Wheat Germ Agglutinin

ISOLATION AND CRYSTALLIZATION*

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SUMMARY

Procedures for the isolation, purification, and crystallization of wheat germ agglutinin are described. The agglutinin was purified 184-fold to homogeneity from commercial wheat germ lipase. A molecular weight of 23,500 was estimated for the protein by means of sedimentation equilibrium and sodium dodecyl sulfate gel electrophoresis. The agglutinin is a glycoprotein. Amino acid and carbohydrate compositions are reported. The protein contains unusually high half-cystine and glycine. Glucose was found to be the major carbohydrate constituent.

A lipase preparation from wheat germ was found by Aub et al., (1) to agglutinate primarily, but not exclusively, malignant cells. The impurity in the lipase preparation which caused agglutination of the transformed cells was found after partial purification to be a glycoprotein (2, 3). Purification of the wheat germ agglutinin was begun with the hope of using the pure material as a tool to study changes in the cell surface structure after viral transformation (4).

This preliminary report is concerned with the isolation and crystallization of the wheat germ agglutinin, as well as its physical and chemical properties.

Assay of Agglutination—Washed L1210 leukemia cells (0.1 ml), suspended at a concentration of about 2 × 10⁶ cells per ml of phosphate-buffered NaCl solution (pH 7.4), were mixed with agglutinin solution (0.01 ml), and a hanging drop of this preparation was examined microscopically after 5 min of mixing on a rocking platform at room temperature. A serological scale of 0 (more than 75% single cells), + (50%), + + (25%), + + + (10%), and + + + + (less than 4% single cells) was used to estimate the degree of clumping.

One unit of agglutinating activity was defined as the reciprocal of the amount of agglutinin (in milligrams per ml of the assay system) which gave half-maximal (+ +) agglutination. It should be noted, however, that this method is not as accurate as the usual enzymatic assay methods, since it is only semi-quantitative and since some experience is required until blind readings agree within 10 to 15%.

Isolation of Wheat Germ Agglutinin—Partially purified agglutinin was prepared according to the procedure already described (5), with the following modifications. Wheat germ lipase (10 g; Pentex or Miles Laboratories, Inc., Kankakee, Illinois) was dissolved in distilled water (500 ml) and treated at 63° for 15 min. The precipitate was removed by centrifugation at 18,000 × g for 15 min. Crystalline ammonium sulfate was added to the supernatant while stirring at 4° to give 38% saturation. The precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed extensively against 1 mM phosphate buffer, pH 7.0. The dialyzed sample was then chromatographed on DEAE-cellulose (Cellex D, Bio-Rad; 2.5 × 20 cm) which was previously equilibrated with the same buffer. Elution was performed with approximately 300 ml of 1 mM phosphate buffer, pH 7.0. All of the agglutinating activity eluted without adsorption on DEAE-cellulose, suggesting that the agglutinin is a relatively basic protein. Chromatography on carboxymethylcellulose (Cellex C, Bio-Rad; 1.0 × 25 cm) was then performed. Agglutinating activity was released around 0.2 M NaCl as shown in Fig. 1. Active fractions were pooled, dialyzed against distilled water, and lyophilized. A summary of the purification of wheat germ agglutinin is presented in Table I. Due to the high stability of the agglutinin, recoveries are relatively high and usually amounted to more than 50% yield.

Crystallization—The lyophilized agglutinin preparation obtained from carboxymethylcellulose chromatography was dissolved in distilled water (about 20 mg per ml) and kept at 4°. Crystals appeared after 1 day (Fig. 2). For recrystallization, the crystals were harvested by centrifugation, dissolved in a small volume of 0.05 N HCl, neutralized with NaOH, and kept at 4°. Many crystals are longer than 1 mm.

The specific activity of crystalline wheat germ agglutinin was 470 units per mg for L1210 leukemia cells. When human red blood cells (Hyland Division Travenol Laboratories, Inc., Costa Mesa, California) were used as test cells, the same amount of the agglutinin gave 700 units for blood group A1 cells and 180 units for blood group B cells.

Analysis of Purity and Estimation of Molecular Weight—Crystalline wheat germ agglutinin gave a single band on polyacrylamide gel electrophoresis (Fig. 3A). In a Spinco model E ultracentrifuge, the agglutinin gave a single symmetrical peak with a sedimentation coefficient of 2.1 S. An equilibrium run was performed in 0.05 N HCl at 20° and 9,341 rpm, and a molecular weight of 23,500 was computed. The partial specific volume of

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Fig. 1. Chromatography of wheat germ agglutinin on carboxymethylcellulose. Active fractions from a DEAE-cellulose column were pooled (92 mg of protein), dialyzed against 1 mM phosphate buffer, pH 6.0, and applied to the column. After two-step elution, first with 500 ml of 1 mM phosphate buffer, pH 6.0, and then with 500 ml of 0.08 M NaCl in the same buffer, a linear gradient of NaCl from 0.08 to 0.4 M was applied. Fractions (15 ml) were collected and assayed for optical density at 280 nm (O-O-O) and agglutinating activity (●-●-●).
TABLE I

<table>
<thead>
<tr>
<th></th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Yield</th>
</tr>
</thead>
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<tr>
<td>Lipase extract</td>
<td>9600</td>
<td>1.9 (1.0)(^a)</td>
<td>1.86</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>3150</td>
<td>5.9 (3.1)</td>
<td>1.85</td>
<td>99</td>
</tr>
<tr>
<td>(NH(_4))SO(_4)</td>
<td>665</td>
<td>20 (13.7)</td>
<td>1.73</td>
<td>93</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>92</td>
<td>140 (73.7)</td>
<td>1.29</td>
<td>69</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>31</td>
<td>350 (184)</td>
<td>1.09</td>
<td>59</td>
</tr>
</tbody>
</table>

\(^a\) Purification factor starting with the commercial lipase preparation.

Fig. 2. Crystals of wheat germ agglutinin photographed through a polarizing microscope (Carl Zeiss, West Germany). The crystal in the inset (B) is about 2-mm long.

the agglutinin was assumed to be 0.69 as calculated from its amino acid composition (5).

Another molecular weight approximation was made on sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Inouye (6). Wheat germ agglutinin gave a single band, and a molecular weight of 23,000 to 24,000 was estimated. Earlier data on partially purified wheat germ agglutinin gave a molecular weight of 26,000 (2).

**Amino Acid and Carbohydrate Compositions**—Crystalline wheat germ agglutinin (1 mg) was hydrolyzed in 6 N HCl at 105° for 24 or 72 hours in evacuated sealed tubes and aliquots (150 µg eq) were analyzed on a Beckman amino acid analyzer. Two independent analyses were made, and weight recoveries were 93 and 96%, respectively, in the first analysis and 90 and 93%, respectively, in the second. These values seem to be reasonable, since wheat germ agglutinin contains about 4.5% by weight of carbohydrate as described below. Results of the two independent analyses expressed as residues per 23,500 g of wheat germ agglutinin were as follows: lysine 9, 10; histidine 2, 2; arginine 6, 6; aspartic acid 20, 20; threonine 6, 6; serine 16, 16; glutamic acid 22, 22; proline 8, 8; glycine 50, 52; alanine 15, 14; half-cystine 40, 40; valine 4, 2; methionine 3, 3; isoleucine 3, 3; leucine 7, 6; tyrosine 9, 10; phenylalanine 5, 5; and tryptophan 4. Tryptophan was determined from the absorption spectrum of an unhydrolyzed wheat germ agglutinin solution in 0.1 N NaOH (7). The major unusual feature was the presence of very high amounts of half-cystine and glycine. Contents of aspartic acid and glutamic acid were also high, although several of these residues are probably present in the amide form since, based on its elution behavior from ion exchange columns and its mobility in disc gel electrophoresis, wheat germ agglutinin seems to be a relatively basic protein.

The role of the carbohydrate prosthetic group in agglutinating activity was emphasized earlier by the fact that the activity was sensitive to periodate oxidation (1–3). Reducing sugar tests (8) indicated that about 4.5% of the wheat germ agglutinin was carbohydrate, using glucose as standard. Preliminary experiments on the sugar composition were made by paper chromatography in two different solvent systems (butanol-1-pyridine-water, 6:4:3 (8), butanol-1-ethanol-water, 10:1:2 (10)). After hydrolyzing the agglutinin in 2 N H\(_2\)SO\(_4\) at 100° for 4 hours, the neutral sugars were separated from amino acids by passage.
through a mixed-bed ion exchange resin (AG 501-X8 (D), Bio-Rad), while the amino sugars were liberated from the glycoprotein in 4 N HCl at 100° for 6 hours. Glucose was found to be the major carbohydrate component, whereas xylose and hexosamine occurred to a lesser degree. The ratios of the three sugars varied from 3 to 5 moles of glucose and 1 to 2 moles of xylose and hexosamine per mole of the agglutinin. The mannose content decreased gradually with the degree of purity of the wheat germ agglutinin. The crystalline wheat germ agglutinin probably has no mannose or at best 1 mole per mole of the agglutinin.

More detailed analytical data on the amino acid and carbohydrate composition will be reported elsewhere.

Wheat germ agglutinin preparations obtained from a Sephadex G-75 (2) or a DEAE-cellulose chromatography gave two bands on polyacrylamide gel electrophoresis (Fig. 3C). The two bands were always of about the same relative intensity. However, a single band, corresponding to the slower moving band of the two (Fig. 3, A and C), was observed upon electrophoresis of the active material eluted from a carboxymethylcellulose column. The faster moving band (Fig. 3B), which was also obtained in pure form after chromatography on a carboxymethylcellulose column, was found to have no agglutinating activity for either L1210 leukemia cells or human red blood group A or B cells. Amino acid analysis showed a clear difference between the two proteins, e.g. wheat germ agglutinin contained 40 residues of half-cystine and 3 residues of valine, whereas the faster moving band contained 14 residues of half-cystine and 22 residues of valine per 23,500 g. Therefore, the main impurity associated with wheat germ agglutinin up to the DEAE-cellulose step is probably not a subunit of wheat germ agglutinin or a similar kind of phytohemagglutinin.

A comparison with other agglutinins, concanavalin A and soybean agglutinin, both known to agglutinate also primarily but not exclusively transformed cells, indicates that these two agglutinins do not contain any cysteine, whereas carbohydrates have been observed in soybean agglutinin (mannose and glucosamine) but not in concanavalin A (11-13). On the other hand, only the nonagglutinating, inactive subunit of concanavalin A (active molecule, 55,000 and 100,000; inactive subunit, 27,000) (14, 15) has a molecular weight in the range of wheat germ agglutinin.

An x-ray crystallographic study on wheat germ agglutinin is now in progress in Dr. R. Langridge's laboratory at Princeton University, and preliminary results will be reported (16).

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REFERENCES


The Stimulation of Rat Liver Phenylalanine Hydroxylase by Phospholipids

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SUMMARY

The maximum velocity of rat liver phenylalanine hydroxylase is stimulated by the following phospholipids, in order of decreasing potency: lysolecithin, lysophosphatidylserine, phosphatidylserine, and sphingomyelin. Lysolecithin stimulates the maximum velocity 50-fold when the naturally occurring pterin, tetrahydrobiopterin, is used as cofactor, and only 1.5-fold when the synthetic cofactor, 6,7-dimethyl-tetrahydropterin, is employed. In the presence of tetrahydrobiopterin, lecithin also converts the initial velocity-phenylalanine concentration relationship from a sigmoidal to a hyperbolic curve.

Rat liver phenylalanine hydroxylase requires a reduced pterin cofactor as a source of electrons for the conversion of phenylalanine to tyrosine (1). When a synthetic pterin, 6,7-dimethyltetrahydropterin, is used as cofactor, the initial velocity-phenylalanine concentration curve is of the classical Michaelis-Menten hyperbolic type (2). When the naturally occurring pterin, tetrahydrobiopterin (3), is employed as cofactor, phenylalanine gives a sigmoidal saturation curve and a maximum velocity much lower than that obtained with the synthetic cofactor (2).

It was recently reported that when DMHP, or is used as the cofactor and tryptophan as the substrate for crude rat liver phenylalanine hydroxylase, the velocity-tryptophan concentration curve is sigmoidal (4). It was also reported that high concentrations (i.e. 0.3 %) of propanol can convert the kinetics from sigmoidal to the Michaelis-Menten hyperbolic form. We were interested in determining whether a similar effect of propanol could be observed with phenylalanine hydroxylase in the presence of its normal substrate, phenylalanine, and the naturally occurring cofactor, BH4. We found that not only does propanol change the kinetics from sigmoidal to hyperbolic under these conditions, but it also increases the maximum velocity several-fold. In a search for a naturally occurring compound that might be a more potent activator of the hydroxylase, we found that lyssolecithin and lysophosphatidylserine at low concentrations are capable of greatly stimulating the
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