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Simultaneous clarification of *Escherichia coli* culture and purification of extracellularly produced penicillin G acylase using tangential flow filtration and anion-exchange membrane chromatography (TFF-AEMC)

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ABSTRACT

Downstream purification often represents the most cost-intensive step in the manufacturing of recombinant proteins since conventional purification processes are lengthy, technically complicated, and time-consuming. To address this issue, herein we demonstrated the simultaneous clarification and purification of the extracellularly produced recombinant protein by *Escherichia coli* using an integrated system of tangential flow filtration and anion exchange membrane chromatography (TFF-AEMC). After cultivation in a bench-top bioreactor with 1 L working volume using the developed host/vector system for high-level expression and effective secretion of recombinant penicillin G acylase (PAC), the whole culture broth was applied directly to the established system. One-step purification of recombinant PAC was achieved based on the dual nature of membrane chromatography (i.e. microfiltration-sized pores and anion-exchange chemistry) and cross-flow operations. Most contaminant proteins in the extracellular medium were captured by the anion-exchange membrane and cells remained in the retentate, whereas extracellular PAC was purified and collected in the filtrate. The batch time for both cultivation and purification was less than 24 h and recombinant PAC with high purity (19 U/mg), yield (72% recovery), and productivity (41 mg of purified PAC per liter of culture) was obtained. Due to the nature of the non-selective protein secretion system and the versatility of ion-exchange membrane chromatography, the developed system can be widely applied for effective production and purification of recombinant proteins.

1. Introduction

Traditional downstream processing for recombinant protein production includes several major preparative and purifying steps, i.e. clarification, capture, intermediate purification, polishing, and formulation [1,2]. It often accounts for the major portion of protein manufacturing costs. Technological experience accumulated over the past few decades has highlighted the importance of more systematic and integrated approach for optimal bioprocess development. In light of this, tackling technical issues in the upstream (i.e. strain construction) and/or midstream (i.e. cultivation) stages might become an effective solution to the reduction of the high costs associated with the downstream processing stage. However, practical demonstration of this well-perceived knowledge is still uncommon up to now because most recombinant proteins in *E. coli* are produced intracellularly and, as a result, bioprocessing operations in the cultivation and downstream processing stages are rather independent. While strategies for extracellular production of recombinant proteins [3] have been proposed, downstream processing might remain tedious without excluding extensive preparative steps due to the composition incompatibility of the spent culture medium and chromatographic buffer.

Among various techniques associated with downstream processing for recombinant protein production, membrane filtration and chromatography are commonly performed [2]. While traditional chromatographic columns are well characterized and widely used, they have many limitations, such as lengthy cleaning and

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packing steps, low diffusive binding of materials, low flow rate, high pressure drop, and long processing time [4]. Chromatographic membranes, on the other hand, offer numerous advantages over chromatographic columns, including high convective binding of materials, high flow rate, low pressure drop, short processing time, being disposable rendering regeneration and cleaning unnecessary, and being easy to scale up [4–6]. Moreover, recent improvements in membrane chemistry have yielded better membrane products with various chromatographic functionalities, such as ion-exchange, affinity, and hydrophobic interaction, as well as high binding capacities comparable to resins [4,6]. In particular, ion-exchange membrane chromatography is gaining popularity as a polishing step for the removal of viral particles and endotoxin upon the production of monoclonal antibodies [7]. Additionally, chromatographic membranes can be manufactured in a non-conventional format of cross-flow geometry, such as hollow-fiber, plate and frame or spiral wound devices, which was originally designed to reduce fouling during ultrafiltration [1,4,6]. These tangential flow devices give chromatographic membranes an extra dimension of applicability that has not been fully explored.

Chromatography is usually the key step to increase the purity of recombinant protein of the process stream during downstream processing. Prior to this step, extensive preparative steps are required, which may be as time-consuming and costly as chromatographic operations. For recombinant proteins produced intracellularly in *E. coli*, the preparative steps typically include centrifugation, filtration, precipitation, and desalting to formulate the lysate in a buffer compatible with the subsequent chromatographic operation (Fig. 1). While the strategies for extracellular production of recombinant proteins have the major advantage of the recombinant protein product being separated from most intracellular contaminant proteins, further purification of the secreted recombinant protein product will most likely require several of the above preparative steps (Fig. 1). Accordingly, simplification or even elimination of these preparative steps would substantially reduce the processing time and manufacturing costs.

In this study, we demonstrated a novel and effective bioprocess for extracellular production of recombinant protein in *Escherichia coli* and its immediate purification through the seamless integration of all cultivation and downstream processing steps. The integrated system combined simultaneous operations of *tangential flow filtration* for culture clarification and *anion exchange membrane chromatography* (TFF-AEMC) for PAC harvest/purification. Penicillin G acylase (PAC), an industrial enzyme for the production of β-lactam antibiotics [8,9], was used as a target protein for the demonstration because it is normally expressed in the periplasm of *E. coli* with a heterodimeric structure consisting of a 23-kDa α-subunit and a 63-kDa β-subunit through rather unique post-translational processing [10,11]. The industrial importance of PAC also leads to the development of various expression strategies for its large-scale production. Extracellular production of recombinant PAC was achieved through the use of an outer-membrane mutant with a defective murein lipoprotein [12–14] as an expression host. The mutation resulted in the leakage of periplasmic proteins, including PAC, through the compromised outer-membrane into the extracellular medium with a minimal effect on cell growth. Compared to the traditional downstream processing for purification of intracellularly produced PAC using anion-exchange chromatography as the key purification step, the developed integrated system greatly reduced the length, complexity, and manufacturing costs of this bioprocess (Fig. 1). The demonstration of TFF-AEMC for simultaneous culture clarification and recombinant protein purification significantly extends the benefits and applicability of the biochemical and genetic strategies for extracellular production of recombinant proteins.

2. Materials and methods

2.1. Plasmids and strains

The strain used for PAC expression in this study were *E. coli* JE5505 (Coli Genetic Stock Centre, Yale, USA) genotype (*F*$^-$, *Δ(gpt-proA)62*, *lacY1*, *tsx-29*, *glnV44(AS)*, *galK2(Oc)*, and λ$^-$, *Δlpp-254*, *pps-6*,*hisG4(Oc)*, *xylA5*, *mtl-1*, *argE3(Oc)*, *thi-1)* [15]. Transformation was done by electroporation using the *E. coli* Pulser (Bio-Rad, Hercules, CA). The plasmid pTrcKnPAC2902 containing the pac operon from *E. coli* ATCC11105 regulated by the trc promoter was previously constructed [10].

2.2. Cultivation methods

The recombinant strain was stored at −80 °C in a lysogeny broth (LB)/glycerol (85% (v/v) LB broth described below, 15% (v/v) glycerol) stock. The cells were revived by plating on LB agar (15 g/L agar dissolved in LB broth) supplemented with kanamycin (50 μg/mL). A single colony was inoculated into a flask of 50 mL of LB broth (5 g/L yeast extract, 10 g/L tryptone, 0.5 g/L NaCl, and 50 μg/mL kanamycin) and incubated in a rotary shaker for 16 h at 30 °C and 200 rpm. The 50 mL seed culture was inoculated into a bench-top bioreactor (Omni-Culture, VirTis, Gardiner, NY) containing 1 L working volume of LCM3 medium (5 g/L casamino acids, 2.5 g/L yeast extract, 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, 2 mM NaCl, 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM K_2HPO_4, 0.2 mM $CaCl_2 \cdot 2H_2O$, 1.5 mg/L $FeSO_4 \cdot 7H_2O$, 0.6 mg/L of $MnSO_4$, 0.6 mg/L $CuSO_4 \cdot 5H_2O$, 0.6 mg/L $ZnSO_4 \cdot 7H_2O$) with 20 μL/L Antifoam 204 (Sigma, St. Louis, MO). The bioreactor was sparged with filter-sterilized air at 2 vvm and agitated at 250 rpm. The pH was regulated using a combination pH electrode (Mettler-Toledo, Switzerland), a pH controller (PC310, Suntex, Taipei, Taiwan), and two MasterFlex peristaltic pumps (Cole-Palmer, Vernon Hills, IL, USA) at pH = 7.0 ± 0.1. The reactor was operated at 28 °C. Protein expression was induced with 0.15 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after approximately 4 h when the cell density reached 0.5 OD600. The broth was harvested after 12 h of induction for purification.

2.3. Chromatography materials

The strong anion-exchange (Q) membranes were provided by Natrix Separations Inc. (Burlington, ON, Canada). The typical mean dynamic binding capacity for bovine serum albumin (BSA) is 200 mg/mL of membrane volume at 10% break-through. Membranes were installed into either a 25 or 47 mm stainless steel holder NX9100 series (Natrix Separations Inc., Burlington, Canada). One 25 mm membrane comprises 0.1 mL of membrane volume, two 47 mm membranes comprise 0.75 mL of membrane volume, and one 0.02 m^2 cross-flow cassette comprises 5 mL of membrane volume. The loading/wash buffers were composed of 25 mM Tris–HCl (pH 8) or 25 mM Bis-Tris–HCl (pH 7). The elution buffer consisted of the appropriate loading buffer containing 1 M NaCl. All buffers were prepared with 18 MΩ deionized water and filtered with 0.45 μm polypropylene filters (VWR, Radnor, PA, USA).

2.4. Dead end filtration anion-exchange membrane chromatography (DEF-AEMC)

DEF-AEMC, a process where the anion-exchange filter membrane is oriented perpendicular to the flow direction of the feed solution passing through the membrane, was performed using low pressure liquid chromatography system (Bio-Logic LP, Biorad, Hercules, CA) with online UV absorbance at 280 nm and conductivity meters. These metrics were recorded using the accompanying

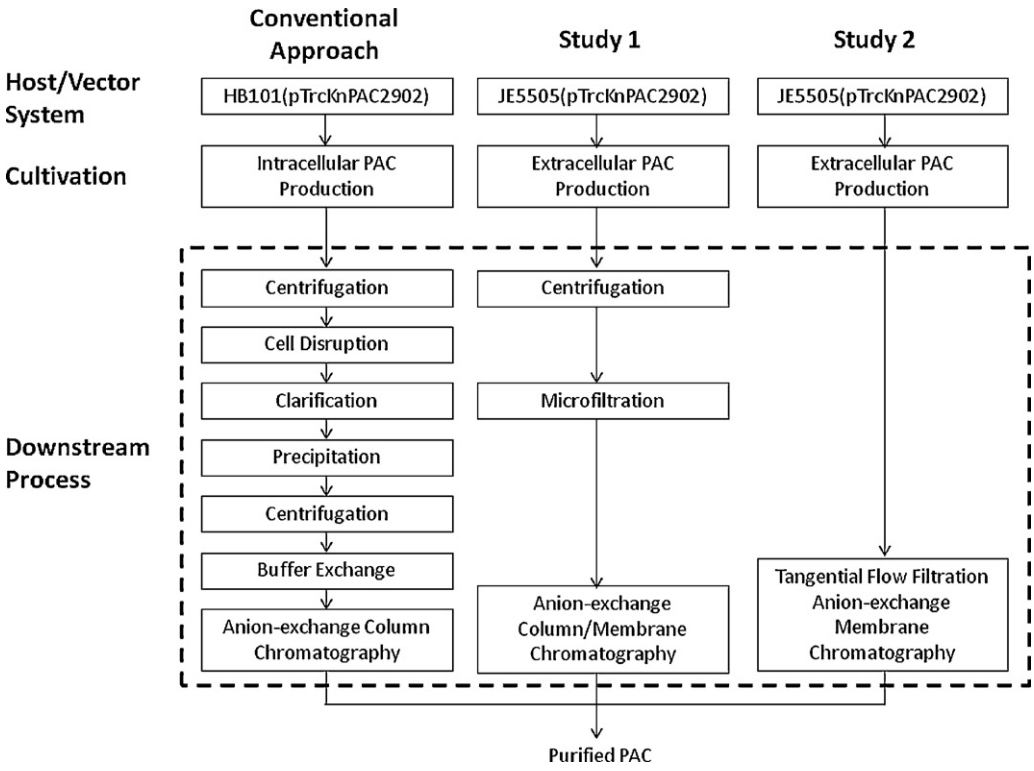

Fig. 1. The proposed purification scheme investigated herein using tangential flow filtration anion-exchange membrane chromatography (TFF-AEMC) to simultaneously clarify and purify extracellular PAC (study 2) compared to an equivalent purification scheme using a traditional AEC column or DEF-AEMC (study 1) [17]. These developed bioprocesses are contrasted with a conventional industrial process for the large-scale production of PAC.

software LP Logic (Biorad, Hercules, CA). Two 47 mm Q membrane discs in the stainless steel holder were attached upstream of the indicators and the inlet was downstream of a sample injection device. All runs were operated at a flow rate of 4 mL/min. The membrane was equilibrated for 15 min with the appropriate loading buffer described in Section 2.3. Culture broth was centrifuged at $6000 \times g$ for 10 min and adjusted to the appropriate pH then filtered using 0.22 μm polypropylene syringe filters (VWR, Radnor, PA, USA) prior to loading. Twenty milliliters of filtered cell free medium was loaded onto the membrane at a flow rate of 4 mL/min. After loading, 60 mL of loading buffer was used to wash weakly bound protein from the membrane. After washing, a 60 mL linear salt elution gradient was applied to the membranes. Eight millilitre fractions were collected with a fraction collector (BioLogic BioFrac, Biorad, Hercules, CA). Four fractions were pooled as loading flow-through, washing flow-through, and two elution peaks. All fractions were assayed using the bicinchoninic acid assay and PAC enzymatic assay and analyzed for protein composition by SDS-PAGE. All dead-end experiments were performed in duplicate.

2.5. Determination of dynamic binding capacity

Determination of the dynamic binding capacity was performed using the low pressure chromatography BioLogic system described previously. One 25 mm Q membrane disc in a stainless steel holder was equilibrated with 15 mL of loading buffer (25 mM Bis-Tris–HCl pH 7.0) at 1 mL/min prior to sample loading. One hundred milliliters of clarified medium with unadjusted pH (7.0 ± 0.1) was loaded to the membrane at 1 mL/min. Fractions were collected every 1 min and analyzed for the relative purity of PAC in the flow-through by SDS-PAGE stained with silver nitrate and analyzed using Image J software (National Institute for Health). Effluent UV absorbance at 280 nm and conductivity were monitored as previously described.

2.6. TFF-AEMC

The cross-flow system was assembled using TangenX pressure gauges, TangenX cross flow apparatus (TangenX, Shrewsbury, MA, USA), Masterflex L/S 25 silicone tubing, Masterflex 7550-60 computerized peristaltic pump (Cole-Palmer, Vernon Hills, IL, USA), 1 L pyrex reservoir bottle with bottom hose outlet, 1-L pyrex flask (Corning, Corning, NY, USA), and Explorer Pro EP2102 balance (Parsippany, NJ, USA) as seen in Fig. 2. A 0.02 m^2 membrane cassette was hydrated by pumping 500 mL of 25 mM Bis-Tris–HCl pH 7 at a feed flow rate (F_F) = 600 L/h m^2 (LHM) and permeate flow rate (F_P) = 60 LHM for 15 min. The membrane back-pressure was maintained at 5 psi for all subsequent steps using a pressure valve. After equilibration, the buffer from the reservoir and permeate was removed and discarded. One litre of non-clarified culture broth was added to the reservoir. The loading stage was conducted at F_F = 600 LHM until 750 mL of permeate was collected. The remaining sample was removed from the reservoir and 500 mL of 25 mM Bis-Tris–HCl pH 7 was used to wash the membrane for 30 min at F_F = 600 LHM. The wash buffer was removed and 250 mL of 1 M NaCl in 25 mM Bis-Tris–HCl pH 7 was used to elute the contaminating proteins from the membrane for 30 min at F_F = 600 LHM. The permeate and reservoir protein content was monitored by the BCA assay and PAC assay approximately every 30 min. The permeate flow rate was monitored during the loading stage using the balance. The UV absorbance at 280 nm was recorded using the Biologic LPLC system.

2.7. Ultrafiltration

Oligopeptides were removed by diafiltration giving over 25 fold reduction using 3 kDa nominal molecular weight cut-off (NMWCO) centrifugal devices and samples were resuspended in loading buffer to their original volume and analyzed using BCA assay, PAC assay and SDS-PAGE (Pall's Corp., St. Louis, USA). Permeate was also

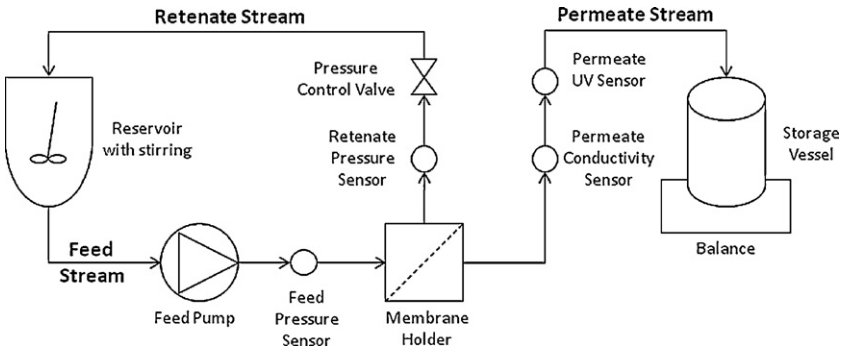

Fig. 2. Schematic of cross-flow system.

subjected to 25 fold reduction by diafiltration using a 10 kDa NMWCO cellulose membrane and a 200 mL stirred cell device (Millipore, Billerica, USA). Liquid was driven through the membrane by nitrogen which was used to pressurize the device to 25 psi.

2.8. Enzymatic activity

Penicillin G acylase was assayed at 37 °C using penicillin G as a substrate. The amount of enzymatic reaction product of 6-aminopenicillanic acid (6-APA) was quantified using a colorimetric method developed previously [16]. All assays were conducted in duplicate. One unit was defined as the amount of enzyme that hydrolyzed 1.0 μmol penicillin G per minute at 37 °C.

2.9. Total protein determination

The BCA assay for total protein concentration was performed using a kit (Pierce Biotechnology, Rockford, IL, USA) in the microplate assay format. Samples were all performed in duplicate and were appropriately diluted using the appropriate loading buffer to fall in the linear range of the kit; 20–2000 μg/mL. The standard curve was performed in duplicate on each plate with BSA. Absorbance was measured at 562 nm with Thermo Labsystems Multiskan Ascent photometric plate reader (Thermo Scientific, Wilmington, USA).

2.10. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA, USA) using a 12.5% polyacrylamide separating gel stacked by a 4% polyacrylamide stacking gel. The loading amount was 10 μL for chromatography fractions. Electrophoresis was conducted under a constant voltage of 200 V for 60 min. Gels were stained using silver nitrate and dried. Dried gels were scanned using an HP laserjet scanner 3020.

3. Results and discussion

3.1. Binding of PAC on anion-exchange membrane

The rationale of the present study was to create an integrated downstream processing scheme with minimal preparative steps for direct harvest and purification of the extracellularly produced recombinant PAC using TFF-AEMC. Traditional protein purification using column chromatography, cells and large debris would be first eliminated from the crude broth to prevent clogging and fouling of the resin. For direct loading of the spent medium containing the target protein for chromatographic operation, the high salt content must be removed by a desalting operation and, if possible, the sample volume should be properly reduced. To eliminate these preparatory steps and to ensure the functional operation of anion-exchange membrane chromatography, the spent medium should have a sufficiently low conductivity and a permissible pH to allow its direct processing. Since a buffer with a low salt concentration is normally inadequate for *E. coli* cultivation, medium composition and cultivation conditions were previously developed to drive effective extracellular production of recombinant PAC with minimum growth impairment as well as to facilitate protein binding onto the anion-exchange membrane during the subsequent chromatographic operation [17]. Using a bench-top bioreactor, JE5505 harboring pTrcKnPAC2902 was cultivated in LCM3 medium for extracellular production of PAC. The spent cell-free medium was collected at 12 h post-induction as protein expression had reached a plateau and extended cultivation would result in significant cell lysis which complicated downstream processing. At this time, the culture had a cell density of 2.8 ± 0.3 OD_{600} with a specific extracellular PAC activity of 434 ± 86 U/L/OD_{600}. Most importantly, the secretion efficiency was as high as 90% and the spent medium had a sufficiently low conductivity (5.9 ± 0.8 mS/cm) to allow its direct loading for subsequent anion-exchange chromatography. Recombinant PAC in the spent medium was purified based on a non-retentive separation through which the majority of

Table 1
Performance of PAC purification by AEMC by direct loading of 20 mL of clarified culture supernatant to two 47 mm dead-end Q membranes operated at pH 7 and pH 8 with a flow-rate of 4 mL/min.

		Protein concentration [mg/mL]	Volumetric PAC activity [U/mL]	Specific activity [U/mg]	PAC recovery	Purification [fold]
Cell free medium	Total protein including oligopeptides	0.63 ± 0.05	0.67 ± 0.04	1.1 ± 0.1	–	–
	Total protein excluding oligopeptides	0.133 ± 0.007	0.69 ± 0.02	5.2 ± 0.3	–	–
Anion-exchange (pH 8)	Total protein including oligopeptides	0.357 ± 0.009	0.50 ± 0.01	1.40 ± 0.05	75%	1.3
	Total protein excluding oligopeptides	0.03 ± 0.01	0.516 ± 0.008	17 ± 5	103%[a]	3.3
Anion-exchange (pH 7)	Total protein including oligopeptides	0.396 ± 0.001	0.503 ± 0.004	1.27 ± 0.01	75%	1.2
	Total protein excluding oligopeptides	0.03 ± 0.01	0.45 ± 0.02	15 ± 6	89%[a]	2.9

[a] PAC recovery during ultrafiltration step.

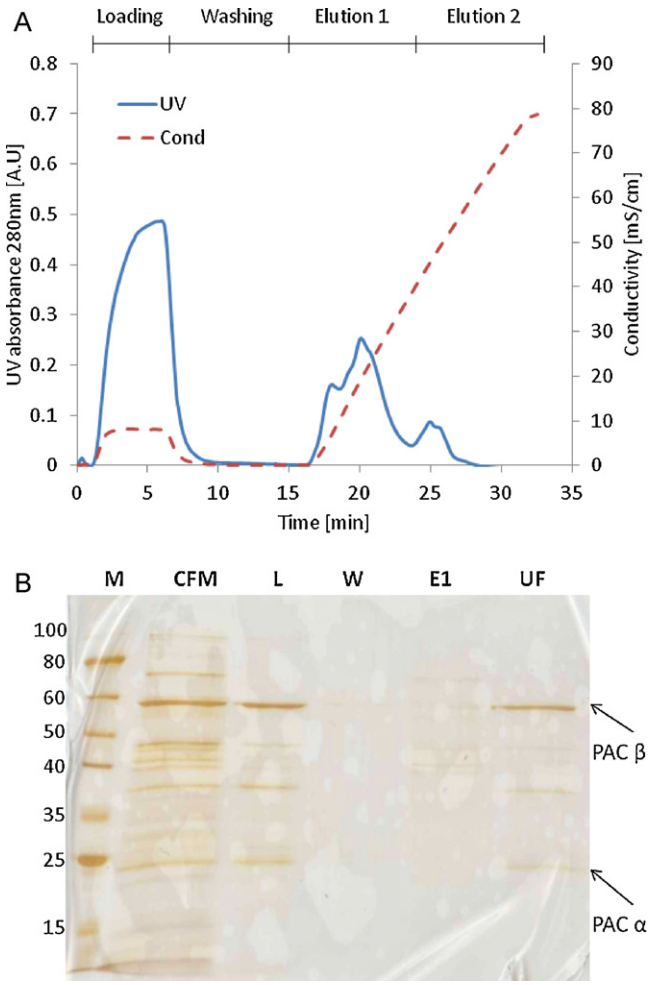

Fig. 3. Purification of extracellular PAC from 20 mL of cell free medium using Q membranes in dead-end flow format at pH 7 at 4 mL/min. (A) Elution profile indicating the UV absorbance in a solid line and conductivity in a dashed line. Fractions were pooled according to the 4 steps shown above the plot. (B) Pooled fractions from the separation in panel A were analyzed using SDS-PAGE. Lanes contain protein markers (M), cell free medium (CFM), loading flow-through (L), washing flow-through (W), elution 1 (E1) and the ultrafiltrate of the loading flow-through (UF). PAC subunits are indicated with arrows.

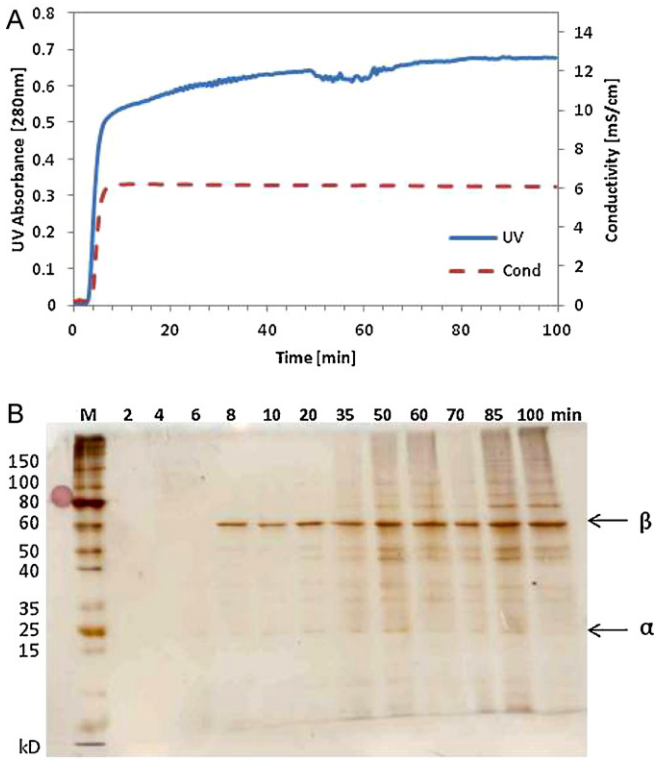

Fig. 4. Determination of dynamic binding capacity of one 25 mm Q membrane in a dead-end device with a system residence volume of 3 mL. One hundred milliliters of clarified spent medium was loaded at a volumetric flow rate of 1 mL/min and pH of 7.0. (A) Elution profile of protein breakthrough. (B) SDS-PAGE analysis of corresponding protein breakthrough. PAC subunits, the protein marker molecular weights (M) and the time at which the chromatography fraction was taken are indicated.

contaminant proteins bound to the anion-exchanger and PAC was recovered in the flow-through fraction with a significantly increased purity (Table 1).

Theoretically, the operating conditions for ion-exchange chromatography should not differ remarkably between a column and a membrane. Nevertheless, binding of PAC onto the anion-exchange membrane chromatography was characterized in this study with a DEF-AEMC device to ensure its functional operation and optimize the purification performance. Using anion-exchange membrane discs in a stainless steel holder with a low hold-up volume, the spent cell-free medium at pH 7 containing recombinant PAC was loaded for chromatographic operation and the results are summarized in Fig. 3. Various fractions were collected, pooled, and analyzed, including the loading flow-through, washing flow-through, and elution. These samples were also subjected to ultrafiltration (3 kDa NMWCO) to remove residual oligopeptides in order to properly quantify the protein concentration. It was observed that recombinant PAC hardly bound to anion-exchange membrane and the majority of it was collected in the flow-through fraction with significantly improved purity (Fig. 3B). Two distinct peaks were observed upon the elution of bound molecules with a linear salt gradient. The contents of these two peaks were pooled (designated as elution 1 and elution 2 in Fig. 3) and were determined to primarily consist of contaminant proteins and RNA, respectively. The results suggest that most contaminant proteins were removed through their binding onto the anion-exchange membrane and, as a result, PAC purity was significantly increased. The low level of RNA detected in the spent medium suggest minor cell lysis during the cultivation and the released RNA was completely adsorbed by the anion-exchange membrane and eluted at a high salt concentration (data not shown) without contaminating the purified PAC. PAC purification performance for anion-exchange membrane chromatography was found to be excellent under both pH 7 and pH 8 operating conditions (Table 2), implying a precise pH control during the cultivation might not be critical. Because of the high costs associated with extensively purified enzymes, industrial processes tend to use crude extracts or partially purified enzymes for catalysis. Hence, the purified PAC based on anion-exchange membrane chromatography in this study should be eligible for industrial applications even though it is not completely purified.

In addition, the dynamic binding capacity of the anion-exchange membrane for binding of the contaminant proteins was evaluated by continuous loading of 100 mL spent cell-free medium through a 25 mm disc membrane and the results are summarized in Fig. 4. No protein breakthrough occurred after loading the first 3 mL (lane 6-min). SDS-PAGE analysis indicates that PAC was the first major protein to break through the membrane with a few minor contaminant proteins at the loading volume of 5 mL (lane 8-min), suggesting that PAC might carry the least surface charge under this loading condition. As expected, the break-through of the contaminant proteins increased with the loading volume and the PAC purity dropped slightly after a loading of 17 mL (lane 20-min) of the spent medium, resulting in approximately 85% PAC purity

Table 2
Summary of purification performance by TFF-AEMC.

		Volume [L]	Total protein [mg/mL]	PAC activity [U/mL]	Specific activity [U/mg]	Purification [fold]	Step recovery[a]	Overall recovery[b]
Cell free medium	Total protein including oligopeptides	1.00	0.734 ± 0.002	1.07 ± 0.03	1.46 ± 0.04	–	–	–
	Total protein excluding oligopeptides	1.00	0.15 ± 0.01	1.07 ± 0.03	7.1 ± 0.5	–	–	–
Anion-exchange permeate	Total protein including oligopeptides	0.75	0.60 ± 0.03	1.03 ± 0.03	1.7 ± 0.1	1.2	96%	72%
	Total protein excluding oligopeptides	0.75	0.055 ± 0.008	1.05 ± 0.04	19 ± 3	2.7	102%	74%

[a] Recovery of PAC based on the actual volume processed.
[b] Recovery of PAC based on the original volume harvested.

according to the densitometric analysis of the SDS-PAGE gel. The purity decreased significantly when the loading volume was more than 32 mL (lane 35-min). However, complete protein breakthrough was never achieved due to a serious membrane fouling which developed upon a loading of more than 50 mL of the spent medium. The results suggest that the loading volume for AEMC critically affected the PAC purity and must be carefully assessed.

3.2. Simultaneous culture clarification and PAC purification with TFF-AEMC

Using the developed system of TFF-AEMC (Fig. 2), one liter of the whole *E. coli* culture broth at pH 7 containing the extracellularly secreted recombinant PAC was processed and the processing results are summarized in Fig. 5. The culture in the reservoir was circulated at a rate of 600 LHM for approximately 280 min with a back-pressure being maintained at 5 psi. The flow rate of the permeate was monitored to ensure no fouling of this system. The elution profile associated with the TFF-AEMC operation is shown in Fig. 5A. The total protein concentration of the permeate, in terms of the UV absorbance at 280 nm, peaked upon the initial loading of the culture, then dropped slightly and remained relatively constant up to the washing step. The peak upon the breakthrough of proteins into the permeate at approximately 15 min was hypothesized to represent less charged molecules, including PAC. Additionally, upon an initial loading of approximately 50 mL of the spent medium onto the membrane, no PAC activity was detected in the permeate fractions, suggesting that PAC initially bound to the membrane but was later replaced by other impurities with a higher binding affinity during the loading phase. The constant total protein concentration in the permeate leads to at least two observations. First, cell lysis was not aggravated during the TFF-AEMC processing. This is important, in particular for this case where a slight cell lysis appeared to be unavoidable during the cultivation, since further cell lysis would contaminate the extracellular medium and complicate the PAC purification. Second, the binding of the extracellular contaminant proteins onto the membrane was effective without exceeding the chromatographic capacity of the membrane. Because PAC was unable to bind onto the anion-exchange membrane under the spent-medium conditions, the majority of it was collected in the permeate with a small amount of contaminant protein. As a result, its purity has been significantly increased through this non-retentive purification.

Reduction in the volume of the retentate due to the draining of the permeate resulted in an increase in the cell density of the retentate. Out of the 1-L whole culture broth being processed, approximately 750 mL was collected as the permeate containing purified PAC and the cell density in the retentate was significantly increased by 4 fold. Because of the non-retentive separation mode associated with PAC purification, the permeate containing the purified product was continuously drained from the processing stream. Hence, the system required a minimum operating volume (~100 mL in this study to cover the tubing volume and minimum working volume of the reservoir) for continuous operation. Such a minimum volume is not required for retentive separations, for which the product to be purified binds onto the membrane and the processing is typically operated in a recycling mode through which the permeate is recycled back to the reservoir without being drained from the process stream [1,18]. A major technical issue for the TFF-AEMC processing, particularly for that with a non-retentive separation mode, is that the increasing cell density of the retentate tends to aggravate the membrane fouling and consequently prevents the product harvest/purification. The circulation rate of the whole culture broth has to be high enough to minimize fouling without causing cell lysis. In this study, while the flow rate of the permeate began at a reasonably high level of approximately 38 LHM, it quickly decreased to 10 LHM within the first 50 min of the processing and eventually reached a constant flow rate of approximately 6 LHM till the end (Fig. 5B), suggesting that membrane fouling was minimal.

3.3. Analysis of protein compositions

The total protein concentrations (analyzed by the BCA assay) for both cell-free retentate and permeate were maintained relatively constant at 700 μg/mL and 570 μg/mL, respectively, before 200 min (Fig. 5B), suggesting the processing resulted in minimum cell lysis during this time period. Note that oligopeptides were included when these two concentrations were analyzed with the BCA assay and, after excluding oligopeptides based on the data in Table 2, the actual total protein concentrations for both cell-free retentate and permeate were estimated to be 140 μg/mL and 50 μg/mL, respectively. The difference between these two concentrations, i.e. 90 μg/mL, reflects the amount of contaminant proteins binding onto the membrane. We believe that such binding behavior was the major cause to reduce the flow rate of the permeate since a similar decrease in the flow rate of the permeate was observed when clarified broth was applied under the same operating conditions and a final constant flow rate of approximately 6 LHM was reached after 100 min of processing (data not shown). While the total protein concentration of the permeate was rather constant according to the UV monitoring (Fig. 5A), the BCA assay revealed a low-level cell lysis towards the end of the processing, particularly after 200 min, since there was a slight increase in the total protein concentrations for both the permeate and cell-free retentate during that time period (Fig. 5B). The protein composition of the cell-free retentate was also monitored by SDS-PAGE and a low-level cell lysis could be detected by visualizing new bands in the late samples (i.e. 240 and 280 min) (Fig. 5C). While no major difference in the protein composition of the cell-free retentate was observed, the volumetric PAC activity in this fraction increased over the time, i.e. from 1.07 U/mL at 0 min to 1.56 U/mL at 280 min.

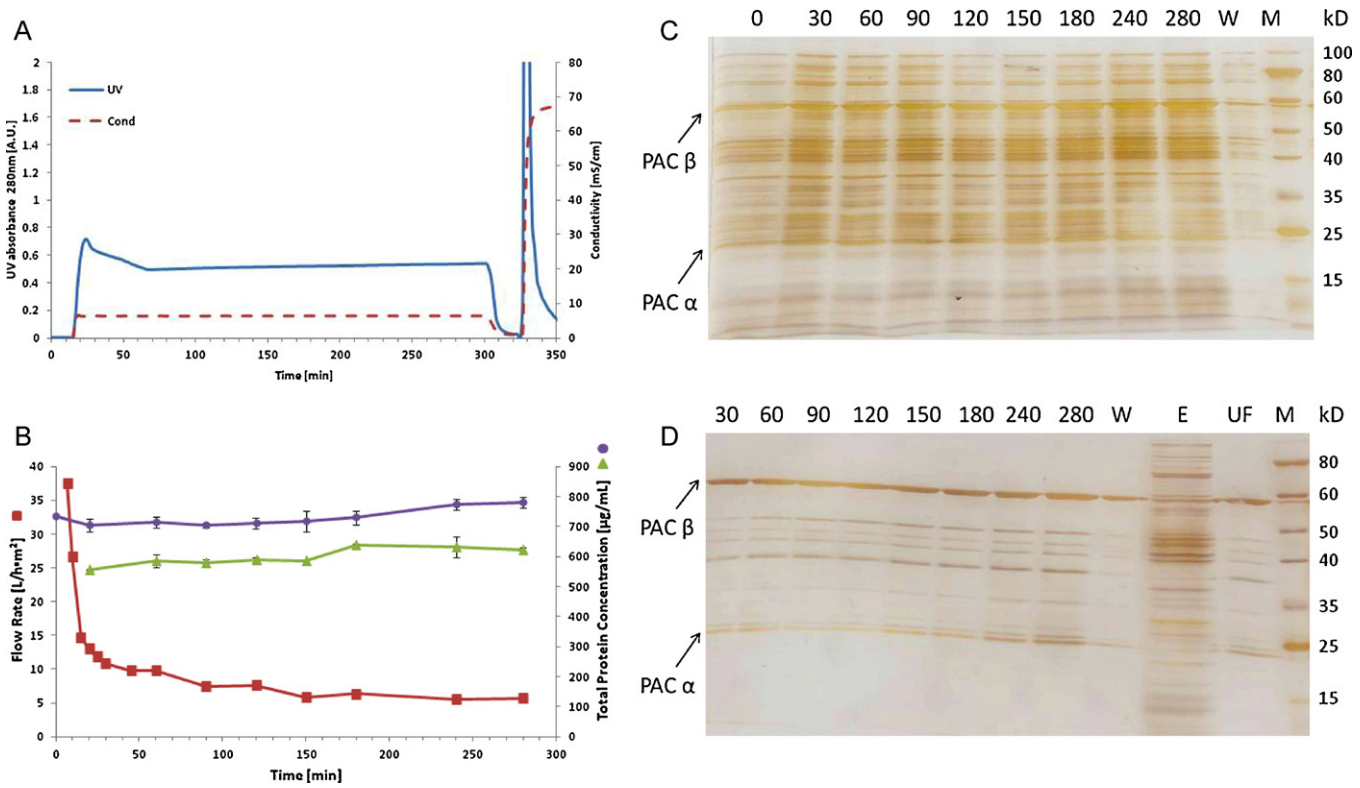

Fig. 5. Purification of extracellular PAC using non-clarified cultivation broth and Q anion-exchange cross-flow membranes. (A) Typical elution profile of PAC purification using non clarified cultivation broth containing extracellular PAC. UV absorbance is indicated with a solid line, conductivity is indicated with a dashed line. (B) Monitoring for fouling and cell lysis during loading stage of cross-flow purification. Permeate flow rate in squares was used to monitor fouling, retentate total protein in circles monitors the amount of lysis, and permeate total protein concentration in triangles was used to monitor the final product. (C) SDS-PAGE analyses of retentate protein content during cross-flow purification. Samples were collected from the reservoir at the times indicated from 0 to 280 min. The washing step retentate (W) and a protein marker (M) were also analyzed. (D) SDS-PAGE analyses of permeate samples taken during cross-flow operation. Samples were collected from the permeate effluent at times indicated from 30 to 280 min. A protein marker (M), a sample of the washing step permeate (W), the elution step permeate (E) and the ultrafiltrate of the permeate (UF) were also analyzed.

Such concentration polarization is not unusual for ultrafiltration and is indicative of membrane fouling [1,19] though the flow rate of the permeate was not fully stopped. If necessary, the fouling can be relieved by reversing the retentate flow direction or increasing the tangential flow rate to improve the flux of the permeate [1].

Compared to the crude protein composition of the cell-free medium (i.e. the 0-min lane in Fig. 5C), all permeate samples contained a relatively pure PAC, for which both α and β subunits were clearly visible as the major bands (Fig. 5D). Such a high PAC purity in the permeate was stably maintained throughout the entire period though there appears to be a slight additional cell lysis towards the end of the processing. After approximately 750 mL of permeate was drained, the remaining culture of the retentate was collected and a wash buffer was used for brief circulation to remove any unbound proteins and cells from the system. All bound proteins on the anion-exchange membrane were eluted through brief circulation of the loading buffer supplemented with 1 M NaCl. A large elution peak was visible on the elution profile and the eluted proteins were collected from the permeate side. Both washing and eluted fractions contained minimum PAC activities (less than 8% and 1% of the total PAC activity respectively) and were analyzed by SDS-PAGE (lanes W and E, Fig. 5D). The results suggest that most contaminant proteins bound onto the anion-exchange membrane, resulting in a significant increase of PAC purity in the permeate. Consequently, the purity of PAC in the permeate could be potentially affected by the conductivity of the spent medium, which determined the binding efficiency of contaminant proteins.

3.4. PAC purification performance

The performance for the one-step purification of PAC by TFF-AEMC is summarized in Table 2. A minor technical issue associated with the purification of PAC in this study (i.e. a non-retentive separation mode) was the presence of oligopeptides in the spent medium. Because oligopeptides hardly bound onto the anion-exchange membrane, they remained with purified PAC in the permeate. Such a technical issue will not exist for a retentive separation mode, for which oligopeptides could be easily separated from the target protein binding onto the ion-exchange membrane. While the oligopeptides will not affect the catalytic activity of PAC upon industrial applications, if necessary, they can be easily removed by ultrafiltration during the concentration step given that the permeate was already pre-filtered by microfiltration upon the TFF-AEMC processing. The specific PAC activity for the crude cell-free spent medium was already as high as 7.1 U/mg protein (excluding oligopeptides). With the TFF-AEMC processing, it reached an extremely high level of 19 U/mg protein (excluding oligopeptides), resulting in a 2.6-fold purification. Note that the specific activity of PAC from Sigma–Aldrich (catalog no. 76427) was only 5–10 U/mg protein. In addition to the high purification, the recovery of PAC activity for the TFF-AEMC processing was as high as 96%. This means 72% of PAC in the 1-L cell-free medium was harvested and purified in the 750-mL permeate. This overall recovery was limited by the volume of permeate that could be separated/drained from the whole culture during the TFF-AEMC processing. Regardless, 72% is still considered an excellent yield and is often higher than the yields associated with the traditional

multi-step processes for purifying intracellular PAC. One such process employing ammonium sulfate precipitation and anion-exchange chromatography as the main purification steps had a low overall recovery of only 48% and a 5.7 fold increase in purity, a factor which due to the high level of host protein contamination in cellular lysates may result in a product of lower purity than that achieved in this study [20]. Also importantly, the overall batch cycle time involved in the developed bioprocess, including both cultivation and TFF-AEMC processing, was less than 24 h, significantly enhancing the overall productivity of this bioprocess.

4. Conclusions

A bioprocess with an integrated scheme for all upstream, midstream, and downstream stages was developed for effective production of recombinant PAC. Using the constructed *E. coli* host/vector system and the optimized medium for cultivation, high-level extracellular production of recombinant PAC was conducted. One-step downstream processing was conducted for harvest and purification of recombinant PAC based on the dual nature of the anion-exchange membrane as a filter for culture clarification and a chromatographic medium for PAC purification. The decreased number of steps for downstream processing and excellent purification performance could represent a significant cost saving for manufacturing of recombinant PAC. Due to the non-selective nature of the secretion system (i.e. any periplasmic proteins can be potentially secreted) and the versatility of ion-exchange chromatography, the developed strategy could be easily implemented for the production of any recombinant proteins. In addition, the overall bioprocess could be modified for continuous operation through the development of continuous cultivation for extracellular production of recombinant proteins with the effluent of the chemostat being concurrently processed by TFF-AEMC.

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