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Selective affinity of L-histidine immobilized onto poly(ethylene–vinyl alcohol) hollow-fiber membranes for various oligoglucuronans Influence of the degree of polymerization and the degree of substitution by acetate

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Abstract

A new species of oligoglucuronan was previously extracted from a polysaccharide crude fraction produced by a *Sinorhizobium meliloti* mutant strain, by chromatography on L-histidine immobilized onto poly(ethylene–vinyl alcohol) (PEVA) hollow-fiber membranes. Until now, only one specific species of oligoglucuronan was selectively adsorbed on the membranes with immobilized histidine. In this work, the specific membranes, commonly used to purify proteins, were tested on different non-ionic and anionic carbohydrate components. We determined only anionic oligosaccharides are selectively retained on these membranes. The affinity between the L-His–PEVA membranes and oligoglucuronans varying in the degree of polymerization and the degree of substitution by acetyl residues was studied.

Keywords: Membranes; Hollow-fiber membranes; *Sinorhizobium meliloti*; Immobilized histidine; Poly(ethylene–vinyl alcohol) membranes; Oligoglucuronans; Carbohydrates

1. Introduction

The mutant strain *Sinorhizobium meliloti* M5N1CS (NCIMB 40472) produces, during fermentation, high-molecular-mass exopolysaccharides (HMW EPSs) (M_r>100 000) and medium-molecular-mass (MMW) EPSs (20 000<M_r<100 000); the HMW and MMW EPS fractions contain only homopolymers of glucuronic acid residues partially acetylated on the C2 and/or C3 position [1,2]. During fermentation, low-molecular-mass (LMW) EPSs (M_r<20 000) are also synthesized; this fraction contain three different oligoglucuronan species which differ on the degree of substitution (DS) by acetate and on the degree of polymerization (DP) [3], and cyclic β-(1→2)-glucans unsubstituted or substituted with *sn*-glycerol-1-phosphate residues (unpublished results) varying on the DS and on the DP.

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The LMW glucuronan fraction is mainly composed of molecules containing an unsaturated residue at the non-reducing end formed by degradation (β elimination) of the glucuronan with a glucuronan lyase [4,5]. Molecules produced de novo, containing a glucuronic residue at the non-reducing end were detected [3].

In a previous work we have shown [6] that L-histidine immobilized onto poly(ethylene–vinyl alcohol) (L-His–PEVA) hollow-fiber membranes can be used at pH 4.8 for the separation of a species of LMW acetylated glucuronans from a crude LMW EPS fraction produced by *S. meliloti* M5N1CS. The pH conditions were retained due to the pK_a of polyglucuronic acid solutions: $\approx 4 < pK_a < \approx 5$ [7]. The glucuronan species retained on the L-His–PEVA membranes was identified as a LMW glucuronan (DP>16) without unsaturated residue at the non-reducing end, weakly acetylated especially at the C3 position.

In order to determine either the L-His–PEVA membranes are able to retain only one species of anionic component with a specific acetylation degree or they present a degree of affinity variable for various saccharidic components, non-ionic and ionic polymers were tested on the specific membranes as well as oligoglucuronans with different DP and acetylation degrees.

2. Experimental

2.1. Monomers and dextrans tested

Glucuronic acid and glucose were from Sigma. Dextran standard polymers (M_r 12 000 and 25 000) were from Fluka.

2.2. Preparation of glucuronan and succinoglycan

The *S. meliloti* M5N1CS strain (NCIMB 40472) (1) was cultivated 91 h at 30°C in a 2-l batch fermentor containing RC medium [8] supplemented with sucrose (RCS) [3].

The *S. meliloti* M5N1 wild type strain producing a succinoglycan [9] was grown in 2-l Erlenmeyer flasks containing 1 l of RC medium supplemented with fructose (1% w/v) (RCF). The flasks were incubated at 30°C for 240 h on a rotary shaker (100 rpm); the inoculum was 100 ml of a *S. meliloti* M5N1 culture in RCF medium first incubated 20 h on a rotary shaker (100 rpm) at 30°C.

After incubation, the culture media were centrifuged, and the supernatants containing the EPSs were fractionated into HMW and MMW EPSs (M_r> 20 000) and LMW EPSs (20 000>M_r>5000) by ultrafiltration as described previously [1,3].

2.3. Chromatography on DEAE-Sepharose column

The polysaccharide samples in AcOH–$AcONH_4$ ($5 \cdot 10^{-2}$ *M*, pH 4.8) were applied on a DEAE-Sepharose CL6B column (10.5×1 cm) equilibrated with the same buffer (0.5 ml/min). First, the column was rinsed with AcOH–$AcONH_4$ buffer ($5 \cdot 10^{-2}$ *M*, pH 4.8), then, the retained polysaccharides were eluted with a linear gradient of AcOH–$AcONH_4$ buffer from 0.5 to 1 *M*, finally, the elution was completed with a 2 *M* buffer. The column was connected to an ultraviolet detector (240 nm) and an evaporative light scattering detection (ELSD) system (from Alltech). The light scattering detector was used to detect all the saccharidic components while the UV detector was used only to detect oligoglucuronans [3]. For further experiments, the fractions were collected, desalted on a Sephadex G-10 column and dried by lyophilization [3].

2.4. Preparation of pure acetylated and deacetylated LMW glucuronans

Acetylated glucuronans were obtained from LMW glucuronan fractions collected after chromatography on a DEAE-Sepharose column of a crude LMW EPS fraction [3]. Deacetylated glucuronans were obtained from the previous LMW glucuronan fractions incubated for 5 h 30 min at 30°C at pH 10 (with NaOH), desalted and dried as described previously [3].

The separation of the different acetylated and deacetylated glucuronans according to their degree of polymerization was performed on a Bio-Gel P6 column (100×2.5 cm) [10].

2.5. Chromatography on L-His–PEVA hollow-fiber membranes

Membranes made of L-His–PEVA hollow-fibers were prepared as described [11]. Two modules were used, a small surface His–PEVA membrane (70 cm^2 total surface) and a larger (1000 cm^2).

The small surface L-His–PEVA module was equilibrated with AcOH–$AcONH_4$ ($5 \cdot 10^{-2}$ *M*, pH 4.8) buffer, the flow-rate was 0.4 ml/min in tangential mode. Different samples (2 mg) were dissolved in the same AcOH–$AcONH_4$ buffer (3 ml) and applied on the chromatographic membranes on closed circuit during 1 h [6]. Then, the module was connected to a peristaltic pump in line with an ELSD system (from Alltech); the connection to the peristaltic pump allowed a flow-rate stabilized at 0.4 ml/min.

Elution was performed first with 24 ml of the previous buffer and followed by a linear gradient of AcOH–$AcONH_4$ from $5 \cdot 10^{-2}$ *M* to 1 *M* (28 ml), finally, the elution was completed with 8 ml of the 2 *M* AcOH–$AcONH_4$ buffer.

The large surface module was used for scaled up operation. The tested sample was a LMW EPS fraction (M_r<20 000) obtained from 1 l of *S. meliloti* culture medium. The pH in the permeate obtained by ultrafiltration on a M_r 20 000 cut-off membrane of the bacterial free culture medium was stabilized at 4.8 with H_3PO_4 (1 *M*). Samples were applied to the column on a closed circuit mode during 3 h at a flow-rate of 5 ml/min. The module was then rinsed with 1.5 l of AcOH–$AcONH_4$ ($5 \cdot 10^{-2}$ *M*, pH 4.8) buffer and the elution was performed by 700 ml of 1 *M* AcOH–$AcONH_4$ buffer [1]. The LMW EPS fractions retained on the column and the non-retained fraction were collected, purified by ultrafiltration on a M_r 5000 cut-off membrane. The LMW EPS fractions (5000<M_r<20 000) were dried by lyophilization.

3. Results and discussion

In a previous work, we described how the L-His–PEVA hollow-fiber membranes can be considered as a new chromatographic method useful for the purification of specific LMW glucuronans. LMW glucuronans with a DP>16 without unsaturated residue at the non-reducing end and weakly acetylated especially at the C3 position were separated from a LMW EPS crude fraction (20 000> M_r>5000) containing substituted and unsubstituted cyclic glucans, and different LMW glucuronan species varying on the DP and the DS by acetate.

In order to determine if only a specific glucuronan species is selectively adsorbed on the membranes, or different carbohydrate components can be retained, different saccharidic components were chromatographed on the L-His–PEVA module.

3.1. Chromatography of non-ionic saccharidic components on the L-His–PEVA module

Two dextrans (M_r: 12 000 and 25 000) dissolved (2 mg) in $5 \cdot 10^{-2}$ *M* AcOH–$AcONH_4$ (pH 4.8) buffer (3 ml) were chromatographed on closed circuit during 1 h on the L-His–PEVA prepared cartridge (70 cm^2 total surface). The module was rinsed with the same buffer (24 ml), then the elution was performed with a linear gradient of AcOH–$AcONH_4$ buffer from $5 \cdot 10^{-2}$ *M* to 1 *M* (28 ml) and finally the elution was completed with 2 *M* buffer (8 ml). The saccharidic content in the eluted fractions was analyzed by ELSD connected to the cartridge. No dextran was retained on the L-His–PEVA module (data not shown). This result is similar to that obtained with cyclic β-(1→2)-glucans solutions chromatographed on the L-His–PEVA membranes [6]. Glucose solutions (2 mg) in $5 \cdot 10^{-2}$ *M* AcOH–$AcONH_4$ (pH 4.8) buffer (3 ml), chromatographed in the same conditions did not show any affinity to the specific membranes (data not shown).

These results indicate that glucose and linear or cyclic glucans show no affinity for the L-His–PEVA membranes. Probably, the retention of the specific glucuronan species on the same membranes obtained previously was not due to the hydroxyl functions present on the glucuronan.

3.2. Chromatography of non-ionic saccharidic components substituted

In order to determine if the presence of anionic substituent on a non-ionic saccharidic polymer in-

fluence the affinity between the polymer and the specific membranes, a succinoglycan [12] sample was tested. The tested sample was a solution of a crude LMW succinoglycan fraction (M_r<20 000), obtained from 1 l of a *S. meliloti* M5N1 medium cultivated during 240 h. The LMW succinoglycan fraction adjusted to pH 4.8 with 1 *M* PO_4H_3 was applied to the L-His–PEVA module (1000 cm^2 total surface) on a closed circuit mode during 3 h in the same conditions as previously [6]. We determined (data not shown) that in the tested conditions the succinoglycan presented no affinity for the membranes. This result is similar to that obtain with solutions of cyclic β-(1→2)-glucans substituted with *sn*-glycerol-1-phosphate residues [6] chromatographed on the L-His–PEVA membranes. We concluded that the presence of anionic substituent as the *sn*-glycerol-1-phosphate or the pyruvyl and succinyl residues on the cyclic glucan or the succinoglycan, respectively are not sufficient to induce the retention of the substituted polysaccharides on the L-His–PEVA chromatographic membranes. The retention of the specific glucuronan species on the membranes obtained previously may be attributed to the anionic character of the molecule and to the presence of acetyl residues at specific positions.

3.3. Chromatography of unsubstituted oligoglucuronans (oligoglucuronates)

3.3.1. Chromatography of oligoglucuronate fractions

In order to determine the influence of the carboxyl and the acetyl functions of oligoglucuronans on the affinity between the specific glucuronan species and the L-His–PEVA membranes, pure deacetylated oligoglucuronans of DP 3 and 4 were tested on the L-His–PEVA membranes as previously described. The analyses of the eluted fractions by a light scattering detector revealed that oligoglucuronates were retained on the specific membranes (Fig. 1a). The same experiment realized with a pure glucuronic acid (2 mg) solution in $5\cdot10^{-2}$ *M* AcOH–$AcONH_4$ (pH 4.8) buffer (3 ml) indicated this component was not retained on the membranes (data not shown). These results indicate that in the conditions applied, pure oligoglucuronates present an affinity with the L-His–PEVA membranes, but only one acidic func-

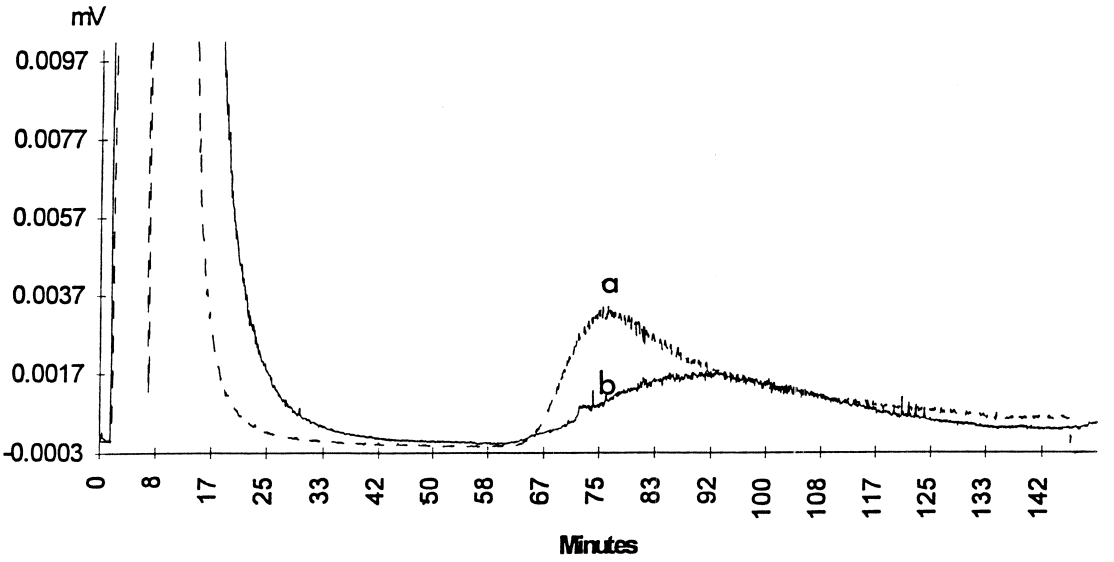

Fig. 1. Elution profiles of deacetylated oligoglucuronans (2 mg) in $5\cdot10^{-2}$ *M* AcOH–$AcONH_4$ (3 ml) chromatographed on L-His–PEVA membranes (70 cm^2). Samples were applied 1 h on L-His–PEVA membranes, the flow-rate was 0.4 ml/min. The eluent consisted of first $5\cdot10^{-2}$ *M* AcOH–$AcONH_4$ then a linear gradient of the same buffer from $5\cdot10^{-2}$ *M* to 1 *M*, finally, the elution was completed with a 2 *M* buffer. Detection by ELSD. (a) Deacetylated oligoglucuronans DP 3 and DP 4. (b) Complete LMW glucuronan fraction deacetylated by NaOH treatment.

tion is not sufficient to establish a stable interaction between the monomer and the membranes. Probably, in the conditions applied, the presence of several carboxylic functions is necessary for the interaction between the chromatographic membranes and the biomolecule, but the presence of acetyl residues may influence the affinity between the two elements.

3.3.2. Determination of the selectivity of adsorption of glucuronates

The LMW glucuronan fractions purified on a DEAE-Sepharose column [3] were gathered deacetylated and desalted before injection on the L-His–PEVA membranes. The DP of the molecules was estimated between 1 and about 20. In order to determine the selectivity of adsorption on the specific membranes of oligoglucuronates with different DPs, the non-retained fraction after chromatography on the module and the retained fraction (Fig. 1b) were collected, desalted and chromatographed on a DEAE-Sepharose column (Fig. 2). We determined the ratio of the different species of oligoglucuronans in the two fractions was different. The oligoglucuronate with a DP 4 was mainly retained. We concluded that several carboxylic functions are necessary to allow the association between the L-His–PEVA membranes and the saccharidic component, but this condition is not sufficient because a deacetylated glucuronan with a DP 4 is more retained than higher DP molecules, while the LMW glucuronan with a DP>16 lowly acetylated especially at the C3 position was separated in the same conditions as above from a LMW EPS crude fraction containing all the species of acetylated oligoglucuronan. Probably, the presence of acetate residues at specific position on the polyanionic molecule confer a specific conformational structure which will increase the affinity between the chromatographic support and the oligoglucuronan.

3.4. Chromatography of substituted oligoglucuronans

In order to determine the effect of acetyl substituents on the affinity of oligoglucuronans to the L-His–PEVA membranes, a mixture of pure acetylated oligoglucuronans DP 3 and DP 5 with DS values corresponding to 22% and 12.5%, respective-

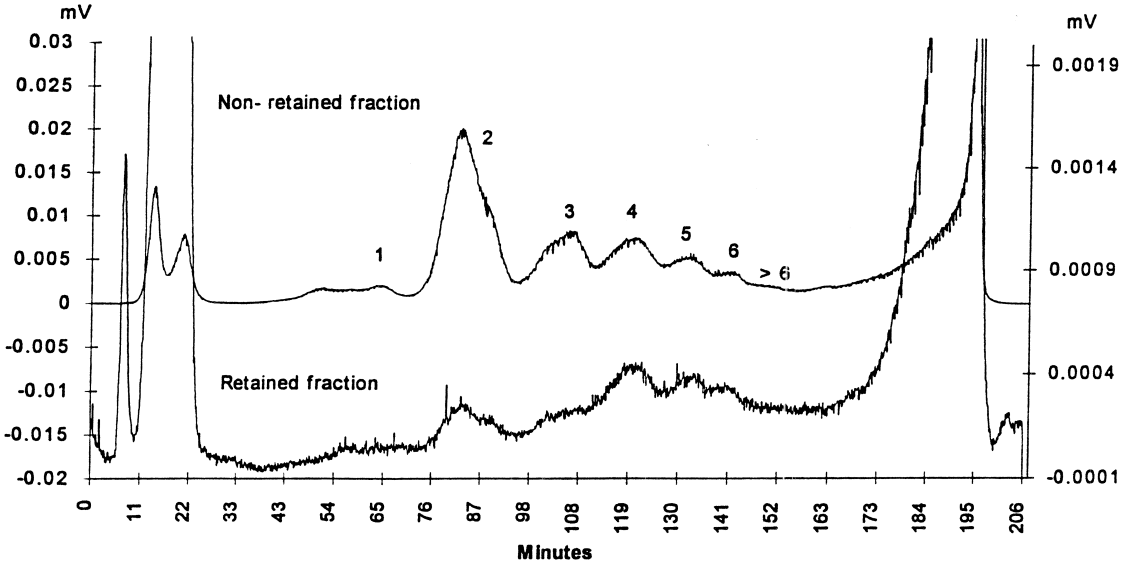

Fig. 2. Elution profile on a DEAE-Sepharose column (10.5×1 cm) of the complete deacetylated LMW glucuronan fractions retained and non-retained after chromatography on the L-His–PEVA membranes (70 cm^2). The flow-rate on the DEAE column was 0.5 ml/min. The eluent was $5 \cdot 10^{-2}$ *M* AcOH–$AcONH_4$ then a linear gradient of the same buffer from 5.10^{-2} *M* to 1 *M*, at last, the elution was completed with 2 *M* AcOH–$AcONH_4$ buffer. Detection by ELSD. The degree of polymerization of glucuronates is indicated by numbers under the peaks

ly, were injected on the chromatographic membranes. After chromatography on a DEAE-Sepharose column of the non-retained and retained fractions as previously, we observed the weakly acetylated oligoglucuronan was mainly retained on the L-His–PEVA membranes, however, the DP 3 deacetylated was less retained than the same glucuronic molecule acetylated (Fig. 3). We confirmed a high level of substitution by acetate reduces the affinity between the oligoglucuronan and the L-His–PEVA membranes, while a low degree of substitution increases the affinity between the chromatographic support and the pure glucuronan sample; probably the presence of numerous acetyl residues reduces the interactions between the glucuronan and the chromatographic membranes, but their presence probably at specific positions confers a conformational structure of the glucuronan allowing higher interactions.

In order to determine if the LMW glucuronan (DP>16) lowly acetylated at the C3 position is the sole acetylated oligoglucuronan in a LMW EPS crude fraction able to be retained by the specific chromatographic membranes at pH 4.8 [6], a LMW EPS crude fraction not retained after chromatography on the L-His–PEVA membranes [6] was chromatographed once more on closed circuit during 1 h on the small area (70 cm^2) L-His–PEVA membranes. The elution profile (Fig. 4) indicates that acetylated glucuronans presenting a specific affinity with the L-His–PEVA membranes were still present in the sample previously chromatographed on the same membranes. The retained molecules after a second chromatography on the L-His–PEVA membranes may correspond to the same glucuronan species (LMW glucuronan DP>16, lowly acetylated at the C3 position) remaining in the chromatographed sample or correspond to other species. In order to identify the molecules retained on the membranes, both the retained and the non-retained fractions were collected, desalted, dried and dissolved in 500 μl of the $5 \cdot 10^{-2}$ *M* AcOH–$AcONH_4$ buffer and then applied to a DEAE-Sepharose column. The elution profile of the previous fraction retained on the L-His–PEVA membranes compared to that of the non-retained sample (Fig. 5) indicates oligoglucuronans belonging to the three glucuronan families presenting different acetylation degree average (A=38%; B=24%; C=17%) [3] are retained on the chromato-

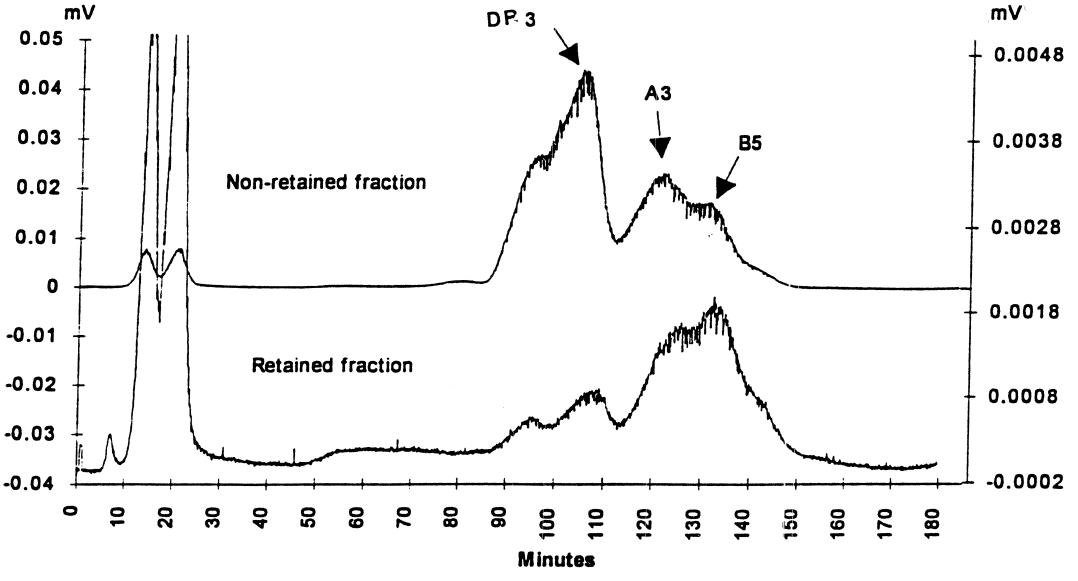

Fig. 3. Elution profile on a DEAE-Sepharose column (10.5×1 cm) of the non-retained and retained fractions after chromatography on L-His–PEVA membranes (70 cm^2) of a mixture of oligoglucuronans. DP3: Deacetylated oligoglucuronan with a DP 3; A3 and B5: acetylated oligoglucuronans with a DP 3 and a DS=22%, and a DP 5 and a DS=12.5%, respectively, obtained as previously described [3]. The flow-rate on the DEAE column was 0.5 ml/min. The eluent was $5 \cdot 10^{-2}$ *M* AcOH–$AcONH_4$ then a linear gradient to 1 *M*, finally, the elution was completed with 2 *M* AcOH–$AcONH_4$ buffer. Detection by ELSD.

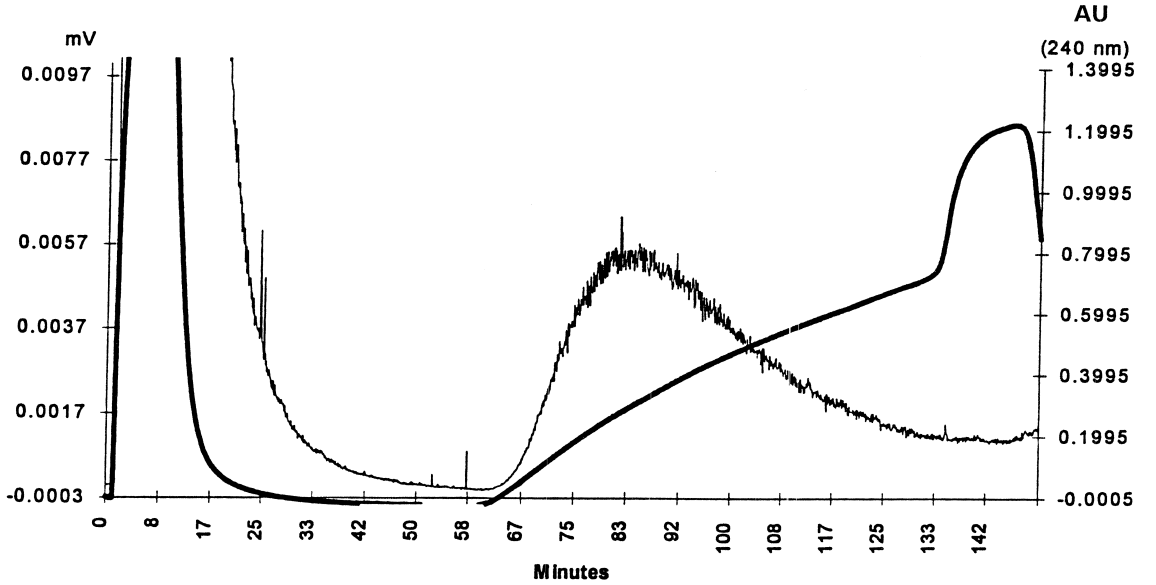

Fig. 4. Elution profile on L-His–PEVA membranes (70 cm^2) of the non-retained LMW EPS crude fraction from the *S. meliloti* M5N1CS strain, first chromatographed on a L-His–PEVA module. Samples (3 mg) in $5 \cdot 10^{-2}$ *M* $AcOH–AcONH_4$ (3 ml) were applied 1 h on L-His–PEVA membranes, the flow-rate was 0.4 ml/min. The eluent consisted of first $5 \cdot 10^{-2}$ *M* $AcOH–AcONH_4$ (19 ml) then a linear gradient to 1 *M* (11 ml), finally, the elution was completed with 2 *M* $AcOH–AcONH_4$ buffer. Detection by ELSD (—) and UV detection at 240 nm (-).

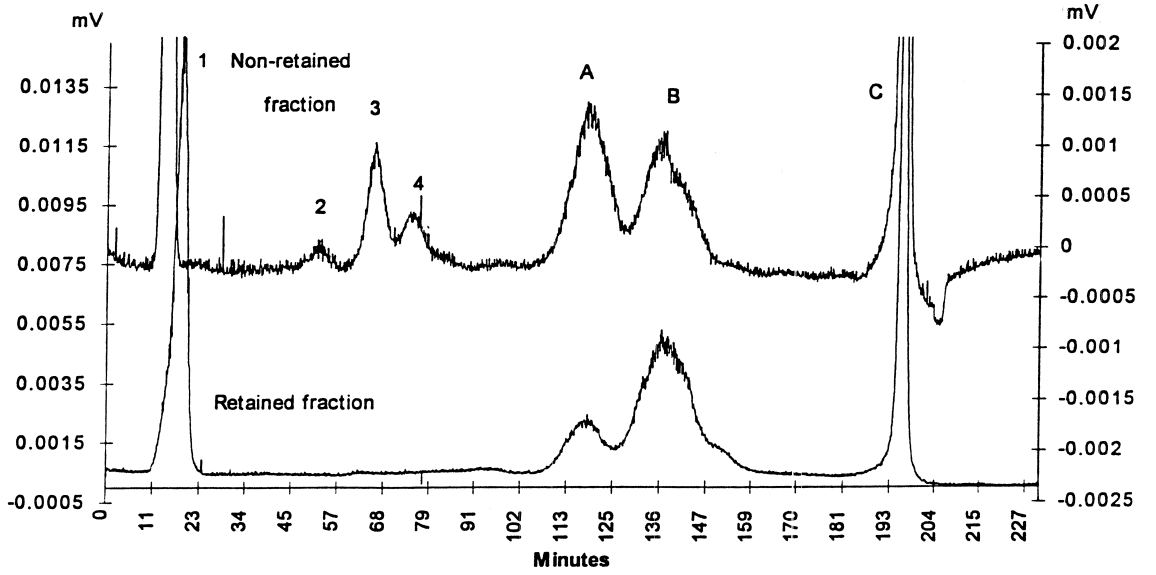

Fig. 5. Chromatography on a DEAE-Sepharose column (10.5×1 cm) of the retained and non-retained fractions after chromatography on L-His–PEVA membranes (70 cm^2) of a LMW EPS crude fraction remaining after a first chromatography on the same L-His–PEVA membranes. The flow-rate on the DEAE column was 0.5 ml/min. The eluent was $5 \cdot 10^{-2}$ *M* $AcOH–AcONH_4$ then a linear gradient to 1 *M*, finally, the elution was completed with 2 *M* $AcOH–AcONH_4$ buffer. Detection by ELSD. A, B, C: Oligoglucuronan families with DS of 38%, 24% and 17%, respectively; 1, 2, 3, 4: cyclic β-(1→2)-glucan families with differing degrees of substitution.

graphic membranes after extraction of the LMW oligoglucuronan lowly acetylated at the C3 position. This result indicates the molecules retained on the specific membranes after a first cycle of chomatography of a crude LMW EPS fraction [6] are different of that retained after a second cycle of chromatography. The ratio of each acetylated glucuronan families retained on the membranes varied, the more retained corresponding to the less acetylated. However, pure glucuronans belonging to the A and to the B family in $5 \cdot 10^{-2}$ *M* AcOH–$AcONH_4$ buffer, injected separately on the L-His–PEVA membranes are retained similarly on the membranes (data not shown); this results confirm that in the conditions applied, the L-His–PEVA membranes present a higher affinity for oligoglucuronans lowly acetylated, but probably the position of the acetate may influence the association between the chromatographic support and the glucuronan.

4. Conclusions

L-His–PEVA hollow-fiber membranes were previously characterized as a new chromatographic method useful to separate LMW glucuronans lowly acetylated especially at the C3 position from a LMW EPS crude fraction. In this work, we determined various glucuronans presenting different degree of polymerization and degree of substitution by acetyl residues may be obtained successively from a crude LMW EPS fraction by chromatography on the L-His–PEVA hollow-fiber membranes. The retention of the biomolecules on the chromatographic support is dependent of the carboxyl group, however, acetyl residues at the C2 or the C3 position influence the glucuronan interactions with the membranes. We determined, the number of acetyl residues influences the membrane affinity for the glucuronan, molecules lowly acetylated present a higher affinity for the L-His–PEVA hollow-fiber membranes than the higher acetylated; this result is similar to those obtained previously for the separation of oligoglucuronans by anion-exchange chromatography [3]. Probably, the tri-dimensional conformation of the oligoglucuronans related to the substitution by acetate influences the retention on the chromatographic support.

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