expressed in the cytoplasm, but also confirmed to possess identical (authentic) primary structures as those of their native counterparts [25]. Moreover, both bFGF and hEGF were shown to be auto-cleavable from their fusion partner, the Sce VMA intein, to form soluble products in the cytosol of *E. coli* [25]. Since then, employing an amplification-based approach, which involved the application of an efficient expression construct, designated pWK311RompAd, and working in conjunction with a refined fed-batch fermentation protocol, we achieved a 5-fold increase in the production of bFGF, thereby resulting in an impressive yield of 610 mg l^{-1} of the polypeptide in *E. coli*. More importantly, the product was confirmed to be authentic bFGF (abFGF), thus sharing the same primary structure with its native counterpart [38].

In this communication, the efficacies of different purification methods to purify recombinant abFGF from both the growth medium and cell lysate of JM101 [pWK311RompAd] were investigated. Implementation and optimization of the purification steps, systematic removals of protein contaminants, and subsequent fine-tuning of the steps involved were reported and discussed. The abFGF product was characterized to be homogeneously pure and authentic in structure.

Materials and Methods

Bacterial Strain and Plasmid

E. coli JM101 [39] was used as the expression host. Plasmid pWK311ROmpAd employed for abFGF expression was described previously [38].

Chemicals and Anti-abFGF Antibody

All the chemicals used were from Sigma Aldrich unless otherwise specified. Small quantities of abFGF previously attained [26] were used to immunize rabbits. The primary antibody used for Western blot analysis was purified from the antiserum using recombinant Protein G-Sepharose 4B resins [Life Technologies, Rockford, IL, USA] as recommended by the manufacturer.

Expression of abFGF in Fermentors

The seed culture employed for fermentation was prepared in two stages using MMBL medium [29]. Cultivation of JM101 [pWK311ROmpAd] was performed in 2-liter fermentors using conditions essentially the same as described previously [38]. The efficacies of purification of abFGF expressed in both the cytoplasm and culture supernatant were studied.

Precipitation of abFGF from Culture Medium Using Ammonium Sulphate

Ammonium sulphate was added gradually with rapid mixing into the culture supernatant for 3 h to attain equilibrium [40]. The amounts of salt required to attain various levels of saturation were calculated with the help of the encorbio tool [http://www.encorbio.com/protocols/AM-SO4.htm]. During the process of precipitation, the mixture was kept at pH 8.5 by adding 1 M Tris-HCl. Precipitated abFGF was harvested by centrifugation at 12,000 ×g for 40 min at 4°C. The abFGF pellet was saved and dissolved in 50 ml of 50 mM Tris, pH 7.4 buffer, followed by extensive dialysis against buffer B (1 mM Na_2HPO_4, 0.17 mM KH_2PO_4, pH 7) at 4°C and filtering through a 0.45-µm filter.

Cell Lysis

The cell pellet was dissolved in 400 ml of phosphate-buffered saline (PBS; 50 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl and 1 mM EDTA, pH 6.4) and breakage was achieved using a pressure cell homogenizer (model FPG12800, Stansted Fluid Power Ltd; Harlow, Essex) as described previously [41]. The cell lysate was clarified using 0.45-µm filters.

Cation Exchange Chromatography

The column (XK 26/40; Pharmacia biotech, Uppsala, Sweden; bed height of 14 cm and bed volume of 74 ml) packed with CM Sephadex C-25 gel (GE Healthcare, Sweden) was first equilibrated with PBS. Then 400 ml of the filtered cell lysate were loaded onto the column at a rate of 1 ml min^{-1}, followed by washing the column thoroughly with PBS. Bound proteins were eluted with elution buffer (HS-PB; 50 mM Na_2HPO_4/NaH_2PO_4, 600 mM NaCl, 1 mM EDTA, pH 7.4) using a single step gradient, with the effluent collected in 4.0 ml fractions at a rate of 1 ml min^{-1}. Altogether 70 fractions were collected and they were assayed for abFGF activity by SDS-PAGE and/or Western blot analysis. The active fractions were pooled and subjected to further purification by heparin chromatography.

Affi-gel Heparin Affinity Chromatography

The column (XK 16/20; Pharmacia biotech, Uppsala, Sweden; bed height of 8 cm and bed volume of 16 ml) packed with Affi-gel heparin gel (Bio-Rad Laboratories, USA) was first equilibrated exhaustively with HS-PB at a flow rate of 1 ml min^{-1}. Then 96 ml of abFGF sample collected from cation purification were loaded onto the column at a rate of 0.5 ml min^{-1}, followed by washing the column thoroughly with HS-PB. Bound proteins were eluted with buffer A (50 mM Na_2HPO_4/NaH_2PO_4, 2.5 M NaCl, 1

mM EDTA, pH 7.4) in a single step, with the effluent collected in 1.5 ml fractions at a rate of 0.75 ml min^{-1}. Altogether 64 fractions were collected and they were analysed for abFGF activity by SDS-PAGE and/or Western blot analysis.

Size-exclusion Chromatography

Sephadex G-25 (Amersham Pharmacia biotech, Uppsala, Sweden) chromatography was used to remove salt from the abFGF-containing fractions collected from heparin purification. The column (XK 26/40; Pharmacia biotech, Sweden; bed height of 30 cm and bed volume of 160 ml) was equilibrated thoroughly with a modified PBS (LS-PBS; 150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) at a flow rate of 2.0 ml min^{-1}. A volume of 22.5 ml of heparin eluate sample was loaded onto the column at a rate of 1 ml min^{-1}. Bound abFGF was eluted as 2 ml fractions at 1 ml min^{-1} using LS-PBS.

Protein Analysis

Protein bands separated on a 10% tricine SDS-PAGE gel [42] were stained with Coomassie Brilliant Blue [43] or silver nitrate solution [44] according to standard operating procedures. Pre-stained protein markers (Broad range; Bio-Rad) were used as molecular weight markers. Western blot analysis was performed as described previously [43]. Verification of the identity of the purified abFGF was achieved using LC-MS/MS as described previously [25,26].

Results

Purification of abFGF from Culture Supernatant

Ammonium Sulphate Precipitation

Our previous work indicated that 20% of abFGF, comprising ~110 mg l^{-1} of the polypeptide, expressed in transformant JM101 [pWK311ROmpAd] was detected in the growth medium [38]. Thus the culture supernatant appeared to present a decent source for the purification of abFGF. Initially, ammonium sulphate precipitation was employed to reduce the volume of excreted abFGF.

It was found that when ammonium sulphate had reached 25% saturation, precipitation of abFGF began to occur (data not shown). The precipitation process took effect until salt saturation had reached a level of 70%, after which further salt addition would not result in more precipitation of abFGF. Further study showed that as high as 92% of abFGF could be effectively precipitated from the medium if ammonium sulphate was added to a level of 68% saturation.

Heparin Affinity Chromatography

Around 100 mg of abFGF were salt precipitated from 1.3 litres of the culture medium of JM101 [pWK311ROmpAd]. Subsequent to extensive dialysis against buffer B (Materials and Methods), 90.3% of abFGF activity (equivalent to 98.6 mg) was recovered. The pH value of the dialysed sample was adjusted to 7.4, followed by loading the entire volume onto the heparin column (Materials and Methods). Bound abFGF was eluted effectively with a linear gradient of NaCl (0.5 - 2.5 M). The active fractions, 19-46 (Figure1(a) and 1(b)), in which 80% of abFGF activity was recovered (Table 1(a)), were pooled for dialysis and subsequent analysis.

Analysis of Purified abFGF

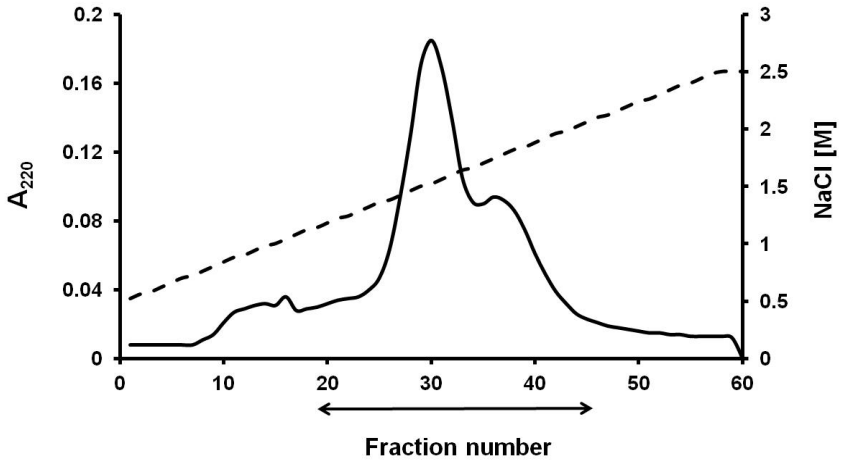
Subsequent to desalting by dialysis, a total yield of 76.5 mg of abFGF was recovered (Table 1(a)). The purified product was revealed to be homogeneously pure as substantiated by silver staining (Figure 2) and immunoblotting analysis (data not shown).

Sample	Total protein[a] (mg)	abFGF[b] (mg)	Specific activity[c] (mg/mg)	Purification factor[d]	Recovery[e] (%)
Supernatant	4097.2 ± 171.6	109.1 ± 2.9	0.03	1.0	100.0
Ammonium Sulphate precipitate	2005.7 ± 60.8	100.6 ± 2.4	0.05	1.9	92.2
Desalted precipitate	1891.2 ± 71.9	98.6 ± 6.0	0.05	2.0	90.3
Heparin eluate	87.2 ± 0.9	87.2 ± 0.9	1	37.6	80.0
Dialysed eluate	76.5 ± 1.2	76.5 ± 1.2	1	37.6	70.2

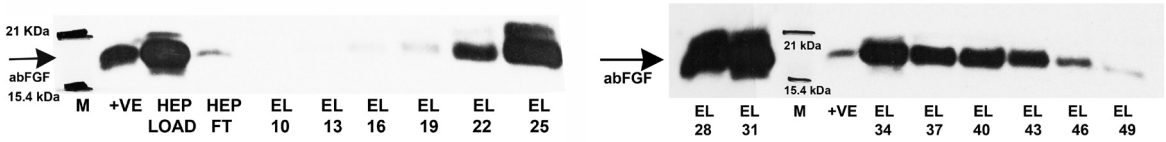
[a, b] Total protein and abFGF were estimated using immunoblot techniques. All the values were represented as mean ± SEM obtained from three independent experiments.
[c] Specific activity measured as amount of abFGF[(b)] / amount of total protein[(a)].
[d] Purification factor measured as specific activity of individual step / specific activity of initial step.
[e] Recovery of abFGF obtained in individual stage in comparison with initial stage.

Table 1(a): Summary of purification of abFGF from culture supernatant of JM101 [pWK311RompAd].

(a)



(b)



Figures 1(a-b): Purification of abFGF from culture supernatant of JM101 [pWK311RompAd] by heparin chromatography. The conditions of the purification were described in Materials and Methods. (a) In the chromatogram, A_{220} values (——), NaCl elution gradient (-----) and the fractions containing abFGF activities (⟷) are denoted. (b) Western blot analysis revealing abFGF activities distributed in various eluted fractions. The lanes marked HEP LOAD (material loaded onto the heparin column) and HEP FT (flow-through) were each loaded with 2 µl of sample, whereas lanes EL 10-60 were each loaded with 8 µl of fractionated eluate. M denotes molecular weight markers; +VE denotes abFGF; the position of abFGF is indicated by the arrow (⟶).

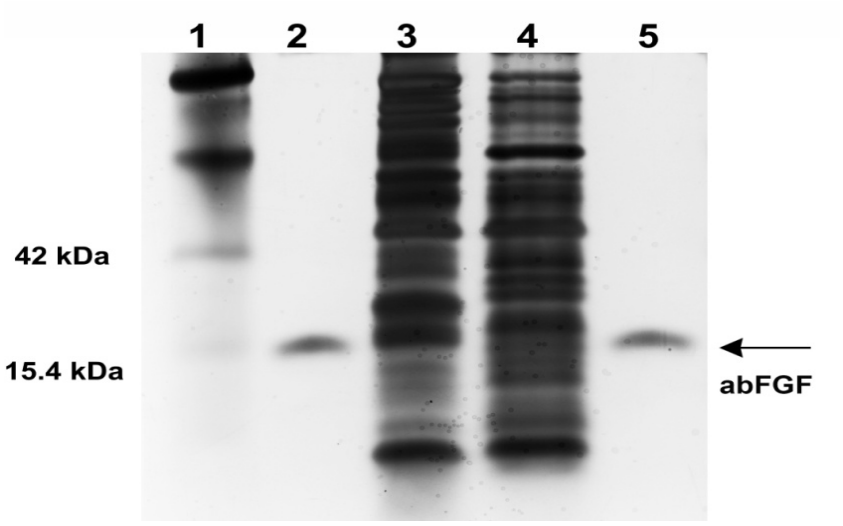


Figure 2: SDS-PAGE analysis of samples collected from different stages of purification of abFGF from culture supernatant of JM101 [pWK311RompAd]. Samples were run on a 10% gel and analysed with silver staining. Lanes: (1) protein molecular weight markers; (2) 1 µg abFGF standard; (3) culture supernatant sample; (4) sample collected after ammonium sulphate precipitation; (5) dialysed sample of eluate collected from the heparin column. The position of abFGF is indicated by the arrow (⟶).

Purification of abFGF from the Cell Lysate

CM Sephadex C-25 Chromatography

In purifying abFGF from the lysate of JM101 [pWK311ROmpAd], in view that a relatively small sample size was handled and that a major portion, over 80% (>500 mg l^{-1}) of abFGF was confined in the cytoplasm [38], ion exchange chromatography was considered to be suitably employed as the first method of choice. Moreover, since abFGF possesses a high pI value of 9.6 [9], a cation exchange protocol was expected to work more efficiently than an anion exchange method in separating abFGF from endogenous proteins. Consequently, CM Sephadex C-25 ion-exchange chromatography (Materials and Methods) was exploited as the first step to purify abFGF. The suitability of three different buffer systems composed of MESNA, Tris-HCl and phosphate, of which the pH values were set from 6 to 7.4, in the equilibration of the cation exchange column was evaluated. It was found that the phosphate buffer (PBS; Materials and Methods) provided the best conditions for binding abFGF to the resins.

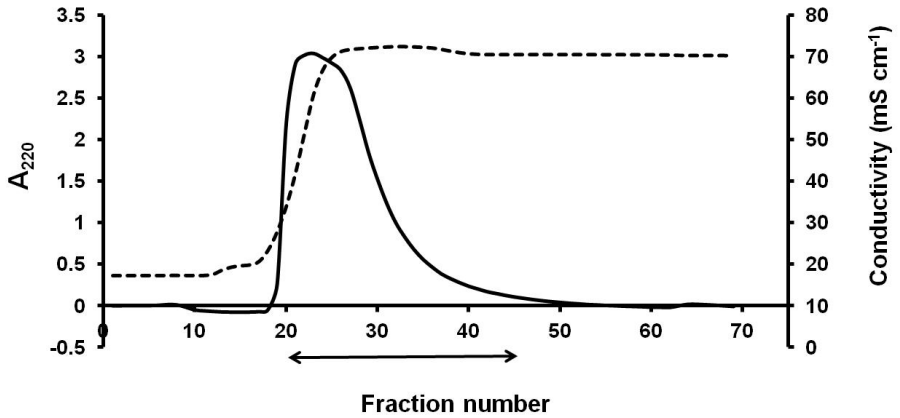
Subsequent to equilibration with PBS, the column was loaded with 360 ml of filtered cell lysate containing 321 mg of abFGF. With a step gradient and a high salt buffer, HS-PB (Materials and Methods), the bound proteins were eluted efficiently. SDS-PAGE analysis (Figure 3(b)), followed subsequently by Western blotting (data not shown), revealed that abFGF elution began when the conductivity increased up to around 40 mS cm^{-1} (fraction 21) and continued until fraction 45, where the conductivity, 70 mS cm^{-1}, was determined to be maximum (Figure 3(a)). Fractions 21-45, in which 71.7% of abFGF activity was recovered (Table 1(b)), were pooled for heparin chromatography.

Sample	Total protein[a] (mg)	abFGF[b] (mg)	Specific activity[c] (mg/mg)	Purification factor[d]	Recovery[e] (%)
Cell lysate	35788.6 ± 2327.6	321 ± 8.3	0.01	1	100
CM eluate	1075.2 ± 42	230.2 ± 10.8	0.21	23.7	71.7
Heparin eluate	206.6 ± 1.5	206.6 ± 1.5	1	111.1	64.4
Desalted eluate	196.4 ± 3.2	196.4 ± 3.2	1	111.1	61.2

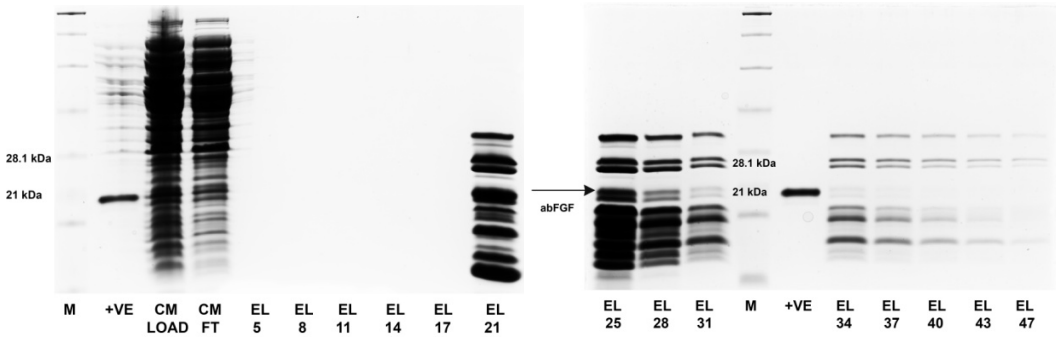
[a, b] Total protein and abFGF were estimated using immunoblot techniques. All the values were represented as mean ± SEM obtained from three independent experiments.
[c] Specific activity measured as amount of abFGF[(b)] / amount of total protein[(a)].
[d] Purification factor measured as specific activity of individual step / specific activity of initial step.
[e] Recovery of abFGF obtained in individual stage in comparison with initial stage.

Table 1(b): Summary of purification of abFGF from cell lysate.

(a)



(b)

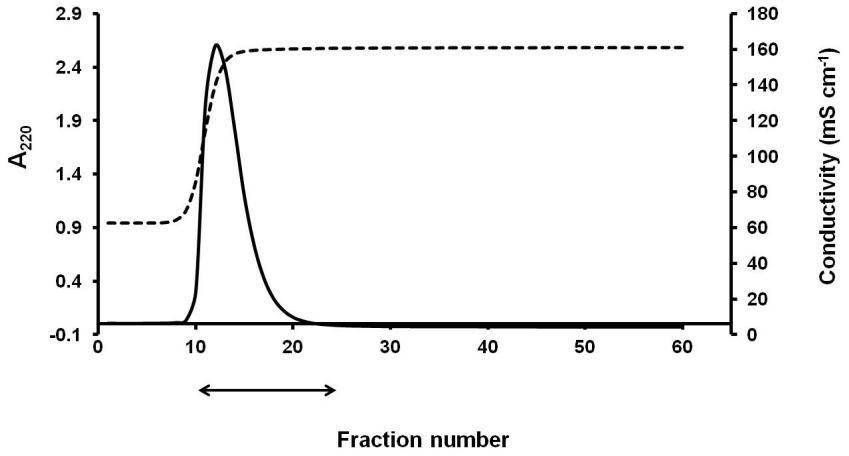


Figures 3(a-b): Purification of abFGF from lysate of JM101 [pWK311ROmpAd] by cation exchange chromatography. The conditions of the purification were described in Materials and Methods. (a) Elution of the bound proteins was achieved using a single step gradient as shown. In the chromatogram, A_{220} values (——), the conductivity profile (-----) and the fractions containing abFGF activities collected for the next stage of purification (◄——►) are denoted. (b) SDS-PAGE analysis of eluted fractions collected from the cation exchange column. The lanes marked CM LOAD (material loaded onto the cation exchange column) and CM FT (flow-through) were each loaded with 10 µl of sample, whereas lanes EL 5-47 were each loaded with 16 µl of fractionated eluate. Fractions EL 21-45 analysed by Western blotting to contain abFGF activities (data not shown) were pooled for heparin chromatography. M denotes molecular weight markers and +VE denotes standard abFGF which is indicated by the arrow (——►).

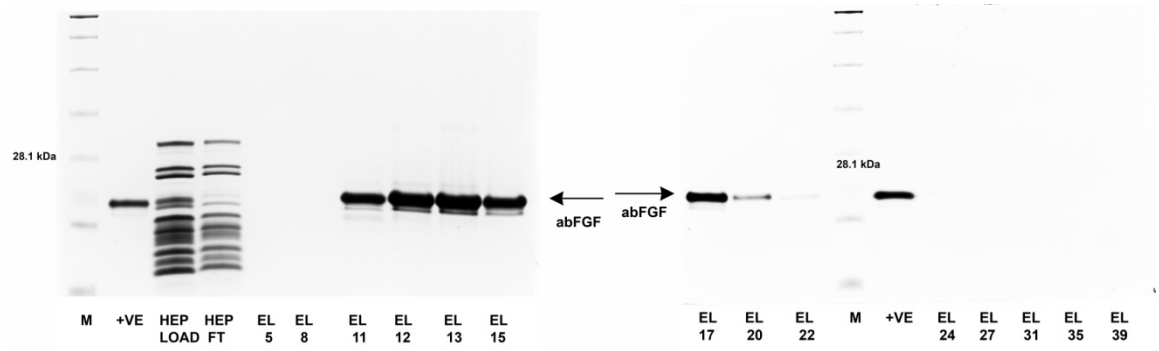
Heparin Chromatography

Trial runs were conducted to identify conditions that might facilitate maximum binding of abFGF to heparin. Variations in pH and temperature did not have a significant impact on the binding. However, it was noted that supplementing increased concentrations of NaCl to PBS (HS-PB; Materials and Methods) helped minimize non-specific binding of contaminants to heparin. To initiate heparin affinity chromatography, 96 ml of active sample containing 230 mg of abFGF eluted from the cation exchange column were loaded onto the heparin column (Materials and Methods). Despite using HS-PB, abFGF bound well to heparin and there was no apparent loss of it in the flow-through. Thus, a rather high concentration of NaCl, 2.5 M (buffer A; Materials and Methods), was employed to facilitate abFGF elution (Figure 4). It was noted that elution of abFGF began when the conductivity increased up to around 110 mS cm^{-1} (fraction 11) and lasted until fraction 23, where the conductivity, 160 mS cm^{-1}, was determined to be maximum (Figure 4(a) and 4(b)). The active fractions, 11-23, were collected for size-exclusion chromatography.

(a)



(b)

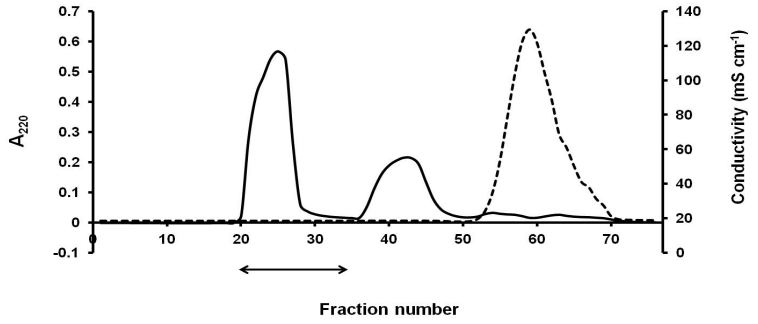


Figures 4(a-b): Purification of abFGF by heparin chromatography. (a) The fractions containing abFGF activities denoted in (Figure 3) were collected and loaded onto the heparin column as described in Materials and Methods. A single-step elution was employed to elute the bound proteins as shown. In the chromatogram, A_{220} values (——), the conductivity profile (-----) and the fractions containing abFGF activities collected for size-exclusion purification (⟷) are denoted. (b) SDS-PAGE analysis of eluted fractions collected from the cation exchange column. The lanes marked HEP LOAD (material loaded onto the heparin column) and HEP FT (flow-through) were each loaded with 10 µl of sample, whereas lanes EL 5-39 were each loaded with 16 µl of fractionated eluate. Fractions EL 11-23 analysed by Western blotting to contain abFGF activities (data not shown) were pooled for size-exclusion chromatography. M denotes molecular weight markers; +VE denotes abFGF standard; the position of abFGF is indicated by the arrow (⟶).

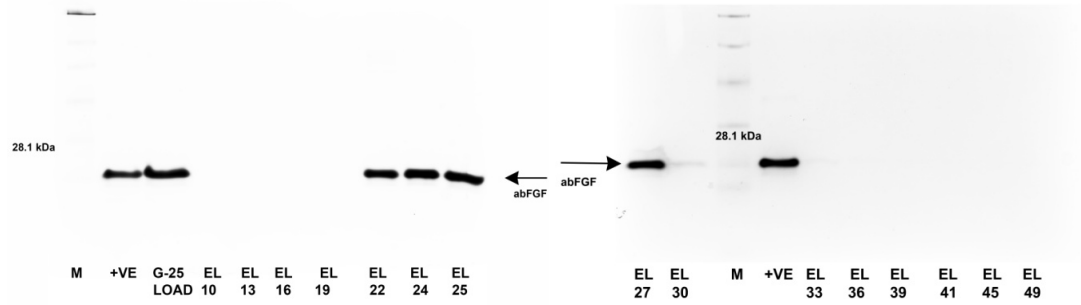
Size-exclusion Chromatography

A pool of 22.5 ml containing 206 mg of abFGF was then desalted using a Sephadex G-25 desalting column (Materials and Methods). At low NaCl concentrations, abFGF was found to interact weakly with the gel matrix, thus retarding its elution from the column (data not shown). Therefore, in equilibrating the column, 1X PBS supplemented with 150 mM NaCl (LS-PBS; Materials and Methods) was used. Eventually, elution of abFGF was achieved with continuous application of LS-PBS. A good recovery of over 196 mg of abFGF was retrieved from fractions 20 to 34 covering the first protein peak (comprising 60 to 88 ml of the void volume) of the elution profile (Figure 5).

(a)



(b)



Figures 5(a-b): Purification of abFGF by size-exclusion chromatography. (a) The fractions containing abFGF activities denoted in (Figure 4) were pooled and desalted using a Sephadex G-25 column as described in Materials and Methods. In the chromatogram, A_{220} values (——), the conductivity

profile (-----) and the fractions containing abFGF activities collected for lyophilisation (⟷) are denoted. (b) SDS-PAGE analysis of eluted fractions collected from the size-exclusion column. The lanes marked G-25 LOAD (eluted sample collected from the heparin column) was loaded with 10 µl of sample, whereas lanes EL 10-49 (fractionated eluates) were each loaded with 16 µl of sample. M denotes molecular weight markers; +VE denotes abFGF standard; the position of abFGF is indicated by the arrow (⟶).

Analysis of abFGF Products Purified from Culture Medium and Lysate of JM101 [pWK311ROmpAd]

SDS-PAGE

Protein products purified from culture medium and cell lysate preparations of JM101 [pWK311ROmpAd] were resolved on SDS-PAGE gels, followed by silver staining. The results supported the view that the abFGF products obtained from both purification protocols were homogeneously pure (Figure 2 and figure 6). Immunoblotting and scanning densitometry further substantiated the conclusion that both products were free of detectable protein contaminants (data not shown).

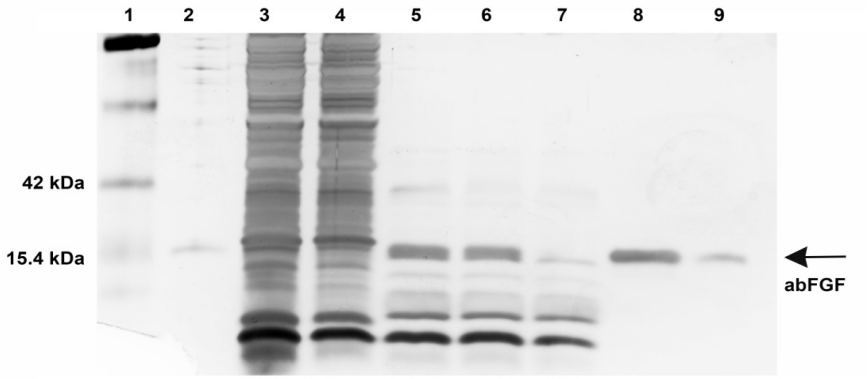


Figure 6: SDS-PAGE analysis of samples collected from different stages of purification of abFGF from cell lysate of JM101 [pWK311RompAd]**.** Samples were run on a 10% gel and analysed with silver staining. Lanes: (1) protein molecular weight markers; (2) 1 µg of abFGF standard; (3) cell lysate sample; (4) and (5) flow-through and eluate samples collected from the CM column, respectively; (6) sample loaded onto the heparin column; (7) and (8) flow-through and eluate samples collected from the heparin column, respectively; (9) eluate sample collected from the size-exclusion column. The position of abFGF is indicated by the arrow (⟶).

LC-MS/MS

Last but not least, analysis by LC-MS/MS (Materials and Methods) substantiated the above results that the purified abFGF products were homogeneously pure. Moreover, the products were also shown to share an identical primary structure comprising 146 amino acid residues (Table 2) with native abFGF [12].

Peptide[b]	Mr(Expt)[c]	Mr(calc)[d]
$^{NH2-}$PALPEDGGSGAFPPGHFK	1779.605	1779.858
LYCKNGGFFLR	1374.645	1373.691
IHPDGRVDGVR	1219.538	1219.642
SDPHIKLQLQAEER	1663.025	1662.869
LLASKCVTDECFFFER	2021.918	2020.939
LESNNYNTYR	1272.405	1272.573
KYTSWYVALK	1257.505	1257.676
SVTDECFFFER	1435.025	1435.608
TGPGQKAILFLPMSAK	1657.785	1657.923
AILFLPMSAKS^{-COOH}	1192.765	1192.653

[a] Purified cell lysate sample was tryptic digested and analysed by LC-MS/MS

[b] A total of 442 peptides were identified by Mascot search engine and the sequences covering the N-terminal and C-terminal regions of abFGF were presented.

[c] Experimental mass to charge ratio of peptide transformed to relative molecular mass.

[d] Relative molecular mass calculated from matched peptide sequence.

Table 2: Analysis of purified abFGF sample of cell lysate[a] by liquid chromatography tandem mass spectrometry.

Discussion

Expression of recombinant bFGF using various approaches and host systems have been reported for many years [17-21]. However, probably due to the difficulties in implementing cost-effective mass production of bFGF, the market price of this polypeptide, even though it is essentially available in the form of isoforms possessing different structural compositions and

molecular sizes, has been set incredibly and unreasonably high, amounting to over US$ 1 million per gram [45,46]. A primary goal of the research activities undertaken in our laboratory has been the engineering of efficient host systems for cost-effective production of quality proteins. A prominent example has been the application of an excretory approach to the cost-effective production of authentic hEGF (ahEGF) in *E. coli* [29]. Recombinant ahEGF was not only shown to possess the same primary structure as its native counterpart, but also demonstrated to be superbly stable and potent [27]. Moreover, it is available at a much reduced price, which is as low as only 25-30% of that of its commercial counterparts [16].

On the other hand, the fine quality of ahEGF has been shown to play a crucial role in effective treatments of chronic or hard-to-heal wounds including diabetic foot ulcers, bedsores and Steven Johnson syndrome [33-37]. In this regard, it has been speculated that ahEGF is as safe as native hEGF [27,47], and that hEGF derivatives might exhibit unusual interaction with hEGF receptor mutants possessing increased ligand affinities, thus potentially causing malignant transformation when applied to promote cell growth [37,48].

In view of the fine performance of ahEGF and the fact that mass production of this authentic polypeptide had been achieved cost-effectively, in attempting to express recombinant bFGF, we planned to devise a protocol to facilitate expression of authentic bFGF (abFGF) and subsequently its production on a large scale. Recently, using an innovative intein mediated gene expression approach, abFGF has been successfully expressed as a soluble product in both the cytoplasm and culture medium of *E. coli* [25]. Subsequent to structural optimization and gene copy number increment, the new construct, pWK311ROmpAd, was shown to be able to express an unusually high level, over 600 mg l^{-1}, of abFGF in *E. coli* [38]. It was 5 times better than that obtained from the prototype plasmid, pWK3R, constructed in the initial study [25].

Since high levels of abFGF, ca. 500 and 110 mg l^{-1}, were detected in the cytoplasm and growth medium of JM101 [pWK311ROmpAd], respectively, it was wondered how effective it might be if abFGF was purified from both fractions. In view of the large volume of the medium to be handled, it was decided that it would be beneficial if the volume was largely reduced first, and that salt precipitation using $(NH_4)_2SO_4$ might be an appealing choice. Initially, although it was quite time consuming and labour intensive to achieve maximum precipitation of abFGF, which would still maintain a high specific activity, the study was worthwhile since the resulting process was highly reproducible, effective (Table 1(a)) and easier to operate in subsequent rounds of abFGF precipitation. After dissolving the precipitate in a reduced volume of buffer, abFGF was conveniently and efficiently purified using the conventional method, heparin affinity chromatography (Figure 1). Subsequent to the removal of salt using dialysis, a good rate of ca. 70% of abFGF originally precipitated from the culture medium was recovered from the entire process (Table 1(a)).

A totally different approach was taken as the first step to purify abFGF from the lysate of JM101 [pWK311ROmpAd]. Despite a much smaller sample volume to be handled, large quantities of endogenous proteins in the lysate required an efficient method for their removal. The exceptionally high pI value, 9.6, of abFGF [9] suggested that cation exchange chromatography might be applicable to the separation of the polypeptide from the majority of contaminating proteins. With the use of weakly acidic PBS, abFGF bound readily onto the negatively charged cation exchange matrix, and was effectively eluted from the column using a high salt buffer. The protocol resulted in a well recovered (ca. 72%) and a much less contaminated (ca. 24-fold purer) intermediate abFGF product (Table 1(b)).

Subsequent to the removal of a major fraction of the host cell proteins using the cation exchange procedure (Table 1(b)), further purification of abFGF by first heparin affinity chromatography and then desalting or size-exclusion chromatography was readily accomplished. The overall recovery of abFGF was decently high, reaching a rate of over 61%. More importantly, the final abFGF product was analysed to be homogeneously pure (Table 1(b); Figure 6).

Despite the large differences in composition and volume between the crude samples derived from the culture medium and the cytoplasm, purification of abFGF was shown to be essentially successful and effective in both cases (Tables 1(a) and 1(b)). In retrospect, the first step implemented in the two purification protocols: salt precipitation of the culture medium and cation exchange chromatography of the cell lysate, played a crucial role in establishing the suitability, which concerned not only high levels of activity and specific activity but also a manageable size, of the crude samples for further processing. Accordingly, salt precipitation helped largely reduce the working volume of the culture medium, whereas cation exchange chromatography effectively removed contaminating proteins from the lysate (Figure 6). Consequently, the following heparin purification step commonly employed in both schemes was facilitated to be effectively undertaken to remove residual host cell proteins and result in homogeneously pure abFGF (Figure 2 and 6; Tables 1(a) and (b)). On the whole, around 37 and 111 folds of abFGF purification were productively achieved using culture supernatant and lysate preparations of JM101 [pWK311ROmpAd] as starting materials, respectively (Tables 1(a) and (b)).

Notwithstanding the effective application of salt precipitation to the concentration of culture medium on a small scale, in handling precipitation of a large volume, the operation might not be technically convenient or cost-effective. On the other hand, in view that: i) cation exchange chromatography is a relatively facile process to implement; ii) over 61% of purified

abFGF was retrievable from the cell lysate (Table 1(b)); iii) the DNA construct, pWK311ROmpAd, being capable of mediating both cytoplasmic and extracellular expression of abFGF in *E. coli* [38], could be modulated to facilitate essentially the former mode of expression, it is possible to implement a facile protocol for cost-effective production and purification of abFGF essentially using the lysate of JM101 [pWK311ROmpAd] as the starting material. The protocol may prove to be beneficial to the development of a large-scale production process, through which abFGF may become commercially available at a much reduced price for use in a wide range of applications.

Acknowledgments

This study was funded by RGC project: GRF16101515 and Research Contracts: 13142580CLIL07W011 and 1617219-0. We thank Biosciences Central Research Facility at HKUST for their mass spectrometry services. This article does not contain any studies with human participants or animals undertaken by any of the authors.

References

1. Andres C, Hasenauer J, Ahn HS, Joseph EK, Isensee J, et al. (2013) Wound-healing growth factor, basic FGF, induces Erk1/2-dependent mechanical hyperalgesia. PAIN® 154: 2216-2226.
2. Ono I, Akasaka Y, Kikuchi R, Sakemoto A, Kamiya T, et al. (2007) Basic fibroblast growth factor reduces scar formation in acute incisional wounds. Wound Repair Regen 15: 617-623.
3. Gospodarowicz D, Mescher AL (1980) Fibroblast growth factor and the control of vertebrate regeneration and repair. Ann N Y Acad Sci 339: 151-174.
4. Virag JAI, Rolle ML, Reece J, Hardouin S, Feigl EO, et al. (2007) Fibroblast growth factor-2 regulates myocardial infarct repair. Am. J. Pathol. 171: 1431-1440.
5. Gospodarowicz D, Bialecki H, Thakral TK (1979) The angiogenic activity of the fibroblast and epidermal growth factor. Exp Eye Res 28: 501-514.
6. Coutu DL, Galipeau J (2011) Roles of FGF signaling in stem cell self-renewal, senescence and aging. Aging 3: 920.
7. Morrison RS, Sharma A, De Vellis J, Bradshaw RA (1986) Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. Proc Natl Acad Sci 83: 7537-7541.
8. Woodbury ME, Ikezu T (2014) Fibroblast growth factor-2 signaling in neurogenesis and neurodegeneration. J Neuroimmune Pharmacol 9: 92-101.
9. Gospodarowicz D, Cheng J, Lui GM, Fujii DK, Baird A, et al. (1985) Fibroblast growth factor in the human placenta. Biochem Biophys Res Commun 128: 554-562.
10. Gospodarowicz D, Cheng J, Lui GM, Baird A, Böhlent P (1984) Isolation of brain fibroblast growth factor by heparin-sepharose affinity chromatography: identity with pituitary fibroblast growth factor. Proc Natl Acad Sci USA 81: 6963-6967.
11. Böhlen P, Baird A, Esch F, Ling N, Gospodarowicz D (1984) Isolation and partial molecular characterization of pituitary fibroblast growth factor. Proc Natl Acad Sci USA 81: 5364-5368.
12. Esch F, Baird A, Ling N, Ueno N, Hill F, et al. (1985) Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. Proc Natl Acad Sci U S A 82: 6507-6511.
13. Mascarelli F, Raulais D, Counis MF, Courtois Y (1987) Characterization of acidic and basic fibroblast growth factors in brain, retina and vitreous chick embryo. Biochem Biophys Res Commun 146: 478-486.
14. Gospodarowicz D, Cheng J, Lui GM, Baird A, Esch F, et al. (1985) Corpus luteum angiogenic factor is related to fibroblast growth factor. Endocrinology 117: 2383-2391.
15. Shimojo M, Nakajima K, Takei N, Hamanoue M, Kohsaka S (1991) Production of basic fibroblast growth factor in cultured rat brain microglia. Neurosci Lett 123: 229-231.
16. Wong WKR, Fu Z, Wang YY, Ng KL, Chan AKN (2012) Engineering of efficient *Escherichia coli* excretion systems for the production of heterologous proteins for commercial applications. Recent Pat Chem Eng 5: 45-55.
17. Barr PJ, Cousens LS, Lee-Ng CT, Medina-Selby A, Masiarz FR, et al. (1988) Expression and processing of biologically active fibroblast growth factors in the yeast *Saccharomyces cerevisiae*. J Biol Chem 263: 16471-16478.
18. Garke G, Deckwer WD, Anspach FB (2000) Preparative two-step purification of recombinant human basic fibroblast growth factor from high-cell-density cultivation of *Eschericia coli*. J Chromatogr B Biomed Sci App 737: 25-38.
19. Mu X, Kong N, Chen W, Zhang T, Shen M, et al. (2008) High-level expression, purification, and characterization of recombinant human basic fibroblast growth factor in *Pichia pastoris*. Protein Expr Purif 59: 282-288.
20. An N, Ou J, Jiang D, Zhang L, Liu J, et al. (2013) Expression of a functional recombinant human basic fibroblast growth factor from transgenic rice seeds. Int J Mol Sci 14: 3556-3567.
21. Mirzahoseini H, Mehraein F, Omidinia E, Razavi MR (2004) Differential expression of human basic fibroblast growth factor in *Escherichia coli*: potential role of promoter. World J Microbiol Biotechnol 20: 161-165.
22. Alibolandi M, Mirzahoseini H (2011) Purification and refolding of overexpressed human basic fibroblast growth factor in *Escherichia coli*. Biotechnol Res Int 2011:973741.
23. Wong WK (2008) Application of recombinant microbial systems to the production of commercially valuable proteins. Res J Biotech 3(3): 3.
24. Wong WKR, Lam E, Huang RC, Wong RSC, Morris C, et al. (2001) Applications, and efficient large-scale production of recombinant human epidermal growth factor. Biotechnol Genet Eng Rev 18: 51-71.
25. Kwong KWY, Wong WKR (2013) A revolutionary approach facilitating co-expression of authentic human epidermal growth factor and basic fibroblast growth factor in both cytoplasm and culture medium of Escherichia coli. Appl Microbiol Biotechnol 97: 9071-9080.
26. Kwong KWY, Ng KL, Lam CC, Wang YY, Wong WKR (2013) Authentic human basic fibroblast growth factor produced by secretion in *Bacillus*

subtilis. Appl Microbiol Biotechnol 97: 6803-6811.

27. Huang RC, Lam E, Chen YH, Hackett J, Lam TL, et al. (1999) Human epidermal growth factor excreted by recombinant *Escherichia coli* K-12 has the correct N-terminus and is fully bioactive. Process Biochem 35: 1-5.

28. Wong DKH, Lam KHE, Chan CKP, Wong YCV, Wong WKR, et al. (1998) Extracellular expression of human epidermal growth factor encoded by an *Escherichia coli* K-12 plasmid stabilized by the ytl2-incR system of *Salmonella typhimurium*. J Ind Microbiol Biotechnol 21: 31-36.

29. Sivakesava S, Xu ZN, Chen YH, Hackett J, Huang RC, et al. (1999) Production of excreted human epidermal growth factor (hEGF) by an efficient recombinant *Escherichia coli* system. Process Biochem 34: 893-900.

30. Kwong KWY, Ng AKL, Wong WKR (2016) Engineering versatile protein expression systems mediated by inteins in *Escherichia coli*. Appl Microbiol Biotechnol 100: 255-262.

31. Wong WKR (2014) Means and method for expression of authentic human epidermal growth factor and/or basic fibrolast growth factor in cytoplasm and/or culture medium of *Escherichia coli*. US patent: 9580484B2.

32. Wong WKR (2014) Method and composition for treating skin wounds with epidermal growth factor. European patent: EP1482966B1.

33. Tsang MW, Wong WKR, Hung CS, Lai KM, Tang W, et al. (2003) Human epidermal growth factor enhances healing of diabetic foot ulcers. Diabetes Care 26: 1856-1861.

34. Wong WKR, Lam KH, Tsang MW (2009) Method and composition for treating skin wounds with epidermal growth factor. US Patent: 7517528B2.

35. Tsang MW, Tsang KY, Wong WKR (2004) The use of recombinant human epidermal growth factor (rhEGF) in a gentleman with drug-induced Steven Johnson syndrome. Dermatol Online J 10: 25.

36. Wong WKR, Kwong KWY, Ng KL (2016) Application of recombinant human epidermal growth factor to effective treatment of scalds. J Anal Pharm Res 3: 1-7.

37. Wong WKR (2015) Effective treatment of an unhealed incision of a diabetic patient with recombinant human epidermal growth factor. Mod Chem Appl 3: 166.

38. Kwong KWY, Sivakumar T, Wong WKR (2016) Intein mediated hyperproduction of authentic human basic fibroblast growth factor in *Escherichia coli*. Sci Rep 6: 33948.

39. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York..

40. Burgess RR (2009) Protein precipitation techniques. Methods Enzymol 463: 331-342.

41. Ng KL, Lam CC, Kwong KWY, Dik WN, Lai CY, et al. (2016) Enhancement of fish growth employing feed supplemented with recombinant fish growth hormone expressed in *Bacillus subtilis*. Res J Biotechnol 11: 1-11.

42. Schägger H, Von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368-379.

43. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, et al. (1987) Current protocols in molecular biology, John Wiley and Sons, New York.

44. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68: 850-858.

45. Proteins (Human Source) (2018) Biocompare.com, (n.d.).

46. Basic fibroblast growth factor (Human, rDNA derived) WHO International Standard (2018), (n.d.).

47. Wong WKR, Ng KL, Lam CC, Hu XH, Nelson CYL, et al. (2017) Review article: reasons for underrating the potential of human epidermal growth factor in medical applications. J Anal Pharm 4: 00101.

48. Berlanga-Acosta J, Govilondo-Cowley J, del Barco-Herrera D, Martin-Machado J, Guillen-Nieto G (2011) Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) as tissue healing agents: clarifying concerns about their possible role in malignant transformation and tumor progression. J Carcinog Mutagen 2: 115.