PROTEIN–SUGAR INTERACTION: PURIFICATION BY AFFINITY CHROMATOGRAPHY OF *SOLANUM TUBEROSUM* AGGLUTININ (STA-LECTIN)

F. DELMOTTE, C. KIEDA and M. MONSIGNY

Centre de Biophysique Moléculaire du C.N.R.S. and Laboratoire de Chimie Biologique de l'Université d'Orleans, 45045 Orléans Cedex, France

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1. Introduction

Solanum tuberosum tubers contain a lectin which agglutinates erythrocytes [1]. Several procedures of purification of this lectin have been proposed: Singh et al. [2] used an acetone fractionation, Marinkovich [3] used chromatography on an SP-cellulose column and electrophoresis on starch block; recently Allen and Neuberger [4] used ammonium sulfate precipitation and several column chromatography steps on DEAE-cellulose, CM-cellulose, Sephadex G-100 and SP-Sephadex.

The material isolated by Singh et al. [2] was not a protein, the procedure of Marinkovich [3] did not yield a pure product, and the procedure of Allen and Neuberger [4] was time consuming. In order to overcome these difficulties, we have tried to develop an easier procedure using affinity chromatography.

2. Materials and methods

Ammonium sulfate and buffer salts were obtained from Merck. DEAE-cellulose DE 52 was from Whatman (Maidstone, England) and Sepharose 4B and Sephadex G-100 from Pharmacia (Uppsala, Sweden). All reagents used were analytically pure of the best grade available. Di-*N*-acetyl-chitobiose, tri-*N*-acetyl-chitotriose, tetra-*N*-acetyl-chitotetraose, *p*-nitrophenyl tri-*N*-acetyl-β-chitotrioside were prepared as described previously [5]. The potato tuber extract was obtained from Roquette Frères (Lille, France). The agglutinating activity of the lectin was tested using rabbit red blood cells.

2.1. *Glycosylhydrolase activities*

The glycosylhydrolase activities were monitored using *p*-nitrophenyl glycosides according to Conchie [6]. The following glycosides were used: *p*-NP-β-GlcNAc, *p*-NP-α-Man, *p*-NP-β-Glc, *p*-NP-αGlc, *p*-NP-β-Gal, *p*-NP-α-Gal, *p*-NP-α-Fuc and *p*-NP-β-Fuc (Koch-Light Laboratories, Colnbrook, England).

2.2. *Affinity chromatography columns*

Sepharose substituted by ovomucoid and Sepharose substituted by *p*-aminobenzyl 1-thio-*N*-acetyl-β-D-glucosaminide were prepared as described previously by Privat et al. [7] and Rafestin et al [8] respectively.

Sepharose substituted by *p*-aminophenyl di-*N*-acetyl-β-chitobioside. The *p*-nitrophenyl-β-chitobioside (546 mg), prepared by the method of Zurabyan et al. [9] dissolved in methanol-water (9:1, v/v) (100 ml) in the presence of HCl (12 N, 0.1 ml) was reduced by hydrogen (0.1 atm, 6 hr) with palladium charcoal (0.5 g) as catalyst. The suspension was filtered on a celite pad which was washed with methanol. The solution was concentrated by evaporation under diminished pressure. The sirup was diluted with water (5 ml) and this colored solution was applied to a column (50 cm × 2 cm) of Sephadex G-10 and eluted with water. The fractions containing the *p*-amino-phenyl chitobioside were combined and freeze-dried (314 mg).

Sepharose 4B (25 ml) activated with cyanogen bromide [10] was coupled with ε-aminocaproic acid,

and the free carboxyl groups were then activated with CMCI [*N*-cyclohexyl-*N*-(2-(4-β-morpholinyl)-ethyl) carbodiimide-methyl-*p*-toluene sulfonate] (Fluka) [11].

The *p*-aminophenyl-β-chitobioside (276 mg) was linked to this Sepharose 4B. In order to avoid any ion exchange effects, unreacted carboxyl groups in the extension arm were blocked with 0.5 mmol of Tris–[2-amino-2-hydroxymethyl-propane-1,3-diol] and with 1.6 mmol of CMCI. The substituted Sepharose was washed with a 0.05 M Tris–HCl, 0.5 M NaCl buffer (pH 7.2) and poured into a column (7 cm × 1.5 cm).

3. Results and discussion

The purification scheme is shown in table 1. The first two steps (ammonium sulfate precipitation and DEAE-cellulose column) are slight modifications of the procedure described by Allen and Neuberger [4]. All operations were performed at 4°C.

The potato extract from potato tuber was centrifuged at 8000 *g* for 20 min in order to eliminate fine particules. Crystalline ammonium sulfate (901 g) was added to the combined supernatants (3.5) to produce 50% saturation at 0°C. The precipitate was left to settle overnight and collected by centrifugation at 8000 *g* for 20 min. The precipitate was suspended in water, dialyzed against distilled water (2 × 24 hr), and the suspension centrifuged at 8000 *g* for 20 min. The supernatant (612 ml) included all the lectin activity.

DEAE-cellulose column

The supernatant (612 ml) from the previous step was applied directly to a column of DEAE-cellulose (35 cm × 4 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 8.6) and washed with the same buffer. Fractions (20 ml) were collected. The active material passed through the column, as shown in fig.1.

As several authors have claimed that the

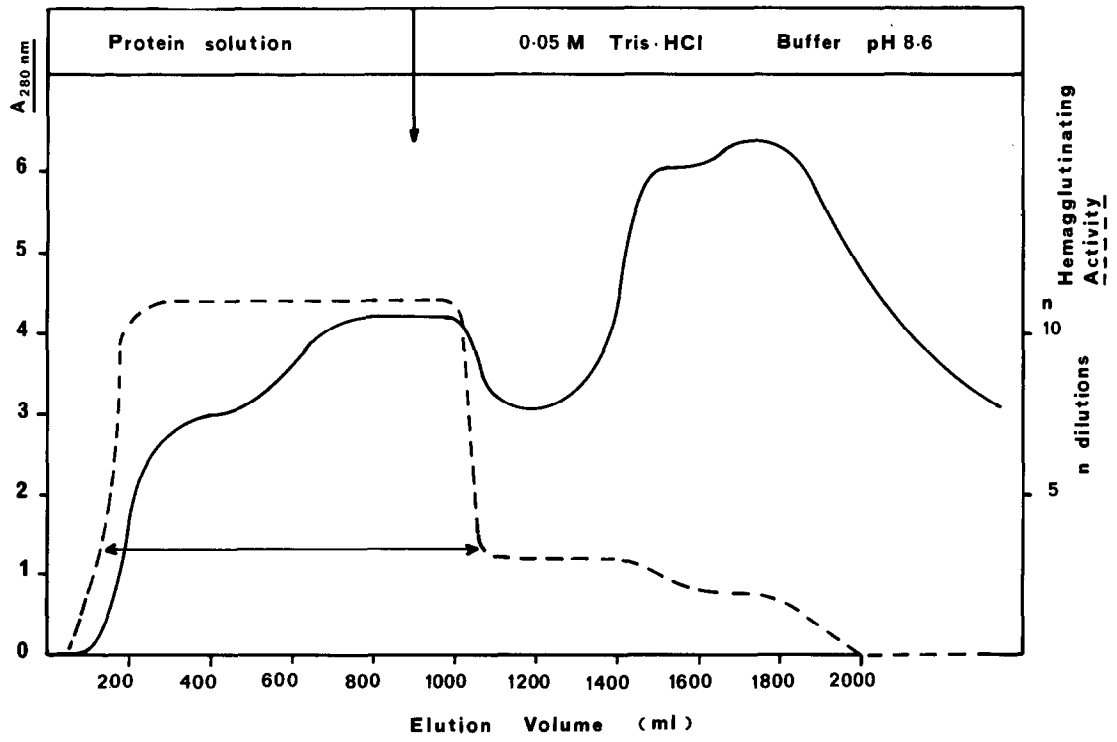

Fig.1. DEAE-cellulose chromatography of dissolved 50% ammonium sulfate precipitate (612 ml). The column (35 cm × 4 cm) was eluted with 0.5 M Tris–HCl buffer (pH 8.6). (———) Absorption at 280 nm; (– – – –) agglutinating activity; flow rate 50 ml/hr; fractions (20 ml) were collected. (←——→) Frations with agglutinating activity were pooled.

Table 1
Purification of potato lectin

Procedure	Volume (ml)	$A^{280\ nm}_{1\ cm}$	Total protein content (g)	Agglutination titre**	Total activity (agglutination units)	Specific activity (units/mg)	Yield %
Potato tuber extract after centrifugation	3500	44	128.3	0.4×10^6	2.7×10^{10}	0.2×10^6	100
50% $(NH_4)_2SO_4$ precipitate after dialysis and centrifugation	612	26	13.3	2.1×10^6	2.6×10^{10}	1.9×10^6	96
DEAE-cellulose column	575	3.3	1.6	2.1×10^6	2.4×10^{10}	7.3×10^6	89
Affinity chromatorgraphy on *p*-aminobenyl-1-thio-*N*-acetyl-β-D-glucosaminide	35	2.0	0.058*	33×10^6	2.35×10^{10}	11.7×10^6	87

The procedure started with 3.5 l of potato tuber extract. The protein content was estimated photometrically assuming a specific absorbance: $A^{280\ nm}_{1\ cm,\ 1\%} = 12$.

* Weight after freeze drying

** Rabbit red blood cells.

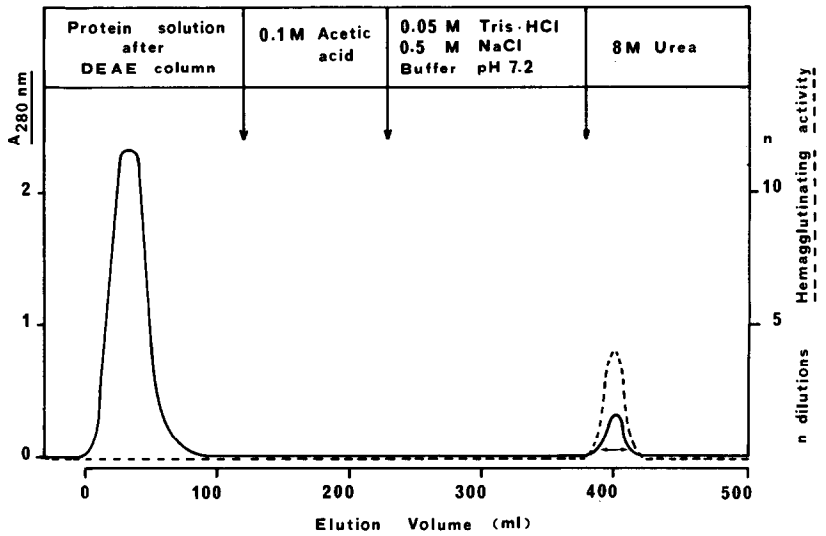

Fig.2. Affinity chromatography on ovomucoid substituted-Sepharose column (9 cm × 1.5 cm; flow rate 30 ml/hr). Pooled frations (30 ml) from DEAE-cellulose chromatography column were applied; the column was washed with 0.05 M Tris–HCl, 0.5 M NaCl buffer, pH 7.2, with 0.1 M acetic acid; with 0.05 M Tris–HCl, 0.5 M NaCl buffer, pH 7.2 and with 8 M urea, successively. (———) Absorption at 280 nm; (– – – –) agglutinating activity after exhaustive dialysis.

agglutination of cells by *Solanum tuberosum* extracts or agglutinin (STA) is not inhibited by mono-saccharides or their derivatives [4, 12–14] but is inhibited by di-*N*-acetyl chitobiose or the β-1-4 linked oligomers of *N*-acetylglucosamine [4,13], we started using Sepharose substituted by ovomuciod which has already been used to isolate wheat-germ agglutinin [7,15], another di-*N*-acetyl-chitobiose binding lectin.

An aliquot (30 ml) of the active material from the DEAE column was applied to the ovomucoid substituted Sepharose column equilibrated with a 0.05 M Tris–HCl, 0.5 M NaCl buffer, pH 7.2. The column was washed with the same buffer, to remove inactive protein. The lectin could not be eluted with 0.1 M acetic acid, 0.05 M HCl, or 0.1 M sodium borate, pH 10.05, but only by 8 M urea solution (fig.2). Unfortunately, the protein isolated in this way had almost quantitatively lost its activity. This result can be explained by the high affinity of the lectin for linked ovomucoid, and only a drastic denaturation of the lectin can allow the elution of the protein.

In order to develop a non-denaturing procedure, we tried using *p*-aminophenyl di-*N*-acetyl-β-chitobioside substituted Sepharose. Unfortunately the lectin was also strongly bound to the matrix and only 8 M urea solution was able to elute the protein which had lost its activity.

Finally, we used *p*-aminobenzyl 1-thio-*N*-acetyl β-D-glucosaminide substituted Sepharose as follows:

The clear active material (575 ml) obtained from the DEAE-column was applied directly at 4°C to this conjugated Sepharose column (7 cm × 1.5 cm) which had been equilibrated with a 0.05 M Tris–HCl buffer, pH 7.2. The column was eluted at 60ml/hr, washed with 0.05 M Tris–HCl, 1 M NaCl buffer, pH 7.2, until no significant amount of material absorbing at 280 nm was detected in the effluent ($A^{280\ nm}_{1\ cm} < 0.05$). The lectin was then eluted with 0.1 M acetic acid. Under these conditions, the protein was fully active. As a slight impurity was detected by polyacrylamide gel electrophoresis and by column chromatography on Sephadex G-100, the procedure was therefore slightly modified: before elution with 0.1 M acetic acid, the column was washed further with sodium acetate 0.1 M, pH 4.1. Under these conditions, the impurity was eluted and the acetic acid eluate contained only the pure lectin (fig.3).

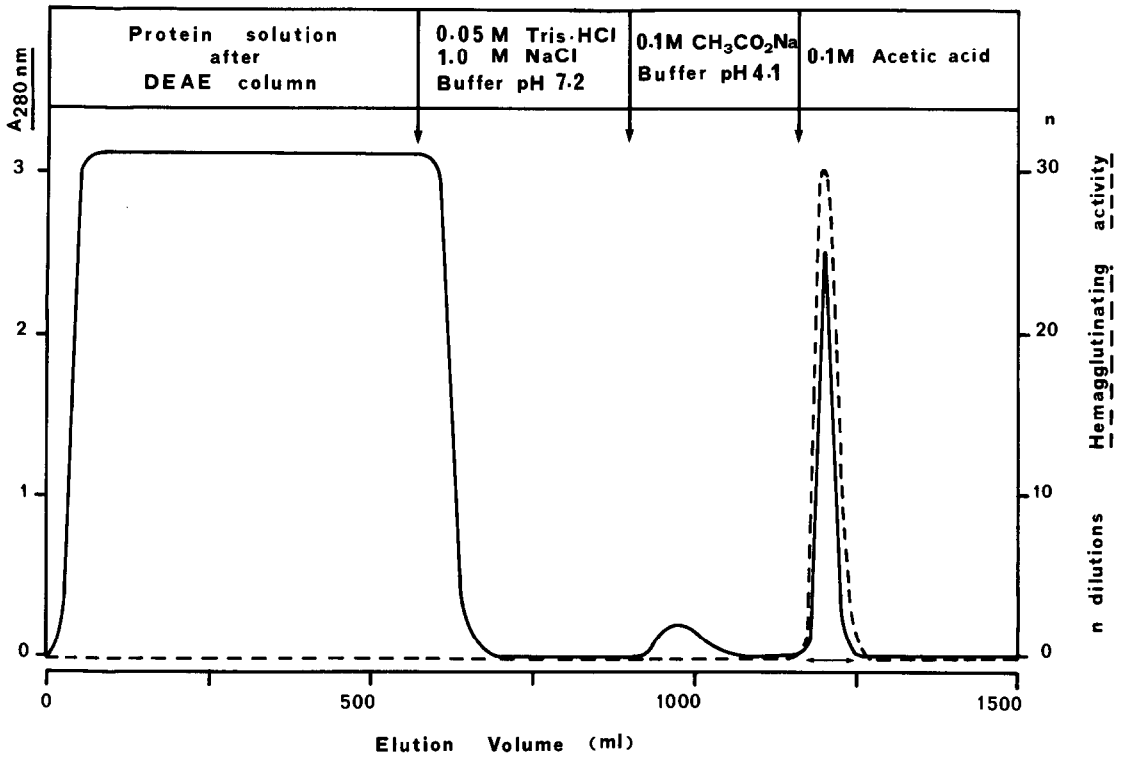

Fig. 3. Affinity chromatography of pooled fractions (575 ml) from DEAE cellulose on Sepharose *p*-aminobenzyl-1-thio-β-D-1-2-dideoxy-β-D-glucopyranoside column (7 cm × 1.5 cm, flow rate 60 ml/hr). The column was washed with 0.1 M NaCl buffer, pH 7.2, then with sodium acetate 0.1 M buffer, pH 4.6, and the protein was eluted by 0.1 M acetic acid. Fractions of 12 ml were collected. (———) Absorption at 280 nm; (– – – –) agglutinating activity; (←———→) Fractions with agglutinating activity were pooled.

Table 2
Enzymatic activities (nmol × min^{-1} × mg^{-1} protein)
(assuming a protein absorbance of $A^{280\,nm}_{1\,cm,\,1\%} = 12$)

p-Nitrophenyl glycosides	Potato tuber extract	50% $(NH_4)_2SO_4$ precipitate	Eluate from DEAE-cellulose column	Pure STA
β-GlcNAc	5.54	12.02	0	0
α-Man	2.24	0.98	2.93	0
β-Glu	5.21	0.51	0	0
α-Glu	0.03	0	0	0
β-Gal	1.93	0.67	0	0
α-Gal	1.47	7.05	0	0
α-Fuc	0.15	0.21	0	0
β-Fuc	0	0	0	0

The acetic acid eluate was dialysed against distilled water and freeze dried.

The purification procedure described above involves only three steps: ammonium sulfate fractionation, filtration on DEAE-cellulose and affinity chromatography. The yield at each step is high, and the overall yield is better than 85%. The DEAE-cellulose was an important step, since it allowed the elimination of the glycosylhydrolases, specially of the *N*-acetyl-β-D-glucosaminidases (table 2). Washing the affinity column with 1 M NaCl buffer removed the α-D-mannosidase and the lectin was then devoid of any glycosylhydrolase activity.

It is worth pointing out that a monosaccharide derivative (*p*-aminobenzyl-1-thio-β-D-glucosaminide) can be used to purify this lectin though the free *N*-acetyl-D-glucosamine is a very poor inhibitor, and though 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine substituted Sepharose [16] does not bind the lectin (N. Sharon, personal communication). The aromatic ring seems to play an important role in enhancing the affinity of the ligand towards the lectin. This point was confirmed by experiments on the inhibition of agglutination of rabbit erythrocytes; the concentration of sugar required to inhibit one hemagglutinating dose [17] was: 700 μmol D-GlcNAc; 150 μmol $(GlcNAc)_2$; 50 μmol $(GlcNAc)_3$; 10 μmol $(GlcNAc)_4$; 200 μmol *p*-NP-β-D-GlcNAc; 100 μmol S-*p*-NBz-β-D-GlcNAc; 60 μmol *p*-NP-β-$(GlcNAc)_2$; 20 μmol *p*-NP-$(GlcNAc)_3$. A similar conclusion was reached in the case of the wheat-germ lectin [18]. In contrast, with high affinity ligands linked to the matrix (such as the di-*N*-acetyl-chitobiose derivative or ovomuciod) the binding of the lectin was so strong that the protein could be eluted only with a denaturing agent.

The purified STA migrated as a single band in 10% polyacrylamide gel at pH 9.2, in 10% polyacrylamide gel containing 0.1% SDS and 0.1% mercapto-ethanol at pH 7.2 and in polyacrylamide gel electrofocusing with a pH_i of 8.4. In immunodiffusion and immunoelectrophoresis using rabbit antibodies, only one precipitation band could be detected. Amino acid analysis of acid hydrolysates of STA indicated the presence of hydroxyproline [4] but the complete absence of cystathionine and of ornithine, in constrast with the results of Marinkovich [3] and Allen and Neuberger [4]. A minute amount of $GlcNH_2$ was detected on the amino-acid analyzer, and the amount of neutral sugar was 43.6% (tested by the orcinol sulphuric acid reaction [19]) showing that STA is a glycoprotein, in agreement with the report of Allen and Neuberger [4].

The same procedure with the *p*-amino-benzyl-1-thio-β-D-*N*-acetyl-glucosamine substituted Sepharose column, can be used to isolate pure wheat germ agglutinin. This procedure is now being tested for the isolation of other *N*-acetyl-glucosamine specific lectins, e.g. from *Ulex Europeus* and *Cytisus sessilifolius*.

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