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Purification of oligouronides by immobilized L-histidine pseudoaffinity chromatography ☆

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Abstract

Selective purification of α-(1,4)-oligogalacturonides was investigated using several pseudobioaffinity chromatography matrix with aminoacid L-histidine as pseudobiospecific ligand: (1) sepharose 4B-bisoxyran-histidine, (2) sepharose 4B-epoxy-histidine, (3) silica-oxyran-histidine and (4) CIM-disk-EDA-histidine. These anionic oligosaccharides prepared by enzymatic and chemical cleavage of polygalacturonic acid were used as models sugar in order to optimize the adsorption and elution parameters for a selective purification of bioactive oligouronides. Monolithic CIM-disk chromatography is one of the fastest liquid chromatographic method using for separation and purification of biomolecules thanks to high mass transfer rate. In this way, this new monolithic CIM-disk system with L-histidine immobilized: immobilized histidine affinity chromatography (IHAC) constitutes a good tool allowing the fast and selective purification of bioactive oligouronides.
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Keywords: Oligouronides; Purification; Pseudobioaffinity; L-Histidine; Monolith chromatography; CIM-disk

1. Introduction

Oligosaccharides possess interesting biological properties [1–12]. Among these compounds, oligogalacturonides which are linear α-(1,4)-linked D-galactopyranuronosyl oligosaccharides, obtained by the partial depolymerization of polygalacturonic acid (PGA) may be considered as good models to investigate biological activities. Their structures are relatively homogeneous and they are available after pectin depolymerization by enzymatic or other procedures [2]. Degree of polymerization (dp) of poly- and oligogalacturonic acids appears to control physiological roles [2,13]. However, the high degree of purification required for these applications is still a major obstacle for their development. Therefore, it is necessary to develop extraction and purification processes which are fast, effective, and suitable for large-scale production of oligosaccharides over the widest possible range of dp values and at a reasonable cost. Consequently, specific purification of oligosaccharides can be investigated by bioaffinity. In fact, these last decades, pseudobioaffinity chromatography using L-histidine immobilized onto poly-(ethylene-vinyl alcohol) hollow fiber membranes were developed to separate and purify protein in large amount as immunoglobulin (IgG) from human serum [14,15]. It was shown that oligosaccharides from polyglucuronic acid could be retained and fractionated by pseudobioaffinity on L-histidine immobilized [16,17]. Consequently, it appears interesting to investigate new affinity chromatographic matrix with L-histidine ligand for the selective purification of oligogalacturonides.

These last years, a new polymeric macroporous material based on radical co-polymerization of glycidyl methacrylate and ethylene glycol dimethacrylate under the trademark Convective Interaction Media (CIM®) was introduced. CIM monolithic supports represent a novel generation of stationary phase used for liquid chromatography and bioconversion [18–21]. Contrary to other usual chromatographic supports consisting of macroporous particles, where the void volume between individual porous particles is unavoidable, CIM-disk technology is

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a monolithic macroporous material where there are predominantly convective transports of liquid within the pores and the molecules transport and transfer to active sites is not limited by diffusion resulting in a very fast separation due to the low mass transfer resistance of the CIM-disk support.

Then, in this present paper, selective and fast purification of oligogalacturonides using pseudobioaffinity chromatography were investigated with several L-histidine immobilized chromatographic supports and notably monolith CIM-disk. The purpose of this study was to propose a new approach for the fast and selective purification of oligogalacturonides obtained by enzymatic or chemical cleavages of polygalacturonic acid.

2. Materials and methods

2.1. Production of oligouronides

Two families of oligogalacturonides were generated. Unsaturated oligogalacturonides (ΔOGA) were produced by enzymatic cleavage of commercial polygalacturonic acid (Sigma) using pectolyase activity from *Aspergillus japonicus* (Sigma). Polygalacturonic acid (PGA) solution (3%, w_t/v) in 50 mM phosphate buffer at 25 °C was incubated with enzymatic preparation during 24 h. After incubation, enzymatic β-elimination was stopped by dipping the reaction medium into 95 °C water bath during 5 min. Mixture of oligomers was then centrifuged at 15,000 × *g* for 20 min at 20 °C and the supernatant was recovered and freeze dried. Saturated oligogalacturonides (OGAs) were produced by thermal degradation of PGA according to Simms et al. [22]. PGA solution in water (10 g/L) is first autoclaved at 120 °C for 40 min. After cooling at room temperature, the solution was centrifuged at 14000 × *g* for 10 min at 4 °C. The supernatant was then adjusted to pH 2 by addition of diluted HCl to precipitate the high molecular weight polysaccharides remained in the PGA heated solution. After a second centrifugation, the supernatant called S_2 was collected and adjusted to pH 7 by addition of diluted NaOH and finally freeze dried.

2.2. Preparation of matrix

In order to study the specific absorption between oligogalacturonides and L-histidine, different chromatographic supports with L-histidine immobilized on sepharose gel were investigated. (1) Sepharose 4B-bisoxyran-histidine, (2) sepharose 4B-epoxy-histidine and (3) silica-oxyran-histidine gels, were prepared as described by Kanoun et al. [23].

The monolithic support CIM-disk-EDA-histidine was prepared according to the following procedure: the support for the L-histidine immobilization was a CIM-EthylenDiAmine (EDA) disk (BIA Separations, Slovenia) with an I.D. of 12 mm, a thickness of 3 mm and a volume of 0.34 mL. After the immobilization step, the disk was thoroughly washed with water and the working buffer. Structures of L-histidine matrices are described in Fig. 1.

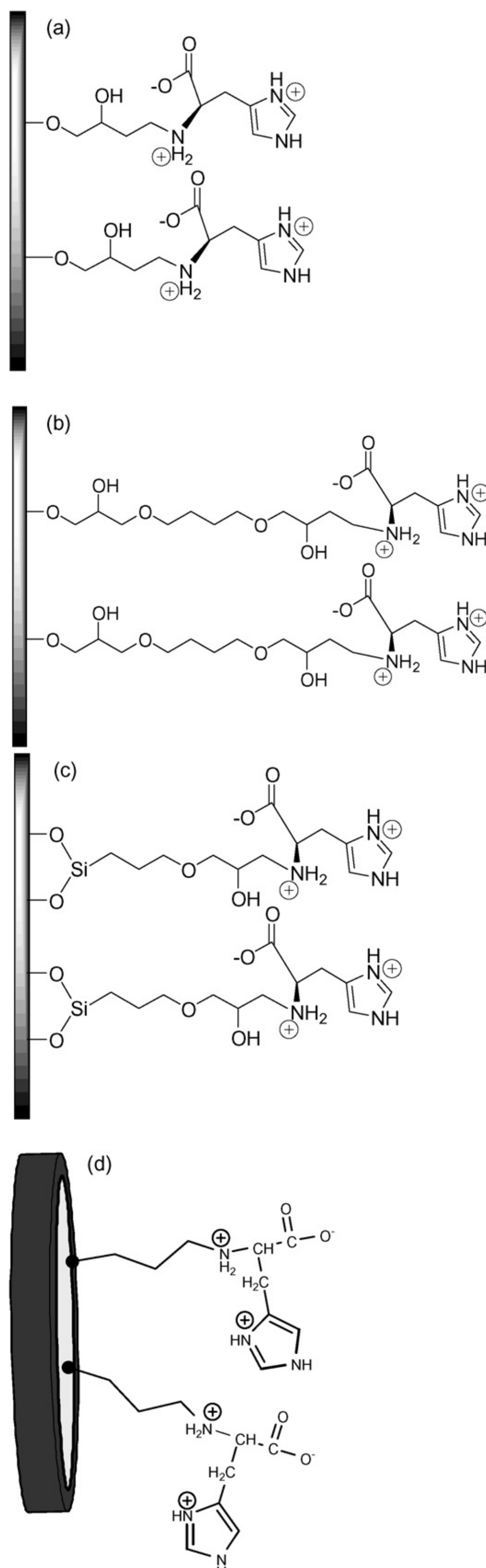

Fig. 1. Schematic structure of different immobilized L-histidine chromatographic matrix. (a) Sepharose 4B-epoxy-histidine, (b) sepharose 4B-bisoxyran-histidine, (c) silica-oxyran-histidine and (d) CIM-disk-EDA-histidine.

2.3. Purification of oligouronides

Purifications were performed at room temperature using low-pressure liquid chromatography. Chromatographic experiments were performed on 3 mL of histidyl-sepharose gel (at room temperature, flow rate: 0.5 mL/min). Equilibration of the column was performed by passing three column volumes of the adsorption buffer before injection of oligogalacturonides (10 mg). Oligogalacturonides were then eluted with increasing ionic strength of buffer (NaCl 0.25, 0.50, 1 M in same buffer). Fractions of 1 mL were collected for colorimetric assay.

CIM-disk-EDA-histidine was performed using a gradient HPLC system equipped with UV detector for analysis of unsaturated oligogalacturonides (ΔOGA) at 230 nm. Fractions of 0.5 mL were collected for colorimetric assay.

2.4. Oligogalacturonide assays

Concentration of oligogalacturonides was determined by colorimetric assay according to the procedure of Van den Hoogen et al. [24] where uronic acids were quantified at 490 nm ($A_{490\,nm}$) after reaction with *m*-hydroxybiphenyl (*m*-HBP). The results were expressed in D-galacturonic acid g/L equivalent.

3. Results and discussion

As we had previously observed on the interaction hypotheses proposed for the fractionation and purification of human IgG and oligoglucuronides by PEVA-histidine chromatography [15–17], the selective purification of oligogalacturonides with L-histidines ligands immobilized onto different chromatographic support was investigated. After cleavage of PGA by thermal and enzymatic processes, oligogalacturonides (OGAs and ΔOGAs) were fractionated by immobilized histidine affinity chromatography (IHAC).

3.1. Selection of specific soft gel matrices for L-histidine immobilization

As it was described in literature [14], many matrices containing hydroxyl function (soft gel or hollow fiber membranes) have been used as specific support matrices for L-histidine immobilization. Silica or agarose beads were usually used as support matrices and these soft gels were mostly used to purify lot of proteins. Moreover, for a given protein or given biomolecules, larges differences in capacity were observed for these different supports [14]. In fact, in histidine ligand affinity chromatography, histidine can be coupled to solid matrices like sepharose, or silica, either through its NH_2 or COOH functional groups with a spacer arm as illustrated in Fig. 1, and the nature of the buffer's ions can influence adsorption capacity of biomolecules adsorbed [14,17]. Then, in this present work we investigated these pseudobiospecific chromatographic supports to study the selectivity of these matrices for the fractionation and purification of oligogalacturonides.

Previously [17], it was shown that oligoglucuronides adsorption onto hollow fiber membranes was efficient at pH near to pK_a of oligoglucuronides. This adsorption was found to strongly depend on the nature of the buffer's ions at low ionic strength where better adsorption was found using acetate buffer (50 mM, pH 4.8) contrary to positive (Tris–succinate) or negative (phosphate) buffer where adsorption capacity was lowest. Consequently, introduction of positive charges on the pseudobiospecific ligand immobilized onto matrix by buffer modulation created a cationic environment that increase stability and biomolecules adsorption showing a predominant role of the carboxylates (COO−) in oligoglucuronides retention. Therefore, interactions were essentially governed by induced ionic force between the biomolecules and ligand residual charge. Others thermodynamics and structural parameters, such as hydrogen bond and/or hydrophobic associations cannot be ignored in retention mechanism [14].

According to these observations, we have selected the same buffer condition for the fractionation and the purification of oligogalacturonides with dp up to 10. In these experimental conditions, a simple one-step procedure utilizing: histidyl-epoxy-sepharose, histidyl-bisoxyran-sepharose and histidyl-oxyran-silica pseudobioaffinity chromatography, allowed the selective separation and purification of different families of oligogalacturonides.

Samples of oligomers mixture: OGA and ΔOGAs, respectively, were loaded on histidyl soft gels column to purify oligogalacturonides. As shown in Figs. 2 and 3, all these pseudobioaffinity matrices constitute good tools for the selective fractionation of ΔOGAs and OGAs. As observed for each oligogalacturonides family (OGAs and ΔOGAs) the elution profiles were globally similar with all the gels for the same elution gradient. Then for the ΔOGAs family (Fig. 2), four distinct retaining oligosaccharidic fractions were observed in the same elution buffer condition. Concerning OGAs fractions obtained according to the experimental procedure developed by Simms et al. [22], the elution profiles (Fig. 3) revealed the presence of oligogalacturonides with degree of polymerization up to 7 in accordance with data of Simms et al. [22]. For each chromatography analysis, galacturonic acid content of purified oligogalacturonides was determined by colorimetric assay. Table 1 resumes the final amounts of oligomers retained for 1 mL of histidyl-sepharose/silica soft gel.

As expected and by comparison with the reported data with protein retentions [14,25] the present observation indicated a high importance of the arm spacer. It was interesting to note that the nature of the spacer seems influence the adsorption capacity of oligogalacturonides on L-histidine immobilized. In fact, many authors have already suggested that the introduction of spacer arm should increase the specific interaction between biospecific ligand and bio molecule to be separated. This hypothesis was effectively confirmed by Ezzedine et al. [25] with the purification of IgG_1 and Factor VIIIc using histidyl-sepharose 4B pseudobioaffinity gel. Increasing in ligand density (due to the presence of spacer arm) was then correlated with an increased capacity for the specific proteins. According to structure presented in Fig. 1, the length of spacer arms: short (epichlorhydrin spacer arm: three carbons) and long (1,4-butanediol-diglycidyl-ether: bisoxyrane spacer arm: seven carbons) influence the retention

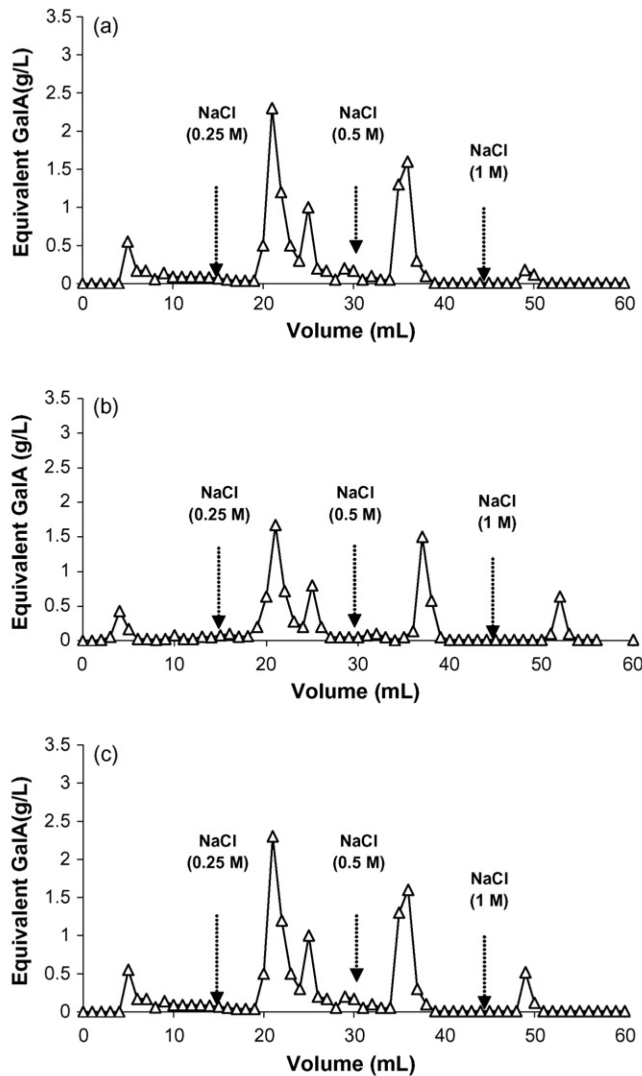

Fig. 2. Purification of ΔOGA onto 2 different chromatographic gels. (a) Sepharose-4B-epoxy-histidine, (b) sepharose-4B-bisoxyran-histidine and (c) silica-oxyran-histidine. Columns were equilibrated with acetate buffers (25 mM, pH 5) at the flow rate of 0.5 mL/min. Anionic oligosaccharides (10 mg) were injected. Oligomers were eluted with increasing the ionic strength of buffer (NaCl 0.25, 0.50, 1 M in starting buffer). Fractions of 0.5 mL collected were assayed by colorimetric method for their sugar content.

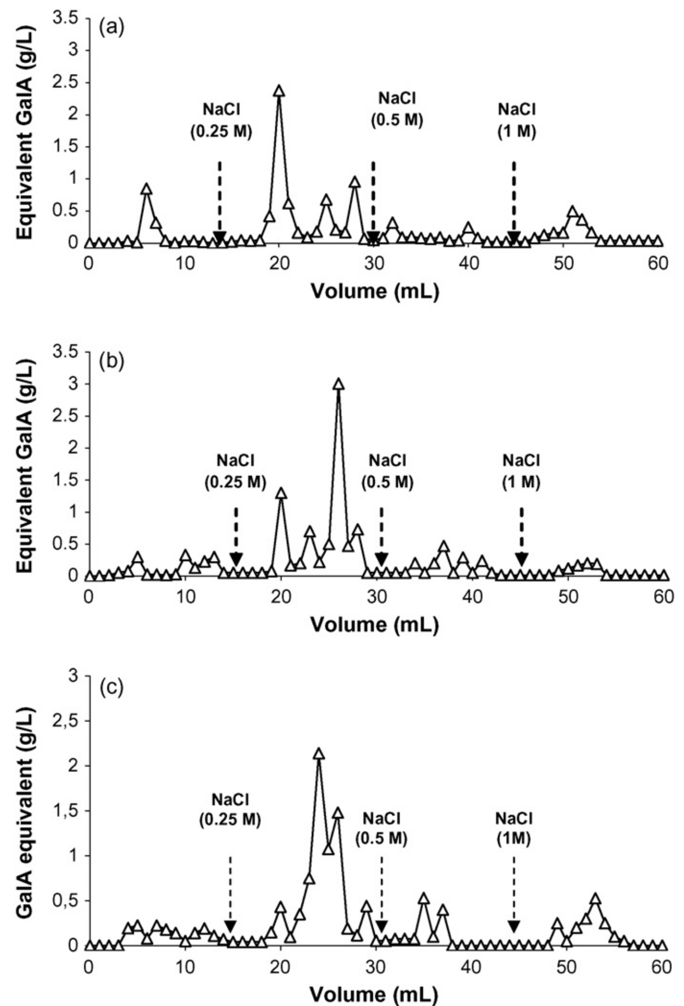

Fig. 3. Purification of OGAs onto 3 different chromatographic gels. (a) Sepharose-4B-epoxy-histidine, (b) sepharose-4B-bisoxyran-histidine and (c) silica-oxyran-histidine. Columns were equilibrated with corresponding buffers (25 mM, pH 5) at the flow rate of 0.5 mL/min. Anionic oligosaccharides (10 mg) were injected. Oligomers were eluted with increasing the ionic strength of buffer (NaCl 0.25, 0.50, 1 M in starting buffer). Fractions of 0.5 mL collected were assayed by colorimetric method for their sugar content.

of biomolecules. Then, increase the distance ligand from the matrix (sepharose or silica) seems to be of great importance in the purification of oligogalacturonides. As indicated in Table 1, L-histidine-bisoxyran-sepharose presented a higher oligogalacturonides binding capacity (∼3 mg/mL of gel) than L-histidine-epichlorhydrin-sepharose (∼2 mg/mL of gel), despite its lower ligand density. Then, in case of L-histidine-bisoxyran-sepharose 4B, the adsorption capacity of oligosaccharides was 90% whereas in the case of L-histidine-epoxy-sepharose 4B the adsorption capacity was significantly lower (60%). On the other hand, with silica-oxyran histidine the adsorption capacity (75%) was near to adsorption capacity found with bisoxyran as arm spacer. As can been observed, maximal specific anionic oligosaccharide retention was obtained for a long

Table 1
Maximum adsorption capacity of oligogalacturonides separated and purified by histidine ligand affinity chromatography (HLAC)

	Maximum adsorption capacity on different matrices			
	Silica histidyl (mg/mL gel)	Histidyl bisoxyran (mg/mL gel)	Histidyl epoxy (mg/mL gel)	Histidyl CIM-EDA disk (mg/0.34 mL)
OGA	2.50	3.30	2.00	0.70
ΔOGA	2.30	3.00	2.10	0.85

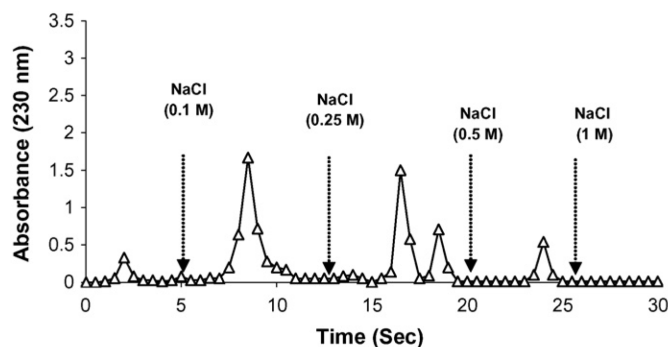

Fig. 4. Adsorption of ΔOGA onto CIM-disk-EDA-histidine. Columns were equilibrated with corresponding buffers (25 mM, pH 5) at the flow rate of 5 mL/min. Anionic oligosaccharides (1 mg) were injected. Oligomers were eluted with increasing the ionic strength of buffer (NaCl 0.1, 0.25, 0.5 and 1 M in starting buffer) and measured by UV detector at 230 nm.

arm spacer, such as 1,4 butanediol-diglycidyl-ether (bisoxyran), which allows highest ligand density and then better anionic oligosaccharide retention.

3.2. *CIM-disk-EDA-histidine*

At the light of the first results obtained with soft gel matrices, we investigated the development of a new histidine immobilized matrices using monolith technology. This last decade a lot of work described the use of monolith matrix in biotechnology for very fast separation and efficient purification of biomolecules. In this context, short monolithic CIM disc columns were performed for purification of proteins in different modes, such as hydrophobic interaction, ion exchange, or affinity mode [18–21,26].

Due to the advantages of monolith matrix, we investigated the purification of anionic oligosaccharides using L-histidine immobilized on the monolithic support CIM-EDA disk. Thus, we have tested affinity of histidyl-ethylenediamine for the purification of unsaturated oligogalacturonides (ΔOGA). In this experiment, all elution was carried out by a discontinuous gradient of 0.1; 0.25; 0.5 and 1 M NaCl. By comparison with the experiments done with soft gel, the separation quality of oligogalacturonides from PGA depolymerization extract did not change with the speed of the separation. The working flow rate of the monolith column was maintained at 5 mL/min and the total experiment starting from the injection of the sample to regeneration with NaCl took less than 5 min comparatively to experiment done with histidyl soft gel which took 30 min.

In fact, from the data shown in Fig. 4, we can observe four peaks in CIM-disk EDA-histidine chromatogram. Thus, this CIM-disk system makes possible to obtain the same result as the soft gel chromatography but with an important gain of time since after 30 s, ΔOGA were separated and purified (Fig. 4).

The efficiency of monolith system comparatively to other histidyl-immobilized matrix (Table 1) allows to propose this new chromatographic tool for specific purification of anionic oligosaccharides in fast scale up since the retention capacity of histidyl CIM-EDA disk was 0.85 mg of oligogalacturonides for a column volume of 0.34 mL.

According to previous studies on oligouronides purification using pseudobioaffinity chromatography histidine [17], monolithic CIM-disk immobilized histidine affinity chromatography (IHAC) systems offer advantage of fast separation and purification of oligogalacturonides.

4. Conclusion

This pseudobioaffinity chromatographic support has many advantages in terms of speed and handling and proved to be very efficient for the separation and purification of oligogalacturonides. Consequently, this study with CIM-disk immobilized histidine affinity chromatography opens the way to the development of promising chromatographic support for large-scale purification of oligouronides allowing industrial applications.

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