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Studies on *Solanum tuberosum* Agglutinin

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Abstract

Potato lectin (*Solanum tuberosum* agglutinin, STA) was purified by affinity chromatography on $(GlcNAc)_3$-Sepharose 6B. The molecular weight of STA was estimated to be about 100,000 by gel filtration on Sephadex G-150 in 0.05 M phosphate buffered saline, pH 7.2. By sedimentation equilibrium analysis, it was shown that STA was an aggregating system with a monomer molecular weight of 54,000. Equilibrium dialysis showed that STA (dimer) has two binding sites for a specific sugar per molecule. STA had a high content of sugar, most of which was arabinose, and was rich in Hyp and Cys. Upon interaction with specific sugars, STA induced a UV-difference spectrum having positive peaks at 292 nm and 285 nm, which seemed to be characteristic of the tryptophyl residue. The association constants of STA with chitin oligosaccharides were determined from the intensities of the difference spectra at various concentrations of sugars. They increased with increasing chain-length of the sugar. Frontal affinity chromatography of STA was performed on columns of immobilized chitin oligosaccharides and that of chitin oligosaccharides on a column of immobilized STA. The association constants obtained with STA-Sepharose were in good agreement with those obtained by difference spectra, whereas those obtained with $(GlcNAc)_{2,3}$-Sepharose were much higher, presumably owing to the effect of multivalency of ligands. The analyses of CD spectra of STA in the far UV region indicated the presence of approximately 40% of β and 60% of unordered form, and no evidence of α-helix conformation. This confirms the structure suggested by the unusual amino acid composition and by the high content of sugar.

Introduction

Lectins are proteins or glycoproteins of plant and animal origin that bind specific carbohydrates and agglutinate cells of various types [2]. Blood-group-nonspecific potato lectin (*Solanum tuberosum* agglutinin, STA) was first described by Marcusson-Begun [3] and its partial purification was first reported by Marinkovich [4].

STA is specifically inhibited in its hemagglutination reaction by oligosaccharides that contain β-linked *N*-acetyl-D-glucosamine residues [5, 6]. STA has a specificity similar to but not identical with WGA, which is also inhibited by oligosaccharides containing *N*-acetyl-D-glucosamine. WGA, but not STA, binds with free sialic acid [7] or sialyl residues in glycoprotein [8] and glycolipid [9]. Recently, we have found that STA, but not WGA, binds specifically with keratan sulfate and chitin sulfate, despite the presence of sulfate groups [10].

The complete purification of STA accomplished by Allen and Neuberger [11] required time-consuming steps, including gel-filtration and ion-exchange chromatographies. Purified STA was found to be an unusual glycoprotein with a high content of half-cystine and hydroxyproline residues, and with L-arabinose as the major sugar component. Allen *et al.* [12], and Muray and Northcote [13] reported that almost all hydroxyproline residues in STA are substituted with L-arabinose residues, that the average hydroxyproline-to-arabinose ratio is 1 : 3-4, and the L-arabinose is present in the β-linked furanoside form.

Delmotte *et al.* [14] first described an effective adsorbent for the affinity chromatography of STA by coupling 4-aminobenzyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside to Sepharose by a long spacer-group. However, the preparation of this adsorbent required a five stage synthesis. A more convenient method [15, 16] for preparing an affinity adsorbent of STA has been described: Tri-*N*-acetylchitotriose, a sugar specific to STA, was coupled with epoxy-activated Sepharose prepared by treatment with epichlorohydrin [15, 16]. Desai and Allen [17] reported a similar method using bis(oxirane) as the reagent for epoxy-activation. Subsequently two methods of purification of STA, by affinity chromatography on fetuin-Sepharose [18] and on glutaraldehyde-fixed erythrocyte, [19] have been reported. The properties of STA purified by $(GlcNAc)_3$-Sepharose, especially the spectroscopic properties and the specific interaction between STA and oligosaccharides, is described herein.

Experimental Procedures

Materials: Potatoes were obtained from a local market. 5,5'-

Dithiodi(2-nitrobenzoic acid) (DTNB) and 2,2'-dithiopyridine (2-PDS) were obtained from Wako Pure Chemical Industry Ltd. (Osaka, Japan). *N*-Acetyl-D-glucosamine (GlcNAc), di-*N*-acetylchitobiose $(GlcNAc)_2$, tri-*N*-acetylchitotriose $(GlcNAc)_3$, and tetra-*N*-acetylchitotetraose $(GlcNAc)_4$ were prepared by the method of Rupley [20], and purified by gel filtration on Sephadex G-25 after *N*-acetylation. Collagenase from *Clostridium histolyticum* was obtained from Seikagaku Kogyo Co., Ltd. (Japan). Collagen type I from bovine achilles tendon was obtained from Sigma Chemical Company.

SDS-Polyacrylamide-Gel Electrophoresis: This procedure was performed by the method of Fairbanks *et al.* [21].

Gel Filtration: Gel filtration was performed on a column (3.0 × 90 cm) of Sephadex G-150, equilibrated with 0.15 M NaCl-5 mM phosphate buffer (pH 7.2). The following substances were used for the estimation of molecular weight; Dextran T-110 (110,000), Dextran T-40 (40,000), human γ-globulin (160,000), bovine serum albumin (67,000), and D-glucose.

Sedimentation Experiments: Sedimentation experiments were performed at 8,766 r.p.m. with a Beckman Spinco Model E Analytical Ultracentrifuge. All experiments were carried out at 19.8°C on solutions in 0.1 M NaCl. Protein concentration varied from 10–3.3 mg/ml. The value of 0.65 reported by Allen *et al.* [12] was used for the partial-specific volume.

Amino Acid Composition: The amino acid analyses were performed with a JEOL JLC-5AH Amino Acid Analyzer. The standard system for acidic and neutral amino acids involved successive elution of a 70-cm column with sodium citrate buffer at pH 3.25 (0.2 M), pH 4.25 (0.2 M), and pH 5.28 (0.35 M) from 45-75°C, at a flow rate of 0.83 ml/min, and with $SnCl_2$-ninhydrin as reagent for color development. Basic amino acid analysis was performed by elution of a 15-cm column with sodium citrate buffer, pH 5.28 (0.35 M), at 45°C at a flow rate of 1.22 ml/min. For the analysis of ornithine and lysine, sodium citrate buffer, pH 4.85 (0.35 M), was used. Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton [22].

Samples of protein were hydrolyzed for 24, 48, and 72 h, at 110°C *in vacuo*, and correction factors were obtained for the destruction of serine and threonine. The content of half-cystine was determined by the method using DTNB [23] and 2-PDS [24], after reduction with $NaBH_4$ in 8 M urea [25].

Analyses of Neutral Sugar: Neutral sugar composition of STA was determined by g.l.c. with a Shimazu Gaschromatograph GC-4CM, after methanolysis and trimethylsilylation [26], mannitol being the

internal standard.

Amino Sugar Analyses: Samples were hydrolyzed with 3 M HCl for 15 h at 100°C. The amino sugars were determined with an amino acid analyzer and were eluted from the 15-cm column with a sodium citrate buffer (0.35 M, pH 5.28) at 45° at a flow rate of 0.52 ml/min.

Equilibrium Dialysis: Binding of $(GlcNAc)_3$ to STA was measured by equilibrium dialysis. A sample (200 or 300 μl) of STA solution (2.04 mg/ml of 0.15 M NaCl, pH 7.0) was introduced into one compartment, and a solution (200 or 300 μl) of various amounts of $(GlcNAc)_3$ (from 560-6.4 μM) into the other compartment of the dialysis cell. The cells were shaken for 24 h at 4°C, and samples were removed for assay. The concentration of the free sugar was determined by the Park-Johnson method [27] with $(GlcNAc)_3$ as standard.

UV-difference Spectroscopy: The difference spectra (lectin-sugar *vs.* lectin) were measured at room temperature with a Hitachi type 340 spectrophotometer, equipped with a recorder and a microcomputer. For the determination of equilibrium-binding parameters, the intensities of difference spectra were determined as a function of the increasing sugar concentration. The peak-trough difference was used as a measure of the association [28].

Preparation of STA-Sepharose 4B: Sepharose 4B was activated with epichlorohydrin [16] and aminated with a concentrated solution of ammonium hydroxide [29]. The concentration of amino groups introduced into agarose was 55 μmol/ml of gel according to the sodium 2,4,6-trinitrobenzene-sulfonate (TNBS) color test [29]. The coupling of STA to amino-Sepharose was performed as follows: Suction-dried amino-Sepharose 4B (1 g) and 20 mg of STA were dissolved in a solution containing 20 mg of $(GlcNAc)_2$ as the haptenic sugar. The coupling reaction was performed with (3-dimethylaminopropyl)-1-ethylcarbodiimide (EDC) at pH 4.75 [30], and the mixture was kept overnight at 4°C. The remaining amino groups were treated with acetic anhydride [30] in the presence of $(GlcNAc)_2$. The concentration of immobilized STA was estimated at 7 mg/ml of gel from the concentration of STA in the supernatant solution after coupling.

Frontal Analysis on STA-Sepharose: The frontal analysis was performed at 4°C according to the method of Kasai *et al.* [31-33]. An STA-Sepharose column (0.59 × 5.12 cm) was previously equilibrated with a 0.15 M NaCl solution, and a solution of $(GlcNAc)_{2-4}$ in 0.15 M NaCl (concentration: $[A]_0$) was applied continuously. Elution of $(GlcNAc)_{2-4}$ was monitored by the Park-Johnson method [27]. The elution volume was calculated from the equation (1), where a is the volume of one fraction, n is the tube number of a certain fraction at the plateau, and $[A]_i$ is the concentration of fraction i. After

being used, the column was washed with 0.15 M NaCl (about 500 times the bed volume) before the next operation. As V_0, the elution volume of Glc that does not interact with STA was used. The association constant was calculated according to the equation (2) [32] where K_a is the association constant and B_t is the total amount of the immobilized ligand in the column.

$$(1) \qquad v = na - \frac{a \sum_{i=1}^{n} [A]_i}{[A]_0}$$

$$(2) \qquad \frac{1}{[A]_0(V - V_0)} = \frac{1}{B_t \cdot K_a} \cdot \frac{1}{[A]_0} + \frac{1}{B_t}$$

Frontal Analysis on $(GlcNAc)_{2,3}$-*Sepharose*: $(GlcNAc)_{2,3}$-Sepharose 6B was prepared according to the method previously reported [16]. The chromatography was carried out at 4°C and the column of $(GlcNAc)_{2,3}$-Sepharose (0.2×1.5 cm) was previously equilibrated with 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. The STA solution was applied continuously, and the elution of STA was monitored at A_{280}. The V_0 value was obtained from the elution volume of bovine serum albumin, and calculated according to equation (2).

CD Spectra: CD spectra were recorded with a JASCO J-20 Dichrograph for solutions in a 1.0-cm cell for the region above 250 nm, and in a 0.2-cm cell for the region below 250 nm. The data are expressed as mean-residue ellipticities $[\theta]$ in $deg \cdot cm \cdot dmol^{-1}$, the mean residue weight being taken as 120. Calculation of the secondary structure by the least-square method was made by a computer using the standard value reported by Chen *et al.* [34], Chang *et al.* [35] and Hayashi *et al.* [36].

Collagenase Treatment of STA: To 1.5 mg of STA, dissolved in 1 ml of 0.05 M Tris-HCl, pH 7.5, containing 0.36 M $CaCl_2$ was added 100 μl of *Clostridium histolyticum* collagenase solution (0.21 mg/ml of the Tris buffer). After incubation for 19 h at 37°C, the increase of amino terminal residues by the cleavage with collagenase was measured by the ninhydrin color reaction at 570 nm. Collagen from bovine achilles tendon was also treated with collagenase as a control experiment.

Results

Purification of STA: All procedures were carried out at 4°C unless otherwise stated. The potato tubers (2 kg) were washed, finely grated, and suspended in 3 M acetic acid. After 12 h, the extract was centrifuged for 30 min at 15,240 *g*, and $(NH_4)_2SO_4$ was

Table I. Purification of STA[a]

Procedure	Total activity ×10 (units)	Weight (g)	Specific activity[b] (units/mg)	Yield (%)
0–55% sat. $(NH_4)_2SO_4$ ppt	3,000	3	1,000	100
$(GlcNAc)_3$-Sepharose 6B	2,900	0.017	179,000	96

[a] From 2 kg of potato tubers.

[b] Units are expressed as the amount of lectin required to cause half-maximum agglutination.

added to the supernatant solution until a concentration of 55% was reached. The precipitate was allowed to settle overnight, and then collected by centrifugation at 15,240 *g* for 30 min. The precipitate was dissolved in water, the solution dialyzed extensively against water, and then clarified by centrifugation at 15,240 *g* for 30 min. The supernatant solution was applied to an affinity column of tri-*N*-acetylchitotriose-Sepharose, prepared as described previously [16], and the chromatography was performed at room temperature. The column was washed successively with PBS, 1 M NaCl, and water, and then the lectin was eluted with 0.2 M ammonium hydroxide

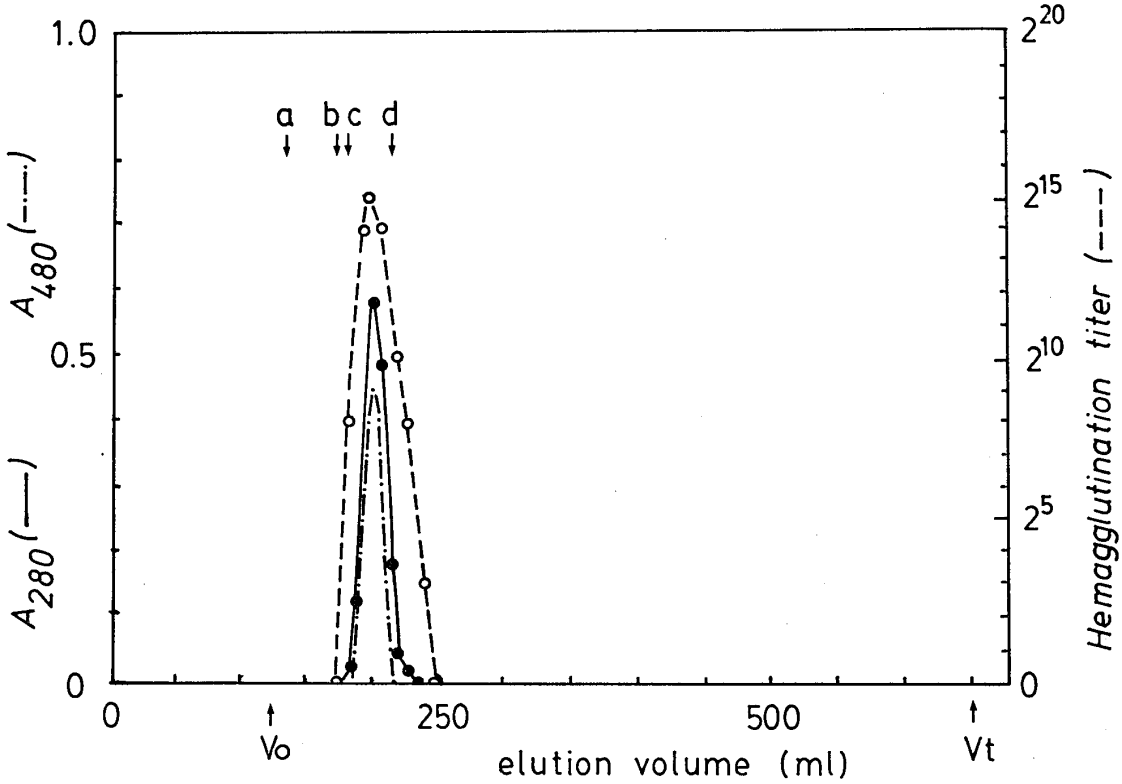

Fig. 1. Gel chromatography of purified STA on Sephadex G-150 column. The purified STA was applied to the column (3.0×90 cm) equilibrated with PBS and eluted with the same buffer at a flow rate of 10 ml/h. The standards for molecular weight determination were: (a) Dextran T-110 (mol wt 110,000); (b) T-40 (mol wt 40,000); (c) globulin (mol wt 160,000); and (d) bovine serum albumin (mol wt 67,000).

solution. The active fractions were directly lyophilized. Almost all of the agglutinating activity was recovered (see Table I).

Purity and Molecular Weight of STA: After electrophoresis in 5% polyacrylamide gel at pH 8.0 in the presence of 1% SDS and 6% mercaptoethanol, the purified STA gave a single band on staining with Coomassie brilliant blue. In this system and by use of markers, a molecular weight of 100,000 was estimated.

Gel chromatography of STA was performed on Sephadex G-150 with 0.015 M PBS (pH 7.2). As shown in Fig. 1, the peak of protein determined by its absorbance at 280 nm coincided with those of neutral sugar, determined by the phenol-sulfuric acid method [37], and of hemagglutinating activity. The molecular weight was estimated to be 100,000.

STA was also analyzed by sedimentation-equilibrium ultracentrifugation. Plots of $\ln fr$ *vs.* r^2 for STA (3.3 mg/ml) are shown in Fig. 2. The molecular weight increased with the concentration of STA as previously reported by Allen *et al.* [12], suggesting the aggregation of the lectin. The molecular weight of the monomer was estimated to be 54,000 by extrapolation to zero concentration and use of $v=0.65$.

The results of a typical equilibrium dialysis experiment are presented in Fig. 3. The results were plotted according to the

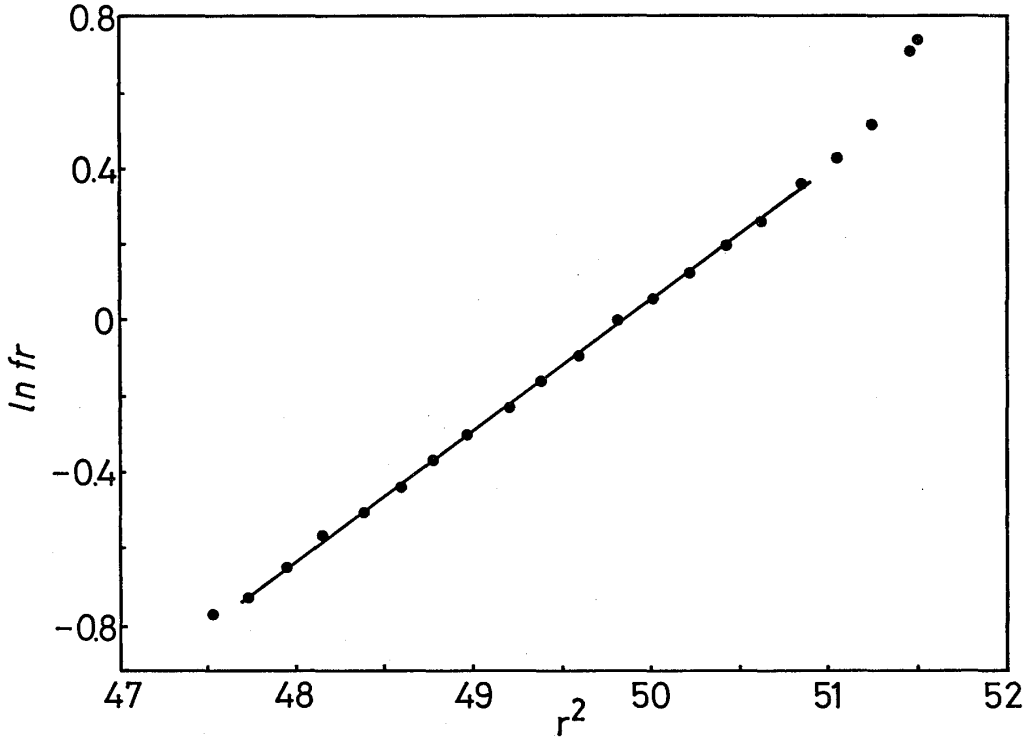

Fig. 2. Sedimentation equilibrium $\ln fr$ *versus* r^2 plots. The solution of the lectin in 0.1 M NaCl (3.3 mg/ml) was centrifuged at 8,766 rpm at 19.8°C.

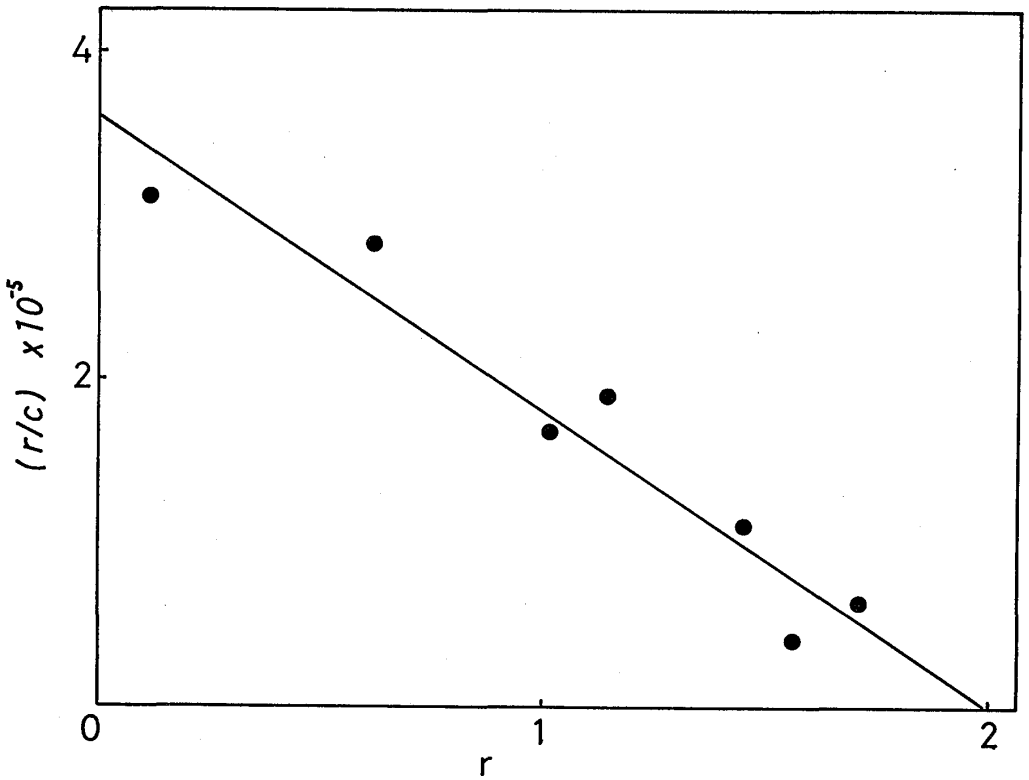

Fig. 3. Equilibrium dialysis data for the binding of $(GlcNAc)_3$ to STA, plotted according to Scatchard [35]. Experimental details are described under "Methods".

Table II. Amino acid and carbohydrate composition of purified STA

Amino acid	wt (%)		Carbohydrate	wt (%)
Hyp	11.2	(50)[a]	Ara	44.0
Asp	2.1	(9)	Gal	4.0
Thr	2.2	(11)	Glc	0.6
Ser	3.4	(19)	Xyl	0.6
Glu	4.0	(16)	Man	trace
Pro	3.9	(20)	GlcN	0.3
Gly	2.9	(22)		
Ala	1.2	(8)		
(1/2)Cys	2.9	(14)		
Val	0.2	(0.9)		
Met	0.3	(1.3)		
Ile	0.8	(3.7)		
Leu	0.7	(3.3)		
Tyr	1.6	(5.3)		
Phe	0.1	(0.5)		
Orn	0.3	(0.9)		
Lys	2.0	(8)		
His	0.3	(1)		
Arg	0.6	(2.1)		
Trp	1.4	(4.1)		
Total	42.1		Total	49.5

[a] Values in parenthesis are expressed as molar ratios relative to Hyp (=50).

Scatchard equation $r/c=nK-rK$ (38), where r is the number of $(GlcNAc)_3$ bound per STA, c is the concentration of free sugar, n is the maximal value for r, and K is the association constant. In this plot, extrapolation yielded a value of $n=2.0$, indicating two binding sites for $(GlcNAc)_3$ per molecule of STA. From the linear plot, the binding constant was calculated as $1.8\times10^4\ M^{-1}$.

Amino Acid and Carbohydrate Composition of STA: The amino acid composition of STA (see Table II) is remarkable for the extremely high content of Hyp, Gly, Pro, and Cys residues. The contents of Phe, Val, His, and Met residues were low.

The existence of Orn residues was supported by a peak coinciding with that of standard ornithine, and its content was estimated to be about 1 residue per STA monomer (mol. wt. 50,000).

The state of the half-cystine residues detected by the amino acid analyzer was investigated by the methods using the disulfide exchange reagents DTNB and 2-PDS. After denaturation with 8 M urea and

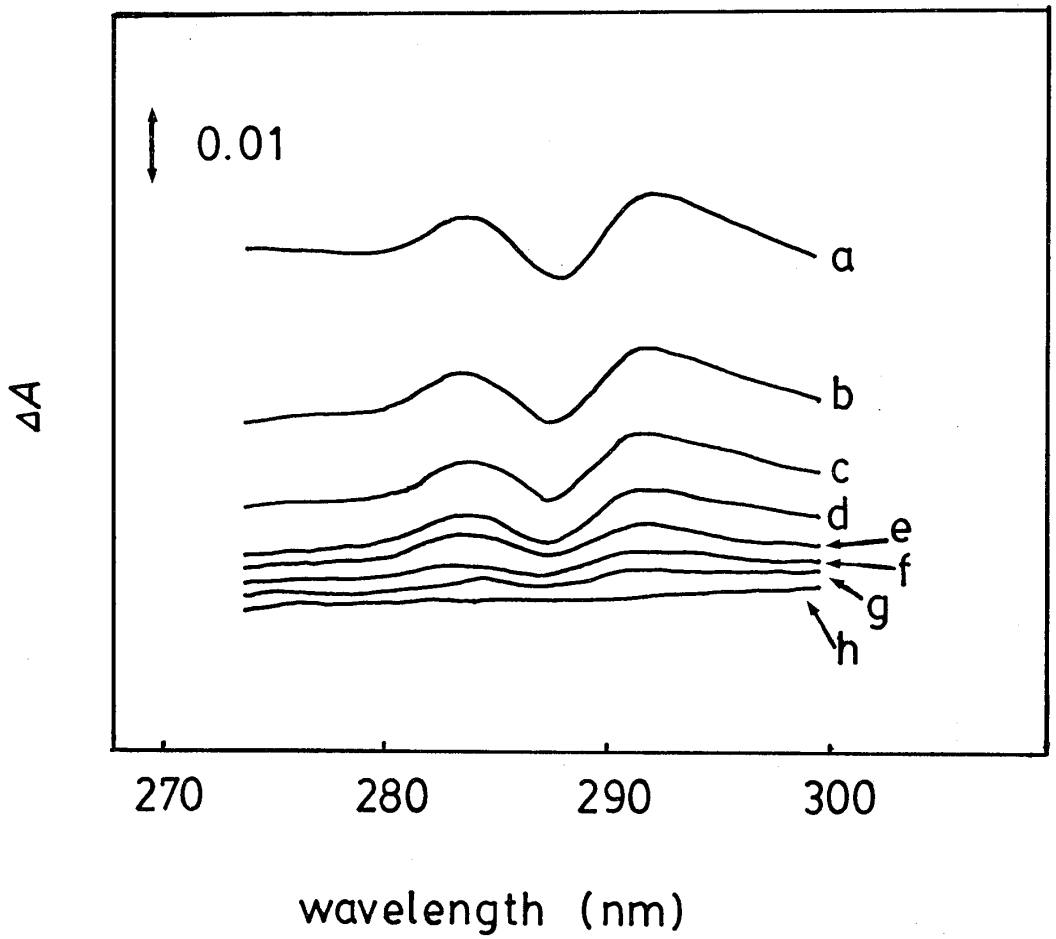

Fig. 4. Difference spectra of STA induced by $(GlcNAc)_2$. The concentration of STA was 0.49 mg/ml, and that of $(GlcNAc)_2$: (a) 6.71, (b) 3.35, (c) 1.68, (d) 0.84, (e) 0.42, (f) 0.21, (g) 0.111, and (h) 0.05 mM.

reduction with $NaBH_4$, about 13 cysteine residues were detected per mole of STA monomer, but no cysteine was detected in the absence of $NaBH_4$, regardless of the existence of 8 M urea.

STA contained 44% (w/w) of carbohydrate, 89% of which was arabinose. Very low but significant proportions of Gal, Glc, Xyl, and GlcN were detected. These results are slightly different from those reported by other authors. Allen and Neuberger (11) reported no Xyl and Man, Muray and Northcote (13) no Xyl and GlcN, and 7% of Man.

UV-Difference Spectra: The difference spectra of STA induced by the addition of $(GlcNAc)_2$ are shown in Fig. 4. Compounds $(GlcNAc)_{1-4}$ showed no absorbance at wavelength between 300 nm and 250 nm. Difference spectra have peaks at 292 nm and 285 nm, and are typical of the perturbation of the tryptophan residue. These peaks were also observed with $(GlcNAc)_3$ and $(GlcNAc)_4$, but not with GlcNAc at the concentration used. The intensity of the difference spectrum of STA was dependent on the concentration of $(GlcNAc)_2$, as shown in Fig. 5. Plots of $\Delta A_{292-288}$ *vs.* the concentration of $(GlcNAc)_2$ in the mixture showed a hyperbolic curve. Plots according to equation (3) are shown in Fig. 6.

$$\frac{[S]}{\Delta A} = \frac{1}{\Delta A_{max}}[S] + \frac{1}{\Delta A_{max} \cdot K} \quad (3)$$

In this equation, K=binding constant of the STA-$(GlcNAc)_{2-4}$ complex, $[S]$ is the concentration of $(GlcNAc)_{2-4}$, and A_{max} is the maximum

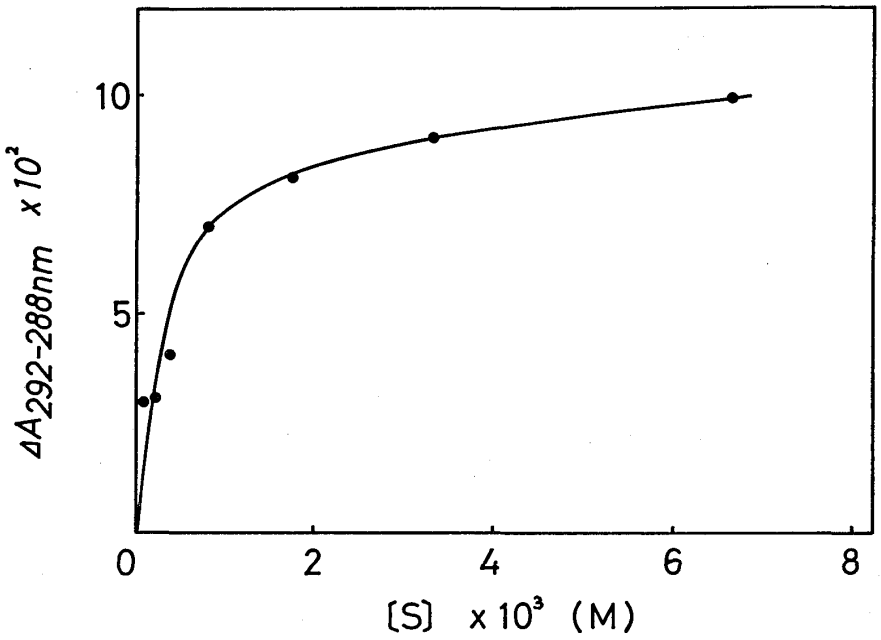

Fig. 5. Effect of the concentration of $(GlcNAc)_2$ on the difference spectra of STA.

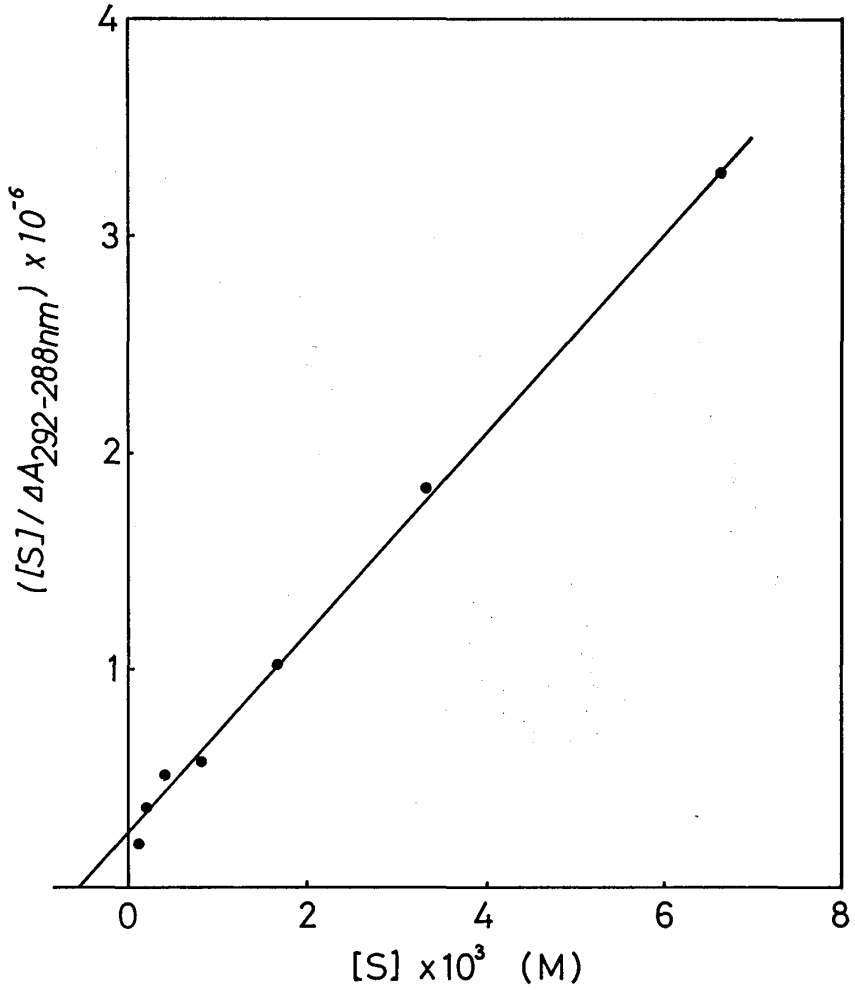

Fig. 6. Plot of $[S]/\Delta A_{292-288}$ *vs.* $(GlcNAc)_2$ concentration. The solid line was calculated from a least-square fit to the data. The dissociation constant K_d was calculated from the intercept on the abscissa.

absorption difference obtained when all of the STA are complexed with $(GlcNAc)_{2-4}$ molecules. The solid line is a least-squares plot of the data to the equation.

Affinity Chromatography of Crude STA on $(GlcNAc)_{1-3}$*-Sepharose 6B*: A solution of crude STA having a titer of 16 was continuously applied to each column until STA began to pass through the column. This was reached with less than 2 ml of STA solution for a GlcNAc-Sepharose column, as shown in Fig. 7. On the other hand, $(GlcNAc)_2$-Sepharose and $(GlcNAc)_3$-Sepharose columns required much larger amounts of STA solution, 600 and 2600 ml, respectively. The amounts of STA adsorbed on the column increased with the increase of chain-length of the sugar ligand. These amounts may be used as a measure of the adsorption capacity of the column, although each column was not completely saturated with STA.

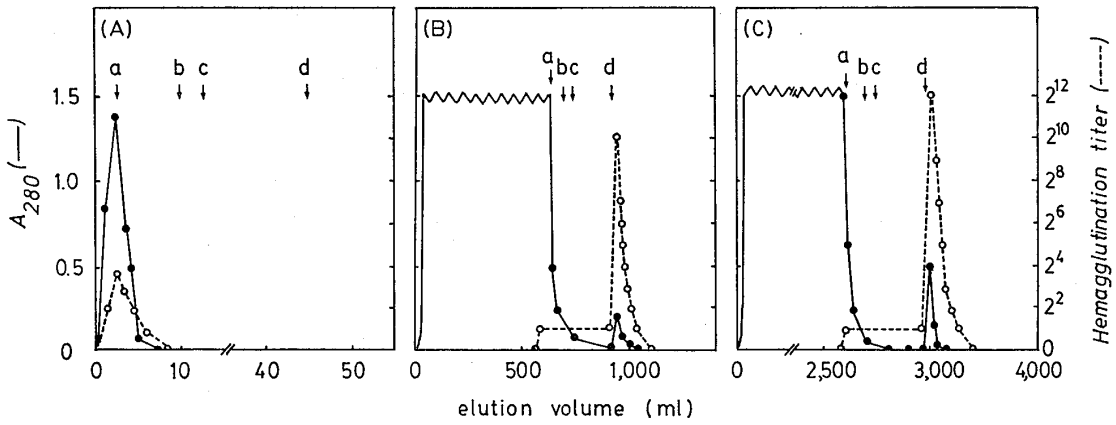

Fig. 7. Affinity chromatography of crude STA on $(GlcNAc)_{1-3}$-Sepharose 6B. Affinity chromatography of crude STA was performed on column (0.9×1.7 cm) of: (A) GlcNAc-; (B) $(GlcNAc)_2$-; and (C) $(GlcNAc)_3$-Sepharose 6B. A crude STA solution (1%) in PBS (titer 64) was continuously applied to each column until the hemagglutinating activity was detected in the effluent, and then the column was washed with PBS (a), 1 M NaCl (b), and H_2O (c). The elution of STA adsorbed was started with 0.2 M NH_4OH (d).

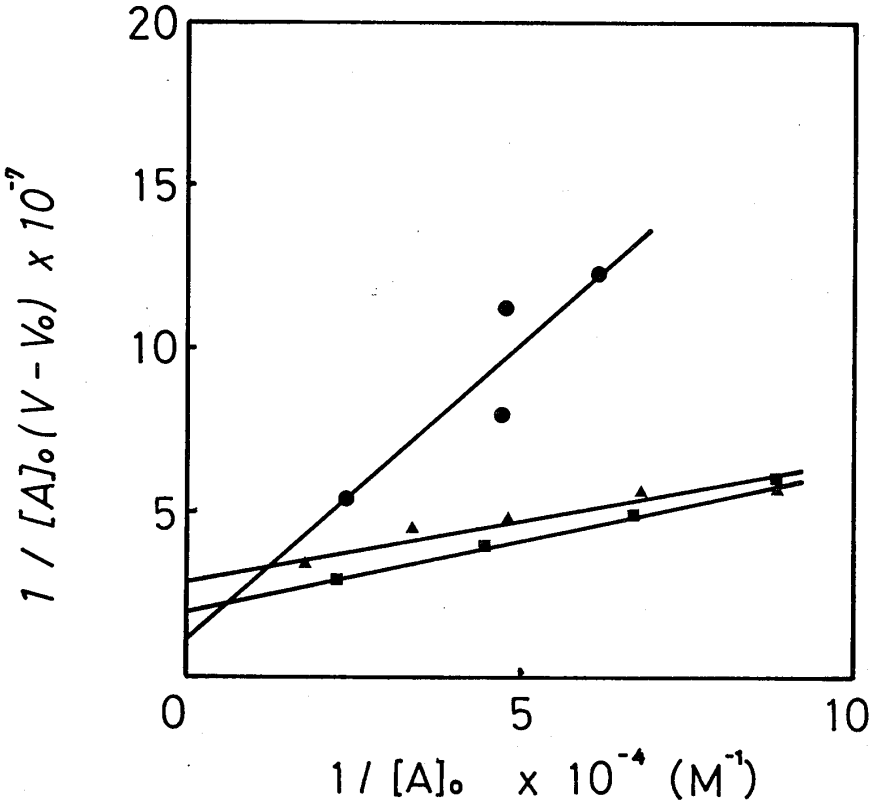

Fig. 8. Frontal-affinity chromatography of $(GlcNAc)_{2-4}$ on STA-Sepharose. Plots of $1/[A]_0$ *vs.* $1/[A]_0(V-V_0)$. The affinity chromatography of $(GlcNAc)_{2-4}$ was performed on a column of STA-Sepharose (0.6×5.1 cm) equilibrated with 0.15 M NaCl at 4°C. The data were plotted according to equation (2): ●, $(GlcNAc)_2$; ■, $(GlcNAc)_3$; and ▲, $(GlcNAc)_4$.

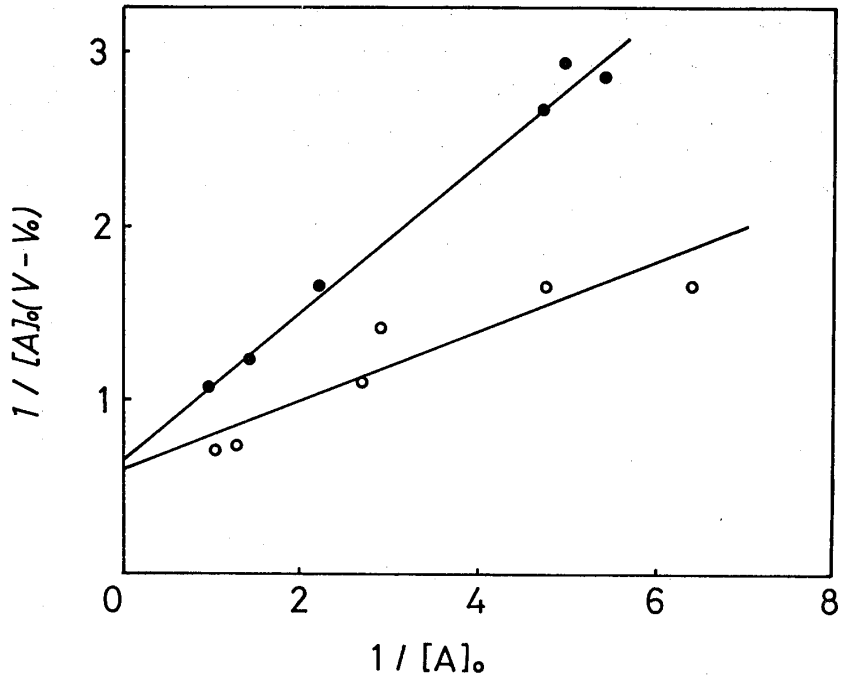

Fig. 9. Plots of $1/[A]_0$ *vs.* $1/[A]_0(V-V_0)$. Elution volume was obtained by the equation (1) and the data were plotted according to the equation (2): ●, $(GlcNAc)_2$-Sepharose; and ○, $(GlcNAc)_3$-Sepharose.

Frontal Affinity-Chromatography: The elution of chitin oligosaccharides from a column of STA-Sepharose was retarded with decreasing sugar concentration. Plots of $1/[A]_0$ *vs.* $1/[A]_0(V-V_0)$ for the frontal affinity chromatography of $(GlcNAc)_{2-4}$ on STA-Sepharose are shown in Fig. 8. The association constant of STA for $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ was calculated to be 6.3×10^3, 4.1×10^4, and $8.3\times10^4\ M^{-1}$, respectively. The adsorption capacity of STA-Sepharose for $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ was calculated to be 90, 50, and 34 nmol/ml, respectively. The values of adsorption capacity calculated were 40-70% of that obtained from the concentration of immobilized STA, and they increased with a decrease in the size of the sugar molecule.

Plots of $1/[A]_0$ *vs.* $1/[A]_0(V-V_0)$ for $(GlcNAc)_2$-Sepharose and $(GlcNAc)_3$-Sepharose are shown in Fig. 9. From the intercept of abscissa, the association constant of STA was calculated to be $6.5\times10^4\ M^{-1}$ for $(GlcNAc)_2$, and $1.5\times10^5\ M^{-1}$ for $(GlcNAc)_3$. From the intercept of ordinate, the adsorption capacity of $(GlcNAc)_2$-Sepharose was calculated to be 0.18, and that of $(GlcNAc)_3$-Sepharose 0.17 μmol STA/ml of gel. These values correspond to only 1/150 of the amount (30 μmol/ml of gel) of immobilized $(GlcNAc)_{2\text{ or }3}$, which was determined by hexosamine analysis.

CD Spectra: STA showed a negative CD band centered at 200 nm

and a positive band at 224 nm (solid curve in Fig. 10). A computer analysis of the secondary structure using standard data for α-helix, β-structure, and unordered structure given by Chen *et al.* [34] gave values (see Table III) that were compared with values calculated for lysozyme and values reported by Chen *et al.* [34]. STA had 40% of β-form and 60% of unordered form, but no α-helix. When com-

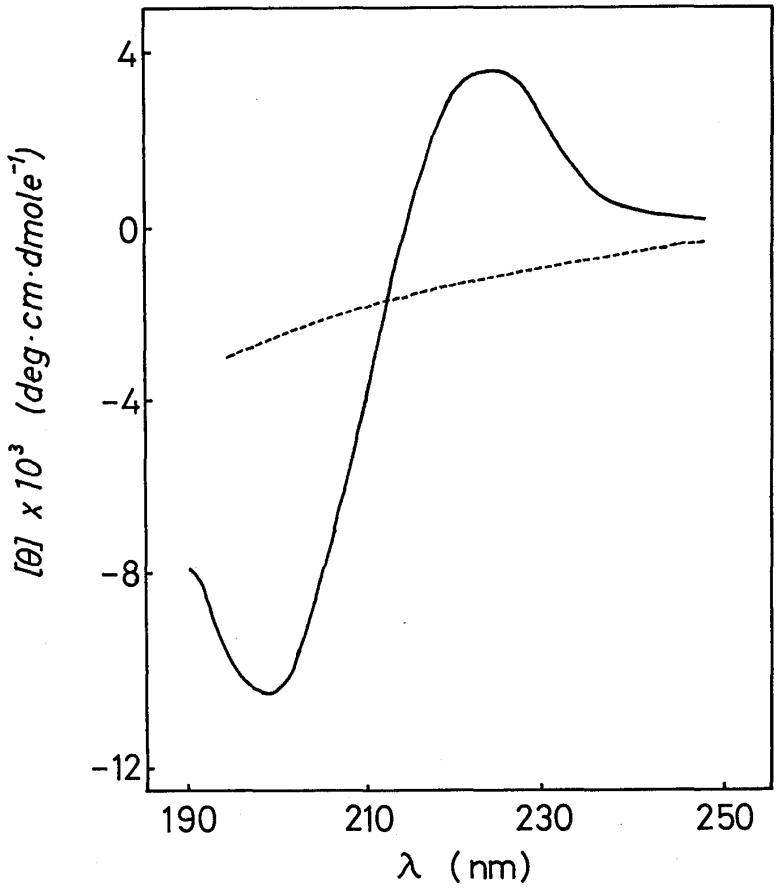

Fig. 10. Far ultraviolet CD spectrum of STA. The light path was 2 mm and the concentration of STA was 0.12 mg/ml in water. The dotted line shows the spectrum of the solvent (H_2O) measured in the same manner.

Table III. Fractions of α-helix, β and unordered form in STA and lysozyme determined by CD spectrometry

	α-Helix (%)	β-Form (%)	Unordered form (%)
STA	0	40	60
Lysozyme	40	13	47
From CD spectra[a]	37	11	
X-ray[b]	41	16	

[a] Ref. 34.
[b] Ref. 35.
$[\theta]_\lambda = f_\alpha[\theta]_{\alpha,\lambda} + f_\beta[\theta]_{\beta,\lambda} + f_R[\theta]_{R,\lambda}$

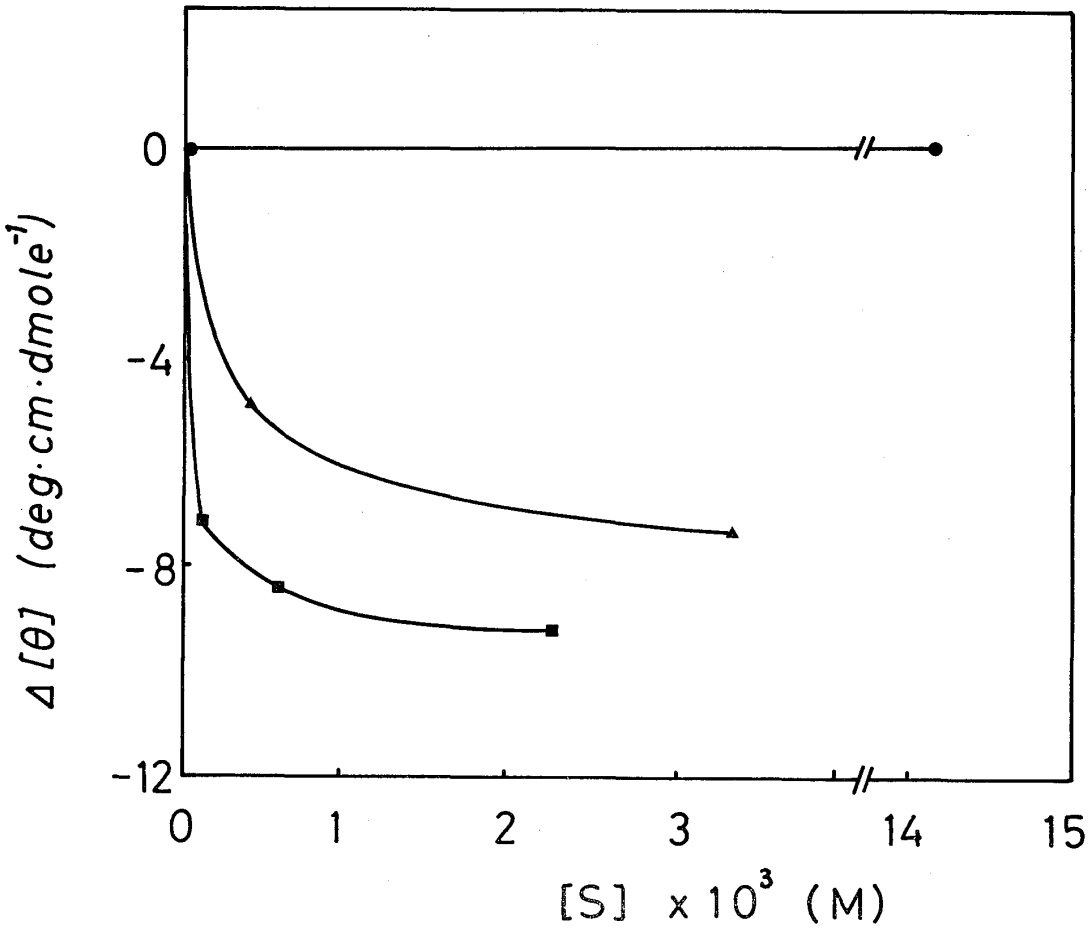

Fig. 11. Change in values of $[\theta]$ at 291 nm with the concentrations of GlcNAc, $(GlcNAc)_2$, and $(GlcNAc)_3$. CD was measured for STA solution (0.49 mg/ml water) with various concentrations of sugars at room temperature. The optical path-length was 1 cm. The values of $\Delta[\theta]$ at 291 nm were plotted against the concentration of sugars: ●, GlcNAc; ▲, $(GlcNAc)_2$; and □, $(GlcNAc)_3$.

puter analysis of secondary structure was made using data for β-turn [35] and collagen helix [36], it was suggested that STA had 10–15% β-turn and 20–40% collagen helix. Under the conditions of collagenase treatment, where 700 mol of amino terminal was produced per mol of the collagen, no amino acid terminals were produced from STA. The CD spectra of STA at 250–300 nm were modified by the addition of $(GlcNAc)_2$ and $(GlcNAc)_3$, but not of GlcNAc (final concentration was 3 mg/ml). Changes in the values of $[\theta]$ at 291 nm by various concentrations of sugars are shown in Fig. 11. The intensities of changes induced by $(GlcNAc)_3$ were larger than those by $(GlcNAc)_2$.

Discussion

Most lectins are usually extracted with PBS at a pH of about 7.

When potatoes were extracted with PBS, the color of the extract turned rapidly to brown, and a black precipitate finally formed. Allen *et al.* [12] added sodium hydrogensulfite to a PBS extract of potato to prevent browning. However, we found that STA is unstable under reductive or oxidative conditions, and is easily inactivated by sodium hydrogensulfite. On the other hand, STA is very stable under acidic or alkaline conditions. When potatoes were extracted with 3 M acetic acid, the browning was prevented, and the hexosaminidase in the extract was inactivated, but not STA. The crude STA fraction obtained by ammonium sulfate fractionation of potato extract was free from browning, even if it was thereafter solubilized in PBS. When crude STA was applied to a $(GlcNAc)_3$-Sepharose column, it was completely adsorbed and could be eluted with 0.2 M NH_4OH. Purified STA was easily obtained when active, eluted fractions were directly lyophilized. Since the total purification procedures were short and simple, the overall recovery of STA from the extract was very high, as shown in Table I, when compared with those reported earlier [11, 12, 14, 39, 40].

The purified STA was found to be homogeneous on SDS-PAGE and gel filtration, and its molecular weight was estimated to be about 100,000. This value is in good agreement with the value of 120,000, previously reported by Allen and Neuberger [11] for STA purified by conventional chromatographies. Sedimentation-equilibrium centrifugation showed that STA exists in an aggregating system, and the molecular weight of the STA monomer was estimated to be 54,000, which is slightly higher than the values of 50,000 [12] and 46,000 [11] reported earlier. Equilibrium dialysis showed that STA has two binding sites per molecule (dimer), assuming the molecular weight to be 100,000. These results suggest that STA exists in a monomer-dimer system, and acts as a dimer in hemagglutination reaction.

The STA described herein has a high content of carbohydrate (*ca.* 50%), most of which (89%) being arabinose, and a high content of hydroxyproline and half-cystine as reported by Allen *et al.* [11, 12]. The stability of STA, which is resistant to heat, base, and acid, may depend on its high content of carbohydrate residues and disulfide bonds. Inactivation of STA by sodium hydrogensulfite may be due to the cleavage of disulfide bonds, which are essential for the conformation of STA.

Analysis of the CD spectrum gave various results, depending upon the method applied. The calculation by the method of Chen *et al.* [34] showed that STA has 40% of β-form, 60% of random coil, and no α-helix. The analysis of the CD spectrum of lysozyme by

the same methods showed results similar to those obtained by X-ray crystallography [40]. The content of β-form can be calculated from the value of $[\theta]$ at 195 nm, using a value of 30,000 [40] or 40,000 [41] for a model substance presumably having 100% pleated sheets. Since STA has a $[\theta]$ value of 10,000 at 195 nm, it may be assumed that STA contains 25–35% of β-form structure. The content of α-helix was also calculated by the method of Brahms *et al.* [42]; $H=[\theta]_{222}/(-40{,}000)=0$, $H=\{[\theta]_{208}-(-40{,}000)\}/[33{,}000-(-4{,}000)]<0.06$. These results also showed the high content of β-form and no α-helix in STA. As shown in Table II, STA has a high content of Hyp, Pro, Gly, Ser, and Cys residues, which do not form easily an α-helix. These results also support the unusual conformation suggested by the analysis of the CD spectrum of STA. Though analysis of the CD spectrum using standard data for collagen helix given by Hayashi *et al.* [36] showed the existence of collagen helix, STA was not digested by the collagenase from *Clostridium histolyticum*. The results suggest that STA has no sequences corresponding to X-Gly-Pro-Y-Gly-Pro. (Hyp+Pro)/Gly in STA was about 3.2 which is higher than that of collagen 1, but is close to the values for proline-rich proteins, 2–3, [43–48]. CD spectra of these proline-rich proteins were reported to be similar to the pattern of poly-proline II type [45–48].

The CD spectra of many lectins have been reported. RCA contains 13% of α-helix and 5% of β-form [49]; *Bandeiraea simplicifolia* lectin I 30–40% of β-form [50]; *Pisum sativum* lectin a β and unordered structure [51]; and WGA no α-helix, 12% of β-pleated structure, and probably a β-turn structure [52]. From the mean-residue ellipticity at 195–197 nm, the lectins of *Dolichos biflorus*, *Helix pomatia*, *Lotus tetragonolobus*, *Phaseolus vulgaris* (erythro-agglutinin), *Sophora japonica*, and *Ulex europeus* (I) were estimated to have 28–48% of β-form structure. Therefore, many plant lectins contain a rather large amount of β-form, and STA is not the exception in this respect.

Some lectins, such as *Arachis hypogaea* agglutinin (peanut lectin) [53, 54], Con A [55], *Lens culinaris* hemagglutinin [53], *Sophora japonica* agglutinin [54], and *Vicia cracca* agglutinin [56], induce UV-difference spectra upon binding with specific sugars. Spectrophotometers having a high sensitivity have recently made possible to obtain the association constants of lectins with specific sugars [53, 54]. In the case of STA, difference spectra with two positive peaks at 292 and 285 nm, and a shoulder at 275 nm were induced by chitin oligosaccharides (Fig. 4). The highest peak was at 292 nm, and only one shoulder was observed at 275 nm, in contrast with the spectra obtained with WGA, where peaks at 285 (highest), 292, and

275 nm have been observed [53]; this suggested that the microenvironment of the tryptophan residues was perturbed by the binding to specific sugars [57] and that the contribution of tyrosine residues is low. The value of $\Delta\varepsilon_{max}$ reflects the number of tryptophan residues perturbed by the binding [58]. The number of perturbed tryptophan residues in STA was calculated to be 2–4, and the value of $\Delta\varepsilon_{max}$ increased with the chain length of the sugar, and with the binding constant (Table III), as observed for lysozyme with chitin oligosaccharides [59].

The modification of near-UV-CD spectra by the binding of a specific sugar has been observed for some lectins; AHA [60], RCA [49], and WGA [52] induced the change of near-UV-CD bands upon interaction with a specific sugar, which indicated the alteration of the asymmetric environment mainly at some aromatic side-chain chromophores at the surface of the lectin molecule. The modification of the CD spectrum of WGA at 272 and 285 nm probably involves tyrosine residues and, at 290 nm, tryptophan residues. In the case of STA, $(GlcNAc)_2$ and $(GlcNAc)_3$, but not GlcNAc, induced the decrease of the CD intensity at 250–300 nm. The intensity of the CD band at 291 nm involving tryptophan residues was decreased more strongly by $(GlcNAc)_3$ than by $(GlcNAc)_2$, as shown in Fig. 11. These results indicate that tryptophan residues in STA are perturbed more strongly by $(GlcNAc)_3$ than $(GlcNAc)_2$ but not by GlcNAc.

As shown in Table IV, the binding constants of STA with chitin oligosaccharides obtained by UV-difference spectroscopy were in good agreement with those obtained by frontal analysis on STA-Sepharose, and much lower than those obtained by frontal analysis on $(GlcNAc)_n$-Sepharose. The binding capacity of STA-Sepharose was calculated to be 40–70% of that calculated from the amount of immobilized STA, while the capacities of $(GlcNAc)_n$-Sepharose were about 1/150 of those estimated from the amount of immobilized ligands. Since the concentration of the immobilized sugars is very high (30 μmol/ml of gel), not all ligands may interact with a large molecule of STA owing to steric hindrance, but they may contribute to the very high,

Table IV. Association constants of STA with chitin oligosaccharides[a]

	$(GlcNAc)_2$	$(GlcNAc)_3$	$(GlcNAc)_4$
UV-Difference spectrum	2.0×10^3 (2,200)	4.1×10^4 (3,000)	5.4×10^4 (3,600)
Frontal analysis by STA-Sepharose column	6.3×10^3	4.1×10^4	8.3×10^4
Frontal analysis by oligosaccharide-Sepharose coulmn	6.5×10^4	1.5×10^5	

[a] Values are association constants (M^{-1}).

[b] Values in parentheses are $\Delta\varepsilon_{max}$.

apparent binding-constant by the so-called multivalent effect. The binding constant between STA and chitin oligosaccharides increased with the chain length of oligosaccharides, as was observed on WGA. However, the binding constants of $(GlcNAc)_3$ and $(GlcNAc)_4$ for STA were larger than those for WGA, respectively, and the binding constant of $(GlcNAc)_2$ for STA was smaller than for WGA [61]. This tendency was shown also by hemagglutination studies [11, 62, 63, 64]. These results suggest that STA has a larger binding site than WGA.

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