PURIFICATION OF WHEAT GERM AGGLUTININ USING AFFINITY CHROMATOGRAPHY ON CHITIN

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Summary: Chitin, the naturally occurring polymer of N-acetyl-glucosamine, has been used as a ligand matrix for affinity chromatography of wheat germ agglutinin in a new purification method which is well suited for large scale preparations. The agglutinin obtained is homogeneous with respect to polypeptide chain molecular weight, has a blocked amino terminus and is free of proteolytic and β-N-acetyl-glucosaminidase activities.

Wheat germ agglutinin is a plant lectin which has been used extensively to study the role of carbohydrates in the structure and functions of cell membranes (1-5). Its specificity for N-acetyl-glucosamine has been amply demonstrated by equilibrium dialysis (6) and by the ability of this sugar and its oligosaccharides to inhibit agglutination activity (2,3,7). This specific binding has been the basis for several procedures for purification of wheat germ agglutinin (WGA) by affinity chromatography. Two of these use ovomucoid, a glycoprotein rich in N-acetyl-glucosamine (GlcNac), as the immobilized ligand (3,8), while two others use GlcNac linked to Sepharose through an inert hydrocarbon bridge (9,10). We report here that chitin, the naturally occurring polymer of GlcNac, when ground and packed into a column serves both as matrix and ligand in affinity chromatography of WGA. The simplicity, speed and economy of chitin affinity chromatography suggest that it should be useful for large scale purification of WGA.

Materials and Methods: Wheat germ (Ekra, Bern, Switzerland) was purchased from a local health food shop, and GlcNac was obtained from Merck. Chitin, in the form of ground clam shells, was from Sigma. It was ground further in a coffee grinder for a few minutes, and washed extensively with water, 0.05 N HCl, 1% Na_2CO_3 and ethanol until the wash solution had an optical density less than 0.05

at a wavelength of 280 nm. It was then packed into a column to give a bed volume of 4 ml for preliminary experiments, or of 800 ml for large scale preparations. To obtain a fraction enriched in oligosaccharides of GlcNac, chitin was hydrolyzed and partially fractionated on activated charcoal (11). Paper chromatography of the enriched fraction showed it to be composed mainly of di- and trisaccharides, with smaller amounts of monosaccharide and larger oligomers.

Proteolytic activity was measured by the method of Rosenbusch (12), β-N-acetyl-glucosaminidase activity by the method of Agrawal and Bahl (13), and protein according to Lowry et al (14). The concentration of a solution of pure WGA was determined using an extinction coeffecient ($A^{1\%}_{280\ nm}$) of 14.3 (6). Amino acid analysis was done on performic acid oxidized protein (15) according to Spackman, Stein and Moore (16) using a Beckman model 4255 liquid column chromatograph. Agglutinin activity was determined using a minor modification of a method previously described (17) with type A+ erythrocytes from out of date blood provided by the Swiss Red Cross.

Results and Discussion: Because the purification scheme presented here relies upon the specific interaction of WGA with chitin, we first determined how much WGA could be bound per gram of chitin. This was investigated in two days: (a) by titrating a solution of WGA with increasing amounts of chitin; (b) by saturating a small amount of chitin with a concentrated solution of WGA, washing the chitin free of unbound WGA, and then eluting the bound protein with 0.05 N HCl. The results of these experiments indicate that 1 g of chitin binds approximately 10 mg of WGA (Table I). We have used this binding capacity as the basis of an affinity chromatography step in the following purification procedure.

One kg of crude wheat germ is ground in a mill and extracted with 10 l of 0.05 N HCl for one hour at room temperature. The mixture is centrifuged at 2,000 x g for 10 min. and the extract is brought to 35% saturation with ammonium sulfate. The precipitate is collected at 10,000 x g for 15 min. suspended in one l of 0.05 N HCl and dialyzed extensively against water and then

Table I
WGA Binding to Chitin

	chitin samples *	
experiment	1	2
	(mg WGA bound/g chitin)	
I. Titration	-	8
II. Saturation and Elution	11	12

I. To a solution containing 10 mg of WGA in 20 ml of phosphate-buffered saline, pH 7.4, 250 mg quantities of chitin were added. 10 minutes after each addition, the suspension was centrifuged briefly and the absorbance at 280 nm of the supernatant was measured. The control value, the absorbance of the supernatant from a suspension of chitin in the absence of WGA, was subtracted from the experimental value. The difference was then used to calculate the amount of WGA bound.

II. 25 mg chitin were placed into a small disposable Eppendorf pipet tip plugged with glass wool. 1 ml of a concentrated WGA solution (3 mg/ml) in phosphate-buffered saline, pH 7.4, was passed through the pipet three times. The chitin was washed with buffer (4 volumes of 0.5 ml each) and then the WGA was eluted with 0.05 N HCl (4 volumes of 0.5 ml each). The absorbance at 280 nm of the elutant was determined and used to calculate the amount of WGA bound. If the WGA solution is passed through the same pipet tip with chitin seven times, the same amount of WGA is bound.

* The two chitin samples used were of different purity and were ground to different extents. The close agreement between the two suggest that these factors do not greatly affect the capacity of chitin for WGA.

against 0.01 M Tris-HCl, pH 8.5. The soluble material is mixed with 400 ml of DEAE-cellulose contained in a sintered glass funnel. Shortly thereafter eluant is collected by application of moderate negative pressure. The resin is washed repeatedly with 0.01 M Tris-HCl, pH 8.5, until only negligible agglutinating activity can be eluted. All fractions containing activity are pooled and applied to a chitin column.

The chitin column is first developed with 0.01 M Tris-HCl, pH 8.5, containing 1 M NaCl. This solution elutes protein which either does not bind to the column, or binds nonspecifically.

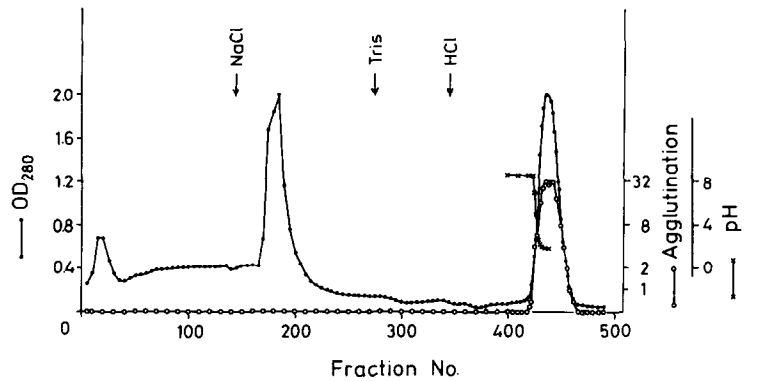

Figure 1. Affinity chromatography of partially purified WGA on a chitin column. The 2.4 l effluent from DEAE-cellulose was applied to a column (5.4 x 35 cm; about 250 g) of chitin buffered with 0.01 M Tris-HCl, pH 8.5. The column was developed first with 2 l 0.01 M Tris-HCl, 1 M NaCl, pH 8.5, then with one l 0.01 M Tris-HCl, and finally with 2 l 0.05 N HCL. The flow rate was approximately one l/hour, and fractions of about 15 ml were collected. The symbols are: ●, absorbance at 280 nm; o, agglutinating activity, expressed as the factor by which 0.01 ml of the sample in question must be diluted to give 2+ agglutination of erythrocytes (17); x, pH.

The column is then washed with 0.01 M Tris-HCl to remove the NaCl. If this step is omitted, very little WGA is recovered in the following step, presumably because the protein precipitates on the matrix. Finally, the column is eluted with 0.05 N HCl: WGA elutes as the pH decreases (Figure 1). The fractions containing agglutinating activity are pooled.

The protein in these fractions is concentrated by ammonium sulfate precipitation (see above). It is then resuspended in and dialyzed overnight against 0.05 N HCl and applied to a Sephadex G-50 column equilibrated with the same solvent. This gel filtration step separates WGA from several non-agglutinating high molecular weight contaminants that remain after elution from chitin (Figure 2). A summary of the purification is given in Table 2.

Alternate ways of eluting the WGA from chitin have been attempted. Elution with several column volumes of a solution of 0.05 M GlcNac does not yield a sharp peak of WGA, nor does preincubation of the chitin matrix and the WGA with 0.1 M GlcNac prevent WGA from binding to the column. However, if the column is developed with oligosaccharides of GlcNac (chitin hydrolyzate, 1% w/v) WGA readily elutes and

Table II

Summary of the Purification

step	specific activity (units/mg)	total protein[a] (mg)	total activity (units)
HCl Extraction	11.4	1.47×10^5	1.68×10^6
DEAE-Cellulose	310	4.67×10^3	1.45×10^6
Chitin	1800	4.91×10^2	8.85×10^5
Sephadex G-50	1790[b]	3.34×10^2	5.98×10^5

a Protein concentration in this experiment was determined from the absorption at 280 nm of each sample, assuming an extinction coefficient equal to that of WGA.

b The apparent decrease in specific activity is within the error of the agglutination assay.

is free of any plypeptide contaminants, as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (18,19). Agglutinin activity can be found in this fraction after extensive dialysis against 0.05 N HCl. Although this procedure gives pure WGA samples, large scale preparations using elution with chitin hydrolyzate are impractical due to the tedious preparation of large amounts of oligosaccharide-enriched material.
Elution with acid is therefore our method of choice.

We have characterized our purified agglutinin in the following ways. (a) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gives a single polypeptide chain of molecular weight 17,000 (Figure 2, insert), in agreement with previous reports (6,7,9,10). (b) The specific activity of the preparation is as high as that for crystalline WGA: 2+ agglutination of type A+ erythrocytes is achieved at 5 μg WGA per ml. (c) An attempt to determine the amino-terminal residue by reaction with dimethylaminonaphthalene sulfonyl chloride (dansyl chloride) (20) has shown no alpha-amino dansyl derivatives. This suggests that the amino terminus is blocked, as also reported by Shaper et al (10). Nagata, in this laboratory, has shown by three independent methods that the amino terminus is

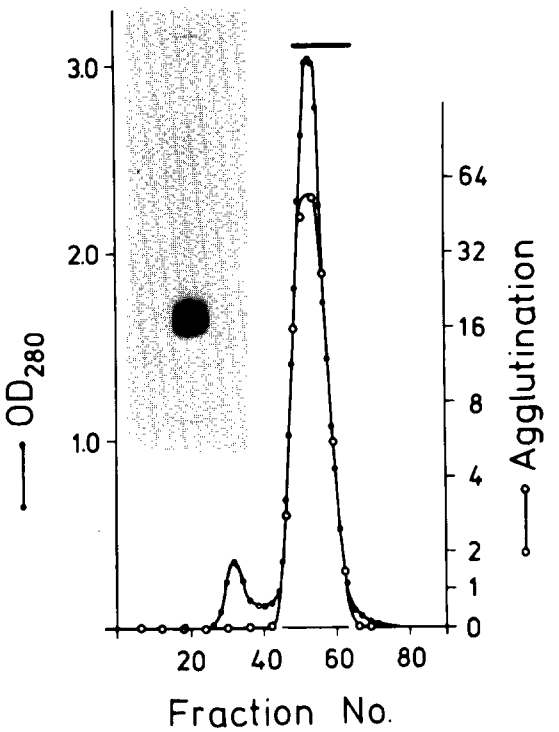

Figure 2. Gel filtration chromatography of WGA at low pH. The pooled fractions from the chitin column (Fig. 1) were concentrated and applied to a Sephadex G-50 column (5 x 75 cm) in 0.05 N HCl. 17 ml fractions were collected. The symbols are as in Figure 1; the fractions indicated by the bar were pooled. Insert: polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of the pooled sample. 60 μg of protein was applied to a gel containing 10% acrylamide and 0.1% methylene bisacrylamide. Electrophoresis was from top to bottom.

blocked (unpublished data). (d) Attempts to find either β-N-acetyl-glucosaminidase or protease activity in the purified protein have shown no such contamination. We consider this an important control because chitin affinity chromatography might also purify hexosaminidases and proteases (our chitin preparation probably still contains protein which could bind proteolytic enzymes). (e) The amino acid composition is identical within the expected errors to those already reported for WGA (6-10). (f) The WGA is easily crystallized, as previously reported (17). In all these respects, then, the WGA obtained by the procedure described here is identical to purified WGA described earlier.

We feel that this procedure has several advantages over those previously published. It does not rely on crystallization as a final purification step. It entails neither the preparation of ligands nor their coupling to a resin, and is considerably more economical. It is quite rapid, and can easily be adapted for large scale purifications. Chitin affinity chromatography is also used in our laboratory for the separation of inactive protein and modifying reagents from modified but active WGA. Iodinated and fluorescein-labelled WGA have been prepared using this technique, and further applications are anticipated.

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