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Effect of chain length of oligosaccharide in high-performance affinity chromatography of a lectin on oligosaccharide-immobilized columns

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SUMMARY

Homologous oligosaccharides ranging from bioses to tetraoses were immobilized to 3-(β-aminoethoxy)propylsilica by reductive amination, and the chromatographic behaviour of concanavalin A (Con A) with the resultant gels was examined. Con A showed a strong affinity to immobilized mannose oligomers having mainly α1→2 interglycosidic linkages, giving broadened peaks when phosphate buffers containing glucose as a displacing sugar were employed as eluents. The association constant (K_a) calculated from elution volume increased with increasing degree of polymerization, implying that not only the non-reducing monosaccharide residue but also the interior portion of the sugar chain took part in complexation. Con A showed a similar tendency to glucose oligomers linked through α1 →6 bonds, although the K_a values were much smaller than those of the corresponding mannose oligomers.

INTRODUCTION

Affinity chromatography based on specific interactions between proteins and carbohydrates has been widely applied to the isolation and purification of the biological substances involved. Especially chromatography on protein-[1] or carbohydrate-immobilized[2] agarose gels is convenient and is capable of affording highly purified components on a preparative scale by a simple procedure of specific adsorption followed by desorption. However, the use of such soft gels is inappropriate for analytical purposes, because of the low reproducibility of analysis and the poor durability of the matrices. In a previous paper[3] we reported the attempted immobilization of a few popular disaccharides to a methacrylate polymer and demonstrated the usefulness of the resulting resins for high-performance affinity chromatography (HPAC) of lectins. In a subsequent paper[4] we reported the efficient separation of proteins on a resin carrying a number of oligosaccharides derived from a glycoprotein, by stepwise elution with buffers containing various displacing sugars. Such HPAC techniques are valuable for the separation and determination of specific proteins of biological interest in minute amounts. They are also useful for the investigation of

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structure–affinity correlations. In continuation of such HPAC studies, we have now examined the effect of the chain length of homologous oligosaccharides on their affinity to protein. This paper reports investigations of the interactions of mannose and glucose oligomers immobilized to silica with concanavalin A (Con A) as a specific protein.

EXPERIMENTAL

Chemicals

Con A from *Canavalia ensiformis* was obtained from Hohnen Seiyu (Tokyo, Japan) and used as a $1.0 \cdot 10^{-3}$% solution in the eluent used for HPAC. Homogeneity of Con A was confirmed by electrophoresis on polyacrylamide gel containing $1.0 \cdot 10^{-2}$% sodium dodecyl sulphate (SDS) according to the procedure of Laemmli[5]. Yeast α-glucosidase was purchased from Sigma (St. Louis, MO, U.S.A.) and used after dialysis against 120 m*M* phosphate buffer (pH 6.8).

Glucose oligomers (isomaltooligosaccharides) were obtained by partial acid hydrolysis of dextran, followed by separation on a column of Bio-Gel P-4, by the method of Yamashita *et al.*[6]. The peracetates of mannose oligomers were obtained by acetolysis of yeast mannan, followed by fractionation of the product on a silica gel column by stepwise elution with benzene–ethyl acetate. Each of the purified acetates was dissolved in 100 m*M* sodium methoxide in methanol[7], and the reaction mixture was deionized by passing it through a column of Amberlite CG-120 (H^+). Evaporation of the combined eluate and the water washings to dryness gave a pure sample of a mannose oligomer. Based on ^{1}H NMR analysis, the deacetylated products were concluded to have the structures Manα1 → 2Man, Manα1 → 2Manα1 → 2Man and Manα1 → 3Manα1 → 2Manα1 → 2Man.

Dihydroxysilica gel (LiChrosorb DIOL, 5 μm, pore size 6 nm), which has a 3-(β,γ-dihydroxypropoxy)propyl group on a polysilicate base, was obtained from Merck (Darmstadt, F.R.G.). Other reagents and solvents were of the highest grade commercially available.

Immobilization of oligosaccharides to dihydroxysilica gel

Dihydroxysilica gel (3 g) and sodium metaperiodate (3 g) were added to 90% aqueous acetic acid (60 ml) and the mixture was gently swirled for 2 h at room temperature. The gel was collected by centrifugation at 1800 *g*, washed thoroughly with water and suspended in methanol (20 ml). Sodium cyanoborohydride (0.63 g) and ammonium acetate (1.0 g) were added to the suspension and the mixture was kept overnight at room temperature with swirling. By this series of treatments the 3-(β,γ-dihydroxypropoxy)propyl group was converted, via the aldehyde form, to a 3-(β-aminoethoxy)propyl group. The resulting aminosilica gel was collected and washed thoroughly with water.

The aminosilica gel was stirred in an aqueous solution containing an oligosaccharide sample (0.10 g), sodium cyanoborohydride (0.63 g) and acetic acid (0.10 ml) in methanol (10 ml) for 24 h at 37°C. The oligosaccharide-immobilized silica gel thus obtained was further treated with glutaraldehyde (0.10 ml), to block the remaining free amino group. The gel finally obtained was collected and washed thoroughly with water.

HPAC

A sample of oligosaccharide-immobilized silica gel was packed in a stainless-steel column (100 mm × 4 mm I.D.) by the slurry method using water as a packing solvent. The column temperature was maintained at 29°C throughout the work. Elution of HPAC was performed with 50 m*M* phosphate buffer (pH 6.8) containing sodium chloride at a concentration of 200 m*M* at a flow-rate of 0.50 ml/min. Con A (200 ng per 20 μl) was injected via a Rheodyne 7125 injector. Con A in the eluates was monitored by measuring the fluorescence at 340 nm, generated from the tryptophan residue in the polypeptide core, with irradiation at 300 nm.

Determination of the total amounts of immobilized oligosaccharides

The total amounts of immobilized oligosaccharides were determined spectrophotometrically by the direct phenol–sulphuric acid method as follows. A sample of oligosaccharide-immobilized silica (*ca.* 10 mg), weighed accurately, was suspended in distilled water (0.50 ml). A 5.0% aqueous solution (0.50 ml) of phenol was added and the mixture was shaken vigorously. Concentrated sulphuric acid (5.0 ml) was added in one portion and the mixture was vortexed. The developed colour was measured at 490 nm and the total amount of the immobilized oligosaccharide was calculated using a calibration graph constructed using standard solutions of mannose or glucose.

The absorbance obtained for the isomaltose-immobilized gel by this method was almost the same as that for the acid hydrolysate (2 *M* trifluoroacetic acid, 100°C, 5 h) of the same amount of the isomaltose-immobilized gel. This result provided evidence that hydrolysis was complete during the above operation. The values obtained (m*M* in the gel) were as follows: glucobiose (isomaltose), 25.7; glucotriose (isomaltotriose), 21.1; glucotetraose (isomaltotetraose), 7.93; mannobiose, 25.0; mannotriose, 10.4; and mannotetraose, 5.51. The concentration of the 3-(β-aminoethoxy)propyl group in the gel, as calculated from elemental analysis of carbon (3.2%), was 0.10 *M*. Based on this value, the efficiencies of immobilization of oligosaccharides ranged from 6.3 to 17%.

Determination of the molar fractions of the oligosaccharides immobilized to the particle surface

A 50-mg portion of maltose-immobilized silica gel, prepared as described above for other oligosaccharides, was incubated for 24 h at 37°C, with constant stirring, with yeast α-glucosidase (*ca.* 10 mU) in 120 m*M* phosphate buffer (pH 6.8). The reaction mixture was centrifuged at 1800 *g* and the combined supernatant and water washings were desalted by passage through a small column of Amberlite CG-120 (H^+) and Amberlite CG-400 (CH_3COO^-). The combined eluate and water washings were evaporated to dryness and the residue was reconstituted with water (100 μl). High-performance liquid chromatography (HPLC) of a 20-μl aliquot on a column of Interaction CHO-620 (300 mm × 7.8 mm I.D., 80°C) with water as eluent (0.5 ml/min), with UV detection at 280 nm after post-column labeling with 2-cyanoacetamide by the method of Honda *et al.*[8], gave a glucose peak equivalent to 2.79 nmol. From this value the amount of maltose immobilized to the particle surface was calculated to be 270 nmol/g.

The total amount of immobilized maltose was determined by acid hydrolysis with 2 *M* trifluoroacetic acid for 6 h at 100°C, followed by HPLC of the liberated glucose as described above. The value obtained was 43.0 μmol/g.

From the foregoing two values of the enzyme- and acid-released glucose, the molar fraction was calculated to be 0.0063. This value is considered to be universally applicable to all oligosaccharide-immobilized gels, as immobilization was assumed to have occurred at an equal rate to both the particle surface and the inner walls of the pores. The values obtained for the effective concentration of oligosaccharides were as follows: glucobiose (isomaltose), $162 \cdot 10^{-6}$ *M*; glucotriose (isomaltotriose), $132 \cdot 10^{-6}$ *M*; glucotetraose (isomaltotetraose), $50.0 \cdot 10^{-6}$ *M*; mannobiose, $158 \cdot 10^{-6}$ *M*; mannotriose, $65.8 \cdot 10^{-6}$ *M*; and mannotetraose, $34.8 \cdot 10^{-6}$ *M*.

RESULTS AND DISCUSSION

Among various methods for the determination of the magnitude of protein–carbohydrate interactions, HPAC is one of the most convenient and provides reproducible data. In HPAC methods, either protein- or carbohydrate-immobilized gels may be used. Immobilization of protein involves coupling of the free amino group in a protein sample to a supporting gel. During this chemical reaction, care must be taken to avoid three-dimensional structural changes of the protein molecule, and to leave the amino groups at and near the binding site of the protein intact. In addition, protein-immobilized gels are generally unstable to heat and prolonged storage. In contrast, immobilization of carbohydrate eliminates the problem of denaturation, and the stability of immobilized carbohydrate is greater than that of immobilized protein. For this reason the latter mode was adopted in this work.

The oligosaccharides used were of homologous glucose or mannose series, ranging from biose to tetraose. All glucosyl residues in the glucose oligomers were $\alpha 1 \rightarrow 6$ linked, and all mannosyl residues in the mannobiose and mannotriose were $\alpha 1 \rightarrow 2$ linked. The mannosyl residue at the non-reducing end of the mannotetraose was exceptionally $\alpha 1 \rightarrow 3$ linked. All reducing monosaccharide residues in these oligosaccharides were derivatized, on immobilization, to the glycamines having open-chain structures which served as spacers. As a result, every oligosaccharide immobilized to silica gel functioned as a saccharide having a degree of polymerization (DP) one less than that of the starting oligosaccharide.

Fig. 1 shows the changes in the efficiency and shape of the Con A peak for each column packed with an oligosaccharide-immobilized silica gel, with varying concentrations of glucose as a displacing sugar in the mobile phase.

With both series of oligosaccharides, the number of theoretical plates (N) increased with increase in DP and with increase in the concentration of glucose (Fig. 1a and b for glucose and mannose oligomers, respectively). The increment of N corresponding to the change in DP from 2 to 3 was small, whereas that for the change in DP from 3 to 4 was large, especially in the mannose series. The mannose oligomers gave broader peaks than the corresponding glucose oligomers with all DP values, giving N values approximately one fifth (DP 2–3) or half (DP 4) of those of the corresponding glucose oligomers. These results indicate that mannose oligomer-immobilized gels showed a stronger affinity to Con A, giving broadened peaks, and accordingly smaller values of N. Fig. 1c and d show the changes in the tailing factor (T) of the Con A peak. All columns showed large T values, gradually increasing with increasing glucose concentration, but the variation was not large in either series. The T values became slightly larger as DP increased. In every system Con A interacts with both immobilized

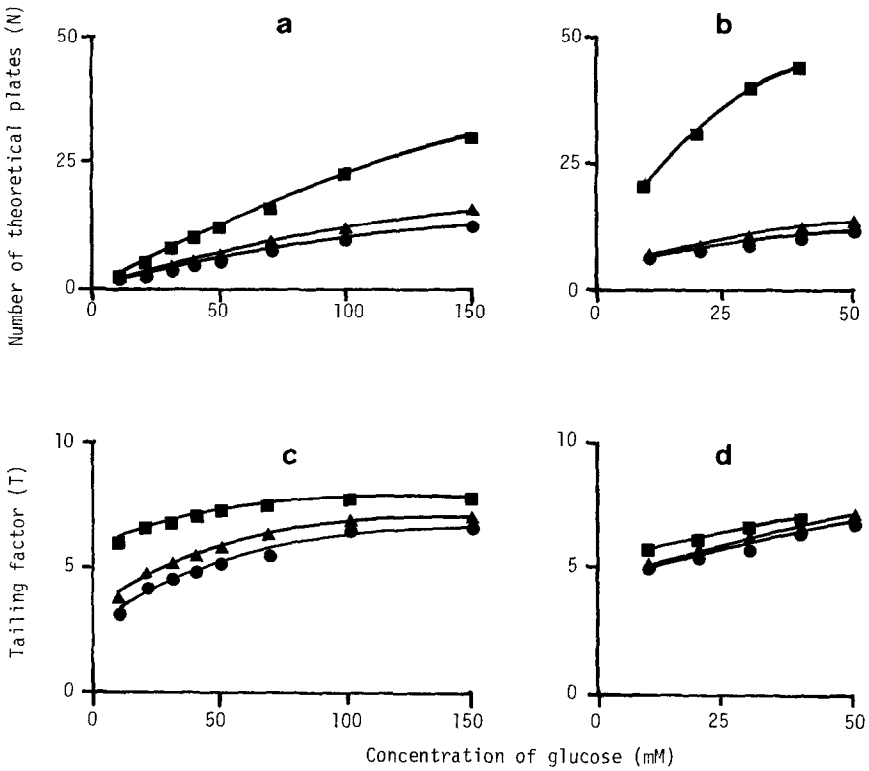

Fig. 1. Effects of glucose concentration on the number of theoretical plates [N; (a) glucose oligomer-immobilized silica; (b) mannose oligomer-immobilized silica] and the tailing factor [T; (c) glucose oligomer-immobilized silica; (d) mannose oligomer-immobilized silica] of the Con A peak. Column, 100 mm × 4 mm I.D.; column temperature, 29°C; eluent, 50 mM phosphate buffer (pH 6.8) containing sodium chloride at a concentration of 200 mM and glucose at a concentration of either 10, 20, 30, 40, 50, 70, 100 or 150 mM; flow-rate, 0.5 ml/min; detection, fluorescence at 340 nm with irradiation at 300 nm. Amount of sample (Con A) injected: 200 ng per 20 μl. ● = Biose; ▲ = triose; ■ = tetraose.

oligosaccharide and glucose in the eluent. If Con A interacts in the monovalent mode, the equation proposed by Swaisgood and Chaiken[9] can be applied:

$$\frac{1}{V - V_0} = \frac{K_d}{(V_0 - V_m)\,[M]} + \frac{[L]\,K'_d}{(V_0 - V_m)\,[M]\,K'_d} \quad (1)$$

where V, V_m and V_0 are elution volume of Con A, the volume of mobile phase in the column and the elution volume of unretained species, respectively. The values of V_m and V_0 could be obtained as the peak volumes of injected water (detected as a solvent shock by UV absorption at 254 nm) and Con A eluted with a sufficiently high concentration (500 mM) of glucose. At this concentration, the binding of Con A to glucose was so strong that its binding to the immobilized oligosaccharide was almost negligible, hence Con A was not retained at all. [L] and [M] designate the concentration of glucose as a displacing sugar and the effective concentration of immobilized oligosaccharide, respectively. The pores in the supporting gel were too small (average

diameter 10 nm) for the Con A molecule to enter them. In order to obtain the effective concentration of oligosaccharide immobilized to the particle surface, ready to interact with Con A, we determined the amounts of enzyme- and acid-released glucose from maltose-immobilized silica gel. The ratio of these amounts (0.0063) can be regarded as the molar fraction of oligosaccharides on the particle surface. Thus [M] was obtained by multiplying the total number of moles of immobilized oligosaccharides by a factor of 0.0063 and dividing the product by the volume of stationary phase, which was obtained by subtraction of V_m from the column volume. K_d and K'_d are the equilibrium dissociation constants of immobilized oligosaccharide–Con A and glucose–Con A complexes, respectively.

Eqn. 1 indicates that the relationship between [L] and $(V - V_0)^{-1}$ is first order. However, the plot of [L] *vs.* $(V - V_0)^{-1}$ in the range 10–100 m*M* gave concave curves for all oligosaccharide-immobilized gels, as exemplified by Figs. 2 and 3. On the other hand, non-linear least-squares regression analysis by using the MULTI system[10] showed an excellent fit to the equation applied to bivalent system also proposed by Swaisgood and Chaiken[9]:

$$\frac{1}{V - V_0} = \frac{1 + 2 \cdot \frac{[L]}{K'_d} + \left(\frac{[L]}{K'_d}\right)^2}{(V_0 - V_m)\left[2 \cdot \frac{[M]}{K_d} + \left(\frac{[M]}{K_d}\right)^2 + 2 \cdot \frac{[L][M]}{K_d K'_d}\right]} \quad (2)$$

The association constant (K_a) of an immobilized oligosaccharide to Con A could be calculated as the reciprocal of K_d. The values of K_a obtained are summarized in Table I. The results indicate that K_a became larger as DP increased, and the rate of increase accelerated with increase in DP. The K_a value for tetraose was by

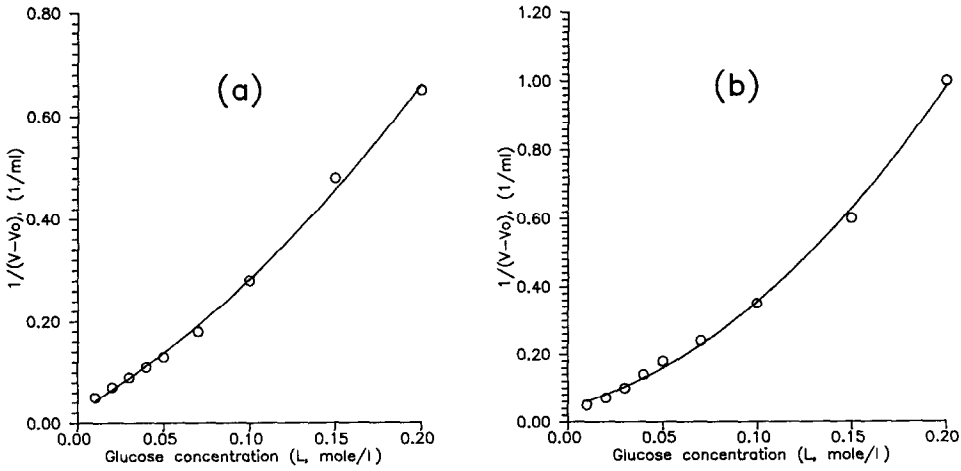

Fig. 2. Relationship between glucose concentration (L) and $(V - V_0)^{-1}$ for (a) glucobiose-immobilized and (b) glucotriose-immobilized columns. Analytical conditions as in Fig. 1.

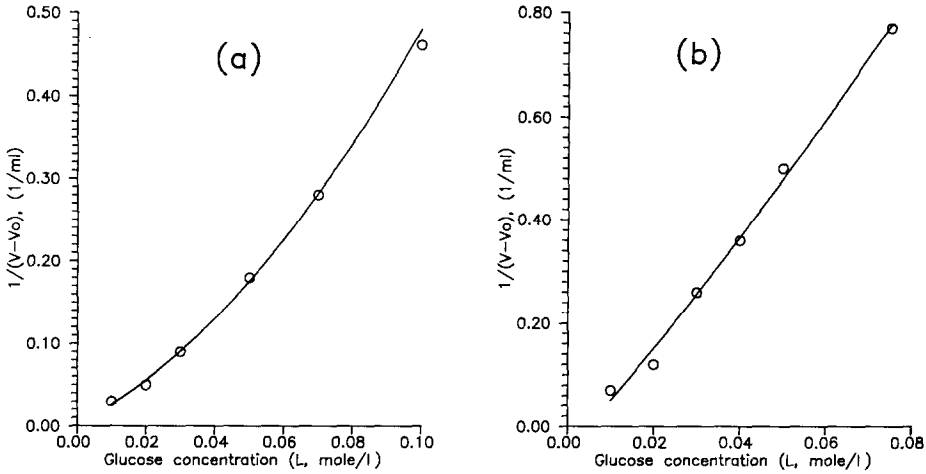

Fig. 3. Relationship between glucose concentration (L) and $(V - V_0)^{-1}$ for (a) mannobiose-immobilized and (b) mannotriose-immobilized columns. Analytical conditions as in Fig. 1.

approximately one order of magnitude higher than that of biose in both series of oligosaccharides. Also, the K_a values for the immobilized mannose oligomers were several times larger than those of the corresponding glucose oligomers having the same DP values.

The K_a value for mannobiose ($4.82 \cdot 10^5$ l mol^{-1}) is approximately 60 times larger than the literature value for methyl α-mannopyranoside ($8.3 \cdot 10^3$ l mol^{-1} at 27°C), obtained by a spectrophotometric method based on displacement of Con A-bound *p*-nitrophenyl α-mannopyranoside with methyl α-mannopyranoside in

TABLE I

ASSOCIATION CONSTANTS OF CON A TO VARIOUS IMMOBILIZED OLIGOSACCHARIDES

Immobilized oligosaccharide	*Association constant (K_a, 10^5 l mol^{-1})*	*Square of decision coefficient (r^2)*[a]
Glucose oligomer:		
Biose	1.30	0.92
Triose	2.04	0.97
Tetraose	14.4	0.94
Mannose oligomer:		
Biose	4.82	0.90
Triose	5.33	0.91
Tetraose	41.0	0.89

[a] $r^2 = \dfrac{[\Sigma(\overline{\text{obs}} - \text{obs}_i)^2 - \Sigma(\text{cal}_i - \text{obs}_i)^2]}{\Sigma(\overline{\text{obs}} - \text{obs}_i)^2}$, where

$\overline{\text{obs}}$ = average of observed values; obs_i = the *i*th observed value and cal_i = the *i*th calculated value.

solution[11]. The immobilized saccharide in this work had mannamine as aglycone, which is bound to silica via the ethoxypropyl group. The variation of the aglycone from the small methyl group in methyl α-mannopyranoside to the bulky aglycone in the immobilized saccharides may be the main cause of the observed difference in the K_a values, although the difference in assay method should also be taken into account.

Although HPAC may not give genuine association constants of free oligosaccharides to proteins, comparison of K_a values among immobilized saccharides obtained under identical conditions offers useful information on structure–affinity relationships. The increase in K_a with increasing DP implies that elongation of the carbohydrate chain increases the binding sites to Con A, *i.e.*, the binding site is not only at the non-reducing monosaccharide residue but also in the interior portion of the carbohydrate chain. It is plausible to assume that the hydroxyl groups in the non-reducing and interior monosaccharide residues were bound to the specific amino acid residue(s) in Con A, and elongation of the oligosaccharide chain resulted in an increase in the number of such bonds, by which Con A–oligosaccharide complexes were stabilized. The effect of the disposition of hydroxyl groups on the affinity to Con A is a problem to be investigated further. A comparative study by using positional isomers of oligosaccharides is now in progress. Considering the Con A part, it is well known that Con A exists as a tetramer of α-subunits under physiological conditions[10]. It may be considered that there are two pairs of subunits and the two subunits of each pair behave identically on binding to an oligosaccharide. However, further structural studies of Con A are necessary before unequivocal conclusions can be drawn.

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