Volume 330, number 2, 133–136 FEBS 12952 September 1993

Modification by site-directed mutagenesis of the specificity of *Erythrina corallodendron* lectin for galactose derivatives with bulky substituents at C-2

Rafael Arango[a], Esperanza Rodriguez-Arango[a], Rivka Adar[a], David Belenky[a], Frank G. Loontiens[b], Shmuel Rozenblatt[c], Nathan Sharon[a,*]

[a]*Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel*
[b]*Department of Biochemistry, Physiology and Microbiology, Faculty of Science, State University of Ghent, B-9000 Ghent, Belgium*
[c]*Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel*

Received 23 July 1993

Examination of the three-dimensional structure of *Erythrina corallodendron* lectin (ECorL) in complex with a ligand (lactose), the first of its kind for a Gal/GalNAc-specific lectin [(1991) Science 254, 862–866], revealed the presence of a hydrophobic cavity, surrounded by Tyr^{108} and Pro^{134}–Trp^{135}, which can accommodate bulky substituents such as acetamido or dansylamido (NDns) at C-2 of the lectin-bound galactose. Comparison of the primary sequence of ECorL with that of soybean agglutinin, specific for galactose and its C-2 substituted derivatives, and of peanut agglutinin, specific for galactose only, showed that in soybean agglutinin, Tyr^{108} is retained, and Pro^{134}–Trp^{135} is replaced by Ser–Trp, whereas in peanut agglutinin, the former residue is replaced by Thr and the dipeptide by Ser–Glu–Tyr–Asn. Three mutants of ECorL were therefore constructed: L2, in which Pro^{134}–Trp^{135} was replaced by Ser–Glu–Tyr–Asn; Y108T, in which Tyr^{108} was replaced by Thr and the double mutant L2; Y108T. They were expressed in *Escherichia coli*, as done for recombinant ECorL [(1992) Eur. J. Biochem. 205, 575–581]. The mutants had the same hemagglutinating activity as native or rECorL. Their specificity for galactose, GalNAc and MeβGalNDns was examined by inhibition of hemagglutination and of the binding of the lectin to immobilized asialofetuin; in addition, their association constants with MeαGalNDns and MeβGalNDns were measured by spectrofluorimetric titration. The results showed that Y108T had essentially similar specificity as the native and recombinant lectins. The affinity of L2 and L2;Y108T for galactose was also the same as ECorL, but they had a lower affinity for GalNAc and markedly diminished affinity for the dansyl sugars (up to 43 times, or 2 kcal, less). This appears to be largely due to steric hindrance by the two additional amino acids present in the cavity region in these mutants. Our findings also provide an explanation for the inability of PNA to accommodate C-2-substituted galactose derivatives at its primary subsite.

Binding site; *N*-Acetylgalactosamine; *N*-Dansylgalactosamine; Peanut agglutinin; Sugar specificity; Three-dimensional structure

1. INTRODUCTION

Legume lectins exhibit a considerable degree of sequence homology [1,2] and, to the extent examined, possess nearly superimposable tertiary structures [3–5], but they differ in their carbohydrate specificities. For example, lectins of this family that are specific for galactose differ considerably in their relative affinity for 2-substituted galactose derivatives. Thus, *Erythrina corallodendron* lectin (ECorL) binds *N*-acetylgalactosamine slightly better than galactose and shows a strong preference for *N*-dansylgalactosamine [6,7]; soybean agglutinin (SBA) binds the former monosaccharide considerably better than galactose, and shows also pronounced preference for *N*-dansylgalactosamine [8], whereas peanut agglutinin (PNA) does not bind *N*-acetylgalactosamine or *N*-dansylgalactosamine in its primary subsite [9,10].

Examination of the three-dimensional structure of the ECorL–lactose complex [3] gives us some clues to understand the structural basis of the above mentioned differences. An open space or cavity can be seen, close to the 2-OH of the bound galactose, that could readily accommodate bulky substituents (Fig. 1). This space is bordered by Gly^{107}–Tyr^{108} (loop 1) and Asn^{133}–Pro^{134}–Trp^{135}–Asp^{136} (loop 2). Asn^{133} and Asp^{136} are invariant in all the known primary sequences of the legume lectins and Gly^{107} in most of them [1,2,11,12]. Moreover, these residues occupy identical positions in the three-dimensional structures examined to date [3,5]. It is therefore unlikely that they are responsible for the differences in sugar specificity. Although the three-dimensional structures of SBA and PNA are not yet known, comparison of their primary sequences [11–13] with that of ECorL [14,15] shows differences in the regions of the above mentioned loops (Fig. 1). In loop 1, Tyr^{108} in ECorL is retained in SBA, but in PNA it is replaced by threonine. In loop 2, the main difference is the presence of two

*Corresponding author. Fax (972) (8) 468 256.

Abbreviations: ECorL, *Erythrina corallodendron* lectin; rECorL, recombinant ECorL; PNA, peanut agglutinin; SBA, soybean agglutinin; MeαGalNDns and MeβGalNDns, methyl α- and β-*N*-dansylgalactosaminide; PCR, polymerase chain reaction.

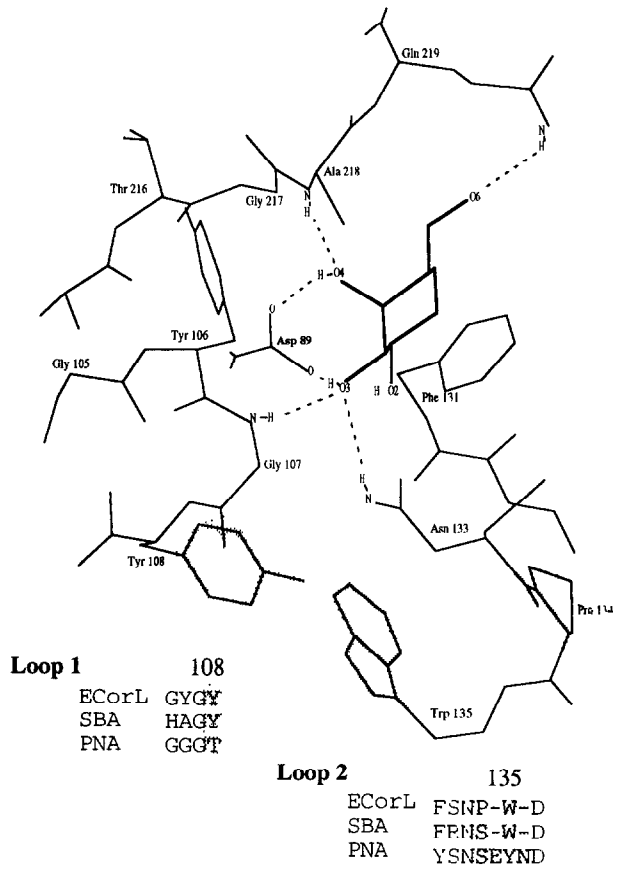

Fig. 1. Region of the combining site of ECorL with bound galactose, based on the 3D structure of the lectin–lactose complex [3]. Hydrogen bonds are indicated by dashed lines. Side chains of residues that may be in contact with buky substituents at the C-2 of galactose are shadowed. The insets present the sequences of loop 1 and loop 2 in ECorL, where mutations were done, and the homologous residues in SBA [13] and PNA [11,12] (shadowed).

extra amino acids in PNA. In order to test which of these may account for the differences in specificity of these lectins for galactose derivatives carrying bulky substituents at C-2, we constructed two single point mutants and a double mutant that mimic the binding site of PNA, expressed them in *E. coli*, as done by us for the recombinant lectin [16], and studied their specificity.

2. MATERIALS AND METHODS

2.1. *Materials*

Native ECorL from mature dry seeds was prepared by affinity chromatography on lactose–Sepharose [6]. Vent DNA Polymerase was from New England Biolabs; pCR-1000 was from Invitrogen (San-Diego, CA) and other enzymes were from Amersham Corp. (Buckinghamshire, England), Pharmacia (Uppsala, Zweden) and New England Biolabs (Beverly, MA). Polyclonal antibodies against ECorL were from rabbits. Goat anti-rabbit alkaline phosphatase conjugate was from BioMakor (Rehovot, Israel). DNA sequencing reagents were from US Biochemical Corp. (Cleveland, OH). Synthetic oligonucleotides were prepared by the Chemical Services of the Weizmann Institute of Science. Sugars were from commercial sources, of the highest purity available. Methyl α- and β-*N*-dansylgalactosaminide were a gift from Dr. Willy Kinzy (CIBA-GEIGY, Basel).

2.2. *Lectin mutants*

Mutations were done using PCR according to standard methods [17], with pUC-ECorL as target [14]. Primary PCRs were performed in pairs containing the mismatched oligonucleotide and the corresponding 3′ or 5′ oligonucleotide. The primer at the 5′ end was the standard M13 reverse primer [18] and that at the 3′ end was **TGGGATCC**ACATGACAGATAATAACTAACGAC located at positions 872–896 of the ECorL cDNA [15]. The latter primer contains 8 additional nucleotides (bold letters) that provide a *Bam*HI restriction site. Reactions were performed in a volume of 100 μl containing a PCR buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), dNTPs (200 μM each), 50 pmol of 3′ and 5′ primers, 200 ng plEcL-C (a plasmid containing ECorL cDNA) [16], 2.5 U of Vent DNA Polymerase and two drops of mineral oil. Twenty five cycles were performed, each consisting of denaturation at 95°C for 30 s, annealing at 42°C for 40 s, and extension at 72°C for 1 min. The extension time of the last cycle was 10 min to ensure that all single-stranded DNA had been copied or re-annealed.

After completion of the primary PCRs, aliquots of 20 μl were size fractionated on 1% low melting agarose gel. Each pair of fragments of the expected size was cut out from the gel, mixed and heated at 65°C in order to melt the agarose. The melted agarose (5 μl) containing the primary fragments served as target DNA for the secondary PCR with the 3′ and 5′ oligonucleotides mentioned above as primers, under the same conditions as for the primary reactions. The final mutated PCR product was cloned into the vector pCR-1000 [19] following the suppliers protocol. Confirmation of the mutations was done by sequencing of the mutated cDNAs in the corresponding region [20]. The mutant cDNAs were cloned into pET-3d by standard ligation methods using *Nco*I–*Bam*HI restriction sites [21]. Expression and re-folding of lectin mutants were performed as described for rECorL [16]. Additional purification of the bacterially produced lectins was by gel-filtration on a Sephadex G-150 column (1.5 × 90 cm) equilibrated with 10 mM Tris–HCl, pH 7.5, 0.15 M NaCl; elution was with the same buffer. Only the slow-migrating peak, which was eluted at approximately the same volume as rECorL (~56 kDa), was collected (40–95% of the applied protein). The final yield was 3–7 mg/l.

2.3. *Protein estimation*

Protein was estimated by measurement of absorption at 280 nm, using the optical factor for *Erythrina cristagalli*, which is 1.53 $mg^{-1} \cdot cm^2$ [22].

2.4. *Hemagglutinating activity and inhibition of hemagglutination*

These were assayed by the serial dilution method in microtiter plates using a suspension of sialidase-treated [9] human type-O erythrocytes.

2.5. *Lectin-asialofetuin binding assay*

The method employed was essentially as described for the 14 kDa β-galactoside-binding human lectin [23]. Briefly, the native or bacterially produced lectins (250–500 ng) were reacted with asialofetuin (20 μg) immobilized on a Nunc Maxisorp immunoplate in the presence of various concentrations of different sugars, and the bound lectin was quantitated by a double antibody method using anti-ECorL rabbit antiserum and alkaline phosphatase-conjugated goat anti-rabbit IgG. Development was with *p*-nitrophenyl disodium phosphate. Coating of plates with asialofetuin was overnight at 4°C, blocking was with 0.5% bovine serum albumin in phosphate-buffered saline (PBS; 10 mM KH_2PO_4, 0.04 M Na_2HPO_4, 0.9% NaCl, pH 7.4) and washings after each step were done with PBS. The concentration of sugar required for 50% inhibition (I_{50}) was calculated, taking the quantity of lectin bound in the absence of inhibitor as 100%.

2.6. *Electrophoretic techniques*

SDS-PAGE was performed according to the method of Laemmli [24] with 4% stacking gel and 12% separating gel. Electrophoretic

transfer of proteins from the gel to the nitrocellulose membrane was according to Nielsen et al. [25] and was followed by immunodetection. The polyclonal anti-ECorL antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit antibody, followed by development with 5-bromo-4-chloro-indole-phosphate and nitro-blue tetrazolium.

2.7. *Spectrofluorimetric titrations*

These were performed at 23°C with MeαGalNDns and MeβGalNDns, as described [7].

3. RESULTS AND DISCUSSION

Upon SDS-PAGE (Fig. 2) the L2, Y108T and L2;Y108T mutants (which are devoid of carbohydrate) migrated as single or double bands close to rECorL (apparent M_r 28 kDa), but somewhat slower than the native lectin (apparent M_r 30 kDa) which is a glycoprotein (Fig. 2). It should be noted that purified native *Erythrina* lectins often give two closely migrating bands on SDS-PAGE [6,26]. This is probably due to C-terminal processing of the lectins, which occurs both in the plant and in *E. coli* (N.M. Young, private communication). The mutants reacted with the polyclonal (Fig. 2) as well as with a monoclonal (data not shown) anti-ECorL antibody [27], and bound to immobilized asialofetuin, as did native and recombinant ECorL. They had the same hemagglutinating activity as ECorL or rECorL (Table I). As sialidase-treated human erythrocytes expose galactose residues on their surface, it would appear that no major alterations in the affinity of the mutants for galactose occurred with the amino acid changes. This conclusion is supported by the results of the inhibition of hemagglutination and of the binding of the lectin to asialofetuin, which show that the affinity of the different lectins for galactose was the same, within experimental error (Table II). In both assays Y108T bound *N*-acetylgalactosamine and

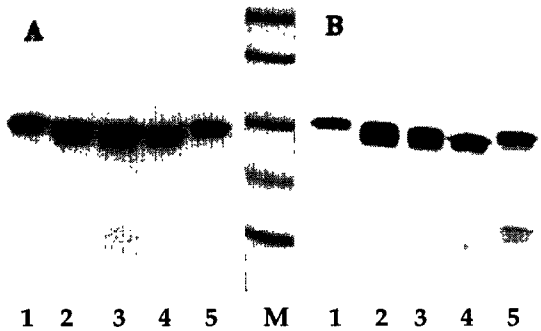

Fig. 2. SDS-PAGE and Western blot of ECorL, rECorL and its mutants. Lectins (3–5 μg), obtained from inclusion bodies and purified on Sephadex G-150, were analyzed by electrophoresis on a 12% SDS-PAGE and stained either with Coomassie brilliant blue (A) or transferred to nitrocellulose membrane and probed with polyclonal rabbit anti-ECorL antibodies (B). Lanes: 1, native ECorL (extracted from seeds); 2, rECorL; 3, L2; 4, Y108T; 5, L2;Y108T; M, molecular weight markers: bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; lysozyme 14.3 kDa.

Table I

Hemagglutinating activity of ECorL and the bacterially produced mutants and their inhibition by different sugars

Lectin	MHC[a]	Gal(MIC)[b]	Relative inhibitor activity[c]	
			GalNAc	MeβGalNDns
ECorL	5.3	25	2	500
rECorL	5.5	25	2	400
Y108T	3.9	12.5	2	400
L2	5.7	12.5	0.5	43
L2;Y108T	5.8	12.5	0.5	20

[a] MHC, minimal lectin concentration (μg/ml) that gives hemagglutination; in different assays this value ranged between 1–6 μg/ml, but was always the same for rECorL and the mutants, as for ECorL.
[b] MIC, minimal sugar concentration (mM) that gives inhibition.
[c] The inhibitory activity of galactose has been arbitrarily set to 1.

MeβGalNDns similarly to ECorL and rECorL. Remarkably, L2 and L2;Y108T bound these compounds about 2- to 4-times and 10-times weaker, respectively. The decrease in affinity of the latter two mutants for dansyl-substituted galactosamine was confirmed in the spectrofluorimetric binding assay (Table III). The results showed that the association constants of MeαGalNDns and MeβGalNDns with Y108T were only 1.3- to 2.7-times lower than with rECorL, whereas with L2 and L2;Y108T they were markedly lower, up to 43-times for L2 and MeαGalNDns. The slight binding preference of rECorL and the mutants for the α-anomer is constant. Also, the molar fluorescence change of the dansyl derivatives seems to be unaffected by the mutations, indicating that the environment in the cavity remained hydrophobic [7], and is similar to that observed in *Erythrina cristagalli* lectin [22].

We have thus been able to modifiy the specificity of ECorL for GalNAc and GalNDns by site-directed mutagenesis. The results also show that loop 2 of the combining region of ECorL is responsible for the enhanced binding of galactose derivatives with bulky groups (acetamido or dansylamido) at C-2 displayed by this lectin. Our data give us ground to believe that the differences in sugar specificity of ECorL and SBA on the one

Table II

Comparison of the I_{50}[a] of different sugars with ECorL, rECorL and its mutants, measured by a lectin–asialofetuin binding assay

Lectin	Gal	GalNAc	MeβGalNDns
ECorL	5.70 ± 0.52	2.13 ± 0.85	0.036 ± 0.01
rECorL	4.42 ± 0.66	1.32 ± 0.33	0.016 ± 0.01
Y108T	6.30 ± 0.57	2.60 ± 0.52	0.022 ± 0.01
L2	5.43 ± 1.8	10.13 ± 0.32	0.23 ± 0.07
L2;Y108T	7.10 ± 1.0	4.60 ± 0.5	0.19 ± 0.09

[a] I_{50} = concentration (mM) required for 50% inhibition of the binding of the lectin to asialofetuin.

Table III

Characteristics for binding of MeαGalNDns and MeβGalNDns with ECorL, rECorL and its mutants

Lectin	MeαGalNDns			MeβGalNDns			β/α
	ΔF1	$K_a \times 10^{-5}$ (M^{-1})	ΔΔG′ (kcal·mol^{-1})	ΔF1	$K_a \times 10^{-5}$ (M^{-1})	ΔΔG′ (kcal·mol^{-1})	ΔΔG′
ECorL	3.75	3.58	–	5.36	1.00	–	0.76
rECorL	3.91	2.16	0	5.50	1.16	0	0.37
Y108T	3.92	1.31	0.30	4.90	0.78	0.24	0.31
L2	3.74	0.084	1.94	4.27	0.056	1.81	0.32
L2;Y108T	3.87	0.206	1.10	5.49	0.12	1.35	0.32

The titration parameters, ΔF1 (relative increase in ligand fluorescence) and K_a (association constant) were obtained by simulation of the titration curve, that corresponds to a simple binding equilibrium. $\Delta G'$ (at 23°C) = $-RT\ln K_a$; $\Delta\Delta G'$ is the difference in binding free energies between a mutant lectin and rECorL or, for a given lectin, between the β and α anomers.

hand and of PNA (at the primary subsite) on the other are largely due to steric hindrance by the additional amino acids present in loop 2 of the sugar binding site of the latter lectin.

Acknowledgements: The authors thank Dr. Boaz Shanaan for providing the coordinates of the ECorL–lactose complex; Dr. Willy Kinzy for the gift of MeαGalNDns and MeβGalNDns; Mrs. Hedva Latter for the polyclonal anti-ECorL, and Dr. Halina Lis for her help with the preparation of the manuscript. This research was supported in part by the Forchheimer Center for Molecular Genetics at the Weizmann Institute of Science and by The Basic Research Foundation administered by the Israel Academy of Sciences and Humanities. D.B. thanks FEBS for a short-term fellowship which enabled him to perform the spectrofluorimetric titrations in Ghent.

REFERENCES

[1] Sharon, N. and Lis, H. (1990) FASEB J. 4, 3198–3208.
[2] Konami, Y., Yamamoto, K., Osawa, T. and Irimura, T. (1992) FEBS Lett. 404, 129–135.
[3] Shaanan, B., Lis, H. and Sharon, N. (1991) Science 254, 862–866.
[4] Sharon, N. (1993) Trends Biochem. Sci. 18, 221–227.
[5] Delbaere, L.T.J., Vandonselaar, M., Prasad, L., Quail, J.W., Wilson, K.S. and Dauter, Z. (1993) J. Mol. Biol. 230, 950–965.
[6] Lis, H., Joubert, F.J. and Sharon, N. (1985) Phytochemistry 24, 2803–2809.
[7] Kinzy, W.R., Belenky, D.M., Loontiens, F.G. and Sharon, N. (1992) Glycoconjugate J. 9, 225–227.
[8] De Boeck, H., van Tilbeurgh, H., Sharon, N. and Loontiens, F.G. (1984) J. Biol. Chem. 259, 7067–7074.
[9] Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. (1975) J. Biol. Chem. 250, 8518–8523.
[10] Swamy, M.J., Gupta, D., Mahanta, S.K. and Surolia, A. (1991) Carbohydr. Res. 213, 59–67.
[11] Rodriguez-Arango, E., Arango, R., Adar, R., Galili, G. and Sharon, N. (1992) FEBS Lett. 307, 185–189.
[12] Young, N.M., Johnston, R.A.Z. and Watson, D.C. (1991) Eur. J. Biochem. 196, 631–637.
[13] Vodkin, L.O., Rhodes, P.R. and Goldberg, R.B. (1983) Cell 34, 1023–1031.
[14] Adar, R., Richardson, M., Lis, H. and Sharon, N. (1989) FEBS Lett. 257, 81–85.
[15] Arango, R., Rozenblatt, S. and Sharon, N. (1990) FEBS Lett. 264, 109–112.
[16] Arango, R., Adar, R., Rozenblatt, S. and Sharon, N. (1992) Eur. J. Biochem. 205, 575–581.
[17] Higuchi, R., Krummel, B. and Saiki, R.K. (1988) Nucleic Acids Res. 16, 7351–7367.
[18] Messing, J. (1983) New M13 Vectors for Cloning, Academic Press, New York.
[19] David, A., Mead, N., Pey, K., Herrnstadt, C., Marcil, R.A. and Smith, L.M. (1991) Bio/Technology 9, 657–663.
[20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[21] Ausubel, F.M., Brent, R., Kingstong, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Strhul, K. (1988) Current Protocols in Molecular Biology, Greene and Wiley-Interscience, New York.
[22] De Boeck, H., Loontiens, F.G., Lis, H. and Sharon, N. (1984) Arch. Biochem. Biophys. 234, 297–304.
[23] Hirabayashi, J. and Kasai, K. (1991) J. Biol. Chem. 266, 23648–23653.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Nielsen, P.J., Manchester, K.L., Towbin, H., Gordon, J. and Thomas, G. (1982) J. Biol. Chem. 257, 12316–12321.
[26] Iglesias, J.I., Lis, H. and Sharon, N. (1982) Eur. J. Biochem. 123, 247–252.
[27] Belenky, D. and Sharon, N. (1991) 15th Int. Congr. Biochem. Jerusalem, Israel, Aug. 4–8. Abstract, pp. 331.