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CARBOHYDRATE-BINDING SPECIFICITY OF SILKWORM LECTIN*

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ABSTRACT

The binding specificity of a lectin from the hemolymph of silkworm larvae was examined quantitatively by taking advantage of the fluorospectrophotometric properties of the lectin. On excitation at 280 nm, the lectin fraction gave a fluorescence-emission spectrum centered at 336 nm, which was attributable to tryptophan residues. The fluorescence could be completely quenched by the addition of specific saccharides. The affinity constants of the silkworm lectin with specific saccharides were calculated from the changes in intensities of fluorescence-difference spectra induced by the saccharides. The silkworm lectin had the highest affinity for dermatan sulfate and hyaluronic acid, followed by protuberic acid, heparin, and chondroitin sulfate A. Among monosaccharides tested, only D-glucuronic acid and *N*-acetylneuraminic acid induced weak but significant quenching, and their affinity constants were found to be low. These results indicate that the silkworm lectin has a strong affinity for carboxyl groups, especially α-L-iduronic acid residues, in the saccharides. In most cases, sulfate groups on the saccharides interfere with the specific interactions.

INTRODUCTION

Proteins having hemagglutinating activity are widely distributed in the hemolymphs of invertebrate species[1,2] as well as in the serum of vertebrates[3,4]. Most of the hemolymph lectins from invertebrates are reported to be specific for 2-acetamido-2-deoxy-D-galactose/D-galactose[5–7] or sialic acid[8–10], and are generally considered to be involved with invertebrate immunity[2]. However, the hemolymph of silkworm larvae has been recently reported to contain a high-molecular-weight lectin that is inhibited by heparin[11]. The specific activity of the lectin changes during development from the fifth instar to the adult, with a transient increase just before pupation. This silkworm lectin might thus be involved in cellular adhesiveness during differentiation, rather than immunity.

To understand the biological role of lectins, an understanding of the specificity of lectins for saccharides is essential. As little is known about the specificities

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of lectins for glycosaminoglycans, we studied the specificity of plant lectins and chicken liver lectin for glycosaminoglycans by ultraviolet-difference spectroscopy[12] and fluorescence-difference spectroscopy[13,14], which are more-quantitative and reproducible methods for detecting the interactions between lectins and saccharides than the hemagglutination-inhibition assay. In this work, the specificities of the silkworm lectin were extensively studied by fluorescence-difference spectroscopy and compared with those of chicken liver lectin and fibronectin.

EXPERIMENTAL

Materials. — Dermatan sulfate (DS) from rooster comb (C/O, 0.46; sulfate, 14.7%) was obtained from Seikagaku Kogyo (Tokyo, Japan), and further purified by alcohol fractionation before use. Hyaluronic acid from rat skin was prepared as described previously[15]. Heparin (C/O, 3.83; sulfate, 30.3%) was purchased from Wako Pure Chemicals Ind. (Osaka, Japan). Protuberic acid (L-iduronic acid/D-glucuronic acid = 1:2; molecular weight 100,000) from *Kobayasia nipponica* was a generous gift from Prof. T. Miyazaki (Tokyo College of Pharmacy)[16]. Alginic acid, and heparan sulfate (1.25M NaCl fraction from bovine kidney, total sulfate, 16.0%; *N*-sulfate, 7.29%) were obtained from Seikagaku Kogyo. Chitin sulfate (sulfate, 26.5%) was prepared from the tests of tunicates[17]. Dextran sulfate (S, 17.1%; mol.wt. 500,000) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Chondroitin sulfate A (ChS-A) from whale cartilage (sulfate, 14.1%) and chondroitin from squid skin (sulfate, <0.7%) were prepared as described previously[18,19]. Poly(L-glutamic acid) sodium salt was purchased from Sigma Chemical Co. (U.S.A.) and polyphosphoric acid was from Wako Pure Chemicals Ind.

Preparation of lectin. — *Bombyx mori* larvae were reared on mulberry leaves until about 0–2 days before pupation, and then hemolymph was collected from the abdominal legs. Silkworm lectin was purified according to the method of Suzuki and Natori[11] with a slight modification. To suppress the tyrosinase activity, 1/10 vol. of toluene or 1/4 vol. of bleeding buffer (75mM Na_2HPO_4/KH_2PO_4–75mM NaCl (pH 7.2)–0.5mM *p*-nitrophenyl *p*-guanidinobenzoate–5mM sodium diethyldithiocarbamate) was added beforehand to the collecting tube. The combined hemolymph was centrifuged at 3000*g* to remove hemocytes, and the supernatant solution (1 mL) was applied to a column (3.4 × 50.5 cm) of Toyopearl HW-75 equilibrated with 10mM Tris-HCl (pH 7.2)–4mM 2-mercaptoethanol (MTB).

Hemagglutination assay. — Lectin activity was assayed with trypsinized and glutaraldehyde-treated rabbit or sheep erythrocytes[20]. Hemagglutinating activity was determined by using serial 2-fold dilutions of the hemolymph or the lectin fraction in microtiter V-plates (Cooke Engineering). A 4% erythrocyte suspension in MTB (25 μL) and 25 μL of a sample solution were mixed in each well and incubated for 1 h at room temperature. The titer was taken as the reciprocal of the highest dilution of the sample causing hemagglutination, and the specific activity is expressed as the titer divided by the protein concentration (mg/mL) of the sample.

The inhibitory activity of the haptens was determined by incubation of the lectin solution (titer = 4) with serial 2-fold dilutions of the hapten solutions for 2 h at room temperature, and then adding an erythrocyte suspension. The lowest concentration that decreased the hemagglutination titer by one step was taken as the concentration of hapten inhibitor that caused 50% inhibition of the lectin activity.

Fluorescence-difference spectroscopy. — Fluorescence data for the lectin fraction were obtained with a Hitachi type 650-60 fluorescence spectrophotometer equipped with a chart recorder. Correction of emission spectra with rhodamine B and measurements of fluorescence-difference spectra were performed with a microcomputer interfaced to the fluorescence spectrophotometer. The lectin solution in MTB (A_{280} = 0.1) and various saccharide solutions dissolved in the same buffer (0.3–40 mg/mL) were used. All measurements were performed at room temperature (22–25°), from 300 to 400 nm, at a scan speed of 180 nm/min with excitation at 280 nm. For determination of the affinity constants (K_a), the peak intensities of the difference spectra were determined as a function of saccharide concentration. Corrections were made for the fluorescence of polysaccharides as necessary.

RESULTS AND DISCUSSION

Lectin preparation. — When the hemolymph from the larvae just before pupation was subjected to gel filtration on a column of Toyopearl HW-75, hemagglutinating activities were excluded, as shown in Fig. 1(A). The separation of the lectin from other proteins was greatly improved by use of a column of Toyopearl HW-75 instead of one[11] of Sephacryl S-300. The specific activity increased 1600-fold over that of the original hemolymph and the recovery of the lectin activity was 90%. The combined lectin fractions showed a u.v. absorption maximum at 275 nm, and were considered to be free from nucleic acid, unlike chicken liver lectin[14]. When the hemolymph from immature larvae was used, the lectin activities were included in the gel, as shown in Fig. 1(B), suggesting that the molecular weight of the lectin increases just before pupation. This might explain the drastic elevation of the lectin activity at that stage[11], probably caused by molecular aggregation.

The SDS–polyacrylamide gel electrophoresis of this fraction showed one major band at mol.wt. 85,000, with several minor bands. Further purification by concanavalin A–Sepharose or gelatin–Sepharose was unsuccessful because the lectin was not adsorbed on these columns, suggesting that it differs from the lectin reported by Kato *et al.*[21] and fibronectin[22].

Hemagglutination-inhibition assay. — Concentrations of about 1–10μM of protuberic acid, DS, chitin sulfate, chondroitin, and heparin produced 50% inhibition of the hemagglutination by the silkworm lectin. None of the monosaccharides tested, including *N*-acetylneuraminic acid (0.01M), and D-glucuronic acid and other neutral sugars (0.1M) had inhibitory activity. These results show

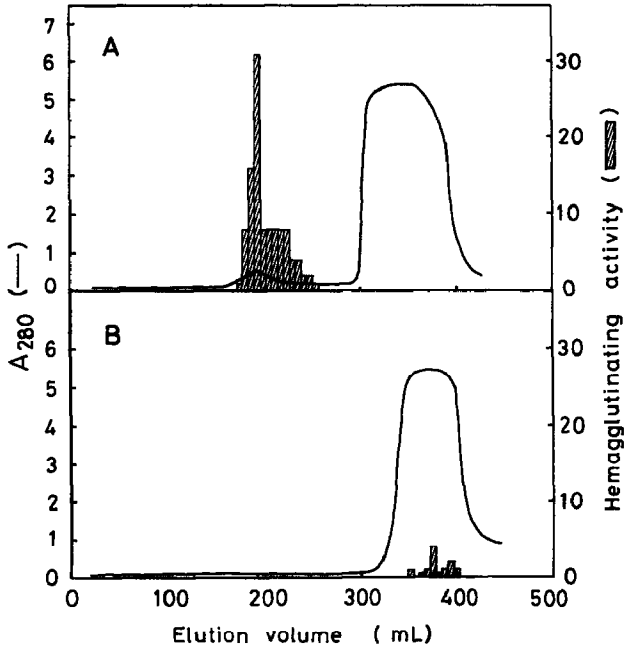

Fig. 1. Gel chromatography on Toyopearl HW-75. A, the hemolymph obtained from larvae just before pupation; B, the hemolymph obtained from immature larvae (4 days before pupation). Gel chromatography was performed as described in the text.

some discrepancy from those obtained by Suzuki and Natori[11]. However, this lectin seems to be essentially the same as the one reported by Suzuki and Natori, whose hemagglutination-inhibition activity fluctuated depending on the batch of erythrocytes treated.

Fluorescence-spectroscopic studies. — On excitation at 280 nm, the lectin fraction showed a fluorescence-emission spectrum with a maximum at 336 nm, which was attributable to tryptophan residues. The fluorescence-emission intensity was markedly quenched by the addition of DS (<1mM) and other acidic polysaccharides, whereas these changes were not observed on addition of *N*-acetyl-D-glucosamine or neutral sugars (>30mM), as shown in Fig. 2. It was therefore assumed that the quenching was due to a specific interaction between the acidic saccharides and the lectin. The quantitative and reproducible analysis of the interaction by fluorescence-difference spectroscopy is, therefore, applicable to the lectin sample despite the fact that it still contains contaminants. The fluorescence-quenching depended on the concentration of DS, and finally reached perfect quenching, as shown in Fig. 3. Hill's plots[14] were linear with a slope of 1 ±0.05, as shown in Fig. 4. The K_a value of this lectin with DS was calculated from the intercept on the abscissa.

The K_a values obtained in this way and molecular weights of the saccharides are shown in Table I. Molecular weights of the polysaccharides were estimated from their reducing values. Among monosaccharides tested, *N*-acetylneuraminic

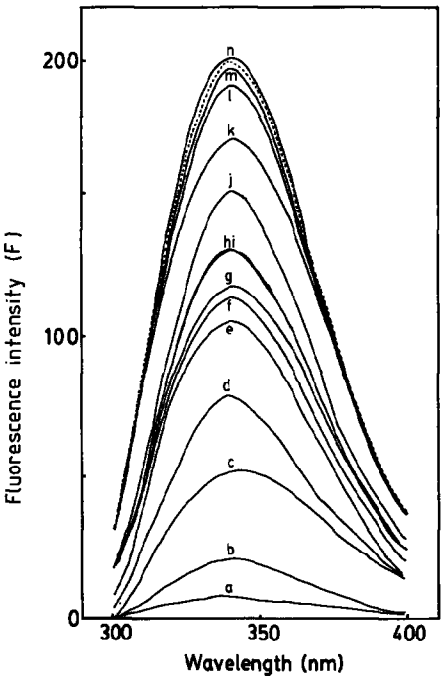

Fig. 2. Fluorescence-emission spectra of DS–lectin in the presence of various saccharides. ----, Spectrum of DS–lectin alone; ——, spectra in the presence of saccharides. The final concentrations of various sugars are indicated in parentheses. Corrections were made for the fluorescence of polysaccharides. a, DS (0.10mM); b, chondroitin (0.19mM); c, chitin sulfate (0.19mM); d, hyaluronic acid (0.024mM); e, protuberic acid (0.030mM); f, *N*-acetylneuraminic acid (23mM); g, Alginic acid (0.19mM); h, heparin (0.20mM); i, ChS-A (0.22mM); j, D-glucuronic acid (28mM); k, dextran sulfate (0.015mM); l, 2-acetamido-2-deoxy-D-galactose (44mM); m, D-glucose (35mM); n, D-galactose (500mM), 2-acetamido-2-deoxy-D-glucose (500mM), poly(L-glutamic acid) (1.4mM), and polyphosphoric acid (1.5mM).

acid and D-glucuronic acid induced weak but significant quenching, but neutral sugars did not, as shown in Fig. 2. These results indicate that the lectin can interact with the sugar residues having carboxyl groups. Acidic polysaccharides have generally high K_a values, and DS, which consists of α-L-iduronic acid and 2-acetamido-2-deoxy-4-*O*-sulfo-D-galactose residues, has the highest K_a value of the saccharides tested. As the K_a value of silkworm lectin with DS is higher than those with protuberic acid, which contains α-L-iduronic acid and β-D-glucuronic acid in the molar ratio of 1:2, and chondroitin sulfate A, which contains β-D-glucuronic acid and 2-acetamido-2-deoxy-4-*O*-sulfo-β-D-galactose residues, the lectin seems to have a higher affinity for α-L-iduronic acid residues, than for β-D-glucuronic acid. Among acidic polysaccharides containing β-D-glucuronic acid, the highest K_a values were observed for hyaluronic acid, this might reflect a multivalent effect, as this glycan has the highest molecular weight. The results of the fluorescence-difference spectroscopy and hemagglutination-inhibition assay were essentially in agreement, and saccharides having a high affinity to the lectin induced strong quenching of the fluorescence and could inhibit the hemagglutinating activity of the lectin.

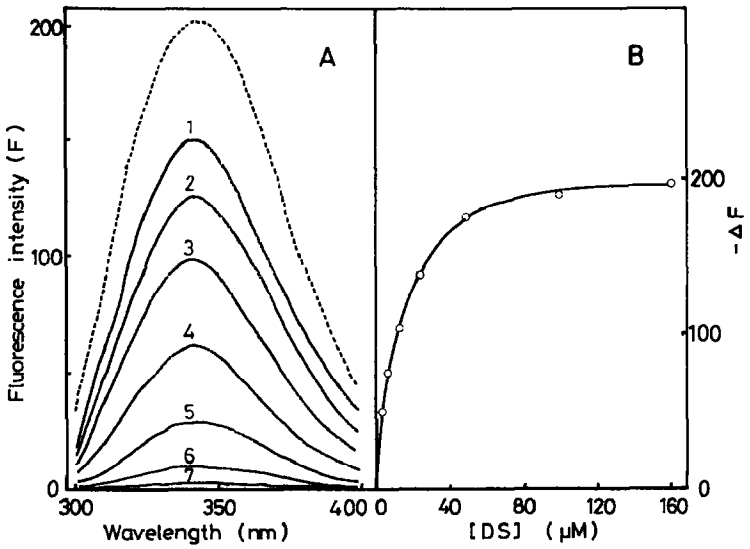

Fig. 3. Fluorescence-emission spectra of silkworm lectin induced by DS (A) and the effect of DS concentration on the difference spectra (B). The concentrations of DS were (1) 1, (2) 6, (3) 13, (4) 25, (5) 51, (6) 101, and (7) 160μM. ΔF is the difference between the fluorescence intensity at 336 nm at the saccharide concentration indicated and that of lectin alone (----).

TABLE I

VALUES OF K_a WITH VARIOUS CARBOHYDRATES

Carbohydrate	*Mol. wt.*	K_a	
		Silkworm	*Chicken liver*
Dermatan sulfate	44,000	8.4×10^4	4.2×10^4
Hyaluronic acid	104,000	5.5×10^4	1.9×10^4
Protuberic acid	100,000	4.6×10^4	3.2×10^4
Chondroitin	18,800	2.3×10^4	4.5×10^3
Chitin sulfate	35,000	1.4×10^4	—
Heparan sulfate	18,000	1.0×10^4	9.8×10^2
Heparin	30,000	3.0×10^3	1.8×10^3
Alginic acid	11,000	3.0×10^3	5.0×10^3
Chondroitin sulfate A	29,000	2.8×10^3	2.9×10^3
N-Acetylneuraminic acid	309	1×10^2	9×10^1
D-Glucuronic acid	194	9×10^0	6×10^1
N-Acetyl-D-galactosamine	221	$<3 \times 10^0$	0
D-Glucose, D-Galactose	180	0	0
N-Acetyl-D-glucosamine	221	0	0
Poly(L-glutamic acid)	14,000	0	—
Polyphosphoric acid	338	0	—
Dextran sulfate	100,000	incomplete quenching	0

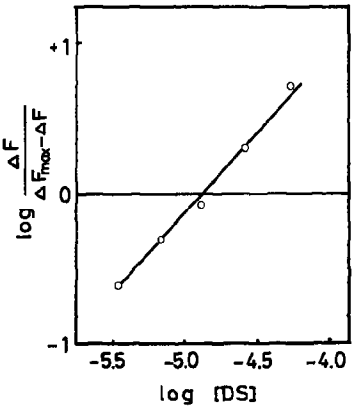

Fig. 4. Plots of $\log\Delta F/(\Delta F_{max} - \Delta F)$ versus log[DS]. The data of fluorescence difference spectra were plotted according to the equation: $\log \Delta F/(\Delta F_{max} - \Delta F) = \log[DS] + \log K_a$, where [DS] is the concentration of DS, and ΔF_{max}, the maximum intensity-difference obtained when all the lectin was in the form of a complex with DS.

The sugar specificity of silkworm lectin closely resembles that of chicken liver lectin in the affinity for carboxyl groups, especially L-iduronic acid residues, in the saccharides; but some differences are found in the reactivity toward sulfate groups in the saccharides. Correlation between the affinity constants and sulfate contents of heparin and heparan sulfate or that of chondroitin sulfate A and chondroitin, suggests that sulfate groups in the polysaccharides interfere with the specific binding to the lectin, as they do with chicken liver lectin. However, almost complete fluorescence quenching was observed by addition of chitin sulfate (>240μM). Moreover, weak fluorescence-quenching (at the maximum, 20% of perfect quenching) was caused by dextran sulfate, which was not so with chicken liver lectin. Poly(L-glutamic acid) and polyphosphoric acid failed to induce any change in the fluorescence spectrum of the lectin. This result rules out the possibility that the fluorescence changes observed were due to non-specific electrostatic interactions. The results thus suggest that sulfate groups in the polysaccharides contribute to weak binding with silkworm lectin only when there are no carboxyl groups in the saccharides.

The hemolymph of the horseshoe crab contains another type of glycosaminoglycan-reactive lectin[23]. Horseshoe crab lectin has a high affinity for *N*-acetyl groups, a weak affinity for carboxyl groups, and no affinity for sulfate groups, whereas silkworm and chicken liver lectins have a high affinity for carboxyl groups in the saccharides and no affinity for *N*-acetyl groups. The horseshoe crab lectin seems to contribute to a host-defense mechanism against bacterial infection by becoming attached to *N*-acetyl groups in the external polysaccharides and peptidoglycans on the bacterial cell-wall[23].

A class of storage proteins or glycoproteins is also reported to reach a peak hemolymph concentration just prior to pupation, to supply amino acids for the formation of adult structures[24–26]. However, from the binding ability of silkworm

lectin toward various glycosaminoglycans as mentioned here, it may be presumed that this lectin acts *in vivo* not merely as a storage protein, but as a specific recognition molecule like a vertebrate fibronectin during adult development, by binding with extracellular or cell-surface glycosaminoglycans, such as hyaluronate in the larvae[27]. To examine this possibility, the specificity of the silkworm lectin toward biomolecules other than glycosaminoglycans must be studied in more detail.

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