ADSORPTION AND ELUTION OF LECTINS BY AFFINITY MEMBRANES

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Abstract

Affinity membranes suitable for the purification of lectins were prepared by chemical modifications of a cellulose matrix. As a model protein a lectin obtained by chromatographic techniques from *Momordica charantia* seeds was mainly used; *Peanut agglutinin* and *Ricinus communis agglutinin* were also considered. Different ligands were tested according to the different affinity towards the lectins used. Among the various ligands tested arabinogalactan and N-acetyl-D-galactosamine gave the best performances. The ligand binding reaction onto the epoxy groups of the activated matrices has been optimized with respect to concentration of ligand, temperature and reaction time. The ligand immobilized on the membrane surface is quantified indirectly by measuring the amount of protein bound to the membrane. The kinetics of adsorption and desorption of the purification process has been studied in detail for the different supports. Modified membranes have been used in separation process of lectins with good binding capacity towards the protein of interest.

1. Introduction

Lectins are glycoproteins endowed with many interesting properties, as they bind carbohydrates, agglutinate cells and precipitate polysaccharides [1], [2]. Due to those properties lectins are used to determine carbohydrates either in solution or on cell surfaces. They have multiple sugar binding sites, therefore, upon reaction with cells, for example erythrocytes, they will not only combine with the sugars on their surfaces, but will also cause cross-links of the cells and their subsequent precipitation, a phenomenon referred to as cell agglutination [3].

Lectins are normally purified from crude vegetable extracts by chromatographic techniques. Objective of this work is the development of an analogous process for the purification of *Momordica charantia* and other galactose binding lectins with affinity membranes [4].

Affinity membranes were prepared by chemical modification of two supports: a filter paper made of native cellulose and an epoxy pre-activated stabilized reinforced cellulose membrane. Arabinogalactan and N-acetyl-D-galactosamine were tested as affinity ligands due to their different affinity with respect to the protein used. Adsorption experiments were performed in batch and in continuous mode in order to determine the binding capacities of the affinity membranes. Adsorption isotherms of *Momordica charantia* lectin were obtained for all the different membranes investigated and the relevant parameters were calculated. As a comparison, *Ricinus communis agglutinin* and *Peanut agglutinin*, that show specificity for galactose like *Momordica charantia* lectin, were also tested. The different affinity membranes

were compared with respect to the results obtained in terms of binding capacity, kinetic behaviour and selectivity. The feasibility of the process has been demonstrated.

2. Materials and methods

Proteins: Lectin extracted from *Momordica charantia* seeds (MCL) was kindly provided by the Department of Experimental Pathology of the University of Bologna [5],[6]. In this work other galactose-specific lectins have been tested: *Ricinus communis agglutinin* (RCA) and *Peanut agglutinin* (PNA), purchased from Vector Laboratories, USA. These glycoproteins were chosen among the commercially available lectins because they have similar structure and they show the same specificity towards carbohydrates as MCL; their characteristics are reported in Table 1.

Table 1. Lectin properties

LECTIN	MW(kDa)	SUBUNITS	SPECIFICITY SUGAR
Momordica Charantia Lectin **(MCL)**	**120**	**4**	**β-Gal, GalNAc**
Ricinus Communis Agglutinin **(RCA)**	**120**	**4**	**Gal, GalNAc**
Peanut Agglutinin **(PNA)**	**110**	**4**	**GalNAc**

in which **Gal**: Galactose, **β-Gal**: β-galactose, **GalNAc**: N-acetyl-D-galactosamine.

Membranes: Two different matrices were used as affinity support: cellulose filter paper purchased from Whatman, with thickness of 150 μm, pores capable to retain particles of 20-25 μm diameter and porosity of about 62% and Sartobind epoxy-activated membrane with thickness of 250 μm, average pore size of 0.45 μm, a porosity of about 64% and a minimum static binding capacity for the target protein of 30 μg/cm^2, kindly provided by Sartorius AG.

Ligands: The affinity of lectins towards polysaccharides can be measured by haemagglutination tests [7], [8], [9]. Different ligands were chosen according to their affinity to the target proteins [5]. MCL has affinity for β-anomers of D-galactose and among those arabinogalactan and N-acetyl-D-galactosamine were tested as affinity ligands. All ligands were purchased from Sigma-Aldrich, Italy.

Chemicals and solutions: Citric acid was purchased from Carlo Erba Reagents, Italy. Sodium thiosulfate, $NaBH_4$, lactose,1,4-butanediol diglycidyl ether, sodium azide, glycine and sodium acetate were ACS reagent grade purchased from Sigma-Aldrich, Italy. BCA protein assay reagents was purchased from Pierce.
The loading buffer, PBS, was prepared from a ten fold solution: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 mL deionised water, brought to pH 7.4 and additioned with deionised water up to 1 L. Before use, dilution 1:10 with deionised water is applied. The elution buffer was 0.1M lactose in PBS.
All buffers were filtered prior to use through 0.45 μm nitrocellulose membrane filter purchased from Millipore.
Molecular weight markers for SDS–PAGE electrophoresis (Standards Broad Range) and Blue Coomassie were purchased from Bio-Rad Laboratories.

Equipment: Absorbance readings were performed using an UV-Vis spectrophotometer: Shimadzu UV-1601. SDS–PAGE analysis of the protein solutions was performed with Criterion electrophoresis system from Bio-Rad Laboratories, using precast gels. HPLC analysis was performed with a Waters Alliance 2695, with a size exclusion chromatography column Bio-Sep SEC-S4000 purchased from Phenomenex.
Dynamic experiments have been performed using an Amicon dead end membrane holder that can allocate up to 20 flat sheet membranes of 2.5 cm diameter in a stack. A peristaltic pump, Gilson Minipuls 3 was used, with a head of eight channels, ten rollers that can accept tube sizes from 0.2 mm to 4 mm (internal diameter). The maximum back pressure is 500 kPa (75 psi).

Membrane modification: Whatman filters 541 have been modified following the two stage protocol described in ref. [10]. First a spacer arm, 1,4-butanediol diglycidyl ether, was bound to the surface. The concentration of active epoxy groups onto the membranes was measured by titration with sodium thiosulfate [11] and then the ligand was bound to the epoxy active groups present.
Sartobind epoxy is a regenerated cellulose membrane pre-activated for ligand coupling. The different ligands were then coupled using the same protocol for the two different matrices, and different procedures depending on the ligand chosen.

Ligand coupling: Arabinogalactan activation: 4.5 g of arabinogalactan were dissolved in 150 mL 0.3N NaOH. Epoxy activated membranes were immersed in the solution and the coupling reaction started by adding 1 mg/mL of $NaBH_4$. The system was shaken 24 hours at 40°C. The membranes were thoroughly washed with deionised water before use [12].
N-acetyl-D-galactosamine activation: 7.5 g of N-acetyl-D-galactosamine were dissolved in 150 mL 0.1N NaOH. Epoxy activated membranes were immersed in the solution and shaken 24 hours at 40°C. The membranes were thoroughly washed alternatively with deionised water and 0.1M sodium borate buffer before use [13]. The affinity membrane obtained are:

- Whatman 541-Arabinogalactan (W541-AraGal)
- Sartobind Epoxy-Arabinogalactan (SarEpo-AraGal)
- Sartobind Epoxy-N-Acetyl-D-Galactosamine (SarEpo-GalNAc)

The affinity membranes can then be stored in 0.02% sodium azide at 4°C.

Protein Concentration Measurement: Lectin concentration was measured either by reading the absorbance at 280 nm or by using the bicinchoninic acid assay (BCA assay, from Pierce). The BCA assay is used for the colorimetric detection and quantitation of total protein; this method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (Biuret reaction) with the highly sensitive and selective colorimetric detection of cuprous cation using a reagent containing BCA. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with protein concentrations over a broad working range from 20 µg/mL to 2000 µg/mL.
Calibration curves were prepared for both methods by using pure lectin solutions at known concentration values.

3. Experimental and results

3.1 Batch adsorption experiments

Affinity membranes were tested for adsorption in batch experiments in order to measure static binding capacity. The membranes, cut in discs of 2.5 cm diameter, were immersed in a beaker containing pure protein solution and gently agitated in an orbital shaker. The duration of the adsorption step depends on the concentration of the protein solution; in the range of protein concentration used, in all cases the equilibrium is reached within 2 hours. This has been verified with detailed kinetic studies presented in previous work [4]. After adsorption, the membranes were washed with PBS buffer at pH 7.4. Elution was performed with different buffers with the aim of finding optimal conditions for protein recovery. The concentration of lectin in solution has been monitored with two different techniques: absorbance reading at 280 nm and BCA protein assay with absorbance reading at 562 nm. The results are in good agreement with one another, indicating that both techniques can be equivalently used for this purpose [4]. The experiments were repeated for the different matrices and for all the ligands tested.

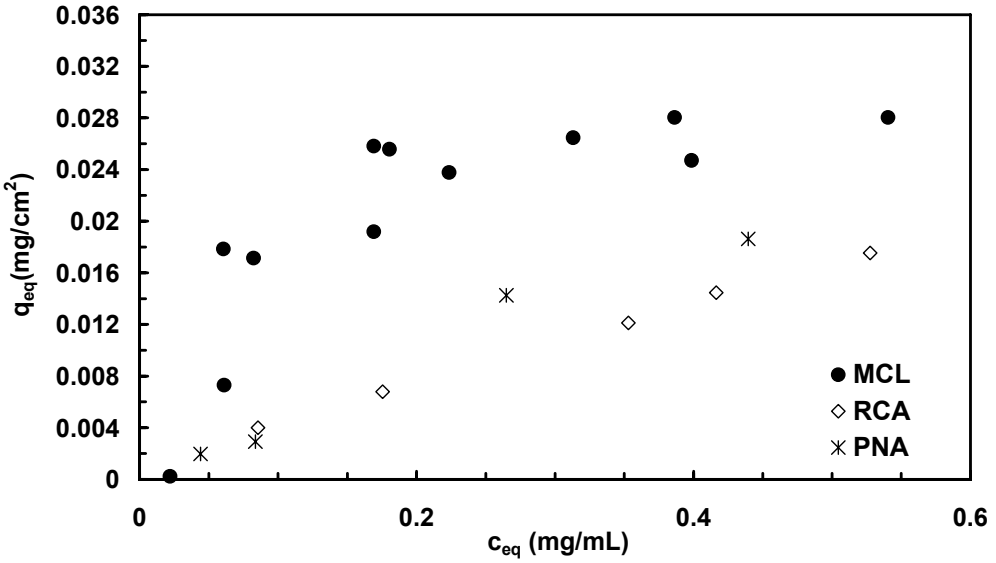


Fig. 1. Static binding capacity of Sartobind-epoxy-arabinogalactan towards different proteins: MCL, PNA, RCA.

Different lectins, MCL, PNA and RCA, with similar sugar specificity were adsorbed onto affinity membranes obtained with Sartobind epoxy modified with arabinogalactan, and their static binding capacity is reported in Fig. 1. From the equilibrium data, it can be noted that MCL shows the highest binding capacity whilst RCA and PNA have similar adsorptive performances. Other affinity membranes were tested for adsorption of MCL: Whatman 541 coupled with arabinogalactan and Sartobind-epoxy modified with N-acetyl-D-galactosamine, the affinity chromatography ligand normally used for the purification of lectins with specificity for galactose containing sugars. The experimental data points are reported in Fig. 2 together with the adsorption isotherms interpolated with the Langmuir equation. The Langmuir parameters, q_m and K_d, were calculated and are reported in Table 2; in this table the static

binding capacity has been reported also in mg/mL of membrane volume in order to better account for the different thickness of the supports used.

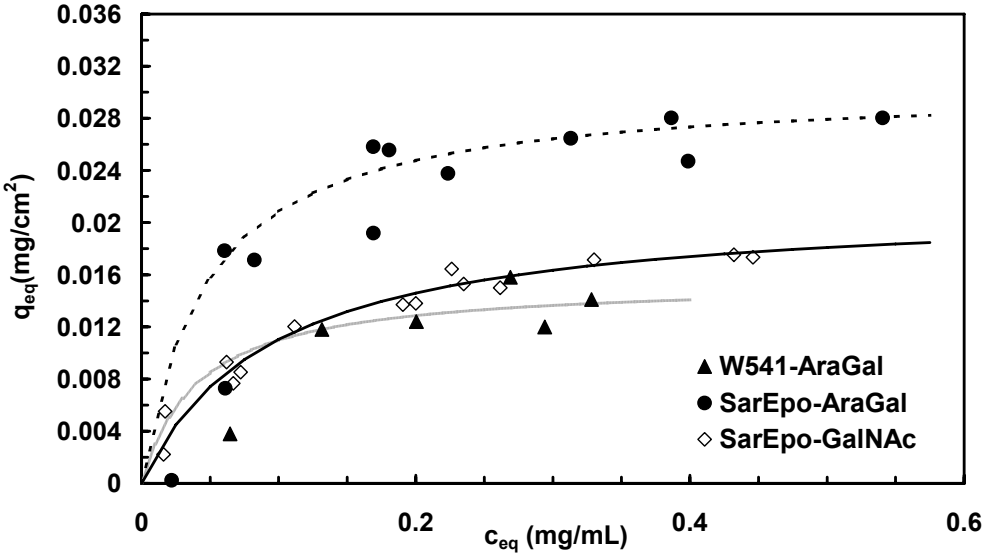


Fig. 2. Adsorption isotherms of MCL onto different affinity membranes.

Table 2. Membrane capacity for the different supports tested with MCL

Membrane	Ligand	K_d (mg/mL)	q_m (mg/cm^2)	q^*_m (mg/mL)
W541	AraGal	0.041	0.016	1.067
SarEpo	AraGal	0.046	0.031	1.240
SarEpo	GalNAc	0.095	0.022	0.880

The static binding capacity is of the same order of magnitude for all the membranes used in spite of the different properties of the two supports considered, and arabinogalactan gives higher binding capacity than N-acetyl-D-galactosamine; this result, together with the fact that arabinogalactan is cheaper than N-acetyl-D-galactosamine, makes arabinogalactan the preferred ligand for the separations considered.

3.2 Dynamic experiments

A second series of experiments has been performed in a continuous flow system configuration with two different operating modes: at total recycle, as illustrated in Fig. 3a and without recycle as in the typical breakthrough experiment set-up, as shown in Fig. 3b. Experiments with total recycle were performed with MCL, which is a lectin difficult to obtain and is not commercially available, whereas for the flow through configuration RCA was used. In both cases PBS was the buffer of choice for the adsorption and washing stages, while elution buffers were varied for the different experiments. The effects of the number of membranes in the stack, concentration of the feed solution and flow rate were investigated.

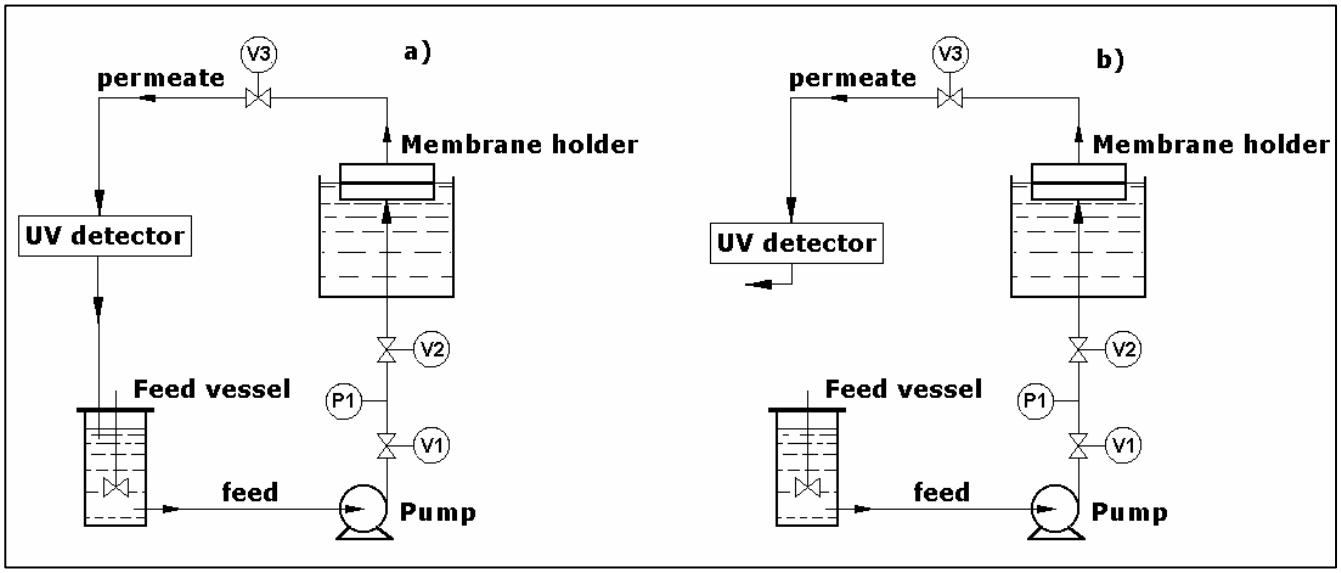


Fig. 3. Experimental set-up used for dynamic experiments: **a)** with total recycle, **b)** in flow through configuration.

In Fig. 4 a typical concentration profile for a dynamic experiment of RCA is reported. The small elution peak represents one of the main drawbacks of this process and the quantity of protein that was eluted is not comparable to the amount of protein adsorbed. A similar behaviour in terms of protein recovered in the elution step has been observed with MCL as shown in Fig. 5. A detailed description of the experiments at total recycle is presented in [4].

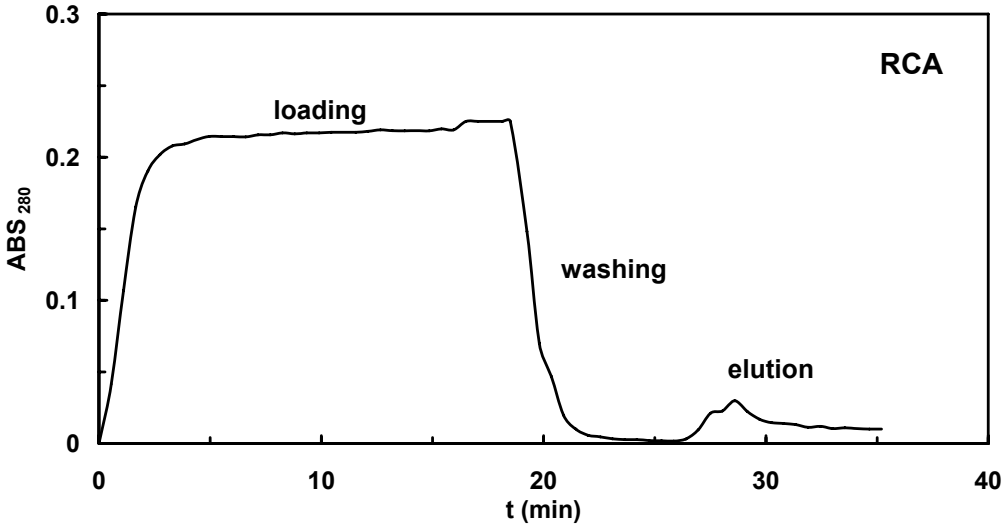


Fig. 4. Concentration profiles of adsorption, washing and elution curves during a dynamic experiment of RCA at the following conditions: c_0=0.2 mg/mL, V=19.5 mL, Q= 1 mL/min, total membrane area 38 cm^2, elution with acetate buffer.

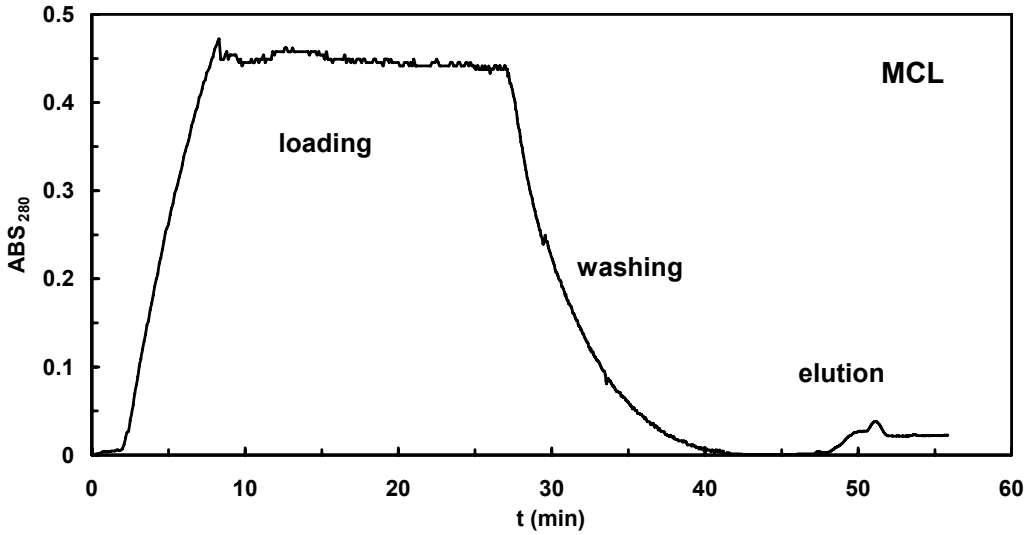
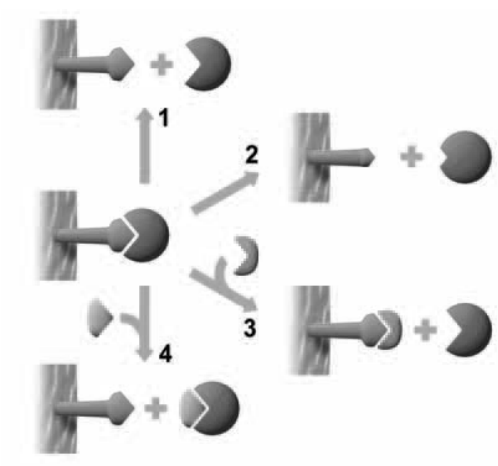


Fig. 5. Concentration profiles of adsorption, washing and elution curves during a dynamic experiment of MCL at the following conditions: c_0=0.5 mg/mL, V=1 mL, total membrane area 23 cm^2, elution buffer 0.2M lactose.

3.3 Elution

In purification processes the elution step is very important when the objective of the process is the recovery of a target protein. The correct choice of elution conditions to break the affinity interaction is often as important as the optimization of binding conditions. There are different elution techniques, that may be either selective or non-selective, as shown in Fig. 6, [14].



Method 1
The simplest case. A change of buffer composition elutes the bound substance without harming either the protein or the ligand.

Method 2
Extreme values of pH or high concentrations of chaotropic agents are required for elution, but these may cause permanent or temporary damage.

Methods 3 and 4
Specific elution by addition of a substance that competes for binding. These methods can enhance the specificity of media that use group-specific ligands.

Fig. 6. Elution methods.

The different elution methods tested in this work are summarized in Table [4] reported below.

Table 4. Eluents and elution methods.

Eluent	Elution method
Glycine 0.1M (pH 2.8)	**Method 2: pH elution**
Citric acid (pH 2.5)	**Method 2: pH elution**
Acetate buffer (pH 3.0)	**Method 2: pH elution**
Sodium dodecyl sulfate	**Method 2: chaotropic eluent or detergent** [15]
Lactose 0.2M	**Method 3: competitive elution** [7], [12], [16],[17]
β-Lactose 0.2M	**Method 3: competitive elution** [16]
D-Galactose 0.1M	**Method 3: competitive elution** [18]
Arabinogalactan	**Method 3: competitive elution** [13]

For batch experiments, the membranes were immersed in a beaker containing 3 mL of elution buffer and gently agitated in an orbital shaker for 2 hours. While for the second series of experiments performed in a continuous flow system configuration, elution has been performed in two steps: in the first one 1 mL of eluent solution has been re-circulated to the membrane cell for 1 hour. Then the membranes were taken off the membrane holder and immersed in a beaker containing 3 mL of elution buffer and gently agitated in an orbital shaker overnight.

Because of the small amount of immobilized protein, we were not able to quantify the recovery of the target proteins through absorbance techniques. We have obtained some indications on the efficiency of elution through SDS-PAGE analysis and size exclusion chromatography.

Among the buffers used best results were obtained with 0.2M lactose in PBS, both with SarEpo-AraGal and SarEpo-GalNAc membranes.

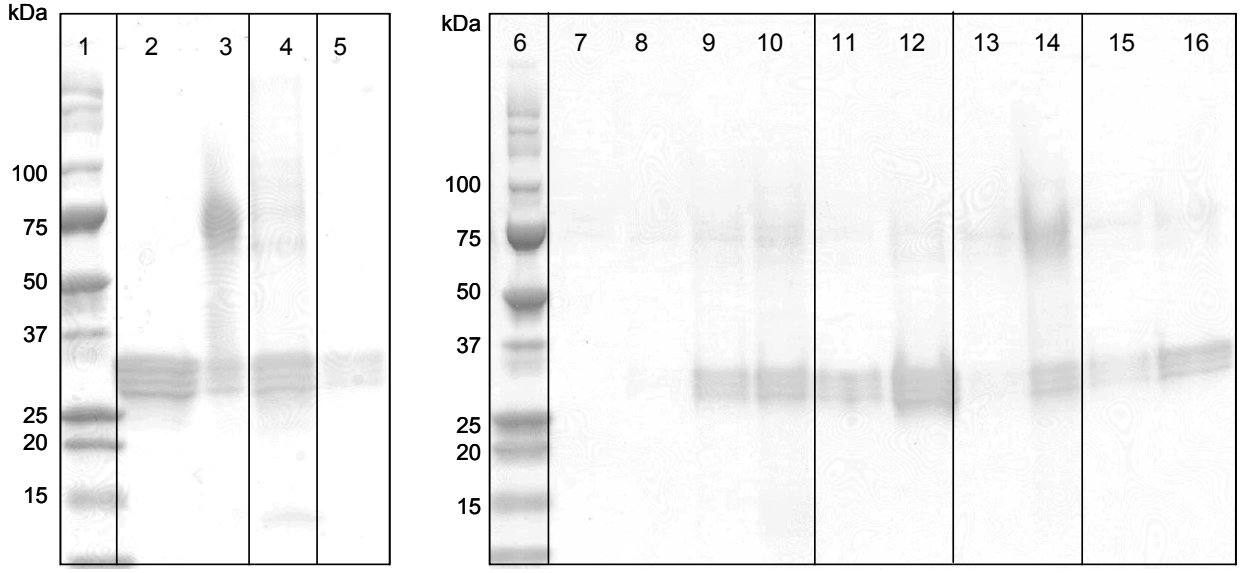


Fig. 7. Gel electrophoresis results. Lanes 1 and 6, MW markers; lanes 2 and 3, MCL feed solution (lane 2 reducing conditions, lane 3 non reducing conditions); lanes 4-5 and 7-16, concentrated eluted fractions with lactose 0.2M; lanes 4-5 and 7-12, adsorption experiments of MCL on SarEpo-AraGal membranes; lanes 13-16,

adsorption experiments of MCL on SarEpo-GalNAc membranes; lanes 4 and 5, experiments performed in a continuous flow system configuration; lanes 7-16, batch experiments.

Through SDS-PAGE analysis we have checked the ability of the eluent to remove the immobilized protein. The characteristic bands of the sub-units (28-30 kDa) are visible in all the fractions eluted with lactose. No improvements have been obtained using β-lactose. Probably because both the galactose of lactose and the galactose of β-lactose have β configuration. Moreover this disaccharide gives strong interferences in reading absorbance at 280 nm or with colorimetric assays, as we can see in the data reported in Table 4

Table 4. Comparison between absorbance of eluent and eluted fractions read against PBS buffer.

ABS_{280}	time (min)			
	0	150	1110	1620
lactose 0,2M	**0,0356**	**0,0366**	**0,0662**	**0,0828**
eluted fraction	**0,0356**	**0,0549**	**0,0898**	**0,0696**
β-lactose 0,2M	**0,0745**	**0,0718**	**0,0741**	**0,0776**
eluted fraction	**0,0745**	**0,0786**	**0,0830**	**0,0791**

Table 4 represents a comparison between two elutions and their relative blank solutions. This experiment has been performed with SarEpo-AraGal membranes adsorbed with a solution of RCA. After the adsorption and washing step the membranes were divided in two parts and eluted with 3 mL of lactose and of β-lactose. Absorbance reading were taken against PBS for the two elutions and for blanks of the elution buffers without membranes. In both cases the absorbance at 280 nm increases with time, but this trend is most likely due to lactose. Conversely the absorbance of the β-lactose solution does not increase with time, but the interference of the eluent is too high to quantify with accuracy the amount of eluted protein.

Size exclusion chromatography confirmed SDS-PAGE analysis. First of all, the possibility to elute the immobilized lectins with lactose [Figs.8,9,10]. Indeed, the chromatogram of the eluted protein shows the presence of the eluent (peak with retention time of 11.5 minutes) and of the protein (peak with retention time of 9.5 minutes). Moreover, size exclusion chromatography confirmed the possibility to reduce the eluent interference of with dialysis: the comparison between an eluted fraction before and after dialysis is reported in Fig. 10. As expected, the effect of dialysis is to reduce eluent concentration, but also to induce a dilution of the protein. Although dialysis has reduced the interference of the eluent, the protein concentration remains too low to obtain a reliable quantification of the lectin amount recovered.

Since elution proved to be a critical step, different buffers were tested in order to obtain improvements, including 0.1M glycine pH 2.8 and 0.1M citric acid pH 2.5. Among the buffers used for elution best results were obtained with 0.2M lactose in PBS.

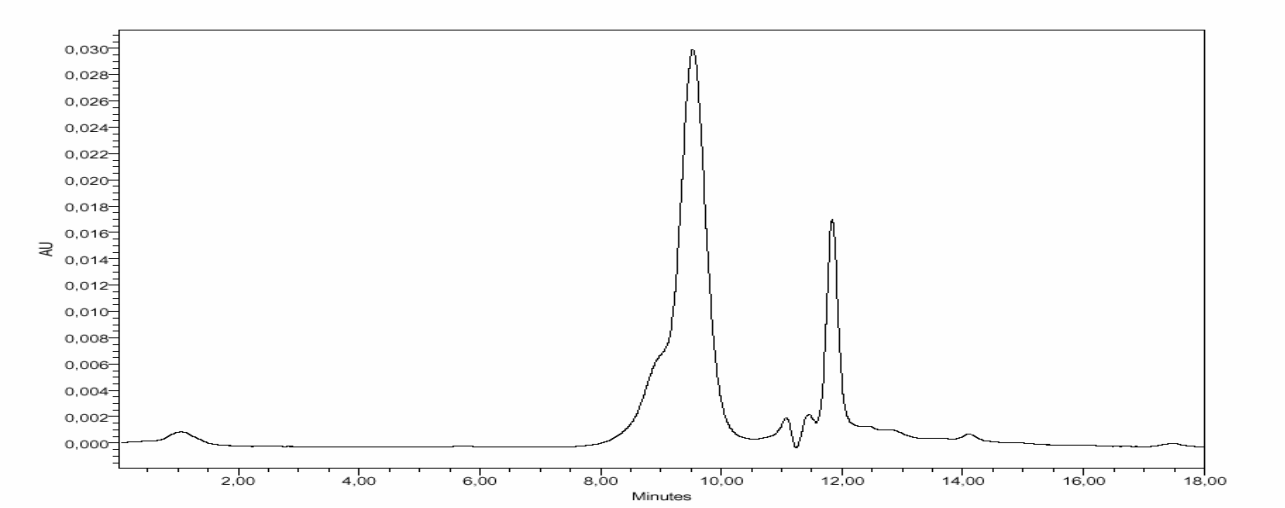


Fig. 8. Size exclusion chromatogram of MCL in PBS buffer

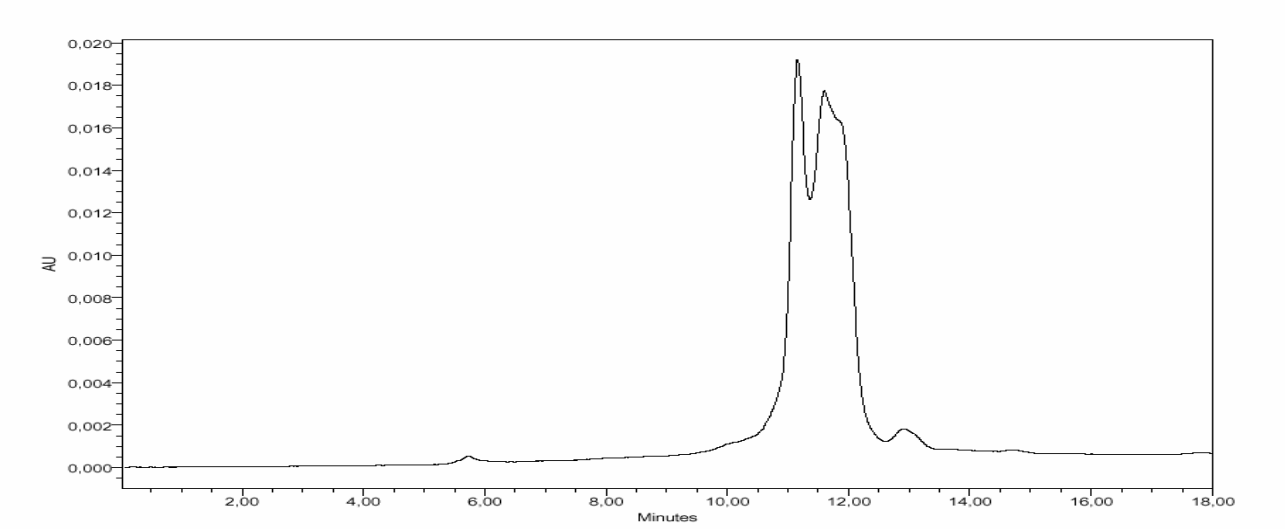


Fig. 9. Size exclusion chromatogram of elution buffer containing 0,2M lactose

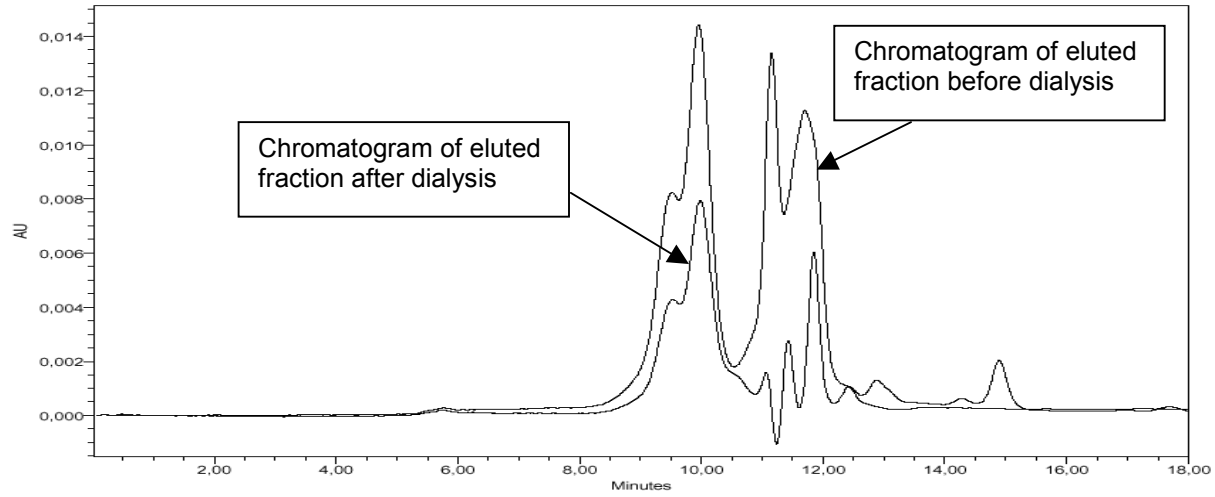


Fig. 10. Size exclusion chromatogram of eluted fractions, before and after dialysis against PBS to remove lactose.

4. Conclusions

Affinity membranes for the adsorption of *Momordica charantia, Ricinus Communis Agglutinin, Peanut Agglutinin* lectins were obtained starting from two different cellulosic matrices, i.e. Whatman 541 and Sartobind epoxy activated membranes. The ligands coupled to the supports were arabinogalactan and N-acetyl-D-galactosamine.
The resulting affinity membranes were tested for adsorption in batch and dynamic experiments and were characterised in terms of binding capacities and kinetic parameters. Among the carbohydrates tested, N-acetyl-D-galactosamine gave the worst binding capacity performance Arabinogalactan was the ligand of choice due to its good binding capacity, easiness of use and price.

Elution remains the critical step of the process and still needs to be optimised. The affinity membranes presented in this work should be tested directly with the aqueous extract from *Momordica charantia* seeds and this is the first objective for future work.

Acknowledgements

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