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Systematic Comparison of Oligosaccharide Specificity of *Ricinus communis* Agglutinin I and *Erythrina* Lectins: a Search by Frontal Affinity Chromatography†

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***Ricinus communis* agglutinin I (RCA120) is considered a versatile tool for the detection of galactose-containing oligosaccharides. However, possible contamination by the highly toxic isolectin 'ricin' has become a critical issue for RCA120's continued use. From a practical viewpoint, it is necessary to find an effective substitute for RCA120. For this purpose, we examined by means of frontal affinity chromatography over 100 lectins which have similar sugar-binding specificities to that of RCA120. It was found that *Erythrina cristagalli* lectin (ECL) showed the closest similarity to RCA120. Both lectins prefer Galβ1-4GlcNAc (type II) to Galβ1-3GlcNAc (type I) structures, with increased affinity for highly branched *N*-acetyllactosamine-containing *N*-glycans. Their binding strength significantly decreased following modification of the 3-OH, 4-OH and 6-OH of the galactose moiety of the disaccharide, as well as the 3-OH of its *N*-acetylglucosamine residue. Several differences were also observed in the affinity of the two lectins for various other ligands, as well as effects of bisecting GlcNAc and terminal sialylation. Although six other *Erythrina*-derived lectins have been reported with different amino acid sequences, all showed quite similar profiles to that of ECL, and thus, to RCA120. *Erythrina* lectins can therefore serve as effective substitutes for RCA120, taking the above differences into consideration.**

Key words: *Erythrina* lectins, frontal affinity chromatography, galactose-binding lectin, *Ricinus communis* agglutinin, sugar-binding specificity.

Abbreviations: ASF, asialofetuin; ASL, Asialolactoferrin; ECafL, *Erythrina caffra* lectin; ECL, commercial *Erythrina cristagalli* lectin; ECorL, *Erythrina corallodendron* lectin; ECriL, *Erythrina cristagall*i lectin; EFlaL, *Erythrina flabelliformis* lectin; ELysL, *Erythrina lysistemon* lectin; EVesL, *Erythrina verseptillo* lectin; FAC, frontal affinity chromatography; PA, pyridylaminated; pNP, *p*-nitrophenyl; RCA120, *Ricinus communis* agglutinin I; SBA, *Glycine max* agglutinin.

Seeds of *Ricinus communis*, the common castor bean, contain two kinds of R-type lectins, designated RCA60 and RCA120 (*1–3*). The former is a dimeric protein with a molecular weight of 60,000, consisting of a single α- (29,500 Da) and a single β- (34,000 Da) chain (*4*, *5*). This isolectin, also known as ricin, is a deadly poison, whose usage is therefore severely restricted. The latter lectin, a non-toxic protein characterized as a hemagglutinin with a molecular weight of 120,000, is a tetramer comprising two ricin-like dimers held together by non-covalent forces (*2*, *4*, *6*). Due to its high affinity for glycans containing non-reducing terminal βGal residues, RCA120 has been widely used as a versatile tool to detect sugar structures such as those found in complex-type *N*-linked glycans (*7–9*). Despite the extreme usefulness of RCA120, costs of tests for its possible contamination by ricin constitute a critical issue for its future commercial production. Introduction of an effective substitute for RCA120 is therefore a necessity from a practical viewpoint. From a glycobiological viewpoint, it is also necessary to compare the fine specificities of the related lectins in a quantitative manner.

With the aim of discovering a substitute for RCA120, detailed sugar-binding specificities for over 100 lectins were systematically investigated. Ninety-six pyridylaminated (PA)-oligosaccharides were examined by means of frontal affinity chromatography (FAC) (*10*).

†Carbohydrate-binding specificity data for *Erythrina* lectins using glycan array are available in the Consortium for Functional Glycomics database, *i.e.* CFG-glycan database, (www.functionalglycomics.org/static/index.shtml).

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FAC, originally developed by Kasai *et al*. (*11*) and refined by Hirabayashi *et al*. (*12*), was shown to apply to comparative analysis of many lectins using a set of fluorescently labelled glycans (*13–17*). FAC is known to provide excellent results in the determination of dissociation constants (K_d's) especially for relatively weak lectin-oligosaccharide interactions, *i.e*. $K_d > 10^{-6}$ M. As a result of screening, *Erythrina cristagalli* lectin (ECL) (*18–22*), a readily available galactose-(*e.g*. Gal/GalNAc) specific legume lectin, was found to have the most similar specificity to RCA120, together with other *Erythrina*-derived lectins (*23–28*). Although physiological functions of such legume lectins remain to be elucidated, ECL has been shown to have various biological activities toward mammalian cells: ECL specifically agglutinates untreated human erythrocytes of all blood types as well as rabbit erythrocytes (*20*). It is also mitogenic for human peripheral blood T lymphocytes, but not for mouse thymocytes or splenocytes. In an ECL staining experiment, great enhancement of Galβ1-4GlcNAc-terminated chains has been shown after neuraminidase treatment of the surfaces of embryoid bodies (*29*).

In this paper, we describe detailed, comparative studies of oligosaccharide specificity between RCA120 and *Erythrina* lectins. The results demonstrate that the *Erythrina* lectins are the most suitable substitutes for RCA120, although the differences in specificity between the two should be considered in the evaluation of data.

MATERIALS AND METHODS

NHS-activated Sepharose 4 Fast Flow was purchased from Amersham Biosciences (Little Chalfont, Bucks, UK). All chemical reagents used in this study were of analytical grade. Asialofetuin (ASF) from fetal calf serum (48,000 Da) and RNase B from bovine pancreas (15,000 Da) were purchased from Sigma. *Glycine max* agglutinin (SBA) (30,000 Da) was purchased from Seikagaku Co. Asialolactoferrin (ASL) was prepared by acid-treatment of lactoferrin from bovine milk (86,000 Da) obtained from Wako (Osaka, Japan).

Oligosaccharides—*p*-Nitrophenyl (pNP) glycosides of βGal, βGalNAc, αMan, Galβ1-4Glcβ (βLac) and αFuc were purchased from Sigma (St Louis, MO, USA); pNP-αGlc was from Calbiochem (San Diego, CA, USA). Other pNP-glycosides [pNP-αGal, αGalNAc, βMan, Galβ1-4GlcNAcβ (βLacNAc) and Galβ1-3GalNAcα (core1)] were obtained from Toronto Research Chemicals, Inc. (North York, Canada).

Pyridylaminated (PA) oligosaccharides used in this study are listed in Fig. 1. PA-*N*-linked glycans numbered **002-014**, **103**, **105**, **107**, **108**, **307**, **313**, **314**, **323**, **405**, **410**, **418–420**, **501–504** and **506** were purchased from Takara Bio Inc. (Kyoto, Japan); **304**, **403** and **404** were from J-Oil Mills Inc. (Tokyo, Japan); **505**, **509** and **510** were generous gifts from T. Angata (AIST, Tsukuba, Japan); and the others were from Seikagaku Co. (Tokyo, Japan). The sources of non-labelled glycans (**601** and **602**) were from Dextra Laboratories, Ltd (Reading, UK). Glycolipid- and other-type glycans numbered **701–703**, **705–713**, **715–718**, **720**, **721**, **724**, **726** and **728–731** were obtained from Takara Bio., Inc. The sources of non-labelled glycans were as follows: **727** from Funakoshi Co., **704**, **733** and **734** from Dextra Laboratories, Ltd; **725**, **909**, **910** and **911** from Calbiochem; **906** and **907** from Seikagaku Co. Oligo-lactosamines numbered **901–903** and **905** and milk oligosaccharides numbered **722**, **723**, **732** and **735–739** were generous gifts from K. Yoshida (Seikagaku Co.) and from T. Urashima (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan), respectively. The non-labelled glycans were pyridylaminated with Glyco Tag (Takara Bio., Inc.).

Preparation of lectin columns—RCA120 from J-Oil Mills Inc. was dissolved in 100 mM $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl, and was coupled to NHS-activated Sepharose 4 Fast Flow according to the manufacturer's instructions (Amersham Biosciences). After deactivation of excess NHS groups by 0.5 M monoethanolamine, the resin was washed twice with 0.1 M CH_3COONa buffer, pH 4.0, and then with 0.1 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl. The resultant lectin-Sepharose was suspended in 10 mM Tris–HCl, pH 7.4, containing 0.8% NaCl. The slurry was packed into a capsule-type miniature column (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 μl; Shimadzu Co., Kyoto, Japan). The lectin-column was slotted into a stainless-steel holder, and was connected to an automated FAC instrument (see later). ECL-agarose was purchased from Vector Lab. (Burlingame, CA, USA), and was similarly packed into a miniature column. Lectins from six other *Erythrina* species (*23*, *24*, *27*), containing *E. cristagalli*, were purified as described previously (*20*, *24*) and immobilized as above.

Frontal affinity chromatography (FAC)—FAC was performed using an automated system, FAC-1 [Shimadzu Co. (*10*, *14*, *15*)]. The principle and basic procedures of FAC have been described previously (*12*). Lectin-immobilized columns were connected to the FAC-1, and were equilibrated with 10 mM Tris–HCl, pH 7.4, containing 0.8% NaCl (TBS). The flow rate and column temperature were kept at 0.125 ml/min and 25°C, respectively. After equilibration of the miniature columns, either PA- (2.5 or 5.0 nM) or pNP-oligosaccharides (5.0 μM) dissolved in TBS were successively injected into a pair of lectin-columns by the auto-sampling system. Elution of PA- and pNP-oligosaccharides was monitored by fluorescence (excitation and emission wavelengths of 310 and 380 nm, respectively) and UV (280 nm), respectively. The volume of the elution front (V) of each oligosaccharide is calculated as described previously (*12*), and V_0 is that of a reference substance [*e.g*. Manα1-6(Manα1-2)Manβ1-4GlcNAcβ1-4GlcNAc, **003**]. Retardation of the elution front relative to that of the appropriate standard oligosaccharide, *i.e*. $V-V_0$, was then determined. K_d values for dissociation of lectins and oligosaccharides were obtained from $V-V_0$ and B_t (effective ligand content) according to the basic equation of FAC: $K_d = B_t/(V-V_0) - [A]_0$, where $[A]_0$ is the initial concentration of PA- or pNP-oligosaccharides. Since $[A]_0$ is, in most cases, much smaller than K_d, the equation can be simplified to be $K_d = B_t/(V-V_0)$.

It is often useful to discuss lectin–oligosaccharide interactions in terms of affinity constant (K_a) instead

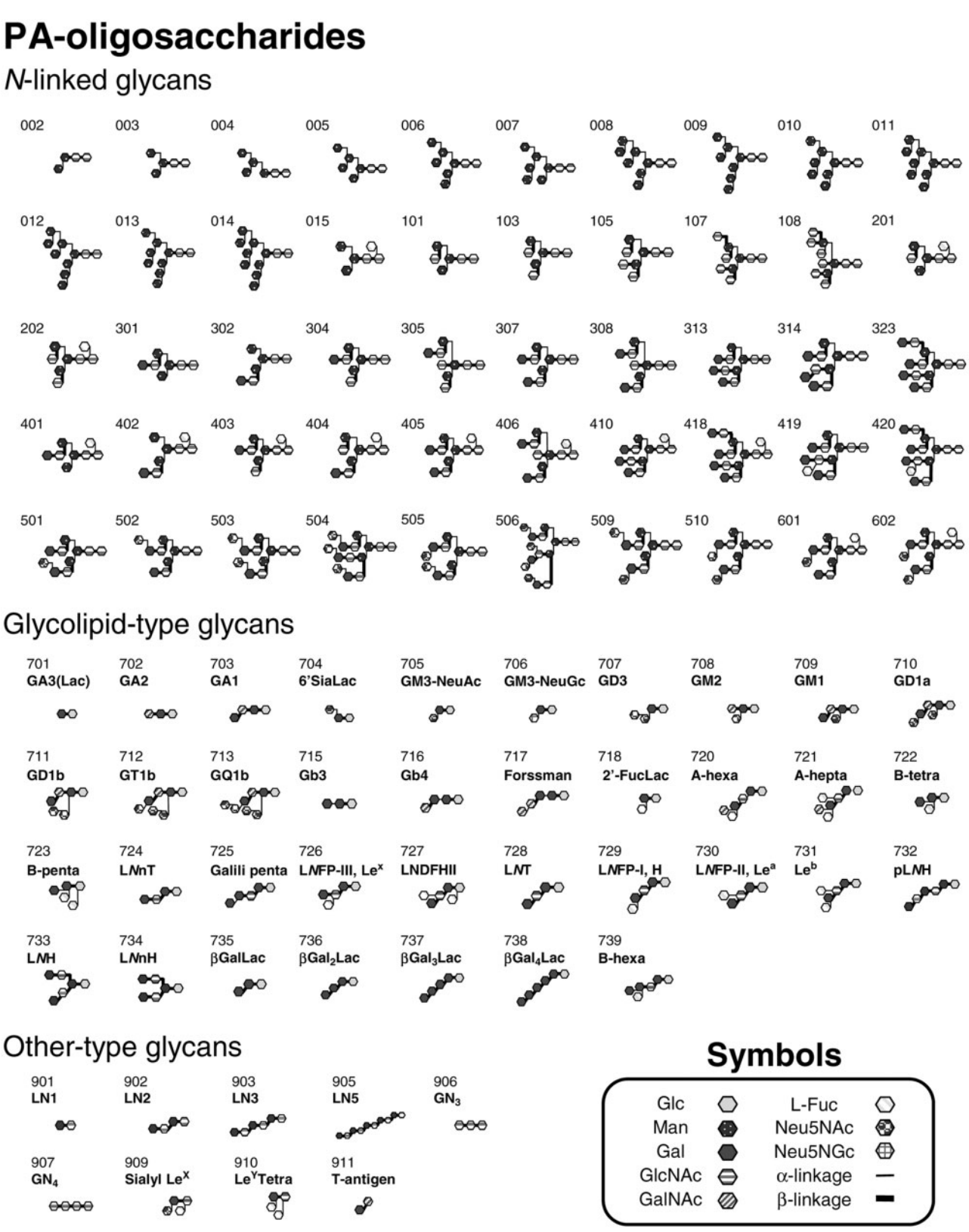

Fig. 1. **Schematic representation of oligosaccharide structures.** Note that the reducing terminal is fluorescently labelled by 2-aminopyridine for FAC analysis. Each coloured hexagon represents a pyranose ring of different monosaccharides shown in inset. Anomeric carbon, *e.g.* C1 in aldohexoses, is placed at the right side, and C2, C3, C4 . . . are placed clockwise. Thin and thick bars represent α- and β-linkages, respectively.

of K_d. The two equilibrium constants are related by: $K_d = 1/K_a$.

Concentration-dependence analysis—For determination of B_t and K_d values for the lectin columns described earlier, βLacNAc-pNP was diluted to various concentrations (5–100 μM) and was applied to each column. Using the resulting V values, Woolf–Hofstee-type plots, *i.e.* $(V-V_0)$ vs. $(V-V_0)[A]_0$, were constructed as described previously (*12*, *14*). The plots were used to determine B_t and K_d values from the intercept and the slope of the fitted curve, respectively.

Calculation of correlation coefficient—To quantify the extent of similarity between RCA120 and six *Erythrina* lectins, correlation coefficients were calculated as described previously (*30*, *31*). The K_a values for 46 glycans, *i.e.* **301–504**, **506**, **601**, **701**, **702**, **716–718**, **724**, **725**, **728** and **732–905** used for comparison of these lectins, were determined. The correlation coefficient was defined as: $\sum(x_i\text{-}x)(y_i\text{-}y)/\sqrt{(\sum(x_i\text{-}x)^2\sum(y_i\text{-}y)^2)}$, $X=(x_1, x_2, \ldots, x_{46})$, $Y=(y_1, y_2, \ldots, y_{46})$, where X and Y denote glycans to be compared, and x and y are the average K_a values of RCA120 and each of *Erythrina* lectins, respectively.

Enrichment experiment—In order to compare the performance of RCA120 and ECA, the following glycoprotein-enrichment experiment was performed. Glycoproteins, ASF, RNase B, SBA and ASL dissolved in 10 mM Na-phosphate, pH 7.4, containing 0.8% NaCl (PBS) were mixed at a final concentration of 0.4 mg/ml. To columns (0.5 ml bed volume) of RCA120-agarose (4.0 mg/ml, Vector Lab.) and ECL-agarose (4.2 mg/ml, J-Oil Mills Inc.) equilibrated with PBS, the above glycoprotein mixture (0.5 ml) was applied. After washing of the columns with 2.5 ml of PBS, bound glycoproteins were eluted with 2.5 ml of PBS containing 0.1 M lactose. Fractions (0.5 ml) were collected and elution of protein was monitored by absorption (280 nm). Representative fractions 2 (flow-through), 8 and 12 (elution) were also subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using gradient gel (10–20%), and the gel was stained for protein with Coomassie Brilliant Blue by a standard procedure.

Lectin-microarray analysis—To validate the flow-through and adsorbed fractions obtained by the above enrichment test, glycan profiling was performed by means of lectin-microarray (*32*, *33*). Briefly, 60 μl of Cy3-labelled glycoproteins from representative fractions 2 (flow-through) and 8 (adsorbed) obtained by chromatography on RCA120- and ECL-agarose columns was applied to the lectin microarray (ver. 4.5). After the microarray was incubated for interaction at 20°C for 6.5 h, the glass slide was rinsed twice with the probing buffer for lectin microarray, and fluorescence image of the microarray was acquired by using an evanescent-field activated fluorescence scanner, SC-profiler (Moritex Co., Yokohama, Japan) essentially as described previously (*32–34*). All data were analysed with the Array Pro analyzer, version 4.5 (Media Cybernetics, Inc.). The net intensity value for each spot was calculated as the signal intensity minus a background value. Three spots of the signal net intensity values were averaged.

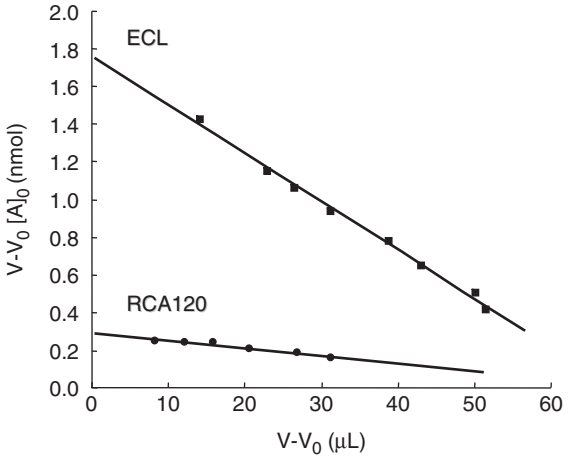

Fig. 2. **Woolf–Hofstee-type plots for RCA120- (circles) and ECL-immobilized (squares) columns.** For determination of B_t and K_d values for the immobilized lectins, pNP-βLacNAc was diluted to various concentrations (5–100 μM), and was applied to each column. Woolf–Hofstee-type plots were made by using the obtained $V-V_0$ values and $[A]_0$.

RESULTS AND DISCUSSION

Evaluation of the lectin columns—For evaluation of the columns prepared for RCA120 and ECL, the effective ligand contents (B_t values) were determined based on the so-called 'concentration-dependence analysis' (*12–14*). For this purpose, commercially available *p*-nitrophenyl (pNP) derivatives of simple saccharides, *i.e.* αGal, βGal, αGalNAc, βGalNAc, αMan, βMan, αGlc, βLac, βLacNAc, αFuc and Galβ1-3GalNAcα (core1), were tested. When they were applied to the RCA120 and ECL columns at the minimal concentration of detection (*i.e.* 5 μM), the strongest affinity was observed for pNP-βLacNAc with both lectins. However, in case of RCA120, it also showed significant affinity for βGal, followed by βLac and βGalNAc, while ECL showed lower affinity to βLac than βGalNAc and βGal. These results agree with the previous reports in that RCA120 and ECL have affinity for galactose-containing glycosides (*2*, *4*, *21*, *35*, *36*). Concentration-dependence analysis was conducted with pNP-βLacNAc in the range 5 to 100 μM (Fig. 2). As a result, B_t and K_d values were determined to be 0.29 nmol and 4.1×10^{-6} M, respectively, for the RCA120 column, and 1.76 nmol and 2.6×10^{-5} M, respectively, for the ECL column. Using these data, availabilities (proportion of B_t/immobilized amount) of the RCA120 and ECL columns were calculated to be 38.2 and 56.0%, respectively. Specifications of the columns used in this study are summarized in Table 1.

For the systematic comparison of 11 pNP-sugars, a bar graph representation of affinity constant (K_a) is shown in Fig. 3A. Notably, RCA120 recognized pNP-βGal with almost comparable affinity ($K_d = 5.8 \times 10^{-6}$ M) to pNP-βLacNAc (4.1×10^{-6} M), whereas ECL showed four times higher affinity for pNP-βLacNAc (2.6×10^{-5} M) than pNP-βGal (1.1×10^{-4} M). ECL is therefore considered to require a full disaccharide unit of LacNAc to show its full

Table 1. **Specifications of RCA120- and *Erythrina* lectin-immobilized columns used in this study.**

Lectin name	Origin	Lectin family	Immobilized (mg/ml resin)	B_t (nmol)	r^2 [b]	K_d for pNP-βLacNAc (M)	Correlation coefficient
RCA120	*Ricinus communis*	R-type lectin	2.9	0.29	0.95	4.1×10^{-6}	–
ECL	*Erythrina cristagalli*[a]	Legume lectin	3.0	1.76	1.00	2.6×10^{-5}	0.92
ECafL	*Erythrina caffra*	Legume lectin	2.0	0.56	0.99	2.7×10^{-5}	0.90
ECorL	*Erythrina corallodendron*	Legume lectin	2.0	0.16	0.99	1.3×10^{-5}	0.91
ECriL	*Erythrina cristagalli*[a]	Legume lectin	2.0	0.64	0.99	2.0×10^{-5}	0.91
EFlaL	*Erythrina flabelliformis*	Legume lectin	2.0	0.29	0.99	1.2×10^{-5}	0.94
ELysL	*Erythrina lysistemon*	Legume lectin	2.0	0.14	0.99	9.3×10^{-6}	0.94
EVesL	*Erythrina verseptillo*	Legume lectin	2.0	0.21	1.00	1.7×10^{-5}	0.92

[a]Two *Erythrina cristagalli* preparations are used in this study: one designated ECL is a commercial product from Vector Lab., and the other designated ECriL is a preparation in this study (*20*).
[b]Reliability of lines obtained as a result of Woolf–Hofstee-type plot in each concentration analysis.

affinity [*cf.* galectins, representative β-galactoside-specific animal lectins (*13*)], whereas RCA120 recognized predominantly terminal βGal residues.

Overall features of oligosaccharide specificities of RCA120 and ECL—In order to compare detailed specificities of RCA120 and ECL, their fine sugar-binding specificities were investigated by FAC using 96 PA-oligosaccharides. The two lectins were found to show similar profiles for PA-oligosaccharides, although the overall affinity of RCA120 is approximately 10 times higher than that of ECL (Fig. 3B). The overall similarity between them is 0.92 in terms of correlation coefficient, which is often used for comparative glycan profiling (*31*, *34*, *37*). The similarity is considered to be 'high' enough if the correlation coefficient is >0.9, whereas it is considered 'low' if the coefficient is below 0.5. Both RCA120 and ECL showed affinity for galactosylated (**301**–**420**) and sialylated (**501**–**602**), complex-type *N*-linked glycans, galactose-containing glycolipid-type (**701**–**739**) glycans and others (**901**–**911**), whereas no detectable affinity was observed for high-mannose type (**002**–**015**) and agalactosylated *N*-linked glycans (**101**–**202**).

Common and distinct properties of RCA120 and ECL—As described, both RCA120 and ECL showed significant affinity for galactosylated, complex-type *N*-linked glycans. Among completely galactosylated, non-fucosylated glycans (**301**, **302**, **307**, **313**, **323**), RCA120 showed the highest affinity for **323** (tetra-antennary glycan, $K_d = 1.6 \times 10^{-6}$ M), followed by **313** (tri-, 1.7×10^{-6} M), **307** (bi-, 2.3×10^{-6} M), **302** (mono-, 5.0×10^{-6} M) and **301** (mono-, 9.2×10^{-6} M) (Fig. 3B, left). Almost the same feature was observed for ECL, *i.e.* **323** (1.5×10^{-5} M), **313**, (1.3×10^{-5} M), **307** (1.7×10^{-5} M), **301** (3.1×10^{-5} M), **302** (4.0×10^{-5} M) (Fig. 3B, right). In summary, the affinity was found to increase as the branching number increases. Core (α1-6) fucosylation did not substantially affect the affinity to either lectin. In contrast, α1-3 fucosylation (as in Le^x) significantly decreased the affinity of both RCA120 and ECL (**313** vs. **419**, and **323** vs. **420**). This feature was more evident for glycolipid-type glycans, *i.e.* **724** (L*N*nT, $K_d = 5.9 \times 10^{-6}$ M for RAC120 and 3.7×10^{-5} M for ECL) vs. **726** (Le^x, not detected).

The binding affinity was also reduced by modification of terminal Gal with α2-3- or α2-6-linked NeuAc (*e.g.* **503**–**506**, **509**, **510** and **602**). However, these effects were significantly different for RCA120 and ECL. Thus, both α2-3 and α2-6 sialylation abolished the affinity for ECL, while the effects were apparently partial with RCA120 (compare data for fully sialylated glycans, **503**–**510**). This observation is consistent with the previous reports (*4*, *6*, *32*).

Another characteristic of ECL is preference for type II (Galβ1-4GlcNAc) over type I (Galβ1-3GlcNAc) structures. This was reported originally by Kaladas *et al.* (*38*). Though reported by ourselves for RCA120 (*37*), it was strongly confirmed in the present study that both RCA120 and ECL distinguished type I/II structural isomers, *e.g.* **313** and **314**, **724** and **728**, and **733** and **734** (Fig. 3B). Most typically, RCA120 showed significant affinity for **724** ($K_d = 5.9 \times 10^{-6}$ M), whereas it showed barely detectable affinity for its linkage isomer, **728** (not detected). Similarly to RCA120, ECL consistently preferred type II structures (*39*), but its preference was never absolute; *e.g.* compare data for **724** ($K_d = 3.7 \times 10^{-5}$ M) with **728** (3.0×10^{-4} M). Such a feature has not been described previously in a quantitative sense.

When oligo-*N*-acetyllactosamines, **901**, **902**, **903** and **905**, were compared, repetition of the LacNAc unit had no apparent effect on affinity of RCA120 and ECL (Fig. 3B). This observation suggests that they can recognize only non-reducing terminal LacNAc structures, and modification of the 3-OH group of Gal abolishes their affinity, as is also the case for α2-3 sialylation described earlier (Fig. 4). This is not surprising, since X-ray crystallography of saccharide complexes of both ECL and ECorL (*18*, *19*, *25*, *26*, *28*, *40*, *41*) revealed that C3, 4 and 6 in particular of the galactose are H-bonded to the lectins, while the C2-OH points into a cavity and can accommodate bulky substituents.

RCA120 and ECL showed significant difference in the effect of bisecting GlcNAc. RCA120 showed significantly higher affinity for *N*-linked glycans containing bisecting GlcNAc, *i.e.* **305** ($K_d = 4.2 \times 10^{-6}$ M), **308** (2.0×10^{-6} M) and **406** (2.0×10^{-6} M), than those lacking it, *i.e.* **304** (7.1×10^{-6} M), **307** (2.3×10^{-6} M) and **405** (2.5×10^{-6} M). In contrast, the affinity of ECL was significantly reduced by the presence of bisecting GlcNAc, *i.e.* **304** ($K_d = 3.3 \times 10^{-5}$ M) > **305** (4.2×10^{-5} M), **307** (1.7×10^{-5} M) > **308** (2.2×10^{-5} M) and **405** (2.0×10^{-5} M) > **406** (2.4×10^{-5} M).

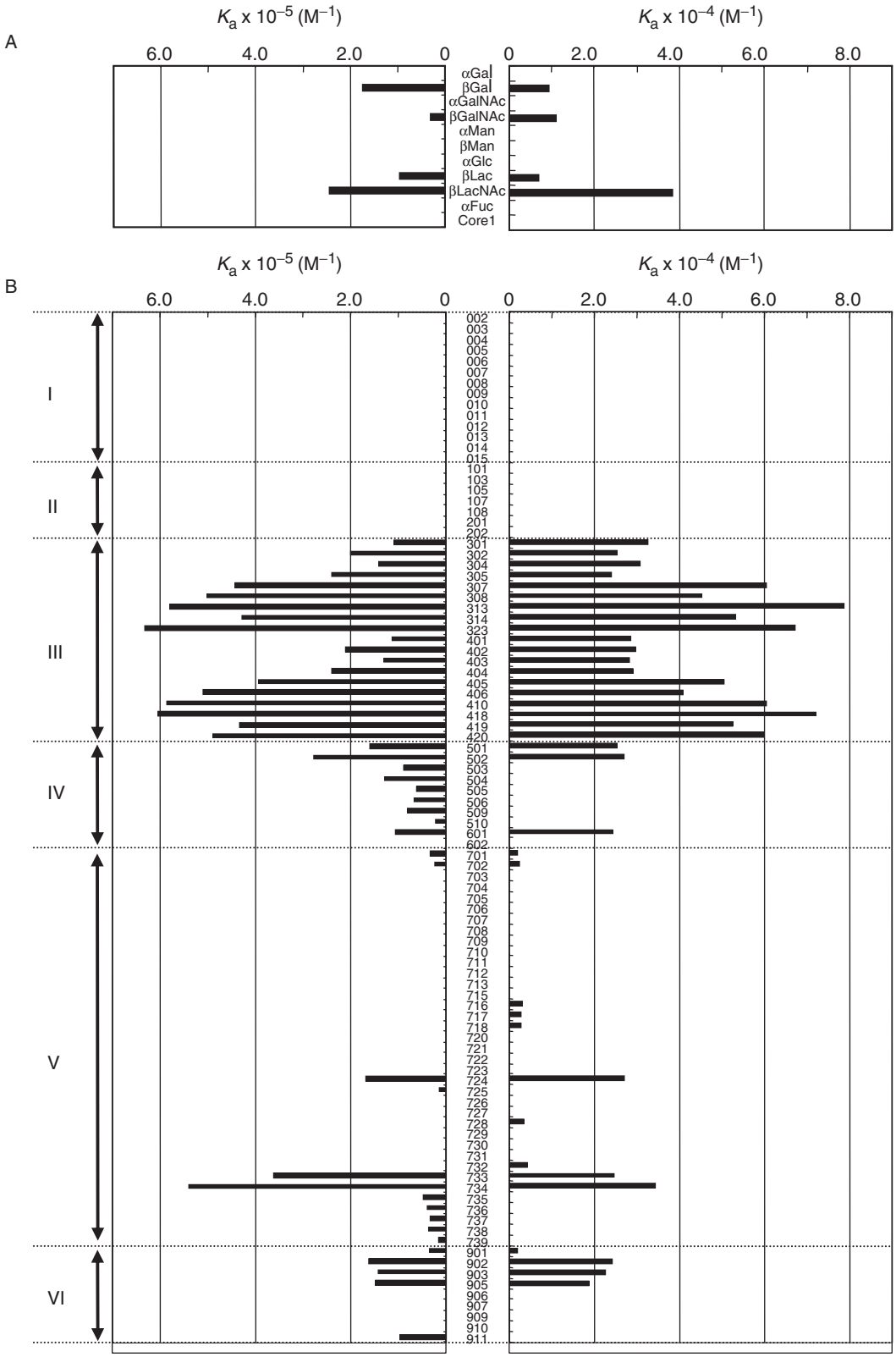

Fig. 3. **Bar graph representation of affinity constants (K_a's) of RCA120 (left) and ECL (right) toward pNP- (A) and PA-oligosaccharides (B).** The small Arabic figures in the centre correspond to sugar numbers indicated in Fig. 1; whereas larger Roman figures on the left side of graphs represent types of glycans; *i.e.* high-mannose-type (I), agalacto-type (II), galactosylated-type (III) and sialylated-type (IV) *N*-linked glycans, glycolipid-type glycans (V) and others (VI). For structures of oligosaccharides and specifications of RCA120 and ECL columns, see Fig. 1 and Table 1, respectively.

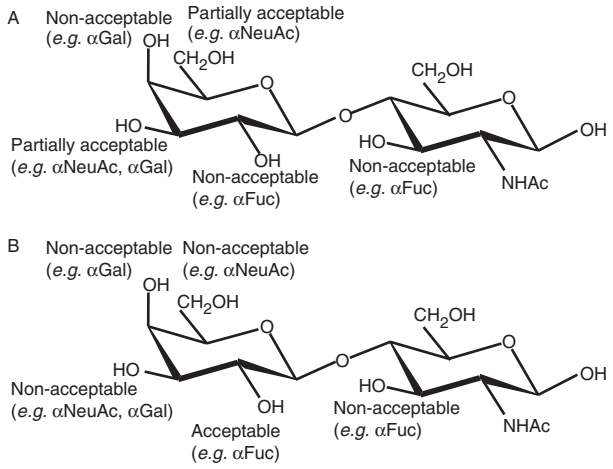

Fig. 4. **Effect of substitution in the LacNAc unit for RCA120 (A) and ECL (B)**. In the case of RCA120, substitutions of hydroxyl groups at C2 and 4 on Gal and C3 on GlcNAc are not acceptable and those of C3 and 6 on Gal are partially acceptable. In the case of ECL, those of C3, 4 and 6 on Gal are not acceptable, that of C3 on GlcNAc is partially acceptable and that of C2 on Gal is fully acceptable.

From a practical viewpoint, it is important to know performance of ECL in comparison with RCA120. For this purpose, we carried out an enrichment test using model glycoproteins, lactoferrin and fetuin having sialylated complex-type *N*-linked glycans together with soybean agglutinin (SBA) and RNase B having high-mannose-type *N*-linked glycans. For the sake of simplicity, the former two glycoproteins were used after desialylation, *i.e*. asialolactoferrin (ASL) and asialofetuin (ASF), for which both RCA120 and ECA have high affinity. A mixture of these model glycoproteins was applied to RAC120- and ECL-agarose columns, and representative fractions (*i.e*. 2, 8 and 12) were subjected to SDS–PAGE and lectin microarray analyses as described under 'Materials and methods' section. As a result, closely similar elution profiles were obtained (Fig. 5A). As expected, RNase B and SBA having only high-mannose-type *N*-linked glycans were eluted at the void volume (fraction 2) of the columns with no apparent interaction with immobilized RCA120 and ECL (lanes 2 and 5 in Fig. 5B). On the other hand, both ASL and ASF were adsorbed on the columns, and were specifically eluted with 0.1 M lactose (lanes 3 and 6 in Fig. 5B). Consistently lectin microarray data indicated the absence of signals of βGal-binders, RCA120 and ECA, in the flow-through fraction 2 (upper panels of Fig. 5C), while they were evident in the adsorbed fraction 8 obtained from both RCA120 and ECL columns (lower panels of Fig. 5C). These results clearly showed that practical performance of ECL to enrich glycoproteins having non-reducing terminal β-galactosides is almost the same as RCA120.

Analysis of other Erythrina lectins—As an extension of the above study, other *Erythrina* lectins, which have been reported previously by one of the authors (*20*, *23*, *24*), were analysed with the expectation that some of these ECL-related lectins would show still higher similarity to RCA120. For this purpose, six *Erythrina* lectins purified from *E. caffra*, *E. corallodendron*, *E. cristagalli*, *E. flabelliformis*, *E. lysistemon* and *E. verseptillo* were analysed (designated in this study ECafL, ECorL, ECriL, EFlaL, ELysL and EVesL, respectively; note that commercial ECL and purified ECriL are from the same species). It has been reported that glycopeptides isolated from human transferrin and hen ovalbumin were successfully purified by using some of these lectins (*27*). As expected, ECL and ECriL showed almost identical specificity profiles (correlation coefficient, 0.98). Since their structures are assumed to be identical, the observed difference should correspond to the experimental error of the analysis. However, other *Erythrina* lectins also showed similar specificities (Fig. 6), despite the different origins, and thus, having different structures (*23*). Though absolute K_d values are not available, relative carbohydrate-binding specificity of *Erythrina* lectins obtained by glycan array are available in the CFG database, which showed basic agreement with the present result in that the lectins have high affinity for terminal Gal/GalNAc residues and preferred type II structures. In addition, all of the *Erythrina* lectins showed almost the same extents of similarity to RCA 120 (*i.e*. 0.90–0.94). The calculated correlation coefficient values, 0.90–0.94 (Table 1), are sufficiently high to demonstrate close resemblance in the sugar-binding specificity between RCA 120 and the *Erythrina* lectins.

CONCLUDING REMARKS

At present, the use of RCA120 involves a serious risk of contamination by a toxic isolectin, ricin. As we have demonstrated, RCA120 and *Erythrina* lectins including commercial ECL, show similar specificities for a panel of oligosaccharides in terms of lowered affinity by modification with α2-3/2-6 NeuAc, preferred recognition of type II over type I structures, and the detrimental effect of fucosylation at 3-OH of the GlcNAc residue. In fact, an ECL-immobilized column proved to be fully applicable for enrichment of certain glycoproteins carrying non-reducing terminal *N*-acetyllactosamine with almost the same enrichment efficiency as an RCA120-column when serum glycoproteins were targeted (data not shown). Despite overall similarities, significant differences have also been revealed as summarized in Fig. 4. The differences as regards both affinity strength and detailed specificity should therefore be taken into account for the evaluation of experimental results obtained with the *Erythrina* lectins in comparison to those obtained with RCA120.

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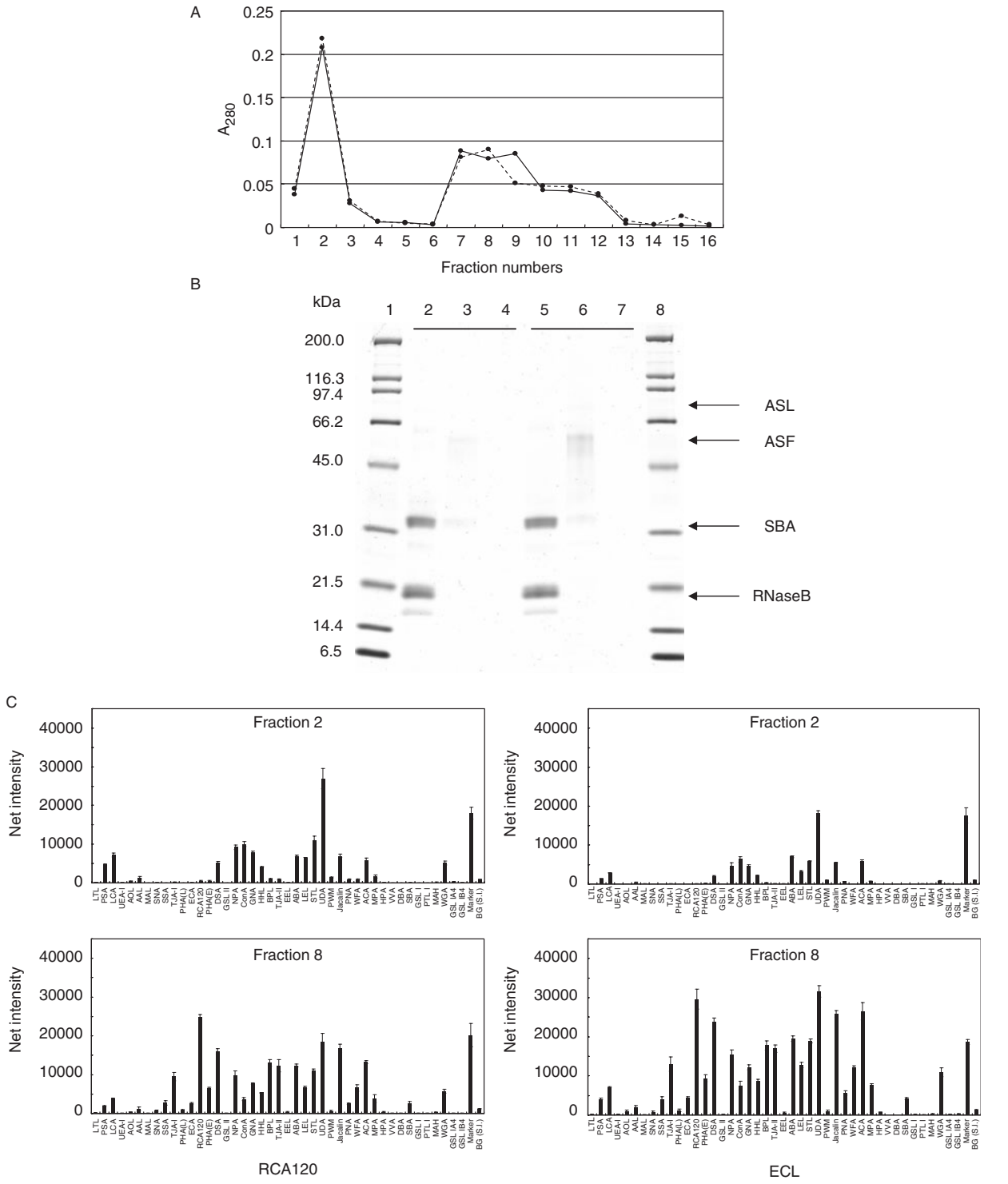

Fig. 5. **Enrichment of representative glycoproteins by using RCA120- and ECL-agarose columns.** (A) Elution of glycoproteins (ASL, ASF, SBA and RNase B) was monitored by absorption at 280 nm (A_{280}), and representative fractions (2, 8 and 12) were subjected to SDS–PAGE and lectin microarray analyses as described under 'Materials and methods' section. A_{280} of each fraction form RCA120- and ECL-agarose are shown in solid and dotted lines, respectively. (B) Samples from fractions (2, 8 and 12) were analysed by SDS–PAGE. Lanes 1 and 8, molecular weight markers with indicated sizes; lanes 2 and 5, flow-through fraction (2) from the RCA120 and ECL-columns, respectively; lanes 3 and 6, adsorbed and 0.1 M lactose-eluted fraction (8) from the RCA120 and ECL-columns, respectively; lanes 4 and 7, adsorbed and PBS-washed fraction (12) from the RCA120 and ECL-columns, respectively. (C) Bar graph representation of the result of lectin microarray of flow-through fraction (2, upper panels) and adsorbed and 0.1 M-lactose eluted fraction (8, lower panels). The fluorescence signals for fractions obtained form the RCA120 (left panels) and ECL-columns (right panels) were obtained by an evanescent-field activated fluorescence scanner, SC-Profiler as described under 'Materials and methods' section.

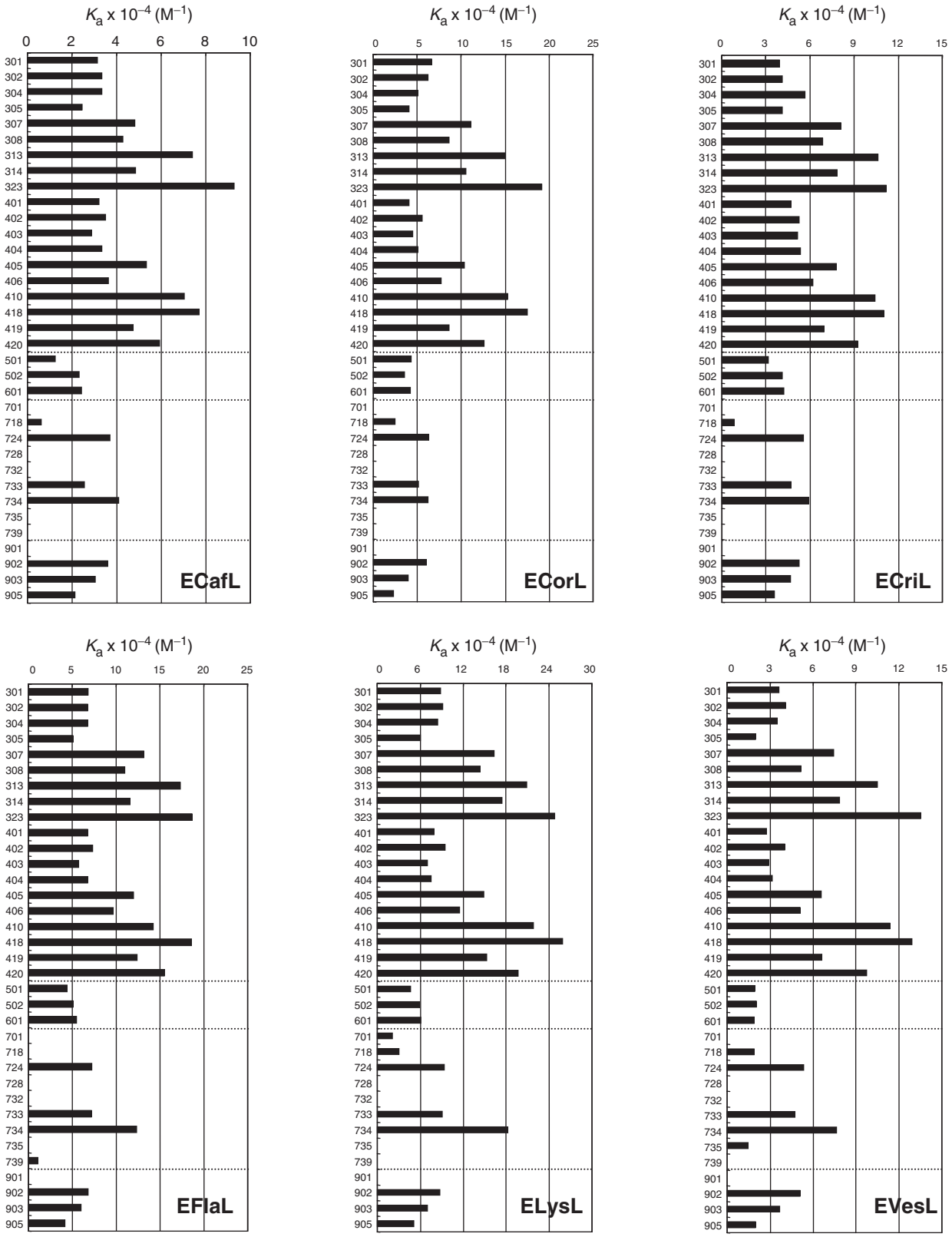

Fig. 6. **Bar graph representation of affinity constants (K_a's) between 6 *Erythrina* lectins and 35 PA-oligosaccharides.** Arabic figures on the left side of each graph correspond to sugar numbers indicated in Fig. 1. For structures of oligosaccharides and specifications of *Erythrina* immobilized-columns, see Fig. 1 and Table 1, respectively. Note that abscissa scales are variable depending on overall binding strengths of *Erythrina* lectins.

REFERENCES

1. Citores, L., Ferreras, J.M., Iglesias, R., Carbajales, M.L., Arias, F.J., Jiménez, P., Rojo, M.A., and Girbés, T. (1993) Molecular mechanism of inhibition of mammalian protein synthesis by some four-chain agglutinins: proposal of an extended classification of plant ribosome-inactivating proteins (rRNA *N*-glycosidases). *FEBS Lett.* **329**, 59–62
2. Sweeney, E.C., Tonevitsky, A.G., Temiakov, D.E., Agapov, I.I., Saward, S., and Palmer, R.A. (1997) Preliminary crystallographic characterization of ricin agglutinin. *Proteins* **28**, 586–589
3. Baenziger, J.U. and Fiete, D. (1979) Structural determinants of *Ricinus communis* agglutinin and toxin specificity for oligosaccharides. *J. Biol. Chem.* **254**, 9795–9799
4. Green, E.D., Brodbeck, R.M., and Baenziger, J.U. (1987) Lectin affinity high-perfomance liquid chromatography. *J. Boil. Chem.* **262**, 12030–12039
5. Wu., J.H., Shigh, T., Herp, A., and Wu, A.M. (2005) Carbohydrate recognition factors of the lectin domains present in the *Ricinus communis* toxic protein (ricin). *Biochimie* **88**, 201–217
6. Hegde, R. and Podder, S.K. (1998) Evolution of tetrameric lectin *Ricinus communis* agglutinin from two variant groups of ricin toxin dimers. *Eur. J. Biochem.* **254**, 596–601
7. Yamashita, K., Umetsu, K., Suzuki, T., Iwaki, Y., Endo, T., and Kobata, A. (1988) Carbohydrate binding specificity of immobilized *Allomyrina dichotoma* lectin II. *J. Biol. Chem.* **263**, 17482–17489
8. Abdul-Rahman, B., Ailor, E., Jarvis, D., Betenbaugh, M., and Lee, Y.C. (2002) β-(1→4)-Galactosyltransferase activity in native and engineered insect cells measured with time-resolved europium fluorescence. *Carbohydr. Res.* **337**, (21–23), 2181–2186
9. Medina-Bolivar, F., Wright, R., Funk, V., Sentz, D., Barroso, L., Wilkins, T.D., Petri, W.Jr, and Cramer, C.L. (2003) A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. *Vaccine* **21**, 997–1005
10. Hirabayashi, J. (2004) Lectin-based structural glycomics: glycoproteomics and glycan profiling. *Glycoconj. J.* **21**, 35–40
11. Kasai, K., Oda, Y., Nishikata, M., and Ishii, S. (1986) Frontal affinity chromatography: theory, for its application to studies on specific interaction of biomolecules. *J. Chromatogr. A.* **376**, 33–47
12. Hirabayashi, J., Arata, Y., and Kasai, K. (2003) Frontal affinity chromatography as a tool for elucidation of sugar recognition properties of lecions. *Methods Enzymol.* **362**, 352–368
13. Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W.E.G., Yagi, F., and Kasai, K. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochem. Biophys. Acta.* **1572**, 232–254
14. Nakamura, S., Yagi, F., Totani, K., Ito, Y., and Hirabayashi, J. (2005) Comparative analysis of carbohydrate-binding properties of two tandem repeat-type Jacalin-related lectins: *Castanea crenata* agglutinin and *Cycas revolute* leaf lectin. *FEBS J.* **272**, 2784–2799
15. Nakamura-Tsuruta, S., Uchiyama, N., and Hirabayashi, J. (2006) High-throughput analysis of lectin-oligosaccharide interactions by automated frontal affinity chromatography. *Methods Enzymol.* **415**, 311–325
16. Nakamura-Tsuruta, S., Kominami, J., Kamei, M., Koyama, Y., Suzuki, T., Isemura, M., and Hirabayashi, J. (2006) Comparative analysis by frontal affinity chromatography of oligosaccharide specificity of GlcNAc-binding lectins, *Griffonia simplicifolia* lectin-II (GSL-II) and *Boletopsis leucomelas* lectin (BLL). *J. Biochem.* **140**, 285–291
17. Nakamura-Tsuruta, S., Kominami, J., Kuno, A., and Hirabayashi, J. (2006) Evidence that *Agaricus bisporus* agglutinin (ABA) has dual sugar-binding specificity. *Biochem. Biophys. Res. Commun.* **347**, 215–220
18. Turton, K., Natesh, R., Thiyagarajan, N., Chaddock, J.A., and Acharya, K.R. (2004) Crystal structures of Erythrina cristagalli lectin with bound *N*-linked oligosaccharide and lactose. *Glycobiology* **14**, 923–929
19. Svensson, C., Teneberg, S., Nilsson, C.L., Kjellberg, A., Schwarz, F.P., Sharon, N., and Krengel, U. (2002) High-resolution crystal structures of *Erythrina cristagalli* lectin in complex with lactose and 2'-α-L-fucosyllactose and correlation with thermodynamic binding data. *J. Mol. Biol.* **321**, 69–83
20. Iglesias, J.L., Lis, H., and Sharon, N. (1982) Purification and properties of a D-galactose/*N*-acetyl-D-galactosamine-specific lectin from *Erythrina cristagalli*. *Eur. J. Biochem.* **123**, 247–252
21. Stancombe, P.R., Alexander, F.C.G., Ling, R., Matheson, M.A., Shone, C.G., and Chaddock, J.A. (2003) Isolation of the gene and large-scale expression and purification of recombinant *Erythrina cristagalli* lectin. *Protein Expr. Purif.* **30**, 283–292
22. Whitehurst, C.E., Day, N.K., and Gengozian, N. (1990) Sugar competition assays reveal high affinity receptors for *Erythrina cristigalli* lectin on feline monocytes. *J. Immunol. Methods* **131**, 15–24
23. Bonneil, E., Young, N.M., Lis, H., Sharon, N., and Thibault, P. (2004) Probing genetic variation and glycoform distribution in lectins of the *Erythrina* genus by mass spectrometry. *Arch. Biochem. Biophys.* **426**, 241–249
24. Lis, H., Joubert, F.J., and Sharon, N. (1985) Isolation and properties of *N*-acetyllactosamine-specific lectins from nine *Erythrina* species. *Phytochemistry* **24**, 2803–2809
25. Sharon, N. and Lis, H. (2002) How proteins bind carbohydrates: lessons from legume lectins. *J. Agric. Food Chem.* **50**, 6586–6591
26. Surolia, A., Sharon, N., and Schwarz, F.P. (1996) Thermodynamics of monosaccharide and disaccharide binding to *Erythrina corallodendron* lectin. *J. Biol. Chem.* **271**, 17697–17703
27. Derbray, H., Montreuil, J., Lis, H., and Sharon, N. (1986) Affinity of four immobilized *Erythrina* lectins toward various N-linked glycopeptides and related oligosaccharides. *Carbohydr. Res.* **151**, 359–370
28. Gupta, D., Cho, M., Cummings, R.D., and Brewer, C.F. (1996) Thermodynamics of carbohydrate binding to galectin-1 from Chinese hamster ovary cells and two mutants. A comparison with four galactose-specific plant lectins. *Biochemistry* **35**, 15236–15243
29. Werane, K.A., Winter, H.C., O'Shea, K., and Goldsteon, I.J. (2006) Use of lectins for probing differentiated human embryonic stem cells for carbohydrates. *Glycobiology* **16**, 981–990
30. Takegawa, Y., Ito, S., Yoshioka, S., Deguchi, K., Nakagawa, H., Monde, K., and Nishimura, S. (2004) Structural assignment of isomeric 2-aminopyridine-derivatized oligosaccharides using MS^n spectral matching. *Rapid Commun. Mass Spectrom.* **18**, 385–391
31. Takegawa, Y., Deguchi, K., Ito, S., Yoshioka, S., Sano, A., Yoshinari, K., Kobayashi, K., Nakagawa, H., Monde, K., and Nishimura, S. (2004) Assignment and quantification of 2-aminopyridine derivatized oligosaccharide isomers coeluted on reversed-phase HPLC/MS by MS^n spectral library. *Anal. Chem.* **76**, 7294–7303
32. Kuno, A., Uchiyama, N., Koseki-Kuno, S., Ebe, Y., Takashima, S., Yamada, M., and Hirabayashi, J. (2005) Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nat. Methods* **2**, 851–856
33. Uchiyama, N., Kuno, A., Koseki-Kuno, S., Ebe, Y., Horio, K., Yamada, M., and Hirabayashi, J. (2005) Development of

a lectin microarray based on an evanescent-field fluorescence principle. *Methods Enzymol.* **415**, 341–351
34. Ebe, Y., Kuno, A., Uchiyama, N., Koseki-Kuno, S., Yamada, M., Sato, T., Narimatsu, H., and Hirabayashi, J. (2006) Application of lectin microarray to crude samples: differential glycan profiling of lec mutants. *J. Biochem.* **139**, 323–327
35. Critchley, P. and Clarkson, G.J. (2003) Carbohydrate-protein interactions at interfaces: comparison of the binding of *Ricinus communis* lectin to two series of synthetic glycolipids using surface plasmon resonance studies. *Org. Biomol. Chem.* **1**, 4148–4159
36. Bhattacharyya, L., Haraldsson, M., and Brewer, C.F. (1988) Precipitation of galactose-specific lectins by complex-type oligosaccharides and glycopeptides: studies with lectins from *Ricinus communis* (agglutinin I), *Erythrina indica*, *Erythrina arborescens*, *Abrus precatorius* (agglutinin), and *Glycine max* (soybean). *Biochemistry* **27**, 1034–1041
37. Kamekawa, N., Hayama, K., Nakamura-Tsuruta, S., Kuno, A., and Hirabayashi, J. (2006) A combined strategy for glycan profiling: a model study with pyridylaminated oligosaccharides. *J. Biochem.* **140**, 337–347
38. Kaladas, P.M., Kabat, E.A., Iglesias, J.L., Lis, H., and Sharon, N. (1982) Immunochemical studies on the combining site of the D-galactose/*N*-acetyl-D-galactosamine specific lectin from *Erythrina cristagalli* seeds. *Arch. Biochem. Biophys.* **217**, 624–637
39. Teneberg, S., Angstrom, J., Jovall, P.A., and Karlsson, K.A. (1994) Characterization of binding of Galβ4GlcNAc-specific lectins from *Elythrina cristagalli* and *Erythrina coralodendron* to glycosphingolipids. *J. Biol. Chem.* **269**, 8554–8563
40. Kulkarni, K.A., Srivastava, A., Mitra, N., Sharon, N., Surolia, A., Vijayan, M., and Suguna, K. (2004) Effect of glycosylation on the structure of *Erythrina corallodendron* lectin. *Proteins* **56**, 821–827
41. Elgavish, S. and Shaanan, B. (1998) Structures of the *Erythrina corallodendron* lectin and of its complexes with mono- and disaccharides. *J. Mol. Biol.* **277**, 917–932