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Specificities of *Ricinus communis* agglutinin 120 interaction with sulfated galactose

Yufeng Wang [a], Guangli Yu [a,*], Zhangrun Han [a], Bo Yang [a], Yannan Hu [a], Xia Zhao [a], Jiandong Wu [a], Youjing Lv [a], Wengang Chai [a,b,*]

[a] Shandong Provincial Key Laboratory of Glycoscience and Glycoengineering, and Key Laboratory of Marine Drugs, Ministry of Education, Ocean University of China, Qingdao 266003, China
[b] Glycosciences Laboratory, Department of Medicine, Imperial College London, Northwick Park & St Mark's Campus, Harrow, Middlesex HA1 3UJ, United Kingdom

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ABSTRACT

Lectins are used extensively as research tools to detect and target specific oligosaccharide sequences. *Ricinus communis* agglutinin I (RCA_{120}) recognizes non-reducing terminal β-D-galactose (Galβ) and its specificities of interactions with neutral and sialylated oligosaccharides have been well documented. Here we use carbohydrate arrays of sulfated Galβ-containing oligosaccharide probes, prepared from marine-derived galactans, to investigate their interactions with RCA_{120}. Our results showed that RCA_{120} binding to Galβ1–4 was enhanced by 2-*O*- or 6-*O*-sulfation but abolished by 4-*O*-sulfation. The results were corroborated with competition experiments. *Erythrina cristagalli* lectin is also a Galβ-binding protein but it cannot accommodate any sulfation on Galβ.

1. Introduction

Carbohydrate chains of glycoproteins, glycolipids, proteoglycans, and polysaccharides mediate many biological processes through their interactions with functional proteins. Unraveling these interactions is important in biomedical sciences [1]. Lectins (the carbohydrate-binding proteins) are a diverse family of proteins that recognize oligosaccharides. They are present in almost all living organisms, from viruses, plants, to mammals, and exhibit a variety of functions. Plant lectins of different specificities are particularly useful tools and have been used extensively to detect and target specific oligosaccharide sequences in cell typing, tissue staining, characterization of carbohydrates, and enrichment and purification of glycoproteins. *Ricinus communis* agglutinin I (RCA_{120}) and *Erythrina cristagalli* lectin (ECL) recognize carbohydrate chains with non-reducing terminal β-D-galactose (Galβ) and both lectins prefer Galβ1-4GlcNAc to Galβ1-3GlcNAc sequence [2,3]. Increased affinity for highly branched Galβ1-4GlcNAc containing *N*-glycans has been observed [4,5], but repetition of the Galβ1-4GlcNAc unit has no apparent effect on its binding ability to RCA_{120} [2,4] and ECL [2].

RCA_{120} is purified from seeds of castor plant and is a tetrameric hemagglutinin with two α (29.5 kD) and two β (37 kD) subunits. Only the β subunits of RCA_{120} display binding activity to Galβ1-terminating oligosaccharides [6]. RCA_{120} has been one of the most widely used lectins [7,8] and has been applied to study of cell surface glycoproteins [9,10] and used in affinity chromatography for separation of glycoproteins with Galβ-terminating oligosaccharide chains from glycolipids [11]. RCA_{120} has a preference for a Galβ1–4 rather than Galβ1–3 or Galβ1–6 terminal sequences. However, there seems no specific requirement for the sub-terminal residue, e.g. Galβ1-4GlcNAc, Galβ1-4Glc and Galβ1-4Man have shown similar activities [4]. Modification of the terminal Galβ has significant effect on its binding ability to RCA_{120}. For example, when the 2-OH of Galβ was replaced by 2-NAc, as in the case of GalNAcβ, the activity was reduced [4]. Similarly, binding was reduced or abolished by modification of the terminal Galβ by an acidic sugar sialic acid (NeuAc) at either the 3-*O*- or 6-*O*-positions [2,4]. Although ECL, purified from the seed of *E. cristagalli*, is also a Galβ1-binding lectin [12], some differences in its binding specificity have been observed [2]. For example, addition of an α-L-fucose (Fuc) to the 2-*O*-position of the terminal Galβ can enhance its binding ability to ECL [5]. However,

Abbreviations: RCA_{120}, *Ricinus communis* agglutinin; ECL, *Erythrina cristagalli* lectin; DHPE, 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine; NGL, neoglycolipid; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Fuc, fucose; NeuAc, *N*-acetylneuaminic acid; anGal, 3,6-anhydro-α-galactopyranose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; G4, galactotetraose; LSTc, LS-tetrasaccharide C; A4, neoagar-tetrasaccharide; dsK, desulfatedκ∂-carrageenan; dsL, desulfated λ-carrageenan; HBS, HEPES buffered solution; HBST, HBS containing 0.05% Tween 20

* Corresponding authors. Address: Glycosciences Laboratory, Department of Medicine, Imperial College London, Northwick Park & St Mark's Campus, Harrow, Middlesex HA1 3UJ, United Kingdom. Fax: +44 20 8869 3455 (W. Chai).

E-mail addresses: glyu@ouc.edu.cn (G. Yu), w.chai@imperial.ac.uk (W. Chai).

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ECL binding to Galβ1-4GlcNAc is also abolished by sialyl substitution of the 3-OH or 6-OH of the terminal Galβ [2,5].

For both lectins, substitution of Galβ by sialic acid abolishes the binding ability, but there has been very limited information on the effect of sulfate substitutions. It has been recently reported, using two commercial disaccharides based on the Galβ1-4GlcNAc sequence with different sulfate substitutions, that 6-*O*-sulfation of the Gal enhanced the binding whereas 3-*O*-sulfation on the GlcNAc did not [3]. The effect of other sulfation patterns (e.g. 2-*O*-, 3-*O*- and 4-*O*-sulfation) of the important terminal Gal on their interactions with RCA_{120} has not been investigated, presumably due mainly to the difficulty in obtaining such oligosaccharides. In several carbohydrate recognition systems, e.g. in the case of E-selectin, sialyl in the recognition motif Lewis^a or Lewis^x sequences can be substituted by a sulfate, while the same activities were maintained [13,14]. The importance of sulfation in oligosaccharide sequences has been implicated in many biological processes. It would be important to know the effect and to define the specificity of sulfation on Galβ interactions with the two lectins in order to explore the wider scope of their applications, e.g. in the rapid developing field of marine biology of polysaccharides. Here we use variously sulfated Galβ-containing oligosaccharides, obtained from marine-derived galactans (Fig. 1), to investigate their interactions with RCA_{120} and ECL. Sulfated galactans have shown a wide range of pharmacological activities including antiviral, antitumoral, immunoregulatory, antiangiogenic, anti-inflammatory, anti-coagulant and anti-thrombotic properties [15] and have become of considerable biomedical interest. Definition of the specificities and the detailed knowledge of the lectin binding activities to sulfated non-reducing terminal Gal will be important for their potential application, as a very usful tool, in marine carbohydrate research.

Sulfated galactan-oligosaccharides were prepared from red algae polysaccharide carrageenans and agarose (Fig. 1) by controlled mild acid hydrolysis [16–18]. These oligosaccharides were converted into neoglycolipid (NGL) probes by reductive amination [19–21] for their immobilization on solid surfaces by hydrophobic interaction. NGLs were arrayed on nitrocellulose membranes for binding assays and on microtiter well surface for inhibition experiments. Carbohydrate microarray has recently emerged as a promising tool for high-throughput analysis of carbohydrate–protein interactions as only small amounts of sample are required and many compounds can be screened in parallel in a single operation [1,22–24]. In the present study we use the array format for binding experiments and the results obtained were corroborated by inhibition assays for investigation of sulfated galactan oligosaccharide interactions with RCA_{120} and ECL.

2. Materials and methods

2.1. Materials

Odd-numbered κ-carrageenan- and agarose-derived tri- to undecasaccharides (K3–K11 and A3–A11, respectively), even-numbered ι-carra-tetra- to decasaccharides I4–I10 (see Table for structures), and 6-*O*-sulfated agarose polysaccharide (from *Gloiopeltis furcata*) were prepared in our laboratory as described previously [16,17]. Lacto-N-neotetraose (LNnT), lacto-N-tetraose (LNT), galactotetraose (G4), neoagar-tetrasaccharide (A4) and LS-tetrasaccharide C (LSTc) were purchased from Dextra Laboratories (Reading, UK). Marine-derived galactans κ- and λ-carrageenan, glucose, lactose, primulin, orcinol, tetrabutylammonium cyanoborohydride, fluorescein isothiocyanate-conjugated streptavidin (streptavidin-FITC), Tween 20, streptavidin-peroxidase, 3,3′,5,5′-tetramethylbenzidine, urea hydrogen peroxide and 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) were purchased from Sigma (Shanghai, China). Biotinylated RCA_{120} (biotin-RCA_{120}) and ECL (biotin-ECL) were purchased from Vector Laboratories (Peterborough, UK). Nitrocellulose membrane was from Bio-Rad (Hemel, Hempstead, UK) and SPE Silica columns from Waters (Manchester, UK). All other reagents and solvents used were analytical grade.

2.2. Preparation of 6-*O*-sulfated agaro- and λ-carra-oligosaccharides

Mild acid hydrolysis of sulfated agarose was performed essentially as previously described [17], with some modifications. In brief, the polysaccharide (20 mg/ml) was treated with 0.1 M H_2SO_4 at 60 °C for 2 h. The hydrolytic product was neutralized, concentrated and subjected to gel filtration chromatography on an ÄKTA-FPLC system (Pharmacia Biotech, Sweden) using a Superdex 30 column. Hydrolysate of sulfated agarose was eluted with 0.1 M NH_4HCO_3 at a flow of 0.1 ml/min with detection by refractive index. Oligosaccharide fractions were pooled and the volatile NH_4HCO_3 was removed by lyophilization.

Oligosaccharides of λ-carrageenan were similarly prepared, but the polysaccharide concentration was at 10 mg/ml and the hydrolysis was at 80 °C for 3 h. Gel filtration chromatography was carried out with 0.3 M NH_4HCO_3.

2.3. Preparation of desulfated κ- and λ-carra-oligosaccharides

Desulfation of λ-carrageenan polysaccharide was carried out essentially as described [25,26]. In brief, λ-carrageenan (50 mg) was dissolved in 9 ml of dry dimethyl sulfoxide before addition of pyridine (6 ml), pyromellitic acid (65 mg) and NaF (60 mg). The mixture was stirred at 120 °C for 3 h. The reaction was stopped by cooling down to room temperature and mixing with aqueous $NaHCO_3$ solution (3%, 5 ml). The reaction mixture was dialyzed (MWCO 3.5 KD) and the retained fraction was concentrated by rotary evaporation before lyophillization to obtain the desulfated λ-carrageenan polysaccharide.

For hydrolysis, the desulfated λ-carrageenan (10 mg/ml) was dissolved in 0.1 M H_2SO_4 and the mixture was incubated at 80 °C for 6 h. The reaction was stopped by neutralization with 1 M NaOH and the salt was removed by a Sephadex G10 column (1.0 × 100 cm). The desalted sample was then passed through a Q-Sepharose Fast Flow anion exchange column (1.0 × 5 cm), with washing by de-ionized water, to ensure a completely removal of any possible residual sulfated oligosaccharides. The neutral oligosaccharide mixture obtained was then fractionated by gel filtration with a Superdex Peptide column (Tricorn 10/300 GL) to obtain desulfated λ-carra-di-, tri- and tetrasaccharides dsL2, dsL3 and dsL4 (Table 1, #28–30). Desulfated κ-carra-trisaccharide dsK3 (Table 1, #7) was similarly prepared.

2.4. Preparation, purification and quantitation of oligosaccharide neoglycolipids

Conjugation of oligosaccharides to DHPE, by reductive-amination, and their purification and quantitation were performed as previously described [19–21]. NGLs were purified by mini-silica columns (3 ml) and quantified before stored in glass vials at −20 °C. Stock solutions of NGLs were diluted to 10 pmol/μl for binding assay and 4 pmol/μl for inhibition assay before use.

2.5. Binding assays of oligosaccharide NGLs with RCA_{120} and ECL

The conditions for binding experiments were initially tested at a range of probe concentrations (2–50 pmol/μl) using NGLs of selected oligosaccharides, including the negative and positive controls, and also at different lectin concentrations (1–10 μg/ml for