PURIFICATION OF WHEAT GERM AGGLUTININ BY AFFINITY CHROMATOGRAPHY ON A SEPHAROSE-BOUND N-ACETYLGLUCOSAMINE DERIVATIVE

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SUMMARY: Sepharose-2-acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine was prepared by a reaction of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine and N-(benzyloxycarboxyl)-ε-aminocaproic acid, removal of the O-acetyl and the benzyloxycarboxyl groups and coupling to Sepharose. The product was used for the purification of wheat germ agglutinin, by adsorption from a crude wheat germ extract and elution with 0.1M acetic acid. The purified agglutinin was homogeneous on SDS-polyacrylamide gel electrophoresis and had a specific hemagglutinating activity of 3000 u/mg when tested on trypsinized rabbit erythrocytes. It was rich in cysteine, cystine and glycine, and contained no sugar.

INTRODUCTION: Cell-agglutinating and sugar-specific proteins, known as lectins, can be purified either by conventional methods of protein chemistry, or by affinity chromatography (for reviews see refs. 1 and 2). We have recently reported the preparation of Sepharose-ε-aminocaproyl-β-D-galactopyranosylamine and its use for the purification of soybean agglutinin, a lectin specific for N-acetyl-D-galactosamine and D-galactose (3).

Here we describe the synthesis of the analogous derivative of N-acetyl-D-glucosamine, and its use for the purification of the N-acetyl-D-glucosamine-specific wheat germ agglutinin (WGA) (4-7) from commercial wheat germ.

The purification of WGA from wheat germ lipase by affinity chromatography on columns of insolubilized ovomucoid, a glycoprotein rich in N-acetyl-D-glucosamine has been previously described, (5,6).

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EXPERIMENTAL: N-Acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucopyranose) was a gift from Pfizer Inc. (New York). Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden), and cyanogen bromide from Eastman Kodak Co. (Rochester, N.Y.). Commercial wheat germ was used as source of WGA.

Melting points were determined with a Fisher-Johns apparatus. Rotations were determined, in 1-dm, semimicro tubes, with a Perkin-Elmer No. 141 Polarimeter; i.r. spectra of KBr pellets (1 mg compound in 100 mg KBr) were recorded with a Perkin-Elmer Spectrophotometer Model 237,and n.m.r. spectra with a Varian T-60 apparatus. Evaporations were performed in vacuo, the bath temperature was below 50°. Homogeneity of compounds was tested by ascending t.l.c. on precoated plates of silica gel G (Merck). Spots were detected by spraying with 20% sulfuric acid and heating at 200° for a few minutes. Microanalyses were performed by the laboratory of microanalysis of the Department of Organic Chemistry at the Weizmann Institute of Science.

Hemagglutinating activity was determined by a spectrophotometric method (8,9) using trypsinized rabbit erythrocytes.

2-Acetamido-3,4,6-tri-O-acetyl-N-[N-(benzyloxycarbonyl)-ε-aminocaproyl]-2-deoxy-β-D-glucopyranosylamine (I).

Isobutylchloroformate (12 mmoles), and triethylamine (12 mmoles) were added to a solution of N-(benzyloxycarbonyl)-ε-aminocaproic acid (12 mmoles) (10) in N,N-dimethylformamide (20 ml), kept at - 5°. The mixture was stirred for 20 min. at -5° and then filtered. The filtrate was immediately added to a solution of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine, (10 mmoles in 20 ml, N,N-dimethylformamide) [synthesized from 2-acetamido-2-deoxy-D-glucopyranose according to literature (11, 12)]. The mixture was stirred at room temperature for two hours and then allowed to stand for 16 h after which the solvent was evaporated to dryness. The residue was crystallized twice from methanol:ether. Yield - 62% (3.68 g), m.p. 203°-205°; $[\alpha]_D^{20}$ + 11.9° (c, 1.0, N,N-dimethylformamide); i.r. data: ν_{max}^{KBr} 1530 cm^{-1} (peptide Amide I), 1655 (CONH), 1700 (benzyloxycarbonyl C=O) and 1745 (O-acetyl); n.m.r. data:τ 2.72 (5H, phenyl), 3.05, 3.55 (2 NH), 7.96,

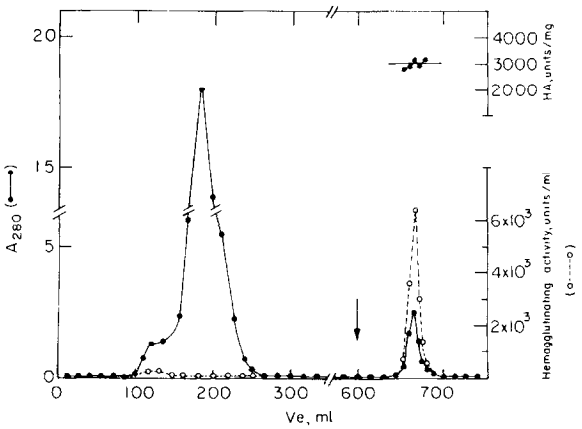

Figure 1. Affinity chromatography of WGA on a column of Sepharose-2-acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine. An extract from 100 g defatted wheat germ (90 ml) was applied to a column (2.8 x 17 cm) and after washing with 600 ml saline (15 ml.fractions), elution (arrow) was performed with 0.1M acetic acid (6 ml.fractions). The flow rate was 100 ml/h and chromatography was carried out at 4°. HA - Hemagglutinating activity.

8.05 (9H, three acetoxy groups), 8.12 (3H, acetamido group). Found: C, 56.89; H, 6.51; N, 7.15. $C_{28}H_{39}O_{11}N_3$ requires C, 56.68; H, 6.57; N, 7.08.

2-Acetamido-N-[N-(benzyloxycarbonyl)-ε-aminocaproyl]-2-deoxy-β-D-glucopyranosylamine (II).

Freshly prepared 0.1M methanolic sodium methoxide (13) was added dropwise to a solution of compound I (5 mmoles in 50 ml absolute methanol) to give pH 8. The mixture was stirred for 5 h at room temperature. The progress of deacetylation was monitored by t.l.c. in acetone-methanol (9:1 v/v) (for R_fs. see Table 1). The solution was neutralized by the addition of solid CO_2 and the solvent was evaporated to dryness. The residue was crystallized from methanol:ether. Yield - 90% (2.1 g); m.p. 218°-220°; $[\alpha]_D^{20}$ + 12.9° (c, 1.0, N,N-dimethylformamide); i.r. data: 1530 cm^{-1} (peptide amide I); 1655 (CONH), 1705 (benzyloxycarbonyl C=O). Found: C, 56.67; H, 7.07; N, 9.17. $C_{22}H_{33}O_8N_3$ requires C, 56.52; H, 7.12; N, 8.99.

2-Acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine (III).

A solution of compound II (4.5 mmoles in 75 ml methanol:water, 8:2 v/v) was hydrogenated in the presence of 10% palladium-on-charcoal (50 mg) at atmospheric pressure at room temperature for 8 h. The catalyst was filtered off and the

TABLE I

R_f values of N-acetylglucosamine derivatives on t.l.c. in different solvent systems

T.l.c. was performed on precoated silica gel plates (20x20 cm) CAMAG (Muttenz/Schwiez).

Compound*	Ethyl Acetate:Acetone (4:1 v/v)	Acetone:Methanol (9:1 v/v)	Methanol:Acetone (9:1 v/v)
I	0.55	1.0	1
II	0	0.57	0.95
III	0	0	0.3

*For description of I, II and III, see text.

filtrate was evaporated to dryness. The residue was crystallized from methanol: ether. Yield 80% (1.2 g); m.p. 193°-195° (dec.). $[\alpha]_D^{20}$ + 23.3° (c, 1.0 water); i.r. data: 1530 cm^{-1} (peptide Amide I), 1655 (CONH). Found C, 50.32; H, 7.93; N, 12.78. $C_{14}H_{27}O_6N_3$ requires: C, 50.43; H, 8.17; N, 12.6.

Binding of the ligand (III) to Sepharose 4B

Sepharose 4B was activated with cyanogen bromide and then reacted with 2-acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine, by the general method of Axén et al (14) following the detailed procedure described before (3). The conjugated resin was stirred gently for 3 h in 1M ethanolamine, pH 8, to block unreacted active groups on the Sepharose. The amount of ligand covalently bound to the Sepharose was determined by acid hydrolysis of the conjugated resin and estimation of the liberated glucosamine on a Beckman-Spinco amino acid analyzer. The conjugated resin was found to contain 2 μmoles of covalently bound ligand per ml packed Sepharose.

Purification of WGA.

Extraction of the agglutinin from defatted commercial wheat germ, and

isolation of the 55% ammonium sulfate fraction were done essentially as described by Allen et al. (7). After dialysis and centrifugation, the clear supernatant obtained from 100 g of defatted wheat germ was applied at 4° to a column (2.8 x 17 cm) containing 100 ml of the conjugated Sepharose which had been equilibrated with saline. The column, kept at 4°, was eluted at the rate of 100 ml/h, first with saline (15 ml.fractions), until no significant amount of material absorbing at 280 nm was detected in the effluent (A280 <0.05), and then with 0.1 M acetic acid (6 ml. fractions). The effluent was monitored by its absorbance at 280 nm.

The fractions, eluted with acetic acid and containing U.V. absorbing material were pooled, neutralized by dropwise addition of 1M NaOH, dialyzed extensively against water, centrifuged and lyophilized.

RESULTS AND DISCUSSION

The purification procedure described above involves only three steps: water extraction, ammonium sulfate fractionation and affinity chromatography (Table II). Ammonium sulfate precipitation resulted in a 4-fold purification of WGA, with about 50% yield. The bulk of the proteins present in the active ammonium sulfate fraction was not adsorbed to the column (Fig. 1), but the hemagglutinating activity of these proteins was negligible. The agglutinin that was adsorbed to the column could not be eluted with N-acetyl-D-glucosamine (0.1M, 200 ml); it was however eluted with 0.1M acetic acid. The final product had the same activity as that described in the literature (7), and was obtained in a comparable yield.

The purified WGA migrated as a single band in 10% polyacrylamide gel containing 0.1% SDS. Amino acid analysis of acid hydrolysates of WGA gave a composition very similar to that reported in the literature; it was rich in cysteine,cystine and glycine (6,7,15). No indication for the presence of amino sugars was observed on the amino acid analyser trace, and there was no neutral sugar (<0.01%) tested by the phenol-sulfuric acid reaction (16), showing that WGA is not a glycoprotein. This result is in agreement with the report of Allen et al. (7) but it contradicts other reports (4,6,15).

TABLE II

Purification of WGA from Wheat Germ

Fraction	Total protein (g)	Total activity (hemagglutinating units)	Specific activity (units/mg)	Yield %
Water extract from 100 g defatted wheat germ	28	280,000	10	100
Ammonium sulfate precipitate (55%) dialyzed and centrifuged	3.5	140,000	40	50
Purified WGA after affinity chromatography	0.03	90,000	3000	32

Table II. Protein in the first two fractions was determined according to Lowry et al. (17) and in the last step by direct weighing of the lyophilized agglutinin. Hemagglutinating activity was determined spectrophotometrically (8,9).

Ion exchange chromatography of the purified WGA on a SP-Sephadex column according to the method of Allen et al. (7) gave three protein peaks each having the same hemagglutinating activity (~3000 u/mg). The amount of protein in each peak was different from that reported previously (7). This may reflect strain differences in the wheat used for the isolation of the agglutinin.

The use of the column described in this communication for the isolation of other N-acetylglucosamine-specific lectins (e.g. from *Ulex europeus* and potato) is now being tested.

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