COMPARISON OF PURIFICATION OF RECOMBINANT *N*-DEMTHYLASE B (NdmB) USING SIMULATED MOVING BED AFFINITY CHROMATOGRAPHY (SMB) AND FPLC

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Abstract

Simulated moving bed (SMB) is a well-established technology based on simulating a countercurrent contact between the solid (stationary) phase and liquid phase. It is widely used in separation of sugars, racemic drugs and drug intermediates at commercial scale. SMB has been shown to be more beneficial in terms of productivity; such as product concentration as well reduced use of solvents/buffers, while retaining product purity. Use of SMB in protein purification in biopharma industry is not common. This is in spite of the fact that most of the work reported in the area of FPLC-based protein purification is on separation of binary mixtures. Protein purification is ideally suited for SMB where two components are separated as raffinate and extract. There are a couple of reports on the protein purification of complex mixtures using SMB. We have developed a four-zone SMB method (Bind, Wash, Elute and Regenerate) using Nickel (HIS-Select) affinity chromatography to purify recombinant N-demethylase B (NdmB). NdmB (Mol.wt: 35 kDa) from *Pseudomonas putida* CBB5 catalyzes the N-3-demethylation of caffeine. This enzyme has been cloned and expressed *E. coli* as his-tag protein. We carried out 30-L fermentation of *E. coli* expressing NdmB and the cell paste obtained from this fermentation was used for purification of NdmB using SMB (Octave 100, Semba Biosciences) and FPLC (Akta). About 24 grams of cells paste was used for each method of purification. For SMB we used 8 columns of 5-mL size and for FPLC, one 40-mL (XK26) column. Equal amount of protein was loaded on to both systems. After washing the column with Buffer (25mM KPi, 300mM NaCl, 10mM Imidazole, pH 7.5), NdmB was eluted with Elution Buffer (25mM KPi, 300mM NaCl, 250mM Imidazole, pH 7.5). The recovery of NdmB from SMB and FPLC was 30% and 23% respectively. Based on band intensity on SDS-PAGE of NdmB, it was assessed that SMB achieved 95 % purity whereas from FPLC it was 91%. Measuring the overall productivity in terms of (a) time taken for purification, 150 minutes vs. 350 minutes (SMB vs FPLC), (ii) buffer use, 1 vs 1.5 times (SMB vs FPLC), and (iii) column regeneration (no additional unit operation for SMB), SMB method was better than the FPLC (batch chromatography). SMB being a continuous process is more suitable for large scale manufacturing of proteins/therapeutics. CBB will continue to explore SMB application for protein purification and for chiral molecule separations with the intent of developing this technology for industrial applications.

Introduction

Simulated Moving Bed (SMB) chromatography was first introduced by Universal Oil Product (UOP) in 1960's. The SMB concept is based on the simulation of a true countercurrent operation between the solid and the liquid phase, by valve switching over a series of columns. The countercurrent operation allows a more efficient use of the adsorbent and liquid phase yielding higher productivity, reduced solvent consumption and higher purity of product of interest[1,2]. Since then SMB is widely used in petrochemical industry, sugar processing industry, in chiral separations and resolution of fine chemicals at multi ton scale. However, separation of proteins is still predominantly done by conventional packed bed column (batch) chromatography, use of SMB technology for protein purification in biopharmaceutical industry may have some regulatory issues like batch control etc. There are a few reports on the purification of proteins by SMB.

Different methods for SMB optimization and process development have been suggested in literature. We have implemented a simplified approach of four-zone SMB method (Bind, Wash, Elute and Regenerate) (**Figure 1**) using Nickel (HIS-Select) affinity chromatography. We have purified a recombinant N-demethylase B (NdmB). NdmB (Mol.Wt: 35 kDa) from *Pseudomonas putida* CBB5 catalyzes the N-3-demethylation of caffeine. We have used Semba Octave 100 Chromatography System, (**Figure 2**) a versatile small footprint system capable of performing SMB and other continuous automated separation protocols. This equipment is suitable for gram scale purification of proteins with eight columns positions. This study compares purification of NdmB using Semba Octave 100 Chromatography System and GE Akta FLPC System (**Figure 3).**

Figure 1: SMB Method

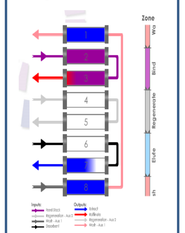


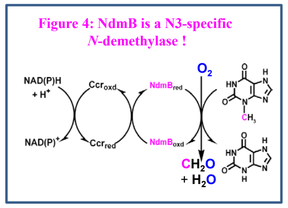
Figure 2: Octave 100

Figure 3: Akta FPLC

Introduction (NdmB)

- We chose to compare purificatino of recombinant N-demethylase (NdmB) using both batch column chromatography and SMB. Ndm B was chosen since Octave 100 doesn't have a UV detector and this protein is red in color. It facilitated easy monitoring of this his-tagged protein.
- NdmB contains conserved Rieske [2Fe-2S] and mononuclear ferrous domains (Figure 4).
- Phylogenetic analysis grouped NdmB with several Rieske Oxygenases that cleaved C-N bonds (carbazole oxygenases) and C-O bonds (*O*-demethylases).

Figure 4: NdmB is a N3-specific *N*-demethylase !

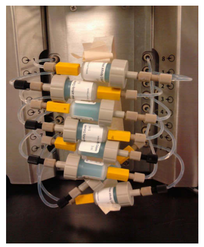


- NdmB are the first reported Rieske Oxygenases with N-demethylation activity.

Results

Cell paste was obtained from 30-L fermentation of *E.* coil expressing NdmB. The cell paste was frozen at -80C until processing. Initial column chromatography process was optimized using batch chromatography. As part of process development. Small scale purification was carried out on Akta FPLC, where a 5-mL His-select column was used. Three batches of NdmB purification was carried out without any regeneration of resin and a fourth batch after regeneration. Results in **Table 1** show that recovery of NdmB was low without regeneration of the resin. Based on these result we introduced a regeneration step in SMB, where each column is regenerated after elution in a continuous fashion. **Figure 5,** shows the SMB process design.

Figure 5: SMB CONTINUOUS PROCESS.
USED FOR THIS STUDY
8 COLUMN x 5mL (10mL/zone)



ZONE I: BINDING / 2 columns / 10mL/ Flow rate=3ml/min / T= 4mins

ZONE II: WASHING / 2 columns / 10mL/ Flow rate=6ml/min/ T= 4mins

ZONE III: ELUTION / 2 columns / 10mL/ Flow rate=2,5ml/min/ T= 4mins

ZONE IV: CLEANING / 2 columns / 10mL/ Flow rate=2,5ml/min/ T= 4mins

Table 1: NdmB Purification onAkta FPLC with and without regeneration
Four separate purification was performed to optimize the process

Sample	Protein Conc. (mg/ml)				Volume (ml)				Total Protein (mg)			
	1st 3g	2nd 3g No RG	3rd 3g No RG	4th 3g RG	1st 3g	2nd 3g No RG	3rd 3g No RG	4th 3g RG	1st 3g	2nd 3g No RG	3rd 3g No RG	4th 3g RG
Load	19.9	19.9	19.9	19.9	15	15	15	15	298.5	298.5	298.5	298.5
Load F.T.	13.6	13.3	12.0	10.9	15	15	15	15	204.0	199.5	180	163.5
Wash F.T.1	1.1	1.5	1.5	1.4	35	35	35	35	38.5	52.5	52.5	49.0
Wash F.T.2	0.0	0.0	0.0	0.0	25	25	25	25	0.0	0.0	0.0	0.0
Ni Pool	2.09	1.91	1.75	2.42	35	35	35	35	73.2	66.9	61.3	84.7
Recovery									24.5%	22.4%	20.5%	28.4%

No RG: No Regeneration, RG: Regeneration, FT: Flow Thru

Comparative Study on FPLC and SMB

Comparative purification study was carried out on FPLC and SMB using same column volume and same resin. In case of FPLC, XK26/10 column with 40-mL His-Select Resin was used . For SMB, 8 x 5-mL columns were used. The Nickel column load sample was prepared as described below under identical conditions.

* Amount of cell paste: 24g
* Lyses Buffer: 200ml of Binding/Washing Buffer (25mM KPi, 300mM NaCl, 10mM Imidazole, pH 7.5)
* Cell breaking method: Microfluidization Two passes at 18.5Kpsi
* Centrifuge: Beckman rotor No. 14, 12Krpm (22Kfg) for 30min at 4°C
* Filter the Ni load through 0.8um then 0.45um filter.
* About 360-mL of cell lysate with protein concentration of 7.74 mg/mL was loaded onto FPLC and SMB columns.
* **Table 2** shows the recovery of Ndm B on FPLC column . **Figure 6** is the elution profile from the Akta FPCL chromatography . **Table 3** shows the recovery of Ndm B from SMB

Table 2: NdmB Recovery on Akta FPLC

Sample	Protein Conc. (mg/ml)	Volume (ml)	Total Protein (mg)
Ni Load	7.74	360	2786.4
Load F.T.	4.29	360	1544.4
Wash F.T.	0.55	400	220
Elution (Pool)	4.91	130	638.2
Recovery	-	-	23 %

Figure 6: NdmB Elution Profile on Akta FPLC

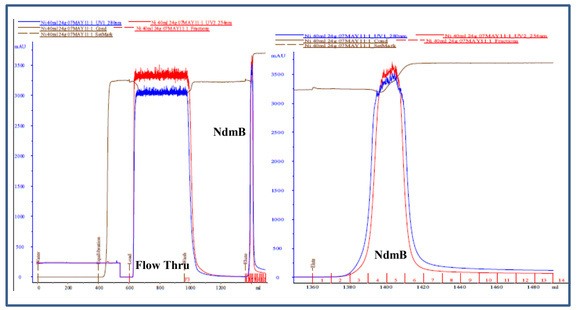
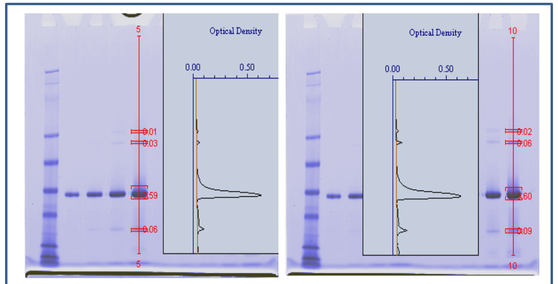


Table 3: NdmB Recovery on SMB (Octave 100)

Sample	Protein Conc. (mg/ml)	Volume (ml)	Total Protein (mg)
Ni Load	7.14	360	2570.40
Load F.T.	3.18	450	1431.00
Wash F.T.	0.63	520	327.60
Elution	2.89	260	751.40
Recovery	-	-	30 %

Figure 7: Comparison of NdmB Purity from SMB and FPLC



Lane 5 Name: #5

Band #	Lane Name	Peak OD	Relative Qty
5 - 1	#5	0.59	95.4
5 - 2	#5	0.03	1.4
5 - 3	#5	0.01	0.7
5 - 12	#5	0.06	2.5

Lane 10 Name: #10

Band #	Lane Name	Peak OD	Relative Qty
10 - 1	#10	0.60	91.8
10 - 2	#10	0.06	2.1
10 - 3	#10	0.02	0.9
10 - 4	#10	0.09	5.2

Figure 3: Key Parameters Comparison from SMB and FPLC

Process	Productivity (mg/mL.min)	Total Ndm B Recovered (mg)	Protein Production (mg / min)	*Specific Activity (nmol/min-mg)
Batch ÄKTA 40mL Column 24g Cells load	0.046	638.2	1.824	22.4 ± 3.7
SMB Continuous System Semba Bio-8x5mL 24g Cells load	0.131	787.02	5.247	27.5 ±8.4

* One unit activity = 1 µmole 3-Methyl Xanthine consumed per minute, in the presence of saturating amounts of Cytochrome C Reductase, NADH and 50 µM Fe^{2+}

Conclusions

* The recovery of NdmB from SMB is higher than FPLC.
* Based on band intensity on SDS-PAGE of NdmB, it was assessed that SMB achieved 95 % purity whereas from FPLC it was 91%. (**Figure 7**)
* Productivity in terms of time taken for purification, on SMB was150 minutes whereas for FPLC it took 350 minutes
* Column regeneration (no additional unit operation for SMB), SMB method was better than the FPLC (batch chromatography). SMB being a continuous process is more suitable for large scale manufacturing of proteins/therapeutics.
* CBB will continue to explore SMB application for protein purification and for chiral molecule separations with the intent of developing this technology for industrial applications.

References

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Semba Biosciences