Purification of Surface Layer Protein of Lactic Acid Bacteria Isolate from Indigenous Fermented Bambangan by Monolithic Chromatography

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Abstract

Monolithic adsorbent is a novel technology for high throughput downstream processing of biomolecules. It offers high yield, cost-effective and unparalleled preparation or regeneration in the long run. The building block of monolithic polymer is polymethacrylate, which is an organic polymer that exhibits tuneable surface and pore characteristics. Currently, we are facing challenges in eradicating the wall channel effect caused by monolith shrinkage to create a functionalised monolith with high binding capacity in large volume. Any efforts we did to eliminate the wall channels in the conical column will be implemented and the result of wall channel elimination will be reflected based on the backpressure and elution time of bovine serum albumin (BSA). Elution time of BSA will also be used as a parameter to determine the binding capacity of the functionalized monolith. For the characterisation of the target bio-molecules, phosphate buffered saline and lithium chloride will be used to detach the s-layer protein followed by zeta-potential analysis to determine its pI value. This review paper is an overview on the features of polymethacrylate monoliths, preliminary studies on the characteristic and application of s-layer protein, wall channel elimination methods and various parameters that serve as reference point for monolithic column optimisation.

Keywords: Monolithic adsorbent, Surface Layer Protein, Fermented Bambangan

INTRODUCTION

In downstream processing, liquid chromatography still represents the most powerful and reliable technique to achieve high yield and high purities for biomolecules separation. This technique involves the use of particulate based
supports consisting of many beads with small internal pores that contributes to its ability to manipulate hydrophobicity, charge, size, and affinity interaction. However, these supports are designed for efficient separation of micro molecules which poses an issue when it comes to separation of larger molecules. The tiny pores sizes of the bead impede the entry of larger molecules and allow interactions to occur only on the outer surface which results in poor binding. The low usage rate of the available capacity of the resin may also results in high levels of non-specific binding in the intra-particle (Lyddiatt, 2002).

Monolithic supports are a novel developing technology with high ceiling of potential, more so than conventional particulate supports. A lot of research and developments have been conducted in the past decade to utilize monolithic supports as the stationary phase in chromatographic separation. Unlike the conventional particulate supports, monolithic supports comprise a single structure with a highly interconnected network of channels that resemble honeycomb. Large pores size (>200 nm) has been the major inherent advantage of monolithic support which allows convective mass transport to take place that is much superior to mass transfer by diffusion found in particulate support (Jungbauer et al., 2002). With the exception of affinity chromatography, mass transfer has generally been considered as the rate limiting factor in chromatographic separation. The monolithic structures predominated by convective transport mechanism removed the limiter casted on conventional chromatographic techniques for larger molecules that are unable to penetrate into the internal structure of particulate support.

However, there are some inherent shortcomings of monolithic support as it tends to shrink post-polymerization which results in formation of wall channel in between the column hardware and stationary phase. Such phenomenon will induce leaking of sample due to lower pressure region compared to the monolith region (Siouffi, 2003).

This review is a general overview on characteristic of the polymethacrylate monolith and target protein (S-Layer Protein) and various methods of optimizing the monolithic adsorbent by eliminating the wall channels and purification processes in order to create a high binding capacity functionalized monolith to purify s-layer protein from a novel strain of Lactobacillus plantarum.

**CHARACTERISTIC OF MONOLITHIC ADSORBENT**

**Advantages of monolithic support**

Monolithic support features lower pressure drop that vary with different pore
structure orientations (Mihelič et al., 2005; Yang et al., 2005). Such feature allows higher flow rates of the mobile phase to be applied which enhanced the capacity of separation. Despite low absolute surface area, the increase in flow rate makes up for the lost capacity for larger molecules due to smaller specific surface area (Vlakh and Tennikove, 2009). The comparison of physical characteristics between monolithic supports and particulate supports extend much further than pore size alone.

Mechanical strength of monolithic supports plays an important role. It only takes a single crack in solid structure to cause column failure and good loss of valuable product (Podgornik et al., 2004). Generally, monolithic structures have more often than not exhibit good mechanical stability and rely heavily on the materials of construction (Vlakh and Tennikove, 2009). Such attribute allows larger monolithic supports to be cast, currently up to 8 L in volume (Jungbauer et al., 2002). Greater capacity for separation is made possible by increased volume which is a limiting factor for particulate supports.

Building block of monolithic polymer

The prevalent building block for monolithic polymer is the polymethacrylate resin which is an organic polymer that exhibit flexible surface and pore characteristics modification via accurate alteration in synthesis conditions. Polymethacrylate monolithic polymer is synthesised upon activation by initiator via a free radical polymerisation process in an unstirred mould, thus, resulting in a shape that conforms to that of the mould as well as a porous structure which allows direct flow of a liquid through it. Temperature is vital in controlling the porous characteristics. By adjusting the polymerization temperature, we can fine tune the average pore size within a wide range spanning 2nd order of magnitude (Yang et al., 2005). Collectively, the interconnected globules that are partly aggregated give shape to polymethacrylate monoliths. Irregular voids existing between clusters of globules inter-globules or even intra globules make up the pores in polymer. The internal organization of both globules and clusters within the polymer matrix can be reflected by the pore size distributions that are mainly dependent on the composition of the polymerization mixture and reaction conditions which offer freedom for fine tuning the pores size.

Repeated process of elution and regeneration is possible owing to the chemical stability of polymethacrylate monoliths and this unique feature is what differentiates it from other types of monoliths. During elution, without any damage to the ligand or support, the retained biomolecules can be easily separated from polymethacrylate monoliths. Such vital feature will come
in handy when the ligand is expensive, which is often the case for affinity chromatography (Jungbauer et al., 2002; Yang et al., 2005). Preventing degradation of ligands is vital as it could result in leaching of moieties into purified products and may be unfavourable in the case of therapeutic or food products. A strong basic solution (sodium hydroxide) is normally used to complete the regeneration process. Analogous to elution, the ligands remain bonded to polymethacrylate support during regeneration and not significantly degraded over time (Jungbauer et al., 2002; Yang et al., 2005). Pore structure of the monolith is dictated by the chemical composition of polymethacrylate monolith. For instance, other than altering the polymerization temperature the pore characteristics of the monolith can change dramatically by adjusting the composition of the porogen, ratio of monomers to porogen or both in the initial polymerization mixture. There are others significant advantages associated with the preparation of the support apart from chemical and physical characteristic of the polymethacrylate monolithic structure (Freitag and Allington, 2002). Owing to the synthesis technique of polymethacrylate monoliths, the supports can be produced in various conformations for example disks, rods and tubes, to satisfy a wide range of distinctive chromatographic purifications (Vlakh and Tennikove, 2009).

**Monolithic chromatography of biomolecules**

Small molecules differ greatly from protein and DNA due to their non-uniform charge distribution followed by irregular adsorption sites, peptides and oligonucleotides are less predictable than other biomolecules. Sýkora et al. (1999) demonstrated that molded monolithic columns can be used in an ion-exchange mode for the separation of oligonucleotides. They also tested a wide range of flow rate which hardly changes the separation ability of monolithic column due to its intrinsically low pressure drop characteristic. Podgonik et al. (1999) partitioned four deoxynucleotides by using Convective Interaction Media (CIM) disks that are methacrylate based. The separation was enhanced by elevating column length.

Broad ranges of proteins have been experimented on by using monolithic supports. Luo et al. (2001) have tried to separate lysozyme from egg white and commercially available human serum albumin by using metal immobilized iminodiacetic acid-bound molded monolithic rods of methacrylate based support. Riberiro et al. (2013) tested different chromatographic support to perform anion-exchange purification of recombinant factor IX from cell culture supernatant. Monolithic support top the chart for highest binding capacity which was then further investigated. Podgonik et al. (1999) and Podgonik et al.
(2001) have demonstrated the use of disks to separate manganese peroxidase and lignin peroxidase which results in faster separation than it was achieved with a packed columns.

**Characteristic of s-layer protein**

Surface Layer Protein of *Lactobacillus plantarum* are among the smallest known ranging from 25 to 71 kDa and are highly basic with calculated pI values ranging from 9.35 to 10.4 (Johnson *et al.*, 2013).

**APPLICATION OF LACTOBACILLUS S-LAYER PROTEIN**

**Anti-adhesive and anti-infectious effects**

Study done by Li *et al.* (2011) showed that the chromatographically purified S-layer protein SlpA from *L. acidophilus* ATCC 4356/NCFM is able to elicit the ERK1/2 signaling pathway that inhibit caspase-3 activity in *Salmonella*-infected Caco-2 cells, thereby decreasing *Salmonella*-induced Caco-2 cell apoptosis and cell damage (Li *et al.*, 2011).

**Vaccine development: immunoglobulin binding fusion protein**

In order to prevent neonatal diarrhoea, purified epithelial cell binding S-layer protein of *L. brevis* KCTC 3102 (ATCC 8287) was used by Khang *et al.* (2009) to guide antibodies to the calves’ intestinal surfaces. It was found out that by administering both antiviral and antibacterial drugs simultaneously with fusion protein, higher recovery of calves from diarrhoea was obtained compared to administering the antibodies alone. However, the exact mechanism of protection remains speculative (Khang *et al.*, 2009).

**WALL CHANNEL ELIMINATION**

A lot of wall channels elimination methods has been proposed and published. From the literature review, we could device the best method to remove the wall channel without disrupting its inherent characteristic.

**Post-polymerization packaging**

Shrinkage of monolith with any monomers as feedstock is a prevalent phenomenon. This method involves the preparation of preformed monolith in a mold with wider dimension than the column. Post polymerization followed by monolith washing, the bed support will therefore shrink to fit the dimension of the column. Hence, the proper design prior to the experiment based on the expected degree of shrinkage is vital so that the most optimal dimension of mold is selected. The resulting monolith will be removed and encased in the column for use (Shi *et al.*, 2011).
Covalent bonding in-between monolith and the column’s wall
This method involves the pre-treatment of the column. Monolith shrinkage can be reduced or eliminated by covalent attachment of the monolith to the capillary or tubing walls. Thus, it can be directly synthesized in situ without moving the monolith from mold to column (Shi et al., 2011).

Swollen the monolith itself by chemical means
Post polymerization functionalization or rinsing with 100% acetonitrile is able to induce swelling of the bed support which seal against the wall of the column (Pohl and Saini, 2007).

PARAMETERS THAT REFLECT COLUMN EFFICIENCY

Backpressure profiling
Whatever method employed to reduce the wall channels, we need an indicator to inform us about the status of wall channeling. Backpressure is a good indicator with increment value showing progress of wall channel reduction (Pohl and Saini, 2007).

Dynamic binding capacity
Such analysis provides insight on the efficiency of our column. Any biomolecules for example Bovine Serum Albumin can be used to load the column without using elution buffer. Once the column has reach its capacity of binding the biomolecules, the rest of the sample will be eluted out which will be reflected on the chromatogram. The area of the chromatogram prior to the breakthrough point indicates the binding capacity of our column.

Flow rate optimisation (Van Deemter Plot)
Columns with high number of theoretical plates (N) generally have higher efficiency than the one with lower N. Narrower peak is also observed in those columns with higher N. Columns with lower HETP (Height equivalent to theoretical plate) indicate having more N, and hence, much efficient than columns with higher HETP. With all the data available (N, HETP), the optimum linear velocity of mobile phase can be obtained by plotting the Van Deemter plots (Plate height vs Linear Velocity of Mobile phase). The point (linear velocity) at which the minimum plate height is obtained would be the optimum flow rate for the sample purification, in this case, s-layer protein. Under this condition, the resulting peak is more symmetrical, sharper and band broadening is minimized (Ongkudon and Danquah, 2010).
\[ N = 5.55 \left( \frac{t_R}{w_{1/2}} \right)^2 \quad \text{HETP} = \frac{L}{N} \]

\[ t_R = \text{Retention Time} \]

\[ w_{1/2} = \text{Peak width at half height} \]

\[ L = \text{Length of column} \]

**CONCLUSION**

A proper devised method in wall channel elimination is critical for the purification of s-layer protein to be successful. Methods that imposed little or no risk to the monolith’s inherent characteristic will be favourable. From the literature, cation exchange chromatography method is favourable due to the highly basic nature of the *Lactobacillus plantanum* s-layer protein. The expected range of its molecular weight is 25 to 71 kDa, allowing us to narrow the possible range on SDS-PAGE analysis. Zeta potential analyzer will be used to determine the exact pI value of the s-layer protein on the narrowed range of potential slps purified from crude extract.

**REFERENCES**


