Purification and Characterization of the Agglutinins from the Sponge *Aaptos papillata* and a Study of Their Combining Sites†

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ABSTRACT: The lectins from the sponge *Aaptos papillata* were isolated by affinity chromatography using polyleucyl blood group A + H substance from hog stomach linings as an absorbent and eluting with 3 M $MgCl_2$. Further separation on diethylaminoethylcellulose and preparative disc electrophoresis on polyacrylamide gave the three fractions, *Aaptos* lectins I, II, and III. They were essentially homogeneous in polyacrylamide electrophoresis and sedimentation analysis; a small second component was seen in lectins I and II in immunoelectrophoresis at high concentration. The $s_{20,w}^0$ values for *Aaptos* lectins I, II, and III were 3.5, 6.0, and 5.5. By electrophoresis in sodium dodecyl sulfate with and without β-mercaptoethanol *Aaptos* lectin I showed two bands corresponding to molecular weights of 12 000 and 21 000; *Aaptos* lectins II and III gave only one band of molecular weight of 16 000. In isoelectric focusing, *Aaptos* lectin I showed bands at pH 4.7 and 5.4 and in the range between 6.8 and 7.6, while *Aaptos* lectins II and III were almost identical with bands at pH 3.8, 4.7 to 4.9, and 5.3. *Aaptos* lectin I differed from II and III in amino acid composition but the latter two were very similar. They contained no significant carbohydrate. *Aaptos* lectin I reacted best with blood group substances with terminal nonreducing *N*-acetyl-D-glucosamine residues precipitating about two-thirds of the lectin N added while blood group substances with terminal nonreducing DGalNAc were almost inactive. However, *Aaptos* lectin II was completely precipitated by blood group substances and glycoproteins containing terminal DGalNAc, DGlcNAc, or sialic acid residues. *Aaptos* lectin III had a precipitation pattern similar to *Aaptos* lectin II. DGlcNAc but not DGalNAc inhibited precipitation of *Aaptos* lectin I by blood group substances and *N,N′,N″,N‴*-tetraacetylchitotetraose was the best inhibitor and was 2000 times more active than DGlcNAc. Precipitin reactions with *Aaptos* lectin II were inhibited by equal amounts of DGlcNAc and by sialic acid which were four times more potent than DGalNAc. *N,N′,N″*-triacetylchitotriose was the best inhibitor and was 13 times better than DGlcNAc. At 37 °C three to four times higher amounts of inhibitor were necessary to inhibit precipitation of *Aaptos* lectin II than were needed at 4 °C, indicating higher affinity of blood group substance for *Aaptos* lectin II with increasing temperature. *Aaptos* lectin I was precipitated by the monofunctional hapten *p*-nitrophenyl-αDGalNAc, while *p*-nitrophenyl-βDGalNAc did not precipitate and was a good inhibitor. Both phenomena indicate involvement of hydrophobic bonds.

Though hemagglutinins seem to be very common in sponges, only few have been described and studied in detail (Dodd et al., 1968, Khalap et al., 1970, 1971; Bretting and Kabat, 1976). Most sponge lectins agglutinate human A, B, O, and AB erythrocytes to the same extent but may discriminate among animal red blood cells by titer differences. They are considered to be relatively nonspecific. The basis for such broad specificity is an important area for study; numerous plant lectins also show similar broad specificities while others may have relatively sharp specificities. Hemagglutination of human erythrocytes by crude extracts of *Cliona celata, Axinella spec.*, and *Axinella polypoides* could be inhibited by DGal and by oligosaccharides with nonreducing terminal linked DGal (Khalap et al., 1970; Bretting, 1973).

Gold et al. (1974) purified an *Axinella spec.* lectin by ion-exchange chromatography and gel filtration showing the agglutinin to be an acid protein with an isoelectric point of 3.8 to 3.9 and with a molecular weight of 15 000–18 000. In hemagglutination inhibition lacto-*N*-tetraose was the best inhibitor. Bretting and Kabat (1976) purified the lectins of *Axinella polypoides* by affinity chromatography on Sepharose 4B, using D-galactose for elution. Three immunologically unrelated lectins were separated from this material by ion-exchange chromatography on DEAE[1]-cellulose and by preparative polyacrylamide gel electrophoresis. The two main fractions had isoelectric points of 3.8 and 3.9 and molecular weights of 21 000 and 15 000, respectively. Both agglutinins precipitated with human blood group A, B, H, and precursor I substances but to different extents. Precipitin inhibition experiments revealed that both are inhibited best by terminal nonreducing DGal glycosidically linked β1→6 or by *p*-nitrophenyl-βDGal.

The properties of other sponge lectins and their specificities have not been as clearly defined. Bretting (1973) showed that two proteins could be adsorbed from the crude extract of *Aaptos papillata* by human A, B, and O red blood cells, but only one of these gave hemagglutination. This fraction was isolated by gel filtration on Sephadex G-75; its specificity remained unknown (Bretting and Renwrantz, 1974).

In this study we report the isolation and characterization of three lectins from *Aaptos papillata* as well as information on the nature and specificity of their combining sites.

Experimental Section

Materials. The sponge *Aaptos papillata* was collected in the Mediterranean near Banyuls sur Mer (France). The sponge

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[1] Abbreviations used: DEAE, diethylaminoethyl; CEA, carcinoembryonic antigen; OSM, ovine submaxillary mucin.

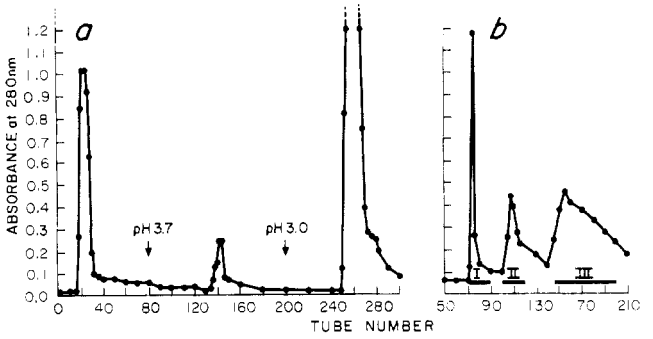

FIGURE 1: Purification of *Aaptos* lectins I, II, and III. (a) Three fractions eluted from a DEAE-cellulose column (18 × 4.5 cm) equilibrated with 0.07 M barbital buffer, pH 6.2; 250 mg was applied eluting stepwise with 0.07 M barbital buffer pH 3.7 and 3. The first peak (tubes 18–30) is *Aaptos* lectin I fraction which passed through the column. The last peak (tubes 250–290), *Aaptos* DEAE III, was separated further on polyacrylamide gel into *Aaptos* lectin II and *Aaptos* lectin III. (b) Preparative electrophoresis of the 80 mg of *Aaptos* DEAE III on 10% polyacrylamide gel. Peak I (tubes 72–80) contained unpolymerized acrylamide, peak II (tubes 100–120) is *Aaptos* III, and peak III (tubes 148–210) is *Aaptos* lectin II.

tissue was cut into small pieces and dried at 35–40 °C. The dry material was homogenized in a blender and stored at −20 °C until used. Saline extracts were prepared by stirring 90 g of sponge tissue with 1.25 l. of phosphate-buffered saline for 16 h at 4 °C. The extract was centrifuged at 2000 rpm for 30 min and then for 5 h at 15 000 rpm in the cold. The violet-red supernatant was Millipore filtered and is referred to as crude extract; 0.02% NaN_3 was added as a preservative. Blood group substances used were the previously described preparations from human ovarian cysts, hog gastric mucins, and horse stomach linings (Kabat, 1956; Schiffman et al., 1964; Lloyd and Kabat, 1968; Vicari and Kabat, 1969). The materials with blood group A or H activity from hog gastric mucin A + H were those described by Pereira and Kabat (1976), the Tij-fractions with B, I-Ma, and I-Step activity were those studied by Maisonrouge-McAuliffe and Kabat (1976), and pneumococcal S XIV polysaccharide before and after β-galactosidase treatment are those studied by Howe et al. (1958). The blood group H substance Morgan standard was from Dr. W. T. J. Morgan (1960). P_1 glycoprotein from hydatid cyst fluid was provided by Dr. W. M. Watkins (Cory et al., 1974). Ovine submaxillary mucin (OSM) was from Dr. W. Pigman (Pigman and Gottschalk, 1966); dextran N236 has been used in this laboratory. Carcinoembryonic antigen (CEA) was provided by Dr. P. Gold (Fuks et al., 1975), streptococcal group A and C polysaccharides were from Dr. R. M. Krause (Krause and McCarty, 1961, 1962), and a synthetic antigen of DGlcNAcβ1→4DGlcNAcβ1→Npoly(Asn) (called antigen A) was from Dr. T. W. Shier (1971); three preparations made on different dates were provided and stated to react differently with wheat germ agglutinin; the reason is not understood. Monosaccharides were obtained commercially (Mann Research Laboratories or Nutritional Biochemicals Corp.). The blood group oligosaccharides used were those isolated and characterized in this laboratory (Lloyd et al., 1966; Etzler et al., 1970; Maisonrouge-McAuliffe and Kabat, 1976). DGlcNAcβ1→6DGal, DGlcNAcβ1→3DGal, and DGlcNAcβ1→3[DGlcNAcβ1→6]DGal were from Dr. Z. Yosizawa (1962), *N,N'*-diacetylchitobiose was from Dr. N. Sharon (Allen et al., 1973), and *N,N',N''*-triacetylchitotriose and *N,N',N'',N'''*-tetraacetylchitotetraose were from Dr. I. J. Goldstein (Goldstein et al., 1975). Lacto-N-tetraose was provided by the late Professor Dr. Kuhn (Kuhn and Gauhe, 1962). The streptococcal group C oligosaccharide was from Drs. T. J. Kindt and J. E. Coligan (Coligan et al., 1975).

Physicochemical Methods. Analytical procedures and their sources are given in the previous paper on the lectin of *Axinella polypoides* (Bretting and Kabat, 1976). They comprise sugar and amino acid analysis, disc electrophoresis in polyacrylamide, analytical isoelectric focusing, molecular weight determination in sodium dodecyl sulfate with and without β-mercaptoethanol, immunoelectrophoresis, and hemagglutination. Sedimentation velocity was measured in a Spinco Model E ultracentrifuge equipped with ultraviolet optics and automatic temperature control through the courtesy of Dr. H. Rosenkranz and Mr. S. Rosenkranz. The sedimentation coefficient was calculated at lectin concentration with an extinction of 0.500 at 280 nm in phosphate-buffered saline (pH 5.9) from measurements at 50 740 rpm and 8.8 °C.

Separation Procedures. The immunoadsorbent polyleucyl hog A + H (PL hog A + H) was prepared by the technique of Tsuyuki et al. (1956) as described by Kaplan and Kabat (1966). Elution was carried out batchwise with 3 M $MgCl_2$. DEAE-Cellulose (Figure 1) and Bio-Gel chromatography was performed as stated previously (Bretting and Kabat, 1976). Preparative disc electrophoresis was in a Uniphor 7900 column designed for preparative purposes by LKB. Separations were achieved with a 10% acrylamide gel. Separation conditions were the same as those for the analytical runs, except that the buffer concentrations were lowered to 10 mM. After application of the 2-ml sample (80 mg of protein), electrophoresis was run at 400 V and 6 mA. After the protein had entered the gel, the voltage was increased to 800 V. Bromophenol blue was added as a marker. Elution was set to about 16 ml/h and fractions of 1.6 ml were collected. Preparative isoelectrofocusing was performed according to the directions of the manufactuer (LKB-Productor A B Sweden) with a 110-ml column at 6 °C using 2% carrier ampholytes (Ampholine), pH 3.5–10, and a sucrose gradient for 72 h at 3 W or less. Concentration was by pressure filtration with a Diaflow UM-10 membrane (Amicon Corporation, Lexington, Mass.)

Immunochemical Methods. Quantitative precipitin analyses were done by a microprecipitin technique (Kabat, 1961) employing a final volume of 250 μl, unless otherwise stated. The tubes were incubated for 1 h at 37 °C and kept for 1 week at 4 °C. Nitrogen in washed precipitates was determined by the ninhydrin procedure (Schiffman et al., 1964). Antiserum to a crude extract of *Aaptos papillata* was produced in a rabbit as described earlier (Bretting, 1973).

Results

Purification of the Lectin. The clear violet-red crude extract (500 μg of N/ml) agglutinated red blood cells of groups A_1, A_2, B, and O each to a titer of 64 and gave ten bands in immunoelectrophoresis. Quantitative precipitin analysis showed 12% of the total nitrogen to be precipitable by hog gastric mucin blood group A + H substance. Purification was carried out batchwise by affinity chromatography on PL-hog A + H. One part PL-hog A + H (300 mg) plus 5 parts of washed Celite are mixed and saturated with lectin; 100 ml of crude extract was required. After extensive washing (*A* at 280 nm less than 0.035), the lectin was extracted with 15 ml of 3 M $MgCl_2$ and separated from the insoluble immunoadsorbent by centrifugation. This was repeated five to six times until the *A* of the last supernatant was less than 0.100. The supernatants were combined and dialyzed for 3 days (six changes) against 0.05 M barbital buffer (pH 8.0) and subsequently concentrated to 3 to 4 ml. Lectin was separated from the nitrogen-containing

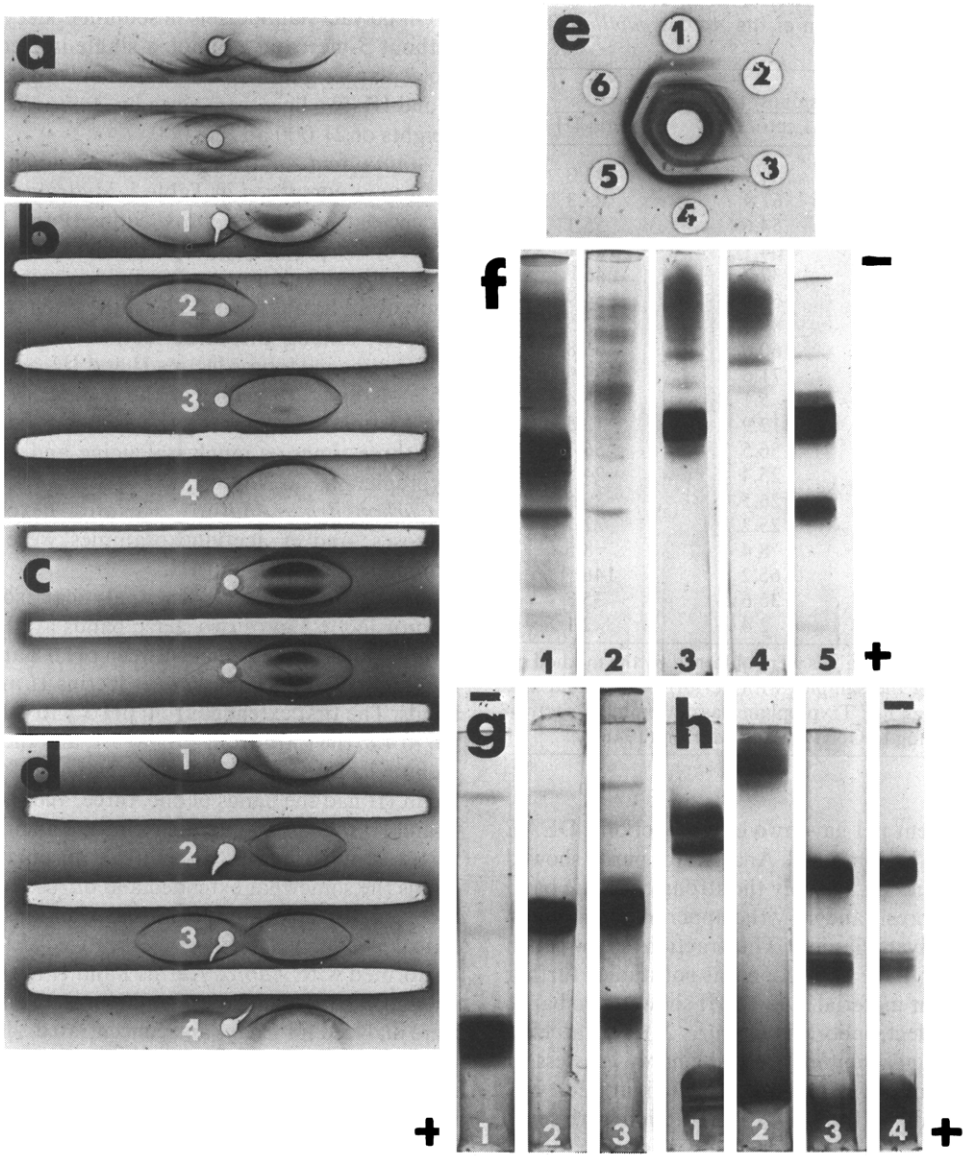

FIGURE 2: Immunoelectrophoretic, immunodiffusion, and disc electrophoresis patterns of the *Aaptos* lectins. Immunoelectrophoresis of (a) crude extract (1350 μg of N/ml) before (upper well) and after (lower well) absorption on PL-hog H + A (650 μg of N/ml); (b) the PL-hog H + A eluate (240 μg of N/ml, well 1), *Aaptos* lectin I (87 μg of N/ml, well 2), *Aaptos* lectin II (180 μg of N/ml, well 3), and *Aaptos* lectin III (115 μg of N/ml, well 4); (c) *Aaptos* lectin II (730 μg of N/ml, upper well) and *Aaptos* lectin III (350 μg of N/ml, lower well); (d) the PL-hog H + A eluate (450 μg of N/ml, well 1); the supernatant of this fraction after adding *p*-nitrophenyl-αDGalNAc and removing the precipitate (well 2); the precipitate dissolved in saline after washing three times with *p*-nitrophenyl-αDGalNAc (well 3); and the supernatant of the PL-hog H + A eluate after adding *p*-nitrophenyl-αDGlc (well 4). All troughs contain rabbit antiserum against *Aaptos* crude extract. (e) Ouchterlony test: [1] crude extract (1350 μg of N/ml); [2] crude extract after absorption on PL-hog H + A (650 μg of N/ml); [3] *Aaptos* lectin I (87 μg of N/ml); [4] *Aaptos* lectin II (180 μg of N/ml); [5] *Aaptos* lectin III (175 μg of N/ml); [6] PL-hog H + A eluate (450 μg of N/ml). The center well contains rabbit antiserum against *Aaptos* crude extract. Disc electrophoresis: (f) [1] crude extract (32 μg of N); [2] crude extract absorbed on PL-hog H + A (16 μg of N); [3] PL-hog H + A eluate (27 μg of N). (*Aaptos* lectin II is the darkest band. *Aaptos* lectin III was very faint and is not seen in f3 but is the bottom dark band in f5); [4] *Aaptos* lectin I (8.7 μg of N); [5] *Aaptos* DEAE III containing *Aaptos* lectins II and III (21 μg of N); (g) [1] *Aaptos* lectin II (18 μg of N); [2] *Aaptos* lectin III (18 μg of N); [3] *Aaptos* DEAE III (21 μg of N); (h) [1] fraction I_1 from *Aaptos* lectin I (12 μg of N); [2] fraction I_2 from *Aaptos* lectin I (21 μg of N); [3] fraction II_1 from *Aaptos* lectin II (21 μg of N); [4] fraction II_3 from *Aaptos* lectin II (16 μg of N).

buffer on a Bio-Gel P-10 column 2 × 70 cm equilibrated with 0.01 M phosphate buffer pH 8.0; 20 mg of protein of purified lectin was recovered.

The eluted material, PL-hog A + H fraction, was assayed for purity by double diffusion in agar and by immuno- and polyacrylamide electrophoresis. As seen in Figure 2, the PL-hog A + H fraction gave at least three bands in the Ouchterlony test and five bands in immunoelectrophoresis (Figure 2b) among them two strong bands, one moving toward the anode and another one moving toward the cathode. In polyacrylamide gel, separation into three major and several minor bands was obtained with 10% acrylamide at pH 8.6. Separations were poor in gels with lower or higher acrylamide concentrations or at acid pH (4.5). Thus the PL-hog A + H fraction seemed to contain two or more lectins along with some contaminating proteins.

By ion-exchange chromatography on DEAE-cellulose, the PL-hog A + H fraction gave three fractions. A typical separation was achieved with a column (18 × 4.5 cm) equilibrated with 0.07 M barbital–acetate–HCl (pH 6.2). Most of the protein applied (250 mg) was bound; a minor fraction (DEAE I) eluted unretarded. As seen in Figure 1a, stepwise elution

TABLE I: Amino Acid Composition of the *Aaptos papillata* Lectins.

Amino Acid	Residues/100 000 g		
	Lectin I	Lectin II	Lectin III
Asp	83.8	104.9	93.0
Thr	49.5	61.9	63.3
Ser	68.9	54.6	55.1
Glu	74.6	104.1	104.1
Pro	39.6	37.3	46
Gly	72.9	62.2	54.9
Ala	59.5	89.8	91.5
Val	37.9	60.6	65.8
Cys[a]	0.0	71.6	63.9
Met[a]	2.8	5.1	5.4
Ile	36.0	12.9	13.5
Leu	65.3	56.5	56.7
Tyr	65.6	25.4	24.8
Phe	31.8	26.5	29.5
Lys	18.5	25.2	22.7
His	19.0	8.4	8.5
NH_3	108.0	65.2	146
Arg	33.3	35.6	35.8
Trp[b]	24.8	2.4	2.1

[a] Cysteine and methionine values were obtained by the method of Moore (1963) oxidizing the samples with performic acid and hydrolysis in 6 N HCl for 24 h. [b] Tryptophan was determined spectrophotometrically according to Goodwin and Morton (1946).

with buffer at different pH gave two other fractions: DEAE II at pH 3.7 and DEAE III at pH 3. Analysis for purity showed that the DEAE I fraction gave only the strong cathodic band in immunoelectrophoresis and only the upper main band and two minor ones in polyacrylamide. This fraction is now called *Aaptos* lectin I. The DEAE II fraction was not studied further because of insufficient material. DEAE III showed two bands in polyacrylamide electrophoresis (Figure 2f) and at high concentrations two bands in immunoelectrophoresis possessing the same mobility, but diffusing differently (Figure 2c).

The two components in the DEAE III fraction were separated by preparative polyacrylamide electrophoresis (Figure 1b). The slower migrating component is *Aaptos* lectin II, the faster *Aaptos* lectin III; completeness of separation when both components were reexamined analytically on polyacrylamide gels is seen in Figure 2g. However, both agglutinins showed the two bands in immunoelectrophoresis as found in the DEAE III fraction at high concentrations. In Ouchterlony tests, *Aaptos* lectin I showed one band; *Aaptos* lectins II and III showed two. While the lines of *Aaptos* lectins II and III fused completely, *Aaptos* lectin I crossed the inner lines of the other lectins, producing spurs on each side demonstrating absence of immunological cross reactivity (Figure 2e).

Aaptos lectins II and III agglutinated 2% suspensions of human A, B, and O erythrocytes to the same extent, an equal volume of a solution of 2 μg of N/ml giving minimal hemagglutination. Lectin I even at 170 μg of N/ml did not agglutinate human erythrocytes. However, if the erythrocytes were incubated for 30 min with *Aaptos* lectin I, washed three times, and subsequently treated with rabbit serum against crude extract, which by itself did not agglutinate, strong agglutination occurred showing the *Aaptos* lectin I was bound to the cell surface.

Physicochemical Analysis. In the analytical ultracentrifuge using ultraviolet optics *Aaptos* lectins I, II, and III at concentrations of 250, 600, and 650 μg of protein/ml, respectively, sedimented with $s_{20,w}^0$ of 3.5, 6.0, and 5.5.

On polyacrylamide gels in sodium dodecyl sulfate with and without β-mercaptoethanol, a single line corresponding to a molecular weight of 16 000 was seen with both *Aaptos* lectins II and III; two lines were given by *Aaptos* lectin I of molecular weights of 21 000 and 12 000.

Amino Acid Analysis. The amino acid analyses of the three lectins are compared in Table I. Hydrolysis in 6 N HCl was carried out for 24 h. *Aaptos* lectins II and III resemble each other, but *Aaptos* lectin I is different. All three lectins are high in aspartic and glutamic acids and have similar proportions of some other amino acids. The most outstanding differences, however, among the three lectins are the high content of half-cystine in *Aaptos* lectins II and III, accounting for about 7.5% of the total weight of the protein, while lectin I lacks half-cystine and contains twice as much histidine and tyrosine and three times as much isoleucine and ten times as much tryptophan as do *Aaptos* lectins II and III.

Carbohydrate Analysis. Hexoses, hexosamines, methylpentoses, and *N*-acetylhexosamines were not detected in *Aaptos* lectins I, II, and III.

Isoelectric Focusing. In analytical isoelectric focusing *Aaptos* lectin I gave two sharp bands at pH 4.7 and 5.0 and several bands in the region between pH 6.8 and 7.6. The DEAE III fraction containing *Aaptos* lectins II and III shows four bands. The first extended from pH 3.3 to 4.1, the second from 4.6 to 4.8, the third from 4.85 to 5.1, and the fourth at pH 5.3. *Aaptos* lectin II had bands one, two, and four, while *Aaptos* lectin III had the bands of one, three, and four. *Aaptos* lectins II and III precipitated in the vicinity of their isoelectric points; this was noted in DEAE-cellulose chromatography and explains the somewhat extended and diffuse bands in isoelectric focusing. To separate the various fractions observed in analytical isoelectric focusing, preparative isoelectrofocusing was performed with *Aaptos* lectins I and II in the 110 ml of LKB column. The results are seen in Figures 3b and 3a. All fractions were dialyzed for 5 days and concentrated. *Aaptos* lectin I gave three fractions: I_1, I_2, I_3. When the separated fractions were reapplied for polyacrylamide electrophoresis (10%), fraction I_1 gave two bands both of which were clearly separated from the single band obtained with fractions I_2 and I_3 which behaved identically (Figure 2h). A large amount of *Aaptos* lectin II precipitated during isoelectric focusing. One-milliliter fractions were collected. The precipitate dissolved in phosphate-buffered saline (pH 8) and absorption at 280 nm was determined. Three fractions, II_1, II_2, and II_3, were obtained. Only II_3 was well separated from II_1 and II_2. No precipitation was observed when fraction II_3 was collected. The three fractions were reexamined by polyacrylamide electrophoresis; each gave three bands (Figure 2h). The slowest moving band corresponded to *Aaptos* lectin II, the somewhat faster moving band corresponded to *Aaptos* lectin III, and the fastest moving was close to bromophenol blue, indicating a low molecular weight. Fractions II_1 and II_2 at high concentrations showed two bands in immunoelectrophoresis migrating identically but diffusing differently as did all preparations of *Aaptos* lectin II. Fraction II_3 gave only one band, but it was examined only at much lower concentration.

Immunochemical Specificity. The blood group A_1 and A_2 substances from human ovarian cysts MSS 10% 2×, Cyst 9 and Cyst 14, as well as hog mucin blood group A + H substance precipitated close to 6.5 μg of N with *Aaptos* lectin II, essentially all of the 6.7 μg of N lectin added, and for 50% precipitation 5 μg of eachwere required. MSM 10% and WGØOH insoluble, human blood group A_1 and A_2 substances, and an A-specific fraction separated from hog mucin A + H,

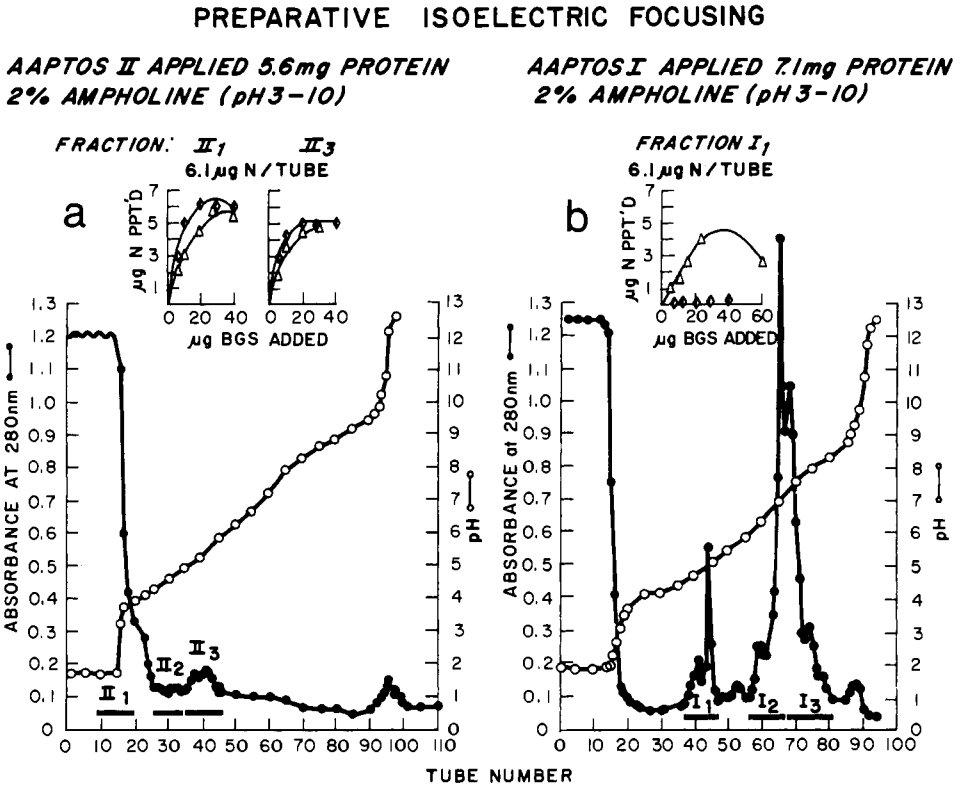

FIGURE 3: Preparative isoelectric focusing of *Aaptos* lectin II and *Aaptos* lectin I. (a) *Aaptos* lectin II. (b) *Aaptos* lectin I. Insets show precipitin curves of the isolated fractions with human blood group A_1 substance MSS 10% 2× (◆) and with horse 4 25% (△). Peaks I_2 and I_3 did not precipitate.

were somewhat less active, requiring 6 to 14 µg of blood group substances for 50% precipitation and reaching a maximum at 5.5 µg of N (Figure 4a). Two blood group B substances, Beach ϕOH insoluble from a human ovarian cyst and PM ϕOH insoluble from human saliva were almost inactive, less than 1 µg of N being precipitated even with large amounts of these blood group substances. However, horse 4 25%, a blood group B substance from horse stomach linings, precipitated 6 µg of N of lectin II and was as active as the blood group A substances (Figure 4b). Tij ϕOH insoluble, a blood group B substance from a human ovarian cyst with high I-Ma but low I-Step activity, precipitated only 2.5 µg of N, even with 50 µg of blood group substance. Tij 20% 2× having low B and I-Ma but high I-Step activity precipitated *Aaptos* II almost as well as did horse 4 25%. Tij 10% 2× having the same I-Ma potency as Tij ϕOH insoluble and being intermediate in B and I-Step activity behaved almost identically with Tij ϕOH insoluble. Blood group A and B substances, after one cycle of Smith degradation, MSS 1st IO_4^-/BH_4^-, and Beach 1st IO_4^-/BH_4^-, precipitated 4 µg of N, about two-thirds of the maximum. JS ϕOH insoluble, an H-active material and OG 20% 2×, a precursor I substance, both from human ovarian cysts, required 20 to 40 µg of blood group substance for 50% precipitation of their maximum (5 µg of N). Cyst Tighe and Morgan standard H, two other H-active materials, and Le^a active substances, N-1 10% 2× and N-1 ϕOH insoluble, all derived from human cyst material, reacted poorly, precipitating only 1 to 2 µg of N of the lectin. An H-active fraction from hog mucin A + H precipitated 5.5 µg of N of 6.1 µg of lectin N. Fifty percent precipitation required 14 µg of blood group substance (Figure 4c).

P_1 glycoprotein from hydatid cyst fluid and pneumococcal polysaccharide S XIV were almost inactive (Figure 4d and e). However, JS ϕOH insoluble after periodate oxidation and Smith degradation as well as S XIV after treatment with β-galactosidase (*Cl. tertium* 1259; Howe et al., 1958) to expose DGlcNAc residues showed substantially increased reactivity. JS ϕOH insoluble IO_4^-/BH_4^- 1st stage precipitated 7 µg of N of 6.7 µg of *Aaptos* lectin II N added and S XIV β-galactosidase treated gave a maximum of 5.5 µg of N of 6.1 µg of N *Aaptos* lectin II added. JS ϕOH insoluble after two cycles of complete Smith degradation in which most of the terminal DGlcNAc residues are destroyed showed a substantial decrease in activity, precipitating only 3.5 µg of N maximally. Ovine submaxillary mucin (OSM), containing 80% sialyl-*N*-acetylgalactosamine and approximately 20% unsubstituted DGalNAc residues, and one antigen A preparation containing DGlcNAcβ1→4DGlcNAcβ→N poly(Asn) residues reacted well with *Aaptos* lectin II, giving 6.1 µg of N specific precipitate. Two other antigen A preparations precipitated less well giving 2.5 µg of N and 1 µg of N precipitate maximally. CEA reacted strongly, requiring 45 µg of CEA to yield 6 µg of N precipitate. Streptococcal group C polysaccharide reacted weakly with *Aaptos* lectin II, precipitating only 1.5 µg of N. Streptococcal group A polysaccharide was inactive (Figure 4f).

Precipitin reactions between subfractions II_1 and II_3 obtained by isoelectrofocusing and blood group substances MSS 10% 2× and horse 4 25% are shown in Figure 3a (inset). Fraction II_1 behaved identically with *Aaptos* lectin II; fraction II_3 was less active precipitating 5 µg of N of the 6.1 µg of N added.

Inhibition experiments (Figure 5a) were carried out with monosaccharides, methyl-, ethyl-, and *p*-nitrophenyl glycosides, and a variety of di- and oligosaccharides. DGlcNAc, DGalNAc, and sialic acid were the only active monosacchar-

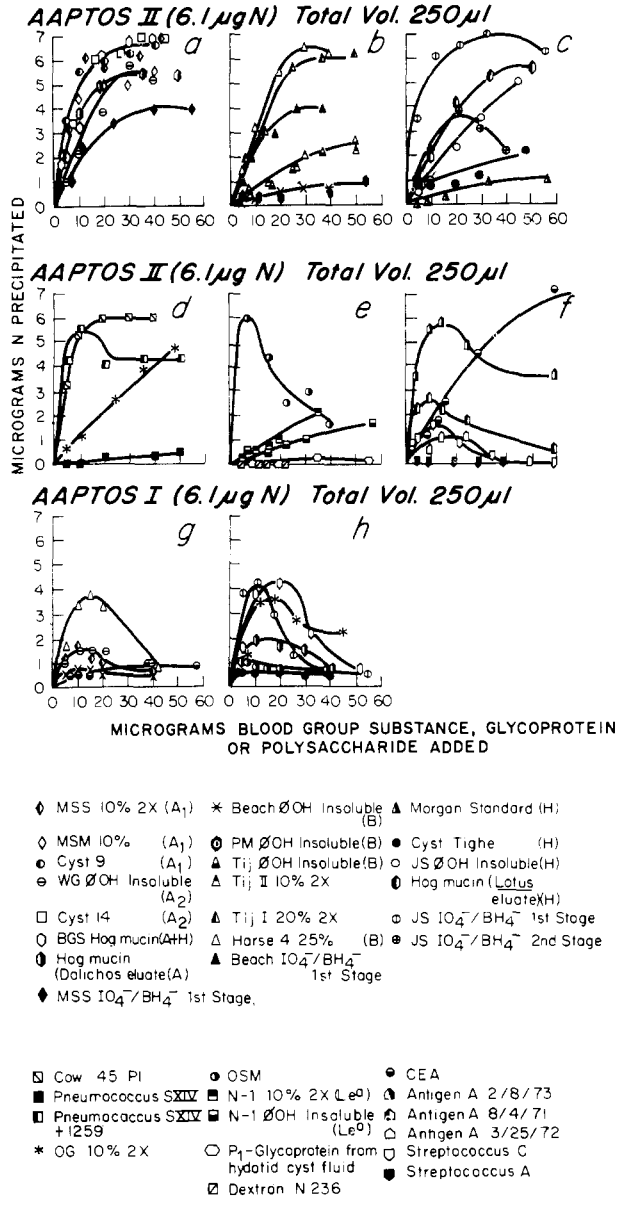

FIGURE 4: Quantitative precipitin curves of *Aaptos* lectin II(a–f) and *Aaptos* lectin I (g, h) with blood group substances, glycoproteins, and polysaccharides; 6.7 μg of N of lectin was used with MSS 10% 2× cyst 14, JS 1st IO_4^-/BH_4^-, and Tij 20% 2× with others 6.1 μg of N of lectin was used.

ides among those tested; 370 nmol of DGlcNAc, 500 nmol of sialic acid, and 1500 nmol of DGalNAc were required for 50% inhibition of the precipitin reaction between *Aaptos* lectin II and the blood group A substance MSS 10% 2×. D-Fucose, L-fucose, D-mannose, D-glucosamine, and D-glucose were inactive up to 22 000 nmol. DManNac was inactive up to 500 nmol, the highest amount used. Methyl-β-DGlcNAc was slightly more active and *p*-nitrophenyl-β-DGlcNAc was five times more active than DGlcNAc. Methyl-α-DGlcNAc was only as potent as DGalNAc. Ethyl-β-DgalNAc was three times as active as DGalNAc, and *p*-nitrophenyl-β-DGalNAc about twice as active as DGlcNAc. Methyl-α-DGalNAc and DGalNAcα1→3DGal did not differ from DGalNAc; however, *p*-nitrophenyl-α-DGalNAc was inactive up to the amount (4500 nmol) tested. *N,N'*-Diacetylchitobiose showed a sixfold increase in activity; *N,N',N''*-triacetylchitotriose and *N,N',N'',N'''*-tetraacetylchitotetraose both being of equal potency were 15 times more active than DGlcNAc. DGlcNAcβ1→6DGal and DGalβ1→3[DGlcNAcβ1→6]-*N*-acetyl-D-galactosaminitol were almost as active as methyl-β-DGlcNAc but less potent than *N,N'*-diacetylchitobiose. DGlcNAcβ1→3DGal inhibits the precipitation to 90% at a concentration of 389 nmol, being as active as DGlcNAc in β1→6 linkage or methyl-β-DGlcNAc. DGlcNAcβ1→3-D-galactitol and oligosaccharides obtained from streptococcal group A containing DGlcNAcβ1→3LRha residues were inactive at 600 nmol and 200 μg, respectively, the highest amounts used. DGlcNAcβ1→3-[DGlcNAcβ1→6]-D-galactitol with two terminal nonreducing DGlcNAc residues, one in β1→6 and the other one in β1→3 linkage, was somewhat more active than methyl-β-DGlcNAc. DGlcNAcα1→4DGalβ1→3DGalNAc was only as potent as methyl-α-DGalNAc and DGlcNAcα1→4DGalβ1→4DGlcNAc was inactive even at the largest amount used (200 nmol) showing that α-linked terminal nonreducing DGlcNAc is less potent than β-linked DGlcNAc residues. Lacto-*N*-tetraose and DGalβ1→3DGlcNAc, both having a subterminal DGlcNAc residue, are inactive in concentrations higher than required for 90% inhibition with DGlcNAc.

Using DGlcNAc, methyl-β-DGlcNAc, *N,N'*-diacetylchitobiose, *N,N',N''*-triacetylchitotriose, and *N,N',N'',N'''*-tetraacetylchitotetraose to inhibit the precipitin reaction between *Aaptos* lectin II and MSS 10% 2× at 37 °C, a remarkable displacement to higher amounts of inhibitor needed was observed. A three- to fourfold increase was seen, but the relative inhibiting power of these four sugars to one another was almost unchanged (Figure 6). Precipitin studies with MSS 10% 2× and *Aaptos* lectin II at 4 and 37 °C showed no differences in the amount of *Aaptos* lectin N precipitated and in the quantity of blood group substance used to reach 50% of the maximum (data not shown).

Aaptos lectin III showed precipitin reaction with three blood group substances MSS 10% 2×, N-1 ØOH insoluble, and horse 4 25% almost identical with those obtained with lectin II (data not shown). Because of these findings and the immunochemical and physicochemical similarities to *Aaptos* lectin II, it was not studied further.

Only a limited number of precipitin studies (Figure 4g,h) were carried out with *Aaptos* lectin I, because of the small amounts of purified material available. None of the 11 blood group substances and polysaccharides tested completely precipitated the 6.1 μg of lectin N added. Hog mucin blood group A + H substance, and JS ØOH insoluble after one cycle of Smith degradation precipitated 4.2 μg of N. Horse 4 25% and Cyst OG 20% 2× were somewhat less active, giving 3.7 and 3.5 μg of specific precipitate N. The human blood group A_1 and A_2 substances MSS 10% 2× and WG ØOH insoluble precipitated 1.5 μg of N and the A-active material separated from hog mucin A + H 1.8 μg of N. Ovine submaxillary mucin, Beach ØOH insoluble, Tighe, and CEA were almost inactive giving 1 μg of precipitate N or less.

Precipitin studies with subfractions I_1, I_2, and I_3 obtained by isoelectric focusing with blood group substances MSS 10% 2× and horse 4 25% are shown in Figure 3b (inset). Horse 4 25% precipitated 4.3 μg of N of the 6.1-μg fraction I_1 N added, but did not react with I_2 and I_3, and MSS 10% 2× did not react with any of the three subfractions. Since fraction I_1 made up only about 10% of the total N of the original lectin I, the reaction of lectin I with horse 4 25% which gave 3.7 μg of N cannot be due solely to its content of I_1. Mixing fractions I_1, I_2, and I_3 in the proportions in which they occur in *Aaptos* lectin I (1:2:7) did not result in significant precipitation with

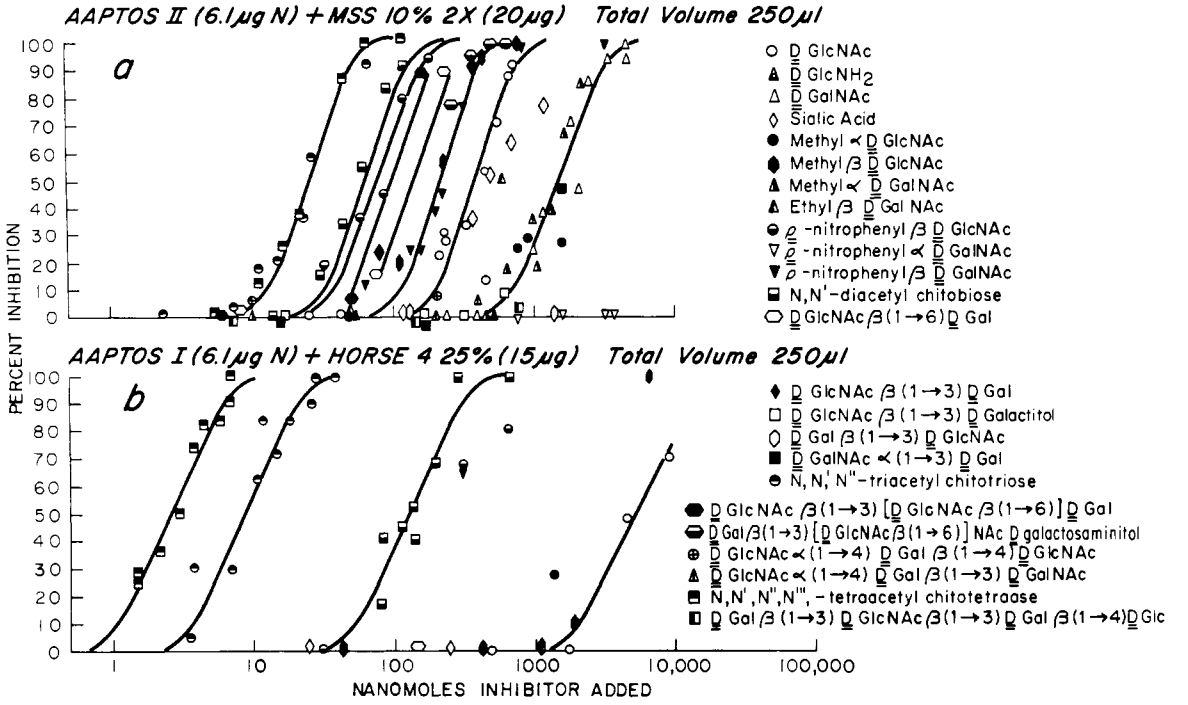

FIGURE 5: Inhibition by monosaccharides and various oligosaccharides of precipitation of human blood group A substance MSS 10% 2× with *Aaptos* lectin II (a) and of blood group B substance horse 4 25% with *Aaptos* lectin I (b).

horse 4 25%, suggesting that fractions I_2 and I_3 might have been inactivated by the separation procedure.

The ability of several monosaccharides, glycosides, and oligosaccharides to inhibit the precipitation of *Aaptos* lectin I with horse 4 25% is shown in Figure 5b. Among the monosaccharides tested, only DGlcNAc and DGlcNH were active. DGlcNAc (5000 nmol) was required for 50% inhibition, while $DGlcNH_2$ gave 60% inhibition at 22 000 nmol (not shown). DGlc, DMan, DGal, and DGalNAc were inactive up to 22 000 nmol. Methyl-βDGlcNAc and methyl-αDGlcNAc were roughly twice as potent as DGlcNAc; *p*-nitrophenyl-βDGlcNAc and *p*-nitrophenyl-βDGalNAc were 20 times more active than DGlcNAc and only about one-half as active as *N,N'*-diacetylchitobiose which gave 50% inhibition at 130 nmol. *N,N',N''*-Triacetylchitotriose and *N,N',N'',N'''*-tetraacetylchitotetraose requiring 9 and 2.5 nmol for 50% inhibition were 600 and 2000 times more potent than DGlcNAc, respectively. DGlcNAcβ1→6DGal was inactive up to 150 nmol, the highest amount used and, thus, was much less potent than *N,N'*-diacetylchitobiose or *p*-nitrophenyl-βDGlcNAc.

Inhibition studies with *Aaptos* lectin I were carried out using horse 4 25% for precipitation, while those with *Aaptos* lectin II were with MSS 10% 2×. For better comparison between the two lectins, inhibition studies with *Aaptos* lectin II were repeated with DGlcNAc, *N,N'*-diacetylchitobiose, *N,N',N''*-triacetylchitotriose, and *N,N',N'',N'''*-tetraacetylchitotetraose using horse 4 25% for precipitation. The resulting inhibition curves (not shown) were displaced so that smaller amounts of material were needed. Only 45 nmol of DGlcNAc was required compared with 370 nmol of DGlcNAc when precipitation was performed with MSS 10% 2×. However, the relative inhibiting potencies of these four sugars was unchanged, the tetra- and trisaccharides being equal and about 13 times more active than DGlcNAc.

An unusual observation was made, namely, that *p*-nitrophenyl-αDGalNAc itself precipitated up to 2.3 μg of the 6.1 μg of *Aaptos* lectin I N added. The precipitate formed slowly

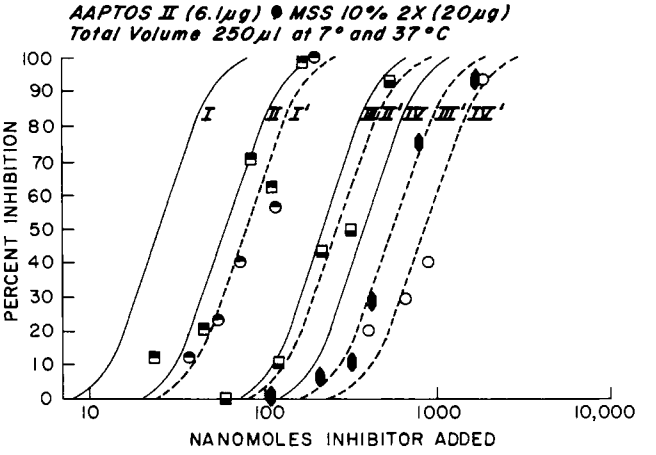

FIGURE 6: Inhibition of precipitin reaction of human blood group A substance MSS 10% 2× with *Aaptos* lectin II at 4 °C (solid lines) and at 37 °C (dotted lines). Curves I and I' trimer and tetramer of β1→4 linked DGlcNAc, II and II' DGlcNAcβ1→4DGlcNAc, III and III' methyl βDGlcNAc, IV and IV' DGlcNAc. Symbols are as those in Figure 5.

and became much less soluble even in larger amounts of saline after incubation overnight at 4 °C; 250 μg of *p*-nitrophenyl-αDGalNAc (50 μl) also gave strong precipitates, which formed immediately with 25 μl of concentrated crude extract (980 μg of N/ml) and 25 μl of the purified material (850 μg of N/ml) eluted from PL hog A + H (50 μl) and containing *Aaptos* lectins I with II and III. They were easily resolubilized by addition of 100 μl of saline shortly after formation, but fter placing at 4 or at 37 °C overnight they became almost insoluble in saline but could be readily solubilized with 6 M guanidine hydrochloride. The immunoelectrophoretic patterns of the supernatants and of the redissolved precipitates of purified material and *p*-nitrophenyl-αDGalNAc are seen in Figure 2d. The supernatants after 2 and 48 h at 37 °C with *p*-nitrophenyl-αDGalNAc contained substantial amounts of *Aaptos* lectin

II, but no *Aaptos* lectin I. The dissolved precipitates contained both *Aaptos* lectin I and *Aaptos* lectin II. The purified *Aaptos* lectin II fraction was not precipitated by *p*-nitrophenyl-αDGalNAc even if high concentrations of lectin (730 μg of N/ml) were used. It only coprecipitated from the material purified on PL hog H + A, containing *Aaptos* lectins I, II, and III; precipitates also formed at 37 °C after 48 h of incubation with 50 μl of *p*-nitrophenyl-βDGlcNAc (4 mg/ml) or *p*-nitrophenyl-αDGlc (3 mg/ml). They formed slowly and to a much lesser extent. The supernatants contained *Aaptos* lectins I and II, showing that the precipitation of *Aaptos* lectin I was incomplete. In immunoelectrophoresis the solubilized precipitates obtained with *p*-nitrophenyl-αDGlc and *p*-nitrophenyl-βDGlcNAc showed only the cathodic migrating band of *Aaptos* lectin I.

Discussion

The lectins of *Aaptos papillata* were isolated by affinity chromatography using PL hog A + H as absorbent and 3 M $MgCl_2$ for elution. Further separation on DEAE-cellulose resulted in three fractions: *Aaptos* I eluting first at pH 6.2, DEAE II at pH 3.7, and DEAE III at pH 3. DEAE III could be separated on preparative polyacrylamide electrophoresis into *Aaptos* lectin II and *Aaptos* lectin III.

In analytical disc electrophoresis, *Aaptos* lectin I migrated slowly showing a major and two minor bands. *Aaptos* lectins II and III were separated completely each showing one fast main band and a slowly moving minor band. The main band of *Aaptos* lectin II migrated faster than *Aaptos* lectin I but not as fast as *Aaptos* lectin III; the minor bands of *Aaptos* lectins II and III moved at the same rate. In gel diffusion with rabbit antiserum against crude extract, the precipitin lines of *Aaptos* II and III fused completely, while *Aaptos* lectin I crossed the lines of *Aaptos* lectins II and III and spurs could be seen on both sides. In immunoelectrophoresis, *Aaptos* lectins II and III migrated with equal speed toward the anode, and *Aaptos* lectin I migrated toward the cathode, showing that *Aaptos* lectin I differed from *Aaptos* lectins II and III and that II and III are related immunochemically.

Ultracentrifugation showed $s_{20,w}^0$ of 6.0 for *Aaptos* lectin II, 5.5 for *Aaptos* lectin III, and 3.5 for *Aaptos* lectin I. Polyacrylamide electrophoresis in sodium dodecyl sulfate with and without β-mercaptoethanol showed one band corresponding to a molecular weight of 16 000 for *Aaptos* lectins II and III, and two bands corresponding to molecular weights of 21 000 and 12 000 for *Aaptos* lectin I. Thus *Aaptos* lectins II and III seem to be composed of identical subunits, and *Aaptos* lectin I is composed of two different subunits, neither of which corresponds to those from *Aaptos* lectins II and III. The differences in sedimentation velocity and mobility in polyacrylamide gel between *Aaptos* lectins II and III may be explained by differing numbers of the same subunit. Both fractions at high concentrations gave a second line in immunoelectrophoresis and gel diffusion which could be due to small amounts of impurities.

A molecular weight of 65 000 was found by Bretting and Renwrantz (1974) for the *Aaptos* agglutinin by gel filtration on Sephadex G-75. This is lower than generally expected from a sedimentation coefficient of $s_{20,w}^0$ 6.0 or 5.5; the difference may be accounted for if there was some interaction with the Sephadex or if the molecule has an unusual shape. The two different subunits of *Aaptos* I gave molecular weight of 33 000 consistent with the $s_{20,w}^0$ of 3.5.

The amino acid data are very similar for *Aaptos* lectins II and III but differ substantially from those for *Aaptos* lectin I. High values of aspartic and glutamic acid as found in *Aaptos* lectins I, II, and III are general features of lectins; high half-cystine values are rare but occur in lectins from *Triticum vulgaris* (Allen et al., 1973), *Solanum tuberosum* (Allen and Neuberger, 1973), *Helix pomatia* (Hammarström and Kabat, 1969), nurse shark (Harisdangkul et al., 1972), and eel (Springer and Desai, 1971). The high content of tyrosine in *Aaptos* lectin I is also very unusual. The amino acid composition of *Axinella polypoides* lectins, the only other sponge lectin studied, differs considerably from those of *Aaptos*, being low in half-cystine, tyrosine, and histidine (Bretting and Kabat, 1976). Carbohydrates were not detected in the *Aaptos* lectins.

Aaptos lectin II was almost completely precipitated by all human blood group A_1 and A_2 substances, by hog A + H as well as the A-active fraction from hog A + H, indicating a specificity for terminal DGalNAc. OSM which contains both DGalNAc and sialic acid as terminal residues also reacted well; it is not clear whether one or both of these residues are involved.

The failure to precipitate with Beach ØOH insoluble and PM ØOH insoluble, two human blood group B substances, the P_1-active material from hydatid cyst fluid and pneumococcal polysaccharide S XIV, suggested that no cross-reactivity with terminal nonreducing DGal occurred. This was supported by the very weak reaction found with the two human blood group Le^a substances N-1 ØOH insoluble and N-1 10% 2× and two other B-active materials Tij 10% 2× and Tij ØOH insoluble. An interaction of *Aaptos* lectin II with the H determinant was excluded by the weak reaction of the two human blood H substances Cyst Tighe and Morgan standard. However, the large amount of N precipitated with JS ØOH insoluble after one cycle of Smith degradation and with *Pneumococcus* S XIV after β-galactosidase treatment exposing DGlcNAc in both substances suggested that *Aaptos* lectin II could also react with terminal nonreducing DGlcNAc. The good reactivity with at least one preparation of the synthetic antigen A containing DGlcNAcβ1→4DGlcNAcβ→poly(Asn) and with Tij 20% 2×, a fraction containing group III blood groups I determinants (reacting with anti-I Step) which is expected to have terminal DGlcNAc residues (Feizi et al., 1971; Maisonrouge-McAuliffe and Kabat, 1976), supports this inference. The complete precipitation of *Aaptos* lectin II with the B-active material horse 4 25% and with the H-active fraction of hog A + H and the considerable reactivity with the blood group H substance JS ØOH insoluble and the precursor I substance OG 20% 2× may be ascribed to their heterogeneity in content of side chains with terminal βDGlcNAc. Oligosaccharides with terminal nonreducing DGlcNAc were isolated after Carlson degradation from JS ØOH insoluble (Rovis et al., 1973) and from Tij ØOH insoluble and 20% 2× (Maisonrouge-McAuliffe and Kabat, 1976). The main differences found with *Aaptos* lectin I in precipitin reactions with blood group substances and polysaccharides as compared with *Aaptos* lectin II may be summarized as follows. (1) None of the materials tested precipitated *Aaptos* lectin I completely; JS ØOH insoluble after a first stage of Smith degradation and hog H + A which are expected to have terminal nonreducing DGlcNAc react best but precipitate only two-thirds of the total N added. (2) The precipitates with *Aaptos* lectin I are highly soluble in excess polysaccharide. (3) Blood group A_1 and A_2 substances with terminal nonreducing DGalNAc react very poorly with *Aaptos* lectin I. (4) CEA which reacts very well with *Aaptos* lectin II is almost inactive with *Aaptos* lectin I. Thus it appears that *Aaptos* lectin I may react with terminal DGlcNAc but not

DGalNAc. Precipitin reactions with MSS 10% 2× and *Aaptos* lectin II could be inhibited by DGlcNAc and DGalNAc, whereas the precipitin reaction of *Aaptos* lectin I and horse 4 25% was only affected by high concentrations of DGlcNAc and not by DGalNAc, in accord with the findings from precipitin reactions with blood group substances. Acetylated amino groups were important for the inhibition of *Aaptos* lectin II, as seen by the failure of $DGlcNH_2$ and $DGalNH_2$ to inhibit even at concentrations up to 22 000 nmol, while with *Aaptos* lectin I comparable inhibition by $DGlcNH_2$ required only five times more than were needed with DGlcNAc. The differences between *Aaptos* lectins I and II were even more pronounced when the relative inhibitory power of N-acetylated chitobiose, -triose, and -tetraose were compared. *N,N'*-Diacetylchitobiose is 6 times more active than DGlcNAc with *Aaptos* lectin II, but 38 times more active with *Aaptos* lectin I. *N,N',N''*-Triacetylchitotriose and *N,N',N'',N'''*-tetraacetylchitotetraose are of equal potency and 15 times more effective than DGlcNAc on a molar basis with *Aaptos* lectin II, while these two oligosaccharides differ in activity against *Aaptos* lectin I, being 600 times and 2000 times more potent than DGlcNAc. These findings suggest combining sites for *Aaptos* lectins II and I of sizes complementary to a tri- and tetrasaccharide, respectively. Of four lectins wheat germ agglutinin (Goldstein et al., 1975), potato lectin (Allen and Neuberger, 1973), the lectin of *Helix pomatia* (Hammarström and Kabat, 1969), and one of the lectins from *Ulex europeus* (Matsumoto and Osawa, 1970) reacting with DGlcNAc residues in polysaccharides, only wheat germ agglutinin and the potato lectin have combining sites at least as large as a trisaccharide. Both show huge differences between DGlcNAc and their best inhibitors as found with *Aaptos* lectin I. Ratios of inhibitory power of oligosaccharides to DGlcNAc with *Aaptos* lectin II are much lower (Figure 5a).

Aaptos lectin II is better inhibited by β-linked DGlcNAc and DGalNAc than by the α-linked anomers. DGlcNAc linked β1→3 or β1→6 to DGal were equal in potency and as active as methyl-βDGlcNAc, but DGlcNAcβ1→3-D-galactitol and oligosaccharides containing DGlcNAcβ1→3Rha in terminal nonreducing linkage were inactive, showing that the penultimate sugar could cause steric hindrance and render the β-linked DGlcNAc inaccessible, accounting for the failure of streptococcal group A polysaccharide to precipitate *Aaptos* lectin II and the weak reaction with streptococcal group C polysaccharide. Wheat germ agglutinin reacts very well with CEA (Goldstein et al., 1975; Hammarström et al., 1975), while *Aaptos* lectin I reacts weakly. Since periodate oxidation and methylation studies (Hammarström et al., 1975; Fuks et al., 1975) indicate the absence of terminal nonreducing DGlcNAc in CEA, the reactive DGlcNAc must be internal and the findings of Anderson et al. (1975) that *N,N',N''*-triacetylchitotriose was better than the dimer or *p*-nitrophenyl-βDGlcNAc could indicate specificity for an internal sequence of one or more β-linked DGlcNAc residues. Thus the poor reaction of CEA with *Aaptos* lectin I supports the inference that the *Aaptos* lectin I site is directed against terminal nonreducing chains of β1→4 linked DGlcNAc.

Aaptos lectin II shows only moderate differences between DGlcNAc and *N,N',N''*-triacetylchitotriose and reacts very well with CEA, suggesting some spcificity for subterminal or internal DGlcNAc. However, lacto-*N*-tetraose and DGalβ1→3DGlcNAc at molar concentrations three and four times higher than that required for 50% inhibition with DGlcNAc in the precipitin reaction with MSS 10% 2× were inactive and oligosaccharides with internal *N,N'*-diacetylchitobiose were not available. Sialic acid is almost as potent as DGlcNAc in inhibition of precipitation of blood group substances with *Aaptos* lectin II and might contribute to the reactivity of CEA with *Aaptos* lectin II.

Inhibition studies at 37 °C showed a three- to fourfold increase in amount of inhibitor for 50% inhibition without significant changes in relative inhibiting activity among the oligosaccharides used. This suggests higher affinity of *Aaptos* lectin II for blood group substances MSS 10% 2× at 37 than at 4 °C so that higher concentrations of monovalent inhibitor are required to compete with the multivalent blood group substance for the combining sites of the lectin. The basis for this unusual observation, not previously reported for other lectins, is not clear but may be related to the increased strength of hydrophobic bonds at higher temperatures. Hydrogen bonds and hydrophobic bonds both contribute to the stability of a protein or a protein complex, but show opposite dependency on temperature (Scheraga et al., 1962). Usually the decrease in the contribution of hydrogen bonds to total binding outweighs the increase in binding of hydrophobic bonds as temperature increases. In some rare cases, however, increased stability of proteins with higher temperatures could be related to decreased entropy due to an increase in hydrophobic bond-strength within the molecule (Nalbandian et al., 1971; Lanyi and Stevenson, 1970). Breakage of hydrophobic bonds was also assumed when some enzymes disintegrated at 0 °C into subunits with a subsequent loss of activity, which was fully regained at 38 °C (Scrutton and Utter, 1965; Havir et al., 1965).

Thus formation of larger *Aaptos* aggregates at 37 °C may contribute to the increased stability of the *Aaptos* II-MSS 10% 2× complex at this temperature. Alternative explanations may relate to a change in the combining site of *Aaptos* lectin II creating better complementarity or to an increase in hydrophobic binding between receptor and combining site at higher temperatures plus a cooperative effect of the multivalent blood group substance giving a relative advantage over that of the monovalent inhibitor. It might be possible to distinguish between these possibilities by studying dependence of the association constant on temperature.

Because of the small amounts of lectin, inhibition of precipitin reactions with *Aaptos* lectin I were not studied at different temperatures. However, another unusual effect was observed. *Aaptos* lectin I could be precipitated by *p*-nitrophenyl-αDGalNAc, whereas *p*-nitrophenyl-βDGalNAc did not precipitate but was a good inhibitor. Springe and Desai (1971) found that the lectin in eel serum was precipitated by 3-*O*-methyl-DFuc and by 3-*O*-methyl-DGal, while LFuc and its glycosides were inhibitors; nonpolar interactions were thought to be involved. The relation of the two phenomena is not clear since precipitation of the *Aaptos* lectin occurred with glycosides, while the latter occurred with sugars containing a free reducing group. Insufficient data are available to evaluate why the *Aaptos* materials are precipitated by *p*-nitrophenyl-DGalNAc.

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