Jurnal Teknologi

Full Paper

MULTIPLE INTERACTIONS MIXED MATRIX MEMBRANE CHROMATOGRAPHY USING ANION AND CATION EXCHANGER RESIN FOR WHEY PROTEIN FRACTIONATION

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Article history
Received
30 October 2014
Received in revised form
17 January 2015
Accepted
15 June 2015

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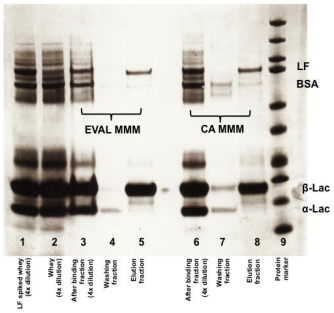
Graphical abstract



Abstract

Membrane chromatography can overcome the limitation of packed bed chromatography in terms of high processing speed, low pressure drop and acceptable protein binding capacity. In the current study, multiple interactions mixed matrix membrane (MMM) chromatography was prepared for batch fractionation of whey protein. Lewatit CNP105 cation exchanger resin and Lewatit MP500 anion exchanger resin were mixed into two different membrane polymer solutions of ethylene vinyl alcohol (EVAL) and cellulose acetate (CA). The membranes were test to bind lactoferrin (LF)-spiked whey. The binding capacity for acidic whey proteins to the MMM follows the order of β-lactoglobulin (β-Lac) > BSA > α-lactalbumin. The binding capacity of EVAL MMM is higher than CA based MMM, more prominently for β-Lac. The average binding capacity of β-Lac in the EVAL and CA MMM are 50.827 mg β-Lac/ g MMM and 19.174 mg β-Lac /g MMM, respectively. High purity of β-Lac and LF were recovered from the MMM after the elution as shown by the SDS PAGE gel.

Keywords: Mixed matrix membrane, membrane chromatography, whey, Lewatit MP500, Lewatit CNP105

Abstrak

Membran kromatografi dapat mengatasi had kromatografi turus terpadat dari aspek kelajuan pemprosesan yang tinggi, kejatuhan tekanan yang rendah dan kapasiti penjerapan protein yang berpatutan. Di dalam kajian ini, membran campuran matrik (MMM) dengan pelbagai interaksi telah disediakan untuk pemisahan whey protein yang beroperasi secara berkelompok. Lewatit CNP105 penukar kation resin dan Lewatit MP500 penukar anion resin telah dicampurkan ke dalam dua larutan polimer membran berbeza iaitu ethylene vinyl alcohol (EVAL) dan cellulose acetate (CA). Membran tersebut diuji untuk menjerap larutan whey yang telah ditambah dengan lactoferrin (LF). Kapasiti penjerapan untuk whey protein yang berasid adalah mengikut turutan β-lactoglobulin (β-Lac) > BSA > α-lactalbumin. Kapasiti penjerapan untuk EVAL MMM adalah tinggi berbanding CA MMM terutamanya untuk β-Lac. Purata kapasiti penjerapan β-Lac dalam EVAL dan CA MMM adalah 50.827 mg β-Lac / g MMM dan 19.174 mg β-Lac / g MMM, masing-masing. Tahap ketulenan yang tinggi untuk β-Lac dan LF telah diperolehi dari MMM selepas elution seperti yang ditunjukkan oleh SDS PAGE gel.

Kata kunci: membrane campuran matrik (MMM), membrane kromatografi, whey, Lewatit MP500, Lewatit CNP105

1.0 INTRODUCTION

Various techniques are available for protein separation such as packed bed chromatography, membrane filtration, precipitation and most recently membrane chromatography. Chromatography based process is a preferable method that can give high resolution protein separation [1]. However, protein purification by chromatography which normally operated in packed bed configuration, had several limitations such as high pressure drop across the column and unable to operate at high flow rate [1–3]. Membrane chromatography, that combined the principle of membrane and chromatography in single format, is an alternative for packed bed chromatography. It shows several advantages such as high flow rate operability without the loss of adsorption capacity, simple module preparation, less complicated scale up process and low pressure drops [1, 4, 5].

The simple procedure for preparing membrane chromatography material is through mixed matrix membrane (MMM) preparation concept [6, 7]. MMM chromatography was prepared by physical blending of ground adsorptive resin into the membrane polymer solution. This homogenous dope solution was then transformed to the membrane form through a phase inversion process. Unlike in chemical modification approach, MMM approach is safer and any potential damage to the membrane due to the use of harsh or excessive chemicals can be avoided. Another advantage of this method is resins with differing adsorptive functionalities can be conveniently embedded within a single membrane at any desired ratio to suit the expected protein profile of a raw feed stream.

Saufi and Fee [6, 8] have successfully prepared multiple interactions or multi-mode MMM chromatography for whey protein fractionation. They had incorporated Lewatit MP500 anion exchange resin and SP Sepharose cation exchange resins in ethylene vinyl alcohol (EVAL) matrix [6]. The multiple interactions MMM was able to bind basic and acidic whey protein simultaneously during batch fractionation of whey. However, the cation SP Sepharose resin used in their study is expensive and costly. It is favorable to find a low cost cation resin but shows similar efficiency to that SP Sepharose resin. In the current study, the multiple interactions MMM was developed using low cost Lewatit CNP 105 cation exchanger resin and Lewatit MP500 anion exchanger resin. Both resins are use extensively at heavy metal removal industrial and cheap compare to Sepharose based ion exchanger resin. The resins were incorporated in two different membrane polymer solution of EVAL and cellulose acetate (CA). The performance of the EVAL and CA multiple interactions MMM chromatography for batch binding of lactoferrin (LF) spiked - whey protein were compared. A combination of anion and cation resin in the single membrane matrix can simultaneously capture acidic whey proteins (β-Lactoglobulin (β-Lac), α-lactalbumin (α-Lac), bovine serum albumin (BSA)) and basic whey protein (lactoferrin (LF)) in single adsorption step.

2.0 EXPERIMENTAL

2.1 Materials

EVAL, a random copolymer of ethylene and vinyl alcohol with an average content of 44 mol% and CA were used as a based matrix for preparing MMM. Dimethysulfoxide (DMSO) and 1-octanol were used as solvent and non-solvent additive respectively in EVAL casting solutions. PEG 400 in liquid form was used as the pore forming agent and NMP as the solvent in CA casting solution. The adsorptive resins incorporated in casting solution are Lewatit MP500 anion exchange resin and Lewatit CNP105 cation exchange. All the chemicals were purchased through Sigma Adrich, Malaysia.

The buffer solution was prepared from sodium phosphate dibasic heptahydrate, sodium dihydrogen orthophosphate 1-hydrate and sodium chloride. β-Lac, α-Lac, LF and BSA were purchased from Sigma and used without further purification. Two mobile phase solutions were used in HPLC analysis were buffer A of 0.1% (w/v) trifluoroacetic acid (TFA) in ultrapure water and buffer B of 10% (v) of solution 0.085% (w/v) TFA in ultrapure water: 90% (v) HPLC-grade acetonitrile.

2.2 Preparation of LF-Spiked Whey

Fresh milk was bought from nearby dairy farm. It was centrifuged at 12 000 rpm at 4°C for 10 min in Eppendorf centrifuge model 5810R for defatting. Defatted milk was then heated to 40°C in a water bath under gentle mixing. The pH was adjusted between 4.6-4.8 using 0.5 M HCl to precipitate the casein from the milk. Precipitated casein was discarded and whey supernatant was centrifuged at 12 000 rpm at 4°C for 10 min. Whey was filtered several times with filter paper with the final filtration was achieved with a 0.45 μm membrane filter. The final pH of whey was adjusted to pH 6 using 0.5 M NaOH. 6.5 mg LF pure protein was added into the 20 ml whey to prepare a LF-spiked whey.

2.3 Preparation of Mixed Matrix Membrane

MMM was prepared in two different membrane based polymer solution. First solution consist of 15 wt% EVAL polymer and 15 wt% 1-octanol in DMSO solvent. Second solution is 10 wt% CA and 20 wt% PEG 400 in NMP solvent. The polymer was dissolved in a solvent under continuous stirring at 60°C for several hours. The adsorbent resins (Lewatit MP500 and Lewatit CNP105) to be incorporated into the polymer solution were grinded into small size about 45 μm using an ultracentrifugal mill. Ground resin was added to the prepared polymer solution at certain weight fraction relative to the polymer content in the solution. This mixture was stirred again until homogeneous MMM

casting solution was formed. The total resin loading relative to the mass of based polymer was fixed at 50 wt% which consists of 42.5 wt% of Lewatit MP500 and 7.5 wt% Lewatit CNP105 [6].

Flat sheet MMM was casted using conventional casting method as described detail in previous publication [6]. MMM casting solution was poured onto a glass plate support and spread to form a thin film using a stainless steel casting block. Immediately after casting, the glass plate with the film on the surface was immersed to the coagulation water bath at room temperature until the membrane is completely solidified and detached from the glass. The resulting MMM was washed with water several times and left in the water bath overnight in order to ensure the trace of solvent is completely removed from the membrane structure. Finally, the wet membrane was freeze dried to remove water without damaging the membrane structure.

2.4 Batch Fractionation of Whey

MMM sheet was cut into a rectangular shape with a dimension of 10 mm x 20 mm. The membrane was undergone the following step: 1) equilibration in 20 mM sodium phosphate pH 6 binding buffer for 1 hour; 2) binding with 2 ml LF-spiked whey solution for 3 hour; 3) washing with binding buffer for 30 minutes and 4) desorption with elution buffer of 1M NaCl in 20 mM sodium phosphate pH 6 for 3 hours. The membrane was taken out from the solution at the end of each steps and was lightly dried by patting with tissue before proceed to the next step. All the solution in each step was further analyzed by HPLC and SDS-PAGE analysis.

2.5 Gel Electrophoresis

Whey protein fractions were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using pre-casted gel, NuPAGE 4-12% Bis-Tris Gel (Invitrogen (Carlsbad, USA). A recommended protocol by manufacturer was followed. Protein sample was mixed with NuPAGE LDS buffer 4X (Invitrogen) and NuPAGE 10X reducing agent (Invitrogen). The gel was slotted into XCell SureLock Electrophoresis Cell (Invitrogen), filled with NuPAGE MES SDS running buffer (Invitrogen) and connected to the PowerPac Universal (Bio-Rad, CA, USA) power supply. Proteins were stained with Coomassie Brilliant Blue R 250, 0.125% (w/v) in 10% (v/v) acetic acid and 40% (v/v) methanol. Destaining was carried out in a solution of 10% (v/v) acetic acid and 20% (v/v) methanol in water.

2.6 High Performance Liquid Chromatography

The amount BSA, α-Lac and β-Lac were quantified by C4 Jupiter column (300 Å, 250 × 4.6 mm i.d., 5 μm particle size; Phenomenex, Cheshire, UK) according to the methods described by Casal *et al*. [9]. The column was attached to the Waters ACQUITY UPLC H-Class system. Mixed proteins standard (β-Lac, α-Lac, BSA) were used to quantify the unknown concentrations in the sample. The samples were diluted with appropriate amount of mobile phase buffer A within the range of peak area in standard curve.

3.0 RESULTS AND DISCUSSION

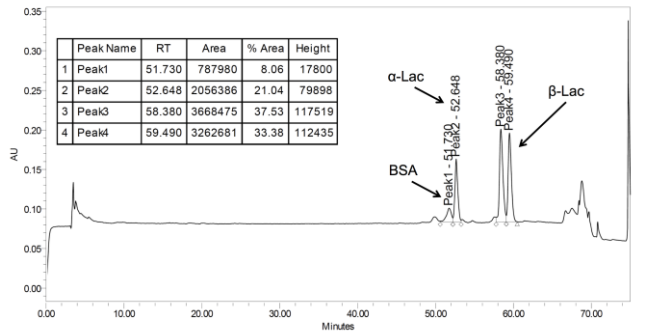
3.1 HPLC Whey Protein Analysis

Typical HPLC chromatogram for protein fractions from batch fractionation was shown in Figure 1. HPLC analysis was able to detect and quantify only acidic whey proteins of β-Lac, α-Lac and BSA. Basic whey protein, LF, was not able to detect using C4 Jupiter column HPLC but was analyzed qualitatively using SDS-PAGE. The retention time for BSA and α-Lac are around 51 and 52 minutes, respectively. β-Lac shows two peaks between 57 to 59 minutes that are belong to the β-Lac A and β-Lac B fraction. All acidic whey proteins were adsorbed by the MMM as can be seen by the decreasing of the peak area for each protein.

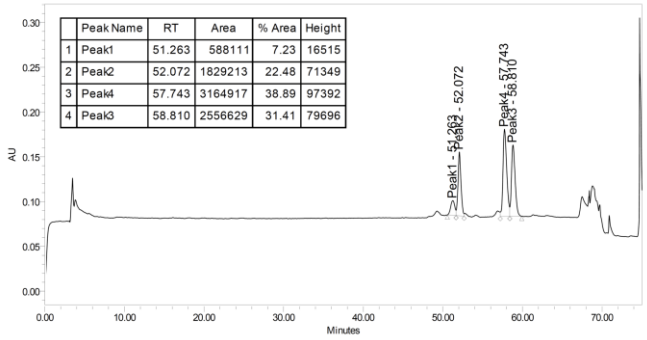
3.2 Batch Fractionation of Whey

Data for the batch fractionation of whey using EVAL and CA based MMM chromatography was showed in Table 1. The weight of CA MMM is higher than EVAL MMM for the same membrane area due to the density factor. The density of CA and EVAL are 1.30 and 1.14 g/cm^{-3}, respectively (Sigma). The binding capacity for acidic proteins to the MMM follows the order of β-Lac> BSA >α-Lac which is in an agreement with the previous publications [10, 11]. The binding capacity of EVAL MMM is higher than CA based MMM, more prominently for β-Lac. The average binding capacity of β-Lac to the EVAL MMM is 50.827 mg β-Lac/ g MMM which is more than double of CA MMM.

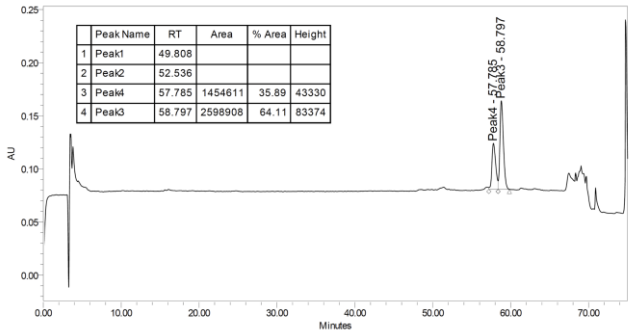
Depending on the types of functional groups exist on the membrane matrix, the protein binding can be affected in several ways such as repelling of the protein due to the same charge molecules or nonspecific binding. Repelling of protein only resulted in low protein binding but nonspecific binding should be avoided. Nonspecific binding by hydrophobic interactions tends to be very strong and often an irreversible process. This will contribute to low protein recovery during the elution and harsh elution protocol is required if the membrane to be reuse. It is seem that CA MMM somehow show a relatively low elution recovery compared to the EVAL MMM.



(a)



(b)



(c)

Figure 1 HPLC chromatogram for protein fraction from batch fractionation of whey using EVAL MMM. (a) feed (LF-spiked whey) - 4X dilution, (b) whey after binding - 4X dilution, (c) elution fraction

Both negative (BSA, α-Lac, β-Lac) and positive (LF) whey proteins were bound simultaneously to the multiple interactions MMM chromatography due to the presence of cation (Lewatit CNP105) and anion (Lewatit MP 500) resin in the membrane matrix. At pH 6, BSA, α-Lac and β-Lac become negatively charge (i.e. the pH of solution is higher than the pI of proteins) and interact with the positive quaternary amine group in MP500 resin through ionic interaction. LF becomes positively charged at pH 6 because the pH of solution is less than the pI of LF. LF was bound with the negative carboxylic group in the CNP105 resin.

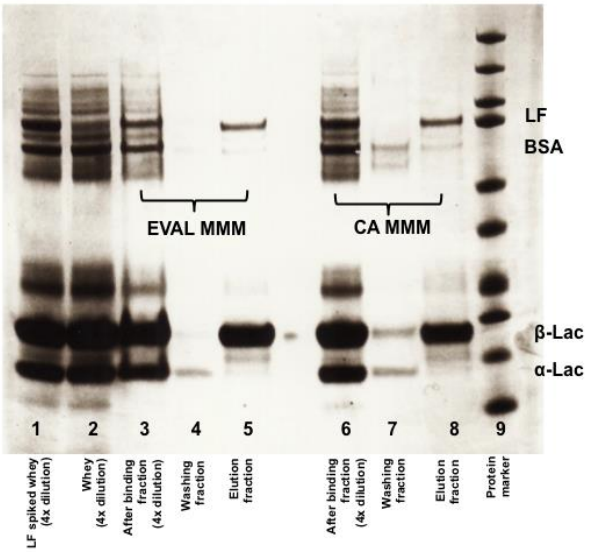


Figure 2 SDS-PAGE of several fractions from batch fractionation of LF-spiked whey using EVAL MMM and CA MMM

The data in Table 1 presented a binding for the negative whey proteins only since the LF cannot be detects by the HPLC method. The binding of LF to the MMM can be confirmed and visualized by the SDS-PAGE gel as shown in Figure 2. Strong band of LF was detected in the elution fraction together with the band of β-Lac for both type of MMMs. Very fade band of BSA can also be detected in the elution fraction especially for CA MMM. However the amount is too small and not detectable by the HPLC method. This is the reason of zero elution recovery of BSA in Table 1. Meanwhile, most of the α-Lac bound was lost during the washing step as shown in lane 4 and 7 of the gel. No α-Lac band was presents in the elution fraction which is consistent with the zero recovery showed by the HPLC analysis. Other types of proteins were also leached out from the CA MMM (lane 8) which is consistent to the low binding capacity of CA MMM compared to the EVAL MMM.

4.0 CONCLUSION

Multiple interaction MMM chromatography using low cost ion exchanger resin was investigated for whey protein fractionation in the current study. The type of membrane matrix used influenced the protein binding capacity. MMM prepared from EVAL polymer had a higher binding capacity than CA membrane. Nevertheless, both type of membrane were able to bind simultaneously acidic and basic whey protein in single run. This multiple interactions MMM was able to produce almost pure protein mixture of β-Lac and LF after the elution step.

Table 1 Whey protein batch fractionation using MMM. (a) EVAL based MMM and (b) CA based MMM

(a) EVAL MMM

Protein	BSA				α-Lactalbumin				β-Lactoglobulin			
MMM sample set	A	B	C	Ave	A	B	C	Ave	A	B	C	Ave
Mass of MMM, mg	27.1	28.8	30.4	28.8	27.1	28.8	30.4	28.8	27.1	28.8	30.4	28.8
Protein in feed, mg	0.486	0.486	0.486	-	1.847	1.847	1.847	-	8.112	8.112	8.112	-
Protein bound on MMM, mg	0.370	0.362	0.359	0.364	0.130	0.069	0.097	0.099	1.314	1.429	1.653	1.465
Binding percentage, %	76.1%	74.5%	73.9%	74.8%	7.0%	3.7%	5.3%	5.3%	16.2%	17.6%	20.4%	18.1%
Capacity, mg protein bound/g MMM	13.653	12.569	11.809	12.677	4.797	2.396	3.191	3.461	48.487	49.618	54.375	50.827
Protein eluted, mg	0	0	0	0	0	0	0	0	1.219	1.275	1.414	1.303
Elution recovery, %	0	0	0	0	0	0	0	0	92.8%	89.2%	85.5%	89.2%

(b) CA MMM

Protein	BSA				α-Lactalbumin				β-Lactoglobulin			
MMM Set	A	B	C	Ave	A	B	C	Ave	A	B	C	Ave
Mass of MMM, mg	42.0	41.2	39.2	40.8	42.0	41.2	39.2	40.8	42.0	41.2	39.2	40.8
Protein in feed, mg	0.486	0.486	0.486	-	1.847	1.847	1.847	-	8.112	8.112	8.112	-
Protein bound on MMM, mg	0.363	0.371	0.361	0.365	0.087	0.149	0.120	0.119	0.787	0.796	0.763	0.782
Binding percentage, %	74.7%	76.3%	74.3%	75.1%	4.7%	8.1%	6.5%	6.4%	9.7%	9.8%	9.4%	9.6%
Capacity, mg protein bound/g MMM	8.643	9.005	9.209	8.952	2.071	3.617	3.061	2.916	18.738	19.320	19.464	19.174
Protein eluted, mg	0	0	0	0	0	0	0	0	0.592	0.651	0.585	0.609
Elution recovery, %	0	0	0	0	0	0	0	0	75.2%	81.8%	76.7%	77.9%

Acknowledgement

The authors gratefully acknowledge the financial support from the Ministry of Higher Education Malaysia under the Research Acculturation Grant Scheme (RAGS – RDU121415).

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